Worm 2:1, e23415; January/February/March 2013; © 2013 Landes Bioscience

Co-occurrence of tetraspanin and ROS generators

Conservation in protein cross-linking and other developmental processes

Hiroki Moribe[1,*] and Eisuke Mekada[2]

[1]Department of Biology; Kurume University School of Medicine; Fukuoka, Japan; [2]Department of Cell Biology; Research Institute for Microbial Diseases; Osaka University; Osaka, Japan

Keywords: reactive oxygen species, cuticle, cross-linking, dual oxidase, BLI-3, tetraspanin, TSP-15, convergent evolution

Abbreviations: ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; DUOX, dual oxidase; DUOXA, dual oxidase activator/dual oxidase maturation factor; NOX, NADPH oxidase; TGase, transglutaminase; TEM/TERM, tetraspanin-enriched microdomain; ECM, extracellular matrix

Submitted: 12/09/12

Accepted: 12/21/12

http://dx.doi.org/10.4161/worm.23415

*Correspondence to: Hiroki Moribe; Email: himoribe@med.kurume-u.ac.jp

Commentary to: Moribe H, Konakawa R, Koga D, Ushiki T, Nakamura K, Mekada E. Tetraspanin is required for generation of reactive oxygen species by the dual oxidase system in Caenorhabditis elegans. PLoS Genet 2012; 8:e1002957; PMID:23028364; http://dx.doi.org/10.1371/journal.pgen.1002957.

The nematode exoskeleton, commonly called the cuticle, is a highly structured extracellular matrix mainly composed of collagen. Secreted collagen molecules from the underlying epidermal cells are cross-linked via their tyrosyl residues. Reactive oxygen species (ROS) are required for the cross-linking reaction to produce tyrosyl radicals. The conserved ROS generator enzyme in *C. elegans*, BLI-3/CeDUOX1, a homolog of dual oxidases (DUOXs), is responsible for production of hydrogen peroxide. The ROS generation system must be properly controlled since ROS are highly reactive molecules that irreversibly inhibit the functions of cellular components such as nucleic acids and proteins. We recently reported that the ROS generation system directed by BLI-3 requires the tetraspanin protein, TSP-15. Herein we outline the process of cuticle development with a focus on the molecular roles of TSP-15 in the BLI-3 system. We also propose the co-occurrence of tetraspanin and ROS generators by convergent evolution.

Collagen Biosynthetic Pathway in Cuticle Development

Nematode cuticle possesses both toughness and flexibility, which protects internal tissues from adverse environments, maintains body morphology and mechanically supports locomotion via attachment to body wall muscle. Cuticle is predominantly composed of collagens encoded by over 170 genes in the *C. elegans* genome. Collagens are synthesized in underlying epidermal cells, the hypodermis and seam cells, covering almost the entire body. The collagen biosynthetic pathway is a multistep processes involving modification, folding, cleavage and secretion directed by a number of enzymes and molecular chaperons.[1] The importance of the co- and post-translational processing of collagen proteins has been supported by a comprehensive study of their mutants.[2] Gain- or reduction/loss of function of the genes relevant to collagen processing are often associated with body morphological defects represented as dumpy (Dpy), roller (Rol), squat (Sqt), blister (Bli) and molting defect (Mlt). In the final events of processing, collagen triple helices are secreted from the epidermis and are extracellularly cross-linked. Each collagen helix is covalently cross-linked via tyrosine residues resulting in the formation of dityrosine and trityrosine, which are unusual non-reducible bonds in vertebrates (see below). In *C. elegans*, tyrosine cross-linking of collagens is established by ROS-mediated catalysis driven by the BLI-3/CeDUOX1 system.[3-5] BLI-3 is a homolog of vertebrate dual oxidases that are members of the NADPH oxidase (NOX) family comprising NOX1–5 and DUOX1–2. These proteins are ROS generators that deliberately produce ROS for cellular signaling and anti-microbial responses.[6-9]

Identity and Difference of the Cross-Linking Process in Mammalian and Nematode Skin

Both nematode and mammalian skin layers share common structural

characteristics, but mammalian skin is a more complex structure. It is composed of multilayered live and dead keratinocytes that constitute a cornified layer that faces the external side.[10] Cross-linking of proteins such as involucrin and loricrin in the cornified layer is also essential for the function of mammalian skin as an external barrier. Transglutaminases (TGases) play a primary role in the cross-linking process, which catalyzes the formation of non-reducible covalent bonds between lysine and glutamate.[11] Although tyrosine cross-linkages are found in other structural proteins at low frequency and are known as biomarkers of oxidative stress and aging, little is known about their physiological role in mammals.[12] Hydroxyl lysine and lysine between collagen helices are cross-linked by lysyl oxidase in vertebrates. Lysyl oxidase-mediated cross-linkages were not found in the *C. elegans* cuticle, but they do contribute to cross-linking of type IV collagen in the basement membrane.[13,14] Interestingly, both TGases- and ROS-mediated cross-linking is observed in the formation of the fertilization envelope in sea urchin egg.[15]

Tetraspanin is a New Component of the BLI-3/CeDUOX1-ROS-Generating System

Historically, ROS have been considered deleterious by-products produced by aerobic metabolism or by exogenous stresses such as UV light and radiation, which inflict oxidative damage to organisms. The physiological role of ROS was originally believed to provide an "oxidative burst" that kills invading microbes in phagocytes. H_2O_2 produced by DUOXs also has an essential role in non-phagocytic anti-microbial defense in mucosal epithelia such as the airway and gastrointestinal tract in a wide-range of animals, including mammals, fish and insects.[16,17] The critical role of H_2O_2 produced by BLI-3/CeDUOX1 in *C. elegans* innate immunity was also demonstrated.[18-21] Besides host defense, ROS act as an intracellular redox signaling molecule by modulating target proteins via modification of their free thiol groups.[22,23] In both cases, the ROS production must be strictly regulated so that it does not damage the host. The activity of the catalytic core of NOX1-3 is regulated by the recruitment of regulatory subunits to the plasma membrane.[9,24] NOX5 and DUOX1-2 contains EF-hand motifs in the cytoplasmic region and calcium (Ca^{2+}) stimulation is essential for activation. In addition, DUOXs require interaction with their maturation factor, DUOXAs, for H_2O_2 production.[25] Dual oxidase maturation factors (DUOXA1/2) dimerize with DUOXs to target DUOXs to the cell surface.[26-28]

We preciously reported that a tetraspanin protein TSP-15 is required for cuticle development for functioning as an external barrier.[29] We recently clarified that TSP-15 functions in collagen cross-linking as a component of the DUOX system.[5] Similar to mammalian DUOXs, the BLI-3 system also requires a maturation factor and cooperates with a neighboring heme peroxidase, which corresponds to DOXA-1 and MLT-7 in *C. elegans*, respectively.[4,30] *bli-3*, *doxa-1* and *mlt-7* mutants displayed the same cuticle deficiency as a *tsp-15* mutant. Cuticle disorganization in the *tsp-15* mutant is due to impaired tyrosine cross-linking during cuticle development. In addition, the *tsp-15* mutant was restored by exogenous expression of both *bli-3* and *doxa-1*, implying that these three genes are part of the same genetic pathway. We also showed requirement of TSP-15 for BLI-3 activity by heterologous reconstitution of BLI-3, TSP-15 and DOXA-1 in mammalian cells. Finally, we showed that TSP-15 forms protein complexes with BLI-3 and DOXA-1 in vitro and in vivo.

Speculation of the Molecular Role of Tetraspanin in the BLI-3 System

Despite our contributions to the field, the molecular role of TSP-15 in H_2O_2 generation by the BLI-3 system remains elusive. By immunoblot assay, TSP-15 did not alter the protein expression level of BLI-3 at the cell surface, leading us to question what molecular switch occurs within the BLI-3 system upon association with TSP-15. The tetraspanin family comprises a large group of integral membrane proteins with common secondary and tertiary structures, including four transmembrane regions, small and large extracellular loops (LEL) and conserved cysteine residues in the LEL contributing to the formation of disulfide bonds.[31,32] It is known that tetraspanins laterally associate with each other as well as numerous membrane "partner" proteins such as adhesion molecules, growth factor receptors, membrane-bound proteases, immunoglobulin superfamily proteins. Tetraspanins also interact with intracellular signaling/cytoskeletal proteins and lipids, resulting in the formation of a highly ordered lipid-protein unit referred to as a tetraspanin-enriched microdomain (TEM/TERM) or "tetraspanin web." TEM is a distinct class of membrane microdomain and a new type of signaling platform involved in cell-cell communication.[33-36] Association with tetraspanins may properly tune functions of partner proteins. However, this modulation and facilitation process is not consistent – it may differ from partner to partner. The most likely role of TEM is in spatial assembly and clustering of specific molecules that contribute to accelerating the reaction cascade and enabling additional interactions and linkage with other key molecules and substrates. If the partner proteins are relevant to cell adhesion, antigen presentation or matrix degradation, it is convincible that the compartmentalization of these responsible molecules at specialized membrane microdomains will efficiently support their function in adhesion strengthening, cell-cell communication at immune synapses or ECM degradation in tumor invasion. It may also facilitate accessibility to substrates, while segregation of partner proteins to the microdomain may prevent non-specific reactions.[37] We currently do not have enough information to provide mechanistic insights into the TSP-15 role in the BLI-3 system, however, we have several hypotheses. First, association with TSP-15 or targeting to TEM may support the subsequent recruitment of unknown factors, or it may facilitate other forms of post-translational modification on BLI-3 that is essential for BLI-3 activation. Or more directly, as reported for other NOX isozymes and their subunits, it might induce a conformational change in BLI-3 to activate it. Conversely, interaction with TSP-15 or engagement to TEM may

replace/exclude an inhibitory factor preventing non-specific activation of BLI-3. Further analysis will be required to define the molecular role of TSP-15.

Conservation of Tyrosine Cross-Linking Machinery in Other Developmental Processes in Different Species

Although it is still not known whether tetraspanin is also crucial for the mammalian DUOX pathway, conservation of involvement of tetraspanin in the ROS generation system is, at least in part, confirmed by genetic studies of pathogenic fungi. During the infection process of the rice pathogenic fungus *Magnaporthe grisea*, the attached conidium differentiates to appressorium, a specialized structure for infection. Then the appressorium develops a penetration peg and perforates the cell wall of the host tissues. It was independently demonstrated that the mutant of *M. grisea* tetraspanin (*MgPLS1*) and the ROS generator (*MgNOX2*) was non-pathogenic owing to impairment of penetration peg formation.[38,39] The pathogenicity of other parasitic fungi with an appressoria-mediated penetration strategy in different clades was also dependent on *PLS1* and *NOX2*.[40,41] Both *PLS1* and *NOX2* were identified in other types of fungi with non-pathogenic lifestyles lacking appressorium. Furthermore, the consistency of the mutant phenotype was also observed in different developmental processes in these saprophytic fungi. In *Podospora anserina* and *Neurospora crassa*, both of their corresponding mutants of *PLS1* and *NOX2* showed the same defects in the process of germination from the ascospore.[42,43] Although the molecular mechanisms of requirement of PLS1 and NOX2 in these processes are still uncertain, the recurrent involvement of tetraspanin and ROS generators in the same cellular processes in a wide range of species makes it possible that convergent evolution is responsible for the co-occurrence of this molecular machinery.[44,45]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by a Grant-in-Aid for Young Scientists 18770171 to H.M. and by the Global COE Program (Frontier Biomedical Science Underlying Organelle Network Biology), Ministry of Education, Culture, Sports, Science and Technology (Japan).

References

1. Page AP, Johnstone IL. The cuticle. In: The C. elegans Research Community, ed. WormBook: WormBook. http://www.wormbook.org
2. Myllyharju J, Kivirikko KI. Collagens, modifying enzymes and their mutations in humans, flies and worms. Trends Genet 2004; 20:33-43; PMID:14698617; http://dx.doi.org/10.1016/j.tig.2003.11.004.
3. Edens WA, Sharling L, Cheng G, Shapira R, Kinkade JM, Lee T, et al. Tyrosine cross-linking of extracellular matrix is catalyzed by Duox, a multidomain oxidase/peroxidase with homology to the phagocyte oxidase subunit gp91*phox*. J Cell Biol 2001; 154:879-91; PMID:11514595; http://dx.doi.org/10.1083/jcb.200103132.
4. Thein MC, Winter AD, Stepek G, McCormack G, Stapleton G, Johnstone IL, et al. Combined extracellular matrix cross-linking activity of the peroxidase MLT-7 and the dual oxidase BLI-3 is critical for post-embryonic viability in *Caenorhabditis elegans*. J Biol Chem 2009; 284:17549-63; PMID:19406744; http://dx.doi.org/10.1074/jbc.M900831200.
5. Moribe H, Konakawa R, Koga D, Ushiki T, Nakamura K, Mekada E. Tetraspanin is required for generation of reactive oxygen species by the dual oxidase system in *Caenorhabditis elegans*. PLoS Genet 2012; 8:e1002957; PMID:23028364; http://dx.doi.org/10.1371/journal.pgen.1002957.
6. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 2007; 87:245-313; PMID:17237347; http://dx.doi.org/10.1152/physrev.00044.2005.
7. Lambeth JD. NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol 2004; 4:181-9; PMID:15039755; http://dx.doi.org/10.1038/nri1312.
8. Leto TL, Morand S, Hurt D, Ueyama T. Targeting and regulation of reactive oxygen species generation by Nox family NADPH oxidases. Antioxid Redox Signal 2009; 11:2607-19; PMID:19438290; http://dx.doi.org/10.1089/ars.2009.2637.
9. Sumimoto H. Structure, regulation and evolution of Nox-family NADPH oxidases that produce reactive oxygen species. FEBS J 2008; 275:3249-77; PMID:18513324; http://dx.doi.org/10.1111/j.1742-4658.2008.06488.x.
10. Candi E, Schmidt R, Melino G. The cornified envelope: a model of cell death in the skin. Nat Rev Mol Cell Biol 2005; 6:328-40; PMID:15803139; http://dx.doi.org/10.1038/nrm1619.
11. Iismaa SE, Mearns BM, Lorand L, Graham RM. Transglutaminases and disease: lessons from genetically engineered mouse models and inherited disorders. Physiol Rev 2009; 89:991-1023; PMID:19584319; http://dx.doi.org/10.1152/physrev.00044.2008.
12. Feeney MB, Schöneich C. Tyrosine modifications in aging. Antioxid Redox Signal 2012; 17:1571-9; PMID:22424390; http://dx.doi.org/10.1089/ars.2012.4595.
13. Cox GN, Kusch M, Edgar RS. Cuticle of *Caenorhabditis elegans*: its isolation and partial characterization. J Cell Biol 1981; 90:7-17; PMID:7251677; http://dx.doi.org/10.1083/jcb.90.1.7.
14. Norman KR, Moerman DG. The *let-268* locus of *Caenorhabditis elegans* encodes a procollagen lysyl hydroxylase that is essential for type IV collagen secretion. Dev Biol 2000; 227:690-705; PMID:11071784; http://dx.doi.org/10.1006/dbio.2000.9897.
15. Wong JL, Wessel GM. Free-radical crosslinking of specific proteins alters the function of the egg extracellular matrix at fertilization. Development 2008; 135:431-40; PMID:18094022; http://dx.doi.org/10.1242/dev.015503.
16. Allaoui A, Botteaux A, Dumont JE, Hoste C, De Deken X. Dual oxidases and hydrogen peroxide in a complex dialogue between host mucosae and bacteria. Trends Mol Med 2009; 15:571-9; PMID:19913458; http://dx.doi.org/10.1016/j.molmed.2009.10.003.
17. Bae YS, Choi MK, Lee WJ. Dual oxidase in mucosal immunity and host-microbe homeostasis. Trends Immunol 2010; 31:278-87; PMID:20579935; http://dx.doi.org/10.1016/j.it.2010.05.003.
18. Chávez V, Mohri-Shiomi A, Garsin DA. Ce-Duox1/BLI-3 generates reactive oxygen species as a protective innate immune mechanism in *Caenorhabditis elegans*. Infect Immun 2009; 77:4983-9; PMID:19687201; http://dx.doi.org/10.1128/IAI.00627-09.
19. Jain C, Yun M, Politz SM, Rao RP. A pathogenesis assay using *Saccharomyces cerevisiae* and *Caenorhabditis elegans* reveals novel roles for yeast AP-1, Yap1, and host dual oxidase BLI-3 in fungal pathogenesis. Eukaryot Cell 2009; 8:1218-27; PMID:19502579; http://dx.doi.org/10.1128/EC.00367-08.
20. Hoeven Rv, McCallum KC, Cruz MR, Garsin DA. Ce-Duox1/BLI-3 generated reactive oxygen species trigger protective SKN-1 activity via p38 MAPK signaling during infection in *C. elegans*. PLoS Pathog 2011; 7:e1002453; PMID:22216003; http://dx.doi.org/10.1371/journal.ppat.1002453.
21. van der Hoeven R, McCallum KC, Garsin DA. Speculations on the activation of ROS generation in *C. elegans* innate immune signaling. Worm 2012; 1:160-3; http://dx.doi.org/10.4161/worm.19767.
22. Brown DI, Griendling KK. Nox proteins in signal transduction. Free Radic Biol Med 2009; 47:1239-53; PMID:19628035; http://dx.doi.org/10.1016/j.freeradbiomed.2009.07.023.
23. Finkel T. Signal transduction by reactive oxygen species. J Cell Biol 2011; 194:7-15; PMID:21746850; http://dx.doi.org/10.1083/jcb.201102095.
24. Lambeth JD, Kawahara T, Diebold B. Regulation of Nox and Duox enzymatic activity and expression. Free Radic Biol Med 2007; 43:319-31; PMID:17602947; http://dx.doi.org/10.1016/j.freeradbiomed.2007.03.028.
25. Grasberger H, Refetoff S. Identification of the maturation factor for dual oxidase. Evolution of an eukaryotic operon equivalent. J Biol Chem 2006; 281:18269-72; PMID:16651268; http://dx.doi.org/10.1074/jbc.C600095200.
26. Grasberger H, De Deken X, Miot F, Pohlenz J, Refetoff S. Missense mutations of dual oxidase 2 (*DUOX2*) implicated in congenital hypothyroidism have impaired trafficking in cells reconstituted with DUOX2 maturation factor. Mol Endocrinol 2007; 21:1408-21; PMID:17374849; http://dx.doi.org/10.1210/me.2007-0018.
27. Luxen S, Noack D, Frausto M, Davanture S, Torbett BE, Knaus UG. Heterodimerization controls localization of Duox-DuoxA NADPH oxidases in airway cells. J Cell Sci 2009; 122:1238-47; PMID:19339556; http://dx.doi.org/10.1242/jcs.044123.

28. Morand S, Ueyama T, Tsujibe S, Saito N, Korzeniowska A, Leto TL. Duox maturation factors form cell surface complexes with Duox affecting the specificity of reactive oxygen species generation. FASEB J 2009; 23:1205-18; PMID:19074510; http://dx.doi.org/10.1096/fj.08-120006.
29. Moribe H, Yochem J, Yamada H, Tabuse Y, Fujimoto T, Mekada E. Tetraspanin protein (TSP-15) is required for epidermal integrity in *Caenorhabditis elegans*. J Cell Sci 2004; 117:5209-20; PMID:15454573; http://dx.doi.org/10.1242/jcs.01403.
30. Donkó A, Péterfi Z, Sum A, Leto T, Geiszt M. Dual oxidases. Philos Trans R Soc Lond B Biol Sci 2005; 360:2301-8; PMID:16321800; http://dx.doi.org/10.1098/rstb.2005.1767.
31. Boucheix C, Rubinstein E. Tetraspanins. Cell Mol Life Sci 2001; 58:1189-205; PMID:11577978; http://dx.doi.org/10.1007/PL00000933.
32. Hemler ME. Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain. Annu Rev Cell Dev Biol 2003; 19:397-422; PMID:14570575; http://dx.doi.org/10.1146/annurev.cellbio.19.111301.153609.
33. Charrin S, le Naour F, Silvie O, Milhiet PE, Boucheix C, Rubinstein E. Lateral organization of membrane proteins: tetraspanins spin their web. Biochem J 2009; 420:133-54; PMID:19426143; http://dx.doi.org/10.1042/BJ20082422.
34. Hemler ME. Tetraspanin functions and associated microdomains. Nat Rev Mol Cell Biol 2005; 6:801-11; PMID:16314869; http://dx.doi.org/10.1038/nrm1736.
35. Levy S, Shoham T. The tetraspanin web modulates immune-signalling complexes. Nat Rev Immunol 2005; 5:136-48; PMID:15688041; http://dx.doi.org/10.1038/nri1548.
36. Yáñez-Mó M, Barreiro O, Gordon-Alonso M, Sala-Valdés M, Sánchez-Madrid F. Tetraspanin-enriched microdomains: a functional unit in cell plasma membranes. Trends Cell Biol 2009; 19:434-46; PMID:19709882; http://dx.doi.org/10.1016/j.tcb.2009.06.004.
37. Yáñez-Mó M, Gutiérrez-López MD, Cabañas C. Functional interplay between tetraspanins and proteases. Cell Mol Life Sci 2011; 68:3323-35; PMID:21687991; http://dx.doi.org/10.1007/s00018-011-0746-y.
38. Clergeot PH, Gourgues M, Cots J, Laurans F, Latorse MP, Pepin R, et al. *PLS1*, a gene encoding a tetraspanin-like protein, is required for penetration of rice leaf by the fungal pathogen *Magnaporthe grisea*. Proc Natl Acad Sci U S A 2001; 98:6963-8; PMID:11391010; http://dx.doi.org/10.1073/pnas.111132998.
39. Egan MJ, Wang ZY, Jones MA, Smirnoff N, Talbot NJ. Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease. Proc Natl Acad Sci U S A 2007; 104:11772-7; PMID:17600089; http://dx.doi.org/10.1073/pnas.0700574104.
40. Segmüller N, Kokkelink L, Giesbert S, Odinius D, van Kan J, Tudzynski P. NADPH oxidases are involved in differentiation and pathogenicity in *Botrytis cinerea*. Mol Plant Microbe Interact 2008; 21:808-19; PMID:18624644; http://dx.doi.org/10.1094/MPMI-21-6-0808.
41. Veneault-Fourrey C, Lambou K, Lebrun MH. Fungal Pls1 tetraspanins as key factors of penetration into host plants: a role in re-establishing polarized growth in the appressorium? FEMS Microbiol Lett 2006; 256:179-84; PMID:16499604; http://dx.doi.org/10.1111/j.1574-6968.2006.00128.x.
42. Lambou K, Malagnac F, Barbisan C, Tharreau D, Lebrun MH, Silar P. The crucial role of the Pls1 tetraspanin during ascospore germination in *Podospora anserina* provides an example of the convergent evolution of morphogenetic processes in fungal plant pathogens and saprobes. Eukaryot Cell 2008; 7:1809-18; PMID:18757568; http://dx.doi.org/10.1128/EC.00149-08.
43. Malagnac F, Lalucque H, Lepère G, Silar P. Two NADPH oxidase isoforms are required for sexual reproduction and ascospore germination in the filamentous fungus *Podospora anserina*. Fungal Genet Biol 2004; 41:982-97; PMID:15465387; http://dx.doi.org/10.1016/j.fgb.2004.07.008.
44. Malagnac F, Bidard F, Lalucque H, Brun S, Lambou K, Lebrun MH, et al. Convergent evolution of morphogenetic processes in fungi: Role of tetraspanins and NADPH oxidases 2 in plant pathogens and saprobes. Commun Integr Biol 2008; 1:180-1; PMID:19704887; http://dx.doi.org/10.4161/cib.1.2.7198.
45. Moribe H, Mekada E. Tetraspanins in lower eukaryotes. In: Berditchevski F, Rubinstein E, eds. Tetraspanins: Springer, 2013; In press.