Response to Reviewer 1 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?	Yes	
Is the research design appropriate?	Can be improved	
Are the methods adequately described?	Yes	
Are the results clearly presented?	Yes	
Are the conclusions supported by the results?	Must be improved	

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

Comments: The manuscript "Pygo-F773W Mutation Reveals Novel Functions Beyond Wnt 2 Signaling in Drosophila" raises a relevant question concerning the possible roles of Pygo independent of its participation in the Wingless signaling pathway. To analyze this aspect of Pygo function, the authors generate the mutation F773W in the pygo locus, making a protein that resemble in this position the human Pygo orthologs. This is supposed to increase the binding of the mutant protein to Histone H3 di- and trimethylated in lysine 4, but this expectation does not appear to hold true. The new mutant pygo F773W turns out to be viable in homozygosis, and the authors made a very comprehensible effort to characterize the developmental effects of this mutant on organism viability, fertility, mobility and gene expression in adult flies. There is by no means a clear way to connect the array of phenotypes observed in this mutant background with the possible normal functions of pygo acting independently of wingless signaling. This would require some comparison between this allele and other pygo alleles or RNAi approach. Without this set of data, it is not possible to identify functions of pygo altered by this mutation, and the authors should discuss this. An identification of pygo requirements in adult flies may be accomplished by expressing pygo RNAi in specific tissues such as cardiac cells, neurons, muscles, germ cells. This will allow at least to identify if any of the pygo

F773W phenotypes is also observed upon a clean reduction of pygo expression in particular tissues.

In this manner, the phenotypes of pygo F773W, which clearly are not related to wingless signaling, are still a bit of a curiosity that is difficult to relate to normal functions of pygo. Still, this manuscript has value in the sense that it may represent an interesting starting point to further dissect pygo function. The authors identify that the greatest number of genes differentially expressed in pygo F773W correspond to posttranslational modification, protein turnover and chaperones. This makes me wonder to what extent the mutant cause some level of unfolded protein response, which may be the cause of the observed phenotypes.

Response: Thank you for your insightful comments and suggestions. We wholeheartedly agree with your feedback, as it provides valuable guidance for our ongoing research. Our laboratory has been dedicated to investigating the influence of Pygo on Drosophila heart function and aging. In our 2013 publication titled "Pygopus Maintains Heart Function in Aging Drosophila Independently of Canonical Wnt Signaling," we discovered that cardiac-specific Pygo knockdown operates independently of the Canonical Wnt pathway, playing a role in the regulation of Drosophila heart function and aging without a clear explanation of the specific mechanism. The advancement of CRISPR/Cas9 gene editing technology now enables us to delve deeper into the effects of point mutations in the Pygo gene.

The *Pygo* gene mutants that have been reported so far all have abnormal development of cuticles and denticles, which is a hallmark phenotype of abnormal Wnt signaling. However, we did not observe relevant abnormalities in Pygo-F773W mutant flies. We have also explored how whole-body knockdown of Drosophila Pygo leads to developmental delays and hindered eclosion. Transcriptome analysis of surviving larvae revealed a significant impact on the Wnt signaling pathway following Pygo whole-body knockdown (unpublished). And the cardiac phenotype of Pygo-F773W mutant flies is similar to the phenotype caused by specific knockdown of Pygo in the Drosophila heart in our laboratory, which is independent of the Canonical Wnt pathway. Therefore, we speculate that Pygo-F773W may mainly function through pathways other than Wnt signaling.

We have discussed it in paragraph 2 of the Discussion section. According to the reviewer's comments, we have added a description of the results of our Pygo whole-body knockdown Drosophila transcriptome experiment in the Discussion(line395-399). 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. We are in the process of analyzing Pygo-related genes in the Drosophila heart through various experiments. Thank you for your careful review.