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Sensing and Responding to Stress Stimuli in Caenorhabditis elegans: Implications for Aging and Immunity

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Sensing and Responding to Stress Stimuli in *Caenorhabditis elegans*: Implications for Aging and Immunity

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Sensing and Responding to Stress Stimuli in *Caenorhabditis elegans*:
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Abstract

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Animals have evolved sophisticated mechanisms to perceive stress or even anticipate it before it has caused damage. Ideally, stress responses are controlled to ensure that they are specific and not excessive lest they cause collateral damage to the host. Here I describe my work in *Caenorhabditis elegans* examining the initiation and consequences of stress responses. In chapters 2 and 3, I examine the mechanisms of initiation of *C.elegans* immune responses to *Pseudomonas aeruginosa,* focusing on pathogen recognition. In chapters 4 and 5 I describe a project that was initiated within the realm of host-pathogen interactions, but evolved to focus on age as a context determining the outcome of both biotic and abiotic stress responses.

Despite extensive knowledge of conserved signaling pathways in the *C.elegans* immune response, a question that has remained elusive is whether *C.elegans* can directly recognize pathogens, or if alternatively, they detect cellular damage caused by infection. I have shown that the immune response to *P. aeruginosa* can be dissociated from colonization and that potential damage from secreted molecules cannot induce a marker of the early immune response; in contrast, a non-pathogenic *Pseudomonad* can. This suggests that the response can be initiated at least in part by structural features of the bacteria. Furthermore, I have identified a family of genes in *C.elegans* encoding LysM domain proteins which are known to participate in recognition of microbial envelope components. Genetic and functional analyses suggest a potential role for family members in pathogen recognition in *C. elegans*.

In the second half of this dissertation, I show that while the *C.elegans* JNK homolog KGB-1 is stress-protective during development, its activation in adults compromises stress resistance as well as general lifespan. I go on to show that this phenomenon is mediated in part by KGB-1's age-dependent antagonistic modulation of the conserved FOXO transcription factor, DAF-16. Genome-wide analysis of the KGB-1 transcriptome revealed that KGB-1 regulates several genes in an age-specific manner, and pointed to another conserved transcription factor, FOS-1 as a mediator of KGB-1's effects. Interestingly, the phenomenon of the same protein having opposite effects dependent on age is reminiscent of the Antagonistic Pleiotropy theory for the evolution of aging. Our results shed light on molecular mechanisms underlying this long-standing theory.

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Chapter 1

Introduction: Stress Responses

Introduction

Stress, broadly defined is any deviation from homeostasis. In biology, there are a number of factors, both abiotic and biotic which may give rise to stress. These include relatively straightforward factors like oxidative damage, temperature, disruptions in protein folding, and infection. They may also include more indirect effects such as increased reproduction diverting resources away from somatic maintenance and stress resistance. All of these things are not mutually exclusive, and animals must find ways to integrate information on stress stimuli to maintain homeostasis. On a broader time-scale, the types of stress an organism experiences, and its ability to respond to these stressors are also shaped by its evolutionary history. This is why different microbes have different sensitivities to antibiotics; why seemingly similar plants or animals may differ in a trait such as thermotolerance; or why an individual native to Sub-Saharan Africa might fare better than an individual of European descent when confronted with a malaria infection. Understanding stress responses as a whole requires the examination of three specific questions. What initiates them? How are they controlled? What are the consequences of their activation? These are important questions because of the critical nature of stress responses in biology. Simply put, stress responses are required for survival, and therefore often trump other aspects of an organism's biology. Therefore, understanding stress responses not only reveals mechanisms for maintaining homeostasis, but it provides an opportunity to understand the constraints that have been placed on events in an organism's life history.

In this chapter, I review current knowledge on stress responses in as they relate to innate immunity as well as more general stress responses. I then conclude with a discussion on how the environment or context in which these responses are activated has important consequences that extend beyond dealing with the original stressor.

Innate Immunity

Every plant and animal has had to evolve in the presence of a variety of microbes. For example, it is estimated that the human microbiota comprises more than ten times as many cells as human cells [1, 2]. These microbes have played a significant role in shaping the evolution of the host. Some of them are beneficial or even essential to the host such as the bacteria *Bacteroides thetaiotaomicron,* a microbe residing in the human intestine that encodes a plethora of enzymatic activities that humans are lacking to digest dietary polysaccharides [3]. Another example comes from plants, where Rhizobial bacteria secrete factors necessary for the formation of plant nodules that fix nitrogen [4]. Still, certain microorganisms are pathogenic, in some cases causing serious disease. This is the challenge for the innate immune system – how to recognize pathogenic microbes among the many that are harmless, and how to mount an appropriate response to them.

The general paradigm of innate immunity is based on recognition of conserved structural features of pathogens known as Pathogen Associated Molecular Patterns or PAMPs by host proteins known as Pattern Recognition Receptors (PRRs). PAMPs are

molecules that are often essential or very important to the microorganism and therefore cannot be lost in order to evade the immune system. Furthermore, their conserved nature allows the immune system to recognize a wide range of pathogens, with a relatively limited repertoire of receptors [5]. While this strategy is very effective, many pathogens can modify their PAMPs as a means to escape immune detection. Examples include *P. aeruginosa* degradation of flagellin monomers [6], peptidoglycan modification by many pathogens [7], and modification of the LPS O-antigen by *Salmonella* [8]. However since a given pathogen expresses many PAMPs, it is almost impossible to fully evade the immune system. To date, there have been dozens of PRRs identified that recognize microbial constituents such as, LPS, peptidoglycan, flagellin, and double stranded RNA among others. Many of these PRRs belong to the well-studied Toll-Like Receptor (TLR) family of proteins. In mammals, when PRRs are engaged by PAMPs, they signal through a select group of adaptor proteins ultimately resulting in the activation of protective transcription factors and the secretion of a variety of inflammatory mediators [9].

However even within the animal kingdom there are variations on the mechanisms of how immune responses are initiated. For example in vertebrates, TLRs bind to PAMPs directly [10]. In *Drosophila*, where Toll Receptors were first discovered, the situation is different, with the Toll protein acting as an intermediary in the bacterial pathogen recognition process. The proteins that directly detect the pathogen are called Peptidoglycan Recognition Proteins (PGRPs), and they set off a proteolytic cascade of host proteins that ultimately activate Toll. Therefore in Drosophila, the Toll ligand is host, not pathogen-derived. In another invertebrate, *C.elegans*, which is the model of choice in this dissertation, the process of immune response initiation is much less understood.

C.elegans is peculiar in that it lacks orthologs for almost all of the known PRR receptors. There is a single Toll ortholog, *tol-1,* but its role in infection resistance does not appear to be crucial. One report suggested that *tol-1* contributes to infection resistance through a behavioral mechanism, while another suggests it has a direct role in defense against certain pathogens [11, 12]. The lack of definitive evidence showing *tol-1* is involved in pathogen recognition in the worm has led some to suggest that Toll-mediated immunity arose after the evolutionary split between coelomates and pseudocoelomates (like *C.elegans*) [13]. One theme that has emerged in *C.elegans* in that the nervous system is important in controlling innate immunity. Studies have demonstrated roles for the neuro-immune modulation of insulin and TGF-Beta signaling in resistance to bacterial and fungal pathogens respectively [14, 15]. Additional data has raised the suggestion that neuronal G protein coupled receptors could be PRR candidates. It has been shown that an ortholog of the mammalian neuropeptide Y receptor encoding a GPCR called *npr-1* is involved in both pathogen avoidance as well as the regulation of immune genes [16, 17]. Another neuronal GPCR named OCTR-1 has been shown to regulate innate immunity by modulating the unfolded protein response [18], and finally the GPCR encoded by *fshr-1* is expressed in both neuronal and intestinal tissues and contributes to the infection response and resistance [19]. However the ligands that might be sensed by the nervous system with regards to innate

immunity are unknown and it is curious that in several of the above cases the immune system acts to suppress rather than activate immunity.

Furthermore, the ostensible lack of PRRs in general in the worm has raised the possibility that *C.elegans* might not utilize direct pathogen recognition at all. Nevertheless, there are many suggestions that the worm can in fact discriminate between pathogens. These include the fact that immune responses to bacteria are in part specific to the pathogen [20, 21], which in itself could simply indicate that different pathogens cause different types of damage. However, in some cases even dead pathogens, where no damage would be expected can induce an immune response [22, 23]. Exploring how immune responses are initiated in *C.elegans* is the focus of the first half of this dissertation.

JNK signaling in stress responses

Stress-activated protein kinases are highly conserved signaling modules in animals. One of the best studied stress-activated protein kinases is the c-Jun N-terminal kinase (JNK) protein. JNK proteins are activated by and participate in the response to diverse stress stimuli including ER stress, oxidative stress and infection. Typically, these proteins are activated through a three-tiered phosphorylation cascade that requires the presence of adaptor proteins [24, 25]. JNK proteins then go on to modify the activity of diverse proteins such as transcription factors, microtubule components, and ubiqutin ligases [26]. In addition to their roles in responding to exogenous stress, JNK often plays crucial roles in development, as demonstrated by the fact that mice that are double mutants for the Jnk1 and Jnk2 isoforms are not viable [27]. In invertebrate models such as *C.elegans* and *Drosophila*, JNK signaling has also been demonstrated to be important for normal lifespan, and this effect is due in large part to the ability of JNK activity to promote nuclear localization of stress-protective FOXO transcription factors [28-30].

Since they have a wide range of targets, and influence features of normal host physiology, the study of JNK signaling provides a unique opportunity to address the question of how stress responses impact an animal's health in general. As detailed in a later section of this chapter, and in chapters 4 and 5, JNK activation can have profound effects on aspects of an animal's health that are not necessarily related to the events that initiated the activation in the first place.

Stress responses in context – Innate Immunity

In a previous section, I introduced how PRRs are able to recognize specific microbial factors that might signal stress, and how downstream signaling can translate these initiating events into a stress response. However there are additional layers of specificity and the concept of the 'context' of stress responses has not been addressed. First, consider the example of innate immunity to bacterial pathogens. Animals use PRRs to recognize PAMPs, but in fact the term 'PAMP' is a misnomer as the molecules they refer to are present on all microbes, not just pathogens and are therefore

sometimes referred to as Microbe Associated Molecular Patterns or MAMPs. This then presents a problem – the human body harbors trillions of bacteria, and most of them are either innocuous or beneficial. If the host immune response relied on the presence of MAMPs alone, it would not only destroy a number of organisms that provide key functions, but it would also set off a persistent storm of inflammation that would cause extensive tissue damage.

There are at least three mechanisms in vertebrates that have been suggested to prevent such events from occurring. In the case of the intestine, one is that PRRs are physically sequestered from MAMPs derived from non-pathogens thus preventing their activation. This idea is supported by the fact that several PRRs are intracellular or are located on the basolateral side of membranes while commensals might be primarily confined to the intestinal lumen. Therefore only microbes that could cause tissue damage or invade cells would engage PRRs. However this idea may be too simplistic, as it has been shown that TLRs can recognize commensals under normal conditions, and this interaction is actually important for maintaining intestinal integrity and homeostasis [31]. A second possibility is that while commensal bacteria may be recognized, host factors modulate the immune response to prevent its over-activation. Evidence for this hypothesis has been provided by experiments in mice raised in microbe-controlled environments and/or mice deficient in immune modulators such as IL-10. In short, animals lacking IL-10 are healthy when raised in germ-free environments, however they develop serious inflammatory conditions when raised solely in the presence of commensals (i.e. no pathogens present) [32] . This inflammation is initiated by TLR recognition of the commensals [33]. A third mechanism to prevent unwarranted immune responses would be for the host to detect 'damage' caused by the pathogen rather than the direct detection of the pathogen itself. In mammals a variety of such host-derived 'danger' signals have been shown to function in the immune responses. Danger Associated Molecular Patterns or DAMPs encompass molecules diverse as ATP, uric acid, nucleic acids, heat shock proteins, and polysaccharides like heparan sulphate and hyaluronan [34]. Many microbial pathogens alter the levels and/or localization of such molecules, and sensing these changes can be a good indication of infection. In *C.elegans*, where there are still no identified pattern recognition receptors, some have proposed that sensing damage is the dominant mode of initiating immune responses. Recent reports demonstrating that inhibition of 'core processes' like translation can induce immune responses have lent support for this idea [35-37].

While the above concepts provide valuable insight into innate immunity, each one of them on their own gives an unsatisfactory explanation as to how the specificity and control of immune responses is achieved. Consider the following three questions: If sequestration prevents immune over-activation, how does an animal deal with situations where tissue damage might cause a breach in this sequestration? If immune-modulatory factors dampen the recognition response to commensals, how are pathogens released from this level of control? And finally, how does the animal avoid mounting a pathogen-specific response when DAMPs are released by processes that are not mediated by pathogens? The most foolproof way for the immune system to

function properly would be to integrate *all* of the information available *in context*, before making a 'decision'. For example, engagement of PRRs does not necessarily signal the presence of a pathogen, but engagement of PRRs simultaneously with the presence of DAMPs probably does. Similarly, the presence of DAMPs alone is much more likely to indicate wounding than it is to indicate infection.

In light of such concerns, some have proposed a more integrative view of innate immunity referred to as 'two signal models of innate immunity' and 'patterns of pathogenesis' [38, 39]. The two signal model is well established in the field of adaptive immunity with the most prominent example being T cell activation. During T cell activation 'signal one' refers to the T cell receptor binding MHC-antigen complexes and 'signal two' refers to binding of additional receptors on the T cell to 'co-stimulatory' molecules on the Antigen Presenting Cell (APC). Since co-stimulatory molecules are induced in the APC only when it has been activated by a microorganism, the requirement for two signals ensures the T cell response is specific and does not lead to problems like autoimmunity [40]. It is reasonable to think that some aspects of innate immunity might have such built in controls as well. Patterns of pathogenesis also suggest that the context is a vital factor in tailoring the activities of the innate immune system [41]. For example,this could occur at the level of initial pathogen recognition. Since a single MAMP often has multiple receptors, that may be located in distinct cellular compartments, this may help to distinguish between different classes of microbes / pathogens, for example intracellular vs. extracellular. Tailoring of the response could also occur at later stages of infection. Since different pathogens may disrupt different host processes, the DAMPs they elicit may convey information about the type of infection in progress.

To summarize, in the ideal situation there would be two signals responsible for the induction of an immune response by the innate immune system. One signal should be mediated by PRRs indicating the presence of a microbe. Another signal should indicate that the microbe is pathogenic. The perception of these two signals should occur sequentially, but also should have some overlap in time in order to ensure the most optimal response. In this way, initiation of the response can be fast since it is mediated by recognition. This response can then be amplified and sustained, but only if necessary via perception of the second signal.

Stress responses in context – the example of JNK signaling

JNK signaling influences numerous biological processes such as cell death/survival, development, and insulin signaling. Interestingly, sometimes it can affect the same process in completely opposite ways. How such a small set or proteins can have so many different effects is an intriguing question. While all of the details are far from being filled in, the picture that is emerging is that the context in which JNK proteins are activated is one of the principal determinants of what the outcome of that activation will be. Three context-themed proposals for how JNK specificity is achieved are briefly outlined here. The first proposes that it is the activity of additional signaling pathways that can collaborate with JNK which determines what the outcome of JNK activation will

be. Some of the support for this idea comes from data showing that the collaboration of JNK with the JunD protein can promote cell survival in contrast to the better known role of JNK in promoting cell death [42]. Another study showed that reactive oxygen species induced by TNF signaling are required for JNK to promote cell death [43]. Secondly, it has been suggested that it is the duration, and strength of JNK activation that determines its outcome, with short activation promoting cell survival, and sustained activation promoting cell death [44, 45]. Finally, the third idea proposes that the tissue in which a JNK protein is activated plays an important role in its effects will be [46].

When Stress responses go awry

While there are several mechanisms to control stress responses, these mechanisms are imperfect. When they malfunction, it can lead to a variety of serious, acute and chronic illnesses. One of the classic examples of the immune system reacting inappropriately to stress stimuli is in the case of so called 'sterile inflammation'. Sterile inflammation occurs when tissues experience injury in the absence of a microbial infection. Tissue injury releases a number of DAMPs, particularly when cells die by necrosis rather than the highly controlled process of apoptosis. This in turn results in the recruitment of innate immune cells that are involved in the resolution of the injury – particularly phagocytic cells like neutrophils that can remove debris [47]. However, activated immune cells like nuetrophils also carry a variety of destructive capabilities such as the production of pore-forming proteins, generation of reactive oxygen species, and hydrolytic activities [48]. These activities are designed to kill pathogens; however an activated immune cell does not always 'know' that it is responding to injury and not infection. Consequently, if these potent activities are left unchecked, the immune system can actually mediate pathologies such as ischemic-reperfusion injury [49],and liver toxicity caused by common drugs such as acetaminophen [50, 51].

JNK signaling is another example of the double-edged nature of stress responses. While JNK signaling is generally stress-protective, there are a number of instances in which it can contribute to pathology. These pathologies are often age-associated and include Alzheimer's disease, ischemia reperfusion injury in the heart and brain, and obesity [46, 52-55]. Unlike the case of innate immunity, the details of how and why JNK signaling can become detrimental are much less clear. One thing that has become apparent however is that prolonged or excessive JNK activation can be deleterious. Much of the data supporting this has come from studies in invertebrate models. In *Drosophila*, JNK activity can contribute to the disruption of intestinal homeostasis [56, 57], and in the extreme case, flies that are homozygous mutants for the phosphatase that inhibits JNK are not viable [58]. This is similar to the case in *C.elegans* where mutants for the phosphatase, VHP-1 which inhibits the JNK orthologs, KGB-1 are developmentally lethal [59].

In this dissertation I explore two aspects of stress responses in *C.elegans*. First, how they are initiated and secondly what the context-dependent consequences of their activation are. My data provides evidence for the existence of specific pathogen recognition in the worm, and in a surprising discovery, identifies age as a dominant

factor determining the outcome of JNK signaling. Interestingly, the latter finding may represent new experimental evidence for a 55 year-old theory on the evolution of aging.

References

1. Whitman, W.B., D.C. Coleman, and W.J. Wiebe, *Prokaryotes: the unseen majority.* Proc Natl Acad Sci U S A, 1998. **95**(12): p. 6578-83.

2. Ley, R.E., D.A. Peterson, and J.I. Gordon, *Ecological and evolutionary forces shaping microbial diversity in the human intestine.* Cell, 2006. **124**(4): p. 837-48.

3. Xu, J., et al., *A genomic view of the human-Bacteroides thetaiotaomicron symbiosis.* Science, 2003. **299**(5615): p. 2074-6.

4. Oldroyd, G.E., et al., *The rules of engagement in the legume-rhizobial symbiosis.* Annu Rev Genet, 2011. **45**: p. 119-44.

5. Medzhitov, R. and C. Janeway, Jr., *Innate immune recognition: mechanisms and pathways.* Immunol Rev, 2000. **173**: p. 89-97.

6. Bardoel, B.W., et al., *Pseudomonas evades immune recognition of flagellin in both mammals and plants.* PLoS Pathog, 2011. **7**(8): p. e1002206.

7. Wolfert, M.A., A. Roychowdhury, and G.J. Boons, *Modification of the structure of peptidoglycan is a strategy to avoid detection by nucleotide-binding oligomerization domain protein 1.* Infect Immun, 2007. **75**(2): p. 706-13.

8. Duerr, C.U., et al., *O-antigen delays lipopolysaccharide recognition and impairs antibacterial host defense in murine intestinal epithelial cells.* PLoS Pathog, 2009. **5**(9): p. e1000567.

9. Kumar, H., T. Kawai, and S. Akira, *Pathogen recognition by the innate immune system.* Int Rev Immunol, 2011. **30**(1): p. 16-34.

10. Medzhitov, R., *Toll-like receptors and innate immunity.* Nat Rev Immunol, 2001. **1**(2): p. 135-45.

11. Pujol, N., et al., *A reverse genetic analysis of components of the Toll signaling pathway in Caenorhabditis elegans.* Curr Biol, 2001. **11**(11): p. 809-21.

12. Tenor, J.L. and A. Aballay, *A conserved Toll-like receptor is required for Caenorhabditis elegans innate immunity.* EMBO Rep, 2008. **9**(1): p. 103-9.

13. Kanzok, S.M., et al., *Origin of Toll-like receptor-mediated innate immunity.* J Mol Evol, 2004. **58**(4): p. 442-8.

14. Zugasti, O. and J.J. Ewbank, *Neuroimmune regulation of antimicrobial peptide expression by a noncanonical TGF-beta signaling pathway in Caenorhabditis elegans epidermis.* Nat Immunol, 2009. **10**(3): p. 249-56.

15. Kawli, T. and M.W. Tan, *Neuroendocrine signals modulate the innate immunity of Caenorhabditis elegans through insulin signaling.* Nat Immunol, 2008. **9**(12): p. 1415-24.

16. Styer, K.L., et al., *Innate immunity in Caenorhabditis elegans is regulated by neurons expressing NPR-1/GPCR.* Science, 2008. **322**(5900): p. 460-4.

17. Reddy, K.C., et al., *Caenorhabditis elegans NPR-1-mediated behaviors are suppressed in the presence of mucoid bacteria.* Proc Natl Acad Sci U S A, 2009. **108**(31): p. 12887-92.

18. Sun, J., et al., *Neuronal GPCR controls innate immunity by regulating noncanonical unfolded protein response genes.* Science, 2011. **332**(6030): p. 729-32.

19. Powell, J.R., D.H. Kim, and F.M. Ausubel, *The G protein-coupled receptor FSHR-1 is required for the Caenorhabditis elegans innate immune response.* Proc Natl Acad Sci U S A, 2009. **106**(8): p. 2782-7.

20. Wong, D., et al., *Genome-wide investigation reveals pathogen-specific and shared signatures in the response of Caenorhabditis elegans to infection.* Genome Biol, 2007. **8**(9): p. R194.

21. Alper, S., et al., *Specificity and complexity of the Caenorhabditis elegans innate immune response.* Mol Cell Biol, 2007. **27**(15): p. 5544-53.

22. Irazoqui, J.E., et al., *Distinct pathogenesis and host responses during infection of C. elegans by P. aeruginosa and S. aureus.* PLoS Pathog, 2010 **6**: p. e1000982.

23. Pukkila-Worley, R., F.M. Ausubel, and E. Mylonakis, *Candida albicans infection of Caenorhabditis elegans induces antifungal immune defenses.* PLoS Pathog, 2011. **7**(6): p. e1002074.

24. Kyriakis, J.M. and J. Avruch, *Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation.* Physiol Rev, 2001. **81**(2): p. 807-69.

25. Morrison, D.K. and R.J. Davis, *Regulation of MAP kinase signaling modules by scaffold proteins in mammals.* Annu Rev Cell Dev Biol, 2003. **19**: p. 91-118.

26. Weston, C.R. and R.J. Davis, *The JNK signal transduction pathway.* Curr Opin Cell Biol, 2007. **19**(2): p. 142-9.

27. Kuan, C.Y., et al., *The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development.* Neuron, 1999. **22**(4): p. 667-76.

28. Oh, S.W., et al., *JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16.* Proc Natl Acad Sci U S A, 2005. **102**(12): p. 4494-9.

29. Wang, M.C., D. Bohmann, and H. Jasper, *JNK extends life span and limits growth by antagonizing cellular and organism-wide responses to insulin signaling.* Cell, 2005. **121**(1): p. 115-25.

30. Wang, M.C., D. Bohmann, and H. Jasper, *JNK signaling confers tolerance to oxidative stress and extends lifespan in Drosophila.* Dev Cell, 2003. **5**(5): p. 811-6.

31. Rakoff-Nahoum, S., et al., *Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis.* Cell, 2004. **118**(2): p. 229-41.

32. Davidson, N.J., et al., *Chronic colitis in IL-10-/- mice: insufficient counter regulation of a Th1 response.* Int Rev Immunol, 2000. **19**(1): p. 91-121.

33. Rakoff-Nahoum, S., L. Hao, and R. Medzhitov, *Role of toll-like receptors in spontaneous commensal-dependent colitis.* Immunity, 2006. **25**(2): p. 319-29.

34. Chen, G.Y. and G. Nunez, *Sterile inflammation: sensing and reacting to damage.* Nat Rev Immunol, 2010. **10**(12): p. 826-37.

35. Dunbar, T.L., et al., *C. elegans detects pathogen-induced translational inhibition to activate immune signaling.* Cell Host Microbe, 2012. **11**(4): p. 375-86.

36. Melo, J.A. and G. Ruvkun, *Inactivation of conserved C. elegans genes engages pathogen- and xenobiotic-associated defenses.* Cell, 2012. **149**(2): p. 452-66.

37. McEwan, D.L., N.V. Kirienko, and F.M. Ausubel, *Host translational inhibition by Pseudomonas aeruginosa Exotoxin A Triggers an immune response in Caenorhabditis elegans.* Cell Host Microbe, 2012. **11**(4): p. 364-74.

38. Vance, R.E., *Cutting edge: cutting edge commentary: a Copernican revolution? Doubts about the danger theory.* J Immunol, 2000. **165**(4): p. 1725-8.

39. Fontana, M.F. and R.E. Vance, *Two signal models in innate immunity.* Immunol Rev, 2011. **243**(1): p. 26-39.

40. Sharpe, A.H. and G.J. Freeman, *The B7-CD28 superfamily.* Nat Rev Immunol, 2002. **2**(2): p. 116-26.

41. Vance, R.E., R.R. Isberg, and D.A. Portnoy, *Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system.* Cell Host Microbe, 2009. **6**(1): p. 10-21.

42. Lamb, J.A., et al., *JunD mediates survival signaling by the JNK signal transduction pathway.* Mol Cell, 2003. **11**(6): p. 1479-89.

43. Kamata, H., et al., *Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases.* Cell, 2005. **120**(5): p. 649-61.

44. Ventura, J.J., et al., *Chemical genetic analysis of the time course of signal transduction by JNK.* Mol Cell, 2006. **21**(5): p. 701-10.

45. Wei, J., et al., *c-Jun N-terminal kinase (JNK-1) confers protection against brief but not extended ischemia during acute myocardial infarction.* J Biol Chem, 2011. **286**(16): p. 13995-4006.

46. Sabio, G., et al., *Role of muscle c-Jun NH2-terminal kinase 1 in obesity-induced insulin resistance.* Mol Cell Biol, 2010. **30**(1): p. 106-15.

47. Kono, H. and K.L. Rock, *How dying cells alert the immune system to danger.* Nat Rev Immunol, 2008. **8**(4): p. 279-89.

48. Segal, A.W., *How neutrophils kill microbes.* Annu Rev Immunol, 2005. **23**: p. 197-223.

49. Tsung, A., et al., *The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion.* J Exp Med, 2005. **201**(7): p. 1135-43.

50. Imaeda, A.B., et al., *Acetaminophen-induced hepatotoxicity in mice is dependent on Tlr9 and the Nalp3 inflammasome.* J Clin Invest, 2009. **119**(2): p. 305-14.

51. Liu, Z.X., et al., *Neutrophil depletion protects against murine acetaminophen hepatotoxicity.* Hepatology, 2006. **43**(6): p. 1220-30.

52. Baines, C.P. and J.D. Molkentin, *STRESS signaling pathways that modulate cardiac myocyte apoptosis.* J Mol Cell Cardiol, 2005. **38**(1): p. 47-62.

53. Morishima, Y., et al., *Beta-amyloid induces neuronal apoptosis via a mechanism that involves the c-Jun N-terminal kinase pathway and the induction of Fas ligand.* J Neurosci, 2001. **21**(19): p. 7551-60.

54. Kuan, C.Y., et al., *A critical role of neural-specific JNK3 for ischemic apoptosis.* Proc Natl Acad Sci U S A, 2003. **100**(25): p. 15184-9.

55. Hirosumi, J., et al., *A central role for JNK in obesity and insulin resistance.* Nature, 2002. **420**(6913): p. 333-6.

56. Seisenbacher, G., E. Hafen, and H. Stocker, *MK2-dependent p38b signalling protects Drosophila hindgut enterocytes against JNK-induced apoptosis under chronic stress.* PLoS Genet, 2011. **7**(8): p. e1002168.

57. Biteau, B., C.E. Hochmuth, and H. Jasper, *JNK activity in somatic stem cells causes loss of tissue homeostasis in the aging Drosophila gut.* Cell Stem Cell, 2008. **3**(4): p. 442-55.

58. Martin-Blanco, E., et al., *puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in Drosophila.* Genes Dev, 1998. **12**(4): p. 557-70.

59. Mizuno, T., et al., *The Caenorhabditis elegans MAPK phosphatase VHP-1 mediates a novel JNK-like signaling pathway in stress response.* Embo J, 2004. **23**(11): p. 2226-34.

Chapter 2

Dissociation of immune responses from pathogen colonization supports pattern recognition in *C. elegans*.

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Dissociation of immune responses from pathogen colonization supports pattern recognition in *C. elegans*

Kwame Twumasi-Boateng and Michael Shapira

Abstract

Caenorhabditis elegans has been used for over a decade to characterize signaling cascades controlling innate immune responses. However, what initiates these responses in the worm has remained elusive. To gain a better understanding of the initiating events we delineated genome-wide immune responses to the bacterial pathogen *Pseudomonas aeruginosa* in worms heavily-colonized by the pathogen versus worms visibly not colonized. We found that infection responses in both groups were identical, suggesting that immune responses were not correlated with colonization and its associated damage. Quantitative RT-PCR measurements further showed that pathogen secreted factors were not able to induce an immune response, but exposure to a non-pathogenic *Pseudomonas* species was. These findings raise the possibility that the *C.elegans* immune response is initiated by recognition of microbe-associated molecular patterns. In the absence of orthologs of known pattern recognition receptors, *C. elegans* may rely on novel mechanisms, thus holding the potential to advance our understanding of evolutionarily conserved strategies for pathogen recognition.

Introduction

The soil nematode *Caenorhabditis elegans* has been used for over a decade to study host-pathogen interactions. Such studies provided detailed information on pathogen-specific innate immune responses, and the signal transduction pathways and transcription factors that activated them (reviewed in [1]). However, what initiates innate immune responses in the worm remains unknown.

In vertebrates, innate immune responses are initiated mainly by recognition of pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide, peptidoglycan, or microbially-modified nucleic acids; PAMPs are occasionally referred to as MAMPs - microbe-associated molecular patterns, to acknowledge their existence in non-pathogenic microbes. In addition, recognition of damage-associated molecular patterns (DAMPs), such as ATP and monsodium urate crystals, can enhance activation by PAMPs, but also may be sufficient for initiation of innate immune responses [2]. The pattern recognition receptors (PRRs) responsible for recognizing PAMPs or DAMPs include members of several protein families including the Toll/Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like nucleotide recognition receptors (RLRs) [3]. Innate immune recognition is conserved through evolution, both in terms of its general strategy as well as the proteins involved: The first TLR, Toll, originally identified as essential for *Drosophila's* development, was subsequently shown to also have a crucial role in innate immunity [4, 5], NLRs take part in antimicrobial defenses both in animals and in plants [6] and a RIG-I homolog was recently shown to function in *C. elegans* anti-viral defenses [7]. Although *C. elegans* mounts specific responses to infections with

different pathogenic bacteria, manifested in a robust gene induction [8], it is yet unknown how it discriminates between different bacteria. *C. elegans* has one Toll homolog gene, *tol-1*, which appears to be is largely dispensable for immune protection ([9, 10], but see [11])).; Additionally, FSHR-1, a heterotrimeric G protein with leucine-rich repeats (a motif shared among all vertebrate TLRs and NLRs), is necessary for immune protection, but is equally protective against Gram-negative and -positive pathogens, suggesting that it might not function in the proximal events of pathogen recognition. [12]. The inability to identify *C. elegans* PRRs based on orthology raises the possibility that *C. elegans* may use novel modes of pathogen recognition. Alternatively, *C. elegans* may respond to pathogen-specific damage caused in the course of infection. To discriminate between these two possibilities we used an infection model of *C. elegans* intestinal colonization by *Pseudomonas aeruginosa* [13].

Results

The PA14 strain of *Pseudomonas aeruginosa*, made to express GFP (PA14-GFP from here on), can be followed as it colonizes the worm intestine, leading to death within three days. This colonization requires live bacteria and depends on bacterial regulators of virulence [14]. Furthermore, once reaching a significantly visible level of colonization (e.g. following 18 hours of exposure), most worms remain colonized, even when transferred to *E. coli* (65% of N=96). This suggests active interactions between the pathogen and its host, which enable the pathogen to persist in most cases. When a genetically-homogenous and age-synchronized population of worms is exposed to PA14-GFP, significant heterogeneity is seen in colonization by the pathogen (Fig. 1A). We reasoned that if colonization-associated damage elicited immune responses in *C. elegans*, then immune responses would be correlated to the degree of colonization. To test this, we chose the extreme case of comparing significantly colonized worms with worms that were not visibly colonized. We exposed wild-type worms to PA14-GFP under standard conditions (or to *E. coli* control) and following 18 hours, separated significantly colonized worms (green) from visibly non-colonized (dark). Subsequently, gene expression was examined using microarrays in both groups as well as in those exposed to the *E. coli* control. Genes responding to *P. aeruginosa* were identified using a multi-class t-test with a false discovery rate of 10%. This analysis identified gene classes previously reported as being induced by *P. aeruginosa,* including lysozymes, lectins [15, 16], several neuropeptide-like genes, and detoxification genes [16](Fig. 1B and supplementary table 1). The analyzed dataset is attached as supplementary table 2. Importantly, infection responses were largely independent of the degree of colonization. Thus, although worm death from PA14 infection is correlated with colonization, the responses against it were not.

Several possible explanations may account for immune responses without visible colonization. The first that we wished to be able to rule out was that worms that are not visibly colonized were previously colonized, but managed to clear the infection. To test this possibility under similar conditions as those of the experiment we picked visibly colonized worms (N=80) following a 12-hour exposure to PA14-GFP, and continuing the exposure to PA14-GFP on new plates, we examined whether any of these worms

appeared dark by the time of collection (6 hours later); this turned out not to be the case, as all worms remained green. This does not mean that 'dark' worms are completely non-colonized, as they may be colonized with an undetectably small number of bacteria, but the similarity between their immune responses and those of fully-colonized worms implies that the immune response is not correlated with the degree of colonization.

A second possibility is that molecules secreted by *P. aeruginosa* cause damage to the worm, which independent of colonization can induce the immune response. This is particularly plausible since *P. aeruginosa* is known to secrete a wide array of small-molecule and proteinaceous exotoxins [17]. To address this possibility, we used quantitative (q)RT-PCR to follow the expression of F55G11.2, a gene with a yet uncharacterized function, which was previously identified as part of the earliest responses to *P. aeruginosa* [15]. A closer examination showed that F55G11.2 was strongly induced within two hours of exposure to the pathogen (Fig. 2B) and as early as one hour following exposure (not shown). However, F55G11.2 was not induced when worms were exposed to a conditioned solution from a *P. aeruginosa* 24-hour culture (the 'secretome'), laced onto dead *E. coli* (Fig. 2A). Induction of F55G11.2 was similarly missing when worms were grown on plates conditioned with *P. aeruginosa* grown on a filter, which was removed prior to transfer of worms and replaced with dead *E. coli* serving as food (Fig. 2B). Similar lack of induction was observed for *lys-2* and *pgp-5*, two additional infection response genes that respond to *P. aeruginosa*, but with a slower time course than F55G11.2 (Fig. 2C and results not shown). Thus, secreted factors are not sufficient to induce immune responses against *P. aeruginosa*.

A third possibility that we are unable to rule out is that volatile toxins released by *P. aeruginosa* cause damage to the worm, which induces immune responses. *P. aeruginosa* has a distinctive smell produced by a combination of volatile compounds. Of these, one is hydrogen cyanide, a potent toxin. However, cyanide production was not found to take part in PA14 pathogenicity in *C. elegans* (unlike the PA01 strain [18]) and without any additional known toxic volatile compounds released from *P. aeruginosa*, this is unlikely.

The fourth possible explanation for induction of immune responses prior to detectable colonization is that *C. elegans* can recognize molecules associated with the pathogen (i.e. PAMPs/MAMPs), with a sensitivity that allows it to respond to a small number of bacteria. The failure of the *P. aeruginosa* 'secretome' to induce immune responses, supported this possibility. To decouple structural features of *Pseudomonas* from its pathogenicity, we examined immune responses to *Pseudomonas mendocina,* a recently identified *C. elegans* commensal that shows no pathogenicity, both in terms of survival/lifespan as well as with regards to early symptoms of infection (i.e. muscle function and movement)(Fig. S1 and Montalvo-Katz, unpublished results). Exposure to intact *P. mendocina* lead to F55G11.2 gene induction, smaller than the response to *P. aeruginosa*, but reproducible (Fig. 2D). This is consistent with the hypothesis that *C. elegans* can recognize cell-associated moieties that are shared between *P. aeruginosa* and *P. mendocina,* and that such recognition plays a role in the initiation of early

immune responses. As further evidence that *C.elegans* is capable of responding to very small numbers of bacteria, we reasoned that if this was the case then the response to *P. aeruginosa* should be very rapid, occurring once the worm has ingested a small number of bacteria. Indeed, within 15 minutes of exposure to *P. aeruginosa*, F55G11.2 was induced ~7.5 fold (Fig 3.) Altogether, our data suggest that *C. elegans* immune responses against *P. aeruginosa* are initiated by PAMP recognition, or, since the recognized pattern is shared with the non-pathogenic *P. mendocina*, MAMP recognition.

Discussion

We found that *C. elegans* immune response occurs prior to any visible colonization. Death, and presumably damage due to *P. aeruginosa* infection, is correlated with the extent of colonization. That this correlation does not hold for immune responses suggests sensitive detection of molecular patterns, apparently cell-associated and furthermore, shared among pathogenic and non-pathogenic *Pseudomonas* species. Recognition of MAMPs by *C. elegans* does not exclude the possibility that it can also respond to additional types of stimuli. In fact, the smaller magnitude of the response to *P. mendocina* compared to *P. aeruginosa* may be indicative of multiple signals, some cell-associated, but others associated with pathogenesis (perhaps DAMPs), leading to a full-blown immune response. The results described here provide evidence for MAMP recognition in *C. elegans*, but the nature of these MAMPs remains to be identified. Furthermore, since no PRR orthologs have been identified to date in *C. elegans*, these results further encourage a search for *C. elegans* PRRs, as they may represent novel mechanisms of pathogen recognition.

Methods

Strains. Worms were of the N2 wild-type strain. Bacterial strains included *E. coli* OP50-1, the clinical isolate *Pseudomonas aeruginosa* strain PA14, or a PA14 derivative expressing GFP off a stable plasmid [13]. *Pseudomonas mendocina*, a non-pathogenic environmental *Pseudomonad* was isolated from worms grown on soil (Montalvo-Katz, unpublished).

Worm infection and sorting. Synchronized populations of wild-type worms, grown under standard conditions, were transferred at day two of adulthood either to *E. coli*, or to PA14-GFP. After eighteen hours, worms presented a wide-range of colonization reflected by accumulation of GFP-expressing bacteria in their intestine. Colonized (intensely green) and non-colonized (dark) worms were separated, either using the COPAS^{TM} BIOSORT worm sorter ((Union Biometrica; two experiments) or by picking >100 worms of each group under a fluorescent stereoscope (one experiment, serving as a control for the automatic sorting).

Testing the effects of the *P. aeruginosa* secretome on *C. elegans* immune responses. Three approaches were employed for testing potential contribution of *P. aeruginosa* secreted factors independently of the secreting bacteria, results of the first and third methods are presented in Fig. 2: in the first, *P. aeruginosa* was grown on a 0.2

μM mixed cellulose esters filter (Millipore) placed on modified NGM plates at 37^{o}C for 24 hours, at the end of which the underlying agar was blue due to secreted pyocyanin; the filter (containing bacteria) was then removed, *E. coli* added as food, and worms laid on plates; since filters may absorb some of the secreted molecules, particularly proteins, the second approach involved exposing worms to supernatants obtained from saturated *P. aeurigonsa* cultures; supernatants were cleared of bacteria by repeated centrifugation/transfer (microcentrifuge, 14K RPM, 10 minutes each, 8 times), and following testing for absence of bacteria, 100 μl supernatant was added to lawns of dead *E. coli;* to account for molecules possibly secreted only on solid medium, in the third approach we submerged *P. aeruginosa* lawns in 1.2 ml M9 solution, let it sit for an hour at room temperature to allow secreted factors to diffuse out of the agar and lawn, then collected supernatant, removed bacteria by repeated centrifugation as above, and added to lawns of dead *E. coli.* All three methods resulted in the same results.

RNA extraction, microarrays and qRT-PCR. RNA was extracted from 100-700 worms per group/time-point using Trizol (Invitrogen). For microarray experiments, RNA was amplified using the MessageAmp™ II aRNA Amplification Kit (Ambion), labeled with the ULS™ aRNA Labeling Kit (Kreatech) and co-hybridized to Epoxy (Corning) microarrays spotted with 60-mer oligonucleotides (Washington University Genome Sequencing Center) with a similarly amplified and labeled reference RNA sample [15].
For (q)RT-PCR measurements, gene-specific threshold cycle (Ct) values were normalized to the respective actin values, and presented as fold change over the time = 0 point.

PCR Primers

pan-actin forward	TCGGTATGGGACAGAAGGAC
pan-actin reverse	CATCCCAGTTGGTGACGATA
F55G11.2 forward	TGGTTCTCCAGACGTGTTCA
F55G11.2 reverse	CAGCCTTGCCTTTACTGACA
lys-2 forward	CCAATATCAAGCTGGCAAGG
lys-2 reverse	GTTGGATTGTTTGGCCAGTT

Statistical analysis
Gene expression profiles obtained with microarrays were analyzed by a multi-class t-test using Significance Analysis of Microarrays (SAM; [19]), implemented as part of the TMEV software package. Based on T statistics the test retrieves genes with a T value above a cutoff score estimated to give the desired false discovery rate (selected to be 10%). This analysis was used to identify *C. elegans* genes differentially expressed in either one of the three analyzed groups: worms exposed to *E. coli*, worms exposed and colonized by *P. aeruginosa* PA14-GFP, or worms exposed to PA14-GFP, but not colonized. Since no difference was observed between expression profiles in worms collected manually or with the worm sorter, data from the three repeats for each of the three experimental groups were pooled. The resulting list of genes responding to PA14 contained 359 genes.

References

1. Irazoqui, J.E., J.M. Urbach, and F.M. Ausubel, *Evolution of host innate defence: insights from Caenorhabditis elegans and primitive invertebrates.* Nat Rev Immunol, 2010. **10**(1): p. 47-58.

2. Chen, G.Y. and G. Nunez, *Sterile inflammation: sensing and reacting to damage.* Nat Rev Immunol, 2010. **10**(12): p. 826-37.

3. Kumar, H., T. Kawai, and S. Akira, *Pathogen recognition by the innate immune system.* Int Rev Immunol, 2011. **30**(1): p. 16-34.

4. Rosetto, M., et al., *Signals from the IL-1 receptor homolog, Toll, can activate an immune response in a Drosophila hemocyte cell line.* Biochem Biophys Res Commun, 1995. **209**(1): p. 111-6.

5. Lemaitre, B., et al., *The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults.* Cell, 1996. **86**(6): p. 973-83.

6. Ting, J.P., S.B. Willingham, and D.T. Bergstralh, *NLRs at the intersection of cell death and immunity.* Nat Rev Immunol, 2008. **8**(5): p. 372-9.

7. Lu, R., et al., *An RIG-I-Like RNA helicase mediates antiviral RNAi downstream of viral siRNA biogenesis in Caenorhabditis elegans.* PLoS Pathog, 2009. **5**(2): p. e1000286.

8. Wong, D., et al., *Genome-wide investigation reveals pathogen-specific and shared signatures in the response of Caenorhabditis elegans to infection.* Genome Biol, 2007. **8**(9): p. R194.

9. Kanzok, S.M., et al., *Origin of Toll-like receptor-mediated innate immunity.* J Mol Evol, 2004. **58**(4): p. 442-8.

10. Pujol, N., et al., *A reverse genetic analysis of components of the Toll signaling pathway in Caenorhabditis elegans.* Curr Biol, 2001. **11**(11): p. 809-21.

11. Tenor, J.L. and A. Aballay, *A conserved Toll-like receptor is required for Caenorhabditis elegans innate immunity.* EMBO Rep, 2008. **9**(1): p. 103-9.

12. Powell, J.R., D.H. Kim, and F.M. Ausubel, *The G protein-coupled receptor FSHR-1 is required for the Caenorhabditis elegans innate immune response.* Proc Natl Acad Sci U S A, 2009. **106**(8): p. 2782-7.

13. Tan, M.W., S. Mahajan-Miklos, and F.M. Ausubel, *Killing of Caenorhabditis elegans by Pseudomonas aeruginosa used to model mammalian bacterial pathogenesis.* Proc Natl Acad Sci U S A, 1999. **96**(2): p. 715-20.

14. Tan, M.W., et al., *Pseudomonas aeruginosa killing of Caenorhabditis elegans used to identify P. aeruginosa virulence factors.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 2408-13.

15. Shapira, M., et al., *A conserved role for a GATA transcription factor in regulating epithelial innate immune responses.* Proc Natl Acad Sci U S A, 2006. **103**(38): p. 14086-91.

16. Troemel, E.R., et al., *p38 MAPK regulates expression of immune response genes and contributes to longevity in C. elegans.* PLoS Genet, 2006. **2**(11): p. e183.

17. Lau, G.W., D.J. Hassett, and B.E. Britigan, *Modulation of lung epithelial functions by Pseudomonas aeruginosa.* Trends Microbiol, 2005. **13**(8): p. 389-97.

18. Gallagher, L.A. and C. Manoil, *Pseudomonas aeruginosa PAO1 kills Caenorhabditis elegans by cyanide poisoning.* J Bacteriol, 2001. **183**(21): p. 6207-14.

19. Tusher, V.G., R. Tibshirani, and G. Chu, *Significance analysis of microarrays applied to the ionizing radiation response.* Proc Natl Acad Sci U S A, 2001. **98**(9): p. 5116-21.

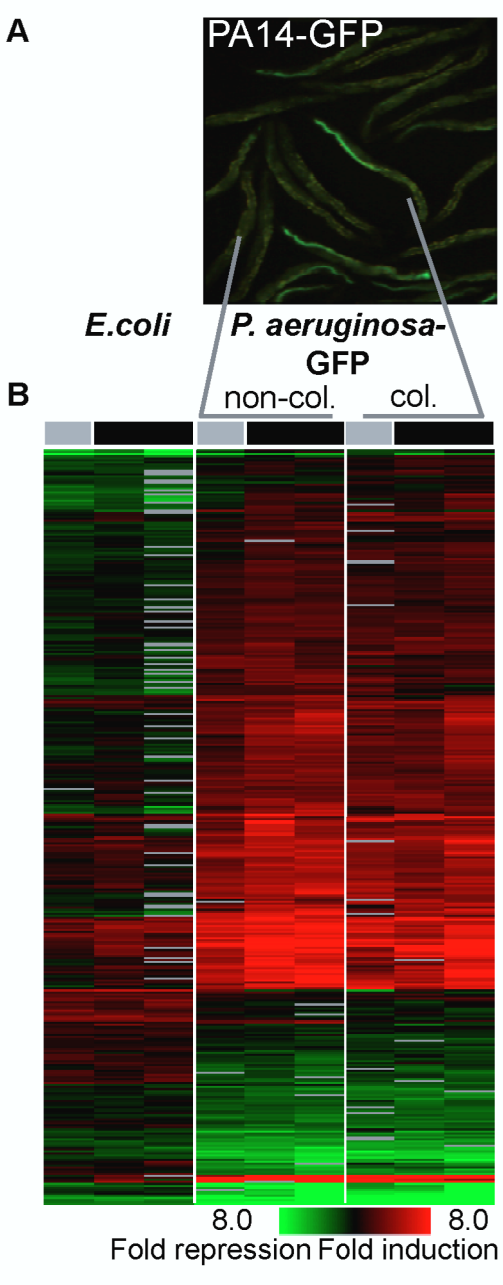

***Figure 1. C. elegans* immune responses to *Pseudomonas aeruginosa* are independent of colonization.** A) 2-day old adult *C.elegans* exposed to GFP-expressing *P. aeruginosa* for 18 hrs show variability in colonization, allowing isolation of colonized and non-colonized worms. B) Gene expression profiles of *C. elegans* fed with *E. coli* or with GFP-expressing *P. aeruginosa* (colonized and non-colonized) for 18 hours; separation was achieved either by picking under a fluorescent stereoscope (1 experiment; grey bar), or using the COPAS™ Worm Sorter (two independent experiments ; black bar). Shown are genes responding to the pathogen, as identified with a multi-class t-test analysis (10% false discovery rate).

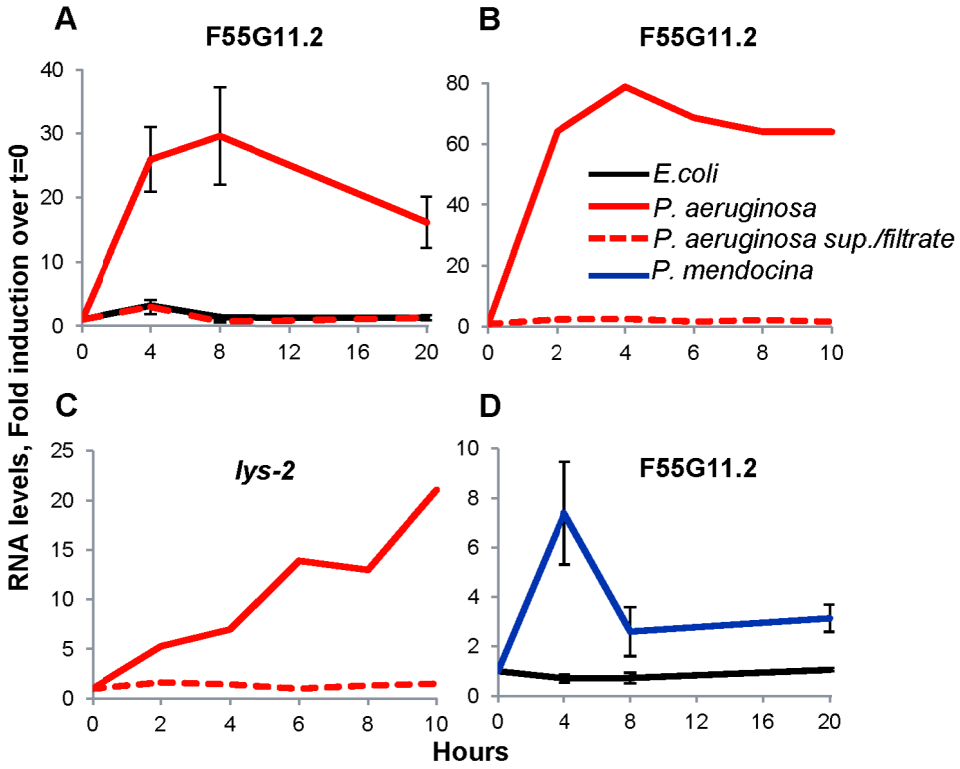

***Figure 2. Pseudomonas* secreted factors are not sufficient to induce immune responses, while conserved cell-associated factors are.** Gene expression measured by qRT-PCR, following exposure of young-adult *C. elegans* (at T_0) to *E. coli*, alone or with *P. aeruginosa* supernatant (A) or *P. aeruginosa* filtrate (B,C), or to the non-pathogenic *P. mendocina* (D)

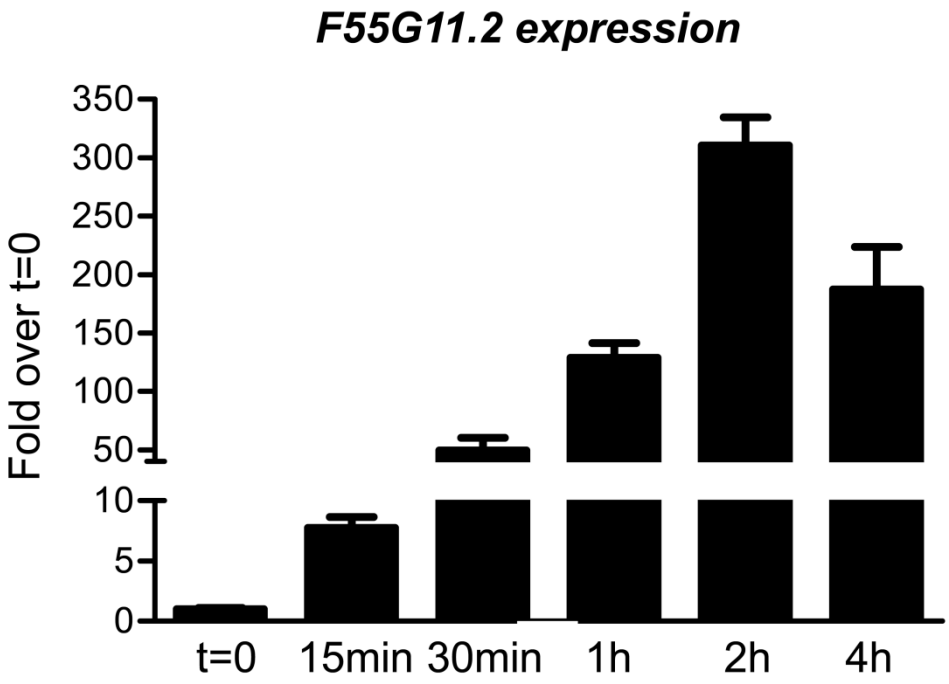

Figure 3. A representative of the immune response to *P. aeruginosa* can be induced within minutes of pathogen exposure. Gene expression measured by qRT-PCR, following exposure of young-adult *C. elegans* to *P. aeruginosa* for the indicated times

Appendix: Supplemental Information

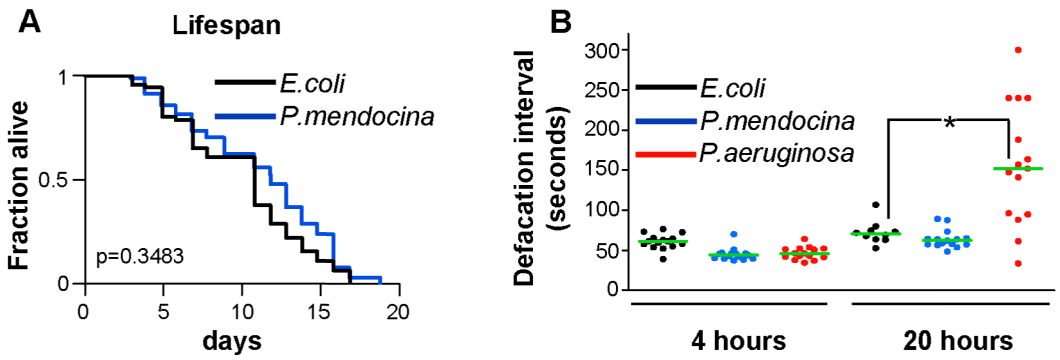

Figure S1. *Psuedomonas mendocina* is a non-pathogenic species. (A) Lifespan analysis of worms grown on *P. mendocina* shows comparable lifespan to that of worms grown on the normal food bacteria *E. coli* (N=90-93 worms for each group). Differences between curves were evaluated statistically using Kaplan Meier survival analysis followed by the Logrank test (p=0.3483). (B) Muscle function decline, represented by the rate of defecation, a coordinated muscle program, becomes apparent following 20 hours of exposure to the pathogen *P. aeruginosa*, but not to *E. coli* or *P. mendocina* (at 25^{o}C). Dots represent average interval between defecations (n=10 cycles, or less, when intervals exceeded four minutes) in individual young-adults; green bars represent medians. *p=0.002 (t-test). The general speed of worm movement also decreased in *P. aeruginosa* but not in *P. mendocina* (not shown).

Chapter 3

A candidate *C. elegans* pathogen recognition gene

Introduction

The *C.elegans* transcriptional response to pathogens comprises some elements that are shared between pathogens, but also includes elements that are highly specific to a given pathogen [1]. In some cases such as with the *Staphylococcus aureus* [2] and *Candida albicans* [3] a significant portion of the transcriptional response to the live pathogen can be recapitulated with a heat-killed one. These data provide evidence that there is a component other than damage, presumably the recognition of PAMPs which contributes to the immune response. However the questions of what these PAMPs are and the identity of the Pattern Recognition Receptors (PRRs) that sense them remain unanswered.

In the case of bacterial pathogens, peptidoglycan (PG) is an excellent candidate to be a PAMP sensed by *C.elegans*. PG is an essential component of the bacterial envelope, made of alternating units of N-acetylglucosamine and N-acetylmuramic acid cross-linked by short peptide chains. The structure of the peptide chain differs between Gram-negative and Gram-positive bacteria providing one level of specificity for detecting bacteria. In the case of Gram-positive bacteria, PG is displayed cell surface which would make it readily accessible to a theoretical *C.elegans* PRR. In Gram-negative bacteria, PG is buried beneath the outer membrane, however free PG is released as a normal part of growth and could be detected by worm surface receptors. Additionally a variety of mechanisms exist to deliver PG into host cells. These include bacterial Type IV secretion [4], outer membrane vesicles [5] host peptide transporters [6], and endocytosis [7]. Therefore PG could be a ligand for extracellular and/or intracellular receptors. Finally, in invertebrates, where innate immunity has been studies extensively such as in insects, the detection of PG appears to be the dominant mode of pathogen recognition [8, 9].

In *Drosophila*, PG is detected by a family of proteins called Peptidoglycan Recognition Proteins (PGRPs). The *Drosophila* genome contains 13 such genes which can be spliced to generate 19 different proteins [8]. Unlike mammalian peptidoglycan recognition proteins (PGLYRPs) which are directly bactericidal effectors [10], many of the insect PGRPs are signaling proteins. In *Drosophila* the interaction of PGRPs with Gram-positive or negative PG triggers the Toll or IMD respectively proteolytic signaling cascades, both of which activate NF-kB transcription factors [11, 12]. Another protein family involved in PG binding are the LysM proteins, named for the Lysin Motif protein domain. The structure of the LysM domain has been solved in bacterial species which revealed two alpha helices packed onto the same side of a two-strand anti-parallel beta sheet [13, 14] The LysM domain was first discovered in a phage [15], but has subsequently been shown to be present in bacteria, fungi, animals and plants [16]. Depending on the specific protein, LysM domains can bind PG or chitin-like molecules which unlike PG are polymers of only N-acetylglucosamine . In microorganisms, these proteins often contain an enzymatic activity that modifies the microbial envelope and in some cases serve as virulence factors by generating PG fragments that are inflammatory modulators [17]. LysM proteins of multicellular organisms are most studied in plants. Interestingly plant LysM proteins are involved in the perception of both

mutualistic microbes such as in the case of rhizobial NOD factors [18, 19] as well as in the perception of pathogenic microorganisms. These LysM domains are found in proteins with kinase domains or interact with kinases to elicit appropriate responses.

We have found five LysM-encoding genes in *C.elegans*. Microarray analysis indicated that one of them was down-regulated by *P. aeruginosa*. Intriguingly, this family member is required for full protection from *P. aeruginosa* as well s the early immune response to *P. aeruginosa*. Given the known functions of LysM proteins, this gene is a promising candidate to play a role in pathogen recognition in the worm.

Results

B0041.3 – a LysM domain-containing gene is regulated by *P. aeruginosa*.

In examining the known and predicted functions of genes significantly regulated by *P. aeruginosa* in our colonized vs non-colonized microarrays (Chapter 2) one of the genes that stood out was B0041.3. B0041.3 is a gene with uncharacterized function in *C.elegans* but is interesting because it encodes a LysM domain which has been shown to be involved recognition of microbial envelope components in plants [18]. Examination of B0041.3 expression from the microarray revealed that it was repressed by 18hr exposure to *P. aeruginosa* in both colonized and non-colonized conditions (Fig 1A). This was initially surprising if one considered B0041.3 as a candidate to be involved in defense against the pathogen. Nevertheless, this type of regulation is not unheard of, especially in light of the fact that the time point for the microarray data was approximately 18 hours, and *P. aeruginosa* is known to employ immunosuppressive mechanisms in other animals [20-22]. We therefore assessed B0041.3 expression at an earlier time point (4 hours) following *P. aeruginosa* exposure when less suppressive activities would be expected. Interestingly, at 4 hours, B0041.3 was marginally induced ~1.5 fold by *P. aeruginosa* (Fig 1B). In summary, B0041.3, a LysM containing gene is regulated by *P. aeruginosa* exposure.

Several LysM domain-containing genes in *C.elegans* contribute to infection resistance.

The suppression of B0041.3 by *P. aeruginosa* led us to search for all *C.elegans* LysM domain-containing genes and to examine whether they were important for the ability to resist infection. A search for genes in *C.elegans* encoding a LysM domain protein identified a total of five genes, all of which are uncharacterized. We used RNAi to individually knock-down expression of each of the five LysM genes and then assessed the susceptibility of animals to *P. aeruginosa* infection. Knock-down of all five genes caused a modest increase in infection susceptibility (Fig. 2A-E), and in the case of B0041.3 RNAi, this effect was statistically significant (Fig. 2A). These effects were not dependent on reproduction, since experiments were performed in infertile worms. We next looked at one of the earlier measures of the ability to resist infection which is the rate of bacterial colonization. In this case, RNAi of all five LysM caused a substantial increase in colonization by *P. aeruginosa* following 18 hours of exposure (Fig. 3). In the case of B0041.3, we tested whether this effect was specific to the pathogen by following colonization of B0041.3(RNAi) animals fed the normal *E. coli* food source. Unlike *P.*

aeruginosa, B0041.3 RNAi did not increase colonization by *E. coli* (Fig. S1). Together, these results suggested that at least B0041.3 provides specific protection against *P. aeruginosa*.

Knock-down of LySM domain-containing genes does not compromise general health.

We considered the possibility that knock-down of LysM genes may adversely affect some general aspect of *C.elegans* health, indirectly leading to increased pathogen sensitivity. To address this possibility, we examined the lifespan of LysM(RNAi) animals on dead *E. coli.* As shown in (Fig. 4), knock-down of LysM genes, did not shorten lifespan, and in most cases, actually extended lifespan. This result indicates that LysM RNAi does not compromise general health, and that its effects are infection-specific.

B0041.3 contributes to the induction of the early immune response to *P. aeruginosa.*

Given that several of the LysM genes in *C.elegans* played a role in the ability to resist colonization by and/or survive infection with *P. aeruginosa*, we asked if they were important for the induction of the early immune response. We knocked-down expression of all five LysM genes, exposed animals to *P. aeruginosa*, and then assessed their ability to induce expression of F55G11.2. RNAi of B0041.3 impaired the induction of F55G11.2, an early immune response gene, while RNAi of the other LysM genes did not (Fig. 5). Therefore, B0041.3 contributes to both protection from infection, as well as induction of the early immune response. We next asked whether the participation of B0041.3 in the early immune response was specific to *P. aeruginosa* or extended to other bacteria as well. Since B0041.3 is predicted to bind peptidoglycan, we assessed its contribution toward the immune response of a Gram-negative pathogen, *Enterococcus faecalis* which differs in the structure of its peptidoglycan. Interestingly, unlike *P. aeruginosa*, B0041.3 was not required for induction of a marker of the response to *E. faecalis*. This result indicates that B0041.3 may participate in discriminating between Gram-positive and Gram-negative pathogens.

Expression pattern of two LysM genes

In order to better understand where LysM genes might contribute to pathogen resistance we generated GFP reporters for the two of the LysM genes – B0041.3 and T01C4.1. B0041.3 was expressed in the pharynx, vulva, rectal epithelial cells and the ventral nerve cord (Fig. 1A). T01C4.1 was expressed exclusively in the pharynx. The significance of B0041.3 expression in the ventral nerve cord motor neuron is not known, however one thing that unifies the other three areas of expression is that they are at 'openings' of the worm body to the environment. For a protein that might function as a pattern recognition receptor, such a positioning would be advantageous, as it places the protein at the first points of entry for a bacterial pathogen.

Discussion

C.elegans first came into use as a model organism to study innate immunity in the late nineties. In subsequent years a great deal of knowledge has been garnered on the

interaction of *C.elegans* with bacterial, fungal, and viral pathogens. Many studies identified key signaling pathways in the worm that were important for innate immunity, as well as virulence factors in the corresponding pathogens. Several of these defense mechanisms have been conserved throughout evolution, including in humans [23]. However one piece of information that has remained conspicuously absent in the worm is how immune responses are initiated. Although there are suggestions (such as in chapter 2 of this dissertation) that *C.elegans* can specifically recognize pathogens, detailed molecular evidence is lacking. Additionally, unlike their insect and vertebrate counterparts, *C.elegans* is devoid of almost all of the well-studied pattern recognition receptors and the key proteins that they signal through. These observations have led some to suggest that initiation of immune responses in the worm stem from the detection of DAMPs (damage associated molecular patterns) or disruptions in host physiology.

Recently, three papers have been published that lend support to this theory. Both McEwan et al and Dunbar et al have shown that inhibition of translation mediated by the *P. aeruginosa* virulence factor Exotoxin A is sufficient to induce a marker of the *C.elegans* immune response named *irg-1.* [24, 25]. Furthermore, they show that this induction can be mimicked by pharmacological translation inhibition, indicating that the response is a property of a perturbation in host physiology rather than a direct response to the pathogen. It is worth noting that in contrast to our marker of the early immune response (F55G11.2), *irg-1* is induced independently of the conserved p38 MAPK pathway, and is not induced by virulence-attenuated *Pseudomonas* [26]. It is reasonable to speculate then that *irg-1* and F55G11.2 report on different processes – i.e. recognition vs. damage. Similarly Melo and Ruvkun have shown that disruption of core host processes such as transcription, translation and respiration through RNAi or pharmacologically can induce the transcriptional responses normally seen to pathogens [27]. This work provides strong evidence for a damage-mediated component of the immune response; however it is important to consider the time scale of events. In the studies above, immune responses were frequently assessed 24 hours following treatment, and in some cases, after 2 days of RNAi. Therefore, these studies focus on the late immune response, which one would presume to be mediated at least in part by damage, but do not address aspects of the early immune response which we have shown can occur within 15 minutes of pathogen exposure. Given the rapidity with which the immune response to *P. aeruginosa* can be induced, and the fact that the immune response can be induced by a related non-pathogenic microbe, our goal was to identify regulators of the early immune response.

From our microarray data, we identified an uncharacterized gene named B0041.3 which is predicted to encode a peptidoglycan binding protein. B0041.3 was slightly induced by *P. aeruginosa* early in the course of infection, but was suppressed later. Therefore, it is possible that B0041.3 is initially induced as a protective measure, but is also a target for immune suppression by *P. aeruginosa*. A subsequent search identified four additional *C.elegans* genes encoding a LysM domain(s).

Knock-down of all five LysM-encoding genes resulted in a moderate decrease in survival following infection with *P. aeruginosa*, with the effect being significant only in the case of B0041.3 knock-down. It is possible that some of the LySM genes function redundantly, and simultaneous knock-downs will be required to resolve this issue. However, all five of the LysM genes were required for protection from colonization by *P. aeruginosa*, as well as another Gram-negative pathogen *Salmonella* (data not shown) The pathogen sensitivity resulting from LysM RNAi appears to be a bona fide immune defect, as lifespan is not shortened in LysM RNAi animals. The fact that in many cases lifespan was actually extended by LysM RNAi may suggest that reducing expression of immune genes in the absence of infection is beneficial. Interestingly, studies in mammalian systems have shown that in aged animals, the expression and secretion of a number of 'pro-inflammatory' molecules with roles in the immune response can actually contribute to age-related pathology [28, 29]

Of the five LysM genes, only B0041.3 was required for induction of our marker of the early immune response, F55G11.2. It remains possible that the other LysM genes contribute to induction of different immune genes, possibly in response to other pathogens and/or as suggested above the LysM genes may function redundantly. It is also possible that some of the LysM genes, particularly T01C4.1 and F07G11.9 which are predicted to be chitinases are not involved in immunity per se, but rather play roles in the deposition of chitin which is known to be present in the worm eggshell and pharynx [30]. We therefore focused on B0041.3 since it had the strongest effects both in terms of resisting infection, and on induction of F55G11.2. Given that B0041.3 is predicted to have peptidoglycan binding activity, we wondered whether it was equally required for the immune response to Gram-negative and Gram-positive pathogens, as several peptidoglycan binding proteins in other animals bind only to gram-positive or gram negative peptidoglycan or strongly to one type and weakly to the other. Interestingly, while B0041.3 was required for the immune response to *P. aeruginosa*, which contains DAP-type peptidoglycan, it was not required to induce marker of the response to *E. faecalis* which contains Lys-type peptidoglycan. In fact, the response of B0041.3(RNAi) animals to *E. faecalis* was higher than controls. This phenomenon of transcriptional responses to different classes of microbial pathogens being reciprocally regulated has been observed by others [2, 3, 31]. Together, our data provides encouraging support for the possibility that B0041.3 is a regulator of the early immune response to *P. aeruginosa*, and suggests that this regulation may occur through the recognition of specific forms of peptidoglycan.

How might B0041.3 contribute to the immune response? B0041.3 encodes a single LysM domain, is not predicted to have any transmembrane domains, and is not predicted to have a signal sequence. Presumably then, B0041.3 is an intracellular cytoplasmic protein. B0041.3 does not encode any enzymatic activity, and unlike some plant LysM proteins, is not coupled to signaling kinase domains. Therefore, B0041.3 may bind ligands, possibly peptidoglycan that have been delivered into the cell, and then interact with other signaling proteins to regulate the immune response. This type of mechanism would be analogous to that of the mammalian NOD proteins. NOD proteins are intracellular proteins that are presumed to bind peptidoglycan fragments

(although direct binding between peptidoglycan and NODs has not been shown [32]) and then subsequently oligomerize and recruit signaling proteins, ultimately resulting in NF-κB activation [33].

Future studies should be biochemically oriented, and directed at demonstrating a physical interaction between B0041.3 and peptidoglycan, as well as co-immunoprecipitation to identify B0041.3 interacting proteins. The B0041.3 translational GFP fusion we have generated provides a tool to pursue both of these avenues. In conjunction with our current data, if peptidoglycan-binding capability can be demonstrated, B0041.3 would be the first pattern recognition receptor identified in *C.elegans*.

Experimental Procedures

Strains. Worm strains used were the N2 wild-type strain and strain BA837, *spe-26(it112)*. Bacterial strains included *E. coli* OP50-1, the clinical isolate *Pseudomonas aeruginosa* strain PA14, or a PA14 derivative expressing GFP off a stable plasmid. *Pseudomonas mendocina*, a non-pathogenic environmental *Pseudomonad* was isolated from worms grown on soil (Montalvo-Katz, *Infection & Immunity*, in press), and *Enterococcus faecalis* strain V583.

RNAi by feeding for LysM genes was performed for at least 4 days (egg stage to D2 adult), and in some cases for two generations using clones from the Ahringer RNAi library.

RNA extraction and qRT-PCR. RNA was extracted from ~100 worms per group/time-point using Trizol (Invitrogen). For (q)RT-PCR measurements, gene-specific threshold cycle (Ct) values were normalized to the respective actin values, and presented as fold change over the time = 0 point.

PCR Primers

pan-actin forward	TCGGTATGGGACAGAAGGAC
pan-actin reverse	CATCCCAGTTGGTGACGATA
F55G11.2 forward	TGGTTCTCCAGACGTGTTCA
F55G11.2 reverse	CAGCCTTGCCTTTACTGACA
abf-2 forward	GTGCCAGAATGGATGTTCCT
abf-2 reverse	CCGGTAACACACACAAGTCG
B0041.3 forward	CCATGAATGATGACGATCCA
B0041.3 reverse	CTTTGGGTTGTGCCGTATCT
B0041.3(A)	ATTTTTTGGTATTTTTCCAAA
B0041.3(A*)	CAAGTTTGCACCCCCTGAAATG

B0041.3(B-transcriptional)
AGTCGACCTGCAGGCATGCAAGCTCATTCATGGTTTAACGTCGTTC
B0041.3(B-translation)
AGTCGACCTGCAGGCATGCAAGCTAATATGATCATAAGCTGAGCTGCT
T01C4.1(A) TTACATTTTTTGAAAGTTTTAG

T01C4.1(A*) TTGTGGTTTCAAAATAAATTTT
T01C4.1(B-promoter)
AGTCGACCTGCAGGCATGCAAGCTGGAACCTAAATTATTTGAAAGT
T01C4.1(B-translation)
AGTCGACCTGCAGGCATGCAAGCTGCCATTTGTAGTTCTGTAATTAAC

Survival assays were performed at 25^{o}C in triplicate with approximately 100 animals per group, per experiment. Animals were transferred to experimental plates immediately following RNAi. Lifespan assays were performed on NGM plates with kanamycin-killed *E. coli* as the food source. *P. aeruginosa* infection experiments were performed using the slow killing protocol as described elsewhere.

Generation of Transgenic worms. Transgenic worms were generated using the fusion PCR method described elsewhere. Briefly, for generation of the B0041.3 translational reporter, the 624 bp region immediately upstream of the B0041.3 start codon along with the full B0041.3 gene up until the stop codon was cloned from genomic DNA. This PCR fragment was fused in frame to a GFP PCR fragment derived from the plasmid pPD95.75 which contains the full length *gfp* gene as well as the *unc-54* 3' UTR. B0041.3 and T01C4.1 transcriptional reporters were generated in a similar manner by fusing the 624 bp and 1162 bp respectively regions immediately upstream of the start codon to the aforementioned GFP PCR product. All transgenes were injected into wild type worms at a concentration of 75ng/µl together with the dominant *rol-6* co-injection marker at 30ng/µl, and the empty vector, Bluescript at a concentration of 45ng/µl.

References

1. Wong, D., et al., *Genome-wide investigation reveals pathogen-specific and shared signatures in the response of Caenorhabditis elegans to infection.* Genome Biol, 2007. **8**(9): p. R194.

2. Irazoqui, J.E., et al., *Distinct pathogenesis and host responses during infection of C. elegans by P. aeruginosa and S. aureus.* PLoS Pathog, 2010 **6**: p. e1000982.

3. Pukkila-Worley, R., F.M. Ausubel, and E. Mylonakis, *Candida albicans infection of Caenorhabditis elegans induces antifungal immune defenses.* PLoS Pathog, 2011. **7**(6): p. e1002074.

4. Viala, J., et al., *Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island.* Nat Immunol, 2004. **5**(11): p. 1166-74.

5. Kaparakis, M., et al., *Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells.* Cell Microbiol. **12**(3): p. 372-85.

6. Vavricka, S.R., et al., *hPepT1 transports muramyl dipeptide, activating NF-kappaB and stimulating IL-8 secretion in human colonic Caco2/bbe cells.* Gastroenterology, 2004. **127**(5): p. 1401-9.

7. Marina-Garcia, N., et al., *Clathrin- and dynamin-dependent endocytic pathway regulates muramyl dipeptide internalization and NOD2 activation.* J Immunol, 2009. **182**(7): p. 4321-7.

8. Royet, J. and R. Dziarski, *Peptidoglycan recognition proteins: pleiotropic sensors and effectors of antimicrobial defences.* Nat Rev Microbiol, 2007. **5**(4): p. 264-77.

9. Charroux, B., et al., *Bacterial detection by Drosophila peptidoglycan recognition proteins.* Microbes Infect, 2009. **11**(6-7): p. 631-6.

10. Kashyap, D.R., et al., *Peptidoglycan recognition proteins kill bacteria by activating protein-sensing two-component systems.* Nat Med, 2011. **17**(6): p. 676-83.

11. Kurata, S., S. Ariki, and S. Kawabata, *Recognition of pathogens and activation of immune responses in Drosophila and horseshoe crab innate immunity.* Immunobiology, 2006. **211**(4): p. 237-49.

12. Royet, J., *Infectious non-self recognition in invertebrates: lessons from Drosophila and other insect models.* Mol Immunol, 2004. **41**(11): p. 1063-75.

13. Bateman, A. and M. Bycroft, *The structure of a LysM domain from E. coli membrane-bound lytic murein transglycosylase D (MltD).* J Mol Biol, 2000. **299**(4): p. 1113-9.

14. Bielnicki, J., et al., *B. subtilis ykuD protein at 2.0 A resolution: insights into the structure and function of a novel, ubiquitous family of bacterial enzymes.* Proteins, 2006. **62**(1): p. 144-51.

15. Garvey, K.J., M.S. Saedi, and J. Ito, *Nucleotide sequence of Bacillus phage phi 29 genes 14 and 15: homology of gene 15 with other phage lysozymes.* Nucleic Acids Res, 1986. **14**(24): p. 10001-8.

16. Buist, G., et al., *LysM, a widely distributed protein motif for binding to (peptido)glycans.* Mol Microbiol, 2008. **68**(4): p. 838-47.

17. Lenz, L.L., et al., *SecA2-dependent secretion of autolytic enzymes promotes Listeria monocytogenes pathogenesis.* Proc Natl Acad Sci U S A, 2003. **100**(21): p. 12432-7.

18. Antolin-Llovera, M., et al., *Receptor kinase signaling pathways in plant-microbe interactions.* Annu Rev Phytopathol, 2012. **50**: p. 451-73.

19. Willmann, R., et al., *Arabidopsis lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection.* Proc Natl Acad Sci U S A, 2011. **108**(49): p. 19824-9.

20. Diaz, M.H., et al., *Pseudomonas aeruginosa induces localized immunosuppression during pneumonia.* Infect Immun, 2008. **76**(10): p. 4414-21.

21. Hooi, D.S., et al., *Differential immune modulatory activity of Pseudomonas aeruginosa quorum-sensing signal molecules.* Infect Immun, 2004. **72**(11): p. 6463-70.

22. Pritchard, D.I., *Immune modulation by Pseudomonas aeruginosa quorum-sensing signal molecules.* Int J Med Microbiol, 2006. **296**(2-3): p. 111-6.

23. Irazoqui, J.E., J.M. Urbach, and F.M. Ausubel, *Evolution of host innate defence: insights from Caenorhabditis elegans and primitive invertebrates.* Nat Rev Immunol, 2010. **10**(1): p. 47-58.

24. Dunbar, T.L., et al., *C. elegans detects pathogen-induced translational inhibition to activate immune signaling.* Cell Host Microbe, 2012. **11**(4): p. 375-86.

25. McEwan, D.L., N.V. Kirienko, and F.M. Ausubel, *Host translational inhibition by Pseudomonas aeruginosa Exotoxin A Triggers an immune response in Caenorhabditis elegans.* Cell Host Microbe, 2012. **11**(4): p. 364-74.

26. Estes, K.A., et al., *bZIP transcription factor zip-2 mediates an early response to Pseudomonas aeruginosa infection in Caenorhabditis elegans.* Proc Natl Acad Sci U S A, 2010. **107**(5): p. 2153-8.

27. Melo, J.A. and G. Ruvkun, *Inactivation of conserved C. elegans genes engages pathogen- and xenobiotic-associated defenses.* Cell, 2012. **149**(2): p. 452-66.

28. Campisi, J., *Aging, Cellular Senescence, and Cancer.* Annu Rev Physiol, 2012.

29. Laberge, R.M., et al., *Glucocorticoids suppress selected components of the senescence-associated secretory phenotype.* Aging Cell, 2012. **11**(4): p. 569-78.

30. Zhang, Y., et al., *The chitin synthase genes chs-1 and chs-2 are essential for C. elegans development and responsible for chitin deposition in the eggshell and pharynx, respectively.* Dev Biol, 2005. **285**(2): p. 330-9.

31. Kim, Y. and E. Mylonakis, *Caenorhabditis elegans immune conditioning with the probiotic bacterium Lactobacillus acidophilus strain NCFM enhances gram-positive immune responses.* Infect Immun, 2012. **80**(7): p. 2500-8.

32. Tanabe, T., et al., *Regulatory regions and critical residues of NOD2 involved in muramyl dipeptide recognition.* Embo J, 2004. **23**(7): p. 1587-97.

33. Kanneganti, T.D., M. Lamkanfi, and G. Nunez, *Intracellular NOD-like receptors in host defense and disease.* Immunity, 2007. **27**(4): p. 549-59.

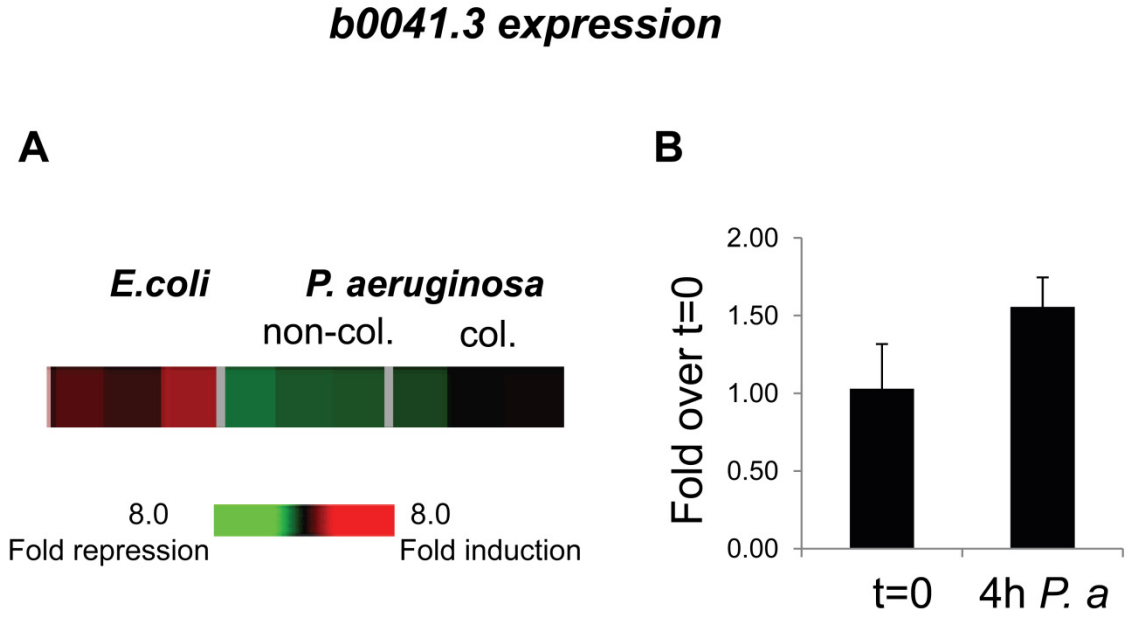

Figure 1. B0041.3, a LysM-domaing containing gene is regulated by exposure to *P. aeruginosa*. Gene expression measured by microarray following ~ 18hrs of exposure of young adult *C.elegans* to *P. aeruginosa* (A) or by qRT-PCR, following 4 hours of exposure to *P. aeruginosa* (B). Shown in (A) are values for three independent experiments, in (B) are average values for two independent experiments.

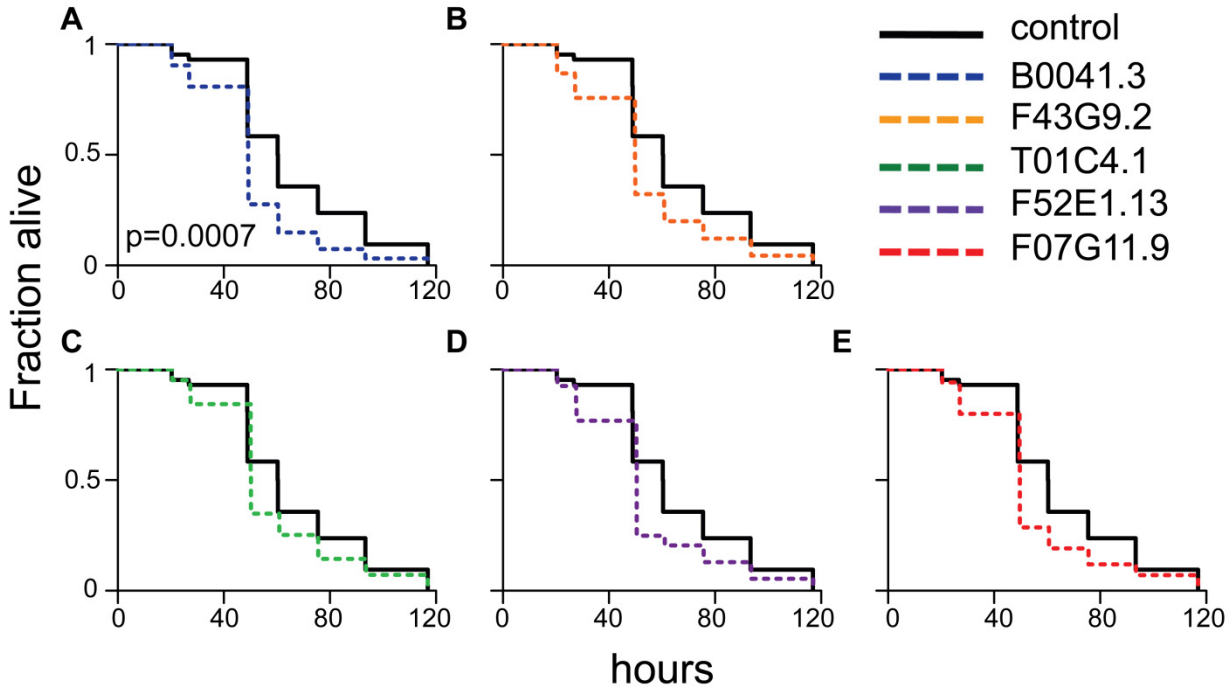

Figure 2. RNAi of LysM domain-containing genes causes moderate sensitivity to *P. aeruginosa* infection. Survival curves for spe-26(it112) mutants treated with RNAi from the egg-stage until day-two of adulthood (4 days total), then exposed to *P. aeruginosa*. (B-E) were not statistically significant.

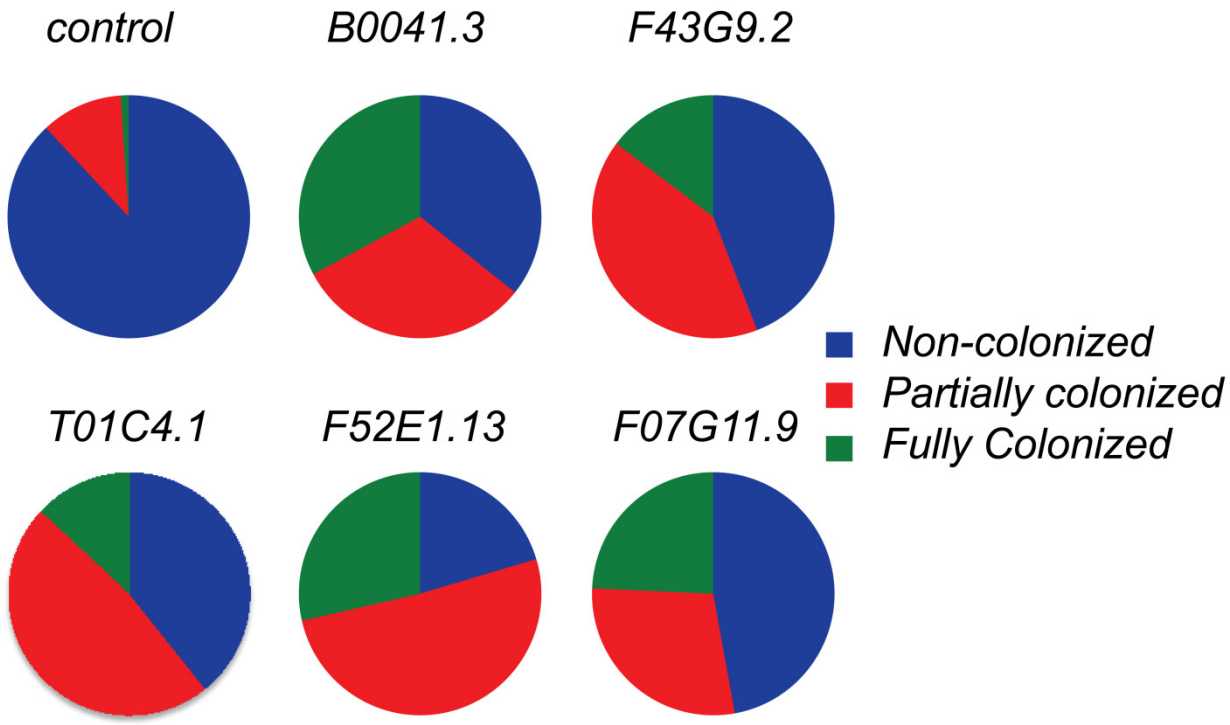

Figure 3. RNAi of LysM-domain containing genes increases the rate of colonization by *P. aeruginosa*. spe-26(it112) mutants were treated with RNAi from the egg-stage until day-two of adulthood (4 days total), then exposed to GFP-expressing *P. aeruginosa* for approximately 18 hours. Pie charts represent the percentage of animals in each designated group. N=34-92 animals total per RNAi condition.

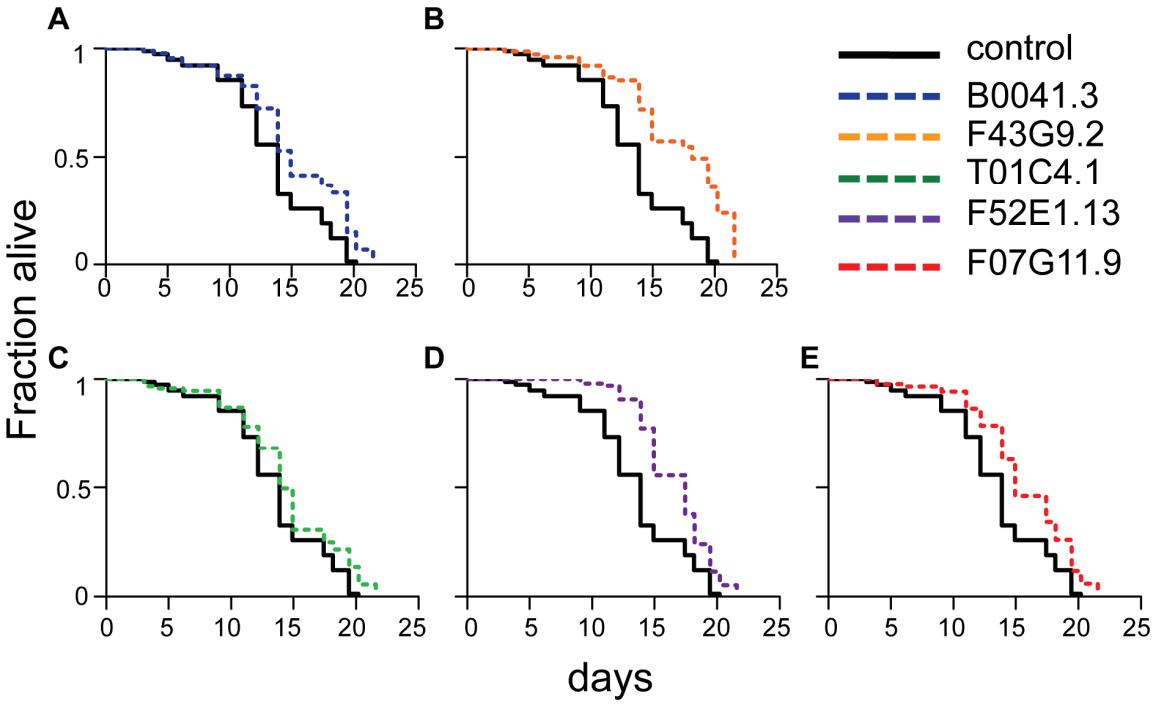

Figure 4. RNAi of LysM-domain containing genes does not shorten lifespan. Survival curves for spe-26(it112) mutants treated with RNAi from the egg-stage until day-two of adulthood (4 days total), then transferred to lifespan plates (with dead *E. coli* as a food source).

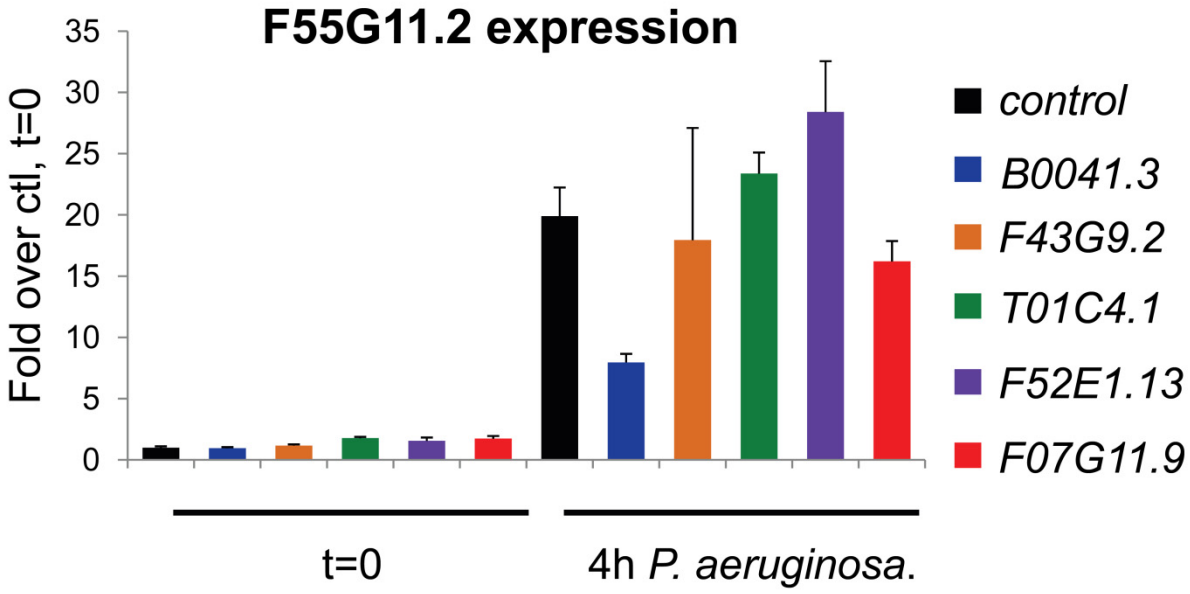

Figure 5. B0041.3 is required for the full induction of a representative of the response to *P. aeruginosa*. F55G11.2 expression measured by qRT-PCR, following exposure of young-adult LysM(RNAi) *C. elegans* to *Pseudomonas aeruginosa.*

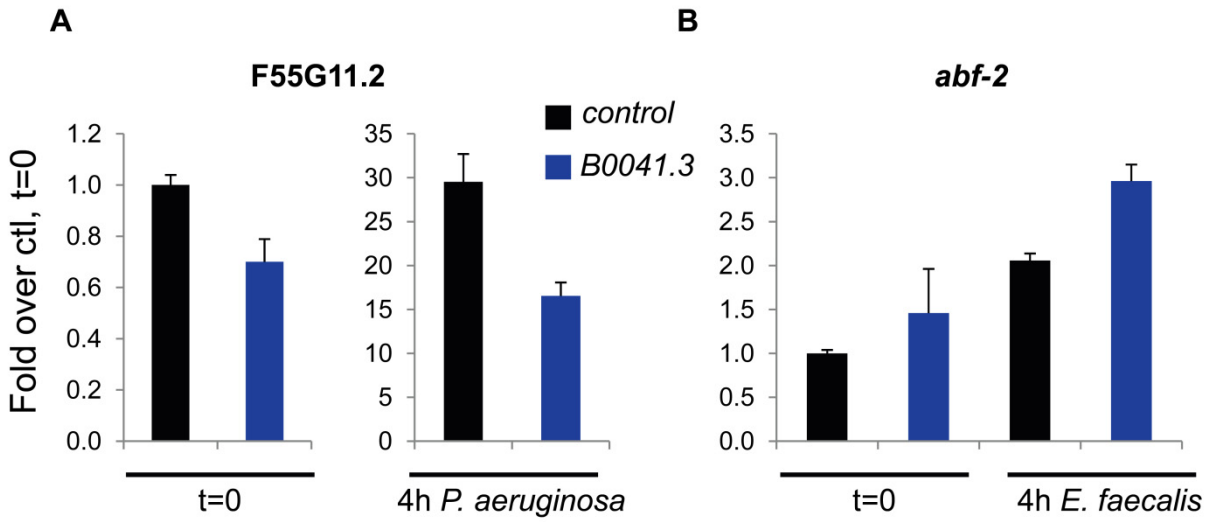

Figure 6. B0041.3 contributes to induction of the response to *P. aeruginosa*, but not *E. faecalis*. Gene expression measured by qRT-PCR, following exposure of young-adult *C. elegans* to *Pseudomonas aeruginosa* (A) or *Enterococcus faecalis* (B).

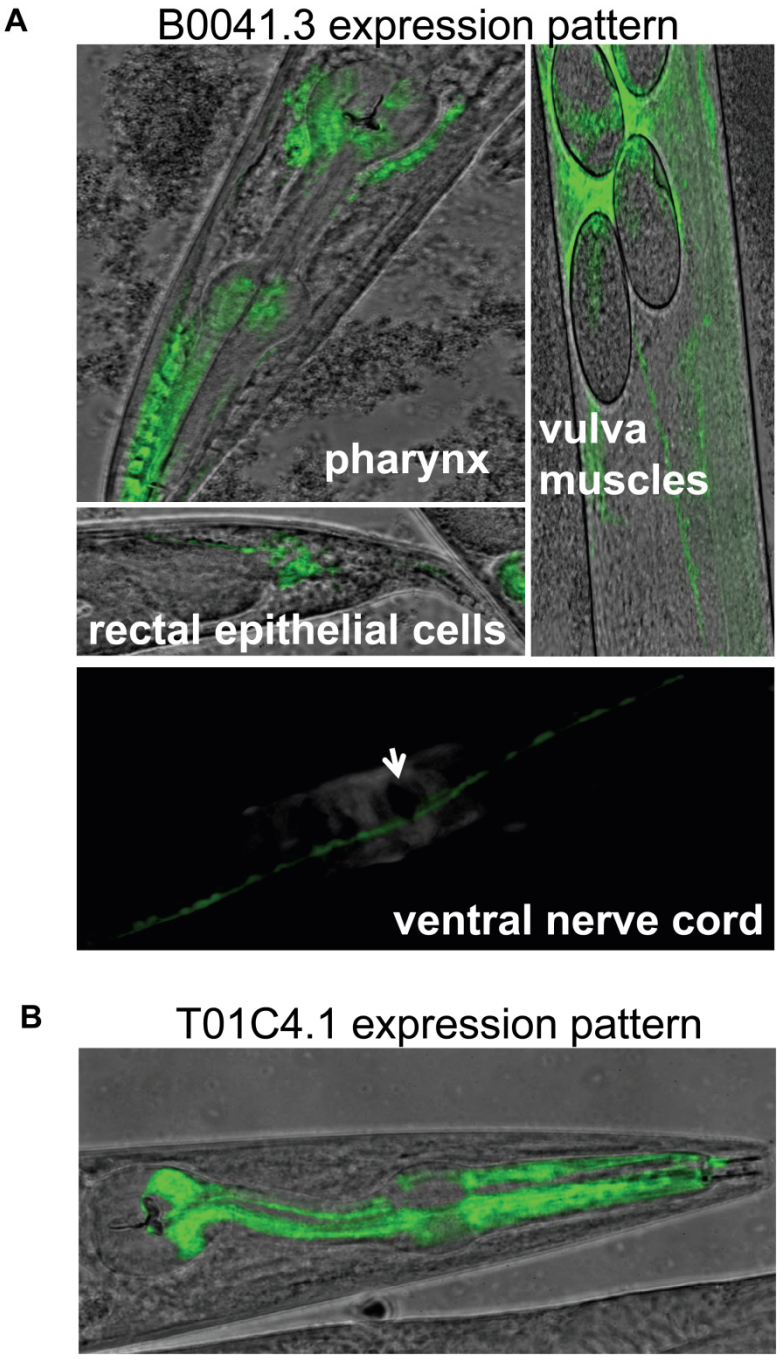

Figure 7. Expression pattern of two LysM-domain containing genes. Images show expression patters for gfp -fusions in young adults. (A) Translational fusion (pB0041.3::B0041.3::GFP), (B) transcriptional fusion pT01C4.1::GFP)

Appendix: Supplemental Figures

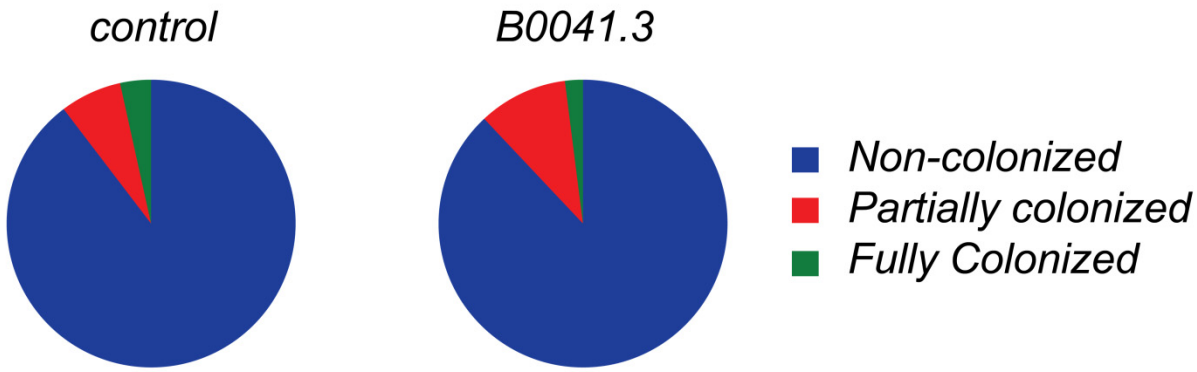

Figure S1. B0041.3 knock-down does not increase the rate of colonization by *E. coli*. wild-type animals were treated with RNAi for two generations, then exposed to GFP-expressing *E.coli* for approximately 20 hours. Pie charts represent the percentage of animals in each designated group. N=29 for control and N=60 for B0041.3 RNAi.

Chapter 4

An age-dependent reversal in the protective capacities of JNK signaling shortens *C. elegans* lifespan.

Results presented here were published as part of the paper:

Twumasi-Boateng K, Wang TW, Tsai L, Lee KH, Salehpour A, Bhat S, Tan MW, and Shapira M. An age-dependent reversal in the protective capacities of JNK signaling shortens *C. elegans* lifespan. Aging Cell 2012;11(4):659-667

An age-dependent reversal in the protective capacities of JNK signaling shortens *C. elegans* lifespan.

Kwame Twumasi-Boateng, Tim W. Wang, Linda Tsai, Kuang-Hui Lee, Ali Salehpour, Sudarshan Bhat Man-Wah Tan, and Michael Shapira

Abstract

Stress Activated Protein Kinase (SAPK) pathways are evolutionarily conserved signaling modules that orchestrate protective responses to adverse environmental conditions. However, under certain conditions, their activation can be deleterious. Thus, activation of the c-Jun N-terminal kinase (JNK) SAPK pathway exacerbates a diverse set of pathologies, many of which are typical of old age. The contexts determining whether the outcome of JNK signaling is protective or detrimental are not fully understood. Here, we show that the age of an animal defines such a context. The *Caenorhabditis elegans* JNK-homolog, KGB-1, provides protection from heavy metals and protein folding stress in developing animals. However, we found that with the onset of adulthood, KGB-1 activity becomes detrimental, reducing stress resistance and lifespan. Genetic analyses coupled with fluorescent imaging linked this phenotypic switch to age-dependent antagonistic modulation of DAF-16/FOXO: KGB-1 activation enhanced DAF-16 nuclear localization and transcriptional activity during development, but decreased it in adults. Epistasis analyses showed that DAF-16 was necessary and sufficient to explain some of the *kgb-1*-dependent detrimental phenotypes, but not all. The identification of early adulthood as a point following which the contribution of KGB-1 activity reverses from beneficial to detrimental sheds new light on the involvement of JNK signaling in age-related pathologies. Furthermore, the age-dependent reversal has intriguing implications for our understanding of aging.

Introduction

Dealing with adverse environmental perturbations is a fundamental necessity for all organisms. It is therefore not surprising that stress-activated protein kinase pathways are among the most ancient and conserved signaling modules in metazoa. The two archetypical pathways comprising this category are the p38 and the c-Jun N-terminal kinase (JNK). They are activated by a range of adverse environmental conditions, including protein folding stress, oxidative stress and infection, and function in a typical MAPK three-tier, scaffold-protein-aided hierarchy, in which a mitogen-activated protein (MAP) kinase kinase kinase phosphorylates and activates a MAP kinase kinase, which activates either the p38 or the JNK MAP kinases, which modify the activity of numerous and diverse targets through phosphorylation [1, 2].
JNK proteins were shown in mammals to respond to various stress conditions, as well as to inflammatory cytokines, and to modify proteins as diverse as ubiquitin ligases, microtubule-associated proteins and several transcription factors (reviewed in [3]). In their protective capacity JNK proteins contribute to microtubule stabilization, neurite growth, autophagy and induction of cytoprotective gene expression [4-7]. JNK signaling also plays pivotal roles in development, as demonstrated by embryonic lethality in mice

lacking both of the two ubiquitously-expressed isoforms JNK1 and JNK2 (in contrast to mice lacking the neuronal-specific JNK3, which are viable)[8]. In contrast to these beneficial contributions, JNK signaling has also been shown to have detrimental consequences. JNK activation can be pro-apoptotic and is associated with exacerbation of a diverse set of pathologies, many of which are typical of old age. These include Alzheimer's disease, ischemia/reperfusion-induced tissue damage in the heart and brain, and insulin resistance [9-12]. Accordingly, *jnk3* disruption protects mice from ischemia-associated damage and *jnk1* disruption improves insulin sensitivity in a mouse model of obesity [11, 13]. What determines the outcome of JNK activation is not fully known, but a recurrent theme is that the mode/context of activation plays an important role. It has been shown that interactions with other signaling pathways can modulate the activation, and/or contribution, of JNK signaling [14, 15]; others have suggested that the duration of activation determined the outcome of JNK signaling - short activation leading to protection, a prolonged one leading to cell death [16, 17]; and yet another hypothesis, referring to the whole organism, suggested that JNK activation may have different outcomes depending on tissue-specificity of this activation [12].

Invertebrate model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* are useful for understanding the effects and interactions of JNK proteins, in particular by allowing dissection of cytoprotective gene expression and analyzing tissue-specific contributions [18]. Work in these organisms also enabled studying the interactions between the JNK pathway and insulin/insulin-like-growth-factor (IGF) signaling (IIS), arguably the most significant mechanism of aging regulation. IIS plays a pivotal role in coordinating metabolic homeostasis and determining longevity in a variety of organisms [19]. Best characterized in *C. elegans,* the main contribution of IIS is in antagonizing nuclear localization of the DAF-16/FOXO transcription factor, which controls the expression of a robust stress-protective transcriptional program [20]. Thus, by restricting nuclear localization of DAF-16, IIS limits resistance to oxidative stress, infection, and ultimately reduces lifespan [21, 22]. JNK proteins were shown to phosphorylate several central and auxiliary components of the IIS pathway (reviewed in [18]. The outcome of these interactions is typically the promotion of FOXO nuclear localization and an increase in stress resistance and lifespan [23, 24]. However, similar to mammals, JNK activation may also have detrimental consequences in invertebrates. In drosophila, uncontrolled JNK activity impairs epithelial gut integrity [25], and causes improper differentiation in the aging intestine [26]. Also, flies heterozygous for disruption of a dual specificity phosphatase, which negatively regulates JNK signaling, show increased oxidative stress resistance and lifespan, but homozygotes are developmentally lethal [27, 28]. Similarly, loss of the orthologous *C. elegans* phosphatase, VHP-1, results in developmental lethality that is rescued by disruption of the cognate JNK homolog KGB-1 [29]. Thus, in invertebrates, as in vertebrates, JNK activation can have contrasting outcomes. However, as in mammals, what determines these outcomes is not fully understood.

Using *C. elegans*, we show that age defines a context determining the outcome of JNK activation. *C. elegans* has three JNK homologs, similar to mammals. The neuronal JNK-1 provides protection from oxidative and heat stress and interacts with the IIS pathway leading to nuclear localization of DAF-16 [23, 30]. Of the other two JNK homologs, KGB-2 is uncharacterized; and KGB-1, was shown to be necessary both for germline

proliferation and for protection from heavy metals and protein folding stress [29, 31, 32]. Both KGB-1 as well as the p38 homolog PMK-1, which provides protection from infection and oxidative stress [33, 34], are negatively regulated by the dual specificity phosphatase VHP-1, a ubiquitously-expressed MKP-7 homolog (Fig. 1A). Previous work showed that knocking down the expression of *vhp-1* during larval development increased phosphorylation of PMK-1, and downstream to it resistance of worms to infection with the bacterial pathogen *Pseudomonas aeruginosa* [35]. However, we found that knocking down *vhp-1* past development had the opposite effect - decreased resistance to infection, which depended on *kgb-1*. We show that the contribution of *kgb-1* to stress resistance reverses with age – from a protective role in dealing with heavy metals and protein folding stress in developing larvae, to being generally detrimental in adults, causing a decrease in resistance to heavy metals and protein folding stress, in addition to infection resistance, and shortening lifespan under normal conditions. The age-dependent switch in KGB-1's function was linked to age-dependent antagonistic modulation of DAF-16 - promoting DAF-16 activity during development, but attenuating it in adults. Our results demonstrate that age can be a context determining the outcome of JNK activation, and describe a molecular mechanism underlying this phenomenon.

Results

Age reverses the protective effect of *vhp-1* knock-down

Our original intention was to study the contribution of PMK-1 activation to infection resistance. By knocking down the expression of *vhp-1* during the two days of larval development (RNAi-dev, Fig. 1B), we increased the resistance of worms to infection, as previously reported (Fig. 1C)[35]. However, to our surprise, the same knock-down in young adult animals, only two-days older (RNAi-ad), caused the opposite effect, rendering worms more susceptible to infection (Fig. 1D). Thus, a two-day difference in the onset of RNAi treatment reversed the consequences of *vhp-1* knock-down from a median of 55% increase in infection resistance to a 34% decrease. This observation led us to focus our efforts on trying to understand the mechanism underlying such a switch.

The age-dependent reversal in the vhp-1 RNAi phenotype is independent of reproductive status or growth

Trade-offs between stress-resistance and growth, or reproduction, are well-documented [36]. Development of *vhp-1* mutants was previously reported to arrest at the L3 larval stage [29], and *vhp-1(RNAi-dev)* animals showed a high percentage of small animals that produced either no, or few progeny. While *vhp-1(RNAi-ad)* animals showed no gross defects in fertility, minor effects might still have existed. This raised the possibility that the reversal in the effect of vhp-1 RNAi might be an indirect consequence of age-dependent effects on growth and reproduction. To test this possibility, we examined wild-type animals rendered sterile prior to adult-stage *vhp-1* knock-down, by *cdc-25.1* knock-down. We found that these animals showed the same decreased infection resistance phenotype as fertile animals (Fig. 1D, orange lines). Furthermore, both increased infection resistance in *vhp-1(RNAi-dev)* animals and decreased resistance in *vhp-1(RNAi-ad)* animals were replicated in sterile *glp-4* and *spe-26* mutants, which lack gonads, or sperm, respectively (Fig. S1).

Effects on animal size were also not the cause of the vhp-1 RNAi phenotype reversal: animals exposed to vhp-1 RNAi throughout development, plus the first two days of adulthood (four days instead of two), showed stunted growth and reduced fecundity, similar to *vhp-1(RNAi-dev)* animals, but were less resistant to infection, similar to their age-matched *vhp-1(RNAi-ad)* animals (Fig. S2). Together, these experiments rule out involvement of reproductive status, or size, in the age-associated reversal in the *vhp-1* knock-down infection-resistance phenotype.

The age-dependent phenotype reversal represents a more general shift in the ability to resist environmental stress

In addition to infection resistance, we found that an age-dependent phenotype reversal also appeared in *vhp-1(RNAi)* animals challenged with heavy metals. Cadmium resistance changed from a 40% increase in *vhp-1(RNAi-dev)* animals (Fig. 1E) to a 50% decrease in *vhp-1(RNAi-ad)* animals (Fig. 1F), compared to age-matched control-treated animals. Because stress resistance is tightly linked to lifespan, we examined the effects of *vhp-1* knock-down on lifespan. The median lifespan of *vhp-1(RNAi-dev)* animals on dead *E. coli* was 84% of that of age-matched control-treated animals (Fig. 1G); more prominently, median lifespan of *vhp-1(RNAi-ad)* animals was 69% of controls (Fig. 1H). Thus, the long-term consequences of *vhp-1* knock-down are detrimental, even in the absence of exogenous stress.

***kgb-1* plays a central role in the age-dependent reversal in the effects of *vhp-1* knock-down**

Ruling out dependence on growth and reproduction led us to hypothesize that the reversal in the *vhp-1* knock-down phenotype might have reflected age-associated changes in signaling by the two stress activated protein kinases regulated by VHP-1. We therefore examined *pmk-1* and *kgb-1* mutants to identify which of the two genes was necessary for which of the age-dependent phenotypes. In agreement with previous reports, *pmk-1* disruption suppressed the increased infection resistance following development-stage *vhp-1* knock-down (Fig. 2A)[35]. On the other hand, increased cadmium resistance following the same RNAi treatment was suppressed instead by *kgb-1*-disruption (Fig. 2D). As for the detrimental effects of *vhp-1* knock-down in adults, all depended on *kgb-1* alone (Fig. 2B,C, E-G)(and in both fertile and sterile animals (Fig. 2B and C)). This was observed in animals carrying two different mutant *kgb-1* alleles, *km21* and *um3*, ruling out allele-specific effects (Fig. S3). On the other hand, mutants for the two other *C. elegans* JNK homologs, *jnk-1* and *kgb-2*, responded to *vhp-1* knock-down as wild-type animals, ruling out their involvement (not shown). Thus, a previously-unknown detrimental contribution of *kgb-1* in adult animals was identified that contrasts with its early-life protective role against cadmium toxicity. Similarly opposing contributions were also observed for the role of *kgb-1* in protection from protein folding stress caused by the N-glycosylation inhibitor tunicamycin (Fig. S4). Together, these results demonstrated that the reversal in the vhp-1 RNAi phenotype is *kgb-1*-dependent and further suggested a post-developmental switch in the contribution of *kgb-1*, from beneficial (for cadmium and tunicamycin protection) to generally detrimental.

***kgb-1* knock-down corroborates age-dependent reversal in *kgb-1*'s contribution to cadmium resistance**.

One observation that appears to conflict with the hypothesis that KGB-1's function switches to detrimental in adulthood is that lifespan of *kgb-1* mutants is often shorter than that of wildtype animals (Fig. 2G). However, the discrepancy could be resolved if it is assumed that *kgb-1* had an early-life beneficial contribution that was greater than its late-life detrimental one. Supporting a bimodal contribution we noticed that survival of *kgb-1* mutants on cadmium showed an initial fast phase of dying, followed by survival of the remaining animals (50% of the initial number) to a maximum lifespan that exceeded that of wildtype animals (Fig. 2E). To better resolve the effect of *kgb-1* disruption on stress resistance we examined *kgb-1(RNAi)* animals, expecting that residual *kgb-1* may be sufficient to provide essential functions, yet would allow testing age-dependent contributions. When exposed to cadmium as larvae, animals treated with kgb-1 RNAi (beginning *in utero*) showed reduced survival compared to controls, supporting an early-life beneficial contribution (Fig. 3A). In contrast, *kgb-1(RNAi)* animals exposed to cadmium at day two of adulthood showed increased survival, supporting a late-life detrimental contribution (Fig. 3B). Quantitative RT-PCR measurements showed comparable knock-down of *kgb-1* expression in both ages, with 'residual' levels amounting to 30-50% of control levels (Fig. 3 insets and Fig. S5). Together, these results supported the hypothesis of an age-dependent switch in *kgb-1*'s contribution to survival. Furthermore, by producing essentially the reverse image of the effects of *vhp-1* knock-down on cadmium resistance, the outcome of *kgb-1* knock-down demonstrated that the switch is a characteristic of the normal KGB-1 physiology and not merely a product of uncontrolled activation.

Loss of *kgb-1*'s larval-protective capacities in adults.

Our survival analyses followed the effects of the preceding age-dependent knock-down, which determined the state of animals at the beginning of the analysis. However, the longer the survival analysis, the more diluted the effects of the initial knock-down might be. To better dissect *kgb-1*'s age-dependent contributions we employed *kgb-1* mutants and knock-down animals in assays limited to defined life phases. We began by comparing the development of *kgb-1* and wildtype animals. Development of the two strains was indistinguishable under normal conditions. However, when exposed to cadmium at the egg stage, wild-type larvae showed delayed development; nevertheless, within three days around 40% reached adulthood (Fig. 3C). In contrast, the majority of *kgb-1* larvae exposed to cadmium died during development, with only 4% of total reaching adulthood. Thus, in larvae, *kgb-1* is essential for survival and development under the adverse conditions of cadmium exposure. Next, we evaluated the short-term contribution of *kgb-1* to stress resistance in larvae. This was performed in the context of an acute exposure to high cadmium concentration (5mM), and was compared to that of two-day old adults. Within ten hours of exposure 75% of *kgb-1* larvae were dead, compared to 45% among wild-type larvae, supporting a short-term protective role for KGB-1 in developing animals (Fig. 3D). In contrast, *kgb-1* disruption had no effect on cadmium resistance in adults, suggesting that in adults the contribution of *kgb-1* to cadmium resistance was negligible. Finally, further focusing on *kgb-1*'s contribution in adults, we found that knocking down its expression only after

development was completed resulted in lifespan extension (Fig. 3E). Thus, as the beneficial contribution of *kgb-1* to cadmium resistance wanes, it becomes a negative factor, compromising animal survival.

Upstream activation of KGB-1 does not change with age

The age-associated changes in the outcome of KGB-1 activation could have been due either to changes in events upstream or downstream to KGB-1 activation. Since VHP-1 modulates phosphorylation of both KGB-1 and PMK-1, we examined if changes occurred in the overall balance of this network. Basal RNA levels of *kgb-1*, *pmk-1*, and the *vhp-1*a isoform at day two of adulthood were higher than those in L4 larvae, while *vhp-1b* levels did not change with age (Fig. S6 and data not shown). Since the functional output of this network depends not simply on expression levels, but on levels of the phosphorylated active proteins, we analyzed phosphorylation levels for both PMK-1 and KGB-1. Immunoblotting showed that basal levels of phosphorylated proteins did not differ significantly between worms of the two examined ages for either PMK-1 or KGB-1 (Fig. 4A, B). *vhp-1* knock-down increased phosphorylation of both PMK-1 and KGB-1 irrespective of age, suggesting that both were similarly activated during development and adulthood (Fig. 4A, B). In addition, increases in PMK-1 phosphorylation were found to be associated with increased expression (also age-invariable) of two downstream targets of PMK-1, the infection-protective genes *lys-2* and F08G5.6 [37, 38], suggesting that PMK-1 retained its infection-protective contribution in adults (Fig. 4C). Nevertheless, the constitutive beneficial contribution of PMK-1 appears to be superseded in adults by the overriding detrimental effects of KGB-1 (Fig. 1D), and becomes apparent only once *kgb-1* is disrupted (Figures 2B, C and Fig. S3B).

The MAPK kinase MEK-1 is the main activator of KGB-1 and requires the scaffold protein SHC-1 for this activation. Both were previously shown to be essential for KGB-1's ability to provide protection from heavy metal stress during development [32]. Disruption of either *mek-1* or *shc-1* also suppressed the detrimental consequences of adult-stage *vhp-1* knockdown (Fig. 4D), demonstrating that the machinery required for activation of KGB-1 is equally important for its development-stage beneficial and adult-stage detrimental contributions. Thus, the switch in *kgb-1*'s contribution cannot be explained by changes in KGB-1 activation, and instead points to events downstream to this activation as those that change with age.

Age-dependent modulation of DAF-16 downstream to KGB-1 activation

Given *kgb-1's* contribution to lifespan determination and stress resistance, we wondered if KGB-1 interacted with the IIS pathway. Insulin signaling restricts lifespan and stress-resistance mainly by inhibiting nuclear localization of the conserved FOXO transcription factor DAF-16 [21]. We therefore examined the effect of *vhp-1* knock-down on DAF-16. Using a transgenic strain expressing a functional GFP-tagged DAF-16 [21], we found that *vhp-1* knock-down in developing animals significantly increased DAF-16::GFP nuclear localization in intestinal cells (independently of its effects on development itself, see Fig. S7), while its knock-down in adults reduced it (Fig. 5A, B). Both the larval stage increase in DAF-16 nuclear localization or the adult-stage decrease were suppressed by *kgb-1* disruption, observed in mutants carrying either the *kgb-1(um3*) allele (not

shown) or the *kgb-1(km21)* allele (Fig. 5B black columns). Furthermore, presenting the mirror image to *vhp-1* knock-down, knock-down of *kgb-1* (for two generations) caused approximately a 2-fold increase in the prevalence of intestinal DAF-16::GFP nuclear localization in adults, from 3% in control-treated animals to 8% in *kgb-1(RNAi)* adults (N=100 and 87, respectively). Together, these results support a role for KGB-1 in age-dependent antagonistic modulation of DAF-16.

To examine the contribution of *kgb-1* to modulation of endogenous DAF-16 activity we employed a transgenic strain with *gfp* expression controlled by the promoter of the DAF-16 target gene, *sod-3*. This expression was prominent in the pharynx, intestine, and in adults also in the vulva. Whereas pharyngeal expression remained unchanged, intestinal expression increased in *vhp-1(RNAi-dev)* animals and decreased in *vhp-1(RNAi-ad)* animals (Fig. 5C, D); vulval expression in adults similarly decreased. Again, *kgb-1* disruption suppressed the effects of vhp-1 RNAi (Fig. 5D black columns). Together, these analyses support the notion that KGB-1 enhanced DAF-16 function in developing animals, but attenuated it in adults.

DAF-16 modulation accounts for some, but not all, of the *kgb-1*-dependent phenotypes

The age-dependent contribution of *kgb-1* to both DAF-16 regulation as well as stress resistance and lifespan led us to hypothesize that modulation of DAF-16 was responsible for the observed *kgb-1*-dependent phenotypes. If true, *daf-16* mutants should not be affected by KGB-1 activation or disruption. This was the case with regards to *kgb-1*'s detrimental contribution to infection resistance and lifespan in adult animals: *vhp-1* knock-down only marginally decreased the lifespan of *daf-16* animals (Fig. 6A) and did not sensitize them to infection (Fig. 6B). Furthermore, knock-down of *kgb-1* itself did not increase the lifespan of *daf-16* mutants, in contrast to its effects in wild-type animals (Fig. 6C). It should be noted that increased infection resistance following development-stage *vhp-1* knock-down, which depends on *pmk-1,* was still apparent in *daf-16* mutants (Fig. S8). Our model further predicted that *daf-16* knock-down should phenocopy *vhp-1* knock-down in adults. Previous work indeed showed that *daf-16(RNAi-ad)* animals had shortened lifespan [39]. We further found that such animals also had decreased infection resistance (Fig. 6D). This is an adult-specific phenomenon, as *daf-16* disruption in larvae does not affect infection resistance (e,g, [22]).

Our results demonstrated that *daf-16* regulation underlies adult-specific detrimental effects of *kgb-1* on infection resistance and lifespan. However, we found that the contribution of *kgb-1* to cadmium resistance was independent of *daf-16,* with both vhp-1 and kgb-1 RNAi treatments having largely the same effects as in wildtype worms (Fig. S9). Since MEK-1 and SHC-1, KGB-1's activator and its scaffold protein, respectively, were previously shown to be required in the epidermis for their contribution to cadmium resistance [32] and as our data showed *kgb-1*-dependent regulation of DAF-16 prominently in intestinal cells, it is possible that KGB-1 contributes to cadmium resistance in a *daf-16*-independent manner in the epidermis, whereas its detrimental effects on infection-resistance and lifespan depend on DAF-16 modulation in intestinal cells (Fig. S9). Overall, the experiments in *daf-16* mutants demonstrated involvement of *daf-16* in *kgb-1*-dependent detrimental effects in adults. However, the contribution of

intestinal *kgb-1*-dependent DAF-16 nuclear localization in developing animals remains an open question.

Discussion

The dichotomy in the contribution of JNK signaling, stress protective vs. tissue damaging, is a fundamental feature of JNK signaling described in both mammals and drosophila. It is accepted that context has a dominant role in determining the outcome of JNK activation, but what defines this context(s) is not fully understood. Here, we show that age defines a context determining the outcome of JNK activation. We found that the *C. elegans* JNK homolog, KGB-1, protected developing larvae from heavy metals and protein folding stress, but sensitized young adults to the same stressors (as well as to bacterial infection), and shortened lifespan under normal conditions. The reversal in *kgb-1*'s contribution was manifested following inactivation (*kgb-1* knock-down) and hyperactivation (*vhp-1* knock-down) alike, demonstrating that the degree of activation is secondary to the phenomenon itself and pointing at the relevance of the reversal of *kgb-1* contribution to the normal *C. elegans* physiology.

Underlying the reversal in *kgb-1*'s contribution, we found a reversal in the effects of KGB-1 on DAF-16's output, suggesting that DAF-16 is a mediator of KGB-1's age-dependent opposing contributions to stress resistance. Although *daf-16* was indeed found by epistasis analysis to be necessary for the detrimental effects of *kgb-1* on infection resistance and lifespan, it was not involved in the effects of *kgb-1* on cadmium resistance in either age; furthermore, the significance of its *kgb-1*-dependent nuclear localization in the larval intestine is still unknown (Fig. 7). Knock-down experiments targeting specifically intestinal gene expression showed that unlike infection resistance, cadmium resistance was not affected by intestinal KGB-1 activation. Together with a previous study reporting that cadmium resistance depended on epidermal-specific JNK signaling [32], this suggests that KGB-1 activation in different tissues contributes to distinct stress phenotypes and that DAF-16 is an intestine-specific mediator, while other proteins may mediate the contribution of *kgb-1* outside of the intestine, e.g. in the epidermis. Lastly, how KGB-1 interacts with DAF-16 is yet unknown, but previous work focusing on the neuronal homolog JNK-1 suggests that KGB-1 may physically interact with DAF-16 [23]. .

Our results suggest that the dichotomy in JNK signaling goes beyond tissue-specificity, as opposite effects of KGB-1 activation are observed in the same tissue in different ages. Furthermore, *vhp-1* knock-down in different ages results in similar increases in phosphorylated KGB-1, suggesting that the extent of activation does not change with age. Our results are consistent with the hypothesis that age itself (onset of adulthood) determines a set-point that reverses the outcome of JNK signaling in the same tissue. They further suggest that past a certain developmental stage the contribution of JNK signaling becomes mainly detrimental. If the trend identified in *C. elegans* extends to mammals, which is quite possible based on the known parallels, it would help to explain the detrimental involvement of JNK signaling in various age-related pathologies. Furthermore, it would suggest JNK proteins as potential targets for inhibition to ameliorate such pathologies, with minimal detrimental consequences.

The model emerging for *kgb-1*, describing a beneficial contribution during development, but detrimental contribution past development is reminiscent of the characteristics predicted by the Antagonistic Pleiotropy theory for the evolution of aging. This theory proposes that aging has evolved as a consequence of an age-associated decline in selection pressure, permitting genes with late-life deleterious effects to be positively-selected if they confer an advantage early in life [40]. While this theory proposes a framework to explain how traits causing aging could be selected, it does not offer concrete underlying mechanisms. Any protein/pathway that is important early in life – for development, or reproduction - and is detrimental later, as evidenced by lifespan extension following disruption in adults, may represent a mechanism of antagonistic pleiotropy. A prominent example of this is the conserved nutrient-sensing protein kinase TOR (Target of Rapamycin), which positively regulates mRNA translation; its disruption during *C. elegans* development causes growth arrest or lethality, but its inhibition in adults prolongs lifespan [41]. TOR is the more pivotal of several genes involved in protein synthesis that show similar trends [42, 43]. Whereas such observations support Antagonistic Pleiotropy and point to the important role played by regulation of protein synthesis in lifespan determination, they do not explain how this occurs. In the absence of such mechanistic details, it remains unclear what would make a mechanism that is beneficial early in life become detrimental later. Two possible scenarios (of several possible ones) include a switch in protein function, or tipping a balance between co-existing beneficial and detrimental contributions; neither could be evaluated without a mechanistic example of Antagonistic Pleiotropy. In fact, it was argued that convincing examples of Antagonistic Pleiotropy would be hard to find due to the difficulty of knowing the contribution of a gene in different parts of the life cycle [40]. However, current methods for conditional gene knock-out or age-specific knock-down enable just that. The age-dependent switch that we identified in the contribution of *kgb-1* to stress resistance, particularly in the contribution to cadmium resistance, supports a model in which a developmental program changes the beneficial contribution of a protein to detrimental.

One of the implications of the Antagonistic Pleiotropy theory was assumed to be the great number of pleiotropic genes contributing to aging, as any large negative contribution of a gene variant to late adult life could be offset by a relatively small advantage conferred early in life, potentially contributing to high variability in such genes among individuals. This may be taken to signify that the chance of singling out common causes of aging was very small. Our findings, however, present a case of a pleiotropic gene with a significant positive contribution early in life. Whether this is the rule or the exception for pleiotropic aging genes remains to be seen, but if the former, it may suggest that some causes of aging could be by-products of essential processes making them more likely to be shared among individuals and therefore increasing the chances of identifying targets for the treatment of aging.

Our results provide evidence that age is an important factor determining the role JNK signaling plays in contributing to stress protection and health. Furthermore, they provide tangible evidence moving the phenomenon of antagonistic pleiotropy from an abstract driving force in the evolution of aging to a mechanism that contributes to proximal features of aging, and by doing so elaborates on the original model. A better

understanding of KGB-1's detrimental effects has the potential to shed more light on the evolution of senescence and on aging itself.

Methods

Strains used appear in the relevant sections. A complete list appears as supporting material.

RNAi by feeding was performed at 25°C, typically for two days - from egg stage to the terminal L4 stage (RNAi-dev), or from L4 to second day of adulthood (RNAi-ad). Sterilization by *cdc-25.1* knockdown was performed as described elsewhere [44].

Survival assays were performed at 25°C in triplicates with approximately 100 animals per group, per experiment. Animals were transferred to experimental plates immediately following RNAi. Lifespan, cadmium and tunicamycin assays were performed on NGM plates, NGM supplemented with 100 □M $CdCl_2$, or with 10ug/ml tunicamycin, respectively; in all cases, worms were fed kanamycin-killed *E. coli.* Experiments with high (5mM) cadmium were performed in liquid K medium (53 mM NaCl, 32 mM KCl) without food. *P. aeruginosa* infection experiments were performed using the slow killing protocol described elsewhere [45].

Development assays. Animals were grown on NGM plates containing 50μM $CdCl_2$ (at 25°C) or 1μg/ml tunicamycin (at 20°C). Following three days, percentage of animals reaching adulthood was counted. 50-170 eggs per strain/treatment were assayed, and experiments were performed twice.

RNA extraction and Quantitative (q)RT-PCR. RNA was extracted using Trizol (Invitrogen) from 100-200 worms per sample and treated with Turbo DNAse (Ambion). For qRT-PCR measurements, gene-specific threshold cycle (Ct) values were normalized to the respective actin values, and presented as fold changes over the appropriate control samples. For a list of primers see supporting information.

Immunoblotting was performed based on standard procedures (see supporting experimental procedures for details) using the following antibodies at the indicated dilutions: anti-pKGB-1 [32], gratefully received from Dr. Kunihiro Matsumoto, Nagoya, Japan, 1:300; anti-pPMK-1 (Cell Signaling Technology 9215, 1:1000); and anti-actin (Santa Cruz Biotechnology sc-10731, 1:200). Secondary antibodies were peroxidase-conjugated donkey anti-rabbit (Jackson Immunoresearch 711-035-152, 1:2500). Band intensities were measured with Photoshop, and normalized to their local background, as well as to band densities in actin immunoblots.

GFP imaging employed *daf-16::gfp* or P_{sod-3}::*gfp* transgenic animals. For cadmium experiments, animals were placed on NGM plates or NGM+100□M $CdCl_2$ at the L4 stage for one day at 25°C. Animals were washed off, paralyzed with 25mM levamisole (Sigma) and mounted on slides, altogether taking less than five minutes, to minimize 'slide stress'. Images were acquired using identical settings for control and experimental

samples. Whole-worm GFP intensity was quantified in P_{sod-3}::*gfp* animals using Metamorph (Molecular Devices), subtracting local background intensity for each worm. 20-30 animals were quantified per group, per experiment. Nuclear localization in DAF-16::GFP worms was measured as percentage of animals with intestinal nuclear localization. Each experiment included 30-200 animals per group.

Statistical analyses

Differences between survival curves were evaluated using Kaplan Meier analysis followed by Logrank test, or when indicated, the Wilcoxon test; the latter allocates more weight to early time points. Differences between one time point measurements performed in several occasions (i.e. survival on high $CdCl_2$, qRT-PCR) were assessed using two-way ANOVA with the experimental group as one factor, and time of experiment as the other. Differences in DAF-16 nuclear localization were assessed using a paired t-test.

References

1. Kyriakis, J.M. and J. Avruch, *Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation.* Physiol Rev, 2001. **81**(2): p. 807-69.

2. Morrison, D.K. and R.J. Davis, *Regulation of MAP kinase signaling modules by scaffold proteins in mammals.* Annu Rev Cell Dev Biol, 2003. **19**: p. 91-118.

3. Weston, C.R. and R.J. Davis, *The JNK signal transduction pathway.* Curr Opin Cell Biol, 2007. **19**(2): p. 142-9.

4. Chang, L., et al., *JNK1 is required for maintenance of neuronal microtubules and controls phosphorylation of microtubule-associated proteins.* Dev Cell, 2003. **4**(4): p. 521-33.

5. Podkowa, M., et al., *Microtubule stabilization by bone morphogenetic protein receptor-mediated scaffolding of c-Jun N-terminal kinase promotes dendrite formation.* Mol Cell Biol, 2010. **30**(9): p. 2241-50.

6. Ogata, M., et al., *Autophagy is activated for cell survival after endoplasmic reticulum stress.* Mol Cell Biol, 2006. **26**(24): p. 9220-31.

7. Hayakawa, J., et al., *The activation of c-Jun NH2-terminal kinase (JNK) by DNA-damaging agents serves to promote drug resistance via activating transcription factor 2 (ATF2)-dependent enhanced DNA repair.* J Biol Chem, 2003. **278**(23): p. 20582-92.

8. Kuan, C.Y., et al., *The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development.* Neuron, 1999. **22**(4): p. 667-76.

9. Baines, C.P. and J.D. Molkentin, *STRESS signaling pathways that modulate cardiac myocyte apoptosis.* J Mol Cell Cardiol, 2005. **38**(1): p. 47-62.

10. Morishima, Y., et al., *Beta-amyloid induces neuronal apoptosis via a mechanism that involves the c-Jun N-terminal kinase pathway and the induction of Fas ligand.* J Neurosci, 2001. **21**(19): p. 7551-60.

11. Kuan, C.Y., et al., *A critical role of neural-specific JNK3 for ischemic apoptosis.* Proc Natl Acad Sci U S A, 2003. **100**(25): p. 15184-9.

12. Sabio, G., et al., *Role of muscle c-Jun NH2-terminal kinase 1 in obesity-induced insulin resistance.* Mol Cell Biol, 2010. **30**(1): p. 106-15.

13. Hirosumi, J., et al., *A central role for JNK in obesity and insulin resistance.* Nature, 2002. **420**(6913): p. 333-6.

14. Lamb, J.A., et al., *JunD mediates survival signaling by the JNK signal transduction pathway.* Mol Cell, 2003. **11**(6): p. 1479-89.

15. Kamata, H., et al., *Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases.* Cell, 2005. **120**(5): p. 649-61.

16. Ventura, J.J., et al., *Chemical genetic analysis of the time course of signal transduction by JNK.* Mol Cell, 2006. **21**(5): p. 701-10.

17. Wei, J., et al., *c-Jun N-terminal kinase (JNK-1) confers protection against brief but not extended ischemia during acute myocardial infarction.* J Biol Chem, 2011. **286**(16): p. 13995-4006.

18. Biteau, B., et al., *Regulation of Drosophila lifespan by JNK signaling.* Exp Gerontol, 2011. **46**(5): p. 349-54.

19. Tatar, M., A. Bartke, and A. Antebi, *The endocrine regulation of aging by insulin-like signals.* Science, 2003. **299**(5611): p. 1346-51.

20. Murphy, C.T., et al., *Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans.* Nature, 2003. **424**(6946): p. 277-83.

21. Henderson, S.T. and T.E. Johnson, *daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans.* Curr Biol, 2001. **11**(24): p. 1975-80.

22. Garsin, D.A., et al., *Long-lived C. elegans daf-2 mutants are resistant to bacterial pathogens.* Science, 2003. **300**(5627): p. 1921.

23. Oh, S.W., et al., *JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16.* Proc Natl Acad Sci U S A, 2005. **102**(12): p. 4494-9.

24. Wang, M.C., D. Bohmann, and H. Jasper, *JNK extends life span and limits growth by antagonizing cellular and organism-wide responses to insulin signaling.* Cell, 2005. **121**(1): p. 115-25.

25. Seisenbacher, G., E. Hafen, and H. Stocker, *MK2-dependent p38b signalling protects Drosophila hindgut enterocytes against JNK-induced apoptosis under chronic stress.* PLoS Genet, 2011. **7**(8): p. e1002168.

26. Biteau, B., C.E. Hochmuth, and H. Jasper, *JNK activity in somatic stem cells causes loss of tissue homeostasis in the aging Drosophila gut.* Cell Stem Cell, 2008. **3**(4): p. 442-55.

27. Wang, M.C., D. Bohmann, and H. Jasper, *JNK signaling confers tolerance to oxidative stress and extends lifespan in Drosophila.* Dev Cell, 2003. **5**(5): p. 811-6.

28. Martin-Blanco, E., et al., *puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in Drosophila.* Genes Dev, 1998. **12**(4): p. 557-70.

29. Mizuno, T., et al., *The Caenorhabditis elegans MAPK phosphatase VHP-1 mediates a novel JNK-like signaling pathway in stress response.* Embo J, 2004. **23**(11): p. 2226-34.

30. Neumann-Haefelin, E., et al., *SHC-1/p52Shc targets the insulin/IGF-1 and JNK signaling pathways to modulate life span and stress response in C. elegans.* Genes Dev, 2008. **22**(19): p. 2721-35.

31. Smith, P., et al., *The GLH proteins, Caenorhabditis elegans P granule components, associate with CSN-5 and KGB-1, proteins necessary for fertility, and with ZYX-1, a predicted cytoskeletal protein.* Dev Biol, 2002. **251**(2): p. 333-47.

32. Mizuno, T., et al., *Role of the Caenorhabditis elegans Shc adaptor protein in the c-Jun N-terminal kinase signaling pathway.* Mol Cell Biol, 2008. **28**(23): p. 7041-9.

33. Kim, D.H., et al., *A conserved p38 MAP kinase pathway in Caenorhabditis elegans innate immunity.* Science, 2002. **297**(5581): p. 623-6.

34. Inoue, H., et al., *The C. elegans p38 MAPK pathway regulates nuclear localization of the transcription factor SKN-1 in oxidative stress response.* Genes Dev, 2005. **19**(19): p. 2278-83.

35. Kim, D.H., et al., *Integration of Caenorhabditis elegans MAPK pathways mediating immunity and stress resistance by MEK-1 MAPK kinase and VHP-1 MAPK phosphatase.* Proc Natl Acad Sci U S A, 2004. **101**(30): p. 10990-4.

36. Harshman, L.G. and A.J. Zera, *The cost of reproduction: the devil in the details.* Trends Ecol Evol, 2007. **22**(2): p. 80-6.

37. Troemel, E.R., et al., *p38 MAPK regulates expression of immune response genes and contributes to longevity in C. elegans.* PLoS Genet, 2006. **2**(11): p. e183.

38. Shapira, M., et al., *A conserved role for a GATA transcription factor in regulating epithelial innate immune responses.* Proc Natl Acad Sci U S A, 2006. **103**(38): p. 14086-91.

39. Dillin, A., D.K. Crawford, and C. Kenyon, *Timing requirements for insulin/IGF-1 signaling in C. elegans.* Science, 2002. **298**(5594): p. 830-4.

40. Williams, G.C., *Pleiotropy, Natural Selection, and the Evolution of Senescence.* Evolution, 1957. **11**(4): p. 398-411.

41. Hansen, M., et al., *Lifespan extension by conditions that inhibit translation in Caenorhabditis elegans.* Aging Cell, 2007. **6**(1): p. 95-110.

42. Chen, D., et al., *Longevity determined by developmental arrest genes in Caenorhabditis elegans.* Aging Cell, 2007. **6**(4): p. 525-33.

43. Curran, S.P. and G. Ruvkun, *Lifespan regulation by evolutionarily conserved genes essential for viability.* PLoS Genet, 2007. **3**(4): p. e56.

44. Shapira, M. and M.W. Tan, *Genetic analysis of Caenorhabditis elegans innate immunity.* Methods Mol Biol, 2008. **415**: p. 429-42.

45. Tan, M.W., S. Mahajan-Miklos, and F.M. Ausubel, *Killing of Caenorhabditis elegans by Pseudomonas aeruginosa used to model mammalian bacterial pathogenesis.* Proc Natl Acad Sci U S A, 1999. **96**(2): p. 715-20.

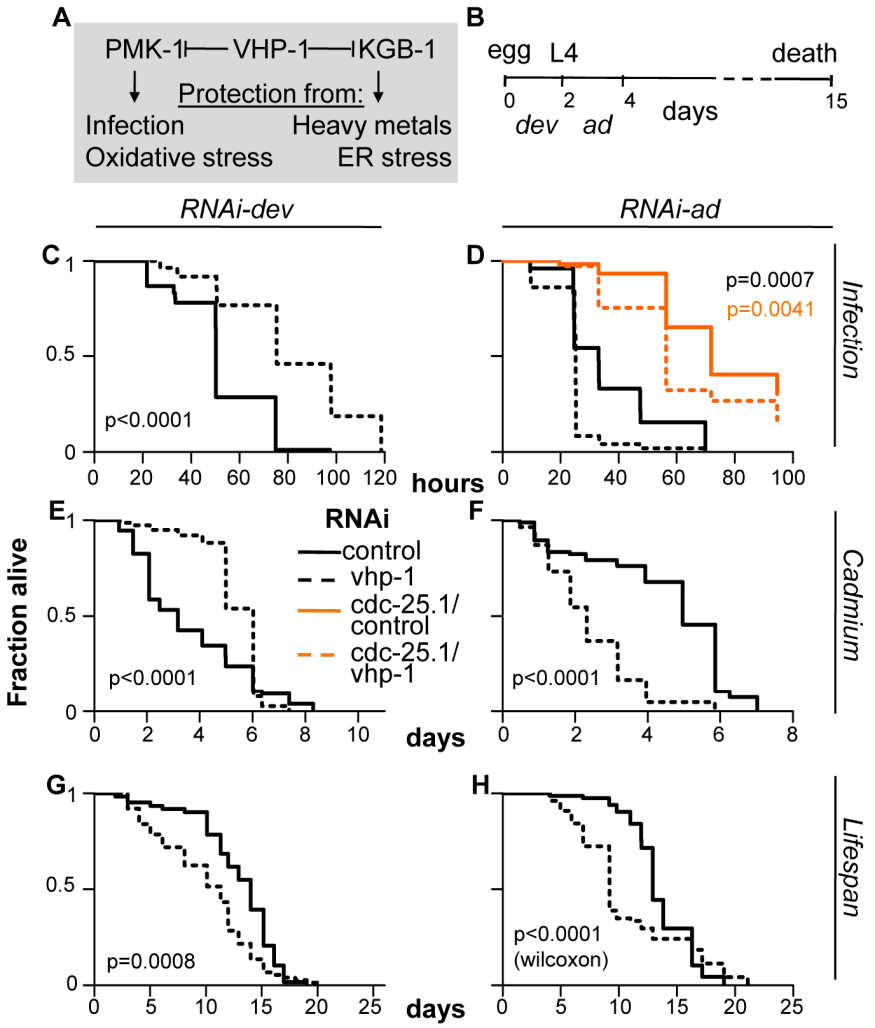

Figure 1. An age-associated reversal in the vhp-1 RNAi phenotype (A) VHP-1 negatively regulates both PMK-1 and KGB-1. (B) Timeline for the life of wild-type *C. elegans* at 25°C. (C-H) Survival curves for wild type animals fed with RNAi-expressing *E. coli*, as designated, and subsequently exposed to *P. aeruginosa* (C,D), 100 μM cadmium (E,F) or dead food bacteria (G,H). Knock-down was performed during development (dev = egg until L4 stage) (C,E,G), or early adulthood (ad = L4 stage for two days), (D,F,H); or, following sterilization achieved by a development-stage exposure to cdc-25.1 RNAi (orange curves in (D)). Graphs are representatives of ≥2 experiments showing similar results.

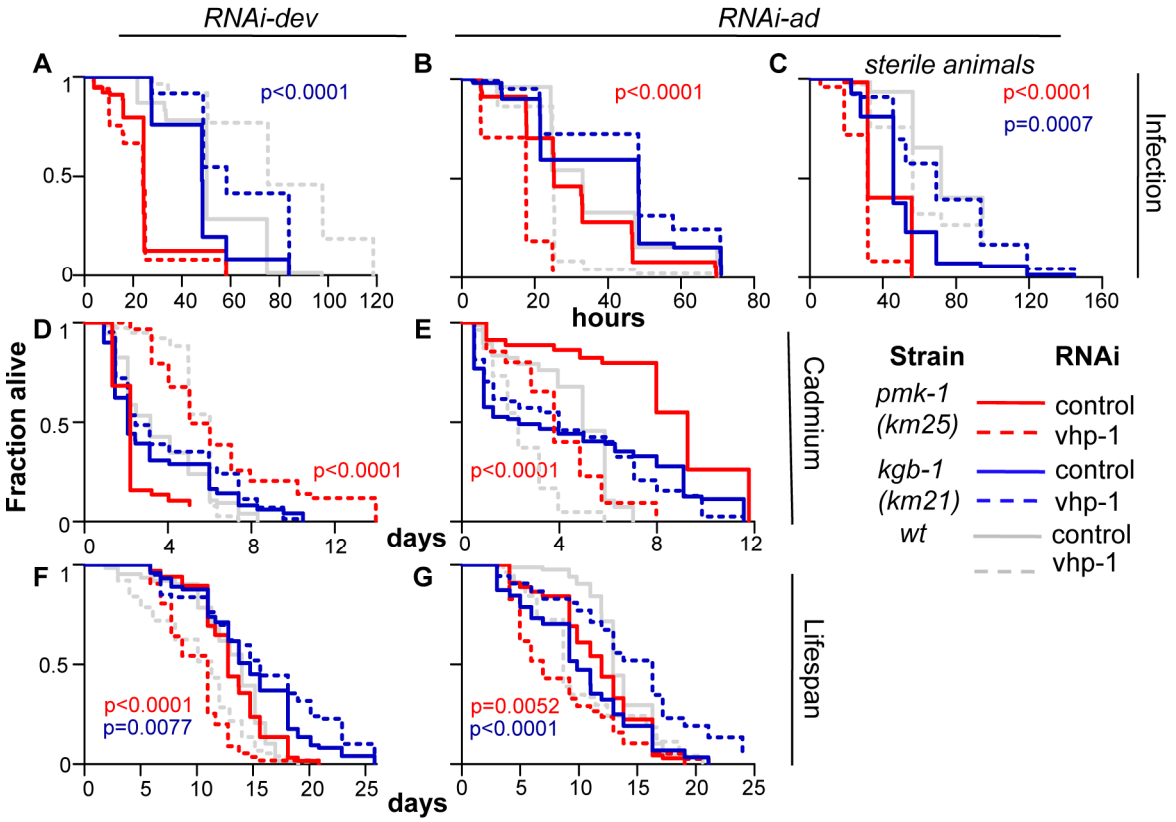

Figure 2. *kgb-1* plays a central role in the age-associated reversal in the vhp-1 RNAi phenotype. Survival curves for mutant animals fed with RNAi-expressing *E. coli*, as designated, and subjected to conditions as in Fig. 1. Gray curves, representing survival of *w*ild-type animals are the same as in Figure 1 and are shown for comparison. Knock-down was performed during development (dev) (A,D,F), or early adulthood (ad), (B,C,E,G); in C, adult-stage *vhp-1* knock-down was performed following development-stage knock-down of *cdc-25.1* . Graphs are representatives of ≥2 experiments showing similar results. Curves without designated p-values are not significantly different (p>0.05)

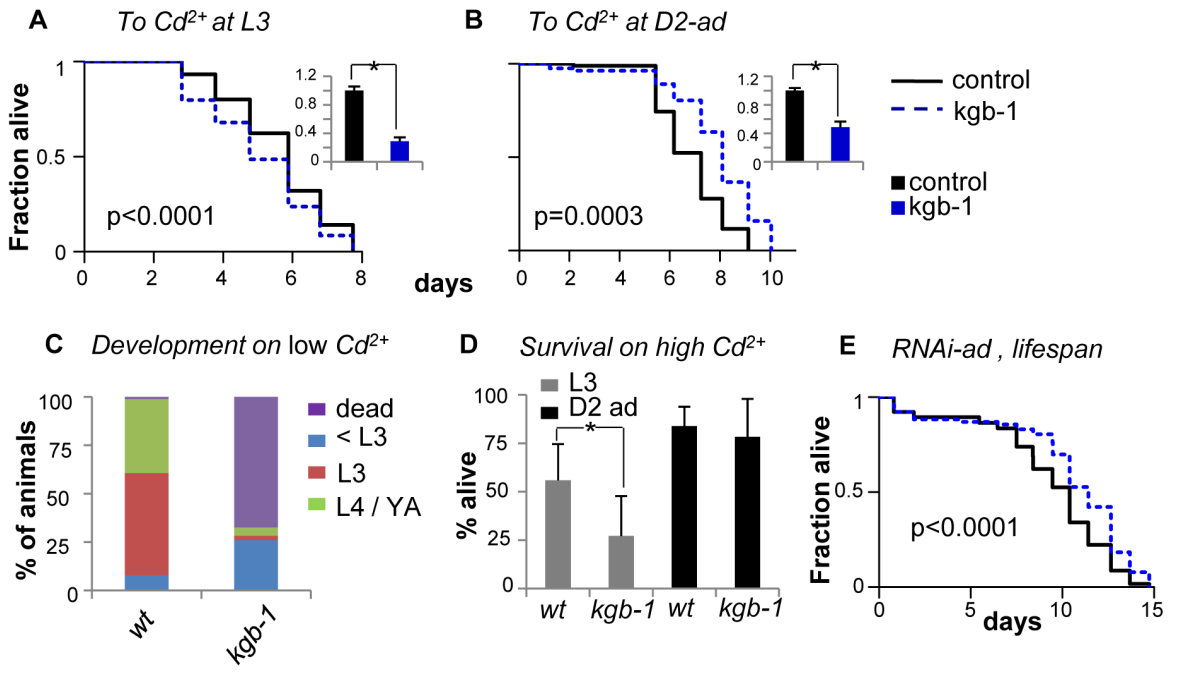

Figure 3. *kgb-1* protects larvae from cadmium, but is detrimental in adults. (A,B) Survival curves for animals treated for 2 generations with kgb-1 RNAi and exposed to 100 µM $CdCl_2$, as L3 larvae (A) or as 2-day old adults (B); shown are representatives of three experiments. Insets present qRT-PCR evaluation of the extent of *kgb-1* knock-down in the respective ages, presented as average±SD (of two experiments) of fold change over control treatment: *p<0.001. (C) Developmental status and survival of untreated wild-type and *kgb-1(km21)* worms following a 3–day exposure to 50 µM cadmium (at 25°C). Shown is a representative of two independent experiments, each containing at least 50 worms per strain. (D) Survival following acute exposure to cadmium (5 mM, in liquid). Animals exposed to cadmium at the indicated stages were assayed for survival 10 hours later (L3 larvae) or between 11 and 17 hours later (D2 adults). Shown are means ± SD of two independent experiments, *p= 0.00260. N=60 per strain per experiment. (E) *kgb-1* knock-down in wild-type adults (beginning at L4 for two days) extends lifespan. Shown is a representative of three experiments.

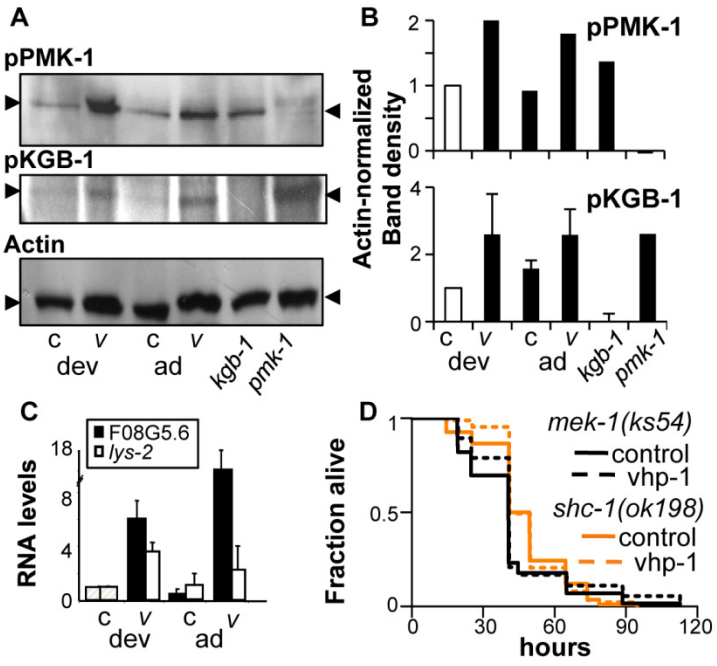

Figure 4. PMK-1 and KGB-1 activation following *vhp-1* knock-down does not change with age. (A) Immunoblot of wild-type or indicated mutant animals (negative controls) treated with control (c) or *vhp-1* (v) RNAi as designated. (B) Actin-normalized band densities in 1 experiment for pPMK-1 or means±SD of 4 gels representing two experiments for pKGB-1 presented as fold over values in *control(RNAi-dev)* animals (empty columns). (C) RNA levels, measured by quantitative (q)RT-PCR, of PMK-1's downstream targets *lys-2* and F08G5.6. Means±SD of two independent experiments, presented as fold changes over values in striped columns. (D) Infection survival curves following adult-stage *(RNAi-ad)* treatment.

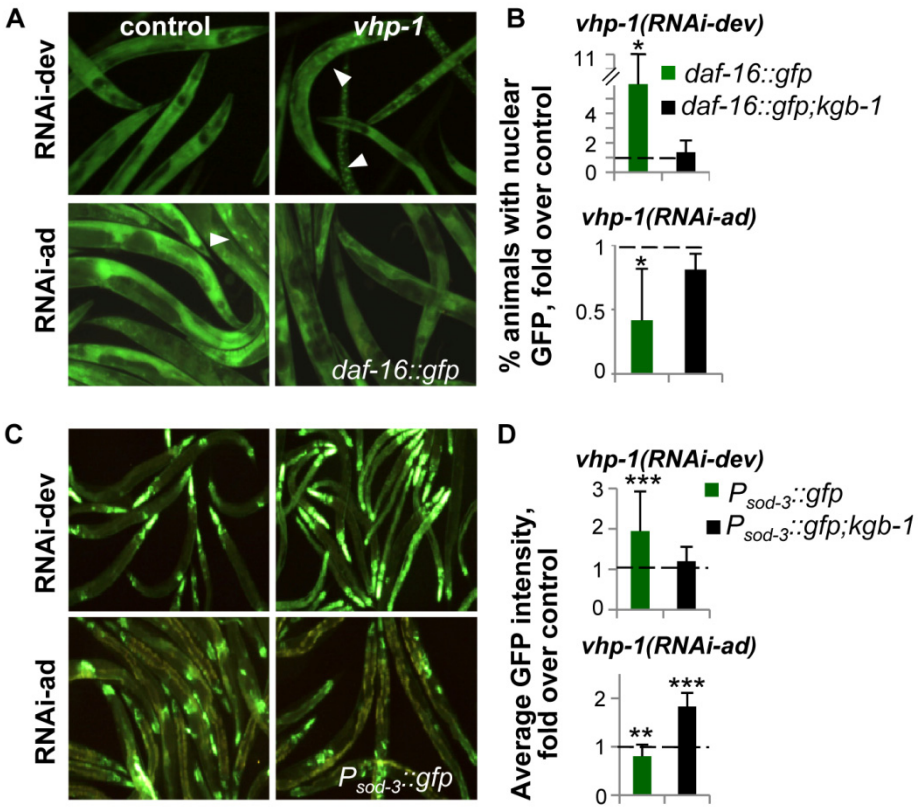

Figure 5. *kgb-1* contributes to age-dependent modulation of DAF-16 nuclear localization. (A) Representative images of DAF-16::GFP-expressing animals exposed to RNAi as designated; arrowheads mark localization in intestinal nuclei. (B) Quantification of DAF-16::GFP nuclear localization in images as in A , shown as the percentage of *vhp-1(RNAi)* animals with intestinal nuclear localization of DAF-16::GFP, and as fold over control-treated animals ; Means ± SD of 4-5 experiments in wild-type animals, or 3, in *kgb-1(km21)* mutants. *p<0.05, **p<0.0001. Basal prevalence of DAF-16 nuclear localization (in animals treated with control RNAi) was 0.6-11% in wildtype *RNAi-dev* animals, 0-6% in *kgb-1;RNAi-dev* animals, 14-23% in wildtype *RNAi-ad* animals and 2-9% in *kgb-1;RNAi-ad* animals. (C) Representative images, and their quantification (D) of GFP levels in P_{sod-3}*::gfp* or P_{sod-3}*::gfp;kgb-1(um3)* animals, shown as in B. Means±SD of 3 experiments (P_{sod-3}*::gfp*) or 2 experiments (P_{sod-3}*::gfp;kgb-1*) with 20-30 animals per group; **p= 0.0007, ***p<0.0001.

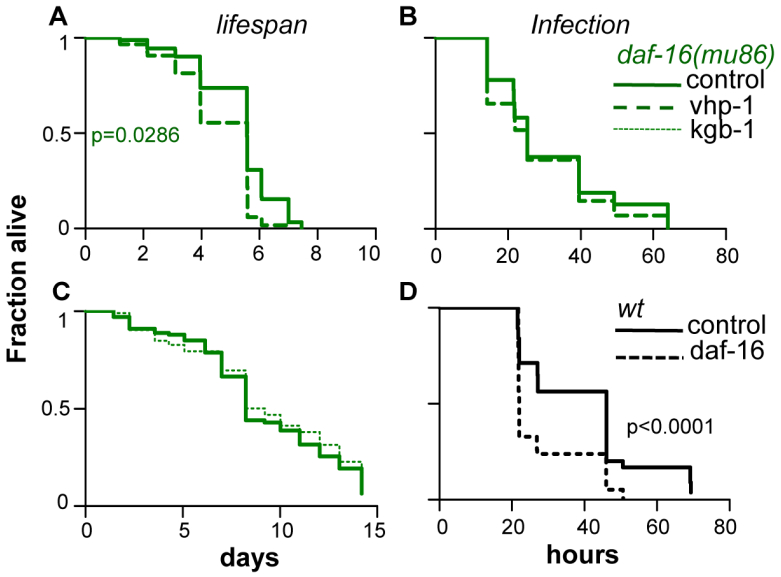

Figure 6. *daf-16* is necessary for some *kgb-1*-dependent detrimental phenotypes in adults. Survival curves for wild-type animals (black) and *daf-16(mu86)* mutants (green) exposed to RNAi, as designated, during early adulthood (L4 to day 2) and followed by lifespan analysis (A,C) or infection survival (B,D). When not shown, p-values were not significant ($p>0.05$). Each panel shows a representative experiment of two independent experiments with similar results; each experiment in *daf-16* mutants was performed alongside a wild-type animal control (not shown) verifying that typical trends (as in Figures 1 and 4) were recapitulated.

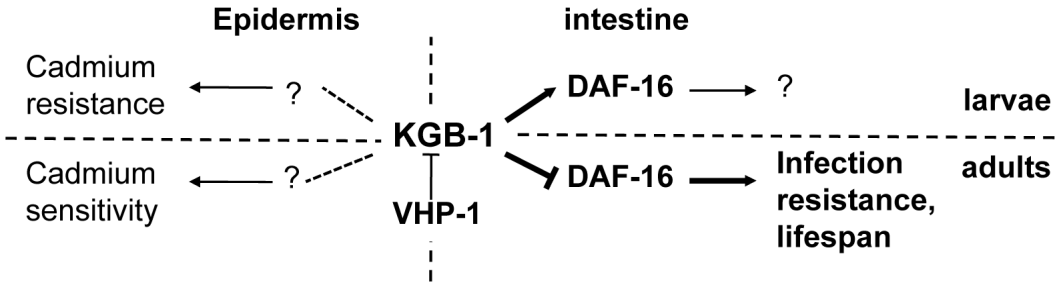

Figure 7. A model for tissue specific and age-dependent contributions of KGB-1. Boldface represents aspects of the mechanism that were worked out, capitals represent gene-products. KGB-1 enhances nuclear localization of DAF-16 in the intestine of larvae (with yet unknown consequences), but attenuates this localization in adult animals, leading to infection susceptibility and, in the long term, shortening lifespan. For cadmium resistance, KGB-1 activation is protective in larvae, but sensitizes adults. Both contributions are likely to be in the epidermis (Mizuno, 2008), but the mediator(s) are yet unknown. Not shown in the model is PMK-1, activation of which is invariably infection protective.

Appendix: Supplemental Information

Supporting Experimental Procedures

Worm strains. Worm strains were obtained from the *Caenorhabditis* Genetics Center and maintained using standard procedures. Strains included are: N2, as the wild-type strain; KU25, *pmk-1(km25);* KU21, *kgb-1(km21*), carrying a deletion spanning exons 4 and 5 of *kgb-1*, which was previously shown to suppress the larval arrest phenotype caused by *vhp-1* disruption [1]*;* KB3, *kgb-1(um3)*, carrying a deletion spanning exons 6-9 of *kgb-1*, including the entire active site [2]; SS104, *glp-4(bn2); glp-4(bn2);rrf-3(pk1426*)[3]*;* BA837, *spe-26(it112);* NH3119*, shc-1(ok198);* FK171*, mek-1(ks54);* CF1038, *daf-16(mu86)*; CF1553, muIs84*[P_{sod3}::gfp];* TJ356, zIs356*[P_{daf-16}::daf-16::gfp;rol-6]*; and HC75, *sid-1(qt2);*ccIs4251, with a disruption in the gene encoding a dsRNA channel necessary for systemic RNAi ,thus restricting knock-down to the intestine [4]. Strains *kgb-1(um3)*;*P_{daf-16}::daf-16::gfp* and *kgb-1(um3)*;*P_{sod3}::gfp* were generated by crossing strain KB3 with TJ356 and CF1553 respectively, and strain *kgb-1(km21)*;*P_{daf-16}::daf-16::gfp* was generated by crossing strain KU21 with TJ356.

RNAi. *vhp-1* was targeted by two RNAi clones showing similar results in infection assays. The identity of the Open Biosystems library clone, [5] was confirmed by sequencing and used throughout this study. All other RNAi clones were from the Ahringer library[6].

PCR Primers.

F08G5.6 forward	CACAATGATTTCAATGCGAGA
F08G5.6 reverse	GTTTCGACCGAGAAATCGAG
lys-2 forward	CCAATATCAAGCTGGCAAGG
lys-2 reverse	GTTGGATTGTTTGGCCAGTT
kgb-1 forward	TTGCGCACAAACTCTGGTAG
kgb-1 reverse	CACCGGACACTTCACTTTCA
pmk-1 forward	GCCAATGTTTCCACAGACAA
pmk-1 reverse	TCAGCACAAACAGTTCC
vhp-1 forward	TCTCGAAACTCATCAGAAGACG
vhp-1 reverse	TCCATTTTTGTGCAACCTGA
pan-actin forward	TCGGTATGGGACAGAAGGAC
pan-actin reverse	CATCCCAGTTGGTGACGATA

Immunobloting. Approximately 500-600 worms were harvested with M9 buffer and re-suspended in Radio-Immunoprecipitation Assay (RIPA) buffer containing protease and phosphatase inhibitor cocktails (Calbiochem and Roche, respectively). Homogenization was achieved using a combination of pestle homogenization, freeze-thaw cycles and sonication. The resulting homogenate was spun briefly and total protein content in the supernatant was determined using the Coomassie (Bradford) Protein Assay Kit (Pierce Biotechnology). Fifty micrograms of total protein were loaded per lane, separated on a 10% Tris-HCl polyacrylamide gel (Bio-Rad) and transferred in 20% methanol buffer at 4^{o}C to Amersham Hybond-P PVDF membranes (GE Healthcare). Prior to incubation

with primary antibodies, membranes were blocked with 5% BSA in TBST (for pKGB-1 antibodies, gratefully received from Dr. Kunihiro Matsumoto, Nagoya, Japan [7], 1:300), or Western Breeze® Blocking solution (Invitrogen) (for pPMK-1 (Cell Signaling Technology, 9215, 1:1000), and actin (Santa Cruz Biotechnology, sc-10731, 1:200). Secondary antibodies were peroxidase-conjugated donkey anti-rabbit (Jackson Immunoresearch, 711-035-152, 1:2500). Signal was detected using Western Lightning ECL kit (Perkin Elmer). Band intensities were measured with Adobe Photoshop, and normalized to their local background, as well as to band densities in actin immunoblots.

References

1. Mizuno T, Hisamoto N, Terada T, Kondo T, Adachi M, et al. (2004) The Caenorhabditis elegans MAPK phosphatase VHP-1 mediates a novel JNK-like signaling pathway in stress response. Embo J 23: 2226-2234.

2. Smith P, Leung-Chiu WM, Montgomery R, Orsborn A, Kuznicki K, et al. (2002) The GLH proteins, Caenorhabditis elegans P granule components, associate with CSN-5 and KGB-1, proteins necessary for fertility, and with ZYX-1, a predicted cytoskeletal protein. Dev Biol 251: 333-347.

3. Shapira M, Hamlin BJ, Rong J, Chen K, Ronen M, et al. (2006) A conserved role for a GATA transcription factor in regulating epithelial innate immune responses. Proc Natl Acad Sci U S A 103: 14086-14091.

4. Winston WM, Molodowitch C, Hunter CP (2002) Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1. Science 295: 2456-2459.

5. Reboul J, Vaglio P, Rual JF, Lamesch P, Martinez M, et al. (2003) C. elegans ORFeome version 1.1: experimental verification of the genome annotation and resource for proteome-scale protein expression. Nat Genet 34: 35-41.

6. Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, et al. (2003) Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature 421: 231-237.

7. Mizuno T, Fujiki K, Sasakawa A, Hisamoto N, Matsumoto K (2008) Role of the Caenorhabditis elegans Shc adaptor protein in the c-Jun N-terminal kinase signaling pathway. Mol Cell Biol 28: 7041-7049.

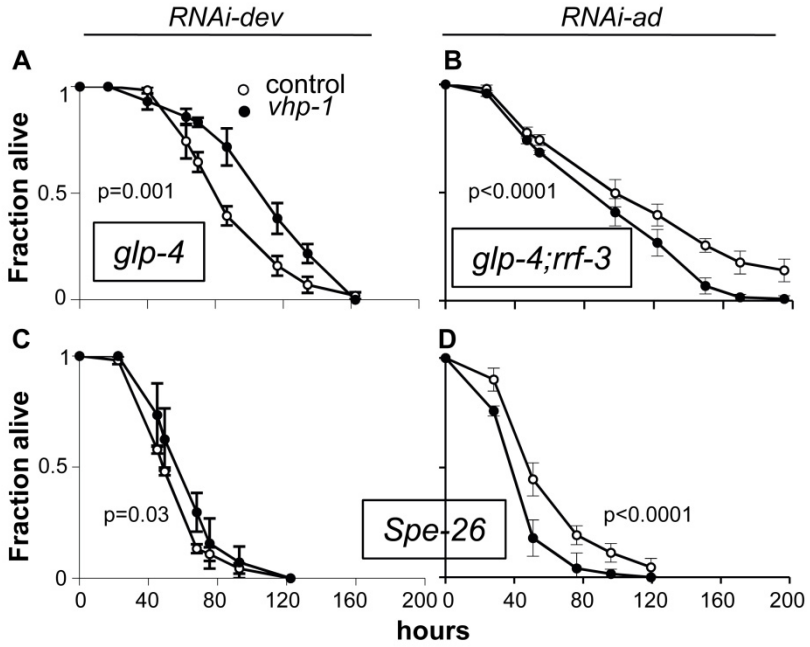

Figure S1. Changes in infection resistance following vhp-1 knock-down are not associated with changes in reproduction. Survival curves for gonad-less glp-4(bn2) mutants treated with RNAi from egg stage until L4 (RNAi-dev) (A), glp-4(bn2);rrf-3(pk1426) mutants treated with RNAi from the L4 stage for two days (RNAi-ad) (B), or sperm-defective spe-26(it112) mutants treated with RNAi from the egg (C) or L4 stage (D). In all cases, the effect of vhp-1 RNAi is the same as that obtained in wild-type fertile worms. Shown are means ± SD of fraction of live animals on each of 3 plates

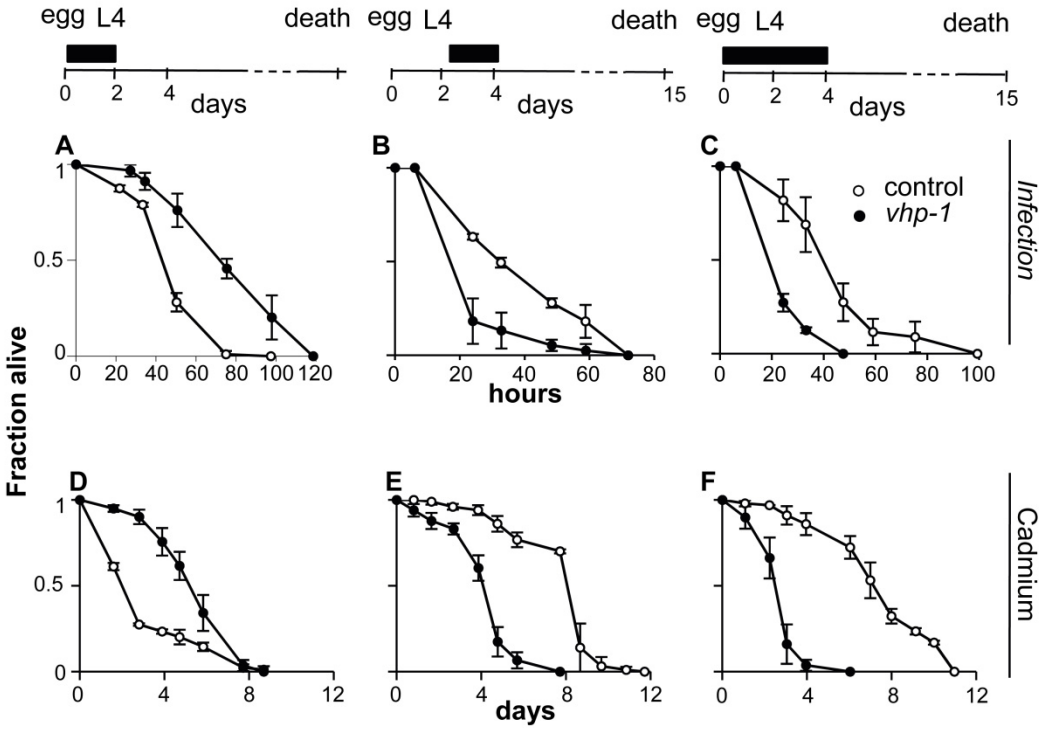

Figure S2. Effects of development-stage *vhp-1* knock-down are outweighed by its effects in adults. Survival curves for wild-type animals treated with the designated RNAi clones and infected with *P. aeruginosa* (A-C)or exposed to 100 µM cadmium chloride (D-F). Time course of RNAi treatment was as depicted in the accompanying schemes (bars). In both infection and cadmium assays, animals exposed to vhp-1 RNAi from the egg stage for 4 days develop into small adults, yet present decreased stress resistance (C and F)**,** similar to the *vhp-1(RNAi-ad)* animals shown in (B and E) and in contrast to egg RNAi for 2 days (A and D). $p<0.0001$ for all panels. This suggests dominance of the effects caused by vhp-1 RNAi in adults over those caused by development-stage knock-down. Furthermore, since the treatment in B adds the treatments of RNAi-dev and RNAi-ad, yet results in the same phenotype as in A, this phenotype is not likely to be due to decreased RNAi efficiency in adults compared to developing larvae.

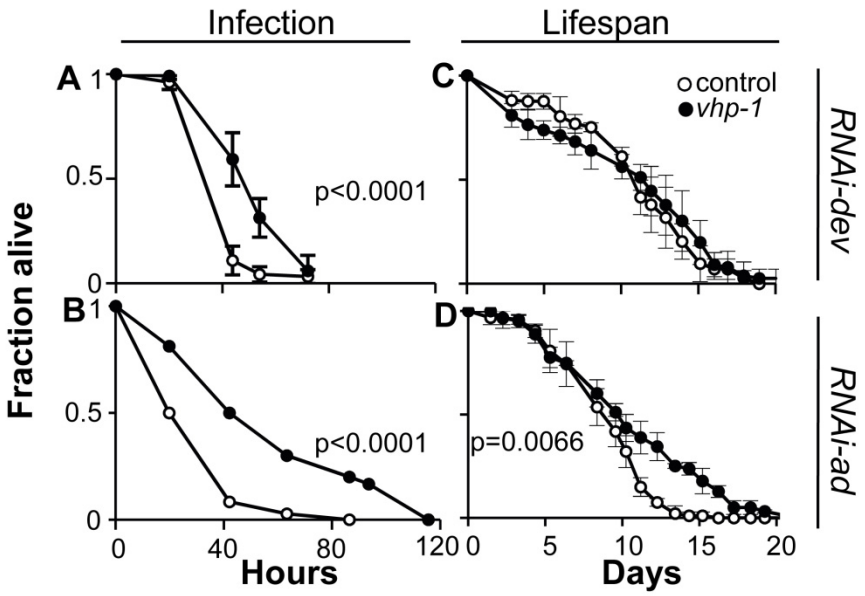

Figure S3. *kgb-1(um3)* mutants are immune to the detrimental effects of adult *vhp-1* knock down. Survival curves of *kgb-1(um3)* mutants treated with designated RNAi from the egg stage until L4 (*RNAi-dev*) or from the L4 stage for two days (*RNAi-ad*) and assayed for either survival on *P. aeruginosa* (A, B) or lifespan on dead *E.*coli (C, D).

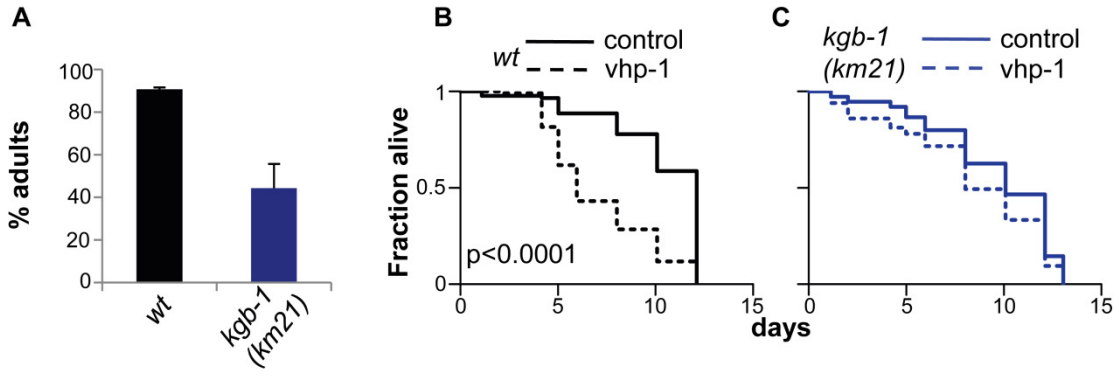

Figure S4. Age-dependent reversal in *kgb-1*'s contribution to tunicamycin resistance. (A) Percentage of animals reaching adulthood within 3 days of development on 1µg/ml tunicamycin at 20°C. Shown are averages of two independent experiments, each with >100 animals per strain. (B,C) Survival on 10µg/ml tunicamycin following vhp-1 knock-down in adults.

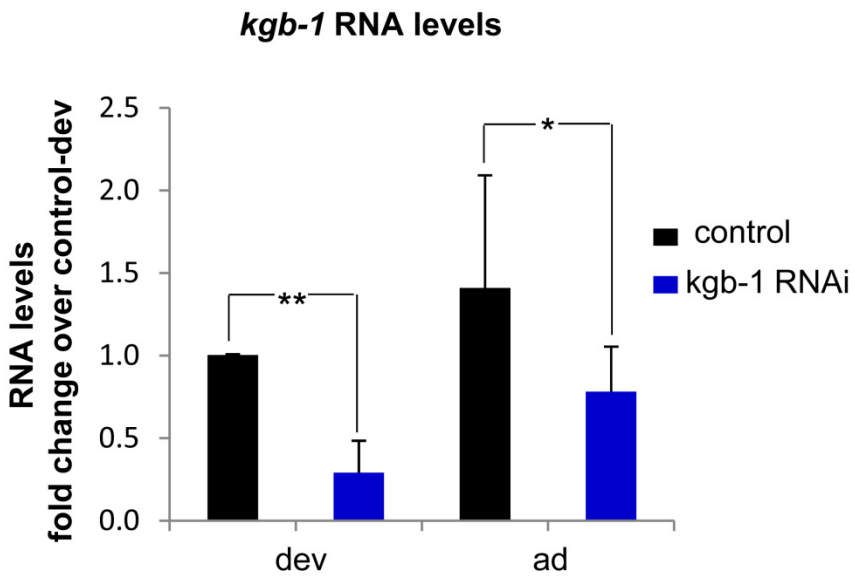

Figure S5. RNAi knock-down efficiency in larvae and adult animals is comparable. RNA levels measured by qRT-PCR in wildtype animals treated with RNAi from egg to L4 stage (dev) or from L4 to two-day old adult (ad). Shown are means and SD of data from two experiments. **p<0.0001, *p=0.0018 (2-way ANOVA for effects of experiment and treatment).

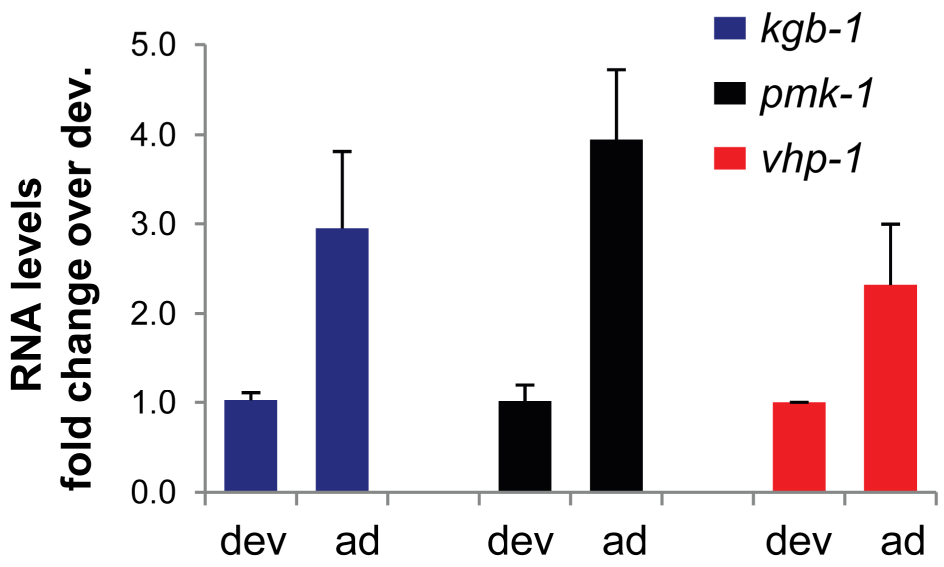

Figure S6. **Increases in *kgb-1*, *pmk-1* and *vhp-1* expression with age.** mRNA levels measured by qRT-PCR in wildtype L4 stage (dev) or two-day old adult (ad) animals. *vhp-1* data is for the a-isoform. Shown are means and SD of duplicate measurements from three experiments (*kgb-1 and pmk-1*) or two experiments (*vhp-1*). $p<0.001$ for each gene

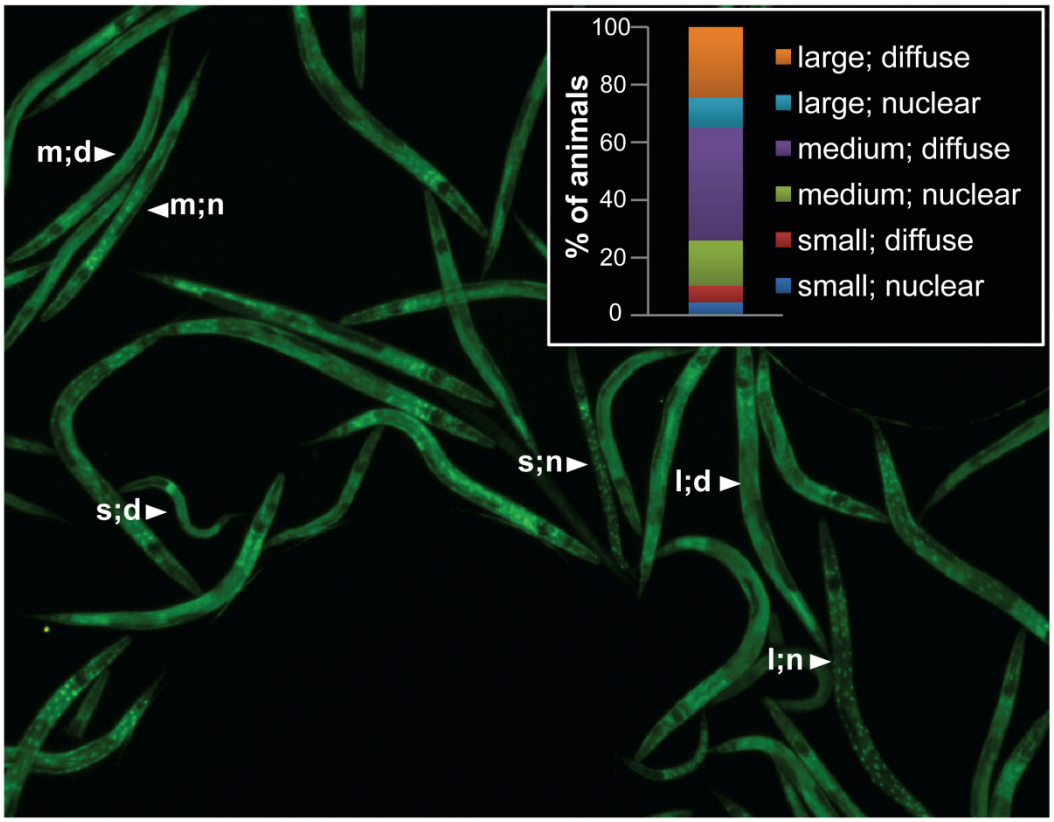

Figure S7. DAF-16 nuclear localization in *vhp-1(RNAi-dev)* animals is not correlated with its effects on their development. Representative image of DAF-16::GFP-expressing animals treated with vhp RNAi from the egg stage, showing nuclear localization in worms of different sizes/developmental stages (s, small; m, medium sized; l, large; n, nuclear; d, diffused DAF-16). **Inset,** break-down of all worms in similar images according to size and DAF-16 localization. N=1066 animals. The relative proportion of animals with nculear localization was similar in all size groups.

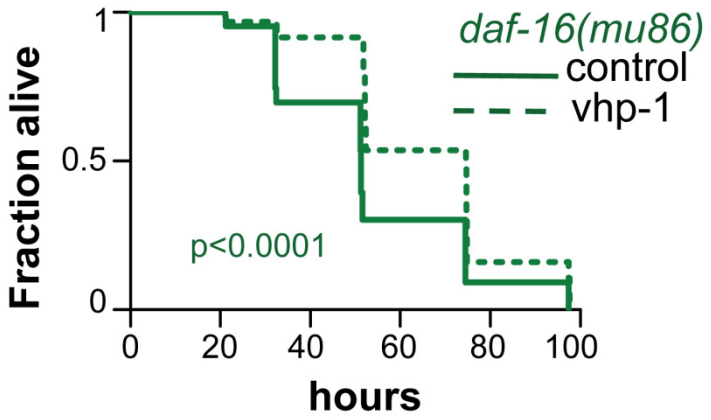

Figure S8. *daf-16* disruption does not prevent increased infection resistance in animals exposed to vhp-1 RNAi during development. Survival curve of *daf-16* mutants treated with vhp-1 RNAi during development and subsequently exposed to *P. aeruginosa*.

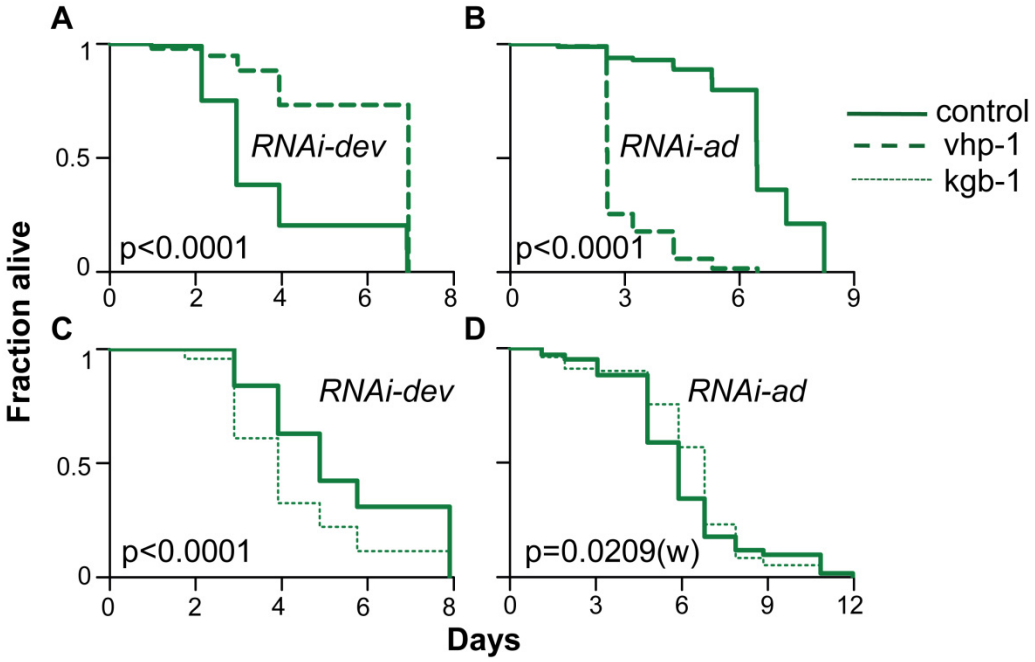

Figure S9. The age-dependent contribution of *kgb-1* to cadmium resistance is *daf-16*-independent. Survival curves for *daf-16* mutants treated with vhp-1 (A,B) or kgb-1 RNAi (C,D), in the designated stages, followed by exposure to 100μM $CdCl_2$.

Chapter 5

Downstream mediators of KGB-1's age-dependent effects

Introduction

In chapter 4, I presented data demonstrating that the outcome of activating the *C.elegans* JNK homolog, KGB-1 is dependent upon the age of activation. KGB-1 serves a stress-protective function during development, but in adults its activation decreases stress resistance and shortens general lifespan. Some of these phenotypes (infection resistance and lifespan in adults) depended on KGB-1's modulation of DAF-16; however the cadmium survival phenotypes in either age did not. In this chapter, I describe data seeking to expand on our mechanistic understanding of KGB-1's age-dependent effects. Two directions were pursued: First, characterization of KGB-1's interactions with insulin-signaling in regulating DAF-16. Secondly, I used microarray gene expression analysis as an unbiased approach to identify downstream targets of KGB-1 that operate independently of DAF-16, and used this data to identify additional mediators of *kgb-1*-depndent phenotypes.

Results

Disruptions in insulin signaling can prevent some of KGB-1's detrimental effects

In the previous chapter I showed that KGB-1 can modulate the FOXO transcription factor, DAF-16 in an age-dependent manner. While the relevance of this regulation in larvae remains to be seen, KGB-1's negative regulation of DAF-16 in adults was responsible for the infection susceptibility and lifespan reduction phenotypes following vhp-1 RNAi. This was demonstrated through epistasis analyses using *daf-16* mutants (Chapter 4, Fig. 6). We therefore reasoned that increasing DAF-16 activity in adults might ameliorate the detrimental effects of KGB-1. To this end we employed two strains (*daf-2* and *age-1*) with mutations in the IIS pathway, resulting in increased DAF-16 nuclear localization and activity [1]. Both strains are more resistant to *P. aeruginosa* than wild type animals [2]. Remarkably, treatment of either mutant with vhp-1 RNAi during adulthood resulted in a complete reversal of the increased susceptibility to infection seen in wild type animals (Fig. 1), while IIS mutants treated with vhp-1 RNAi during development retained the increased resistance to infection seen in wild type animals (Fig. S1).

Since the increased infection susceptibility of wild type vhp-1(RNAi-ad) animals is associated with the ability of KGB-1 to decrease DAF-16 nuclear localization, we wondered what effect if any KGB-1 had on DAF-16 in IIS mutants which are protected from the detrimental effect of vhp-1 RNAi on infection resistance. We therefore introduced the *age-1* mutation into the DAF-16::GFP expressing strain and assessed the effects of vhp-1 RNAi. As expected, *age-1;daf-16::gfp* animals displayed extensive DAF-16 nuclear localization under control conditions. However, unlike in animals with intact IIS, vhp-1 RNAi was unable to decrease DAF-16 nuclear localization in *age-1;daf-16::gfp* worms (Fig. 2 A, B). In order to determine whether this effect was specific to IIS we used cdc-25.1 RNAi which prevents germline proliferation [3, 4]. Removal of the germline has been shown to increase DAF-16 nuclear localization and lifespan [5-7]. Similar to IIS mutation, cdc25.1 RNAi drove DAF-16 into the nucleus; however unlike

the case of IIS mutation, vhp-1 RNAi was fully capable of driving DAF-16 out of the nucleus (Fig. 2C).

Together, these results indicate that disruptions in IIS can protect from detrimental effects of KGB-1, and that this protection is associated with the inability of KGB-1 to negatively modulate DAF-16.

vhp-1 knock-down results in robust, *kgb-1*-dependent transcriptional changes
While the detrimental effects of KGB-1 activation on infection resistance and lifespan could be attributed to negative modulation of DAF-16 activity, neither of KGB-1's effects on cadmium were dependent on *daf-16*. In order to identify additional regulators of *kgb-1*-dependent phenotypes, we used microarray analysis to determine the *kgb-1* dependent transcriptome. Our age-specific analysis allowed us to determine which elements of this transcriptome were age-dependent. On a global level, vhp-1 knock-down resulted in robust, reproducible effects on transcription. In the majority of cases, genes were induced by vhp-1 RNAi, were *kgb-1* dependent, and *daf-16*- independent (Fig. 3).

While most of the *kgb-1* regulated genes were affected similarly in larvae and adults, we identified gene subsets that were regulated by *kgb-1* in an age-specific manner (Fig. 4). Several of the genes whose expression changed following development-stage KGB-1 activation have roles in protein folding and/or ER homeostasis (GO terms protein isomerase activity, p = 4.27e-05; endoplasmic reticulum, p = 0.023). This is consistent with the KGB-1's known role in providing protection from protein folding stress [8]. Of the genes regulated by *kgb-1* specifically in adults, a subset were found to be targets of DAF-16 and some have roles in lifespan determination
(Go term determination of adult lifespan, p = 0.017). In summary, KGB-1 activation causes a number of transcriptional changes which are largely *daf-16*-independent, and some of these changes are age-specific.

Regulation of transthyretin-like genes by KGB-1 may contribute to KGB-1-dependent detrimental phenotypes
One group of genes that stood out in our microarray data were the transthyretin-like (TTR) genes. In *C.elegans* TTR is a highly expanded gene family with almost 60 members. Of the ones present in our microarray, many were induced by vhp-1 RNAi and / or increased in expression with age. In cases where TTR genes were induced by vhp-1 RNAi, the effect appeared to be stronger in adulthood or in some cases entirely limited to adulthood. In the majority of cases, the effects of age and vhp-1 RNAi on *ttr* gene expression were dependent upon *kgb-1* (Fig. 5A). The expression patterns for several genes were confirmed by qRT-PCR (Fig. 5B-D and data not shown). Given this striking pattern, we wondered whether TTRs might play a role in *kgb-1*-dependent detrimental phenotypes. To begin to address this question, we looked at stress resistance following vhp-1(RNAi-ad) in an available mutant for one of the *ttrs*, *ttr-1*. Similarly to several other members, *ttr-1* mRNA expression increased with age, and was induced by vhp-1 RNAi specifically in adults (Fig. 6A). Interestingly, *ttr-1* mutation was able to fully suppress the effect of vhp-1 (RNAi-ad) on infection susceptibility (Fig. 6B),

and to greatly attenuate the effect of vhp-1(RNAi-ad) on cadmium resistance (Fig. 6C), but was unable to suppress the effect of vhp-1 RNAi on lifespan (not shown). These results suggest that *ttr-1* and possibly other TTRs might be mediators of some of KGB-1's detrimental effects in adults.

Several transthyretin genes contain an AP-1 binding site in their promoters

The fact that many of the *ttr* genes were regulated in similar manner suggested that they might be under the control of the same transcription factor(s). We reasoned that such a transcription factor might be a target of KGB-1 and may mediate some of its effects since the expression pattern of several *ttr's* was dependent on *kgb-1*. We looked for common sequences in the promoters of all *C.elegans* TTR genes and identified the motif TGAGTCAT which is the AP-1 binding site [9] (Fig. 7A). AP-1 is a heterodimeric transcription factor typically composed of the FOS and JUN proteins. We next used RNAi to determined whether AP-1 was in fact involved in the regulation *ttr* genes. As shown in (Fig. 7B-E) knock-down of the gene encoding the *C.elegans* FOS ortholog, *fos-1* resulted in the induction of several *ttr* genes, with the effect being more pronounced in adults versus larvae. This data indicates that AP-1 is one of the mediators of KGB-1 dependent gene expression, and at least in the case of the *ttr*'s suggests that FOS-1 may act as a transcriptional repressor.

***fos-1* contributes to *kgb-1* dependent resistance to cadmium in larvae**

Given the effects of *fos-1* knock-down on kgb-*1*-dependent gene expression, we wondered whether *fos-1* was functionally important for any of the *kgb-1*-dependent phenotypes. While *fos-1* knock-down did not affect *kgb-1*-dependent infection and lifespan survival phenotypes (not shown), fos-1 RNAi had a marked effect on cadmium resistance. We found that removal of fos-1 during development resulted in increased cadmium resistance, and that this resistance could not be further increased with vhp-1 RNAi (Fig 8A). Interestingly, this effect was restricted to larvae, as *fos-1(RNAi-ad)* animals behaved similarly to controls (Fig. 8B). One explanation that is consistent with these observations is that FOS-1 is a repressor of protective responses to cadmium, and that KGB-1 acts as a repressor of FOS-1. Other possibilities which we cannot rule out at this time are addressed in the discussion section. Nevertheless, these results combined with those from the previous section demonstrate a clear involvement of AP-1 in *kgb-1*-dependent gene expression and resistance to cadmium.

Discussion

The age-dependent effect of KGB-1 activation is an intriguing phenomenon with implications for how we think about stress resistance, aging, and a variety of age-associated diseases which have been shown to be associated with JNK activity [10]. In this chapter we have sought to understand the downstream mediators and mechanisms of KGB-1's protective effects during development, and its detrimental effects in adulthood. As a starting point, we built upon data from Chapter 4 demonstrating that KGB-1's negative modulation of DAF-16 activity could explain its detrimental effects on infection resistance and lifespan.

We wondered whether increasing DAF-16 activity might be able to ameliorate some of KGB-1's detrimental effects. One way in which this can be achieved is through the mutations in the IIS pathway which results in nuclear retention of DAF-16. Strikingly, disrupting either *daf-2* or *age-1* not only suppressed the infection susceptibility of vhp-1 RNAi(ad) animals, but reverted the phenotype to increased resistance. One explanation that could account for the prevention of KGB-1's detrimental effect is that the increased DAF-16 activity caused by IIS mutation can compensate for KGB-1's detrimental effect on DAF-16. Alternatively, IIS mutations might prevent KGB-1's detrimental effect on DAF-16 altogether. However one peculiar aspect of *age-1* and *daf-2* ;vhp-1 RNAi(ad) animals was their extreme resistance to infection. While the resistance might be attributable to concomitant activation of the p38 pathway following vhp-1 RNAi, the magnitude of the increase was surprising. It has been shown that in addition to providing protection from infection, p38 activation can cause ER stress [11]. Therefore, it is possible that reduction in IIS which is known to protect against ER stress [12, 13], may 'unlock' the full protective potential of p38 by counteracting deleterious aspects of its activation.

Our subsequent experiments in adult *age-1;daf-16::gfp* animals where DAF-16 is constitutively nuclear revealed that unlike in wild-type animals, KGB-1 activation was unable to decrease DAF-16 nuclear localization. This was in contrast to germline-less animals where DAF-16 is also constitutively nuclear, but KGB-1 was able to decrease DAF-16 nuclear localization. While we cannot rule out the possibility that the signals sending DAF-16 into the nucleus following IIS disruption are simply stronger than the signals of germline removal, it is nevertheless interesting to note that these effects correlate with KGB-1's effects on infection resistance. In germline-less worms KGB-1 activation decreases survival, whereas in IIS mutants it does not. These experiments provide further evidence for the central role of KGB-1's modulation of DAF-16 in its detrimental effects in adults. Additionally, they suggest that KGB-1 may work through the IIS pathway to exert its effects on DAF-16 although additional experiments will be needed to clarify this idea.

In order to identify additional mediators of *kgb-1*-dependent phenotypes, particularly the effects on cadmium resistance which were *daf-16*-independent, we employed whole genome transcriptional analysis. The majority of *kgb-1*-dependent transcription was independent of *daf-16*, and the function of genes which were regulated by *kgb-1* in an age-specific manner correlated well with our knowledge of age-specific *kgb-1* dependent phenotypes. One group of genes, the transthyretins or *ttrs* had many members that were induced by KGB-1 activation, in several cases specifically in adulthood. In mammals the transthyretin protein's normal function is to act as regulators of thyroid hormone and retinol transport and homeostasis [14, 15]. However the misfolding and aggregation of human transthyretin leads to amyloid disease [16, 17]. Since many *ttr's* were induced by KGB-1 in *C.elegans*, we wondered if they might contribute to KGB-1's detrimental effects. *ttr-1* (one of the genes for which there was an available mutant) mutation was able to completely suppress the detrimental effect of KGB-1 on infection resistance and largely suppress the effect on cadmium resistance. These results are interesting in light of the role of human TTR in disease. Further

studies will be required to determine the mechanism(s) of *ttr-1*'s contribution to *kgb-1*-dependent phenotypes and whether this represents a general feature of the *ttr* family in *C.elegans*.

Since so many *ttr* genes were regulated by *kgb-1*, they provided an opportunity to look for downstream regulators of *kgb-1*-dependent transcription, and we identified an AP-1 binding site in the promoters of several ttr's. Furthermore we found that RNAi directed against the AP-1 subunit, *fos-1*, resulted in increases in *ttr* gene expression and in terms of *kgb-1*-dependent phenotypes, increased cadmium resistance in larvae. There are two main possibilities which could account for these effects. One is that FOS-1 acts alone as a transcriptional repressor, and is itself a target for repression by KGB-1. However the fact that AP-1 is typically a heterodimer and that FOS is not known to homodimerize or bind AP-1 sites on its own [18, 19] raise another possibility, which is that fos-1 RNAi simply 'unmasks' the activity of other AP-1 components. In this scenario, *fos-1* removal would result in gene induction by allowing the formation of alternative AP-1 complexes with stronger transcription-activating abilities than complexes containing FOS-1. One of the prominent candidates to consider would be the typical FOS partner, JUN (which has been shown to homodimerize [19]). *jun-1* is also an interesting candidate since it is a downstream target of KGB-1 in the response to bacterial pore-forming toxins [20]. Additional potential partners include some members of the ATF family which also form AP-1 complexes [21, 22]. Experiments are ongoing to determine the role of AP-1 transcription factors besides FOS-1 in *kgb-1*-dependent gene expression and phenotypes.

In summary, data from this chapter suggest that KGB-1 may act on IIS to regulate DAF-16 activity, that the transthyretin gene family may be additional mediators of KGB-1's detrimental effects and that AP-1 is likely to mediate some *kgb-1*-dependent transcription and phenotypes. Experiments aimed at identifying proteins that physically interact with KGB-1 are underway, and will provide further detailed molecular insight into KGB-1's age-dependent effects.

Experimental Procedures

Worm strains. Worm strains were obtained from the *Caenorhabditis* Genetics Center and maintained using standard procedures. Strains included are: N2, as the wild-type strain; TJ1052, *age-1(hx546)*; CB1370, *daf-2(e1370)*; KU21, *kgb-1(km21*) ; SS104, *glp-4(bn2);*VC1791*, ttr-1(ok2250); daf-16(mu86);pmk-1(km25)* double mutants were generated by mating strains KU25 and CF1038*;* TJ356, zIs356*[P_{daf-16}::daf-16::gfp;rol-6]*; *age-1(hx546)*;*P_{daf-16}::daf-16::gfp* and was generated by mating strains TJ1052 and TJ356.

RNAi by feeding was performed as described in Chapter 4.

Survival assays were performed at 25^{o}C in triplicate with approximately 100 animals per group, per experiment. For details see Chapter 4.

RNA extraction and Quantitative (q)RT-PCR was performed as in Chapter 4.

GFP imaging of *daf-16::gfp* and *age-1*;*daf-16::gfp* transgenic animals was performed as described in Chapter 4.

Statistical analyses of survival curves were performed as described in Chapter 4.

Microarrays. RNA was extracted from several hundred worms per group using Trizol (Invitrogen). After extraction, RNA was treated with Turbo DNAse (Ambion). Samples were co-hybridized with a similarly treated reference RNA sample without amplification to Epoxy (Corning) microarrays spotted with 60-mer oligonucleotides (Washington University Genome Sequencing Center). Static hybridizations were done under M-series Lifterslips (Erie Scientific). Hybridizations were done at 43^{o}C overnight (16-20hrs) in formamide-based buffer using the Genisphere Array350 labeling system in chambers humidified with 4X SSC buffer. Three biological replicates were performed for each condition in wild-type and *kgb-1* mutant animals, and two biological replicates were performed in *daf-16;pmk-1* mutants.

For Global gene expression profiles obtained with microarrays, samples from wild-type animals were analyzed using a two-way ANOVA (with RNAi and age as the two factors) with data permuted within an age group (RNAi-dev or RNAi-ad) for 200 iterations. Analysis was implemented in MATLAB. Genes that were statistically significantly regulated (with a false discovery rate of 10%) in wild-type animals were identified, and corresponding gene expression profiles from mutant animals were appended to this data. Genes regulated by vhp-1 RNAi in an age-specific manner were identified using Pearson correlation. Briefly, the expression profile for a gene demonstrating an expression pattern of interest (for example, induced only during development) was selected and the expression profiles of all other genes from the original dataset were tested against it for their Pearson correlation with the original gene. Genes used for 'benchmark' patterns were: induced in larvae: C14C6.5, repressed in larvae: Y45F10C.4; induced in adults: F41E6.6; repressed in adults: D1086.3. Cutoffs for correlation were determined by visual inspection of heat maps for each expression pattern of interest.

PCR Primers.

ttr-1 forward	CTGACTCGGCTGGATTCTTT
ttr-1 reverse	TGGAATGTCGACACGGAGT
ttr-18 forward	CTTGCCGGAAACAAGAAAGA
ttr-18 reverse	AGTCTCTCCCTCGCTGATGA
ttr-20 forward	CGGCTGGAGTGAAGGTTAAG
ttr-20 reverse	TCACCTTCGGGTCGATAGTC
ttr-52 forward	TTCCAAGTGACTGGTTGTGC
ttr-52 reverse	TTGATGGTAGGAACAGGGGTA
pan-actin forward	TCGGTATGGGACAGAAGGAC
pan-actin reverse	CATCCCAGTTGGTGACGATA

References

1. Mukhopadhyay, A., S.W. Oh, and H.A. Tissenbaum, *Worming pathways to and from DAF-16/FOXO.* Exp Gerontol, 2006. **41**(10): p. 928-34.

2. Garsin, D.A., et al., *Long-lived C. elegans daf-2 mutants are resistant to bacterial pathogens.* Science, 2003. **300**(5627): p. 1921.

3. Ashcroft, N. and A. Golden, *CDC-25.1 regulates germline proliferation in Caenorhabditis elegans.* Genesis, 2002. **33**(1): p. 1-7.

4. Kim, J., et al., *A mutation of cdc-25.1 causes defects in germ cells but not in somatic tissues in C. elegans.* Mol Cells, 2009. **28**(1): p. 43-8.

5. Lin, K., et al., *Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling.* Nat Genet, 2001. **28**(2): p. 139-45.

6. Yamawaki, T.M., et al., *Distinct activities of the germline and somatic reproductive tissues in the regulation of Caenorhabditis elegans' longevity.* Genetics, 2008. **178**(1): p. 513-26.

7. Berman, J.R. and C. Kenyon, *Germ-cell loss extends C. elegans life span through regulation of DAF-16 by kri-1 and lipophilic-hormone signaling.* Cell, 2006. **124**(5): p. 1055-68.

8. Mizuno, T., et al., *Role of the Caenorhabditis elegans Shc adaptor protein in the c-Jun N-terminal kinase signaling pathway.* Mol Cell Biol, 2008. **28**(23): p. 7041-9.

9. Lee, W., P. Mitchell, and R. Tjian, *Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements.* Cell, 1987. **49**(6): p. 741-52.

10. Sabapathy, K., *Role of the JNK pathway in human diseases.* Prog Mol Biol Transl Sci, 2012. **106**: p. 145-69.

11. Richardson, C.E., T. Kooistra, and D.H. Kim, *An essential role for XBP-1 in host protection against immune activation in C. elegans.* Nature. **463**(7284): p. 1092-5.

12. Henis-Korenblit, S., et al., *Insulin/IGF-1 signaling mutants reprogram ER stress response regulators to promote longevity.* Proc Natl Acad Sci U S A, 2010. **107**(21): p. 9730-5.

13. Morley, J.F., et al., *The threshold for polyglutamine-expansion protein aggregation and cellular toxicity is dynamic and influenced by aging in Caenorhabditis elegans.* Proc Natl Acad Sci U S A, 2002. **99**(16): p. 10417-22.

14. Schreiber, G., *The evolutionary and integrative roles of transthyretin in thyroid hormone homeostasis.* J Endocrinol, 2002. **175**(1): p. 61-73.

15. Monaco, H.L., *The transthyretin-retinol-binding protein complex.* Biochim Biophys Acta, 2000. **1482**(1-2): p. 65-72.

16. Kelly, J.W., et al., *Transthyretin quaternary and tertiary structural changes facilitate misassembly into amyloid.* Adv Protein Chem, 1997. **50**: p. 161-81.

17. Saraiva, M.J., *Transthyretin mutations in hyperthyroxinemia and amyloid diseases.* Hum Mutat, 2001. **17**(6): p. 493-503.

18. Mason, J.M., et al., *Semirational design of Jun-Fos coiled coils with increased affinity: Universal implications for leucine zipper prediction and design.* Proc Natl Acad Sci U S A, 2006. **103**(24): p. 8989-94.

19. Halazonetis, T.D., et al., *c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities.* Cell, 1988. **55**(5): p. 917-24.

20. Kao, C.Y., et al., *Global functional analyses of cellular responses to pore-forming toxins.* PLoS Pathog, 2011. **7**(3): p. e1001314.

21. Hess, J., P. Angel, and M. Schorpp-Kistner, *AP-1 subunits: quarrel and harmony among siblings.* J Cell Sci, 2004. **117**(Pt 25): p. 5965-73.

22. van Dam, H. and M. Castellazzi, *Distinct roles of Jun : Fos and Jun : ATF dimers in oncogenesis.* Oncogene, 2001. **20**(19): p. 2453-64.

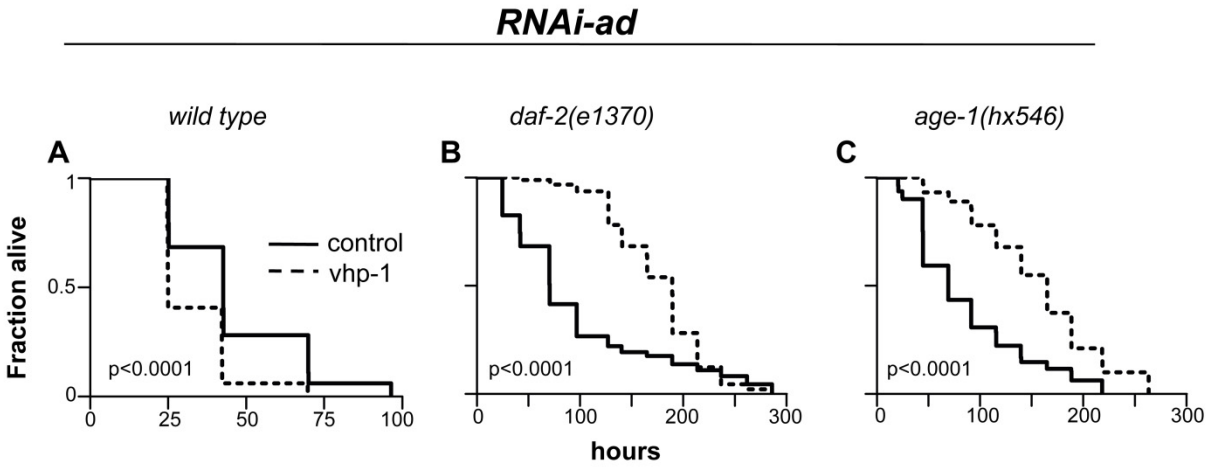

Figure 1. Mutations in insulin signaling prevent the detrimental effects of KGB-1 on infection resistance. Survival curves for indicated strains treated with RNAi from the L4-stage until day-two of adulthood, and then transferred to *P. aeruginosa plates.*

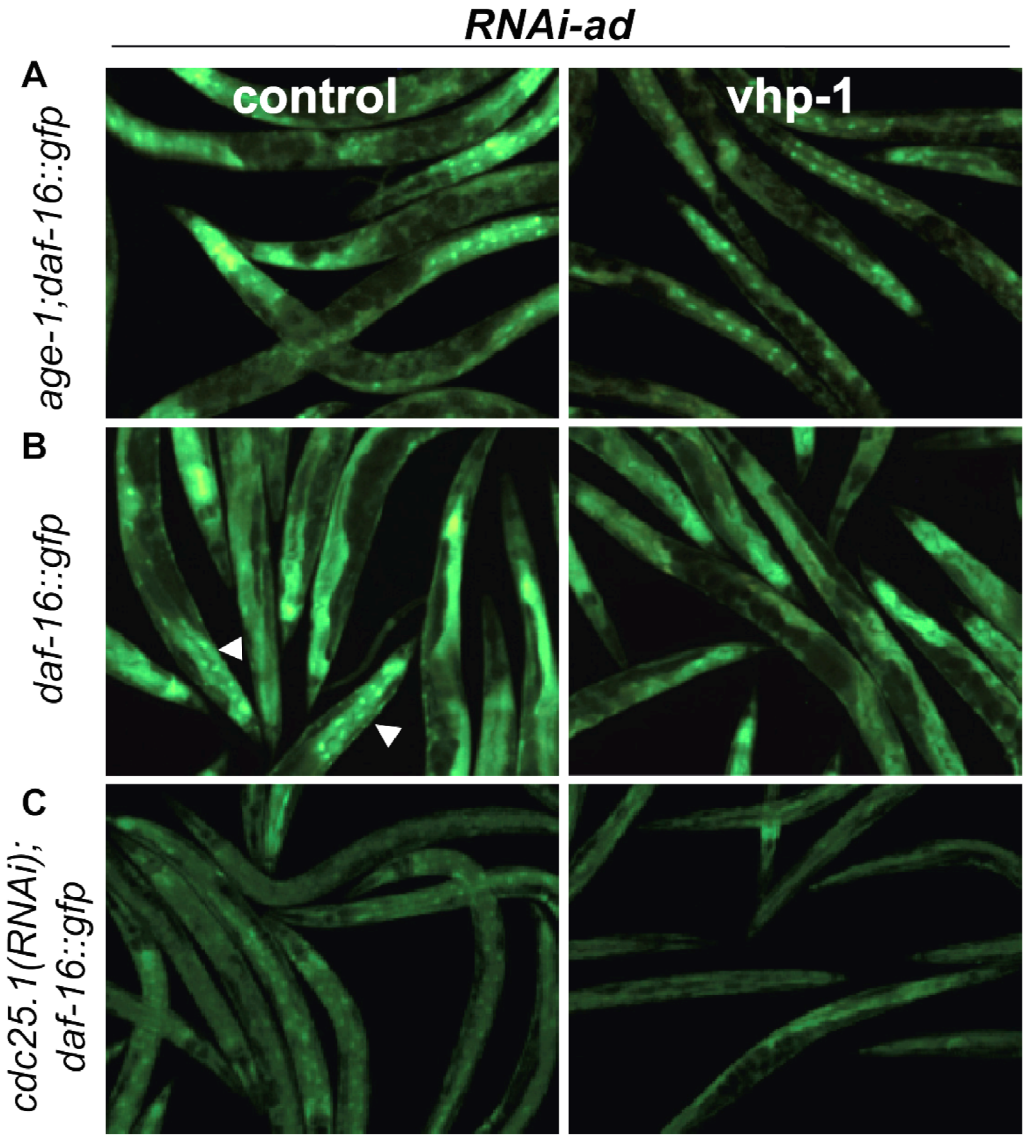

Figure 2. A disruption in insulin signaling prevents KGB-1's effect on DAF-16 nuclear localization in adults. Images show worms containing a *daf-16::gfp* translational fusion and the *age-1(hx546)* mutation (A) , the non-manipulated strain (B) or treated with cdc25.1 RNAi maternally and throughout development (C). In all panels, control or vhp-1 RNAi was performed from the L4-stage until day two of adulthood.

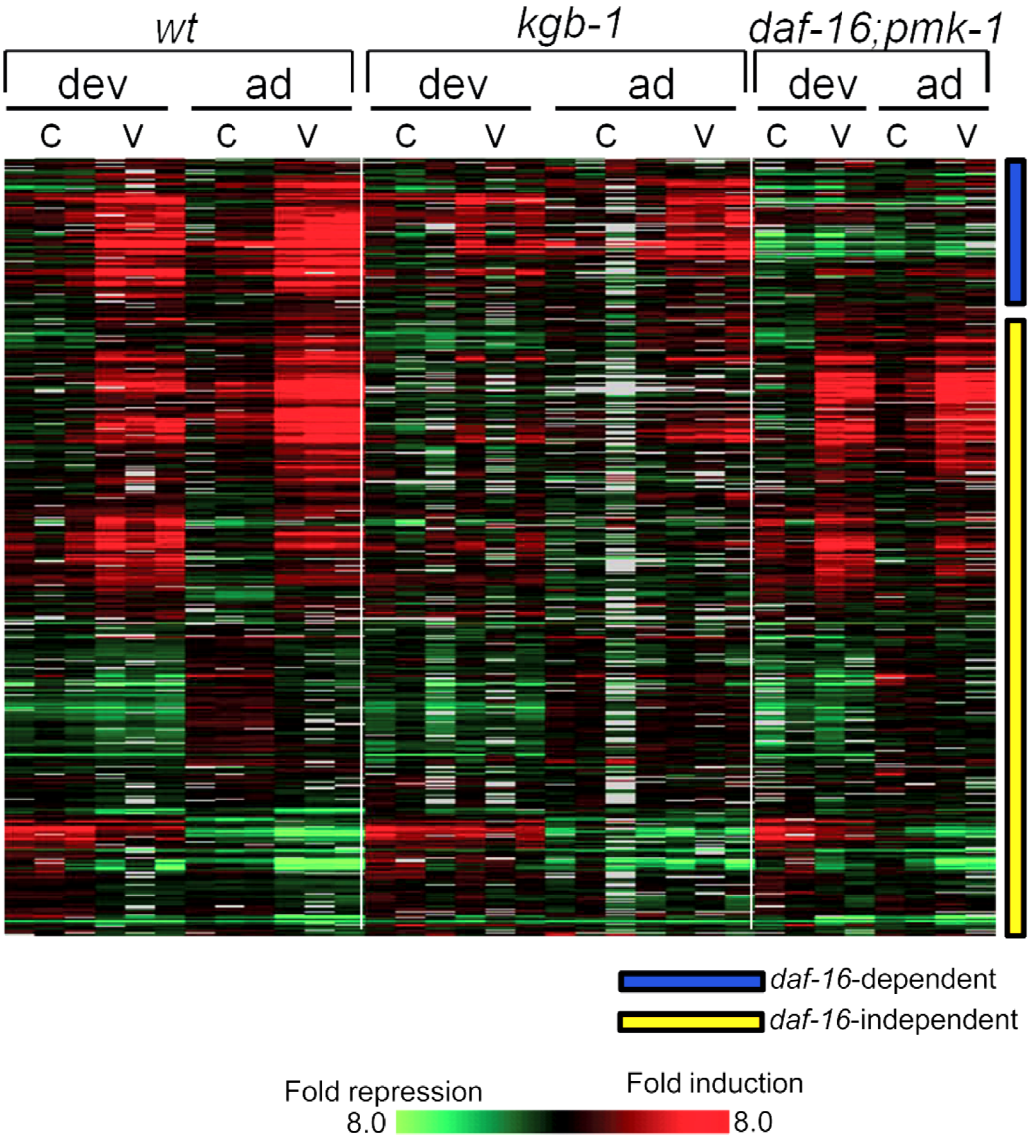

Figure 3. Genome-wide transcriptional changes following vhp-1 RNAi. Heat map of microarray data showing genes differentially regulated by vhp-1 RNAi in wild type animals, and their expression in the indicated mutants.

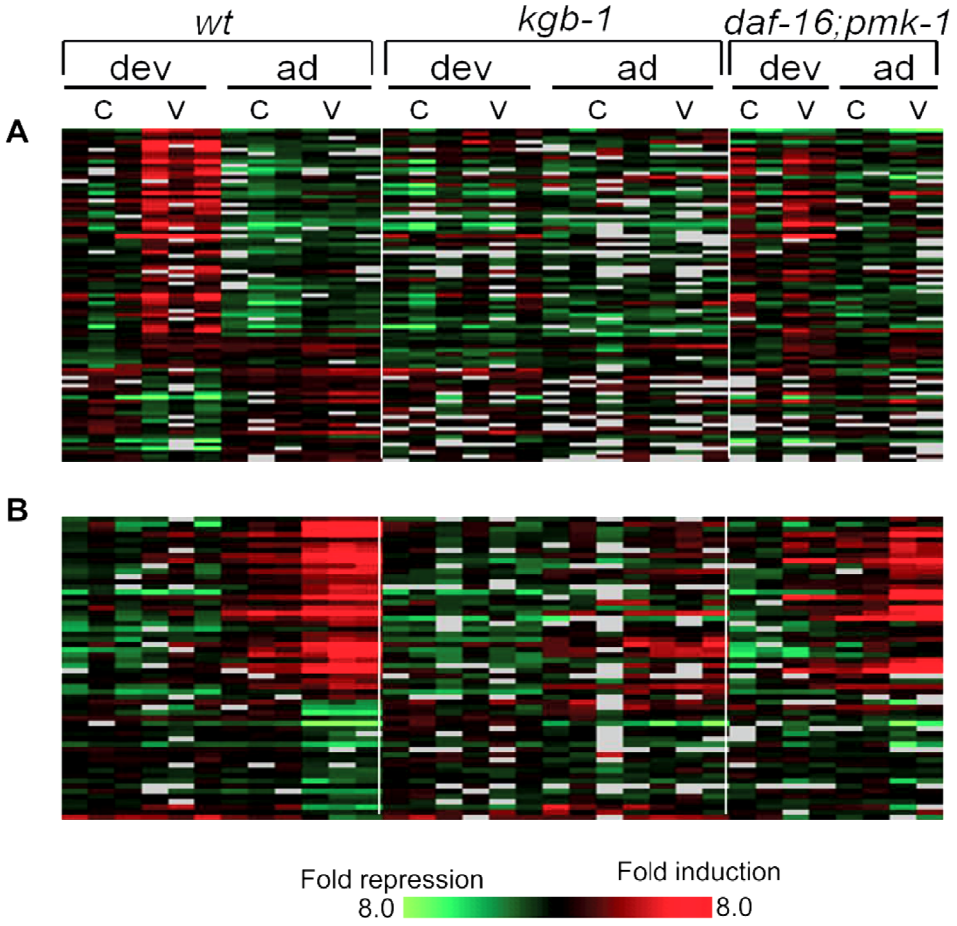

Figure 4. Age-specific effects of vhp-1 RNAi. Heat map of microarray data showing genes regulated by vhp-1 RNAi specifically during development (A) or during adulthood (B)

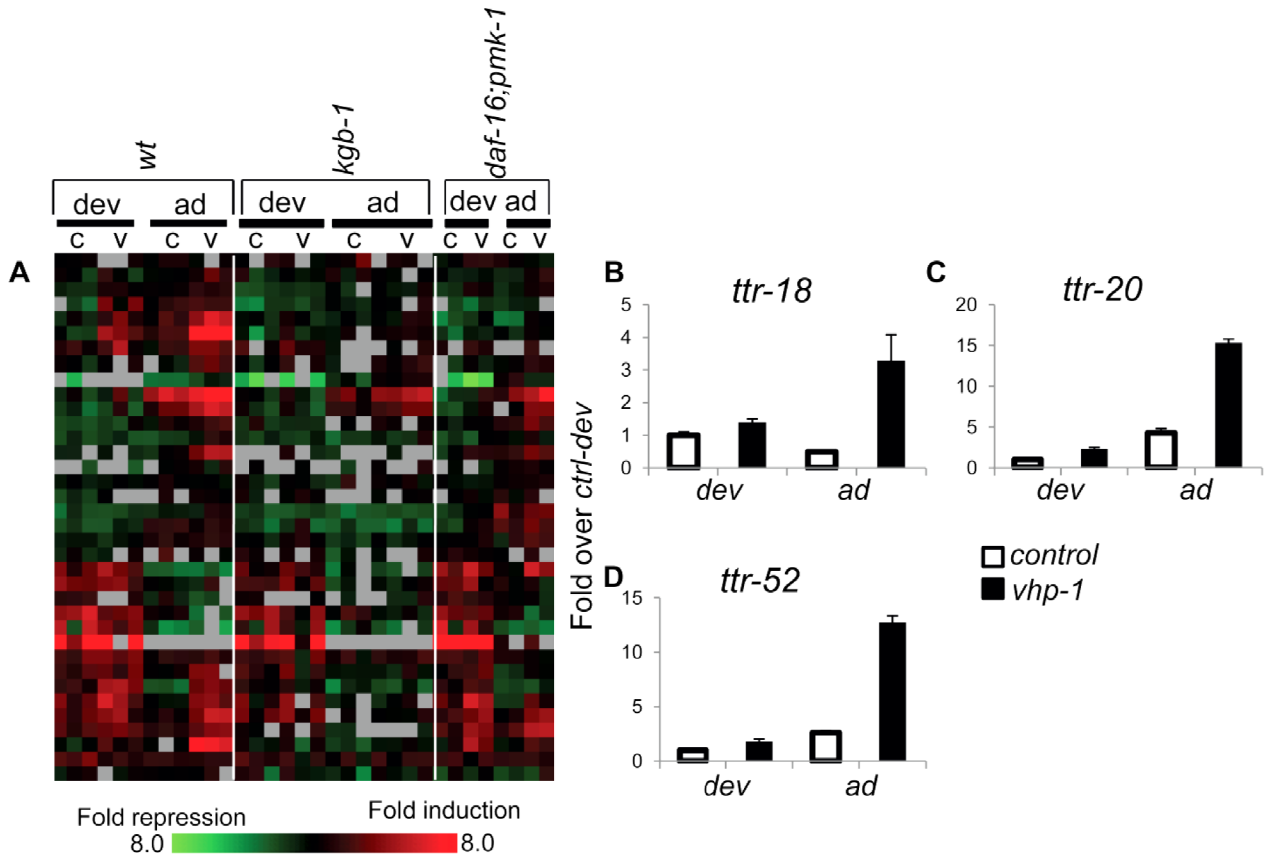

Figure 5. Several *C.elegans* transthyretin genes are regulated by KGB-1. (A) Heat map showing *C.elegans* TTR Gene expression measured by microarray following control (c) or vhp-1 (v) RNAi in the indicated strains. (B-D) qRT-PCR confirmation of the expression of three TTR genes in wild-type animals.

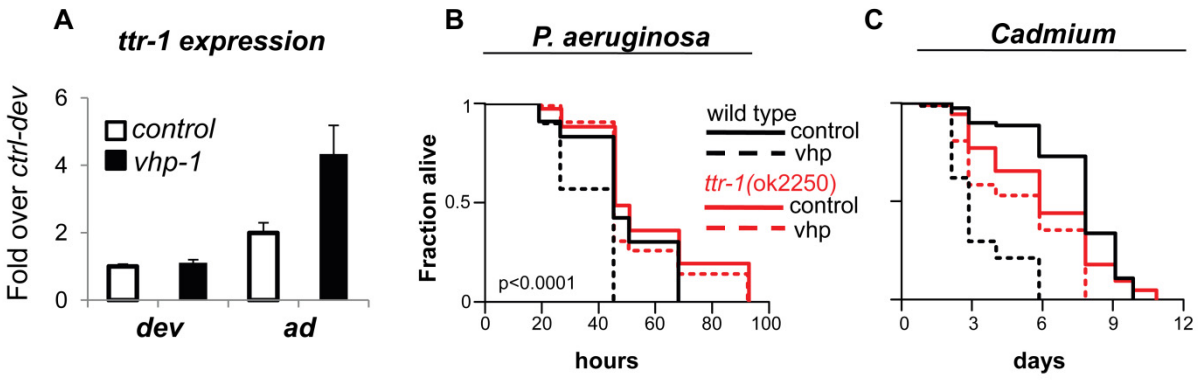

Figure 6. *ttr-1* is induced by vhp-1 RNAi and its disruption prevents some of the KGB-1-dependent detrimental phenotypes. (A) *ttr-1* genes expression measured by qRT-PCR in wild-type animals. (B-C) Survival curves for wild-type or *ttr-1* mutants treated with RNAi as indicated from the L4 stage until day-two of adulthood and then transferred to *P. aeruginosa* (B) or cadmium plates (C)

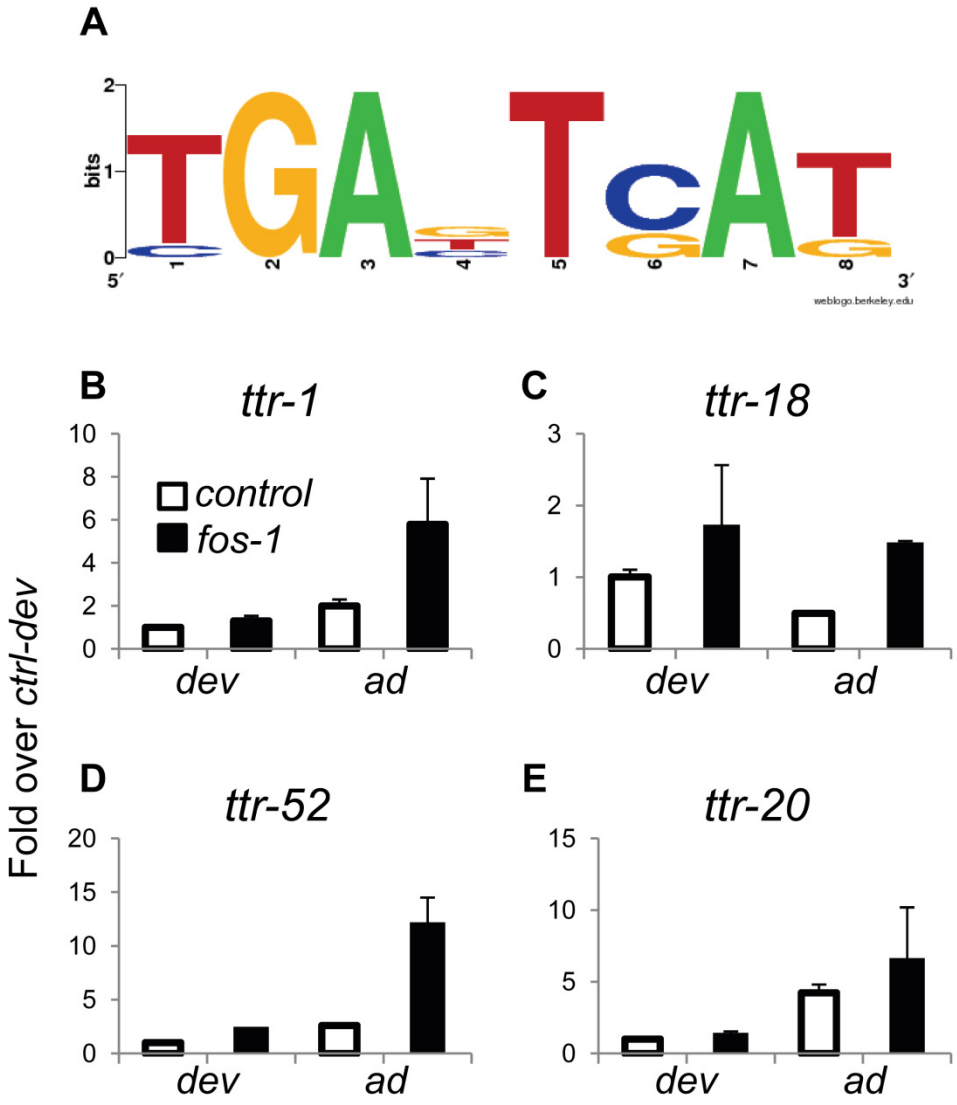

Figure 7. *fos-1* contributes to *kgb-1*-dependent TTR gene expression. (A) An AP-1 motif identified with Bioprospector by searching the 500bp immediately upstream of all *C.elegans* TTR genes. The motif logo was generated using weblogo.berkeley.edu. (B-E) *ttr-1* genes expression measured by qRT-PCR in wild-type animals following control or fos-1 RNAi.

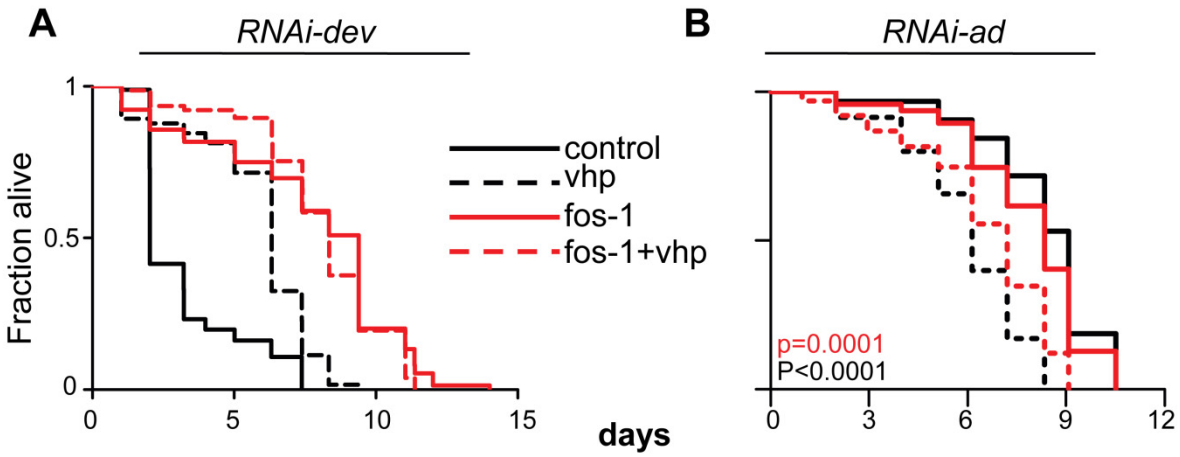

Figure 8. *fos-1* contributes to *kgb-1*-dependent protection from cadmium in larvae. Survival curves for wild-type animals treated with the indicated RNAi during development (A) or adulthood (B) and then transferred to cadmium plates.

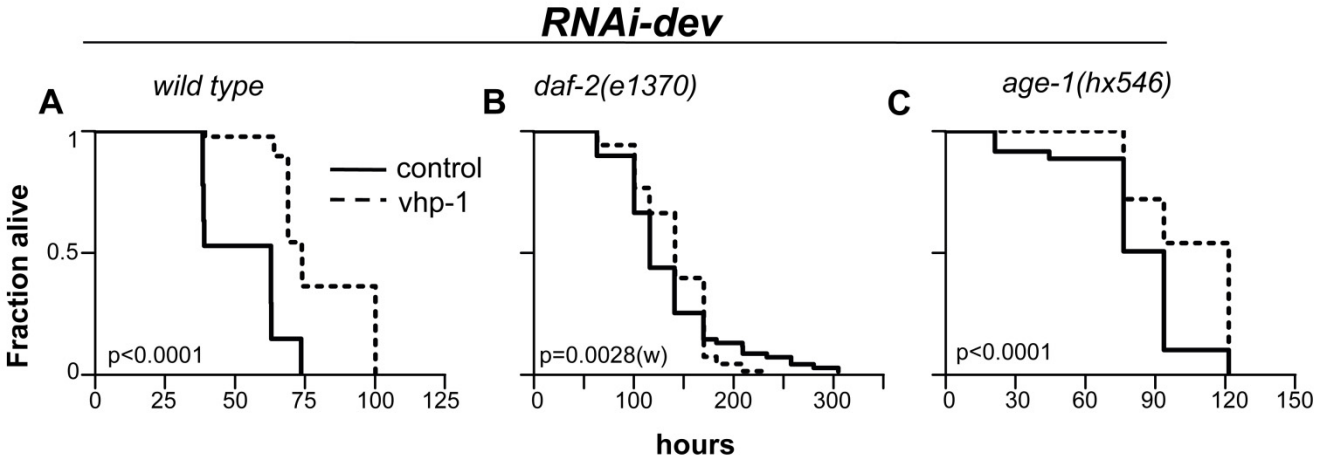

Figure S1. vhp-1 RNAi provides protection from infection in insulin signaling mutants. Survival curves for wild-type or *daf-2* mutant animals treated with the indicated RNAi during development and then transferred to *P. aeruginosa* plates.

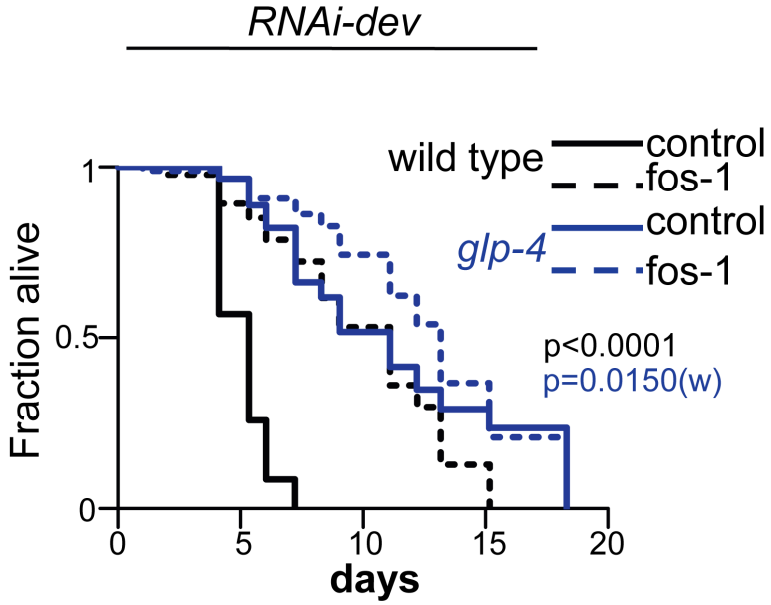

Figure S2. The contribution of *fos-1* cadmium protection is not solely dependent on reproduction. Survival curves for wild-type or *glp-4(bn2)* animals treated with the indicated RNAi during development and then transferred to cadmium plates.