Tetraspanin protein (TSP-15) is required for epidermal integrity in *Caenorhabditis elegans*

Hiroki Moribe[1,*], John Yochem[2,*], Hiromi Yamada[1], Yo Tabuse[3], Toyoshi Fujimoto[4] and Eisuke Mekada[1,‡]

[1]Department of Cell Biology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan
[2]Department of Genetics, Cell Biology, and Development, University of Minnesota, 420 Washington Avenue, SE Minneapolis, MN 55455, USA
[3]Fundamental Research Laboratories, NEC Corporation, 34 Miyukigaoka, Tsukuba, Ibaraki 305-8501, Japan
[4]Department of Anatomy and Molecular Cell Biology, Graduate School of Medicine, Nagoya University, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

*These authors contributed equally to this work
‡Author for correspondence (e-mail: emekada@biken.osaka-u.ac.jp)

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Summary

Epidermal integrity is essential for animal development and survival. Here, we demonstrate that TSP-15, a member of the tetraspanin protein family, is required for epithelial membrane integrity in *Caenorhabditis elegans*. Reduction of *tsp-15* function by mutation or by RNA interference elicits abnormalities of the hypodermis, including dissociation of the cuticle and degeneration of the hypodermis. Lethality during molting often results. Examination of GFP transgenic animals, genetic mosaic analysis and rescue assays revealed that TSP-15 functions in hyp7, a large syncytium that composes most of the hypodermis. Assays with a membrane-impermeable dye or leakage analysis of a hypodermal-specific marker indicate that the barrier function of the hypodermal membrane is impaired owing to the loss or reduction of TSP-15. These results indicate that TSP-15 functions in the maintenance of epithelial cell integrity.

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Key words: Tetraspanin, TSP-15, Epidermis, Cuticle, Integrity

Introduction

Epidermis is important not only as a barrier between the external environment and the underlying inner tissues, but also for maintenance of body morphology. The integrity of the epidermis is established by highly organized adhesion between polarized epithelial cells and by adhesion of epithelial cells to an extracellular matrix (ECM). Thus, weakness of the cell-cell or cell-ECM connections can result in fragility of the skin, as represented by inherited skin disorders such as epidermolysis bullosa and epidermolytic hyperkeratosis (Korge and Krieg, 1996). Although a number of molecules are known to be involved in the maintenance of epidermal integrity, the complexity of the problem suggests that many more molecules are involved. Genetic analysis in model organisms is a potentially powerful approach for elucidating these molecules and their interactions with each other (Hahn and Labouesse, 2001).

In the nematode *Caenorhabditis elegans*, the epidermis, which is called the hypodermis, is a classic polarized epithelium (White, 1988). Most of the hypodermis is composed of a large syncytium termed hyp7, with smaller epithelia composing the lateral parts of the midbody, the anterior and posterior ends of the animal, and other specialized parts of the body (White, 1988). The cuticle, which is the exoskeleton of the worm, is produced by secretion of collagenous and other material from the apical surface of the hypodermis (Kramer, 1997). A cuticle composed of collagen rather than of chitin favors *C. elegans* as a genetic model for the epidermis, and many mutations have been isolated that cause abnormality in either the hypodermis, cuticle, or fibrous organelles (FOs) which are functionally identical to mammalian hemidesmosomes, resulting in severe epidermal defects. Mutations in the genes encoding cuticle collagen or collagen-processing proteins result in cuticular deficiency, causing blistering of the cuticle (Bli, Blister) or conferring a short and fat shape on the animal (Dpy, dumpy; Sqt, squat) (Johnstone, 2000). Mutations in *mua-3*, *mup-4*, or *vab-10* can affect connections of the FOs to the muscle or cuticle, resulting in detachment of the cuticle from the hypodermis and body muscles from the hypodermis (Bercher et al., 2001; Bosher et al., 2003; Hong et al., 2001; Plenefisch et al., 2000). In addition, depletion of intermediate filament proteins by RNA interference (RNAi) can cause abnormal morphology of the epidermis (Karabinos et al., 2001).

Here we report that reduction of function of *tsp-15*, one of twenty tetraspanin genes in *C. elegans*, affects the integrity of the epidermis in a striking manner. This was a surprising result, because tetraspanins have not before been associated with epithelial integrity in vivo. In mammals, the tetraspanins constitute a widely expressed protein family of more than 30 members, including CD9, CD63, CD81, CD82 and CD151 (Boucheix and Rubinstein, 2001; Hemler, 2003). Members of this family are characterized by four transmembrane regions, short cytoplasmic tails at the N- and C-termini, one small extracellular loop, and one large one with a conserved CCG motif as well as other conserved cysteines (Stipp et al., 2003). The physiological roles of tetraspanins are largely unknown. Several lines of evidence indicate that tetraspanins are involved

in cell adhesion, migration, tumor metastasis and neurite outgrowth (Boucheix and Rubinstein, 2001; Hemler, 2003). Another major cellular function of certain tetraspanins is involvement in membrane fusions, which occur during fertilization (Le Naour et al., 2000; Miyado et al., 2000), infection of host cells by microbes (Clergeot et al., 2001; Pileri et al., 1998; Silvie et al., 2003), formation of muscle and fusions of macrophage and monocytes (Tachibana and Hemler, 1999; Takeda et al., 2003). Tetraspanins also have been associated with membrane fusions within cells, suggesting a role in the trafficking of intracellular vesicles (Kobayashi et al., 2000; Sincock et al., 1999).

A feature of tetraspanins is that they associate with, or form complexes with, a number of other transmembrane proteins, such as integrins, Ig superfamily proteins, membrane-anchored growth factors and tetraspanins themselves (Berditchevski, 2001; Boucheix and Rubinstein, 2001; Hemler, 2003; Nakamura et al., 1995). These lateral interactions of tetraspanins with other membrane proteins may be achieved through their large extracellular domain and the transmembrane domains (Kazarov et al., 2002; Yauch et al., 2000). In contrast to the lateral interaction, there is a cytoplasmic association of tetraspanins with the intracellular signaling molecules phosphatidylinositol 4-kinase and protein kinase C (Berditchevski et al., 1997; Yauch and Hemler, 2000; Zhang et al., 2001a). Thus, through these lateral and cytoplasmic interactions, tetraspanins are thought to play a role in assembling membrane proteins for intercellular signaling, cell adhesion and other cellular functions as described above (Berditchevski, 2001; Hemler, 2003).

The effect of a tetraspanin deficiency on the integrity of the *C. elegans* hypodermis provides a model both for investigating the function of tetraspanins in general in an organism and for investigating the development and maintenance of the hypodermis, which can be viewed as a model for skin. The phenotype of the deficiency is discussed with respect to models for the function of tetraspanins.

Materials and Methods

Nematode strains and genetics

Unless otherwise stated, the Bristol isolate of the N2 strain was used as the wild type, and worms were grown at 20°C on NGM plates containing lawns of the *Escherichia coli* strain OP50 as described (Brenner, 1974). *sv15* was isolated following standard mutagenesis of N2 worms with 50 mM ethyl methanesulfonate in a screen for recessive mutations that affect the completion of molting. Extraneous mutations were minimized by eight outcrosses before establishing SP2275 [*tsp-15(sv15)*]. Standard genetic mapping placed *sv15* very near *unc-29* on LGI (descriptions of and references for the genetic markers and deficiencies used in this work can be found at www.wormbase.org). Three-point mapping using a strain with the genotype *sv15/unc-29(e1072) lin-11(n566)* indicated that *sv15* is probably a mutation in a gene residing one or two cosmids to the right of *unc-29*: of 104 Lin non-Unc recombinants, three crossovers occurred between *e1072* and *sv15* and 101 between *sv15* and *n566*.

Deficiency crosses made use of the following three strains: MT2138 (*unc-13 lin-11/nDf29*), MT2179 (*unc-13 lin-11/nDf25*) and MT2180 (*unc-13 lin-11/nDf23*). Males from a mating of N2 and SP2275 were mated with heterozygotes from each of the deficiency strains, and the mating plates were examined for the presence of progeny displaying aspects of *sv15*. Such animals were followed for their ability to reach adulthood. Effects on embryogenesis were not examined as the deficiency strains themselves produce significant numbers of dead embryos.

Genetic mosaic analysis

An organism that is genetically mosaic has some cells that have a wild-type version of a gene and some cells with only a mutant version of the gene. Genetic mosaics can be generated in *C. elegans* because of occasional nondisjunction of minichromosomes during mitosis. A strain is constructed such that the version of the gene on the homologous chromosomes is mutant, but the strain also harbors a minichromosome that has a wild-type copy of the gene. The minichromosome can be either a chromosomal fragment that behaves as a free duplication (Herman, 1984) or an extrachromosomal array that can form in vivo following microinjection of DNA (Lackner et al., 1994). If nondisjunction occurs during division of a cell, the daughter cell that inherits the minichromosome will generate a clone of cells that have a wild-type version of the gene; the daughter that fails to inherit the minichromosome will produce a clone of cells that only have the mutant version of the gene. The clones can be detected independently of the gene being analyzed if the minichromosome also harbors a cell-autonomous marker than is expressed in most cells of the organism that inherit the minichromosome. Once mosaic organisms are detected based on the presence of these clones, one can then determine whether certain patterns of mosaicism have phenotypic consequences for the organism. In this way, it can be determined which cells must express a wild-type copy of a gene in order to prevent a mutant phenotype. The analysis can also be used to determine whether a gene functions cell-autonomously or non-autonomously with respect to the mutant phenotype. *C. elegans* is well suited to mosaic analysis because the cell lineage has been described in detail and is nearly invariant (Sulston et al., 1983). Thus, one can often determine which particular cell experienced nondisjunction of a minichromosome during development. These principles have been discussed in detail (Yochem and Herman, 2003).

For the mosaic analysis described here, an extrachromosomal array was created that harbors both wild-type copies of the *tsp-15* gene and copies of *sur-5::gfp*, a cell-autonomous marker for genetic mosaics (Yochem et al., 1998). First, a strain having the genotype *tsp-15(sv15)/unc-29(e1072)* was microinjected (Mello and Fire, 1995) with a mixture of two plasmids: pTG96 for expression of SUR-5::GFP and SP#jy410a, which derives from the cosmid F53B6 and rescues *tsp-15(sv15)*. A segregation analysis of transgenic progeny of the injected worms led to the identification of *mnEx140*, an extrachromosomal array that clearly derived from both plasmids: both *tsp-15(+)* and *sur-5::gfp* always co-segregated for this array. This led to the establishment of SP2392, a strain with the genotype *tsp-15(sv15); mnEx140[tsp-15(+); sur-5::gfp]*, which was the source of the mosaics. For the detection of the mosaics, several thousand non-mutant L4 larvae or young adults were examined with a dissecting microscope equipped with a Hg lamp for large clones that lacked SUR-5::GFP fluorescence, indicating nondisjunction of *mnEx140* during early embryogenesis. Based on the cell lineage (Sulston et al., 1983), the cell division at which the nondisjunction occurred was deduced following an examination of GFP-positive and negative cells at high magnification with a compound microscope.

Total RNA preparation, *tsp-15* cDNA identification and DNA sequencing

Total RNA was prepared from mixed-stage populations of liquid-cultured worms or from mixed-stage populations grown on NGM plates using TRIzol reagent (Invitrogen). The 5′ and 3′ ends of the *tsp-15* mRNA were determined by RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE, Ambion). Primers specific for internal parts of the *tsp-15* coding region were designed based on the genomic DNA sequence present in WormBase. All exons were

confirmed by direct DNA sequencing of PCR products following reverse transcription.

For determination of the nature of the transcripts produced by the *sv15* mutation, total RNA of single N2 or *tsp-15(sv15)* larvae was isolated as described above, followed by DNase I (Roche) treatment. PCR was performed using the primers HM018 (5′-GCTGAACTC-GTTTCACCGTCAC-3′) and HM044 (5′-GCCTCTAGATTTTCGT-ATGAGCTTGGAATACC-3′). The PCR product was subjected to DNA sequencing and to Southern blot analysis with 3′-labeled HM018 as the probe. For identification of the *sv15* mutation, DNA was isolated from the homozygous mutant and from the parental strain and used as templates for PCR products based on the *tsp-15* genomic sequence. The sequence of all of the exons and adjacent parts the introns and flanking DNA was determined for both strands.

Construction of reporter genes

To construct the TSP-15::GFP translational fusion vector, pHM032 [*tsp-15::gfp*], the cosmid clone F53B6, which includes the *tsp-15* coding region at its extreme left, was modified as follows: the 5′ end of F53B6 was extended by PCR using the primer HM027 (5′-TGAGGTACCCAGAATGAGGATCAGGGTAACTGACTTGACC-3′) to have a *Kpn*I site for connection with 5′-neighboring T08G11 sequence; the 3′ end of the *tsp-15* coding region was engineered by PCR using the primer HM040 (5′-GCTCTAGAAAATTGTGATTTT-TTTCGTATGAGCTTGGAATACC-3′) to abolish the stop codon of TSP-15 for in-frame fusion of green fluorescent protein (GFP). A 1.1 kb *Hind*III-*Kpn*I fragment of T08G11 and a 3.8 kb *Kpn*I-*Xba*I fragment of the modified F53B6 was conjugated and then subcloned into pPD95.77 (kindly provided by A. Fire). This plasmid finally contained a 3.7 kb 5′ flanking region of *tsp-15* ORF, all exons and introns, and a fused GFP at the 3′ end.

A 0.4 kb minimal essential region for hypodermis-specific expression of the *dpy-7* gene (Gilleard et al., 1997) was isolated by genomic PCR using the primers HM094 (5′-GGCAAGCTTTGATC-GAAAGTCTCTCTCCGGTAGC-3′) and HM095 (5′-GCGGATCC-TTTATCTGGAACAAAATGTAAG-3′). This region was inserted into pPD95.77 or used to replace the *Hind*III-*Bam*HI fragment of pPD49.78. These products were designated pHM078 [P_{dpy-7}*::gfp*] and pHM080, respectively. Human CD151, monkey CD9, human CD82 and human CD63 cDNAs were subcloned into pHM080. The *tsp-15* coding region (amplified with the primers HM069, 5′-GCC-GGTACCTATGGGAGCATTAGGCGACAG-3′; and HM103, 5′-GCCTCTAGATTATTTTCGTATGAGCTTGGAATACC-3′) and *tsp-15*^{sv15} (amplified with the primers HM069 and HM135, 5′-GC-TCTAGATCAACAGGACTCTGGAATGAGC-3′) were connected to a (His)^6 tag sequence at their N-termini and then subcloned into pHM080. Tsp74F, a putative *Drosophila* homologue of TSP-15 (Todres et al., 2000), was isolated from Schneider's 2 cells by RT-PCR using the primers HM114 (5′-GCCAAGCTTATGGGCT-TCAGTTCGCGAATGGAC-3′) and HM115 (5′-GCCTCTAGAT-TCGATCATATTGAATAGTAGACAGG-3′) and cloned into pHM080.

RNA interference (RNAi)

RNAi feeding was performed as described (Timmons et al., 2001) with the following minor modifications. Briefly, after IPTG induction and 4 hours of culture, *E. coli* suspensions were concentrated in 0.1 volume of M9 buffer, and then spread and allowed to dry on NGM plates containing antibiotics. These plates were immediately stored at 4°C without further incubation until use in experiments. This treatment improved the efficiency of the RNAi. Young adult hermaphrodites (P_0) were fed on the plates and transferred to new plates at 12 hour intervals for four days. The phenotypes of the F_1 progeny were determined 60 to 72 hours after the parental worms were seeded.

Transformation of nematodes and rescue assays

Germline transformation was performed by standard methods (Mello and Fire, 1995). Fifty ng/μl pHM032 [*tsp-15::gfp*] was injected into the syncytial gonads of young adult hermaphrodites. Fifty ng/μl pRF4 [*rol-6 (su1006)*] was co-injected as a marker. For rescue analyses, 1 ng/μl *dpy-7* promoter (P_{dpy-7})-driven tetraspanin constructs pHM090 [P_{dpy-7}*::tsp-15His*], pHM164 [P_{dpy-7}*::tsp-15*^{sv15}*His*], pHM083 [P_{dpy-7}*::CD9*], pHM085 [P_{dpy-7}*::CD63*], pHM084 [P_{dpy-7}*::CD82*], pHM082 [P_{dpy-7}*::CD151*], pHM118 [P_{dpy-7}*::Tsp74FHA*] or pHM032 was injected into *tsp-15(sv15)* mutants. Fifty ng/μl p*lin-44::gfp* (Herman et al., 1995) was used as an injection marker, and the total amount of DNA was 100 ng/μl with pBluescript SK II. The phenotypes of more than 100 GFP-positive progeny derived from three independent germline transformants were observed for the rescue analyses. pHM078 [P_{dpy-7}*::gfp*] was used to mark the hypodermis in *tsp-15(sv15)* and *bli-2(e768)* mutants.

Immunohistochemistry

Cryosectioning of worms was performed as described (Graham et al., 1997). Five-μm-thick sections of *Ex[tsp-15::gfp]* worms were prepared and incubated in 1 μg/ml rhodamine phalloidin (Molecular Probes) or in 1:300 dilutions of MH4 or MH5 antibodies that recognize a component of FOs (Francis and Waterston, 1991; Hresko et al., 1994). Goat Cy3-conjugated anti-mouse IgG (Chemicon) was used as the secondary antibody. The sections were analyzed by LSM 510 confocal microscopy (Carl Zeiss) with a 63× water-immersion objective.

Hoechst staining

Worms were washed off plates with M9 buffer and then incubated in M9 buffer containing 1 μg/ml Hoechst 33258 (Sigma) at room temperature for 15 minutes with gentle agitation, followed by several washes with M9 buffer.

Electron microscopy

Worms were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for two days, osmicated, stained en bloc with uranyl acetate, dehydrated and embedded in Quetol 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed under a JEOL 1200EX electron microscope.

Results

The *tsp-15* gene product

A BLAST search of the *C. elegans* genomic database using human and mouse tetraspanin protein sequences identified 20 tetraspanin-related genes, a finding consistent with a previous prediction (Todres et al., 2000). To examine the physiological functions of the tetraspanins, we isolated cDNAs for each of these genes and performed RNAi analyses. As described below, double strand RNA (dsRNA) from one of these genes, *tsp-15*, confers a dramatic effect on epidermal morphology. In contrast to *tsp-15*, dsRNA from the other 19 tetraspanin genes did not appear to affect the hypodermis, suggesting that *tsp-15* has a specialized function, which may be within the hypodermis.

An analysis of cDNA by PCR techniques has revealed that the *tsp-15* transcripts contain the trans-splicing leader sequence SL1 at the 5′ end and a poly(A) sequence at the 3′ end. Comparison of the genomic and cDNA sequences revealed that the *tsp-15* gene is composed of six exons (Fig. 1A). The 258 amino acid product (Swiss Prot No. Q9XVM9) predicted for

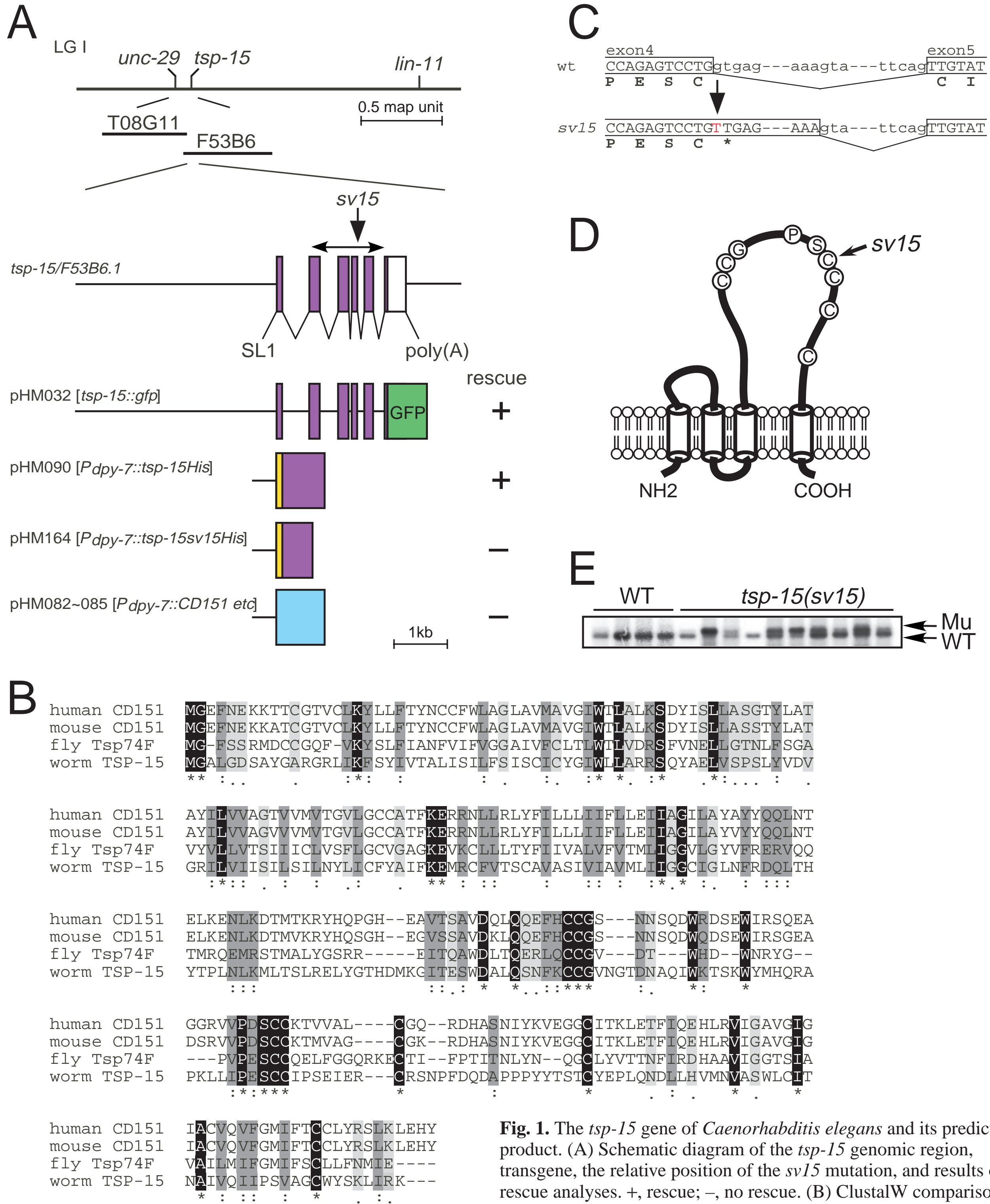

Fig. 1. The *tsp-15* gene of *Caenorhabditis elegans* and its predicted product. (A) Schematic diagram of the *tsp-15* genomic region, transgene, the relative position of the *sv15* mutation, and results of rescue analyses. +, rescue; –, no rescue. (B) ClustalW comparison of TSP-15 (Q9XVM9) with human CD151 (P48509), mouse CD151 (Q35566) and fruit fly Tsp74F (Q9VVM5), which are the tetraspanins from other species that most closely resemble TSP-15. Identical and similar amino acids are indicated. The identities of the sequences between human and worm or fly and worm are both ~24%. (C) Representation of the position of the *sv15* mutation (a G-to-T transversion), and the effect of the mutation on splicing of intron 4. The mutation alters the splice donor site, and a cryptic splice donor in the middle of intron 4 is often used (upper versus lower case). (D) Schematic representation of the putative structure of TSP-15 and the relative position of the *sv15* mutation. Well-conserved amino acid residues among the tetraspanin family are indicated. (E) Expression of the wild-type transcript is reduced in *tsp-15(sv15)* mutants and a cryptic donor site is often used. RT-PCR and subsequent Southern blotting of *tsp-15* transcripts obtained from single mutant larvae. The region of *tsp-15* examined by the RT-PCR is indicated with a double arrow in A. *tsp-15(sv15)* mutants produced a PCR product of the same length as that of the wild type and the ratio of correct and irregular transcripts differs for each individual mutant.

the *tsp-15* gene has four hydrophobic stretches that can potentially delineate a small extracellular loop from a large extracellular loop. A BLASTP search revealed that TSP-15 is similar to the mammalian tetraspanin CD151 (Fig. 1B). Although the identity is not high (24%), the residues that are hallmarks of tetraspanins, including CCG and SCC triplets, are conserved in the large extracellular loop (Fig. 1B,D).

tsp-15(RNAi) animals have epidermal defects

When wild-type worms are fed dsRNA derived from the *tsp-15* gene, more than 95% of the progeny die as larvae and have abnormal epidermal morphology (Fig. 2B,E). Most of the *tsp-15(RNAi)* animals arrest growth in the fourth (L4) larval stage, during which the body swells and internal tissue gradually becomes colorless. Moreover, small and large blisters of the cuticle are occasionally seen. About 1% of *tsp-15(RNAi)* animals develop into adults showing a distended body shape, epidermal blistering, or both.

Mutations in a least six genes, *bli-1* to *bli-6*, can cause blistering of the *C. elegans* cuticle (WormBase). Usually seen only in adulthood, the morphology of the blisters implies that they are filled with fluid and that they result from a separation of layers of the cuticle. The blistered cuticle observed with *tsp-15(RNAi)* differs, however, from that seen with a typical *bli* mutation, such as *bli-2(e768)* (Fig. 2C,F). Unlike the *bli* mutant, unidentified material accumulates within the blisters of the *tsp-15(RNAi)* animals (Fig. 2B,E). Furthermore, in contrast to the few large blisters that appeared in *bli* mutants, a number of small blisters appear around the whole body wall in *tsp-15(RNAi)* animals. These observations, together with observations described below, indicate that the phenotype of *tsp-15(RNAi)* animals is distinct from that caused by the *bli* mutations.

Isolation of a *tsp-15* mutation exhibiting multiple epidermal defects

During a search for recessive mutations that affect the completion of molting, a mutation, *sv15*, was isolated that displays an unusual phenotype. The most obvious consequence of the mutation is that homozygotes are nearly always short and fat (Dpy; Fig. 2H). The homozygotes can also have blistering of the cuticle, which can be one of two types. One type is rare and usually is seen only at adulthood. The blisters appear to be filled with clear fluid and resemble the blisters seen with *bli-2(e768)*, the typical *bli* mutation described above and in Fig. 2G. In contrast, 25/31 L3 and 26/26 L4 homozygotes have at least a few small blisters. As for RNAi of *tsp-15*, the blisters are filled with material, which in some cases is clearly cellular (Fig. 3). Three of 35 L1 larvae, on the other hand, lacked blisters, but all were dumpy.

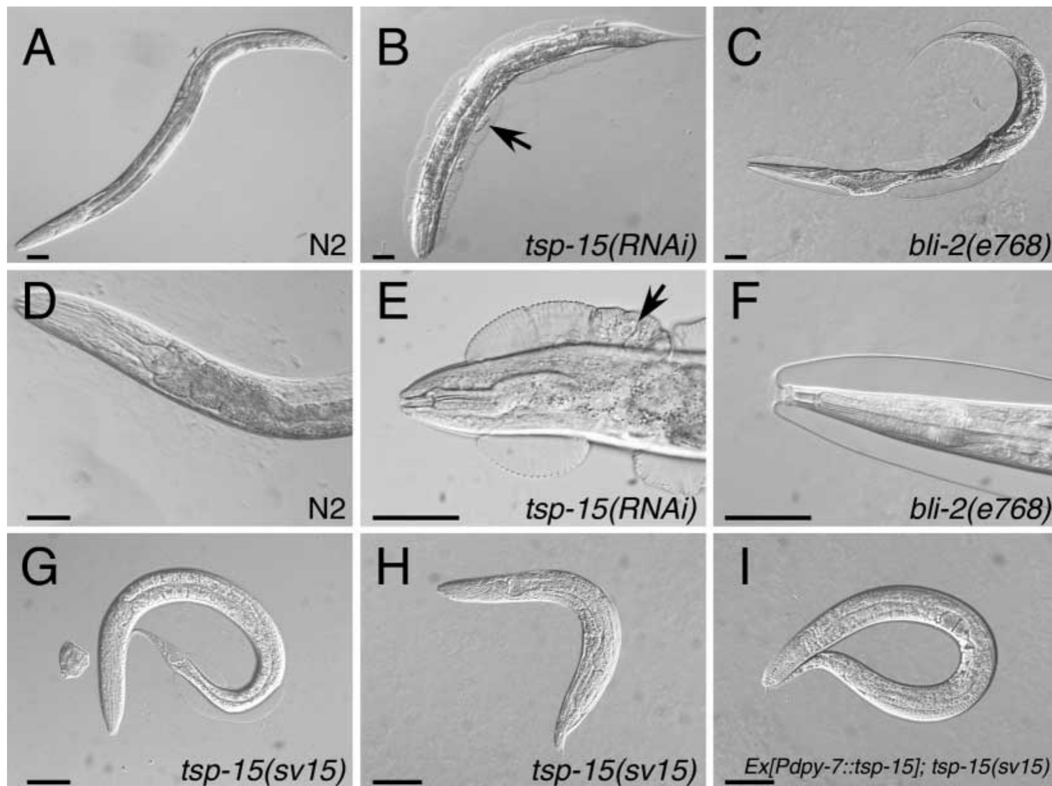

Fig. 2. Abnormal epidermal morphology caused by loss or reduction of *tsp-15* in *C. elegans*. (A-I) Nomarski images of N2 L4 larva (A,D), *tsp-15(RNAi)* L4 larva (B,E), *bli-2(e768)* adult (C,F), *tsp-15(sv15)* L3 (G) and L4 larva (H), and *Ex[P_{dpy-7}::tsp-15His]; tsp-15(sv15)* L4 larva (I) are shown. *tsp-15(RNAi)* animals (B,E) show numerous epidermal blisters and a swollen body morphology. Unlike *bli-2(e768)* mutants (C,F), unidentified material accumulates within the blisters in *tsp-15(RNAi)* animals (arrows in B and E). *tsp-15(sv15)* mutants typically have milder defects than *tsp-15(RNAi)* animals (compare G with B and E) and sometimes only exhibit a dumpy shape (H). (I) Rescue of *sv15* by expression of the wild-type *tsp-15* gene in the hypodermis. Bars, 50 μm.

As described in the next section, the *sv15* mutation can have a dramatic effect on epidermal morphology (Figs 2 and 3). The mutation can also confer retarded growth and slow movement. Over half the *sv15* mutants can develop into adults. These are Dpy (Fig. 2H) and occasionally have a trail of old cuticle attached to the body. About 35% of the mutants die during one of the four larval stages, and the death is usually accompanied by epidermal blistering (Fig. 2G,H), a distended body morphology, or both. These effects of the mutation are for the most part reminiscent of *tsp-15(RNAi)*. Genetic mapping, which placed *sv15* just to the right of *unc-29* on LGI; rescue experiments (Fig. 1A), including expression of a *tsp-15* cDNA from a heterologous promoter (Fig. 2I); and DNA sequence analysis, described below, demonstrate that *sv15* is indeed a mutation in *tsp-15*. One difference between the mutation and RNAi is that larval lethality caused by the RNAi usually occurs in the later stages. In addition, although embryonic lethality was not observed in *tsp-15(RNAi)* worms, approximately 10% of the *sv15* mutants die during embryogenesis. The eggshells of the affected embryos were spherical rather than oval, and the embryos died prior to the completion of morphogenesis (data not shown). Deficiency and RNAi studies indicate that *sv15* is a hypomorphic mutation: it does not cause a complete loss of function.

DNA sequence analysis of *tsp-15(sv15)* homozygotes revealed a G-to-T transversion of the first nucleotide of the splice donor of intron 4 (Fig. 1C). RT-PCR analysis from single *tsp-15(sv15)* mutant larvae (note that they have still not held eggs) demonstrated that the irregular transcripts were incorrectly spliced in the middle of intron 4 and connected to exon 5, resulting in a transcript that was 65 nucleotides longer than that of wild-type *tsp-15* mRNA (Fig. 1C,E). The mutant *tsp-15* product harbors a stop codon in place of cysteine 190 (Fig. 1C,D), which is a conserved residue for the disulphide bond-mediated structure of tetraspanin proteins (Stipp et al., 2003). In *tsp-15(sv15)* mutants, some *tsp-15* transcripts are correctly spliced, and the ratio of correct and irregular transcripts is different for individual animals (Fig. 1E), which may explain the variable penetrance and expressivity of *tsp-15(sv15)*. Regarding the severity of the *tsp-15(sv15)* mutant phenotype, we performed *tsp-15(RNAi)* in the *tsp-15(sv15)* mutant background. All the *tsp-15(RNAi);tsp-15(sv15)* animals died in the L2 or L3 stages, showing that *tsp-15(RNAi)* can synergistically augment the phenotype of *tsp-15(sv15)* (data not shown). This suggests that the severity of the phenotype dependents on a reduction in the level of the *tsp-15* product.

Although the RNAi of *sv15* animals indicates that *sv15* is probably a hypomorphic mutation, it is still possible that the truncated TSP-15 protein produced from the *sv15* allele when splicing of intron 4 fails might have a gain of normal or aberrant function whose manifestation requires at least two copies of the mutant gene. To examine this possibility, we made use of *nDf29*, a large chromosomal deficiency that should delete the wild-type copy of *tsp-15*, to generate animals that have one copy of *tsp-15(sv15)* but no other copies of the gene. Consistent with the RNAi, *sv15*/*nDf29* animals are less healthy than animals that are homozygous for *sv15*: although half of the homozygotes can become fertile adults, none of the hemizygous animals reached adulthood. Moreover, the dumpiness conferred by *sv15* appeared to be enhanced by the deficiency. Similar results were seen with *nDf25* and *nDf23*, which are other large deficiencies of the relevant region of LGI. In addition, expression of the mutant TSP-15 protein (TSP-15^{sv15}) in *tsp-15(sv15)* or N2 animals results in no obvious effects (data not shown). Thus, *tsp-15(sv15)* is a hypomorphic (i.e. reduction-of-function) mutation.

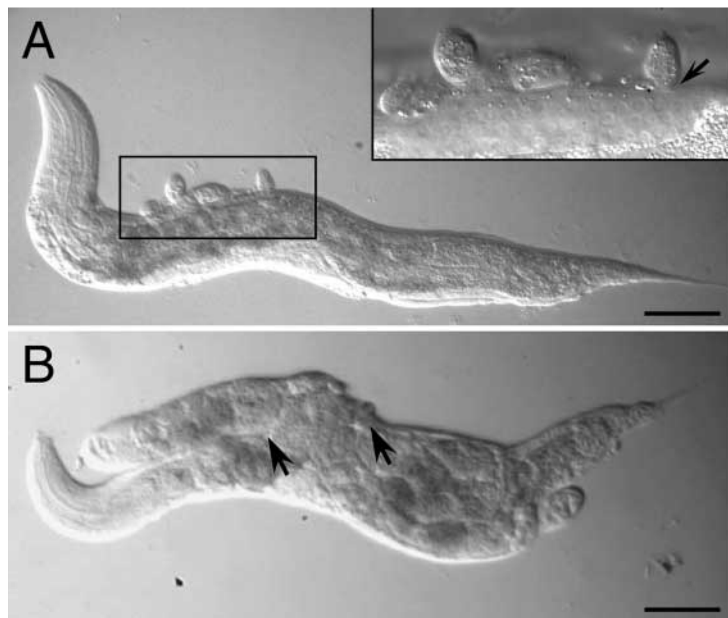

Fig. 3. Protrusions in *C. elegans* associated with *tsp-15(sv15)*. (A) An L4 larva from the SP2275 strain with small protrusions of the hypodermis. A Nomarski image of the cluster of protrusions is shown at higher magnification in the inset. Nuclei can be seen in several of them, and the posterior-most protrusion is connected to the main body by a narrow stalk (arrow), a common feature of small protrusions. (B) An L4 larva from SP2275 with two large protrusions. The dorsal protrusion is connected to the main body in the region delineated by the arrowheads. The protrusion clearly contains part of the intestine. Bars, 50 μm.

Unusual effects of *sv15* on the hypodermis

Closer examination of *sv15* homozygotes revealed that at least some and perhaps all of the small blisters are actually outgrowths of the hypodermis perpendicular to the long axis of the body (Fig. 3A). Present at low penetrance (2%), are animals with highly unusual protuberances that can become quite large relative to the body (Fig. 3B). The protuberances are sometimes connected to the main body by a narrow stalk and are often perpendicular to the main body, like the small blisters. Finally, preliminary observations indicate that the seam cells, which compose the lateral hypodermis and make adherences junctions with hyp7 (Francis and Waterston, 1991), are often aberrant, including large gaps in the seam and regions of forking and branching.

Requirement of TSP-15 protein in the hypodermis

To investigate the expression pattern of the gene, GFP, lacking a specific protein-sorting signal, was fused in-frame to the C-terminus of the full length TSP-15 protein

(Fig. 1A). The *tsp-15::gfp* transgene was found to rescue *tsp-15(sv15)*, indicating that the TSP-15::GFP protein can substitute functionally for native TSP-15 and that this fusion gene is probably expressed at the same sites as the native *tsp-15* gene. TSP-15::GFP expression begins in the middle of embryogenesis (around the comma stage) and continues through adulthood (Fig. 4A,B). Expression was detected in the pharynx and body wall from the larva to the adult and in the vulva at the adult stage (Fig. 4B). Owing to the epidermal defects of the *tsp-15* mutant animals, we examined the expression pattern of TSP-15::GFP in the body wall in detail by histochemical analysis. In transverse sections, TSP-15::GFP appears to be present in hyp7, including the thin regions between the cuticle and the body muscle quadrants, which are visualized with fluorescent phalloidin in Fig. 4C. Expression in the thin layers of hyp7 was confirmed by co-staining with MH4 (data not shown) or with MH5 (Fig. 4D). Both are monoclonal antibodies that stain the fibrous organelles present in the region for attachment of the body muscles to the cuticle (Bosher et al., 2003; Francis and Waterston, 1991; Hresko et al., 1994; Karabinos et al., 2001). In addition, TSP-15::GFP is expressed in a punctate pattern in the thick region of the hypodermis (Fig. 4E).

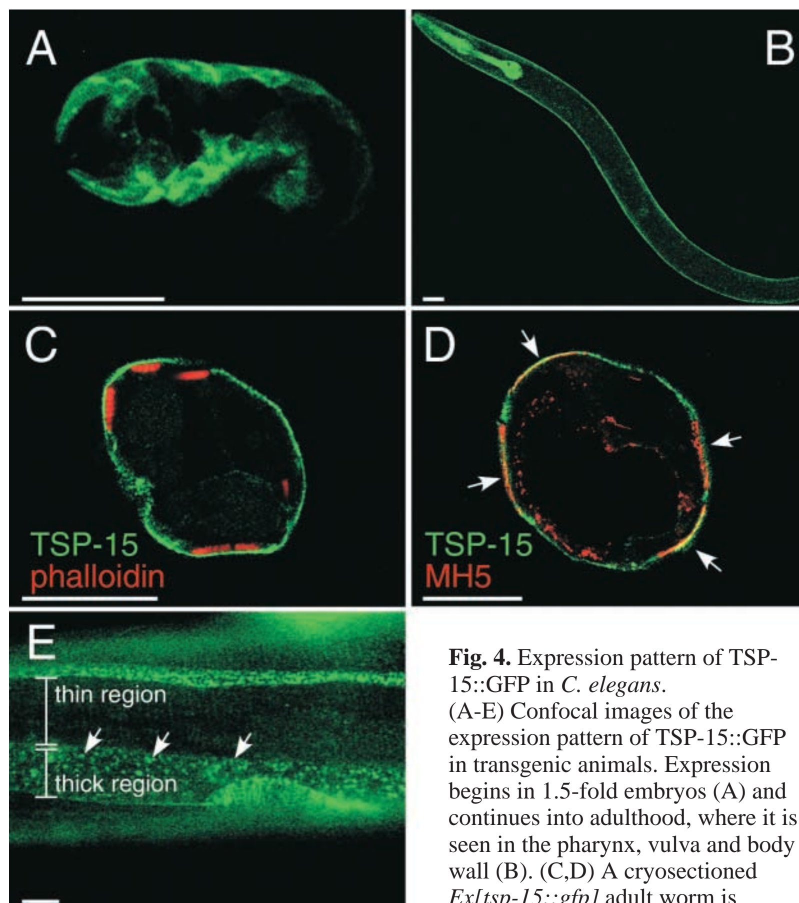

Fig. 4. Expression pattern of TSP-15::GFP in *C. elegans*. (A-E) Confocal images of the expression pattern of TSP-15::GFP in transgenic animals. Expression begins in 1.5-fold embryos (A) and continues into adulthood, where it is seen in the pharynx, vulva and body wall (B). (C,D) A cryosectioned *Ex[tsp-15::gfp]* adult worm is shown. TSP-15::GFP is not seen in the body muscle quadrants, which are visualized by rhodamine-phalloidin staining (C), but is present in the hypodermis, as confirmed by colocalization with MH5 antigen (D, arrows), a component of the FOs. (E) A punctate localization of TSP-15::GFP (arrows) in thick region of hypodermis. Bars, 25 μm.

In order to determine which cells must express the wild-type *tsp-15* gene in order to prevent the *tsp-15* mutant phenotype, genetic mosaics were generated from a strain, SP2392, having the genotype *tsp-15(sv15); mnEx140 [tsp-15(+); sur-5::gfp]*. The mosaic analysis was based on the generation of *tsp-15(–)* clones within an animal that otherwise has wild-type copies of the *tsp-15* gene following nondisjunction of the extrachromosomal array *mnEx140* during embryonic cell divisions. Cells in a mosaic animal that failed to inherit the array were determined by examining nuclei for the presence or absence of green fluorescence, which is conferred cell-autonomously by *sur-5::gfp* DNA also present on the array. Although a zygote must inherit *mnEx140* in order to be classed as wild type, there was a striking plasticity in inheritance of the array in mosaic animals that lacked all aspects of the *tsp-15* mutant phenotype: inheritance of the array either by AB or by P_1, the daughters of the zygote, is sufficient to rescue the mutant phenotype fully (Fig. 5). This pattern of mosaicism indicates that the gene is required in hyp7, which is a large syncytium composed of nuclei that descend from both AB and P_1 (Sulston et al., 1983). With the exception of one body muscle and two neurons, no other extensive cell type or tissue has progenitors from both AB and P_1. It might be argued that inheritance of wild-type *tsp-15* by any cell is sufficient to prevent the mutant phenotype, but there were mosaic animals (for instance, mosaics in which only the intestinal cells expressed the array) that had the *tsp-15* mutant phenotype, indicating that they failed to inherit the array in critical cells or tissue. Additional evidence that hyp7 is the focus of *tsp-15* activity derives from rare mosaics in which inheritance by either of the blastomeres P_2, Ca, C, ABa, ABp, ABpl or ABar was sufficient for rescue of the mutant phenotype (Fig. 5): each of these blastomeres contributes nuclei to hyp7, but they have nothing else in common. Thus, the mosaic analysis provides independent evidence that the only critical tissue for the expression of *tsp-15* is the syncytium hyp7. Because *tsp-15(RNAi)* and *tsp-15(sv15)* affect the hypodermis and cuticle, it is reasonable to assume that *tsp-15* acts cell-autonomously within hyp7, because hyp7 is the major hypodermis and is the source of most of the body cuticle of *C. elegans* (White, 1988).

tsp-15(sv15) rescue assays

Of the mammalian tetraspanins, TSP-15 is most similar to CD151. Although the similarity is low, we nevertheless tested whether expression of cDNA clones of human CD151 and other tetraspanins could rescue *tsp-15(sv15)*. The tetraspanin genes were expressed in a hypodermis-specific manner using a minimal *dpy-7* promoter (Gilleard et al., 1997). Rescue was determined by scoring the progeny derived from three independent germline transformants, and the expression of each tetraspanin in transgenic animals was confirmed by western blotting (data not shown). Under the expression driven

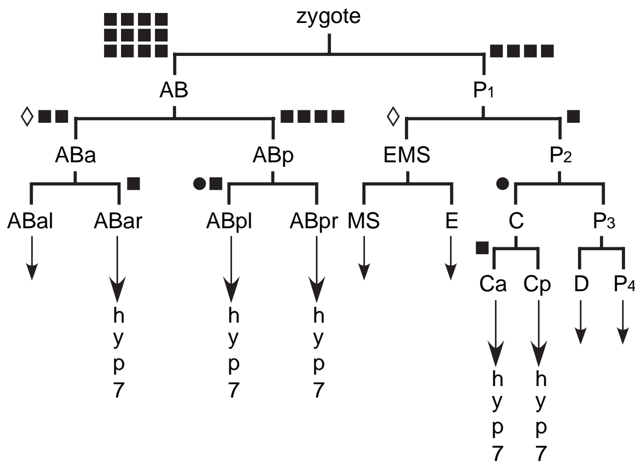

Fig. 5. Genetic mosaics of *C. elegans* implicate hyp7 as the focus of *tsp-15* activity. A total of 28 mosaics were identified that had had early losses of *mnEx140* but displayed no aspects of the *sv15* mutant phenotype, indicating where in the early cell lineage inheritance of wild-type *tsp-15* DNA is sufficient to prevent the mutant phenotype. Based on expression of *sur-5::gfp* in descendents of the early cells of an embryo (Sulston et al., 1983), cells establishing a positive clone in an individual mosaic are indicated with a square next to the name of the cell. For two mosaics, one depicted with circles and the other with diamonds, two cells of the embryo independently established positive clones. The early cells of an embryo that contribute to the hyp7 syncytium are indicated.

by the *dpy-7* promoter, *tsp-15(sv15)* can be rescued by the wild-type *tsp-15* gene itself (Fig. 1A and 2I) or by the *tsp-15::gfp* fusion gene described above, which further confirms the requirement for TSP-15 in the hypodermis. In contrast, neither CD151, CD9, CD63, CD82 nor *Tsp74F* (CG5492), a *Drosophila* tetraspanin (Fradkin et al., 2002), could rescue *tsp-15(sv15)* (data not shown).

Cuticle degeneration and loss of hypodermis membrane integrity in *tsp-15(RNAi)*

Renewed at each of the four molts, the cuticle is a multi-layered structure that is composed in part of highly crosslinked collagen (Kramer, 1997). Cortical, medial, fiber and basal layers are identified in the adult cuticle (Fig. 6A) (Cox et al., 1981). The ultrastructure of *tsp-15(RNAi)* animals showing epidermal blisters was observed by transmission electron microscopy. Severe splits within the cuticle were observed in *tsp-15(RNAi)* animals. The splits seemed to occur in the cortical layer, while the fiber and basal layers were unaffected. Granular and membranous materials, reminiscent of degenerated cellular organelles, accumulated within the split cuticle (Fig. 6B-D). In addition to the unusual cuticle structure, abnormal gaps were also observed underneath the cuticle between the hypodermis and musculature (Fig. 6B,D). The hypodermis was severely distorted with the basement membrane exhibiting inconsistent thickness (Fig. 6C,D). FOs, which are responsible for attachment of the body muscles to

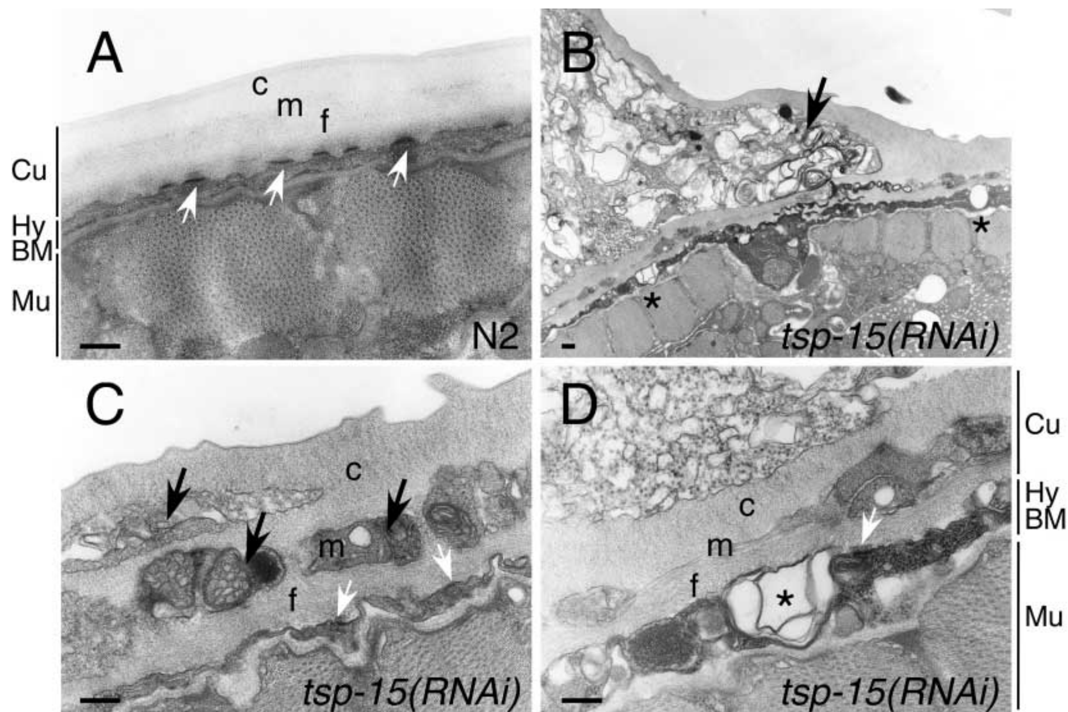

Fig. 6. Cuticular and hypodermal abnormalities in *tsp-15(RNAi) C. elegans*. (A-D) Transmission electron micrographs of the epidermis of N2 and *tsp-15(RNAi)* animals. C and D are higher magnification images of *tsp-15(RNAi)* animals. In *tsp-15(RNAi)* animals, the cuticle is dissociated in the cortical layer (c). Unidentified material accumulates within the cortical and medial layers (m) of the cuticle (black arrows in B and C; compare with A), but the fiber layer (f) remains intact. There are some abnormal gaps within the hypodermis (asterisks in B and D). Note that the hypodermis and basement membrane are severely distorted, and the basement membrane has inconsistent thickness (C,D). The FOs, represented as electron-dense structures are indicated by white arrows. Cu, cuticle; Hy, hypodermis; BM, basement membrane; Mu, muscle. Bars, 200 nm.

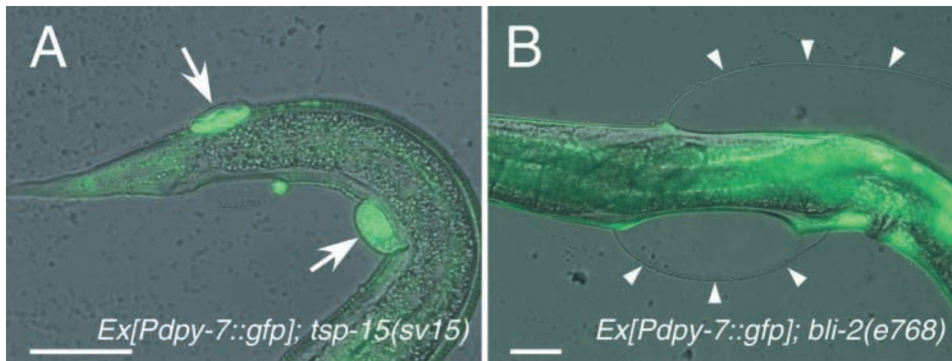

Fig. 7. Functional deficiency of epidermal membrane integrity in *tsp-15* hypomorph *C. elegans*. (A,B) P_{dpy-7}*::gfp* expression patterns merged with the Nomarski images in the *tsp-15(sv15)* and *bli-2(e768)* backgrounds. In *tsp-15(sv15)* mutants, but not in *bli-2(e768)* mutants, GFP fluorescence is observed within blisters (arrows), suggesting that cellular components have leaked into the blisters in *tsp-15* mutants. Small arrowheads indicate the edge of the blisters in *bli-2(e768)* mutants. Bars, 50 μm.

the cuticle, were still observed in the hypodermis of *tsp-15(RNAi)* animals as electron-dense structures (Fig. 6C, white arrow), but the number of FOs was reduced in *tsp-15(RNAi)* animals compared with that of wild-type controls (Fig. 6A,C).

The integrity of the hypodermal membrane was examined based on its ability to act as a barrier to either GFP or Hoechst 33258, a hydrophilic and therefore membrane-impermeable dye that stains nuclei. GFP lacking a signal sequence was expressed in hyp7 using the promoter from the *dpy-7* gene, and leakage of GFP from the cytoplasm to the outside of the plasma membrane was measured. GFP fluorescence accumulated within the blisters in *tsp-15(sv15)* mutants (Fig. 7A), but such leakage of GFP was not observed in *bli-2(e768)* mutants (Fig. 7B) or in wild-type worms (N2) (data not shown). These results suggest that the plasma membrane of the hypodermis in *tsp-15* mutants has lost its barrier function at that time or is too fragile to maintain constant integrity, although an alternative explanation based on the micrographs in Fig. 3 is that the GFP seen in the blisters represents outgrowth of hyp7 orthogonal to the body.

The barrier function of the hypodermis membrane in *tsp-15(sv15)* animals was further examined by staining from the outside by suspending living worms in buffer containing Hoechst 33258, which fluoresces when bound to DNA. As expected for a membrane-impermeable dye, the nuclei of living cells fail to stain in more than 90% of wild-type worms (N2) (Fig. 8A, G). Under the same conditions, *tsp-15(RNAi)* and *tsp-15(sv15)* worms often show nuclear staining (Fig. 8B,D,E).

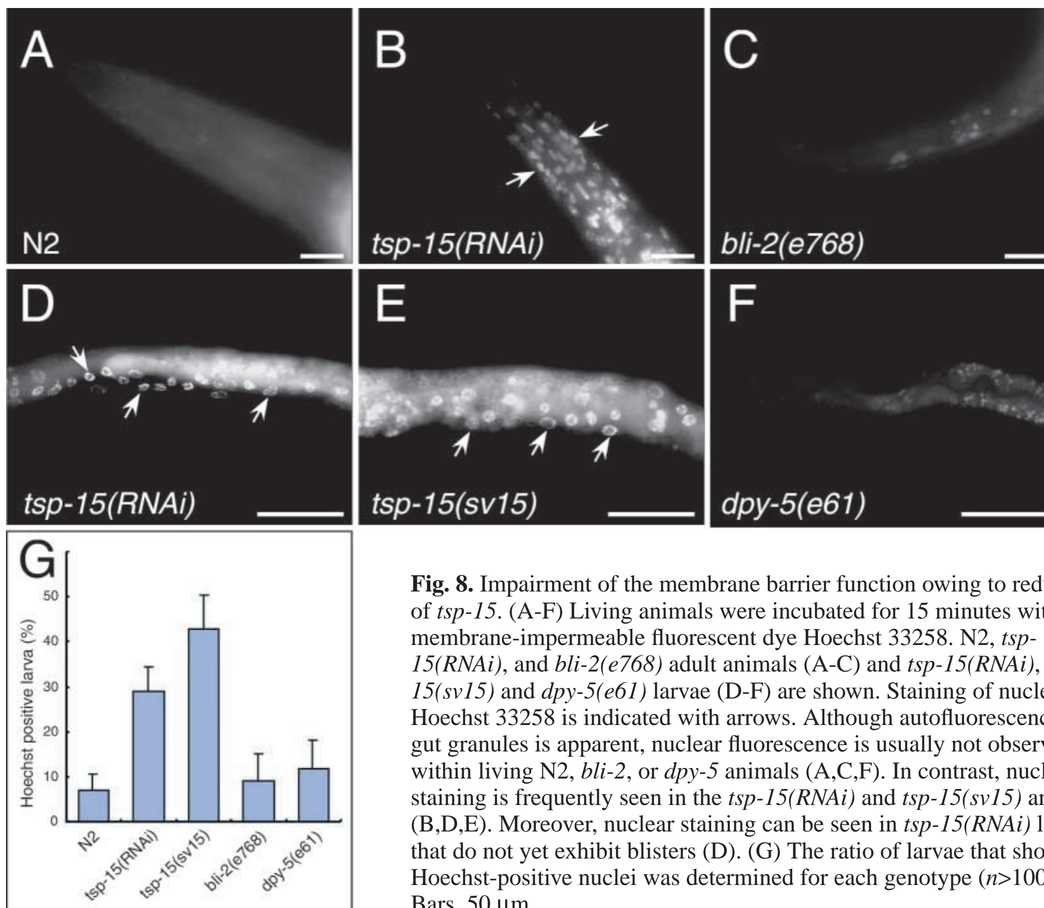

Fig. 8. Impairment of the membrane barrier function owing to reduction of *tsp-15*. (A-F) Living animals were incubated for 15 minutes with the membrane-impermeable fluorescent dye Hoechst 33258. N2, *tsp-15(RNAi)*, and *bli-2(e768)* adult animals (A-C) and *tsp-15(RNAi)*, *tsp-15(sv15)* and *dpy-5(e61)* larvae (D-F) are shown. Staining of nuclei by Hoechst 33258 is indicated with arrows. Although autofluorescence of gut granules is apparent, nuclear fluorescence is usually not observed within living N2, *bli-2*, or *dpy-5* animals (A,C,F). In contrast, nuclear staining is frequently seen in the *tsp-15(RNAi)* and *tsp-15(sv15)* animals (B,D,E). Moreover, nuclear staining can be seen in *tsp-15(RNAi)* larvae that do not yet exhibit blisters (D). (G) The ratio of larvae that showed Hoechst-positive nuclei was determined for each genotype (n>100). Bars, 50 μm.

Emphasizing the unusual nature of the *sv15* defect, staining is not often seen with other mutations that affect the cuticle, including the blister mutation *bli-2(e768)* or the dumpy mutation *dpy-5(e61)* (Fig. 8C,F). In addition, nuclear staining was observed in *tsp-15(RNAi)* and *tsp-15(sv15)* animals that were lacking blisters at the L3 and L4 stages, suggesting that membrane fragility precedes appearance of the blisters (Fig. 8D,E,G). Considering all these results together, we conclude that the loss or reduction of TSP-15 results in impairment of the membrane integrity.

Discussion

Complex epidermal abnormality in *C. elegans* deficient for TSP-15

We have demonstrated that a tetraspanin, TSP-15, is essential for the integrity of the hypodermis of *C. elegans*. Reduction of function of *tsp-15* by RNAi or by mutation results in complex defects in epidermal morphology, including a dumpy shape and blistering of the cuticle. Although these defects are seen with mutations in other genes in *C. elegans*, the *tsp-15* mutants also show severe abnormalities in epithelial cell function in the hypodermis, including detachment of the hypodermis cell membrane from the cuticle, a reduced number of fibrous organelles (FOs), and possible loss of the barrier function of the hypodermis membrane. Consistent with these hypodermal defects, *tsp-15* is required in hyp7, the major component of the hypodermis.

What is the primary defect in *tsp-15* mutants? Previously isolated mutants with Bli and Dpy phenotypes have been shown to have deficiencies in cuticle collagen or collagen-processing proteins (Johnstone, 2000). Thus, it is conceivable that *tsp-15* mutants may have a defect in a step of cuticle biogenesis that makes the animals dumpy and makes the cuticle prone to separation into layers. The occasional large blisters of *tsp-15* that appear to be filled with clear fluid in particular resemble those seen with the *bli* mutations. Perhaps more indicative, though, of the epidermal pathology of *tsp-15* are the small blisters that occur frequently and pervasively and are seen even in larval stages. The presence of material, which can include nuclei and other cellular components, within the blisters might be explained by a loss of the integrity of the cuticle. Perhaps the hydrostatic pressure of the animal forces internal parts and tissues into a cuticle that lacks proper integrity and is therefore prone to forming internal pockets that are manifested externally as small blisters. The strength of the defective cuticle is sufficient, though, to maintain the overall integrity of most of the animals, at least during early larval stages, but makes them vulnerable to stresses. Molting might be a particular strain for the *tsp-15* animals, because the cuticle that is being synthesized may be compromised, and because the old cuticle, which may also be defective, must continue to maintain the integrity of the animals until the new cuticle succeeds this function.

Another explanation of the complex phenotype of *tsp-15* mutants is deficiency in the function of the hypodermis itself rather than in the cuticle it synthesizes. In contrast to cuticle biogenesis, the contribution of the hypodermis to epidermal morphology is largely unknown except that rearrangement of its cytoskeletal elements during late embryogenesis is essential for formation of the final overall shape of the exoskeleton, and the cytoskeletal elements of the hypodermis are transiently connected to the cuticle during each postembryonic molt (Costa et al., 1997; Priess and Hirsh, 1986). Based on leakage of GFP from hyp7 and on the uptake of Hoechst, the present study indicates that fragility of the hypodermal membrane precedes the appearance of blisters, suggesting a key role of *tsp-15* in generating or maintaining the integrity of the membrane, though a defective cuticle may not always be apparent with the light microscope. If the defect is in the apical membrane of hyp7, TSP-15 may function in membrane stability through interaction with ECM components, connection with cytoskeletal proteins, biogenesis and transport of plasma membrane components, or formation of membrane protein complexes with other membrane proteins. It is also possible that TSP-15 is required for repair of the plasma membrane, a process that involves exocytosis of lysosomes in mammalian tissue culture cells (Bi et al., 1995; Reddy et al., 2001). Deficiency in such a repair system could result in impaired membrane integrity and leakage of hypodermal components. Impaired stability of the hypodermis membrane may synergistically affect the tight adhesion with the cuticle and cuticle biogenesis. Furthermore, especially in the molting process, irreversible rupture of the fragile membrane may occur through mechanical damage. Further analyses of *tsp-15* mutants are needed to investigate this hypothesis.

Possible molecular function of TSP-15 in the hypodermis

TSP-15 is a transmembrane protein that is required in hyp7. Since tetraspanin proteins generally do not have either enzymatic activity or any ligand activity, the molecular roles of TSP-15 in the hypodermis remain speculative. Several lines of evidence have suggested the involvement of tetraspanins in cell adhesion, and it was demonstrated that CD151 is a component of hemidesmosomes along with $\alpha6\beta4$ integrin (Sterk et al., 2000). Loss of hemidesmosomal components results in skin diseases such as epidermolysis bullosa in humans and mice (Borradori and Sonnenberg, 1996). As for hemidesmosomes in vertebrates, FOs in the hypodermis of *C. elegans* play a role in the maintenance of epidermal integrity as a cell attachment machinery, at least in the regions of the body muscle quadrants (Bosher et al., 2003; Hresko et al., 1999; Karabinos et al., 2001; Plenefisch et al., 2000). Hence, based on the sequence similarity of TSP-15 with CD151, a probable explanation is that loss of TSP-15 molecules results in functional or structural defects in the cell attachment machinery. In *tsp-15(RNAi)* animals, however, electron microscopic analysis did not show such severe detachment in the cuticle-hypodermis or hypodermis-muscle boundary as shown in *mua-3* and *mup-4* mutants, which are known to have defective FOs (Bercher et al., 2001; Hong et al., 2001). It was also shown that CD151 failed to rescue the *tsp-15(sv15)* mutants and that the localization of TSP-15::GFP was distinct from the circumferential bundle pattern characteristic of the molecules composing FOs. Regarding this issue, we were unable to demonstrate by co-immunoprecipitation (see supplementary material, Fig. S1) an association of TSP-15 with PAT-3, the integrin β subunit in *C. elegans* (Gettner et al., 1995). Although an association of TSP-15 with the cell

attachment machinery remains to be demonstrated, a role in the attachment of the hypodermis to its ECM, the cuticle, should still be considered.

Another explanation is that TSP-15 is involved in the transport and fusion of intracellular vesicles. In mammals, several classes of tetraspanins function in endocytosis: CD82 binds with EGFR and desensitizes it (Odintsova et al., 2000), and CD63 accumulates within the endocytotic compartment (Kobayashi et al., 2000). As shown for several other tetraspanins (Berditchevski, 2001; Bonifacino and Dell'Angelica, 1999), a tyrosine internalization motif, YxxΦ (where Φ represents a hydrophobic amino acid), which is a conserved binding sequence for the endocytotic adaptor molecule adaptin, is found in the C-terminal cytoplasmic domain of TSP-15. It has been shown that deficiency in endocytosis and mutations in the endocytotic molecules result in defects in epidermal morphology and molting in *C. elegans* (Grant and Hirsh, 1999; Greener et al., 2001; Shim and Lee, 2000; Zhang et al., 2001b), suggesting that endocytosis is intimately connected with the maintenance and development of the epidermis, but these deficiencies do not cause blistering of the cuticle. The *tsp-15* defect may instead be in the opposite process, secretion of components of the cuticle. Perhaps there is a loss of regulation of fusion of secretory vesicles to the apical plasma membrane of hyp7, such that there are regions with insufficient fusion and regions with excess fusion. The regions with excess fusion could be where blebs are seen to enter the cuticle. Perhaps the large protuberances that are occasionally seen result from excess fusion on a grand scale. The extreme demand on the hypodermis for proper secretion of components during formation of a new cuticle may also explain why molting is a particular strain for the mutants.

In conclusion, we have reported that a tetraspanin, TSP-15, is essential for epidermal integrity and molting in *C. elegans*. This study provides the first direct evidence of functional involvement of a tetraspanin in epidermal integrity in vivo and the first demonstration of a functional requirement for a tetraspanin in *C. elegans*. Tetraspanin genes have also been investigated in another model organism, *Drosophila melanogaster*. P-element insertions in one of them, *late bloomer*, are known to retard the rate but not the accuracy of the formation of certain synapses (Kopczynski et al., 1996), and a genome-wide analysis of the genes has recently been reported (Fradkin et al., 2002). Thus, investigation of the enigmatic membrane molecules termed tetraspanins is now established in two model organisms, and further analysis of TSP-15 should enhance a molecular understanding of the cellular signaling and genetic hierarchy that contribute to the establishment or maintenance of epidermal cells.

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