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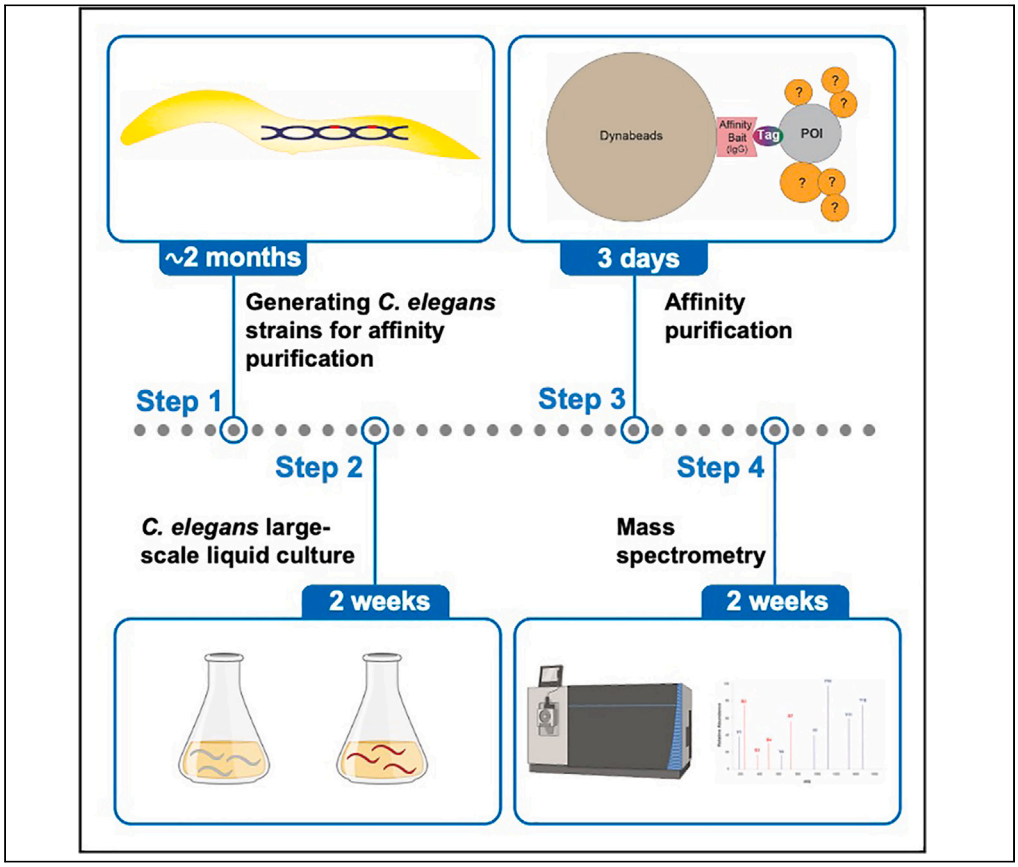
Optimized protocol for *in vivo* affinity purification proteomics and biochemistry using *C. elegans*



We present an optimized protocol for *in vivo* affinity purification proteomics and biochemistry using the model organism *C. elegans*. We describe steps for target tagging, large-scale culture, affinity purification using a cryomill, mass spectrometry, and validation of candidate binding proteins. Our approach has proven successful for identifying protein-protein interactions and signaling networks with verified functional relevance. Our protocol is also suitable for biochemical evaluation of protein-protein interactions *in vivo*.

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

Muriel Desbois, Joseph S. Pak, Karla J. Opperman, Andrew C. Giles, Brock Grill

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Highlights

Affinity purification proteomics in *C. elegans* identifies protein interactions *in vivo*

C. elegans offers large-scale liquid cultures and versatile genetic toolkit

Protocol facilitates *in vivo* biochemistry to test protein-protein interactions

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Optimized protocol for *in vivo* affinity purification proteomics and biochemistry using *C. elegans*

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SUMMARY

We present an optimized protocol for *in vivo* affinity purification proteomics and biochemistry using the model organism *C. elegans*. We describe steps for target tagging, large-scale culture, affinity purification using a cryomill, mass spectrometry and validation of candidate binding proteins. Our approach has proven successful for identifying protein-protein interactions and signaling networks with verified functional relevance. Our protocol is also suitable for biochemical evaluation of protein-protein interactions *in vivo*.

For complete details on the use and execution of this protocol, please refer to Crawley et al.,[1] Giles et al.,[2] and Desbois et al.[3]

BEFORE YOU BEGIN

C. elegans is an invertebrate nematode that has been an essential model for deciphering gene function *in vivo* and has enabled tremendous advances in understanding basic biology and tackling important questions in biomedical research. This most often occurs via unbiased forward and candidate genetic screens. However, genetic approaches have some notable limitations as a starting point for discovery science. Essential genes are less likely to be investigated as mutations in these genes result in lethality. Functional redundancy between genes might prevent or reduce phenotypic outcomes. Compensation between genes might reduce the effect of a loss-of-function mutation. Affinity purification (AP) proteomics overcomes these limitations because it does not rely on the use of mutants. Rather, AP-proteomics identifies the putative interactome for a protein of interest (POI) thereby presenting opportunities to identify novel protein complexes, signaling pathways and functional interactions.

To date, AP-proteomics has been underutilized in *C. elegans* relative to more traditional genetic screening approaches. Nonetheless, *C. elegans* is particularly well-suited and offers several advantages as a platform for large-scale proteomics. *C. elegans* are easily grown in large quantities, which is essential for isolating considerable amounts of a POI and its interactome. Numerous tools are available that can be used to genetically tag almost any POI.[4–7] It is feasible to transgenically express a POI using a native, physiologically relevant promoter on a protein null mutant background, thereby increasing affinity enrichment of protein complexes of interest. Finally, rapid genetics facilitates experiments to evaluate how an affinity tag and its location affect POI function. These advantages heighten the likelihood of AP-proteomics identifying bona fide binding proteins while

decreasing the risk of false-positive hits. Importantly, there is substantial evidence for the utility of *in vivo* AP-proteomics in *C. elegans*.[8–16]

At present, relatively few labs use AP-proteomics in *C. elegans* to study POIs expressed exclusively or primarily in the nervous system. This is potentially because it has been difficult to obtain sufficient amounts of POI from the nervous system, which comprises a relatively small portion of the worm's total mass. Here, we describe an optimized AP-proteomics protocol that we previously used to study two gigantic E3 ubiquitin ligases with prominent expression and function in the nervous system, RPM-1 and EEL-1.[1–3] Ubiquitin ligases catalyze conjugation of ubiquitin to substrates often leading to substrate degradation by the proteasome. In the case of RPM-1 (MYCBP2) and EEL-1 (HUWE1), prominent links to neurodevelopmental disorders have been identified.[17–19] Thus, AP-proteomics has and will continue to yield important advances in understanding how these molecules regulate nervous system development and why altering their function leads to neurological disorders.

Identifying both the ubiquitination substrates and interactome for ubiquitin ligases presents further challenges. This includes the transient nature of interactions between ubiquitin ligases and their substrates, and the number of binding proteins associated with physically large ubiquitin ligases such as RPM-1 and EEL-1. Our prior work has shown that AP-proteomics is valuable for identifying binding proteins that mediate RPM-1 and EEL-1 function, but are not ubiquitination substrates.[2,12,20–22] These findings have underpinned the concept that RPM-1 acts as both a signaling hub and ubiquitin ligase.[18] In our most recent studies, we leveraged AP-proteomics and a biochemical 'trap' that enriches RPM-1 ubiquitin ligase substrates.[1,3] CRISPR-based native biochemistry and genetic interactions were then used to validate substrates.

Thus, over several studies with multiple POIs, we have arrived at optimized methods for *in vivo* AP-proteomics using *C. elegans*. While our studies focused on neuronal ubiquitin ligases, we anticipate our approach could be deployed to target other POIs expressed within and outside the nervous system.

Before beginning the protocol:

1. Check the protocol overview and summary shown in Figure 1.
2. Prepare all stock solutions beforehand.
3. Make sure all *C. elegans* strains are healthy before starting.
4. Make sure to have liquid nitrogen readily available.
5. Establish a connection with a mass spectrometry facility.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-FLAG (use at 1:1000 dilution)	Cell Signaling	Cat # 2368S
Mouse monoclonal anti-GFP (use at 1:1000 dilution)	Roche	Cat # 11814460001
Mouse monoclonal anti-SBP (use at 1:1000 dilution)	MilliporeSigma	Cat # MAB10764
HRP-conjugated sheep IgG anti-mouse (use at 1:20 000 dilution)	GE Healthcare Life Sciences	Cat # 45000679
HRP-conjugated donkey IgG anti-rabbit (use at 1:20 000 dilution)	GE Healthcare Life Sciences	Cat # 45000682

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
OP50 *E. coli*	Caenorhabditis Genetics Center (CGC) – University of Minnesota	Cat # OP50
HB101 *E. coli*	Fermenter service (University of Minnesota – Biotechnology Institute)	https://bti.umn.edu/biotechnology-resource-center/
Chemicals, peptides, and recombinant proteins		
Nematode growth medium (NGM) (NaCl, Bacto-Peptone, Agar, Cholesterol, $CaCl_2$, $MgSO_4$, KH_2PO_4, K_2HPO_4, ddH_2O)	This paper	Materials
TMP (4,5′,8-Trimethylpsoralen) 1 mg/mL	Sigma	Cat # T6137-100mg
S basal and S complete (NaCl, K_2HPO_4, KH_2PO_4, $CaCl_2$, $MgSO_4$, Cholesterol, $C_6H_8O_7.H_2O$, $C_6H_5K_3O_7 \cdot H_2O$, Disodium EDTA, $FeSO_4 \cdot 7\ H_2O$, $MnCl_2 \cdot 4\ H_2O$, $ZnSO_4 \cdot 7\ H_2O$, $CuSO_4 \cdot 5\ H_2O$)	This paper	Materials
M9 (Na_2HPO_4, KH_2PO_4, NaCl, $MgSO_4$)	This paper	Materials
Sucrose	Sigma	Cat # S0389
Roche cOmplete mini protease inhibitor cocktail EDTA free tablet	Sigma	Cat # 11836170001
NP-40 Surfact-Amps, 10% stock solution	Thermo Fisher	Cat # 28324
CHAPS powder	MP Biomedicals	Cat # 04808196
Tris/HEPES lysis buffer (Tris or HEPES, NaCl, $MgCl_2$, Glycerol)	This paper	Materials
HALT Protease inhibitor Cocktail (100×)	Thermo Fisher	Cat # 78429
HALT Phosphatase Inhibitor Cocktail (100×)	Thermo Fisher	Cat # 78420
DTT	Fisher	Cat # BP1725
PMSF	Sigma	Cat # P7626-5G
NuPage LDS sample buffer (4×)	Invitrogen	Cat # NP0007
NuPage Sample Reducing Agent (10×)	Invitrogen	Cat # NP0009
High-molecular-weight standards	Cytiva	Cat # 17061501
HiMark Prestained Protein Standard	Invitrogen	Cat # LC5699
NuPage 3–8% Tris-Acetate Gel	Invitrogen	Cat # EA03785BOX
NuPage 4%–12% Bis-Tris Gel	Invitrogen	Cat # NP0323BOX
NuPage Tris-Acetate SDS Running Buffer (20×)	Invitrogen	Cat # LA0041
NuPage MOPS SDS Running Buffer (20×)	Invitrogen	Cat # NP0001
NuPage Antioxidant	Invitrogen	Cat # NP0005
TAC transfer buffer (Bicine, Bis-Tris, EDTA, Methanol)	This paper	Materials
TBS (Tris, NaCl)	This paper	Materials
Tween 20	Sigma	Cat # P7949
4× Laemmli Sample Buffer	Bio-Rad	Cat # 161-0747
2-Mercaptoethanol	Bio-Rad	Cat # 161-0710
7.5% Mini-Protean TGX Tris-Glycine Precast Gels	Bio-Rad	Cat # 4561024
Porcine trypsin	Sigma	Cat # T6567
Critical commercial assays		
Pierce BCA protein assay kit	Thermo Fisher	Cat # 23225
Pierce Silver Stain Kit	Thermo Fisher	Cat # 24612
ECL, SuperSignal West Pico/Femto	Thermo Fisher	Cat # 34580 and Cat # 34095
Experimental models: Organisms/strains		
C. elegans: Strain N2 (hermaphrodite, all life stages)	Caenorhabditis Genetics Center (CGC) – University of Minnesota	Cat # N2
C. elegans strains of interest that will be generated for affinity purification (hermaphrodite, all life stages)		

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Orbitrap Fusion/Lumos (instrument control software)	Thermo Fisher	
Xcalibur (data collection software)		https://www.thermofisher.com/order/catalog/product/OPTON-30965
Proteome Discoverer (peptide identification software)		https://www.thermofisher.com/us/en/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-software/multi-omics-data-analysis/proteome-discoverer-software.html
Scaffold (data analysis software)		https://www.proteomesoftware.com/
Other		
Pipette pump	Bel-Art	Cat # F378980000
CryoMill (with liquid nitrogen tank)	Retsch	Cat# 20.749.0001
Stainless-steel grinding jar, 50 mL (ordered with CryoMill)	Retsch	Cat # 01.462.0332
Stainless-steel grinding ball, 20 mm (ordered with CryoMill)	Retsch	Cat # 05.368.0062
Dynabeads M-280 Sheep anti-Rabbit IgG	Invitrogen	Cat # 11204D
DynaMag-2	Thermo Fisher	Cat # 12321D
DynaMag-15	Thermo Fisher	Cat # 12301D
Tube Revolver Rotator	Thermo Fisher	Cat # 88881001
High-speed centrifuge (fixed angle rotor; 20,000 × *g*)	Beckman	Varies with model
Nalgene Oak Ridge High Speed Centrifuge Tube 50 mL	Thermo Fisher	Cat # 3119-0050
Benchtop centrifuge (swinging bucket rotor; 1,500 × *g*)	Hermle	Varies with model
Porcelain mortar	Sigma	Cat # Z247499
Orbital flask shaker, 2-inch orbit, 185 rpm	VWR	Cat # 10027-162
Low retention 1.7 mL microcentrifuge tube	Costar	Cat # 3207
Protein mini gel tank	Thermo Fisher	Cat # A25977
Mini-PROTEAN gel transfer tank	Bio-Rad	Cat # 1658029
Analytical RP column, 0.075 × 250 mm Acclaim PepMap RLSC nano Viper (via proteomics core)	Thermo Fisher	Cat # 164569
ZipTip with 0.2 µL C18 resin (via proteomics core)	MilliporeSigma	Cat # ZTC18M096
EASY-nLC 1000 system (via proteomics core)	Thermo Fisher	Cat # LC120
Orbitrap Fusion/Lumos Tribrid Mass Spectrometer (via proteomics core)	Thermo Fisher	Varies with instrument
6 cm and 10 cm plates (disposable Petri dishes)	VWR	Cat # 25384-090 and 25384-088
Spectroline XL1000 UV crosslinker	Fisher Scientific	Cat # 11-992-89
2 L Erlenmeyer flasks	VWR	Cat # 10545-844
15 mL and 50 mL conical tubes	Genesee Scientific	Cat # 28-103 and 28-108
Vacuum filter systems, 0.22 µm PES membrane, 250 mL and 500 mL	Genesee Scientific	Cat # 25-225 and 25-227
Dry ice	Provided by your institute	Provided by your institute
Liquid nitrogen	Provided by your institute	Provided by your institute
Liquid nitrogen dewar	Thermo Fisher	Cat # S34074C

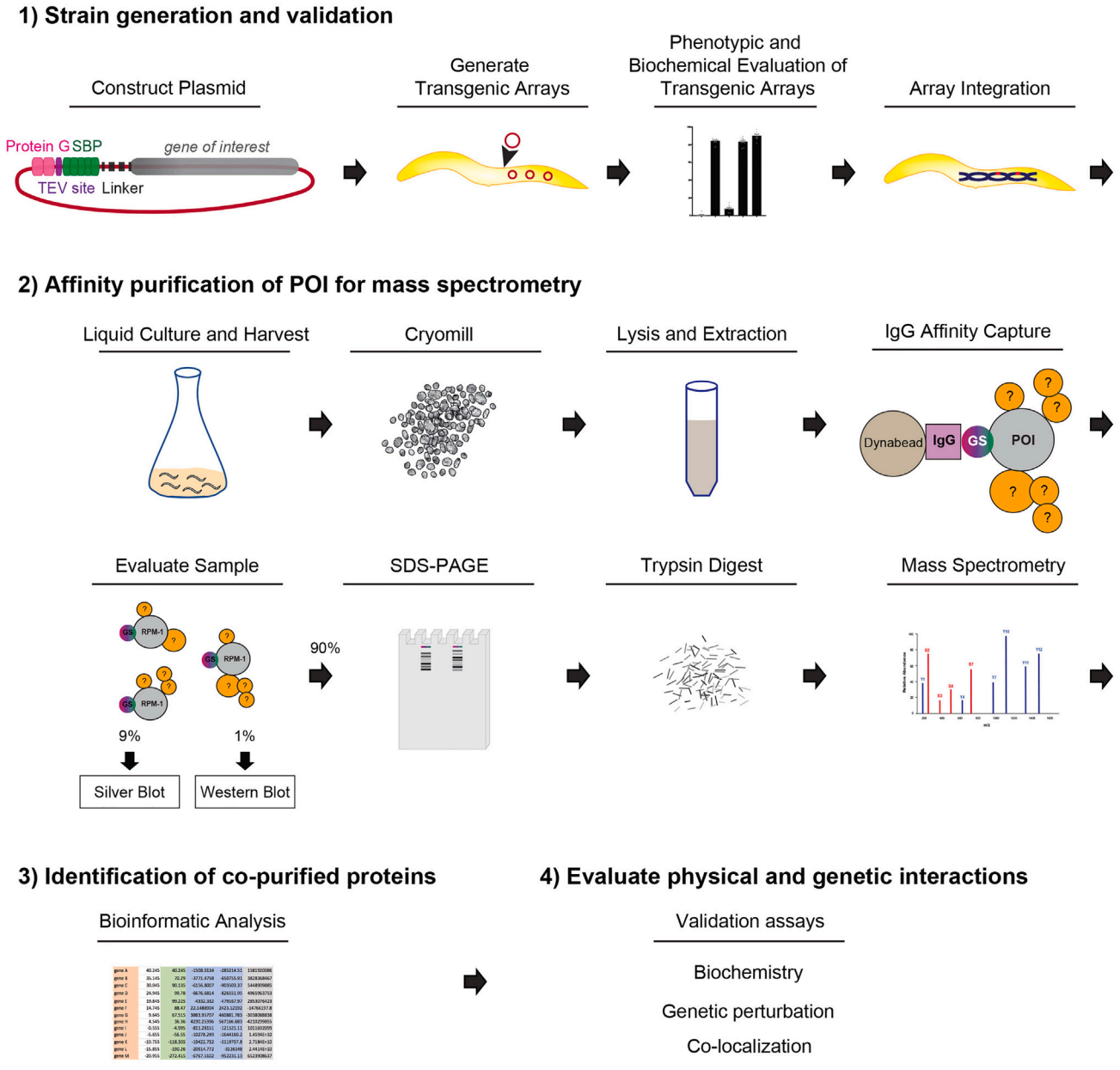

Figure 1. Experimental workflow for AP-proteomics in *C. elegans*

Schematic illustrating full workflow for AP-proteomics using *C. elegans*. Workflow is divided into four main sections: 1) *C. elegans* strain building and validation, 2) affinity purification and mass spectrometry, 3) bioinformatic analysis of proteomic hits, and 4) validation of putative binding proteins.

MATERIALS AND EQUIPMENT

Strains

C. elegans strains (wild-type N2, strains of interest that will be generated for affinity purification).

Culturing *C. elegans* on solid media in preparation for liquid culture

6 and 10 cm Nematode growth medium (NGM) plates (store for up to 2 months at 4°C):

Reagent	Final concentration	Amount
NaCl	3 g/L	3 g
Bacto-Peptone	2.5 g/L	2.5 g
Agar	16 g/L	16 g
ddH_2O	N/A	To 1 L
Total	**N/A**	**1 L**

Autoclave, then add ingredients below in the order specified on a stir plate. Use autoclave/filter sterilize (0.22 μm filter) solutions except cholesterol which is in ethanol, store solutions at 20°C–23°C for 6 months:

Reagent	Final concentration	Amount
Cholesterol (5 mg/mL in 95% ethanol)	5 mg/L	1 mL
$CaCl_2$ (0.5 M)	10 mM	2 mL
$MgSO_4$ (1 M)	1 mM	1 mL
Potassium phosphate buffer pH 6.0 (see below)	25 mM	25 mL
ddH_2O	N/A	To 1 L
Total	**N/A**	**1 L**

Pour 10 mL per 6 cm plate, or 30 mL per 10 cm plate.

Potassium phosphate buffer (1 M, pH 6.0):

Reagent	Final concentration	Amount
KH_2PO_4	0.8 M	108.3 g
K_2HPO_4	0.2 M	35.6 g
ddH_2O	N/A	To 1 L
Total	**N/A**	**1 L**

Seeded NGM plates (store at 20°C–23°C for 15 days or 4°C for 2 months):

Inoculate 6 cm plates with 200 μL OP50. Let the OP50 dry for 24 h at 20°C–23°C before using.

Inoculate 10 cm plates with 500 μL OP50. Spread bacteria across entire plate using sterilized bacteria spreader, leaving ~1 cm around the rim of agar without bacteria. Let dry for 24 h at 20°C–23°C before using.

OP50 E. coli liquid culture (store at 4°C for 1 month).

Inoculate 100 mL LB media with 1 colony of OP50. Incubate for ~18 h without shaking at 37°C.

Transgene integration

TMP (4,5′,8-Trimethylpsoralen) 1 mg/mL (Sigma T6137-100mg) dissolved in DMSO (Sigma D2650) (store at −20°C for 1 month).

Concentrated OP50:

Spin down 15 mL of OP50 liquid culture in 15 mL conical tube for 5 min at 3,000 × *g*. Aspirate supernatant using vacuum filtration system leaving 200 μL liquid media. Resuspend pellet in remaining media.

C. elegans liquid culture

S Basal (store at 20°C–23°C for 1 year):

Reagent	Final concentration	Amount
NaCl	100 mM	5.85 g
K_2HPO_4	1 g/L	1 g
KH_2PO_4	6 g/L	6 g
ddH_2O	N/A	To 1 L
Total	**N/A**	**1 L**

Sterilize by autoclaving.

Potassium citrate buffer (1 M, pH 6.0) (store at 20°C–23°C for 1 year):

Reagent	Final concentration	Amount
Citric acid monohydrate	95 mM	20 g
Tri-potassium citrate monohydrate	905 mM	293.5 g
ddH_2O	N/A	To 1 L
Total	**N/A**	**1 L**

Adjust pH to 6.0. Sterilize by autoclaving.

Trace metals solution (store at 20°C–23°C for 1 year, keep away from light):

Reagent	Final concentration	Amount
disodium EDTA	1.86 g/L	1.86 g
$FeSO_4 \cdot 7\ H_2O$	0.69 g/L	0.69 g
$MnCl_2 \cdot 4\ H_2O$	0.2 g/L	0.2 g
$ZnSO_4 \cdot 7\ H_2O$	0.29 g/L	0.29 g
$CuSO_4 \cdot 5\ H_2O$	0.025 g/L	0.025 g
ddH_2O	N/A	To 1 L
Total	**N/A**	**1 L**

S complete (use on the same day, do not store):

Add components using sterile technique. Do not autoclave.

Reagent	Final concentration	Amount
S Basal (1×)	1×	1 L
potassium citrate buffer pH 6	1 M	10 mL
trace metals solution	N/A	10 mL
$CaCl_2$	0.5 M	1 mL
$MgSO_4$	1 M	3 mL
cholesterol (5 mg/mL in 95% ethanol)	5 mg/L	1 mL
Total	**N/A**	**1 L**

M9 buffer (store at 20°C–23°C for 1 year):

Reagent	Final concentration	Amount
Na_2HPO_4	6 g/L	6 g
KH_2PO_4	3 g/L	3 g
NaCl	5 g/L	5 g
ddH_2O	N/A	To 1 L

Aliquot in 500 mL bottles and sterilize by autoclaving. Once cool, add the following:

$MgSO_4$ (1 M)	1 mM	500 µL
Total	**N/A**	**500 mL aliquots**

HB101 suspension (store at 4°C for 3 months):

5 g HB101 (grown in a fermenter, see key resources table) resuspended in 50 mL M9.

2 L Erlenmeyer Flasks.

Harvesting liquid culture

60% filter sterilized sucrose (store at 4°C for 1 year):

Dissolve 600 g sucrose in 500 mL ddH_2O. Bring up final volume with ddH_2O to 1 L. Sterilize solution with 0.22 µm filter.

0.1 M NaCl (store at 4°C for 1 year):

Dissolve 5.84 g NaCl in 800 mL ddH_2O. Bring up final volume with ddH_2O to 1 L.

Generating *C. elegans* grindates using a cryomill

Roche cOmplete mini protease inhibitor cocktail EDTA free tablet (Sigma 11836170001).

Dry ice.

Liquid N_2 in dewar.

Whole worm lysate generation and affinity purification

NP-40 Surfact-Amps, 10% stock solution (ThermoFisher 28324).

CHAPS powder (MP Biomedical 04808196) dissolve in sterile ddH_2O to generate 10% stock solution.

Tris/HEPES stock lysis buffer (store at 20°C–23°C for up to 2 months):

Reagent	Final concentration	Amount
Tris or HEPES (pH 7.5) (1 M)	50 mM	25 mL
NaCl (3 M)	150 mM	25 mL
$MgCl_2$ (1 M)	1.5 mM	750 µL
Glycerol (100%)	10%	50 mL
ddH_2O	N/A	To 500 mL
Total	**N/A**	**500 mL**

Filter sterilize

Add fresh for each experiment:

Reagent	Final concentration	Amount
Stock buffer	N/A	~43 mL
DTT (1 M)	1 mM	45 μL
Detergent (NP40 or CHAPS, 10%)	0.1% or 0.3%	450 μL (0.1%) or 1.35 mL (0.3%)
HALT Protease inhibitor (100×)	1×	450 μL
HALT Phosphatase Inhibitor (100×)	1×	450 μL
PMSF (100 mM) (soluble at 37°C)	1 mM	450 μL
Total	**N/A**	**45 mL**

Pierce BCA protein assay kit (ThermoFisher 23225).

Dynabeads M-280 Sheep anti-Rabbit IgG (Invitrogen 11204D).

HALT Protease inhibitor Cocktail (100×) (ThermoFisher 78429).

HALT Phosphatase Inhibitor Cocktail (100×) (ThermoFisher 78420).

DTT (Fisher BP1725).

PMSF (Sigma P7626-5G).

Silver stain and Western blot

Pierce Silver Stain Kit (ThermoFisher 24612).

LDS sample buffer (1×): Use 20 μL 1× LDS sample buffer for every 100 μL of Dynabeads slurry used (or 10 μL of agarose beads)

Reagent	Final concentration	Amount
NuPage LDS sample buffer (4×) (Invitrogen NP0007)	1×	5 μL
NuPage Sample Reducing Agent (10×) (Invitrogen NP0009)	1×	2 μL
ddH_2O	N/A	13 μL
Total	**N/A**	**20 μL per reaction**

High Molecular Weight Standards (Cytiva 17061501).

HiMark Prestained Protein Standard (Invitrogen LC5699).

NuPage 3–8% Tris-Acetate Gel (Invitrogen EA03785BOX).

NuPage 4%–12% Bis-Tris Gel (Invitrogen NP0323BOX).

NuPage Tris-Acetate SDS Running Buffer (20×) (Invitrogen LA0041).

NuPage MOPS SDS Running Buffer (20×) (Invitrogen NP0001).

NuPage Antioxidant (Invitrogen NP0005).

TAC transfer buffer (1× use fresh, do not store):

Reagent	Final concentration	Amount
Bicine	25 mM	4.08 g
Bis-Tris	25 mM	5.23 g
EDTA (0.5 M, pH 8.0)	0.5 mM	2 mL
Methanol	5%	50 mL
ddH_2O (cold)	N/A	To 1 L
Total	**N/A**	**1 L**

TBS (1×): 140 mL 1 M Tris pH7.5, 350 mL 3 M NaCl, add water to 7 L.

TBS-T (1×): 1 L of TBS + 1 mL of 100% Tween-20 (Sigma, P7949).

Antibodies (see key resources table).

ECL, SuperSignal West Pico/Femto (ThermoFisher 34580/34095).

Sample preparation for MS

4× Laemmli Sample Buffer (Bio-Rad 161-0747).

2-mercaptoethanol (Bio-Rad 161-0710).

7.5% Mini-Protean TGX Tris-Glycine Precast Gels (Biorad 4561024).

Porcine trypsin (Sigma T6567).

STEP-BY-STEP METHOD DETAILS

Generating *C. elegans* strains for affinity purification (part I)

Timing: ~2 months

In this section, we explain how to create the *C. elegans* strains needed to perform affinity purification. We advise on protein tag usage and on experiments to test affinity tag construct functionality.

1. Design transgenic constructs to express tagged POI and tagged control for affinity purification:
   a. Design a genetic construct for tagged POI expression in *C. elegans* according to the type of affinity purification that will be executed, biochemical properties of the POI, and potential follow-up experiments.

   Note: See Part VIII, step 29 for examples.

   Note: As a negative control, we used an affinity-tagged GFP (GS::GFP) *C. elegans* strain.

      i. Test all genetic constructs with phenotypic assays to ensure that the tagged-POI is functional (see step 3).
      ii. Common parameters to test include tag identity, tag size, tag location (e.g., N-/C-terminus or internal), linker sequence(s), promoter sequence, and genomic DNA versus cDNA for the POI.
      iii. Our prior studies[1–3] used a 2× protein G - 5× streptavidin binding peptide tag (GS) separated by a TEV protease cleavage site (Table S1). We anticipate our protocol would work well for other single-step affinity purification tags such as GFP, FLAG or HA. However, we note that optimization of affinity purification conditions should be performed for all tags. While the GS tag we used was originally designed for tandem purification,[23] we do

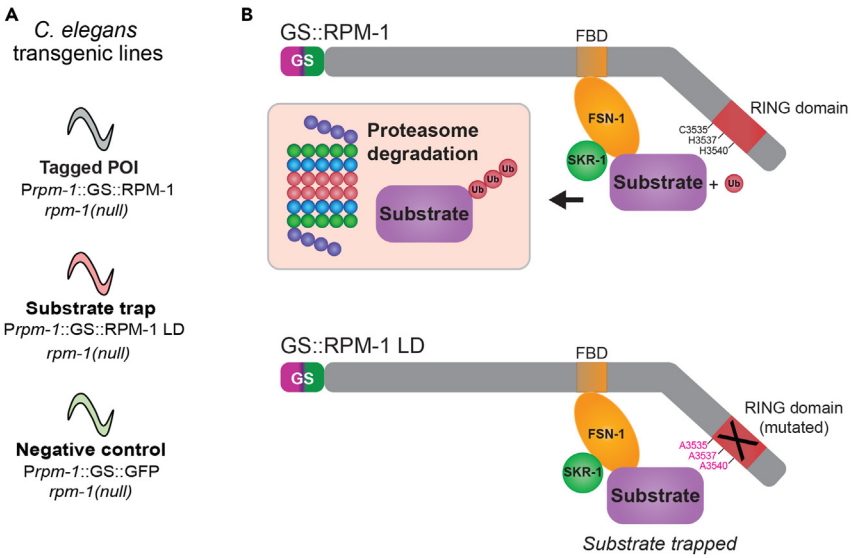

Figure 2. Designing strains and constructs for ubiquitin ligase substrate enrichment

(A) Example of three transgenic strains required for AP-proteomics experiments with ubiquitin ligase as POI. Note RPM-1 RING family ubiquitin ligase is used as example.

(B) Schematic depicting RPM-1 which functions in a multi-component E3 ubiquitin ligase complex to target substrates for ubiquitination and degradation via the proteasome (upper diagram). RPM-1 LD construct generated by mutating three catalytic residues (C3535A, H3537A, H3540A). Substrates are biochemically 'trapped' by RPM-1 LD/FSN-1/SKR-1 ubiquitin ligase complex which enriches substrate interactions (lower diagram).

not recommend tandem affinity purification. In our hands, this takes more optimization, results in lower amounts of POI, and extended time frames/steps for tandem purification reduce intact protein complexes.

2. Generate transgenic worms expressing extrachromosomal arrays (see Figure 2 for example):
   a. Once transgene designs are ready, clone expression constructs containing the native gene promoter, gene (cDNA or ORF), and native 3′ UTR.

   Note: Native promoters and integrated transgenes ensure physiologically relevant expression while still reaping benefits of more POI and associated complexes from mild overexpression. Negative control tags should also be driven by the native promoter for the POI.

   b. Microinject POI protein null mutants (P0) with plasmids containing transgene and co-injection markers (see Part VIII, step 30 for example).

   Note: The use of POI protein null mutant is critical to ensure no competition with endogenous POI, and to promote formation of protein complexes with tagged POI. We generally start with 20ng/mL of plasmid for injection, but this amount might need to be optimized for function based on rescue (see step 3).

   c. Select transgenic strains with 40%–60% transmission of the co-injection marker (percentage of total worms on a plate carrying the marker).

   Note: This moderate transmission frequency facilitates isolation of integrated transgenic strains at later steps.

3. Test for functionality of affinity-tagged POI:

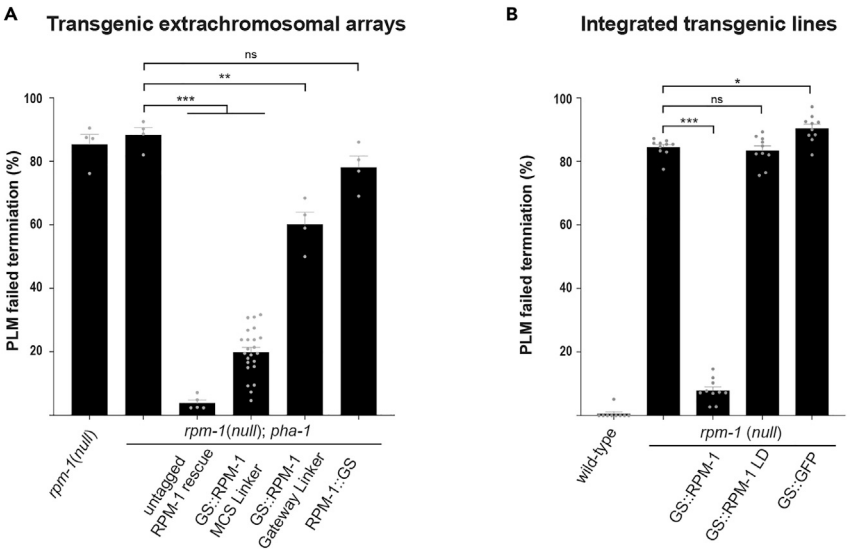

Figure 3. Evaluation of GS::RPM-1 function using transgenic rescue

(A) *rpm-1* mutants display high frequency axon termination defects. Defects are not altered by *pha-1* ts mutant which is used as transgenic selection cassette. GS::RPM-1 with MCS linker is fully functional and robustly rescues *rpm-1* (lf) defects. In contrast, GS::RPM-1 with Gateway linker and RPM-1 with C-terminal tag (RPM-1::GS) do not rescue efficiently. Untagged RPM-1 is positive control for rescue.

(B) Evaluation of integrated transgenes showing GS::RPM-1 (MCS linker) is functional while GS::RPM-1 LD is not. GS::GFP is included as negative control for GS affinity tag. Histograms represent means, dots represent average for single count (35–40 worms/count), error bars are SEM. Significance was tested using Student's *t*-test with Bonferroni correction. ***$p < 0.001$, **$p < 0.01$ and ns = not significant.

a. Test strains isolated in step 2.c for expression and function of the affinity tagged POI using phenotypic rescue experiments (see Figure 3A for example).
b. If strains carrying extrachromosomal arrays do not successfully rescue the null phenotype, return to step 2.b and adjust injection concentrations higher or lower. Alternatively, return to step 1.a and alter the location of the tag on the POI, and/or test different linkers.
c. Biochemically test the transgenic strains isolated in step 2.c for affinity tagged-POI. For detailed methodologies, see procedures below (Part III and IV) for affinity purification and western blotting which can also be used for biochemical evaluation of transgenic lines.
d. Transgenic extrachromosomal arrays that display the best phenotypic rescue and have biochemically validated expression of tagged-POI should be integrated into the genome (see below in step 4).

4. Integrate transgenic arrays to create stable transgenic lines:
   a. Place 200 late L4 (larval stage 4) extrachromosomal array-carrying worms in 200 μL of M9.
   b. Mix 20 μL of 1 mg/mL TMP (dissolved in DMSO (store −20°C for 1 month)) in 380 μL of M9 and add the solution to the tube with worms.
   c. Incubate at 20°C–23°C for 15 min on a rotator with gentle mixing.
   d. In the dark, transfer TMP treated worms to an unseeded 10 cm NGM plate. With the lid off, expose worms to 350 μJ (X100)/cm^3 UV using Spectroline UV crosslinker.
   e. After UV exposure, add concentrated OP50 (see Materials) and let worms recover at 15°C.
   f. The next day, pick 10 healthy looking young adult transgenics (P0s) to 10 plates each (total of 100 P0s).
   g. In the next generation, pick 25 F1 plates from each of the P0 plates.
   h. In the next generation, pick 3–5 F2s to individual plates from each F1 plate.
   i. Screen F2s for 100% transmission indicating that the transgenic array is integrated into the genome. Outcross transgenic integrants at least 4×.

j. Cross integrated transgene onto POI null mutant (preferably protein null). Secondary check for rescue (see Figure 3B for example) and confirm affinity tagged POI expression by immunoprecipitation and western blot.

Note: Usually 1–2 integrants will be isolated. For more integrants, increase the number of P0, F1, and F2 worms picked. Other integration techniques can potentially be used, such as irradiation or bombardment.

Large-scale liquid culture (part II)

Timing: 2 weeks

The following protocol will describe how to harvest 2 × 250 mL liquid cultures of *C. elegans*. The protocol can be scaled up or down based on how much POI is present.

5. Prepare worms for culture:
   a. Day 1 plates: On one 10 cm plate containing OP50, transfer enough young adult worms to have a fully starved plate (L1 synchronization) after 72–80 h at 23°C. Several generations of worms will grow on the plate before starvation and L1 synchronization happens.

   Note: For wild-type N2, ~13–15 young adult worms per plate.

   b. Day 2 plates: the next day, in four 10 cm plates, transfer enough worms for crowded but not starved plate after 72 h at 23°C.

   Note: For N2 worms, ~8–10 worms per plate.

6. Set up the liquid culture:
   Begin when Day 1 plates are fully starved. Starvation occurs 72–80 h after setup when OP50 bacterial lawn is gone, worms are at the L1 stage, and no dauers are present.

   Note: Presence of dauers on plates must be avoided as they will induce further dauer formation during liquid culture.

   a. Prepare 500 mL of S Complete media (use same day, do not store) using sterile procedures.
   b. Add 225 mL of S Complete to each of the 2 × 2 L flasks.
   c. Add 5 mL of prepared liquid HB101 culture (See Materials) to each flask.

   Note: To perform many experiments with different strains, large quantities of HB101 will be needed. While this can be grown in house, our group usually grows HB101 using a fermenter service (see key resources table section).

   d. Transfer worms from Day 1 plate into 15 mL conical tube. Pour M9 onto the plate, swirl and remove liquid using a glass pipette and add to the conical tube. Wash 3 times: for each wash add 10 mL of M9, spin worms down (350 × *g*, 1 min 30 s), aspirate supernatant and repeat. After the final wash, resuspend in 6 mL of M9.
   e. Divide resuspended worms equally (3 mL) into 2 Erlenmeyer flasks prepared above (steps 6.b and 6.c).
   f. Place flasks on orbital shaker and set at 185 RPM (calculated for a 2inch orbit shaker) at 20°C–23°C .
   g. The next day (16–24 h), harvest Day 2 plates by washing with M9 as describe above at step 6d. Divide the resuspended worms equally between the flasks set up at step 6e, and feed by adding 7.5 mL of HB101 to each flask.

h. On the 3rd day of culture add 10 mL of liquid HB101 culture to each of the flasks to provide more food for the worms.
i. After 24 h (day 4 of culture), check health and density of worms:
   i. Pipet 1 mL of liquid culture onto an unseeded NGM plate.
   ii. Let the liquid evaporate and observe the worms' health and density.

Note: Some HB101 bacteria should still be present showing that the worms still have enough food to eat and worms should have visible gut granules indicating good health.

Note: With successful liquid culture, you will see all developmental stages and very few dauers or pre-dauer L2d. See troubleshooting section if there are large numbers of dauers.

7. Harvest liquid cultures:

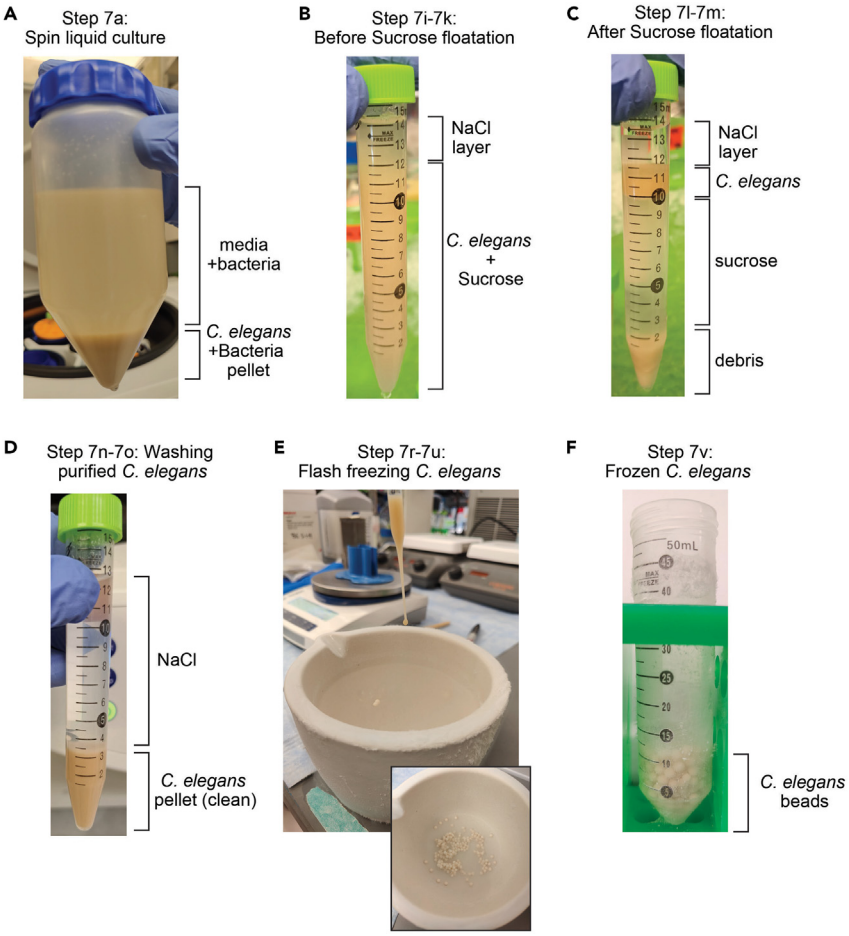

Figure 4. Images for key steps in harvesting *C. elegans* from large-scale liquid cultures (step 7)
(A) Photograph of *C. elegans* pellet after spinning culture from one flask into 250 mL conical tube.
(B) *C. elegans*-sucrose mixture with 2 mL layer of NaCl that facilitates separation of layers during sucrose flotation.
(C) Different layers found after sucrose floatation. Note layer of enriched, cleaned *C. elegans*.
(D) *C. elegans* pellet after 3 washes post sucrose floatation.
(E) Example of flash freezing process where Pasteur pipette is used to place drops of *C. elegans* into mortar with liquid N_2.
(F) Flash frozen beads of *C. elegans* in 50 mL tube before storage at −80°C.

Note: See Figure 4 for illustration of key steps.

a. Distribute liquid culture from the two flasks into 10 × 50 mL conical tubes or 2 × 250 mL conical bottles.
b. Put tubes on ice to let worms settle for 5 min.

⚠ CRITICAL: all following steps must be done on ice with ice-cold solutions.

c. While tubes are on ice, prepare 250 mL 0.1 M NaCl (keep on ice) and aliquot 10 mL into 4 × 15 mL conical tubes on ice.
d. Spin worms down (750 × *g*, 4 min, 20°C–23°C).
e. Aspirate off supernatant with a vacuum leaving 5 mL of liquid on top of the worm pellet. Be careful not to dislodge the pellet.
f. Consolidate the worms into two 50 mL conical tubes. Adjust volume to 50 mL with 0.1 M NaCl and mix by inverting.
g. Spin down (750 × *g*, 4 min) and aspirate supernatant leaving 10 mL of liquid on top of the worm pellet.
h. Resuspend worm pellet and transfer into 4 × 15 mL tubes. Adjust volume to 6 mL of worms per tube using 0.1 M NaCl.

⚠ CRITICAL: The next 6 steps should be done as quickly as possible (less than 5 min), as worms will not survive in sucrose for long.

i. Add 6 mL of ice-cold sterile 60% sucrose to each tube of worms. The final concentration of sucrose in the tube will be 30%.
j. Mix tubes well by inverting several times.

Note: if the solution is not completely mixed, the sucrose floatation may fail.

k. With a 5 mL glass pipet, carefully overlay the suspension with 2 mL of 0.1 M NaCl.

Note: NaCl layer should be visibly distinct from the sucrose layer. Worms will float to the top of sucrose while bacteria and debris remain in the sucrose solution and pellet.

l. Spin the tubes (1,450 × *g*, 2 min 30 s) with **no or minimal** deceleration (see troubleshooting).
m. Using a glass pipette, carefully collect worms in NaCl (less than 5 mL) and bring as little of the sucrose layer as possible. Deposit worms into 15 mL tubes containing 10 mL of NaCl (prepared in step 7.c).
n. Mix the tubes well by inverting several times.
o. Spin the tubes (350 × *g*, 1 min 30 s) and aspirate supernatant.
p. Wash the tubes 2× to remove sucrose: add 15 mL 0.1 M NaCl to tubes, spin down (350 × *g*, 1 min 30 s) and aspirate the supernatant.
q. After the final wash, aspirate supernatant leaving a small (<200 μL) amount of liquid over the worm pellet for resuspension.
r. Prechill ceramic mortar with liquid N_2 and fill to 75% full.
s. With a pipette pump and Pasteur pipette, resuspend worms. The solution will be very thick; add slightly more 0.1 M NaCl if you have trouble pipetting.
t. Transfer a small drop of clean animals to an unseeded NGM plate to determine sample quality.

Note: The prep should not contain dauers or bacteria and the worms should be healthy and contain a mixture of developmental stages (L1-adult).

Note: worms grown in liquid culture will look longer and thinner than when grown on plates.

u. Pipet resuspended worms dropwise into liquid N_2 and flash freeze into small beads. Repeat for each tube of worms.
v. Carefully pour frozen worm beads into a 50 mL conical tube (prechilled with liquid N_2). When liquid N_2 has fully evaporated, measure the mass of beads.

Note: Typical yield per 250 mL culture is 2–3g.

w. Store frozen worms at −80°C.

Pause point: Frozen pellets can be stored up to 2 months.

Preparing whole worm grindates using a cryomill (part III)

Timing: 1–2 h

Our published studies in *C. elegans*[1–3] and previous AP-proteomics studies using yeast[24] indicate that liquid nitrogen flash freezing and cyromilling is key for enhancing protein complex isolation and AP-proteomics outcomes. Here, we provide instruction on how to use a cryomill with *C. elegans*.

⚠ CRITICAL: Prechill all conical tubes on dry ice and metal spatulas in liquid N_2. All tubes must be kept on dry ice at all times.

8. Measure 5 g of worm pellets per strain into prechilled 50 mL conical tubes and keep on dry ice.
9. Add $^1/_2$ of a Roche cOmplete mini protease inhibitor cocktail EDTA free tablet to each tube.
10. Assemble the cryomill, the grinding jar, and the grinding ball according to manufacturer's instructions. Run pre-cool setting for 5 min to cool jars with liquid N_2.

Note: Use 50 mL jars to grind because smaller jars make it more difficult to completely remove ground worms.

11. Pour contents of conical tube (from steps 8 and 9) into the grinding jar with grinding ball and load onto the cryomill.
12. For 5 g of worms, pre-cool the jar for 2 min at 5 Hz (s^{-1}) then grind for 5 min at 30 Hz (s^{-1}).
13. When worms are ground, pour liquid N_2 into the prechilled mortar and add the ground worms. Use the prechilled metal spatula to remove any grindate stuck to the jar wall and grinding ball.
14. Pour ground worms and liquid N_2 mix into the prechilled conical tube. Let the liquid N_2 evaporate and return the tube to dry ice.
15. Repeat steps 10–14 for each *C. elegans* strain.

Note: Grind strains in the appropriate order (i.e., control first) to limit cross-contamination if reusing the grinding jar.

16. Store conical tubes with *C. elegans* grindates at −80°C or proceed immediately to the next step.

Pause point: Frozen grindates can be stored for 2–3 days at −80°C. Long-term storage is not recommended.

Affinity purification of POI protein complexes from whole worm lysates (part IV)

Timing: 2–3 days

The following protocol will outline the steps for affinity purification of proteins using 80 mg of total protein starting material as determined by a BCA assay. The amount of *C. elegans* lysate used will

vary depending on POI expression levels. For mass spectrometry, you will need 0.4–1 μg of purified POI.

⚠ CRITICAL: To reduce keratin and other common contaminants, clean work surfaces fresh for each experiment with 70% isopropanol and use gloves, surgical mask, hair net and lab coat while handling samples. Only use single use, sterile plastics. High speed centrifuge tubes, cryomill grinding jars and cryomill grinding balls should be washed with detergent (Contrad 70, Decon Labs 1002) and wiped dry with filtered 70% isopropanol using low particulate cleanroom pads (Contec AMEC0001).

17. Perform affinity purification on the protein of interest.

Note: Perform the following steps at 20°C–23°C.

⚠ CRITICAL: Steps a to c need to be performed quickly (less than 1 min).

a. Remove tube with *C. elegans* grindates stored at −80°C, and quickly dislodge aggregates by tapping the tube on a bench top or using a sterile spatula.
b. Immediately add room temperature (20°C–23°C) lysis buffer to ground worms in 1:4 w/v ratio (e.g., resuspend 5 g worms in 20 mL lysis buffer). Keeping lysis buffer at 20°C–23°C helps dissolve the grindates.
c. Use a sterile, plastic spatula to mix worm lysate (mix less than 1 min).
d. Mix on rotator for 5 min at 4°C.

⚠ CRITICAL: Perform the following steps at 4°C or on ice. All tubes must be prechilled on ice.

e. Transfer lysates to a prechilled centrifuge tube.
f. Spin (20,000 × *g*) for 10–15 min at 4°C.
g. Pipet supernatant into prechilled 50 mL conical tube.

⚠ CRITICAL: Do not freeze lysate after this step. Keep on ice.

h. Perform BCA assay following manufacturer's instructions to assess total protein concentration in lysate. Calculate amount of lysate needed for 80 mg total protein. Typical yield from 5 g grindate in 20 mL lysis buffer is a concentration of 3–5 mg/mL of total protein.

Note: the amount of lysate required for a successful proteomics experiment should be optimized for your POI.

i. Prepare 2 × 250 μL of Dynabeads (10 mg/mL stock) in 2 × 15 mL tubes by washing with cold lysis buffer 3 times. Collect beads with a DynaMag-15 magnet and discard the supernatant using a serological pipet after each wash.
j. Transfer volume of lysate equivalent to 40 mg of total protein (about 10 mL) to each 15 mL conical tube of Dynabeads for a total of 80 mg total protein.
k. Incubate with rotation at 4°C for 1–4 h. The antibody-POI complexes will bind to the affinity resin present on the Dynabeads. For example with a GS tagged POI, IgG is coupled to Dynabeads.

Note: Length of incubation time should be optimized based on amount of POI in a set amount of lysate. We found shorter incubation times are better because they minimize degradation of POI and protein complexes. This is supported by previous studies in yeast that demonstrated short incubations times are key for enriching intact protein complexes.[25]

l. Use the DynaMag-15 magnet to collect Dynabeads for 2 min. Invert the tube twice while on the magnet to collect any Dynabeads in the tube cap.
m. Wash Dynabeads:
    i. Add 8 mL of cold lysis buffer to the tube, remove the magnet, invert several times.
    ii. Collect beads with a DynaMag-15 magnet and discard the supernatant using a serological pipet after each wash.
n. Repeat washes 5 times.
o. Transfer the beads to a 1.7 mL low retention tube.

Note: Low retention tubes limit the amount of protein that sticks to the plastic of the tube and reduces false positive hits.

p. Add 500 μL of cold lysis buffer to the Dynabeads and gently resuspend.
q. Transfer 50 μL (10%) of suspension to a new tube and collect the beads using DynaMag-2 and aspirate supernatant. Set samples aside and use it for quality control analysis (see step 18 below).
r. Collect beads from the 450 μL (90%) resuspension using the DynaMag-2 and aspirate supernatant. Store beads at −80°C until ready to perform mass spectrometry (see Part V below).

Pause point: Samples can be stored at −80°C for up to 2 weeks.

18. Perform quality control for affinity purification:
Preparing diagnostic sample:
    a. Use quality control samples set aside at step 17.q.
    b. Add 25 μL of 1× LDS sample buffer directly to Dynabeads.
    c. Heat samples at 70°C for 10 min. Agitate tubes by flicking halfway through incubation to mix beads.
    d. Collect the beads with a DynaMag-2 magnet to separate them from the supernatant which will be used for silver staining (steps 18.e–18.j) and western blotting (steps 18.k–18.r).
    Silver stain: used for quality control only to determine sample purity and POI quantity (for example see Figure 5A).
    e. Place NuPAGE gel into an Invitrogen running apparatus. Add the appropriate buffer according to the manufacturer's recommendations.
        i. Higher molecular weight POI (150 kDa or larger) use 3–8% Tris-acetate gel.
        ii. Lower molecular weight POI (under 150 kDa) use 4%–12% Bis-Tris gel.
    f. Load 90% of the diagnostic sample (equivalent to 9% of total from step 17.p) into NuPAGE gel.
    g. Alongside samples, load protein molecular weight standards (Cytiva) for analysis of protein size and quantity in samples. At least 3 concentrations of standard should be run to accurately determine amount of POI.

Note: Protein molecular weight standards mass range and amount of standard loaded will depend on the size and amount of POI. Evaluate amount of POI using a similar size standard.

h. Run NuPAGE gel (see manufacturer's instructions).

Note: For GS::RPM-1, we run the gel at 150 volts for 1 h and 30 min.

i. To perform a silver stain, use the Pierce silver stain kit (follow manufacturer's instructions).
j. Determine the quality of affinity purification by comparing bands present in POI sample with the negative control sample.

Note: The control should have relatively sparse labeling compared to test POI.

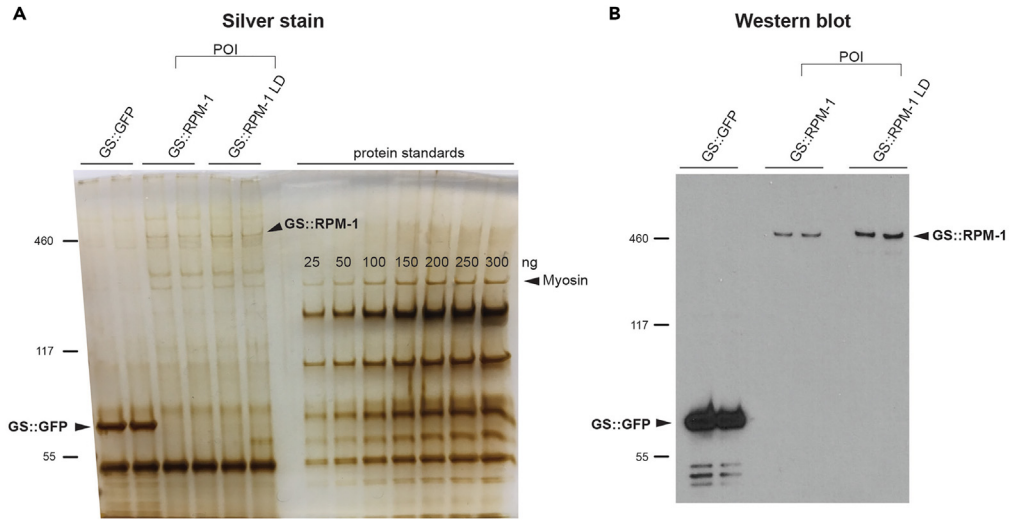

Figure 5. Example of quality control for affinity purification using RPM-1 ubiquitin ligase as POI

(A) Silver staining used to visualize affinity purified samples for POI (GS::RPM-1 and GS::RPM-1 LD) and negative control (GS::GFP). Shown are two independent replicates for each affinity purification target. Affinity purification samples were generated from whole *C. elegans* extracts (Tris 0.1% NP-40 lysis buffer) using IgG-Dynabeads. Note numerous enriched silver stained species in GS::RPM-1 and GS::RPM-1 LD test samples compared to GS::GFP negative control. Right lanes show serial dilution of Cytiva high molecular weight (HMW) protein standards where Myosin is used to estimate POI quantity.

(B) Western blot with anti-SBP antibody used to confirm expression and size of POI (GS::RPM-1 and GS::RPM-1 LD) in affinity purified samples.

Western blot: used to confirm expression of POI (for example see Figure 5B).

k. Load 10% of the diagnostic sample (equivalent to 1% of total from step 17p) into a NuPAGE gel and run.

l. Transfer to PVDF membrane using wet transfer in TAC transfer buffer at 4°C.

Note: For proteins under 150 kDa, 90 V for 1 h is sufficient. For proteins greater than 150 kDa, transfer at 30 V for 16 h. Block with 5% milk in TBS-T for 1 h at 20°C–23°C.

m. Incubate blot in primary antibody diluted in 5% milk and TBS-T 16–20 h at 4°C.

n. Wash blot 3× with TBS-T for 5 min each.

o. Incubate blot in secondary antibody diluted in TBS-T for 1 h at 20°C–23°C.

p. Wash blot with 3× with TBS-T then 3× with TBS.

q. Incubate blot with chemiluminescent substrate (SuperSignal West Pico/Femto) and develop using x-ray film or digital bioimager (Azure).

Note: We use SuperSignal Pico for biochemistry with transgenic strains and Femto (diluted 1:2 in TBS) for CRISPR-based native biochemistry.

Identification of proteins co-precipitating with POI using MS (part V)

Timing: 1–2 weeks depending upon mass spectrometry facility lead time

Please note, this section is only intended as a guideline for labs and mass spectrometry facilities. Details on preparing samples and settings for mass spectrometry will vary depending on the mass spectrometry facility and individual samples. We recommend talking carefully with your mass spectrometry facility/director/collaborator to define this process.

19. Prepare sample for MS.
    a. Add 50 μL of Laemmli sample buffer to frozen Dynabeads from step 17.r (90% of total purified sample).
    b. Boil samples at 95°C for 15 min.

    Note: Many MS facilities will prepare samples for MS. This protocol will briefly describe sample preparation that yielded high-quality MS results.

    c. Run boiled samples on Mini-Protean TGX Tris-Glycine Precast Gel (Biorad) at 120 V for 10 min until entire sample just enters top of gel.
    d. Coomassie stain gel at 20°C–23°C for 1 h with shaking followed by de-staining to reveal protein samples.

    Note: Individual protein bands will not be observed because samples are run for a short period of time. This is done to clean sample not to separate individual protein species.

    e. Excise compressed gel bands containing entire sample using new razor blade and individual Eppendorf tube for each test strain and control. While we do not analyze individual bands by mass spectrometry, readers might still find the following protocol for in-gel trypsin digestion of protein samples for mass spectrometry a valuable resource.[26]
    f. Treat with 10 mM DTT.
    g. Treat with 50 mM iodoacetamide.
    h. In-gel digest sample with 60 ng/μL trypsin adding enough volume to cover gel pieces.
    i. Peptide pools were acidified and desalted using a Zip-Tip C18 column (5 μg capacity).
    j. Resuspend samples in 0.1% formic acid.
20. Perform liquid chromatography tandem mass spectrometry (LC-MS/MS).
    a. Load sample into EASY-nLC 1000 system coupled to Nanospray Flex Ion Source and Orbitrap Fusion (or Lumos) Tribrid Mass Spectrometer.
    b. Elute peptides in analytical RP column (0.075 × 150 mm Acclaim PepMap RLSC nano Viper) operated at 300 nL/min using the following gradient:
        i. 2%–25% solvent B for 40 min.
        ii. 25%–44% solvent B for 20 min.
        iii. 44%–80% solvent B for 10 s.
        iv. 80% solvent B for 5 min.
        v. 80-5% solvent B for 10 s.
        vi. 5% solvent B for 20 min.

    Note: Solvent A: 0.1% formic acid (v/v) and Solvent B: 0.1% formic acid (v/v), 80% CH_3CN (v/v)).

    c. Operate Orbitrap Fusion in data-dependent MS/MS mode using ten most intense precursors detected in survey scan from 380 to 1400 m/z performed at 120K resolution.
    d. Perform Tandem MS by HCD fragmentation with normalized collision energy (NCE) of 30%.
    e. Identify proteins using Mascot and Sequest algorithms (Proteome Discoverer software). Also include as settings:
        i. Carbamidomethylation of Cys as fixed modification.
        ii. Oxidation (Met) and deamidation (Asn, Gln) as variable modifications.
        iii. Three trypsin missed cleavages.
        iv. Mass tolerance of 10 and 20 ppm for precursor and fragment ions, respectively.
    f. Search MS/MS raw files against the *C. elegans* proteome database downloaded from UniProt (https://www.uniprot.org/proteomes). Also database search for common contaminants, such as human keratin and porcine trypsin. Consider using database of common contaminants from the global proteome machine (cRAP database: https://www.thegpm.org/crap/).

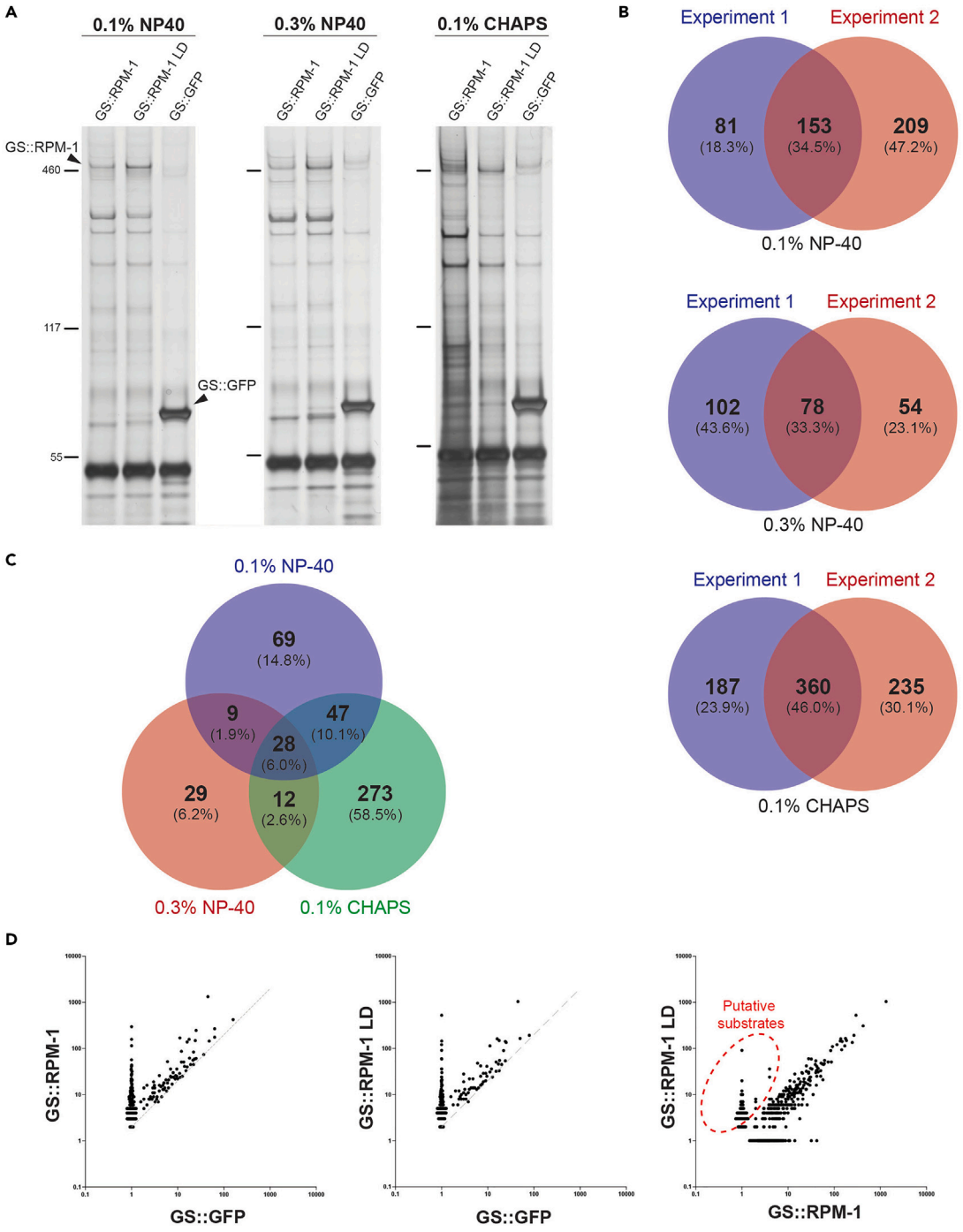

Figure 6. Detergent type and concentration affect AP-proteomic profiles

(A) Silver stain showing POI (GS::RPM-1 and GS::RPM-1 LD) and negative control (GS::GFP) across three extraction conditions.

(B) Venn diagrams comparing AP-proteomic hits for GS::RPM-1 from different independent experiments under similar extraction conditions.

(C) Venn diagram comparing AP-proteomic hits for GS::RPM-1 across different extraction conditions. Note that only hits identified in both experiments for a given condition were evaluated.

(D) Scatter plots comparing single AP-proteomic experiments between specified genotypes. Proteomic hits are compared between GS::GFP negative control and POI test samples, GS::RPM-1 and GS::RPM-1 LD. Proteomic hits represent individual proteins and gray lines delineate 2-fold enrichment over GS::GFP control. Putative substrates (dashed red oval) are enriched in GS::RPM-1 LD versus GS::RPM-1 sample. Total spectral count value is plotted on log10 scale.

Note: While we cite the latest versions of the global proteome machine database of contaminants and the *C. elegans* proteome database users are advised to use the latest database versions.

Bioinformatic analysis to identify proteins copurifying with POI (part VI)

Timing: 1–2 weeks

This section highlights the bioinformatic pipeline used to create a list of proteomic candidates that represent putative POI binding proteins. The analysis includes steps and criteria for eliminating false positives.

Note: Prior to performing initial data analysis, at least two independent proteomics experiments should be performed. For robust large-scale results, we recommend 4–8 proteomics experiments total with at least two independent experiments for a given extraction condition. Variable extraction using different lysis buffer conditions (e.g., varying detergent type and/or concentration) is highly recommended. In our studies we used 2 different detergents: NP-40 at two concentrations (0.1% and 0.3%) and CHAPS (0.1%). In our experience, protein complexes and binding proteins identified by proteomics can vary with extraction conditions (Figures 6B and 6C). Empirical evaluation of different extraction buffer conditions for a given POI is recommended. Note that higher detergent concentrations are more stringent and could destabilize protein complexes.

21. Process MS/MS results using Scaffold Proteome Software to identify proteins associated with peptide MS/MS spectra.
22. Create spreadsheet for each experiment, strain and purification condition tested indicating the corresponding database *protein database ID, protein name, protein size, percent coverage, total spectral count (peptide), exclusive spectral count, and unique spectral count* (Figure 7).
    a. Total spectral count indicates total number of times a given peptide was identified.
    b. Exclusive spectral count indicates peptides that can be found only in one *C. elegans* protein.
    c. Unique spectral count indicates the number of specific peptide sequences found for each candidate protein.
23. Candidate POI binding proteins are identified based on the following criteria:
    a. Detected in at least two experiments.
    b. Peptide identifications established at >5.0% probability to achieve a false discovery rate (FDR) < 1.0% by Scaffold Local FDR algorithm.
    c. Protein identifications are established at >98.0% probability to achieve an FDR <1.0% and contained at least two identified peptides. Assign protein probabilities using the Protein Prophet algorithm.
    d. Candidate shows 2× or greater enrichment of total spectra samples compared to negative control.
    e. Candidate is not commonly found in proteomics experiments. Examples of common proteins identified that are not usually functionally relevant binding proteins for a POI are ribosomal proteins and vitellogenin. These proteins are removed because they are generally found in *C. elegans* proteomic samples due to their roles in translation and post-embryonic development, respectively.
24. Compare outcomes for a given candidate with different extraction conditions. For an example, see Part VIII, step 33 (Figures 6A and 6B).
25. Calculate normalized total spectral count for candidate protein based on size of candidate and numbers of peptides identified for POI by MS:

				% Coverage			Total Spectral Count			Exclusive Spectral Count			Unique Peptide Count		
Experiment	ID name	Protein Name	Protein size	GS::RPM-1	GS::RPM-1 LD	GS::GFP	GS::RPM-1	GS::RPM-1 LD	GS::GFP	GS::RPM-1	GS::RPM-1 LD	GS::GFP	GS::RPM-1	GS::RPM-1 LD	GS::GFP
Tris 0.1% NP40 replicate 1	E3 ubiquitin-protein ligase rpm-1 OS=Caenorhabditis elegans GN=rpm-1 PE=1 SV=1	rpm-1	418 kDa	40.00%	48.50%	0	263	439	0	263	439	0	109	138	0
Tris 0.1% NP40 replicate 2	E3 ubiquitin-protein ligase rpm-1 OS=Caenorhabditis elegans GN=rpm-1 PE=1 SV=1	rpm-1	418 kDa	44.10%	55.30%	0	297	525	0	297	525	0	125	154	0
Tris 0.3% NP40 replicate 1	E3 ubiquitin-protein ligase rpm-1 OS=Caenorhabditis elegans GN=rpm-1 PE=1 SV=1	rpm-1	418 kDa	35.20%	52.70%	0	206	449	0	206	449	0	97	149	0
Tris 0.3% NP40 replicate 2	E3 ubiquitin-protein ligase rpm-1 OS=Caenorhabditis elegans GN=rpm-1 PE=1 SV=1	rpm-1	418 kDa	28.40%	51.00%	0	171	421	0	171	421	0	81	142	0
Tris 0.1% Chaps replicate 1	E3 ubiquitin-protein ligase rpm-1 OS=Caenorhabditis elegans GN=rpm-1 PE=1 SV=1	rpm-1	418 kDa	22.30%	43.00%	0	112	398	0	112	398	0	61	131	0
Tris 0.1% Chaps replicate 2	E3 ubiquitin-protein ligase rpm-1 OS=Caenorhabditis elegans GN=rpm-1 PE=1 SV=1	rpm-1	418 kDa	20.80%	46.20%	0	101	355	0	101	355	0	56	125	0
Hepes 0.1% NP40 replicate 1	E3 ubiquitin-protein ligase rpm-1 OS=Caenorhabditis elegans GN=rpm-1 PE=1 SV=1	rpm-1	418 kDa	24.50%	42.50%	0	115	287	0	115	287	0	62	119	0
Tris 0.1% NP40 replicate 1	F-box/SPRY domain-containing protein 1 OS=Caenorhabditis elegans GN=fsn-1 PE=1 SV=1	fsn-1	37 kDa	17.80%	23.80%	0	10	44	0	10	44	0	5	7	0
Tris 0.1% NP40 replicate 2	F-box/SPRY domain-containing protein 1 OS=Caenorhabditis elegans GN=fsn-1 PE=1 SV=1	fsn-1	37 kDa	28.60%	22.00%	0	11	20	0	11	20	0	7	6	0
Tris 0.3% NP40 replicate 1	F-box/SPRY domain-containing protein 1 OS=Caenorhabditis elegans GN=fsn-1 PE=1 SV=1	fsn-1	37 kDa	24.40%	24.40%	0	14	21	0	14	21	0	7	7	0
Tris 0.3% NP40 replicate 2	F-box/SPRY domain-containing protein 1 OS=Caenorhabditis elegans GN=fsn-1 PE=1 SV=1	fsn-1	37 kDa	13.00%	22.00%	0	9	18	0	9	18	0	4	6	0
Tris 0.1% Chaps replicate 1	F-box/SPRY domain-containing protein 1 OS=Caenorhabditis elegans GN=fsn-1 PE=1 SV=1	fsn-1	37 kDa	0	24.40%	0	0	17	0	0	17	0	0	7	0
Tris 0.1% Chaps replicate 2	F-box/SPRY domain-containing protein 1 OS=Caenorhabditis elegans GN=fsn-1 PE=1 SV=1	fsn-1	37 kDa	6.93%	22.00%	0	2	13	0	2	13	0	2	6	0
Hepes 0.1% NP40 replicate 1	F-box/SPRY domain-containing protein 1 OS=Caenorhabditis elegans GN=fsn-1 PE=1 SV=1	fsn-1	37 kDa	0	24.40%	0	0	14	0	0	14	0	0	7	0
Tris 0.1% NP40 replicate 1	Skp1-related protein OS=Caenorhabditis elegans GN=skr-1 PE=1 SV=1	skr-1	20 kDa	0	11.90%	0	0	5	0	0	5	0	0	2	0
Tris 0.1% NP40 replicate 2	Skp1-related protein OS=Caenorhabditis elegans GN=skr-1 PE=1 SV=1	skr-1	20 kDa	0	34.10%	0	0	10	0	0	10	0	0	6	0
Tris 0.3% NP40 replicate 2	Skp1-related protein OS=Caenorhabditis elegans GN=skr-1 PE=1 SV=1	skr-1	20 kDa	11.90%	19.90%	0	4	7	0	4	7	0	2	4	0
Tris 0.1% Chaps replicate 1	Skp1-related protein OS=Caenorhabditis elegans GN=skr-1 PE=1 SV=1	skr-1	20 kDa	19.30%	0	0	2	0	0	2	0	0	2	0	0
Tris 0.1% Chaps replicate 2	Skp1-related protein OS=Caenorhabditis elegans GN=skr-1 PE=1 SV=1	skr-1	20 kDa	0	32.40%	0	0	5	0	0	5	0	0	3	0
Hepes 0.1% NP40 replicate 1	Skp1-related protein OS=Caenorhabditis elegans GN=skr-1 PE=1 SV=1	skr-1	20 kDa	0	15.30%	0	0	3	0	0	3	0	0	2	0
Tris 0.1% NP40 replicate 1	X-linked retinitis pigmentosa GTPase regulator homolog OS=Caenorhabditis elegans GN=glo-4 PE=1 SV=2	glo-4	153 kDa	15.70%	21.20%	0	35	49	0	35	49	0	18	23	0
Tris 0.1% NP40 replicate 2	X-linked retinitis pigmentosa GTPase regulator homolog OS=Caenorhabditis elegans GN=glo-4 PE=1 SV=2	glo-4	153 kDa	15.30%	26.70%	0	32	59	0	32	59	0	20	30	0
Tris 0.3% NP40 replicate 1	X-linked retinitis pigmentosa GTPase regulator homolog OS=Caenorhabditis elegans GN=glo-4 PE=1 SV=2	glo-4	153 kDa	11.00%	17.50%	0	23	40	0	23	40	0	13	20	0
Tris 0.3% NP40 replicate 2	X-linked retinitis pigmentosa GTPase regulator homolog OS=Caenorhabditis elegans GN=glo-4 PE=1 SV=2	glo-4	153 kDa	13.30%	23.40%	0	26	52	0	26	52	0	14	26	0
Tris 0.1% Chaps replicate 1	X-linked retinitis pigmentosa GTPase regulator homolog OS=Caenorhabditis elegans GN=glo-4 PE=1 SV=2	glo-4	153 kDa	0	12.40%	0	0	28	0	0	28	0	0	14	0
Tris 0.1% Chaps replicate 2	X-linked retinitis pigmentosa GTPase regulator homolog OS=Caenorhabditis elegans GN=glo-4 PE=1 SV=2	glo-4	153 kDa	0	15.90%	0	0	31	0	0	31	0	0	19	0
Hepes 0.1% NP40 replicate 1	X-linked retinitis pigmentosa GTPase regulator homolog OS=Caenorhabditis elegans GN=glo-4 PE=1 SV=2	glo-4	153 kDa	0	9.60%	0	0	19	0	0	19	0	0	11	0
Tris 0.1% NP40 replicate 1	mRNA export factor rae-1 OS=Caenorhabditis elegans GN=rae-1 PE=1 SV=1	rae-1	41 kDa	23.30%	53.60%	0	22	46	0	22	46	0	7	16	0
Tris 0.1% NP40 replicate 2	mRNA export factor rae-1 OS=Caenorhabditis elegans GN=rae-1 PE=1 SV=1	rae-1	41 kDa	57.60%	62.70%	0	28	60	0	28	60	0	14	18	0
Tris 0.3% NP40 replicate 1	mRNA export factor rae-1 OS=Caenorhabditis elegans GN=rae-1 PE=1 SV=1	rae-1	41 kDa	40.80%	50.70%	0	28	47	0	28	47	0	12	15	0
Tris 0.3% NP40 replicate 2	mRNA export factor rae-1 OS=Caenorhabditis elegans GN=rae-1 PE=1 SV=1	rae-1	41 kDa	30.00%	62.70%	0	27	54	0	27	54	0	10	18	0
Tris 0.1% Chaps replicate 1	mRNA export factor rae-1 OS=Caenorhabditis elegans GN=rae-1 PE=1 SV=1	rae-1	41 kDa	19.00%	50.70%	0	9	43	0	9	43	0	4	15	0
Tris 0.1% Chaps replicate 2	mRNA export factor rae-1 OS=Caenorhabditis elegans GN=rae-1 PE=1 SV=1	rae-1	41 kDa	21.70%	57.10%	0	12	35	0	12	35	0	6	15	0
Hepes 0.1% NP40 replicate 1	mRNA export factor rae-1 OS=Caenorhabditis elegans GN=rae-1 PE=1 SV=1	rae-1	41 kDa	10.70%	39.10%	0	4	22	0	4	22	0	3	10	0
Tris 0.1% NP40 replicate 1	Probable protein phosphatase 2C T23F11.1 OS=Caenorhabditis elegans GN=ppm-2 PE=3 SV=2	ppm-2	39 kDa	12.10%	5.90%	0	4	4	0	4	4	0	3	2	0
Tris 0.1% NP40 replicate 2	Probable protein phosphatase 2C T23F11.1 OS=Caenorhabditis elegans GN=ppm-2 PE=3 SV=2	ppm-2	39 kDa	5.90%	8.43%	0	4	4	0	4	4	0	2	2	0
Tris 0.3% NP40 replicate 1	Probable protein phosphatase 2C T23F11.1 OS=Caenorhabditis elegans GN=ppm-2 PE=3 SV=2	ppm-2	39 kDa	5.90%	8.43%	0	2	3	0	2	3	0	2	2	0
Tris 0.3% NP40 replicate 2	Probable protein phosphatase 2C T23F11.1 OS=Caenorhabditis elegans GN=ppm-2 PE=3 SV=2	ppm-2	39 kDa	9.27%	15.40%	0	5	7	0	5	7	0	3	4	0
Tris 0.1% Chaps replicate 2	Probable protein phosphatase 2C T23F11.1 OS=Caenorhabditis elegans GN=ppm-2 PE=3 SV=2	ppm-2	39 kDa	0	12.10%	0	0	5	0	0	5	0	0	3	0
Tris 0.1% NP40 replicate 1	Cyclin-dependent-like kinase 5 OS=Caenorhabditis elegans GN=cdk-5 PE=1 SV=1	cdk-5	33 kDa	0	12.70%	0	0	6	0	0	6	0	0	4	0
Tris 0.3% NP40 replicate 1	Cyclin-dependent-like kinase 5 OS=Caenorhabditis elegans GN=cdk-5 PE=1 SV=1	cdk-5	33 kDa	0	9.25%	0	0	5	0	0	5	0	0	3	0
Tris 0.3% NP40 replicate 2	Cyclin-dependent-like kinase 5 OS=Caenorhabditis elegans GN=cdk-5 PE=1 SV=1	cdk-5	33 kDa	0	5.48%	0	0	4	0	0	4	0	0	2	0
Tris 0.1% NP40 replicate 1	Serine/threonine-protein kinase unc-51 OS=Caenorhabditis elegans GN=unc-51 PE=1 SV=1	unc-51	95 kDa	0	21.40%	0	0	22	0	0	22	0	0	11	0
Tris 0.1% NP40 replicate 2	Serine/threonine-protein kinase unc-51 OS=Caenorhabditis elegans GN=unc-51 PE=1 SV=1	unc-51	95 kDa	3.27%	15.20%	0	4	24	0	4	24	0	2	9	0
Tris 0.3% NP40 replicate 1	Serine/threonine-protein kinase unc-51 OS=Caenorhabditis elegans GN=unc-51 PE=1 SV=1	unc-51	95 kDa	4.91%	21.30%	0	4	20	0	4	20	0	3	12	0
Tris 0.3% NP40 replicate 2	Serine/threonine-protein kinase unc-51 OS=Caenorhabditis elegans GN=unc-51 PE=1 SV=1	unc-51	95 kDa	3.74%	19.50%	0	3	22	0	3	22	0	2	9	0
Tris 0.1% Chaps replicate 1	Serine/threonine-protein kinase unc-51 OS=Caenorhabditis elegans GN=unc-51 PE=1 SV=1	unc-51	95 kDa	0	11.80%	0	0	11	0	0	11	0	0	7	0
Tris 0.1% Chaps replicate 2	Serine/threonine-protein kinase unc-51 OS=Caenorhabditis elegans GN=unc-51 PE=1 SV=1	unc-51	95 kDa	0	11.10%	0	0	14	0	0	14	0	0	6	0
Hepes 0.1% NP40 replicate 1	Serine/threonine-protein kinase unc-51 OS=Caenorhabditis elegans GN=unc-51 PE=1 SV=1	unc-51	95 kDa	0	10.90%	0	0	10	0	0	10	0	0	5	0

Figure 7. Example of proteomics data organization (step 22)

Each experiment is indicated in the first column and color code highlights varying detergent and buffer conditions. Later columns indicate: database ID, protein name, protein size, percent coverage, total spectral count (peptide), exclusive spectral count, and unique spectral count for samples (GS::GFP control, GS::RPM-1 and GS::RPM-1 LD). Shown are functionally validated RPM-1 binding proteins (FSN-1, SKR-1, GLO-4, RAE-1, PPM-2) and ubiquitination substrates (UNC-51, CDK-5).

$$\frac{((\text{Candidate total spectra in experiment} - \text{Candidate total spectra in negative control})/\text{Candidate size})}{\text{POI total spectra in experiment}/\text{POI size}}$$

Note: To quantify outcomes, we perform statistical analysis using Student's t-test of normalized spectral counts over 3 or more independent experiments. We use a p-value of $p < 0.05$ to limit false negatives. False positives are still possible but should be reduced by the criteria for protein identification above and by candidate validation (see Part VII below). Importantly, statistical significance can increase confidence in a putative binding protein, but lack of significance does not rule out the validity of a putative hit that meets criteria. This is particularly relevant when dealing with protein complexes where individual components are likely to show varying levels of identification by mass spectrometry.

Validation of putative POI binding proteins identified by LC-MS/MS (part VII)

Timing: 6–12 months but varies with number and type of validation experiments

Proteomics only reveals putative binding proteins for a POI. We consider it important to perform further experiments to confirm a proteomic candidate. Here we list the typical experiments we execute to validate a putative binding protein.

26. Validate protein-protein interactions (PPIs) with biochemistry:

Note: Putative POI binding proteins can be validated biochemically using co-immunoprecipitation (co-IP) as well as other biochemical or optical approaches such as Fluorescence Lifetime IMaging (FLIM)-FluoRescence Energy Transfer (FRET).

a. Design construct for tagged candidate protein using a distinct tag from POI.
b. Generate transgenic or CRISPR-engineered worms expressing tagged candidate protein.
c. Perform co-IP (or other interaction assay) to verify physical interaction between candidate and POI.

Note: The protocol for growing *C. elegans*, generating grindates and performing affinity purification (Part I-IV, above) is also used for co-IP to test PPIs. To do so, we use between 1 and 10 mg of total protein from whole worm extracts. We use 10–15 μL of protein G agarose beads (Roche) coupled to appropriate primary antibody to immunoprecipitate tagged POI protein complexes. Dynabeads were only needed for high-quality AP-proteomics.
For examples of successful *in vivo* biochemistry in *C. elegans* using coIP with both transgenic approaches or CRISPR-based native constructs see the following studies.[1–3,17,27] FLIM-FRET is an imaging approach that tests whether two proteins are in close molecular proximity at subcellular locations. For examples of using FLIM-FRET in *C. elegans* to evaluate PPIs we suggest the following studies.[28,29]

27. Evaluate genetic interactions between POI and proteomic candidates:

Note: To explore the biological relevance of putative PPIs, we recommend genetic studies between a candidate and POI.

a. Evaluate if the single mutant for candidate phenocopies the mutant for POI.
b. Generate and test a strain with null mutations in both candidate and POI to examine their genetic interactions.

Note: We have found proteins in the same complex display both genetic enhancer effects and same pathway genetic outcomes. Genetic suppression has been observed when the POI inhibits a binding protein or enzymatic substrate. Phenotypic suppression is a common genetic relationship between ubiquitin ligase POIs and their substrates.

28. Test for colocalization between POI and proteomic candidates:

Note: Another criterion for biological relevance is localization of a candidate and the POI to the same cellular or subcellular compartment.

a. Engineer different fluorescent tags onto the POI and candidate using either transgenics or CRISPR engineering.
b. Evaluate colocalization with confocal microscopy or other high-resolution microscopy techniques.

Note: Colocalization of a candidate with the POI in a subcellular compartment is consistent with the candidate being a POI binding protein.

Example of experimental setup and analysis (part VIII)

For the example below, we provide extra details and data to fully illustrate the strategy and design underpinning our previous proteomic studies.[1,3]

29. Design *rpm-1* transgenic lines.

Note: For RPM-1 proteomics, we utilized a GS affinity tag (Table S1) which was previously used for tandem affinity purification (TAP) proteomics in mammalian cells.[23] In our hands with the *C. elegans* model, rapid single-step affinity purification is superior to TAP because it yields more POI, less deterioration of PPIs and requires no optimization for TEV protease cleavage. The importance of rapid purification for AP-proteomics has also been supported by prior

studies using yeast.[25] We tested two linker sequences (Table S2) between GS and RPM-1 and observed differing effects on RPM-1/POI function (Figure 3A).

Note: We used a native *rpm-1* promoter to drive GS::RPM-1 expression exclusively in the nervous system. GS::RPM-1 was expressed using genomic DNA (including native promoter and 3′UTR) on an *rpm-1* protein null mutant background (Figure 2A).[17,30] Thus, we ensured physiologically relevant GS::RPM-1 expression, and prevented competition for binding proteins with endogenous RPM-1.

Note: In order to distinguish substrates from non-substrate binding proteins, we introduced point mutations to create GS::RPM-1 ligase dead (LD), which lacks catalytic ubiquitin ligase activity (Figure 2). We have shown that GS::RPM-1 LD acts as a biochemical 'trap' to enrich substrates (Figure 2B).[1,3] In our experience, substrates were enriched in GS::RPM-1 LD samples while binding proteins were present in both GS::RPM-1 and GS::RPM-1 LD samples.

Note: As a control strain, we used the *rpm-1* promoter to express GS-tagged GFP (GS::GFP). GS::GFP was subjected to the same experimental conditions as our POI constructs (Figure 2A). This negative control strain was used to identify false positive hits during bioinformatic analysis of mass spectrometry data (see Part VI).

30. Generate RPM-1 transgenic worms.

Note: Microinjections were done on a *pha-1(e2123); rpm-1(ju44)* protein null mutant background using a concentration of RPM-1 plasmid previously shown to rescue mutant phenotypes and a PHA-1 rescue cassette.[27] While co-injection marker could vary, we chose to use PHA-1 positive selection to facilitate biochemical screening for GS::RPM-1 transgenic lines. Since the *pha-1* mutant is temperature sensitive, non-transgenic *pha-1; rpm-1* mutants arrest at the L1 larval stage at 23°C. In contrast, *pha-1; rpm-1* mutants expressing PHA-1 and RPM-1 transgenes do not arrest. As a result, PHA-1 selection ensures that only transgenic worms are present in large-scale liquid cultures, which is highly advantageous for biochemistry with transgenic extrachromosomal arrays. Note that another visible co-injection marker is also necessary for the subsequent integration of extrachromosomal arrays.

31. Test functionality of GS tagged RPM-1 through phenotypic rescue.

Note: We used axon termination defects that occur in the mechanosensory neurons of *rpm-1* mutants as a phenotypic readout to test GS::RPM-1 constructs. We evaluated variable GS tag placement (N- and C-terminus) and different linkers between the GS tag and RPM-1 (Figure 3A).

Note: We first confirmed that *pha-1* did not interfere with the *rpm-1* phenotype (Figure 3A, *rpm-1; pha-1*). As a positive control for rescue, we used untagged RPM-1. We observed robust rescue for GS::RPM-1 with a multiple cloning site (MCS) linker but rescue was substantially impaired by a linker generated using Gateway cloning (Figure 3A and Table S2). RPM-1::GS (C-terminal tag location) did not rescue (Figure 3A), which is likely due to GS tag proximity to the catalytic RING domain (Figure 2B). Therefore, we selected GS::RPM-1 with an N-terminal tag and MCS linker for our studies, and generated both RPM-1::GS LD and GS::GFP control constructs similarly. A flexible linker might also be considered for fusing an affinity tag with the POI (Table S2).

32. Integrate extrachromosomal arrays.

Note: We tested GS::RPM-1, GS::RPM-1 LD and GS::GFP control for expression using western blot (not shown). With function and expression confirmed, one extrachromosomal array was integrated for each construct. Transgenic arrays were integrated into the genome using the TMP/UV protocol described above.

Note: After outcrossing to wild-type, integrants were isolated on an *rpm-1*(*ju44*) protein null mutant background and again evaluated for rescue. We confirmed rescue with integrated GS::RPM-1, while GS::RPM-1 LD and GS::GFP did not rescue (Figure 3B).

Note: Affinity purifications were done from integrated transgenic strains using IgG coupled Dynabeads, and quality control diagnostics were performed by silver staining (Figure 5A, 9% of sample) and Western blotting (Figure 5B, 1% of sample). This demonstrated two key points. 1) We successfully purified POIs, GS::RPM-1 and GS::RPM-1 LD, as well as the GS::GFP negative control. 2) We observed relatively clean GS::GFP samples, and the presence of numerous silver-stained bands in test samples (GS::RPM-1 and GS::RPM-1 LD) compared to negative control (GS::GFP) (Figure 5A).

33. Analyze RPM-1 AP-proteomics results.

Note: Scaffold (Proteome Software) was used to validate peptides and protein identifications detected by LC-MS/MS. RPM-1 binding proteins were assessed using the criteria described above in step 23. Ribosomal proteins and vitellogenins were removed as they are found in samples because of their roles in translation and post-embryonic development, respectively. Keratin, a contaminant from sample preparation, was removed as was GFP as it is present only in the negative control.

Note: We also tested how two detergents (NP-40 and CHAPS) and varied detergent concentration (0.1% and 0.3% NP-40) affect proteomic profiles. We found that silver stain profiles (Figure 6A) and proteomic hits (Figures 6B and 6C) varied substantially with different extraction conditions. Two independent proteomics experiments for each extraction condition yielded both common and unique proteomic hits. These findings support two points: 1) Extraction conditions influence protein complexes and binding proteins associated with the POI. 2) At least two independent proteomics experiments should be performed for a single extraction condition due to increased hit identification.

Note: To present data for individual proteomics experiments, we plotted total spectral counts for individual hits that were enriched two-fold or more in test samples versus GS::GFP negative control (Figure 6D). Hits enriched in GS::RPM-1 and GS::RPM-1 LD samples compared to GS::GFP samples largely represent putative RPM-1 binding proteins. To further differentiate between putative RPM-1 binding proteins and possible substrates, we highlight a much smaller number of proteomic hits that were enriched in GS::RPM-1 LD compared to GS::RPM-1 (Figure 6D). To evaluate data for a given proteomic hit, we normalized based on two considerations. 1) POI amount, because the amount of GS::RPM-1 was consistently a bit lower than GS::RPM-1 LD (Figures 5B and 7). 2) Protein size of the proteomics candidates, as larger proteins have a greater probability of being identified than smaller proteins (See step 25 for calculation).

EXPECTED OUTCOMES

Expression levels will vary for different POIs and optimization for an individual POI is key. Generally, 500 mL of liquid culture will yield 4–6 g of frozen worm pellets. 5 g of cryomilled worm pellets yielded approximately 400–500 ng of GS::RPM-1 POI which was a sufficient quantity for AP-proteomics (~0.4–1 μg POI recommended).[1,3] Known biological and biochemical interactors of a POI serve as

AP-proteomics positive controls. As shown by the RPM-1 example, we isolated a few hundred putative hits (Figures 6B and 6C). This is quite high and indicative of a signaling hub. A much smaller number of hits were identified with AP proteomics for the EEL-1 ubiquitin ligase.[2] We consider a validation experiment (Part VII) essential for establishing a bona fide binding protein for a POI. AP-proteomics is likely to yield unexpected putative binding proteins, and these should not be ruled out. To the contrary, they might be the most interesting candidates because they hint at the molecular frontier that AP-proteomics is designed to probe.

LIMITATIONS

While our protocol is capable of identifying *in vivo* binding proteins for a POI, the success of the protocol will vary based on the expression levels and stability of the POI. Highly unstable proteins or proteins expressed at very low levels may not be good candidates for AP-proteomics. Additionally, detecting weak or transient interactions could be challenging.

We prefer to perform proteomics using mixed stage worms so as not to bias our AP-proteomics. However, it might be necessary to use a specific development stage when certain POIs are expressed or known to function. Using synchronized populations of worms could affect total protein yield compared to what we observe with mixed stage worms. For example, synchronized L1s would yield much less total material. However, we do think it would be plausible to use this protocol with synchronized worms if sufficient amounts of POI (~0.4–1 μg) can be purified.

Our validated POIs (RPM-1 and EEL-1)[1–3] are expressed in the nervous system and found outside the nucleus. However, if a POI is largely localized to the nucleus, it would be reasonable to adjust detergent conditions to lyse the nuclear envelope in order to study nuclear protein-protein interactions. We remind the reader that the harsher the detergent lysis conditions the more damaging to PPIs.

We have not attempted AP-proteomics in *C. elegans* using POIs expressed in a relatively limited subset of neurons. We anticipate that this could create issues purifying enough POI and associated binding proteins for proteomics. Nonetheless, our protocol sets all optimization parameters that one would follow to determine whether it would be possible to work with a given POI even if expressed in relatively few neurons.

TROUBLESHOOTING

Problem 1

Affinity tag interferes with POI expression or function (Part I).

Potential solution

- Test for different tags or tag locations (e.g., N-terminal, C-terminal, etc). We recommend all steps in design, tag testing and validation of POI function regardless of which affinity tag is used. While we have used a GS tag (Table S1)[23] for rapid, single-step AP-proteomics, our method should be compatible with a variety of affinity tags (GFP, MYC, FLAG, HA, etc).
- Linker length and flexibility between the affinity tag and the POI can affect function (Figure 3A). Thus, some optimization could be required.

Problem 2

Low worm yield or unhealthy worms from liquid culture (Part II).

Potential solution

- Details on setting up liquid culture might vary for different *C. elegans* strains, especially in the case of slow growing or less healthy transgenic strains.

- Preparing worms for mixed-stage culture: The amount of worms should be experimentally tested before committing to large-scale culture. Prior to adding Day 1 plates to liquid culture, they should be just starved with most worms having hatched into L1s and few remaining eggs. This will maximize the number of adults grown in culture.
- Day 2 plates should be crowded but not starved. Starvation will increase risk of dauer formation. Day 2 plates are ideally mixed stage. Additional second spike of worms grown on plates is meant to stagger life stages in liquid culture.
- Maximum culture volume for a 2 L flask is 250 mL. Using higher volumes will decrease aeration and cause worms to grow slowly and increase dauer formation.
- If flasks are not shaken at proper speed, aeration is reduced and worms grow slower. If you observe slower growth rate in liquid culture compared to plates consider increasing shaker speed We normally use 185 RPM for 2-inch orbit shaker which could be increased to 220 RPM.

Problem 3

Worms do not separate or do not survive during the sucrose floatation (steps 7.i–7.m).

Potential solution

- Always use lowest possible deceleration setting to prevent worms from mixing into sucrose. High deceleration will cause mixing of layers.
- Since sucrose is toxic to worms, deceleration should not take longer than 2 min even on the lowest setting.
- The solutions used are not ice-cold and/or the tubes were not kept in ice.

Problem 4

Protein samples are ''dirty''; many non-specific bands are observed in the control sample using silver stain detection (step 18).

Potential solution

In a successful scenario, silver stained gels will show a clear difference between POI lanes and negative control lanes (see Figure 5A).

- Change affinity bead matrix. Dynabeads are magnetic particles with a surface that minimizes chemical agglutination and non-specific binding. We compared protein purification profiles of IPs executed with IgG coupled Dynabeads to IgG coupled agarose beads. We observed less background in control samples for Dynabeads compared to agarose beads, while the quality of bait protein did not change (Figures 8A and 8B).

Problem 5

POI yield is poor or known binding proteins are not detected.

Potential solution

- The ratio of lysis buffer to ground worms can also be optimized for protein extraction. We tested 3 ratios - 1:1, 1:2 and 1:4 (grams ground worms/mL lysis buffer). We found a ratio of 1:4 yielded the highest protein extraction. A ratio higher than 1:4 is not recommended as protein concentration drops too low for efficient affinity purification.
- Different protease inhibitor cocktails also affect POI degradation during extraction (Figures 8C and 8D). We achieved best results with Pierce HALT protease inhibitors (Figure 8C).
- While it is possible to manually grind frozen *C. elegans* under liquid nitrogen using mortar and pestle, a cryomill yields superior results. A cryomill facilitates automated, homogeneous generation of submicron grindates under liquid nitrogen cooling (Figure 9). It generates superior

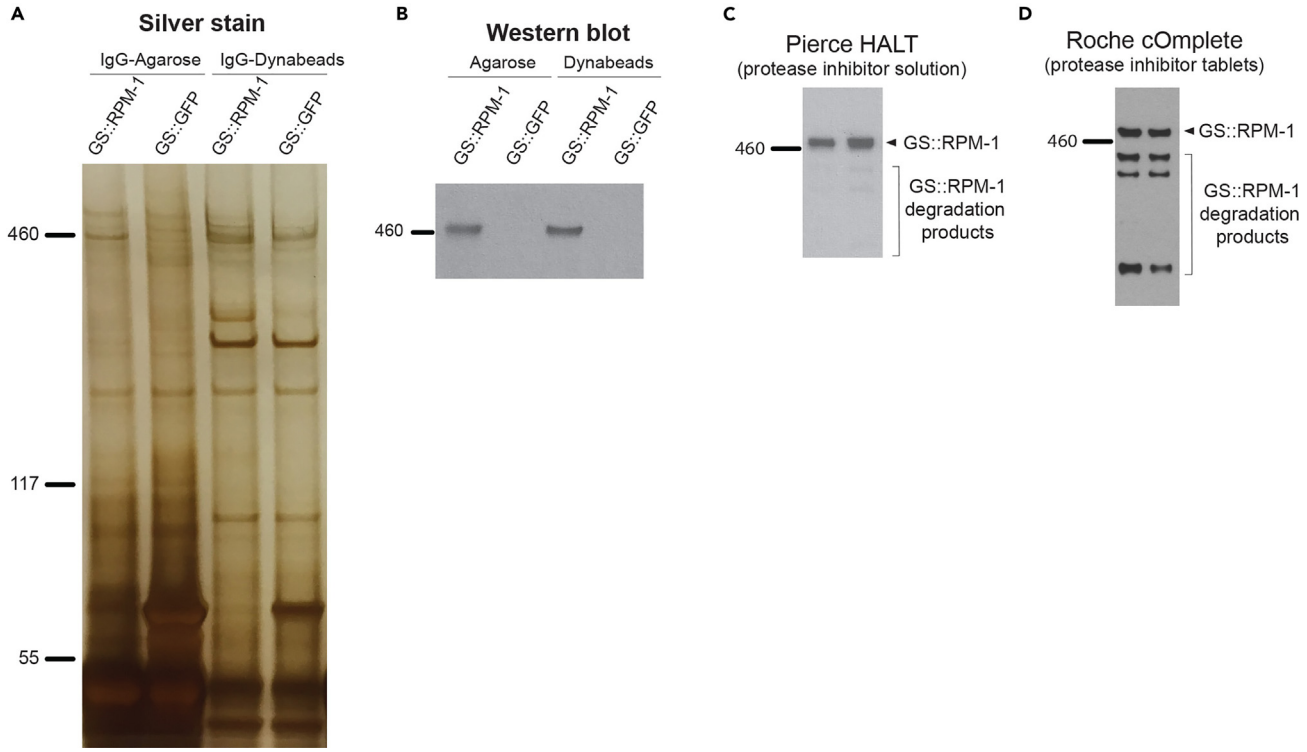

Figure 8. Dynabead matrix and HALT protease inhibitors improve POI affinity purification and reduce degradation

(A) Silver stain for POI (GS::RPM-1) and negative control (GS::GFP). We observed cleaner background in GS::GFP control sample and more enrichment of silver stained species in GS::RPM-1 test sample when using IgG-Dynabeads compared to IgG-Agarose beads.

(B–D) Anti-SBP Western blot to detect GS::RPM-1. (B) Shows similar capture of GS::RPM-1 with IgG-Dynabeads and IgG-Agarose. (C) Shows primarily intact full-length GS::RPM-1 when Pierce HALT protease inhibitors are added to lysis buffer. (D) Shows extensive GS::RPM-1 degradation products occur with Roche cOmplete protease inhibitors.

grinding compared to mortar and pestle (Figure 9). In our experience, a cryomill yields increased extraction of POI, better preserves protein complexes, requires less time, and yields more consistent results between experimentalists.

- In our protocol, we describe the amount of whole worm lysate (total protein) and beads that we used. However, these quantities will depend on the specific POI and affinity tag. We advise testing small-scale IPs to optimize affinity purification, sample cleanliness and amount of POI purified prior to mass spectrometry. Initially test several small amounts of total protein to determine the quantity of POI. We initially start with 1–10 mg total protein. Use a constant volume of Dynabeads (50 µL of slurry) for comparisons and bring each tube to the same total volume using cold lysis buffer. Use IP and Western blotting to determine the amount of POI in a given amount of lysate. Quantify the amount of POI using protein standards on a silver stain by comparing the signal of POI to a similar size molecular weight standard (for example see Figure 5A). Optimal amounts of sample lysate to matrix occurs when the silver stain shows the greatest quantity of enriched POI and cleanest negative control sample. Once this ratio of lysate to matrix is established, affinity purification should be scaled up to yield ~0.4–1 µg of POI.

Problem 6

Co-immunoprecipitation to validate PPI is not working (step 26).

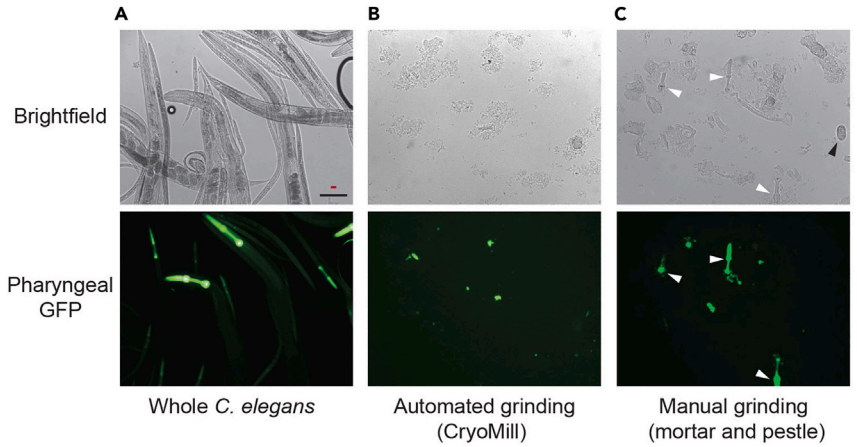

Figure 9. Automated cyromill grinding of *C. elegans* is superior to manual grinding by mortar and pestle

(A–C) Visual comparison using brightfield (upper panels) and epifluorescence microscopy (lower panels) of (A) whole unground *C. elegans*, (B) automated grinding with cryomill under liquid nitrogen cooling, and (C) manual grinding by mortar and pestle. Note automated cyromill consistently grinds *C. elegans* to low micrometer particles without any visible intact pharynxes, larvae or embryos. In contrast, manual grinding is inconsistent leaving intact portions of worms (white arrowheads denote pharynxes) and eggs (black arrowhead). Transgenic P*myo-2*::GFP used to visualize pharynx. Scale bars are 100 μm (black) and 20 μm (red).

Potential solution

- The GS tag we used for AP-proteomics is not ideal for biochemical validation studies because Protein G will bind to secondary antibodies in a Western blot. We typically use 3×FLAG, MYC and GFP tags for CRIPSR-based native biochemistry or biochemistry using PHA-1 positive transgene selection. However, other tags will potentially work with our protocol.
- If the POI is expressed at low levels, overexpression using transgenic extrachromosomal arrays and PHA-1 positive selection could be necessary to test PPIs. We have validated PPIs using two approaches. 1) CRIPSR-based native-biochemistry.[1,3] 2) Transgenic *in vivo* biochemistry using PHA-1 temperature sensitive selection to enrich for worms carrying transgenic extrachromosomal arrays expressing tagged POI and binding protein.[2,27] The CRISPR approach has the advantage of being entirely endogenous and physiological but a low expressing POI or candidate could make co-IP experiments challenging. Biochemistry with transgenic arrays will increase expression of the POI and/or candidates, and PHA-1 temperatures selection further facilitates high sensitivity biochemistry.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Brock Grill (brock.grill@seattlechildrens.org).

Materials availability

Reagents used in this protocol have been published and are available upon request to the lead contact.

Data and code availability

Protocol presents all data used in this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102262.

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AUTHOR CONTRIBUTIONS

M.D., J.S.P., and B.G. wrote the manuscript. M.D. and J.S.P. generated figures and performed data analysis. A.C.G. and K.J.O. drafted initial protocol. K.J.O. and A.C.G. performed optimization testing for AP-proteomics, protein extraction conditions, affinity matrices, and protease inhibitors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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