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Speculations on the activation of ROS generation in *C. elegans* innate immune signaling

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We recently published work demonstrating that ROS (reactive oxygen species) generated by the dual oxidase, Ce-Duox1/BLI-3, in response to infection in *Caenorhabditis elegans* activates the transcription factor SKN-1, initiating a protective response. Moreover, we showed that the crucial innate immune pathway, p38 MAPK signaling, was responsible for relaying the activating signal. In this commentary, we speculate on the signaling pathway upstream of Ce-Duox1/BLI-3 that triggers its activity. Specifically, we hypothesize that a G-protein signaling pathway comprising Gαq - PLCβ - TPA-1 - DKF-2 activates Ce-Duox1/BLI-3. Our rationale is based on work showing that these components are connected to p38 MAPK signaling and innate immunity in the worm, and investigations in other organisms demonstrating that some of these components are involved in dual oxidase activation.

Keywords: dual oxidase, reactive oxygen species, innate immunity, *C. elegans*, signaling

Abbreviations: ROS, reactive oxygen species; NOX, NADPH oxidase; DUOX, dual oxidase

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Coordinated regulation of immunity is not only crucial for fighting invading pathogens, but also vital for safeguarding host tissues from injury due to excessive reactions. Unchecked immune responses can lead to tissue damage, disease and sometimes death of the host. An example of an immune response that can potentially harm host tissue is the purposeful generation of reactive oxygen species (ROS) by NADPH oxidase enzymes.

The first characterized NADPH oxidase was the phagocytic gp91phox/Nox2. It produces ROS in the phagolysozomes of neutrophils and other immune cells, contributing to the destruction of invading microbes.[1,2] The robust consumption of molecular oxygen and the generation of superoxide anions by this enzyme was termed the oxidative burst. Six other homologs of Nox2 (Nox1, Nox3, Nox4, Nox5, Duox1 and Duox2) were subsequently identified in the human genome and found to be expressed in a wide range of tissues, giving rise to the Nox/Duox (NADPH oxidase/dual oxidase) family of proteins. All members of this family of proteins retain a catalytic C-terminal domain comprising NADPH and FAD binding sites and two, membrane-bound heme moieties. In addition to the C-terminal catalytic domain, calcium binding EF hand motifs are found in dual oxidases and Nox5. Dual oxidases also possess an N-terminal heme-containing peroxidase domain. Nox and Duox enzymes occur in plants, algae, fungi, amoeba, nematodes, echinoderms, urochordates, insects, fish, reptiles, birds and mammals, but are absent in prokaryotes.[1] Therefore, a variety of systems can be used to study these proteins.

The genome of *C. elegans* contains two genes that encode for dual oxidases, Ce-Duox1/BLI-3 and Ce-Duox2, but lacks genes encoding for Nox enzymes.[3] However, Ce-Duox2 may be a pseudogene because it does not appear to be expressed, and a deletion mutant has no phenotype.[4,5] In initial studies, a functional role for Ce-Duox1/BLI-3 in the biogenesis of the cuticle was described, and it was localized to the hypodermis.[3] Ce-Duox1/BLI-3 was postulated to generate hydrogen peroxide by the C-terminal catalytic domain for use by the N-terminal perioxidase domain. Specifically, the model proposes that the peroxidase domain uses hydrogen peroxide as an electron donor to

generate radical tyrosine molecules that react with one another, creating protein crosslinks that stabilize and strengthen the cuticle. Consistent with this model, loss of Ce-Duox1/BLI-3 results in blistering and bubbling of the cuticle.[3]

Another role for Ce-Duox1/BLI-3, which others and we have established, is the protective generation of ROS in the intestine in response to infection.[4,6] Though overall this response was beneficial, there was evidence that the elevated levels of host-generated ROS caused cellular damage.[7,8] We speculated that to maintain redox homeostasis during infection, the worm might simultaneously engage oxidative stress response programs, such as the phase II detoxification response, to scavenge free radicals and other reactive molecules using glutathione.[9] Phase II detoxification of xenobiotic and chemically induced oxidative stress in *C. elegans* has been extensively studied and shown to be regulated by SKN-1, which is distantly related to the human Nuclear factor erythroid related factor (Nrf).[10-12] In mice, Nrf2 mediated regulation of redox status was shown to modulate immune responses during inflammation.[13] However, a role for SKN-1 in responding to infection in the worm had not been demonstrated. Additionally, a link between ROS produced by NOX/DUOX enzymes and the activation of the Nrf2/SKN-1 family of transcription factors had not been established in any organism.

In van der Hoeven et al., we demonstrated, using a variety of techniques, that SKN-1 is activated in the intestine of the worm in response to the human pathogens *Enterococcus faecalis* and *Pseudomonas aeruginosa*.[9] For example, we observed increased transcription of several SKN-1 dependent genes, such as *gcs-1, gst-4, gst-5, gst-7* and *gst-10,* by qRT-PCR and promoter fusions to *gfp,* in some cases. Moreover, we established that ROS produced by Ce-Duox1/BLI-3 activates SKN-1 through the p38 MAPK signaling pathway, similarly to that previously shown for chemically induced oxidative stress.[14] The p38 MAPK signaling pathway is central to *C. elegans* innate immune response and is comprised of the Toll/IL-1 receptor domain protein, TIR-1, the MAPKKK, NSY-1, the MAPKK, SEK-1, and the MAPK, PMK-1.[15,16] Our analysis of the p38 MAPK pathway revealed that components NSY-1, SEK-1 and PMK-1 are required for activation of SKN-1, while TIR-1, which is essential in responding to pathogens, is not required.[9] Finally and most importantly, we showed that SKN-1 positively impacts survival during infection. Loss of *skn-1* decreased resistance to the pathogens, whereas overexpression resulted in enhanced survival. Overall, SKN-1 is activated via signaling through the p38 MAPK pathway in response to the oxidative burst generated by Ce-Duox1/BLI-3 during infection of the worm intestine.[9]

Currently, we are focused on understanding how Ce-Duox1/BLI-3 is triggered to produce ROS in response to pathogen. Interestingly, the p38 MAPK pathway was shown to upregulate *DUOX* gene expression in Drosophila.[17] However by western analysis, we see no increase in Ce-Duox1/BLI-3 protein levels in infected, as compared with uninfected, *C. elegans* (Garsin lab, unpublished data), leading us to speculate that activation of Ce-Duox1/BLI-3 happens post-translationally. Genetic evidence in Drosophila has shown the Gαq-phospholipase Cβ (PLCβ) pathway utilizes secondary messengers, Inositol triphosphate (Ins(1,4,5)P_3) and Ca^{2+}, to regulate DUOX activity.[18] It was proposed that a microbe-derived ligand triggers a G-protein coupled receptor, leading to the release of Gαq, which subsequently activates PLCβ to hydrolyze phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) into inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG). Next, Ins(1,4,5)P_3 binds to the Ins(1,4,5)P_3 receptor (Ins(1,4,5)P_3R), located on the endoplasmic reticulum (ER) membrane, causing the induction and release of intracellular calcium. The released calcium is thought to bind to the EF hands, modulating the activity of DUOX.[17,18] In another study using mammalian cell lines derived from the intestinal epithelium, Duox activity was shown to be dependent on intracellular calcium levels and to be regulated by protein kinase C and protein kinase A.[19] DAG released by the hydrolysis of PtdIns(4,5)P_2 binds to protein kinase C, while protein kinase A is activated by the binding of cAMP synthesized by adenyl cyclase. The study highlights that phosphorylation of Duox at specific serine residues enhances its sensitivity to calcium, further modulating its activity.[19] Boots et al. demonstrated activation of Duox1 in immortalized human bronchial epithelial (HBE1) cells by extracellular ATP and purinergic receptor stimulation.[20] Based on this information, we postulate that these components also may regulate Ce-Duox1/BLI-3.

Though no evidence currently supports a role for the above-mentioned components in ROS generation in the worm, many do appear to affect susceptibility to pathogen, as would be predicted if they did regulate Ce-Duox1/BLI-3. For example, work by Kawli et al. showed that Gαq (EGL-30) and PLCβ (EGL-8), in *C. elegans,* regulated both pathogen immune responses and oxidative stress responses through the p38 MAPK pathway.[21] DAG, released by PLCβ hydrolysis of membrane lipids, also regulates p38 MAPK activity in the intestine. DAG can additionally interact with C1 domain containing proteins TPA-1 (protein kinase Cδ) and DKF2 (protein kinase D2), which are expressed in the *C. elegans* intestine.[22-24] Interestingly, the TPA-1 - DKF2 module induces the production of immune effectors and the oxidative stress response, via the p38 MAPK pathway. Furthermore, worms lacking DFK-2 and TPA-1 were hypersensitive to killing by pathogenic bacteria.[23] Taken together, there is good evidence that the Gαq - PLCβ - TPA-1 - DKF-2 pathway activates the p38 MAPK pathway by DAG signaling. Considering that we have established ROS generated by Ce-Duox1 works through the p38 MAPK pathway to activate the SKN-1-dependent oxidative stress response, it is plausible that a Gαq - PLCβ - TPA-1 - DKF-2 pathway could regulate Ce-Duox1/BLI-3 activity in the worm, and that the ROS generated by BLI-3 acts as a signaling molecule to link the upstream G-protein signaling pathway to the downstream p38 MAPK signaling module (**Fig. 1**). Validating this hypothesis would enable us to explore the possibility of identifying G-protein coupled receptors that regulate this response by screening for differences in pathogen-triggered ROS production.

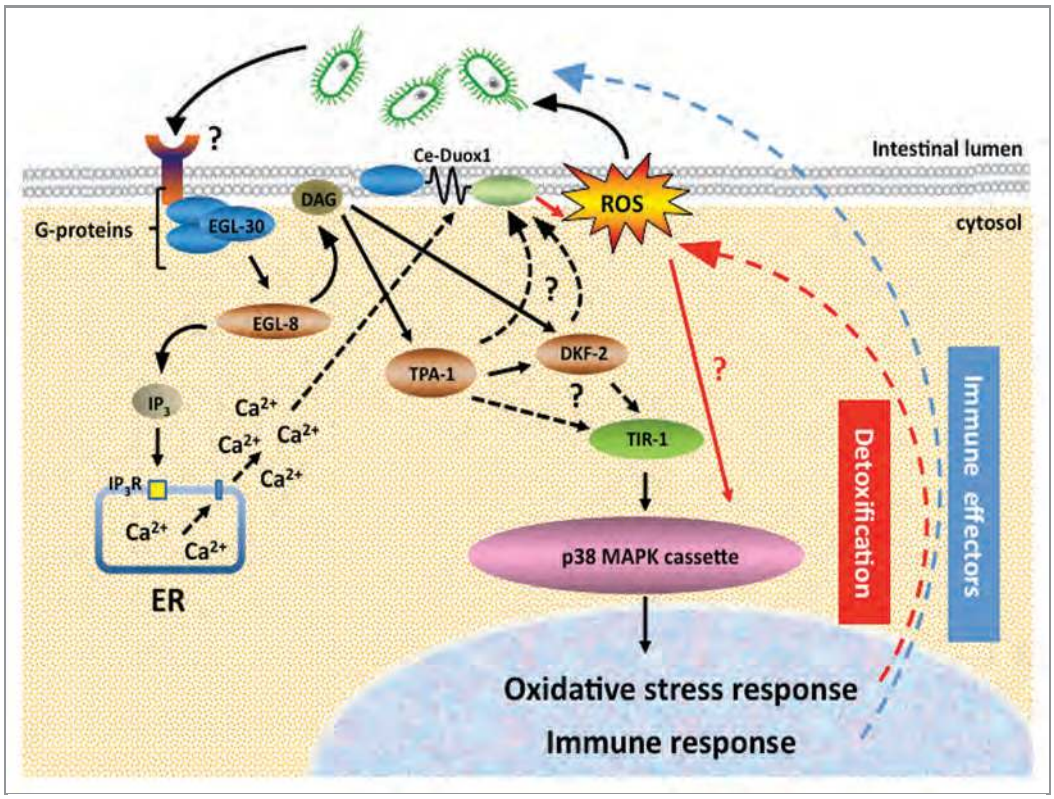

Figure 1. Hypothetical model depicting the activation of Ce-Duox1/BLI-3 and the p38 MAPK pathway in *C. elegans* during infection.

In conclusion, we have established a connection between ROS produced by Ce-Duox1/BLI-3 and the activation of the oxidative stress transcription factor SKN-1, in a p38 MAPK dependent manner, during *C. elegans* infection. The next challenge is to identify the regulatory network that controls Ce-Duox1/BLI-3 activity in response to pathogen invasion.

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