The *C. elegans* TspanC8 tetraspanin TSP-14 exhibits isoform-specific localization and

function

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research and wrote the paper.

Abstract

Tetraspanin proteins are a unique family of highly conserved four-pass transmembrane

proteins in metazoans. While much is known about their biochemical properties, the in vivo

functions and distribution patterns of different tetraspanin proteins are less understood. Previous

studies have shown that two paralogous tetraspanins that belong to the TspanC8 subfamily, TSP-

12 and TSP-14, function redundantly to promote both Notch signaling and bone morphogenetic

protein (BMP) signaling in *C. elegans.* TSP-14 has two isoforms, TSP-14A and TSP-14B, where

TSP-14B has an additional 24 amino acids at its N-terminus compared to TSP-14A. By

generating isoform specific knock-ins and knock-outs using CRISPR, we found that TSP-14A

and TSP-14B share distinct as well as overlapping expression patterns and functions. While TSP-

14A functions redundantly with TSP-12 to regulate body size and embryonic and vulva

development, TSP-14B primarily functions redundantly with TSP-12 to regulate postembryonic

mesoderm development. Importantly, TSP-14A and TSP-14B exhibit distinct subcellular

localization patterns. TSP-14A is localized apically and on early and late endosomes. TSP-14B is

localized to the basolateral cell membrane. We further identified a di-leucine motif within the N-

terminal 24 amino acids of TSP-14B that serves as a basolateral membrane targeting sequence,

and showed that the basolateral membrane localization of TSP-14B is important for its function.

Our work highlights the diverse and intricate functions of TspanC8 tetraspanins in *C. elegans*,

and demonstrates the importance of dissecting the functions of these important proteins in an

intact living organism.

Author summary

Tetraspanin proteins are a unique family of highly conserved four-pass transmembrane proteins

in higher eukaryotes. Abnormal expression of certain tetraspanins is associated with various

types of diseases, including cancer. Understanding the functions of different tetraspanin proteins

in vivo is crucial in deciphering the link between tetraspanins and their associated disease states.

We have previously identified two tetraspanins, TSP-12 and TSP-14, that share redundant

functions in regulating multiple aspects of *C. elegans* development. Here we show that TSP-14

has two protein isoforms. Using CRISPR knock-in and knock-out technology, we have found

that the two isoforms share unique, as well as overlapping expression patterns and functions.

Furthermore, they exhibit distinct subcellular localization patterns. Our work highlights the

diverse and intricate functions of tetraspanin proteins in a living multicellular organism, and

demonstrates that protein isoforms are another mechanism *C. elegans* uses to increase the

diversity and versatility of its proteome.

Introduction

Tetraspanin proteins are a unique family of four-pass transmembrane proteins that are

highly conserved in metazoans [1, 2]. There are 33 tetraspanins in humans, 37 in *Drosophila* and

21 in *C. elegans*. All tetraspanin proteins contain four transmembrane domains and two

extracellular (EC) loops, including the small EC1 loop and the large EC2 loop. The large EC2

loop contains a conserved Cys-Cys-Gly (CCG) motif and additional cysteine residues that

mediate disulfide bond formation. Based on the number of cysteine residues in the EC2 loop,

tetraspanins can be classified into distinct subfamilies. One of them is the TspanC8 subfamily.

There are six members of TspanC8 tetraspanins in mammals, Tspan5, Tspan10, Tspan14.

Tspan15, Tspan17 and Tspan33 [3, 4]. TspanC8 tetraspanins are known to bind directly to

ADAM10 (A Disintegrin and Metalloprotease 10) to regulate its trafficking and function [5-7].

Evidence from recent studies showed that different TspanC8s localize ADAM10 to different

subcellular compartments and differentially affect the ability of ADAM10 to cleave distinct

substrates [8-12]. However, the large number of TspanC8 proteins in mammals makes it

technically challenging to comprehensively decipher the endogenous localization and functions

of each of these TspanC8 tetraspanins in live animals during development.

With the availability of molecular genetic tools to manipulate its genome and the

possibility to perform high resolution imaging on live animals, *C. elegans* provides a unique

model system for dissectting the functions of TspanC8 tetraspanin proteins in vivo. Unlike

mammals, *C. elegans* has only one TspanC8 tetraspanin, TSP-14, which has a paralog TSP-12.

Despite being a C6 tetraspanin, TSP-12 functions redundantly with TSP-14 to promote both

Notch signaling and BMP signaling [13-15]. Specifically, while animals lacking either TSP-12

or TSP-14 do not exhibit any overt phenotypes, animals lacking both TSP-12 and TSP-14 are

small (Sma), vulvaless (Vul), exhibit maternal effect embryonic lethality (EMB), and have

suppression of the *sma-9(0)* coelomocyte defects (Susm) [16]. The Sma and Susm phenotypes of

tsp-12(0); tsp-14(0) double mutants are due to defects in BMP signaling, a pathway known to

regulate body size and postembryonic mesoderm development in *C. elegans* [17]. We have

previously shown that endogenous TSP-12 and TSP-14 are both localized to the plasma

membrane and to various intracellular vesicles that include early, late and recycling endosomes

[16]. In the early embryo, TSP-12 is required for the cell surface localization of the *C. elegans*

ADAM10 ortholog SUP-17 [15]. In hypodermal and intestinal cells in the developing larvae,

TSP-12 and TSP-14 function redundantly to regulate the recycling of the type II BMP receptor

DAF-4 to the cell surface [16].

During the course of these studies, we noticed that the *tsp-14* locus encodes two major

protein isoforms, TSP-14A and TSP-14B, that differ by only 24 amino acids at the N-termini.

Using a combination of CRISPR knock-in/knock-out technology and high-resolution

microscopy, we assessed the endogenous expression, localization, and function of the two

different TSP-14 isoforms in live animals. We show here that TSP-14A and TSP-14B share

unique and overlapping functions in *C. elegans* development. Moreover, while TSP-14A is

localized to apical, intracellular vesicles, TSP-14B is localized to the plasma membrane on the

basolateral side. We further identified the basolateral membrane localization signal within the 24

amino acids unique to TSP-14B, and showed that this sequence is essential for both the

localization and function of TSP-14B. Our work highlights the diverse and intricate functions of

TspanC8 tetraspanins in an intact living organism.

Results

The *tsp-14* locus produces two protein isoforms, TSP-14A and TSP-14B, which differ by 24

amino acids at their N-termini

Sequencing data from existing cDNA clones showed that the *tsp-14* locus produces two

major TSP-14 isoforms, *tsp-14a* and *tsp-14b*, which share the same 3' ends, but differ at their 5'

ends (wormbase.org). Specifically, *tsp-14b* uses an upstream alternative 35bp first exon. While

the second exon of *tsp-14b* and the first exon of *tsp-14a* share the same 3' ends, *tsp-14b* has an

additional 163bp segment upstream of the shared 126bp sequences (Figure 1A). Thus the *tsp-14*

locus is predicted to produce two protein isoforms, TSP-14A and TSP-14B, which differ at their

N-termini, with TSP-14B having an extra 24 amino acids at its N-terminus compared to TSP-

14A (Figure 1B-1C). Both isoforms share the same four transmembrane domains, two

extracellular loops that include eight conserved cysteine residues in the second extracellular

loop, and an intracellular C-terminal tail. Whether there is any functional significance regarding

the presence of the two TSP-14 isoforms was not yet known.

TSP-14A and TSP-14B have distinct and shared functions during *C. elegans* development

To determine the functional significance of the two TSP-14 isoforms, we used

CRISPR/Cas9 to generate isoform specific knock-outs, *tsp-14a(0)* and *tsp-14b(0)* (Figure 2A,

Supplemental Figure 1A). Because the start codon of TSP-14A also encodes a methionine

residue in TSP-14B, we mutated it from methionine (ATG) to isoleucine (ATA) to generate the

tsp-14a null allele *jj304(tsp-14a(0))*. We reasoned that this methionine to isoleucine change can

prevent the translation of TSP-14A without significant disruption of the TSP-14B coding

sequence (Figure 2A, Supplemental Figure 1A). To generate the *tsp-14b* null allele *jj317(tsp-*

14b(0)), we mutated the ATG start codon of TSP-14B and a downstream in-frame ATG codon to

TTG and TTA respectively (Figure 2A, Supplemental Figure 1A). Because *tsp-14* null mutants

have no phenotype on their own, but display multiple phenotypes in the *tsp-12(0)* background

due to the functional redundancy shared by *tsp-14* and *tsp-12* [13, 15], we tested the functionality

of *tsp-14a(0)* and *tsp-14b(0)* by introducing each allele into the *tsp-12* null background. For this

purpose, we used a known deletion allele of *tsp-12*, *ok239*, as well as a new deletion allele *jj300*

that we generated via CRISPR (Supplemental Figure 1B). Both alleles will be denoted as *tsp-*

12(0), unless specified. As shown in Figure 2, *tsp-12(0); tsp-14(0)* double null mutants are small

(Sma), vulvaless (Vul), and exhibit 100% maternal effect embryonic lethality (EMB) and a high

penetrance (80%) of suppression of the *sma-9(0)* coelomocyte defects (Susm) ([16], Figure 2B,

2F-H, 2P). Like *tsp-12(0); tsp-14(0)* double null mutants, *tsp-12(0); tsp-14a(0)* mutants are also

Sma, Vul, and 100% EMB (Figure 2B, 2I-K). But *tsp-12(0); tsp-14a(0)* mutants only exhibit a

19.4% penetrance of the Susm phenotype (Figure 2P), a very slight increase of penetrance than

that of *tsp-12(0)* single null mutants (16.5%). Conversely, *tsp-12(0); tsp-14b(0)* mutants have a

normal body size, do not exhibit any vulva defects or embryonic lethality, yet exhibit a 37%

penetrance of the Susm phenotype (Figure 2B, 2L-N, 2P). These results suggest that TSP-14A

and TSP-14B have distinct functions during *C. elegans* development: TSP-14A shares redundant

functions with TSP-12 in regulating body size, embryonic and vulva development, while TSP-

14B functions redundantly with TSP-12 in regulating postembryonic mesoderm development.

Because *tsp-12(0); tsp-14(0)* double null mutants exhibit a 80% penetrance of the Susm

phenotype, higher than that of either *tsp-12(0); tsp-14a(0)* or *tsp-12(0); tsp-14b(0)* mutants

(Figure 2P), TSP-14A and TSP-14B must share redundant functions with each other, and with

TSP-12, in regulating postembryonic mesoderm development.

Single-copy transgene analysis supports distinct and overlapping functions of TSP-14A and

TSP-14B

One caveat with the CRISPR-mediated knock-out experiments is that certain editing

manipulations of one isoform could unavoidably affect the other one. To overcome this problem,

we expressed TSP-14A or TSP-14B as single copy transgenes into the same neutral genomic

environment [18], which allows for direct comparison of the functionality of the two isoforms.

We chose the *ttTi4348* locus on Chromosome I as the insertion site and examined the function of

each isoform on its own in the *tsp-12(0); tsp-14(0)* double null background. We used two

different *tsp-14* promoter elements to drive the expression of cDNAs encoding either TSP-14A

or TSP-14B: a 3.3kb promoter element, which is immediately upstream of the ATG of TSP-14A,

and a 5.2kb promoter element, which includes another 1.9kb sequence upstream of the ATG of

TSP-14B (Supplemental Figure 2, see also materials and methods). Both promoter elements can

drive GFP reporter expression in multiple cell types, including hypodermal cells (hyp 7 and seam

cells), although we detected stronger and less mosaic reporter expression under the 5.2kb

promoter (Supplemental Figure 2B, 2C). Using both the 3.3kb and the 5.2kb promoter elements

to drive the expression of *tsp-14* as single copy transgenes, TSP-14A failed to rescue, while TSP-

14B partially rescued, the Susm phenotype of *tsp-12(0); tsp-14(0)* double mutants (Figure 3A,

3B). This is consistent with the notion that TSP-14B plays a major role in postembryonic

mesoderm development, but normal postembryonic development requires both TSP-14A and

TSP-14B as well as TSP-12. Intriguingly, TSP-14A and TSP-14B each on its own can

significantly rescue the body size defects of *tsp-12(0); tsp-14(0)* double mutants, with more

efficient rescue observed when expression was driven by the longer 5.2kb *tsp-14* promoter as

compared with the 3.3kb promoter (Figure 3C). Taken together, the above results support the

notion that TSP-14A and TSP-14B exert distinct and overlapping functions in regulating body

size and mesoderm development.

Endogenous TSP-14A and TSP-14B exhibit distinct expression and localization patterns

To understand the basis underlying the distinct and shared functions of TSP-14A and

TSP-14B, we used CRISPR/Cas9-mediated homologous recombination to tag each of the

isoforms and examined their expression and subcellular localization patterns. We used two

different approaches to tag each of the isoforms.

First, we inserted GFP and three copies of the FLAG tag (3xFLAG) at their respective N-

termini after the ATG start codons. This led to the generation of N-terminally tagged TSP-14B

(*jj192*) and TSP-14A (*jj183)* (Figure 4A, materials and methods). We verified the successful

tagging of each isoform by western blot analysis, which also indicated that TSP-14A is

expressed at a much higher level compared to TSP-14B (Figure 4D). As expected, both *jj183* and

jj192 animals are healthy and fertile, although *jj183* worms are slightly longer than wild-type

worms at the same developmental stage (Figure 4B). We then introduced *jj183* and *jj192* into the

tsp-12(0) null background, and examined their phenotypes. *tsp-12(0); tsp-14(jj183)* and *tsp-*

12(0); tsp-14(jj192) animals are both viable and fertile, without any embryonic lethality (Figure

4B-C, Supplemental Figure 3), suggesting that neither tag significantly disrupts TSP-14 function.

tsp-12(0); tsp-14(jj183) animals have a normal body size (Figure 4B, Supplemental Figure 3A),

but exhibit a slight increase in penetrance of the Susm phenotype compared to *tsp-12(0)* single

mutants (Figure 4C, Supplemental Figure 3B). Because the first coding exon of TSP-14A is part

of the second coding exon of TSP-14B (Figure 4A), we suspect that the slight increase in the

penetrance of the Susm phenotype in *tsp-12(0); tsp-14(jj183)* animals is likely due to the GFP

tag affecting the proper expression or splicing of TSP-14B. Surprisingly, *tsp-12(0); tsp-14(jj192)*

mutants have a significantly lower penetrance of the Susm phenotype compared to that of *tsp-*

12(0) single mutants (Figure 4C, Supplemental Figure 3B). This improved Susm phenotype may

be due to the higher expression level of GFP::3xFLAG::TSP-14B compared to the endogenous

level of TSP-14B (Figure 4D, see below).

We have previously tagged TSP-14 endogenously at its C-terminus with GFP::3xFLAG

(*jj219*, Figure 3A) and showed that *jj219*, which has both TSP-14A and TSP-14B tagged, is fully

functional [16]. Therefore, as an alternative approach to specifically tag TSP-14A or TSP-14B,

we introduced the same GFP::3xFLAG tag as in *jj219* into either *jj304(tsp-14a(0))* or *jj317(tsp-*

14b(0)), and generated *jj304 jj319*, which has C-terminally tagged TSP-14B without any TSP-

14A, and *jj317 jj377*, which has C-terminally tagged TSP-14A without any TSP-14B (Figure

4A). Both *jj304 jj319* and *jj317 jj377* animals behaved similarly as their respective untagged

counterparts, *jj304(tsp-14a(0))* and *jj317(tsp-14b(0))*. Western blots confirmed the specific

tagging of TSP-14 isoforms in *jj304 jj319* and *jj317 jj377* animals (Figure 4D). Notably, *jj192*

animals (N-terminally tagged TSP-14B + untagged TSP-14A) appear to have an elevated amount

of TSP-14B protein than both *jj304 jj319* animals (C-terminally tagged TSP-14B without

endogenous TSP-14A) and *jj219* animals (C-terminally tagged TSP-14A + C-terminally tagged

TSP-14B) (Figure 4D). The underlying basis is currently unknown.

Using the various strains generated above. we conducted high resolution imaging using

the Aryscan imaging system (materials and methods). We found that TSP-14A and TSP-14B

exhibit distinct expression and localization patterns. First, only TSP-14A, but not TSP-14B, is

detectable in the germline and sperm cells, as well as at the tip of the anterior sensory cilia

(Figure 5A, 5B), while TSP-14B is detectable in the pharynx (Figure 5F). Both TSP-14A and

TSP-14B are found in hypodermal cells (Figure 5C, 5G, 5K, 5P, 5S, 5V) and the developing

vulva (Figure 5E, 5I, 5M, 5O, 5R, 5U). However, the two isoforms exhibit different subcellular

localization patterns. In hypodermal cells, TSP-14A is primarily localized in intracellular

vesicles, while TSP-14B is mainly localized on the cell surface (compare Figures 5C vs. 5G, and

Figures 5S vs. 5V). In the developing vulva, TSP-14A is localized on the apical side, while TSP-

14B is localized on the basolateral membrane (compare Figures 5E vs. 5I, and Figures 5R vs.

5U, for worms at the L4 Christmas-tree stage). Similar localization patterns hold true in the

pharynx (Figures 5A, 5F, 5J). Consistent with the higher levels of TSP-14A protein that we

detected on western blots (Figure 4E), the TSP-14A signals are significantly brighter than TSP-

14B under the microscope (see Figure 5J, 5K, 5M, 5O when both TSP-14A and TSP-14B are

present). Thus, TSP-14A and TSP-14B exhibit distinct expression and localization patterns.

TSP-14A is localized to early and late endosomes

We have previously shown that TSP-14 is localized to early, late, and recycling

endosomes, that it co-localizes with TSP-12 and shares functional redundancy with TSP-12 in

promoting BMP signaling [16]. Because TSP-14A is localized to intracellular vesicles, we

further examined its localization relative to various endosomal markers and to TSP-12. As shown

in Figure 6, TSP-14A co-localizes with TSP-12 (Figure 6B-6H). Furthermore, TSP-14A co-

localizes with both the early endosome marker RAB-5 and the late endosome marker RAB-7, but

shows little co-localization with the recycling endosome marker RAB-11 (Figure 6I-6R). Given

that we previously detected recycling endosome localization of TSP-14 using *jj219*, which labels

both TSP-14A and TSP-14B [16], our findings suggest that the very faint vesicular signal from

TSP-14B may come from recycling endosomes. However, due to the very weak signal of TSP-

14B and the limited number of TSP-14B-positive vesicles, we have not been able to address this

question using the reagents that we generated. Nevertheless, our findings suggest that TSP-14A

is localized to different endosomal vesicles.

TSP-14B is sufficient on its own to localize to the basolateral membrane of polarized cells

Our finding that TSP-14B::GFP::3xFLAG in *tsp-14a* null *(jj304 jj319)* animals is

localized to the basolateral surface of vulval and hypodermal cells (Figure 5R-5T) suggests that

the basolateral localization of TSP-14B does not require the presence of TSP-14A. To directly

test this hypothesis, we forced the expression of C-terminally-tagged TSP-14B as a single copy

transgene (*jjSi395*) in *tsp-14(0)* null background using a heterologous *snx-1* promoter. As a

control, we also forced the expression of C-terminally TSP-14A (*jjSi393*) using the same

approach. In the *tsp-14(0)* null background, TSP-14B (*jjSi395)* is localized to the basolateral

membrane of pharyngeal and intestinal cells, while the TSP-14A (*jjSi393*) signal is only weakly

detectable in intracellular vesicles in the intestine (Figure 7A,7B). Thus, in the absence of TSP-

14A, TSP-14B alone is sufficient to localize to the basolateral membrane of polarized cells. This

result suggests that the basolateral membrane targeting sequence resides in the coding region of

TSP-14B.

TSP-14B contains a basolateral membrane targeting signal in its first 24 amino acids

Since the only difference between TSP-14A and TSP-14B is the N-terminal 24 amino

acids present in TSP-14B, we reasoned that these 24 amino acids may contain signal(s) that

targets TSP-14B to the basolateral membrane. Indeed, we found an EQCLL motif (Figure 8A)

within these 24 amino acids in TSP-14B, similar to the well characterized di-leucine basolateral

targeting sequence [DE]xxxL[LI] [19, 20]. Using the CRISPR/Cas9 system, we mutated the

EQCLL sequence into AQCAA in the *jj192*(GFP::3xFLAG::TSP-14B) background (Figure 8B)

and obtained *tsp-14(jj322 jj192)* (Figure 8E, Supplemental Figure 1C). We found that the GFP-

tagged AQCAA mutant TSP-14B protein in *jj322 jj192* worms becomes localized to the apical

side of the developing vulva (compare Figure 8C vs 8F) and in intracellular vesicles of

hypodermal cells (Figure 8D vs. 8G), just like TSP-14A. These results demonstrate that TSP-

14B's localization to the basolateral membrane depends on the EQCLL motif.

275   To examine the functional consequences of having TSP-14B mis-localized, we

introduced the AQCAA mutation into wild-type worms via CRISPR and obtained *tsp-14(jj368)*

(Figure 8A, Supplemental Figure 1C). We then introduced *jj368* into the *tsp-12(0)* background

and examined the phenotypes of *tsp-12(0); tsp-14(jj368)* double mutants. We found that *tsp-*

12(0); tsp-14(jj368) double mutants [*tsp-12* null mutants with mis-localized TSP-14B] exhibit

more severe EMB, body size and and more penetrant Susm phenotypes compared to *tsp-12(0);*

tsp-14(jj317) double mutants [*tsp-12* null mutants without any TSP-14B] (compare Figure 8H vs.

Figure 2B, and compare Figure 8I vs. Figure 2P). Moreover, *tsp-12(0); tsp-14(jj368)* double

mutants exhibit a significantly reduced brood size (Figure 8J) and severe embryonic lethality

(77% EMB), phenotypes not displayed by *tsp-12(0); tsp-14(jj317)* double mutants. We reasoned

that these phenotypes seen in the *tsp-12(0); tsp-14(jj368)* double mutants cannot simply be due

to the mis-localization of TSP-14B. Because the EQCLL motif is located right upstream of the

ATG codon of TSP-14A (Figure 8A), mutating this motif may have affected a cis-regulatory

element(s) important for the proper expression of TSP-14A. To test this hypothesis, we tagged

both TSP-14A and TSP-14B in the *jj368* background at their C-terminal ends using CRISPR,

and generated *tsp-14(jj368 jj378*, Figure 8K). As suspected, *tsp-14(jj368 jj378*) worms have very

little TSP-14A or TSP-14B protein that is detectable on western blots (Figure 4D) and via

imaging (Figure 8L). Any detectable TSP-14 protein in *tsp-14(jj368 jj378)* animals appears to be

apical and intracellularly localized (Figure 8L). We noticed that *tsp-14(jj368)* single mutants

exhibit a 20% Susm phenotype, while *tsp-14(0)* null mutants do not show any Susm phenotype

(Figure 8I), suggesting that mis-targeting TSP-14B to the apical side may have a dominant

negative effect on TSP-14 and TSP-12 function.

Discussion

Despite extensive biochemical studies of the tetraspanin family of proteins, there are

significant gaps in our understanding of how different tetraspanin proteins function in vivo in

different cellular and developmental contexts. A major challenge for dissecting the in vivo

functions of multi-member families of proteins, such as the tetraspanins, in vertebrates is the

functional redundancy shared by different members of the same family or subfamily, or by

different isoforms of the same protein (for example, [21, 22]). In this study, we used CRISPR-

mediated knock-in and knock-out technology and showed that two isoforms of a single *C.*

elegans TspanC8 tetraspanin, TSP-14A and TSP-14B, exhibit distinct subcellular localization

patterns, and share overlapping as well as unique expression patterns and functions. Our work

highlights the diverse and intricate functions of TspanC8 tetraspanins in a living organism. It

also adds another example to the existing literature that shows protein isoforms are another way

for *C. elegans* to increase the diversity and versatility of its proteome.

Our isoform specific knock-in experiments showed that TSP-14B is localized to the

basolateral membrane, while TSP-14A is localized to the early and recycling endosomes on the

apical side of polarized epithelial cells. We further identified a basolateral membrane targeting

sequence (EQCLL) within the N-terminal 24 amino acids unique to TSP-14B, and showed that

this ExxLL motif is critical for the basolateral membrane localization of TSP-14B. The ExxLL

di-leucine motif in TSP-14B differs slightly from the canonical [DE]xxxL[LI] di-leucine motif,

which has been previously found to serve as a basolateral membrane targeting sequence (for

reviews, see [19, 20]). Basolateral sorting of proteins with the canonical [DE]xxxL[LI] di-

leucine motif is primarily mediated by the AP-2 clathrin adaptor, although it can also be

mediated by the AP-1 adaptor (for reviews, see [19, 20]). Both AP-1 and AP-2 adaptor

complexes exist in *C. elegans* [23-26]. Future work will determine whether the AP-1 or the AP-2

adaptor is involved in sorting TSP-14B to the basolateral membrane.

To date, most TspanC8 tetraspanins have been reported to be either cell surface or

intracellularly localized in cultured cells, most of which are non-polarized [5, 8, 12, 27]. Our

findings suggest that some TspanC8 proteins may also exhibit distinct localization patterns in

polarized cells. Indeed, a recent report showed apical junction localization of the TspanC8

member Tspan33 in polarized mouse cortical collecting duct (mCCD) cells [9]. In the same

study, the authors reported that another TspanC8 protein, Tspan15, is not on the apical plane, but

is instead localized along lateral contacts of polarized mCCD cells [9]. Whether other TspanC8

tetraspanins or their specific isoforms also exhibit distinct subcellular localization patterns in

polarized epithelial cells remains to be determined.

How the apically localized TSP-14A and basolateral membrane-localized TSP-14B exert

their distinct, as well as overlapping, functions in vivo is currently not well understood.

Nevertheless, our studies provide clues for the cellular basis underlying the functional

redundancy shared by TSP-12 and TSP-14. TSP-14 is known to function redundantly with its

paralog TSP-12 to promote both Notch signaling and BMP signaling [13, 15, 16]. Specifically,

tsp-12(0); tsp-14(0) double null mutants exhibit maternal-effect embryonic lethality (Emb) and

are egg-laying defective (Egl) due to defects in vulva development, two processes known to be

regulated by Notch signaling [13]. *tsp-12(0); tsp-14(0)* double null mutants are also small and

exhibit a suppression of the *sma-9(0)* postembryonic mesoderm defect (Susm), processes

regulated by BMP signaling [17, 28]. Like TSP-14, TSP-12 is localized to various endosomes, as

well as on the basolateral membrane of polarized vulval and intestinal epithelial cells [16].

Animals lacking TSP-12 and TSP-14A share the same penetrance (100%) of Emb and Egl

phenotypes as *tsp-12(0); tsp-14(0)* double null animals, suggesting that endosome-localized

TSP-12 and TSP-14A are critical for most, if not all, of the biological processes regulated by

Notch signaling. In contrast, BMP-regulated body size and postembryonic mesoderm patterning

appear to require both TSP-14A and TSP-14B to function together with TSP-12. Yet the

contributions of TSP-14A and TSP-14B towards these two BMP-regulated processes are not

equal. Three lines of evidence suggest that TSP-14B, but not TSP-14A, is the major player that

functions redundantly with TSP-12 in patterning the postembryonic mesoderm. First, animals

lacking TSP-12 and TSP-14B (but not TSP-14A) exhibit a higher penetrance of the Susm

phenotype than that of *tsp-12(0)* single mutants (Figure 2). Second, when introduced as a single

copy transgene, TSP-14B, but not TSP-14A, could partially rescue the Susm phenotype of *tsp-*

12(0); tsp-14(0) double null mutants (Figure 3). Third, a moderate increase in the level of TSP-

14B in *jj192* animals (GFP::3xFLAG::TSP-14B) was sufficient to partially compensate for the

lack of TSP-12 in *tsp-12(0)* null mutants in patterning the postembryonic mesoderm (Figure 4,

Figures S3). Consistently, when *tsp-14b* was overexpressed as a transgene in the *tsp-12(0); tsp-*

14(0) double null background, the penetrance of the Susm phenotype is also lower than that seen

in *tsp-12(0)* single mutants (Figures S2). Despite this shared redundancy between TSP-12 and

TSP-14B in mesoderm patterning, TSP-14A also plays a role in this process, as the penetrance of

the Susm phenotype in *tsp-12(0); tsp-14(0)* double null mutants is higher than that of *tsp-12(0);*

tsp-14b(0) animals (Figure 2).

The roles of TSP-14A and TSP-14B in body size regulation appear more complicated. On

the one hand, knockout experiments suggest that TSP-14A plays a major role in regulating body

size, as *tsp-12(0); tsp-14a(0)* double mutants have a smaller body size, although they are not as

small as the *tsp-12(0); tsp-14(0)* double null mutants (Figure 2). On the other hand, forced

expression of either TSP-14A or TSP-14B can partially rescue the small body size phenotype of

tsp-12(0); tsp-14(0) double null mutants (Figure 3). Furthermore, increased amount of TSP-14B

in *tsp-12(0); tsp-14(jj192)* mutants [with endogenous TSP-14A but without TSP-12] led to both

a slight decrease in body size and a less penetrant Susm phenotype (Figure 4B, Supplemental

Figure 3A), suggesting possible antagonistic roles of TSP-14B in the regulation of body size vs.

mesoderm development. These functional differences of TSP-14A and TSP-14B may be due to a

combination of their unique expression and localization patterns, and ultimately, to the distinct

molecular interactions at their specific cellular and subcellular environment.

Tetraspanins can have homotypic and heterotypic interactions to organize membranes

into tetraspanin-enriched microdomains, and regulate the trafficking or clustering of different

membrane or membrane-associated proteins [2, 29-31]. For example, TspanC8 tetraspanins are

known to bind and directly regulate the maturation and cell surface localization of ADAM10 [5-

7], which is a metalloprotease that cleaves the Notch receptor in *C. elegans*, Drosophila, and

mammals. We have previously shown that the *C. elegans* ADAM10, called SUP-17, is also

involved in regulating BMP signaling, and that the neogenin homolog UNC-40 may be one of

the SUP-17/ADAM10 substrates in regulating BMP signaling [15]. We further showed that TSP-

12, but not TSP-14, is required for the cell surface localization of SUP-17/ADAM10 in the early

embryo [15]. In addition, TSP-12 and TSP-14 function redundantly to promote the trafficking

and cell surface localization of the BMP type II receptor DAF-4 in the developing larvae [16]. It

will be important to determine the specific contributions that TSP-14A and TSP-14B each makes

towards these TSP-14-mediated functions, and how each of the isoforms works at the molecular

level. Finally, both TSP-12 and TSP-14 are also expressed in tissues beyond the sites where

BMP signaling and Notch signaling act. They could in principle regulate the trafficking and/or

localization of other proteins in addition to SUP-17/ADAM10 and DAF-4/BMPRII. The

identification of additional proteins trafficked by TSP-12 and TSP-14 (either TSP-14A or TSP-

14B or both), and subsequent determination on how TSP-12 and TSP-14 regulate their

trafficking/localization will elucidate how these two tetraspanin proteins function during the

development of a multicellular living organism like *C. elegans*. The information obtained could

shed light on the myriad of complex functions of the various TspanC8 proteins in mammals, and

ultimately aid in the development of therapeutic strategies for the treatment of various diseases

caused by abnormal expression or function of certain tetraspanins [32-34].

Materials and Methods

C. elegans **strains and molecular reagents**

C. elegans strains used and generated in this study are listed in Supplemental Table S1.

All *C. elegans* strains used in this study are derived from the Bristol N2 strain, maintained at

20°C (unless otherwise noted). Oligonucleotides and plasmids used and generated in this study

are listed in Supplemental Table S2.

406 **Body size measurement and *sma-9(0)* suppression assays**

407 Body size measurement and the *sma-9(0)* suppression assays were performed following

408 the methods described in [35]. Hermaphrodite worms at the L4 Christmas-tree stage (based on

409 the developing vulva) were used for body size measurement. Images of worms were taken using

410 a Leica DMR2 compound microscope equipped with a Hamamatsu Orca-03G camera using the

411 iVision software (BioVision Technologies). The length of each worm was then measured

412 using segmented lines in the open-source software Fiji. Body length of at least 30 worms per

413 genotype were measured. For the *sma-9(0)* coelomocyte suppression (Susm) assays, strains

414 expressing the *CC::gfp* marker *arIs37(secreted CC::gfp)* or *ccIs4438(intrinsic CC::gfp)* in

415 specific mutant backgrounds were generated (Table S1). Hermaphrodite worms with GFP-

416 labeled coelomocytes (CCs) were scored at the young adult stage under a Nikon SMZ1500 stereo

417 zoom microscope equipped with a Sola light engine (Lumencor). For each genotype, two

418 independent isolates were used and worms from three to five plates per isolate were scored. For

419 each plate, the number of worms with 5-6 CCs was divided by the total number of worms

420 examined to determine the penetrance of the Susm phenotype. For statistical analysis, an

421 ANOVA and Tukey's honestly significant difference (HSD) test were performed to test

422 differences in body size and Susm penetrance between different genotypes using R.

423

424 **CRISPR/Cas9 experiments**

425 For all CRISPR/Cas9 experiments, Cas9 target sites were chosen using the CRISPR

426 online design tool CHOPCHOP (http://chopchop.cbu.uib.no). Oligos with the single-guide

427 (sg)RNA sequences used to generate the sgRNA-expressing plasmids (using method described in

428 [36]) are listed in Table S2. For the knockout experiments, two sgRNA targeting sites, one

around the start codon, and the other around the stop codon, of the gene, were chosen in order to

completely knockout the gene of interest. To generate point mutations using the CRISPR/Cas9

system, single stranded DNA oligos (listed in Table S2) were used as the homologous repair

templates. To endogenously tag the different TSP-14 isoforms, plasmids containing the

homologous repair templates were generated by following the method described by Dickinson et

al [37]. Specifically, ~600-bp homology arms for each target locus were amplified from N2

genomic DNA and then inserted into the GFP^SEC^3xFlag vector pDD282 or the

TagRFP^SEC^3xMyc vector pDD286 via Gibson Assembly (New England BioLabs).

Hygromycin was used to select the knock-in candidate lines based on the strategy described in

Dickinson et al [37].

For the single-copy insertion rescue experiments, we followed the strategy described by

Pani and Goldstein [38] and chose *ttTi4348* on chromosome I as the insertion site. pAP082 was

the plasmid used for the expression of Cas9 and sgRNA [38]. Repair templates were generated

by replacing the ClaI and AvrII fragment in pAP088 with various fragments containing the

specific promoter, cDNA or chimeric cDNA-gDNA of the specific gene, and the specific 3'UTR.

All plasmids used in CRISPR/Cas9 experiments are listed in Table S2.

Live imaging and image analysis

L4 stage or young adult worms were mounted on 2% agarose pads with 10 mM

levamisole (CAS 16595-80-5; Sigma-Aldrich). A Zeiss LSM i880 microscope with Airyscan

equipped with 40× Fluar objective (N.A. 1.3) or 60× Plan-apochromat objective (N.A. 1.4) using

Immersol 518F oil (Carl Zeiss) was used to capture both the fluorescent and differential

interference contrast (DIC) images at super-resolution. Images were viewed and processed with

ZEN software. GFP was excited at 488 nm, and RFP or TagRFP was excited at 561 nm. GFP

emission was captured with the BP495-550 filter, and RFP or TagRFP emission was captured

with the LP570 filter. To determine the subcellular localization of GFP::TSP-14A, and the co-

localization of TSP-14A with TSP-12, quantitative colocalization analysis was performed using

the JACoP plugin of the open-source Fiji software [39]. For each image, the Costes threshold

regression was used as the reference to establish a threshold, and three randomly selected square

regions were chosen for each image pair. For each genotype, more than 10 worms were imaged

and analyzed. Co-localization analysis was conducted by calculating both Pearson's correlation

coefficient (PCC) and Mander's overlap coefficient (MOC) [40].

Western blot analysis

For the western blotting experiments, 100 L4-worms were hand-picked into 20 µL

ddH$_2$O, immediately flash-frozen in liquid nitrogen, and kept frozen for more than 30 minutes.

Then 5 µL 5×SDS sample buffer (0.2 M Tris·HCl, pH 6.8, 20% glycerol, 10% SDS, 0.25%

bromophenol blue, 10% β-mercaptoethanol) was added to each sample. The samples were then

boiled at 95°C for 10 min, centrifuged at 13k rpm for 20 min, and stored at -20°C until gel

electrophoresis. Proteins were separated using 7.5% Mini-PROTEAN TGX Precast Gels from

Bio-Rad Laboratories, Inc., and transferred onto Immobilon-P PVDF membrane

(MilliporeSigma) for 9 min under 1.3A and 25V using the Power Blotter Station (Model:

PB0010, Invitrogen by Thermo Fisher Scientific). The membrane was incubated in EveryBlot

Blocking Buffer (Bio-Rad Laboratories, Inc.) for 5 min at room temperature, and then incubated

at 4°C overnight in primary antibodies diluted in EveryBlot Blocking Buffer. Primary antibodies

used include mouse anti-FLAG IgG monoclonal antibody (Krackler Scientific; 45-F3165; diluted

1:2,000) and mouse anti-actin IgM JLA20 monoclonal antibody (Developmental Studies

Hybridoma Bank; diluted 1:2,000). Secondary antibodies used included peroxidase-conjugated

donkey anti-mouse IgG and peroxidase-conjugated goat anti-mouse IgM (all from Jackson

ImmunoResearch; diluted 1:10,000). Enhanced chemiluminescence was detected using the

Western Blotting Luminol Reagent (Santa Cruz Biotechnology; sc-2048). The Bio-Rad

ChemiDoc MP imaging system was used to capture the chemiluminescence signal. The western

blot experiment was repeated 3 times using different biological samples. Open-source Fiji

software was used to quantify western blotting images.

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Competing interest

The authors declare that no competing interests exist.

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Figure legends

Figure 1. The two TSP-14 isoforms: TSP-14A and TSP-14B.

(A). Schematic representation of the *tsp-14* locus, showing only the alternative and shared

coding exons of *tsp-14a* and *tsp-14b*. Exons are represented by boxes, and introns by lines. ATG

represents the start codon of each isoform. The first coding exon for *tsp-14b* is located 3511bp

upstream of the first coding exon of *tsp-14a*. **(B).** A schematic of TSP-14A and TSP-14B

proteins, showing the four transmembrane domains, and the various extracellular and

intracellular regions. The only difference between TSP-14A and TSP-14B is at their N-termini,

with the red line representing the extra amino acids present in TSP-14B. The CCG and CC

motifs in the second extracellular loop are represented by solid circles, with orange solid circle

representing cysteine, while the yellow solid circle representing glycine. PM, plasma membrane.

Ext, extracellular. **(C).** Alignment of the amino acid sequences of TSP-14A and TSP-14B. TSP-

14B has 24 additional amino acids at its N-terminus (red), which includes the ExxLL motif

(blue). The four transmembrane domains are indicated in green and the cysteine residues in the

extracellular domain are in red.

Figure 2. Isoform-specific knockouts of TSP-14 cause distinct phenotypes.

(A). Schematics of the *tsp-14* locus, showing isoform-specific knockouts generated in this study.

In *jj304,* the TSP-14A start codon ATG (methionine) is mutated to TTG (isoleucine), knocking

out TSP-14A, while introducing a single, although conserved amino acid change in TSP-14B.

The *tsp-14(jj304)* allele is also referred to as *tsp-14a(0)*. In *jj317*, the TSP-14B start codon ATG

and a downstream, in-frame, ATG (see Figure 1C) are mutated to TTG and TTA respectively,

knocking out TSP-14B without affecting TSP-14A. The *tsp-14(jj317)* allele is also referred to as

tsp-14b(0). **(B).** Relative body lengths of synchronized L4 worms of various genotypes. The

mean body length of wild-type worms is normalized to 1.0. Numbers within each bar indicate the

total number of worms measured. For each double mutant, data were pooled from two

independent isolates. Error bars represent 95% confidence interval (C.I.). Tukey's honestly

significant difference (HSD) test following an ANOVA was used to test for differences between

different genotypes. ***$P < 0.0001$. n.s., no significant difference. WT: wild-type. *tsp-12(0)*: *tsp-*

12(jj300). *tsp-14(0)*: *tsp-14(jj95)*. *tsp-14a(0)*: *tsp-14(jj304)*. *tsp-14b(0)*: *tsp-14(jj317)*. **(C-N).**

Differential interference contrast (DIC) micrographs of wild-type (WT, C-E), *tsp-12(0); tsp-*

14(0) (F-H)*, tsp-12(0); tsp-14a(0)* (I-K)*, tsp-12(0); tsp-14b(0)* (L-N) animals at the L4 (C, F, I,

L) or young adult stage (D, G, J, M), or their embryos at mid-embryogenesis (E, H, K, N). L4,

L4 larva. YA, young adult. Scale bars represent 20 μm. **(O).** Diagrams depicting the *sma-9(0)*

suppression (Susm) assay. Mutations in the BMP pathway can suppress the loss of M-lineage-

derived coelomocytes (CCs) in *sma-9(0)* mutants. CCs are depicted as green circles. **P).** Table

summarizing the results of the *sma-9(0)* suppression assay of various *tsp-14* alleles. Percentage

of suppression was calculated by the number of worms with 1-2 M-derived CCs divided by the

total number of worms scored. N represents the total number of worms counted. Data from two

independent isolates were combined for each genotype. * The loss of M-derived CCs phenotype

of *sma-9(cc604)* null mutants is not fully penetrant.

Figure 3. TSP-14A and TSP-14B have distinct and shared functions.

(A) Schematics depicting the single copy transgenes expressing either *tsp-14a* or *tsp-14b* under

the 3.3kb or 5.2kb *tsp-14* promoter at the *ttTi4348* locus on chromosome I. **(B)** Table

summarizing the results of the *sma-9(0)* suppression assay when *tsp-14a* or *tsp-14b* was

expressed as a single copy transgene. Percentage of suppression was calculated by the number of

worms with 1-2 M-derived CCs divided by the total number of worms scored. * The loss of M-

derived CCs phenotype of *sma-9(cc604)* null mutants is not fully penetrant. **(C)** Relative body

lengths of synchronized L4 worms of various genotypes at the Christmas tree stage. The mean

body length of wild-type (WT) worms is normalized to 1.0. Numbers within each bar indicate

the total number of worms measured. For each double mutant, data were pooled from two

independent isolates. Error bars represent 95% C.I. Tukey's HSD test following an ANOVA was

used to test for differences between different genotypes. ***$P < 0.0001$. WT: wild-type. *tsp-*

12(0): *tsp-12(jj300)*. *tsp-14(0)*: *tsp-14(jj95)*.

Figure 4. Endogenous TSP-14A ad TSP-14B can be respectively and functionally tagged.

(A). Schematic representations of wild-type and GFP-tagged TSP-14 isoforms. In *jj192* animals,

TSP-14B is specifically tagged at its N-terminus with GFP::3xFLAG. In *jj183* animals, TSP-14A

specifically tagged at its N-terminus with GFP::3xFLAG. However, if spliced properly, the tag is

also inserted into TSP-14B after the first 24aa. Both TSP-14A and TSP-14B are tagged at their

C-termini with GFP::3xFLAG in *jj219* animals. The same GFP::3xFLAG tag as in *jj219* is also

in *jj304 jj319* and *jj317 jj377* animals when one of the respective *tsp-14* isoforms is knocked out.

(**B).** Relative body lengths of synchronized L4 worms of various genotypes. The mean body

length of wild-type worms is normalized to 1.0. Numbers within each bar indicate the total

number of worms measured. For each double mutant, data were pooled from two independent

isolates. Error bars represent 95% C.I. Tukey's HSD test following an ANOVA was used to test

for differences between different genotypes. ***$P < 0.0001$. n.s., no significant difference. As

shown, *tsp-12(0); tsp-14(jj192)* worms are slightly smaller than wild-type (WT) worms, but not

as small as *tsp-12(0); tsp-14(jj95)* worms. **(C).** Tables summarizing the results of the *sma-9(0)*

suppression assay of various *tsp-14* alleles, in combination with the *tsp-12(jj300)* null allele.

Percentage of suppression was calculated by the number of worms with 1-2 M-derived CCs

divided by the total number of worms scored. N represents the total number of worms counted.

Data from two independent isolates were combined for each genotype. * The loss of M-derived

CCs phenotype of *sma-9(cc604)* null mutants is not fully penetrant. **(D).** Western blot showing

the various tagged forms of TSP-14A and/or TSP-14B detected by anti-FLAG antibodies. Anti-

actin antibodies were used as a loading control. For each lane, 100 synchronized L4 animals

were used to prepare the samples. The genotypes of *jj322 jj192* and *jj368 jj378* are described in

Figure 7.

Figure 5. TSP-14A and TSP-14B exhibit distinct localization patterns.

(A-M) Airyscan confocal images showing the localization of TSP-14A (A-C' and E), TSP-14B

(F-G' and I), and both TSP-14A and TSP-14B (J-K' and M) in the head region (GFP: A, F, J,

and DIC: A', F', J'), hypodermal cells (GFP: C, G, K, and DIC: C', G', K'), and the developing

vulva (E, I, and M). GFP::TSP-14A is detectable in the tips of anterior sensory cilia (arrow in A),

and in sperm cells (arrows in B). * in A and B marks autofluorescence signals. Scale bar in A-M,

10 μm. Panels D, E and L are schematic representations of the different knock-ins. **(O-W)**

Airyscan confocal GFP (O, P, R, S, U, V) and corresponding DIC (O', P', R', S', U', V') images

of the developing vulva (O, R, U) and hypodermal cells (P, S, V) from worms expressing

different tagged versions of TSP-14. Panels Q, T and W are schematic representations of the

different knock-ins. Scale bars in O-W, 20 μm.

Figure 6. TSP-14A is co-localized with TSP-12 and is localized to endosomes.

(**A)** Schematics of GFP- or TagRFP-tagged TSP-14A. (**B-G**) Airyscan confocal images of late

L4 hypodermal cells expressing both tagged TSP-14A (B, GFP-tagged TSP-14A and E,

TagRFP-tagged TSP-14A) and tagged TSP-12 (C, TagRFP-tagged TSP-12 and F, GFP-tagged

TSP-12). The corresponding merged images are shown in D and G, respectively. (**H**) The

colocalization between TSP-14A and TSP-12 was quantified using two different methods. PCC,

Pearson's correlation coefficient; MOC, Mander's overlap coefficient. PCC indicates the

correlation of GFP::TSP-14A with TagRFP::TSP-12 or TagRFP::TSP-14A with GFP::TSP-12.

MOC indicates the fraction of TagRFP::TSP-12 that overlaps with GFP::TSP-14A (the second

bar), or the fraction of GFP::TSP-12 that overlaps with TagRFP::TSP-14A (the forth bar). (**I-Q**)

Airyscan confocal images of L4 stage hypodermal cells expressing N-terminally GFP-tagged

TSP-14A (J, M, P) and different endosomal markers, TagRFP::EEA-1 (I, early endosomes),

TagRFP::RAB-7 (L, late endosomes), and TagRFP::RAB-11 (O, recycling endosomes). K, N

and Q are the corresponding merged images. (**R**) Bar graphics showing the correlation of

TagRFP (EEA-1, RAB-7 or RAB-11) with GFP::TSP-14A (PCC), and the fraction of TSP-

14A::GFP that overlaps with TagRFP (MOC). Error bars in H and R represent SEM. Statistical

analysis was done using a 2-tailed *t* test with 95% CIs. Scale bar, 5 μm.

Figure 7. TSP-14B is sufficient to localize to the basolateral membrane upon forced

expression.

Airyscan confocal images of L4 worms expressing either TSP-14A (**A**) or TSP-14B (**B**) in the

tsp-14(0) background under the *snx-1* promoter as single copy transgenes in the *ttTi4348* locus

on chromosome I. Images shown are of the head region and intestine cells. Arrows point to TSP-

14A or TSP-14B signals. Asterisks mark intestinal autofluorescence. TSP-14B::GFP is

concentrated on the membrane in the head region and in the basolateral membrane of the

intestinal cells, while the TSP-14A::GFP signal is not detectable on cell membranes. Scale bars,

20 μm.

Figure 8. The 24aa unique to TSP-14B contains a basolateral membrane targeting

sequence.

(A, B, E) Schematics of the wild-type (WT) and *jj368* mutant *tsp-14* loci, either untagged (A), or

N-terminally GFP-tagged (B, E), showing the EQCLL sequence in WT TSP-14B (or in *jj192*, B)

mutated to AQCAA in *jj368* (A) or *jj322 jj192* (E). **(C-D, F-G)** Airyscan confocal images of L4

larvae showing that wild-type (WT) TSP-14B is localized to the basolateral membrane in the

developing vulva (C) and in hypodermal cells (D), while mutated TSP-14B carrying the AQCAA

mutation is localized to the apical side of the developing vulva (F) and in intracellular vesicles in

hypodermal cells (G). Scale bars, 10 μm. **(H)** Relative body lengths of synchronized L4 worms

at the Christmas tree stage. The mean body length of wild-type worms is normalized to 1.0. For

each double mutant, data were pooled from two independent isolates. Numbers within each bar

indicate the total number of worms measured. Error bars represent 95% C.I. Tukey's HSD

following an ANOVA was used to test for differences between different genotypes. ***P* <

0.0001. WT: wild-type. *tsp-12(0)*: *tsp-12(jj300)*. *tsp-14(0)*: *tsp-14(jj95)*. As shown, *tsp-12(0);*

tsp-14(jj368) worms are slightly smaller than WT worms, but not as small as *tsp-12(0); tsp-*

14(jj95) worms. **(I)** Table summarizing the results of the *sma-9(0)* suppression assay. Percentage

of suppression was calculated by the number of worms with 1-2 M-derived CCs divided by the

total number of worms scored. N represents the total number of worms counted. Data from two

independent isolates were combined for each genotype. * The loss of M-derived CCs phenotype

of *sma-9(cc604)* null mutants is not fully penetrant. (**J**) Graph summarizing the brood sizes of

different strains. *tsp-12(0)*, *tsp-14(0)* and *tsp-14(jj368)* single mutants have the same brood size

as wild-type (WT) worms, while *tsp-12(0); tsp-14(0)* and *tsp-12(0); tsp-14(jj368)* double

mutants have significant reduced brood sizes compared to WT. *tsp-12(0)*: *tsp-12(jj300)*. *tsp-*

14(0): *tsp-14(jj95)*. Error bars represent 95% C.I. Tukey's HSD following an ANOVA was used

to test the differences between different genotypes. ***P < 0.0001. (**K**) Schematic

representations of the N-terminally GFP-tagged TSP-14A and N-terminally GFP-tagged TSP-

14A with EQCLL mutated to AQCAA (*jj368 jj378*). (L) Airyscan confocal images of L4 larvae

showing the developing vulva and hypodermal cells. The GFP signal in *jj368 jj378* worms is

very faint and not localized to the basolateral cell membrane. Scale bars, 10 μm.

Figure 1

A

Wild-type
TSP-14A
TSP-14B

jj304 (tsp-14a(0))
No TSP-14A
TSP-14B

jj317 (tsp-14b(0))
TSP-14A
No TSP-14B

B

Relative Body Length

N=71 N=70 N=61 N=59 N=64 N=80 N=136 N=87

WT
tsp-12(0)
tsp-14(0)
tsp-12(0); tsp-14(0)
tsp-14a(0)
tsp-14b(0)
tsp-12(0); tsp-14a(0)
tsp-12(0); tsp-14b(0)

*** *** n.s.

WT | **tsp-12(0); tsp-14(0)** | **tsp-12(0); tsp-14a(0)** | **tsp-12(0); tsp-14b(0)**

C L4 | F L4 | I L4 | L L4

D YA | G YA | J YA | M YA

E Embryo | H Embryo | K Embryo | N Embryo

O

sma-9(0) suppression assay

M-derived coelomocytes
WT
sma-9(0)
sma-9(0); BMP pathway(0)

P

Genotype	Suppression(%)	N
sma-9(cc604)	1.4±0.2*	1359
tsp-12(jj300); sma-9(cc604)	16.5±0.8	3343
tsp-14(jj95) sma-9(cc604)	1.9±0.3	2347
tsp-12(jj300); tsp-14(jj95) sma-9(cc604)	80.0±1.3	3952
tsp-14(jj304) sma-9(cc604)	2.0±0.3	1274
tsp-12(jj300); tsp-14(jj304) sma-9(cc604)	19.4±1.9	1938
tsp-14(jj317) sma-9(cc604)	5.4±0.6	958
tsp-12(jj300); tsp-14(jj317) sma-9(cc604)	37.0±2.9	1413

Figure 2

A

jjSi388

jjSi390

jjSi401

jjSi402

B

Genotype	Suppression(%)	N
sma-9(cc604)	1.3±0.3*	834
tsp-12(jj300); tsp-14(jj95) sma-9(cc604)	79.9±6.5	900
tsp-12(jj300); tsp-14(jj95) sma-9(cc604); jjSi388	79.2±6.7	873
tsp-12(jj300); tsp-14(jj95) sma-9(cc604); jjSi401	76.9±6.2	522
tsp-12(jj300); tsp-14(jj95) sma-9(cc604); jjSi390	55.2±7.8	707
tsp-12(jj300); tsp-14(jj95) sma-9(cc604); jjSi402	46.2±9.5	648

C

Figure 3

A

Wild-type

jj192

jj183

jj219

jj304 jj319

jj317 jj377

B

*** p < 0.0001

In panel B, *tsp-12(0): jj300*

C

Genotype	Suppression(%)	N
sma-9(cc604)	1.5±0.2*	1359
tsp-12(jj300); sma-9(cc604)	16.5±0.8	3343
tsp-14(jj95) sma-9(cc604)	1.9±0.3	2347
tsp-12(jj300); tsp-14(jj95) sma-9(cc604)	80.0±1.3	3952
tsp-14(jj183) sma-9(cc604)	1.9±0.1	2808
tsp-12(jj300); tsp-14(jj183) sma-9(cc604)	20.8±0.8	2979
tsp-14(jj192) sma-9(cc604)	0.7±0.1	3327
tsp-12(jj300); tsp-14(jj192) sma-9(cc604)	2.4±0.4	2140

D

Figure 4

Figure 5

Figure 6

Figure 7

A

WT

TSP-14A   ---------------*MGSGEPTRARAVV

TSP-14B   MPHRAPRRFMKTAPGACDWEQCLLMGSGEPTRARAVV

jj368

TSP-14A*   ---------------*MGSGEPTRARAVV

TSP-14B   MPHRAPRRFMKTAPGACDWAQCAAMGSGEPTRARAVV
(EQCLL-AQCAA)

B
jj192
TSP-14A
GFP::3xFLAG::TSP-14B

C L4 developing vulva — WT (EQCLL)

D Early L4 hyperdemal cells — WT (EQCLL)

E
jj322 jj192
TSP-14A* (EQCLL-AQCAA)
GFP::3xFLAG::TSP-14B (EQCLL-AQCAA)

F Mut (AQCAA)

G Mut(AQCAA)

H
Relative Body Length

WT, tsp-12(0), tsp-14(0), tsp-12(0); tsp-14(0), tsp-14(jj368), tsp-12(0); tsp-14(jj368)

N=64 N=46 N=61 N=55 N=64 N=175

***

I

Genotype	Suppression(%)	N
sma-9(cc604)	1.4±0.2*	1359
tsp-12(jj300); sma-9(cc604)	16.5±0.8	3343
tsp-14(jj95) sma-9(cc604)	1.9±0.3	2347
tsp-12(jj300); tsp-14(jj95) sma-9(cc604)	80.0±1.3	3952
tsp-14(jj368) sma-9(cc604)	20.3±0.3	3078
tsp-12(jj300); tsp-14(jj368) sma-9(cc604)	68.5±2.4	2700

J
Brood Size

WT, tsp-12(0), tsp-14(0), tsp-12(0); tsp-14(0), tsp-14(jj368), tsp-12(0); tsp-14(jj368)

***

K
jj368 jj378
TSP-14A::GFP::3xFLAG*
TSP-14B (EQCLL-AQCAA)::GFP::3xFLAG

L L4 developing vulva / Early L4 hyperdemal cells

jj368 jj378

Figure 8