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## A new transkingdom dimension to NO signaling

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### Abstract

Bacterial-derived metabolites profoundly influence the host's cellular and organismal physiology. Seth *et al.* reports that via interspecies S-nitrosylation, microbiota-derived nitric oxide directly alters the host's Argonaute family protein activity, and consequently impinges on the overall post-transcriptional gene silencing program through the microRNA (miRNA) machinery.

### Text

At the end of his life, Alfred Nobel considered it the “irony of fate” that for angina treatment, the doctors handed him a prescription of nitroglycerine, the very explosive compound that brought Nobel his vast fortune. Fast forward a hundred years, Nobel might delight in another irony that part of his extraordinary largess would be awarded to three scientists for their “discoveries concerning nitric oxide as a signaling molecule in the cardiovascular system”. We now know that nitroglycerine releases nitric oxide (NO) into to the blood stream, and this diatomic, freely diffusible gas acts as a potent vasodilator, a neurotransmitter, and a versatile signalling molecule that governs many fundamental cellular processes that are universal and essential for life.

In the current issue of *Cell*, a study of Seth *et al.* uncovers a new dimension to NO signaling, in that microbial-derived NO can also alter host biology through direct S-nitrosylation, namely the covalent attachment of a -NO group to the thiol moiety of a conserved cysteine residue within a protein (**Figure 1**). First described in 1992 (Stamler *et al.*, 1992), S-nitrosylation is now recognized as a major post-translational modification mechanism and a principle NO signaling transduction modality. In the past few years, the reported number of S-nitrosylated proteins rapidly increased from 3,000 to 7,000 in literature. Dysregulation in the S-nitrosylation is implicated in a broad range of human pathologies (Hess and Stamler, 2012).

Virtually all living organisms produce their own intercellular NO during the conversion of L-arginine to L-citulline, via the organisms' intrinsic nitric oxide synthase. *C.elegans*, a bacteriophage, however, is an exception. Lacking nitric oxide synthase in its genome, *C.elegans* relies exclusively on the NO derived from their closely associated microbes for relevant physiological and cellular functions. NO produced by *B.subtilis*, a natural bacteria food substrate and a constituent member of *C.elegans* microbiota, can prolong worm life (Gusarov *et al.*, 2013). These observations raise the hypothesis that in worms, all NO-dependent processes, including S-nitrosylation, must critically depend on bacteria provision.

Seth *et al.* first surveyed the S-nitrosylation profile of the proteomes derived from developing worms, and detected a clear S-nitrosylation signal in over 900 host proteins in animals grown on wild-type bacteria. However, in worms fed on bacteria mutant for NO production ( $\Delta nos$  in *B. subtilis* or  $\Delta narG$  in *E. coli*), the signal strength is dampened by 60%. This provides a first glimpse into how extensively a host's proteome can be modified by a single bacteria-derived metabolite. Among the 900 candidates, Seth *et al.* zeroed in on one candidate, the Argonaute (Ago)-related protein (ALG-1), a homologue of the human Ago2, a core component of the RNA-induced silencing complex (RISC).

Ago family proteins, along with the miRNA-RISC complex interaction, are highly conserved across the eukaryotic kingdoms. They are dedicated to orchestrate gene silencing programs via the microRNA (miRNA) machinery. Once docked onto RISC, the miRNAs guide the RISC to find their cognate mRNA. Upon the formation of miRNA and target mRNA complex, Ago2 proteins interacts with a conserved GW family protein, such as GW182, which further recruits downstream effectors to ultimately mediate post-transcriptional translation repression and mRNA decay (Treiber *et al.*, 2019). Seth *et al.* observed that ALG-1 is S-nitrosylated in worms fed with wild-type bacteria, the signal is again decreased by 60% simply by feeding worms with  $\Delta narG$  *E. coli* or  $\Delta nos$  *B. subtilis*, confirming that bacteria-derived NO plays a key role to S-nitrosylate Ago.

To search for the specific S-nitrosylated cysteine residue, Seth *et al.* employed HEK293 cells, whose endogenous Ago2 is basally S-nitrosylated, and can be further augmented by exogenous addition NO. Among the 22 cysteine residues in Ago2, Seth *et al.* determined that the primary S-nitrosylation site is Cys691, corresponding to Cys855 in ALG-1. Replacing this particular cysteine residue with a serine dampens the nitrosylation signal in both HEK293T cells and worms.

Depending on the cellular context, Ago2 protein undergoes extensive post-translational modifications that regulate its function and turnover, which in turn alters the activities and dynamics of its associated miRNAs (Jee and Lai, 2014). Proxyl-4 hydroxylation and ubiquitination affect Ago protein stability. Phosphorylation of Ago2 at serine S387 by the human protooncogene Akt3 can strengthen the binding between Ago2 and GW182 (Horman *et al.*, 2013). Interestingly, AGO2 and GW182 are reciprocally co-immunoprecipitated in cultured cells and in worms, but their binding is significantly weakened by exogenous NO treatment, or by the C691S mutation. Furthermore, in developing *C. elegans* that have consumed  $\Delta nos$  *B. bacillis*, greater quantity of AIN-1 remains bound to ALG-1. Therefore, S-nitrosylation at the conserved cysteine residue disrupts the protein complex formation between Ago proteins and GW182, a prerequisite for post-transcriptional mRNA silencing. Overall, S-nitrosylation on Argonaute protein via microbial provision of NO can serve as a molecular signal to relieve translational repression on a plethora of host target genes.

What are the cellular consequences of interfering with Ago2-GW182 binding by S-nitrosylation? In *C. elegans*, ALG-1 specifically binds the 3' end of let-7 miRNA, one of first "founder" microRNAs discovered in worms (Reinhart *et al.*, 2000). The Ago-let7 interaction is also highly evolutionarily conserved across the eukaryotic kingdom. Seth *et al.* utilized

the well-characterized Hmga2 luciferase assay to assess let-7 activity, while modifying the S-nitrosylation status of Ago-2. In the presence of NO, S-nitrosylation on Ago2 effectively de-represses the luciferase activity. Notably, the C691S Ago2 mutant also de-represses the luciferase activity, but such derepression is refractory to NO treatment. This observation confirms that S-nitrosylation on C691S interferes Ago2 and GW182 interaction to relieve translational inhibition, but the cysteine residue itself is necessary for such binding for silencing mRNA target. Consistent with this observation, Cys691 resides in the vicinity of the GW182 interaction site of Ago2, away from the miRNA-mRNA complex binding motif. Therefore, the aberrant reporter activity mediated by the C691S mutation is unlikely to be attributed to the potential interruption of Ago-miRNA binding.

The let-7 miRNA is conserved from worms to humans. Mutations in let-7 itself, or in the let-7 target sequence of mRNAs can lead to developmental defects and poor disease prognosis (Bussing et al., 2008). Since S-nitrosylation on Ago potentially interferes with let7-mediated gene silencing, Seth *et al* searched for conspicuous developmental or physiological consequences at the organismal level. *let-7* mutant worms die of vulva rupture during development, due to the failure to repress a single target: lin41, which results in vulva morphogenesis defect (Ecsedi et al., 2015). In a lin-7 temperature sensitive mutant (n2853), consuming wild-type *B. subtilis* leads to ectopic increase of lin-41 mRNA and 35% animal lethality at restrictive temperature, but feeding the worms with  $\Delta$ nos *B. subtilis* can decrease lin-41 expression level and increase worm viability by 2-fold. Furthermore, worms bearing the C885S mutation in ALG-1 are lethal, but such lethality is rescued in the n2853 mutant. However, as the S-nitrosylation site is abolished in the C855S mutant, the vulva bursting phenotype can no longer be rescued by ingesting  $\Delta$ nos bacteria. These observations provide strong evidence that microbiota-mediated S-nitrosylation on worm AGO protein can alter vulva bursting phenotype and have profound developmental consequences.

It is estimated that up to 10% circulating mammalian serum metabolites can be attributed to bacterial origin (Wikoff et al., 2009), and trans-kingdom communications between bacteria and their eukaryotic associates have long been observed from yeast to man. For example, lactate produced by *S.gallinurum* robustly induces the heritable expression of the [gar+] prion in *Saccharomyces cerevisiae*, and so that the yeast overcomes glucose repression (Garcia et al., 2016); short-chain fatty acids (SCFAs), such as acetate and propionate derived from microbial digestion of dietary fibers can activate signal transduction events downstream of a defined set of orphan G-protein coupled-receptors (Tan et al., 2014). However, the molecular mechanisms explaining how these microbial-derived molecules can alter the host's biology remain largely unexplored. By narrowing down the chemical action of a particular microbial compound to a specific S-nitrosylation event, on a specific cysteine residue within a host protein, Seth *et al.* unravels the mechanism of such interspecies communication with unprecedented precision in the host-microbe interaction field. This study unequivocally demonstrates that the resident microbiota can directly control host protein function through a small metabolite, and such modification on a single residue can profoundly alter the entire transcriptional and translational landscape, along with the developmental trajectory of a host. Apart from Argonaute, Seth et al. found >900 S-nitrosylated proteins in worms fed exclusively on wildtype bacteria. They account for almost 5% of the worm genome and

comprise a treasure trove of metabolic and redox enzymes, stress-sensors, proteasome subunits and other important regulators. Future studies should be devoted to understand how microbiota-mediated S-nitrosylation impact the function of these proteins and the biological processes that implicate them. In retrospect, can the developmental defects and physiological deficits observed in different germ-free animal models be attributed to microbial S-nitrosylation? If so, how and to what extent? Moreover, in the eukaryotic animals that generate their own intracellular NO, what proportion of S-nitrosylation in the overall proteome can be traced to microbial origin? Can microbial-derived NO impose regulatory control on the host's own NO synthesis pathway? Answers to these questions will enhance our understanding how mechanistically our microbial partners shapes different aspects of our biology, and the pathologies associated with such interspecies exchange of metabolites.

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**Figure 1. Interspecies S-nitrosylation of host Ago protein affects worm development**

As reported by Seth et al, the aerobic *E.Coli* and the anaerobic *B. Subtilis* are closely associated with their *C.elegans* host. Nitric oxide (NO) derived from these bacteria directly S-nitrosylates the worm Ago homologue on a conserved cysteine residue. S-nitrosylation weakens the Ago-GW182 interaction, and consequently relieves the let-7 microRNA mediated inhibition on lin-41. Such S-nitrosylation event thus impacts vulva morphogenesis and worm development. By inference, other yet unknown host cellular and physiological events can also be affected by microbial S-nitrosylation (colored boxes).

