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Reduced insulin/IGF1 signaling prevents immune aging via ZIP-10/bZIP-mediated feedforward loop

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Re: JCB manuscript #202006174

Prof. Seung-Jae Lee
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Dear Prof. Lee,

Thank you for submitting your manuscript entitled "Reduced insulin/IGF1 signaling prevents immune aging via ZIP-10/bZIP-mediated positive feedback loop". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. As you will see from their comments they both find the work extremely interesting. Both of them however, have a series of specific and precise questions that needs to be addressed. So even though the paper is not acceptable in the JCB in its present form we would be happy to re-consider a revised manuscript that would address each points raised by the reviewers. The paper will then be re-reviewed by the original reviewers. Please bear in mind that the JCB allows only one round of revision so I would strongly encourage you to address all points made by the reviewers. It would also be useful for the journal if you could let us know how long you think the revisions will take.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

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Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

I look forward to seeing a revised manuscript.

Sincerely,

Gerard Karsenty, MD, PhD
Monitoring Editor

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Reviewer #1 (Comments to the Authors (Required)):

This is a very interesting story. The authors provide data showing that reduced IIS rejuvenates immunity at older age via a feedback circuit involving FOXO and bZIP transcription factors. The results could have important implications to understand immune aging and how to delay this process. Nevertheless, I have a few questions/points that I hope the authors will be able to address:

- In Fig. 1A and S1D, the authors conclude that old isp-1 mutant worms display susceptibility to PA14 infection comparable to young isp-1 mutant animals. However, although to a lesser extent than daf-2 mutants, Fig. S1D indicates that old isp-1 worms also display enhanced survival upon PA14 when compared with young isp-1 worms. This is not well explained/noted in the main text. It is important to statistically test whether the differences between old and young isp-1 are significant and comment on this in the main text. At least, this seems to be the case for one of the independent replicates. Likewise, the authors mention regarding isp-1 worms that the "overall survival rate was lower than that of wild-type worms". The data presented in Table S1 indicate that this might be true for young isp-1 compared to young WT, but not when old isp-1 worms are

compared with old WT worms. Actually, old isp-1 worms appear to be even more resistant to PA14 infection than old WT in one replicate experiment.
- In Fig. S1J, the results clearly show that daf-2 treatment does not prevent age-associated decline in resistance against oxidative stress, especially when compared with day 4 wild-type worms. However, it is surprising that daf-2 RNAi does not confer resistance to oxidative stress when directly compared with control worms of the same age. Resistance to oxidative stress is one of the well-studied phenotypes induced by reduction of IIS signalling. The lack of effects on daf-2 RNAi treatment could be due to its application at an older age than previous studies, which it could be discussed in the Discussion section. I could not find how the authors perform the analysis of survival rates under oxidative conditions in the Methods section. Please include this information. If the authors performed these assays in liquid media, they might consider to do again the assay in agar plates which give more reliable results for oxidative stress resistance than liquid media.
- It is not clear to me why the authors conclude that the expression of selected DAF-16 targets not related with immunity such as mtl-1 and hsp-16.2 displayed age-dependent increase in wild-type worms and a further increase in aged daf-2 mutants, whereas known PMK-1 targets show decrease during aging in both wild-type and daf-2 support the following conclusion: "These data are consistent with our results showing the requirement of DAF-16/FOXO for the enhanced immunity observed in old daf-2 mutants".
- In Fig. 4, the authors perform a targeted screen using mutants that carry mutation in 13 genes selected among the 72 candidates. If there are not available mutants for the other genes, the authors should may consider a targeted RNAi screen against each of the 72 candidates, as this could provide novel regulators of immune aging and increase the implications of their findings. If the authors decide to keep only the data in the 13 genes analyzed and not to examine the other candidates, they should explain in detail the rationale to select these 13 genes in particular.
- After the screen of the 13 mutants, the authors focused on ins-7, which is an agonist of the IIS pathway and it has been previously described as a modulator of resistance to pathogens in young age. Then, they find that another of their targets regulates ins-7 levels. The link between ins-7 and zip-10 -which eventually regulates the immune response via a feedback circuit involving the modulation of IIS itself- provides a mechanistic explanation for the model studied here. Besides ins-7 and zip-10, the authors identified potential novel regulators of immune response in aged animals. It would be interesting to gain further insights on how these factors regulate/delay immune aging, or comment on this in the Discussion.
- Since some of the conclusions rely on RNAi experiments, the authors should present data on the knockdown efficiency for the distinct RNAis used through the manuscript.
- Given that zip-10 is a transcription factor, the authors should assess whether zip-10 mutant worms exhibit decreased mRNA levels of endogenous ins-7 to strengthen the link between zip-10 and ins-7 regulation. In these lines, the author should analyse whether mutations in isy-1 regulate the levels of ins-7:GFP reporter and mRNA levels of endogenous ins-7.
- An important question raised by the authors' findings that should be assessed is whether zip-10 also regulates the impact of HSF-1 on immunity induced by reduced IIS.

Reviewer #2 (Comments to the Authors (Required)):

Lee and colleagues performed an very interesting study looking at the insulin receptor mutant daf-2 in C. elegans, which were previously known to have pathogen resistance compared to wildtype animals, but in this new study it is revealed that pathogen resistance is present at old age and more interesting older daf-2 mutants are even more resistant if compared to younger aged daf-2 worms.

I believe that this study is appropriate for the Journal of Cell Biology, but requires significant editing before acceptance. The Lee lab is well established for lifespan and healthspan studies and the methodologies are appropriate, but in several instances the data presented do not clearly justify the conclusions. There are also several contradictions with the current literature, which might be very interesting, but at present leave the reader confused since they are not addressed.

Major concerns:

The authors should make and present a model for the positive feedback loop. I've read the text sevral times and it is complicated by the fact that you are inactivating inhibitors that increase activators, etc. Along these lines is there a way to show that INS-7 levels are indeed reduced? With this in mind, if daf-2 is absent, how can the absence of ins-7 have any impact (I think it is a positive regulator?). As this is the major conclusion of the paper this needs to be presented in a clear manner.

In Figure S3, are these data from animals exposed to PA14 at the ages indicated? If not, is this data in the submission? It should be included to better justify the conclusions made about the role of each gene in immunocompetency.

The manuscript needs a better description of how statistical comparisons were performed. This is absent from the methods section. Also there are references to "Star methods"? It is not clear what these are.

In figure 3 the authors select two insulin pathway and two p38 pathway genes to examine. This data looks great, but these pathway regulate 100s of genes, what is the importance of these genes?

The authors pick day 9 of adulthood as "old". IN reality this is at best middle age? And if compared to daf-2 is still a very young adult. This should be explained.

What happens if daf-2 RNAi is initiated during development and removed when the animals reach adulthood? Alternatively what happens if you test adult animals that were previously released from dauer?

Dear Drs. Karsenty and Marat,

We appreciate this opportunity to revise our manuscript entitled “Reduced insulin/IGF1 signaling prevents immune aging via ZIP-10/bZIP-mediated positive feedback loop” for publication in *Journal of Cell Biology*. We thank both reviewers for their valuable comments that have been extremely helpful for improving our manuscript.

Our point-by-point responses to the reviewers’ comments are described below. We hope our revised manuscript is suitable for publication in *Journal of Cell Biology*.

Best regards,

Seung-Jae V. Lee (On behalf of all the authors)

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> We agree with the reviewer's comment. In our previous manuscript, we had the data showing that day 9 *isp-1(–)* mutants survived longer than day 1 *isp-1(–)* mutants on PA14 in one trial but did not in the other trial. Because the data were inconclusive, we performed two additional PA14 survival assays using day 1 and day 9 *isp-1(–)* mutants for this revision. We found that *isp-1(–)* mutants at day 9 adulthood exhibited decreased survival on PA14 compared with day 1 adult *isp-1(–)* mutants in both of the additional trials. We included these additional data in the revised manuscript, and described the results as follows.

Results and Discussion, page 6, line 89: We examined whether the rates of immune aging were proportionally affected by genetic mutations that promote longevity. Similar to the wild-type counterparts (Figure 1A-B) (Laws et al., 2004, Youngman et al., 2011), long-lived mutants such as sensory-defective *osm-5*, dietary restriction mimetic *eat-2*, mitochondrial respiration-impaired *isp-1*, and germline-deficient *glp-1* mutants exhibited an age-dependent increase in susceptibility to *Pseudomonas aeruginosa* (PA14) infection (Figure 1A and Figure S1A-E).

- In Fig. S1J, the results clearly show that daf-2 treatment does not prevent age-associated decline in resistance against oxidative stress, especially when compared with day 4 wild-type worms. However, it is surprising that daf-2 RNAi does not confer resistance to oxidative stress when directly compared with control worms of the same age. Resistance to oxidative stress is one of the well-studied phenotypes induced by reduction of IIS signalling. The lack of effects on daf-2 RNAi treatment could be due to its application at an older age than previous studies, which it could be discussed in the Discussion section. I could not find how the authors perform the analysis of survival rates under oxidative conditions in the Methods section. Please include this information. If the authors performed these assays in liquid media, they might consider to do again the assay in agar plates which give more reliable results for oxidative stress resistance than liquid media.

> We appreciate the reviewer's valuable comment. As the reviewer pointed out, in the trial that we included in our previous manuscript, treatment with *daf-2* RNAi from day 4 adulthood did not substantially alter the resistance of day 8 worms against oxidative stress compared with day 8 control RNAi-treated animals. However, in the other set of oxidative stress resistance assay, day 8 animals treated with *daf-2* RNAi from day 4 adulthood exhibited increased resistance to oxidative stress compared with day 8 control RNAi-treated worms. We therefore performed one additional set of the experiment for our revision. In this third trial, worms treated with *daf-2* RNAi from day 4 to day 8 adulthood displayed enhanced resistance against oxidative stress compared with day 8 control RNAi-treated worms. Therefore, worms treated with *daf-2* RNAi from day 4 adulthood exhibited increased survival compared with day 8 control worms (2 out of 3 trials) but decreased survival compared with day 4 control worms (3 out of 3 trials) under oxidative stress conditions. We included these additional data in the revised manuscript. We also included methods for oxidative stress assay in Materials and Methods.

Figure S1 legends, page 6, line 129: (**P**) Worms treated with *daf-2* RNAi from day 4 to day 8 adulthood survived longer under oxidative stress conditions than day 8 control RNAi-treated worms, but survived shorter than day 4 control worms.

Materials and Methods, page 23, line 447: For pathogen resistance assays, day 4 or day 8 adult worms were moved to plates with PA14. For oxidative stress resistance assays, day 4 or day 8 adult worms were transferred to OP50-seeded NGM plates with 7.5 mM tert-butyl hydroperoxide (t-BOOH, Sigma, St Louis, MO, USA) solution. For heat stress resistance assays, day 4 or day 8 adult worms on OP50-seeded plates were placed in a 35°C incubator. The numbers of live worms were scored over time, and worms that did not respond to a gentle touch with a platinum wire were counted as dead.

- It is not clear to me why the authors conclude that the expression of selected DAF-16 targets not related with immunity such as mtl-1 and hsp-16.2 displayed age-dependent

increase in wild-type worms and a further increase in aged daf-2 mutants, whereas known PMK-1 targets show decrease during aging in both wild-type and daf-2 support the following conclusion: "These data are consistent with our results showing the requirement of DAF-16/FOXO for the enhanced immunity observed in old daf-2 mutants".

> We thank the reviewer for the valuable comment. We measured the mRNA levels of *mtl-1* and *hsp-16.1/11* as representative DAF-16 targets but not as DAF-16-dependent immunity factors. We included additional references to clarify this issue. In addition, for our revision, we measured the mRNA levels of three additional targets of DAF-16 and/or HSF-1, *lys-7*, *dod-6* and *sod-3* (Hesp et al., 2015, Hsu et al., 2003, Murphy et al., 2003, Barsyte et al., 2001, Sural et al., 2019), to support our model. Among them, *lys-7* and *dod-6* were further upregulated in *daf-2(–)* mutants during aging compared with wild-type worms. We added the qRT-PCR data to the revised manuscript. In addition, we altered the conclusion sentence to clarify the context.

Results and discussion, page 9, line 154: We then showed that the mRNA levels of four representative DAF-16 and HSF-1 common targets, *mtl-1*, *lys-7*, *dod-6*, and *hsp-16.1/11* (Hesp et al., 2015, Hsu et al., 2003, Murphy et al., 2003, Barsyte et al., 2001, Sural et al., 2019), increased age-dependently in *daf-2(–)* animals under OP50 and PA14-exposed conditions (Figure 3C-F and Figure S3B, S3D-G). In contrast, the mRNA levels of *sod-3*, a DAF-16/FOXO target, or any of three selected PMK-1 targets, T24B8.5, C17H12.8, and K08D8.5 (Troemel et al., 2006, Shivers et al., 2010, Youngman et al., 2011, Wu et al., 2019, Hsu et al., 2003, Murphy et al., 2003), did not increase with age (Figure 3G-I and Figure S3C, S3H-L). These data raise the possibility that common targets of DAF-16/FOXO and HSF-1 contribute to age-dependent increases in immunity observed in *daf-2(–)* adults.

Figure 3 legends, page 16, line 307: (**C**-**F**) qRT-PCR analysis data showing the mRNA levels of *mtl-1* (**C**), *lys-7* (**D**), *dod-6* (**E**), and *hsp-16.1/11* (**F**) in day 1 and day 9 *daf-2(–)* and WT adults on PA14 (n = 3); these four genes were selected as representative common targets of DAF-16 and HSF-1 (Barsyte et al., 2001, Hsu et al., 2003, Murphy et al., 2003, Hesp et al., 2015, Sural et al., 2019), but not necessarily as immune-regulating factors. All these four genes were upregulated with age in *daf-2(–)* animals, but the degree of age-dependent increase in the mRNA level of *hsp-16.1/11* was not larger in *daf-2(–)* mutants than in WT worms. Additional data regarding changes in the mRNA levels of the selected genes on OP50, and *sod-3* on OP50 and PA14 are shown in Figure S3D-I.

Figure S3 legends, page 7, line 158: (**B**) mRNA levels of five selected DAF-16 and/or HSF-1 targets, *mtl-1*, *dod-6*, *lys-7*, *hsp-16.2*, and *sod-3* (Hesp et al., 2015, Hsu et al., 2003, Murphy et al., 2003, Barsyte et al., 2001, Sural et al., 2019), in WT and *daf-2(–)* worms at day 1 and day 9 adulthoods in our RNA seq data (n = 3). … (**D**-**G**) qRT-PCR data showing changes in the mRNA levels of *mtl-1* (**D**), *lys-7* (**E**), *dod-6* (**F**), and *hsp-*

16.1/11 (**G**) in WT and *daf-2(–)* mutants cultured on OP50 (n = 7 for mRNA levels of *mtl-1,* n = 7 for the mRNA levels of *hsp-16.1/11* in WT and *daf-2(–)* animals at day 1, n = 6 for the mRNA levels of *hsp-16.1/11* in WT and *daf-2(–)* animals at day 9, n = 3 for the mRNA levels of *lys-7* and *dod-6*). (**H**-**I**) qRT-PCR data showing changes in the mRNA level of *sod-3* in WT and *daf-2(–)* animals at day 1 and day 9 adulthoods on OP50 (**H**) or PA14 (**I**). Different from the common targets of DAF-16 and HSF-1 (Figure 3C-F and Figure S3D-G), the mRNA level of *sod-3* was not age-dependently increased in WT or *daf-2(–)* animals (n = 3). …. Error bars represent SEM (two-tailed Student's *t*-test, *, †*p*<0.05, **, ††*p*<0.01, ***p*<0.001, black asterisks: *p*-value for day 1 WT 1 vs. each condition, pink asterisks: *p*-value for day 1 *daf-2(–)* mutants vs. day 9 *daf-2(–)* mutants, black cross: *p*-value for day 9 WT vs. day 9 *daf-2(–)* animals). *ama-1* was used as a normalization control.

- In Fig. 4, the authors perform a targeted screen using mutants that carry mutation in 13 genes selected among the 72 candidates. If there are not available mutants for the other genes, the authors should may consider a targeted RNAi screen against each of the 72 candidates, as this could provide novel regulators of immune aging and increase the implications of their findings. If the authors decide to keep only the data in the 13 genes analyzed and not to examine the other candidates, they should explain in detail the rationale to select these 13 genes in particular.

> We thank the reviewer for the critical comment. In our previous studies, we often experienced that survival phenotypes caused by RNAi knockdown of genes were not recapitulated with loss of function mutations (Son et al., 2018, Park et al., 2020). We therefore focused on available mutants instead of testing all the RNAi clones after we obtained the gene list from our RNA seq analysis. Nevertheless, we agree with the reviewer's point, and explicitly described the limitation of our study that did not cover all the 72 candidates for our genetic screen. We revised the manuscript as follows.

Supplemental discussion, page 4, line 80: Second, instead of testing all 72 candidate genes obtained from RNA seq analysis by using RNAi, we focused on 13 available mutants. That is because our previous reports indicate that survival phenotypes caused by RNAi often are not recapitulated with loss of function mutations (Son et al., 2018, Park et al., 2020). It will be important to characterize the roles of the remaining 59 candidate genes in immune aging using genome editing methods in future research.

- After the screen of the 13 mutants, the authors focused on ins-7, which is an agonist of the IIS pathway and it has been previously described as a modulator of resistance to pathogens in young age. Then, they find that another of their targets regulates ins-7 levels. The link between ins-7 and zip-10 -which eventually regulates the immune response via a feedback circuit involving the modulation of IIS itself- provides a

mechanistic explanation for the model studied here. Besides ins-7 and zip-10, the authors identified potential novel regulators of immune response in aged animals. It would be interesting to gain further insights on how these factors regulate/delay immune aging, or comment on this in the Discussion.

> We appreciate the reviewer's valuable comment. Based on several previous studies, we now discuss how an additional potential immune regulator gene, *valv-1*, identified from our genetic screen, may affect immune aging. Similar to *ins-7*, *valv-1* is induced by PA14 infection (Jeong et al., 2020; Shapira et al., 2006; Ma et al., 2020) and *hsf-1* RNAi (Figure 4E), and *valv-1* mutations increased the survival of worms on PA14 (Figure 4A). *valv-1* is expressed in multiple valves including the pharyngeal-intestinal valve (Tong and Buechner, 2008). Because bacterial accumulation caused by defective pharynx activates innate immunity (Kumar et al., Dev Cell., 2019), we speculate that *valv-1* RNAi increases resistance to PA14 by causing defects in pharyngeal-intestinal valve. We describe these possibilities in the Supplemental discussion.

Supplemental discussion, page 3, line 46: **Mutations in *valv-1* may delay immune aging by impairing pharyngeal structure**

By performing a survival screen using available mutants on PA14, we identified several immune regulators, including *valv-1* (valve cell defective-1). Similar to *ins-7*, the expression of *valv-1* is increased by PA14 exposure (Jeong et al., 2020; Shapira et al., 2006; Ma et al., 2020) and *hsf-1* RNAi (this study). *valv-1* is expressed in multiple tissues including pharyngeal-intestinal valves in *C. elegans* (Tong and Buechner, 2008). Bacterial accumulation caused by impaired pharyngeal structure activates innate immune response, which in turn promotes bacterial avoidance and increases lifespan (Kumar et al., 2019). Therefore, exposure to live *E. coli* bacteria may render *valv-1* mutants with impaired pharyngeal structure to be resistant to PA14 during aging.

- Since some of the conclusions rely on RNAi experiments, the authors should present data on the knockdown efficiency for the distinct RNAis used through the manuscript.

> Following the reviewer's suggestion, we performed qRT-PCR analysis to examine the knockdown efficiency. In Figure S1H and S1N, we showed that *daf-2* RNAi during whole life or from day 4 adulthood substantially decreased the mRNA levels of *daf-2*. In Figure S1L-M, we included the data showing the mRNA levels of *daf-2* and *dcr-1* upon knocking down *daf-2* only during development. We found that worms transferred from *daf-2* RNAi to *dcr-1* RNAi displayed significant decreases in *daf-2* mRNA levels at day 1 adulthood but did not at day 9 adulthood. *dcr-1* RNAi substantially decreased the mRNA levels of *dcr-1* at both day 1 and day 9 adulthoods. In Figure S2G-H, we showed that *hsf-1* RNAi and *skn-1* RNAi efficiently reduced the mRNA levels of *hsf-1* and *skn-1*, respectively. We added the data and description to our revised manuscript.

Figure 1 legends, page 14, line 271: (**B-D**) Shown are survival curves of WT (**B**), *daf-2(–)*

(**C**) and *daf-2(RNAi)* worms (Figure S1H) (**D**) transferred from *E. coli* to PA14 at day 1 and 9 adulthoods.

Figure S1 legends, page 5, line 108: (**H**) qRT-PCR analysis indicates that *daf-2* RNAi significantly decreased the mRNA level of *daf-2* (n = 3). Error bars represent standard error of the mean (SEM) (two-tailed Student's *t*-test, ***p<0.001). *pmp-3* was used as a normalization control.

Figure 1 legends, page 14, line 276: (**H**) Temporal *daf-2* RNAi (Figure S1N) treatment from day 4 adulthood increased the PA14 resistance of day 8 adults. Different from *daf-2* RNAi-treated worms during development (Figure S1K-M), adult worms recovered from dauer, which are also expected to experience decreased IIS (Fielenbach and Antebi, 2008), exhibited immune aging (Figure S1O; see Supplemental discussion).

Figure S1 legends, page 6, line 117: (**L**-**M**) Changes in the mRNA levels of *daf-2* (**L**) and *dcr-1* (**M**) at day 1 and day 9 adulthoods (n = 3). Treatment with *daf-2* RNAi during development decreased the mRNA level of *daf-2* at day 1 but did not at day 9 adulthood; this is consistent with previous reports showing that knockdown of *dcr-1*, which encodes DICER1, decreases the effects of *daf-2* RNAi in a time-dependent manner (Dillin et al., 2002, Durieux et al., 2011). Error bars represent SEM (two-tailed Student's *t*-test, *p<0.05, **p<0.01, ***p<0.001). *ama-1* and *pmp-3* were used as a normalization control. (**N**) Changes in the mRNA level of *daf-2* by treatment with *daf-2* RNAi from day 4 to day 8 adulthood. The temporal *daf-2* RNAi significantly deceased the mRNA level of *daf-2* (n = 3). Error bars represent SEM (two-tailed Student's *t*-test, **p<0.01, ***p<0.001). *pmp-3* was used as a normalization control.

Figure 2 legends, page 15, line 288: (**A**-**C**) Mean survival upon PA14 infection of wild-type (WT), *daf-16(mu86)* [*daf-16(–)*], *daf-2(–)*, and *daf-16(–); daf-2(–)* animals (**A**), and control and *hsf-1* RNAi (Figure S2G) (**B**)-, and control and *skn-1* RNAi (Figure S2H) (**C**)-treated WT and *daf-2(–)* animals at day 1, 6, and 9 adulthoods.

Figure S2 legends, page 7, line 144: (**G**-**H**) Changes in the mRNA levels of *hsf-1* (**G**) and *skn-1* (**H**) by *hsf-1* RNAi and *skn-1* RNAi, respectively (n = 3). *hsf-1* RNAi and *skn-1* RNAi efficiently reduced the mRNA levels of *hsf-1* and *skn-1*, respectively, in wild-type (WT) and *daf-2(–)* animals. Error bars represent standard error of the mean (SEM) (two-tailed Student's *t*-test, *p<0.05, **p<0.01, ***p<0.001). *pmp-3* was used as a normalization control.

Materials and Methods, page 26, line 518: For measuring RNAi efficiency, gravid adults were allowed to lay eggs on RNAi bacteria-seeded NGM plates containing 100 μg/ml ampicillin. The worms were then cultured until reaching day 1 adulthood, and harvested by washing with M9 buffer twice. For temporal RNAi experiments, worms at L4 or prefertile young adult stage were treated with 50 μM FUDR. After culturing for four days, control RNAi-fed worms were harvested with M9 buffer, or transferred onto new control or *daf-2* RNAi-seeded plates for another four days of cultures before harvesting with M9

buffer for qRT-PCR analysis. For measuring knockdown efficiency of *daf-2* RNAi during development, worms were grown on control or *daf-2* RNAi until reaching prefertile young adult stage and transferred to control or *dcr-1* RNAi bacteria-seeded plates. These worms at day 1 or day 9 adulthood were harvested by washing twice with M9 buffer.

- Given that zip-10 is a transcription factor, the authors should assess whether zip-10 mutant worms exhibit decreased mRNA levels of endogenous ins-7 to strengthen the link between zip-10 and ins-7 regulation. In these lines, the author should analyse whether mutations in isy-1 regulate the levels of ins-7:GFP reporter and mRNA levels of endogenous ins-7.

> We appreciate the comment by the reviewer. As the reviewer suggested, we measured *ins-7* mRNA levels by using qRT-PCR, and found that *zip-10(–)* mutations suppressed age-dependent increases in *ins-7* mRNA levels. We next measured the levels of *ins-7* mRNA and *ins-7p::gfp* to determine whether *isy-1(–)* affected *ins-7* expression. We found that *isy-1(–)* mutations further increased the age-dependent upregulation of *ins-7p::gfp*. However, *isy-1(–)* did not increase the mRNA level of *ins-7* during aging, with qRT-PCR analysis. Thus, the link from *zip-10* to *ins-7* is strongly supported by our data, but the pathway from *isy-1* to *ins-7* through *zip-10* may be indirect and needs further validation. We added the data and description to the revised manuscript.

Results and Discussion, page 11, line 202: We found that *zip-10* mutations suppressed the age-dependent increase in *ins-7p::gfp* and *ins-7* mRNA expression (Figure 5C-E), suggesting that ZIP-10 induces *ins-7*.

Figure 5 legends, page 19, line 366: (**E**) *zip-10(–)* mutations reduced the age-dependent increase in mRNA level of *ins-7*. *ama-1* and *pmp-3*: normalization controls.

Figure S3 legends, page 9, line 187: (**U**) Images of *ins-7p::gfp* transgenic worms in WT (control) and *isy-1(dma50)* [*isy-1(–)*] mutant backgrounds at day 1 and 9 adulthoods. Scale bar indicates 500 µm. Images with reduced exposure are included in Figure S3W. (**V**) Quantification of the data shown in panel **U** (n = 3, two-tailed Student's *t*-test, ***$p < 0.001$). (**W**) Fluorescence images of *ins-7p::gfp* in control and *isy-1(–)* mutant backgrounds at day 9 adulthood [Note: for visualizing the difference in fluorescence at day 9 adulthood, the same worms in Figure S3U were intentionally displayed with different exposure times (1500 ms for panel U and 250 ms for panel W)]. (**X**) *isy-1(–)* mutations did not further increase the level of *ins-7* mRNA at day 1 or day 9 adulthood, measured by using qRT-PCR, different from *ins-7p::gfp* (Figure S3U-V, see supplemental discussion). *ama-1* and *pmp-3* was used as a normalization control. Error bars represent SEM (n = 5 for day 1 WT, day 1 *isy-1(–)*, and day 9 WT worms, n = 4 for day 9 *isy-1(–)* worms, two-tailed Student's *t*-test, **$p<0.01$).

Supplemental discussion, page 4, line 86: Third, we found that *isy-1(–)* mutations had different effects on the levels of *ins-7* mRNA and *ins-7p::gfp* expression. Specifically, mutations in *isy-1*, which cause upregulation of *zip-10* (Jiang et al., 2018), further upregulated *ins-7p::gfp* during aging, while not affecting *ins-7* mRNA measured with qRT-PCR (Figure S3U-X). We speculate that the difference in the stability of GFP proteins derived from *ins-7p::gfp* and the level of *ins-7* mRNA measured by using qRT-PCR may have contributed to these seemingly conflicting results. The measurement of INS-7 protein levels, which we were not able to perform in this study, will be crucial for resolving this issue in future research.

- An important question raised by the authors' findings that should be assessed is whether zip-10 also regulates the impact of HSF-1 on immunity induced by reduced IIS.
> We thank the reviewer for the valuable comment. We additionally performed PA14 survival assay using *daf-2(–); zip-10(–)* worms treated with *hsf-1* RNAi as well as *daf-16* RNAi. We found that *hsf-1* RNAi or *daf-16* RNAi suppressed the enhanced pathogen resistance of *daf-2(–); zip-10(–)* animals. Thus, HSF-1 as well as DAF-16 is required for the increased immunity of *daf-2(–); zip-10(–)* animals. These data are consistent with our model (Figure 5I), because genetic inhibition of each of HSF-1 and DAF-16, which act downstream of IIS, would suppress the effect of further downregulation of *ins-7* by *zip-10(–)* in *daf-2(–)* animals on PA14. We added the data and the description to our revised manuscript.

Results and Discussion, page 11, line 205: In addition, *hsf-1* RNAi or *daf-16* RNAi suppressed the extended survival of day 9 *daf-2(–); zip-10(–)* animals on PA14 (Figure S3S-T).

Figure S3 legend, page 9, line 186: (**S**-**T**) Enhanced resistance to PA14 by *daf-2(–); zip-10(–)* mutations was suppressed by *hsf-1* RNAi (**S**) or *daf-16* RNAi (**T**).

Reviewer #2 (Comments to the Authors (Required)):

Lee and colleagues performed an very interesting study looking at the insulin receptor mutant daf-2 in C. elegans, which were previously known to have pathogen resistance compared to wildtype animals, but in this new study it is revealed that pathogen resistance is present at old age and more interesting older daf-2 mutants are even more resistant if compared to younger aged daf-2 worms.

I believe that this study is appropriate for the Journal of Cell Biology, but requires significant editing before acceptance. The Lee lab is well established for lifespan and healthspan studies and the methodologies are appropriate, but in several instances the data presented do not clearly justify the conclusions. There are also several

contradictions with the current literature, which might be very interesting, but at present leave the reader confused since they are not addressed.
> Following the reviewer's suggestion, we revised the manuscript to discuss differences between previous studies and our current study. Because of the character count limit for a Report paper in the *Journal of Cell Biology* (<20,000), we included the additional discussion points in Supplemental discussion.

Supplemental discussion, page 2, line 27: **Genetic inhibition of *daf-2* improves health parameters, including innate immunity, in aged worms**

A previous report suggests that *daf-2* mutations extend lifespan but increase the period of frailty in old age, by measuring motility and resistance against abiotic stresses, such as heat and oxidative stresses (Bansal et al., 2015). In contrast, we previously reported that *daf-2* mutations prolong healthy periods throughout adulthood by measuring maximum physical ability (Hahm et al., 2015). In the current work, we showed that relatively old day 9 *daf-2* mutant adults exhibited enhanced immunity, indicating that genetic inhibition of *daf-2* can enhance resistance against pathogenic bacteria (biotic stress) in old age. Another previous report demonstrated that *daf-2* mutations confer resistance to the colonization by dietary bacteria, *E. coli*, in the digestive tract (Podshivalova et al., 2017). Here, we found that mutations in *daf-2* prevented colonization by pathogenic PA14, but *daf-2* RNAi did not. Despite this difference, both *daf-2* mutations and *daf-2* RNAi enhanced immunocompetence in old age. Thus, enhanced pathogen resistance caused by genetic inhibition of *daf-2* does not seem to result from the elimination of the pathogen PA14. In conclusion, genetic inhibition of *daf-2* appears to increase at least one aspect of healthspan, resistance against pathogens, by increasing innate immunity in old worms.

Major concerns:

The authors should make and present a model for the positive feedback loop.

> We appreciate this valuable comment by the reviewer. We included a schematic model that summarizes the current study (Figure 5I).

Results and Discussion, page 11, line 211: Altogether these data suggest that a hypomorphic *daf-2(e1370)* mutation enhances immunocompetence in old age by downregulating ZIP-10, subsequently decreasing INS-7 expression, which underlies immune aging in wild-type worms (Figure 5I).

Figure 5 legend, page 19, line 375: (**I**) A schematic model showing the summary of the current study. Age-dependent increases in INS-7 levels activate insulin/IGF-1 signaling in WT, and this in turn downregulates DAF-16/FOXO and HSF-1, negative regulators of *ins-7*. In addition, ZIP-10, a positive regulator of INS-7, further increases the level of INS-7 in old WT, leading to immune aging. In contrast, hypomorphic mutations in *daf-2*,

which can be further downregulated (Arantes-Oliveira et al., 2003, Hansen et al., 2005), reduce *ins-7* mRNA levels *via* decreasing the activity of DAF-16/FOXO and HSF-1. Subsequently, decreased INS-7 further downregulates insulin/IGF-1 signaling through a positive feedback loop in *daf-2(–)* mutants for delaying immune aging.

I've read the text sevral times and it is complicated by the fact that you are inactivating inhibitors that increase activators, etc. Along these lines is there a way to show that INS-7 levels are indeed reduced?

> Conventional methods for measuring protein levels in *C. elegans* are western blot analysis and fluorescent imaging using translational fluorescent fusion protein-expressing transgenic animals. To our knowledge western blot analysis measuring INS-7 levels has not been published. A mCherry-fused INS-7 was used for determining the localization of INS-7 (Yoshina and Mitani, 2015), but not for measuring the level of INS-7 in whole body. Because of this technical difficulty for measuring INS-7, we added our data showing the mRNA level of *ins-7* in wild-type and *daf-2(–)* animals at day 1 and day 9 adulthoods. In addition, we described the limitation of our study that did not measure INS-7 protein levels in our revised manuscript.

Results and Discussion, page 10, line 188: Among them, *ins-7* (Figure 4B-C), an agonist of DAF-2 that acts as a positive feedback regulator of IIS (Murphy et al., 2003, Murphy et al., 2007), was negatively regulated by both DAF-16/FOXO (Figure 4D) (Murphy et al., 2003, Murphy et al., 2007, Lee et al., 2009) and HSF-1 (Figure 4E); we confirmed this result by using qRT-PCR assays (Figure 4F-G).

Figure 4 legend, page 18, line 342: (**B**-**C**) The mRNA levels of *ins-7* in WT and *daf-2(–)* mutants at day 1 and day 9 adulthoods from RNA seq analysis (n = 3) (**B**) and by using qRT-PCR (*ama-1* and *pmp-3*: normalization controls, n = 3) (**C**).

Figure S3 legends, page 9, line 187: (**U**) Images of *ins-7p::gfp* transgenic worms in WT (control) and *isy-1(dma50)* [*isy-1(–)*] mutant backgrounds at day 1 and 9 adulthoods. Scale bar indicates 500 μm. Images with reduced exposure are included in Figure S3W. (**V**) Quantification of the data shown in panel **U** (n = 3, two-tailed Student's *t*-test, ***$p < 0.001$). (**W**) Fluorescence images of *ins-7p::gfp* in control and *isy-1(–)* mutant backgrounds at day 9 adulthood [Note: for visualizing the difference in fluorescence at day 9 adulthood, the same worms in Figure S3U were intentionally displayed with different exposure times (1500 ms for panel U and 250 ms for panel W)]. (**X**) *isy-1(–)* mutations did not further increase the level of *ins-7* mRNA at day 1 or day 9 adulthood, measured by using qRT-PCR, different from *ins-7p::gfp* (Figure S3U-V, see Supplemental discussion). *ama-1* and *pmp-3* were used as normalization controls. Error bars represent SEM (n = 5 for day 1 WT, day 1 *isy-1(–)*, and day 9 WT worms, n = 4 for day 9 *isy-1(–)* worms, two-tailed Student's *t*-test, **$p<0.01$).

Figure 5 legends, page 19, line 370: (**H**) *isy-1(–)* mutations reduced the enhanced survival of day 9 *daf-2(–)* animals on PA14. We also measured age-dependent changes in *ins-7* mRNA levels in *isy-1(–)* animals using *ins-7p::gfp* and qRT-PCR, but the data were inconclusive (Figure S3U-X, see Supplemental discussion).

Supplemental discussion, page 5, line 92: The measurement of INS-7 protein levels, which we were not able to perform in this study, will be crucial for resolving this issue in future research.

With this in mind, if daf-2 is absent, how can the absence of ins-7 have any impact (I think it is a positive regulator?). As this is the major conclusion of the paper this needs to be presented in a clear manner.

> We appreciate this critical comment by the reviewer. Because our current study used a hypomorphic *daf-2(e1370)* mutation or *daf-2* RNAi, which does not cause a complete loss of function, we think insulin/IGF-1 signaling can be further reduced in these animals. Indeed, phenotypes in *daf-2(e1370)* mutants have been shown to be further enhanced by *daf-2* RNAi (Arantes-Oliveira et al., 2003; Hansen et al., 2005). We revised our manuscript to specifically mention the hypomorphic nature of the *daf-2(e1370)* mutation.

Results and Discussion, page 6, line 96: Surprisingly, day 9 adult *daf-2(e1370)* (a hypomorphic mutation in insulin/IGF-1 receptor gene, hereafter *daf-2(–)* for simplicity) mutants displayed substantially enhanced survival upon PA14 infection compared with day 1 adult *daf-2(–)* mutants and wild-type worms (Figure 1A-C).

Results and Discussion, page 11, line 195: These data suggest that the upregulation of DAF-16/FOXO and HSF-1 decreases *ins-7* expression and enhances immunocompetence during aging in *daf-2* mutants, containing a hypomorphic *e1370* allele.

Results and Discussion, page 11, line 211: Altogether these data suggest that a hypomorphic *daf-2(e1370)* mutation enhances immunocompetence in old age by downregulating ZIP-10, subsequently decreasing INS-7 expression, which underlies immune aging in wild-type worms (Figure 5I).

Figure 5 legend, page 19, line 379: In contrast, hypomorphic mutations in *daf-2*, which can be further downregulated (Arantes-Oliveira et al., 2003, Hansen et al., 2005), reduce *ins-7* mRNA levels *via* decreasing the activity of DAF-16/FOXO and HSF-1.

In Figure S3, are these data from animals exposed to PA14 at the ages indicated? If not, is this data in the submission? It should be included to better justify the conclusions made about the role of each gene in immunocompetency.

> We appreciate this valuable comment by the reviewer. We obtained RNA seq data

using worms cultured on OP50-seeded plates (Figure S3). That is partly because we thought genes that were induced or repressed before PA14 infection would also contribute to immunity. We agree with the reviewer's comment that RNA seq analysis using PA14-exposed worms will further strengthen our current study. However, we think performing additional RNA seq experiments and subsequent analysis is beyond the scope of this short article (Report). Instead, we performed additional qRT-PCR assays to measure the mRNA levels of selected targets of DAF-16, HSF-1 and PMK-1, using wild-type and *daf-2(–)* worms infected with PA14 at indicated ages (day 1 and day 9 adulthoods). We found that three (*mtl-1*, *lys-7* and *dod-6*) of four (*mtl-1*, *lys-7*, *dod-6* and *hsp-16.1/11*) tested common targets of DAF-16/FOXO and HSF-1, were further upregulated in *daf-2(–)* animals at day 9 compared with wild-type animals under both OP50- and PA14-treated conditions. In contrast, none of *sod-3*, a DAF-16/FOXO target, and T24B8.5, C17H12.8 and K08D8.5, PMK-1 targets, were further induced in day 9 *daf-2(–)* animals compared with those in day 1 *daf-2(–)* worms under OP50- or PA14-treated conditions. These data support our PA14 survival data showing that both DAF-16 and HSF-1 are required for preventing immune aging in *daf-2(–)* animals. We included these data and described the limitation of the lack of RNA seq analysis using PA14-infected conditions in our revised manuscript.

Results and discussion, page 9, line 154: We then showed that the mRNA levels of four representative DAF-16 and HSF-1 common targets, *mtl-1*, *lys-7*, *dod-6*, and *hsp-16.1/11* (Hesp et al., 2015, Hsu et al., 2003, Murphy et al., 2003, Barsyte et al., 2001, Sural et al., 2019), increased age-dependently in *daf-2(–)* animals under OP50 and PA14-exposed conditions (Figure 3C-F and Figure S3B, S3D-G). In contrast, the mRNA levels of *sod-3*, a DAF-16/FOXO target, or any of selected PMK-1 targets, T24B8.5, C17H12.8, and K08D8.5 (Troemel et al., 2006, Shivers et al., 2010, Youngman et al., 2011, Wu et al., 2019, Hsu et al., 2003, Murphy et al., 2003), did not increase with age (Figure 3G-I and Figure S3C, S3H-L). These data raise the possibility that common targets of DAF-16/FOXO and HSF-1 contribute to age-dependent increases in immunity observed in *daf-2(–)* adults.

Figure 3 legends, page 16, line 307: (**C**-**F**) qRT-PCR analysis data showing the mRNA levels of *mtl-1* (**C**), *lys-7* (**D**), *dod-6* (**E**), and *hsp-16.1/11* (**F**) in day 1 and day 9 *daf-2(–)* and WT adults on PA14 (n = 3); these four genes were selected as representative common targets of DAF-16 and HSF-1 (Barsyte et al., 2001, Hsu et al., 2003, Murphy et al., 2003, Hesp et al., 2015, Sural et al., 2019), but not necessarily as immune-regulating factors. All these four genes were upregulated with age in *daf-2(–)* animals, but the degree of age-dependent increase in the mRNA level of *hsp-16.1/11* was not larger in *daf-2(–)* mutants than in WT worms. Additional data regarding changes in the mRNA levels of the selected genes on OP50, and *sod-3* on OP50 and PA14 are shown in Figure S3D-I. (**G**-**I**) The mRNA levels of three selected PMK-1-regulated genes, T24B8.5 (**G**), C17H12.8 (**H**), and K08D8.5 (**I**) were downregulated with age in both WT and *daf-2(–)* animals on PA14 (n = 3). See Figure S3J-L for qRT-PCR using worms

cultured on OP50. Error bars represent SEM (two-tailed Student's *t*-test, *, †*p*<0.05, **, ††*p*<0.01, *** *p*<0.001, black asterisks: *p*-value for day 1 WT vs. other conditions, pink asterisks: *p*-value for day 1 *daf-2(–)* mutants vs. day 9 *daf-2(–)* mutants, black cross: *p*-value for day 9 WT vs. day 9 *daf-2(–)* mutants). *ama-1* was used as a normalization control.

Figure S3 legends, page 7, line 158: (**B**) mRNA levels of five selected DAF-16 and/or HSF-1 targets, *mtl-1*, *dod-6*, *lys-7*, *hsp-16.2*, and *sod-3* (Hesp et al., 2015, Hsu et al., 2003, Murphy et al., 2003, Barsyte et al., 2001, Sural et al., 2019), in WT and *daf-2(–)* worms at day 1 and day 9 adulthoods in our RNA seq data (n = 3). (**C**) Three representative PMK-1-regulated genes, T24B8.5, C17H12.5, and K08D8.5 (Shivers et al., 2010, Troemel et al., 2006), were downregulated with age in WT and *daf-2(–)* worms in our RNA seq data (n = 3, two-tailed Student's *t*-test). Error bars represent standard error of the mean (SEM) from RNA seq data. (**D**-**G**) qRT-PCR data showing changes in the mRNA levels of *mtl-1* (**D**), *lys-7* (**E**), *dod-6* (**F**), and *hsp-16.1/11* (**G**) in WT and *daf-2(–)* mutants cultured on OP50 (n = 7 for the mRNA levels of *mtl-1,* n = 7 for the mRNA levels of *hsp-16.1/11* in WT and *daf-2(–)* animals at day 1, n = 6 for the mRNA levels of *hsp-16.1/11* in WT and *daf-2(–)* animals at day 9, n = 3 for the mRNA levels of *lys-7* and *dod-6*). (**H**-**I**) qRT-PCR data showing changes in the mRNA level of *sod-3* in WT and *daf-2(–)* animals at day 1 and day 9 adulthoods on OP50 (**H**) or PA14 (**I**). Different from the common targets of DAF-16 and HSF-1 (Figure 3C-F and Figure S3D-G), the mRNA level of *sod-3* was not age-dependently increased in WT or *daf-2(–)* animals (n = 3). (**J**-**L**) The mRNA levels of PMK-1 target genes, T24B8.5 (**J**), C17H12.8 (**K**), and K08D8.5 (**L**), in WT and *daf-2(–)* worms at day 1 and day 9 adulthoods on OP50. The PMK-1 target genes were downregulated during aging in WT and *daf-2(–)* animals except T24B8.5 in *daf-2(–)* worms. Error bars represent SEM (two-tailed Student's *t*-test, *, †*p*<0.05, **, ††*p*<0.01, ***p*<0.001, black asterisks: *p*-value for day 1 WT 1 vs. each condition, pink asterisks: *p*-value for day 1 *daf-2(–)* mutants vs. day 9 *daf-2(–)* mutants, black cross: *p*-value for day 9 WT vs. day 9 *daf-2(–)* animals). *ama-1* was used as a normalization control.

Supplemental discussion, page 4, line 76: First, we performed RNA seq using worms cultured on *E. coli* OP50-seeded plates but not on PA14-seeded plates. Although the RNA seq analysis using OP50 allowed us to identify several genes that mediate immune aging, such as *ins-7* and *zip-10*, RNA seq analysis using PA14-exposed worms will further help elucidate underlying molecular mechanisms.

The manuscript needs a better description of how statistical comparisons were performed. This is absent from the methods section. Also there are references to "Star methods"? It is not clear what these are.

> We thank the reviewer for these critical comments. We corrected the mistakes in Materials and Methods.

We previously showed 'Normalized mean survival on PA14 (% changes of day 1 WT)' in Figure 1A, 1E, 1F, 2A-F and S1E. However, because of the reviewer's comments, we noticed that our previous normalization method is not a generally used statistical analysis. We therefore revised the plotting method for full survival assays from 'normalized mean survival time on PA14' to 'mean survival on PA14 (hours)' in Figure 1A, 1E, 1F, 2A-F and S1E. The mean survival time on PA14 shown in Figure 1A, 1E, 1F, 2A-F and S1E was calculated by obtaining averages of the mean survival values from at least two independent trials. We added the method to the statistical analysis part in Materials and Methods.

Figure S1 legend, page 5, line 103: See Materials and Methods for immune aging assays using temperature-sensitive *glp-1(–)* mutants.

Materials and Methods, page 30, line 592: **Statistics.** For quantification of imaging, qRT-PCR and normalized survival on PA14, *p* values were calculated by using two-tailed Student's *t*-test. For semi-quantification of PA14-GFP accumulation, *p* values were calculated by using chi-squared test. Statistical analysis of survival data was performed by using OASIS (http://sbi.postech.ac.kr/oasis), which calculates *p* values using log-rank (Mantel-Cox method) test (Yang et al., 2011). All PA14 survival assays were performed at least twice independently. The mean survival on PA14 was caculated by pooling data from different experimental sets.

In figure 3 the authors select two insulin pathway and two p38 pathway genes to examine. This data looks great, but these pathway regulate 100s of genes, what is the importance of these genes?

> We thank the reviewer for pointing this out. We selected *mtl-1* and *hsp-16.1/11* as DAF-16 targets, and T24B8.5 and C17H12.8 as targets of PMK-1. We selected these genes as representative and established target genes that were used in many previous reports (Hesp et al., 2015, Hsu et al., 2003, Murphy et al., 2003, Barsyte et al., 2001, Troemel et al., 2006, Shivers et al., 2010, Youngman et al., 2011, Wu et al., 2019). For this revision, we determined changes in the expression levels of additional target genes of DAF-16 and HSF-1 to support our model. We measured the mRNA levels of *lys-7*, *dod-6* and *sod-3* and found that *lys-7* and *dod-6* were further upregulated in *daf-2(–)* animals during aging compared to wild-type worms. We added the references to support our selection of these target genes and the new data to the revised manuscript.

Results and discussion, page 9, line 154: We then showed that the mRNA levels of four representative DAF-16 and HSF-1 common targets, *mtl-1*, *lys-7*, *dod-6*, and *hsp-16.1/11* (Hesp et al., 2015, Hsu et al., 2003, Murphy et al., 2003, Barsyte et al., 2001, Sural et al., 2019), increased age-dependently in *daf-2(–)* animals under OP50 and PA14-exposed conditions (Figure 3C-F and Figure S3B, S3D-G). In contrast, the mRNA levels of *sod-3*, a DAF-16/FOXO target, or any of three selected PMK-1 targets, T24B8.5,

C17H12.8, and K08D8.5 (Troemel et al., 2006, Shivers et al., 2010, Youngman et al., 2011, Wu et al., 2019, Hsu et al., 2003, Murphy et al., 2003), did not increase with age (Figure 3G-I and Figure S3C, S3H-L). These data raise the possibility that common targets of DAF-16/FOXO and HSF-1 contribute to age-dependent increases in immunity observed in *daf-2(–)* adults.

Figure 3 legends, page 16, line 307: (**C**-**F**) qRT-PCR analysis data showing the mRNA levels of *mtl-1* (**C**), *lys-7* (**D**), *dod-6* (**E**), and *hsp-16.1/11* (**F**) in day 1 and day 9 *daf-2(–)* and WT adults on PA14 (n = 3); these four genes were selected as representative common targets of DAF-16 and HSF-1 (Barsyte et al., 2001, Hsu et al., 2003, Murphy et al., 2003, Hesp et al., 2015, Sural et al., 2019), but not necessarily as immune-regulating factors. All these four genes were upregulated with age in *daf-2(–)* animals, but the degree of age-dependent increase in the mRNA level of *hsp-16.1/11* was not larger in *daf-2(–)* mutants than in WT worms. Additional data regarding changes in the mRNA levels of the selected genes on OP50, and *sod-3* on OP50 and PA14 are shown in Figure S3D-I. (**G**-**I**) The mRNA levels of three selected PMK-1-regulated genes, T24B8.5 (**G**), C17H12.8 (**H**), and K08D8.5 (**I**) were downregulated with age in both WT and *daf-2(–)* animals on PA14 (n = 3). See Figure S3J-L for qRT-PCR using worms cultured on OP50. Error bars represent SEM (two-tailed Student's *t*-test, *, †p<0.05, **, ††p<0.01, *** p<0.001, black asterisks: p-value for day 1 WT vs. other conditions, pink asterisks: p-value for day 1 *daf-2(–)* mutants vs. day 9 *daf-2(–)* mutants, black cross: p-value for day 9 WT vs. day 9 *daf-2(–)* mutants). *ama-1* was used as a normalization control.

Figure S3 legends, page 7, line 158: (**B**) mRNA levels of five selected DAF-16 and/or HSF-1 targets, *mtl-1*, *dod-6*, *lys-7*, *hsp-16.2*, and *sod-3* (Hesp et al., 2015, Hsu et al., 2003, Murphy et al., 2003, Barsyte et al., 2001, Sural et al., 2019), in WT and *daf-2(–)* worms at day 1 and day 9 adulthoods in our RNA seq data (n = 3). (**C**) Three representative PMK-1-regulated genes, T24B8.5, C17H12.5, and K08D8.5 (Shivers et al., 2010, Troemel et al., 2006), were downregulated with age in WT and *daf-2(–)* worms in our RNA seq data (n = 3, two-tailed Student's *t*-test). Error bars represent standard error of the mean (SEM) from RNA seq data. (**D**-**G**) qRT-PCR data showing changes in the mRNA levels of *mtl-1* (**D**), *lys-7* (**E**), *dod-6* (**F**), and *hsp-16.1/11* (**G**) in WT and *daf-2(–)* mutants cultured on OP50 (n = 7 for the mRNA levels of *mtl-1,* n = 7 for the mRNA levels of *hsp-16.1/11* in WT and *daf-2(–)* animals at day 1, n = 6 for the mRNA levels of *hsp-16.1/11* in WT and *daf-2(–)* animals at day 9, n = 3 for the mRNA levels of *lys-7* and *dod-6*). (**H**-**I**) qRT-PCR data showing changes in the mRNA level of *sod-3* in WT and *daf-2(–)* animals at day 1 and day 9 adulthoods on OP50 (**H**) or PA14 (**I**). Different from the common targets of DAF-16 and HSF-1 (Figure 3C-F and Figure S3D-G), the mRNA level of *sod-3* was not age-dependently increased in WT or *daf-2(–)* animals (n = 3). (**J**-**L**) The mRNA levels of PMK-1 target genes, T24B8.5 (**J**), C17H12.8 (**K**), and K08D8.5 (**L**), in WT and *daf-2(–)* worms at day 1 and day 9 adulthoods on OP50. The PMK-1 target genes were downregulated during aging in WT and *daf-2(–)* animals except

T24B8.5 in *daf-2(–)* worms. Error bars represent SEM (two-tailed Student's *t*-test, *, †*p*<0.05, **, ††*p*<0.01, ***p<0.001, black asterisks: *p*-value for day 1 WT 1 vs. each condition, pink asterisks: *p*-value for day 1 *daf-2(–)* mutants vs. day 9 *daf-2(–)* mutants, black cross: *p*-value for day 9 WT vs. day 9 *daf-2(–)* animals). *ama-1* was used as a normalization control.

The authors pick day 9 of adulthood as "old". IN reality this is at best middle age? And if compared to daf-2 is still a very young adult. This should be explained.

> We agree with the reviewer. We used day 9 adult worms for our study because day 9 adulthood is the limit for comparing the survival of wild-type and *daf-2(–)* worms on PA14; after that time point, wild-type worms are too old and sick to perform survival assays on PA14. We explained this in the Methods. In addition, we changed the term from "old" to "day 6" or "day 9" to clarify the age of the worms used in the majority of places in the text.

Materials and Methods, page 21, line 420: For the majority of immune aging assays, day 9 adult worms were used as aged worms to compare the survival of WT and mutant worms on PA14; after day 9 of adulthood, WT worms died very fast upon treating with PA14, and this caused difficulty in comparison analysis with mutants.

Introduction, page 5, line 78: We then showed that DAF-16/FOXO and HSF-1 increased immunocompetence in day 9 *daf-2* mutants by regulating the expression of various genes. In particular, DAF-16/FOXO and HSF-1 downregulated an agonistic insulin-like peptide, INS-7, *via* decreasing the expression of a bZIP transcription factor, ZIP-10, which in turn reduced IIS further and enhanced immunity at day 9.

Results and Discussion, page 6, line 105: Day 9 *daf-2(–)* mutants also survived longer than did young *daf-2(–)* worms after infection with PAO1, moderately virulent *P. aeruginosa* strain (Figure S1I-J).

Results and Discussion, page 7, line 118: *daf-2* mutants displayed substantially reduced PA14 levels in the intestine in both day 1 (Evans et al., 2008a) and day 9 adults (Figure 1I).

Results and Discussion, page 7, line 121: As both day 9 *daf-2* mutant and *daf-2(RNAi)* adults survived longer on PA14 than did day 1 adults (Figure 1A and 1C-F), genetic inhibition of *daf-2* appears to maintain immunocompetence in relatively old (day 9) age, at least in part, independently of PA14 intake and/or clearance.

Results and Discussion, page 8, line 133: *hsf-1* RNAi also largely suppressed the enhanced immunocompetence of day 6 and day 9 *daf-2(–)* adults (Figure 2B, Figure S2C-D). In contrast, RNAi targeting *skn-1* did not affect the enhanced immunocompetence of *daf-2* mutants (Figure 2C, Figure S2E-F). In addition, PMK-1/p38 MAPK, a major immune-regulatory factor, whose reduction is the critical

determinant of immune aging in wild-type animals (Youngman et al., 2011, Kim et al., 2002) and suppresses immunity in young *daf-2(–)* mutants (Troemel et al., 2006), was not required for the enhanced immunity of day 9 *daf-2(–)* adults (Figure 2D and Figure S2I-J). Mutations in each of *nsy-1*/MAPKKK and *sek-1*/MAPKK, which encode upstream kinases of PMK-1 (Irazoqui and Ausubel, 2010, Kim and Ewbank, 2018), were only partially required for the enhanced immunity of day 9 *daf-2(–)* adults (Figure 2E-F and Figure S2K-N).

Results and Discussion, page 11, line 202: We found that *zip-10* mutations suppressed the age-dependent increase in *ins-7p::gfp* and *ins-7* mRNA expression (Figure 5C-E), suggesting that ZIP-10 induces *ins-7*. We also showed that *zip-10* mutation increased the PA14 resistance at day 9 (Figure 5F), but not at day 1 adulthood (Figure S3R). In addition, *hsf-1* RNAi or *daf-16* RNAi suppressed the extended survival of day 9 *daf-2(–); zip-10(–)* animals on PA14 (Figure S3S-T). We also found that mutations in *isy-1*/ISY splicing factor homolog, which cause upregulation of *zip-10* (Jiang et al., 2018), significantly reduced immunocompetence in day 9 but not in day 1 adults (Figure 5G and Figure S3Y). Moreover, we found that the *isy-1* mutation reduced enhanced immunocompetence in day 9 *daf-2(–)* adults (Figure 5H and Figure S3Z-A').

Supplemental discussion, page 1, line 18: Fourth, *daf-2* RNAi treatment in post-reproductive, middle-aged (day 4), wild-type animals was sufficient to enhance immunocompetence at day 8 adulthood (Figure 1H).

Supplemental discussion, page 2, line 33: In the current work, we showed that relatively old day 9 *daf-2* mutant adults exhibited enhanced immunity, indicating that genetic inhibition of *daf-2* can enhance resistance against pathogenic bacteria (biotic stress) in old age.

What happens if daf-2 RNAi is initiated during development and removed when the animals reach adulthood?

> We tested the effect of *daf-2* RNAi during development on PA14 resistance in adult worms. We transferred worms treated with *daf-2* RNAi from hatching to prefertile young adult stage onto *dcr-1* RNAi bacteria-seeded plates at the young adult stage, following previously published methods (Dillin et al., 2002, Durieux et al., 2011). We found that RNAi knockdown of *daf-2* during development was sufficient to increase PA14 resistance in day 9 adult worms. We added these data to our revised manuscript.

Results and discussion, page 7, line 107: We next asked whether temporal knockdown of *daf-2* was sufficient to suppress immune aging. We found that knockdown of *daf-2* during development was sufficient for increasing immunocompetence against PA14 at day 9 adulthood (Figure S1K; see also Supplemental discussion).

Figure S1 legend, page 6, line 113: (**K**) Survival curves of day 1 and day 9 WT adult

worms treated with *daf-2* RNAi during development. We treated worms with *daf-2* RNAi during development and subsequently blocked the RNAi effect in young adults by using RNAi targeting *dcr-1*/DICER1, an essential ribonuclease for RNAi (Dillin et al., 2002, Durieux et al., 2011). (**L**-**M**) Changes in the mRNA levels of *daf-2* (**L**) and *dcr-1* (**M**) at day 1 and day 9 adulthoods (n = 3). Treatment with *daf-2* RNAi during development decreased the mRNA level of *daf-2* at day 1 but did not at day 9 adulthood; this is consistent with previous reports showing that knockdown of *dcr-1*, which encodes DICER1, decreases the effects of *daf-2* RNAi in a time-dependent manner (Dillin et al., 2002, Durieux et al., 2011). Error bars represent SEM (two-tailed Student's *t*-test, **p*<0.05, ***p*<0.01, ****p*<0.001). *ama-1* and *pmp-3* were used as normalization controls.

Materials and Methods, page 23, line 457: For survival assays on PA14 using worms treated with *daf-2* RNAi during development, WT worms were synchronized on *daf-2* RNAi bacteria-seeded plates and transferred onto fresh *dcr-1* RNAi plates at prefertile young adult stage (Dillin et al., 2002, Durieux et al., 2011). Day 1 or day 9 adult worms were then exposed to PA14. Worms that reached young prefertile adulthood were treated with 50 μM FUDR until finishing the survival assays.

Materials and Methods, page 26, line 526: For measuring knockdown efficiency of *daf-2* RNAi during development, worms were grown on control or *daf-2* RNAi bacteria until reaching prefertile young adult stage and transferred to control or *dcr-1* RNAi bacteria-seeded plates. These worms at day 1 or day 9 adulthood were harvested by washing twice with M9 buffer.

Alternatively what happens if you test adult animals that were previously released from dauer?

> Following the reviewer's suggestion, we performed PA14 survival assays with day 1 and day 9 adult worms that experienced dauer stage by using dauer pheromone. Different from *daf-2* RNAi-treated worms during development, we found that day 9 adult worms released from dauer exhibited decreased survival compared with day 1 adult worms on PA14. These data indicate that experiencing a developmental stage of reduced IIS (e.g. dauer) is not sufficient for preventing immune aging. The different results we obtained from these two alternative approaches for reducing IIS during development are very interesting, but we think solving this issue is beyond the scope of the paper, as we just obtained these results for this revision. We therefore added the data and speculation regarding the difference between *daf-2* RNAi knockdown during development and experiencing dauer to the revised manuscript as follows.

Figure 1 legends, page 15, line 277: Different from *daf-2* RNAi-treated worms during development (Figure S1K-M), adult worms recovered from dauer, which are also expected to experience decreased IIS (Fielenbach and Antebi, 2008), exhibited immune aging (Figure S1O; see Supplemental discussion).

Figure S1 legends, page 6, line 128: (**O**) Adult worms recovered from dauer exhibited age-dependent decreases in their survival on PA14.

Supplemental discussion, page 3, line 59: **Reduction of DAF-2 signaling during development confers variable effects on immune aging in a context-dependent manner**

One very interesting observation that we made in this study was that *daf-2* RNAi during development increased immunocompetence in old adults, but experiencing a dauer stage, which reduces insulin/IGF-1 signaling (Fielenbach and Antebi, 2008), was not sufficient for delaying immune aging. We speculate that *daf-2* RNAi treatment during development may at least partially retain the activity of DAF-16 and HSF-1 during aging for delaying immune aging. In contrast, worms that experience dauer stage may need to completely inhibit DAF-16 and HSF-1 for dauer exit to reach adulthood, leading to normal immunosenescence. Therefore, we speculate that the level of IIS in adult worms that experience dauer stage is similar to that in control adult worms. It will be important to experimentally test this possibility in future studies.

Materials and Methods, page 23, line 464: **Preparation of Dauer-recovered Worms.** Dauer pheromone was used for inducing dauer formation (Neal et al., 2013). Diluted crude dauer pheromone (6 µl crude dauer pheromone + 94 µl autoclaved water) was placed on each of 35 mm plate. Three ml of dauer agar solution (0.3 g NaCl, 2 g Noble agar, 100 µl 1 M $CaCl_2$, 100 µl 1 M $MgSO_4$, 2.5 ml 1 M KPO_4 per 100 ml media) sterilized by autoclave was cooled down to 50-60°C and distributed into each of the 35 mm plates containing 100 µl diluted crude dauer pheromone. Because live bacteria interfere with the formation of dauer, heat-killed OP50 was used for food source. Concentrated OP50 (10X) was resuspended by adding S-basal buffer (5.85 g NaCl, 1 g K_2 HPO_4, 6 g KH_2PO_4, 1 ml cholesterol (5 mg/ml in ethanol) per 1 L buffer). OP50 in S-basal buffer was heated at 95°C for 30 min with brief vortexing every 10 min. The heat-killed OP50 was cooled down at 4°C at least 30 min. WT worms were allowed to lay eggs at 25°C. After 72 hrs, worms that became dauer larvae were transferred onto OP50-seeded NGM plates at 20°C for recovery from dauer. When the recovered worms reached L4 or young adult stage, the worms were transferred onto fresh OP50-seeded NGM plates containing 50 µM FUDR. Day 1 or day 9 adult worms were then infected with PA14 for survival assays.

February 1, 2021

RE: JCB Manuscript #202006174R

Prof. Seung-Jae Lee
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Dear Prof. Lee:

Thank you for submitting your revised manuscript entitled "Reduced insulin/IGF1 signaling prevents immune aging via ZIP-10/bZIP-mediated positive feedback loop". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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The authors adequately addressed my previous concerns

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The authors have thoroughly addressed all concerns.