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Mutagenesis and Imaging studies of BMP Signaling Mechanisms in *C. elegans*

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Abstract

C. elegans has played a central role in the elucidation of the TGF β pathway over the last two decades. This is due to the high conservation of the pathway components and the power of genetic and cell biological approaches applied toward understanding how the pathway signals. In this methods chapter, we detail approaches to study the BMP branch of the TGF β pathway in *C. elegans*.

Keywords

C. elegans; body size; development; mutagenesis; genetic screens; coelomocytes; mesoderm; imaging; trafficking

1. Introduction

1.1 TGF β signaling pathways.

The TGF β pathway is an ancient signaling pathway present in the most primitive animals, sponges and trichoplax, but absent from yeast or plants [1,2]. Members of the vertebrate superfamily number approximately 30 ligands and can be roughly placed into the TGF β , activin, and BMP families. Evolutionary studies suggest that of the three families, the TGF β family is a more recent deuterostome invention, with the activin and BMP families being present in the very earliest animals [3]. In mammals, BMPs are associated with many morphological diseases, such as osteogenesis imperfecta and polydactyly, cardiovascular diseases such as pulmonary arterial hypertension, or with the progression of cancer [4]. Essentially every mammalian tissue has one or more TGF β ligands expressed, where they play essential roles.

Many aspects of TGF β signaling are conserved between *C. elegans* and mammals including the structure and function of the ligands, and the downstream signaling components. In *C. elegans*, the BMP family is represented by the Sma/Mab pathway, while the activin family is represented by the dauer pathway [5–14]. TGF β signaling in *C. elegans* is more complicated than two linear pathways as there are five ligands (*dbl-1*, *daf-7*, *unc-129*, *tig-2*, and *tig-3*), but only two complete signaling pathways with possibly multiple ligands in a given pathway (Figure 1) [15–17]. Signaling begins when the ligand binds to the receptors, which are transmembrane serine-threonine kinases, although they can phosphorylate tyrosine residues as well [18]. Upon binding, the type II receptor phosphorylates the type I receptor on the GS domain, which activates it [19]. The primary target of the type I receptor phosphorylation is the C-terminus of the R-Smads, which allows the R-Smads to bind with the co-Smad. In the Sma/Mab pathway, SMA-2 and SMA-3 are R-Smads, and SMA-4 is the co-Smad. *tag-68* encodes a likely I-Smad, but its function has not been fully determined [16]. This Smad complex moves to the nucleus where it binds DNA and alters transcription with the aid of other transcription factors. One conserved transcription factor used in some developmental contexts is SMA-9/Schnurri, a zinc finger-containing protein [8].

1.2 Genetic screens for components of the Sma/Mab pathway

The Sma/Mab pathway in *C. elegans* was originally described as a regulator of body size and male tail development: small body size phenotype (Sma) and male tail abnormal phenotype (Mab) caused by mutations in this pathway (Figure 2). Over time, many additional roles of the pathway have been described, such as its function in innate immunity, reproductive aging, and mesoderm patterning [5,20–26].

The Mab phenotype is labor-intensive to analyze because it requires a genetic background generating a high incidence of males, and it also requires that animals are screened on slides under a compound microscope, so it has been used in relatively small-scale screens. The small body size and mesoderm development phenotypes have been successfully exploited in genetic screens, as described here. The small body size phenotype is visible under the dissecting microscope and was therefore useful in a large-scale genetic screen for BMP signaling components [11]. From the Sma/Mab screen, all the conserved, known core signaling components (ligand, both receptors, three Smads, and Schnurri) [11,7,8,12] and some important regulatory genes [27,6,28–30] were identified. A variation of this screen using genetic suppression of *lon-2*, which encodes an upstream component in the pathway, yielded mutations in many of the same genes [29].

Another important role for the Sma/Mab pathway is the specification of mesodermal cell fates. The mesoderm gives rise to muscles and some non-muscle tissues. In *C. elegans*, the postembryonic mesoderm derives from the M precursor cell, which produces a subset of bodywall muscles, the sex muscles and the non-muscle coelomocytes [31]. The Zn finger containing transcription factor SMA-9/Schnurri is critical for specifying the two postembryonically-derived coelomocytes (CCs), thus in *sma-9* mutants, there are four CCs instead of the six CCs present in wild-type animals (Figure 3) [5]. This CC defect of *sma-9* mutants can be specifically suppressed by mutations in the Sma/Mab pathway, but not by mutations in any other pathways [5,9]. This suppression of the s*sma-9* CC phenotype, the

Susm phenotype, is highly sensitive to altered levels of Sma/Mab signaling: null alleles for a given Sma/Mab pathway gene exhibited higher penetrance of the Susm phenotype compared to partial loss-of-function alleles of the same gene [9]. A *sma-9* suppressor screen has successfully identified the major Sma/Mab signaling components as well as several new, regulatory genes [5,9,13,14]. More is to be learned from both the Sma and the CC screens, as neither have reached saturation for mutants.

2. Materials

2.1 Ethyl methanesulfonate (EMS) Mutagenesis for Sma mutants

1. *C. elegans* variety Bristol, strain N2 unless noted otherwise. Note, wild-type, mutant, and transgenic strains (strains containing engineered DNA) can be requested from the *C. elegans* Genetics Center (CGC) (<http://www.cbs.umn.edu/cgc/>) or from individual laboratories.
2. 60 mm petri dishes with standard worm normal growth medium (NGM) (50 mM NaCl, 15 g/l agar, 20 g/l peptone, 1 mM cholesterol, 1 mM CaCl₂, 1 mM MgSO₄, 1 mM KPi [pH = 7.0]) and seeded with *E. coli* OP50 [32].
3. Pipettors (P20 and P1000 or equivalent) and sterile pipet tips.
4. 15 ml and 50 ml disposable tubes.
5. 50 mM EMS (see Note 1).
6. M9 buffer: (22mM KH₂PO₄, 42mM Na₂HPO₄, 86mM NaCl, 1mM MgSO₄). Aliquot into 100 ml bottles and autoclave.
7. 1N NaOH.
8. Glass pasteur pipettes.
9. Table-top centrifuge.
10. Stereo dissecting microscope with underneath illumination.

2.2 Body size measurements

1. *C. elegans* variety Bristol, strain N2.
2. Control mutant animals: *dbl-1(wk70)*, *sma-3(wk30)*, or *sma-6(wk7)* (Note 2).
3. 60 mm petri dishes with NGM medium and *E. coli* OP50 [32].
4. Pipettors (P1000 or equivalent) and sterile pipet tips.
5. M9 buffer.
6. 1.5 ml microfuge tubes.

¹1N NaOH solution is used to inactivate EMS which is a potent mutagen. Always wear gloves when handling EMS. Keep EMS, and anything that comes in contact with it, in the hood. Inactivate anything that comes in contact with it by soaking in 1N NaOH overnight.

²The strains *dbl-1(wk70)*, *sma-3(wk30)*, and *sma-6(wk7)* contain premature termination codons generating null alleles in the genes encoding the ligand DBL-1, the Smad SMA-3, and the receptor SMA-6 [7,39,12]. Other mutant backgrounds can be analyzed as desired, but it should be kept in mind that for interpretation of double mutant phenotypes, at least one of the mutations must be a null allele of the corresponding gene.

7. Microcentrifuge.
8. Bleach solution: Mix 5 ml 1N NaOH and 2 ml sodium hypochlorite (commercial bleach). Date and use within 10 days.
9. Stereo dissecting microscope with underneath illumination.
10. Sodium azide.
11. Agarose (electrophoresis grade).
12. Microscope slides and coverslips.
13. Compound microscope equipped with camera.
14. Imaging software, such as Fiji [33].

2.3 Double mutant analysis

1. *C. elegans* variety Bristol, strain N2.
2. Control mutant animals: *dbl-1(wk70)*, *sma-3(wk30)*, *sma-6(wk7)* (Note 2), *lon-2(e678)*, and *dbl-1* overexpressing strain *ctIs40* (Note 3).
3. 60 mm petri dishes with NGM medium and *E. coli* OP50 [32].
4. Stereo dissecting microscope with underneath illumination.

2.4 EMS mutagenesis for *sma-9* suppressors

1. *C. elegans* strain LW0040[*arIs37(secreted CC::gfp)* I; *cup-5(ar465)* III; *sma-9(cc604)* X]. This strain has 4 CCs that are labeled by *arIs37(myo-3p::secreted gfp)*, in which secreted GFP made in the bodywall muscles (BWMs) is taken up by the CCs via endocytosis [34,35]. The *cup-5(ar465)* mutation, which affects the *C. elegans* mucolipin homolog, enhances the ability of the CCs to uptake the secreted GFP, making the CCs easily identifiable under a fluorescence stereo microscope.
2. A standard fluorescence stereo microscope equipped with a GFP filter set, underneath illumination, 10x eyepieces, and a built-in zoom up to 10x.
3. Other reagents as described in Section 2.1.

2.5 Molecular Reporters of BMP Signaling

1. *C. elegans* strains:
 LW2433: *jjIs2433[pCXT51(RAD-SMAD) + LiuFD61(mec-7p::mRFP)]* X
 LW2301: *dbl-1(wk70)* V; *jjIs2433[pCXT51(RAD-SMAD) + LiuFD61(mec-7p::mRFP)]* X

³. *lon-2(e678)* is a loss-of-function mutation in *lon-2*, which encodes a glypican that negatively regulates DBL-1 signaling [29]. *ctIs40* is a transgene carrying extra copies of *dbl-1(+)* that results in overexpression of the ligand [12]. In both genetic backgrounds, the DBL-1 pathway is overactive, leading to a long body size.

LW2290: *lon-2(e678) jJIs2433[pCXT51(RAD-SMAD) + LiuFD61(mec-7p::mRFP)] X*

LW2437: *jJIs2437[pCXT51(RAD-SMAD) + LiuFD61(mec-7p::mRFP)] II*

LW2319: *jJIs2437[pCXT51(RAD-SMAD) + LiuFD61(mec-7p::mRFP)] II ; dbl-1(wk70) V*

LW2394: *jJIs2437[pCXT51(RAD-SMAD) + LiuFD61(mec-7p::mRFP)] II ; lon-2(e678) X*

2. Fluorescence compound microscope equipped with a dry or oil 40x objective, a GFP filter set, and a CCD camera.
3. 0.1 μ M tetramisole in M9 buffer (Note 4).

2.6 Materials for *in vivo* imaging of BMP receptors

1. *C. elegans* strains: Bristol strain N2, *pwIs921[pvha-6::sma-6::GFP]*, *pwIs922[pvha-6::daf-4::GFP]*, *pwIs849[pvha-6::tagRFP::RAB-7]*.
2. M9 buffer.
3. OP50 bacterial strain.
4. M9 buffer with 0.1 μ M tetramisole.
5. NGM media.
6. Agarose (gel electrophoresis grade).
7. Dissecting scope.
8. petroleum jelly.

3. Methods.

Grow *C. elegans* strain N2 under standard conditions in 60 mm petri plates with standard NGM media.

3.1 EMS Mutagenesis to screen for Sma mutants

The EMS screening protocol is based on techniques developed by Brenner and colleagues [36] as performed [11].

1. Harvest worms from three 60 mm plates containing many fourth larval stage (L4; Note 5) and young adult animals by washing off with M9 buffer. Pipette about 1 ml M9 buffer onto each plate, swirl, and pour animals and liquid into a 15 ml disposable tube. Let the worms settle and then adjust the total volume to 3 ml.

⁴.Sodium azide can quench the GFP fluorescence of the RAD-SMAD reporter. Thus tetramisole is used to anesthetize the worms.

⁵.L4 larvae are readily identified due to the process of vulval morphogenesis that is occurring during this stage. In the dissecting microscope, this process is visible as a lighter semi-circle seen on the ventral surface midway between the head and the tail. In the compound microscope under Nomarski DIC optics, L4 larvae are characterized by the “Christmas tree structure” formed during vulval morphogenesis [46,47].

- Gently aspirate off the top liquid if you have too much liquid or add M9 to bring the total to the 3 ml mark.
2. Prepare the EMS solution. Pour about 100 ml of 1N NaOH into a 500 ml beaker in the hood (Note 1). Add 1 ml of M9 buffer to a 50 ml capped disposable tube. In the hood, add 20 μ l of EMS to the M9 in the 50 ml tube. Eject the pipet tip into the beaker containing the 1N NaOH. Cap the 50 ml tube and swirl gently to mix the EMS with the M9 solution.
 3. Swirl the 15 ml tube containing the animals in 3 ml of M9 to resuspend them and then pour them into the 50 ml tube that contains the EMS solution. Leave the animals in the solution in the hood for 4 hours. Mix animals gently at least once an hour to resuspend them.
 4. After 4 hours, prepare a 15 ml disposable tube with 12 ml of M9 buffer. With a glass pasteur pipette, suck up the worms that have settled to the bottom of the 50 ml tube and transfer them to the 15 ml tube that contains M9. Discard the pipette into the 1N NaOH solution. Pour the EMS solution into the 1N NaOH to inactivate the mutagen. Use the pasteur pipette to wash the 50 ml tube with 1N NaOH.
 5. Gently spin the 15 ml tube containing the animals, about 1K for 5 minutes in a table top centrifuge. Using a pasteur pipette, remove pelleted animals from the bottom of the tube. Transfer the animals to the edge of a worm plate spread with bacteria. Leave the plate slightly open in the hood. Animals should crawl around for 30 min. to recover. These are the P0 animals.
 6. Transfer single mutagenized P0 animals to worm plates and allow to segregate self-progeny (F1 animals). After 3–4 days at 20°C, transfer two F1 animals each to new plates to segregate F2 animals (Note 6).
 7. Visually screen F2 progeny using a stereo microscope for a clone of small mutants (~1/8 animals are expected on a plate for a recessive mutation). Pick mutants to new plates to segregate and confirm the phenotype in F3 animals. Retain the segregants from only one animal per F2 plate to ensure independent origin of mutants.
 8. To calculate mutation frequency, each screened F2 plate generated from two F1 parents is considered to contain four mutagenized haploid genomes.
 9. Outcross identified mutations by mating six N2 males with three L4 *sma* mutant hermaphrodites on a plate. In the next generation, observe the phenotype of males to identify X-linked mutations and the phenotype of hermaphrodite cross progeny to identify dominant or semi-dominant mutations (Note 7). Mate heterozygous *sma/+* or hemizygous *sma/0* males with N2 hermaphrodites. In the

⁶Since the *Sma/Mab* signaling mutants are slow-growing compared with controls, it is not possible to screen the F2 progeny on the same plates with other segregants from the P0.

⁷For X-linked mutations, male progeny from the cross N2 males X *sma/sma* hermaphrodites will be hemizygous *sma/0* genotype and all of these males will be small. For dominant and semi-dominant mutations, all of the progeny, including *sma/+* cross progeny, will have a small body size.

following generation, pick individual hermaphrodites (half of which should be *sma/+*) to new plates. Look for the segregation of *sma/sma* progeny from these parents and pick them. These are twice outcrossed mutants. Repeat this process 1–2 additional times.

3.2 Body size measurements

1. Grow *C. elegans* wild-type strain N2, a known BMP pathway mutant, and newly isolated *sma* mutant strains under standard conditions.
2. For each strain, obtain a plate containing a large number of gravid hermaphrodites. Wash worms off plates with 1 ml M9 buffer. Transfer to eppendorf tube.
3. Pellet worms at 1000 rpm for 1 min in microcentrifuge. Remove supernatant.
4. Add 500 μ l bleach solution. Mix by inverting. Monitor digestion of worms under the dissecting microscope.
5. Pellet eggs and worm debris at 1000 rpm for 1 min in microcentrifuge.
6. Transfer pellet to new plate. Under the dissecting microscope, many eggs should be visible in the drop. Embryos will hatch and crawl onto the food.
7. After two days, plates should contain large numbers of L4 animals (Note 5). Pick 20–30 L4 animals or animals at different larval stages (Note 8) into a drop of 10% sodium azide in M9 buffer on a 5% agar pad on a glass microscope slide. Cover with cover slip. Measurements should be done within 30 min after adding sodium azide (a nematode anesthetic) since it is harmful to nematodes after extended exposure.
8. Image worms on a compound microscope at low power (100x magnification) or on a dissecting microscope equipped with a camera. Determine the length of the animals by drawing a line down the center of the animal from the head to the tail (Note 9) and analyze using imaging software such as Fiji software [33].
9. An unpaired, two-tailed t test is used to compare the P value between the control *wild-type* animal and each genotype. Fig. 2 shows typical wild-type and mutant lengths.

3.3 Double mutant analysis

To determine whether new *sma* mutants act within the BMP pathway or independently of it, double mutants should be constructed and analyzed [27,8,9,11,13,14,37,28].

⁸.To measure young adult animals, pick 30 L4 animals to a new plate. After 24 hours, mount the young adults on slides and image. To measure animals at larval stages, harvest worms at 24 hours, 48 hours, and 72 hours after bleaching. For larval stage measurements, keep in mind that animals may develop at different rates and that it may be necessary to take this into account when interpreting results. Compare the average length of experimental animals to a wild-type control grown in the same conditions at the same time.

⁹.Sma/Mab signaling mutants are smaller in both length and width. Measuring body length provides a convenient proxy for growth. Other alternatives can also be used, such as measuring perimeter, cross-sectional area, or calculated volume.

1. Cross N2 males with *sma* mutant hermaphrodites. Pick *sma*^{+/+} males and mate with hermaphrodites of the genotypes *dbl-1(wk70)*, *sma-3(wk30)*, *sma-6(wk7)*, *lon-2(e678)* and *ctIs40[dbl-1⁺⁺]*.
2. Pick individual hermaphrodite progeny from each cross to separate plates. These should have approximately wild-type phenotype if the mutations are recessive. Half of these will be the double heterozygotes (Note 10).
3. In the next generation, pick *sma* and/or *lon* animals individually as potential double mutants. Allow to self-fertilize. Confirm double mutants by complementation testing and/or sequence analysis.
4. Determine body size of double mutants as in Section 3.2.
5. For double mutants with *dbl-1*, *sma-3*, and *sma-6*, if the new mutation functions in the same pathway, then the double mutant body size should be the same as the single mutants. If the new mutation functions independently, then the double mutant should be more severe (*i.e.* smaller) than the single mutants (Figure 4).
6. For double mutants with *lon-2* and *dbl-1(++)*, new mutations that suppress the long body size phenotype likely represent genes encoding components downstream of the ligand. Other outcomes may indicate function at the level of receptor-ligand or independent function (Figure 4).

3.4 EMS mutagenesis and CC screening for *sma-9* suppressors

1. Mutagenize LW0040 animals with EMS using the method described in Section 3.1.
2. Pick the mutagenized P0 animals onto NGM plates seeded with *E. coli* OP50 by putting five L4s per plate. Allow worms to grow at 20°C for 5 days.
3. Pick the F1 progeny of the mutagenized worms to new plates by plating 3 animals of similar developmental stages per plate (Note 11). Allow worms to grow at 20°C for 5 days.
4. When the majority of the F2 progeny develop to the late L4 to young adult stage, screen each plate under the fluorescence stereo microscope and save plates that have between 5% to 25% of animals with 6 CCs (Note 12).
5. From each of the saved plates described in step 4 above, pick all the animals with 6 CCs (up to 20 from each plate) and plate them one worm/plate. Those animals

¹⁰This genetic cross assumes that the mutations are unlinked. If the mutations are linked, there are three alternatives: (1) omit the analysis of the particular genetic interaction if other analyses are sufficiently informative; (2) use RNAi instead of mutation to inactivate one of the genes; or (3) use genetic markers to assist in generating the desired double mutant (www.wormbase.org).

¹¹The 4 CCs phenotype of *sma-9(cc604)* mutants is not 100% penetrant: usually zero to 3% of *sma-9(cc604)* animals have 6 CCs as is found in wild-type animals. Because of this low background and because the *sma-9; susm* double mutant strains are slow growing, it is not possible to screen the F2 progeny on the same plates with other segregants from the P0.

¹²Assuming that all the F1 animals on a given plate are fertile and give similar number of F2 progeny, if none of the F1 animals carries a *sma-9* suppressor mutations, near 100% of the F2 animals will have 4 CCs, rather than 6 CCs. If only one of the three F1s carries a *sma-9* suppressor mutation and the mutation is 100% penetrant, then 8.33% of the F2 animals are expected to have 6 CCs. If all three of the F1s carries *sma-9* suppressor mutations and all of the mutations are 100% penetrant, then 25% of the F2 animals are expected to have 6 CCs. To ensure for the recovery of suppressor mutations with a penetrance of at least 50%, plates that have anywhere between 5% and 25% of animals with 6 CCs are kept as candidates.

that produce high penetrance of progeny with 6 CCs are saved as candidate *sma-9* suppressors (Note 13).

6. The putative *sma-9* suppressors can be subjected to further analysis, which includes determining whether they are recessive or dominant, single locus- or multi-loci- mutation. The corresponding genes can be identified via complementation tests, linkage analysis, and SNP-WGS [38].

3.5 Molecular reporters of the *C. elegans* BMP pathway and RAD-SMAD expression and quantification

As described above, the basic signaling components of the BMP pathway in *C. elegans* are highly conserved with their vertebrate counterparts. However, there are sufficient differences in the protein sequences such that the large pool of available mammalian antibodies often does not work well in nematodes. A critical tool needed in assaying TGF β signaling strength is the ability to monitor the level of phosphorylated Smad (pSmad) in the nucleus. A reporter tag for the R-Smads suffers from possible interference with phosphorylation [39]. The most successful approach was to concatenate Smad binding sites upstream of a minimal promoter, as described below. This reporter allows a readout of signaling strength, with low levels in a null background and higher levels with over-expressed components.

The reporter, named the RAD-SMAD (Reporter acting downstream of Smad) reporter, was generated by placing multiple copies of the Smad boxes (or Smad binding site: GTCT) organized in the RLR orientation (R, rightward and L, leftward) upstream of the minimal *pes-10* promoter and a nuclear localized *gfp::lacZ* fusion protein [13]. The RAD-SMAD reporter showed nuclear GFP expression in intestinal and hypodermal cells, tissues known to be responsive to DBL-1 signaling [15]. The level of RAD-SMAD reporter expression in hypodermal nuclei at the L2 larval stage appears positively correlated with the strength of DBL-1 signaling [13] (Figure 5). As a control, the BAD-SMAD (Bad reporter of Smad) reporters, which contain mutations in the Smad boxes, showed no GFP expression in any tissues [13]. The RAD-SMAD reporter has been used to quantify the level of BMP signaling in mutants of several newly identified Sma/Mab modulators [6,9,13,14].

1. Bleach gravid adult hermaphrodites of the following genotypes: your mutation of interest, wild-type, *dbl-1(wk70)* and *lon-2(e678)*, all carrying the same RAD-SMAD reporter transgene (Note 14), following the protocol described in section 3.2. Wash off the bleach using M9 buffer.
2. Allow embryos to hatch in 1 ml of M9 buffer in a 15ml conical tube with gentle rotation at 16°C for 24hr. The embryos will hatch and arrest as synchronized L1 worms.

¹³ Many of the Sma/Mab pathway mutations exhibit partial dominance in their suppression of the *sma-9* CC phenotype [5,9]. Thus multiple 6 CCs-bearing animals from the same plate should be single plated. The plates that reproducibly give the highest penetrance of the 6 CCs phenotype (wild-type phenotype) are likely from animals that are homozygous for the suppressor mutation.

¹⁴ The RAD-SMAD reporter was integrated into the *C. elegans* genome together with a co-injection marker *mec-7p::mRFP*, which is expressed in the six touch neurons and can be used as a marker in genetic crosses introducing the RAD-SMAD reporter into different mutant background. Two different integrated lines are listed, *jfls2433* maps to the X chromosome and *jfls2437* maps to chromosome II. Because the RAD-SMAD reporter exhibits variability in levels of GFP expression between animals, and also between nuclei in the same animal, we recommend the usage of *dbl-1(wk70)* and *lon-2(e678)*, which exhibit reduced or increased RAD-SMAD expression, respectively, as controls prior to the measurement of your mutant of interest.

3. Plate ~200 synchronized L1 worms onto OP50-seeded NGM plates and allow them to grow at 20°C till the L2 larval stage (Note 15).
4. Wash worms off the plates with M9 buffer.
5. Anesthetize worms with 0.1 μ M tetramisole in M9 buffer and mount them onto 2% agarose pads.
6. Collect images of a total of 40 worms per genotype, making sure that at least 5 hypodermal nuclei per worm are within the focal plane imaged using a 40x objective lens on a compound microscope (Notes 16, 17).
7. For each worm, pick five nuclei, measure the fluorescence intensity of each nucleus and the corresponding background signal right outside of that nucleus, using the Fiji software.
8. Calculate the mean GFP signal value for each nucleus by subtracting the nuclear GFP value with the corresponding background value collected in Step 7) above. The resulting values are used to deduce the mean and standard deviation of RAD-SMAD signal for each genotype. Subsequent statistical analysis can be carried out using any standard software package.

3.6 *In vivo* imaging of BMP receptors

The *C. elegans* BMP pathway is expressed in several tissues, most notably in the hypodermis [28,7,10,12] and in the intestine [22,24,40]. The expression levels of many BMP pathway components are sufficiently low in the hypodermis so that tagged receptors are difficult to detect. However, expression levels in the intestine are much more robust, and for this reason, most of the BMP subcellular imaging is done in the intestine.

The intestine houses the nematode innate immunity system, which protects it from environmental pathogens [41,42,23,26,43]. Genetic screens have identified signaling pathways that respond to infection by inducing peptides against bacteria [44]. Some of the induced peptides are regulated by the *C. elegans* BMP pathway making the intestine an appropriate tissue for examination of BMP signaling [22,24].

The intestine is comprised of 20 large epithelial cells (ideal for imaging) that are positioned as bilaterally symmetric pairs to form a long tube around the lumen with distinct apical and basolateral regions (Figure 6). It is anchored to the pharyngeal and rectal valves and as an adult has a left-handed 180° twist around the gonad. As a result, the twist causes the displacement of the intestine slightly left of the midline in the anterior half and slightly right

¹⁵ Expression level of the RAD-SMAD reporter is highly dynamic during development, with stronger intestinal expression in embryos and L1s but fainter in adults, strong hypodermal expression in L2 larvae, and even stronger hypodermal expression in L3 larvae. Thus it is critical that the worms are synchronized when collecting images for the RAD-SMAD reporter.

¹⁶ Take images of hypodermal nuclei in the mid-body region of the worm. Avoid the head and tail hypodermal nuclei because they are smaller. Also avoid those hypodermal nuclei that are located adjacent to the most anterior or posterior intestinal nuclei, as those intestinal nuclei tend to exhibit very bright GFP expression.

¹⁷ Briefly examine through the slides with WT, *dbl-1(wk70)* and *lon-2(e678)* worms to ensure that an appropriate exposure time is chosen for collecting the RAD-SMAD fluorescence images in all genotypes so that the RAD-SMAD fluorescence signals in *lon-2(e678)* worms are not saturated, while the RAD-SMAD fluorescence signals in *dbl-1(wk70)* worms are slightly above background.

of the midline in the posterior half. Care must be taken to find animals positioned correctly to obtain clear images of the intestinal cells (as described below). Imaging in the intestine can be simplified by expressing genes under an intestine-specific promoter so there is less interference from dispersed expression. Several different promoters are available for expression in the intestine, but the *vha-6* promoter works well and is commonly used [6,45,40].

3.6.1 Instruments and software

1. Leica SP5 confocal microscope system or similar.
2. Image analysis and quantification software iVision (BioVision Technologies), Image J (Fiji), or Metamorph.
3. Spinning-disk confocal microscope (Upright Zeiss Axioimager Z1) with Photometrics Evolve 512 EMCCD camera and C-apochromat water-immersion lens or similar and deconvolution software AutoQuant X3 (Media Cybernetics, Rockville, MD) or similar.

3.6.2 Preparation of an agarose pad and staging animals for live imaging

1. Mix agarose powder in deionized H₂O with a final concentration between 2–8% (wt/vol). Heat solution in a microwave oven until agarose is completely dissolved but do not over boil the solution as it will increase the final concentration of the agarose gel.
2. Using a small pipet bulb and a glass Pasteur pipet place a small drop of heated agarose solution onto a 25 × 75 × 1.0 mm microscope slide. To ensure a flat, uniform surface of the agarose pad place two support slides directly alongside the first slide one on either side. Also, place a piece of tape along the top of the support slide.
3. Quickly place another 25 × 75 × 1.0 mm microscope slide over the drop of agarose perpendicular to the first slide.
4. Allow the agarose to solidify, then slide the bottom slide or top slide out from under the agarose, leaving the agarose pad stuck to one slide.
5. Place 5 µl of M9 with tetramisole on the agarose pad.
6. Using a wire worm pick and a dissecting microscope, select and transfer animals to the M9 with tetramisole drop. Select and analyze animals at the same developmental stage. To restrict analysis of the samples to young adults one day past the L4 stage, L4 stage animals are selected a day before imaging and allowed to grow on NGM medium and *E. coli* OP50 plates overnight.
7. Place a cover slip on top of the agarose pad and sample (Note 18).
8. The coverslip can be sealed with petroleum jelly. Heat the petroleum jelly on a 55°C heat block, and apply with a brush.

¹⁸. An alternative method for nanoparticle immobilization on agar has also been reported using submicron polystyrene beads [48].

9. To observe animals on the Confocal laser scanning microscope, place your slide with the agarose pad and sample onto the stage of the microscope.
10. Locate and focus on an animal under low magnification (10x). To observe GFP expression in a transgenic animal, switch the fluorescence source on and switch off bright field.
11. Switch to 40x, 63x, or 100x objectives to observe your cells of interest. For most samples, we use a 63x water immersion lens.
12. To monitor subcellular localization of a protein of interest (*i.e.*, the BMP receptors), green fluorescent protein (GFP), or another fluorescent protein, is routinely expressed as a fusion protein with a gene product of interest (Note 19). This approach enables the observation of the subcellular localization and dynamics of the tagged protein. This article will focus on GFP excitation and emission, but can be adapted to other fluorescent protein fusions of interest. GFP, specifically enhanced GFP (eGFP), has a single excitation peak centered around 488nm with an emission peak wavelength of 509nm.
13. To reduce variability of expression of the fusion protein, analysis of protein localization should be restricted to the anterior half of the animal, specifically the second and third pair of cells. As a result, only animals laying with the anterior half of the intestine unobstructed by the gonad are selected for imaging.
14. For imaging the intestinal cells, use the lowest laser power that gives a reasonable signal to avoid damaging the worms. While viewing be aware of photobleaching.
15. Next, optimize the signal-to-noise ratio in order to establish operating parameters that maximize image quality and minimize specimen damage. Common values to consider include the pinhole, the laser unit or the percent of the laser illumination source, as well as the detector, frequently a photomultiplier tube (PMT). Also, use Leica HyD™ detectors and/or Zeiss GaAsP™ detectors if available (Note 20). The size of the confocal pinhole aperture determines the thickness of the optical section. Generally, a pinhole of 1 airy unit (AU) is determined based on wavelength and numerical aperture and modern confocal microscopes automatically set the pinhole diameter to 1AU. To adjust the sensitivity and background of the detectors, fine-tune the gain (also called the high voltage) and the offset (also called the black level). Gain is the relative measure of the application to the photon detection system, higher gains will result in brighter images (Note 21). To eliminate background, adjust the offset such that the lowest true values of your sample are collected and background signal is dispensed.

¹⁹The ability of the fusion transgene to rescue a mutant phenotype is an excellent test to validate the fusion protein is functional and demonstrates proper subcellular localization. Both SMA-6::GFP and DAF-4::GFP can rescue *sma-6(wk7)* and *daf-4(m72)* mutant phenotypes [6,49].

²⁰Leica HyD (Hybrid detectors) or Zeiss GaAsP (Gallium arsenide phosphide) detectors are both optimized for live cell imaging when compared to traditional photo-multiplier tubes (PMTs). Sensitive live samples, such as *C. elegans*, need to be imaged under low-light conditions to prevent phototoxicity, as well as photobleaching of the fluorophore. Both the HyD and GaAsP offer much higher sensitivity than a standard PMT detector. Furthermore, the high sensitivity of both these detectors allows reduction of light delivered to sample, further reducing potential background signal due to scattering of the light through tissue slices.

16. A unique concern while imaging in the *C. elegans* intestine is the broad spectrum autofluorescence of lipofuscin-containing gut granules (lysosome-related organelles). This can be removed through spectral imaging and subsequent linear unmixing allowing the separation of highly overlapping fluorophores into pure signals. Autofluorescence in other tissues is minimal. For example, the emission spectrum of GFP and autofluorescence substantially overlap. To be able to calculate the fluorophore and auto-fluorescent contributions, linear unmixing requires knowledge of the reference spectra. Predefined and custom fluorescent profiles can be utilized for unmixing. Ideally, a user will collect spectral profiles of the fluorophores as well as the auto fluorescent spectra for the tissue being sampled. For accuracy, the reference spectra are taken from samples containing only the fluorophore of interest, or in the absence of the fluorophore in the case of autofluorescence. For example, control animals with no GFP expressed are scanned under lambda mode (multispectrum mode) ranging from 480 nm to 700 nm to collect the broad autofluorescent spectral profile. GFP expressing control animals, are also imaged in lambda mode to collect the GFP emission spectra from 480 nm to 700 nm. Note, it is preferable to collect the GFP spectra from a tissue with little to no autofluorescence such as oocytes or neuronal expressed GFP. The collected spectra are saved into the spectral database. Intestinal autofluorescence is particularly strong in the DAPI channel. Therefore, objects that appear in both the GFP and DAPI channels are considered autofluorescence and canceled out, while true GFP signal appears in the GFP channel but does not colocalize with the DAPI channel (Note 22).
17. Data sets are collected for 10–12 animals of each genotype or condition being tested (Note 23).
18. Quantification of images are performed with open source Fiji software [33].
19. Regions of interest (ROI) are selected in Fiji software using the “Specify” function. Square pixel boxes are set to a predefined area of 100×100 pixels. Three regions are analyzed for GFP intensity per sample using the “Measure function”. When comparing intensity, an average of six samples are quantified for a total of 18 ROIs.
20. An unpaired, two-tailed *t* test is used to compare the *p* value between the control *wild-type* animal and each genotype.

3.6.3 Colocalization of BMP receptor protein fusions with subcellular markers

1. To test the localization of GFP-tagged BMP signal transduction components, subcellular compartments are marked with another fluorophore fusion protein

²¹Note, as one increases the gain, pixels can be saturated and noise may become evident in the image.

²²An alternative approach if spectral profiling is not available, is to capture a second set of images using a standard commercial DAPI filter and overlay with the GFP image.

²³It is important when comparing samples to image under the same conditions as the control. For example, objectives, pinhole, gain, offset, and laser units should all be kept identical between samples, as well as the age of the animals.

that localizes to the distinct compartment of interest. RFP- or mCherry-tagged compartment markers are typically used to measure colocalization of the GFP-tagged receptors. For example, SMA-6::GFP normally colocalizes to the plasma membrane as well as intracellular endosomal compartments including the late endosome [6]. To label the late endosome a fusion protein of RAB-7 and RFP is expressed in the intestine and measured for colocalization with SMA-6::GFP.

2. To collect the image, place your slide with the agarose pad and sample onto the stage of the microscope, as described above. Locate and focus on an animal under low magnification (10x). To observe GFP expression in a transgenic animal, switch the fluorescence source on and switch off bright field.
3. Optimize the signal-to-noise ratio for each individual channel as described in the previous section. Typically, settings are adjusted for the control sample for optimal GFP and RFP, signal-to-noise, and also DAPI if using this channel for auto fluorescent detection (Note 22, 23).
4. After the samples have been acquired, reference spectra are selected, and channel and color are assigned for each reference spectrum. Unmixing of the images is performed, and background channels are turned off to obtain the true GFP signal.
5. Regions of interest (ROI) are selected in Fiji software using the polygon selections function. Individual cells outlined by an ROI are analyzed for GFP colocalization with RFP using the Analyze -> Colocalizations -> Colocalization thresholds function. Select the image that contains the ROI as Channel 1. Percent of GFP and RFP overlap is determined per volume area, as well as both Mander's overlap coefficient (MOC) and Pearson correlation coefficient (PCC), which are both used to quantify the degree of colocalization between fluorophores. When comparing colocalization of fluorophores, an average of 6 samples are quantified for a total of 6–12 ROIs (Note 24).
6. An unpaired, two-tailed *t* test is used to compare the *p* value between the control *wild-type* animal and each genotype.

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5. References

1. Srivastava M, Begovic E, Chapman J, Putnam NH, Hellsten U, Kawashima T, Kuo A, Mitros T, Salamov A, Carpenter ML, Signorovitch AY, Moreno MA, Kamm K, Grimwood J, Schmutz J, Shapiro H, Grigoriev IV, Buss LW, Schierwater B, Dellaporta SL, Rokhsar DS (2008) The

²⁴Deconvolution software can significantly enhance image quality. Software from Autoquant (X3) or iVision (BioVision Technologies) can be used to process image stacks. Stacks of 10 or more images taken at 0.2 mm intervals along the Z-axis are ideal for deconvolution in either program.

Trichoplax genome and the nature of placozoans. *Nature* 454 (7207):955–960. doi:10.1038/nature07191 [PubMed: 18719581]

2. Srivastava M, Simakov O, Chapman J, Fahey B, Gauthier ME, Mitros T, Richards GS, Conaco C, Dacre M, Hellsten U, Larroux C, Putnam NH, Stanke M, Adamska M, Darling A, Degnan SM, Oakley TH, Plachetzki DC, Zhai Y, Adamski M, Calcino A, Cummins SF, Goodstein DM, Harris C, Jackson DJ, Leys SP, Shu S, Woodcroft BJ, Vervoort M, Kosik KS, Manning G, Degnan BM, Rokhsar DS (2010) The *Amphimedon queenslandica* genome and the evolution of animal complexity. *Nature* 466 (7307):720–726. doi:10.1038/nature09201 [PubMed: 20686567]
3. Huminiecki L, Goldovsky L, Freilich S, Moustakas A, Ouzounis C, Heldin C-H (2009) Emergence, development and diversification of the TGF- β signalling pathway within the animal kingdom. *BMC Evol Biol* 9:28 [PubMed: 19192293]
4. Massagué J (2008) TGF β in Cancer. *Cell* 134 (2):215–230. doi:S0092–8674(08)00878–7 [pii] 10.1016/j.cell.2008.07.001 [PubMed: 18662538]
5. Foehr ML, Lindy AS, Fairbank RC, Amin NM, Xu M, Yanowitz J, Fire AZ, Liu J (2006) An antagonistic role for the *C. elegans* Schnurri homolog SMA-9 in modulating TGF β signaling during mesodermal patterning. *Development (Cambridge, England)* 133 (15):2887–2896
6. Gleason RJ, Akintobi AM, Grant BD, Padgett RW (2014) BMP signaling requires retromer-dependent recycling of the type I receptor. *Proc Natl Acad Sci U S A*
7. Krishna S, Maduzia LL, Padgett RW (1999) Specificity of TGF β signaling is conferred by distinct type I receptors and their associated SMAD proteins in *Caenorhabditis elegans*. *Development (Cambridge, England)* 126 (2):251–260
8. Liang J, Lints R, Foehr ML, Tokarz R, Yu L, Emmons SW, Liu J, Savage-Dunn C (2003) The *Caenorhabditis elegans* schnurri homolog sma-9 mediates stage- and cell type-specific responses to DBL-1 BMP-related signaling. *Development (Cambridge, England)* 130 (26):6453–6464
9. Liu Z, Shi H, Szymczak LC, Aydin T, Yun S, Consta K, Schaeffer A, Ranjan S, Kubba S, Alam E, McMahon DE, He J, Shwartz N, Tian C, Plavskin Y, Lindy A, Dad NA, Sheth S, Amin NM, Zimmerman S, Liu D, Schwarz EM, Smith H, Krause MW, Liu J (2015) Promotion of bone morphogenetic protein signaling by tetraspanins and glycosphingolipids. *PLoS Genet* 11 (5):e1005221. doi:10.1371/journal.pgen.1005221 [PubMed: 25978409]
10. Savage C, Das P, Finelli AL, Townsend SR, Sun CY, Baird SE, Padgett RW (1996) *Caenorhabditis elegans* genes sma-2, sma-3, and sma-4 define a conserved family of transforming growth factor- β pathway components. *Proc Natl Acad Sci USA* 93 (2):790–794 [PubMed: 8570636]
11. Savage-Dunn C, Maduzia LL, Zimmerman CM, Roberts AF, Cohen S, Tokarz R, Padgett RW (2003) Genetic screen for small body size mutants in *C. elegans* reveals many TGF β pathway components. *Genesis* 35 (4):239–247 [PubMed: 12717735]
12. Suzuki Y, Yandell MD, Roy PJ, Krishna S, Savage-Dunn C, Ross RM, Padgett RW, Wood WB (1999) A BMP homolog acts as a dose-dependent regulator of body size and male tail patterning in *Caenorhabditis elegans*. *Development (Cambridge, England)* 126 (2):241–250
13. Tian C, Sen D, Shi H, Foehr ML, Plavskin Y, Vatamaniuk OK, Liu J (2010) The RGM protein DRAG-1 positively regulates a BMP-like signaling pathway in *Caenorhabditis elegans*. *Development (Cambridge, England)* 137 (14):2375–2384
14. Tian C, Shi H, Xiong S, Hu F, Xiong WC, Liu J (2013) The neogenin/DCC homolog UNC-40 promotes BMP signaling via the RGM protein DRAG-1 in *C. elegans*. *Development (Cambridge, England)* 140 (19):4070–4080
15. Gumienny TL, Savage-Dunn C (2013) TGF- β signaling in *C. elegans*. *WormBook*:1–34 doi: 10.1895/wormbook.1.22.2
16. Padgett RW, Patterson GI (2008) TGF β Family Signaling in the Nematode, *C. elegans*. The TGF β Family Cold Spring Harbor Press,
17. Patterson GI, Padgett RW (2000) TGF β -related pathways. Roles in *Caenorhabditis elegans* development. *Trends in Genetics* 16 (1):27–33 [PubMed: 10637628]
18. Lawler S, Feng XH, Chen RH, Maruoka EM, Turck CW, Griswold-Prenner I, Derynck R (1997) The type II transforming growth factor- β receptor autophosphorylates not only on serine and threonine but also on tyrosine residues. *J Biol Chem* 272 (23):14850–14859 [PubMed: 9169454]

19. Shi Y, Massagué J (2003) Mechanisms of TGF- β signaling from cell membrane to the nucleus. *Cell* 113 (6):685–700 [PubMed: 12809600]
20. Luo S, Kleemann GA, Ashraf JM, Shaw WM, Murphy CT (2010) TGF- β and insulin signaling regulate reproductive aging via oocyte and germline quality maintenance. *Cell* 143 (2):299–312 [PubMed: 20946987]
21. Luo S, Shaw WM, Ashraf J, Murphy CT (2009) TGF- β Sma/Mab signaling mutations uncouple reproductive aging from somatic aging. *PLoS Genet* 5 (12):e1000789 [PubMed: 20041217]
22. Mallo GV, Kurz CL, Couillault C, Pujol N, Granjeaud S, Kohara Y, Ewbank JJ (2002) Inducible antibacterial defense system in *C. elegans*. *Current Biology* 12 (14):1209–1214 [PubMed: 12176330]
23. Nicholas HR, Hodgkin J (2004) Responses to infection and possible recognition strategies in the innate immune system of *Caenorhabditis elegans*. *Mol Immunol* 41 (5):479–493 [PubMed: 15183927]
24. Roberts AF, Gumienny TL, Gleason RJ, Wang H, Padgett RW (2010) Regulation of genes affecting body size and innate immunity by the DBL-1/BMP-like pathway in *Caenorhabditis elegans*. *BMC Dev Biol* 10:61 [PubMed: 20529267]
25. Sinha A, Rae R, Iatsenko I, Sommer RJ (2012) System wide analysis of the evolution of innate immunity in the nematode model species *Caenorhabditis elegans* and *Pristionchus pacificus*. *PLoS ONE* 7 (9):e44255 [PubMed: 23028509]
26. Tan MW, Rahme LG, Sternberg JA, Tompkins RG, Ausubel FM (1999) *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. *Proc Natl Acad Sci USA* 96 (5):2408–2413 [PubMed: 10051655]
27. Fernando T, Flibotte S, Xiong S, Yin J, Yzeiraj E, Moerman DG, Meléndez A, Savage-Dunn C (2011) *C. elegans* ADAMTS ADT-2 regulates body size by modulating TGF β signaling and cuticle collagen organization. *Dev Biol* 352 (1):92–103 [PubMed: 21256840]
28. Gumienny TL, Macneil L, Zimmerman CM, Wang H, Chin L, Wrana JL, Padgett RW (2010) *Caenorhabditis elegans* SMA-10/LRIG is a conserved transmembrane protein that enhances bone morphogenetic protein signaling. *PLoS Genet* 6 (5):e1000963. doi:10.1371/journal.pgen.1000963 [PubMed: 20502686]
29. Gumienny TL, Macneil LT, Wang H, de Bono M, Wrana JL, Padgett RW (2007) Glypican LON-2 is a conserved negative regulator of BMP-like signaling in *Caenorhabditis elegans*. *Curr Biol* 17 (2):159–164 [PubMed: 17240342]
30. Gleason RJ, Vora M, Li Y, Kane NS, Liao K, Padgett RW (2017) *C. elegans* SMA-10 regulates BMP receptor trafficking. *PLoS One* 12 (7):e0180681. doi:10.1371/journal.pone.0180681 [PubMed: 28704415]
31. Sulston JE, Horvitz HR (1977) Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev Biol* 56 (1):110–156 [PubMed: 838129]
32. Stiernagle T (2006) Maintenance of *C. elegans*. *WormBook*:1–11 doi:10.1895/wormbook.1.101.1
33. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9 (7):676–682. doi:10.1038/nmeth.2019 [PubMed: 22743772]
34. Fares H, Greenwald I (2001) Genetic analysis of endocytosis in *Caenorhabditis elegans*: coelomocyte uptake defective mutants. *Genetics* 159 (1):133–145 [PubMed: 11560892]
35. Fares H, Greenwald I (2001) Regulation of endocytosis by CUP-5, the *Caenorhabditis elegans* mucolipin-1 homolog. *Nat Genet* 28 (1):64–68. doi:10.1038/88281 [PubMed: 11326278]
36. Brenner S (1974) The genetics of *Caenorhabditis elegans*. *Genetics* 77 (1):71–94 [PubMed: 4366476]
37. Maduzia LL, Roberts AF, Wang H, Lin X, Chin LJ, Zimmerman CM, Cohen S, Feng XH, Padgett RW (2005) *C. elegans* serine-threonine kinase KIN-29 modulates TGF β signaling and regulates body size formation. *BMC Dev Biol* 5 (1):8 [PubMed: 15840165]
38. Doitsidou M, Poole RJ, Sarin S, Bigelow H, Hobert O (2010) *C. elegans* mutant identification with a one-step whole-genome-sequencing and SNP mapping strategy. *PLoS One* 5 (11):e15435. doi: 10.1371/journal.pone.0015435 [PubMed: 21079745]

39. Savage-Dunn C, Tokarz H, Wang H, Cohen S, Giannikas C, Padgett RW (2000) SMA-3 Smad has specific and critical functions in DBL-1/SMA-6 TGF β -related signaling. *Dev Biol* 223 (1):70–76 [PubMed: 10864461]
40. Wang J, Tokarz R, Savage-Dunn C (2002) The expression of TGF β signal transducers in the hypodermis regulates body size in *C. elegans*. *Development* 129 (21):4989–4998 [PubMed: 12397107]
41. Ermolaeva MA, Schumacher B (2014) Insights from the worm: The *C. elegans* model for innate immunity. *Semin Immunol* 26(4):303–309 [PubMed: 24856329]
42. Malone EA, Thomas JH (1994) A screen for nonconditional dauer-constitutive mutations in *Caenorhabditis elegans*. *Genetics* 136 (3):879–886 [PubMed: 8005442]
43. Thomas JH, Birnby DA, Vowels JJ (1993) Evidence for parallel processing of sensory information controlling dauer formation in *Caenorhabditis elegans*. *Genetics* 134 (4):1105–1117 [PubMed: 8375650]
44. Mahajan-Miklos S, Tan MW, Rahme LG, Ausubel FM (1999) Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa*-*Caenorhabditis elegans* pathogenesis model. *Cell* 96 (1):47–56 [PubMed: 9989496]
45. Shi A, Sun L, Banerjee R, Tobin M, Zhang Y, Grant BD (2009) Regulation of endosomal clathrin and retromer-mediated endosome to Golgi retrograde transport by the J-domain protein RME-8. *EMBO J* 28 (21):3290–3302. doi:10.1038/emboj.2009.272 [PubMed: 19763082]
46. Eisenmann DM, Kim SK (2000) Protruding vulva mutants identify novel loci and Wnt signaling factors that function during *Caenorhabditis elegans* vulva development. *Genetics* 156 (3):1097–1116 [PubMed: 11063687]
47. Mok DZ, Sternberg PW, Inoue T (2015) Morphologically defined sub-stages of *C. elegans* vulval development in the fourth larval stage. *BMC Dev Biol* 15:26. doi:10.1186/s12861-015-0076-7 [PubMed: 26066484]
48. Kim E, Sun L, Gabel CV, Fang-Yen C (2013) Long-term imaging of *Caenorhabditis elegans* using nanoparticle-mediated immobilization. *PLoS One* 8 (1):e53419. doi:10.1371/journal.pone.0053419 [PubMed: 23301069]
49. Patterson GI, Kowalik A, Wong A, Liu Y, Ruvkun G (1997) The DAF-3 Smad protein antagonizes TGF- β -related receptor signaling in the *Caenorhabditis elegans* dauer pathway. *Genes Dev* 11 (20):2679–2690 [PubMed: 9334330]
50. Massagué J, Blain SW, Lo RS (2000) TGF β signaling in growth control, cancer, and heritable disorders. *Cell* 103 (2):295–309 [PubMed: 11057902]

Figure 1. The *C. elegans* BMP pathway.

The ligand (DBL-1) binds to the type I and type II receptors (SMA-6 and DAF-4, respectively), stimulating phosphorylation events on the receptors and on the R-Smads. The two R-Smads of this pathway, SMA-2 and SMA-3, associate with SMA-4, the co-Smad, and translocate to the nucleus. One characterized transcription factor, SMA-9 (SCHNURRI), aids in transcription of downstream target genes. The putative I-Smad, TAG-68, is the only I-Smad homolog in *C. elegans*. It is hypothesized to function similarly to the mammalian I-Smads (Smad6 and Smad7) and would therefore be a target of the pathway and attenuate signaling by interacting with the receptor and/or R-Smads [50].

Figure 2. *C. elegans* body size mutants.

Mutations in the *C. elegans* BMP pathway components produce small animals (top panel), while an increase in signaling produces longer animals (bottom panel). Wild-type animals are shown in the middle panel.

Figure 3. The coelomocyte (CC) phenotypes of wild-type and *sma-9* mutant worms.

(A) Diagrams depicting the CC phenotypes of wild-type, *sma-9*, and *sma-9; susm* animals in the LW0040 strain which bears *arls37* that encodes CC localized GFP. (B-C) Merged DIC and GFP images of a wild-type worm (B) and a *sma-9(cc604)* worm (C). * points to the four CCs born during embryogenesis. The two postembryonic M-derived CCs are missing in *sma-9* mutants.

Figure 4. Double mutant analysis flowchart.

Analysis of double mutant phenotypes provides genetic evidence that two gene products, X and Y, act together in a linear pathway or independently in parallel pathways. (A) Genes with similar mutant phenotypes. (B) Genes with opposite mutant phenotypes.

Figure 5. The RAD-SMAD reporter used to measure the level of Sma/Mab signaling in various mutants.

(A) A schematic of the RAD-SMAD reporter construct. SBE stands for Smad binding element. (B-D) Representative GFP fluorescence images showing the expression of the reporter in *lon-2(e678)* (B), wild-type (C), and *dbl-1(wk70)* (C) animals, respectively.

Figure 6. Schematic depiction of the *C. elegans* intestine to demonstrate focal planes captured to study tagged BMP receptor subcellular localization.

An example of DAF-4::GFP localization is depicted in both focal planes. White arrowheads indicate lateral membrane, and yellow arrowheads indicate apical lumen of the intestine.

Figure is modified from Gleason *et al*, 2014 [6].