Fungal phytochrome chromophore biosynthesis at mitochondria

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DOI: 10.15252/embj.2021108083

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Review Timeline:

Submission Date:	22nd Feb 21
Editorial Decision:	25th Mar 21
Revision Received:	21st May 21
Editorial Decision:	17th Jun 21
Revision Received:	18th Jun 21
Accepted:	21st Jun 21

Editor: Ieva Gailite

Transaction Report:

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As you will see from the comments, all reviewers find the topic of the study interesting and novel. However, they also indicate multiple substantial concerns that affect the core conclusions of your study and indicate that substantial further characterisation of the purified HoxA and HoxB, their biochemical properties, enzymatic activity and mitochondrial localisation mechanism would be required before they can recommend publication of the manuscript.

Based on the overall interest expressed by the reviewers, I would like to invite you to submit a revised version of your manuscript, in which you address the comments of all referees. Please note that a strong referee support will be required for the acceptance of the revised manuscript.

I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. Since extensive additional work would be needed to fulfill all referee requests, please also let me know if you find that particular issues will not be addressable in the revised version, in which case I would be happy to discuss alternative publication possibilities within EMBO Press journals.

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When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to receiving your revised manuscript.

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2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. Please note that the Data Availability Section is restricted to new primary data that are part of this study. If no data deposition in external databases is needed for this paper, please then state in this section: This study includes no data deposited in external repositories.
*** Note - All links should resolve to a page where the data can be accessed. ***

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

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9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be

typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

10 When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen: http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

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Referee #1:

This work identified two heme oxygenases, HoxA and HoxB, from A. alternata. The authors proposed that the proteins form metabolon on mitochondria. The result is important.

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As the HoxA and HoxB are very low in activity, is it possible that they or associated factor(s) are only the (minor) components of the BV-generating complex? The deletion and phenotype experiments cannot exclude the possibilities. For in vitro activity, did the authors optimize conditions such as factors, buffers, metal ions, pH, temprature, etc.?

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Fig. 6, No fitted curve(s) for KD?

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The authors describe the interesting finding that mitochondria in the fungus Alternaria alternata have a two HOX enzymes linked to phytochrome activity. This is an interesting and important finding, however, in its current form the manuscript has several weaknesses listed below:

1. The biochemical characterization of HoxA and HoxB have significant questions. First, the titration studies are not well described. Why for example do the authors start with a 1 uM heme solution and titrate in the protein to 10 uM and then further titrate with protein? Following purification are both Hox proteins purified as apo-proteins or do they have either heme or biliverdin bound? Heterologous expression of HO proteins either bacterial or mammalian routinely have biliverdin bound following purification. The comment that not all Hox proteins are capable of binding heme would suggest that the protein is not homogenous and has either a ligand bound or is not folded correctly. If the latter is the case this compromises all of the biochemical experiments. Furthermore, given the lack of significant purification of HoxB interpretation of the data is also a concern (Fig S3)

2. The comment that the appearance of the shoulder in HoxB appears more pronounced and increased faster (pg. 5 lines 152-156) is a measure of differences in affinity is an over interpretation of the data. As stated above issues with protein purity and integrity are a concern.

3. The difference in retention of the biliverdin produced by combination of HoxA and HoxB despite a spectrum that shows biliverdin requires confirmation. Is this a different isomer given that biliverdin alpha is the standard? Is this a modified biliverdin? Mass spectrometry should be performed to

confirm the product and isomer. The product does bind to FphA but again the reconstitution compared to that of BphO would suggest some confirmation of the product by MS would be desirable. Also as HoxA is sufficient for assembly the authors should characterize the biliverdin product ie. the retention time and structure of the HoxA product alone.

4. In Fig 3C the expression of HoxA appears to be sufficient for phytochrome assembly so what is the role of HoxB. Again in Fig4 the auhtors present data regarding the location of HoxA and HoxB but the data on HoxB is lacking. This raises more questions as to the role of HoxB and if it is indeed a HO.

5. The BLI (Fig 6) data again raises some questions as the association phase for the 3.5 and 4.7 uM concentration are not consistent with the others leaving some doubt as to the binding affinity as calculated. One would determine form the data in Fig 6B that tehre is no binding.

Overall the data while supportive of HoxA being a HO enzyme lacks the rigor in the data especially as it pertains to the role of HoxB and the nature of the biliverdin product. I believe these significant gaps require addressing prior to consideration for publication.

Referee #3:

Critique Streng et al
This manuscript describes the identification of putative heme oxygenase proteins that are involved in inserting a tetrapyrrole into the FphA protein in fungi. These HO proteins have remained elusive thus far and make this an important exciting finding if true. However, to this reviewer, the experiments lack important controls to permit interpretation of the data as currently presented. Thus, the manuscript needs major revisions before it is acceptable for publication.

Major concerns
First, the English throughout the manuscript needs improvement as it is missing articles and the phrasing is awkward.

Line 132 refers to ccgA induction and Fig 2B, however, the figure is 2C. It would make more sense to rearrange the order of the figures (e.g. A is fine but describe it. Make B the ccgA graph and then C the plate growth.
There is no mention that hoxB is more sensitive to tBooH or why this may be. Also, for the lay reader, a description of what the plate stresses induce would help guide the reader as to what this plate phenotype is testing. That the deletions look like FphA is not sufficient for the novice reader to understand these subtleties. Many deletion strains in yeast show similar plate phenotypes. In the quantification of ccgA what was the n and what gene was used as a control gene for the RTPCR analysis?

Fig 3 this figure description seems incomplete. A more complete description of the data would be helpful. Does HoxB addition "help"? the scales from A to B make the interpretation hard.
a. there is no mention of the control of BphO in the results text. This is necessary to explain the increased ab at 700+ when Hox proteins are added to imply they act as heme oxygenases like BphO.

Fig S3 - the authors reference that the resulting peak from A + B addition resembled biliverdin, yet no example of what biliverdin looks like is shown.

line 176-177 "suggesting that the activity of HoxA and HoxB together is higher than the sum of both". Would the authors expect that a double KO would have a more dramatic phenotype (Fig 2). This is easily tested. What about double FphA and Hox? Are the phenotypes the same or additive? This might suggest that there are other HOX yet to be discovered or that these are the only ones.

Fig 4. the descriptions of the lanes are not clear. The description says "mitochondria were treated with proteinaseK for 20 min" but no further descriptions are provided to know why the authors have two S and two P fractions (this is found in the methods but would be helpful to a reader if more detail of the fractions is provided in the legend to understand that gels). Why is there no visible CitA-GFPi in the CE or P2 and why would they "enrich" in P2 + PK? It seems like it should just be the same if the amounts being loaded are controlled. Also, what is a GFP trap experiment and why is it provided for cytosolic CitA instead of the HoxA protein. Not sure if this is helpful as described. The introduction to this figure says Hox A and HoxB were GFP tagged yet no data for HoxB is provided. Similarly, the figure legend says HoxA and HoxB. Why is HoxB not shown?
Line 188-189 suggests that cit synth additional band could be free GFP. This is easily determined as the molecular weights of these proteins are known. Molecular weight standards should be shown.

Fig 5. All examples are positive. Is this overexpression or endogenous levels? could it be possible that any mitochondrial proteins, when overexpressed with split YFP tagged show BiFC? It would be nice to show a split YFP tagged mitochondrial protein that is not predicted to interact with a Hox. A nice control might be one that is in the matrix vs the outside of the mitochondria.
B- no molecular weight standards are shown in the SEC. These should be provided.
C- the "purification of PGP is not compelling as shown as an "induced" band cannot be appreciated in any lanes and PGP as indicated is very weak in the elution. A confirming western would be useful. Further, it still looks like the 70kDa band is present in the purification. Perhaps this is the best purification. The authors could refer back to Fig 3 where there is activity shown. Perhaps this reviewer missed it but no description of how big PFP is predicted to be is provided.
C lower panel - How do we know this is specific to PFP/FphA and Hox? Is FphA or the strep tag "sticky"? Again, a negative control would be nice, another strep tagged molecule or mutated FphA that does not show Hox associated. One could interpret the gel as having poorly washed resin....perhaps showing the "washes" would provide that needed control. no description is provided about how the Hox bands were illuminated in the gel only that western blots were run.

Fig 6 description lines 207-214....It is unclear to this reviewer why the authors focused on HoxA. In the data of Figure 5, it looks like HoxB is the more prominent band "binding" to FphA. No reasoning for this was provided. Further, the authors spend time in the manuscript suggesting that HoxA and B interact. Is it possible to do the BLI with HoxA, HoxB or both to determine if the FphA interaction Kd or Ka changes? What is HoxB doing? HoxB deletion phenotype looks more dramatic than HoxA (fig 2)

Discussion - the authors provide a model suggesting that the Hox proteins generate holoprotein FphA, which now interacts with the phosphotransfer protein YpdA to induce the HOG pathway and that holo FphA is imported into the nucleus to control chromatin remodeling enzymes. This is extremely speculative and goes beyond what the data provide. The experiments performed test if Hox proteins can interact with FphA and that FphA shows increased tetrapyrrole insertion. No

experiments are shown regarding Fph (apo or holo) interactions with YpdA, or that there are changes in chromatin remodeling. Further, no experiments are provided that show that holoFphA vs apo FphA is imported into the nucleus. The model should be modified to support what is tested in the manuscript.

Minor concerns
1. Fig 1 mentioned asterisk but the symbol is a star
2. No mention of Fig 2A
3. Line 205 sentence seems incomplete as it is missing the protein name ...."photosensory domain" of FphA

Referee #1:

This work identified two heme oxygenases, HoxA and HoxB, from A. alternata. The authors proposed that the proteins form metabolon on mitochondria. The result is important.

The major concerns:
As the HoxA and HoxB are very low in activity, is it possible that they or associated factor(s) are only the (minor) components of the BV-generating complex? The deletion and phenotype experiments cannot exclude the possibilities. For in vitro activity, did the authors optimize conditions such as factors, buffers, metal ions, pH, temprature, etc.?

The reviewer is right that the activities are low. However, we spent about one year working closely together with the lab in Kaiserslautern to optimize the reaction and the activity we show is the best we could get. However, one has to consider that we don't know the electron donor system in *A. alternata* and that the protein for activity measurements were heterologously expressed. In addition, we show that the reaction *in vivo* occurs at the outer mitochondrial membrane in a protein complex. These conditions are also not easy to mimic *in vitro* and are for sure also not found during the expression in *E. coli*. In sum, the activities are low but obviously sufficient *in vivo*. We added a short chapter in the Discussion.

For biliproteins detetion, why did the authors not detect them via Zinc-induced fluorescence? If they did not, it was not shown or cannot be detected, why?

We added a Zn blot in Fig. 3A.

Other minor concerns:

The authors obseved that not all HoxA or HoxB bound hemin. Is it ascribed to the oligomerization of the proteins, so shielding the binding sites? If yes, did the oligomerization affect the activity? If yes, why with the activity assay, they used the 5-fold higher concentration of the proteins (50 microM vs. 10 microM on oligomerization detection)?

The interpretation of the binding data have to be taken with care, because the proteins are not pure enough to do valid stoichiometric calculations. However, the experiments clearly showed binding, which is a prerequisite for the activity.

Line 161, "retention time was about 2.5 min longer", it is not a small difference, the authors should explain. Is the experiment, HPLC, repeated, how many times?

We did repeat the experiment several times and the difference of the retention time varied between 1 and 2.5 minutes. However, we overlaid the spectrum of the compound in that peak with BV and they perfectly match (suppl. Fig. S3D). We tried hard to do mass spectrometry, but failed. In any case the identification of the chromophore produced in *E. coli* or *in vitro* is only of limited value, because ultimately, one has to purify phytochrome from *A. alternata* and analyze the structure of the endogenous chromomphore.

Is there a tag/motif for mitochondria from HoxA, HoxB or Phy? Why they are attached at mitochondria? Is the attachment specific or unspecific to receptor(s) or similar factor(s)?

Thank you very much for this comment. We have meanwhile characterized the binding and found that the C-terminal anchor is necessary and sufficient for mitochondrial targeting. See new Fig. 4 C, D.

Line 381-382, French press is only for breaking cells. corrected

Line 386, what is "1 VVM air"; there are some too specific abbreviations for readers to understand. Explained.

Line 396, the proteins are not well purified! corrected

Line 404, why is written as "400 microgram"? If the authors insisted, please indicate the volume. Added.

Line 417, Why did the detection set at 650 nm instead of 690-700 nm? Which bilin does absorb maximally at 650 nm in this HPLC conditions?
The spectrum in Fig. S3C shows the maximum of free biliverdin at 656 nm.

Fig. 6, No fitted curve(s) for KD? We added a new Fig. S4 and extended the explaining text in the results section.

Referee #2:

The authors describe the interesting finding that mitochondria in the fungus Alternaria alternata have a two HOX enzymes linked to phytochrome activity. This is an interesting and important finding, however, in its current form the manuscript has several weaknesses listed below:

1. The biochemical characterization of HoxA and HoxB have significant questions. First, the titration studies are not well described. Why for example do the authors start with a 1 uM heme solution and titrate in the protein to 10 uM and then further titrate with protein?

After the addition of protein, the titration was done with hemin.

Following purification are both Hox proteins purified as apo-proteins or do they have either heme or biliverdin bound? Heterologous expression of HO proteins either bacterial or mammalian routinely have biliverdin bound following purification.

We have no evidence for heme binding during the expression. Information added in the Results section.

The comment that not all Hox proteins are capable of binding heme would suggest that the protein is not homogenous and has either a ligand bound or is not folded correctly. If the latter is the case this compromises all of the biochemical experiments. Furthermore, given the lack of significant purification of HoxB interpretation of the data is also a concern (Fig S3)

The binding was only the first step for the enzymatic analysis and was not meant to be quantitative. The determination of the exact stoichiometry, binding affinities etc. would need further purification of the enzymes, which is beyond the scope of this paper. The main focus here is to show that both

HOs are enzymatically active, that they interact at the mitochondrial outer membrane and provide the chromophore for phytochrome.

2. The comment that the appearance of the shoulder in HoxB appears more pronounced and increased faster (pg. 5 lines 152-156) is a measure of differences in affinity is an over interpretation of the data. As stated above issues with protein purity and integrity are a concern.

Changed.

3. The difference in retention of the biliverdin produced by combination of HoxA and HoxB despite a spectrum that shows biliverdin requires confirmation. Is this a different isomer given that biliverdin alpha is the standard? Is this a modified biliverdin? Mass spectrometry should be performed to confirm the product and isomer. The product does bind to FphA but again the reconstitution compared to that of BphO would suggest some confirmation of the product by MS would be desirable. Also as HoxA is sufficient for assembly the authors should characterize the biliverdin product ie. the retention time and structure of the HoxA product alone.

See above.

4. In Fig 3C the expression of HoxA appears to be sufficient for phytochrome assembly so what is the role of HoxB. Again in Fig4 the auhtors present data regarding the location of HoxA and HoxB but the data on HoxB is lacking. This raises more questions as to the role of HoxB and if it is indeed a HO.

We added data showing that HoxB really linearizes hemin and hence works as HO. The activity is much lower than the one of HoxA. We discussed the possibility that HoxB could act as chaperone to stimulate the HoxA activity. In addition, there is evidence that human HO-1 may be processed under stress conditions and fulfill additional roles in nuclei. Our observation that the *hoxB*-deletion strain appears to be more sensitive towards TBooH points into that direction.

5. The BLI (Fig 6) data again raises some questions as the association phase for the 3.5 and 4.7 uM concentration are not consistent with the others leaving some doubt as to the binding affinity as calculated. One would determine form the data in Fig 6B that tehre is no binding.

The problem is that the chromophore in the loaded PGP interferes with the measurement, since it absorbs in the visible spectrum. We explained it in the results section. Still, the differences in affinity are huge, if there is no binding or very weak binding.

Overall the data while supportive of HoxA being a HO enzyme lacks the rigor in the data especially as it pertains to the role of HoxB and the nature of the biliverdin product. I believe these significant gaps require addressing prior to consideration for publication.

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The information was added. It could well be that HoxB is involved in stress resistance. We added a Discussion for this point. *h2b* was used as housekeeping gene.

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BphO is mentioned in the text.

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line 176-177 "suggesting that the activity of HoxA and HoxB together is higher than the sum of both". Would the authors expect that a double KO would have a more dramatic phenotype (Fig 2). This is easily tested. What about double FphA and Hox? Are the phenotypes the same or additive? This might suggest that there are other HOX yet to be discovered or that these are the only ones.

After the establishment of CRISPR/Cas9 in *A. alternata* many manipulations are possible, but still some things are more difficult than in e.g. *A. nidulans*. Despite several attempts to create double-deletion strain, we failed so far. However, the added data about the activity of HoxB and the extended the discussion about a putative chaperone function.

Fig 4. the descriptions of the lanes are not clear. The description says "mitochondria were treated with proteinaseK for 20 min" but no further descriptions are provided to know why the authors have two S and two P fractions (this is found in the methods but would be helpful to a reader if more detail of the fractions is provided in the legend to understand that gels). Why is there no visible CitA-GFPi in the CE or P2 and why would they "enrich" in P2 + PK? It seems like it should just be the

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C lower panel - How do we know this is specific to PFP/FphA and Hox? Is FphA or the strep tag "sticky"? Again, a negative control would be nice, another strep tagged molecule or mutated FphA that does not show Hox associated. One could interpret the gel as having poorly washed resin....perhaps showing the "washes" would provide that needed control. no description is provided about how the Hox bands were illuminated in the gel only that western blots were run.

We added the calibration data for the SEC. As for the interaction results, the referee is right and it could be that is not specific. Therefore, we further studied the interaction with the split YFP system and BLI. The data in *E. coli* were the first indications for protein-protein interaction.

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HoxB is unfortunately not pure enough to do those experiments. We discussed possible roles in the oxidative stress response for HoxB to explain its more dramatic phenotype.

Discussion - the authors provide a model suggesting that the Hox proteins generate holoprotein FphA, which now interacts with the phosphotransfer protein YpdA to induce the HOG pathway and that holo FphA is imported into the nucleus to control chromatin remodeling enzymes. This is extremely speculative and goes beyond what the data provide. The experiments performed test if Hox proteins can interact with FphA and that FphA shows increased tetrapyrrole insertion. No experiments are shown regarding Fph (apo or holo) interactions with YpdA, or that there are changes in chromatin remodeling. Further, no experiments are provided that show that holoFphA vs apo FphA is imported into the nucleus. The model should be modified to support what is tested in the manuscript.

The model summarizes not only our new results but puts them in the frame of the existing data. It was well documented that FphA interacts with YpdA and also that it affects the chromatin structure.

Yu, Z., Armant, O. and Fischer, R. (2016). Fungi use the SakA (HogA) pathway for phytochrome-dependent light signaling. *Nat. Microbiol.* **1**, 16019.

Hedtke, M., Rauscher, S., Röhrig, J., Rodriguez-Romero, J., Yu, Z. and Fischer, R. (2015). Light-dependent gene activation in *Aspergillus nidulans* is strictly dependent on phytochrome and involves the interplay of phytochrome and white collar-regulated histone H3 acetylation. *Mol. Microbiol.* **97**, 733-745.

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2. No mention of Fig 2A added
3. Line 205 sentence seems incomplete as it is missing the protein name ...."photosensory domain" of FphA added

Thank you for submitting a revised version of your manuscript. Your study has now been seen by all original reviewers, who find that their main concerns have been addressed and now recommend publication of the manuscript after a minor revision. Therefore, I would like to invite you to address the remaining referee comments and the following editorial issues before I can extend the official acceptance of the manuscript:

1. Our data editor has done their pre-publication check on your manuscript. I have attached the file here. Please take a look at the word file and the comments in the Figure Legends section and respond to the issues. Please also use this version when you resubmit the revised version.
2. Please add more detailed information in the Author Checklist section B and C or indicate where this information can be found in the manuscript.
3. Please rename the section "Data and materials availability" to "Data availability" and move it to the end of Materials and Methods. To adhere to the journal style, please modify the wording to "This study includes no data deposited in external repositories".
4. Please rename "Competing interests" section into "Conflict of interest".
5. Figure panel 1A is not mentioned in the text, please add a callout.
6. In Fig. 3A, to make clearer that the three sections of the gel are not derived from the same experiment, please add a white space between them.
7. Please rename "Supplementary Material" into "Appendix" and update the nomenclature to Appendix Figure S1, etc. and Appendix Table S1, etc. Please also add a short table of contents at the beginning, of the Appendix file
8. We generally encourage publication of source data for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need one file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labeled with the appropriate figure/panel number and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as supplementary "Source Data". Please let me know if you have any questions about this policy.
9. Papers published in The EMBO Journal are accompanied online by a 'Synopsis' to enhance discoverability of the manuscript. It consists of A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height, jpeg or png format). You can either show a model or key data in the synopsis image. Please note that the size is rather small and the text needs to be readable at the final size. Please send us this information along with the revised manuscript.

Please let me know if you have any further questions regarding any of these points. You can use the link below to upload the revised files.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, and we look forward to receiving the final version.

Referee #1:

This work identified two heme oxygenases, HoxA and HoxB, from A. alternata. The authors proposed that the proteins form metabolon on mitochondria, further they found a C-terminal anchor (CTA) sequence in HoxA for mitochondrial targeting. The result is important.

The authors have improved their manuscript substantially. So this reviewer has only a few minor concerns:

Line 125, "alternataHere"?

Line 245, "last 20? amino acids"?

Line 545, "50 mM Tris-HCl pH 7.8" changes to "50 mM Tris-HCl, pH 7.8".

Line 548, "measured for 60s", s = sec as be mostly written in text?

"Zn-induced red fluorescence" should be written as "Zn2+-induced red fluorescence", where "2+" is superscript.

Referee #2:

This is a resubmission and the concerns raised in the previous submission were in regard to the low enzyme activity of the HOX enzymes and confirmation of the products. The activities of the enzymes are still low however the authors have provided more information to clarify this as well as additional data as to the product verification and confirmation of the biliprotein. The manuscript is acceptable for publication.

Referee #3:

Streng et al
This manuscript is much improved and with minor editing changes is acceptable for publication.

Minor -
Line 156, I think the authors mean slightly higher sensitivity not "resistance" of the hoxB mutant. It grows more poorly, so more sensitive to conditions.

Line 181-3, rewrite to say stoichiometries for heme binding were not determined as the protein preparations were not pure.

Line 314, Chaperon is missing the "e" chaperone

The authors performed the requested changes.

Editor acce;pted the revised manuscript.

EMBO PRESS

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Reinhard Fischer
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2021-108083R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:
➜ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
➜ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
➜ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
➜ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
➜ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

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

➜ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
➜ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
➜ a statement of how many times the experiment shown was independently replicated in the laboratory.
➜ definitions of statistical methods and measures:
• common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
• are tests one-sided or two-sided?
• are there adjustments for multiple comparisons?
• exact statistical test results, e.g., P values = x but not P values < x;
• definition of 'center values' as median or average;
• definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

B- Statistics and general methods

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was chosen by experience from best practices in qPCR.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Three independent biological replicates were used. The person who did perform the experiments did not know about the outcome.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	see 3
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	only standard deviation is shown and no other statistical tests were needed.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	variation was not testet
Is there an estimate of variation within each group of data?	NA

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Anti-GFP Roche 11 814 460 001, Anti-His Thermo Fisher MA1-21315
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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

D- Animal Models

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

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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