# Response to Reviewer 2 Comments

**1. Summary**

Thank you for your extremely valuable and professional comments and suggestions. Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied comments carefully and have made correction which we hope meet with approval.

| **2. Questions for General Evaluation** | **Reviewer's Evaluation** | **Response and Revisions** |
|---|---|---|
| Does the introduction provide sufficient background and include all relevant references? | Yes | |
| Are all the cited references relevant to the research? | Can be improved | |
| Is the research design appropriate? | Can be improved | |
| Are the methods adequately described? | Yes | |
| Are the results clearly presented? | Must be improved | |
| Are the conclusions supported by the results? | Must be improved | |

**3. Point-by-point response to Comments and Suggestions for Authors**

**Major points:**

**Comments 1:** In this study, the authors generated a pygo-F773W mutant using CRISPR/Cas9 technology. To enhance the reader's understanding, please provide the mutation site with domain information of pygo. Describe the reason why the authors generated and analyzed the pygo-F773W mutant. If this mutation information is from humans, please provide the mutation site with alignment results of pygo between Drosophila and humans.

**Response 1**: We are grateful for the suggestion. To be more clearly and in accordance with the reviewer suggestions, we have provided the mutation site with domain information of Pygo-F773W in Figure 1A.

And the selection of this mutation is based on previous research reports：

Compared with mammals, the Drosophila Pygo gene is due to the unconservation of the amino acid at p.773 position in the PHD domain (F773W), which reduces their affinity for histones. Meanwhile, there is some controversy about the functional study of Pygo's histone binding ability. Because of the convenience of gene editing with the arrival of CRISPR/Cas9 technology, we chose to conduct point mutation research in Drosophila. We described the Pygo-F773W mutant in paragraph 3 of the Introduction (line 61-78), and we have made moderate modifications.

**Comments 2:** In the Discussion section, the authors described that the phenotypes are similar between the pygo knockdown and the pygo-F773W mutation. Have the authors

determined if the expression level of pygo was not altered in the pygo-F773W mutant compared with the control?

**Response 2:** The transcriptome results show that the expression level of Pygo in the mutant group is lower than that in the control group (Log2FC=-0.614). But the *Pygo* +/null heterozygous - presumably expressing 50% of wild-type level of Pygo - has been reported with no phenotype. Our previous study of the cardiac phenotype caused by Pygo knockdown was through cardiac-specific Pygo knockdown (greater than 80% reduction compared to control). In addition, whole-body knockdown of Drosophila Pygo leads to developmental delays and hindered eclosion, both of which fail to develop into adults. A subsequent transcriptome analysis was performed on surviving larvae with Pygo whole-body knockdown, revealing a significant impact on the Wnt signaling pathway (unpublished). Therefore, we believe that the phenotypes caused by Pygo-F773W are mainly related to this amino acid change.

**Comments 3:** Is the expression pattern of pygo different across tissues or developmental stages?

**Response 3:** In situ hybridization experiments show that Pygo is ubiquitously expressed during the embryonic stage [3]. We have not yet conducted specific studies on the spatiotemporal expression of Pygo. However, in our laboratory's previous research on the role of Pygo in the heart, we found that Pygo is expressed in the nuclei of adult fruit fly cardiomyocytes, but not in pericardial cells. In addition, Data analysis of GSE221154 of the GEO database showed that the expression of Pygo was slightly higher in the head than in muscles, but there was no significant change in Pygo expression during the development of adult flies (3d, 15d, 30d, 45d).

**Comments 4:** How about the locomotor ability in the larval stage of the pygo-F773W mutant flies? Furthermore, is the significantly shortened post-eclosion lifespan associated with lower locomotor ability (flies may be easily stuck to the food) and/or expression changes of other genes due to the pygo-F773W mutation?

**Response 4:** Thank you for your careful review. During the experiment, it was observed that the time of pygo-F773W mutant fruit flies from embryo to adult was 1-2 days longer than that of the control group. Selecting appropriate fruit fly larvae for locomotor ability experiments proved to be challenging due to varying crawling abilities based on larval size. To ensure consistency, 3-day-old adult fruit flies were chosen for the movement experiments.

Indeed, we found that more mutant fruit flies would stick to the culture medium, possibly due to the low motility of the flies. Therefore, when doing lifespan analysis, we make effort to use relatively dry culture medium tubes. Additionally, after transferring the fruit flies to new culture tubes, we place the tubes horizontally for a period of time and change the culture tubes every three days. We have made every effort to reduce the lethality caused by the fruit flies getting stuck to the food.

**Comments 5:** The authors presented the results of KEGG pathway analysis separately for up-regulated and down-regulated genes. What about the GO enrichment analysis results for both up and down-regulated genes?

**Response 5:** Thank you for your careful review. Since the number of differentially expressed genes screened by transcriptome results was small, up-regulated and down-regulated genes were not distinguished in GO and KEGG analyses. Due to the large number of up-regulated genes, KEGG pathway analysis may enrich a certain pathway with all up-regulated genes, which are represented by solid circles in the figure. The solid triangles indicate that there are up-regulated and down-regulated genes in the pathway.

**Comments 6:** In this study, some results (e.g., Wnt signaling and H3K9ac) are different from the expected results, as mentioned by the authors. Have the authors checked the possibility of other mutations induced by CRISPR/Cas9 technology?

**Response 6:** The possibility of off-target is indeed an unavoidable issue with all gene editing technologies, including CRISPR/Cas9. We can only minimize this possibility. The homozygous mutant fruit flies we obtained have all undergone at least three generations of cultivation. Regarding the reason why the H3K9 acetylation results are inconsistent with predictions, we are conducting further experimental analysis to investigate the reasons.

**Comments 7:** It seems that there are conflicting results. The authors described it like this: "Overall, it was observed that a greater number of genes exhibited upregulation compared to those showing downregulation following the Pygo-F773W mutation, indicating a prevalent trend towards enhanced transcriptional activity." However, the H3K9ac levels were reduced in the embryo stage of pygo-F773W mutants or not changed in the adult stage of pygo-F773W mutants. How can we understand these results? Have the authors checked other histone markers?

**Response 7:** Thank you for your careful review. This description is based on the initial predicted results, but subsequent experimental results do not support it. We also attempted to detect H3K14ac levels, but the antibody specificity in fruit flies was poor, resulting in ineffective experimental data. We will further investigate the effects of this mutation on post-translational modifications through omics analysis. Thank you very much for your thorough review; we have deleted this description from the text.

**Comments 8:** In this study, the authors generated the Pygo-F773W mutants and analyzed their phenotypes. Then, the authors performed transcriptome analysis and western blotting to identify gene expression changes. However, it seems that the molecular results are not sufficient to support the phenotypes induced by the Pygo-F773W mutation.

**Response 8:** The mechanistic study in this paper is indeed not in-depth enough, and the research on Pygo-F773W mutants has primarily provided direction for our subsequent experiments. Further investigation is needed. Currently, we have conducted studies on Pygo and post-translational modifications in the fruit fly heart, and the data are still being organized.

**Minor points:**

**Comments 1:** Page 11 line 300-301: "Because it has been previously reported that the organs where Pygo acts in a Wnt 300 independent manner mainly include eye, teeth, testis, brain, intestine and salivary gland." – please add the proper references to this sentence.

**Response 1:** Thank you very much for your reminder. We have added the corresponding references to that sentence in the text.

**Comments 2:** The labels on the figures are small, making it difficult to read them. Please increase the size of the labels on the figures.

**Response 2:** Thank you for your careful review. We are very sorry for the oversight in this manuscript and inconvenience they caused in your reading. We have already made adjustments or enlargements to all Figures.