RESPONSE TO THE REVIEWERS

We thank the editor for the opportunity to submit a revised version of our manuscript "**All-Trans Retinoic Acid increases DRP1 levels and promotes mitochondrial fission**". We appreciate the time and effort that the editor and the reviewers dedicated to providing suggestions for improvement. We have revised the manuscript, and as a result of the reviewers' suggestions which we addressed, the work has been greatly improved. We highlight in yellow the changes that were made to the manuscript. Please see below for a point-by-point response to the reviewers' comments and concerns.

Reviewer 2

In the manuscript entitled, "All-Trans Retinoic Acid increases DRP1 levels and promotes mitochondrial fission", Chidipi, et al., have investigated the effect of ATRA on mitochondrial dynamics in vitro and in vivo using a variety of tools including confocal microscopy, electron microscopy, and stochastic modeling. The authors conclude that treatment with ATRA results in increased expression and mitochondrial localization of the mitochondrial fission protein, Drp1. The increased Drp1 is further shown to alter mitochondrial ultrastructure which has been validated by confocal microscopy in combination with stochastic modeling. The role of ATRA in regulating one of the most important facets of cardiac cell functioning via mitochondrial remodeling is interesting. In addition, the development of stochastic mitochondrial network remodeling studies would provide an opportunity for studying mitochondrial physiology in other diseases including cancer, aging, and metabolic disorders. The authors should address the following concerns before the manuscript can be accepted for publication.

We grateful for the reviewer's encouraging comments and for the valuable suggestions. Performing the requested corrections and addressing the raised concerns have helped us present a better and stronger work.

Please see below for a point-by-point response to the comments and concerns.

Comment 1- The data for the effect of ATRA on MFN1 and MFN2 should be included.

Response: We included MFN1/2 data as supplementary figure1 and 2

Comment 2- In Figure 2A, although the induction of Drp1 upon Atra treatment is evident, it seems the levels of GAPDH are higher in ATRA-treated samples than

control samples. The authors must cross-check the densitometry-based quantification data and replace the blot with a more representative one.

Response: The blot in figure 2A has been replaced. Thank you

Comment 3- In figure 2, the authors must include the effect of ATRA on other mitochondrial fusion/fission proteins.

Response: We have added western blot data as supplementary figure 1 and 2. These data show that ATRA did not significantly alter other mitochondrial fusion/fission proteins.

Comment 4- In the legend for figure 3, the authors must correct the typographical error in the spelling for ATRA.

Response: Done. Thank you

Comment 5- Figure 3B and 3D, the details of the statistical test applied must be included in the legend. The authors must also include the number of cells that were analyzed to compute the Mander's coefficient.

Response: Done. Thank you

The modified text as follow in ***Page 9***, lines 366 to 372

Figure 3. Quantification of mitochondrial localization of DRP1 and OPA1 by confocal microscopy. (A) Mitochondria were labeled with antibodies against DRP1 and (C) OPA1 in purified cardiac mitochondrial clusters from vehicle (upper panels) and ATRA (lower panels) treated mice. Red channel: MitoTracker™ Red. (B) and (D) Mander's coefficient of colocalization between MitoTracker™ Red (M2; black bars) and DRP1 (M1; gray bars) (B), or OPA1 (M1; gray bars) (D). In ATRA treatment, there was a significant increase in DRP1 colocalization with MitoTracker™ Red (*p < 0.01, vs. vehicle; unpaired t-test; N=3 mice; n=12 images) (B), but no change in OPA1 (N=3 mice; n=11 images) (D). Mitochondria were loaded with MitoTracker™ Red (E) and labeled with secondary antibody alone (F).

Comment 6- Figures 3G, H do not make much sense and must be removed. It would be logical to include the TEM images of mitochondria treated with vehicle or ATRA (which the authors have included in figure 4).

Response: Panels 3G and H from figure 3 have been removed as requested.

Comment 7- The modeling of the mitochondrial network was performed in HL-1 cells instead of the previously used cell line model, HEK293. Is there a particular reason why the authors have switched to the cardiac cell line?

Response: Thank you for pointing this out. Our imaging of the mitochondrial network in HEK293 cells did not result in robust images that could be used for analysis as in Figure 5. Therefore, only HL-1 cells were used. We now mention this in the limitation section.

Comment 8- In figure 5, the details of the statistical test applied must be included in the legend.

Response: We now have a statistics section in methods, and we modified the legend.

The modified text as follows in methods section ***Page 7,*** line 316 to 318

2.9. Statistics
Data are presented as mean ± standard error of the mean. Unpaired 2-sample t-test or one sample t and Wilcoxon test were used for statistical analysis.

The modified figure legend on ***Page 12,*** lines 467 to 472

Figure 5. Microscopic properties of the mitochondrial network in control and ATRA treated cells HL-1 cells. Representative confocal images of the mitochondrial network in (A) control and (B) ATRA treated cells from experiments (left panels) and the network retrieved using image processing (right panels). Distributions of loop sizes (C), branch lengths (D), and cluster sizes (E) (cumulative probability) for control (red) and ATRA treated cells (blue). Mean degree (F), average cluster size (G), giant cluster (H), and the giant cluster normalized to the total network size (I). *p < 0.05; **p < 0.01, vs. control; unpaired 2-sample t-test.

Comment 9- While the authors have developed a very interesting simulation method to compute the mitochondrial fission and fusion rates, they must include if the values for different parameters derived using the simulations are significant in Tables 1 and 2.

Response: The reviewer raised a very good point.

To highlight the difference in control and ATRA treated cells, we now add the following statement on ***Page 11***:
In fact, these results indicate that the ratio between tip-to-tip fusion and fission rates is about 83% smaller in ATRA treated cells as compared to control cells.

We also point this out in the Legend of Table 1.
In the legend of Table 2, we mention:

Notice that X_1, X_2, and X_3 decreases by 25%, increases by 17%, decreases by 10% respectively in ATRA-treated cells as compared to control.

The modified text as follows in results section ***Page 11***

We found a smaller number of X3 species in ATRA treated cells, which further confirms mitochondrial fragmentation in these cells as compared to control cells. In figure 4 E, the number of mitochondria is increased by ATRA treatment (Panel E, *$p < 0.05$ vs. control). Such an increase could be attributed to the increase in fragmentation or fission (lower X3) as suggested by the simulations. Similarly, in figure 5, ATRA decreased the mitochondrial loop size (i.e. decreased number of mitochondria in a loop), branch length (number of edges in a linear branch), and mitochondrial cluster size (number of edges in a cluster, which consist of both linear branches and cyclic loops but is disconnected from the rest of the network). The simulations of Figure 6 assess the structure of the mitochondrial network (is the network fragmented or not) irrespective of the network size. That is, Figure 6 gives a measure of how fragmented the mitochondrial network which was imaged and quantified in Figure 5 is and what are the microscopic fusion and fission rates that can explain such fragmentation. The simulations indicate that the ratio between tip-to-tip fusion and fission rates is about 83% smaller in ATRA treated cells as compared to control cells.

Tabe1 Legend ***page 13*** lines 480 to 484

Table 1. Comparison of microscopic parameters of mitochondrial network obtained from experiments (Exp) and simulations (Theory). Mean degree (columns 2 and 3) and Ng/N (columns 4 and 5) from experiment and theory. Columns 6 and 7 list the values of C1 (tip-to-tip fusion/fission ratio) and C2 (tip-to-side fusion/fission ratio) obtained by fitting the model to the data. Notice that C1 decreases by 83% in ATRA-treated cells as compared to control.

Table 2 Legend ***page 13*** lines 485 to 487

Table 2. The fractions of X1, X2, and X3 species at steady state using C1 and C2 values estimated for control and ATRA treated cells. Notice that X1, X2, and X3 decreases by

25%, increases by 17%, decreases by 10% respectively in ATRA-treated cells as compared to control

Comment 10- The effect of ATRA on mitochondrial dynamics is a very interesting facet to regulate cellular metabolism and physiology. Can the authors comment on a plausible mechanism for ATRA-induced Drp1 expression resulting in mitochondrial fission?

Response: The reviewer raised an excellent point. There is no known pathway linking ATRA to increased DRP1 levels. It could be possible that ATRA induces epigenetic modifications through retinoic acid receptor dependent mechanisms which ultimately modulate DRP1 expression [102].

We included this in the discussion section on ***page 14***, lines 542 to 544 where we state: To our knowledge, there is no known pathway directly linking ATRA to increased DRP1 levels. It could be possible that ATRA induces epigenetic modifications through retinoic acid receptor dependent mechanisms which ultimately modulate DRP1 expression [102].

Comment 11- Can the authors also comment on the effect of heightened mitochondrial fission on the survival of cells upon ATRA treatment as cellular metabolism is also dependent on mitochondria? Also, did the authors observe an increase in autophagy, specifically, mitophagy, upon ATRA treatment?

Response: We did not directly investigate autophagy, and mitophagy in this current work. However, several studies have shown that loss of DRP1 enlarges the heart size by possibly decreasing autophagy and mitophagy [99-101].

This statement is now included on ***page 14,*** lines 533 to 541 of the discussion. We state:

Ashrafian et al. found that alteration in the DRP1 gene leads to enlarge ventricular thickness, contractility, and increased fibrosis in transgenic mice [96]. Tang et al. found a lower level of DRP1 in chronically hypertrophied, hypertensive rat hearts [97], but the pro-hypertrophic neurohormone norepinephrine increases DRP1 levels in cardiomyocyte [98]. Additionally, it was shown that DRP1 downregulation resulted in mitochondrial elongation, impaired mitochondria aggregation, and accelerated apoptosis in cardio-myocytes [93]. Several studies have shown that loss of DRP1 enlarges the heart size by possibly decreasing autophagy and mitophagy [99-101].