RESEARCH ARTICLE

RNAi targeting *Caenorhabditis elegans* α -arrestins marginally affects lifespan [version 1; peer review: 2 approved with reservations]

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Abstract

Background: α -arrestins are a family of proteins that are implicated in multiple biological processes, including metabolism and receptor desensitization. **Methods:** Here, we sought to examine the roles of α -arrestins in the longevity of *Caenorhabditis elegans* through an RNA interference screen. **Results:** We found that knocking down each of 24 out of total 29 *C. elegans* α -arrestins had small or no effects on lifespan. Thus, individual *C. elegans* α -arrestins may have minor effects on longevity. **Conclusions:** This study will provide useful information for future research on the functional role of α -arrestins in aging and longevity.

Keywords

C. elegans, α -arrestin, insulin/IGF-1 signaling, aging, lifespan

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Introduction

α -arrestins are a family of proteins that contain arrestin domains whose sequences and structures have similarities with those of classical visual and β -arrestins¹⁻³. α -arrestins are considered as ancestral forms of arrestins because their orthologs exist in fungi, including yeast, which do not have visual or β -arrestins^{1,4}. Several mammalian α -arrestins modulate metabolism and receptor desensitization^{5,6}, but much remains to be elucidated concerning the functions of α -arrestins in many organisms. The human genome encodes 6 α -arrestins and 4 visual or β -arrestins. Interestingly, the simple roundworm *Caenorhabditis elegans* genome contains 29 α -arrestin and 1 β -arrestin genes^{1,7}. Therefore, the *C. elegans* system provides opportunities for the genetic analysis of α -arrestins in various aspects of physiology both individually and combinatorially. However, information regarding the functions of *C. elegans* α -arrestins is limited^{8,9}.

C. elegans is an excellent genetic model organism that has been exploited for studying conserved biological processes, including apoptosis, behavior, development and aging. In particular, its short lifespan in combination with genetic amenability has made *C. elegans* one of the most popular models for research on aging and longevity^{10,11}. Many factors, including components in insulin/insulin-like growth factor 1 (IGF-1) signaling (IIS), have been identified as lifespan and aging regulators in *C. elegans*¹¹⁻¹³. For example, genetic inhibition of IIS components, such as DAF-2/insulin/IGF-1 receptor, robustly extends lifespan and delays physiological aging through up-regulating transcription factors, including DAF-16/FOXO¹¹⁻¹³. Importantly, the roles of these aging-regulatory factors in *C. elegans* have been shown to be conserved in other species, including *Drosophila* and mammals^{12,13}. One of the powerful ways to identify novel factors that influence aging is by employing genetic screens, such as an RNA interference (RNAi) screen. We previously identified several genetic factors, including RNA helicases, that modulate longevity in *C. elegans*, through targeted or genome-wide RNAi screens¹⁴⁻¹⁷. Because of its robust longevity phenotype that confers sensitivity to changes in lifespan, *daf-2*/insulin/IGF-1 receptor mutants serve as an excellent platform for the identification of novel lifespan-regulating factors^{17,18}.

In this study, we aimed to determine whether any α -arrestins played a role in the lifespan regulation of wild-type or *daf-2* mutants. We performed a lifespan assay-based RNAi screen targeting 24 out of 29 *C. elegans* α -arrestins. We found that knocking down each of the α -arrestins had small or no effects on the lifespan of wild-type and *daf-2* mutants. Thus, *C. elegans* α -arrestins may play minor or modulatory roles in lifespan regulation. Based on our results, it will be important to test the roles of α -arrestins in combinatorial manners and/or by using strong loss-of-function mutations in future research.

Methods

Caenorhabditis elegans strains

All strains were maintained as previously described¹⁹. The following strains were used in this study: N2 wild-type, CF1041 *daf-2(e1370)* III outcrossed six times to N2.

Phylogenetic analysis

The protein sequences of 27 α -arrestins, except *arnd* (arrestin domain protein)-20 and *arnd-21*, were obtained from Wormbase (www.wormbase.org, version WS259). The protein sequences of *arnd-20* and *arnd-21* were obtained from Ensembl (<http://www.ensembl.org>, release 89). The phylogenetic tree of 29 α -arrestins in *C. elegans* was generated using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>)²⁰ and re-visualized using the Dendroscope 3 (version 3.5.9)²¹. For the α -arrestins that have multiple isoforms, isoform a was used for the analysis.

RNAi clones

Twenty one RNAi clones that target *C. elegans* α -arrestin genes were used from two commercial *C. elegans* RNAi feeding libraries. Specifically, RNAi clones targeting *arnd-2*, *arnd-6*, *arnd-7*, *arnd-8*, *arnd-9*, *arnd-10*, *arnd-13*, *arnd-16*, *arnd-18*, *arnd-23*, *arnd-24*, *arnd-25*, *arnd-28* and *ttm-2* (toxin-regulated targets of MAPK 2) were obtained from Ahringer laboratory library (Geneservice Ltd., Cambridge, UK), while those against *arnd-1*, *arnd-3*, *arnd-4*, *arnd-5*, *arnd-14*, *arnd-15* and *arnd-19* were from Vidal laboratory library (Source BioScience, Nottingham, UK). Three RNAi clones targeting *arnd-11*, *arnd-17*, and *arnd-26* were generated by molecular cloning using infusion recombinase (EZ-Fusion™ Cloning Kit, Enzymomics, Daejeon, South Korea). The N2 genomic DNA was obtained through the lysis of worms using proteinase K (Invitrogen, Carlsbad, CA, USA) and N2 complementary DNA was obtained from RNA extraction using RNAiso Plus (Takara, Shiga, Japan) followed by reverse transcription using ImProm-II reverse transcriptase (Promega, Madison, WI, USA, USA). The infusion reaction between PCR products and pL4440 plasmids (Fire lab *C. elegans* vector kit, 1999) digested with HindIII (New England Biolabs, Ipswich, MA, USA) and Acc65I (New England Biolabs) was followed by transformation of in-house competent *E. coli* HT115 cells and by selection of positive clones on ampicillin (USB, Santa Clara, CA, USA)-containing LB plates. Primers (CosmoGenetech, Seoul, South Korea) that were used to amplify coding regions of *arnd-11* from N2 genomic DNA, and those of *arnd-17* and *arnd-26* from N2 complementary DNA are as follows: forward primer 5'-GAATTCGATATCAAGCTC-CCTCGTGCAAATTAGGAAA-3' and reverse primer 5'-CTATAG-GGCGAATTGGGGTTCCTCCCACTCCATACA-3' for *arnd-11*; forward primer 5'-GAATTCGATATCAAGCTATGGTGCAGTTA-GATCGTTTTG-3' and reverse primer 5'-CTATAGGGCGAATT-GGTAAATCGGTATAAAATGG-3' for *arnd-17*; forward primer 5'-GAATTCGATATCAAGCTATGAAGGTCGATTACTTCG-3' and reverse primer 5'-CTATAGGGCGAATTGGCTACTTCTCG-GAGCCATTG-3' for *arnd-26*. The sequences of all these 24 α -arrestin RNAi clones were confirmed using DNA sequencing (Solgent, Daejeon, South Korea) before lifespan assays. RNAi clones targeting remaining five α -arrestin genes were not generated.

RNAi screen using lifespan assay

The RNAi screen employing lifespan assay was performed as previously described¹⁷. Briefly, *E. coli* HT115 bacteria that expressed specific double-stranded RNAs (dsRNAs) were cultured overnight at 37°C in LB media containing 50 µg/ml ampicillin

(USB). The cultured bacteria were seeded onto nematode growth media plates containing 50 µg/ml ampicillin and incubated overnight at 37°C. The seeded bacteria were treated with 1 mM isopropyl β-D-1-thiogalactopyranoside (Gold Biotechnology, St. Louis, MO, USA) and incubated at room temperature for approximately 24 h to induce dsRNAs. Age-synchronized wild-type N2 and *daf-2(e1370)* mutant animals were grown on RNAi plates from embryo to L4 stage at 20°C. Worms were then transferred onto RNAi plates containing 5 µM 5-fluoro-2'-deoxyuridine (FUDR; Sigma, St. Louis, MO, USA), which prevents eggs from hatching, at young (day 1) adult stage, and transferred again to new FUDR-containing RNAi plates after 1 or 2 days. All lifespan assays were performed at 20°C as duplicates by two independent researchers. The survival of worms was determined by gently touching worms with a platinum wire. Worms that did not respond were counted as dead worms and removed

from the plates. Worms that crawled off the plates, burrowed into the agar media, or displayed internal hatching or vulval rupture were censored, but included in the statistical analysis. Lifespan data from two independent lifespan experiments within the experimental sets were pooled and used for statistical analysis (see Dataset 1–Dataset 3). OASIS 2 (Online Application for Survival Analysis 2; <https://sbi.postech.ac.kr/oasis2>)²² was used for the statistical analysis of lifespan results. *P* values were calculated using long-rank (Mantel-Cox method) test.

Results

Knocking down each of several *C. elegans* α-arrestins marginally influenced lifespan

We measured the lifespan of wild-type and long-lived *daf-2* mutant animals upon knocking down each of 24 of 29 genes encoding putative α-arrestin proteins (Figure 1A). We used *daf-16* RNAi

Figure 1. RNAi targeting each of *Caenorhabditis elegans* α-arrestins had small or no effects on lifespan. (A) A phylogenetic tree showing *C. elegans* α-arrestin family members. Asterisks (*) indicate α-arrestins that were not examined in this study and number signs (#) indicate predicted pseudogenes. (B) Each circle represents percent mean lifespan change by treatment with individual α-arrestin gene RNAi in wild-type (WT) and *daf-2(e1370)* (*daf-2(-)*) mutants. Red triangles indicate lifespan changes by *daf-16* RNAi, which was used as a positive control. (C–F) Lifespan curves of WT and *daf-2(-)* animals treated with RNAi targeting *arrd-16* (C), *arrd-1* (D), *arrd-24* (E), and *arrd-3* (F). See Table S1 for statistical analysis.

that largely suppressed the longevity of *daf-2(-)* mutants as a positive control (Figure 1B, Figure S1A, Table S1)^{17,18}. We found that knockdown of each of individual α -arrestin genes tended to slightly reduce lifespan in wild-type or in *daf-2(-)* mutants (Figure 1B, Figure S1B–U, Table S1). Out of the 24 RNAi clones, RNAi targeting *arrd* (arrestin domain protein)-13, *arrd-16*, *arrd-23*, *arrd-24* or *arrd-25* in wild-type, and RNAi against *arrd-1*, *arrd-2*, *arrd-5*, *arrd-24* or *arrd-28* in *daf-2(-)* mutant animals decreased lifespan by more than 5% (Figure 1B–E, Figures S1B, D, K, Q, R, T, Table S1). Specifically, *arrd-16* RNAi decreased lifespan in wild-type by 9%, and *arrd-1* RNAi decreased lifespan in *daf-2(-)* mutants by 7% (Figure 1B and C). In addition, RNAi targeting *arrd-24* decreased the lifespan of wild-type and *daf-2* mutant animals by 11% and 6%, respectively (Figure 1E). In contrast, *arrd-3* RNAi increased the lifespan of wild-type animals by 10% (Figure 1F). Overall, genetic inhibition of individual α -arrestin genes using RNAi appears to have minor effects on *C. elegans* lifespan.

Dataset 1. Kaplan-Meier estimator of RNAi lifespan experiments

<http://dx.doi.org/10.5256/f1000research.12337.d173158>

Kaplan-Meier estimate values were calculated from pooled lifespan data of two independent lifespan experiments using OASIS2 (<https://sbi.postech.ac.kr/oasis2/>). 'At risk' indicates the number of individuals at risk just prior to a specific time point. 'S_hat' indicates Kaplan-Meier estimate of survival function, 'Var S_hat' indicates variance of 'S_hat', and 'SE(S)' indicates standard error of 'S_hat'.

Dataset 2. Mean lifespan and mortality rates of RNAi experiment results

<http://dx.doi.org/10.5256/f1000research.12337.d173159>

Mean lifespan, ages at different percent mortality, linear interpolation of mortality curve at specific mortality rate were calculated from pooled lifespan data of two independent lifespan experiments. Data were obtained using OASIS2 (<https://sbi.postech.ac.kr/oasis2/>).

Dataset 3. Statistical analysis of lifespan data

<http://dx.doi.org/10.5256/f1000research.12337.d173160>

Statistical test results (Chi square, *p* value, Bonferroni *p* value) were calculated between 'condition 1' and 'condition 2'. Test results were obtained using OASIS2 (<https://sbi.postech.ac.kr/oasis2/>).

Discussion

The lifespan-regulatory functions of α -arrestins remain largely unexplored at the organism level. Here we showed that RNAi knockdown of individual *C. elegans* α -arrestins had small or no effects on lifespan in wild-type or *daf-2* mutants. As knocking down each of several α -arrestins resulted in minor changes in lifespan, these *C. elegans* α -arrestins may play modulatory roles in longevity regulation. RNAi targeting some α -arrestins might be insufficient for causing strong lifespan phenotypes for various reasons. This may be because many of *C. elegans* α -arrestins are predicted to be expressed in neurons^{7,9,23–25}, which are refractory to RNAi^{26–28}. Lifespan assays using RNAi-hypersensitive mutants, including *rrf-3(-)* and *eri-1(-)* animals^{28–30}, treated with α -arrestin RNAi, or using α -arrestin null mutants will help address this issue. Another possibility is that *C. elegans* α -arrestins may have

functional redundancy, considering the large number of the α -arrestin family members in *C. elegans* and their sequence similarity¹⁷. In addition, some α -arrestins may mostly function by modulating the action of their interacting proteins⁶. In this case, genetic inhibition of α -arrestins may rather subtly affect the functions of their interacting partners that directly regulate physiology, such as aging and longevity, causing weak or no phenotypes. Thus, it will be interesting to test the effects of simultaneous inhibition of α -arrestins, and to identify and to functionally characterize proteins that bind *C. elegans* α -arrestins.

In mammals, several α -arrestins are implicated in metabolic regulation⁵. TXNIP (thioredoxin-interacting protein), an inhibitor of thioredoxin in mammals^{31–33}, is a crucial negative regulator of glucose uptake^{34,35}. ARRDC4 inhibits glucose uptake in cultured mammalian cells as well³⁵, and ARRDC3 deficiency protects against obesity in male mice through increasing energy expenditure³⁶. Because metabolism is closely associated with aging³⁷, it will be interesting to test whether α -arrestins in complex metazoans play roles in aging via regulating metabolism.

Data availability

Dataset 1. Kaplan-Meier estimator of RNAi lifespan experiments. Kaplan-Meier estimate values were calculated from pooled lifespan data of two independent lifespan experiments using OASIS2 (<https://sbi.postech.ac.kr/oasis2/>). 'At risk' indicates the number of individuals at risk just prior to a specific time point. 'S_hat' indicates Kaplan-Meier estimate of survival function, 'Var S_hat' indicates variance of 'S_hat', and 'SE(S)' indicates standard error of 'S_hat'. doi, [10.5256/f1000research.12337.d173158](https://doi.org/10.5256/f1000research.12337.d173158)³⁸

Dataset 2. Mean lifespan and mortality rates of RNAi experiment results. Mean lifespan, ages at different percent mortality, linear interpolation of mortality curve at specific mortality rate were calculated from pooled lifespan data of two independent lifespan experiments. Data were obtained using OASIS2 (<https://sbi.postech.ac.kr/oasis2/>). doi, [10.5256/f1000research.12337.d173159](https://doi.org/10.5256/f1000research.12337.d173159)³⁹

Dataset 3. Statistical analysis of lifespan data. Statistical test results (Chi square, *p* value, Bonferroni *p* value) were calculated between 'condition 1' and 'condition 2'. Test results were obtained using OASIS2 (<https://sbi.postech.ac.kr/oasis2/>). doi, [10.5256/f1000research.12337.d173160](https://doi.org/10.5256/f1000research.12337.d173160)⁴⁰

Competing interests

No competing interests were disclosed.

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Supplementary material

Figure S1. RNAi knockdown of each *Caenorhabditis elegans* α -arrestin had minor effects on the lifespan of wild-type or *daf-2(-)* mutants. (A–U) Lifespan curves of wild-type (WT) and *daf-2(e1370)* (*daf-2(-)*) mutants treated with RNAi targeting *daf-16* (A), which was used as a positive control, *arrd-2* (B), *arrd-4* (C), *arrd-5* (D), *arrd-6* (E), *arrd-7* (F), *arrd-8* (G), *arrd-9* (H), *arrd-10* (I), *arrd-11* (J), *arrd-13* (K), *arrd-14* (L), *arrd-15* (M), *arrd-17* (N), *arrd-18* (O), *arrd-19* (P), *arrd-23* (Q), *arrd-25* (R), *arrd-26* (S), *arrd-28* (T) and *ttm-2* (U). See [Table S1](#) for statistical analysis and for additional repeats of lifespan data using *daf-16* RNAi.

[Click here to access the data.](#)

Table S1. Lifespan data of *Caenorhabditis elegans* α -arrestin RNAi clones. Lifespan data within the solid lines indicate the pooled experimental datasets that are performed at the same time by two independent researchers. *P* values were calculated within the sets using log-rank (Mantel-Cox) method²². Percent (%) changes and *p* values for the lifespan data of specific α -arrestin RNAi-treated wild-type (WT) and *daf-2(e1370)* worms were calculated against those of control RNAi-treated WT and *daf-2(e1370)* worms within the sets, respectively. Percent (%) changes and *p* values for the lifespan data of control RNAi-treated *daf-2(e1370)* mutants were calculated against those of control RNAi-treated WT worms. SEM represents standard error of the mean, and 75th percentile indicates the age (days) at 75% mortality.

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Version 1

Reviewer Report 06 September 2017

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Roger Pocock

Development and Stem Cells Program, Monash Biomedicine Discovery Institute, Department of Anatomy and Developmental Biology, Monash University, Melbourne, Vic, Australia

In this manuscript Park *et al.* performed RNAi knockdown of 24 of the 29 alpha-arrestin genes in *C. elegans* to ask whether they are important for lifespan regulation in wild type and daf-2 mutant animals. Their data shows that, in their experimental conditions, most alpha-arrestin genes are individually not important for regulating lifespan.

Concerns:

One major issue with the study is whether RNAi knockdown of the alpha-arrestin genes is working. As some alpha-arrestins are expressed in the nervous system, which is refractory to RNAi, this is a concern. To determine whether the RNAi knockdown is working then Q-RTPCR should be performed. However, this is not a small task for 24 genes, therefore, further experiments using sensitized genetic backgrounds for RNAi or elevated temperatures may tease out phenotypes for some alpha-arrestin genes. This could particularly be performed for the alpha-arrestin genes that when knocked down engender a slight effect on lifespan.

In parallel to this, the authors could align the alpha-arrestin gene sequences to ask if there is a common sequence that could be used by RNAi targeting to knockdown multiple alpha-arrestin genes simultaneously.

The authors knockdown of 24 of the 29 alpha-arrestin genes. I wonder why they didn't generate RNAi clones for the remaining 5, especially since they did generate 3 other constructs for their analysis. Potentially, the important alpha-arrestin genes is amongst those not examined.

The authors did not report any other behavioural or morphological phenotypes observed by alpha-arrestin gene knockdown. Any information on observed phenotypes would be of interest to the research community.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response (F1000Research Advisory Board Member) 16 Oct 2017

Seung-Jae V Lee, Department of Biological Sciences, Korea Advanced Institute of Science and Technology, South Korea

Dear Dr. Roger Pocock,

We sincerely appreciate your valuable comments on our manuscript. We agree with all your points and revised our manuscript to describe the limitations of this study. We would like to mention that the main motivation for publishing this manuscript is to provide our negative results to researchers who are interested in characterizing α -arrestins in *C. elegans*. Our point-by-point responses are described below. We hope you find our revised manuscript is suitable for publication in *F1000Research*.

In this manuscript Park et al. performed RNAi knockdown of 24 of the 29 alpha-arrestin genes in *C. elegans* to ask whether they are important for lifespan regulation in wild type and *daf-2* mutant animals. Their data shows that, in their experimental conditions, most alpha-arrestin genes are individually not important for regulating lifespan.

Concerns:

One major issue with the study is whether RNAi knockdown of the alpha-arrestin genes is working. As some alpha-arrestins are expressed in the nervous system, which is refractory to RNAi, this is a concern. To determine whether the RNAi knockdown is working then Q-RTPCR should be performed. However, this is not a small task for 24 genes, therefore, further experiments using sensitized genetic backgrounds for RNAi or elevated

temperatures may tease out phenotypes for some alpha-arrestin genes. This could particularly be performed for the alpha-arrestin genes that when knocked down engender a slight effect on lifespan.

> We agree with the reviewer's comment that the RNAi knockdown efficiency is a major concern in interpreting our screen results. It would have been great if we had performed qRT-PCR analysis. However, as the reviewers pointed out, this is not a small task. We currently do not have any plan for following up this project and want to inform other researchers of our screen data. Therefore, we further emphasized the pitfall of our results in the Discussion as follows,

In Discussion: "The lifespan-regulatory functions of α -arrestins remain largely unexplored at the organism level. Here we showed that RNAi knockdown of individual *C. elegans* α -arrestins had small or no effects on lifespan in wild-type or *daf-2* mutants. Our study has limitations that need to be considered for interpretation. First, RNAi targeting some α -arrestins might be insufficient for causing strong lifespan phenotypes. This may be because many of *C. elegans* α -arrestins are predicted to be expressed in neurons^{7, 9, 24-26}, which are refractory to RNAi²⁷⁻²⁹. Lifespan assays using RNAi-hypersensitive mutants, including *rrf-3(-)* and *eri-1(-)* animals²⁹⁻³¹, treated with α -arrestin RNAi, or using α -arrestin null mutants will help address this issue. In addition, as we did not test whether RNAi targeting each of α -arrestin genes was effective by using quantitative RT-PCR, our negative data should be interpreted with caution."

In parallel to this, the authors could align the alpha-arrestin gene sequences to ask if there is a common sequence that could be used by RNAi targeting to knockdown multiple alpha-arrestin genes simultaneously.

> We appreciate the reviewer's comment. The α -arrestin family members share arrestin-domains (O. Hobert, *Wormbook*, 2013). Thus, it will be intriguing if we could examine whether targeting arrestin domain for RNAi to knock down multiple *arrd* genes simultaneously and if multiple *arrd* gene knock down has effects on lifespan. We modified our manuscript to address this issue in Discussion as follows,

In Discussion: "Second, *C. elegans* α -arrestins may have functional redundancy considering the large number of the α -arrestin family members in *C. elegans* and their sequence similarity^{1, 7}, which may obscure examining the functional importance of each α -arrestin. In addition, some α -arrestins may mostly function by modulating their interacting proteins⁶. In this case, genetic inhibition of α -arrestins may rather subtly affect the functions of their interacting partners that directly regulate physiology, such as aging and longevity, causing weak or no phenotypes. Thus, it will be interesting to test the effects of simultaneous inhibition of α -arrestins possibly through targeting the arrestin domain, and to identify and to functionally characterize proteins that bind *C. elegans* α -arrestins."

The authors knockdown of 24 of the 29 alpha-arrestin genes. I wonder why they didn't generate RNAi clones for the remaining 5, especially since they did generate 3 other constructs for their analysis. Potentially, the important alpha-arrestin genes is amongst those not examined.

> We appreciate the reviewers' comment. We aimed at obtaining all the α arrestin RNAi

clones that are not commercially available, except two predicted pseudogenes, *arrd-20* and *arrd-21* (Wormbase, version WS259). We successfully constructed three RNAi clones targeting *arrd-11*, *arrd-17* and *arrd-26*, but could not obtain the other three RNAi clones, targeting *arrd-12*, *arrd-22* and *arrd-27*. We addressed this issue in Methods for RNAi clones and in Discussion as follows,

In Methods, RNAi clones: "RNAi clones for *arrd-12*, *arrd-22*, and *arrd-27* were not generated because of unknown technical problems and were not included in the lifespan screen. *arrd-20* and *arrd-21* are predicted to be pseudogenes (Wormbase, version WS259) and were excluded from our screen."

In Discussion: "Third, it is possible that three α -arrestins, *arrd-12*, *arrd-22* and *arrd-27*, which were not tested in our screen, may play crucial roles in lifespan regulation. Thus, it will be important to examine if genetic inhibition of each of these three α -arrestin genes affects lifespan in future studies."

The authors did not report any other behavioural or morphological phenotypes observed by alpha-arrestin gene knockdown. Any information on observed phenotypes would be of interest to the research community.

> We thank the reviewer for the valuable comment. One noticeable phenotype we observed was that the eggs laid by worms fed with *tmm-2* RNAi bacteria hatched more frequently than control eggs on FUDR-treated plates, although we did not quantify the results. We described this issue in Methods as follows,

In Methods, RNAi screen using lifespan assays: "Eggs laid by *tmm-2* RNAi-treated worms hatched more frequently than control eggs on FUDR-containing plates in two independent lifespan experiments."

Competing Interests: No competing interests were disclosed.

Reviewer Report 04 September 2017

<https://doi.org/10.5256/f1000research.13357.r25290>

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Alessandro Bitto

Department of Pathology, University of Washington, Seattle, Seattle, WA, USA

Jason Pitt

Department of Pathology, University of Washington, Seattle, WA, USA

In the manuscript *RNAi targeting Caenorhabditis elegans alpha-arrestins marginally affects lifespan* Park and co-workers provide largely negative results from lifespan experiments following RNAi knockdown of most of the worm alpha-arrestins. While we welcome the effort to publish negative

results, there is one major concern with the experimental approach and several minor concerns with the interpretation of the results that should be addressed before the manuscript is fully accepted for indexing in *F1000Research*.

Major concern:

The authors data is almost entirely negative: there were only 4 clones which had a statistically significant effect. The authors attempt to address this concern by providing RNAi data for *daf-16*, which is known to strongly suppress the lifespan extension of *daf-2(e1370)* as well as decreasing N2 lifespan. However, the authors must demonstrate that alpha-arrestin RNAi knockdown is functioning. As suggested by the authors, there is some evidence that alpha-arrestins are expressed in neurons which are notoriously refractory to RNAi. The authors must show that they can use RNAi to knockdown alpha-arrestins by QRTPCR analysis. The authors could also address this concern by performing the RNAi in one of the mutant strains in which RNAi works in neurons and report if the lifespan still does not change, as they suggest in the discussion. We believe that providing these data is necessary to correctly interpret the results from the lifespan analyses.

Minor concerns:

1. In the text the authors speak broadly of individual arrestins having no role in longevity however the authors only tested 24 of the 29 arrestins in the worm. If the authors had limited themselves to only those clones available in commercial RNAi libraries this would be understandable, however the authors did construct 3 new RNAi feeding constructs. This brings up the question to why the authors did not prepare the remaining 5 constructs. If the authors have reason to exclude those genes from their analysis (ie. they are pseudogenes, not expressed, etc) they should discuss these reasons in the text. Besides, there is the possibility that one of the missing constructs may indeed have major effects on lifespan in *C. elegans*.
2. The title of the manuscript implies there are more than one result than was observed. The simpler conclusion from the results provided is that individual alpha-arrestin knockdown has no effect of lifespan. This is the fundamental observation of the paper considering that most of them (20 out of 24) had no effect and that 4 of them had such an extremely small effect that it is arguable that the sample size is insufficient to support any conclusion¹. We understand the authors' motivation to make the manuscript sound more appealing, however, their interpretation is disingenuous, given their results.
3. Because alpha-arrestins share significant sequence homology the authors should make some mention as to the likelihood that RNAi of any one arrestin would have off target effects on other arrestins. The authors could easily address this question experimentally with QRTPCR analysis. This is particularly relevant as one of the main conclusions of the manuscript is that future alpha-arrestin studies should knockdown multiple alpha-arrestins simultaneously.
4. Given that the authors observed the RNAi treated animals for weeks or months (in *daf-2*) it is surprising that no other behavioral or physical phenotypes from the alpha-arrestin RNAi were observed during the course of the analysis. If the authors truly did not observe any other phenotypes they should state it briefly in the text.

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Genet. 2017; 8: 92 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biology of Aging

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Author Response (F1000Research Advisory Board Member) 16 Oct 2017

Seung-Jae V Lee, Department of Biological Sciences, Korea Advanced Institute of Science and Technology, South Korea

Dear Dr. Alessandro Bitto and Dr. Jason Pitt,

We greatly appreciate the reviewers' valuable comments on our manuscript. We agree with all the reviewers' comments and modified our manuscript to describe the limitations of our studies raised by the reviewers. We would like to mention that the main purpose of publishing our current manuscript is to provide our data to other researchers who are interested in the characterization of *C. elegans arrd* genes, instead of keeping our current data unpublished. Our point-by-point responses are described below. We hope you find our revised manuscript is suitable for publication in *F1000Research*.

Reviewers' comments:

In the manuscript RNAi targeting *Caenorhabditis elegans* alpha-arrestins marginally affects lifespan Park and co-workers provide largely negative results from lifespan experiments following RNAi knockdown of most of the worm alpha-arrestins. While we welcome the

effort to publish negative results, there is one major concern with the experimental approach and several minor concerns with the interpretation of the results that should be addressed before the manuscript is fully accepted for indexing in *F1000Research*.

Major concern:

The authors data is almost entirely negative: there were only 4 clones which had a statistically significant effect. The authors attempt to address this concern by providing RNAi data for *daf-16*, which is known to strongly suppress the lifespan extension of *daf-2(e1370)* as well as decreasing N2 lifespan. However, the authors must demonstrate that alpha-arrestin RNAi knockdown is functioning. As suggested by the authors, there is some evidence that alpha-arrestins are expressed in neurons which are notoriously refractory to RNAi. The authors must show that they can use RNAi to knockdown alpha-arrestins by qRT-PCR analysis. The authors could also address this concern by performing the RNAi in one of the mutant strains in which RNAi works in neurons and report if the lifespan still does not change, as they suggest in the discussion. We believe that providing these data is necessary to correctly interpret the results from the lifespan analyses.

> We completely agree with the reviewers for the comment. It would have been great if we had performed qRT-PCR for validating the RNAi efficiency, while we were doing lifespan assays. As our experiments were done as an RNAi screen, our initial plan was validating RNAi efficiency and using RNAi-sensitive mutants if we had identified some positive RNAi clones. However, as the reviewers pointed out, our screen results were negative. We currently do not have any plan for following up this project and just want to inform other researchers of our screen data. Therefore, instead of putting efforts on this project retrospectively, we emphasized the limitations of our data raised by the reviewers to inform the readers of pitfalls by revising the manuscript as follows,

In Discussion: "The lifespan-regulatory functions of α -arrestins remain largely unexplored at the organism level. Here we showed that RNAi knockdown of individual *C. elegans* α -arrestins had small or no effects on lifespan in wild-type or *daf-2* mutants. Our study has limitations that need to be considered for interpretation. First, RNAi targeting some α -arrestins might be insufficient for causing strong lifespan phenotypes. This may be because many of *C. elegans* α -arrestins are predicted to be expressed in neurons^{7, 9, 24-26}, which are refractory to RNAi²⁷⁻²⁹. Lifespan assays using RNAi-hypersensitive mutants, including *rrf-3(-)* and *eri-1(-)* animals²⁹⁻³¹, treated with α -arrestin RNAi, or using α -arrestin null mutants will help address this issue. In addition, as we did not test whether RNAi targeting each of α -arrestin genes was effective by using quantitative RT-PCR, our negative data should be interpreted with caution."

Minor concerns:

1. In the text the authors speak broadly of individual arrestins having no role in longevity however the authors only tested 24 of the 29 arrestins in the worm. If the authors had limited themselves to only those clones available in commercial RNAi libraries this would be understandable, however the authors did construct 3 new RNAi feeding constructs. This brings up the question to why the authors did not prepare the remaining 5 constructs. If the authors have reason to exclude those genes from their analysis (ie. they are pseudogenes, not expressed, etc) they should discuss these reasons in the text. Besides, there is the possibility that one of the missing constructs may indeed have major effects on lifespan in

C. elegans.

> We appreciate the reviewers' comment. We initially aimed at obtaining all the α arrestin RNAi clones that are not commercially available except two predicted pseudogenes, *arrd-20* and *arrd-21* (Wormbase, version WS259). We successfully constructed three RNAi clones targeting *arrd-11*, *arrd-17* and *arrd-26*, but could not obtain the other three RNAi clones for *arrd-12*, *arrd-22* and *arrd-27* because of unknown technical problems. We agree with the reviewers' point that these three genes that were not tested may have major effects on lifespan. We addressed this issue in Methods for RNAi clones as well as in Discussion as follows,

In Methods, RNAi clones: "RNAi clones for *arrd-12*, *arrd-22*, and *arrd-27* were not generated because of unknown technical problems and were not included in the lifespan screen. *arrd-20* and *arrd-21* are predicted to be pseudogenes (Wormbase, version WS259) and were excluded from our screen."

In Discussion: "Third, it is possible that three α -arrestins, *arrd-12*, *arrd-22* and *arrd-27*, which were not tested in our screen, may play crucial roles in lifespan regulation. Thus, it will be important to examine if genetic inhibition of each of these three α -arrestin genes affects lifespan in future studies."

2. The title of the manuscript implies there are more than one result than was observed. The simpler conclusion from the results provided is that individual alpha-arrestin knockdown has no effect of lifespan. This is the fundamental observation of the paper considering that most of them (20 out of 24) had no effect and that 4 of them had such an extremely small effect that it is arguable that the sample size is insufficient to support any conclusion¹. We understand the authors' motivation to make the manuscript sound more appealing, however, their interpretation is disingenuous, given their results.

> We thank the reviewers' comment on the title and changed the title as follows,
Title: "RNAi targeting *Caenorhabditis elegans* α -arrestins has small or no effects on lifespan"

3. Because alpha-arrestins share significant sequence homology the authors should make some mention as to the likelihood that RNAi of any one arrestin would have off target effects on other arrestins. The authors could easily address this question experimentally with QRT-PCR analysis. This is particularly relevant as one of the main conclusions of the manuscript is that future alpha-arrestin studies should knockdown multiple alpha-arrestins simultaneously.

> We agree with the reviewers that the RNAi targeting each of α -arrestin genes may have caused off-target effects on other α -arrestin genes because of sequence similarities. We performed off-target prediction using Clone Mapper (<http://bioinformatics.lif.univ-mrs.fr/RNAiMap>), and at least with the prediction, the RNAi clones used in our screen do not appear to have major off-target effects on any other genes including α -arrestin genes, except *arrd-19* RNAi which may also target *arrd-21* which is a predicted pseudogene. Although qRT-PCR will be a straightforward way as the reviewers suggested, again currently we do not have any plan on following up this project and we therefore instead acknowledged the limitation. We added the prediction results in the Supplementary Table S1 and described this issue in the Methods, RNAi clones part as follows,

In Methods, RNAi clones: "All RNAi clones used in our screen were examined for their potential off-target effects by using Clone Mapper (<http://bioinformatics.lif.univ-mrs.fr/RNAiMap>)²², and no significant off-target was predicted except *arrd-19* RNAi, which may additionally target a predicted pseudogene *arrd-21* (Table S1). Experimental validation by qRT-PCR will be necessary to completely exclude the possible off-target effects by RNAi clones."

In Supplementary material: "Table S1. Possible RNAi target prediction. RNAi clones used in the screen were examined for their possible off-targets by Clone Mapper (<http://bioinformatics.lif.univ-mrs.fr/RNAiMap>)²². For RNAi clone names, 'mv' indicates a RNAi clone from Vidal library and 'sjj' indicates a RNAi clone from Ahringer library. RNAi clones generated by us for this study are labeled as 'Lee lab'. Scores for potential target transcripts were calculated as described previously²² and were rounded off to the second digit after the decimal point."

In Reference: 22. Thakur, N., et al., Clone Mapper: An Online Suite of Tools for RNAi Experiments in *Caenorhabditis elegans*. G3 (Bethesda), 2014. 4(11): p. 2137-2145.

4. Given that the authors observed the RNAi treated animals for weeks or months (in *daf-2*) it is surprising that no other behavioral or physical phenotypes from the alpha-arrestin RNAi were observed during the course of the analysis. If the authors truly did not observe any other phenotypes they should state it briefly in the text.

> We appreciate the reviewers' comment. As our main interests were lifespan changes from a large scale screen, we did not pay attention to other phenotypes. Nevertheless, one noticeable phenotype we observed was that the eggs laid by worms fed with *ttm-2* RNAi bacteria hatched more frequently than control eggs on FUDR-treated plates, although we did not quantitate the results. We described this issue in Methods as follows,
In Methods, RNAi screen using lifespan assays: "Eggs laid by *ttm-2* RNAi-treated worms hatched more frequently than control eggs on FUDR-containing plates in two independent lifespan experiments."

Competing Interests: No competing interests were disclosed.

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