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An Evolutionarily Conserved Innate Immunity Protein Interaction Network*[S]

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Background: Innate immunity affects infectious and inflammatory diseases.
Results: Using RNAi and proteomic data, we identified a novel evolutionarily conserved protein network that modulates innate immunity.
Conclusion: Studies using mutant *C. elegans* and mice demonstrate the utility of this network for disease investigation.
Significance: This innate immunity network provides a novel set of targets for future innate immunity disease studies.

The innate immune response plays a critical role in fighting infection; however, innate immunity also can affect the pathogenesis of a variety of diseases, including sepsis, asthma, cancer, and atherosclerosis. To identify novel regulators of innate immunity, we performed comparative genomics RNA interference screens in the nematode *Caenorhabditis elegans* and mouse macrophages. These screens have uncovered many candidate regulators of the response to lipopolysaccharide (LPS), several of which interact physically in multiple species to form an innate immunity protein interaction network. This protein interaction network contains several proteins in the canonical LPS-responsive TLR4 pathway as well as many novel interacting proteins. Using RNAi and overexpression studies, we show that almost every gene in this network can modulate the innate immune response in mouse cell lines. We validate the importance of this network in innate immunity regulation *in vivo* using available mutants in *C. elegans* and mice.

The innate immune response plays a critical role in fighting infection (1) but can also affect the pathogenesis of numerous diseases with an inflammatory component (2–5). Evidence for the importance of innate immunity in host defense in humans comes from the identification of polymorphisms in innate immunity signaling genes that render those individuals highly susceptible to infection (6). The innate immune response can also affect the risk of many diseases with an inflammatory component ranging from atherosclerosis to arthritis to cancer to sepsis (3, 7–10). Thus, it is critical that the innate immune response be properly modulated, active when needed to fight infection, and inactive when not needed to prevent other diseases. Thus, genes that transduce innate immune signals have become targets for the development of therapeutics for numerous indications (11), and polymorphisms in those genes that affect disease risk could be used to develop diagnostic tests for personalized medicine (3).

We have performed comparative genomics RNAi screens in *Caenorhabditis elegans* and mouse macrophages to identify novel, conserved regulators of innate immunity (12). The nematode *C. elegans* lacks an adaptive immune response, but it does have an innate immune response that involves production of antimicrobial genes to fight infection. Most but not all *C. elegans* pathogens infect the nematode digestive tract and induce the increased expression of antimicrobial genes in intestinal epithelial cells (13, 14). Production of these presumed antimicrobials is controlled by many conserved innate immunity signaling genes (15–23). The pattern of antimicrobial genes induced by different pathogens is specific to each pathogen (15, 18, 23–27), suggesting that *C. elegans* can distinguish between pathogens, although the mechanisms of how it does so are still unclear. Macrophages are key phagocytic innate immune cell that affect many diseases (28). Our hypothesis is that by identifying orthologous genes that affect innate immunity in multiple species, suggesting evolutionary conservation of their function, we can expect that these genes will likewise affect innate immunity and disease in humans.

Using RNAi screens in these simple model systems, we have identified many candidate regulators of innate immunity (12, 29–31). To sort through these candidates and determine their possible function, we examined our candidates for potential protein-protein interactions using a *C. elegans* protein interaction database (12). This led to the discovery of a network of proteins that included several of our candidate innate immunity regulators. Here, we expand the analysis of this innate immunity protein interaction network to include interactions in multiple species (*C. elegans*, *Drosophila*, and humans). Using

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[S] This article contains supplemental Figs. 1 and 2 and Tables 1–3.

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RNAi, we show that almost every gene in this network modulates the response to lipopolysaccharide (LPS) in mouse macrophage cell lines. We then validated the importance of this network *in vivo* using available mutations in *C. elegans* and mice. Thus, this novel innate immunity protein interaction network should provide a valuable resource for further innate immunity and inflammatory disease studies.

EXPERIMENTAL PROCEDURES

RNAi in Mouse Macrophage Cell Lines—RNAi was performed largely as described previously (12). In brief, pools of four siRNA duplexes or individual siRNA duplexes (Dharmacon) were transfected into either of two mouse macrophage cell lines (J774A.1 or RAW264.7) using the Amaxa nucleofector 96-well shuttle according to the manufacturer's instructions. Negative control siRNAs used were either a pool of four siRNA duplexes that do not target any mouse gene (Dharmacon nontargeting pool 1) or a single siRNA duplex not targeting any gene (Dharmacon nontargeting siRNA 1). Cells were then plated at either 100,000 cells/well in a 96-well format for ELISAs or 250,000 cells per well in a 6-well format for qPCR[2] studies. 24 h after plating, the cells were exposed to the indicated pathogen-associated molecular patterns (PAMPs) for 6 h. *Escherichia coli* O111:B4 LPS was from List Biological Laboratories; PAM3CSK4 was from Invivogen. The LPS dose of 20 ng/ml was chosen for RNAi experiments as this dose gave an essentially complete response without overwhelming the system. Six-hour exposures were used as this would capture rapidly induced cytokines such as TNFα and cytokines induced later such as IL-6 (32). Following the exposures, cytokine production was monitored by ELISA (R&D Systems) on cell supernatants. The cells were then either subjected to viability analysis or were used to generate RNA for qPCR.

Viability of the cells was monitored by staining cells with fluorescein diacetate and measuring resultant fluorescence on a plate reader as described (33). For qPCR studies, RLT buffer was added directly to the cells, and RNA was purified using the RNeasy kit (Qiagen). qPCR was then performed using the QuantiTect SYBR Green RT-PCR assay kit (Qiagen) and an ABI 7900 Real Time thermocycler. Primer sequences used for qPCR are listed in supplemental Table 1. Expression levels were normalized using primers for β-actin.

In separate experiments, phagocytosis was monitored using cells subjected to RNAi as described above. Phagocytosis of FITC-labeled *E. coli* particles was measured using the Vybrant phagocytosis assay kit (Molecular Probes) as described (34).

C. elegans Survival Assays—*C. elegans* survival assays were conducted largely as described previously (35). In brief, animals in the late L4 stage were exposed to either pathogenic *Pseudomonas aeruginosa* strain PA14 (36) or nonpathogenic *E. coli* strain OP50 at 25 °C on standard nematode growth medium (37). The sole exception to this was the temperature-sensitive *repo-1* mutant, which was allowed to develop at the permissive temperature (15 °C) and which was subsequently exposed to PA14 at the restrictive temperature of 26 °C at the young adult stage. Life span analysis using *E. coli* was performed in the presence of the sterilizing agent 5-fluoro-2′-deoxyuridine (38). For the survival assays in the presence of heat-killed *E. coli*, bacteria were incubated at 65 °C for 3 h, concentrated 10-fold, and plated on nematode growth medium plates (37) supplemented with 50 μg/ml ampicillin. Strains used were wild type N2, FX05176 *klp-12*(*tm5176*) IV, VC767 *set-18*(*gk334*) I, FX01968 *siah-1*(*tm1968*) IV, VC812 *tag-260*(*ok1339*) V, and *repo-1*(*or430ts*) IV. The *repo-1* strain was outcrossed four times; the *klp-12* and *siah-1* strains were outcrossed twice. The *set-18* and *tag-260* strains were not reported to be outcrossed. The presence of all deletions was verified by PCR on genomic DNA. Deletion mutations have also been isolated in *klp-7*/Kif2a and *ant-1.1*/Slc25a5; both homozygous mutant strains were reported to be lethal or arrest during development and were therefore not tested in pathogen assays. Occupancy of the bacterial lawn and pharyngeal pumping rates of *repo-1* mutant animals were monitored 18 h after shifting the young adults onto *P. aeruginosa* bacteria.

Generation of Macrophages Overexpressing Macf1—For the *Macf1* overexpression experiments, a plasmid containing the *Macf1* cDNA cloned downstream of the CMV promoter (39) was co-transfected with plasmids containing NFκB-AP1-luc (a 133-bp derivative of the IL-8 promoter driving firefly luciferase expression (40)) and SV40-rluc (normalization control from Promega) into RAW264.7 cells using FuGENE-HD (Roche Applied Science) according to the manufacturer's instructions. 24 h after transfection, cells were exposed to LPS for 6 h, and luciferase activity was measured using the Dual-Luciferase reporter assay kit (Promega). Firefly luciferase activity was normalized relative to *Renilla* luciferase activity. As a control, a plasmid driving expression of chloramphenicol acetyltransferase (CAT) using the CMV promoter (pCDNA3.1/CAT, Invitrogen) was transfected in place of Macf1 in some experiments.

Generation and Phenotyping of Bone Marrow-derived Macrophages (BMDM) with Decreased Macf1 Expression—Generation of the $Macf1^{flox/flox}$ mice has been described (41). $Macf1^{flox/flox}$ mice were crossed with B6.129-$Lyzs^{TM1(cre)Ifo}$/J mice (JAX) (42). The *Lyzs* promoter drives cre expression and thus deletion of *Macf1* in the myeloid lineage in this strain. BMDM lacking *Macf1* ($Macf1^{flox/flox}$, $Lyzs^{cre/cre}$) were compared with control BMDM expressing *Macf1* ($Macf1^{+/+}$, $Lyzs^{cre/cre}$); both groups of mice were siblings derived from $Macf1^{flox/+}$, $Lyzs^{cre/cre}$ × $Macf1^{flox/+}$, $Lyzs^{cre/cre}$ matings.

BMDM were generated as described (34). In brief, femur and tibia marrow was harvested, filtered, and plated in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), penicillin and streptomycin (Fisher), and 20 ng/ml mouse M-CSF (R&D Systems). After 6 days, nonadherent stem cells were washed away, and adherent cells were collected by trypsinization for further experiments. Plating was performed similarly to the RNAi experiments described above. The extent of bone marrow stem cells differentiating into macrophages was similar between $Macf1^{flox/flox}$ mice and their wild type siblings (determined by F4/80 staining, wild type BMDM 76 ± 6% F4/80$^+$, 21 ± 3 mean fluorescence intensity; $Macf1^{flox/flox}$ BMDM 83 ±

[2] The abbreviations used are: qPCR, quantitative PCR; PAMP, pathogen-associated molecular pattern; BMDM, bone marrow-derived macrophage.

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3% F4/80$^+$, 21 ± 3 mean fluorescence intensity, $n = 4$, $p = 0.98$, both strains were homozygous for *Lyzs*$^{cre/cre}$).

Statistical Analyses—All data are from a minimum of three biological replicates. Statistical analyses for ELISAs and qPCRs were performed in GraphPad Prism 5 using unpaired *t* tests to determine significance ($p < 0.05$). Analysis of *C. elegans* survival data also was performed using Graphpad Prism 5. We previously presented siRNA data for *Kif2a* siRNA (12); in this prior study, *Kif2a* siRNA's effect on the LPS response did not reach statistical significance, largely due to one outlier in the data (mean = 78% of control, $n = 8$, $p = 0.197$). The data in this study are statistically significant (mean 52%, $n = 7$, $p = 0.003$), and both data sets when considered together are also significant (mean 66%, $n = 15$, $p = 0.0028$).

RESULTS

Identification of an Innate Immunity Protein Interaction Network—Using comparative genomics RNAi screens in *C. elegans* and mouse macrophages, we previously identified a protein interaction network that includes several proteins that modulate the innate immune response (supplemental Fig. 1) (12). RNAi-mediated inhibition of several genes in this network either decreased expression of putative antimicrobial genes in *C. elegans* or production of LPS-induced inflammatory cytokines in murine macrophages or both (12). This protein interaction network was initially identified based largely on published interactions identified using yeast two-hybrid assays on *C. elegans* proteins (43). This proteomic approach has been extensively validated and has a roughly 80% confirmation rate using secondary binding assays (43–46). To further define the members of this protein interaction network, we searched multiple protein-protein interaction databases to identify all published protein-protein interactions in this network in *C. elegans* (43, 47), *Drosophila* (48, 49), or humans (46, 50–54), focusing on homologous proteins in each species (gene homologies in supplemental Table 2). These database searches were used to refine the network and were followed by a comprehensive literature review of each network protein in all three species. The final version of the innate immunity protein interaction network is depicted in Fig. 1; for simplicity, only proteins present in mammals are depicted in Fig. 1, even though this network depicts interactions identified in multiple species. The complete list of protein-protein interactions in Fig. 1 are listed in Table 1. All these interactions are *bona fide* protein-protein interactions (not computational predictions), and many have been identified multiple times in multiple species using several different binding assays.

In addition to many novel candidate proteins, this network contains several proteins in the canonical LPS response pathway. The canonical LPS response pathway in this network includes a MyD88 family member, TRAF6, IκBα, and two IκBα regulators, the E3 ubiquitin ligase SCF$^{\beta\text{-TrCP}}$ and the E2 ubiquitin-conjugating enzyme UBE2d2 (55, 56). We previously showed that RNAi-mediated inhibition of three genes in this network (*Siah1, Macf1,* and *Ube2d2*) affected production of putative *C. elegans* antimicrobial genes, and RNAi-mediated inhibition of two network genes (*Siah1a* and *Macf1*) affected LPS-induced cytokine production in mouse macrophages (12).

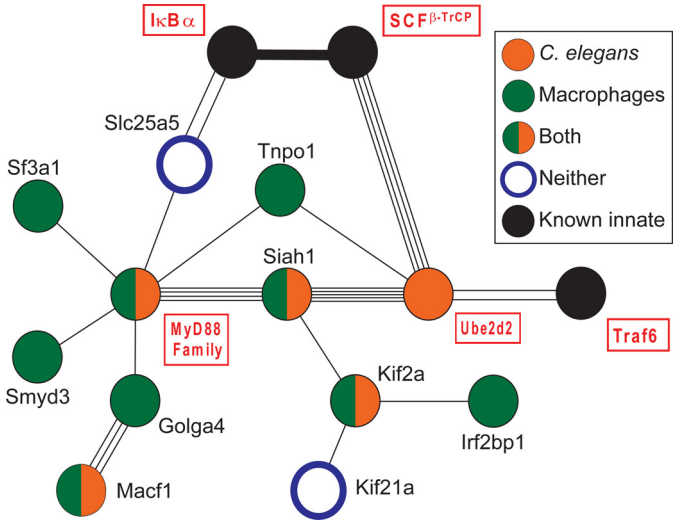

FIGURE 1. **An innate immunity protein interaction network.** The protein interaction map depicts human proteins with known homologous protein-protein interactions in *C. elegans*, humans, or *Drosophila* (list of protein-protein interactions with references is given in Table 1 and a list of gene homologs is provided in supplemental Table 2). Each *line* between proteins indicates a single reported interaction; *multiple lines* indicate multiple reports of that interaction. *Solid black bar* indicates a well established biochemical interaction with multiple reports. Proteins with an RNAi-induced phenotype in *C. elegans* (decreased antimicrobial production (12)) or mouse macrophages (decreased LPS-induced IL-6 production, see Fig. 2 and Ref. 12) are color-coded as indicated. Proteins in the canonical TLR4 LPS response pathway that are already known to regulate innate immunity are labeled with *red boxes* around the protein name.

Although several genes in this network are in the canonical pathway for the LPS response, most genes in this network have not previously been implicated in innate immunity regulation and have not been tested by us or others for an effect on the response to LPS.

Most Genes in This Network Modulate LPS-induced Cytokine Production in Mouse Macrophage Cell Lines—To test the function of the remaining genes in this network, we used RNAi to inhibit 10 genes as follows: nine additional novel candidates as well as the known TLR4 signaling gene MyD88 as a control. Pools of four siRNA duplexes targeting each of these genes were transfected into the J774A.1 mouse macrophage cell line; the cells were stimulated with LPS, and then cytokine production was monitored by ELISA. As a positive control, we showed that inhibition of the LPS receptor TLR4 strongly inhibited IL-6 production (Fig. 2*A*). Inhibition of seven of the 10 network genes led to a statistically significant decrease in LPS-induced IL-6 production (Fig. 2*A*) without affecting cell viability (Fig. 2*B*). qPCR analysis demonstrated that these siRNA treatments were inhibiting expression of the corresponding endogenous gene (Fig. 2*C*).

Sf3a1 and Golga4 Regulate LPS-induced Cytokine Secretion—We performed several further RNAi experiments with the two novel genes that exhibited the strongest phenotypes, *Sf3a1* and *Golga4*. First, we demonstrated that multiple individual siRNA duplexes targeting each of these two genes induced similar phenotypes, a decrease in LPS-induced IL-6 production (Fig. 3). Multiple individual duplexes exhibit the same phenotype, suggesting that the effect on cytokine production is due to inhibition of the corresponding endogenous gene. We also monitored the produc-

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TABLE 1
Published protein-protein interactions in innate immunity protein interaction network

Interaction/species	Interactions[a]	Ref.
Siah1-MyD88		
Human	Y2H	106
Human	Co-IP	106
Siah1-MyD88 family (*tir-1*)[b]		
C. elegans	Y2H	43
C. elegans	Y2H	16
Siah1-Ube2d2		
Human	Y2H	107
Human	Y2H	108
Drosophila	Y2H	109, 110
Human	E2	111
Human	E2	112
Siah1-Kif2a		
C. elegans	Y2H	43
Kif2a-Irf2bp1		
C. elegans	Y2H	43
Kif2a-Kif21a		
C. elegans	Y2H	43
Golga4-MyD88 family (*tir-1*)		
C. elegans	Y2H	43
Golga4-Macf1		
Human	Y2H	102
Human	Co-IP	102
Human	Western overlay	102
Slc25a5-MyD88 family (*tir-1*)		
C. elegans	Y2H	43
Smyd3-MyD88 family (*tir-1*)		
C. elegans	Y2H	43
Tnpo1-MyD88 family (*tir-1*)		
C. elegans	Y2H	43
Tnpo1-Ube2d2		
Drosophila	Y2H	113
Ube2d2-Traf6		
Human	Y2H	108
Human	*In vitro* ubiq	108
Slc25a5-IκBα		
Human	Y2H	114
Human	TAP	115
SCFβ-TrCP-Ube2d2		
Drosophila	Y2H	116
Drosophila	Affin chrom	116
Human	Co-IP	117
Human	*In vitro* ubiq	117
Sf3a1-MyD88 family (*tir-1*)		
C. elegans	Y2H	43
IκBα-SCFβ-TrCP		
Many	Many	117 and references therein

[a] The abbreviations used are as follows: Co-IP = co-immunoprecipitation; Y2H = yeast two-hybrid assay; Affin chrom = affinity chromatography; *In vitro* ubiq = *in vitro* ubiquitination assay; TAP = tandem affinity purification, and E2 ubiq = provides ubiquitin for E3 ubiquitin ligase in biochemical assay.

[b] *tir-1* is the sole *C. elegans* Myd88 family member. Although *tir-1* is most homologous to human Sarm, it functions most like MyD88 in that it is required for resistance to Gram-positive, Gram-negative, and fungal pathogens and functions upstream of the p38 MAPK pathway (15, 16, 118, 119).

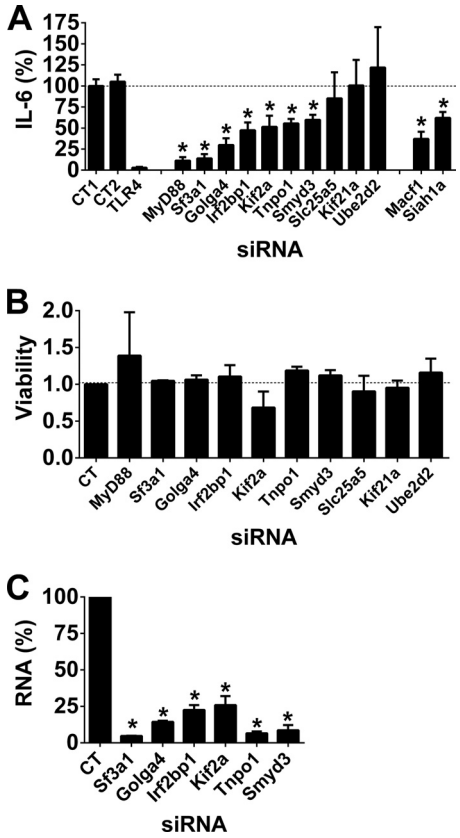

FIGURE 2. **Most genes in the innate immunity protein interaction network affect LPS-induced IL-6 production in the J774A.1 mouse macrophage cell line.** *A*, pools of four siRNA duplexes per gene were transfected into the mouse macrophage cell line J774A.1; cells were stimulated with 20 ng/ml LPS for 6 h, and IL-6 production was monitored by ELISA on cell supernatants. IL-6 production was normalized relative to a control pool of siRNA duplexes (CT1, Dharmacon nontargeting siRNA pool). CT2 is a second negative control (Dharmacon nontargeting siRNA 1). TLR4, the LPS receptor, is presented as a positive control. Two genes in this network (*Macf1* and *Siah1a*) were inhibited previously; the data for these two genes from this prior publication (12) is presented at the end of the panel. *B* depicts the effects on viability of the indicated siRNA treatments normalized so that viability of control siRNA was equal to 1. *C*, depicts the results of qPCR, which was used to monitor RNA knockdown of the indicated genes. *Asterisks* indicate siRNA treatments that induced IL-6 levels (*A*) or gene knockdown (*C*) that were significantly different from the controls ($p < 0.05$). No viability measurements (*B*) were statistically significantly different from control.

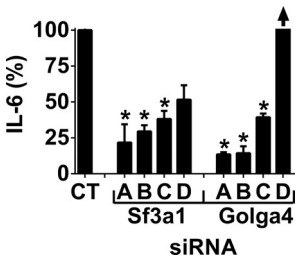

FIGURE 3. **SF3A1 and GOLGA4 regulate LPS-induced IL-6 release in the J774A.1 mouse macrophage cell line.** To confirm the results generated by the pools of siRNA duplexes in Fig. 2 for the two genes that induced the strongest phenotypes, each of four individual siRNA duplexes (labeled *A–D*) were used to inhibit either *Sf3a1* or *Golga4*. The figure depicts LPS-induced IL-6 production following siRNA treatment (20 ng/ml LPS for 6 h). IL-6 is off scale as indicated by the *arrow* above *Golga4* siRNA (*D*) (733 ± 57%). *Asterisks* indicate siRNA treatments that induced IL-6 levels that were statistically different from the control ($p < 0.05$).

tion of several other cytokines and chemokines (TNFα, IL-10, and RANTES) and found that inhibition of *Sf3a1* and *Golga4* decreased their production as well (supplemental Fig. 2), indicating that these two genes have a more general effect on innate immunity.

As an additional test of these two genes, we used RNAi to inhibit them in a second mouse macrophage cell line, RAW264.7 cells. As a control for the RNAi, we found that RNAi-mediated inhibition of genes known to be required for LPS-induced IL-6 production (the LPS receptor *TLR4*, the TLR signaling adaptor *MyD88*, and *IL-6* itself) led to a strong inhibition of IL-6 production, whereas RNAi-mediated inhibition

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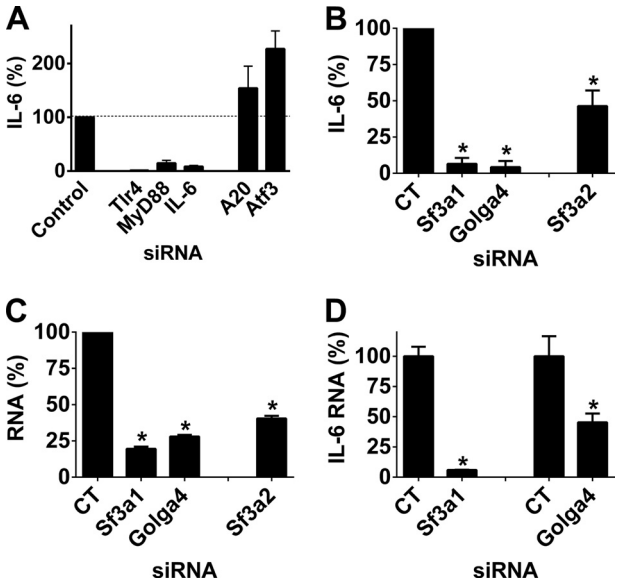

FIGURE 4. **Confirmation that SF3A1 and GOLGA4 regulate LPS-induced IL-6 production using a second mouse macrophage cell line.** *A* and *B,* indicated pools of siRNA duplexes were transfected into the RAW264.7 mouse macrophage cell line; cells were simulated with LPS (20 ng/ml for 6 h), and IL-6 production in cell supernatants was monitored by ELISA. *C* depicts the results of qPCR, which was used to monitor RNA knockdown of the indicated genes in *B*. *D,* indicated pools of siRNA duplexes were transfected into the RAW264.7 mouse macrophage cell line; cells were stimulated with LPS (20 ng/ml for 6 h), and IL-6 RNA production was monitored by qPCR. *Asterisks* indicate siRNA treatments that induced IL-6 protein levels (*B*), gene knockdown (*C*), or IL-6 RNA levels (*D*) that were statistically different from the controls ($p < 0.05$).

of known negative regulators of TLR signaling (*A20* and *Atf3*) (57, 58) led to increased IL-6 release (Fig. 4*A*). Using these same conditions, we found that inhibition of *Sf3a1* and *Golga4* in RAW264.7 cells using a pool of siRNA duplexes targeting each gene strongly inhibited LPS-induced IL-6 production (Fig. 4*B*) and strongly decreased RNA levels for the corresponding endogenous gene (Fig. 4*C*). We also monitored IL-6 RNA levels. Inhibition of *Sf3a1* led to a strong decrease in IL-6 RNA (Fig. 4*D*), much as it did for secreted cytokine (Fig. 4*B*), indicating that *Sf3a1* was likely affecting signaling. In contrast, although *Golga4* inhibition strongly inhibited IL-6 protein secretion (Fig. 4*B*), IL-6 RNA was only moderately decreased when *Golga4* was inhibited (Fig. 4*D*), suggesting that GOLGA4 may be acting differently than SF3A1.

Demonstration of the Importance of This Network in Vivo Using Available Mutations in C. elegans and Mice—To validate these *in vitro* RNAi results and prove that multiple proteins in this network play an important role in regulating innate immunity *in vivo*, we tested the potential immune function of several of these genes in *C. elegans* or mice using available mutants. As outlined below, experiments in both species suggest that many genes in this network affect innate immunity and immunological disease *in vivo*.

Monitoring the Effect of Available C. elegans Network Mutations on Host Defense—Although no viable mutation was available in *prp-21,* the *C. elegans* ortholog of *Sf3a1,* a temperature-sensitive allele of *repo-1,* the *C. elegans Sf3a2* homolog, was available. SF3A1 and SF3A2 (along with SF3A3) form the Sf3a complex. Sf3a interacts with the U2 small nuclear ribonucleoprotein, which in turn interacts with the pre-mRNA branch point near the 3′ splice site in pre-mRNA (59) and facilitates mRNA splicing in conjunction with the rest of the spliceosome (60–63). We first verified that inhibition of *Sf3a2*, like inhibition of *Sf3a1*, diminished LPS-induced IL-6 production in mouse macrophages (Fig. 4*B*), that this inhibition decreased *Sf3a2* RNA levels (Fig. 4*C*), and that viability was not affected by *Sf3a2* inhibition (data not shown). The *C. elegans repo-1/*Sf3a2 allele is a temperature-sensitive allele that may be neomorphic: it is weakly semi-dominant at the restrictive temperature of 26 °C.[3] We also obtained four additional available *C. elegans* mutant strains corresponding to genes in the innate immunity protein interaction network: *klp-12*(*tm5176*), *set-18*(*gk334*), *siah-1*(*tm1968*), and *tag-260*(*ok1339*), orthologs of *Kif21a*, *Smyd3*, *Siah1*, and *Irf2bp1*, respectively. The *klp-12*/*Kif21a*, *set-18/Smyd3*, *siah-1/Siah1*, and *tag-260*/Irf2bp1 alleles are all deletion alleles that should be nulls. All of these mutants other than *Kif21a* correspond to genes that exhibited a phenotype in the macrophage siRNA assay. These five mutant lines were exposed to the nematode and human pathogen *P. aeruginosa* strain PA14 (36, 64, 65), and survival was monitored when compared with the wild type strain N2 (37).

set-18/Smyd3 mutant nematodes survived a slightly shorter length of time than wild type nematodes when exposed to *P. aeruginosa* strain PA14 (Fig. 5*A*). *set-18*/Smyd3 mutant animals also lived slightly shorter lengths of time when grown in the presence of the nonpathogenic *E. coli* strain OP50, the standard laboratory *C. elegans* food source (Fig. 5*B*), raising the possibility that *set-18/Smyd3* could be regulating either host defense or general fitness. Live *E. coli* has been reported to be very slightly pathogenic to *C. elegans* under some conditions (66, 67). We therefore monitored the survival of *C. elegans* strains grown in the presence of heat-killed *E. coli*, and we found that the life span of *set-18/Smyd3* mutant animals was indistinguishable from that of the wild type strain under these conditions (Fig. 5*C*). Thus, the *set-18/Smyd3 m*utant animals exhibited a moderate host defense defect in the presence of both *P. aeruginosa* and live *E. coli*.

Nematodes harboring mutations in either *klp-12*/*Kif21a* or *tag-260*/*Irf2bp1* survived slightly longer in the presence of pathogenic *P. aeruginosa* but not nonpathogenic *E. coli* (Fig. 5, *D–G*), demonstrating that these strains were moderately resistant to pathogens. To monitor the effect of the temperature-sensitive *repo-1/Sf3a2* mutation on host defense, nematodes were allowed to develop at the permissive temperature (15 °C) in the presence of *E. coli* and were shifted to the restrictive temperature (26 °C) and plates containing *P. aeruginosa* as young adults. *repo-1/Sf3a2* mutant animals also survived longer in the presence of pathogenic bacteria (Fig. 5*H*); this resistance to pathogen was all the more striking as the mutant nematodes appeared visibly unhealthy and had a diminished life span in the presence of nonpathogenic *E. coli* (Fig. 5*I*). The pathogen resistance of *repo-1* mutant animals was likely not due to avoidance of the pathogen; 65% of *repo-1* mutant animals ($n = 57$) remained within the pathogenic bacterial lawn compared with

[3] M. R. Keikhaee and B. Bowerman, manuscript in preparation.

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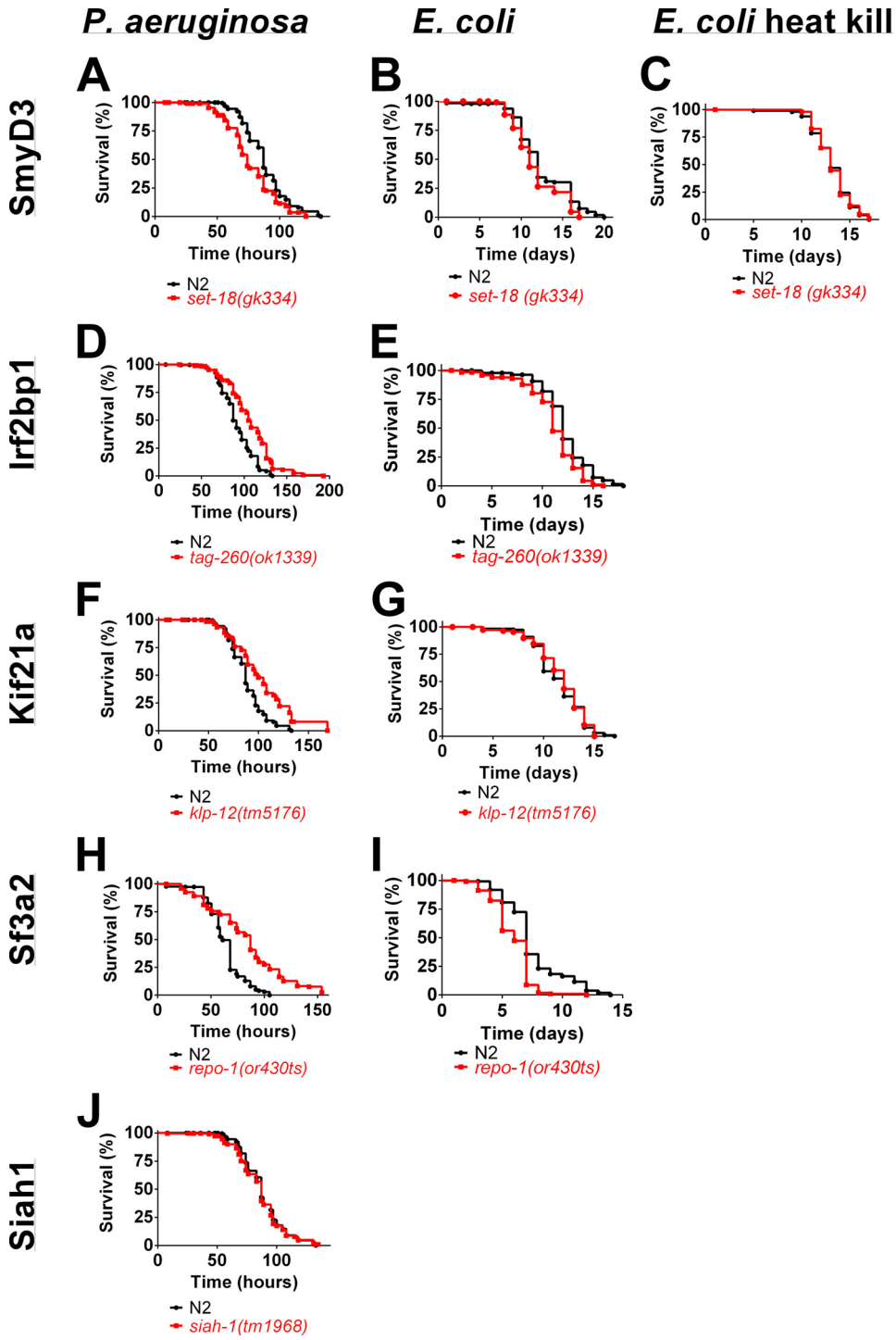

FIGURE 5. **Mutation of innate immunity network genes in *C. elegans* alters nematode host defense.** Depicted are survival curves for the indicated mutant strains exposed to the pathogen *P. aeruginosa* PA14 (*left panels*), nonpathogenic *E. coli* OP50 (*middle panels*), or heat-killed *E. coli* OP50. N2 (wild type nematodes) is depicted in *black*; the indicated mutants are depicted in *red*. The tested alleles and further statistical data (medians, *n*, and *p* values) are listed in supplemental Table 3.

59% of wild type N2 animals ($n = 69$). Similarly, the pathogen resistance of *repo-1* mutant animals also was likely not due to failure to ingest the pathogen as *repo-1* mutant nematodes exhibited normal pharyngeal pumping rates in the presence of *P. aeruginosa* (202 ± 10 pumps/min *repo-1* and 204 ± 10 pumps/min wild type N2, expressed as mean ± S.E., $n = 15$).

Finally, *siah-1/Siah1* mutant animals did not display altered sensitivity to *P. aeruginosa* exposure (Fig. 5*J*). Thus, four of the five innate immunity network mutants tested in *C. elegans* displayed altered nematode host defense.

Macf1 Regulates Cytokine Production in Vivo in Mouse Macrophages—RNAi-mediated inhibition of *Macf1* in the J774A.1 mouse macrophage cell line decreased LPS-induced IL-6 production (Fig. 2*A*) (12). To further explore the role of *Macf1* in the regulation of the LPS response, we used a similar approach to inhibit *Macf1* in a second immortalized mouse

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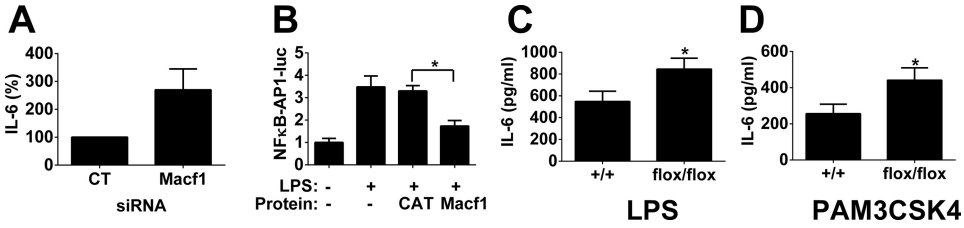

FIGURE 6. **MACF1 inhibits PAMP-induced cytokine production in RAW264.7 cells and *in vivo*.** *A,* pool of four siRNA duplexes targeting either *Macf1* or a nontargeting control siRNA duplex pool were transfected into the mouse macrophage cell line RAW264.7; cells were stimulated with 20 ng/ml LPS for 6 h, and IL-6 production was monitored by ELISA. *B,* RAW264.7 cells were transfected with plasmids overexpressing either *Macf1* or CAT, an NFκB-AP1-luciferase reporter, and the SV40-rluc control plasmid. After transfection, the cells were stimulated (or not) with LPS as indicated. Firefly luciferase activity was monitored and normalized relative to the *Renilla* luciferase control. *C* and *D,* BMDMs from *Macf1*$^{flox/flox}$;*Lyzs-cre* mice and control BMDM from *Macf1*$^{+/+}$;*Lyzs-cre* mice were exposed to either 2 ng/ml LPS or 20 ng/ml PAM3CSK4 for 6 h, and IL-6 production was monitored by ELISA. $n = 9$, $p < 0.05$ for both PAMPs. *Asterisks* indicate data that was significantly different from the control ($p < 0.05$).

macrophage cell line, RAW264.7. Surprisingly, we observed that inhibition of *Macf1* in this second cell line induced the opposite phenotype, an increase in cytokine production (Fig. 6*A*). To further explore the role of *Macf1*, we overexpressed *Macf1* in the RAW264.7 macrophage cell line and monitored the expression of an NFκB-AP1-luciferase reporter (a 133-bp derivative of the IL-8 promoter (40)) using SV40-rluc (Promega) as a normalization control, and we found that overexpression of *Macf1* but not chloramphenicol acetyltransferase (CAT) diminished the response to LPS (Fig. 6*B*), which confirmed the RNAi phenotype in the RAW264.7 macrophages. These opposing results in different immortalized cell lines underlined the importance of investigating the effect of these genes *in vivo*.

To determine the function of *Macf1 in vivo*, we generated BMDM-deficient in *Macf1* expression. To do so, we crossed conditional *Macf1*$^{flox/flox}$ (41) mice with *Lyzs-cre* mice (JAX) (42), which drives deletion of *Macf1* in the myeloid lineage. We generated BMDM from *Macf1*$^{flox/flox}$;*Lyzs-cre* mice and control sibling *Macf1*$^{+/+}$;*Lyzs-cre* mice and found that *Macf1* expression was decreased to 33.0 ± 5.1% of wild type levels in the BMDM from the floxed mice.

BMDM with decreased *Macf1* expression exhibited increased LPS-induced IL-6 production (Fig. 6*C*), confirming the *Macf1* RNAi data in the RAW264.7 cells. In addition to affecting the response to the TLR4 agonist LPS, *Macf1* also affected the response to the TLR2/1 agonist PAM3CSK4 (68) as BMDM with decreased Macf1 expression exhibited increased PAM3CSK4-induced IL-6 production as well (Fig. 6*D*).

DISCUSSION

In this age of systems biology, with high throughput RNAi screens, microarrays, proteomics, and other approaches, it is becoming increasingly important to sift through large datasets to define genes and pathways of interest (69). Many studies have used microarrays or RNAi to identify candidate genes for a phenotype of interest followed by some type of network or interactome analysis to sort through the data. For example, Li *et al.* (70) use a combination of protein interaction studies and RNAi to identify a network that regulates type I interferon production, and Amit *et al.* (71) use similar strategies to understand the transcriptional network underlying mammalian pathogen responses. Several investigators are also using screens in simpler model systems such as *C. elegans* or *Drosophila* to investigate innate immunity, and some of these studies report validation of these model systems data in mammalian cells using RNAi (see for example Refs. 22, 72).

We have performed candidate-based and genomic RNAi screens to identify regulators of the innate immune response to LPS (12) and have incorporated a comparative genomics approach into these studies to facilitate the analysis and strengthen our conclusions. Such cross-species studies offer the opportunity to identify networks not easily identifiable in individual species datasets (73). Additionally, the use of multiple RNAi screens could overcome the reported high false-positive rate in mammalian RNAi screens due to off-target affects (74). Finally, the availability of cheap, rapidly obtainable mutants in model organisms like *C. elegans* (75) allows for rapid testing of *in vitro* results using *in vivo* disease models.

Exposure of *C. elegans* to different pathogenic and nonpathogenic bacteria and fungi induces the expression of different subsets of antimicrobial genes (15, 18, 23–27). This specificity indicates that *C. elegans* can discriminate between different pathogens, even between different Gram-negative bacteria, suggesting that a response to a single PAMP such as LPS cannot explain the complete spectrum of the innate immune response in *C. elegans*. There is evidence for both PAMP and damage-associated molecular pattern-mediated innate immune activation in *C. elegans*, although the relative contribution of each and the specific details are still unclear. Evidence for the PAMP model comes from Aballay *et al.* (76) who show that intact *Salmonella enterica* LPS is required for a robust nematode innate immune response and from Vigneshkumar *et al.* (77) who show that *P. aeruginosa* LPS can alter *C. elegans* antimicrobial gene expression. Evidence for a damage-associated molecular pattern response comes from two studies (78, 79) that show that pathogen-mediated alterations in translation can affect the nematode innate immune response. Thus, it is still unclear what aspects of pathogen recognition are conserved between mammals and nematodes, and thus it remains to be determined whether the genes we identified that function in innate immunity in both models exhibit conservation of overall mechanism or if they function differently.

Using a combination of comparative genomics RNAi screens and protein interaction analysis, we have identified a small network of proteins almost all of whom modulate the innate

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immune response to LPS in mouse macrophage cell lines. The utility of this network for the identification of important innate immune regulators was evidenced by a follow up analysis on the two novel genes whose inhibition generated the largest effects, *Sf3a1* and *Golga4*. Both SF3A1 and GOLGA4 are required for robust LPS-induced IL-6 secretion, because RNAi-mediated inhibition of these genes strongly diminished IL-6 release from macrophages in two different mouse macrophage cell lines. However, the two genes may affect different aspects of the innate immune response.

Sf3a1 and its interacting protein Sf3a2 were required for production of IL-6 protein and IL-6 RNA. Other reports show that RNAi-mediated knockdown of *Sf3a* subunits can affect cell survival in HeLa cells (80); the difference in our data may be due to incomplete (but still very strong) knockdown in macrophages or other cell type-specific differences. The importance of the Sf3a complex in innate immunity regulation is further evidenced by the strong effect of the temperature-sensitive *Sf3a2* mutation on *C. elegans* resistance to pathogenic bacteria, which is all the more striking given that this mutation has the opposite effect on overall cell health. Because inhibition of two different mRNA splicing regulators in this complex diminished the LPS response, we infer that the Sf3a complex is regulating the alternative splicing of a critical innate immunity regulator. Many TLR genes are reported to be alternatively spliced in response to immune cell activation (81). For example, *TNFα*, c-*fos*, *TLR4*, *MyD88*, and *NFκB* have been reported to be differentially spliced in response to LPS or other stimulation (82–86); these alternate splice forms can have very different functions. The interaction of MyD88 family members with Sf3a1 raises the possibility that MyD88 could alter Sf3a1 activity and thus mRNA splicing of an innate immunity regulator gene(s).

In contrast, although inhibition of *Golga4* strongly decreased the amount of IL-6 protein secreted, we found that *Golga4* inhibition had a more moderate effect on IL-6 RNA accumulation. This is consistent with the reported requirement for GOLGA4 in TNFα and IL-10 secretion. GOLGA4 is required for TNFα and IL-10 transport through post-Golgi vesicles (87, 88); because IL-6 and TNFα traffic through a similar initial pathway of secretion (89–91), it is likely that GOLGA4 affects the LPS-induced secretion of both cytokines. Our IL-6 RNA results suggest that GOLGA4 may also have a small effect on signaling as well. The expression of many cytokine trafficking genes is increased in response to LPS stimulation (91), and the reported interaction of a MyD88 family member with GOLGA4 could be a direct protein-protein interaction that facilitates cytokine secretion as well.

In addition to these two genes, we validated several other network genes *in vivo* using available knock-out nematodes or mouse macrophages. In general, mutants with innate immune defects in *C. elegans* are susceptible to pathogens, although there are conditions under which the nematode innate immune response can have a negative impact on survival and thus mutants could enhance pathogen resistance (92).

tag-260/Irf2bp1 mutant nematodes were moderately resistant to pathogen. The fact that IRF2BP1 could affect innate immunity is not surprising given the fact that IRF2BP1 is reported to be a co-repressor for IRF2 (93), which can affect the LPS response in macrophages (94–96). *klp-12/Kif21a* mutant nematodes were also moderately resistant to pathogen. The kinesin KIF21A is a plus-end-directed microtubule motor (97) that has not been implicated in innate immunity previously. *set-18/Smyd3* mutant nematodes were slightly susceptible to pathogenic bacteria but not heat-killed *E. coli*; SMYD3 is a histone methyltransferase that affects transcription (98, 99) and could therefore have many conceivable effects on innate immunity. Although the nematode *siah-1/Siah1* mutation did not affect survival of the presence of *P. aeruginosa,* we note that there are many other pathogens that infect nematodes that we have not tested. Moreover, overexpression of *Siah1* in mammalian cells has been reported to stimulate NFκB activity (100), consistent with our macrophage RNAi data.

We also demonstrated that deletion of another network gene, *Macf1*, from mouse macrophages led to increased PAMP-induced IL-6 production. Unlike *Sf3a1* and *Golga4*, which exhibited similar phenotypes when inhibited in either of two mouse macrophage cell lines, *Macf1* exhibited different effects in these lines, and the effect in RAW264.7 cells but not J774A.1 cells phenocopied the *in vivo* effect. We are uncertain of precisely why the two immortalized lines differ, but one possibility is that because MACF1 affects subcellular trafficking (101–105), perhaps it has both positive and negative effects on different aspects of innate immunity, and differential knockdown could cause different effects. Regardless, it does reinforce the importance of *in vivo* follow-up studies to validate *in vitro* RNAi data.

Roughly one-third of *C. elegans* genes that regulated antimicrobial production in our nematode RNAi screen affected production of LPS-induced cytokine production in mouse macrophages (12). Moreover, 83% (10/12) of the genes in this protein network regulated LPS-induced cytokine production, and 5 of 6 of these network genes affected innate immunity *in vivo*. In contrast, inhibition of only 2 out of 100 other candidate genes (identified using computational approaches examining PAMP-induced gene expression data in the literature) led to an altered LPS-induced cytokine response.[4] Although our comparative genomics and proteomics approach is identifying novel innate immunity regulators at high efficiency, it remains to be determined whether the mechanisms by which these genes act are conserved between species.

In summary, we have identified a network of orthologous interacting proteins in *C. elegans*, *Drosophila*, and mammals, demonstrated that most proteins in this network regulate the response to the TLR4 agonist LPS *in vitro*, demonstrated that the two genes in this network that exhibited the strongest RNAi-induced phenotypes (*Sf3a1* and *Golga4*) do so through different mechanisms, and validated five of these candidates *in vivo* using knock-out nematodes and mice. Thus, this novel evolutionarily conserved protein interaction network will provide very fertile ground for future investigation. Future studies will involve determining the mechanisms by which these genes act and determining what role they play in different diseases that are affected by innate immunity.

[4] S. Alper, unpublished data.

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Supplemental Table 1. Oligonucleotides used for qPCR

	Forward Primer	Reverse Primer
Beta-actin	CTGAACCCTAAGGCCAACCGTG	CCGTCTCCGGAGTCCATCACAATG
IL-6	CTTGGGACTGATGCTGGTGAC	GCCTCCGACTTGTGAAGTGGTATAGACAGG
Sf3a1	GCAGCAGCAAGCTACGCAACAGCAGCTGCC	GCGATGAACTCAAACTCAGGAGGGGGCTCC
Golga4	GTTGCGTGATGCAAAGAACTTAATTGAACAGCTTG	CTCCCTGGGTAGTCATCTGTTTGATATGACTACG
Irf2bp1	GATTCAAGAAGGACCACGGGCTGG	CAGAACCACAGGGGTATTCGGTGA
Kif2a	GGCAAAGAGATTGACTTGGAGAGC	GACGAGGGTGGAGGTAGTTCTGGACTGG
Tnpo1	GAATCTGGCATCTTGGTTTTAGGAGCAATTGCTG	CAAGAGCCTTTTTATCAGAAAGGCACTGAATAAGG
Smyd3	CGCTGAAGAAAATCGAAGAGCTGAAGGCGCACTGG	GATGTCGGGAAGCCGGTTGGAATTGCTGTTTATGATC(
Macf1	GTTCAACGCATTGATTCACCGATACAGGCC	CATCTGGAGATGGCACATCCACATCTTCTGC

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Supplemental Table 2. Homologous genes in Humans, *Drosophila,* and *C. elegans*

Human	*Drosophila*	*C. elegans*	Notes and References
Sf3a1	CG16941	*prp-21*	Homologene
Macf1	shot	*vab-10*	(1,2)*
Golga4	CG3493	*F59A2.6*	*
MyD88	MyD88	*tir-1*	See note about *tir-1* in Table 1
Kif21a	-	*klp-12*	Homologene
Kif2a	Klp10A	*klp-7*	Homologene
Irf2bp1	-	*tag-260*	*
Siah1	sina	*siah-1*	Homologene
Slc25a5	-	*ant-1.1*	*
Tnpo1	Trn	*imb-2*	Homologene
IκBα	cact	*ikb-1*	(3,4)*
Ube2d2	eff	*let-70*	Homologene
Smyd3	Bzd	*set-18*	Homologene
Traf6	traf6	*trf-1*	(3,4)*

*While these *C. elegans* genes are the closest homolog of the human genes, the converse is not necessarily true

Supplementary Table 2 References:

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Supplemental Table 3. *C. elegans* strains and survival data from Fig. 5

Panel in Fig. 5	Genotype	Median survival (hrs)	n	*P* value
P. aeruginosa				
A	N2	87	141	
A	VC767 *set-18(gk334) I*	74	167	< 0.0001
D	N2	87	159	
D	VC812 *tag-260(ok1339) V*	105	190	< 0.0001
F	N2	87	141	
F	FX05176 *klp-12(tm5176) IV*	100	107	< 0.0001
H	N2	61	164	
H	*repo-1(or430ts) IV*	87	178	< 0.0001
J	N2	87	141	
J	FX01968 *siah-1(tm1968) IV*	87	142	0.405

Panel in Fig. 5	Genotype	Median survival (days)	n	*P* value	Notes
E. coli					
B	N2	12	119		
B	VC767 *set-18(gk334) I*	11	107	0.0117	
C	N2	13	98		heat killed *E. coli*
C	VC767 *set-18(gk334) I*	13	102	0.875	heat killed *E. coli*
E	N2	12	124		
E	VC812 *tag-260(ok1339) V*	11	93	0.0004	
G	N2	12	105		
G	FX05176 *klp-12(tm5176) IV*	12	98	0.663	
I	N2	7	105		26ºC
I	*repo-1(or430ts) IV*	6	113	< 0.0001	26ºC

Data is compilation of multiple biological replicates for each strain.
Data for some N2 (wild type) control strains is the same in multiple panels.

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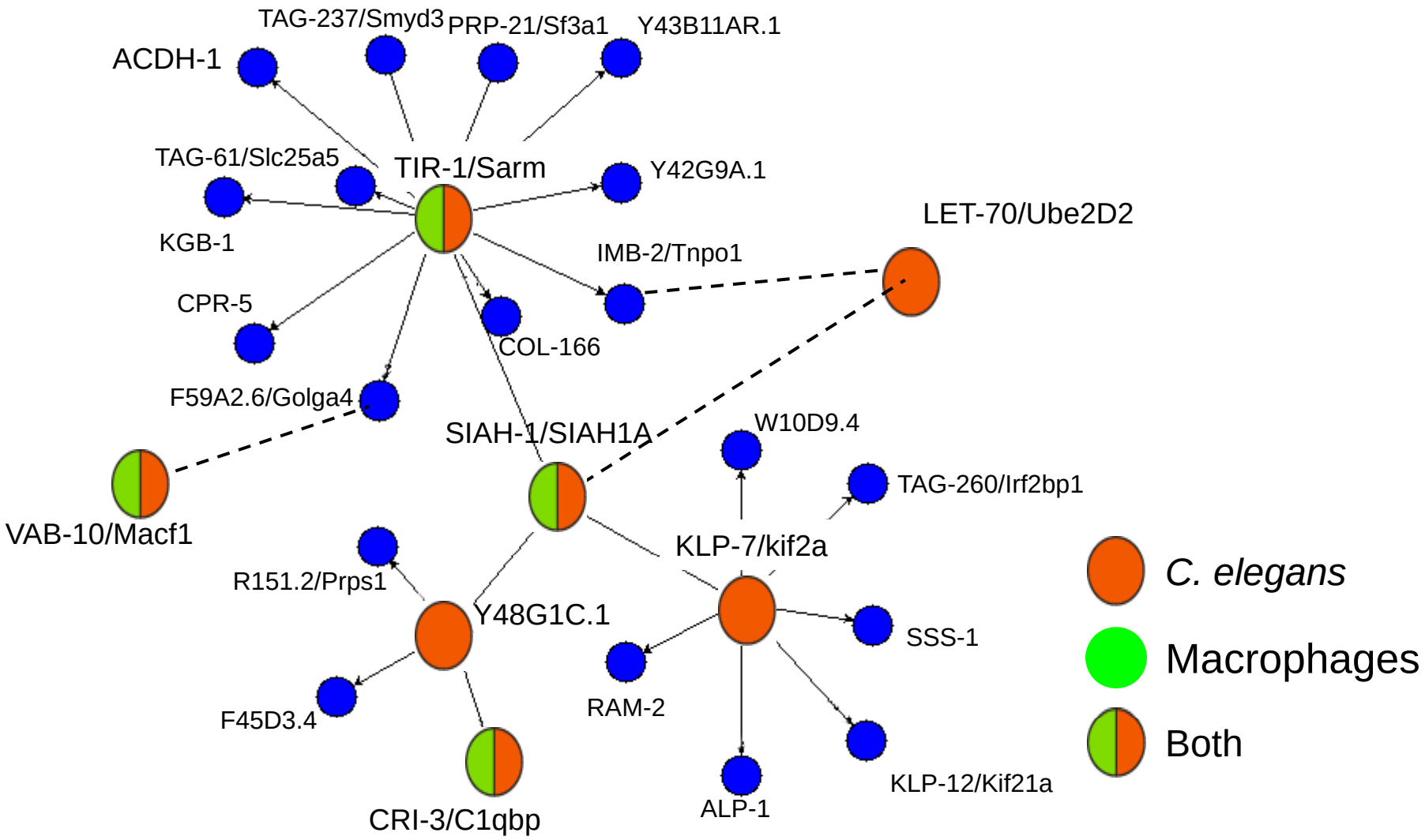

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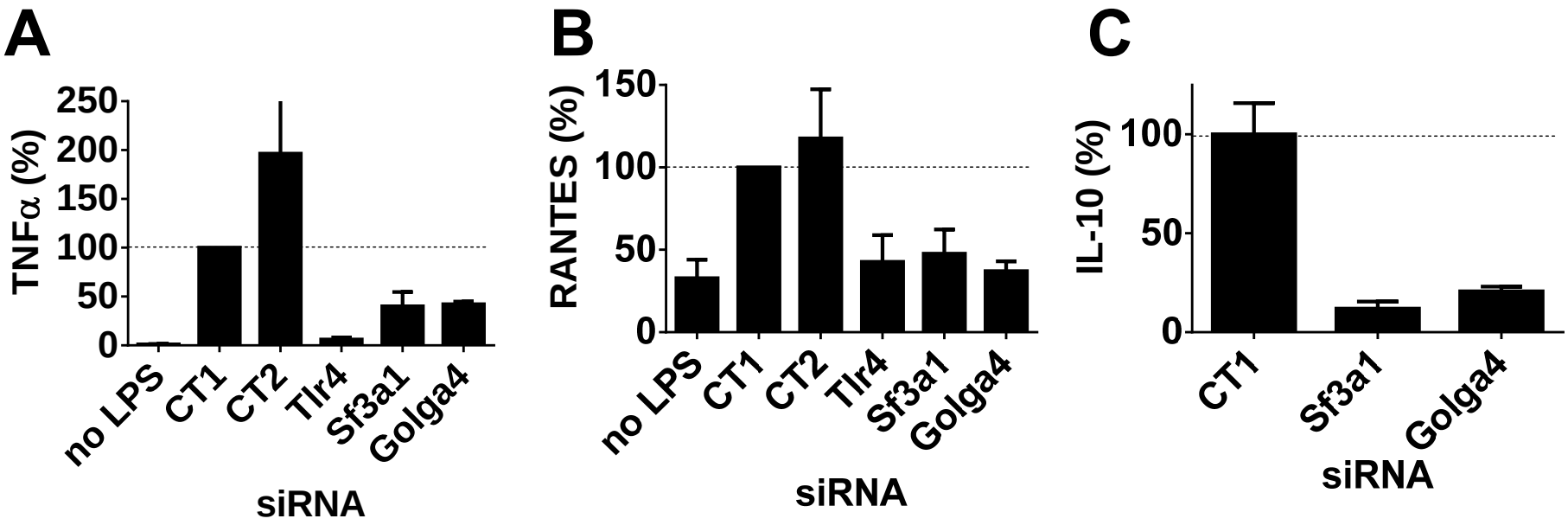

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