Opposing effects of an F-box protein and the HSP90 chaperone network on microtubule stability and neurite growth in *Caenorhabditis elegans*

Chaogu Zheng, Emily Atlas, Ho Ming Terence Lee, Susan Laura Javier Jao, Ken C. Q. Nguyen, David H. Hall and Martin Chalfie
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Original submission

First decision letter

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MS TITLE: F-box protein MEC-15 promotes microtubule stability and neurite growth by antagonizing the HSP90 chaperone network in *Caenorhabditis elegans*

AUTHORS: Chaogu Zheng, Emily Atlas, Ho Ming Terence Lee, Susan Laura Javier Jao, Ken C. Q. Nguyen, David H. Hall, and Martin Chalfie

I have now received reviews of your manuscript from 3 experts. The reviewers' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, all 3 reviewers are very enthusiastic about your study. They do raise a few concerns and offer additional excellent suggestions for improving your study and manuscript. Most notably, Reviewer 1 points out that the current writing suggests a direct molecular antagonism between the UPS ad HSP systems and recommends that you soften your claims, Reviewer 2 points out that PPH-5 is a ser/thr phosphatase and should not dephosphorylate tyr's and suggests that you consider moving the sti-1/pph-5 interaction into the main text, and Reviewer 3 suggests a cullin RNAi experiment that could be informative.

I invite you to consider the reviewers' suggestions and submit a revised manuscript. We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. You are welcome to send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

When you submit your revised manuscript, please clearly HIGHLIGHT all changes made in the revised version. You should avoid using 'Tracked Changes' in Word files as these are lost in PDF conversion. I also request a point-by-point response detailing how you have dealt with the points

raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of the reviewers' criticisms or suggestions, please explain why.

Reviewer 1

Advance summary and potential significance to field

Zheng et al. present a genetic analysis of factors regulating neuronal differentiation in the classic C. elegans touch neuron model. Starting with a neomorphic tubulin mutant that leads to ectopic neurite growth, they show that this phenotype can be suppressed by loss of mec-15, an F-box protein that likely participates in a Skp ligase complex. This group has previously demonstrated that mec-15 plays a number of roles in neuronal differentiation. They then ask what can suppress the mec-15 suppression, and find many components of the Hsp70/90 chaperone system as well as DLK-1, a kinase known to play a central role in neuronal differentiation including in touch neurons. The authors ultimately present data that strongly support the model that mec-15 targets DKL-1 for degradation, and that excess DLK-1 in the mec-15 mutant is able to suppress the ectopic neurite growth as well as some other phenotypes on the tubulin mutant. DLK-1 is stabilized by the Hsp70/90 chaperone system, so loss of these proteins also leads to a loss of DLK-1 and a suppression of some tubulin mutant phenotypes. Prior studies demonstrated that Hsp70 regulates fly and mouse DLK, so this interaction is evolutionarily conserved to worms. In addition, more phenotypes are suppressed by loss of the chaperones , so there are likely additional substrates of mec-15 that are also stabilized by the Hsp70/90 system. The authors present their findings as evidence for an antagonistic relationship between the pro-degradative mec-15 ubiquitin ligase and the pro-stability Hsp70/90 system. Overall this is a solid and careful piece of work that will be of interest to workers in the field. The study is comprehensive and the findings are convincing.

Comments for the author

Zheng et al. present a genetic analysis of factors regulating neuronal differentiation in the classic C. elegans touch neuron model. Starting with a neomorphic tubulin mutant that leads to ectopic neurite growth, they show that this phenotype can be suppressed by loss of mec-15, an F-box protein that likely participates in a Skp ligase complex. This group has previously demonstrated that mec-15 plays a number of roles in neuronal differentiation. They then ask what can suppress the mec-15 suppression, and find many components of the Hsp70/90 chaperone system as well as DLK-1, a kinase known to play a central role in neuronal differentiation including in touch neurons. The authors ultimately present data that strongly support the model that mec-15 targets DKL-1 for degradation, and that excess DLK-1 in the mec-15 mutant is able to suppress the ectopic neurite growth as well as some other phenotypes on the tubulin mutant. DLK-1 is stabilized by the Hsp70/90 chaperone system, so loss of these proteins also leads to a loss of DLK-1 and a suppression of some tubulin mutant phenotypes. Prior studies demonstrated that Hsp70 regulates fly and mouse DLK, so this interaction is evolutionarily conserved to worms. In addition, more phenotypes are suppressed by loss of the chaperones , so there are likely additional substrates of mec-15 that are also stabilized by the Hsp70/90 system. The authors present their findings as evidence for an antagonistic relationship between the pro-degradative mec-15 ubiquitin ligase and the pro-stability Hsp70/90 system. Overall this is a solid and careful piece of work that will be of interest to workers in the field. The study is comprehensive and the findings are convincing.

My only issue with the manuscript is really one of style and emphasis.
The authors present the entire study as evidence for antagonism between the UPS and HSP70/90 chaperones.
While this is true by the definitions of genetic logic, from the perspective of cell biology or molecular mechanism there are no data to support this view. There is no mechanistic link between UPS and HSP systems other than the demonstration that they can regulate the same substrate. As currently presented in the title, abstract and discussion, it sounds as though the authors are claiming a much more direct molecular antagonism. Clarifying what this study actually demonstrates without inadvertently overstating the conclusions will improve the manuscript.

Reviewer 2

Advance summary and potential significance to field

The study by Zheng et al, identifies the Fbox/WD40 repeat domain containing protein MEC-15 to be required for microtubule stability and neurite growth in C. elegans. For this a suppressor screen of the tubulin mec-7(us278) mutant allele was used, that results in hyperstable microtubules and the growth of ectopic neurites. Loss of mec-15 function rescues the mec-7 phenotype, and mec-15 lf by itself results in loss of neurite growth (particularly PML/AN), branching and defects in sensory function of touch-response neurons (TRNs). A further forward genetic screen of mec-15 lf mutants then identified two Hsp90 co-chaperones as suppressors, namely the Hop orthologue sti-1 and the serine/threonine phosphatase pph-5; as well as the MAP3K DLK-1. A further candidate RNAi screen for Hsp70 and Hsp90 family members confirmed pph-5 and sti-1 and also identified the Hsp70 family member hsp-110 and the Hsp90 co-chaperone p23 (daf-41) - and later on cdc-37 - as suppressors of mec-15 lf. The authors provide a very well controlled genetic and functional analysis that makes the compelling case for an inhibitory role of the Hsp90 chaperone family during neurite growth and neuronal development. The inhibitory role of the Hsp90 chaperone machinery is achieved by stabilising the client protein DLK-1, which negatively affects MT stability and TRN development and also synaptic function of GABAergic motor neurons.

Overall, this is a very thorough and well controlled genetic study that very nicely shows how powerful classical genetic analysis can be in identifying novel functions for a well-established chaperone system. Their discovery that the Hsp90 chaperone machinery has an inhibitory effect during neuronal differentiation by stabilising DLK-1 is new and significant. Without doubt, this current study will provide the basis and plenty of playroom for further studies to come that look at the details of chaperone-client function and interaction in a more biochemical manner. There is already a lot of information in this study and the amount of control experiments is massive. The study is likely going to be important for C. elegans neuroscience and the chaperone fields; and I would predict that their findings will spark a lot of interest from the Hsp90 community.
From my perspective, I only have some minor comments and consider the paper almost ready for publication, after clarification of a few points.

Comments for the author

Minor comments:
Intro, line 63: At least one reference would be appropriate here. Suggestions below.

Lindquist S, Craig EA. The heat-shock proteins. Annu Rev Genet. 1988;22:631–677. doi:10.1146/annurev.ge.22.120188.003215

Hartl FU, Bracher A, Hayer-Hartl M. Molecular chaperones in protein folding and proteostasis. Nature. 2011;475(7356):324–332.

line 117: “described elsewhere” – I presume this is submitted elsewhere, but it would be good to know where once published

line 118: mec-15(u1042) is not shown in Figure 1A and 1B as mentioned in the text, only mec-15(u75) is shown.

line 146: The text refers specifically to Figure 1C and Figure S1A-S1C.

line 154: why are two TRN specific promoters used – mec-17 and in the other case mec-18? What is the difference in expression?

line 211 – 218:
I wonder whether the more severe effect at 25C is due to higher turnover of Hsp90 client protein DLK-1 that has the MT-destabilising effect and also which role the ATPase activity of Hsp90 plays with regards to that. Both sti-1 and p23 that were identified as suppressors, are also ATPase inhibitory co-chaperones of Hsp90. For future studies it would be interesting to look whether

increasing the ATPase activity of Hsp90 by e.g. overexpressing Aha1 phenocopies the exacerbated outgrowth phenotype already at 20C.

line 228 – 238:
This is a very compelling discovery, but somehow the authros are trying to hide this beautiful evidence – and which was a lot of work - in the Supplement. It would be worthwhile to at least name the phospho-null and phospho-mimic mutations of STI-1. An interaction between sti-1 and pph-5 has not been published so far, and this will be particularly interesting for the Hsp90 community. Please be aware that Pph-5/pp5 is a serine/threonine phosphatase, so it should not de-phosphorylate tyrosine, as tyrosine mutations are indicated in Figure S5. So this should be acknowledged and clarified in the text.

Reviewer 3

Advance summary and potential significance to field

The core finding of this work by Zheng et al, that Hsp70/90 chaperones and cochaperones can act as negative regulators of neuronal differentiation by stabilising proteins that themselves destabilise microtubules (e.g. DLK-1), is interesting and advances the way in which we commonly think about HSPs. The authors have also found an opposing role of the UPS, primarily represented by mec-15 activity, to demonstrate a balancing act between the protein stabilising effect of chaperones and the protein degradation activity of the UPS, in neuronal differentiation.
These findings are significant both in the context of neuronal development specifically, and with regards to the diverse roles of HSPs more generally.
The study has been carried out meticulously and the quality of the presented data is excellent, with sound statistics and a strong logical flow. The data support the main conclusions drawn by the authors, and I recommend publication in Development with only very minor revisions.

Comments for the author

One more substantial point:

The authors conclude that MEC-15 “likely functions in a SFC complex” (line 39). However, this is not supported by the findings that loss or knockdown of the Skp and Cullin homologs did not mimic the effect of mec-15 loss (according to data not shown in the manuscript, lines 167-173). To overcome the apparent redundancy between these components, the authors could investigate the effect of RNAi of the cullins in the skr-1 mutant background.

Minor points:

- Fig 1C appears to be missing the asterisks for the comparison between the first two bars.
- Line 117: Please write that the mec-7(u278) screen is described in the Methods, not “elsewhere”, which is too vague.
- Line 239: It is not clear where the touch sensitivity data for the hsp-110 and hsp-90 single mutants you refer to are? Similarly, where are the data showing that the single mutants did not have TRN morphology effects? It would be helpful if the location of this data is referred to more clearly in the text.
- Fig S2C: Why were aipr-1, aha-1 and tomm70 used for the RNAi? You did not refer to these in text to explain the rationale.

First revision

Author response to reviewers' comments

Reviewer 1's comments:

My only issue with the manuscript is really one of style and emphasis. The authors present the entire study as evidence for antagonism between the UPS and HSP70/90 chaperones. While this is true by the definitions of genetic logic, from the perspective of cell biology or molecular mechanism there are no data to support this view. There is no mechanistic link between UPS and HSP systems other than the demonstration that they can regulate the same substrate. As currently presented in the title, abstract and discussion, it sounds as though the authors are claiming a much more direct molecular antagonism. Clarifying what this study actually demonstrates without inadvertently overstating the conclusions will improve the manuscript.

Response: We changed the title to "Opposing effects of an F-box protein and the HSP90 chaperone network on microtubule stability and neurite growth in *Caenorhabditis elegans*". We also changed some statements in the abstract and discussion to avoid overstating the conclusion.

Reviewer 2's comments:

1. Intro, line 63: At least one reference would be appropriate here. Suggestions below.

Lindquist S, Craig EA. The heat-shock proteins. Annu Rev Genet. 1988;22:631–677. doi:10.1146/annurev.ge.22.120188.003215

Hartl FU, Bracher A, Hayer-Hartl M. Molecular chaperones in protein folding and proteostasis. Nature. 2011;475(7356):324–332.

Response: We added the two references the reviewer suggested.

line 117: "described elsewhere" – I presume this is submitted elsewhere, but it would be good to know where once published

Response: The paper that describes all mutants isolated from the *mec-7(u278)* suppressors screen is still under preparation. So, that paper is unlikely to be published before the current manuscript.

line 118: mec-15(u1042) is not shown in Figure 1A and 1B as mentioned in the text, only mec-15(u75) is shown.

Response: *mec-15(u1042)* basically showed exactly the same phenotype as *mec-15(u75)* in suppressing the ectopic ALM-PN. The quantification for this suppression is shown in Figure 1C, which included both *u1042* and *u75*. Images of *mec-15(u75) mec-7(u278)* double and *mec-15(u75)* single mutants were shown in Figure 1A and Figure 1D, respectively. Images of *mec-15(u1042) mec-7(u278)* and *mec-15(u1042)* mutants were shown in Figure 2A and Figure 2D, respectively. We corrected the reference to Figures.

line 146: The text refers specifically to Figure 1C and Figure S1A-S1C.

Response: We corrected the reference to Figures.

line 154: why are two TRN specific promoters used – mec-17 and in the other case mec-18? What is the difference in expression?

Response: Both *mec-17* and *mec-18* promoters are only expressed in the six TRNs and their expression level are similar. So, we consider them equivalent and interchangeable. *mec-17* promoter is 1.9-kb long and *mec-18* promoter is 500-bp long. So, for the ease of cloning,

we may pick one over the other if the backbone we used already has the promoter in it.

line 211 – 218:
I wonder whether the more severe effect at 25C is due to higher turnover of Hsp90 client protein DLK-1 that has the MT-destabilising effect and also which role the ATPase activity of Hsp90 plays with regards to that. Both sti-1 and p23 that were identified as suppressors, are also ATPase inhibitory co-chaperones of Hsp90. For future studies it would be interesting to look whether increasing the ATPase activity of Hsp90 by e.g. overexpressing Aha1 phenocopies the exacerbated outgrowth phenotype already at 20C.

Response: We found that RNAi against *aha-1* did not have the same effect as mutations or RNAi of *sti-1* and *p23*, but we did not test overexpression of *aha-1*, which would be an interesting direction to follow up with in a separate study.

line 228 – 238:
This is a very compelling discovery, but somehow the authors are trying to hide this beautiful evidence – and which was a lot of work - in the Supplement. It would be worthwhile to at least name the phospho-null and phospho-mimic mutations of STI-1. An interaction between sti-1 and pph-5 has not been published so far, and this will be particularly interesting for the Hsp90 community. Please be aware that Pph-5/pp5 is a serine/threonine phosphatase, so it should not de-phosphorylate tyrosine, as tyrosine mutations are indicated in Figure S5. So this should be acknowledged and clarified in the text.

Response: We were not trying to hide it. An editor from a different journal suggested us to move that part of the story into supplemental materials. We agreed with the reviewer that we should move these results back to the main text. We pointed out in the text that PPH-5 cannot dephosphorylate tyrosine. In the *mec-7; mec-15; pph-5* triple mutant, we expect STI-1 to be hyperphosphorylated at serine and threonine residues and possibly also phosphorylated at the tyrosine residues at a normal level. We reason that changing some of the tyrosine to non-phosphorylatable phenylalanine can still reduce the overall phosphorylation level of STI-1, therefore increasing its activity. We communicated this clearly in the revised manuscript.

Reviewer 3 Comments for the Author:
One more substantial point:

The authors conclude that MEC-15 "likely functions in a SFC complex" (line 39). However, this is not supported by the findings that loss or knockdown of the Skp and Cullin homologs did not mimic the effect of mec-15 loss (according to data not shown in the manuscript, lines 167-173). To overcome the apparent redundancy between these components, the authors could investigate the effect of RNAi of the cullins in the skr-1 mutant background.

Response: We think the reviewer is suggesting RNAi against other Skp homologs in *skr-1* mutant background. It is a good idea but may not be able to solve the problem of redundancy if more than two Skp homologs interact with MEC-15. In our hands, simultaneously silencing two genes in feeding RNAi is rarely successful. In terms of silencing cullin in *skr*-1 background, it is not clear to us why this can work if there are multiple cullins acting redundantly. In any case, under normal circumstances we may be able to conduct the exploratory RNAi experiments the reviewer requested. But given the current COVID-19 pandemic and the closure of university campuses, it is quite difficult to get the reagents (RNAi colonies and strains) and access the laboratory to perform these experiments.

Moreover, cell-specific silencing of *uba-1* phenocopied the loss of *mec-15*, supporting the idea that ubiquitination is involved in the regulation of neurite growth. Since MEC-15 is a F-box protein, the most likely explanation for the involvement of ubiquitination is that MEC-15 works in a SCF complex that serves as a E3 ubiquitin ligase.

Minor points:

-Fig 1C appears to be missing the asterisks for the comparison between the first two bars.

Response: We added the asterisks for that comparison.

-Line 117: Please write that the mec-7(u278) screen is described in the Methods, not "elsewhere", which is too vague.

Response: We changed it to "see Materials and Methods for details".

-Line 239: It is not clear where the touch sensitivity data for the hsp-110 and hsp-90 single mutants you refer to are? Similarly, where are the data showing that the single mutants did not have TRN morphology effects? It would be helpful if the location of this data is referred to more clearly in the text.

Response: We did not do the touch test for *hsp-110* and *hsp-90* mutants because their homozygotes appeared to be uncoordinated and quite unhealthy. We mentioned that in the revised text. The TRNs in the single mutants of *hsp-90*, *hsp-110*, *pph-5*, *sti-1*, and *p23* are morphologically wild-type. We added images of these mutants to Figure S5 in the supplemental materials.

-Fig S2C: Why were aipr-1, aha-1 and tomm70 used for the RNAi? You did not refer to these in text to explain the rationale.

Response: On line 206, we stated that "Through a candidate RNAi screen of 27 Hsp70/Hsp90-related genes (Table S2), we found that knocking down *daf-41* …" Figure S2C showed a few examples of the RNAi results.

Second decision letter

MS ID#: DEVELOP/2020/189886

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ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. The reviewers' reports are appended below.

Reviewer 1

Advance summary and potential significance to field

none

Comments for the author

none

Reviewer 2

Advance summary and potential significance to field

The authors have addressed all the comments and suggestions in the revised version; and from my perspective I am happy for this manuscript to go forward to publication.

Comments for the author

I don't have any further comments or suggestions.

Reviewer 3

Advance summary and potential significance to field

As stated in the previous round of revision.

Comments for the author

The authors responses to the comments on the previous version of the manuscript, and the revised manuscript itself, are entirely acceptable, and we would now recommend publication with no further revision.
Congratulations to the authors on a very thorough piece of work.