



# Non-coding RNA and the Mitochondrion – Function and Pathology in the Heart

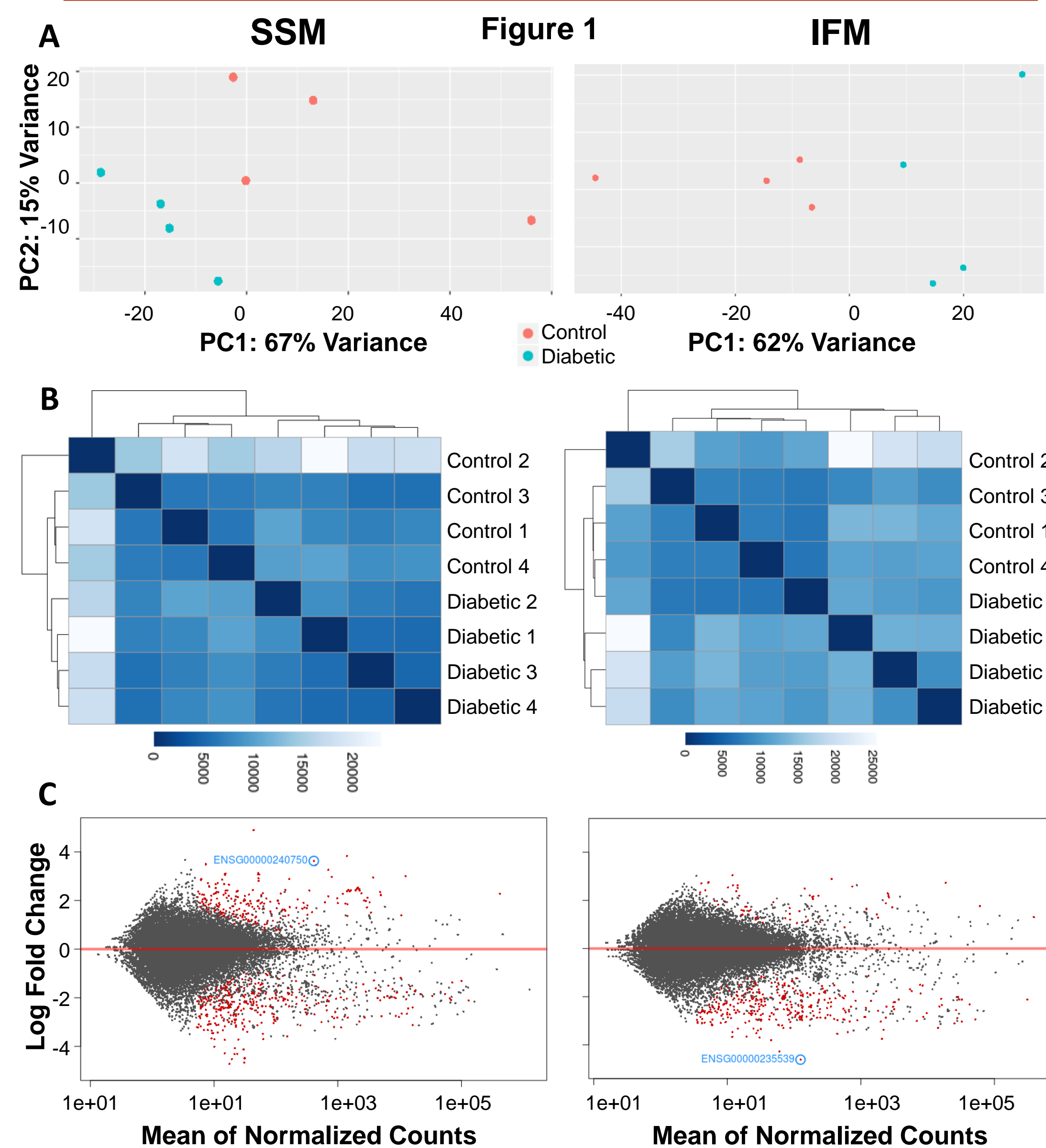
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## Abstract

Mitochondria regulate bioenergetics, apoptosis, cell cycle, calcium handling, and other pathways. In the heart, where energy demands are much greater, preservation of mitochondrial function is vital. Diabetes mellitus significantly impairs cardiac function, with changes in mitochondrial metabolism and integrity. Importantly, the mitochondria contains non-coding RNAs which could act in regulating both mitochondrial and cellular health, but it is currently unknown how regulation occurs.

We wanted to investigate the role of non-coding RNAs in the mitochondria, examining their mechanisms of import/export, binding capacity, and mitochondrial relevance. Understanding the non-coding RNA regulation of mitochondrial and cellular function during diabetes mellitus can profoundly impact our understanding of the pathology. Diabetic and non-diabetic human right atrial tissue and mouse whole heart were used for analysis. Subpopulations of mitochondria (interfibrillar, IFM and subsarcolemmal, SSM) were separated through differential centrifugation. RNA from mitochondrial subpopulations were sequenced through the Illumina HiSeq in two technical replicates. Crosslinking Immunoprecipitation of mitochondrial subpopulations for relevant RNA-binding proteins, Polynucleotide Phosphorylase (PNPase) and Argonaut 2 (AGO2), and the RNA-RNA Interactome were sequenced through the Illumina MiSeq.

In diabetes, the SSM have significantly decreased expression of mitochondrial transcribed mRNA. Additionally, the actions of PNPase (in regulating long-non coding RNAs) and AGO2 (in regulating small non-coding RNA) provides an interesting dynamic for non-coding RNA import. The RNA-RNA interactome of the mitochondria reveals lncRNA-miRNA sponging, suggesting a novel mechanism of non-coding regulation in the mitochondria. lncRNA and miRNA regulation in the mitochondria perpetuates diabetic cardiomyopathy, altering sponging capacity of miRNAs that target the mitochondrial genome.



**Figure 1:** Sample metrics for SSM and IFM of human right atrial mitochondria, differences expressed between diabetic and non-diabetic. (A) Principle Component Analysis (PCA) for sample distribution, revealing both intra- and inter sample variance. (B) Raw count matrices for sample-to-sample distribution implementing the PoiClu package in R. (C) The MA-plot reveals the differentially expressed genes (red,  $P_{adj} \leq 0.05$ ) in comparison to genes with non-significant change between groups (grey). The most differentially expressed gene is circled in blue.

## Introduction

Diabetes mellitus affects 29.1 million Americans and 25.9% of Americans age 65 and older. Adults with diabetes mellitus have two to four times the rate of mortality from heart disease as compared to those without diabetes mellitus, and 68 percent of diabetic patients age 65 and older die of heart disease (Aavik et al. 2015, Alipoor et al. 2017). While transcription factor regulation of gene expression, post-translational modification, and substrate inhibition via negative feedback have shown to be foundational in regulating protein expression and activity, microRNA (miRNA) control of gene expression through inhibition of transcription has become recognized as a significant contributor to homeostasis (Asrih and Steffens 2013).

MiRNA are single-stranded noncoding RNA molecules, ~22 nucleotides long, that associate into a multi-protein RNA-induced silencing complex (RISC) which inhibits its target mRNA species from being translated into a functional protein (Bartel 2009). Differential expression of miRNAs have been characterized in a variety of cardiovascular conditions including atherosclerosis, myocardial infarction, and heart failure to modulate processes such as energy substrate metabolism, fibrosis, and cardiac remodeling (Pinti et al. 2017, Small and Olsen 2011, Zhang and Schulze 2016). Nuclear- and, more recently, mitochondrial-genome-encoded proteins which function in the mitochondria, have been shown to be regulated by miRNA (Baradan et al. 2017, Baseler et al. 2012, Das et al. 2012, Jagannathan et al. 2015). What still remains a highly relevant, and hotly debated, topic is the process involved in miRNA import into the mitochondrion.

While it has been hypothesized that some miRNAs could be transcribed through the mitochondrial genome (Sripada et al. 2012), predominately, miRNA import is favored as the mechanism for miRNA accumulation in the mitochondrion. Research has demonstrated the presence of miRNA within the mitochondrion (Bandiera et al. 2011, Dasgupta et al. 2015, Kren et al. 2009, Shinde and Bhadra 2015, Zhang et al. 2014), and even how fluctuating concentrations within the organelle contributes toward the development of pathologies (Das et al. 2014, Das et al. 2012, Duarte et al. 2015, Shepherd et al. 2017, Sripada et al. 2017). Two currently proposed mechanisms of miRNA import into the mitochondria include the direct movement across the mitochondrial membrane through a chaperone, Argonaut 2 (AGO2) (Das et al. 2012, Zhang et al. 2014), and diffusion through small RNA import machinery, Polynucleotide Phosphorylase (PNPase) (Shepherd et al. 2017). Understanding the significance of miRNA in the mitochondria during diabetes, and how miRNA mitochondrial import occurs, provides the propensity for more completely understanding the pathology.

## Methods and Materials

### Mitochondrial Subpopulations

Subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM) subpopulations were isolated as previously described following the methods of Palmer et al. (Palmer et al. 1977) with minor modifications by our laboratory (Dabkowski et al. 2010; Baseler et al. 2011, 2013; Croston et al. 2013; Thapa et al. 2015). Mitochondrial pellets were resuspended in KME buffer (100mM KCl, 50mM MOPS and 0.5mM EGTA pH 7.4) and utilized for all analyses.

### Cross-linking Immunoprecipitation (CLIP)

CLIP was performed as previously described (Jagannathan et al. 2015), with some modifications. Briefly, human right atrial appendage and animal whole heart mitochondrial subpopulations were irradiated 5 times with 400 mJ/cm<sup>2</sup> (~15 cm distance from the UV source) on ice using a CL-1000 Ultraviolet Crosslinker (UVP, Upland, CA). Anti-Ago2 and anti-PNPase were used for protein pulldown. After RNA 3'-end biotinylation labeling (Thermo Scientific, Pittsburgh, PA), samples were loaded into SDS-PAGE. The membrane above the protein of interest was excised, RNA isolated, samples pooled (n = 5 per group) and submitted for Next-Generation Sequencing.

### RNA Preparation for Next-Generation Sequencing

RNA was purified either from human right atrial appendage total isolated mitochondria or human right atrial appendage and animal whole heart protein-RNA pulldowns, using the miRNeasy Mini Kit (product no.: 217004, Qiagen, Hilden, Germany). Library preparation was performed using NEXTRflex® Small RNA Sequencing Kit v3 (Illumina, Inc., San Diego, CA) for total mitochondria and TruSeq smallRNA Library Prep (Illumina, Inc., San Diego, CA) for protein-RNA pulldowns. Quality of RNA was determined using the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA); degradation of cytosolic ribosomal RNAs (28S and 18S) are used as a measure of the total RNA Integrity Number (RIN). Each sample was run across two lanes of the Illumina Hi-Seq in Rapid Run mode for total mitochondrial miRNA and on one lane of the MiSeq for pooled CLIPed samples.

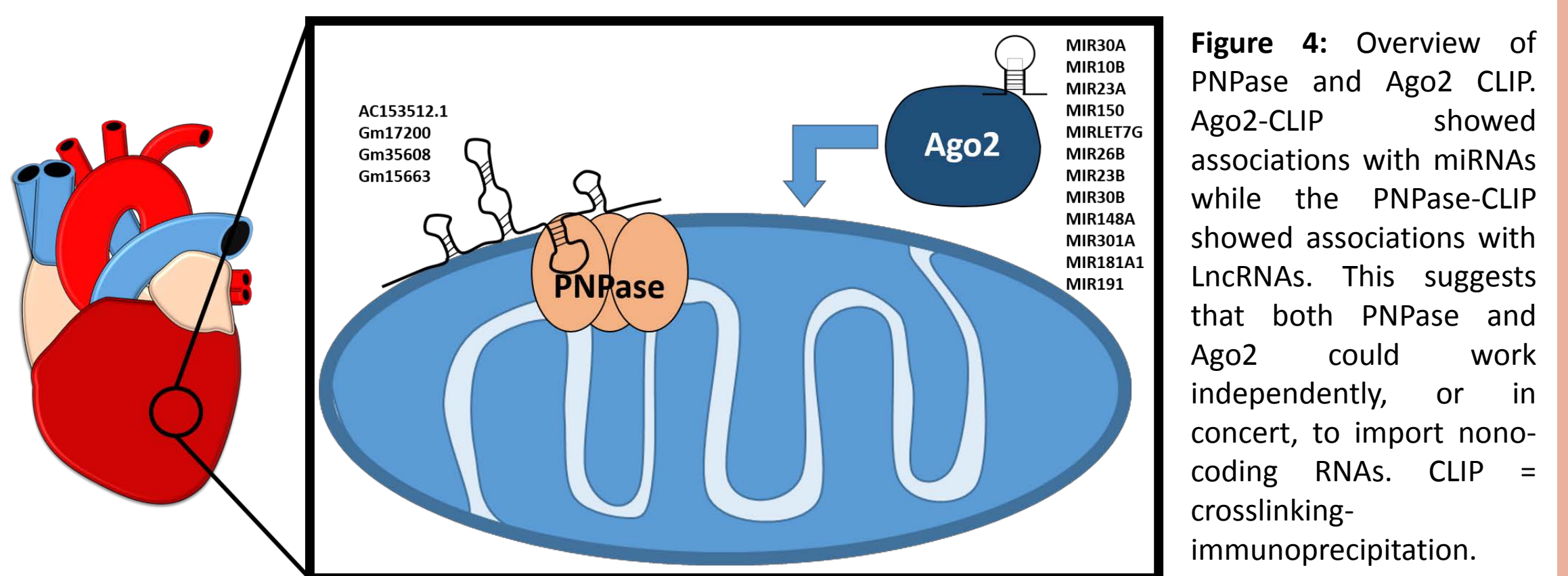
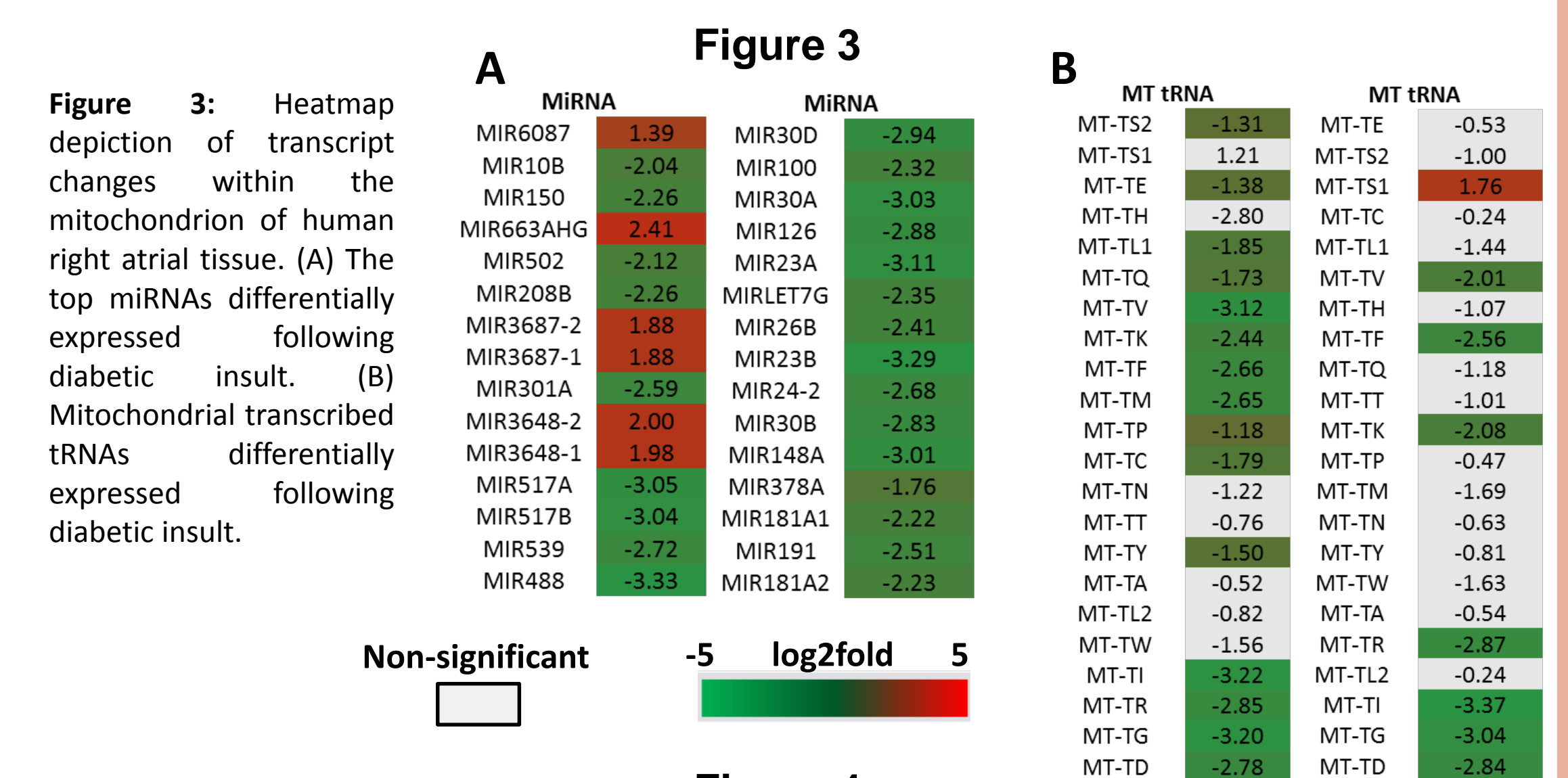
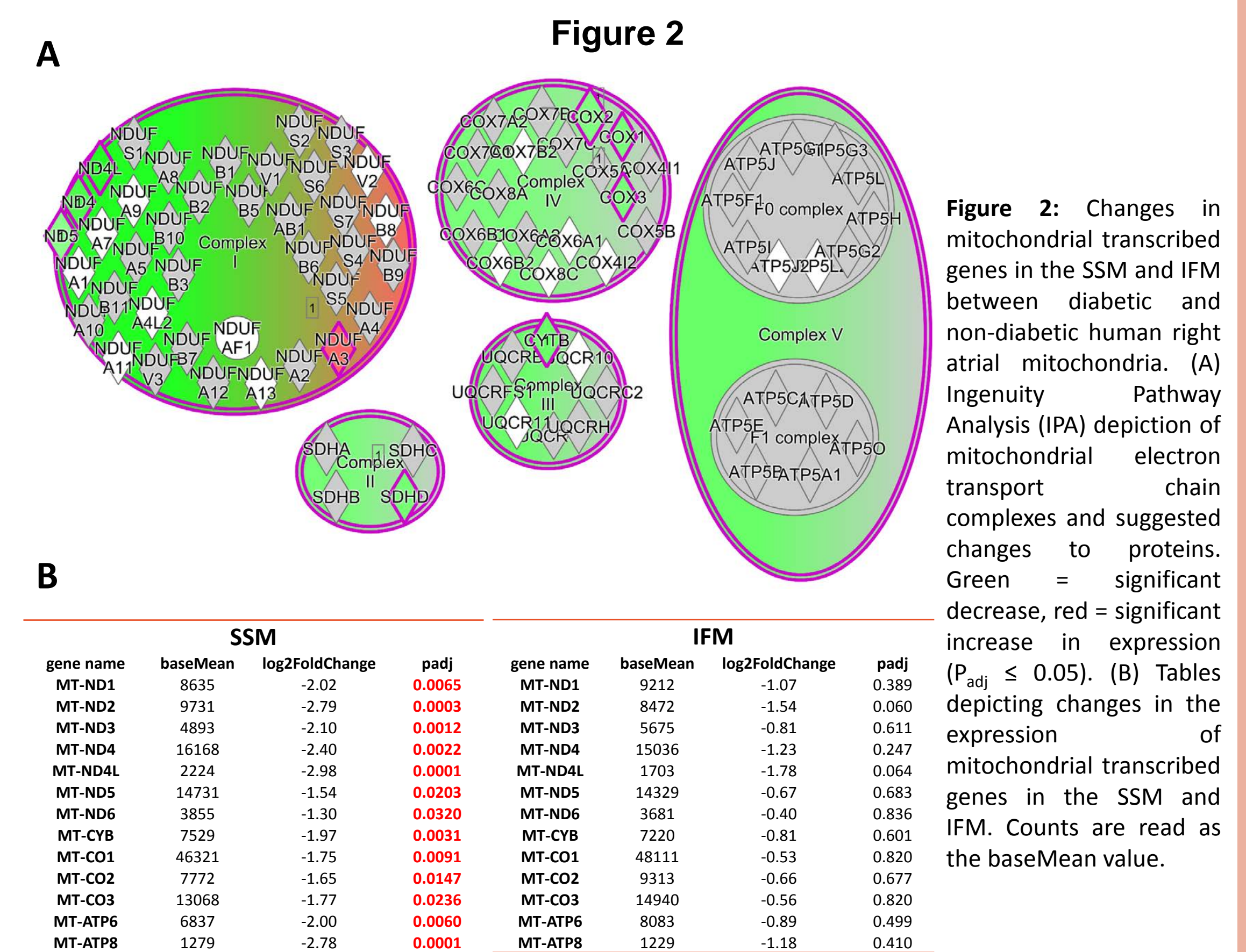
### Bioinformatics

Generated Fastq files were further processed through HISAT2 (total mitochondrial miRNA) and Bowtie (anti-AGO2 and anti-PNPase RNA). Resulting BAM files were aligned to the reference genomes for human (Homo\_sapiens.GRCh38.91.gtf.gz) or mouse (Mus\_musculus.GRCm38.91.gtf.gz) were applicable. The R environment was used to assess sample distribution and differential gene expression through DESeq2.

### Statistics

All measures of significance between the control and diabetic groups for the sequencing data are presented as adjusted P-values. Adjusted P-values are a composition of standard, unadjusted P-values and the stringency of the False Discovery Rate (FDR). Differential expression analysis through DESeq2 implements the Wald Test, using multiple testing against the null hypothesis that P-values are uniformly distributed across a data set, known as the Benjamini-Hochberg procedure. The FDR for this study was set at 0.05.

## Results



## Conclusions

The diabetic heart has been shown to suffer metabolic losses with the progress of the pathology. The mitochondrion, central to this etiology, is still poorly understood as to the regulator mechanisms and processes involved in its non-coding RNA transcriptome. We have identified major differences in mitochondrial transcribed genes and imported non-coding RNAs in the SSM and IFM. This process may be controlled centrally through mitochondrial (PNPase) and nuclear/cytosolic (Ago2) proteins. Further, understanding the transport mechanism of non-coding RNAs into the mitochondrion and how these non-coding RNAs interact with the mitochondrial transcriptome, protein regulation, and other essential processes within the mitochondrion, cell, and cardiac muscle.

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## Disclosures

The authors declare that they have no competing interests, financial or otherwise.