nature portfolio

Peer Review File

Structural basis for the ligand-dependent activation of heterodimeric AHR-ARNT complex

Corresponding Author: Professor Dalei Wu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors present a significant advancement in the structural biology of the aryl hydrocarbon receptor (AhR) by reporting the first X-ray structure of the AhR complex, encompassing the bHLH-PAS region, including the PAS-B domain. In addition, they extend this study to include six known AhR ligands, providing structural insights into ligand binding modes. Finally, the manuscript presents a compelling mechanistic hypothesis regarding the transition from the cytosolic AhR complex with hsp90-XAP2-p23 to the nuclear AhR complex. This is achieved through a combination of structural comparison with cryo-EM data of the AhR-hsp90-XAP2-p23 complex and mutagenesis and Co-IP experiments.

The manuscript is well-written, and the findings are both timely and significant. The AhR research community has long awaited such structural insights. While the cryo-EM structure of the AhR-hsp90-PASB complex and the X-ray structure of the AhR bHLH-PASA dimer addressed part of this need, the present work goes further by providing:
1. Clarification of the positioning and role of the PASB domains within the AhR dimer.
2. Description of the binding modes of six distinct ligands, including the FDA-approved Tapinarof, which could drive future ligand discovery.
3. A mechanistic hypothesis regarding the transition between the cytosolic and nuclear AhR complexes.

However, I believe that certain key aspects, particularly regarding the first of these points, need additional clarification before acceptance.

Major Comments:
1. Position and Role of the PASB Domains: The structural data suggest a dimerization mode where the bHLH regions form stable interfaces, while the PASB domains play a less prominent role in dimerization. This conclusion is further supported by Co-IP experiments, where truncation of AhR residues at the PASB interface does not disrupt dimerization. However, the authors acknowledge that the PASB domains' positioning may be influenced by crystal packing forces, raising concerns about the biological relevance of the PASB interface observed in the structure.This point needs further elaboration. Specifically, the authors should expand on the potential artifacts introduced by crystal packing forces. Several questions remain unanswered:
o Do the authors believe the PASB interface observed in the crystal is biologically relevant, or is it an artifact?
o If crystal packing is responsible for the identification of the PASB domains, what would be the expected or most likely orientation of these domains in the absence of these forces?
o Are the PASB domains inherently dynamic, potentially existing as an ensemble of multiple conformations? If so, how does this influence the interpretation of the PASA-PASB interface and mechanistic hypotheses, particularly the one proposed at lines 275-276?
A more in-depth discussion of these points would help delineate the limitations of the current structure and provide a clearer interpretation of the PASB domain's role.

2. Use of Porcine AhR: While the authors state that porcine AhR was chosen because it was the only form amenable to X-ray crystallography, they should include the percentage sequence identity between porcine and human AhR in the main text. A supplementary figure showing sequence alignments with human AhR, highlighting residue conservation and any gaps or insertions, would be beneficial. This figure should especially focus on the PASB domain to assess any potential impact of sequence divergence on ligand binding or overall domain structure.

3. Clarification of Crystal Packing Forces (Lines 94-99): The manuscript mentions that crystal packing forces stabilize the PASB domains in a unique position, yet it remains unclear how these forces influence the overall structure. Figure 1b does not adequately explain the nature of these forces or their impact. The authors should improve the clarity of this figure and, if necessary, introduce a new supplementary panel to visualize how the crystal packing forces affect domain orientation and stabilization.

4. Controversial Y330A Mutation (Lines 209-212): The mutation of Y330 to alanine, prompted by the different conformation of this residue in the presence of indirubin, shows no differential effect across various ligands in XRE reporter assays. I expected indirubin to behave differently. Could the authors offer an explanation for the uniform response across ligands?

5. XAP2 Displacement Mechanism (Lines 278-281): The Co-IP experiments demonstrate that the ternary ARNT-AhR-XAP2 complex does not form. However, this alone is insufficient to conclude that ARNT displaces XAP2 from the AhR complex. To support this hypothesis, the authors should provide evidence of direct interaction between AhR and XAP2 in their experimental system. Ideally, a Co-IP experiment comparing AhR-XAP2 interaction in the presence and absence of ARNT would conclusively test this hypothesis.

6. For molecular dynamics, the manuscript does not clarify how the authors handled the long PAS-A loops and potential inter-domain linkers that are presumably unresolved in the crystal structure.

Minor Comments:
• Supplementary Figure Order: Supplementary Figure 8 is cited before Supplementary Figure 7. Please correct the figure order for consistency.
• Terminology in Figures: In Figures 4a, Supplementary Figure 7, and related manuscript text, the term "pose" is typically associated with docking studies and could cause confusion. I recommend replacing this term with "binding mode" to more accurately describe the experimental observations.

This work represents a major advance in the structural understanding of AhR, offering novel insights into its ligand-binding properties and mechanistic transitions. While the manuscript is highly relevant, particularly in the context of AhR ligand discovery and mechanistic hypotheses, several critical points need further clarification. I believe that addressing these issues, particularly concerning the PASB domain and the XAP2 displacement mechanism, will significantly strengthen the manuscript.
I recommend acceptance after major revisions, as outlined above.

Reviewer #2

(Remarks to the Author)
The manuscript presents a comprehensive study on the structural mechanisms underlying the activation of the AhR upon ligand binding, with a focus on its PAS-B domain and its interaction with ARNT. The authors employed X-ray crystallography and cryo-EM to elucidate the structural details of the AHR-ARNT-DNA complex in the presence of various ligands. Notably, the structural details of the PAS-B heterodimer of two proteins that have been central to the field have finally been resolved, revealing important interaction mechanisms. The findings are significant and enhance our understanding of AhR's ligand-binding and subsequent transcriptional activation mechanisms. I believe this manuscript can be accepted for publication after some minor revisions.
Comments:
1. Why can the combination of pAHR and hARNT resolve the crystal structure containing the PAS-B heterodimer? Compared to previous work that only resolved the PAS-A heterodimer, are there any interesting findings regarding the crystal cell parameters and protein interface between adjacent asymmetric units?
2. AhR and ARNT from different species binds to each other in this work. Might the binding pattern differ from that of the same species? For instance, are the amino acids at the interface between the two proteins highly conserved across different species?
3. What is the structural basis (or sequence basis) for the major difference between the HIF-2α-ARNT / NPAS4-ARNT architecture and the AhR-ARNT architecture? Is this related to the bridge motif sequence of AhR?
4. Is it possible that the insertion strand formed by the pAhR-PAS-B C-terminal loop is an artificial structural feature resulting from the protein truncation method? Is it speculated that this part of the sequence could also form a similar structure in the full-length AHR protein sequence, and will there be any steric hindrance?
5. How does C-terminal loop-mediated PAS-B heterodimer formation behave in AlphaFold2 or AlphaFold3 predictions?
6. WT cells were selected for the functional experiments throughout the paper, while cells with AhR knockout or knockdown were not included. How should the interference factors of endogenous AhR be taken into account?

Reviewer #3

(Remarks to the Author)
This is an outstanding paper. This is another important contribution from this team that is taking full advantage of structural analysis of the Ah receptor (AHR) bound to ligands, partners and chaperones. This reviewer (Chris Bradfield) does not have the background to challenge any of the structural analyses. Rather, I note a few issues that in my opinion will improve the paper by reflecting some of the science that may have gone underappreciated.

1) The issue of cytosolic vs nuclear forms of AHR is a complicated topic in the AHR field and in the nuclear receptor field in general. Oliver Hankinson, the discoverer of ARNT, still laments his naming of that protein as a translocator and Whitlock and Poellenz and other have battled with these definitions over the years. The best example I can give them is from our lab, where we created a DNA binding domain mutant (dbd) mouse model by inserting a spanner mutation between the last helix and the basic helix. The mutant was shown to be constitutively nuclear by our histo but was still inactive in the absence of ligand Abnormal Liver Development and Resistance to 2,3,7,8-Tetrachlorodibenzo-p-Dioxin Toxicity in Mice Carrying a Mutation in the DNA-Binding Domain of the Aryl Hydrocarbon Receptor | Toxicological Sciences | Oxford Academic (oup.com). The bottom line is you can have a nuclear AHR without activity, nuclear localization can be segregated from activity.
2) The above point may be an important consideration as this team should have the structural insights to explain why this dbd mutant became nuclear. We had postulated we were extruding the NLS away from the protection of Hsp90. Interestingly our NLS mutation was constitutively nuclear and completely devoid of any signaling PIIS002192581954861X.pdf (jbc.org)
3) Again, I will ask the team to read one of our old papers that may also shed insight on their data. Despite the common citation of the Fukunaga paper as the classic demonstration of the domain structure of the AHR, our lab published more complete data on this topic about two years before that manuscript (https://www.pnas.org/doi/pdf/10.1073/pnas.90.18.8566). The reason this paper may be of more value as a citation, despite its provenance, is that we describe the existence of a repressor domain at the exact region of the C terminal loop described in this manuscript. Moreover, in a companion paper, we also functionally map the location of an Hsp90 binding site to this region Mapping the 90 kDa heat shock protein binding region of the Ah receptor - Perdew - 1996 - IUBMB Life - Wiley Online Library .
4) Finally, the structures presented are fantastic. Two issues should be considered in the future or for this ms. One is that value may be gained by reporting failed structures. For example, we have long argued that Kyn is not a ligand, rather a proligand (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5808761/). This goes ignored, but it seems unlikely to me that an acid binds to the AHR pocket. Second, we will ultimately need the structure of a dioxin or dioxin like compounds bound to AHR. One of the primary issues in AHR biology is whether dioxin creates a unique conformation in the AHR leading to a distinct response. While highly unlikely, agencies like EPA may require such a model to support certain risk assessment assumptions.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I thank the authors for their detailed responses and revisions, which have significantly improved the manuscript. However, I still have minor concerns regarding a few key points that require further attention:
1. Residues within the PAS-B Cavity: I appreciate the authors' effort to highlight the similarities between porcine and human AhR, as requested in the previous review. However, as a final point on this topic, I recommend the authors specifically highlight any sequence differences (mutations) in residues located within the PAS-B cavity. This additional analysis would help clarify whether any structural or functional differences in ligand binding could be attributed to sequence variations in this critical region.
2. Interpretation of the Y330A Mutation Results: The authors' discussion of the Y330A mutation and its impact on ligand binding and transcriptional activity remains somewhat unclear and could be misleading. The manuscript states that ligands bind in two distinct modes:
- Mode 1 (Tapinarof, FICZ, BaP, BNF, and Indigo) stabilizes Y330 in a conformation that allows interactions with residues in the C-terminal loop (L398 and L400).
- Mode 2 (Indirubin) alters the orientation of Y330, disrupting its interaction with L398 and L400.
When Y330 is mutated to alanine (Y330A), the results indicate reduced AhR activity across all ligands tested, regardless of their binding mode, and even in the basal state (DMSO). The manuscript concludes that Y330 is generally important for AhR transcriptional activity.
However, this conclusion does not address the discrepancy between the expected differential impact of the mutation on Mode 1 versus Mode 2 ligands. Specifically, since Y330 does not interact with L398/L400 in Mode 2, one might expect the Y330A mutation to have less impact on Indirubin than on Mode 1 ligands. The authors should explicitly discuss this inconsistency.
3. XAP2 Displacement Mechanism: In Supplementary Figure 9e, the bands representing AhR interactions with ARNT and XAP2 appear to be very weak. Could the authors clarify their interpretation of these results? Specifically, it seems that in the absence of Tapinarof, the weakening of the AhR interaction with XAP2 is less evident, while the interaction with ARNT is less pronounced. While this observation aligns with the proposed displacement mechanism, the faintness of the bands makes it challenging to discern meaningful differences in binding under the tested conditions. How do the authors explain the weak bands observed in Supplementary Figure 9e? Could this be due to experimental limitations? A discussion of these points would be valuable to better contextualize these results that are remarkable to understand the effective role of the ligand in the XAP2 displacement mechanism.
4. Handling of PAS-A Loops in MD Simulations: The authors did not provide sufficient details about how the PAS-A loops, unresolved in the experimental structure, were handled in their molecular dynamics (MD) simulations. I understand that they focus the analysis on the PAS-B domains, but the whole system was simulated, and authors should provide details regarding how the unresolved PAS-A loops were modeled. Was any specific software or approach (e.g., loop prediction algorithms) used? The authors should provide these details in the methods section, ensuring transparency in the preparation of the simulation system. This clarification would improve the reproducibility of the study and address questions regarding

the reliability of the starting structure for MD simulations.

Reviewer #2

(Remarks to the Author)
I am pleased to see that the authors have thoroughly addressed the prior concerns and incorporated additional experimental data to strengthen their findings. I believe the revised manuscript meets the standards required for publication in Nature Communications.

Reviewer #3

(Remarks to the Author)
This is a great paper, accept

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have effectively resolved the earlier issues and enriched their study with new experimental data, significantly bolstering their results. I am confident that the updated manuscript is suitable for publication in Nature Communications.

We would like to sincerely thank all three reviewers for their careful evaluation, strong overall encouragement, and constructive guidance in improving our manuscript.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

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o Do the authors believe the PASB interface observed in the crystal is biologically relevant, or is it an artifact?
o If crystal packing is responsible for the identification of the PASB domains, what would be the expected or most likely orientation of these domains in the absence of these forces?
o Are the PASB domains inherently dynamic, potentially existing as an ensemble of multiple conformations? If so, how does this influence the interpretation of the PASA-PASB interface and mechanistic hypotheses, particularly the one proposed at lines 275-276?

A more in-depth discussion of these points would help delineate the limitations of the current structure and provide a clearer interpretation of the PASB domain's role.

Many thanks for these insightful questions. We believe that the observed interaction interface between AHR and ARNT PAS-B domains is biologically relevant, and is conserved among AHR proteins from difference spices. This PAS-B/PAS-B interface was independently observed in isolated dAHR-mARNT PAS-B dimer (**Supplementary Fig. 5a**), and in the multi-domain structures of CLOCK-BMAL1, HIF-α-ARNT, NPAS-ARNT, etc., as summarized by several review articles (Wu et al, *Curr Opin Struct Biol.* 2017; Zhuang et al, *J Mol Biol.* 2024; Rojas et al, *J Mol Biol.* 2024). However, the insertion of the C-ter loop of AHR PAS-B domain into the PAS-B/PAS-B interface, discovered in the current work, is a unique feature for AHR. The other above mentioned bHLH-PAS family members exhibit different conformations at this region, consistent with their non-conserved protein sequences (**Supplementary Fig. 3c**). For AHR, the sequences of this C-ter loop are highly conserved among the vertebrates but not the two invertebrates (**Supplementary Fig. 6**). Therefore, we could not rule out the possibility that the drosophila or nematode AHR PAS-B form a slightly varied interface with ARNT PAS-B in physiological conditions.

Without crystal packing forces, the position of the two PAS-B domains of AHR-ARNT (as a joint unit) would be rather flexible relative to the other domains (bHLH and PAS-A), consistent with our cryo-EM results (**Fig. 1c**). In our multi-domain heterodimeric crystal structure, there is no major interaction interface between the AHR PAS-B domain and its own PAS-A domain or the ARNT PAS-A domain. The spatial positions of PAS-B domains shown in the current structure represent one of the possible orientations and conformations (affected by packing).

So the relative position of the AHR-ARNT PAS-B domains as a whole is dynamic, but the dimerization interface between the two PAS-B domains within this unit is stable. As mentioned in the manuscript (originally in Lines 275-276), the C-ter loop of the AHR PAS-B domain is a key mediator in its interaction with partner proteins. In the AHR-HSP90-XAP2-P23 cytoplasmic complex, this C-ter loop is directly involved in the interaction with XAP2. But in the AHR-ARNT complex, this C-ter loop participates in the interaction with ARNT. Therefore, during the AHR transactivation process in nucleus, the AHR PAS-B C-ter loop undergoes a large conformational change to facilitate the exchange of partner proteins. The relatively isolated positioning of the AHR PAS-B domain, together with the long loop between PAS-A and PAS-B domains (known to be trapped in the lumen of HSP90 dimer), create a spatial opportunity for ARNT to engage the AHR-HSP90-XAP2-P23 complex (**Fig. 6**).

2. Use of Porcine AhR: While the authors state that porcine AhR was chosen because it was the only form amenable to X-ray crystallography, they should include the percentage sequence identity between porcine and human AhR in the main text. A supplementary figure showing sequence alignments with human AhR, highlighting residue conservation and any gaps or insertions, would be beneficial. This figure should especially focus on the PASB domain to assess any potential impact of sequence divergence on ligand binding or overall domain structure.

Thank you for this constructive suggestion. We have compared the sequences of the bHLH, PAS-A and PAS-B domains of porcine and human AHR proteins, noted the sequence identity in the manuscript (**Lines 107-112, 196-204**), and incorporated the alignment as a new figure (**Supplementary Fig. 1b**). It shows that the residues directly involved in the interactions with ARNT and ligands are highly conserved in porcine and human AHR proteins.

3. Clarification of Crystal Packing Forces (Lines 94-99): The manuscript mentions that crystal packing forces stabilize the PASB domains in a unique position, yet it remains unclear how these forces influence the overall structure. Figure 1b does not adequately explain the nature of these forces or their impact. The authors should improve the clarity of this figure and, if necessary, introduce a new supplementary panel to visualize how the crystal packing forces affect domain orientation and stabilization.

As suggested by the reviewer, we have inspected the surrounding symmetric molecules of the AHR-ARNT-DNA complex in the crystal structure, and displayed them as a new supplementary panel (**Supplementary Fig. 2c**). It shows that the AHR-ARNT PAS-B domains form interactions with the PAS-A domains of two surrounding symmetric molecules. These interaction interfaces are generated by crystal packing. We believe that although the relative position of the AHR-ARNT PAS-B domains is dynamic, the packing between these molecules in the crystal constrains the PAS-B domains in a stable position. We have also added the above analysis in the manuscript (**Lines 100-103).**

4. Controversial Y330A Mutation (Lines 209-212): The mutation of Y330 to alanine, prompted by the different conformation of this residue in the presence of indirubin, shows no differential effect across various ligands in XRE reporter assays. I expected indirubin to behave differently. Could the authors offer an explanation for the uniform response across ligands?

We were also a little puzzled when seeing this result (**Fig. 4b**). Therefore, we have further measured the effects of hAHR Y332A mutation (corresponding to Y330 in pAHR) on ligand-induced transcriptional activity in HT1080 and HT1080 AHR-KO cell lines (**Fig. R1a,b**, below). The results showed that the Y332A mutant reduced the agonistic activity of Tapinarof, FICZ, and Indirubin in a similar way, suggesting that this tyrosine residue is an important site for AHR's transcriptional activity. We analyzed the interaction between pAHR Y330 and Jα and following C-ter loop using the Ligplus+ program (**Fig. R1c,d**). The results showed that Y330 forms multiple interactions with residues such as L400 in the AHR-ARNT-FICZ complex (**Fig. R1c**). While in the AHR-ARNT-Indirubin complex, Y330 forms hydrophobic interactions with R396 and L400, although it could not form hydrogen bonds with L398 or L400 (**Fig. R1d**). This indicates that the Y330 residue plays a critical role in maintaining the stability of AHR Jα and C-ter loop, and its mutation will lead to a decrease in AHR transcriptional activity.

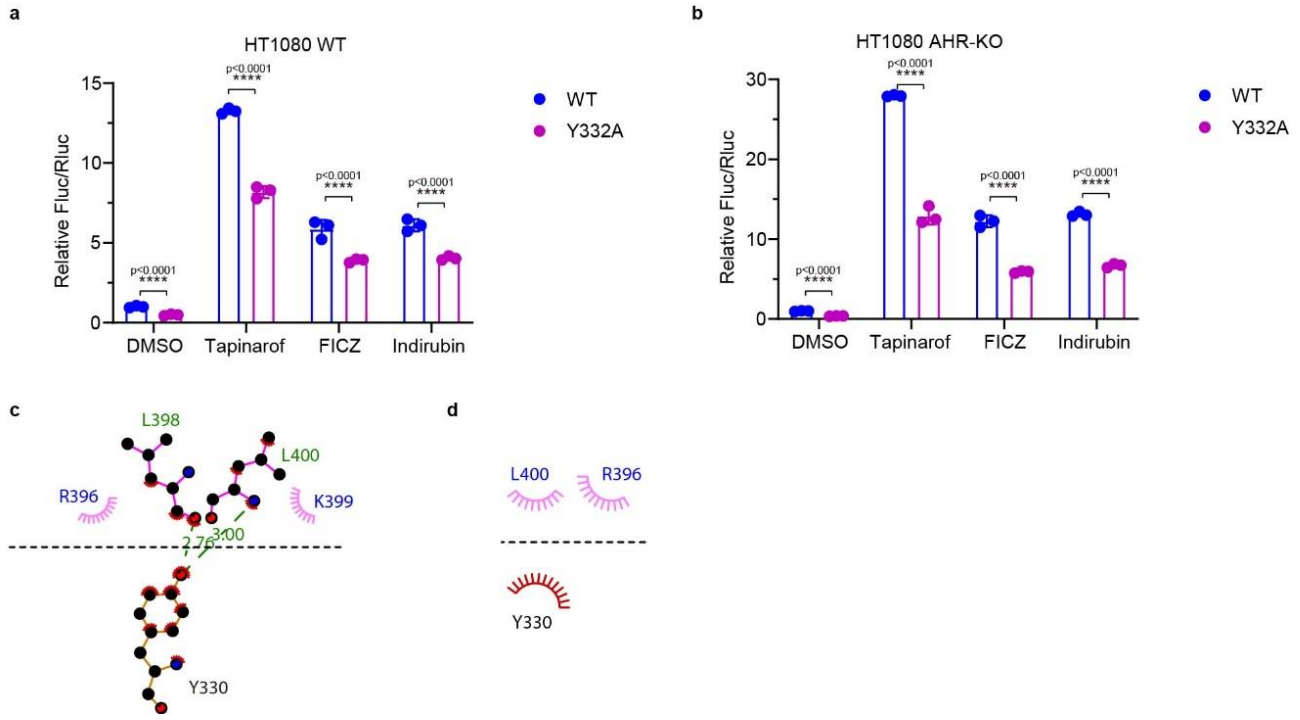

Fig. R1 Potential role of hAHR Y332 or pAHR Y330 residue in modulating the transcriptional activity. a,b, XRE reporter assays evaluating the effects of hAHR Y332A mutation (corresponding to Y330 in pAHR) on transactivation in HT1080 (a) and HT1080 AHR-KO (b) cells. c,d, The detailed interactions between Y330 and Jα and following C-ter loop in the AHR-ARNT-FICZ (c) and AHR-ARNT-Indirubin (d) complex structures. These interactions were analyzed by the Ligplus+ program.

5. XAP2 Displacement Mechanism (**Lines 278-281):** The Co-IP experiments demonstrate that the ternary ARNT-AhR-XAP2 complex does not form. However, this alone is insufficient to conclude that ARNT displaces XAP2 from the AhR complex. To support this hypothesis, the authors should provide evidence of direct interaction between AhR and XAP2 in their experimental system. Ideally, a Co-IP experiment comparing AhR-XAP2 interaction in the presence and absence of ARNT would conclusively test this hypothesis.

Thank you for this constructive suggestion. We performed new Co-IP experiments in HEK293 cells, and compared the AHR-XAP2 interaction in the presence and absence of ARNT (**Supplementary Fig. 9e**). The results showed that the interaction between AHR and XAP2 was indeed weakened in the presence of ARNT. The analysis of experimental results has been updated in the manuscript (**Lines 311-313**).

6. For molecular dynamics, the manuscript does not clarify how the authors handled the long PAS-A loops and potential inter-domain linkers that are presumably unresolved in the crystal structure.

Thank you for raising this question. As the PAS-B domains of the AHR-ARNT heterodimer are relatively independent from the bHLH-PAS-A domains, for MD simulation we only focused on the two PAS-B domains when calculating the binding energy between them, as well as the energy between ligands and the interacting residues in AHR PAS-B domain. We have

added the information of residues used in MD simulation to the Materials and Methods section (**Lines 495-496**).

Minor Comments:
• Supplementary Figure Order: Supplementary Figure 8 is cited before Supplementary Figure 7. Please correct the figure order for consistency.

Thanks for pointing this out. We have added new supplementary figures and confirmed their order in citation.

• Terminology in Figures: In Figures 4a, Supplementary Figure 7, and related manuscript text, the term "pose" is typically associated with docking studies and could cause confusion. I recommend replacing this term with "binding mode" to more accurately describe the experimental observations.

Thank you for this suggestion. We have made according changes in the manuscript.

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I recommend acceptance after major revisions, as outlined above.

Thank you very much for all the above valuable feedback and constructive comments, which are instrumental in enhancing the quality and clarity of our work. We appreciate the time and effort you have dedicated to reviewing our manuscript.

Reviewer #2 (Remarks to the Author):

The manuscript presents a comprehensive study on the structural mechanisms underlying the activation of the AhR upon ligand binding, with a focus on its PAS-B domain and its interaction with ARNT. The authors employed X-ray crystallography and cryo-EM to elucidate the structural details of the AHR-ARNT-DNA complex in the presence of various ligands. Notably, the structural details of the PAS-B heterodimer of two proteins that have been central to the field have finally been resolved, revealing important interaction mechanisms. The findings are significant and enhance our understanding of AhR's ligand-binding and subsequent transcriptional activation mechanisms. I believe this manuscript can be accepted for publication after some minor revisions.

Comments:
1. Why can the combination of pAHR and hARNT resolve the crystal structure containing the PAS-B heterodimer? Compared to previous work that only resolved the PAS-A heterodimer, are there any interesting findings regarding the crystal cell parameters and protein interface between adjacent asymmetric units?

Thank you for the comments and questions. We believe that the high dynamics of the PAS-B domains within the AHR-ARNT heterodimer is one of the key reasons why it is difficult to crystallize this multi-domain complex. In this work, we tested the combination of AHR and ARNT from multiple vertebrate species, and modified the loop regions of AHR and ARNT by truncations in various lengths. Finally, we were able to obtain the well-diffracted crystals of the pAHR-hARNT heterodimer. The other combinations only yielded crystals of much lower quality which did not provide adequate resolution. When analyzing the interactions between this protein-DNA complex and neighboring symmetric molecules, we found that the PAS-B domains of pAHR-hARNT form interaction interfaces with symmetric molecules (shown in **Supplementary Fig. 2c**). Therefore, it is the intermolecular interactions generated by the crystal packing that keep the conformation of the dimeric PAS-B domains stable. This should be the key to our success in obtaining a high-resolution crystal structure of the AHR-ARNT heterodimer containing both PAS-B domains. We added the interaction analysis of AHR-ARNT-DNA with symmetric molecules in the crystal structure in the manuscript (**Lines 100-103**).

2. AhR and ARNT from different species binds to each other in this work. Might the binding pattern differ from that of the same species? For instance, are the amino acids at the interface between the two proteins highly conserved across different species?

As shown in the **Supplementary Fig. 6**, we compared and aligned the protein sequences containing bHLH, PAS-A and PAS-B domains of AHR from human (*Homo sapiens*), porcine (*Sus scrofa*), cattle (*Bos taurus*), mouse (*Mus musculus*), chicken (*Gallus gallus*), frog (*Xenopus laevis*), zebra fish (*Danio rerio*), fruit fly (*Drosophila melanogaster*) and elegans (*Caenorhabditis elegans*). In addition, we added a new panel (**Supplementary Fig. 1b**) to highlight the similarity and difference between hAHR and pAHR. For a better visualization, bellow we have directly aligned the vertebrate AHR and ARNT proteins utilized in our work (**Fig. R2 and R3**), and marked those residues involved in domain-domain interactions. It can be seen that the residues involved in heterodimer interfaces are highly conserved across different vertebrate species. On the other hand, the ARNT residues involved in heterodimerization with AHR are almost fully conserved across these species. Therefore, we believe that the heterodimer mode of pAHR-hARNT would be similar to that of hAHR-hARNT, and represents a universal binding pattern between AHR and ARNT from different species. Detailed description and discussion have been added in the manuscript (**Lines 107-112, 196-204).**

Fig. R2 Sequence alignment of the N-terminal portions of AHR proteins from different species.

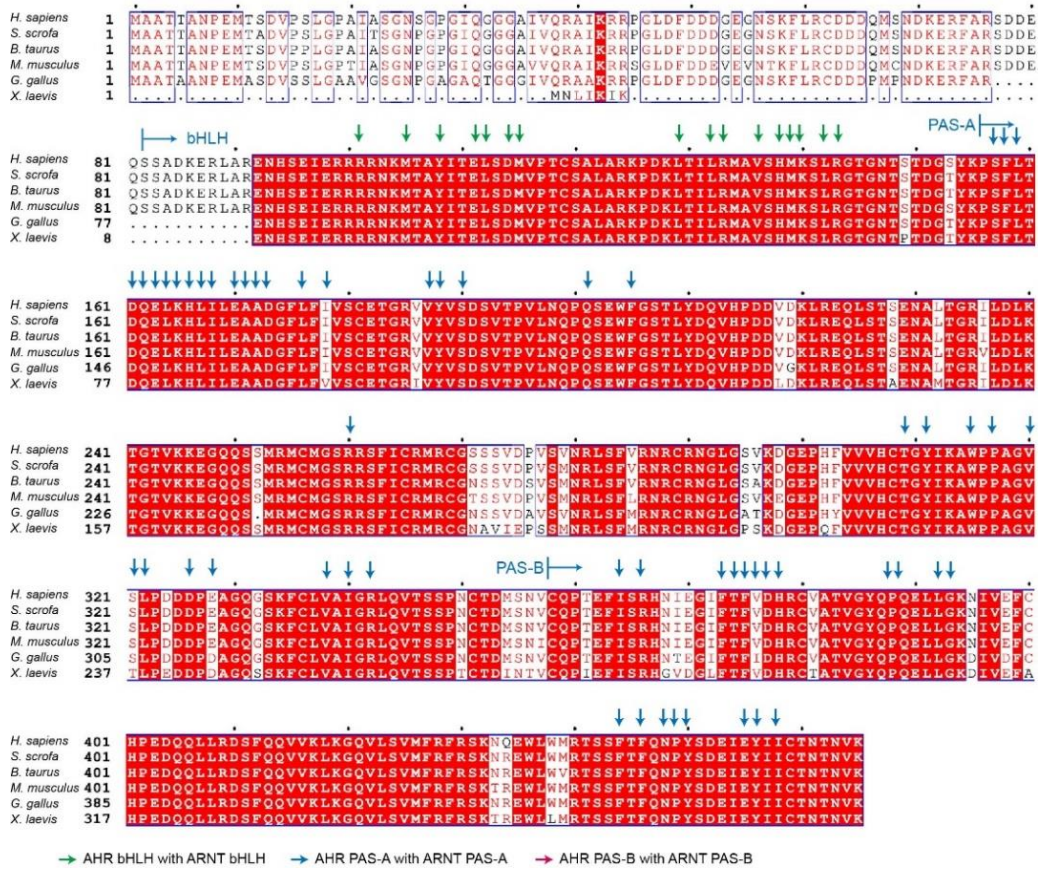

Fig. R3 Sequence alignment of the N-terminal portions of ARNT proteins from different species.

3. What is the structural basis (or sequence basis) for the major difference between the HIF-2α-ARNT / NPAS4-ARNT architecture and the AhR-ARNT architecture? Is this related to the bridge motif sequence of AhR?

Thank you for this insightful question. As shown in **Fig. R4** below, we aligned the sequence of AHR with those of HIF-2α and NPAS4, and highlighted the residues in AHR, HIF-2α and NPAS4 that respectively interact with ARNT. As expected, the residues directly mediating the AHR-ARNT dimerization interfaces in the bHLH and PAS-A domains are highly conserved among AHR, HIF-2α and NPAS4 proteins. However, we found that the PAS-B domain residues of AHR, HIF-2α and NPAS4 that respectively interact with the PAS-B domain of ARNT show clear differences, especially near their C-terminal part. In addition, we further inspected the residues involved in the PAS-A/PAS-B interactions between ARNT PAS-A domain and the HIF-2α or NPAS4 PAS-B domains, and found that those HIF-2α and NPAS4 PAS-B residues exhibit a low similarity to the AHR.

The loops between the PAS-A and PAS-B domains of AHR and HIF-2α are similar in both length and sequence. While the loop between the NPAS4 PAS-A and PAS-B domains is much longer than that of AHR, also with a low sequence similarity. It is possible that the specific sequences of the PAS-B domains of AHR, HIF-2α and NPAS4 define the differences in their dimerization pattern with ARNT. Due to the limited length of this manuscript, we are planning to discuss the above structure and sequence analysis in detail in a separate review article on this topic.

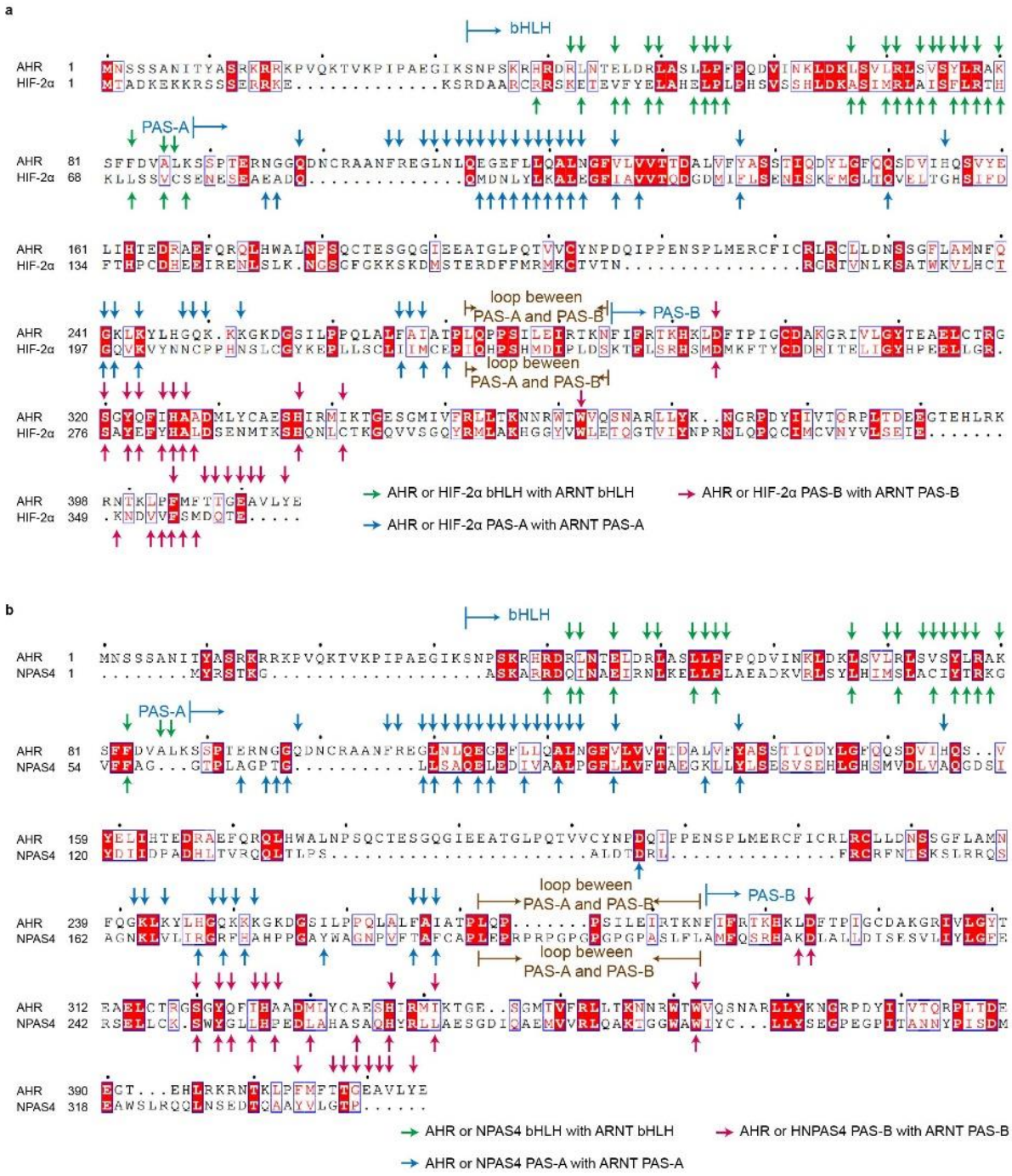

Fig. R4 Sequence alignment of AHR with HIF-2α and NPAS4

4. Is it possible that the insertion strand formed by the pAhR-PAS-B C-terminal loop is an artificial structural feature resulting from the protein truncation method? Is it speculated that this part of the sequence could also form a similar structure in the full-length AHR protein sequence, and will there be any steric hindrance?

Thank you for this critical question. In our crystal structure, the AHR PAS-B C-ter loop is observed to directly mediate the interaction between AHR and ARNT. And our cell-based experiments showed that deletion of the C-ter loop directly reduced the transcriptional activity

of AHR. These results suggest that the C-ter loop is involved in the regulation of AHR functions. Given the conserved sequences of the AHR PAS-B C-ter loop among multiple species (**Supplementary Fig. 6**), we believe that the observed interaction mediated by the C-ter loop in the structure is pointing to a true functional role, rather than being an artificial feature.

5. How does C-terminal loop-mediated PAS-B heterodimer formation behave in AlphaFold2 or AlphaFold3 predictions?

We used AlphaFold3 to predict the structure of the full-length AHR and ARNT proteins. As shown in Fig. R5, the AHR C-ter loop of PAS-B domain also mediates the interaction between AHR and ARNT, in a similar manner to that in our crystal structure. The AlphaFold3 structure does not suggest there would be any interference or impact from the rest of the polypeptide on the C-ter loop.

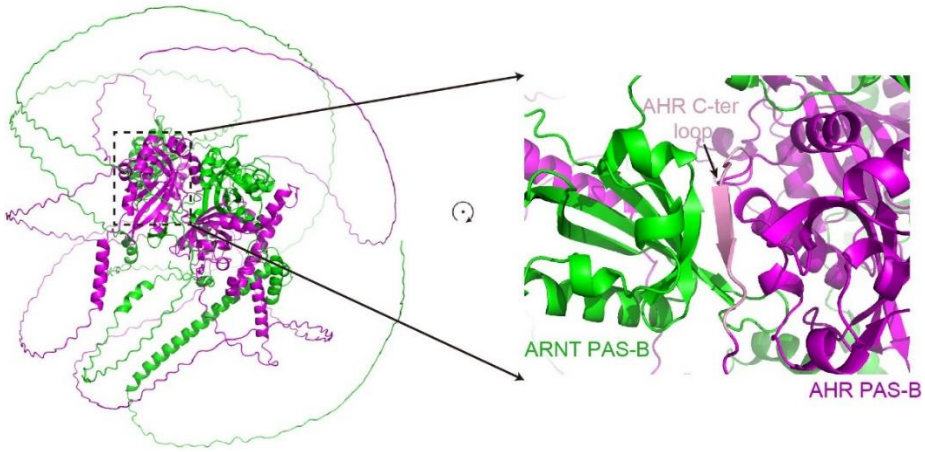

Fig. R5 The full-length AHR-ARNT heterodimer structure predicted by AlphaFold3

6. WT cells were selected for the functional experiments throughout the paper, while cells with AhR knockout or knockdown were not included. How should the interference factors of endogenous AhR be taken into account?

Thank you for this constructive question. Endogenous AHR may indeed interfere with the detection of AHR mutants' activity. Considering that there is equal amount of endogenous AHR in both the experimental and control groups, we believe the difference in activity between the mutant and the wild-type AHR is mainly caused by the mutation itself. But we followed your suggestion and obtained a fibrosarcoma cell line (HT1080) and its corresponding AHR knockout cell line (HT1080 AHR-KO) for further studies. We analyzed most of the mutants involved in the manuscript in both HT1080 and AHR-KO cell lines. The results showed that the effects of all mutants exhibited consistent trends in these two cell lines, which were also similar to the results we obtained in the HEK293 cells. We have revised the manuscript by adding supplementary figures showing the results of deletion of the AHR PAS-B C-ter loop and the H326A mutation (corresponding to H324 of pAHR) in both HT1080 and HT1080 KO-AHR cell lines (**Lines 160-165, 175, Supplementary Fig. 5e-h**).

Reviewer #3 (Remarks to the Author):

This is an outstanding paper. This is another important contribution from this team that is taking full advantage of structural analysis of the Ah receptor (AHR) bound to ligands, partners and chaperones. This reviewer (Chris Bradfield) does not have the background to challenge any of the structural analyses. Rather, I note a few issues that in my opinion will improve the paper by reflecting some of the science that may have gone underappreciated.

1) The issue of cytosolic vs nuclear forms of AHR is a complicated topic in the AHR field and in the nuclear receptor field in general. Oliver Hankinson, the discoverer of ARNT, still laments his naming of that protein as a translocator and Whitlock and Poellenz and other have battled with these definitions over the years. The best example I can give them is from our lab, where we created a DNA binding domain mutant (dbd) mouse model by inserting a spanner mutation between the last helix and the basic helix. The mutant was shown to be constitutively nuclear by our histo but was still inactive in the absence of ligand. Abnormal Liver Development and Resistance to 2,3,7,8-Tetrachlorodibenzo-p-Dioxin Toxicity in Mice Carrying a Mutation in the DNA-Binding Domain of the Aryl Hydrocarbon Receptor | Toxicological Sciences | Oxford Academic (oup.com). The bottom line is you can have a nuclear AHR without activity, nuclear localization can be segregated from activity.

Thank you for these insightful comments and discussion. In the above-mentioned work, Bunger et al. found that after inserting two residues, GS, between R39 and D40 of AHR (AHR^{dbd}), AHR retained its binding ability to ligands and HSP90, but lost its ability to recognize DRE for transcriptional regulation. Interestingly, this AHR^{dbd} mutant was mainly expressed in the nucleus regardless of the presence of ligand. Accordingly, we have cited this paper and modified the description of AHR nuclear translocation (**Lines 56-59**).

2) The above point may be an important consideration as this team should have the structural insights to explain why this dbd mutant became nuclear. We had postulated we were extruding the NLS away from the protection of Hsp90. Interestingly our NLS mutation was constitutively nuclear and completely devoid of any signaling PIIS002192581954861X.pdf (jbc.org)

We agree that the constant nuclear localization of AHR^{dbd} might result from the exposure of AHR nuclear localization signal (NLS) due to the addition of two residues nearby. While in another *jbc* paper, Bunger *et al.* found that after mutating R37, H38 and R39 (related to both the AHR nuclear localization signal and the DRE recognition) to A37, G38, and S39 (AHR^{NLS}), AHR still preserved the ability to bind to ligands and the chaperone protein HSP90, but lost its ability to recognize DRE. Furthermore, unlike the AHR^{dbd} mutant, the AHR^{NLS} mutant was mainly localized in the cytoplasm regardless of the presence or absence of ligands. This may be because mutations in NLS result in the AHR not being recognized by transporter proteins, and thus not being able to translocate into the nucleus. The structural basis of AHR localization in cells would be better known by solving the complex structure of AHR and transporter proteins. We have also citied this *jbc* paper and modified the description of AHR nuclear translocation (**Lines 56-59).**

3) Again, I will ask the team to read one of our old papers that may also shed insight on their data. Despite the common citation of the Fukunaga paper as the classic demonstration of the domain structure of the AHR, our lab published more complete data on this topic about two years before that manuscript (https://www.pnas.org/doi/pdf/10.1073/pnas.90.18.8566). The reason this paper may be of more value as a citation, despite its provenance, is that we describe the existence of a repressor domain at the exact region of the C terminal loop described in this manuscript. Moreover, in a companion paper, we also functionally map the location of an Hsp90 binding site to this region. Mapping the 90 kDa heat shock protein binding region of the Ah receptor - Perdew - 1996 - IUBMB Life - Wiley Online Library.

Many thanks for providing these citations. In the 1993 *PNAS* paper, Dolwick *et al*. expressed a series of AHR C-terminal and N-terminal truncated proteins, and then analyzed the key regions of AHR-ligand binding, ARNT interaction and DRE recognition at the biochemical level. Indeed the "repressor" domain they identified to mediate agonist-induced "depression" upon DNA binding covers the C-ter loop of AHR PAS-B domain we describe in the current work. This interesting coincidence has been mentioned in the discussion section (**Lines 370-372**).

In the 1996 paper, Perdew *et al.* further found that the bHLH and PAS-B regions of AHR played a key role in its interaction with HSP90. These findings provide key information for the complex structures of AHR and its interacting proteins. We have cited this reference and discussed this in our revised manuscript (**Lines 56-57**).

4) Finally, the structures presented are fantastic. Two issues should be considered in the future or for this ms. One is that value may be gained by reporting failed structures. For example, we have long argued that Kyn is not a ligand, rather a proligand (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5808761/). This goes ignored, but it seems unlikely to me that an acid binds to the AHR pocket. Second, we will ultimately need the structure of a dioxin or dioxin like compounds bound to AHR. One of the primary issues in AHR biology is whether dioxin creates a unique conformation in the AHR leading to a distinct response. While highly unlikely, agencies like EPA may require such a model to support certain risk assessment assumptions.

Thank you for the valuable suggestions. In our work, we put significant efforts into trying to obtain co-crystals for many other AHR ligands, including Kyn and TCDD. The major obstacle preventing us from obtaining co-crystals for most of these ligands was either their relatively weak binding capacity (such as Kyn), or their poor solubility (such as TCDD). Because the ligand-binding pocket of AHR is highly hydrophobic, the direct binding of more polar compounds would be diminished. It is indeed possible that certain compounds, such as Kyn, are just pro-ligands. We will continue to work in the future to understand the stereochemical basis for the binding of other AHR ligands. In the meantime, we have added related discussion on AHR pro-ligands in the manuscript **(Lines 355-359).**

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I thank the authors for their detailed responses and revisions, which have significantly improved the manuscript. However, I still have minor concerns regarding a few key points that require further attention:

1. Residues within the PAS-B Cavity: I appreciate the authors' effort to highlight the similarities between porcine and human AhR, as requested in the previous review. However, as a final point on this topic, I recommend the authors specifically highlight any sequence differences (mutations) in residues located within the PAS-B cavity. This additional analysis would help clarify whether any structural or functional differences in ligand binding could be attributed to sequence variations in this critical region.

Thank you for this insightful suggestion on a better clarification of protein sequence similarity. Accordingly, we analyzed the AHR residues directly involved in the cavity formation within the PAS-B domain using the CASTp algorithm (Tian et al., *Nucleic Acids Res*. 2018). There are 26 residues outlining the AHR PAS-B cavity, as marked in Supplementary Fig. 1b and shown in new Supplementary Fig. 1h. Among them, only two residues are different (Y334 and A379 of pAHR versus S336 and V381 of hAHR). Corresponding modification has been made in the manuscript (Lines 196-199).

2. Interpretation of the Y330A Mutation Results: The authors' discussion of the Y330A mutation and its impact on ligand binding and transcriptional activity remains somewhat unclear and could be misleading. The manuscript states that ligands bind in two distinct modes:
- Mode 1 (Tapinarof, FICZ, BaP, BNF, and Indigo) stabilizes Y330 in a conformation that allows interactions with residues in the C-terminal loop (L398 and L400).
- Mode 2 (Indirubin) alters the orientation of Y330, disrupting its interaction with L398 and L400.
When Y330 is mutated to alanine (Y330A), the results indicate reduced AhR activity across all ligands tested, regardless of their binding mode, and even in the basal state (DMSO). The manuscript concludes that Y330 is generally important for AhR transcriptional activity.
However, this conclusion does not address the discrepancy between the expected differential impact of the mutation on Mode 1 versus Mode 2 ligands. Specifically, since Y330 does not interact with L398/L400 in Mode 2, one might expect the Y330A mutation to have less impact on Indirubin than on Mode 1 ligands. The authors should explicitly discuss this inconsistency.

According to the reviewer's constructive comments, we further investigated the influence of this critical Y330 residue by creating more point mutations and measuring the XRE reporter activities in the AHR KO HT1080 cell line (excluding background AHR). As shown in the new Supplementary Fig. 7o, three mutations (Y to A/R/E) decreased the transcriptional activities, while the other two (Y to F/L) did not. Interestingly, the Y330R mutation showed less impact on Indirubin (Mode 2) than on Tapinarof and FICZ (Mode 1). These results together highlight the complex role that Y330 plays in mediating ligand binding and activation. Corresponding

modification has been made in the manuscript (Lines 242-250).

3. XAP2 Displacement Mechanism: In Supplementary Figure 9e, the bands representing AhR interactions with ARNT and XAP2 appear to be very weak. Could the authors clarify their interpretation of these results? Specifically, it seems that in the absence of Tapinarof, the weakening of the AhR interaction with XAP2 is less evident, while the interaction with ARNT is less pronounced. While this observation aligns with the proposed displacement mechanism, the faintness of the bands makes it challenging to discern meaningful differences in binding under the tested conditions. How do the authors explain the weak bands observed in Supplementary Figure 9e? Could this be due to experimental limitations? A discussion of these points would be valuable to better contextualize these results that are remarkable to understand the effective role of the ligand in the XAP2 displacement mechanism.

Many thanks for raising these questions. Accordingly, we have re-performed the experiments and adjusted the sample loading amount to make the bands better visualized. As shown in the new Supplementary Figure 9e, the bands of ARNT and XAP2 detected by WB are clearer. As expected, Tapinarof treatment could enhance the interaction between AHR and ARNT, while reduce that between AHR and XAP2. Overexpression of ARNT could interrupt the interaction between AHR and XAP2 in the presence or absence of Tapinarof. It is possible that the interaction between AHR and XAP2 is weaker than that between AHR and ARNT under physiological conditions. Therefore, the amount of XAP2 precipitated by AHR might be much less than that of ARNT, causing a low WB detection level of XAP2 in our experiments. Corresponding modification has been made in the manuscript (Lines 318-323).

4. Handling of PAS-A Loops in MD Simulations: The authors did not provide sufficient details about how the PAS-A loops, unresolved in the experimental structure, were handled in their molecular dynamics (MD) simulations. I understand that they focus the analysis on the PAS-B domains, but the whole system was simulated, and authors should provide details regarding how the unresolved PAS-A loops were modeled. Was any specific software or approach (e.g., loop prediction algorithms) used? The authors should provide these details in the methods section, ensuring transparency in the preparation of the simulation system. This clarification would improve the reproducibility of the study and address questions regarding the reliability of the starting structure for MD simulations.

We appreciate the reviewer's strictness and apologize for not describing this point clearly in our first round of revision. The two PAS-B domains of AHR-ARNT heterodimer are relatively independent from the bHLH and PAS-A domains. Therefore, for MD simulation we only used the two PAS-B domains when calculating the binding energy between them, as well as the energy between ligands and the interacting residues within the AHR PAS-B domain. We did not include the bHLH and PAS-A domains in the MD simulation. In this current version of manuscript, we have added the information of domains/residues used in MD simulation to the Materials and Methods section (Lines 513-515), and provided the related details in the supplementary MD checklist.