Identification and characterization of protein phosphatases regulating the Sma/Mab pathway in C. elegans

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Identification and characterization of Protein phosphatases modulating Sma/Mab signaling pathway in *C. elegans*

By

Sheng Xiong

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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THE CITY UNIVERSITY OF NEW YORK
Sma/Mab signaling pathway in C. elegans

by

Sheng Xiong

Advisor: Professor Cathy Savage-Dunn

TGFβ signaling is a conserved signaling pathway among eukaryotes, which controls various normal cellular responses from cell proliferation to cell death. The mutations in its components are found in developmental disorders and cancer. Therefore, this signaling pathway is extensively investigated so that new therapeutic targets could be discovered and novel drugs could be developed. Previous studies suggested the involvement of phosphatases in regulation of TGFβ signaling, but these studies were performed in cell culture rather than intact organisms. C. elegans is a tractable organism in which to study signaling in vivo. In C. elegans, growth is controlled by a conserved TGFβ pathway, the Sma/Mab pathway.

We used a C. elegans RNA interference library of phosphatases to identify genes that cause a body size phenotype. Library-wide screening was carried out in an RNAi-hypersensitive mutant background, rrf-3. To further narrow the candidate pool, we analyzed the body size phenotypes of these candidates using different genetic backgrounds. These analyses allow us to narrow the candidate pool down to 80-candidates.

A Sma/Mab pathway reporter, RAD-SMAD reporter (a kind gift from Dr. Jun Liu, Cornell University), was used to assess whether the candidates regulate body size phenotype in a Sma/Mab pathway-dependent manner or not. The reporter assay revealed 22 likely candidates regulate Sma/Mab signaling directly or indirectly. Among them, 11 candidates were verified as protein phosphatases by sequencing. These include homologues of mammalian PPM1A/B/G and PP1.
Our study revealed that *ppm-1* (metal-ion dependent protein phosphatase-1), a human PPM1A/B/G homolog, might act as a potential SMA-3-specific linker phosphatase to regulate the Sma/Mab pathway. This is the first time to show that a homolog of human PPM1A/B/G might act as a potential linker phosphatase of R-Smads to promote TGFβ signaling. Our studies also showed that a homolog of the inhibitory regulatory subunit of human PP1 might synergize with the catalytic subunit in the regulation of TGFβ signaling for the first time. We also discovered the regulatory role of several protein tyrosine phosphatases in this signaling cascade. In summary, this study sheds a light on elucidating the regulatory mechanism of TGFβ signaling pathway, therefore providing insight in various TGFβ signaling-involved human developmental disorders and cancer, and contributing to the development of potential diagnostic markers and therapeutic targets in human diseases.
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Chapter I: Introduction

I.1 C. elegans: a classic genetic model organism

In 1965, Sydney Brenner first introduced Caenorhabditis elegans as a model system to study animal genetics, development and behavior. And it rapidly turned to be a popular tool for scientists to study almost all fundamental biological processes, including embryonic and post-embryonic development, neurobiology, apoptosis, signal transduction, and evolution as well. Meanwhile, it was also used to build disease models to uncover underlying mechanisms, seeking for potential clinical therapy and drug discovery.

Compared to other well developed model organisms, such as Drosophila, zebrafish and mice, C. elegans offers a lot of advantages for genetic studies, especially for understanding developmental processes. It has a rapid life cycle, transparent body and is easy for laboratory cultivation. In addition, its relatively small and fully sequenced genome, well defined cell lineage and easy transformation add credits to its popularity for various study purposes.

I.1.1. Rapid life cycle.

C. elegans has a rapid life cycle: around 3 days for each generation at 20°C and a relatively large number of progeny: each single hermaphrodite can give rise to 300-350 progeny (Wormbook.org). Normal C. elegans has six developmental stages: eggs, L1, L2, L3, L4, and adult (Figure 1.1). At 22°C, embryogenesis requires approximately 14 hours and the post-embryonic development from first larval stage (L1) stage to adult needs 36 hours. In each developmental stage, a stage-specific cuticle is secreted by the hypodermis. As a result, molting the old cuticle and forming a new one is the indication of stage change (Page and Johnstone, 2007).
Under unfavorable living conditions, for example, lack of food, high temperature and overcrowded growth, worms in L2 stage would go into a non-developmental stage, the dauer stage, instead of the L3 stage. Worms that enter the dauer stage change their energy metabolism, accumulating fat and altering their behavior strikingly. In addition, a specific cuticle replaces the one for L2 (Hu, 2007). Dauer larvae can survive for months, approximately ten times their normal life span. When the environment changes back to favorable conditions, they resume development into the L4 stage, bypassing L3 stage.

*C. elegans* has a transparent body throughout development. This is a great advantage for us to examine cells *in vivo* and mutant phenotypes. In addition, the cell number and their position
are consistent among individuals. Adult worms have less than 1,000 cells (excluding germ cells), including 302 neurons in hermaphrodites or 381 neurons in males. The entire cell lineage, from egg to adult, is essentially the same in each animal and is known precisely (Sulston et al. 1977, 1983). This benefits studies on cell fate determination in embryonic and post-embryonic development.

The hermaphrodites and rare males are another key characteristic for *C. elegans*. As a result, self fertilization and cross fertilization coexist in *C. elegans*. The hermaphrodites are protandrous, first producing sperm in late L4 stage, storing them in its spermathecae, and then turning to produce oocytes. Rare males can mate with hermaphrodites, stimulating the production of oocytes by hermaphrodites. Thereby, homozygotic and heterozygotic animals can be easily obtained from mutagenesis or cross. Meanwhile, easy transformation of *C. elegans* by microinjection makes them suitable for genetic analysis. And one can knockdown specific genes using dsRNA through both microinjection and feeding, which facilitates the elucidation of interested target gene function.

Furthermore, *C. elegans* has a small genome, about 97 million base pairs. Approximately, 19,099 genes (The *C. elegans* Sequencing Consortium 1998) are predicted and only 6% of them have been studied genetically or biochemically. There are still a large number of genes whose functions are unknown. This provides a challenging field for researchers to explore.

### I.1.2. Anatomy of *C. elegans*

Basically, like other nematodes, the body of *C. elegans* is made up of two concentric tubes separated by a fluid-filled space, the pseudocoelom. The animal’s shape is maintained by internal hydrostatic pressure. The outer tube contains the nervous system, gonad, coelomeytes,
and excretory/secretory system and is covered by the collagenous, extracellular cuticle, which is secreted by the underlying hypodermis. The inner tube is composed of the muscular pharynx with its nearly autonomous nervous system, intestine, rectum and anus (Figure 1.2).

![Photomicrographs showing major anatomical features of the C. elegans adult hermaphrodite (top) and male (bottom). Shown are lateral views under bright field illumination (Sulston & Horvitz, 1977).](image)

The copulatory bursa in the male tail includes two spicules, a fan, and 18 sensory rays. The fan is an extended acellular structure of cuticle matrix. The sensory rays are distinguished into two identical groups that are one-axis symmetrical along the ventral midline. Each group contains nine different rays numbered 1 through 9 from anterior to posterior (Nguyen et al., 1999). Each ray contains two neurons, RNA and RNB that express different neurotransmitters, and one support cell surrounded by a hypodermal sheath. For example, dopamine is expressed only in the ray5A, ray 7A, and ray 9A neurons (Lints & Emmons, 1999). Rays 1, 5, and 7
localize at the dorsal side of the fan; rays 3, 6, and 9 at the edge; rays 2, 4, and 8 at the ventral side. The Sma/Mab mutants have defects in the male tail, which will be discussed in detail later.

I.2 Transforming growth factor-β (TGF-β) signaling pathway

The transforming growth factor β (TGF-β) superfamily comprises structurally related polypeptide growth factors, including TGF-βs, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), Activins and Nodal (Feng and Derynck, 2005). Each member is capable of regulating a large number of biological processes including cell proliferation, lineage determination, differentiation, motility, adhesion, and death. These TGF-β superfamily ligands, as well as their downstream pathway components, are well conserved during evolution and the signal transduction pathway is relatively simple and linear. Active TGF-β ligand dimer brings together two pairs of transmembrane receptor serine/threonine kinases known as type II and type I receptors. Upon binding TGF-βs, type II receptor phosphorylates and activates type I receptor. The intracellular signal transducers (R-Smads) are then phosphorylated by type I receptor, and in turn, form complexes with Co-Smad. Those Smad complexes accumulate in the nucleus, with the help of other transcriptional cofactors, modulating transcription of target genes (Fig. 1.3). Basically, TGF-β and activins signal their transcription responses through Smad2 and Smad3, whereas BMPs signal through Smad1, Smad5 and Smad8 (Feng and Derynck. 2005).
Figure 1.3 Transforming growth factor-β (TGFβ) signaling pathway (Feng and Derynck, 2005)
I.2.1. TGF-β ligands

Based on structural and functional relatedness, the large TGF-β superfamily is divided into two subfamilies: the TGF-β isoforms, Nodal, and Activins family, and the BMPs and GDFs family. The TGF-β superfamily comprises more than 30 genes in mammals, 7 genes in Drosophila and 5 genes in C. elegans. The ligands are expressed and secreted into the extracellular matrix (ECM) as dimeric pre-proproteins. Dimerization and stabilization require the pro-domain (Gray and Mason, 1990). The mature ligands are cleaved from the prodomain by furin-like convertase (Dubois et al., 1995). Most frequently, ligand dimerizes with itself. However, heterodimerization is also observed, for example in mammals between Nodal and BMP4 or BMP7 (Yeo and Whitman, 2001), and in Drosophila, between Dpp and Scw (Shimmi et al., 2005). A major regulatory step in TGF-β signaling is the regulation of ligand accessibility by extracellular ligand binding proteins.

I.2.2. TGF-β receptors

Compared to the large number of TGF-β ligands, there are only five type II receptors (TβRII) and seven type I receptors (TβRI) identified in humans. They are transmembrane serine/threonine kinases, which contain a short extracellular region (Wells et al., 1997), a transmembrane domain and a cytoplasmic serine/threonine kinase domain. TGF-β binding stabilizes the interaction of the TβRII dimer with two TβRI molecules, forming heterotetrameric, active receptor complexes (Feng and Derynck, 2005). The assembly of the active receptor complexes is different for different ligands. In the case of TGF-β receptors, ligand-bound high affinity type II receptor will recruit type I receptor and this process is facilitated by direct interaction between type II and type I receptor. However, the BMP receptors do not interact
directly. They are linked via the ligands (Groppe et al., 2008).

Considering the relatively small number of TGF-β receptors, it is becoming apparent that different ligand-receptor interactions and type II and type I receptor pairings exist (Feng and Derynck, 2005), which can possibly account in part for the huge diversity of the TGF-β signaling outputs. Indeed, in endothelial cells, TGF-β can activate both ALK1 and ALK5, and a complex has been proposed comprising these two distinct type I receptors (Goumans et al., 2003). Recently, mixed receptor complexes containing TβRII, ALK5, and either ALK2 or ALK3 have been reported to mediate a novel branch of TGF-β signaling in epithelial cells (Daly et al., 2008). In *Drosophila*, a receptor complex comprising the type I receptor Thickveins (Tkv) and Saxophone (Sax) and the type II receptor Punt have also been observed (O’Connor et al., 2006).

Upon ligand binding, the cytoplasmic kinase domain of type II receptor will phosphorylate the GS domain on type I receptor, which in turn activates type I receptor kinase activity. Then the activated type I receptor will phosphorylate Smad proteins and transduce the signal downstream. The nine-amino acid L45 loop in the kinase domain of type I receptor is the critical motif that contributes to the receptor-Smad protein interaction. Huse et al. showed from the crystal structure of type I receptor that the L45 loop is accessible for protein interactions (Huse et al., 2001). Meanwhile, receptors with different signaling specificity have different L45 sequences (Chen et al., 1998; Feng & Derynck, 1997).

**I.2.3. Smad proteins**

Smad family proteins are the well established intracellular effectors in TGF-β signaling. The founding member of the Smad family is the product of the *Drosophila* gene Mad (*mothers against dpp*) (Sekelsky et al., 1995), followed by three Mad homologues identified in *C. elegans,*
called *sma-2, sma-3*, and *sma-4* (Savage et al., 1996, Derynck et al., 1998). Later on, many homologues were discovered in vertebrates and named Smad (merge of MAD and SMA). Smads exist in three subgroups: (1) receptor-regulated Smads (R-Smads), e.g. Smad1, 2, 3, 5, and 8 in vertebrates, Mad in *Drosophila*, SMA-2 and -3, and DAF-8 and -14 in *C. elegans*; (2), common Smads (Co-Smads) e.g. Smad4 in vertebrates, Medea in *Drosophila*, SMA-4, and DAF-3 in *C. elegans*; (3) inhibitory Smads (I-Smads), e.g. Smad6 and 7 in vertebrates and Dad in *Drosophila*.

R-Smads and Co-Smads contains highly conserved MH1 (N-terminal) and MH2 (C-terminal) domains linked by a more divergent linker region. The MH1 domain has approximately 130 amino acids and contributes to the DNA-binding activity. R-Smads can directly interact with type I receptor through the MH2 domain and have a C-terminal SXS motif in which two serines are direct targets for phosphorylation by type I receptor. Phosphorylation of SXS motif in R-Smads leads to a conformational change, resulting in dissociation of R-Smad from type I receptor and formation of a trimeric Smad complex consisting of two R-Smads and a Co-Smad. This trimeric Smad complex then accumulates in the nucleus and regulates gene expression, positively or negatively, usually in association with other transcriptional cofactors (Moustakas & Heldin, 2009; Feng & Derynck, 2005; Derynck & Zhang, 2003; Shi & Massague, 2003).

Since R-Smads localize in the cytoplasm, efficient R-Smad recruitment and activation in response to TGF-β and activin requires other accessory proteins. SARA (Smad Anchor for Receptor Activation), an FYVE domain-containing, plasma membrane localized and EEA-1 positive endosome enriched protein, can interact with type I receptor and Smad2/3 and recruits Smad2 to the receptor for phosphorylation (Wu et al., 2000). Thus, complex formation of the receptors with Smads and SARA at EEA-1 positive endosome can promote TGF-β signaling (Di Guglielmo et al., 2003). Hgs, another FYVE domain protein involved in endosomal trafficking,
may play a role similar to that of SARA, since it also interacts with Smad2 and Smad3 and enhances ligand-induced Smad phosphorylation and gene expression (Miura et al., 2000).

Inhibitory Smads (I-Smads), like Smad6 and Smad7 which are induced by TGF-β family ligands, inhibit TGF-β signaling through various mechanisms (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997). For example, Smad7 is shown to form a stable complex with type I receptors, therefore leading to inhibition of R-Smad phosphorylation and the hetero-complex formation between R-Smads and Co-Smad (Hayashi et al. 1997). Smad7 can also recruit the HECT type of E3 ubiquitin ligases, Smurf1 and Smurf2 to the activated type I receptor ALK5/TbRI, leading to the degradation of the receptor through the proteasomal pathway (Ebisawa et al., 2001; Kavsak et al., 2000).

I.3 Reversible phosphorylation: an essential regulatory mechanism among species

Reversible phosphorylation of proteins is a major mechanism regulating multiple normal biological processes such as metabolism, gene transcription, cell cycle and abnormal biological processes such as carcinogenesis and angiogenesis. It is extremely common in signal transduction, and it is considered the main posttranslational modification mechanism leading to a change in enzyme activity. Phosphorylation state of a certain protein results from a balance of protein kinase and protein phosphatase activity. Alteration of the phosphorylation state of proteins is often a cause of various diseases, such as cancer, diabetes, rheumatoid arthritis, or hypertension. The importance of this regulatory mechanism is evident considering that the number of genes encoding phosphatases and kinases represents 2 to 4% all genes in a typical eukaryotic genome (Manning et al., 2002).

In eukaryotic cells, phosphorylation mainly occurs on three hydroxyl-containing amino
acids, serine, threonine, and tyrosine, of which serine is the predominant target. Proteomic study of 6,600 phosphorylation sites on 2,244 human proteins revealed that phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr) account for 86.4%, 11.8%, and 1.8%, respectively, of the phosphorylated amino acids (Olson et al., 2006). The fully sequenced human genome is thought to contain 518 putative protein kinases (Johnson and Hunter, 2005; Lander et al., 2001; Venter et al., 2001) that can be classified into two families: 90 tyrosine (Tyr) kinases (PTKs) and 428 serine/threonine (Ser/Thr) kinases (PSKs). The exquise specificity of signaling and reversible phosphorylation seems to suggest there would be similar number of corresponding protein phosphatases in the human genome. Surprisingly, there are 107 putative protein Tyr phosphatases (PTPs) (Alson et al., 2004) and far fewer protein Ser/Thr phosphatases (PSPs) (~30). Whereas the PTKs and PTPs are roughly matching each other, the number of catalytic PSPs is an order of magnitude lower than that of PSKs. This dichotomy can be explained by the combinatorial formation of PSP holoenzymes from a shared catalytic subunit and a large number of regulatory subunits (Shi, 2009).

As mentioned before, most phosphorylation events in eukaryotes involve transfer of phosphate to serine (Ser) and threonine (Thr) residues. Removal of this phosphate is catalyzed by Ser/Thr protein phosphatases. Members of the large protein phosphatase family were initially classified, using enzymological criteria, as type 1 (PP1) and type 2 (PP2) phosphatases. PP2 enzymes were subsequently divided into three groups on the basis of the requirements for metal ions: 2A (not requiring metal ions), 2B (activated by calcium), and 2C (Mg$^{2+}$/Mn$^{2+}$-dependent) (Shi, 2009). The molecular cloning of a number of cDNAs and genes from diverse organisms allowed the establishment of three major subfamilies: PPP (phosphoprotein phosphatases), including type 1, 2A, and 2B phosphatases; PPM (metal-dependent protein phosphatases),
including type 2C enzymes (PP2C); and the catalytically aspartate (Asp)-based subfamily, represented by HAD and FCP/SCP (TFIIF-associating component of RNA polymerase II CTD phosphatase/small CTD phosphatase)(Shi, 2009, Figure 1.4).

For several members of the PPP family, the catalytic subunit associates with a great variety of regulatory subunits. Representative members include PP1, PP2A/PP2, PP2B/PP3 (commonly known as calneurin), PP4, PP5, PP6 and PP7 (Figure 1.4). The PPM family includes protein phosphatases dependent on manganese/magnesium ions (Mn$^{2+}$/Mg$^{2+}$), such as PP2C and pyruvate dehydrogenase phosphatase. In contrast to PPP, members of the PPM family do not have regulatory subunits but contain instead additional domains and conserved sequence motifs that may help determine substrate specificity. For both PPP and PPM, metal ions play a catalytic and central role through the activation of a water molecule for the dephosphorylation reaction. In contrast, FCP/SCP uses an aspartate-based catalysis mechanism. The only known substrate of FCP/SCP for a long time is the C-terminal domain (CTD) of RNA polymerase II, which contains tandem repeats of a serine-rich heptapeptide. The conserved structural domain of FCP/SCP is the FCP homology (FCPH) domain. FCPs, But not SCPs, contain a BRCT (BRCA1 C-terminal domain like) domain that is C-terminal to the FCPH domain.
I.3.1 The PPP family of protein phosphatases

Representative members include PP1, PP2A/PP2, PP2B/PP3 (commonly known as calcineurin), PP4, PP5, PP6 and PP7. As mentioned before, most members in the PPP family function as a multiple-subunit holoenzymes regulating diverse biological processes.

Among them, PP1 is a major protein Ser/Thr phosphatase ubiquitously expressed in all eukaryotic cells. Each functional PP1 enzyme consists of a catalytic subunit and a regulatory subunit. The catalytic subunit is highly conserved among all eukaryotes with approximately 70% or greater protein sequence identity in any pair-rise alignment. These identical sequences support...
a conserved fold and a similarly positioned active site for all PPP family members. The catalytic subunit of PP1 adopts a compact \( \alpha/\beta \) fold, with a \( \beta \) sandwich wedged between two \( \alpha \)-helical domains (Egloff et al., 1995; Goldberg et al., 1995). Coordination of the critical metal ions, \( \text{Mn}^{2+} \) and \( \text{Fe}^{2+} \), located in the active site, is provided by three histidines, two aspartic acids, and one asparagine. These residues are highly conserved in all members of the PPP family, suggesting a common mechanism of metal-catalyzed reaction in the protein family.

At least 100 putative PP1-binding regulatory subunits (R subunits) have been identified, with many more expected to be found (Cohen, 2004; Moorhead et al., 2007). These R subunits may target the PP1 catalytic subunit to specific subcellular compartment, modulate substrate specificity, or serve as substrates themselves. Thus the interaction between the catalytic subunit and specific R subunits are central to the function of PP1.

The phosphatase activity of PP1 is regulated by a number of endogenous inhibitory proteins such as inhibitor-1 (I-1) (Nimmo and Cohen, 1978), inhibitor-2 (I-2) (Foulkes and Cohen, 1980), CPI-17 (Eto et al., 1997), and DARPP-32 (Walaas and Greengard, 1991). Despite the sequence conservation, PP2A/PP2 and PP2B/PP3 are not sensitive to inhibition by I-1 or I-2, which forms the basis for classification of type 1 (PP1) versus type 2/3 phosphatases (Ingebritsen and Cohen, 1983).

PP2A/PP2, one of the most abundant enzymes, accounts for up to 1% of the total cellular protein in some tissues. Cellular PP2A exist in two general forms, a heterodimeric core enzyme and a heterotrimeric holoenzyme. The PP2A core enzyme consists of a scaffold subunit (also known as the A or PR65 subunit) and a catalytic subunit (C subunit). Both the A subunit and C subunit have two isoforms, \( \alpha \) and \( \beta \), with the \( \alpha \) isoform being about 10 times abundant than the \( \beta \) isoform (Shi, 2009). The core enzyme interacts with a variable regulatory subunit to assemble
the holoenzyme. The regulatory subunit comprises of four families: B (also known as B55 or PR55), B’ (B56 or PR61), B” (PR48/PR72/PR130), and B’’’ (PR93/PR110). Each family contains two to five isoforms that are encoded by different genes; some isoforms have multiple splice variants. Diverse regulatory subunits are believed to confer exquisite substrate specificity as well as spatially and temporally determined functions to the PP2A holoenzyme.

PP3, (also known as PP2B or calcineurin) is involved in numerous calcium-dependent biological processes. It consists of a catalytic subunit (calcineurin A or CNA) and a regulatory subunit (calcineurin B or CNB). CNA contains an N-terminal phosphatase domain, followed by a CNB-binding helical domain, an autoinhibitory element, and a calcium (Ca\(^{2+}\))-calmodulin-binding domain. To date, three catalytic subunits (α, β, and γ) and two regulatory subunits (B1 and B2) have been identified (Wang et al, 2008). Calcineurin is inactive and only gains phosphatase activity upon association with Ca\(^{2+}\)-calmodulin. Calcineurin recognizes substrate proteins through a consensus recognition motif of PxIxIT (P, proline) (Bultynck et al., 2006; Czirjak and Enyedi, 2006). Sequence variation within the PxIxIT motif results in a wide range of binding affinities-between 0.5 and 250 µM-to calcineurin (Li et al., 2007).

Whereas, most PPP family members have isoforms encoded by different genes, protein phosphatase 5 (PP5) is encoded by a single gene throughout Eukaryota. Another unique feature of PP5 is that its regulatory and catalytic domains are all contained within the same polypeptide. PP5 contains regulatory domain at its N terminus-the tetratricopeptide repeat (TPR), a known protein-protein interaction motif (Shi, 2009). Similar to calcineurin (PP3 or PP2B), the phosphatase activity of free PP5 is suppressed because the TPR domain and a C-terminal helix αJ together maintain PP5 in an autoinhibitory conformation (Shi, 2009). The ability of the TPR
domain to undergo a ligand-induced conformational change allows PP5 to respond to a number of cellular factors and thus it may be crucial to PP5 function.

The structural information of other PSPs in the PPP family is not that clearly elucidated to date as the four predominant members I have just discussed. However, some of them do share considerable sequence similarity with those structurally characterized PSPs. For example, the catalytic subunit of PP4 and PP6 are closely related to the catalytic subunit of PP2A/PP2. Both PP4 and PP6 are believed to play an important role in diverse biological processes independent of PP2A. The catalytic subunit of PP4 associates with its own regulatory subunits R1 and R2 to form distinct complexes (Cohen et al., 2005) whereas the catalytic subunit of PP6 is thought to form a heterotrimeric complex with a Sit4-associated protein (SAP) domain-containing scaffold subunit and an ankyrin repeat subunit that likely serve as a regulatory subunit (Stefansson et al., 2008).

In contrast to PP4 and PP6, PP7 appears to be unique to plants. Unlike other members in PPP family, PP7 has three insertions in its phosphatase domain, and the recombinant PP7 only gains its phosphatase activity by cleavage of its longest insertion, suggesting an autoinhibitory role (Kutuzov et al., 1998).

### I.3.2 The PPM family of protein phosphatases

Although the PPM family members (include PP2C and pyruvate dehydrogenase phosphatase) are distantly related in primary sequence to PPP enzymes, their three-dimensional structure and catalytic mechanisms appear to be quite similar. In sharp contrast with PPP enzymes, in which the catalytic peptide interacts with a large number of distinct regulatory subunits to carry out its specific function, PPMs are normally monomeric enzymes. And PP2C is
insensitive to inhibition by okadaic acid (OA) or microcystin-LR (MCLR) whereas OA inhibit primarily the catalytic subunit of PP2A at an inhibitory constant of approximately 0.1 nM, which is about 100 times potent than its inhibitory constant on PP1 (MacKintosh et al., 1990).

The myriad of functions performed by PPMs is possibly the result of the expression of a large number of catalytic isoforms. For instance, at least 16 PP2C genes are found in humans and probably more than 22 different polypeptides are produced through alternative splicing. The primary function of PP2Cs appears to be the regulation of stress response, although it also plays a role in cell differentiation, growth, survival, apoptosis, and metabolism (Lu and Wang, 2008). Some PP2C members, such as PP2Cα, PP2Cβ, and PH domain leucine-rich repeat protein phosphatase (PHLPP), are candidate tumor suppressor proteins, whereas others, such as PP2Cδ (also known as Wip1), may contribute to oncogenic transformation (Shi, 2009).

The conserved catalytic core domain of human PP2C contains a central β sandwich, with each β sheet flanked by a pair of α helices (Das et al., 1996). This arrangement generates a cleft between the two β sheets, with the two metal ions located at the base of the cleft. Each metal ion is hexa-coordinated by amino acid and water molecules. Three additional α helices, unique to PP2C, associated with the core domain on one side and may contribute to substrate specificity or regulation.

As mentioned above, unlike PPP family, PP2C has many different isoforms encoded by different genes. These isoforms have distinct sequences and domain organizations, which also exhibit distinct functions, expression patterns, and subcellular localization. How these isoforms are regulated during signaling remains largely unknown. In addition to the conserved PP2C phosphatase domain, PHLPP also contains an N-terminal PH domain and a leucine-rich repea
(LRR) domain. How those additional domains may contribute to the phosphatase activity and substrate specificity also remains unclear.

I.1 The FCP/SCP family and Chronophin

In contrast to all other protein Ser/Thr phosphatases discussed herein, members of FCP/SCP family rely on the aspartic acid of the sequence motif DxDxT/V for phosphatase activity. Another unusual feature of this family is that for a long time, there is only one primary known substrate—the CTD of RNA polymerase II, which contains tandem repeat of the sequence YSPTSPS.

The catalytic mechanism of Fcp1/Scp1 may involve two sequential steps (Ghosh et al., 2008; Kamenski et al., 2004). First, an oxygen atom from the carboxyl group from the N-terminal aspartate in the DxDxT motif initiates a nucleophilic attack on the phosphorous atom of a pSer, forming an acylphosphate intermediate. Second, a water nucleophile, likely activated by the second aspartate in this motif, attacks the phosphorous atom in the intermediate, releasing the inorganic phosphate. Mg\(^{2+}\) is thought to facilitate both steps by neutralizing the negative charges of the phosphate group, which is different from that in PPP or PPM family, where the metal ions are directly involved in catalysis through activation of a water nucleophile.

Chronophin, a member of the HAD family, is also an aspartate-based PSP (Gohla et al., 2005). Like FCP/SCP, it contains the signature sequence motif DxDxT and a similar active site (PDB code 2CFR). And it has only one known substrate protein. Chronophin dephosphorylates pSer3 of cofilin, an important regulator of actin dynamics, leading to its activation.
I.4 Protein phosphatases: increasing importance noted in the regulation of TGFβ signaling pathway

As mentioned in Section I.2, phosphorylation of type I receptor in the GS domain by type II receptor upon binding of TGFβ superfamily ligand, and following phosphorylation of R-Smads at their C-terminal SXS domain are key events in TGFβ signal cascade. The discovery of the inhibitory linker region phosphorylation in R-Smads also adds one more essential regulatory point to this signaling cascade. Taken together, kinase activity is essential in the activation of this signaling cascade.

This notion also raises the investigation of protein phosphatases involved in this conserved important signaling pathway since reversible phosphorylation is a well-established essential regulatory mechanism in all living organisms.

Recent studies have identified several phosphatases that dephosphorylate Smads in the nucleus and thus terminate the TGFβ/BMP signal. For example, the pyruvate dehydrogenase phosphatase (PDP) and the small C-terminal domain phosphatases (SCPs) bind and dephosphorylate the SSXS motif of mammalian and *Drosophila* Smad 1/5 (Chen et al., 2006; Knockaert et al., 2006). The phosphatase PPM1A antagonizes both TGFβ and BMP signaling by interacting with and dephosphorylating the C-terminus of all R-Smads (Duan et al., 2006; Lin et al., 2006). Furthermore the SCPs can dephosphorylate the linker region of TGFβ/BMP R-Smads (Sapkota et al., 2007; Sapkota et al., 2006; Wrighton et al., 2006), which has distinct outcomes for TGFβ versus BMP signaling (activation and inhibition, respectively) (Sapkota et al., 2006).

Smads are not only phosphorylated by type I receptor at the C-terminus, but also by a plethora of kinases from cross-talking pathways, such as mitogen-activated protein kinases (MAPKs), cyclin-dependent kinases (CDKs), Jun N-terminal kinases (JNKs) and glycogen
synthase kinase 3 (GSK3) in the linker region. Interestingly, both the C-terminal and the linker phosphorylation of Smads are induced upon stimulation with TGFβ and BMP (Sapkota et al., 2006). The MAPK and GSK3 linker-phosphorylated Smad1 is recognized by the ubiquitin ligase Smurf1, which not only targets the ubiquitintated Smad1 for degradation, but also inhibits its binding to the nucleoporin Nup214. Thus, in order to be efficiently translocated into the nucleus, the Smads have to overcome the inhibitory linker phosphorylation in cytoplasm. It is shown that PP2A/PP2 regulates BMP signaling by interacting with BMP receptor complex and by dephosphorylating both the C-terminus and the linker region. This dephosphorylation is mainly at the linker region, therefore leading to increased nuclear localization and overall amplification of this BMP signal (Bengtsson et al., 2009).

In vertebrates, different B subunits of PP2A demonstrate distinct roles in the regulation of TGFβ/Activin/Nodal signaling: Bα enhances signaling by stabilizing the basal levels of type I receptor, whereas Bδ negatively regulates these pathways by restricting receptor activity (Batut et al., 2008). Dullard, preferentially complexes with the BMP type II receptor (BMPRII) and partially colocalizes with the caveolin-1-positive compartment suggesting that it promotes BMPRII degradation through the lipid raft-caveolar pathway (Satow et al., 2006). Dullard, which contains a conserved catalytic phosphatase motif DxDxT/V (so Dullard is categorized to the FCP/SCP family), also associates with BMPRI and represses the BMP-dependent phosphorylation of BMPRI (Satow et al., 2006).

Despite the increasing data of the protein phosphatases characterized in the regulation of TGFβ signaling pathway, investigators always ask whether there are more protein phosphatase players in the regulation of this important pathway and if so, are those protein phosphatases
identified or characterized show the same function in vivo since most data so far use mammalian cell culture and not tested in an intact model organism.

**I.5 TGF-β signaling pathway in *C. elegans***

In *C. elegans*, five TGF-β-related genes can be identified by sequence homology: *daf-7*, *dbl-1*, *unc-129*, *tig-2*, and Y46E12BL.1. No biological roles have yet been described for *tig-2* or Y46E12BL.1. *daf-7* is a TGF-β-related ligand and *dbl-1* is more related to BMP2 and Dpp. Their downstream signaling components have also been characterized, referred to as the Dauer pathway and the Sma/Mab pathway (Figure 1.4), respectively (Suzuki et al. 1999; Gumienny and Savage-Dunn. 2013).

**Figure 1.5 Dauer pathway and Sma/Mab pathway in *C. elegans* (Gumienny and Savage-Dunn, 2013)**

I.5.1. The Dauer pathway
Under unfavorable living conditions (high population density, low food availability, high temperature), the worms will go into the dauer diapause as mentioned before. The dauer TGF-β pathway is one signaling cascade that regulates this process. DAF-7 acts as the ligand (Ren et al., 1996), which binds to type II receptor DAF-4 (Estevez et al., 1993) and type I receptor DAF-1 (Georgi et al., 1990). Upon ligand binding, DAF-1 phosphorylates R-Smad DAF-8 and DAF-14 (Riddle & Albert, 1997; Inoue & Thomas, 2000). Since DAF-14 does not have identifiable DNA binding domains, it is unlikely to transduce signals directly into the nucleus whereas DAF-8 does have DNA binding activity. Both DAF-8 and DAF-14 negatively regulate Co-Smad DAF-3 (Patterson et al. 1997), which associates with the Ski/SnoN homolog DAF-5 (da Graca et al. 2003) to form a transcription factor complex to control dauer entry (Liu et al. 2004). PDP-1, homologous to human pyruvate dehydrogenase phosphatase (PDP), negatively regulates the Insulin/IGF-1 signaling (IIS), therefore modulating dauer diapauses (Narasimhan et al., 2011).

I.5.2. Sma/Mab pathway

The Sma/Mab pathway was first characterized by Savage et al. in 1996 and regulates body size and the development of male-specific sensory rays and copulatory spicules (Savage et al., 1996). Mutants of components in this pathway have small body size (Sma) phenotype and abnormal male tail (Mab) phenotype. The first three components in this pathway, *sma-2, sma-3* and *sma-4* were cloned by this group (Savage et al., 1996). Shortly after, other related components are discovered. For instance, DBL-1 acts as the ligand (Suzuki et al., 1999) and SMA-6 acts as the type I receptor (Krishna et al., 1999). They share the same type II receptor DAF-4 with the Dauer pathway.
Mutations in Sma/Mab pathway components cause the Sma phenotype. *dbl-1* pathway mutants show decreased seam cell length but normal seam cell nuclei number, suggesting that decreases in cell size rather than cell number are responsible for *dbl-1* mutant phenotype (Wang et al., 2002). Organ size measurements have shown that different organs are reduced in size to different degrees. The degree of reduction in the size of hypodermal seam cells and hyp7 is most proportional to the degree of reduction in body size (Wang et al., 2002; Nagamatsu and Ohshima, 2004), consistent with the defined focus of action of Sma/Mab signaling components in the hypodermis. Loss of *dbl-1* activity results in smaller animals, while *dbl-1* overexpression results in longer animals, suggesting that *dbl-1* acts as a dose-dependent regulator of body size (Morita et al., 1999). In addition, comparison of the growth curves between the wild type and mutant animals shows that the mutant animals have the same body size as wild type at the L1 stage. However, the adult animals show only around 50% body size of the wild type (Savage-Dunn et al., 2000), suggesting that a post-embryonic defect causes the Sma phenotype.

In addition to the Sma phenotype, a Mab phenotype is also found in these mutants. Male tail sensory rays and spicules are essential for mating. Normally in wild type animals, there are nine rays on each side of the male tail. However, *dbl-1, daf-4, sma-2, sma-3, sma-4, and sma-6* mutant males show abnormal ray fusions and crumpled spicules. Ray fusion frequently occurs between rays 4-5, 6-7, and 8-9 (Savage et al., 1996).

In addition to body size regulation and male tail development, the Sma/Mab pathway has been shown to regulate innate immunity of *C. elegans*. *sma-2, -3, -4* and -6 are required for resistance to *Pseudomonas aeruginosa*. The loss of function mutants of those four genes are hypersensitive to infection (Mallo et al., 2002). Liu’s lab shows mutations in the Schnurri homolog *sma-9* cause ventralization of the M lineage and that wild-type SMA-9 antagonizes the
Sma/Mab TGFbeta pathway to promote dorsal M lineage fates (Foehr et al., 2006). More recently, Murphy’s lab shows the involvement of Sma/Mab signaling in reproductive aging. Reduction of Sma/Mab signaling delays reproductive aging by maintaining oocyte and germline quality. Compared to cell-autonomous regulation of body size development, Sma/Mab signaling regulates oocyte and distal germline quality maintenance nonautonomously and this process is temporally and transcriptionally separable from its regulation of growth (Luo et al. 2009; Luo et al. 2010).

To date, few other proteins are found to interact with Sma/Mab pathway components. Padgett’s lab showed that LON-2 can negatively regulate Sma/Mab pathway activity. LON-2 is a conserved member of the glypican family of heparan sulfate proteoglycans and can bind BMP2 in vitro. lon-2(If) mutations result in a long body size phenotype and the Drosophila glypican gene dally rescues the lon-2(If) body size defect (Gumienny et al., 2007). More recently, the sole member of the repulsive guidance molecule (RGM) family of proteins in C. elegans, DRAG-1, is found to positively regulate Sma/Mab pathway activity at the ligand-receptor level (Tian et al., 2010).

In my study, I screened a C. elegans RNA interference (RNAi) library of phosphatases to identify genes that cause a body size phenotype. Since Sma/Mab pathway, a branch of TGFβ signaling in C. elegans, regulates body size and male tail patterning; we propose that protein phosphatases modulating this pathway might contribute to a body size phenotype upon being knocked down by RNAi.

Combining epistatic analyses and reporter assays, 11 candidates were verified as protein phosphatases by sequencing. These include homologs of mammalian PPM1A/B/G and PP1.
Chapter II. Library-wide RNA interference screening to identify protein phosphatases regulating Sma/Mab signaling pathway in *C. elegans*.

II.1 Abstract

RNA interference (RNAi), allows efficient analysis of endogenous gene function in diverse organisms by introduction of double-stranded RNA (dsRNA) with corresponding sequences. In *Caenorhabditis elegans*, it has been previously shown that RNAi can be performed by directly microinjecting dsRNA into the adult hermaphrodite, by soaking the worms into a solution of dsRNA, or by feeding the worms *Escherichia coli* expressing target-gene dsRNA. Because of its ease to use, and high-throughput, the third mode of dsRNA introduction gains more and more attention in large-scale screenings to identify genes involved in complex biological processes.

Here we report a library-wide RNAi screen searching for protein phosphatases modulating Sma/Mab pathway, a TGFβ signaling branch in *Caenorhabditis elegans* regulating the body size and male tail patterning. Some of the methods described in this chapter have been published (Liang et al., 2013). The candidates identified include homologues of mammalian PPM1A/B/G and PP1.
II.2 Introduction

Members of the transforming growth factor β (TGF-β) superfamily regulate a critical array of cellular processes including cell proliferation, lineage determination, differentiation, motility, adhesion, and death (Massague J. 1998). Signal transduction for this family is initiated by formation of the active ligand-receptor complex between TGF-β ligand and two transmembrane ser/thr kinase receptors, type I and type II receptor, and further transduced through the intracellular Smad proteins. In response to TGFβ, receptor-activated Smads (R-Smads) are phosphorylated at their C-terminal SXS motif by type I receptor (Feng and Derynck, 2005; ten Dijke and Hill, 2004). Phosphorylated R-Smads form complexes with common Smad (Co-Smad), Smad4. Smad complexes then accumulate in the nucleus, where Smads regulate downstream target gene expression with or without the cooperation of other specific transcription cofactors.

Signal transduction pathways are often regulated by reversible phosphorylation, a dynamic interplay between protein kinases and phosphatases. TGFβ ligand stimulation results in Smad phosphorylation, inhibition of nuclear export, and persistent accumulation within the nucleus (Schmierer and Hill, 2005), which implies the kinase activity is essential to maintain TGFβ signaling cascade. Recent studies reveal the increasing importance of protein phosphatases involved in the regulation of this canonical signaling cascade. A handful of protein phosphatases have been identified. PPM1A/PP2Cα is shown to terminate TGFβ signaling by dephosphorylating the C-terminal SXS motif of Smad2/3 (Lin et al., 2006) whereas the pyruvate dehydrogenase phosphatase (PDP) and the small C-terminal domain phosphatases (SCPs) modulate BMP signaling by dephosphorylating the SSXS motif of mammalian and Drosophila.
Smad 1/5 (Chen et al., 2006; Knockaert et al., 2006). Furthermore the SCPs can dephosphorylate the linker region of TGFβ/BMP R-Smads (Sapkota et al., 2007; Sapkota et al., 2006; Wrighton et al., 2006), which has distinct outcomes for TGFβ versus BMP signaling (activation and inhibition, respectively) (Sapkota et al., 2006). In vertebrates, different B subunits of PP2A demonstrate distinct roles in the regulation of TGFβ/Activin/Nodal signaling: Bα enhances signaling by stabilizing the basal levels of type I receptor, whereas Bδ negatively regulates these pathways by restricting receptor activity (Batut et al., 2008). Bβ subunit interacts with both BMP type I and II receptors and regulate the BMP signaling by dephosphorylating both the C-terminus and linker region of Smad1 with the linker region as the preferred site, therefore leading to an overall amplification of BMP signaling (Bengtsson et al., 2008). Dullard, which contains a catalytic phosphatase motif DxDxT/V, also associates with BMPRI and represses the BMP-dependent phosphorylation of BMPRI (Satow et al., 2006). The regulatory subunit of PP1, GADD34 interacts with the catalytic subunit of PP1 to dephosphorylate TGFβI with or without the assistance of inhibitory Smads, Smad6 or Smad7, attenuating the TGFβ signaling cascade (Shi et al., 2004).

Despite the increasing number of protein phosphatases identified in the regulation of TGFβ signaling pathways, how those protein phosphatases interact in vivo to modulate TGFβ signaling pathways in diverse tissues and various biological processes and whether there are novel players involved in this well-established simple core signal cascade still remain largely unknown. Most screens reported did not adopt a model organism. Therefore, another major concern is whether those phosphatases function in vivo as well as in vitro. The development of RNA interference technique allow rapid and efficient analysis of gene function in large-scale screens within a relatively simple classic genetic model, Caenorhabditis elegans, by simply
feeding the worms *Escherichia coli* expressing target-gene dsRNA. This method is now widely used to screen players involved in various biological processes such as neuronal specification (Poole et al., 2011), stress response and longevity (Wang et al., 2010), and neuronal dysfunction (Kuwahara et al., 2008).

In the nematode *C. elegans*, a BMP-related signaling pathway regulates body size and patterning of sex-specific tissues of male posterior region (Patterson and Padgett, 2000; Gumienny and Savage-Dunn, 2013). This pathway, which we refer to as the Sma/Mab pathway, consists of ligand *dbl-1* (Suzuki et al., 1999; Morita et al., 1999), type I receptor *sma-6* (Krishna et al. 1999), type II receptor *daf-4* (Estevez et al., 1993), and Smads, *sma-2*, *sma-3* and *sma-4* (Savage et al., 1996). Mutations in any of these core pathway components cause small body size (Sma) phenotype in both hermaphrodites and males, and a male abnormal (Mab) phenotype due to transformations in male sensory ray identity and defective morphogenesis of the male copulatory spicules.

Here we report a systematic RNAi screen for novel protein phosphatases modulating Sma/Mab signaling in *C. elegans*. A *C. elegans* RNAi library of phosphatases including 168 clones was screened under an RNAi-hypersensitive mutant background, *rrf-3*, followed by epistatic analyses and reporter assays. Eight likely candidates were found to regulate Sma/Mab pathway directly or indirectly. These include homologues of mammalian PPM1A/B/G and PP1.
II.3 Materials and methods

Strains and *Caenorhabditis elegans* protein phosphatase RNAi clones

*C. elegans* strains were cultured and maintained using standard methods at 20°C unless otherwise noted (Brenner, 1974). The following strains were used: N2 bristol (wild type), LG II: *rrf-3(pk1426)*, *sma-6(e1482)* (Brenner, 1974), *sma-6(wk7)* (Krishna et al., 1999); LG III: *lin-36(n766)*, *sma-2(e502)*, *sma-3(wk30)*; LG V: *dbl-1(wk70)*; LG X: *dbl-1(ctIs40)*, *eri-1(mg366)IV*; *lin-15B(n744)X*, *lon-2(e678)*.

LW2286 (*lon-2(e678)X; jil5277*), LW2308 (*dbl-1(wk70)V; jil5277*), and LW2436 (*jil5277*) (gifts from Jun Kelly Liu, Cornell University) were used in RAD-SMAD reporter assay.

Bacterial clones from the *Caenorhabditis elegans* phosphatase RNAi library (166 clones) and two other RNAi clones containing *Caenorhabditis elegans* homologues of mammalian SCPs were purchased from Source BioScience Life Science. All bacterial clones were grown on LB agar plates supplemented with carbenicillin (25 µg/ml) and tetracycline (12.5 µg/ml) right before use.

**RNAi screen by feeding**

Large-scale screen was carried out under an RNAi-hypersensitive mutant ground, *rrf-3(pk1426)*, using standard RNAi feeding protocol (Fraser et al., 2000; Kamath et al., 2003). Minor modifications were made and described below.

Day1. A single bacterial colony of each RNAi clone was grown in 1 ml LB broth containing 100
µg/ml of Ampicillin overnight at 37°C, ~280rpm. Bacteria carrying empty RNAi feeding vector L4440 serves as control.

Day2. The 1ml over-night bacterial culture was transferred into 4 ml LB broth with 100 µg/ml of Ampicillin and incubated at 37°C, ~280rpm for another 3-4 hours. Seed the fresh bacterial culture onto EZ RNAi worm plates supplemented with carbenicillin (25 µg/ml) and IPTG (1 mM). Incubate at 37°C, overnight.

Day3. Transfer 10-12 L4 hermaphrodites into a corresponding RNAi bacterial plate obtained from Day2. Incubate at 20°C, overnight until the worms reach young adult stage.

Day4. Transfer 10-12 young adult to a fresh corresponding RNAi bacterial plate. Let those worms lay eggs for 4-5 hours. Then remove the mothers. Leave the RNAi plates with only eggs at 20°C. Incubate for about 72 hours until the progeny reaches young adult stage.

Day7. Mount 35-40 adult worms to standard slides containing 2% Agarose pads. Then photograph using QC Capture 2.73.0 software.

**Analysis of body size measurements:**

The body length of the resulting progeny was measured using Image-Pro Express 5.1.0.12 software. Student t-test was done to analyze the significance of the data (p<0.01).

**Epistatic study**

To investigate the interaction between the protein phosphatase candidates identified and the known core components in Sma/Mab pathway, we carried out epistatic study in the following mutant backgrounds: a strong loss-of-function type I receptor mutant (*sma-6(wk7)*), a weak loss-
of-function type I receptor mutant (*sma-6(e1482)*), a loss-of-function mutant in a negative regulator of this Sma/Mab pathway (*lon-2*), and in a strain that over-expresses the TGFβ ligand, DBL-1 (*dbl-1++*).

First, the mutant worms were fed on appropriate protein phosphatase RNAi clones as described in RNAi screen part. The resulting 72 hour-old progeny were photographed and the body length was analyzed

**RAD-SMAD reporter assay**

To determine whether the protein phosphatase candidates identified regulate body size dependent of or independent of Sma/Mab pathway, RAD-SMAD reporter assays were carried out (Tian et al., 2010).

Briefly, 10-12 L4 animals were transferred to feeding plates seeded with appropriate protein phosphatase RNAi clones, incubated overnight, and transferred to fresh feeding plates to lay eggs for 4 hours. The synchronized progeny were scored at L2 stage when the expression of RAD-SMAD reporter in hypodermis becomes predominant.
II.4 Results

II.4.1 Comparative analysis of RNAi with wild type and different RNAi-hypersensitive mutant strains

First, I determined which RNAi-hypersensitive mutant background is suitable for large-scale RNAi screen. To make the result of an RNAi screen reliable, the starting strain used must be sensitive to RNAi and the experiments carried out under this genetic background must show consistent pattern. Since we screen for a body size phenotype, the brood size should be large enough so that we can obtain enough samples for further analyses. To validate the RNAi effect, I first conducted a trial experiment using two RNAi-hypersensitive mutant stains: *eri-1(mg366)IV; lin-15B(n744)X*, and *rrf-3(pk1426)*, as well as *lin-36(n766)*, a component in the *lin-15* pathway whose RNAi sensitivity is nevertheless similar to N2 (Simmer et al., 2002). I knocked down known components in Sma/Mab pathway: *dbl-1/ligand; sma-2, sma-3, sma-4/Smads*; and *sma-6/receptor* in all three mutant backgrounds (Figure 2.1). All tested strains were small when *dbl-1, sma-2, sma-3, sma-4* were knocked down by RNAi. Whereas, *lin-36* exhibited a significant body length increase upon *sma-6* RNAi, which is opposite to *sma-6(lf)* mutant phenotype. These results suggest that *lin-36* might modulate body size independent of Sma/Mab pathway and cannot be used as an RNAi-hypersensitive strain in our RNAi screen.

Upon *sma-3* RNAi, both wild type (N2) and all three mutant strains show a significant body length decrease, which is consistent with the Sma phenotype exhibited by *sma-3(lf)* genetic mutants. Whereas, the % body length decrease in the N2 background (~10%) is much less than that under RNAi-hypersensitive mutant backgrounds (~15% in *rrf-3* background and ~24% in *eri-1(mg366)IV; lin-15B(n744)X* background, respectively) (Figure 2.1). These data confirmed
that RNAi-hypersensitive mutants are more sensitive than wild type when screening for body size phenotype.

Though our data indicates that \textit{eri-1(mg366)IV; lin-15B(n744)X} demonstrates the strongest phenotype upon RNAi, the animals did not grow well and produced less progeny. Therefore in large-scale screen, I would not get enough worms for further analysis. As a result, we favor the \textit{rrf-3} strain to be the primary mutant background to be used in a large-scale RNAi screen to identify novel protein phosphatases that regulate \textit{C. elegans} body size.

\textbf{Figure 2.1} All three RNAi-hypersensitive mutants tested show body size phenotype upon core Sma/Mab pathway components RNAi. All data were analyzed using student t-test. Error Bar: standard error. *: P<0.01. N (worms scored per RNAi treatment per experiment): ≥30.
II.4.2 Large-scale RNAi screen using *rrf-3* identified novel players involved in body size control

As discussed in Chapter I, Sma/Mab pathway is one of the major pathways regulating *Caenorhabditis elegans* body size. Novel components involved in this pathway and new modifiers modulating the Sma/Mab pathway might affect the body size as well. Based on this hypothesis, I first conducted a pilot screen using *rrf*-3 mutants. The starting RNAi library used in this study includes bacterial clones from the *Caenorhabditis elegans* phosphatase RNAi library (166 clones) and two other RNAi clones containing *Caenorhabditis elegans* homologues of mammalian SCPs were purchased from Source BioScience Life Science. After RNAi, I found that 124 clones contributed to a body size phenotype at 72hrs (Figure 2.2). Among them, 107 resulted in the Lon phenotype whereas 17 led to the Sma phenotype. Though I was focusing on candidates that resulted in body size phenotype upon RNAi treatment, I also noticed that several RNAi clones led to other developmental defects such as Emb (embryonic lethal), or Mor (morphological abnormality such as protruded vulva) were also identified (as summarized in Table 2.1). These data suggest that our screening protocol can also be used to screen other developmental phenotypes than the body size phenotype.
I then carried out in silico analyses to categorize the protein phosphatase candidates using wormbase annotation (http://www.wormbase.org). Among the 107 RNAi clones contributing to the Lon phenotype, 48 belong to the protein tyrosine phosphatase family; 30 are putative members of protein serine/threonine phosphatase family; 1 belongs to the Dual Specificity Protein Phosphatase (DUSP) family; 1 is identified as intestinal acid phosphatase (pho-5); 12 are not annotated as any protein phosphatases, the function of the rest 15 are unclear (as summarized in Table 2.2).

Table 2.1 The RNAi clones cause developmental defects other than body size phenotype in rrf-3 mutant background
<table>
<thead>
<tr>
<th>Phenotype upon RNAi</th>
<th>Gene identifier</th>
<th>Gene function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emb</td>
<td>F26B1.3</td>
<td><em>ima-2, importin-a, nuclear transport factor</em></td>
</tr>
<tr>
<td></td>
<td>F38H4.9</td>
<td><em>let-92, protein serine/threonine phosphatase</em></td>
</tr>
<tr>
<td></td>
<td>F42G8.8</td>
<td>Protein serine/threonine phosphatase</td>
</tr>
<tr>
<td></td>
<td>F44F4.2</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td></td>
<td>F48E8.5</td>
<td><em>paa-1, PP2A A subunit</em></td>
</tr>
<tr>
<td></td>
<td>K07F5.6</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td></td>
<td>Y48G1A_54.b</td>
<td>unknown function</td>
</tr>
<tr>
<td>Mor</td>
<td>F22D6.9</td>
<td>Protein serine/threonine phosphatase</td>
</tr>
<tr>
<td></td>
<td>F26E4.1</td>
<td>Protein serine/threonine phosphatase</td>
</tr>
<tr>
<td></td>
<td>F32E10.4</td>
<td><em>ima-3, importin-a, nuclear transport factor</em></td>
</tr>
<tr>
<td></td>
<td>K02A11.1</td>
<td>PP1 regulatory subunit</td>
</tr>
<tr>
<td></td>
<td>K09F6.3</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td></td>
<td>R03D7.8</td>
<td>PP1 catalytic domain-like</td>
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<td></td>
<td>T15B7.2</td>
<td>Protein tyrosine phosphatase</td>
</tr>
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</table>

Table 2.2 Classification of protein phosphatase candidates that contribute to Lon body size phenotype upon RNAi in *rrf-3* background

<table>
<thead>
<tr>
<th>Protein categories</th>
<th>Gene identifier</th>
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<tr>
<td>Protein tyrosine phosphatase</td>
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<tr>
<td></td>
<td>C05B10.1</td>
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<tr>
<td></td>
<td>C07E3.4</td>
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<td></td>
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<td>C17H12.3</td>
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<td></td>
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<td></td>
<td>F38H4.4</td>
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<td></td>
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<td>Protein serine/threonine phosphatase</td>
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<td></td>
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<tr>
<td></td>
<td>C06G1.5</td>
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<td></td>
<td>C30A5.4</td>
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<td></td>
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<td></td>
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<td></td>
<td>C47A4.3</td>
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<td></td>
<td>F23B12.1</td>
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<td></td>
<td>F23H11.8</td>
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<tr>
<td>Protein serine/threonine phosphatase (cont.)</td>
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<td></td>
<td>F44B9.9</td>
</tr>
<tr>
<td></td>
<td>F49E11.7</td>
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<tr>
<td></td>
<td>F52H3.6</td>
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<td></td>
<td>F58G1.3</td>
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<td></td>
<td>M04C9.6</td>
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<td>Acid phosphatase</td>
<td>B0361.7</td>
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<td>--------------------</td>
<td>---------</td>
</tr>
<tr>
<td>Dual specificity protein phosphatase</td>
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</tr>
<tr>
<td>Function unknown</td>
<td>B0478.2, C25A1.9, CC8.e, F08F3.5, F38E9.3, H02F09.4, H06I04.d, R10E4.9, R17.2, Y18H1A 68.a, Y32H12A.e, Y39B6B.ff, Y62E10A.m, Y110A7A.p, Y119D3 464.f</td>
</tr>
</tbody>
</table>
Among the 17 protein phosphatase candidates that led to a Sma phenotype in the *rrf-3* mutant background upon RNAi, 13 were annotated as protein serine/threonine phosphatase; 1 belongs to the protein tyrosine phosphatase family; 1 was identified as importin-b (*imb-6*); the function of the remaining 2 candidates is yet not clear (as summarized in table 2.3). Taken together, we identified novel players involved in body size control, via a large-scale RNAi screen using *rrf-3*.

<table>
<thead>
<tr>
<th>Protein categories</th>
<th>Gene identifier</th>
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<tbody>
<tr>
<td>Protein tyrosine phosphatase</td>
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<td>Protein serine/threonine phosphatase</td>
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<td>B0511.7</td>
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<td></td>
<td>Y41D4A_3192.b</td>
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</table>

We also noticed that there were some variable results among individual RNAi treatments, which is consistent with the data reported by other laboratories (Simmer et al., 2003). The variable results obtained from individual RNAi treatments were due to the high frequency of false negatives present in each RNAi screen. To minimize the variability issue, we then carried
out a secondary library-wide screen in a *sma-6(wk7)* mutant background, which will be discussed in the following part (II.4.3). A few variable results are shown in Table 2.4.

<table>
<thead>
<tr>
<th>Gene identifier</th>
<th>Gene function</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; <em>rrf-3</em> screen</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; <em>rrf-3</em> screen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% control</td>
<td>P value</td>
</tr>
<tr>
<td>C06A1.3</td>
<td>Protein Ser/Thr phosphatase</td>
<td>114.19±4.55</td>
<td>3.02E-19</td>
</tr>
<tr>
<td>C42C1.2</td>
<td>PP2C-like</td>
<td>89.89±4.96</td>
<td>9.03E-12</td>
</tr>
<tr>
<td>W09C3.6</td>
<td>Protein Ser/Thr phosphatase</td>
<td>108.75±4.00</td>
<td>1.23E-11</td>
</tr>
</tbody>
</table>

II.4.3 RNAi screen in *sma-6(wk7)* background reveals potential modifiers regulating Sma/Mab pathway

When a novel player is identified, it is important to determine the interactions with other known mutants of the same phenotype. This is referred to as an epistatic analysis. By doing this, one can determine that the new component acts in an existing pathway or in a novel pathway for its function. If two separate mutations result in the reduced body length as compared to each mutation alone, this is interpreted as parallel pathway. The homozygous double mutants should be smaller than either single mutant alone, indicating that two mutations have additive effects. If one mutation results in long body size by affecting one pathway, while a second mutation causes small body size affecting another pathway, the double mutant will show additive effect with intermediate body size (Morck et al., 2006) (Figure 2.3).
In our study, it is not practical to make double mutants of all candidates identified with the known components in the Sma/Mab pathway. However, upon RNAi in a single mutant background, we can mimic the phenotype of double mutants, therefore we can get clues of how these candidates interact with the Sma/Mab pathway to regulate the body size in a relatively short time.

**II.4.3.1. Knocking down known components in Sma/Mab pathway indicates the basal activity of this pathway in the absence of ligand activation**

I first carried out experiments to test how the known components in Sma/Mab pathway act in the *sma-6(wk7)* background, a strong loss-of-function allele of the type I receptor, SMA-6.
As is consistent with our expectation, when we knocked the ligand DBL-1 and the type I receptor SMA-6, the RNAi worms exhibited the similar Sma phenotype as *sma-6(wk7)* mutant (p>0.01). In contrast, when we knocked down the expression of SMAs: *sma-2*, and *sma-4*, the RNAi worms show enhanced Sma phenotype (p<0.01) (Figure 2.4). This suggests that without ligand activation, there is basal activity of Sma/Mab pathway which partially promotes growth of *Caenorhabditis elegans*. Whereas when another SMA protein, SMA-3, is downregulated by RNAi, we noticed a similar *sma-6(wk7)* mutant phenotype.

In a previous study done in our lab, Liang et al. identified SMA-9, a *Drosophila* Schnurri homologue, which acts as a transcriptional cofactor to regulate a subset of Sma/Mab pathway targets. Therefore, we also included an RNAi clone of *sma-9* in this study. Upon *sma-9* RNAi, the resulting progeny displayed a similar phenotype as that of the *sma-6(wk7)* mutants.

Taken together, we demonstrated even without ligand activation, there is basal activity of Sma/Mab pathway to promote growth. Our data suggest that this basal activity requires the presence of two intact SMAs, SMA-2 and SMA-4.
II.4.3.2. Comparative analyses of rrf-3 and sma-6(wk7) screening data reveals potential modifiers regulating Sma/Mab pathway

As mentioned in section II.4.2, a primary screen carried out in the rrf-3 RNAi – hypersensitive background, identified a plenty of candidates regulating body size in Caenorhabditis elegans. One of our major concerns is that due to the high frequency of false negatives identified in each RNAi screen, we might have obtained some false positive candidates. So our next goal was to determine whether the candidates were involved in the regulation of Sma/Mab pathway or acted as direct components in this signal cascade. Towards this end, I then screened the whole Caenorhabditis elegans protein phosphatase RNAi library in the sma-6(wk7) background.

I analyzed RNAi phenotypes in the sma-6(wk7) background. 64 out of the 107 RNAi clones that led to the Lon phenotype in the rrf-3 background upon RNAi, exhibited the sma-6(lf) mutant phenotype or suppressed the Sma phenotype in the sma-6(wk7) background (Figure 2.5). There was one RNAi clone that suppressed the Sma phenotype only in the sma-6(wk7) background, but exhibited no body size phenotype in the rrf-3 mutant background upon RNAi. In contrast, 12 RNAi clones identified in the primary rrf-3 screen as potential positive regulators of body size showed no body size change or enhanced Sma phenotype in the sma-6(wk7) background (Figure 2.5). Three RNAi clones resulted in a similar phenotype as that of the sma-
6(1f) mutants, or the enhanced Sma phenotype, whereas had no effect on body size of rrf-3 animals in the primary screen, when knocked down by RNAi (Figure 2.5).

In addition to the body size phenotype, we also noticed other developmental defects while testing the RNAi clones in sma-6(wk7) mutants. 5 out of 6 RNAi clones cause Emb phenotype in both rrf-3 and sma-6(wk7) backgrounds. One RNAi clone displayed the Mor phenotype in sma-6(wk7) mutants but not in rrf-3 worms. However, we did not observe the same Mor phenotype for any of the RNAi clones, as visualized in the rrf-3 background (Table 2.5). The phenotypic differences found between the two genetic mutants might partially be explained

**Figure 2.5 Comparative analysis using rrf-3 and sma-6(wk7) data revealed potential candidates modulating body size in a Sma/Mab pathway-dependent manner.** Left panel: shows the 64 potential negative regulators; Right panel: indicates that the 12 RNAi clones might control body size via positively regulating the Sma/Mab pathway. □: RNAi clones that exhibited expected body size phenotype were identified in rrf-3 screen; ■: RNAi clones identified in sma-6(wk7) screen showed expected body size phenotype.
by the hypersensitivity to RNAi in the \textit{rrf-3} background, since the \textit{sma-6} strain assayed did not contain the \textit{rrf-3} mutation.

<table>
<thead>
<tr>
<th>Phenotype upon RNAi</th>
<th>Gene identifier</th>
<th>Gene function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emb</td>
<td>F26B1.3</td>
<td>\textit{ima-2}, importin-a, nuclear transport factor</td>
</tr>
<tr>
<td></td>
<td>F38H4.9</td>
<td>\textit{let-92}, protein serine/threonine phosphatase</td>
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<td>F44F4.2</td>
<td>Protein tyrosine phosphatase</td>
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<tr>
<td></td>
<td>F48E8.5</td>
<td>\textit{paa-1}, PP2A A subunit</td>
</tr>
<tr>
<td></td>
<td>Y48G1A_54.b</td>
<td>unknown function</td>
</tr>
<tr>
<td>Mor</td>
<td>F08B1.1</td>
<td>Protein tyrosine phosphatase, \textit{vhp-1}</td>
</tr>
</tbody>
</table>

\textbf{II.4.4 Epistatic analyses using \textit{dbl-1(ctls40)} and \textit{lon-2(e678)} further eliminate candidates not involved in regulation of Sma/Mab pathway.}

As discussed in section II.4.3, by comparative analysis using \textit{rrf-3} and \textit{sma-6(wk7)} screening data, we narrowed the candidate pool to 80. I then carried out RNAi experiments using \textit{dbl-1(ctls40)} and \textit{lon-2(e678)} backgrounds. The \textit{dbl-1(ctls40)} worm strain carries an integrated array overexpressing the ligand DBL-1. Therefore, Sma/Mab pathway is hyperactivated, resulting in a Lon phenotype (Figure 2.6). LON-2 is a conserved member of the glypican family of heparin sulfate proteoglycans and negatively regulate Sma/Mab pathway by attenuating the ligand-receptor interaction (Gumienny et al., 2007). \textit{lon-2} mutant therefore exhibits a Lon phenotype (Figure 2.6).

If the protein candidates positively regulate Sma/Mab pathway, upon RNAi, the resulting worms would show the \textit{dbl-1(ctls40)} or \textit{lon-2(e678)} mutant phenotype or suppress the Lon
phenotype. Whereas, if the protein candidates modulate Sma/Mab pathway negatively, the RNAi worms would show the *dbl-1(ctls40)* or *lon-2(e678)* mutant phenotype or enhance the Lon phenotype.

I then compared the *dbl-1(ctls40)* and *lon-2(e678)* data with the previous screening data using *rrf-3* and *sma-6(wk7)*. Based on the data comparison, we narrowed the 80-candidate pool to 69. Taken together, our epistatic analyses showed that the candidate genes identified interact with known components in the Sma/Mab pathway. The epistatic data helped to eliminate some false positives. However, we do not have enough clues to put any single candidate into a precise position in the Sma/Mab pathway yet.
II.4.5 RAD-SMAD reporter assay identified candidates regulating Sma/Mab pathway.

The epistatic study described in section II.4.3 and II.4.4 suggested that we had identified protein phosphatase candidates that regulate Sma/Mab pathway activity to maintain the normal body size of worms. To test this hypothesis, one can demonstrate the direct protein-protein interaction by biochemical assays such as western blot and immunoprecipitation. However, as there are no existing worm specific phospho-SMAD antibodies or anti-SMAD antibodies, it is hard to carry out such experiments. I aligned the primary sequences of SMA proteins with human SMAD proteins, and selected a phospho-SMAD antibody and pan-SMAD antibody binding to the most conserved epitopes. Then I performed immunostaining experiments and western blot analyses. Not one worked, in contrast, when I blotted the same blot with anti-tubulin antibody, I visualized the expected band (data not shown). In short, we cannot use biochemical assays to demonstrate the direct candidate-SMAs interaction due to the lack of available SMA-specific antibodies.

An alternative method to monitor the direct output of Sma/Mab pathway is to use Sma/Mab-responsive reporters. I used the RAD-SMAD reporter (a kind gift from Dr. Jun Kelly Liu, Cornell University) in this study. The RAD-SMAD reporter contains multiple copies of the

![Figure 2.6 Representative figures show the body size phenotype scored in dbl-1(ct1s40) and lon-2(e678) backgrounds, respectively. Panel A, E: dbl-1(ct1s40) worms fed on control RNAi show a Lon phenotype; Panel B: Upon C27B7.6 (a protein Ser/Thr phosphatase) RNAi, dbl-1(ct1s40) exhibited a Suppressed Lon phenotype; Panel F: Upon H02F09.4 (a transposon in origin) RNAi, dbl-1(ct1s40) exhibited an Enhanced Lon phenotype; Panel C, G: lon-2(e678) worms show a Lon phenotype upon control RNAi; Panel D: Upon ppm-1 RNAi, lon-2(e678) worms show a Suppressed Lon phenotype; Panel H: Upon R155.3 (a protein tyrosine phosphatase) RNAi, lon-2(e678) worms show an Enhanced Lon phenotype.](image-url)
SMAD boxes (or SMAD binding site GTCT) organized in the most affinite RLR orientation upstream of the minimal pes-10 promoter and gfp (Tian et al., 2010, Figure 2.7). The expression level of the RAD-SMAD reporter appeared rather dynamic during development, with stronger intestinal expression in embryos and L1s, but fainter in adults and stronger hypodermal expression in L2s and L3s. So we decided to check the reporter activity in the L2 stage upon candidate RNAi depletion.

We proposed that if the candidates regulate body size via the Sma/Mab pathway, the reporter activity would change, upon candidate RNAi depletion. If the candidates positively modulate the Sma/Mab pathway, we would observe weaker expression of RAD-SMAD reporter when these candidates are depleted by RNAi. In contrast, a stronger reporter activity suggests that the candidates tested are negative modifiers of this pathway (Figure 2.7).

I compared the results of reporter assay with the previous screening data and epistatic studies, 22 candidates were identified as the most likely protein phosphatase regulating the Sma/Mab pathway.
II.4.6 Homologues of mammalian PPM1A/B/G and PP1 are identified and verified by sequencing analysis.

As mentioned in section II.4.5, 22 candidates were identified as potential protein phosphatase regulators of the Sma/Mab pathway. We then sent out those RNAi clones for sequencing (as described in materials and methods).

I analyzed the sequencing data by blasting the sequences within the *Caenorhabditis elegans* genome (open database from wormbase.org). 5 out of the 22 candidates did not match any sequence within the whole database (data not shown). 11 are identified as protein phosphatases. One is identified as a member of sri-family chemoreceptor. Another one is annotated as a transposon. The remainders had no known functions (as summarized in Table 2.6).

The sequence verified candidates showed a consistent pattern in primary *rrf-3* screen, epistatic analyses, and the following RAD-SMAD reporter assay (the phenotypes of the protein phosphatase candidates are summarized in Table 2.7).

Table 2.6 Eight RNAi clones are identified as protein phosphatases by sequencing

<table>
<thead>
<tr>
<th>Protein category</th>
<th>Gene identifier</th>
<th>Gene function</th>
</tr>
</thead>
<tbody>
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<td>Chemoreceptor</td>
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<td>7 TM chemoreceptor, sri-family, <em>sri-31</em></td>
</tr>
<tr>
<td>Transposon</td>
<td>H02F09.4</td>
<td>WBTransposon 00000686</td>
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<td>Protein phosphatase</td>
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<td>R155.3</td>
<td>Protein tyrosine phosphatase*</td>
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<tr>
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*: The latest Wormbase release annotates that those genes show protein tyrosine phosphatase activity.
<table>
<thead>
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<th>Functional Category</th>
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<th>RAD-SMAD reporter activity</th>
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<td></td>
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<td>rrf-3</td>
<td>sma-6(wk7)</td>
</tr>
<tr>
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<td>C06A1.3</td>
<td>Lon</td>
<td>NS</td>
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</tbody>
</table>

Lon: long body size; NS: No significant change compared with control RNAi; ND: Not done; Sma: small body size.
*: though show a change in expression level, not agree with other data.
All body size data were analyzed using student t-test, p<0.01.
II.5 Discussion

II.5.1 rrf-3 hypersensitive mutant is highly efficient for large-scale RNAi screen

rrf-3 encodes an RNA-directed RNA polymerase (RdRP) homolog that inhibits somatic RNAi, and thus promotes activity of repeated genes such as multicopy transgenic array. The effect of RRF-3 on RNAi is opposite to that of RRF-1 (which stimulates somatic RNAi). This might arise from the competition by RRF-3 with RRF-1 or EGO-1 in RNAi formation. rrf-3 mutants are hypersensitive to somatic RNAi, which makes rrf-3 a desirable starting strain in various large-scale or genome-wide screens (Simmer et al., 2003).

In our study, we demonstrated that rrf-3 mutants are more suitable to screen for novel players involved in body size control. First, when known components of Sma/Mab pathway are depleted by RNAi, rrf-3 worms show the same Sma phenotype as the known genetic loss-of-function mutants in this pathway. Second, the body length decrease in rrf-3 background is more significant than that in wild type, which increases the sensitivity of the RNAi treatment and enlarges the potential candidate pool. Third, because we need to analyze the body size phenotype of RNAi worms using epistatic analyses, it is important to obtain enough worms for analysis. Though we noticed that eri-1;lin-15B worms were more sensitive to RNAi, the small brood size and decrease in viability limited its usage in such kind of screens.

Due to the high frequency of false negatives existed in each RNAi screen, we cannot rule out the possibility that some candidates tested are missing in the primary rrf-3 screen. And since body size control involves multiple signal pathways, some modifiers might synergize to exert their effect. Therefore, depletion of a single gene probably cannot result in a detectable phenotypic change. Thus in our screen, we might have missed such kind of candidates.
II.5.2 Novel protein phosphatases regulating Sma/Mab pathway are identified

To date, existing evidence has proven the involvement of protein phosphatases in the regulation of TGFβ pathway. Some examples are PPM1A, PDP, SCPs, PP2A, Dullard, and PP1 (Lin et al., 2006; Chen et al., 2006; Knockaert et al., 2006; Sapkota et al., 2007; Wrighton et al., 2006; Batut et al., 2008; Bengtsson et al., 2008; Satow et al., 2006; Shi et al., 2004). These data contribute to elucidate the regulatory mechanism of TGFβ pathway and in some degree explain why this simple signal cascade can regulate a plethora of developmental processes, leading to distinct outcomes in specific cell types.

However, to our knowledge, no one has carried out large-scale screen in a model system to identify novel protein phosphatases involved in this conserved pathway. Whereas, the body of literature suggests that the regulation by protein phosphatases can occur at any level of that signaling pathway, which implies that more protein phosphatases might be involved. That is why we carried out the large-scale screen in *Caenorhabditis elegans*.

There are two branches of the TGFβ pathway within *Caenorhabditis elegans*, the Dauer pathway and the Sma/Mab pathway. The former controls the entrance and exit of dauer stage, a developmental stage equivalent to L3 stage that responds to stress such as food scarcity (Hu, 2007). The latter is required for normal body size and male tail patterning. We proposed that if a protein phosphatase regulates the Sma/Mab pathway, it will contribute to a body size phenotype upon RNAi. If they function in the dauer pathway, we might have observed a dauer-constutive phenotype at 20°C. During our primary *rrf-3* screen, we did not notice any treatments that resulted in the dauer-constitutive phenotype, thus we focused on the body size phenotype.

I then carried out an epistatic analysis in different genetic backgrounds of known components of Sma/Mab pathway. The epistatic data combined with RAD-SMAD reporter assay
results revealed a few protein phosphatase candidates that modulate this pathway. As shown in section II.4.6, we verified 11 out of 22 candidates as protein phosphatase by sequencing.

In summary, we conducted a large-scale RNAi screen by feeding the *Caenorhabditis elegans* with the ds-RNA expressing bacteria. We also demonstrated that the candidates genetically interact with known components in the Sma/Mab pathway by epistatic analyses. The RAD-SMAD reporter assay further identified candidates involved in the regulation of the Sma/Mab pathway. We then verified our results by sequencing. Our screening strategy proved to be successful in revealing novel players involved in the regulation of the Sma/Mab pathway. And this screening strategy can also be modified to search for novel players in other signal pathways.

Among the protein phosphatases identified, we found homologs of mammalian PPM1A/B/G and PP1, which is consistent with previous reports. We also found other three protein serine/threonine phosphatases involved in the regulation of the Sma/Mab pathway. As the TGFβ pathway is conserved among species, our study might shed light on the elucidation of its regulatory mechanisms.

To our surprise, our data revealed that members of the protein tyrosine phosphatase family also modulate the Sma/Mab pathway. One possible explanation is that the tyrosine phosphatases might exert their effects by acting on kinases that modify the linker region of receptor-SMAs, therefore regulating Sma/Mab pathway indirectly. Another possibility is that the protein tyrosine phosphatases might act indirectly through crosstalk with other signaling pathways. We also cannot rule out the possibility that there are some unknown components within this pathway serving as direct substrates of those protein tyrosine phosphatases.
II.5.3 Players other than protein phosphatases are identified to modify the Sma/Mab pathway activity

Though we started our RNAi screen using a Caenorhabditis elegans protein phosphatase RNAi library, we found some of the genes assigned to the library are not real protein phosphatases. For example, we identified *sri-31* as a novel modulator in the Sma/Mab pathway. *sri-31* encodes an ortholog of class I serpentine receptor (or guanine nucleotide-binding protein coupled receptor). It transduces an extracellular signal through interaction with a guanine nucleotide-binding (G) protein and adopts a framework comprising seven transmembrane(TM) helices. Another candidate was H02F09.4, which is annotated as a transposon and believed to have no phosphatase activity.

In short, our screening data and further experiments to characterize the protein phosphatases might supplement or curate the existing information of *Caenorhabditis elegans* genome dataset, therefore providing the investigators some more reliable source to facilitate their study.
Chapter III. PPM-1 (Protein Phosphatase Magnesium^{2+}/manganese^{2+}-dependent-1) regulates body size by modulating Sma/Mab pathway activity

III.1 Abstract

**Background:** The transforming growth factor β (TGFβ) signaling cascade activates diverse cellular responses in normal development events in metazoans. It has also been shown to be involved in the pathogenesis of many human diseases. Reversible phosphorylation is a hot spot to investigate in the last decades, which has proven to be an essential regulatory mechanism to modulate the TGFβ signaling pathway. In *Caenorhabditis elegans*, a conserved TGFβ pathway, the Sma/Mab pathway, controls body size and male tail patterning. To identify and characterize novel protein phosphatases involved in this pathway, we carried out a library-wide RNAi screen using *Caenorhabditis elegans* as a model system followed by genetic and molecular biological studies.

**Results:** Several lines of evidence indicate that the protein phosphatase magnesium^{2+}/manganese^{2+}-dependent-1 (PPM-1) plays a role as a positive regulator of the Sma/Mab pathway in body size control. Epistatic analyses placed it downstream of the DBL-1 ligand and its type I receptors.

**Conclusion:** The PPM-1 protein regulates *Caenorhabditis elegans* body size in a manner dependent on the Sma/Mab pathway.
III.2 Introduction

The transforming growth factor β (TGFβ) superfamily comprises a large number of secreted peptide growth factors: the TGFβ/Activin/Nodal/growth and differentiation factors (GDF) subfamily, and the bone morphogenetic proteins (BMPs) subfamily (Massague and Chen, 2000; Roberts and Sporn, 1993). Members of this superfamily activate a broad range of cellular responses in metazoans, such as cell growth, differentiation, extracellular matrix (ECM) remodeling, and embryonic development (Whitman and Raftery, 2005). Increasing evidence also reveals the involvement of this pathway in the pathogenesis of cancer, fibrotic, cardiovascular, and autoimmune diseases (Akhurst, 2004; Waite and Eng, 2003). In spite of the diverse outcomes of this pathway, the central signaling cascade is rather simple and well established. In response to TGFβ superfamily ligands, a hetero-tetrameric receptor complex is formed at the cell surface comprising the type I receptor and the type II receptor (Liu et al., 1995; Wrana et al., 1992). Type II receptors directly activate type I receptors by phosphorylating key serine residues in the latter’s GS domain. Receptor-regulated Smads (R-Smads) are then phosphorylated in their C-terminal SXS motif by the active type I receptor (Kretzschmar et al., 1997; Macias-Silva et al., 1996). Phosphorylation of R-Smads promotes the heterotrimeric complex formation with Co-Smads and their accumulation in the nucleus to regulate downstream gene expression (Inman and Hill, 2002; Kretzschmar et al., 1997; Lagna et al., 1996; Wu et al., 1997; Zhang et al., 1996). It is thought that the multiple diverse outcomes of this signaling pathway might in part rely on the participation of transcriptional cofactors, such as SHN, a large zinc finger transcription cofactor (Dai et al., 2000; Feng and Derynck, 2005; Marty et al., 2000). Recent studies also demonstrate the cell-specific master transcription factors determine the genes that
Smad1/2/3 bind to and thus regulate the cell-specific responses of TGFβ signaling (Mullen et al., 2011; Trompouki et al., 2011). However, the existence of transcriptional cofactors cannot fully explain the diverse, context-dependent activities of TGFβ pathway.

The Smad proteins consist of two conserved globular domains (MH1 and MH2 domains) connected by a linker region (Shi and Massagué, 2003). The phosphorylation of the two C-terminal serine residues by type I receptor is essential to maintain the activity of TGFβ pathway. In contrast, MAPK phosphorylation of the linker region somehow inhibits Smad1 activity (Aubin et al., 2004; Kretzschmar et al., 1997; Pera et al., 2003). It is further demonstrated that linker phosphorylation restricts Smad1 activity by enabling Smad1 recognition by the HECT-domain ubiquitin ligase Smurf1 (Sapkota et al., 2007). Sapkota and coworkers also showed evidence that the inhibitory Smad1 linker phosphorylation can be induced not only by mitogens and cellular stresses, but also by the BMP pathway itself (Sapkota et al., 2006). Similar findings were made with Smad2 as a target of mitogens and Ras oncogene activation (Grimm and Gurdon, 2002; Kretzschmar et al., 1999). Taken together, the linker phosphorylation of Smad proteins provides feed-back control to TGFβ signaling, therefore modulating this pathway to fulfill its diverse physiological roles.

Reversible phosphorylation, executed by kinases and phosphatases, constitutes an essential regulatory mechanism in all living organisms. Recent studies have identified a plethora of protein serine/threonine phosphatases (PSPs) modulating TGFβ pathway. For instance, the phosphatase PPM1A antagonizes TGFβ signaling by dephosphorylating the very C-terminus of all R-Smads (Duan et al., 2006; Lin et al., 2006). The pyruvate dehydrogenase phosphatase (PDP) and the small C-terminal domain phosphatases (SCPs) modulate BMP signaling by dephosphorylating mammalian and Drosophila Smad 1/5 at the C-terminal SSXS motif (Chen et
al., 2006; Knockaert et al., 2006). In vertebrates, different B subunits of PP2A demonstrate distinct roles in the regulation of TGFβ/Activin/Nodal signalling: Bα enhances signaling by stabilizing the basal levels of type I receptor, whereas Bδ negatively regulates these pathways by restricting receptor activity (Batut et al., 2008). Whereas, the Bβ subunit regulates BMP signaling by dephosphorylating both the C-terminus and linker region of Smad1, with the linker region as the preferred site, therefore leading to an overall amplification of BMP signaling (Bengtsson et al., 2008). The regulatory subunit of PP1, GADD34, interacts with the catalytic subunit of PP1 to dephosphorylate TGFβI, with or without the assistance of inhibitory Smads, Smad6 or Smad7, attenuating the TGFβ signaling cascade (Shi et al., 2004).

In the nematode Caenorhabditis elegans, the BMP-related factor DBL-1 regulates body size and male tail morphogenesis via a conserved receptor/Smad signaling pathway, the Sma/Mab pathway (Gumienny and Savage-Dunn, 2013). The genome of Caenorhabditis elegans is fully sequenced and one can carry out large-scale RNAi screens in Caenorhabditis elegans simply by feeding, Caenorhabditis elegans provides an easy-to-use system to identify novel players within signal pathways in vivo. In our search for novel protein phosphatases modulating the Sma/Mab pathway, we found a protein serine/threonine phosphatase PPM-1 (protein phosphatase Mg\(^{2+}/\)Mn\(^{2+}\)-dependent-1). PPM-1 belongs to the metal-dependent protein phosphatase (PPM) family. The PPM family includes protein phosphatases dependent on magnesium/manganese ions, such as PP2C and PDP. Members of this family do not have regulatory subunits but contain characteristic C-terminal or N-terminal (or both) extensions that may help to determine substrate specificity (Shi, 2009). Here we present genetic and functional evidence that PPM-1 acts as a positive regulator of the Sma/Mab pathway.
III.3 Materials and Methods

Strains and Caenorhabditis elegans protein phosphatase RNAi clones

*C. elegans* strains were cultured and maintained using standard methods at 20°C unless otherwise noted (Brenner, 1974). The following strains were used: N2 Bristol (wild type), LG II: rrf-3(pk1426), sma-6(e1482), sma-6(wk7); LG III: sma-2(e502), sma-3(wk30); LG V: dbl-1(wk70), ppm-1/tag-93(ok578), ppm-1/tag-93(tm653); LG X: ctl1s40 (DBL-1 over-expressing strain), and lon-2(e678).

LW2286 (*lon-2(e678)*X; *jjls2277*), LW2308 (*dbl-1(wk70)*V; *jjls2277*), and LW2436 (*jjls2277*) (gifts from Jun Kelly Liu, Cornell University) were used in RAD-SMAD reporter assay. JY2 (*qcIs54*(col-41p::2xNLS::mCherry; myo-2p::gfp)) (Yin et al., unpublished data) was used in a secondary reporter assay.

Bacterial clones from the *Caenorhabditis elegans* phosphatase RNAi library were purchased from Source BioScience Life Science. All bacterial clones were grown on LB agar plates supplemented with carbenicillin (25 µg/ml) and tetracycline (12.5 µg/ml) right before use.

**RNAi feeding**

RNAi feeding was performed as described in Section II.2. Briefly, 10-12 L4 animals were transferred to feeding plates, incubated overnight, transferred to fresh feeding plates to lay eggs for 4 hours and the stage-synchronized progeny were scored. For the body size phenotype, RNAi hypersensitive *rrf-3* mutants were used and the resulting synchronized progeny were scored at the young adult stage.
Analysis of body size measurements:

The body length of the resulting progeny was measured using Image-Pro Express 5.1.0.12 software. Student t-test was done to analyze the significance of the data (p<0.01).

Epistatic study

To investigate the genetic interaction with the known core components in Sma/Mab pathway, we carried out an epistatic study in the following mutant backgrounds: a strong loss-of-function type I receptor mutant (sma-6(wk7)), a weak loss-of-function type I receptor mutant (sma-6(e1482)), a loss-of-function mutant in a negative regulator of this Sma/Mab pathway (lon-2), a strain that over-expresses the TGFβ ligand, DBL-1, (dbl-1(ctIs40)), and two SMA protein mutant backgrounds: sma-2(e502)III, and sma-3(wk30)III.

The mutant worms were fed on ppm-1/tag-93 RNAi clones as described in RNAi feeding part. The resulting 72 hour-old progeny were photographed and the body length was analyzed.

Reporter assay

To determine whether ppm-1 regulates body size directly through the Sma/Mab pathway or through interactions with other components in this signal cascade, reporter assays were carried out using two reporter constructs: RAD-SMAD reporter (Tian et al., 2010) and col-41p::2xNLS::mCherry (Yin et al., unpublished data). Briefly, 10-12 L4 animals were transferred to feeding plates seeded with the appropriate protein phosphatase RNAi clones, incubated overnight, and transferred to fresh feeding plates to lay eggs for 4 hours. The synchronized progeny were then scored at L2 stage when the expression of RAD-SMAD reporter in hypodermis becomes predominant. The col-41p::2xNLS::mCherry reporter activity was checked
in L3 and early L4 stage when the strongest expression was observed in hyperdermal nuclei.

**Molecular cloning and Sequencing**

To study whether *ppm-1* has conserved function among species, the *Xenopus* homolog of *ppm-1*, *ppm1b*, was identified by blasting the PPM-1 protein sequence in Xenbase (www.xenbase.org). A full-length cDNA clone of *Xenopus tropicalis ppm1b* was purchased from Source Bioscience Lifesciences (Product ID:IRBNp992H1213D). The cDNA insert was amplified using the following primer pair with SalI restriction site introduced in the 5’ end and NotI restriction site added in the 3’ end. The underlined sequences indicate the corresponding restriction sites.

Forward primer: 5’ CGGTCGACATGGGGCATTTTTGGAC
Reverse primer: 5’ GTGCGGCCGCTACCAGGGGTCTCTTCTAG

The amplified PCR products were cloned into an expression vector, pCS2++. The resulting recombinant plasmid was then confirmed by sequencing and subject to further investigation by Dr. Daniel Weinstein’s lab in a collaborative project.
III.4 Results

III.4.1 PPM homologs regulate *Caenorhabditis elegans* body size

In the nematode *Caenorhabditis elegans*, a conserved TGFβ signaling pathway, the Sma/Mab pathway, regulates body size and male tail morphogenesis (Savage-Dunn, 2013). We hypothesized that protein phosphatases modulating this pathway might contribute to a body size phenotype upon RNAi. In our primary screen using the *Caenorhabditis elegans* protein phosphatase RNAi library and *rrf-3* RNAi-hypersensitive mutant, we identified 4 homologs of human PPM1A including *ppm-1* (formerly *tag-93*) that is discussed through this chapter.

*ppm-1*, *ppm-2*, *fem-2*, and the uncharacterized C42C1.2 encode members of the PPM family. When knocked down by RNAi, all four RNAi clones lead to a Sma phenotype in the *rrf-3* hypersensitive background (Figure 3.1).

![Figure 3.1](image_url)

**Figure 3.1** Distinct PPM family members play a role in *C. elegans* body size regulation. *:* tested by student t-test, p<0.01. Error bar: standard error. N (worms scored per RNAi treatment per experiment) ≥30.
Among these four genes, only \( ppm-1 \) regulated a Sma/Mab pathway transcriptional reporter, the RAD-SMAD reporter (Table 3.2), so we focused our studies on the \( ppm-1 \) gene.

### III.4.2 PPM-1 (formerly named as \( tag-93 \)) acts as a potential positive regulator in body size control

In our primary screen, we found that \( ppm-1 \), temporarily named \( tag-93 \), caused a significant body length decrease. In three independent RNAi experiments, the body length of \( rrf-3 \) worms upon \( ppm-1/tag-93 \) RNAi was decreased by 4.9-8%, respectively (\( p<0.01 \), Table 3.1).

The protein product of \( ppm-1/tag-93 \) is conserved with three human PP2C protein phosphatases, \( \text{PP2C}\alpha/\text{PPM1A}, \text{PP2C}\beta/\text{PPM1B}, \) and \( \text{PPM1G} \) (\( \text{F25D1.1a} \): 49% identity and 69% similarity with \( \text{PPM1A} \); 54% identity and 72% similarity with \( \text{PPM1B} \); 40% identity and 61% similarity with \( \text{PPM1G} \)) (Stern et al., 2007; wormbase blast result). Based on this protein phosphatase homology, \( tag-93 \) was renamed as \( ppm-1 \) (\( \text{protein phosphatase magnesium}^{2+}/\text{manganese}^{2+}\)-dependent-1) (Tulgren et al., 2011).

Three open reading frames of \( ppm-1 \) are predicted in the \( \text{Caenorhabditis elegans} \) genome: \( \text{F25D1.1a}, \text{F25D1.1b}, \) and \( \text{F25D1.1c} \) (www.wormbase.org). \( \text{F25D1.1a} \) encodes a protein sequence of 464-amino-acid which has an N-terminal extension besides the predicted catalytic domain and the C-terminal regulatory domain. Whereas \( \text{F25D1.1b} \) and \( \text{F25D1.1c} \) encode a protein sequence of 367-amino-acid and a protein sequence of 385-amino-acid, respectively. Both \( \text{F25D1.1b} \) and \( \text{F25D1.1c} \) lack the N-terminal extension. All predicted ORFs have the two conserved aspartic acid (D) residues which are essential to maintain the protein phosphatase activity. Mutagenesis studies on human PP2C\( \alpha \) showed that mutations of residue D60 and D239 led to 900- and 4000-fold decreases in phosphatase activity, respectively (Jackson et al., 2003).
PPM-1 (protein phosphatase Magnesium$^{2+}$/Manganese$^{2+}$-dependent-1) is a protein serine/threonine phosphatase, belonging to the PPM family. Members of this family do not have regulatory subunits but contain instead additional domains and conserved motifs that may confer substrate specificity. PPM-1 is conserved in diverse species. As seen in Figure 3.1, Caenorhabditis elegans PPM-1 proteins have the highest homology with human PPM1G. Compared with human PPM1B, Caenorhabditis elegans PPM1 is more closely related with the human PPM1A.

We then tested the body size phenotype with two genetic mutant alleles of *ppm-1: ok578* and *tm653*. *ok578* has a 984 bp deletion and two thymidine insertion, which causes a frameshift resulting in a loss of sequence after amino acid 69. Importantly, it also causes the deletion of a key catalytic residue D246 (corresponding to human PP2Cα D239). The deletion allele *tm653* has a 1089 bp deletion which spans a portion of the promoter region, the start codon, and the first 156 amino acids including a catalytic residue D62 (corresponding to human PP2Cα D60). Therefore, both *ok578* and *tm653* are believed to be molecular null mutants. Surprisingly, we found that both alleles did not show any body size change at the young adult stage when compared with wild type (N2 Bristol) worms (data not shown). However, the two deletion alleles we used in our initial body size examination are not outcrossed, therefore we cannot rule out the possibility that other undesirable mutations in the strains masked the effects of the *ppm-1* mutations, leading to an undetectable body size change.

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<th>ppm-1 RNAi, μm</th>
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Table 3.1 *ppm-1* positively regulates body length in *rrf-3* RNAi-hypersensitive mutant background
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<td>1153.04±44.39</td>
<td>-8.02</td>
<td>2.39E-13</td>
</tr>
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</table>

1Body length is measured in µm ± standard deviation at 72 hours after egg collection.
2Control RNAi: *rrf*-3 worms are fed on HT115 bacteria containing only L4440 RNAi feeding vector.
3At least 30 worms are measured per sample.

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**Figure 3.2 ppm-1 is conserved among different species.** The phylogenetic tree is built using Clustal Omega software from EBI website. Protein sequences are obtained from NCBI database. The protein IDs used are listed as below. *C. elegans* PPM-1 isoforms: WP:CE05722, WP:CE36134, WP:CE41642; *C. elegans* PPM homologs: WP:CE02878, WP:CE24009, WP:CE42705; *Homo sapiens* PPM1A isoforms: NP_066283.1, NP_808820.1, NP_808821.2; *Homo sapiens* PPM1B isoforms: NP_002697.1, NP_808907.1, NP_808908.1, NP_001028728.1, NP_028729.1, AAY89642.1, O15355.1; *Xenopus laevis* PPM1B isoforms: NP_001085342.1, NP_001085063.1.
The Sma/Mab pathway plays a major role in body size regulation in *C. elegans*. Mutations in any components of the pathway, *dbl-1* (ligand), *sma-6* (type I receptor), *daf-4* (type II receptor), *sma-2, sma-3, sma-4* (Smad transcription factors), *sma-9* (transcription co-factor) result in smaller bodies than wild type (Estevez et al. 1993; Krishna et al. 1999; Liang et al. 2003; Savage-Dunn 2013; Savage-Dunn et al. 2003; Savage et al. 1996; Suzuki et al. 1999).

In our primary screen for novel players in Sma/Mab pathway, we found that depletion of *ppm-1* by RNAi, the *rrf-3* worms exhibited a Sma phenotype as seen in core Sma/Mab component mutants. I next determined whether the *ppm-1* regulates body size in parallel to the Sma/Mab pathway, or if it directly acts on the Sma/Mab components using epistatic analysis. Epistasis analysis was performed using different genetic backgrounds that were wild-type for *rrf-3*, so we first established whether *ppm-1(RNAi)* has an effect on N2. In our assay conditions, *ppm-1(RNAi)* did not reduce the average length of N2 (Figure 3.3), so negative results obtained in other genetic backgrounds must be considered inconclusive.

I performed epistasis analysis with the *dbl-1* overexpression phenotype using *dbl-1(ctIs40)* transgenic worms. Overexpression of *dbl-1* results in a long (Lon) body phenotype. The Lon phenotype of *dbl-1* overexpression is suppressed in *sma-2, sma-3, sma-4, sma-6* or *daf-4* mutant backgrounds, placing the activity of these small (Sma) genes downstream of the DBL-1 ligand activity (Suzuki et al. 1999). If the *ppm-1* gene activity were downstream of DBL-1, while depleted by RNAi, we would expect a suppression of the Lon phenotype as observed with the known small (Sma) genes in this pathway. I fed *dbl-1* overexpressing worms on *ppm-1* RNAi plates. However, rather than the expected suppression of the Lon phenotype, the body length did not change significantly (Figure 3.3).
I next examined the RNAi effect on the lon-2(e678) mutant background. LON-2 is a conserved member of the glypican family of hepanan sulfate proteoglycans, a family with several members known to regulate growth-factor signaling in many organisms. Epistasis tests with Sma/Mab pathway component mutants: dbl-1(wk70) and sma-6(wk7) placed lon-2 as a negative upstream regulator of this pathway (Gumienny et al., 2007). lon-2 mutations produce the dbl-1 overexpression body size phenotype. Animals are 14-26% longer than wild type worms. Upon ppm-1 RNAi, lon-2 worms show significant body length decrease (3.14% decrease compared with worms fed on control RNAi, Figure 3.3, p<0.01). The suppression of lon-2 Lon body size phenotype placed ppm-1 downstream of lon-2. Since LON-2 acts at the level of ligand-receptor interactions, PPM-1 might act downstream of the ligand-receptor level.

I next examined the effect of ppm-1 on sma-6(wk7), a strong loss-of-function allele of sma-6, by feeding the mutant worms with ppm-1 ds-RNA expressing bacteria, and observed no significant body size change in the resulting progeny (Figure 3.3). This suggests that ppm-1 might be involved directly in the Sma/Mab pathway. Otherwise, we should have observed an additive body size phenotype upon ppm-1 RNAi. The same result was found when tested with sma-6(e1482), a weak loss-of-function allele of sma-6 (Figure 3.3). However, it is a little bit surprising that we did not find any enhanced Sma phenotype under the sma-6(e1482) background upon ppm-1 RNAi, since this allele only partially reduces Sma/Mab signaling.

Recent studies pointed out that human PPM1A/PP2Cα terminates TGFβ signaling through dephosphorylation of Smad2/3 and facilitate the nuclear export of dephosphorylated Smad2/3 (Lin et al., 2006). As ppm-1 is conserved with human PPM1A/PP2Cα (49% identity and 69% similarity), we were curious about the genetic interaction between ppm-1 and SMA proteins in Caenorhabditis elegans. Double knock-down experiments were carried out by
investigating smo-2 and smo-3 mutants upon ppm-1 RNAi. The body size phenotype of the resulting progeny was scored. Surprisingly, RNAi against ppm-1 only led to the enhanced Smo phenotype in smo-2(e502) mutants (p<0.01, Figure 3.3). In contrast, the body size of smo-3(wk30) animals did not exhibit any significant change upon ppm-1 RNAi (Figure 3.3).

Taken together, these epistasis tests suggest that ppm-1 genetically interacts with core Smo/Mab pathway components. One possible regulatory mechanism requires the presence of intact SMA-3.

**Figure 3.3 ppm-1 genetically interacts with Smo/Mab pathway components.** Epistasis studies revealed that ppm-1 regulates *C. elegans* body size via the Smo/Mab pathway. * indicates that the mutant alleles tested showed a significant decrease in body length upon ppm-1 RNAi (analyzed by student t-test, p<0.01). Control worms were fed on HT115 bacteria containing only L4440 feeding vector. Error bar: standard error. N (worms scored per RNAi treatment per experiment) ≥30.
III.4.4 *ppm-1* acts through Sma/Mab pathway to regulate *Caenorhabditis elegans* body size

Our previous epistatic studies have shown that *ppm-1* genetically interacts with Sma/Mab pathway components. The results suggest that *ppm-1* might act as a positive regulator downstream of DBL-1 ligand and its receptors to modulate the Sma/Mab pathway activity. We also figured out that the downregulation of *ppm-1* leads to an enhanced Sma phenotype in a *sma*-2 mutant background but not in *sma*-3 mutants, suggesting the involvement of SMA-3 protein in the normal function of PPM-1.

To further validate our conclusion that *ppm-1* modulates *Caenorhabditis elegans* body size through the Sma/Mab signal cascade, we carried out reporter assays using two reporter constructs: RAD-SMAD reporter (gift from Jun Kelly Liu, Cornell University) and *col-41* promoter-driving fluorescent reporter (*col-41p::2xNLS::mCherry; myo-2p::gfp*) (Yin et al., unpublished data). The RAD-SMAD reporter is an efficient research tool used to identify novel players in the Sma/Mab pathway because of its special design as discussed in Tian’s original research paper (Tian et al., 2010). *col-41* is confirmed as a target of Sma/Mab pathway in previous studies (Liang et al., 2003; Savage-Dunn C, 2011).

We hypothesized that if *ppm-1* really plays as a positive regulator of the Sma/Mab pathway, downregulation of *ppm-1* would result in a decrease of both RAD-SMAD and *col-41* promoter-fused reporter activity. We then carried out the reporter assays. Upon *ppm-1* RNAi, worms integrated with RAD-SMAD reporter or *col-41* promoter-driven reporter demonstrated a significant decrease in reporter activity (Figure 3.4). This is consistent with our previous epistasis study.
III.4.5 Establishment of expression constructs used in Xenopus embryo assay

As we have shown in the phylogenetic tree (Figure 3.2), ppm-1 is a conserved PPM family member among species. We wondered whether Xenopus PPM-1 homologs function in a similar way as we have discovered in Caenorhabditis elegans. To address this question, I constructed a recombinant plasmid using pCS2++ expression vector as a backbone. A full-length Xenopus PPM1B cDNA insert was subcloned into pCS++vector as described in the Materials and Methods part (III.3). This cDNA was selected because when blasting the C. elegans PPM-1 protein sequence in Xenbase, the best hit was Xenopus PPM1B. The resulting plasmid named as pSX01 was then verified via sequencing and being further investigated in a collaborative project with Dr. Daniel Weinstein’s lab.

Figure 3.4 ppm-1 positively regulates RAD-SMAD and col-41 promoter-driving reporter activity. Upper panels: RAD-SMAD reporter expression upon RNAi. Worm strain used: LW2436 (jjls2277). Upper left panel: Upon control RNAi, hypodermic nuclear expression of gfp is clearly visualized. Upper right panel: ppm-1 down-regulation results in a significant decrease in hypodermal nuclear expression. Lower panels: col-41 promoter-fused mCherry reporter activity is downregulated upon ppm-1 RNAi. Lower left panel: Normal expression upon control RNAi show exactly the same pattern as RAD-SMAD reporter. Lower right panel: Weaker hypodermic nuclear expression when worms were fed on ppm-1 RNAi clones.
III.5 Discussion

III.5.1 *ppm-1* might act as a SMA-3 linker phosphatase, therefore positively regulating the body size in *Caenorhabditis elegans*

We have shown several lines of genetic and molecular biological evidence, revealing that the *C. elegans ppm-1* regulates worm body size in a manner dependent on the Sma/Mab pathway activity. Epitatic analyses with the core Sma/Mab pathway components indicate that *ppm-1* is directly involved in this signaling cascade downstream of LON-2 activity.

Whereas studies in a *sma-2* mutant and a *sma-3* mutant suggested that the intact SMA-3 protein is essential for *ppm-1* to carry out its normal physiological function. Though at this moment, we have no direct evidence to show how this PPM-1 acts on SMA-3 to fulfill its regulatory role, an analysis on the linker regions of SMAs (compared with known human Smads, Sapkota et al., 2006) revealed that different from SMA-2, SMA-3 has some putative proline-directed serine residues (Figure 3.5). This suggests that PPM-1 might serve as a Smad linker phosphatase to regulate the Sma/Mab pathway activity. Further studies are needed to elucidate the mechanism by which PPM-1 regulates the Sma/Mab pathway. More biochemical assays could be done to determine the direct substrate of PPM-1 when worm-specific anti-SMA and/or anti-phospho-SMA antibodies are available.

The two available deletion alleles of *ppm-1* (believed as molecular null mutants based on the sequence analysis compared with human PPM1A), *ok578* and *tm653*, did not show a body size phenotype when fed on regular bacterial strain, DA387. There are three possible explanations for this finding. First, these alleles do not delete the entire coding sequence, so possibly the N-terminal and C-terminal sequences of *ppm-1* might confer some compensation role and maintain the basal Sma/Mab signaling, therefore it is possible that the deletion mutants
are not functional null mutants. Second, members of the PPM family have relatively conserved catalytic domains. The RNAi clones we used on our research might also knock down other PPM family members, leading to a more severe phenotype in RNAi experiments than mutant phenotype. A third possible reason is as mentioned in the result part, since the commercially available strains we used in our initial study are not outcrossed, we cannot rule out the possibility that other undesirable mutations in the background masked the body size phenotype caused by *ppm-1* deletion.

The phylogenetic tree (Figure 3.2) indicates that though *Caenorhabditis elegans* PPM-1 is closely related with human PPM1A and PPM1B, it is evolutionarily more related to human PPM1G. Human PPM1G, a nuclear protein serine/threonine phosphatase, is shown to be a regulator of chromatin remodeling, mRNA splicing, and DNA damage (Beli et al., 2012; Kimura et al., 2006). Recent studies suggest that PPM1G is implicated in cellular survival and neural development (Foster et al., 2013). Our results suggest that it may also play a role in regulation of TGFβ signaling activity.

![Figure 3.5 Potential linker phosphorylation sites in SMA-3 but not SMA-2.](image)

: Proline-directed serines, the conserved SP phosphorylation sites, are indicated.
III.5.2 The *Caenorhabditis elegans* homologs of human PPM1A/B/G regulate body size through different mechanisms

In the epistatic studies and reporter assays, the four *C. elegans* PPM homologs exhibit different phenotypes. As summarized in Table 3.2, *fem-2* and *ppm-2* enhanced the Lon phenotype of both *dbl-1* over-expressing allele and *lon-2(e678)* mutant whereas *C42C1.2* and *ppm-1* suppressed the Lon phenotype of *lon-2(e678)* mutant, while exerting no effect on the *dbl-1* over-expressing allele phenotype (Lon by itself). In the RAD-SMAD reporter assay, only *ppm-1* shows a consistent phenotype with the epistatic research. Downregulation of *ppm-1* results in a weaker activity of RAD-SMAD reporter.

Further functional analyses are required to elucidate the roles of the distinct PPM family members in *Caenorhabditis elegans* body size regulation and growth control.

<table>
<thead>
<tr>
<th>Wormbase annotation</th>
<th><em>rrf-3</em></th>
<th><em>sma-6(wk7)</em></th>
<th><em>dbl-1(ct1s40)</em></th>
<th><em>lon-2(e678)</em></th>
<th>RAD_SMAD reporter assay</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>fem-2</em></td>
<td>Sma</td>
<td>NS</td>
<td>Enhanced Lon</td>
<td>Enhanced Lon</td>
<td>NS</td>
</tr>
<tr>
<td><em>ppm-2</em></td>
<td>Sma</td>
<td>NS</td>
<td>Enhanced Lon</td>
<td>Enhanced Lon</td>
<td>NS</td>
</tr>
<tr>
<td><em>C42C1.2</em></td>
<td>Sma</td>
<td>NS</td>
<td>NS</td>
<td>Suppressed Lon</td>
<td>NS</td>
</tr>
<tr>
<td><em>ppm-1</em></td>
<td>Sma</td>
<td>NS</td>
<td>NS</td>
<td>Suppressed</td>
<td>Weaker</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lon</td>
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</table>

| Sma: Small phenotype; NS: no significant change; Lon: Long phenotype, tested by student t-test, p<0.01 |         |         |         |       |
Chapter IV. Distinct subunits of Protein Phosphatase-1 (PP1) negatively regulate *Caenorhabditis elegans* body size partially through the Sma/Mab pathway

IV.1 Abstract

**Background:** The transforming growth factor β (TGFβ) signaling cascade is an essential signaling pathway both involved in various normal developmental events and the pathogenesis of many diseases. Reversible phosphorylation is recently revealed as a crucial regulatory mechanism to modulate this rather simple signal cascade to fulfill its diverse physiological roles. In *Caenorhabditis elegans*, a conserved TGFβ pathway, the Sma/Mab pathway, controls body size and male tail patterning. In our previous large-scale RNA screen searching for novel protein phosphatase players regulating Sma/Mab pathway, protein phosphatase-1 (PP1) was identified. We then studied the multiple PP1 subunits present in the *Caenorhabditis elegans* protein phosphatase RNAi library using genetic and molecular biological techniques.

**Results:** We demonstrate here several lines of evidence indicating that the two *Caenorhabditis elegans* homologues of human PPP1CB (phosphoprotein phosphatase-1, catalytic subunit, β isoform), *C06A1.3* and *F58G1.3*, and one homologue of human PPP1R2 (phosphoprotein phosphatase-1, regulatory subunit, isoform 2), *Y32H12A.4*, act as negative regulators of the Sma/Mab pathway in body size control. Epistatic analyses suggest that SMA-6 acts downstream of the PP1 subunit homologs.

**Conclusion:** The PP1 protein subunits negatively regulate *Caenorhabditis elegans* body size in a manner dependent on the Sma/Mab pathway activity.
IV.2 Introduction

Protein phosphatase activity plays an essential role to determine the extent and duration of the cellular protein phosphorylation, in response to physiological stimuli. As reversible phosphorylation executed by the interplay between protein kinases and protein phosphatases has been firmly established as an essential, fundamental regulatory mechanism in all eukaryotes in the past 50 years; and in the human genome, more than 420 genes encode serine/threonine kinases, which account for 98.2% of all phosphorylation events (Peti et al., 2012); protein serine/threonine phosphatases (PSPs) become extensively studied.

Protein serine/threonine phosphatases have at least three subfamilies, with the largest family being the phosphoprotein phosphatases (PPP). The PPP family members, such as protein phosphatase-1 (PP1) and protein phosphatase-2A (PP2A/PP2), have a structurally related catalytic subunit and identical reaction mechanism but a distinct set of substrates and interacting proteins. In contrast, all PPM family members, for example, protein phosphatase magnesium\(^{2+}\)/manganese\(^{2+}\)-dependent-1A (PPM1A), are single polypeptides, which execute their diverse roles, depending on splice variants and various isoforms. The FCP/SCP family is characterized by a conserved DXDX(T/V) motif that is essential for their activity and that they share with a superfamily of phosphotransferases and phosphohydralases (Chambers et al., 1994; Kamenski et al., 2004). Among them, PP1 is the most extensively studied, as it is responsible for about 30% of all dephosphorylation reactions in eukaryotic cells (Choy et al., 2012).

The metalloenzyme PP1 (~38.5 kDa, 2 Mn\(^{2+}\) are bound in the active site when expressed in bacteria) is exceptionally conserved in eukaryotes from fungi to human (Choy et al., 2012). The mammalian genome contains three different genes that encode four distinct catalytic PP1 subunits: PP1\(\alpha\), PP1\(\beta/\delta\) and the splice variants PP1\(\gamma\)1 and PP1\(\gamma\)2. The primary sequence
differences among PP1 catalytic units are localized to the N- and C-termini. Free PP1 catalytic subunits have essentially no substrate specificity. \( \geq 200 \) PP1-interacting proteins (PIPs) are identified in vertebrates (Heroes et al., 2013). These proteins target distinct PP1 holoenzymes to particular cellular compartments, confer substrate specificity, and sometimes themselves serve as substrates of PP1. PIPs include PP1 regulatory subunits and inhibitor proteins. \( \geq 70\% \) of these regulatory proteins of PP1 belong to the class of IDPs (intrinsically disordered proteins) including I-2 (inhibitor 2, which will be discussed in this study) (Choy et al., 2012).

The transforming growth factor \( \beta \) (TGF\( \beta \)) signaling cascade controls a broad range of cellular responses in metazoans, such as cell growth, differentiation, extracellular matrix (ECM) remodeling and embryonic development (Whitman and Raftery, 2005). This signaling pathway is also involved in the pathogenesis of human diseases, for instance, cancer, fibrotic, cardiovascular, and autoimmune diseases (Akhurst, 2004; Waite and Eng, 2003). The canonical signaling is mediated via TGF\( \beta \)-induced phosphorylation receptor-activated Smads at the C-terminal SXS motif. The TGF\( \beta \) ligand superfamily comprises two families of secreted peptide growth factors: the TGF\( \beta \)/Activin/Nodal/growth and differentiation factors (GDF) subfamily, and the bone morphogenetic proteins (BMPs) subfamily (Massague and Chen, 2000; Roberts and Sporn, 1993). Upon TGF\( \beta \) ligand binding, a hetero-tetrameric receptor complex is formed at the cell surface comprising the type I receptor and the type II receptor (Liu et al., 1995; Wang et al., 1992). Type II receptors directly activate type I receptors by phosphorylating key serine residues in the latter’s GS domain. Receptor-regulated Smads (R-Smads) are then phosphorylated in their C-terminal SXS motif by the active type I receptor (Kretzschmar et al., 1997; Macias-Silva et al., 1996). Phosphorylation of R-Smads promotes heterotrimeric complex formation with Co-Smads and accumulation in the nucleus to regulate downstream gene
expression (Inman and Hill, 2002; Kretzschmar et al., 1997; Lagna et al., 1996; Wu et al., 1997; Zhang et al., 1996). It is thought that the multiple diverse outcomes of this signaling pathway might in part rely on the participation of extra transcriptional cofactors, such as SHN, a large zinc finger transcription cofactor (Dai et al., 2000; Feng and Derynck, 2005; Marty et al., 2000). Recent studies suggest that cell-specific master transcription factors also contribute to the cell-specific responses of TGFβ signaling (Mullen et al., 2011; Trompouki et al., 2011). However, the existence of transcriptional cofactors cannot fully explain the diverse, context-dependent activities of TGFβ pathway.

The well-established kinase-mediated regulatory mechanisms of the TGFβ signaling imply that reversible phosphorylation might play a crucial role in the modulation of the TGFβ pathway activity. Recent studies have identified a plethora of protein serine/threonine phosphatases (PSPs) that modulate the TGFβ pathway, such as protein phosphatase magnesium$^{2+}$/manganese$^{2+}$-dependent-1A (PPM1A), the pyruvate dehydrogenase phosphatase (PDP), and the small C-terminal domain phosphatase (SCP) (Chen et al., 2006; Duan et al., 2006; Knockaert et al., 2006; Lin et al., 2006). Some members of the major family of protein serine/threonine phosphatases, PPP family, are also found to play a role in the regulation the TGFβ pathway, such as Bα, Bβ, and Bδ subunits of PP2A (Batut et al., 2008; Bengtsson et al., 2008). Increasing evidence shows the involvement of various regulatory subunits of another major PPP family member, PP1, in the regulation of TGFβ pathway (Shi et al., 2004; Han et al., 2012). The regulatory subunit, GADD34, interacts with the catalytic subunit of PP1 to dephosphorylate TGFβI with or without the assistance of inhibitory Smads, Smad6 or Smad7, attenuating the TGFβ signaling cascade (Shi et al., 2004). Inhibitor 5 of protein phosphatase 1
(IPP5) enhances transforming growth factor-β (TGF-β)/Smad signaling in a PP1-dependent manner (Han et al., 2012).

In the nematode *Caenorhabditis elegans*, the BMP-related factor DBL-1 regulates body size and male tail morphogenesis via a conserved receptor/Smad signaling pathway, the Sma/Mab pathway (Savage-Dunn, 2013). In our previous large-scale RNAi screen to search for novel protein phosphatases modulating Sma/Mab pathway, we found two orthologs of human PP1 catalytic unit, β isoform (PPP1CB) and one ortholog of various PP1 regulatory (inhibitory) subunit 2 proteins (PPP1R2). Here we present genetic and functional evidence that the orthologs of human PP1 catalytic subunit and that of human PPP1R2 protein negatively regulate *Caenorhabditis elegans* body size partially through the Sma/Mab pathway.
IV.3 Materials and Methods

Strains and *Caenorhabditis elegans* protein phosphatase RNAi clones

*C. elegans* strains were cultured and maintained using standard methods at 20°C unless otherwise noted (Brenner, 1974). The following strains were used: N2 Bristol (wild type), LG II: *rrf-3(pk1426), sma-6(e1482), sma-6(wk7)*; LG III: *sma-2(e502), sma-3(wk30)*; LG V: *dbl-1(wk70)*; LG X: *ctlIs40* (DBL-1 over-expressing strain), *lon-2(e678)*.

LW2286 (*lon-2(e678)X; jjls2277*), LW2308 (*dbl-1(wk70)V; jjls2277*), and LW2436 (*jjls2277*) (gifts from Jun Kelly Liu, Cornell University) were used in RAD-SMAD reporter assay. JY2 (*qcIs54(col-41p::2xNLS::mCherry; myo-2p::gfp*) (Yin et al., unpublished data) was used in a secondary reporter assay.

Bacterial clones from the *Caenorhabditis elegans* phosphatase RNAi library were purchased from Source BioScience Life Science. All bacterial clones were grown on LB agar plates supplemented with carbenicillin (25 µg/ml) and tetracycline (12.5 µg/ml) right before use.

RNAi feeding

RNAi feeding was performed as described in Section II.2. Briefly, 10-12 L4 animals were transferred to feeding plates, incubated overnight, transferred to fresh feeding plates to lay eggs for 4 hours and the stage-synchronized progeny were scored. For the body size phenotype, RNAi hypersensitive *rrf-3* mutants were used and the resulting synchronized progeny were scored at the young adult stage.

Analysis of body size measurements:
The body length of the resulting progeny was measured using Image-Pro Express 5.1.0.12 software. Student t-test was done to analyze the significance of the data (p<0.01).

**Epistatic study**

To investigate the genetic interaction with the known core components in Sma/Mab pathway, we carried out epistatic study in the following mutant backgrounds: a strong loss-of-function type I receptor mutant \((sma-6(wk7))\), a weak loss-of-function type I receptor mutant \((sma-6(e1482))\), a loss-of-function mutant in a negative regulator of this Sma/Mab pathway \((lon-2)\), in a strain that over-expresses the TGFβ ligand, DBL-1, \((dbl-1(ctls40))\), and in two SMA protein mutant backgrounds: \(sma-2(e502)\)\(III\), and \(sma-3(wk30)\)\(III\).

The mutant worms were fed on corresponding \(pp1\) RNAi clones as described in RNAi feeding part. The resulting 72 hour-old progeny were photographed and the body length was analyzed.

**Reporter assay**

To determine whether the subunits of \(pp1\) regulates body size directly through Sma/Mab pathway or through interaction with components in this signal cascade, reporter assays were carried out using two reporter constructs: RAD-SMAD reporter (Tian et al., 2010) and \(col-41p::2xNLS::mCherry\) (Yin et al., unpublished data). Briefly, 10-12 L4 animals were transferred to feeding plates seeded with appropriate protein phosphatase RNAi clones, incubated overnight, and transferred to fresh feeding plates to lay eggs for 4 hours. The synchronized progeny were then scored at L2 stage when the expression of RAD-SMAD reporter in hypodermis becomes predominant. The \(col-41p::2xNLS::mCherry\) reporter activity was checked in L3 and early L4.
stage when the strongest expression was observed in hyperdermal nuclei.

**Molecular cloning and Sequencing**

To study whether the subunits of *pp1* have conserved functions among different species, the *Xenopus* homologs were identified by blasting the corresponding *Caenorhabditis elegans* protein sequence in Xenbase (www.xenbase.org). The full-length cDNA clones of *Xenopus* homologs were purchased from Source Bioscience Lifesciences (Product ID: IRBH90A1229D, IRBH90F022D, IRBH90B0789D). The cDNA inserts were cut with Sall and NotI restriction enzymes, gel-purified and re-ligated into the expression vector, pCS2++. The resulting recombinant plasmids were then confirmed by sequencing and subject to further investigation by Dr. Daniel Weinstein’s lab in a collaborative project.
IV.4 Results

IV.4.1 Subunits of PP1 act as potential negative regulators in body size control

In the nematode *Caenorhabditis elegans*, a conserved TGFβ signaling pathway, the Sma/Mab pathway, regulates body size and male tail morphogenesis (Savage-Dunn, 2013). We hypothesized that protein phosphatases modulating this pathway might contribute to a body size phenotype upon RNAi. In our primary screen using *rrf*-3 RNAi-hypersensitive mutant, we found that three genes, *C06A1.3*, *F58G1.3*, and *Y32H12A.4*, caused a significant body length increase upon RNAi (Figure 4.1). Knocking down *C06A1.3*, *F58G1.3*, and *Y32H12A.4* led to a body size increase in *rrf*-3 hypersensitive mutant worms by 14.2%, 11.1%, and 5.3%, respectively.

Among the three, *C06A1.3* and *F58G1.3* encode orthologs of human PP1 catalytic subunit, β isoform (47% identity and 65% similarity with human PPP1CB; 46% identity and 65% similarity with human PPP1CB; respectively) (Figure 4.2). The protein product of *Y32H12A.4* is conserved with human PP1 regulatory (inhibitory) subunit 2 protein (PPP1R2) (41% identity and 59% similarity with human PPP1R2). As indicated in the phylogenetic tree in Figure 4.2, the catalytic subunits of PP1 identified from our primary large-scale RNAi screen are conserved among metazoans; which suggests that they may function in a conserved manner. The multisequence alignment of *C. elegans*, *Xenopus*, and human IPP-2s suggest the conservation of their physiological function (Figure 4.3).

PP1 (protein phosphatase 1) is a protein serine/threonine phosphatase, belonging to the PPP family. Functional members of this family consist of a catalytic subunit and a regulatory subunit. In contrast to various regulatory subunits, the catalytic subunit of PP1 is highly conserved among all eukaryotes and has relatively fewer isoforms in vivo. At least 200 putative PP1-binding Regulatory (R) subunits have been identified, with many more expected to be found
Analyses of known R subunits in diverse eukaryotic lineages suggest that these R subunits may target the PP1 catalytic subunit to specific cellular compartments, modulate substrate specificity, or serve as substrates themselves (Shi, 2009). Since Y32H12A.4 (RNAi) causes the same mutant phenotype as knocking down C06A1.3 and F58G1.3, our results suggest that this regulatory subunit positively regulates the activity of the catalytic subunits.

Figure 4.1 The homologs of human PPP1CB and PPP1R2 negatively regulate C. elegans body size. C06A1.3 and F58G1.3 are homologs of human PPP1CB, Y32H12A.4 is the homolog of human PPP1R2. *: Data are analyzed by student t-test, p<0.01. Error bar: standard error. N (worms scored per RNAi treatment per experiment) ≥30.
Distinct subunits of PP1 are conserved among different species. The phylogenetic tree is built using Clustal Omega on-line free software at EMBL-EBI website. *Xenopus* and human protein sequences are obtained from NCBI database. *C. elegans* protein sequences are found at wormbase.org. The protein IDs used are as follows. *C. elegans* PPP1CB homologs: WP:CE02116, WP:CE35562; *Homo sapiens* PPP1CA: NP_001008709.1; *Homo sapiens* PPP1CB: NP_002700.1; *Homo sapiens* PPP1CC: NP_001231903.1; *Xenopus* PPP1CA: NP_001120482.1; *Xenopus* PPP1CB: NP_001011467.1.
IV.4.2 The catalytic subunits and regulatory subunit of PP1 directly regulate the output of Sma/Mab pathway in SMA-responsive reporter assay

Our previous studies have shown that all three subunits of PP1 identified regulate *C. elegans* body size negatively and are conserved among species. Thus, we wanted to know whether the regulation is dependent on the Sma/Mab pathway activity or not. To address this question, I carried out reporter assays using two reporter constructs: RAD-SMAD reporter (gift from Jun Kelly Liu, Cornell University) and *col-41* promoter-fused fluorescent reporter (*col-41p::2xNLS::mCherry; myo-2p::gfp*) (Yin et al., unpublished data). RAD-SMAD reporter is an efficient research tool used to identify novel players in the Sma/Mab pathway because of its special design as discussed in Tian’s original research paper (Tian et al., 2010). COL-41 is confirmed as a target of Sma/Mab pathway in previous studies (Liang et al., 2003; Savage-Dunn C, 2011).

We hypothesized that if the three subunits of PP1 really play as negative regulators of the Sma/Mab pathway, downregulation of the subunit genes would result in increase of both RAD-SMAD and *col-41* promoter-fused reporter activity. We then carried out the reporter assays. Upon RNAi of the three subunit genes, worms integrated with RAD-SMAD reporter demonstrated a significant increase in activity (Figure. 4.4, panel B, C, D). This is consistent with these genes acting via the Sma/Mab pathway. However, when assayed with *col-41* promoter-fused reporter activity, the downregulation of all three subunit genes did not exhibit a

**Figure 4.3 Putative IPP-2s are conserved among *C. elegans, Xenopus, and human.*
The multiple sequence alignment is generated using the on-line clustal Omega software at EBI website.
detectable change in the *col-41* promoter-fused mCherry expression (data not shown). This is likely to be explained by the nature of the reporter constructs themselves. RAD-SMAD reporter has five-tandem Smad-binding site repeat that increases the responsiveness to the signal. Another possible explanation is that *col-41* might be an indirect target of Sma/Mab pathway, which is not very sensitive to fine tuning regulation of Sma/Mab pathway executed by subunits of PP1. Finally, it is possible that other compensatory mechanisms result in the normal regulation of *col-41* but not the artificial RAD-SMAD reporter.

**Figure 4.4** All three subunits of PP1 identified from primary large-scale RNAi screen negatively regulates RAD-SMAD reporter activity. Upper left panel: RAD-SMAD reporter expression upon control RNAi. Upper right panel: Upon the *C. elegans* homolog of human PPP1CB, *C06A1.3*, RNAi, hypodermal nuclear expression of *gfp* is increasing. Lower left panel: The downregulation of another *C. elegans* homolog of human PPP1CB, *F58G1.3*, RNAi, a significant increase of hypodermal nuclear expression of *gfp* is clearly increased. Lower right panel: Knocking down the *C. elegans* homolog of human PPP1R2, *Y32H12A.4*, hypodermal nuclear expression of *gfp* is also increasing.

**IV.4.3** The catalytic subunits of PP1 and the homolog of human PPP1R2 genetically interact with Sma/Mab pathway components
The Sma/Mab pathway plays a major role in body size regulation in *C. elegans*. Mutations in any components of the pathway, *dbl-1* (ligand), *sma-6* (type I receptor), *daf-4* (type II receptor), *sma-2, sma-3, sma-4* (Smad transcription factors), *sma-9* (transcription co-factor) result in smaller bodies than wild type (Estevez et al. 1993; Krishna et al. 1999; Liang et al. 2003; Savage-Dunn 2013; Savage-Dunn et al. 2003; Savage et al. 1996; Suzuki et al. 1999).

In our primary screen for novel players in the Sma/Mab pathway, we found that depletion of the homologs of human PPP1CB, C06A1.3 and F58G1.3, respectively, in the *rrf-3* mutant background, resulted in a Lon phenotype as opposed to the major Sma/Mab component mutants. Our RAD-SMAD reporter assay results are consistent with this finding, suggesting that these subunits of PP1 identified regulate body size through a Sma/Mab pathway-dependent manner. I next determined whether those structural subunits regulate *C. elegans* body size in parallel to Sma/Mab pathway or directly acts on the Sma/Mab components using epistatic analysis.

I first examined the effect of these subunits on *sma-6(wk7)*, a strong loss-of-function allele of *sma-6*, by feeding the mutant worms with the corresponding ds-RNA expressing bacteria. I observed no significant body size change in the resulting progeny, even in *F58G1.3(RNAi)*, a knockdown that causes a Lon phenotype in N2 as well as in *rrf-3* (Table 4.1). When tested with *sma-6(e1482)*, a weak loss-of-function allele of *sma-6*, upon *F58G1.3* RNAi, the Sma phenotype of *sma-6(e1482)* mutant was suppressed significantly, though not rescued to the wild-type level (Table 4.1, data not shown). Taken together, the data we obtained suggest SMA-6 is downstream of these PP1 subunits in the Sma/Mab pathway.

To further validate this result, I then looked at the *dbl-1* overexpression phenotype using *dbl-1(ct1s40)* transgenic worms. Overexpression of *dbl-1* results in a long (Lon) body phenotype. The Lon phenotype of *dbl-1* overexpression is suppressed in *sma-2, sma-3, sma-4, sma-6* or *daf-
4 mutant backgrounds, placing the activity of these small (Sma) genes downstream of the DBL-1 ligand activity (Suzuki et al. 1999). I fed dbl-1 overexpressing worms on the corresponding PP1 subunit RNAi plates. Both homologs of human PPP1CB protein, C06A1.3 and F58G1.3, enhanced the Lon phenotype of dbl-1 overexpressing transgenic worms. In contrast, the downregulation of Y32H12A.4 gene did not change the body size phenotype of this strain (Table 4.1). Since we visualized an additive body size phenotype, these data indicate that the regulation of Sma/Mab pathway by PP1 subunits might partially act through a Sma/Mab-independent manner.

I next examined the RNAi effect on lon-2(e678) mutant background. LON-2 is a conserved member of the glypican family of heparan sulfate proteoglycans, a family with several members known to regulate growth-factor signaling in many organisms. Epistasis tests with Sma/Mab pathway component mutants: dbl-1(wk70) and sma-6(wk7) placed lon-2 as a negative upstream regulator of this pathway (Gumienny et al., 2007). lon-2 mutations produce the dbl-1 overexpression body size phenotype. Animals are 14-26% longer than wild type worms. Upon C06A1.3 RNAi, lon-2 worms show significant body length increase (3.05% increase compared with worms fed on control RNAi, data not shown, p<0.01). In contrast, another homolog of human PPP1CB, F58G1.3, did not exhibit any effect on the body size phenotype of lon-2 animals. However, the downregulation of Y32H12A.4, the homolog of human PPP1R2, leads to a similar enhanced Lon phenotype in lon-2 worms. This suggests that these PP1 subunits might act partially independent of LON-2 to modulate the Sma/Mab pathway. These findings are consistent with the previous results we found in the epistatic analyses using sma-6 mutants and ctlIs40 dbl-1-overexpressing transgenic worms.

| Table 4.1 All three subunits of PP1 identified from the primary RNAi screen genetically interact with Sma/Mab pathway components | 90 |
### IV.4.4 Establishment of expression constructs used in *Xenopus* embryo assay

As we have shown in the phylogenetic tree (Figure 4.2), the catalytic subunits and the inhibitory regulatory subunit of PP1 are conserved among the selected species. Both *C06A1.3* and *F58G1.3* share a 47% sequence identity with *Xenopus* PPP1CB protein and have an overall 77% sequence similarity, which suggests their conserved physiological functions in vivo. The *Caenorhabditis elegans* homolog of human PPP1R2, *Y32H12A.4*, also shows conservation in its sequence compared with *Xenopus* counterpart (a 38% identity and an overall 65% similarity).
In our previous studies, we have presented several lines of evidence to show that the three subunits of PP1 modulate *Caenorhabditis elegans* body size in a Sma/Mab pathway-dependent manner. However, due to the inavailability of phosphor-SMA-specific antibodies, we could not show directly on worms whether these PP1 subunits use Sma/Mab components as direct substrates to regulate its activity or through an indirect mechanism. And since we are also curious about whether the protein phoshatase candidates from our screen have a conserved function among species, we have started a collaborative project with Dr. Daniel Weinstein’s lab. As mentioned in the Materials and Methods section, a series of recombinant plasmids were constructed (Table 4.2). The resulting plasmids were sequencing verified and tested in *Xenopus* embryo assay. Surprisingly, microinjection of double-strand RNAs of single PP1 subunit did not exhibit any detectable phenotype. This might be partly explained by the compensation of other catalytic subunit isoforms, which maintains the normal activity of BMP signaling within *Xenopus* embryo (data not shown). Coinjection with both catalytic and regulatory subunit ds-RNA might lead to a more predominant phenotype and is worth trying.

<table>
<thead>
<tr>
<th>Construct name</th>
<th><em>Caenorhabditis elegans</em> homolog</th>
<th><em>Xenopus</em> cDNA insert</th>
</tr>
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<tr>
<td>pCS393</td>
<td>C06A1.3</td>
<td>PPP1CB</td>
</tr>
<tr>
<td>pCS394</td>
<td>F58G1.3</td>
<td>PPP1CA</td>
</tr>
<tr>
<td>pCS395</td>
<td>Y32H12A.4</td>
<td>PPP1R2</td>
</tr>
</tbody>
</table>
IV.5 Discussion

IV.5.1 Two homologs of human PPP1CB act as negative regulators of the Sma/Mab pathway to modulate the body size in Caenorhabditis elegans

We have shown several lines of genetic and molecular biological evidence, revealing that the two C. elegans homologs of human PPP1CB, C06A1.3 and F58G1.3, negatively regulate worm body size in a manner dependent on the Sma/Mab pathway activity. Epitatic analyses with the Sma/Mab pathway components placed the two proteins upstream of SMA-6 receptor.

The suppressed Sma phenotype exhibited by *sma-6(e1482)* mutants upon the *F58G1.3* RNAi suggests that the protein products of this gene executes its growth control role through a SMA-6-dependent manner. In the strong loss-of-function allele, *sma-6(wk7)*, knocking down this gene has no effect on the mutant Sma phenotype. Taken together, intact SMA-6 is required for normal function of the *Caenorhabditis elegans* PPP1CB proteins identified in our study. This is consistent with the findings that PP1 serves as a receptor phosphatase to regulate the TGFβ signaling.

IV.5.2 The *Caenorhabditis elegans* homolog of human PPP1R2 is a negative modulator of the Sma/Mab pathway

The catalytic subunit of PP1 is regulated by the heat-stable protein inhibitor-1, its homologue DARPP-32 (dopamine-and CAMP-regulated phosphoprotein, Mr 32,000) and inhibitor-2 (Watanabe et al., 2001). Wang and his colleague showed in their study that a novel membrane protein KPI-2 (Kinase/Phosphatase/Inhibitor-2) associates with PP1C the protein phosphatase inhibitor-2 form a regulatory complex localized at the membrane (Wang et al, 2002). Neurabins recruit protein phosphatase-1 and Inhibitor-2 to the actin cytoskeleton and regulate
cell morphology (Terry-Lorenzo et al., 2002). The increasing evidence shows the importance of Inhibitor-2 (PPP1R2) in the regulation of PP1-mediated signaling.

In our study, the Caenorhabditis elegans homologue of human PPP1R2, Y32H12A.4, was shown to regulate body size through a Sma/Mab pathway-dependent manner. The epistatic analyses suggest that besides the regulation through the Sma/Mab pathway, Y32H12A.4 might also modulate body size through signaling cascades independent of Sma/Mab pathway. Upon RNAi, Y32H12A.4 enhanced the Lon phenotype of lon-2 mutants while had no effect on the dbl-1 over-expressing phenotype and the Sma phenotype of sma-6(wk7) mutants. These data suggested that in the Sma/Mab pathway, Y32H12A.4 may act upstream of the SMA-6 receptor to regulate its activity.

Although we presented several lines of genetic and molecular biological evidence and revealed the important role of the three subunits of PP1 in the regulation of Caenorhabditis elegans body size as negative modulators, the molecular mechanism of this regulatory event remains largely unkown. For instance, whether the three subunits cooperate to regulate the Sma/Mab pathway or act independently is not addressed yet. The direct substrates or potential interacting partners are not determined.

Our study has just opened a window to investigate the regulatory machinery of PP1 catalytic subunits on the Sma/Mab pathway, which in turn will further the understanding of the TGFβ signaling regulation mechanism among mammalian systems, and provide more therapeutic targets in human diseases.
Chapter V. Perspective

V.1 Functional studies to characterize the roles of PPM-1 and PP1 subunits in the regulation of Caenorhabditis elegans body size via the Sma/Mab pathway

As indicated in our studies described in previous chapters, we carried out a large-scale RNAi screen using Caenorhabditis elegans as a model system and identified a plethora of protein modifiers regulating body size (summarized in Chapter 2, Table 2.2 and Table 2.3). Some of these protein phosphatase candidates are involved in Sma/Mab pathway regulation to modulate worm growth (Chapter 2, Table 2.7). Among them, the protein Ser/Thr phosphatase (PS/TP) family was our first priority to characterize because the core event of TGFβ signaling is the ligand-induced phosphorylation of R-Smad proteins at the C-terminal SXS/T motif. The members of the PS/TP family were therefore studied. ppm-1 was shown in our study as a positive modulator controlling body size via the Sma/Mab pathway; whereas three subunits of PP1, C06A1.3, F58G1.3, and Y32H12A.4, emerged as negative regulators modulating body size in a Sma/Mab-dependent pathway (discussed in Chapters 3 and 4, respectively).

However, our study is just the initiation of the research to elucidate the possible mechanisms by which these protein Ser/Thr phosphatases regulate the Sma/Mab pathway activity. First, the direct substrate(s) of the three PS/TPs are not identified yet. Second, the temporal and spatial expression patterns of the candidates are not investigated and how they interact with the Sma/Mab pathway components require further investigation. Third, whether these PS/TPs work in a discrete manner or somehow through the crosstalk of different signaling pathways to cooperate in the regulation of the Sma/Mab pathway remains unclear and worth of
further research. Fourth, the exact position of these four PS/TPs in the Sma/Mab pathway need to be further studied using the corresponding mutants crossed with known Sma/Mab component mutants.

In summary, we identified protein phosphatases involved in the regulation of the Sma/Mab pathway. Since TGFβ signaling is conserved in all metazoans, our findings will contribute to the elucidation of the fine-tuned regulation of this signaling cascade, therefore facilitating the discovery of possible therapeutic targets in TGFβ-related human diseases, such as cardiovascular diseases, fibrotic diseases, and cancer.

V.2 Protein Tyrosine Phosphatase(s): an emerging role in the regulation of the Sma/Mab pathway

The Sma/Mab pathway is activated by the ligand-induced phosphorylation of of R-Smad proteins at the C-terminal SXS/T motif. In brief, upon DBL-1 ligand binding, the type II receptor DAF-4 phosphorylates the type I receptor SMA-6 at the serine residues in the GS domain. Then the phosphorylated type I receptor SMA-6 in turn phosphorylates R-Smads, SMA-2 and SMA-3. The active SMA-2 and SMA-3 complexes with Co-Smad SMA-4 and translocates into the nucleus to regulate Smad-responsive gene expression with or without transcriptional cofactors, such as SMA-9.

Studies have revealed that the constitutively active type II receptor requires autophosphorylation at Ser213 and Ser409 for full kinase activity and the ability to interact with and activate the type I receptor (Luo et al., 1997). The type II receptor can also
autophosphorylate on Tyr259, 336 and 424, which is thought to play a role in regulating type II kinase activity (Lewler et al., 1997). The type II receptor was also demonstrated to undergo Src-mediated phosphorylation at Tyr284 (Galliher et al., 2006; Galliher et al., 2007). In addition to the long established phosphorylation of serine and threonine residues in the GS domain, the type I receptor has also been shown to be phosphorylated at its tyrosine residues in response to TGFβ (Lee et al., 2007). Taken together, these data suggest the importance of tyrosine phosphorylation in the regulation of the TGFβ pathway.

It is not so surprising when we found out that during our primary screen, a large number of protein tyrosine phosphatases came out (summarized in Table 5.1) contributing to a body size phenotype in *rrf*-3 RNAi-hypersensitive background. With further epistatic analyses and reporter assay, we find a line of protein tyrosine phosphatases involved in the regulation of the Sma/Mab pathway. This might open up a new window of the regulatory mechanism of the TGFβ signaling. As there are many more protein tyrosine phosphatases in the human genome than protein serine/threonine phosphatases (107 and 30, respectively, Shi, 2009), we believe that the further investigation of the regulatory role of protein tyrosine phosphatases can add to the explanation why this rather simple signal cascade can control such a broad range of cellular responses.

<table>
<thead>
<tr>
<th>Table 5.1 The protein tyrosine phosphatase family members contribute to the body size control in <em>C. elegans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Body size phenotype</td>
</tr>
<tr>
<td>Sma</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Lon</td>
</tr>
<tr>
<td></td>
</tr>
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<tr>
<td></td>
</tr>
<tr>
<td>Wormbase annotation</td>
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<td>---------------------</td>
</tr>
<tr>
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</tr>
<tr>
<td>C17H12.3</td>
</tr>
<tr>
<td>C43E11.5</td>
</tr>
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</table>

Sma: small body size; Lon: long body size

Table 5.2 The protein tyrosine phosphatases play a role in the Sma/Mab pathway regulation
<table>
<thead>
<tr>
<th></th>
<th>Lon</th>
<th>NS</th>
<th>NS</th>
<th>NS</th>
<th>Stronger</th>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>Stronger</td>
<td>+</td>
</tr>
<tr>
<td>F54C8.4</td>
<td>Lon</td>
<td>NS</td>
<td>NS</td>
<td>Enhanced Lon</td>
<td>Stronger</td>
<td>ND</td>
</tr>
<tr>
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<td>Lon</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>Stronger</td>
<td>ND</td>
</tr>
<tr>
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<td>Sma</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS/a little bit weaker</td>
<td>ND</td>
</tr>
<tr>
<td>R155.3</td>
<td>Lon</td>
<td>NS</td>
<td>Enhanced Lon</td>
<td>Enhanced Lon</td>
<td>Stronger</td>
<td>+</td>
</tr>
<tr>
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<td>Lon</td>
<td>NS</td>
<td>Enhanced Lon</td>
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<td>+</td>
</tr>
</tbody>
</table>
Appendix

1. *C. elegans* ADAMTS ADT-2 regulates body size by modulating TGFβ signaling and cuticle collagen organization (Fernando et al., 2011)

2. Using RNA-mediated interference feeding strategy to screen for genes involved in body size regulation in the nematode *C. elegans* (Liang et al., 2013)
C. elegans ADAMTS ADT-2 regulates body size by modulating TGFβ signaling and cuticle collagen organization

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**A B S T R A C T**

Organismal growth and body size are influenced by both genetic and environmental factors. We have utilized the strong molecular genetic techniques available in the nematode Caenorhabditis elegans to identify genetic determinants of body size. In C. elegans, DBL-1, a member of the conserved family of secreted growth factors known as the Transforming Growth Factor β (TGFβ) superfamily, is known to play a major role in growth control. The mechanisms by which other determinants of body size function, however, is less well understood. To identify additional genes involved in body size regulation, a genetic screen for small mutants was previously performed. One of the genes identified in that screen was sma-21. We now demonstrate that sma-21 encodes ADT-2, a member of the ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) family of secreted metalloproteases. ADAMTS proteins are believed to remodel the extracellular matrix and may modulate the activity of extracellular signals. Genetic interactions suggest that ADT-2 acts in parallel with or in multiple size regulatory pathways. We demonstrate that ADT-2 is required for normal levels of expression of a DBL-1-responsive transcriptional reporter. We further demonstrate that adt-2 regulatory sequences drive expression in glial-like and vulval cells, and that ADT-2 activity is required for normal cuticle collagen fibril organization. We therefore propose that ADT-2 regulates body size both by modulating TGFβ signaling activity and by maintaining normal cuticle structure.

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**Introduction**

Body size is a fundamental feature of an organism critical to survival and fitness, yet the mechanisms underlying its regulation remain incompletely understood. Existing studies show that body size is in part genetically controlled (Oldham et al., 2000). For example, in Drosophila, the insulin/insulin-like growth factor 1 (IGF1) signaling pathway regulates body size, so that mutants are smaller than wild-type flies (Bohni et al., 1999; Leevers et al., 1996; Neufeld and Edgar, 1998). Flies mutant for Drosophila S6 kinase (DSLK) and d-Myc also have slow growth, reduced cell size and small body size (Gallant et al., 1996; Johnston et al., 1999; Montagne et al., 1999). The Drosophila homolog of the target of rapamycin (TOR) affects growth by modulating the activity of DSLK. Mutant cells are small in size (Neufeld, 2003; Oldham et al., 2000). In contrast, the Salvador–Warts–Hippo (SWH) pathway represses tissue size. Lack of SWH pathway component activity results in overgrowth of adult structures (Badouel et al., 2009; Tyler and Baker, 2007; Willecke et al., 2006).

In Caenorhabditis elegans, there are several known small body size mutants. Components of the DBL-1 Transforming Growth Factor β (TGFβ) pathway play a major role in the regulation of body size. Mutations in any component of the pathway, dbl-1 (ligand), sma-6 (type I receptor), daf-4 (type II receptor), sma-2-3, sma-4 (Smad transcription factors) and sma-9 (transcription co-factor) result in smaller than wild-type body size (Estevez et al., 1993; Krishna et al., 1999; Liang et al., 2003; Savage-Dunn, 2005; Savage-Dunn et al., 2003; Savage et al., 1996; Suzuki et al., 1999).

In addition to the DBL-1 pathway, other genes have been identified that play less critical roles in regulation of body size. One such group is genes that are expressed in sensory neurons, including che-2, che-3, egl-4, tax-6 and cnb-1 (Bandyopadhyay et al., 2002; Fujiwara et al., 2002; Kuhara et al., 2002). The che-2 and che-3 mutations cause small body size due to defects in sensory perception. egl-4 (cAMP-Dependent Protein Kinase) acts downstream of che mutants to regulate body size by repressing the DBL-1 pathway (Fujiwara et al., 2002). tax-6 and cnb-1 encode the catalytic and regulatory subunits of calcineurin, respectively. tax-6 interacts with kin-29 (ser/thr kinase) and mef-2 (MADS box transcription factor) to regulate body size (Singaravelu et al., 2007).

Feeding defective mutants also have small body size. These include pha-2 and pha-3 with abnormal pharyngeal anatomy, eat-1, eat-2, eat-3
with reduced pumping rates and eat-10 with inefficient pharyngeal pumping (Morck and Pilon, 2006). Mutations of components of the TORC2 complex result in small body size (Jones et al., 2009; Soukas et al., 2009). Intriguing recent evidence implicates the cell death machinery in the regulation of cell and body size (Chen et al., 2008). In addition, mutations that affect the structure of the cuticle can change the body size of the animal because the cuticle encapsulates the body. Some examples are dpy-2, dpy-7, dpy-10, dpy-13, sqt-1, sqt-3 and lon-3. All of these genes encode cuticular collagens (Johnstone et al., 1992; Kramer, 1994; Kramer and Johnson, 1993; Kramer et al., 1988; Levy et al., 1993; Nyström et al., 2002; Suzuki et al., 2002; van der Keyl et al., 1994; von Mende et al., 1988). Finally, mutations in a small number of genes which have yet to be assigned to a particular pathway, including sma-1 ([SH-spectrin], sma-5 (MAP kinase BMK1/ERK5 homolog) and rnt-1 (RUNX family transcription factor), also cause small body size (Ji et al., 2004; McKeown et al., 1998; Watanabe et al., 2005).

To understand the genetic basis of body size regulation, a forward genetic screen for small body size mutants was carried out (Savage-Dunn et al., 2003). In that screen, alleles of many of these genes were identified. Furthermore, one of the novel small body size mutants isolated was sma-21, which we now show is allelic with ADAMTS family member adt-2.

ADAMTS (a disintegrin-like and metalloprotease with thrombospondin type I motif) are secreted metalloproteases that bind to the extracellular matrix (ECM) (Kuno and Matsushima, 1998; Porter et al., 2005; Tang, 2001). ADAMTS are related to the ADAM (a disintegrin and metalloprotease) subfamily of transmembrane proteins. Both ADAM and ADAMTS proteins are Zn dependent metalloproteases (Jones and Riley, 2005; Kaushal and Shah, 2000; Stocker et al., 1995). In mammals, the ADAMTS proteases are believed to function in ECM assembly (procollagen N-proteinases: ADAMTS-2, -3 and -14) and ECM degradation (aggrecanases: ADAMTS-1, -4, -5, -8, -9 and -15) (Colige et al., 1995, 1997; Collins-Racine et al., 2004; Fernandes et al., 2001; Kuno et al., 2000; Somerville et al., 2003; Tortorella et al., 2000, 2005; Wang et al., 2003). In C. elegans, some of these proteases are involved in organogenesis by remodeling the ECM. For example, GON-1 and MIG-17 are involved in distal tip cell migration in gonad development (Blelloch and Kimble, 1999; Ihara and Nishiwaki, 2007). Another protease ADT-1 is involved in ray morphogenesis by rapid remodeling of ECM (Kuno et al., 2002).

In this study we describe the role of C. elegans ADAMTS gene adt-2 in the control of body size. Genetic interactions show that adt-2 is likely involved in multiple pathways that regulate body size. We show that ADT-2 is synthesized in glial cells of sensory neurons and in the vulva, and is required to promote DBL-1 signaling activity and for normal cuticle structure.

Results

Isolation of sma-21 mutants

To identify genes required for body size regulation, a forward genetic screen for small body size mutants was carried out (Savage-Dunn et al., 2003). In that screen, N2 hermaphrodites were mutagenized with ethyl methanesulfonate (EMS) and the F2 progeny worms were screened for small body phenotype. In the screen, sma-20 mutants were identified. In the course of mapping, we found that the sma-20 strain has two mutations, sma-20(wk31) and sma-21(wk156). Furthermore, 23 semi-small segregants were isolated from the double mutant strain and it was found that they were all allelic to sma-21. This finding led us to the conclusion that sma-21 regulates body size, and that sma-20 is an enhancer of the sma-21 phenotype with no apparent phenotype on its own. All of the analyses in this paper were performed with a sma-21 (wk156) segregant from which the sma-20 enhancer mutation was outcrossed.

Fig. 1. sma-21 encodes ADT-2. (A,B,C) RNAi inactivation of adt-2 results in small body size. K09F5.1(RNAi) treated worms are indistinguishable from control worms. rrf-3 RNAi hypersensitive strain is used for body size measurements. (D,E,F) Fosmid WRM0636a108 containing adt-2 gene rescues the body size of sma-21(wk156). A,B,D and E are adult worms photographed at the same magnification. Body length was measured in adult worms. Each value represents a mean of 30 – 60 worms. Error bars indicate the standard deviation. Scale bars = 0.2mm. **indicates p<0.01.
**sma-21 encodes an ADAMTS (disintegrin and metalloprotease with thrombospondin repeats) family member ADT-2**

We used single nucleotide polymorphism (SNP) mapping (Davis et al., 2005) to locate *sma-21* on the X chromosome in the region between 7,439,984 (cosmid C01C10) and 7,982,355 (cosmid F45E1). Then, we employed array comparative genomic hybridization (aCGH) to identify any polymorphisms in this region. An oligonucleotide chip spanning this region was designed. This was hybridized with *sma-21* and wild-type genomic DNA at Roche NimbleGen (Maydan et al., 2009). This analysis identified two polymorphisms in the *sma-21* mutant: one in K09F5.1 and one in *adr-2*. Sequence analysis of PCR fragments in *sma-21* verified the two SNPs: one at 7,590,038 bp (G to A) in the *adr-2* gene and the other at 7,740,620 (G to A) in K09F5.1. To determine which of these mutations is responsible for the small body size phenotype in *sma-21*, we used RNAi and transformation rescue. We inactivated the corresponding genes by feeding N2 (wild type) and *rrf-3* (RNAi hypersensitive) worms on *adr-2* RNAi and K09F5.1 RNAi plates (Kamath et al., 2001). *adr-2* RNAi fed worms are small unlike the K09F5.1 RNAi (Fig. 1). Then, we introduced fosmid clones containing *adr-2* wild-type sequences into *sma-21* mutants by microinjection (Mello et al., 1991). *adr-2* RNAi fed worms are small unlike the K09F5.1 RNAi (Fig. 1). Finally, we performed a complementation test between *sma-21* and a deletion allele of *adr-2* (tm975) obtained from the CGC. The complementation test shows that these two mutations fail to complement for body size. Putting all the data together, we can conclude that *sma-21* encodes *adr-2*. We will therefore refer to *sma-21* as *adr-2*.

In order to verify the exon--intron structure of *adr-2*, we sequenced cDNA clones obtained from Dr Yuji Kohara. Sequencing of multiple cDNA clones gave no evidence for alternative splicing. The sequence of wild-type genomic DNA at Roche NimbleGen (Maydan et al., 2009). This analysis identified two polymorphisms in the *sma-21* mutant: one in K09F5.1 and one in *adr-2*. Sequence analysis of PCR fragments in *sma-21* verified the two SNPs: one at 7,590,038 bp (G to A) in the *adr-2* gene and the other at 7,740,620 (G to A) in K09F5.1. To determine which of these mutations is responsible for the small body size phenotype in *sma-21*, we used RNAi and transformation rescue. We inactivated the corresponding genes by feeding N2 (wild type) and *rrf-3* (RNAi hypersensitive) worms on *adr-2* RNAi and K09F5.1 RNAi plates (Kamath et al., 2001). *adr-2* RNAi fed worms are small unlike the K09F5.1 RNAi (Fig. 1). Then, we introduced fosmid clones containing *adr-2* wild-type sequences into *sma-21* mutants by microinjection (Mello et al., 1991). *adr-2* RNAi fed worms are small unlike the K09F5.1 RNAi (Fig. 1). Finally, we performed a complementation test between *sma-21* and a deletion allele of *adr-2* (tm975) obtained from the CGC. The complementation test shows that these two mutations fail to complement for body size. Putting all the data together, we can conclude that *sma-21* encodes *adr-2*. We will therefore refer to *sma-21* as *adr-2*.

### Table 1

<table>
<thead>
<tr>
<th>Amino acid identity of <em>C. elegans</em> of ADT-2 to mammalian and <em>C. elegans</em> ADAMTS. Calculated using Clustalw.</th>
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**Fig. 2.** Structure of the ADT-2 protein. (A) Domain organization of ADT-2 protein. Domain organization of mammalian ADAMTS-2 is shown for comparison (predicted by SBASE and SignalP 3.0 Server). (B) Amino acid sequence of ADT-2. Different domains are highlighted. In red is the signal sequence; in violet, the prodomain; in brown, the catalytic domain. Underlined is the Zn binding motif. In green is the conserved methionine residue downstream of the metal binding motif which forms ‘Met turn’ (Porter et al., 2005). In yellow is the cysteine-rich domain. Thrombospondin type I-like repeats are highlighted in blue. The location of the deletion in tm975 and the missense mutation (Gly 364 - Ser) in wk156 are indicated by a shaded box and an asterisk, respectively. (C) Zinc-binding motif and Met turn of *C. elegans* ADT-2 are aligned with other mammalian and *C. elegans* ADAMTS family members. The conserved zinc-binding motif and methionine residue downstream of the metal binding site are shaded. Asterisks indicate conserved amino acids. The glycine in red is the conserved residue mutated in *adr-2* (wk156). (D) Phylogenetic tree showing relationships between *C. elegans* and human ADAMTS sequences (constructed using BioEdit sequence Alignment Editor and MEGA 4.0.2). The numbers above the nodes indicate the percent bootstrap values in 500 replicates of the data.
Fig. 3. Phenotypes of **adtr-2** mutants. (A) **adtr-2**(*wk156*) mutant has small body size. Growth curve of **adtr-2** showing reduced growth rate compared to wild-type N2 worms. Each time point represents a mean of 25–50 animals. Error bars indicate the standard deviation. Body size of **adtr-2** mutant at each point except 24 h is significantly different from that of wild-type. (B,C) Lethal phenotype of **adtr-2**(*tm975*) deletion homozygotes. Images show an unhatched embryo (three-fold stage; B) and a partially hatched larva (C). (D) Reduced lifespan of **adtr-2** mutants. A Kaplan-Meier survival curve shows the lifespan of wild-type N2 (blue; median survival 16 days), mutant **adtr-2**(*wk156*) (brown; median survival 10 days; logrank statistical comparison *p* < 0.0001 relative to N2), **adtr-2**(*RNAi*) (orange; median survival 8 days; logrank statistical comparison *p* < 0.0001 relative to N2) and **adtr-2**(*RNAi*) initiated in adulthood (green; median survival 14 days; logrank statistical comparison *p* < 0.0001 relative to N2). For each strain, *n* = 120 worms.
EST yk1586e04 contains the full-length transcript revealing that adt-2 consists of 20 exons (exon 1 is non-coding) that encode a protein of 1020 amino acids. ADT-2 belongs to the ADAMTS family of secreted extracellular metalloproteases. These proteins bind to extracellular matrix (ECM) and are believed to be involved in remodeling of the ECM (Porter et al., 2005). As shown in Table 1, the catalytic domain of ADT-2 shows 21% to 41% homology to other ADAMTS family members. These proteins are comprised of several domains. Starting with the N terminus, ADT-2 has a signal peptide (predicted by SignalP 3.0 Server); a catalytic domain with a reprolysin-type zinc-binding motif, HEXH2XXH2X1GX1HD [where X1 is typically hydrophobic aa, X2 is glycine or a hydrophobic aa and X3 is asparagine (http://www. lerner.ccf.org/bme/ape/adamts/domain_organization.php)]; a cysteine-rich domain; a central TS (thrombospondin type I-like) domain and six C-terminal TS repeats (predicted by SBASE release 14, Sept 2006) (Fig. 2A). The domain organization of mammalian ADAMTS-2 is shown for comparison (Fig. 2A). Table 1 shows the amino acid identity in the zinc-binding motif, catalytic domain and in the first thrombospondin type I-like repeat of C. elegans ADT-2 compared to mammalian and C. elegans ADAMTS. The metalloprotease domain of ADT-2 shows the highest similarity to ADAMTS-2, -3, and -14 among mammalian ADAMTS family members (Table 1 and Fig. 2D).

**adt-2(wk156)** is a missense mutation that changes a conserved glycine residue to a serine (highlighted in red in Fig. 2C) within the metal binding motif. Conservation of this glycine residue suggests that it is functionally important. adt-2 (tm9755) has a deletion of 475 bp (WormBase; Fig. 2B) which deletes 92 amino acids within the catalytic domain including the metal binding motif. adt-2 (tm9755) is lethal. Homozygous adt-2 (tm9755) mutants die at the 3-fold stage of embryogenesis or during hatching (Figs. 3B and C).

**Phenotypic characterization of adt-2**

To characterize the body size phenotype of adt-2 (wk156), we created growth curves that indicate the body length of wild-type N2 worms and adt-2 (wk156) worms at various times after egg collection (Fig. 3A). Newly hatched adt-2 mutant larvae have the same body length as control animals, similar to dbi-1 pathway mutants. adt-2 mutant worms grow more slowly after 24 hours resulting in adult worms with about a 20% reduced body length when compared to wild type. Interestingly, adult adt-2 mutants show a significant decrease in body length between 96 and 120 h, whereas wild-type animals continue to grow larger during adulthood. The reduction in body length in adt-2 mutants suggests that the gene is required for maintenance of body length as well as for increase in body length during growth stages.

We have also examined the body width of adt-2 mutants. Typical small mutants, such as those defective in the DBL-1 pathway, have reduced width as well as reduced length. In contrast, dumpy mutants, such as those defective in some cuticle collagens, have reduced length but not reduced width. adt-2 (wk156) mutants show a significantly increased body width, 0.072 mm +/- 0.005 mm vs 0.066 mm +/- 0.005 mm for N2 wild-type controls grown concurrently under the same conditions, p<0.01 (see also Fig. 1E). In contrast, adt-2 (RNAi) animals show a significantly reduced body width, 0.072 mm +/- 0.006 mm vs 0.089 mm +/- 0.006 mm for control animals grown concurrently under the same conditions, p<0.01 (see Fig. 1B). Thus, the partial loss-of-function adt-2 (wk156) mutant resembles a dumpy mutant, whereas more severe loss-of-function due to RNAi inactivation results in a more characteristic small phenotype.

We next evaluated the effects of adt-2 on lifespan. Both adt-2 (wk156) and adt-2 (RNAi) have significantly reduced lifespans compared to wild type, with a median survival of 10 and 8 days from adulthood, respectively, compared with 16 days for wild type (Fig. 3D). Since early lethality could be due to developmental defects associated with adt-2 loss-of-function, we also measured lifespan of animals grown under normal conditions until adulthood and then transferred to adt-2 (RNAi) conditions. These animals also show reduced adult longevity, with a median survival of 14 days and a maximum survival reduced from 21 days to 18 days (Fig. 3D). Overall, the median lifespan of adt-2 mutants and adt-2 (RNAi) under conditions tested is reduced by 13% to 50% relative to wild type. Thus, adt-2 activity is required to promote normal adult longevity.

The reduction in body length of adt-2 mutants could be due to decreased cell number, cell size or alterations in cellular or extra cellular morphology. In order to determine which cause is responsible in this case, we first examined the number of seam cells, the number of intestinal cells, and the number of hypodermal nuclei in wild-type and adt-2 mutant animals. The number of seam cells was measured using the seam cell marker::GFP reporter, and found to be not significantly different in adt-2 (wk156) or adt-2 (RNAi) compared to controls (Table 2). DAPI staining was done in order to count intestinal nuclei, which are the same in adt-2 (wk156) and adt-2 (RNAi) compared with controls (Table 2). We also counted the number of nuclei in the hypodermis of fourth larval stage (L4) worms of adt-2 (wk156), adt-2 (RNAi), and control animals. Hypodermal nuclei were counted using the dpy-7p::Xnls::yfp marker, which is expressed in hypodermal cells beginning at the L1 stage (Myers and Greenwald, 2005). The number of hypodermal nuclei is not significantly different in adt-2 mutants compared to control animals (Table 2). For comparison, we determined the number of hypodermal nuclei in dbi-1 mutants, which is also not significantly different from wild type (114.6 +/- 9.4). These results are consistent with previous reports that DBL-1 pathway mutants have normal numbers of hypodermal nuclei (Nagamatsu and Oshimama, 2004).

We also measured the dimensions of two transcellular tissues (seam cells and the pharynx) in adt-2 and wild type. We crossed an ajm-1:: gfp seam cell marker into adt-2 (wk156) or fed the strain adt-2 (RNAi) bacteria and observed fluorescence in the L3 larval stage. The ajm-1:: gfp marker localizes at the adherens junctions in the seam cells.

| Table 2 | Cell and nuclei measurements of adt-2 and wild-type worms. |
|-----------------|-----------------|-----------------|-----------------|
| N2 | adt-2(wk156) | RNAI control | adt-2(RNAi) |
| **Seam cell number** | 16 +/- 0.0 | 16.1 +/- 0.7 | 16.2 +/- 0.4 |
| (Adult) | (n=30) | (n=30) | (n=30) |
| **Intestinal nuclei number** | 31.4 +/- 1.2 | 31.5 +/- 1.4 | 32.7 +/- 1.4 |
| (Adult) | (n=31) | (n=38) | (n=38) |
| **Hypodermal nuclei number** | 116.3 +/- 11.9 | 114.1 +/- 10.7 | 145.4 +/- 6.3 |
| (L4) | (n=33) | (n=31) | (n=38) |
| **Seam cell perimeter (mm)** | 0.073 +/- 0.011 | 0.069 +/- 0.011 | 0.076 +/- 0.011 |
| (L3) | (n=78) | (n=78) | (n=76) |
| **Pharynx length (mm)** | 0.109 +/- 0.006 | 0.108 +/- 0.009 | 0.108 +/- 0.009 |
| (L3) | (n=18) | (n=18) | (n=18) |
| **Body size (mm)** | 0.651 +/- 0.053 | 0.575 +/- 0.065 | 0.575 +/- 0.065 |
| (L3) | (n=23) | (n=27) | (n=27) |

*p<0.01 compared to control.
The pharynx length and body length of the same L3 worms were also measured. The length measurements for adt-2 (RNAi) animals are not significantly different from controls at the L3 stage (data not shown). The perimeters of individual seam cells and the overall body length of adt-2(wk156), however, are reduced significantly compared to wild-type animals, while the length of the pharynx is not affected (Table 2). These data are consistent with the hypothesis that adt-2 mutants are smaller at least partly due to decreased cell size or altered cell morphology, rather than decreased cell number. Our previous analysis of DBL-1 pathway mutants similarly revealed size reductions in the seam cells but not in the pharynx (Wang et al., 2002). Thus, the adt-2 size defects are similar to those seen in mutants of the DBL-1 signaling pathway.

Genetic interactions of adt-2 and other body size mutants in C. elegans

The DBL-1 pathway plays a major role in body size regulation in C. elegans. Mutations in any components of the pathway, dbl-1 (ligand), sma-6 (type I receptor), daf-4 (type II receptor), sma-2, sma-3, sma-4 (Smad transcription factors), sma-9 (transcription co-factor) will result in smaller bodies than wild type (Estevez et al., 1993; Krishna et al., 1999; Liang et al., 2003; Savage-Dunn, 2005; Savage-Dunn et al., 2003; Savage et al., 1996; Suzuki et al., 1999). Double mutants were created in order to determine the relationship between adt-2 and dbl-1 pathway components. The dbl-1 pathway mutants we used for this are null or strong alleles. Double mutants of adt-2 (wk156) combined with dbl-1(wk70), sma-6(wk7), daf-4(e1364), sma-2(e502), sma-3(wk30), sma-4(e729) or sma-9(wk55) have a smaller body size than the respective single mutants (Fig. 4A), indicating that adt-2 may act in a parallel pathway to regulate body size. To further validate this result, we looked at the dbl-1 over-expression [dbl-1(+)] phenotype in the adt-2 mutant background. dbl-1(++) results in a long (Lon) body phenotype in an otherwise wild-type background, but the small (Sma) phenotypes of sma-2, sma-3, sma-4, sma-6 and daf-4 are epistatic to the Lon phenotype of dbl-1(++) (Estevez et al., 1993; Suzuki et al., 1999). This epistasis places the activity of these sma genes downstream of the DBL-1 ligand activity (Suzuki et al., 1999). We fed dbl-1(++) worms on adt-2 RNAi plates. However, rather than the

Fig. 4. Genetic interactions of adt-2 and other small body size mutants in C. elegans. In each graph first two bars represents N2 and adt-2(wk156). Subsequent bars represent the indicated single mutant and corresponding double mutant with adt-2(wk156) or adt-2(RNAi). Data are shown as a percentage of wild-type body length. Each bar represents a mean of more than 27 adult animals measured at 96 h after embryo collection (except daf-4 and daf-4;adt-2 which were grown at 15°C for 144 h). Error bars indicate the standard deviation. ** indicates p<0.01 for the pairwise comparison between the single mutant and the corresponding double mutant with adt-2.
expected Sma phenotype, if the adt-2 gene activity were downstream, the body length is intermediate (Table 3). This is evidence that adt-2 acts at least partially independently of the DBL-1 pathway.

To determine whether adt-2 interacts with other pathways that regulate body size, we next tested mutants in sensory processing. che-2 and che-3 mutants have impaired sensory cilia and therefore defects in sensory perception. They also have a small body size phenotype. EGL-4 cGMP-dependent protein kinase acts downstream of che genes to regulate body size by repressing the dbl-1 pathway (Fujiwara et al., 2002). The tax-6 and cnb-1 genes encode calcineurin A and B subunits. Both tax-6 and cnb-1 are expressed in sensory neurons. Mutations in these genes cause pleiotropic defects including small body size and defects in sensory neuronal behavior (Bandyopadhyay et al., 2002; Kuhara et al., 2002). cnb-1 also has a transparent appearance because of thinning of the cuticle (Bandyopadhyay et al., 2002). In order to see whether adt-2 functions in the chemosensory pathways, double knockdowns between adt-2 and egl-4, che-2, tax-6, and cnb-1 were made by combining existing chromosomal mutations and RNAi inactivation of adt-2. Alleles chosen were likely molecular nulls or the characterized reference alleles for each gene. The egl-4(n477); adt-2(RNAi) double knockdowns have an intermediate body size compared to egl-4 and adt-2 single mutant animals. The che-2(e1033); adt-2(RNAi), tax-6(ok2065); adt-2(RNAi), and cnb-1(p675); adt-2(RNAi) double knockdowns also show additive effects (Fig. 4B). The feeding defective mutants pha-2 and pha-3 (Morck and Pilon, 2006) were also analyzed, and found to have additive effects on body size with adt-2 (Fig. 4B). A deletion allele of pha-2 is lethal, precluding the possibility of testing for body size phenotypes in the pha-2 homozygous mutant background.

To test whether adt-2 functions in the same pathways as rnt-1 and sma-1, rnt-1(ok351); adt-2(wk156) and sma-1(e30); adt-2(wk156) double mutants were constructed, using a deletion allele of rnt-1 and a nonsense allele of sma-1. The rnt-1 gene is the C. elegans homologue of mammalian RUNX transcription factors (Ji et al., 2004). sma-1 encodes

<table>
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<th>Genotype</th>
<th>Body length in adult animals</th>
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<tr>
<td>N2</td>
<td>1.193 ± /− 0.065</td>
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<td>adt-2</td>
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¹ Value is not significantly different from N2, p > 0.05.
² Value is significantly different from both adt-2 and ctIs40, p < 0.0001.

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<tr>
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<tr>
<td>adt-2; ctIs40[pTG96(sur-5::gfp); dbl-1(++)]²</td>
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Fig. 5. Expression of RAD–SMAD, a DBL-1 pathway transcriptional reporter. GFP expression was assessed at the L2 stage in hypodermal nuclei. (A, B) Wild-type strain fed control or adt-2 (RNAi) bacteria; (C, D) lon-2 mutant fed control or adt-2 (RNAi) bacteria; (E, F) dbl-1 mutant fed control or adt-2 (RNAi) bacteria. Body length of the same strains was measured in adulthood, and the mean length shown in mm in each panel. In each case, the adt-2(RNAi) treatment reduced body size compared to the respective control strain significantly (p < 0.01).
Mutations in these genes cause a small body size phenotype. Double mutants of rnt-1(ok351);adt-2(wk156) and sma-1(e30);adt-2(wk156) show additive effects (Fig. 4C). Thus, we can conclude that adt-2 acts independently of all tested pathways, or is involved in multiple pathways that regulate body size.

Since adt-2 may act in multiple pathways to regulate body size, we tested whether it has an effect on a DBL-1 pathway transcriptional reporter. The RAD–SMAD reporter (a generous gift of Jun Liu) consists of tandem Smad GTCT binding sites driving expression of nuclearily localized GFP (Tian et al., 2010). GFP expression levels from this construct are positively regulated by DBL-1 signaling (Tian et al., 2010) (Figs. 5A, C, E). We tested whether RAD–SMAD expression is regulated by adt-2(RNAi). adt-2(RNAi) reduces the expression of RAD–SMAD in an otherwise wild-type background (Figs. 5A and B). LON-2 is a glypican that negatively regulates DBL-1 activity (Gumienny et al., 2007). In the lon-2 mutant background, RAD–SMAD expression is increased (Tian et al., 2010) (Fig. 5C), and this expression is also reduced by adt-2(RNAi) (Fig. 5D). In the dbl-1 mutant background, RAD–SMAD expression is reduced to a few dimly fluorescing nuclei in the posterior (Fig. 5E) and this expression is not diminished further by adt-2(RNAi) (Fig. 5F). Thus, adt-2 is a positive regulator of a DBL-1-responsive reporter. Furthermore, unlike the additive effect on body size regulation, the effects of dbl-1 and adt-2 on RAD–SMAD regulation are not additive, indicating that ADT-2 regulates this reporter via the DBL-1 pathway. We therefore conclude that ADT-2 directly or indirectly regulates DBL-1 signaling activity.

Our results thus far indicate that ADT-2 may regulate body size in part by modification of the external cuticle.

Fig. 6. Expression pattern of an adt-2 reporter construct adt-2p::gfp. adt-2 is expressed in the glial cells of amphid and labial neurons (A), phasmids (B), and postdeirid (C) and in the vulva (D).
with a deletion allele. The since it causes a mild body size phenotype that is enhanced in trans
The original worms (seam cells. We also detect constrictions of the annuli in
region in which top ridge is discontinued, transitioning from four alae to three alae).

Fig. 7. Aberrant COL-19::GFP localization in adt-2 mutants. (A,C,E) COL-19::GFP expression. (B,D,F) Nomarski microscopy image of cuticular alae. In wild-type animals, COL-19::GFP fusion protein is expressed in annuli extending over most of the dorsal and ventral cuticle (A). The cuticle of adult wild-type hermaphrodite shows lateral alae with three characteristic ridges (B). RNA inactivation of adt-2 causes an expanded region of disruption in the lateral cuticle (double headed arrow; C). Patches of disorganization of the annuli are also evident in the adt-2(tm975/wk156) adult worms (E). Nomarski microscopy images of the cuticle of adt-2(tm975/wk156) hermaphrodites show abnormal numbers of alae (arrowheads; four in D and two in F), as well as discontinuous alae (D; arrows from first asterisk mark region in which third ridge from top is discontinued; second asterisk marks region in which top ridge is discontinued, transitioning from four alae to three alae).

(Thein et al., 2003). As shown in Fig. 7C, animals treated with adt-2 (RNAi) show significant disruptions of the lateral cuticle overlying the seam cells. We also detect constrictions of the annuli in adt-2(RNAi) worms (Fig. 7C and Table 4). We analyzed COL-19::GFP localization in adt-2(tm975/wk156) transheterozygous animals, revealing patches of disorganized annuli (Fig. 7E) and structural defects in the alae. The defects are discontinuous alae and alae with two or four ridges as compared to three ridges in the wild-type worms (Figs. 7B, D and F). These disruptions may partially explain the reduced body size in adt-2 mutants and knockdowns.

Discussion

sma-21, a gene required for normal body size in C. elegans, encodes ADT-2

To characterize the genetic regulation of body size, we undertook the molecular cloning of sma-21, a gene required for normal larval and adult body size in the nematode C. elegans. Four independent pieces of evidence demonstrate that sma-21 encodes ADT-2. First, sma-21 mutants contain a missense mutation in a conserved glycine residue in the catalytic domain of ADT-2. Second, inactivation of ADT-2 by RNAi phenocopies the small body size defect of sma-21 mutants. Third, genomic clones containing adt-2 are sufficient to rescue the body size defect of sma-21 mutants. Fourth, a lethal deletion allele of adt-2 fails to complement sma-21(wk156) for the body size defect.

adt-2 mutants and knockdowns display a graded series of defects. The original wk156 mutation likely results in a partial loss-of-function, since it causes a mild body size phenotype that is enhanced in trans with a deletion allele. The wk156 mutational lesion results in an amino acid substitution for a conserved glycine residue in the zinc-binding motif of the catalytic domain. The resulting mutant protein apparently retains partial activity. Knockdown of adt-2 by RNAi causes a more severe body size phenotype than in the wk156 mutant, as well as some lethality. Finally, the deletion allele tm975, which removes a large part of the catalytic domain including the metal binding motif, causes late embryonic lethality.

The molecular identification of SMA-21/ADT-2 demonstrates a role for ADAMTS function in the regulation of body size in C. elegans. This function in body size regulation may be a conserved activity for ADAMTS proteins. Consistent with this model, ADAMTS mutations and variants are associated with normal and pathological variations in human height. For example, ADAMTS2 is associated with connective tissue disorders such as EDS (Ehlers–Danlos syndrome) in humans and with dermatoparaxis in cattle. Both disorders are characterized by fragility of skin and short stature (Colige et al., 1999; Lenaers et al., 1971). A mutation in ADAMTS10 is associated with Weil–Marchesani syndrome (WMS), a disorder that is characterized by the short body size, short fingers and toes, joint stiffness and eye anomalies (Dagoneau et al., 2004). In addition, a mutation in ADAMTS12 (ADAMTS-like2) leads to geleophysial dysplasia, a condition characterized by short stature and digit abnormalities (Le Goff et al., 2008). Finally, gene variants in human ADAMTS10, ADAMTS17 and ADAMTS13 (ADAMTS-like3) are associated with variation in human height (Gudbjartsson et al., 2008; Lettre et al., 2008; Weedon et al., 2008).

ADT-2 is required for normal cuticle collagen fibril structure and DBL-1 signaling activity

In mammals, ADAMTS proteases can be divided into two classes: those involved in ECM assembly (procollagen N-proteinases: ADAMTS-2, -3 and -14) and those involved in ECM degradation

Table 4

<table>
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<tr>
<th>Genotype</th>
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<th>Number of worms</th>
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<td>None</td>
<td>1.478 +/− 0.14**</td>
<td>688</td>
<td>20</td>
</tr>
<tr>
<td>kabi12[COL-19::GFP]</td>
<td>Empty vector control</td>
<td>1.656 +/− 0.20</td>
<td>459</td>
<td>17</td>
</tr>
<tr>
<td>kabi12[COL-19::GFP]</td>
<td>adt-2(RNAi)</td>
<td>1.437 +/− 0.13**</td>
<td>690</td>
<td>21</td>
</tr>
</tbody>
</table>

**p < 0.01.
(aggrecanases: ADAMTS-1, -4, -5, -8, -9 and -15). ADT-2 is one of four members of the ADAMTS family present in *C. elegans*. Two of these, GON-1 and MIG-17, are more similar to the aggrecanases involved in ECM degradation. These two proteins are involved in cell migrations during organogenesis, and may function by locally degrading the basement membrane ECM. In contrast, ADT-1 and ADT-2 are more similar to the collagenases that are required for ECM assembly. ADT-1 is required for the morphogenesis of male copulatory organs, a process that requires rapid remodeling of the ECM.

In *C. elegans* one ECM tissue, the cuticle, plays a major role in determining body size due to the fact that it encapsulates *C. elegans*. *adt-2* mutants show a series of defects in the cuticle. We have observed the COL-19::GFP collagen fusion protein marker in *adt-2* mutant and RNAi backgrounds, and found that the recruitment of this collagen fusion protein into fibrils in the cuticle is aberrant. We see major disruptions of the COL-19-containing fibrils, particularly in the lateral cuticle underlying the alae. These defects are associated with shorter distances between the rings of annuli indicating excessive longitudinal constriction of the cuticle. Defects in the alae can also be seen by Nomarski DIC microscopy, including the presence of two or four lateral ridges instead of the normal three, and the presence of short discontinuities in the alae ridges. These defects are reminiscent of defects reported in a subset of cuticle collagen mutants such as *dpy-7*, *dpy-13*, and in *col-19(RNAi)* animals. Interestingly, *adt-2* was also previously identified in an RNAi screen for genes required for molting of the cuticle (Craig et al., 2007; Frand et al., 2005). Taken together, these results are consistent with a focus of action for *ADT-2* in the cuticle. Due to the defects in the cuticle of *adt-2* mutants, the cuticle may not be able to maintain its integrity, which leads to the resultant defect in body size. Also, *adt-2(wk156)* mutants lose length rather than growing during adulthood, which could be caused by a lack of cuticle integrity. These defects in the cuticle can also explain the reduced lifespan of *adt-2* mutants as an increased vulnerability to injuries and infection.

To test for an influence of *ADT-2* on the DBL-1/BMP signaling pathway, the major size regulating pathway in *C. elegans*, we employed a DBL-1-responsive transcriptional reporter, RAD–SMAD (Tian et al., 2010). Intriguingly, *adt-2* inactivation leads to a reduction in expression of this reporter, indicating that *ADT-2* also regulates body size in part by direct or indirect modulation of DBL-1 signaling. Since TGFβ ligands are often secreted in inactive forms that must be activated by proteolysis (Lawrence, 2001), it possible that *ADT-2* is directly involved in activation of DBL-1. Based on the other roles of *ADT-2* in the cuticle, however, we find it more likely that *ADT-2* plays an indirect role. Consistent with this hypothesis, ECM is known to influence the activity of signaling pathways; for example, ECM collagens act as regulators of BMP signaling in *Drosophila* (Wang et al., 2008). It is therefore possible that *ADT-2* modification of the ECM leads to changes in DBL-1 bioavailability.

**Materials and methods**

**Strains**

*C. elegans* strains were grown at 20 °C using standard methods (Brenner, 1974), except for *daf-4*, which was grown at 15 °C to prevent constitutive Dauer formation. All of the analyses reported here were performed with a *sma-21(wk156)* segregant from which the *sma-20* enhancer mutation was outcrossed. In addition to strains generated in this study the following strains were used:

- N2 and HA (wild type)
- LG I: *rnt-1(ok351)*
- LG II: *cnb-1(ok276)*
- LG III: *sma-2(e502), sma-3(wk30), sma-4(e729), daf-4(e1364)*
- LG IV: *tax-6(ok2065), egl-4(n477), pha-3(ad687)*

LG V: *dbl-1(wk70), sma-1(e30), him-5(e1490)*

LG X: *adt-2(m975)* obtained from Dr. Shohei Mitani, National Bioresource Project for the nematode, Tokyo Women’s Medical University School of Medicine, Japan; *sma-9(wk55), che-2(e1033), phe-2(ad472)*.

Transgenics: *dbl-1(cts40) (Suzuki et al., 1999)*; *TP12 [COL-19::GFP]* obtained from Dr. Antony Page; *wk51 [SCM::GFP, unc-119(+); jcds1 [ajm-1::gfp]]* obtained from Dr. Jeff Simisko; *arls89 [dp-7p::2Xnls::yfp]* obtained from Dr. Iva Greenwald; *jkl2277 [RAD–SMAD + mec-7p::rfp], lon-2(e678), jkl2277, and sma-6(wk7); jkl2277 (Tian et al., 2010) obtained from Dr. Jun Liu.

**Mapping and cloning of sma-21**

We used SNP mapping (Davis et al., 2005) and aCGH (Maydan et al., 2009) as previously described. Briefly, a microarray was designed for a 0.6 MB region delimited by the standard SNP mapping protocol using an application at http://hokkaido.bcgsc.ca/SNPdetection/. Processing of the microarray was done by Roche NimbleGen. To confirm the polymorphisms identified by aCGH, we generated PCR fragments flanking the mutation sites and directly sequenced them. Primer sequences are available on request. The PCR amplification produces 752 bp fragment for the wild-type allele and a 277 bp fragment for the deletion allele. For rescue, 10 ng/μg fosmid DNA was injected into the gonadal syncytia of *sma-21(wk156)* hermaphrodites with myo-3::mcherry (kindly provided by Dr. Hannes Bülow) as a marker (Mello et al., 1991). Total concentration of the DNA at injection was adjusted to 100 ng/μg using Bluescript SK. *y* cDNAs spanning this region were obtained from Dr. Y. Kohara. The *yk1586e04* was sequenced and the predicted transcript structure was verified. Primers used for sequencing the *yk* clone are available upon request.

**Measurement of nuclear and cell numbers**

We counted the number of nuclei in the hypodermis, seam cells and intestine in wild-type and *adt-2* mutant animals. DAPI staining was used to label the intestinal nuclei. N2, *adt-2* worms were fixed with acetone, washed with PMB (50 mM Na2HPO4 pH 7.5, 1 mM MgCl2) and immersed in 500 μl/ml DAPI solution for 30 min. Worms were then washed twice with PMB and the intestinal nuclei were counted. The number of seam cell nuclei was counted using the transgene *wls51 [SCM::GFP, unc-119(+)]*, a seam cell marker in adult animals (Clucas et al., 2002). The number of nuclei in the hypodermis of L4 animals was counted using the transgene *arls89 [dp-7p::2Xnls::yfp]*, a hypodermal reporter, which is expressed in hyp7 and other hypodermal cells (Myers and Greenwald, 2005). Individual seam cell perimeters were measured in L3 larvae using the *ajm-1::gfp* marker (Mohler et al., 1998) which localizes to adherens junctions. The length of the pharynx and the body size of the same L3 worms were also measured. These markers were introduced into *adt-2(wk156)* by standard genetic crosses. Images were taken using a confocal or epifluorescence microscope.

**RNAi feeding**

RNAi feeding was performed as described in Kamath et al. (2001). Six L4 animals were transferred to feeding plates, incubated overnight, transferred to fresh plates and the progeny were scored.

**adt-2 expression**

We used PCR fusion based approach to create *adt-2p::gfp* construct (Hobert, 2002). We amplified 4492 bp region of genomic DNA.
upstream of \textit{adt-2} to the nearest adjacent gene and fused with a GFP reporter. Primers used for fusion were: \texttt{adt-2A: 5’ GC GGA TCA TAA AAC TAT AGG AAA TTC GGA 3’} \\
\texttt{adt-2B: 5’ GC GGA TCT TTT ATG TAA TAC TAA TACC TTC TGG 3’} \\
\texttt{adt-2C: 5’ GAA AAG TTT TCC TTT ACT CAT AAT GTT GTC GAG TTG} \\
\texttt{gca gaa 3’} \\
\texttt{adt-2D: 5’ GAA AAG TTT TCC TTT ACT ACA Ttg aga atc aga ttt cac acg g 3’} \\
20 ng/µl \texttt{adt-2-GFP} construct was microinjected to N2 hermaphrodites along with \texttt{myo-3::mcherry} as a transformation marker. Total concentration of the DNA at injection was adjusted to 100 ng/µl using Bluescript SK.

Analysis of body size measurements

To characterize the body size phenotype of \textit{adt-2}, we created growth curves by measuring the body lengths of wild-type N2 worms and \textit{adt-2}(wk156) at various times after embryo collection. Worms were grown at 20°C and photographed using AxioVision 3.00 software. The length of each worm was determined by drawing a segmented line along the midline using the same software. To analyze body width, \textit{adt-2}(wk156) and \textit{adt-2}(RNAi) adult worms were photographed at 96 h after embryo collection and the width measured at a position through the vulva in the center of the body.

For body length measurements of the single and double mutants, 96 h old worms (144 h for \textit{daf-4} and \textit{daf-4};\textit{adt-2} double mutant grown at 15°C) were photographed using QC Capture 2.73.0 and the length was measured using Image-Pro Express 5.1.0.12 software.

Lifespan assay

Synchronous animals were obtained by bleeding of gravid hermaphrodites. 120 L4 worms from wild type, \textit{adt-2}(wk156) and \textit{adt-2}(RNAi) were picked and scoring progeny for the expected phenotypes. The \textit{sma-3, sma-4, sma-9, sma-1} mutations were complemented by \texttt{che-2(e1033);adt-2(wk156)}, \texttt{rnt-1} and \texttt{che-2(e1033);adt-2(RNAi)} mutant and \texttt{che-2(e1033);adt-2(wk156)} were grown at 20°C and photographed using Axio Vision 3.00 software. The length of each worm was determined by drawing a segmented line along the midline using the same software. To analyze body length measurements of the single and double mutants, 96 h old worms (144 h for \textit{daf-4} and \textit{daf-4};\textit{adt-2} double mutant grown at 15°C) were photographed using QC Capture 2.73.0 and the length was measured using Image-Pro Express 5.1.0.12 software.

References


Using RNA-mediated Interference Feeding Strategy to Screen for Genes Involved in Body Size Regulation in the Nematode C. elegans

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Abstract

Double-strand RNA-mediated interference (RNAi) is an effective strategy to knock down target gene expression¹-³. It has been applied to many model systems including plants, invertebrates and vertebrates. There are various methods to achieve RNAi in vivo⁴-⁵. For example, the target gene may be transformed into an RNAi vector, and then either permanently or transiently transformed into cell lines or primary cells to achieve gene knockdown effects; alternatively synthesized double-strand oligonucleotides from specific target genes (RNAi oligos) may be transiently transformed into cell lines or primary cells to silence target genes; or synthesized double-strand RNA molecules may be microinjected into an organism. Since the nematode C. elegans uses bacteria as a food source, feeding the animals with bacteria expressing double-strand RNA against target genes provides a viable strategy⁶. Here we present an RNAi feeding method to score body size phenotype. Body size in C. elegans is regulated primarily by the TGF-β - like ligand DBL-1, so this assay is appropriate for identification of TGF-β signaling components⁷. We used different strains including two RNAi hypersensitive strains to repeat the RNAi feeding experiments. Our results showed that mf-3 strain gave us the best expected RNAi phenotype. The method is easy to perform, reproducible, and easily quantified. Furthermore, our protocol minimizes the use of specialized equipment, so it is suitable for smaller laboratories or those at predominantly undergraduate institutions.

Protocol

1. Preparing RNAi Feeding Plates Carrying Target Gene Sequence

1. If using commercially available RNAi libraries (e.g. from Source BioScience LifeSciences), proceed to step 1.4. Alternatively, clone target gene sequences into vector L4440, a commonly used worm RNAi plasmid⁸, by standard cloning protocol.
2. Transform the recombinant plasmid into bacterial strain HT115(DE3), culture them on LB agar plates with 25 μg/ml carbenicillin and 12.5 μg/ml tetracycline, and pick a single clone for future use.
3. Meanwhile, transform the empty vector L4440 into bacterial strain HT115 to use as a control for the experiments.
4. Culture both recombinant and empty L4440 - carrying bacteria in 1 ml LB broth with 100 μg/ml ampicillin overnight at 37 °C. Tetracycline is not added due to the fact that it decreases RNAi efficiency.
5. Add another 5 ml LB broth with 100 μg/ml ampicillin into the overnight culture, incubate for another 4-6 hr at 37 °C.
6. Seed 0.5 ml recombinant or empty L4440 - carrying bacterial culture onto the RNAi worm plates. Label all plates with clone name.
7. Mark the plates and incubate overnight at 37 °C to grow bacterial lawn; these are the RNAi plates with or without target gene sequence. At least 2 plates should be prepared for each condition.

2. Culture Worms on the RNAi Feeding Plates

1. Flame sterilize the tip of a platinum wire worm pick. Use the worm pick to pick 6-10 fourth larval stage (L4) hermaphrodites to each RNAi plate that contains either recombinant or empty L4440; the animals will use the bacteria as a food source.
2. Let hermaphrodites grow at 20 °C (a standard culture condition for C. elegans) overnight, they will become young adults the next day.
3. Transfer 6-10 young adults into a new correspondingly labelled and prepared RNAi plate.
4. Let the adults lay eggs for 4-6 hr, then remove all the adults from the plate; this step is to synchronize the progeny. Once the mothers are removed, start to count time. This is time zero.
5. Incubate the plates at 20 °C to let animals grow to specific developmental stage; in this protocol, we choose young adults for phenotypic analysis. For knockdowns which develop at a normal rate, 72 hr incubation is sufficient for animals to become young adults. However, various genes affect animal development differently. If RNAi causes animals to grow at a different rate, young adults can be identified as those no more than 24 hr past L4 with completed vulval development and 2-6 embryos in the uterus (if fertile). To identify other developmental stages, the investigator should use gonadal and vulval development as a guide.

3. Score Body Size Phenotypes of RNAi Treated Worms

1. Place two layers of colored label tapes on a glass slide. Make two of these glass slides. Then, place a new glass slide in between the two slides with tape. Melt 2% agarose in water; apply one drop to the center of the new glass slide; then press the second glass slide on the top to make a thin layer of 2% agarose as described previously.9
2. Once agarose is solidified, remove the top glass slide; the slide with agarose pad is ready to load worms. Label slide with clone name.
3. Add 10 μl 25 mM NaN₃ to the agarose pad; pick 30-40 animals into the NaN₃ solution to immobilize them; then put coverslip on the top; repeat for both recombinant and empty L4440-carrying RNAi-treated animals.
4. Image the worms under dissecting microscope using 2.5x objective lens; here we use Leica digital camera with supporting software Qcapture.
5. For calibration, first capture an image of a standard micrometer ruler. Then open the image in Image-Pro software, Click on "measure" and choose "calibration" then choose "spatial calibration wizard". With "calibrate the active image" selected, click on "next". Input the name for the calibration and ensure that the spatial reference units are set to micrometer. Then, check the "create a reference calibration" button and click "next". Click on "draw reference line." A reference scale bar will appear. Reposition the scale bar to match the ends of the micrometer, then indicate that the reference indicates 1,000 units. Click "OK", "Next", and "Finish".
6. Once the software is calibrated, open a worm image. Then, select under the "measure" menu, select "calibration" then click on "select spatial". In the pull down menu, select the file name created for the micrometer calibration. Then, under the "measure" menu, select "measurements." In the window that opens, select the free-draw tool and trace a line through the center of the animal body from head to tail with computer mouse (Figure 1). The length will be reported in the window. Now you have two groups of data: body length of animals treated with target gene RNAi and control animals treated with empty L4440 vector.
7. Export the length measurements to a Microsoft Excel file, or other suitable statistical software. Analyze the data by calculating the mean and standard deviation for each sample. Then compare samples using Student's t-test.

Representative Results

In our research, we focus on body size regulation by the DBL-1/TGF-β pathway. The loss of function of the DBL-1 pathway results in small body size, including a shorter body length compared with wild-type animals7,10,11. Thus, screens for C. elegans body size mutants are capable of identifying TGF-β signaling components and modifiers. DBL-1 signaling is mediated by a conserved TGF-β signal transduction pathway that includes cell surface receptors and intracellular Smad signal transducers12. To test the effectiveness of RNAi by feeding for the identification of body size mutants, we used this technique to knock down DBL-1 pathway components: dbl-1/ligand; sma-2, sma-3, sma-4/Smads; and sma-6/ receptor. For our study, we used two different RNAi hypersensitive C. elegans strains to perform the experiment: eri-1;lin-15b and nrf-313,14. Meanwhile, we also used N2 (standard wild-type) strain and lin-36, a component in the lin-15 pathway whose RNAi sensitivity is nevertheless similar to N215. Our results (Figure 2) show that all of these strains displayed the short body size phenotype as expected (p<0.001), except sma-6 RNAi in lin-36 background. In nrf-3 background, body lengths of young adults after RNAi treatment were 84–95% of vector alone. In eri-1;lin-15b background, body lengths of young adults after RNAI were 68–86% of control animals. Compared with lin-36 and N2 strains, both of the RNAI hypersensitive strains nrf-3 and eri-1;lin-15b were more sensitive.

Figure 1. Representative images of animal body length being measured. A, animal image before measurement; B, the center of animal body was traced during the measurement; C, a tracing line along the animal body from head to tail, is measured by software.
Figure 2. Body length of animals in which DBL-1 pathway components have been inactivated by RNAi feeding method. RNAi animals in all of these background strains displayed the short body length phenotype as expected (p<0.001), except sma-6 RNAi in lin-36 background. RNAi of rrf-3 and eri-1; lin-15b strains demonstrated a stronger phenotype than that of lin-36 and N2 backgrounds.

RNAi library clone or clone target gene into L4440 vector and transform into HT115 strain

Culture recombinant or empty vector strain and grow them on RNAi worm plate

Grow L4 animals on these RNAi plates overnight

Pick young adults to new RNAi plates; let them lay eggs for 4-6hrs and then remove all the adults

Incubate plates for 72 hrs; the worms are young adults

Pick 30-40 animals; load them on the slide; take picture and measure body length of each individual

Analyze the data

Figure 3. Schematic description of using RNAi feeding strategy to screen for candidate genes.

Discussion

In this protocol, we describe our method for the identification of body size defective mutants of C. elegans by RNAi feeding. This method is applicable to the identification of TGF-β signaling components. Since such components are highly conserved through evolution, such screens
are relevant to elucidating the molecular mechanisms of TGF-β signaling in all metazoans. An important consideration in designing such a screen is the starting strain. We have demonstrated that both eri-1;lin-15b and rrf-3 are more sensitive to RNAi feeding than the control strains, which is consistent with previous studies. However, even though eri-1;lin-15b demonstrated the strongest phenotype, the animals did not grow well and produced few progeny. Thus, in a large-scale screen, we would not have enough animals for scoring phenotypes from this strain. As a result, we favor the rrf-3 strain to apply the RNAi feeding technique to body size regulation. A second choice to consider in the starting strain is whether to initiate a screen with the DBL-1 pathway intact or to begin with a sensitized strain in which the DBL-1 pathway is either compromised or overactive. These alternative starting points are likely to lead to the identification of overlapping but distinct sets of signaling components. Our protocol can also be modified to the study of other postembryonic phenotypes. In this case, the stage of the animals to be scored for the phenotype of interest may need to be modified by altering the duration of growth in step 2.5. Prior to initiating a large scale screen for other phenotypes, we would recommend a pilot screen with genes known to produce the phenotype of interest such as we describe here for dbi-1, sma-2, sma-3 sma-4, and sma-6.

By using this protocol, knocking down DBL-1 pathway components showed expected body length phenotype. Those RNAi animals were about 68-95% in length compared with control animals. In previous studies, DBL-1 pathway loss of function genetic mutants in adulthood are about 50% shorter than wild-type animals. Therefore, the RNAi feeding technique presented here did not fully eliminate gene activity. Thus, the method may be limited in its ability to identify pathway components that have a weak phenotype. Meanwhile, since there are many genes that regulate body length, genes identified from the screen also might not necessarily fall in DBL-1 pathway. Further experiments should be performed to place candidates in pathways, as is true for any other screen method.

In summary, RNAi feeding screens in C. elegans have proven useful in identifying genes involved in a process of interest. In this protocol, we have optimized existing protocols to be highly effective in the identification of body size defects. The optimized protocol can be further modified for the study of other postembryonic phenotypes.

Disclosures

No conflicts of interest declared.

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