Tetraspanin Is Required for Generation of Reactive Oxygen Species by the Dual Oxidase System in Caenorhabditis elegans

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Abstract

Reactive oxygen species (ROS) are toxic but essential molecules responsible for host defense and cellular signaling. Conserved NADPH oxidase (NOX) family enzymes direct the regulated production of ROS. Hydrogen peroxide (H₂O₂) generated by dual oxidases (DUOXs), a member of the NOX family, is crucial for innate mucosal immunity. In addition, H₂O₂ is required for cellular signaling mediated by protein modifications, such as the thyroid hormone biosynthetic pathway in mammals. In contrast to other NOX isozymes, the regulatory mechanisms of DUOX activity are less understood. Using Caenorhabditis elegans as a model, we demonstrate that the tetraspanin protein is required for induction of the DUOX signaling pathway in conjunction with the dual oxidase maturation factor (DUOXA). In the current study, we show that genetic mutation of DUOX (bli-3), DUOXA (doxa-1), and peroxidase (mlt-7) in C. elegans causes the same defects as a tetraspanin tsp-15 mutant, represented by exoskeletal deficiencies due to the failure of tyrosine cross-linking of collagen. The deficiency in the tsp-15 mutant was restored by co-expression of bli-3 and doxa-1, indicating the involvement of tsp-15 in the generation of ROS. H₂O₂ generation by BLI-3 was completely dependent on TSP-15 when reconstituted in mammalian cells. We also demonstrated that TSP-15, BLI-3, and DOXA-1 form complexes in vitro and in vivo. Cell-fusion-based analysis suggested that association with TSP-15 at the cell surface is crucial for BLI-3 activation to release H₂O₂. This study provides the first evidence for an essential role of tetraspanin in ROS generation.

Introduction

Reactive oxygen species (ROS) are considered deleterious by-products of aerobic metabolism that inflict oxidative damage in organisms, and have been associated with numerous diseases and aging. ROS are produced in phagocytic and non-phagocytic cells and function to eliminate invading microbes [1,2]. The physiological generation of ROS is directed by the NADPH oxidase (NOX) family of enzymes, which are highly conserved integral membrane proteins comprising seven members in mammals (NOX1–NOX5, DUOX1, and DUOX2) [3–5]. Studies of the NOX family have uncovered multiple biological functions of ROS in developmental processes, apoptosis, protein modification, cellular signaling, and are well documented in host defense mechanisms [1,6–8]. Dual oxidases (DUOX) were originally identified as thyroid oxidases, key H₂O₂ generators for the iodination of tyrosine in thyroid hormone precursors during thyroid hormone biosynthesis [9–11]. Whereas most NOX enzymes release superoxide, DUOXs release only H₂O₂ at the cell surface in physiological conditions, by rapid dismutation of intermediate superoxide [12,13]. Mutations in the DUOX2 gene are linked to congenital hypothyroidism in humans and mice [14,15]. DUOX-mediated H₂O₂ production is also crucial for other biological processes, such as extracellular matrix formation [16–18], innate immunity [19–22], and wound healing [23,24]. In C. elegans, BLI-3 encodes a nematode orthologue of DUOXs that is essential for exoskeletal development via tyrosine cross-linking [17,25–27], but which also functions in pathogen-induced ROS production [28–30].

The activity of the catalytic core of NOX enzymes is posttranslationally controlled by the recruitment of regulatory subunits to the plasma membrane [5,31,32]. In contrast to NOX isozymes, the current understanding of the regulation of DUOX proteins is unclear, despite the identification of maturation factors (DUOXA) [33]. Dual oxidase maturation factors (DUOX1 and DUOX2) heterodimerize with DUOX and contribute to its intracellular trafficking [34–36]. In the absence of DUOXA, DUOX is not recruited to the plasma membrane and is inactive [37,38]. DUOX1 preferentially dimerizes with DUOX1A, while DUOX2 preferentially forms
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Author Summary

ROS are highly reactive molecules, which can be inappropriately produced during aerobic metabolism or by exogenous stresses such as exposure to UV light and radiation. ROS interact with cellular components including nucleic acids, lipids, and proteins and irreversibly inhibit their functions. However, ROS are essential for innate host defense and multiple physiological processes and are generated by conserved NADPH oxidase (NOX) family enzymes. The release of ROS by ROS generator enzymes must be properly controlled, as chronic oxidative stress can cause an imbalance of the redox state and is often associated with disease and aging. Using C. elegans as a model, we identified a tetraspanin (TSP-15) protein as a new key component of the ROS generation system controlled by dual oxidase (Bli-3), a unique NOX isozyme in C. elegans. Mutants of both bli-3 and tsp-15 developed the same defects in extracellular matrix cross-linking. Using a combination of genetics and reconstitution experiments in mammalian cells, we have demonstrated a novel requirement of tetraspanin for dual oxidase-dependent ROS generation via complex formation at the cell surface.

Results

Identification of DUOX system mutants resembling the tsp-15 mutant

The splicing error mutation, sv15, within tsp-15 causes a reduction in function of tsp-15 [51]. We characterized other tsp-15 mutants and found that those with deletions within tsp-15 coding regions were lethal to embryos (Table 1, Figure S1, Table S1, Video S1). We screened for novel mutants similar to the tsp-15 hypomorph mutant to obtain clues for the tsp-15 mutant phenotype. We have shown that tsp-15(sv15) mutants have a distinct blister phenotype compared with classical bli mutants, that were classified by dpy-7p::gfp expression (Figure 1B) [51]. Both N2 and OB43 imL1(dpy-7p::gfp) strains were mutated and screened to exclude typical bli mutants [53]. We isolated thirteen alleles classified in four independent complementation groups (CG1–CG4; Table S2). Through single nucleotide polymorphism (SNP) mapping, DNA sequencing, RNAi, and complementation assays, we identified five mutations (Table S2). All identified responsible genes encoded DUOX or related proteins (Figure 1). The im10 mutation in CG1 is a missense mutation in the F56C11.1 gene encoding bli-3/CeDuox-1, a homologue of mammalian dual oxidases (Figure 1A) [17]. A conserved proline at position 1311 in the NOX domain was changed to leucine (P1311L) in im10 mutants. The gk141 was thought to have a deletion in the bli-3 region, and hT2-balanced heterozygotes produced gk141/hT2 adults, indicating that gk141/gk141 homozygotes were embryonically lethal (Table 1, Figure S2A). The im21 and im32 mutations in CG3 were located in the splicing site of the C06E1.3 gene (Figure 1A), possibly causing premature termination. Amino acid comparisons implied that C06E1.3 is a nematode homologue of DUOXA and essential for maturation and membrane targeting of DUOX (Figure S3) [33]; we named this gene doxa-1. Both im38 and im39 in CG4 were identified as missense mutations in ZK430.8, reported as mlt-7 (Figure 1A, Figure S2C) [25]. Mutations im38 and im39 caused a change in the conserved isoleucine at 343 to serine, and phenylalanine at 375 to serine in the peroxidase domain. MLT-7 is a homologue of tetraspanin in combination with BLI-3 [25].

The bli-3, doxa-1, and mlt-7 mutants were rescued by their own cDNA driven by a hypodermis-specific dpy-7 promoter (Table 1, Figure S2). For im21, Venus-tagged doxa-1 at the C-termini (doxa-1::venus, Figure S4A) driven by the doxa-1 promoter effectively rescued the phenotype (Table 1, Figure S2B, Figure S4B). The doxa-1::venus transgene was expressed in the hypodermis, and other tissues such as the pharynx, uterus, gonad, and vulva (Figure S4B).

Involvement of tsp-15 in bli-3 pathway

To investigate the genetic relationship between tsp-15 and newly isolated mutants, we performed mutant rescue assays (Figure 2, Table 1). Over-expression of tsp-15 did not restore the defects of the bli-3 and doxa-1 mutants. Over-expression of bli-3 as well as doxa-1 alone did not rescue the tsp-15 mutant. Co-expression of bli-3 and doxa-1 in the tsp-15 hypomorph mutant effectively rescued the cuticle deficiency (Figure 2, Table 1). In contrast, bli-3 and doxa-1 co-expression in the tsp-15 null mutant resulted in partial rescue of the lethal phenotype. In approximately 1.5% of transgenic animals, embryonic lethality was recovered and the larvae showed a tsp-15 hypomorph mutant-like morphology (Figure 2), which has not been observed in tsp-15 null mutants previously.

Decreased dityrosine levels in tsp-15 mutants

BLI-3 is reported as a key enzyme for the generation of H2O2 for tyrosine cross-linking in the cuticle since the level of di- and
tri-tyrosine formation is reduced in the cuticle of bli-3(RNAi) animals [17]. To examine distribution of cross-linked tyrosine in the exoskeleton of tsp-15 mutants, we carried out immunohistochemical analysis using anti-di-tyrosine antibody. We also checked the endogenous distribution of DPY-7 (a nematode collagen) in tsp-15 mutants by immunostaining. In the normal embryo, di-tyrosine was distributed over the entire cuticle representing the body surface structure, whereas di-tyrosine formation was severely reduced in tsp-15 null embryos (Figure 3A). In contrast, the level of collagen found in tsp-15 null embryos, examined via DPY-7, was comparable to tsp-15(+) embryos, although distribution was severely disturbed (Figure 3B). Thus, cuticle collagen is likely synthesized and secreted from the hypodermis correctly, but a failure of cross-linking of secreted collagen results in fragility of the cuticle in tsp-15 mutants.

Table 1. The tsp-15, doxa-1, and bli-3 genes are in the same genetic pathway.

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WT; wild-type, Emb; embryonic lethal, Bli; blister, Dpy; dumpy.
* 100 animals were counted for each strain.
doi:10.1371/journal.pgen.1002957.t001
BLI-3 activity depends on both TSP-15 and DOXA-1

DUOX was originally identified as a hydrogen peroxide generator for thyroid hormone synthesis [9,11] and DUOXA is essential for DUOX targeting to the plasma membrane [33]. We produced stable transfectants of TSP-15, BLI-3 and DOXA-1 in human HT1080 cells (Figure 4A) to confirm the roles of TSP-15 and DOXA-1 for BLI-3. Release of H$_2$O$_2$ into the culture medium was measured in the absence of other C. elegans proteins.

Extracellular H$_2$O$_2$ from HT1080$^B$ and HT1080$^{TM}$ cells was almost equivalent to basal activity (see Materials and Methods for description of stable transfectants). Unlike the mammalian DUOX system, BLI-3 was only slightly activated by co-expression of DOXA-1 in HT1080$^{DM}$ cells. In addition to DOXA-1, concomitant expression of TSP-15 strongly enhanced production of H$_2$O$_2$ in HT1080$^{DM}$ cells (Figure 4B). The generation of H$_2$O$_2$ was blocked by the flavoprotein inhibitor diphenyleneiodonium (DPI).

Figure 1. bli-3 and doxa-1 mutants are similar to the tsp-15 mutant. (A) The structure of the gene/proteins related to tsp-15 function. Schematic representation of the BLI-3 and MLT-7 protein with functional domain, and the genomic structure of the doxa-1 gene. The im10, im21, im32, im38 and im39 mutations are indicated. Previously identified missense mutations in the bli-3 gene including e767 (glycine to aspartic acid at 246) and n529 (aspartic acid to asparagine at 392) are shown. The bold line indicates the region of the gk141 deletion allele. The im10 mutation has a leucine instead of a proline at position 1311 within the NOX domain. TM and NOX refer to the transmembrane and NOX domains, respectively. The im21 mutation is characterized by a G to A transition in the splice donor site at the fifth intron. The im32 mutation is a G to T transversion in the splice acceptor site at the fourth intron. The im38 and im39 alleles are indicated in the MLT-7 protein. Both alleles contain the missense mutations in the peroxidase domain. (B) bli-3(im10) and doxa-1(im21), but not bli-2(e768) are similar to tsp-15(sv15). Hypodermal expression of GFP driven by dpy-7p::gfp in the mutants revealed an unusual accumulation of cellular materials in the blisters of bli-3, doxa-1 and tsp-15 mutants (indicated by black arrows), but not in bli-2 mutants (indicated by the white arrow). The scale bars represent 50 μm.

doi:10.1371/journal.pgen.1002957.g001
indicating that DUOX was involved in enhanced H$_2$O$_2$ production in TSP-15-transduced cells. We concluded that BLI-3 requires TSP-15 and DOXA-1 for proper function. BLI-3P1311L and BLI-3 G246D identical to the im10 or e767 mutation, respectively, resulted in decreased H$_2$O$_2$ production (Figure 4B). The same results were observed in other independently established stable cell lines, and by transient expression in COS-7 and HeLa cells (data not shown). Regulation of Ca$^{2+}$ characteristically elicits DUOX activity as a thyroid hormone synthesizer. BLI-3 did not require calcium stimulation to produce H$_2$O$_2$ in HT1080TDB cells, and HT1080DB cells were not activated by calcium stimulation either (Figure 4C). This may be due to the fact that the critical amino acid residues for Ca$^{2+}$-binding are poorly conserved in the EF-hand motifs of BLI-3 proteins [17]. Furthermore, BLI-3 was not activated by forskolin (fsk) and phorbol 12-myristate 13-acetate (PMA), which have previously been reported to be mammalian DUOX stimulators (Figure 4C) [54].

**TSP-15 and DOXA-1 associate with BLI-3**

Tetraspanins form protein complexes with a number of other molecules. We performed co-immunoprecipitation assays to determine whether TSP-15 associates with BLI-3 and DOXA-1. BLI-3 was transiently expressed in COS-7 cells where TSP-15 and/or DOXA-1 was stably expressed. As a result, BLI-3 co-immunoprecipitated with DOXA-1 (Figure 5A; lanes 17 and 18) and TSP-15 (Figure 5A, lanes 12 and 14). Co-immunoprecipitation of BLI-3 and DOXA-1 was independent of TSP-15 expression (Figure 5A; lane 17). TSP-15 co-immunoprecipitated with BLI-3 in the absence of DOXA-1 (Figure 5A; lane 12). In addition, TSP-15 and DOXA-1 association was also observed (Figure 5A; lane 14 and 18, Figure S6), indicating that TSP-15, DOXA-1, and BLI-3 form protein complexes. TSP-15 was not co-immunoprecipitated with over-expressed EGF receptor under the same conditions (data not shown). We also verified the same molecular interaction in transgenic animals. In doxa-1::venus transgenic worms (Figure 5B), BLI-3 co-immunoprecipitated with the DOXA-1::Venus fusion protein (Figure 5B; lane 6). Endogenous and tagged TSP-15 associated with BLI-3 (Figure 5B; lanes 2 and 4) and with DOXA-1::Venus (Figure 5B; lane 8). We concluded that TSP-15, DOXA-1 and BLI-3 form a complex.

**BLI-3 activation by TSP-15 at the cell surface**

The molecular requirement of TSP-15 in the BLI-3 system is possibly due to formation of a complex at the plasma membrane, therefore BLI-3 could be activated by TSP-15 localized at the cell surface. We assessed this hypothesis through cell fusion-based analysis (Figure 6A). TSP-15-expressing cells (HT1080T) and DOXA-1/BLI-3-expressing cells (HT1080DB), both of which did not produce H$_2$O$_2$ (Figure 4B), were fused utilizing Sendai virus (HVJ). After the cell fusion reaction, extracellular H$_2$O$_2$ production from fused cells was measured. The fused cells (T::DB) produced H$_2$O$_2$, and the production was inhibited by DPI treatment (Figure 6B). In contrast, BLI-3 carrying the P1311L mutation did not result in H$_2$O$_2$ production. Inhibition of de novo protein synthesis by cycloheximide (CHX) resulted in a slight decrease in H$_2$O$_2$ producing activity in T::DB fusion cells (Figure 6B). These results indicate that TSP-15 did not promote BLI-3 protein expression, but existing TSP-15 at the cell surface was sufficient to activate BLI-3 for H$_2$O$_2$ production. The capability of H$_2$O$_2$ production was rapidly acquired after cell fusion.
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**Figure 3. Deterioration of dityrosine in the tsp-15 mutant.** (A) Representative immunofluorescent images showing the distribution of dityrosine in embryos of the tsp-15(ok854) null mutant. Embryos were obtained from the Ob129 strain, which was the tsp-15(ok854) mutant rescued by a tsp-15p::his15p::tsp-15 extrachromosomal array. Nuclear GFP fluorescence by sur-5:gfp defined the rescued (tsp-15(+)) or spontaneously array-lost (null; tsp-15(0)) embryo. Micrographs on the left show merged Nomarski images exhibiting GFP and dityrosine immunolocalization. Right panels show the reconstruction of confocal images for dityrosine distribution in the same embryo that is displayed on the left. In tsp-15(+) normal embryos, dityrosine localization showed a regular pattern representing the cuticle surface structure. Fluorescence intensity was severely deteriorated in tsp-15(0) embryos. Scale bars indicate 10 µm. (B) DPY-7 localization was compared under the same conditions as in (A). In tsp-15(+) embryos, DPY-7 localized as regular bands in the cuticle. In the tsp-15(0) embryo, the expression of DPY-7 was comparable to the normal embryo despite its disorganized pattern. Scale bars indicate 10 µm.

doi:10.1371/journal.pgen.1002957.g003

Discussion

Organisms have developed regulatory systems to control ROS generation in host defense and cellular signaling. For mammalian DUOX proteins, association with a maturation factor (DUOXA) for targeting to the plasma membrane, Ca2+ regulation via EF-hand motifs, and PKA- or PKC-mediated phosphorylation were identified as regulatory systems. We propose that the tetraspanin protein is a novel component of the DUOX system for ROS generation (Figure 7). Using *C. elegans* as a model organism, we identified a series of genes for ROS generation in which mutants exhibited a phenotype resembling the tetraspanin *tsp-15* mutant. The genes *bli-3*, *doxa-1*, and *mtl-7* were, respectively, the homologues of mammalian DUOX, DUOXA and peroxidase, and mutants of these displayed the same cuticle deficiency (Figure 1, Figure 2, Figure S1, Figure S2). The reason for this cuticle disorganization is deterioration of tyrosine cross-linking in cuticle development as shown in *bb-3* knockout-animals (Figure 3) [17]. We showed that the *tsp-15* mutant was rescued by simultaneous over-expression of *bli-3* and *doxa-1*, implying that these three genes are part of the same genetic pathway (Figure 1, Figure 2). Reconstitution of BLI-3, TSP-15 and DOXA-1 in mammalian cells demonstrated that H2O2 generation by BLI-3 was dependent on TSP-15 as well as DOXA-1 (Figure 4).

It was hypothesized that TSP-15 might enhance BLI-3 protein levels by elevating protein expression or promoting targeting to the cell surface; however, BLI-3 and DOXA-1 protein expression levels were comparable, with or without TSP-15 expression. TSP-15 expression did not affect BLI-3 expression at the cell surface, and did not enhance the association of BLI-3 and DOXA-1. This implies that the role of TSP-15 in the BLI-3 system is not just augmentation of its expression. Our observations support this notion, since over-expression of *bli-3* and *doxa-1* resulted in incomplete rescue of *tsp-15* null mutants. In the *se15* hypomorph mutant, *tsp-15* expression was reduced, and expressed at 10% of wild-type levels (data not shown), therefore the BLI-3 system recovered to produce adequate H2O2 by concomitant over-expression of *bli-3* and *doxa-1*. If the up-regulation of BLI-3 activity by TSP-15 is quantitative, *tsp-15* null mutants should be completely rescued by BLI-3/DOXA-1, however this was not the case. The molecular role of tetraspanin in the DUOX system is likely qualitative. We believe that TSP-15 up-regulates the activity of BLI-3 at the plasma membrane. Cell fusion-based analysis strongly supports this idea, since cells that acquired TSP-15 from other cells rapidly produced H2O2 even when protein synthesis was inhibited. During the HVJ-mediated fusion process, intracellular organelles were morphologically altered and repaired within 30 min [55]. We observed that H2O2 generation was initiated 15 min after recovery, suggesting that individually derived BLI-3/DOXA-1 and TSP-15 rapidly assembled at the cell surface, forming functional complexes. The lipid raft marker protein, flotillin, was rapidly assembled during cell fusion [36]. Inhibition of de novo protein synthesis did not affect H2O2 production in fusion cells, indicating that the existing TSP-15 at the cell surface is sufficient for facilitation of BLI-3 activity.

The molecular mechanisms of up-regulation are still unclear, but we showed that TSP-15, BLI-3 and DOXA-1 form complexes *in vitro* and *in vivo* (Figure 5). BLI-3 directly associates with DOXA-1, as demonstrated in mammalian DUOX and DUOXA. We also demonstrated the association between BLI-3 and TSP-15, and that this was independent of DOXA-1 expression. It is known that tetraspanin associates with a number of membrane proteins and forms large protein complexes at certain membrane microdomains. We speculate that TSP-15 may establish or maintain a specialized membrane microdomain that facilitates generation of H2O2 in conjunction with BLI-3. As reported for other NOX isoforms and their subunits, association with TSP-15 might induce a conformational change in BLI-3 to function properly. Alternatively, TSP-15 may support the recruitment of unknown factors at the membrane microdomain that are essential for BLI-3 activity. Although DOXA-1 is essential for H2O2 production by BLI-3, the role of DOXA-1 in the BLI-3 system is unclear. Unlike mammalian DUOX, BLI-3 was unexpectedly recruited to the plasma membrane in the absence of DOXA-1. DOXA-1 might not regulate BLI-3 trafficking in the DUOX system in *C. elegans*, but we cannot exclude the possibility that expression of *C. elegans* proteins in mammalian cells may cause dysregulation in BLI-3 trafficking. Further investigation is needed to clarify the molecular functions of DOXA-1 in the BLI-3 system. In addition, unlike the mammalian DUOX system, BLI-3 did not respond to various stimuli when reconstituted in mammalian cells (Figure 4C). Absence of negative regulatory factors may explain the constitutive active state of BLI-3 in the heterologous system. For instance, NOXA1 has an inhibitory role in stabilizing the inactive state of mammalian DUOX1 [57]. No NOXA1-like sequence has been found in *C. elegans*, but further investigation would clarify this hypothesis.

Reciprocal homology searches suggested that several human tetraspanins are related to TSP-15 with CD151 (TSPAN24) and TSPAN11 being the most closely related. However, we have not identified any mammalian tetraspanins that could be functionally substituted for TSP-15 in the *tsp-15* mutant [51], or for H2O2 production in the BLI-3/DOXA-1 reconstitution system (data not shown). It is also uncertain whether mammalian tetraspanins have a pivotal role in mammalian DUOX system. Mutations in tetraspanin genes have not been identified in patients suffering from congenital hypothyroidism. In contrast to other NOX isoforms, understanding the regulation of DUOX proteins is emerging. Our data clearly shows that tetraspanin is a new component for directing DUOX activity, contributing to greater understanding of the molecular mechanisms of ROS generation and disorders caused by impairment of ROS generation systems [58,59].
Figure 4. Both TSP-15 and DOXA-1 are required for $H_2O_2$ production by BLI-3 in mammalian cells. (A) Immunoblot analysis of the expression of Xpress-tagged TSP-15, FLAG-tagged DOXA-1, BLI-3 and BLI-3$^{P1311L}$ in HT1080 stable transfectants. Xpress-tagged TSP-15 (30 kDa) is highly glycosylated (Figure S5). (B) Extracellular $H_2O_2$ production from stable transfectants. Fold-activation compared with non-transfected HT1080 cells was determined. Only cells expressing TSP-15, DOXA-1, and BLI-3 (HT1080TDB) significantly generated $H_2O_2$. A 10 µM concentration of DPI inhibited $H_2O_2$ production in HT1080TDB cells. BLI-3 carrying the G246D or P1311L mutation did not release $H_2O_2$. The graph shows the means ± SEM.
Materials and Methods

Worm strains and culture

*C. elegans* was grown at 20°C on NGM plates as described previously [60]. The Bristol N2 strain was used as the wild type. Strains and their genotype used in this study are listed in Table S1.

Mutant screening and identification

N2 or OB43 were mutagenized with 50 mM ethyl methanesulfonate or 5 mM ethyl nitrosourea for 4 h. F2 recessive mutants showing a *wi-15* mutant-like phenotype were screened. SNPs between N2 and Hawaiian CB4856 strains were used for physical mapping of the alleles [61]. Mutations were determined by further DNA sequencing and confirmed by complementation tests, rescue assays by DNA transformation, and RNAi analyses (see Text S1). Mutants were outcrossed at least five times with N2.

cDNA cloning and construction of vectors

Total RNA was isolated from mixed stages of N2 or mutant worms using TRizol reagent (Invitrogen, Carlsbad, CA, USA). First strand cDNA was synthesized by ReverTra Ace (Toyobo, Japan). A 4.6 kb fragment of *bli-3*, 1.2 kb of *doxa-1*, and 2.2 kb of *mtl-7* full length cDNA was prepared by RT-PCR. The *bli-3* fragment and *bli-3* coding vectors were constructed by PCR-based site-directed mutagenesis. *doxa-1::venus* translational fusion construct (Figure S4A) contains a 3.1 kb genomic PCR fragment of the *doxa-1* 5′ flanking region and 2.2 kb *doxa-1* genomic coding region without a termination codon, which was cloned into the Venus translational fusion vector (a gift from Takeshi Ishihara, Kyusyu University). The (His)Xpress-tagged *tp-15* (HisXp:tp-15), *bli-3*, FLAG-tagged *doxa-1* (doxa-1:FLAG) and *mtl-7* were sub-cloned under the control of the *dp-7* promoter for hypodermis-specific expression in the mutant rescue assay [51] or into the pCX4 retroviral vector for transfection into mammalian cells [62]. A 34 kb fosmid clone, WRM065cD06, was purchased from Generescence (Cambridge, UK). A 14.9 kb *HaeII* restriction fragment (nt 16368–31233) which contained the full *bli-3* coding region and also the 5′ flanking region was used for the rescue assay.

Immunohistochemistry and microscopic imaging of embryos

Embryos were collected from gravid hermaphrodites and were immunostained as previously described [63]. Mouse anti-dityrosine (1C3; Nikken Seii Co., Ltd. Shizuoka, Japan), mouse anti-DPY-7 (a gift from Iain L. Johnstone, University of Glasgow) [64] were used at 1:200 and 1:500 dilutions, respectively. Alexa Fluor 488 or Alexa Fluor 546 (Invitrogen) conjugated anti-mouse IgG antibodies were used as secondary antibodies at 1:500 dilutions. Confocal images were acquired with a LSM5 Pascal microscope (Zeiss, Germany). The three-dimensional projections were reconstructed using images of serial Z-section (1–1.5 μm). Micrographs of fluorescence microscopy were captured using a BX50 microscope (Nikon, Tokyo, Japan) equipped with a CoolSnap HQ2 digital camera (Keyence, Osaka, Japan). Image processing and movie construction was performed with Adobe Photoshop CS4 and Image J 1.34, respectively.

Cell culture and transfection

HT1080, HeLa, and COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. Transfection for transient expression was performed using FuGENE HD reagent (Roche, Germany) according to the manufacturer’s protocol. Stable transfectants were obtained by retrovirus transfection [62]. Plasmids were transfected into Plat-E packaging cells with FuGENE 6 reagent (Roche). Culture supernatant was added to HT1080 ecoR, HeLa ecoR and COS-7 ecoR cells which are stable transfectants of the ecotropic retrovirus receptor, mCAT-1 (gifts from Hiroto Mizushima, Osaka University). Transfected cells were selected by incubation with a combination of 1 μg/ml puromycin, 10 μg/ml blasticidin S, and/or 300 μg/ml zeocin for at least two weeks. Stable transfectants of *C. elegans* genes were named after the transgene that was transduced. T, D, and B refer to *tsp-15, doxa-1*, and *bli-3*, respectively (e.g. HT1080TDB cells express *tp-15, doxa-1*, and *bli-3*).

Antibodies

Both BLI-3 and DOXA-1 rabbit antiserum was prepared by SCRM Inc. (Tokyo, Japan). BLI-3 rabbit antiserum was raised against keyhole limpet hemocyanin (KLH)-coupled BLI-3 peptides corresponding to residues 254–269 (N1) and a mixture of residues 1232–1245 and 1478–1490 (Cmix). DOXA-1 rabbit antiserum was raised against a mixture of KLH-coupled DOXA-1 peptides corresponding to residues 170–184 and 326–340. Rabbit serum was purified using a peptide-conjugated sepharose column. Anti TSP-15 monoclonal antibody (2C2) was obtained by immunizing rats with HA-tagged TSP-15 protein into footpads. Hybridoma supernatant was purified by ion-exchange chromatography followed by anti-rat IgG-conjugated Sepharose column chromatography. The 2C2 monoclonal antibody is available for immunoprecipitation.

Western blot, co-immunoprecipitation, and cell surface biotinylation

Cells were harvested and suspended in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, pH 7.5) with 1% CHAPS. For surface labeling, cells were incubated with 0.2 mg/ml sulfo-NHS-LC-biotin (Thermo Fisher Scientific, Rockford, IL, USA) at 4°C for 30 min and then lysed. For immunoprecipitation and pull-down assays, cleared cell lysates were incubated with anti-FLAG M2 beads (Sigma-Aldrich, St Louis, MO, USA), rat anti-TSP-15 antibody (2C2)-conjugated agarose, or streptavidin agarose beads (Thermo Fisher Scientific). Cleared cell lysates and immunoprecipitates were blotted with mouse anti-Omni (Xpress) (D-8; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-FLAG (M2; Sigma-Aldrich), rabbit anti-BLI-3 (N1), rabbit anti-DOXA-1, mouse anti-actin (MAB1501; Millipore, Bedford, MA, USA), or mouse anti-calnexin (AF18; Abcam, Cambridge, UK) antibodies. HRP-conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA, USA), and donkey anti-mouse IgG (Millipore) were used as secondary antibodies. For the immunoblotting of worms, mixed stages of N2, OB129 or OB218 strains were cultured on 150 mm dishes and harvested. Worms were homogenized by sonication and lysed in 1% Triton X-100. Rat anti-TSP-15 or mouse anti-GFP antibody (3E6, Wako chemicals, Osaka, Japan), and anti-rat...
Figure 5. Direct association of BLI-3 with TSP-15 and DOXA-1. (A) Direct association of BLI-3 with TSP-15, and BLI-3 with DOXA-1. BLI-3 was transiently expressed in COS-7 stable transfectants expressing Xpress::TSP-15, DOXA-1::FLAG, or both. Cell surface proteins were labeled with biotin. A 1% CHAPS cell lysate was used for immunoprecipitation or pull-down assay with anti-TSP-15 antibody, anti-FLAG antibody or streptavidin. BLI-3 was co-immunoprecipitated with both TSP-15 (indicated by asterisks) and DOXA-1, and TSP-15 and DOXA-1 were co-immunoprecipitated. Bands above asterisks are non-specific. Cell surface localization of BLI-3 was independent of TSP-15 and DOXA-1. ER-resident calnexin (CANX) was not detected on the cell surface. BLI-3 expression was not affected by TSP-15 and DOXA-1. (B) Direct association of BLI-3 with TSP-15, BLI-3 with DOXA-1, and TSP-15 with DOXA-1 was confirmed in C. elegans. Xpress::tsp-15 was expressed in OB129, and doxa-1::venus is expressed in the OB218 transgenic strain.
IgG- or anti-mouse IgG-conjugated Sepharose was added to the cleared worm lysate. Normal rat IgG or mouse IgG was used as a negative control for specific antibodies. Immunoprecipitates were blotted with anti BLI-3 (Cmix) antibody.

Monitoring H$_2$O$_2$ production in mammalian cells

The H$_2$O$_2$ release into culture supernatants was measured using Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine; Invitrogen), which reacts with H$_2$O$_2$ and is transformed into fluorescent resorufin in the presence of peroxidase [65]. Stable or transient transfectants were plated on 96-well plates at 1–5 $\times$ 10$^4$ cells/well, and cultured for 24–48 h. Cells were incubated in 100 $\mu$l Hanks’ balanced salt solution containing 50 $\mu$M Amplex Red, and 0.1 U/ml HRP (Nacalai Tesque, Kyoto, Japan), with or without 10 $\mu$M diphenyleneiodonium (DPI), 1 $\mu$M ionomycin, 1 $\mu$M forskolin (Fsk), or 1 $\mu$M phorbol 12-myristate 13-acetate (PMA) at 37°C for 1 h. The fluorescence (530 nm excitation, 590 nm emission) was measured by a Power Scan HT (DS Pharma Biomedicals, Osaka, Japan). Fold-increase was compared with the basal activity of non-transfected HT1080 cells and was determined from least four independent experiments.

HVJ–mediated cell fusion

HVJ (Sendai virus)-mediated cell fusion was performed using GenomONE-CF (Ishihara Sangyo Co. Ltd., Osaka, Japan). Venus-tagged DOXA-1 was analyzed with anti-GFP antibody. BLI-3 was co-immunoprecipitated with endogenous and Xpress-tagged TSP-15 and Venus-tagged DOXA-1 in 1% Triton-X100 cell lysates. Endogenous TSP-15 also associated with DOXA-1::Venus. Normal rat and mouse IgG was used as a negative control.

Figure 6. Requirement of TSP-15 for reconstitution of BLI-3 function at cell surface. (A) HVJ-mediated cell fusion. GFP-expressing HT1080 cells and HT1080DB cells labeled with Cell Tracker Orange were fused with HVJ. Under HVJ(+)-conditions, fused cells were large compared with HVJ(−) cells and exhibited yellow/orange fluorescence. Scale bar indicates 50 $\mu$m. (B) HT1080DB cells fused with HT1080 T::DB produced H$_2$O$_2$, which was inhibited by DPI. Mock::DB and T::DB$^{1211L}$ fusion cells did not produce H$_2$O$_2$. Treatment of T::DB fusion cells with 10 $\mu$g/ml cycloheximide (CHX) did not inhibit H$_2$O$_2$ production. The graph shows the means ± SEM. The number of independent experiments is indicated. *P<0.05. (C) Rapid H$_2$O$_2$ production from T::DB fusion cells. The recovery time after the fusion event was examined to determine when fusion cells acquired the ability to produce H$_2$O$_2$. Maximum H$_2$O$_2$ production was observed at 30–60 min, although production was observed at 15 min post-fusion. The graph shows the means ± SEM (n = 3).

References:
were harvested using trypsin. HT1080DB or HT1080DBP1311L cells (8 x 10^5) were mixed with HT1080 ecoR or HT1080T cells (8 x 10^5) in a total volume of 200 μl reaction buffer. A 1 μl volume of inactivated HVJ was then added to the cell mixture. After incubation for 15 min at 37°C, 1 ml of DMEM was added and further incubated for 1 h at 37°C for recovery. Cells were treated with 10 μg/ml cycloheximide (Sigma-Aldrich) during incubation for inhibition of protein synthesis. Cells were washed, and H2O2 production was measured as described above. For the time-course experiment, recovery incubation was examined from 0–120 min, and H2O2 production was measured after a 30 min incubation.

For visualization of the fusion event, HT1080DB cells were pre-stained with 10 μM Cell Tracker Orange (Invitrogen) for 20 min at 37°C, and then fused with GFP-expressing HT1080 cells. Each experiment was tested in duplicate and performed at least three times.

**Statistical analysis**

Data are presented as mean value and error bars indicate the standard error of the mean (SEM) from multiple independent assays. Significance was determined using a two-tailed Student's *t*-test.

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**Figure 7. Molecular regulation of BLI-3 by TSP-15 and DOXA-1.** TSP-15 and DOXA-1 are essential for H2O2 production by BLI-3. TSP-15 associates with BLI-3 at the cell surface or during trafficking. The role of DOXA-1 in BLI-3 targeting to the plasma membrane remains elusive. BLI-3/DOXA-1 complexes at the cell surface are inactive, but recruiting to the tetraspanin-microdomain facilitates the formation of a functional unit for generation of H2O2, that is utilized by innate host immunity, and cross-linking of extracellular matrix with peroxidase (MLT-7).

doi:10.1371/journal.pgen.1002957.g007
Supporting Information

Figure S1 Structure of the tsp-15 gene, allele, expression and mutant phenotypes. (A) Mutation, deletion locus and most frequent splicing pattern from the tsp-15 mutant alleles are shown. The sv15 mutation is a change in the splice donor site of intron 4, as indicated by the arrow. The regions of deletions in gk201, ok854, ok881, and tni1666 alleles are indicated by bold lines. Three of these deletions, with the exception of gk201, result in the lack of most of the second extracellular domain such that these products are no longer functional. In contrast, the gk201 mutation was indistinguishable from wild type animals (data not shown) despite a 425 bp deletion in the 5′ flanking sequence (−703 to −278) of tsp-15, indicating that the deletion sequence does not include an essential element for tsp-15 transcription. (B) The tsp-15 hypomorphic and null mutant, sv15 has the Dpy and Bli phenotype. Small blisters are indicated by arrows. Homozygotes of the tsp-15 deletion alleles show identical recessive embryonic lethal phenotypes. Scanning electron microscopy images show the representative tsp-15 null mutant, ok881. The ok881 homozygote is short and fat, showing croissant-like morphology and also has a wrinkled cuticle. Scale bars indicate 50 μm in larva and 10 μm in the embryo. (C) Time lapse images of TSP-15::GFP [51] expression patterns and tsp-15 null embryos during embryogenesis. Upper panels are confocal images of tsp-15::gfp expression and Nomarski images of the corresponding embryo. Developmental stages are indicated on the top of the micrographs. tsp-15::gfp expression was visible in quadrants of cells along the anteroposterior axis. The expression was decreased and body surface expression was visible around the three-fold stage. TSP-15::GFP expression is prominent in lateral hypodermal cells. Lower panels show representative images of tsp-15(ok854) null mutant embryos. Each developmental stage is the same as shown in the upper panels. The tsp-15(ok854) embryo developed normally, however, at terminal embryogenesis, tsp-15(ok854) embryos shrunk and failed to maintain the vermiciform shape. Inset represents a different focal plane, which depicts an abnormal body protrusion in tsp-15 null embryos. Scale bars indicate 10 μm.

Figure S2 The bli-3, doxa-1 and mlt-7 mutants, RNAi, and rescue assays. (A) The bli-3 deletion mutant, gk141, is lethal to embryos demonstrating developmental arrest with abnormal body shape at late embryogenesis. The gk141 mutant was rescued by hypodermal specific expression of bli-3 cDNA and genomic fragments (a 14.9 kb fragment from fosmid WRM065cD06) containing the bli-3 gene. Scale bars indicate 50 μm. (B) doxa-1(RNAi) animals displayed Bli phenotype as indicated by arrows. The im21 mutant was rescued by hypodermal specific expression of doxa-1 cDNA and the Venus-tagged doxa-1 gene. Scale bars indicate 50 μm. (C) Deficiencies in cuticle development in mlt-7(rolf) animals. Dumpy phenotype in mlt-7(rolf) mutants, and moult defects and blister phenotypes in mlt-7(RNAi) animals are shown. The im39 mutant was rescued by hypodermal-specific expression of mlt-7. Arrows depict blisters in mlt-7(RNAi) animals, and arrowheads indicate body constriction caused by incomplete shedding of old cuticles during the molting process. Scale bars indicate 50 μm.

Figure S3 DOXA-1, the homologue of the mammalian DUOXA protein. Multiple alignment of amino acid sequences corresponding to the human DUOXA1 alpha isofrom (ACH57433.1), human DUOXA2 (NP_997464.2), and nematode DOXA-1 (NP_496886.2). Similar and similar residues are indicated.

Figure S4 Expression pattern of doxa-1. (A) Structure of the doxa-1::venus transgene. Boxes indicate exons. (B) Confocal images of the expression pattern of DOXA-1::Venus. im21 rescued by doxa-1::venus is shown. Higher magnification of pharynx, body surface, gonadal arm and vulval regions were also shown. DOXA-1::Venus was expressed in the terminal bulb of the pharynx (b), hypodermis (especially in seam cells (sc)), distal region of the gonadal arm (g), vulva (v), spermatheca (sp), and uterus (u). Scale bar is indicative of 50 μm.

Figure S5 TSP-15 is highly glycosylated in mammalian cells. Xpress-tagged tsp-15 or tsp-15 carrying a mutation in the N-glycosylation site (N161Q) was transiently expressed in COS-7 cells. Tunicamycin was added at 0.2 μg/ml for 24 h to partially inhibit N-glycosylation. Surface molecules were biotinylated, and cells were lysed with 1% Triton X-100. The lysate was immunoprecipitated with anti-TSP-15 antibody or pull-downed by streptavidin beads. The precipitates were treated with N-glycanase (PNGase F; New England Biolabs.) at 37°C for 24 h. Arrows indicate the deglycosylated form of TSP-15.

Figure S6 Association of TSP-15 with DOXA-1. Co-immunoprecipitation of TSP-15 with DOXA-1. BLI-3 was transiently expressed in COS-7 stable transfectants expressing Xpress::TSP-15 and DOXA-1::FLAG or DOXA-1::FLAG alone. A 1% CHAPS cell lysate was used for immunoprecipitation with anti-FLAG antibody. TSP-15 was co-immunoprecipitated with DOXA-1 irrespective of the presence of BLI-3.

Table S1 Strains and mutants used in this study.

Table S2 Isolated mutants similar to tsp-15(ok15).

Text SI Supplemental Materials and Methods.

Video S1 Time-lapse video microscopy of the tsp-15 null embryo. Confocal time-lapse imaging of the OB129 tsp-15(ok15); imEx89[tsp-15p::HisXp::tsp-15] strain. Nuclear expression of sur-5::gfp injection marker depicts rescued or spontaneously array-rost tsp-15(0) embryos. Two pairs of tsp-15(+)/tsp-15(0) embryos are shown. The tsp-15(0) (i.e. GFP(−)) embryos were elongated and developed normally, but shrunk at the terminal stages and were unable to maintain a vermiciform shape.

Acknowledgments

We would like to thank the Caenorhabditis Genetic Centre, C. elegans Knockout Consortium, and National Bioresource Project Japan for providing C. elegans strains. We are also grateful to I. L. Johnstone (University of Glasgow, UK), A. Fire (Stanford University, USA), H. Mizushima (Osaka University, Japan), and T. Ishihara (Kyushu University, Japan) for their gifts of antibodies and materials. We also thank Takao Inoue (University of Tokyo, Japan) and Asako Sugimoto (Tohoku University, Japan) for technical assistance and helpful discussion.

Author Contributions

Conceived and designed the experiments: HM EM. Performed the experiments: HM RK DK KN. Analyzed the data: HM. Contributed reagents/materials/analysis tools: HM RK DK TU. Wrote the paper: HM.
References


