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Mitochondrial chaperone HSP-60 regulates anti-bacterial immunity via p38 MAP kinase signaling

Dae-Eun Jeong, Dongyeop Lee, Sun-Young Hwang, Yujin Lee, Jee-Eun Lee, Mihwa Seo, Wooseon Hwang, Keunhee Seo, Ara B Hwang, Murat Artan, Heehwa G Son, Jay-Hyun Jo, Haeshim Baek, Young Min Oh, Youngjae Ryu, Hyung-Jun Kim, Chang Man Ha, Joo-Yeon Yoo, Seung-Jae V Lee

Corresponding author: Seung-Jae Lee & Joo-Yeon Yoo, Pohang University of Science and Technology

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 14 June 2016

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and I am afraid that the overall recommendation is not very positive at this stage.

The referees appreciate that we gain new insight into the link between the mitochondria and innate immunity. However they also raise a number of important concerns that are clearly outlined below. They find that some of the conclusions are not sufficiently supported by the data presented. Moreover, they find that we gain too limited molecular insight into how the mitochondrial chaperone Hsp-60 regulates SEK-1 activity. Given the concerns raised and as it is unclear if they can be resolved I am afraid that I can't offer to invite a revised version.

However, should you be able to extend the findings along the lines indicated and importantly add more mechanistic insight into how Hsp-60 regulates SEK-1 activity then I can offer to consider a resubmission. I should point out that for resubmissions that we consider novelty at time of submission.

I am sorry that I can't be more positive on this occasion, but I hope that you find the referees' comments helpful.

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REFEREE REPORTS

Referee #1:

Jeong and coworkers carried out an RNAi screen to identify genes, which modulate C. elegans immunity against P. aeruginosa PA14 from a pool of conserved mitochondrial genes. The authors showed that HSP-60 increases the immunity of worms against PA14 in a p38 MAP kinase-dependent manner. The data is convincing and the results are of broad interest. However, the authors need to address the following points:

1. Figure 6: How do the authors know that the hsp-60 gene is actually overexpressed? The authors may want to consider the quantification of hsp-60 mRNA and/or protein levels in the overexpressing strains, and compare it with the levels of WT worms.

2. Figure EV5: Why don't the hsp-60::gfp OE #4 and #5 strains show any effect on survival on PA14 in comparison to WT worms?

3. The authors showed that overexpression of GFP alone in mitochondria did not affect survival of worms on PA14. However, the HSP-60 overexpression strains have HSP-60 fused with GFP, which may affect the chaperone function. GFP is relatively large in comparison to HSP-60. Can the authors overexpress HSP-60 without GFP fusion, confirm overexpression by mRNA and/or protein levels, and then carry out killing assays on PA14?

4. Figure 7E: The results on HeLa cells are not very convincing. The expression levels of p-p38 MAPK are not different at 0 h and 2 h post PA14 infection in HSPD-1 and HSPD-1-myc containing cells. The only difference is at 1 h post infection. The authors could take more time points and more importantly, should define the robustness of the results by statistical tests.

5. The authors' observation that increased HSP-60 levels increase immunity via pmk-1 pathway is in contrast to Pellegrino et al., Nature, 2014. Pellegrino et al. observed that activation of mitochondrial UPR, which leads to overexpression of HSP-60, increases survival of C. elegans on PA14 independently of pmk-1 pathway. The authors need to discuss the possible reasons for these contrasting results.

Referee #2:

Jeong et al. provide a thorough and clearly presented investigation of the potential role of the mitochondrial chaperone HSP-60 in antimicrobial defense. It extends a number of recent studies and will be of interest to a relatively broad audience, particularly given the preliminary indications of a conservation of the regulatory mechanism. That said, despite the wealth of data, this represents a useful but somewhat incremental advance in the characterization of the link between the UPRmt and host defense.

I have few major concerns. One involves the interpretation of data. The authors write, "Further, knockdown of hsp-60 reduced the level of active phospho-PMK-1 (Fig 4F). These data indicate that activation of PMK-1 upon PA14 infection requires HSP-60".

This, however, does not appear to agree with data shown. In control conditions, infection by PA14 is associated with a 20% increase in PMK-1 phosphorylation (1->1.2); following hsp-60(RNAi) there is a 280% increase (0.28 -> 0.79). Thus although hsp-60 is required for maintaining basal levels of PMK-1 phosphorylation, it is dispensable for activation of PMK-1 upon PA14 infection.

A second major concern is methodological. In their survival experiments, the authors use 5-fluoro-2'-deoxyuridine (FUdR). A series of studies (http://www.ncbi.nlm.nih.gov/pubmed/?term=fudr+elegans) have shown that FUdR has a broad impact on physiology. As a minimum, the key survival experiments performed with PA14 and the other pathogens need to be repeated in the absence of FUdR.

Of more minor concern:

The authors should provide a table summarizing how their results presented in Fig. 1B compare to published data.

And although they write, "selected 220 RNAi clones that did not cause severe developmental delay or lethality for further studies (see Dataset EV1)",
15/16 of the final candidates are associated with WBPhenotype:0000050 (embryonic lethal). They should comment on this.

The paper by Bennett et al, (PMID: 24662282) needs to be cited and discussed, especially since 6/16 RNAi targets identified here were reported to induce hsp-6p::gfp (i.e. the mitochondrial UPR) when knocked down.

Almost as many (5/16) genes are associated with suppressing osm-8 gpdh-1::GFP overexpression when knocked down (PMID: 21253570). How does this fit with the authors' model of a specific link between the UPRmt and resistance to PA14? In a similar vein, 4/16 block epidermal innate immunity (PMID: 27129311).

The 2 recent papers from the Dillin lab (PMID: 27133166 and 27133168) that address the mechanism underlying gene expression changes provoked by the UPRmt need to be cited and discussed.

The authors cite the review Ewbank, 2006; they should also cite the more recent one (PMID: 26694508).

In the discussion, it would also be useful to cite the relevant review, "Local and long-range activation of innate immunity by infection and damage in C. elegans" (PMID: 26517153)

The authors write, "The genetic inhibition of each of the transcription factors significantly increased susceptibility to PA14 (Fig EV3A-C) (Pellegrino et al, 2014).....Together, these data indicate that UPRMT, in general, is required for the PA14 resistance". The authors should make it clear whether this is a novel result, or simply recapitulates what was already known.

Fig 6E: some mitochondria are not labeled with HSP-60::GFP. Is this reproducible? Do they correspond to particular cells?

Dataset EV1A - although more complete than in many studies, the authors need to give the exact names of the selected RNAi clones, in addition to giving their presumed targets. Did they use one of the dedicated tools (e.g. Clone Mapper) to determine targets? If not, they need to, since there have been shown to be deficiencies in the Wormbase predictions (PMID: 25187039). As part of minimal reporting standards, what WS release was used?

What does N/A mean in the column "RNAi source (JA or MV)"? According to Clone Mapper, for many of these genes there are RNAi clones.

In the Abstract, the authors write, "host eukaryotes hijack molecular chaperones generated from bacteria-originated mitochondria". This needs to be reformulated if one assumes that the mechanisms they are describing a normal physiological process.

The authors write, "activates transcription factors including DVE-1/UBL-5". Although UBL-5 forms a complex with DVE-1, it is not a transcription factor.

The authors write, "the mitochondrial chaperone HSP-60/HSPD1, which exhibits exceptional homology across species" - homology is an all-or-none characteristic. The authors mean "sequence conservation". The sequence conservation of HSP-60 is no higher than that of most other chaperones.

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Referee #3:

Jeong and colleagues introduce a novel function of HSP-60/HSPD1, a mitochondrial chaperone associated with UPRmt induction, in the protection of hosts from pathogen infection through the activation of p38 MAPK pathway. By focusing on the C. elegans- Pseudomonas aeruginosa host versus pathogen interaction, the authors show that intestinal and neuronal HSP-60 is important for the resistance of animals upon pathogen infection. HSP-60 is suggested to exert its protective effects through the stabilization of SEK-1, the MAPK kinase which phosphorylates PMK-1/p38 MAPK. Interestingly enough, the protective role of HSP-60 seems to be evolutionarily conserved, since its overexpression is sufficient to protect human cells from P. aeruginosa infection. More specifically, the authors identify several (16) conserved mitochondrial components which affect the survival of C. elegans after P. aeruginosa (PA14) infection. The most robust effect was exhibited by hsp-60 knockdown, and they found that this chaperone alone is necessary and sufficient for the resistance against PA14. HSP-60 protects the animals specifically against PA14 and through the activation of p38 MAP kinase signaling. HSP-60 also stabilizes SEK-1 which then positively regulates p38 which results in increased immunity. Furthermore, this function is conserved: in human cells overexpression of HSPD1 (the ortholog of HSP-60) increases the activity of p38. Overall, the manuscript reports interesting findings, although some of the authors' claims require further experimental support and some issues detailed below need to be addressed.

Comments:
Several previous reports have established the role of HSP-60 and of mito-UPR in immunity, compromising the novelty of the results presented here. For example, the effect of HSP-60 was examined in human monocytes and macrophages upon LPS (lipopolysaccharide from gram-negative bacteria), which is one of the most potent innate immunity activating stimuli known. In this context, which is comparable to the PA14 paradigm used here, HSP-60 was shown to activate the immune response via activation of p38 MAPK signaling (Kol et al., 2000, J. Immunology). More recently, in a publication that is cited in the manuscript (Pellegrino et al., 2014, Nature) it was shown that PA14 infection resulted in an atfs-1-dependnet increase in mitochondrial chaperone Hsp-60 and an increase in pmk-1 activity.

The tissue specific requirement of HSP-60 for the immune response is very interesting and is worthy of further investigation. Recent studies have suggested that HSP-60 is secreted both from tumour and normal cells in detergent-resistant lipid vesicles or exosomes (Gupta and Knowlton, 2006). To what degree this also happens in the worm and whether it underlies the communication between neurons and the intestine are interesting points to consider.

It is also interesting that while knockdown of HSP-60 has an effect on the survival of C. elegans against PA14, it has no effect against other pathogenic bacteria such as E. faecalis. By contrast, knockdown of pmk-1 has an effect against all pathogens tested, suggesting that it is a hub where many signaling pathways of immunity converge. Are other mitochondrial chaperones activated by other pathogens? Is mito-UPR involved or not?

The experiments in HeLa cells are not really novel (see point 1) and they were performed under control conditions, as opposed to using a model of immunity activation (such as the LPS).

The claim that hsp-60 overexpression does not induce UPRMT is not sufficiently supported. The writers should provide the raw data of their quantitative PCR experiments that demonstrate that the lack of significant hsp-6 activation is repeatable and the use of an hsp-6:GFP reporter would constitute even stronger evidence.

The authors have not definitively shown that SEK-1 is the mediator of the effect that HSP-60 has on PMK-1. They need to demonstrate that in a sek-1(-) genetic background the effects of HSP-60 overexpression on PMK-1 activity are completely abolished. Otherwise there could be an additional factor that acts in parallel to the proposed SEK-1>PMK-1 pathway.

An emerging question, which is also addressed to some extent by the authors in the discussion section of the manuscript, is how a mitochondrial chaperone can increase the expression / stabilize the cytoplasmic MAPKK SEK-1. Do the authors have any indication that HSP-60 in nematodes is at least to some extent localized in the cytoplasm or perhaps that its mitochondrial localization changes

upon infection with pathogenic P. aeruginosa bacteria? Figures 6C, D and E do not support a putative cytoplasmic localization for HSP-60. Since commercially antibodies for HSP-60 exist, one possible approach would be the isolation of mitochondrial and cytoplasmic fragments (in nematodes, cells, or ideally both) to analyze the HSP-60 distribution under standard conditions and upon infection. Does HSP-60 directly interact with SEK-1 and protects it from degradation upon pathogen infection? Does the same occur in cell cultures?

Since HSP-60 expression is believed to be upregulated in response to insults or genetic manipulations which trigger UPRmt, a reasonable question would be whether induction of UPRmt (for example through cco-1 or spg-7 RNAi treatments reported in the literature) is detrimental when combined with infection. One could think that induction of UPRmt might sequester a reasonable amount of HSP-60 protein away from the SEK-1-PMK-1 axis, not allowing the activation of a fully functional defense response.

Do the authors have any hypothesis on why depletion of several mitochondrial ribosomal proteins can be beneficial following P. aeruginosa infection (table 1)?

Minor Comments
In figure EV10A, the strain name should be corrected to SEK-1::tagRFP instead of PMK-1::tagRFP (at least according to the figure legend provided).

Furthermore, since the proteins are tagged with fluorescent proteins (GFP, RFP), capital letters should be used on all the figures for the transgenic strains (HSP-60, SEK-1 and PMK-1 overexpressing lines).

The legend of figure EV1B includes the unsupported claim that "mitochondrial translation and respiration appear to confer the susceptibility of C. elegans against bacterial pathogens via modulating SKN-1". The authors should demonstrate that the effect of the cluster III genes in survival is lost in a skn-1(-) background or remove the sentence entirely (since it is not relevant to the rest of the manuscript). That legend also fails to acknowledge that the effect of the cluster III genes in survival is dependent on daf-16.

1st Revision - authors' response 30 November 2016

Referee #1: *Jeong and coworkers carried out an RNAi screen to identify genes, which modulate C. elegans immunity against P. aeruginosa PA14 from a pool of conserved mitochondrial genes. The authors showed that HSP-60 increases the immunity of worms against PA14 in a p38 MAP kinase-dependent manner. The data is convincing and the results are of broad interest. However, the authors need to address the following points:*

1. Figure 6: How do the authors know that the hsp-60 gene is actually overexpressed? The authors may want to consider the quantification of hsp-60 mRNA and/or protein levels in the overexpressing strains, and compare it with the levels of WT worms.

> We thank the reviewer for this helpful comment. We measured the mRNA levels of *hsp-60* in *hsp-60::GFP* transgenic animals by performing qRT-PCR. Our results indicate that the levels of *hsp-60* mRNA are increased in the transgenic animals (Fig EV5A). We now describe these findings in the Results as follows.

Page 9, Line 11: “We then asked whether hsp-60 overexpression affected anti-PA14 immunity by generating transgenic worms that expressed hsp-60 with or without a GFP tag (hsp-60::GFP OE or hsp-60 OE). We first confirmed that these transgenic animals displayed increased levels of hsp-60 mRNA (Fig EV5A and B)…”

Page 62, Line 5, Fig EV5 legend: “**A**, qRT-PCR results indicate that hsp-60::GFP overexpression (hsp-60::GFP OE) increased the level of hsp-60 mRNA.”

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2. Figure EV5: Why don't the hsp-60::gfp OE #4 and #5 strains show any effect on survival on PA14 in comparison to WT worms?

> As we described in Table EV6, *hsp-60::GFP OE* #4 and #5 also significantly increased the survival of animals on PA14 by 15% and 24% respectively (the average of mean survival data obtained from two independent experiments). As the reviewer pointed out, the effects of *hsp-60::GFP OE* #4 and #5 on the survival of worms on PA14 were relatively small compared to those of *hsp-60::GFP OE* #3 (60% increase, the average of mean survival data obtained from two independent experiments); we currently do not know the basis of this variability. We marked % increase in the survival of animals on PA14 and statistical significance in the Figure EV5 legend. We also mention this variability among the transgenic lines by saying,

Page 62, Line 10, Fig EV5 legend: "**C**, Three independent lines (#3, #4 and #5; see Materials and Methods) of transgenic animals that carried extrachromosomal arrays of *hsp-60::GFP* (*hsp-60::GFP OE*) displayed significant increase in resistance to PA14 (p<0.001). We noticed that the effects of *hsp-60::GFP OE* #4 and #5 on the survival of worms on PA14 were smaller than those of *hsp-60::GFP OE* #3. We currently do not know the basis of this variability among the transgenic lines."

3. The authors showed that overexpression of GFP alone in mitochondria did not affect survival of worms on PA14. However, the HSP-60 overexpression strains have HSP-60 fused with GFP, which may affect the chaperone function. GFP is relatively large in comparison to HSP-60. Can the authors overexpress HSP-60 without GFP fusion, confirm overexpression by mRNA and/or protein levels, and then carry out killing assays on PA14?

> We really appreciate this valuable comment. Following this reviewer's suggestion, we generated transgenic animals overexpressing *hsp-60* without any tag (*hsp-60 OE*) and measured the survival of the animals upon PA14 infection. As shown in Fig EV5D, two independent lines of *hsp-60 OE* transgenic animals showed enhanced PA14 resistance. We also confirmed that the mRNA levels of *hsp-60* were increased in these transgenic animals (Fig EV5B). The effects of *hsp-60 OE* on PA14 resistance tend to be smaller than those of *hsp-60::GFP OE*. We found that the levels of *hsp-60* mRNA were significantly increased by 1.2-1.4 folds in *hsp-60 OE* animals and by 2.3 fold in *hsp-60::GFP OE* animals. Thus, it seems likely that the levels of *hsp-60* mRNA correlate with the survival times of animals on PA14. We added these findings to the Results as follows.

Page 9, Line 11: "We then asked whether *hsp-60* overexpression affected anti-PA14 immunity by generating transgenic worms that expressed *hsp-60* with or without a GFP tag (*hsp-60::GFP OE* or *hsp-60 OE*). We first confirmed that these transgenic animals displayed increased levels of *hsp-60* mRNA (Fig EV5A and B), and showed that the GFP-fused HSP-60 was mainly localized to the mitochondria of cells in multiple tissues (Fig 6A-F). Importantly, both *hsp-60::GFP OE* and *hsp-60 OE* significantly increased resistance to PA14 (Fig 6G, EV5C and 5D), which was abolished by *hsp-60* RNAi (Fig 6H)."

Page 62, Line 7, Fig EV5 legend: "**B**, qRT-PCR data indicate that overexpression of *hsp-60* without a tag (*hsp-60 OE*) increased the level of *hsp-60* mRNA in two independent lines of transgenic animals (#1 and #2; see Materials and Methods)."

Page 62, Line 16, Fig EV5 legend: "**D**, Two independent lines of transgenic animals with extrachromosomal arrays of *hsp-60* (*hsp-60 OE* #1 and #2) showed significant increases in PA14 resistance (p<0.01 and p<0.001, respectively). The effects of *hsp-60 OE* on PA14 resistance tend to be smaller than those of *hsp-60::GFP OE* (Fig 6G). Note that the levels of *hsp-60* mRNA were significantly increased by 1.2 to 1.4 folds in *hsp-60 OE* animals and by 2.3 fold in *hsp-60::GFP OE* animals (**A** and **D**). Thus, it seems likely that the levels of *hsp-60* mRNA correlate with the survival time of animals on PA14."

4. Figure 7E: The results on HeLa cells are not very convincing. The expression levels of p-p38 MAPK are not different at 0 h and 2 h post PA14 infection in HSPD-1 and HSPD-1-myc containing cells. The only difference is at 1 h post infection. The authors could take more time points and more importantly, should define the robustness of the results by statistical tests.

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> As the reviewer suggested, we performed Western blot assays measuring both phospho-p38 MAPK (p-p38 MAPK) and total p38 MAPK levels by using HeLa cells at 0, 0.5, 1.0, 1.5, and 2 hours post PA14 infection. We found that PA14 infection significantly increased the p-p38 MAPK level 2 hours after PA14 infection. Importantly, we also found that cells transfected with HSPD1-myc reproducibly displayed higher levels of p-p38 MAPK than control cells (Figures for referees not shown). We added statistical analysis for the quantification (n≥3) (Fig EV8B), and describe these new data as follows.

Page 11, Line 5: "Overexpression of human *HSPD1* accelerated the increase in the level of active phospho-p38 MAPK in cultured human cells 2 hours post PA14 infection (HPI) (Fig 7E and EV8A)."

Page 64, Line 18, Fig EV8 legend: "**B**, Quantification of data in Fig 7E (n≥3). HPI: hours post PA14 infection. Error bars represent SEM. *p* values were calculated by two tailed Student's t test (**p<0.01, ***p<0.001)."

5. The authors' observation that increased HSP-60 levels increase immunity via pmk-1 pathway is in contrast to Pellegrino et al., Nature, 2014. Pellegrino et al. observed that activation of mitochondrial UPR, which leads to overexpression of HSP-60, increases survival of C. elegans on PA14 independently of pmk-1 pathway. The authors need to discuss the possible reasons for these contrasting results.

> We appreciate this valuable comment. As the reviewer mentioned, Pellegrino et al. reported that activation of mitochondrial UPR increases the survival of *C. elegans* on PA14 independently of PMK-1. This conclusion was based on their data showing that *spg-7* RNAi, which induces mitochondrial stress, increased the survival of wild-type animals as well as *pmk-1* mutants on PA14. However, in addition to the induction of *hsp-60*, *spg-7* RNAi up-regulates many other stress-responsive genes and increases lifespan (Curran and Ruvkun, 2007; Chen et al., 2007; Nargund et al., 2012). As we found that *hsp-60 OE* did not increase lifespan, *spg-7* RNAi may increase the survival of *pmk-1* mutants through the activation of longevity pathways, independently of *pmk-1* signaling. Overall, we speculate that *spg-7* RNAi regulates at least two branches of anti-PA14 immune responses; one pathway acts through the induction of anti-microbial genes in a PMK-1-independen manner and the other pathway acts through the induction of mitochondrial chaperones, including HSP-60. The induction of HSP-60 appears to boost immunity by up-regulation of PMK-1 signaling in the cytosol. We now added this point to the Discussion section as described below.

Page 15, Line 5: "In addition, Pellegrino et al. (2014) concluded that activation of UPR^{MT} increases immunity independently of PMK-1, based on the results showing that RNAi targeting *spg-7* (mitochondrial metalloprotease) increases the survival of *pmk-1* mutants as well as wild-type animals on PA14. However, *spg-7* RNAi up-regulates many stress-responsive genes other than mitochondrial chaperones and increases lifespan (Curran and Ruvkun, 2007; Chen et al., 2007; Nargund et al., 2012). Thus, *spg-7* RNAi may increase the survival of *pmk-1* mutants through the activation of longevity and stress-responsive pathways independently of *pmk-1* signaling. Overall, UPR^{MT} appears to regulate at least two branches of anti-PA14 immune responses; one pathway acts through the induction of PMK-1-independent anti-microbial genes and the other pathway acts through the induction of mitochondrial chaperones, including *hsp-60* that regulates immunity via *pmk-1* signaling."

Referee #2:

Jeong et al. provide a thorough and clearly presented investigation of the potential role of the mitochondrial chaperone HSP-60 in antimicrobial defense. It extends a number of recent studies and will be of interest to a relatively broad audience, particularly given the preliminary indications of a conservation of the regulatory mechanism. That said, despite the wealth of data, this represents a useful but somewhat incremental advance in the characterization of the link between the UPRmt and host defense.

I have few major concerns. One involves the interpretation of data. The authors write, "Further,

knockdown of hsp-60 reduced the level of active phospho-PMK-1 (Fig 4F). These data indicate that activation of PMK-1 upon PA14 infection requires HSP-60".

This, however, does not appear to agree with data shown. In control conditions, infection by PA14 is associated with a 20% increase in PMK-1 phosphorylation (1->1.2); following hsp-60(RNAi) there is a 280% increase (0.28 -> 0.79). Thus although hsp-60 is required for maintaining basal levels of PMK-1 phosphorylation, it is dispensable for activation of PMK-1 upon PA14 infection.

> We thank the reviewer for this critical comment and agree with the reviewer's point that HSP-60 is required for maintaining basal levels of phospho-PMK-1 under both control and PA14-infected conditions. Despite genetic requirement of *pmk-1* for anti-PA14 immunity, to our knowledge there has been no experimental evidence showing that PA14 infection increases the level of phospho-PMK-1 in *C. elegans* (Alper et al., 2010; reviewed in Kim and Ewbank, 2015). The quantification of our data also shows that increased phospho-PMK-1 levels were not significantly affected by *hsp-60* RNAi. We now changed the text and added of the quantification of the data (Fig EV4G) in the Results as follows.

Page 8, Line 16: "Further, knockdown of *hsp-60* reduced the level of active phospho-PMK-1 under both control and PA14-infected conditions (Fig 4F and EV4G). These data indicate that *hsp-60* is required for maintaining basal levels of phospho-PMK-1."

A second major concern is methodological. In their survival experiments, the authors use 5-fluoro-2'-deoxyuridine (FUdR). A series of studies (http://www.ncbi.nlm.nih.gov/pubmed/?term=fudr+elegans) have shown that FUdR has a broad impact on physiology. As a minimum, the key survival experiments performed with PA14 and the other pathogens need to be repeated in the absence of FUdR.

> We thank the reviewer for this comment. In our previous manuscript, we followed a standard PA14 slow killing assay protocol that uses FUdR for preventing viable progeny (Reddy et al., 2009); FUdR has been used for this assay because infection with PA14 often results in internal hatching (a bag-of-worm phenotype) in host animals, which leads to rapid deaths of the animals. Following the comments raised by the reviewer, we tested whether *hsp-60* RNAi increased susceptibility to PA14 without FUdR as well, and found that it did (Fig EV2A). In addition, we found that day 6 *hsp-60::GFP OE* animals displayed decreased PA14 susceptibility without FUdR (Fig 6J). Our *E. faecalis* killing assays were also done without FUdR (Fig 2H). Thus, the effects of *hsp-60* RNAi and/or *hsp-60::GFP OE* on pathogen susceptibility were reproduced without FUdR. We added our new data and describe these data to the Fig EV2 legend as follows.

Page 59, Line 20, Fig EV2 legend: "**A**, *hsp-60* RNAi reduced resistance to PA14 without fluoro-2'-deoxyuridine (FUdR) treatment."
Page 51, Line 5, Fig 6 legend: "**J**, Day 6 post-reproductive *hsp-60::GFP OE* animals displayed increased survival on PA14 without fluoro-2'-deoxyuridine (FUdR) treatment."
Page 47, Line 17, Fig 2 legend: "Knockdown of *hsp-60* had little or no effect on the survival of worms infected with *E. faecalis* without fluoro-2'-deoxyuridine (FUdR) treatment (**H**)…"

Of more minor concern:

The authors should provide a table summarizing how their results presented in Fig. 1B compare to published data.

> As the reviewer suggested, we made Table EV1 summarizing RNAi screen data shown in Fig 1B.

And although they write, "selected 220 RNAi clones that did not cause severe developmental delay or lethality for further studies (see Dataset EV1)", 15/16 of the final candidates are associated with WBPhenotype:0000050 (embryonic lethal). They should comment on this.

> We thank the reviewer for this comment. Although 15 out of the 16 RNAi clones are described to cause embryonic lethality in WormBase database, we were still able to use animals that had developed to L4/young adult stage on each of the RNAi clones. Because we knocked down each of mitochondrial components after hatching, we think animals were able to undergo normal embryonic

development and in many cases development to L4/young adults. We now made comments on this to make it clear in the Dataset EV1 legend as follows.

Page 67, Line 11, Dataset EV1 legend: "Although 15 RNAi clones among the 16 RNAi clones were reported to induce embryonic lethal phenotype (WBPhenotype:0000050) in the WormBase database, we were able to perform the survival assays with animals that had developed to L4/young adult stage on each of the RNAi clones. This seems likely to be because we began knocking down each of mitochondrial components after hatching, which allowed animals to undergo normal embryonic development."

The paper by Bennett et al, (PMID: 24662282) needs to be cited and discussed, especially since 6/16 RNAi targets identified here were reported to induce hsp-6p::gfp (i.e. the mitochondrial UPR) when knocked down.

> We thank the reviewer for this suggestion. We cited and discussed the Bennett et al. (2014) paper in the Expanded View Figure EV1 legend as described below.

Page 59, Line 1, Figure EV1 legend: "Among the 16 RNAi clones, we noticed that RNAi clones targeting six genes (*dap-3*, *mrps-5*, *mrpl-22*, *mrps-35*, *mfn-1*, or *hsp-60*) were reported to induce UPR^{MT} (Bennett et al., 2014). As five RNAi clones out of these six clones were included in the cluster III and increased PA14 resistance, these five RNAi clones may activate UPR^{MT} to enhance immunity in *C. elegans*."

Almost as many (5/16) genes are associated with suppressing osm-8 gpdh-1::GFP overexpression when knocked down (PMID: 21253570). How does this fit with the authors' model of a specific link between the UPRmt and resistance to PA14? In a similar vein, 4/16 block epidermal innate immunity (PMID: 27129311).

> We thank the reviewer for this suggestion. We discussed the suggested papers (Rohlfing et al., 2011; Zugasti et al., 2016) in the Figure EV1 legend as follows.

Page 59, Line 6, Figure EV1 legend: "Knocking down each of four genes (*mrps-5*, *mrps-35*, *F53F4.10*, and *mfn-1*) in the cluster III suppresses the induction of *nlp-29*, an epidermal anti-microbial peptide, upon infection with a fungal pathogen (Couillault et al, 2004; Zugasti et al, 2016). RNAi targeting each of five genes (*dap-3*, *mrpl-22*, *F53F4.10*, *mfn-1*, and *lpd-5*) in the cluster III also reduces the induction of an osmotic-response reporter *gpdh-1::GFP* caused by mutations in a mucin-like gene *osm-8* (Rohlfing et al, 2011). Transcriptional changes by osmotic stress and fungal infection are similar (Rohlfing et al, 2010), and resistance to osmotic stress correlates with immunity against fungal infection (Pujol et al, 2008). Therefore, inhibition of genes in the cluster III may promote the resistance against the pathogenic bacteria, PA14, while reducing protective responses to fungal pathogens and osmotic stresses."

The 2 recent papers from the Dillin lab (PMID: 27133166 and 27133168) that address the mechanism underlying gene expression changes provoked by the UPRmt need to be cited and discussed.

> We thank the reviewer for this valuable comment. As the reviewer suggested, we cited these two papers (Tian et al. (2016) and Merkwirth et al. (2016)) as follows.

Page 4, Line 4: "Under stress conditions, UPR^{MT} sends signals to the nucleus through HAF-1, a mitochondrial peptide exporter (Haynes et al, 2010), and up-regulates transcription factors including ATFS-1 (Haynes et al, 2010), DVE-1 (Haynes et al, 2007), its cofactor UBL-5 (Benedetti et al, 2006), and chromatin remodeling factors (Tian et al., 2016; Merkwirth et al., 2016)."

The authors cite the review Ewbank, 2006; they should also cite the more recent one (PMID: 26694508).

> We thank the reviewer for this suggestion and added the citation as follows.

Page 3, Line 8: "*C. elegans* has been used as an excellent model to study organismal innate immunity against pathogenic microbes (Ewbank, 2006; Irazoqui et al, 2010; Kim and Ewbank, 2015)."

In the discussion, it would also be useful to cite the relevant review, "Local and long-range activation of innate immunity by infection and damage in C. elegans" (PMID: 26517153).

> We thank the reviewer for the suggestion. We added the citation (Ewbank and Pujol, 2015) to the Introduction and the Discussion.

Page 3, line 8: "*C. elegans* has been used as an excellent model to study organismal innate immunity against pathogenic microbes (Ewbank, 2006; Irazoqui et al, 2010; Kim and Ewbank, 2015; Ewbank and Pujol, 2016)."

Page 4, Line 1: "Mitochondria also play roles in the resistance of *C. elegans* against pathogenic bacteria by regulating ROS production, mitophagy, and mitochondrial unfolded protein response (UPR^{MT}) (Hwang et al, 2014; Kirienko et al, 2015; Liu et al, 2014; Pellegrino et al, 2014; Ewbank and Pujol, 2015)."

The authors write, "The genetic inhibition of each of the transcription factors significantly increased susceptibility to PA14 (Fig EV3A-C) (Pellegrino et al, 2014).....Together, these data indicate that UPRMT, in general, is required for the PA14 resistance". The authors should make it clear whether this is a novel result, or simply recapitulates what was already known.

> We agree with the reviewer's point. Pellegrino et al. (2014) showed that genetic inhibition of *atfs-1* increased susceptibility to PA14, but they did not determine the effects of other UPR^{MT} factors (*dve-1*, *ubl-5* and *haf-1*) on PA14 resistance. Thus, we changed the sentences related to these results and the location of the citations to make this point clear, by saying,

Page 7, Line 20: "We found that the genetic inhibition of *dve-1* or *ubl-5* significantly increased susceptibility to PA14 (Fig EV3A-B). Mutations in *atfs-1* increased susceptibility to PA14 as well (Fig EV3C), as shown previously (Pellegrino et al, 2014). These data are consistent with the findings that these factors transcriptionally up-regulate HSP-60 (Benedetti et al, 2006; Haynes et al, 2007; Haynes et al, 2010). In contrast, the *haf-1* mutation did not affect the survival following PA14 infection (Fig EV3D)."

Fig 6E: some mitochondria are not labeled with HSP-60::GFP. Is this reproducible? Do they correspond to particular cells?

> We appreciate the reviewer's valuable comment. We obtained and carefully analyzed the pictures of twelve independent animals. We noticed that some of mitochondria that did not seem to be labeled with HSP-60::GFP contained weak GFP signals (Figures for referees not shown). Thus, the GFP signals in some mitochondria appear to be masked by strong red fluorescence signals by Mitotracker staining. Pearson's correlation coefficient analysis further confirmed this conclusion as the co-localization of green and red fuorescence signals was significant (r=0.906, p<0.001, data from all twelve animals). We changed the pictures accordingly and added the quantification data to the Fig 6.

Page 50, Line 14, Fig 6 legend: "HSP-60::GFP (**C**) and Mitotracker that stained mitochondria (**D**) were co-localized (**E**). Boxed areas were magnified in each panel. (**F**) HSP-60::GFP and Mitotracker signals in the boxed area in panel **E** show significant correlation (Pearson's correlation coefficient: r=0.917, p<0.001). We also found that correlation between HSP-60::GFP and Mitotracker signals was significant by using the images of all 12 animals (Pearson's correlation coefficient: r=0.906, p<0.001)."

Dataset EV1A - although more complete than in many studies, the authors need to give the exact names of the selected RNAi clones, in addition to giving their presumed targets. Did they use one of the dedicated tools (e.g. Clone Mapper) to determine targets? If not, they need to, since there have been shown to be deficiencies in the Wormbase predictions (PMID: 25187039). As part of minimal

reporting standards, what WS release was used?

> We thank the reviewer for this valuable comment. As the reviewer suggested, we added the following information to the Dataset EV1A: the exact names of the selected RNAi clones (RNAi clone name column) and expected targets predicted by Clone Mapper with each of the RNAi clone-target scores (Thakur et al., 2014) (Expected target by Clone Mapper and Clone Mapper score columns). For the mitochondrial gene selection, we used HomoloGene database (Wheeler et al., 2006), which is provided by NCBI. We performed *in silico* analysis on December, 2012, and used WS229 release for the selection (ftp://ftp.wormbase.org/pub/wormbase/releases). We added the information for the WS229 release according to the guidelines provided by the WormBase (http://www.wormbase.org/about/citing_wormbase#1320--10) as follows.

Page 66, Line 20: “The Clone Mapper (Thakur et al., 2014; http://www.bioinformatics.lif.univ-mrs.fr/RNAiMap/) was used to determine targets of RNAi clones, using RNAi clone-target scores.”

Page 19, Line 7: “By using the HomoloGene (build 25) database of NCBI (National Center for Biotechnology Information), total 3,147 *C. elegans* genes that have single orthologous human genes were selected (WormBase web site, http;//www.wormbase.org, WS229, released on Dec. 15th, 2011).”

What does N/A mean in the column "RNAi source (JA or MV)"? According to Clone Mapper, for many of these genes there are RNAi clones.

> We thank the reviewer for raising this issue. We meant “N/A” as RNAi clones that were not available in our laboratory. There were three subgroups of “N/A” RNAi clones. First, some of the RNAi bacteria did not grow, for unknown reasons. Second, in rare cases RNAi clones had wrong genes, which were excluded from further analysis. Third, some RNAi clones were not available from either JA or MV library. As we realized that the definition of “N/A” in our previous manuscript was confusing, we specified and defined these RNAi clones as follows; NG (No Growth of RNAi bacteria), IC (Incorrect Clone), and NA (Not Available from JA or MV) in the RNAi source column of the Dataset EV1A.

Page 66, Line 17, Dataset EV1 legend: “RNAi source indicates the origin of commercially available RNAi bacteria (JA: Julie Ahringer, MV: Marc Vidal RNAi library). We were not able to determine the effects of some RNAi clones, which were indicated as NG (No Growth of RNAi bacteria), NA (Not Available from JA or MV), and IC (Incorrect Clone).”

In the Abstract, the authors write, "host eukaryotes hijack molecular chaperones generated from bacteria-originated mitochondria". This needs to be reformulated if one assumes that the mechanisms they are describing a normal physiological process.

> We appreciate this reviewer’s comment. We now replaced the sentence with a more conservative one.

Page 2, Line 15:
(Previous version) “Our study suggests that host eukaryotes hijack molecular chaperones generated from bacteria-originated mitochondria to confer protection against pathogenic bacteria.”
(Modified version) “Our study suggests that molecular chaperones generated from bacteria-originated mitochondria protect host eukaryotes from pathogenic bacteria.”

The authors write, "activates transcription factors including DVE-1/UBL-5". Although UBL-5 forms a complex with DVE-1, it is not a transcription factor.

> As the reviewer pointed out, we changed the sentence as described below.

Page 4, Line 4: “Under stress conditions, UPR^{MT} sends signals to the nucleus through HAF-1, a mitochondrial peptide exporter (Haynes et al, 2010), and up-regulates transcription factors including ATFS-1 (Haynes et al, 2010), DVE-1 (Haynes et al, 2007), and its cofactor UBL-5 (Benedetti et al, 2006)…”

The authors write, "the mitochondrial chaperone HSP-60/HSPD1, which exhibits exceptional homology across species" - homology is an all-or-none characteristic. The authors mean "sequence conservation". The sequence conservation of HSP-60 is no higher than that of most other chaperones.

> We deleted the phrase following the reviewer's thoughtful comment.

Page 16, Line 15: "Overall, these studies and our current work suggest that both *C. elegans* and mammals use the mitochondrial chaperone HSP-60/HSPD1 to boost immunity."

Referee #3:

Jeong and colleagues introduce a novel function of HSP-60/HSPD1, a mitochondrial chaperone associated with UPRmt induction, in the protection of hosts from pathogen infection through the activation of p38 MAPK pathway. By focusing on the C. elegans- Pseudomonas aeruginosa host versus pathogen interaction, the authors show that intestinal and neuronal HSP-60 is important for the resistance of animals upon pathogen infection. HSP-60 is suggested to exert its protective effects through the stabilization of SEK-1, the MAPK kinase which phosphorylates PMK-1/p38 MAPK. Interestingly enough, the protective role of HSP-60 seems to be evolutionarily conserved, since its overexpression is sufficient to protect human cells from P. aeruginosa infection. More specifically, the authors identify several (16) conserved mitochondrial components which affect the survival of C. elegans after P. aeruginosa (PA14) infection. The most robust effect was exhibited by hsp-60 knockdown, and they found that this chaperone alone is necessary and sufficient for the resistance against PA14. HSP-60 protects the animals specifically against PA14 and through the activation of p38 MAP kinase signaling. HSP-60 also stabilizes SEK-1 which then positively regulates p38 which results in increased immunity. Furthermore, this function is conserved: in human cells overexpression of HSPD1 (the ortholog of HSP-60) increases the activity of p38. Overall, the manuscript reports interesting findings, although some of the authors' claims require further experimental support and some issues detailed below need to be addressed.

Comments:
Several previous reports have established the role of HSP-60 and of mito-UPR in immunity, compromising the novelty of the results presented here. For example, the effect of HSP-60 was examined in human monocytes and macrophages upon LPS (lipopolysaccharide from gram-negative bacteria), which is one of the most potent innate immunity activating stimuli known. In this context, which is comparable to the PA14 paradigm used here, HSP-60 was shown to activate the immune response via activation of p38 MAPK signaling (Kol et al., 2000, J. Immunology). More recently, in a publication that is cited in the manuscript (Pellegrino et al., 2014, Nature) it was shown that PA14 infection resulted in an atfs-1-dependnet increase in mitochondrial chaperone Hsp-60 and an increase in pmk-1 activity.

> We thank the reviewer for this valuable comment. As the reviewer mentioned, several reports have shown the potential immune functions of human HSP60 (Chen et al. 1999, J. Immunology; Kol et al., 2000, J. Immunology; Ohashi et al., 2000, J. Immunology). First, Chen et al. (1999, J. Immunology) reported that treatment of macrophages with recombinant human HSP60 (rhHSP60) purified from bacteria induced inflammatory cytokines. Following studies showed that the pro-inflammatory effects rhHSP60 is through Toll-like receptor 4 (TLR4) and p38 MAP kinase signaling in immune cells (Kol et al., 2000, J. Immunology; Ohashi et al., 2000, J. Immunology). However, importantly, these findings were challenged by the reports showing that the pro-inflammatory effects of rhHSP60 were due to contamination with lipopolysaccharide (LPS) during the rhHSP60 purification steps from bacteria (Gao and Tsan, 2003, J Immunology). A subsequent study reported that a domain of HSP60 tightly binds to LPS (Habich et al., 2005, J Immunology). As LPS is a ligand for TLR4 and activates p38 MAP kinase signaling (O'Neill et al., 2013, Nat. Rev. Immunology.), the rhHSP60-dependent regulation of p38 MAP kinase signaling and immune functions has been controversial (reviewed in Tsan and Gao, 2009, J. Leukocyte Biology and Quintana and Cohen, 2011, Trends in Immunology). Our work demonstrates that genetically modulated *hsp-60*, which is free from potential LPS contamination, plays key roles in innate immunity.

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As the reviewer mentioned above, Pellegrino et al. (2014) showed ATFS-1-dependent induction of HSP-60 upon PA14 infection, which is independent of PMK-1. As ATFS-1 is a transcription factor that modulates the expression of multiple anti-microbial genes (Nargund et al., 2012, Science; Pellegrino et al., 2014, Nature), we speculate that ATFS-1 regulates two distinct branches of immune responses; one pathway acts through the induction of anti-microbial genes, which is independent of PMK-1, and the other pathway acts through the induction of mitochondria-protective chaperones such HSP-60 in a PMK-1-dependent manner. Therefore, we believe our study provides important data supporting that HSP-60 in *C. elegans* and human epithelial cells increases anti-bacterial immunity through up-regulation of p38 MAP kinase signaling. We now describe this in the text by saying,

Page 15, Line 15: “The potential regulatory role of HSPD1/HSP60 in p38 MAP kinase-dependent innate immune responses in mammals has been reported. Treatment of immune cells such as macrophages and monocytes with recombinant human HSPD1/HSP60 (rhHSP60) purified from bacteria induces inflammatory cytokines (Chen et al., 1999), whose pro-inflammatory effects act through Toll-like receptor 4 (TLR4) and p38 MAP kinase signaling (Kol et al., 2000; Ohashi et al., 2000). However, several of these findings have been challenged by a report showing that the effects of the rhHSP60 were due to contamination with lipopolysaccharide (LPS) during protein purification processes from bacteria (Gao and Tsan, 2003). This finding was supported by a subsequent study showing that a domain of HSPD1/HSP60 tightly binds to LPS (Habich et al., 2005). As LPS is a ligand for TLR4 and activates p38 MAP kinase signaling (O’Neill et al., 2013), the rhHSP60-dependent regulation of p38 MAP kinase signaling and immune functions remained controversial (Tsan and Gao, 2009; Quintana and Cohen, 2011). Our current study provides crucial in vivo evidence for addressing this issue. First, our data with *hsp-60* RNAi indicate that endogenous HSPD1/HSP60 is required for the maintenance of p38 MAP kinase signaling. Second, we showed that genetically overexpressed HSP60/HSPD1, which is free from LPS contamination, up-regulated p38 MAP kinase signaling both in *C. elegans* and cultured mammalian cells. Thus, our study strongly supports the notion that HSPD1/HSP60 enhances immunity.”

Page 15, Line 5: “In addition, Pellegrino et al. (2014) concluded that activation of UPR^{MT} increases immunity independently of PMK-1, based on the results showing that RNAi targeting *spg-7* (mitochondrial metalloprotease) increases the survival of *pmk-1* mutants as well as wild-type animals on PA14. However, *spg-7* RNAi up-regulates many stress-responsive genes other than mitochondrial chaperones and increases lifespan (Curran and Ruvkun, 2007; Chen et al., 2007; Nargund et al., 2012). Thus, *spg-7* RNAi may increase the survival of *pmk-1* mutants through the activation of longevity and stress-responsive pathways independently of *pmk-1* signaling. Overall, UPR^{MT} appears to regulate at least two branches of anti-PA14 immune responses; one pathway acts through the induction of PMK-1-independent anti-microbial genes and the other pathway acts through the induction of mitochondrial chaperones, including *hsp-60* that regulates immunity via *pmk-1* signaling.”

The tissue specific requirement of HSP-60 for the immune response is very interesting and is worthy of further investigation. Recent studies have suggested that HSP-60 is secreted both from tumour and normal cells in detergent-resistant lipid vesicles or exosomes (Gupta and Knowlton, 2007). To what degree this also happens in the worm and whether it underlies the communication between neurons and the intestine are interesting points to consider.

> Following the reviewer’s valuable suggestion, we tested whether HSP-60 was secreted in *C. elegans*. In *C. elegans*, secreted proteins are taken up by coelomocytes (Fares et al, 2001, Genetics). Thus, we expressed tagRFP-fused HSP-60 in the intestine and neurons by using tissue-specific promoters. We then examined whether intestinal or neuronal HSP-60::tagRFP was taken up by the coelomocytes, but it was not (Fig EV10A-D). Although we still cannot exclude the possible secretion of *C. elegans* HSP-60 based on this negative result, we added our new findings to the Figure EV10 and discussed the results in the Discussion as follows.

Page 14, Line 10: “Intriguingly, a fraction of HSPD1/HSP60 in mammalian cells is shown to be localized in the extracellular space as well (Gupta and Knowlton, 2007; Cappello et al, 2008). Therefore, HSP-60 that is secreted to the extracellular space may also mediate immunity perhaps by acting as a cell-nonautonomous infection signal. We tested this possibility by measuring the

tagRFP-fused HSP-60 signals in the coelomocytes that are known to take up secreted proteins in *C. elegans* (Fares et al, 2001). We therefore expressed HSP-60::tagRFP specifically in the intestine or neurons, but did not detect the RFP signals in the coelomocytes (Fig EV10A-D). Thus, we currently do not have data supporting the secretion of HSP-60 to extracellular space in *C. elegans*."

It is also interesting that while knockdown of HSP-60 has an effect on the survival of C. elegans against PA14, it has no effect against other pathogenic bacteria such as E. faecalis. By contrast, knockdown of pmk-1 has an effect against all pathogens tested, suggesting that it is a hub where many signaling pathways of immunity converge. Are other mitochondrial chaperones activated by other pathogens? Is mito-UPR involved or not?

> We agree with the reviewer's point. We tested whether other pathogenic bacteria, *E. faecalis* and hyper-pathogenic *E. coli*, up-regulated mitochondrial unfolded protein response (UPR^{MT}). We found that infection with *E. faecalis* or pathogenic *E. coli* did not increase the levels of *hsp-6p::GFP* or *hsp-60p::GFP*. We now added the data to Fig EV2D-E and described the data as follows.

Page 60, Line 5, Fig EV2 legend: "**D-E**, Relative intensities for GFP expression were quantified from the animals expressing *hsp-6p::GFP* (**D**) and *hsp-60p::GFP* (**E**) on control bacteria, *E. faecalis*, or pathogenic *E. coli*. Infection with *E. faecalis* or pathogenic *E. coli* did not increase the levels of *hsp-6p::GFP* and *hsp-60p::GFP*."

The experiments in HeLa cells are not really novel (see point 1) and they were performed under control conditions, as opposed to using a model of immunity activation (such as the LPS).

> Kol et al. (2000) previously showed that treatment of immune cells such as monocytes with human recombinant HSP60 (rhHSP60) increased the level of phospho-p38 MAP kinase. As we discussed above, this finding was challenged by Gao and Tian (2003). Our results therefore provide experimental evidence for addressing this issue. First, our *C. elegans* RNAi data demonstrate that endogenous HSPD1/HSP60 is required for the maintenance of p38 MAP kinase signaling. Second, we showed that genetically overexpressed HSPD1/HSP60, which is free from LPS contamination, up-regulated p38 MAP kinase signaling both in *C. elegans* and in human epithelial cells (HeLa cells). We explicitly discuss this point in the Discussion as follows.

Page 15, Line 15: "The potential regulatory role of HSPD1/HSP60 in p38 MAP kinase-dependent innate immune responses in mammals has been reported. Treatment of immune cells such as macrophages and monocytes with recombinant human HSPD1/HSP60 (rhHSP60) purified from bacteria induces inflammatory cytokines (Chen et al., 1999), whose pro-inflammatory effects act through Toll-like receptor 4 (TLR4) and p38 MAP kinase signaling (Kol et al., 2000; Ohashi et al., 2000). However, several of these findings have been challenged by a report showing that the effects of the rhHSP60 were due to contamination with lipopolysaccharide (LPS) during protein purification processes from bacteria (Gao and Tsan, 2003). This finding was supported by a subsequent study showing that a domain of HSPD1/HSP60 tightly binds to LPS (Habich et al., 2005). As LPS is a ligand for TLR4 and activates p38 MAP kinase signaling (O'Neill et al., 2013), the rhHSP60-dependent regulation of p38 MAP kinase signaling and immune functions remained controversial (Tsan and Gao, 2009; Quintana and Cohen, 2011). Our current study provides crucial in vivo evidence for addressing this issue. First, our data with *hsp-60* RNAi indicate that endogenous HSPD1/HSP60 is required for the maintenance of p38 MAP kinase signaling. Second, we showed that genetically overexpressed HSP60/HSPD1, which is free from LPS contamination, up-regulated p38 MAP kinase signaling both in *C. elegans* and cultured mammalian cells. Thus, our study strongly supports the notion that HSPD1/HSP60 enhances immunity."

The claim that hsp-60 overexpression does not induce UPRMT is not sufficiently supported. The writers should provide the raw data of their quantitative PCR experiments that demonstrate that the lack of significant hsp-6 activation is repeatable and the use of an hsp-6:GFP reporter would constitute even stronger evidence.

> We thank the reviewer for raising this issue. We now provide our raw data for quantitative PCR experiments as a supplementary data file for the reviewer. As shown in the raw data file, we used

four different biological replicates, and found that the expression of *hsp-6* was variable and that the difference was not statistically significant. We also used technically duplicated samples for each biological sample to minimize pipetting errors. Because both *hsp-60::GFP OE* and *hsp-6p::GFP* reporter transgenes express the same green fluorescent signals, we were not able to determine whether *hsp-6p::GFP* levels were altered by *hsp-60::GFP OE*.

The authors have not definitively shown that SEK-1 is the mediator of the effect that HSP-60 has on PMK-1. They need to demonstrate that in a sek-1(-) genetic background the effects of HSP-60 overexpression on PMK-1 activity are completely abolished. Otherwise there could be an additional factor that acts in parallel to the proposed SEK-1>PMK-1 pathway.

> We thank the reviewer for the critical comment. We tested whether SEK-1 mediated the effects of *hsp-60* overexpression on PMK-1 activity by measuring the expression of PMK-1 target genes using qRT-PCR. We found that *sek-1* mutation almost completely suppressed the induction of PMK-1-regulated genes conferred by *hsp-60* overexpression (Fig 7J-L). We now added the data in the Fig 7 and described the data as follows.

Page 11, Line 16: "*sek-1* mutations also largely suppressed the induction of PMK-1-regulated genes conferred by *hsp-60::GFP OE* (Fig 7J-L). Thus, HSP-60 appears to act upstream of SEK-1 and PMK-1 to confer anti-PA14 resistance."

Page 52, Line 13: "**J-L**, qRT-PCR analysis showed that *sek-1* mutation largely suppressed induction of three selected PMK-1 target genes, T24B8.5 (J), C17H12.8 (K) and K08D8.5 (L), by *hsp-60::GFP OE* without PA14 infection (n≥3)."

An emerging question, which is also addressed to some extent by the authors in the discussion section of the manuscript, is how a mitochondrial chaperone can increase the expression / stabilize the cytoplasmic MAPKK SEK-1. Do the authors have any indication that HSP-60 in nematodes is at least to some extent localized in the cytoplasm or perhaps that its mitochondrial localization changes upon infection with pathogenic P. aeruginosa bacteria? Figures 6C, D and E do not support a putative cytoplasmic localization for HSP-60. Since commercially antibodies for HSP-60 exist, one possible approach would be the isolation of mitochondrial and cytoplasmic fragments (in nematodes, cells, or ideally both) to analyze the HSP-60 distribution under standard conditions and upon infection. Does HSP-60 directly interact with SEK-1 and protects it from degradation upon pathogen infection? Does the same occur in cell cultures?

> We appreciate the reviewer's valuable comment. As the reviewer suggested, we tested whether HSP-60 is localized in the cytosol and stabilizes SEK-1 through binding. First, we fractionated mitochondria and non-mitochondrial parts, and performed Western blot assays using HSP-60 or GFP antibodies. Interestingly, we detected endogenous HSP-60 and GFP-fused HSP-60 in the post-mitochondrial fraction, which includes cytosolic proteins (Fig 9A-B). In addition, we determined physical interaction between HSP-60 and SEK-1 by using a split GFP system (spGFP) (Ghosh et al, 2000; Hu et al, 2002; Zhang et al, 2004; Feinberg et al., 2007). We generated worms expressing HSP-60 fused with spGFP1-10, an N-terminal GFP fragment (spGFPN), and/or SEK-1 fused with spGFP11, a C-terminal GFP fragment (spGFPC). Importantly, we detected green fluorescent signals in the transgenic animals that expressed both HSP-60::spGFPN and SEK-1::spGFPC (Fig 9G-H) and the expression pattern was cytosolic (Fig 9I). This result suggests that HSP-60 binds SEK-1 in the cytosol. We also overexpressed cytosolic HSP-60::GFP (cytHSP-60::GFP) lacking mitochondria-targeting sequence (MTS) (Fig 9C-E). We found that overexpression of the cytosolic *hsp-60::GFP* (*cythsp-60::GFP OE*) was sufficient for enhancing PA14 resistance (Fig 9F). Together, these results provide mechanisms by which mitochondrial HSP-60 regulates cytosolic SEK-1/PMK-1 signaling. We added our new findings to the Results as follows.

Page 12, Line 7: "We then asked how mitochondrial HSP-60 increased the level of cytosolic SEK-1. A fraction of HSP-60 in yeast and cultured mammalian cells is known to be localized in the cytosol (Chun et al, 2010; Kalderon et al, 2015; Soltys & Gupta, 1996). Thus, we tested the possibility that a fraction of *C. elegans* HSP-60 in the cytosol up-regulated SEK-1. We first found that both endogenous and GFP-fused HSP-60 proteins were detected in the cytosol as well as in the mitochondria (Fig 9A-B). We then determined the functional importance of the HSP-60 in the

cytosol, by overexpressing HSP-60::GFP lacking mitochondria-targeting sequence (MTS), which was indeed localized in the cytosol (cytHSP-60::GFP; Fig 9C-E). Importantly, we found that this *cythsp-60::GFP* enhanced PA14 resistance (Fig 9F)."

Page 12, Line 16: "Next, we asked whether the cytosolic HSP-60 physically interacted with SEK-1 by using a split GFP (spGFP) system (Ghosh et al, 2000; Hu et al, 2002; Zhang et al, 2004; Feinberg et al., 2007). We detected green fluorescent signals in transgenic animals that expressed both N-terminal GFP fragment (spGFPN)-fused HSP-60 and C-terminal GFP fragment (spGFPC)-fused SEK-1 (Fig 9G-H). This result indicates that HSP-60 binds SEK-1 (Fig EV9A). We used spGFPC fused with mitochondrial chaperone HSP-10, whose mammalian homolog interacts with HSP-60 in mitochondria (Bukau and Horwich, 1998) and spGFPC, respectively, as positive and negative controls for HSP-60 binding (Fig. EV9B-D and Fig. 9J-K). We also noticed that the green fluorescence signals were located in the cytosol in the transgenic animals expressing spGFPN-fused HSP-60 and spGFPC-fused SEK-1 (Fig 9I). These results are consistent with the possibility that HSP-60 binds SEK-1 in the cytosol and that stabilizes SEK-1. Altogether, our data suggest that HSP-60 in the cytosol interacts with SEK-1 to confer anti-bacterial defense in the host animals."

Page 14, Line 1: "Our data suggest that HSP-60 located in the cytosol can play a role in immune responses. The effects of cytosolic HSP-60 on immunity led us to speculate that PA14 infection stress might trigger the translocation of HSP-60 from the mitochondria to the cytosol. However, PA14 infection (12 hours) did not alter the level of cytosolic HSP-60 (Fig 9A-B). These data suggest that a fraction of HSP-60 is localized in the cytosol under normal conditions and maintains SEK-1/PMK-1 signaling."

Since HSP-60 expression is believed to be upregulated in response to insults or genetic manipulations which trigger UPRmt, a reasonable question would be whether induction of UPRmt (for example through cco-1 or spg-7 RNAi treatments reported in the literature) is detrimental when combined with infection. One could think that induction of UPRmt might sequester a reasonable amount of HSP-60 protein away from the SEK-1-PMK-1 axis, not allowing the activation of a fully functional defense response.

> We thank the reviewer for raising this intriguing possibility. A previous report showed that *spg-7* RNAi actually enhances resistance to PA14 (Pellegrino et al., 2014, Nature). Therefore we think *spg-7* RNAi-induced mitochondrial stress is not detrimental for the host defense response. Instead, it appears to be sufficient for both increasing mitochondrial protein quality control and host immune responses by inducing mitochondrial chaperones, including HSP-60. We discussed this issue in the Fig EV7 legend by saying,

Page 64, Line 6, Fig EV7 legend: "*hsp-60* expression is known to be increased in response to stresses that trigger UPR^{MT} (Pellegrino et al, 2013). Therefore, the activation of UPR^{MT} upon PA14 infection might sequester a certain amount of HSP-60 away from the SEK-1/PMK-1 signaling pathway, and might not allow the activation of a fully functional defense response. If so, one can expect that the induction of UPR^{MT} is detrimental to animals upon PA14 infection. However, it does not seem to be the case as *spg-7* RNAi, which induces UPR^{MT}, actually enhances resistance to PA14 (Pellegrino et al., 2014)."

Do the authors have any hypothesis on why depletion of several mitochondrial ribosomal proteins can be beneficial following P. aeruginosa infection (table 1)?

> We have two hypotheses regarding this question. First, recent studies have shown that depletion of mitochondrial ribosomal proteins extends the lifespan of *C. elegans* through activating mitochondrial stress responses (Houthkooper et al., 2013). Although enhanced PA14 resistance is not always associated with longevity, many *C. elegans* longevity mutants are resistant against bacterial pathogens (Kim, 2013). Thus, it seems likely that RNAi targeting mitochondrial ribosomal protein genes confers longevity and increases survival on PA14. Second, depletion of mitochondrial ribosomal proteins activates UPR^{MT}, which leads to the induction of mitochondrial chaperones, including HSP-60, and anti-microbial genes. This induction of HSP-60 may then at least in part contribute to PA14 resistance in animals treated with RNAi targeting mitochondrial ribosomal protein genes. We added description regarding these hypotheses to the Fig EV1 legend.

Page 58, Line 12, Fig EV1 legend: "Interestingly, five RNAi clones targeting mitochondrial ribosomal proteins increased PA14 resistance. Recent studies have shown that depletion of mitochondrial ribosomal proteins extends *C. elegans* lifespan through activating mitochondrial stress responses (Houthkooper et al., 2013). Although enhanced PA14 resistance is not always linked to longevity, many *C. elegans* longevity mutants are resistant to bacterial pathogens (Kim, 2013). Thus, it seems likely that RNAi targeting mitochondrial ribosomal protein genes promotes longevity, which may increase survival on PA14. Furthermore, depletion of mitochondrial ribosomal proteins activates UPR^{MT}, which leads to the induction of anti-microbial genes (Nargund et al., 2012; Pellegrino et al., 2014) and the induction of mitochondrial chaperones, including HSP-60. This induction of HSP-60 may then contribute to PA14 resistance, as we showed in this study."

Minor Comments
In figure EV10A, the strain name should be corrected to SEK-1::tagRFP instead of PMK-1::tagRFP (at least according to the figure legend provided).

> We thank the reviewer for the comment, and changed the label in the Figure 8A (previously, it was Fig EV10A) accordingly.

Furthermore, since the proteins are tagged with fluorescent proteins (GFP, RFP), capital letters should be used on all the figures for the transgenic strains (HSP-60, SEK-1 and PMK-1 overexpressing lines).

> We thank the reviewer for this comment. As the reviewer suggested, we used capital letters for the labeling of proteins in the figures.

The legend of figure EV1B includes the unsupported claim that "mitochondrial translation and respiration appear to confer the susceptibility of C. elegans against bacterial pathogens via modulating SKN-1". The authors should demonstrate that the effect of the cluster III genes in survival is lost in a skn-1(-) background or remove the sentence entirely (since it is not relevant to the rest of the manuscript). That legend also fails to acknowledge that the effect of the cluster III genes in survival is dependent on daf-16.

> We appreciate the reviewer for this critical comment. We removed "mitochondrial translation and respiration appear to confer the susceptibility of *C. elegans* against bacterial pathogens via modulating SKN-1" entirely. In addition, we changed the legend for Figure EV1B to include the interpretation regarding the DAF-16 dependency.

Page 58, Line 6, Figure EV1 legend: "In contrast, the expression of PMK-1/p38 MAPK target reporter *T24B8.5p::GFP* was largely unaffected (Fig 1C and Dataset EV1). The effects of the RNAi clones on PA14 resistance decreased or disappeared in *zip-2* or *daf-16* mutant animals (Fig 1B). Together, mitochondrial components that influence immunity against PA14 appear to form functional modules to regulate the activities of immune proteins."

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Additional Correspondence 9 January 2017

Thank you for submitting your manuscript to The EMBO Journal. The manuscript has now been seen by the original 3 referees.

Before I will get back to you with a decision and the full set of referee reports, I would like to ask for your clarification on the comments raised by referee#1 (see below) especially the ones pertaining to the reproducibility of the findings in Figure 7E. I would like to ask if you could to send me the source data for figure 7E and the replicates. You can send me the information via email. I understand that the antibody used might be a bit finicky to work with, but it is important to show representative findings in the figures.

REFEREE REPORT

Referee #1

As explained in more detail below, there are some remaining concerns regarding the overexpression and the robustness/reproducibility of the p38 activation assay.

The authors have failed to explain a possible correlation between survival and overexpression of hsp-60::gfp. Only fold changes for line #1 are shown. The changes in gene expression for animals "overexpressing" hsp-60 are almost insignificant (~1.2 fold).

The results on HeLa cells are still not very convincing, and taking into account the new data, very hard to reproduce. Previous figure 7E showed high increase levels of p-p38 MAPK in response to infection, which are not seen now. Also, changes previously observed at earlier time points, are not seen in the latest experiments. The quantification is also unclear: how can you get a "1.3" fold from "0"? Shouldn't it be "1"?

As a proof of the robustness of the results, the authors showed in their rebuttal letter, 3 experiments, but the image in experiments 1 and 3 seems to be the same.

2nd Editorial Decision 12 January 2017

Thank you for sending me the source data and the replicates for figure 7E. I agree with you that while there is variability in the kinetics of p-p38 induction that one can see increased levels of p-p38 after PA14 infection from the provided blots. The question is how to show the data best and I think showing this in a quantification format is a good suggestion. I am not so sure that you need to show all 6 trials in the figure. Maybe a good way is to show one representative example (gel and quantification) and show the other examples as source data (http://emboj.embopress.org/authorguide#sourcedata). You can refer to the other trials in the figure legend and point the reader to the source data.

I have also provided the other referee comments below. As you can see the referees appreciate that the manuscript has been extended. They have a few remaining points that shouldn't involve too much further work to resolve. I would therefore like to invite you to submit a finally revised manuscript that addresses the last remaining points. You can use the link below to upload the revised version.

Don't hesitate to contact me if you have any questions.

REFEREE REPORTS

Referee #1:

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As a proof of the robustness of the results, the authors showed in their rebuttal letter, 3 experiments, but the image in experiments 1 and 3 seems to be the same.

Referee #2:

The authors have fully answered my concerns and are to be commended for strengthening their study so completely.

I have only very minor remarks:

In Figure EV9 panel B, the worms of the different genotypes should all be of the same age/size.
In the Abstract, "bacteria-originated" should be dropped from the sentence, "Our study suggests that molecular chaperones generated from bacteria-originated mitochondria protect host eukaryotes from pathogenic bacteria" because it has the potential to confuse readers, and there is currently no evidence for any sort of significance in the evolutionary origin of hsp-60.

"Several lines of evidence are against this possibility" should be, "Several lines of evidence argue against this possibility".

In the references, "Gene Ontology C (2015)", should be "The Gene Ontology Consortium (2015)".

Referee #3:

In their revised manuscript, Jeong and colleagues provide additional experimental evidence supporting the role of mitochondrial HSP60 in anti-bacterial immunity. The novelty of the study lies in the findings indicating that genetic inhibition or overexpression of HSP-60 leads to attenuated or enhanced resistance, respectively against P. aeruginosa PA14 both in C. elegans and mammalian cells. Furthermore, HSP-60 -mediated immunity requires the activity of p38 MAP kinase. Notably, the authors suggest that cytosolic HSP-60 associates and stabilizes SEK-1/MPA kinase kinase 3 enhancing innate immunity via PMK-1, the homolog of the mammalian p38 MAP kinase, stimulation.
Indeed the authors addressed approximately all of the issues raised by the reviewers. Moreover, the study provides new mechanistic insights into how HSP60 regulates immune responses both in C. elegans and mammalian cells. However, a point of consideration is that two out of three reviewers (reviewers #2 and #3) challenge the novelty of the study at least regarding the data derived from experiments in HeLa cells.
The novelty of this work is mainly based on findings suggesting that the mechanism through which HSP-60 performs its antibacterial role is through p38 MAP kinase activation. Particular attractive is the fact that the suggested regulatory mechanism of HSP-60 anti-bacterial activity depends on PMK-1 stimulation.
Notably, Pellegrino et al (Nature 2014) had shown that the relative immune mechanism is independent of p38 MAPK signaling. They found that mtUPR activation, which leads to HSP-60 upregulation, enhances survival of C. elegans independently of PMK-1 activity. Thus, the section on the involvement of HSP-60 in the regulation of PMK-1 activity ultimately leading to increased protection against P. aeruginosa needs further consideration. Nevertheless, both studies underline the impact of mitochondrial homeostasis in pathogenic situations. Furthermore, it is indicated that different signaling cascades are promoted in response to diverse stimuli (mitochondrial homeostasis

collapse spg-7 knockdown and pathogens invasion P. aeruginosa), emphasizing the complexity of immune response.

Other comments:
The authors propose that HSP-60 is both mitochondrial and cytoplasmic (based on biochemical experiments presented in figures 9A,B). One should note however, that in figures 6C-E HSP-60:GFP shows a completely dotty expression pattern, reminiscent of proteins which are exclusively localized in the mitochondrial matrix. No sign of diffuse cytoplasmic expression is visible in these confocal images (as shown in figures 9C-E). However, overexpression of the cytoplasmic version of HSP-60 is sufficient to promote survival upon PA14 infection (figure 9F). One question that was raised in the first round of review was whether HSP-60 distribution changes post-infection (in other words whether the chaperone reallocates to the cytoplasm or its mitochondrial/cytoplasmic ratio is affected to serve its proposed role in SEK stabilization). This as an important point, which should be clarified.

In figure 9B, the blot for VDAC-1 (long) is saturated. Please provide a better image.

2nd Revision - authors' response 01 February 2017

Referee #1

As explained in more detail below, there are some remaining concerns regarding the overexpression and the robustness/reproducibility of the p38 activation assay.

The authors have failed to explain a possible correlation between survival and overexpression of hsp-60::gfp. Only fold changes for line #1 are shown.

> We thank the reviewer for raising this concern. We measured *hsp-60* mRNA level in the line #2 transgenic animals that expressed *hsp-60::GFP* as well. We found that the line #2 transgenic animals displayed significantly higher levels of *hsp-60* mRNA compared to that of wild-type animals (n=4). To confirm that *hsp-60::GFP* transgenes increased the level of HSP-60 proteins, we performed Western blot analysis. We used anti-HSP-60 antibody that can detect both endogenous (about 60 kDa) and GFP-fused HSP-60 (about 93 kDa). Two independent lines (#1 and #2) of transgenic animals expressed exogenous HSP-60::GFP, and increased total HSP-60 protein pools. We now included the additional qRT-PCR and Western blot data in the Fig EV5A-C and discussed in the result section of the manuscript as follows.

Page 9, Line 12: "We first confirmed that these transgenes increased the levels of *hsp-60* mRNA (Fig EV5A and D) and HSP-60::GFP protein (Fig EV5B-C). We then showed that the GFP-fused HSP-60 was mainly localized to the mitochondria of cells in multiple tissues (Fig 6A-F)."

The changes in gene expression for animals "overexpressing" hsp-60 are almost insignificant (~1.2 fold).

> Although the change was small as the reviewer pointed out, 1.2-1.4 fold increases in *hsp-60* mRNA levels were statistically significant and enhanced survival on our killing assay. However, we do agree with the reviewer's point that it is misleading to say "overexpression" based on 1.2-1.4 fold increases in the expression. Thus, we will downplay "overexpression" in the main text and write "increased expression" or simply "transgene (Tg)". Following are examples (changes are highlighted with bold and underline).

Page 9, Line 13: "We then asked whether **increased expression of *hsp-60*** affected anti-PA14 immunity by generating transgenic worms that expressed *hsp-60* with or without a GFP tag (*hsp-60::GFP* ***Tg*** or *hsp-60* ***Tg***).

Page 63, Line 1, Fig EV5 legend: "D, qRT-PCR data indicate that **transgenic expression** of *hsp-60* without a tag (*hsp-60* ***Tg***) **slightly but significantly** increased the level of *hsp-60* mRNA in two independent lines (#1 and #2; see Materials and Methods)."

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In addition, perhaps too high level of *hsp-60* is toxic, based on our experience in generating the transgenic animals expressing *hsp-60*. That is because we microinjected the *hsp-60* transgenic DNA constructs even with a low concentration (5 ng/µl) into over 150 animals, which is a very high number, but we were able to obtain only two different lines of transgenic animals, which displayed slight induction. We described this as a potential pitfall for our *hsp-60* transgenesis experiments in the Materials and Methods.

Page 29, Line 9, Materials and Methods: "Only two different lines of transgenic *hsp-60p::hsp-60cDNA* animals, which displayed slight induction of *hsp-60* (Fig EV5B), were obtained over 150 microinjected animals with a low concentration of DNA (5 ng/µl). We therefore speculate that too high level of *hsp-60* may be toxic, and that may be the reason why obtaining *hsp-60*-overexpressing animals was difficult."

The results on HeLa cells are still not very convincing, and taking into account the new data, very hard to reproduce. Previous figure 7E showed high increase levels of p-p38 MAPK in response to infection, which are not seen now. Also, changes previously observed at earlier time points, are not seen in the latest experiments.

We completely agree with the reviewer's point. In our initial submission of figure 7E, HSPD1-myc appeared to increase p38 MAP kinase (p-p38) level at 1 hours after infection (trial 2, in the Source Data 1). However, in the other two separate experiments we performed, significant induction of p-p38 was observed at 2 hours after infection (trial 1 and trial 3 in the Source Data 1). Since the reviewer had suggested that we need to examine additional time points, we added two more time points (0.5 hr and 1.5 hr) and performed triplicate experiments (trials 4-6). Again, we observed the induction of p-p38 at 2 hr after infection.
From these multiple trials, we reached following conclusions.

1. Overexpression of HSPD1-myc increased the levels of p-p38. From the six Western blot data, what we reproducibly observed was that p-p38 level was increased after PA14 infection compared to non-infected control.
2. However, kinetics of p-p38 induction after infection does not seem to be consistent under our experimental condition, as we observed p-p38 induction at 1 hr or 2 hr post-infection.

We think identification of the biological source that influenced the variability of the p-p38 kinetics among samples is beyond our current research scope. Therefore, instead of showing a representative figure (Fig 7E) in the main text, we reached the conclusion that all six trials should be presented as a quantification format (Fig EV8B). In addition, we included all six blots as source data (Source Data 1) in an honest and unbiased manner, as the editor suggested.

The quantification is also unclear: how can you get a "1.3" fold from "0"? Shouldn't it be "1"?

> We thank the reviewer for raising this issue. We previously used an arbitrary unit for band intensities in each blot after normalizing the band intensities of p-p38 with the corresponding band intensities of p38 (previous Figure 7E). We also agree with the reviewer that "relative intensity" in the figure legend was misleading as the quantification was not based on relative intensity; this happened because of miscommunication between the author who did the quantification (Jee-Eun Lee) and the authors who wrote the figure legends (Dae-Eun Jeong and Seung-Jae V. Lee). We quantified the blot data again using relative intensity (see the attached "5. Quantification data for Fig. 7E and EV8B.xls" file). We normalized the band intensities and set the control values (Empty vector control, 0 h) as "1". Although the *p* value is not statistically significant perhaps because of big error bars, all six Western blot data reproducibly show that transfection of *HSPD1-myc* up-regulated p-p38 level 2 hrs post PA14 infection.

As a proof of the robustness of the results, the authors showed in their rebuttal letter, 3 experiments, but the image in experiments 1 and 3 seems to be the same.

> We really thank the reviewer for pointing out our mistake and apologize for the mistake. Regarding the Figure 7E, I checked the source data with my co-authors, including Jee-Eun Lee who did the HeLa cell experiments, and Prof. Joo-Yeon Yoo, her advisor. As the reviewer #1 correctly pointed out, trials 1 and 3 in our rebuttal letter were duplicated data with different exposure times. It was unintentionally included when we assembled the data during our previous preparation. There was no false intention at all, because we do have performed multiple experiments and had six full

sets of data. We showed the representative blot in the Fig 7E and provided quantification data (Fig EV8B) and all six blot data as source data (Source Data 1), as the editor suggested.

Referee #2

The authors have fully answered my concerns and are to be commended for strengthening their study so completely.

I have only very minor remarks:

In Figure EV9 panel B, the worms of the different genotypes should all be of the same age/size.

> As the reviewer suggested, we replaced the previous Fig EV9B images with the ones that show the worms with same age/size.

In the Abstract, "bacteria-originated" should be dropped from the sentence, "Our study suggests that molecular chaperones generated from bacteria-originated mitochondria protect host eukaryotes from pathogenic bacteria" because it has the potential to confuse readers, and there is currently no evidence for any sort of significance in the evolutionary origin of hsp-60.

> We agree with the referee's point. We changed the last sentence in the Abstract as the referee suggested.
Previous version: "Our study suggests that molecular chaperones generated from bacteria-originated mitochondria protect host eukaryotes from pathogenic bacteria"
New version: "Our study suggests that mitochondrial chaperones protect host eukaryotes from pathogenic bacteria by up-regulating cytosolic p38 MAPK signaling."

"Several lines of evidence are against this possibility" should be, "Several lines of evidence argue against this possibility".

> We changed the sentence as the referee suggested.
Page 10, line 1: "Several lines of evidence argue against this possibility."

In the references, "Gene Ontology C (2015)", should be "The Gene Ontology Consortium (2015)".

> We changed "Gene Ontology C (2015)" to "The Gene Ontology Consortium (2015)" in the references.

Referee #3

In their revised manuscript, Jeong and colleagues provide additional experimental evidence supporting the role of mitochondrial HSP60 in anti-bacterial immunity. The novelty of the study lies in the findings indicating that genetic inhibition or overexpression of HSP-60 leads to attenuated or enhanced resistance, respectively against P. aeruginosa PA14 both in C. elegans and mammalian cells. Furthermore, HSP-60 -mediated immunity requires the activity of p38 MAP kinase. Notably, the authors suggest that cytosolic HSP-60 associates and stabilizes SEK-1/MPA kinase kinase 3 enhancing innate immunity via PMK-1, the homolog of the mammalian p38 MAP kinase, stimulation. Indeed the authors addressed approximately all of the issues raised by the reviewers. Moreover, the study provides new mechanistic insights into how HSP60 regulates immune responses both in C. elegans and mammalian cells. However, a point of consideration is that two out of three reviewers (reviewers #2 and #3) challenge the novelty of the study at least regarding the data derived from experiments in HeLa cells.

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> We thank the referee for raising this critical issue. We further tested whether PA14 infection promoted translocation of HSP-60 from mitochondria to the cytosol, and concluded that PA14 infection did not influence the subcellular distribution of HSP-60. Specifically, we infected the wild-type N2 animals with PA14 for 2, 4, 8, and 12 hours and measured the level of cytoplasmic HSP-60 by using subcellular fractionation assay followed by Western blot analysis. We found that PA14 infection did not alter the level of cytosolic HSP-60. We also quantified the ratio of cytoplasmic/mitochondrial HSP-60 and found that 12 hours of infection did not change the ratio. In addition, we found that GFP signal driven by physical interaction between HSP-60 and SEK-1 in the cytoplasm was not affected by PA14 infection. Altogether, our data suggest that PA14 infection does not appear to change the subcellular distribution of HSP-60. We now included these data in the Fig EVXX and explained the data as follows.

Page 13, Line 19: "Our data suggest that HSP-60 located in the cytosol can play a role in immune responses. The effects of cytosolic HSP-60 on immunity led us to speculate that PA14 infection stress might trigger the translocation of HSP-60 from mitochondria to the cytosol. However, it does not seem to be the case, because PA14 infection did not alter the level of cytosolic HSP-60 or the ratio of cytosolic/mitochondrial HSP-60 levels (Fig 9A-B and Fig EV10A-C). In addition, cytosolic GFP signals obtained from the interaction between HSP-60::spGFPN and SEK-1::spGFPC were not increased by PA14 (Fig EV10D). These data suggest that a fraction of HSP-60 is localized in the cytosol under normal conditions and maintains SEK-1/PMK-1 signaling."

In figure 9B, the blot for VDAC-1 (long) is saturated. Please provide a better image.

> We replaced the previous Fig 9B blot data with better images that show clear GFP, alpha-tubulin, and VDAC-1 blots as the referee suggested.

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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
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- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
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2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
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  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

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Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Optimal sample size was chosen based on the method information in previous literature in which similar experiments were performed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample sizes for all the experiments were mentioned in Figure legends (pages 46-55) and/or in Materials and Methods (pages 16-32).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We did not exclude any experimental data.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	For survival analyses, two independent researchers independently measured the survival of aniamals to reduce subjective bias.
For animal studies, include a statement about randomization even if no randomization was used.	We did not use randomization.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No.
4.b. For animal studies, include a statement about blinding even if no blinding was done	We did not do blinding.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	No.
Is there an estimate of variation within each group of data?	Variation in each experimental group was shown in the data figures by using standard error of the mean (SEM) represented as error bars.
Is the variance similar between the groups that are being statistically compared?	We did not perform the statistical test for the variance.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Materials and Methods: page 25-27
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Materials and Methods: page 16-32
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NO.