Supplemental Material for:

Functional changes in the neural retina occur in the absence of mitochondrial dysfunction in a rodent model of diabetic retinopathy

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Running title: DR changes occur with no mitochondrial damage

Supplemental Results

To further investigate whether differences in rarer mtDNA deletions and variants could be identified, the sequencing depth was increased for a subset of samples per experimental group. The average number of mapped reads per sample to the mtDNA was 1,320,490 in ND controls (n=5), 828,758 in D controls (n=4), and 1,151,676 in D+I (n=6) animals, with the average depth of coverage of 9,607 ± 6490, 4,598 ± 1686, and 8,408 ± 3001, respectively. Variant and deletion detection was performed again and no differences in deletion frequencies were detected between the diabetic groups relative to the ND controls (Supplemental Figure 6A). Similarly, no differences with diabetes were found in the total variant frequency (ND $7.79x10^{-5} \pm 5.34x10^{-5}$/bp, D $7.04x10^{-5} \pm 3.01x10^{-5}$/bp, D+I $2.99x10^{-5} \pm 2.22x10^{-5}$/bp, $p = 0.112$) (Supplemental Figure 6B-F). Additionally, further analysis of amino acid altering mutations and silent mutations revealed no significant differences with diabetes (Supplemental Table 8). Finally, there were no significant differences in the variant frequencies across any of the mtDNA annotations with diabetes (Supplemental Table 8).

To validate that no increase in mtDNA mutations or deletions occurs with diabetes, sequencing was performed on mtDNA amplified, using an independent set of primers (Supplemental Table 1), from total genomic DNA isolated from whole retinas of a separate set of rats collected previously (Masser *et al.* 2014). The average mapped reads per sample to the mtDNA was 753,719.6 in ND controls (n=10), 1,041,597 in D controls (n=8), and 638,038.9 in D+I (n=10) animals, with the average depth of coverage of 2,706 ± 810, 3,305 ±1,739, and 2,769 ± 1,153, respectively. There were no differences in the level of deletions between any groups (Supplemental Figure 7A). Similarly, variant analysis revealed no differences in total variant frequency per basepair (ND $4.84x10^{-5} \pm 2.44x10^{-5}$/bp, D $4.10x10^{-5} \pm 1.87x10^{-5}$/bp, D+I $6.84x10^{-5} \pm 5.35x10^{-5}$/bp, $p = 0.504$, n=8-10/group), variants where there was a predicted amino acid change, or silent variants (Supplemental Table 9 and Supplemental Figure 7B-F). Additionally, no differences were found between D or D+I animals compared to ND controls from variant analysis of each annotated mitochondrially-encoded gene (Supplemental Table 9).

Supplemental Methods

Insulin administration

A subset of diabetic rats (n=12/cohort) received insulin replacement beginning 6 weeks post-STZ injection delivered by subcutaneous implantation (26 mg human insulin pellet, 7 mm x 2 mm; LinShin Canada) via trocar under

transient isoflurane vapor anesthesia. 16 gauge needles, trocar, and insulin pellets were rinsed with 10% and 2% betadine solution prior to implantation. The implant site was swabbed with 10% betadine prior to and after implant. Rats were allowed to fully recover from anesthesia prior to returning to bedded cages. Insulin pellets provide a continual dose of 2U of insulin per 24 hours for the duration for the pellet. Endogenous rat c-peptide and exogenous human insulin levels were measured by enzyme-linked immunosorbent assays (Mercodia) (Masser et al. 2014) using plasma collected from trunk blood at the time of euthanization.

Electroretinography and optical coherence tomography

One week prior to euthanasia (12-13 weeks diabetes duration), rats were assessed for retinal function by electroretinography (ERG). The animals were weighed and dark adapted in clean cages for 12 hours overnight prior to the ERG protocol. Preparation of the rats was performed under a dim red light. Rats were anesthetized using a combination of ketamine and xylazine at dosages of 100mg/kg and 5 mg/kg, respectively. Ak-dilate and Mydriacyl drops were applied to right and left corneas to maintain dilation throughout the protocol. Gonak was also applied to each cornea to facilitate electrode attachment. A ColorDome and Espion E3 ERG machine (Diagnosys LLC) was used to stimulate and record responses. The rat was placed on a warm rodent platform. A steel needle electrode was placed under the skin at the base of the tail to serve as a ground and a curved steel needle electrode was hooked in the right cheek to serve as a reference. Looped gold electrodes were centered on both the right and left corneas to record responses. The 11 step protocol consisted of a zero stimulus for the purpose of removing the baseline trace, then a 6 step serial scotopic range of -4.87 log cd*s/m^2 to -1.4 log cd*s/m^2, and finally a 4 step photopic series ranging from 0.34 log cd*s/m^2 to 1.3 log cd*s/m^2 paired with a rod suppressing background illumination (10 cd/m^2) to isolate cone function. Oscillatory potentials (OP) were recorded for both scotopic and photopic readings using a digital filter of 75 – 300 Hz on virtual channels. Raw data from right and left eyes were isolated for each rat from scotopic and photopic steps and responses were averaged for each rat. Time to peak (TTP) for each OP were isolated and averaged per group.

Retinal thickness was measured using optical coherence tomography (OCT) on the Bioptigen SD OCT Envisu R4300 (Leica Microsystems) immediately following ERG measurements. Ak-dilate and Mydriacyl drops were applied to right and left corneas to maintain dilation. Anesthetized rats were placed on the scope platform and the light source scope aimed at each eye subsequently by positioning the animal where the optic nerve head was in view and centered

for both horizontal and vertical planes. OCT images were then analyzed manually in the Bioptigen InVivoVue Diver Software 2.4. A 5x5 grid retinal template was used for depth analysis centered on the optic nerve head for each image. Boundary markers were manually assigned as described by the manufacturer's instruction (Bioptigen). Briefly, boundary marks were placed at the following locations of the retinal cross section image; Inner-retinal nerve fiber layer, outer-retinal nerve fiber layer, outer-inner plexiform layer, outer-inner nuclear layer, outer-outer plexiform layer, outer-outer nuclear layer, inner-inner segment ellipsoid, outer-inner segment ellipsoid, end tips, and retinal pigmented epithelium. Measurements from each layer were averaged across eyes from each animal.

Synaptosome isolation

Retinal synaptosomes were isolated as previously described (VanGuilder *et al.* 2008) and with modifications. Right and left retinas were excised, combined per animal, and placed in 15 mL of ice-cold sucrose buffer (0.32 M sucrose, 4 mM HEPES, 1 mM sodium orthovanadate, pH to 7.4). Retinas were washed in ice-cold sucrose buffer three times allowing retinas to settle on ice after each wash. Retinas were homogenized by mechanical dounce homogenization in 3 mL of ice-cold sucrose buffer (approximately 10-15 strokes). The 3 mL of homogenate was then placed in a 10 mL polycarbonate ultracentrifuge tube (Beckman Coulter) with an additional 2 mL ice-cold sucrose buffer. Homogenates were centrifuged at 4 °C using an Optima L-80 XP ultracentrifuge (Beckman Coulter) and SW 32.1 Ti swinging bucket rotor at 800g (2,500 RPM) for 10 minutes to pellet nuclei and large cell fragments. Supernatant was decanted into a clean ultracentrifuge tube and placed on ice. Pellets were placed on ice while the subsequent spin was performed. Supernatant fractions were centrifuged at 4 °C and 25,000g (14,000 RPM) for 12 minutes to pellet synaptosomes. Pellets were maintained on ice for subsequent use.

Oxygen consumption rate measurement

Oxygen consumption rates (OCR) for retinal synaptosomes were measured using the Seahorse XFe96 extracellular flux analyzer and XF Cell Mito Stress Test (Agilent) as outlined previously (Choi *et al.* 2009) with modifications and according to manufacturer's instructions. One day prior to OCR measurement, Seahorse XF96 microplates were treated overnight with 0.0033% polyethyleneimine solution (Sigma Aldrich) to facilitate synaptosome attachment. Wells for OCR blank measurements were treated identically to assay wells throughout the procedure. Isolated retinal synaptosome pellets from each animal were resuspended in 300 µl ionic media (20 mM HEPES, 10 mM

D-glucose, 1.2 mM disodium phosphate, 1 mM magnesium chloride, 5 mM sodium bicarbonate, 140 mM sodium chloride, pH to 7.4), and 9 µl of the resuspended synaptosomes were aliquoted (20 µg of protein/well quantified after OCR measurements) in duplicate or triplicate wells containing 50 µl of ice-cold ionic media. Plated synaptosomes were adhered to the Seahorse microplate by full speed centrifugation at 4 °C for 1 hour. Ionic media was replaced with 175 µl incubation media (3.5 mM potassium chloride, 120 mM sodium chloride, 1.3 mM calcium chloride, 0.4 mM potassium phosphate, 1.2 mM sodium sulfate, 2 mM magnesium sulfate, 15 mM D-glucose, 4 mg/mL BSA, 10 mM pyruvate) and plates were equilibrated to 37 °C. Oligomycin, FCCP (Carbonyl cyanide-4-trifluoromethoxy phenylhydrazone), and antimycin A/rotenone drugs were dissolved and diluted in incubation media to final loading concentrations of 8 µM, 18 µM, and 10 µM respectively, for final assay well concentrations of 1 µM oligomycin, 2 µM FCCP, and 1 µM rotenone/antimycin a (R/AA). The assay run protocol was as follows; Initialization- calibration and equilibration for 12 minutes, basal rate- 5 steps of 2 minute and 30 seconds OCR measurements with 30 second wait time between measurements, oligomycin injection- 3 steps of mixing for 1 minute, 30 second wait, and 2 minute and 30 seconds measurement, FCCP injection- 3 steps of mixing for 1 minute, 30 second wait, and 2 minute and 30 seconds measurement, R/AA injection- 3 steps of mixing for 1 minute, 30 second wait, and 2 minute and 30 seconds measurement, for a total assay run time of 1 hour and 3 minutes. All steps of the protocol were performed at 37 °C.

Primary OCR data analysis was carried out in the Seahorse Wave 2.3.0 software (Agilent). OCR data from each experimental well with background correction was normalized to the immunoreactivity of COXIV/µg protein from that well, as described in the immunoblot methods section. Wells without discernable OCR measurements were omitted from analysis. Normalized OCR measurements per sample per time point were averaged across replicate wells and per group. Averaged measurements per group were carried out for basal OCR rates, OCR rates after oligomycin treatment, OCR rates after FCCP treatment, and OCR rates after R/AA treatment. Basal OCR was calculated by taking the difference of untreated OCR measurement and R/AA treated OCR measurement. ATP production OCR was calculated by the difference of untreated OCR and oligomycin treated OCR measurement. Proton leak OCR was calculated by the difference of oligomycin treated OCR and R/AA treated OCR. Maximal respiration OCR was calculated by the difference of FCCP treated OCR and R/AA treated OCR. Spare capacity OCR was calculated by the difference of FCCP treated OCR and untreated OCR. Non-mitochondrial respiration OCR was the OCR rate after R/AA treatment. A schematic for the

above explanations is provided in Figure 3B. Outliers from each measurement within a group were removed if they were greater than two sd from the group mean.

Immunoblot and mass spectrometry

Protein was isolated from Seahorse microplate wells and pellet fractions homogenized using protein lysis buffer (100 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 2 mM EDTA, 10 mM HEPES, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, and Complete Mini EDTA-free protease inhibitor cocktail) (VanGuilder et al. 2008). Soluble protein was quantified by bicinchoninic acid (BCA) assay (ThermoFisher Scientific). Immuno dot-blots were performed by reducing 10 µg soluble protein with 1X Laemmli sample buffer with 50 mM DTT at 95 °C for 5 minutes. 2 µl of reduced protein per sample was applied to a dry nitrocellulose membrane with a 96-well die as a guide to prevent spot bleeding. The membrane was dried at room temperature and blocked for 1 hour in 5% BSA 1X PBST at room temperature. Primary antibody (1:1000 in 5% BSA, COXIV, abcam, RRID: AB_301443) was incubated overnight at 4 °C. The membrane was washed three times for 15 minutes with 1X PBST. Secondary antibody (1:1000 in 5% BSA, HRP-conjugated goat anti-mouse, Rockland,RRID: AB_2610851) was incubated for 1.5 hours at room temperature, and washed three times with 1X PBST. Dot-blots were then incubated for 1 minute in ECL substrate (ThermoFisher) and wrapped in plastic wrap for X-ray film exposure and development. COXIV dot-blot intensities were determined by densitometry using ImageQuant (GE, RRID: SCR_014246) image analysis software. Immunoblots were probed using Lamin B1 (1:5000 in 5% non-fat dry milk, Lamin B1, abcam, RRID: AB_2616597), COXIV (1:1000 in 5% BSA, COXIV, abcam, RRID: AB_301443), and synaptophysin (1:500 in 5% BSA, synaptophysin, abcam, RRID: AB_2198854) antibodies, and visualized using HRP-conjugated secondary antibodies (1:1000 in 5% BSA, HRP-conjugated goat anti-mouse, Rockland, RRID: AB_2610851; (1:1000 in 5% BSA, HRP-conjugated rabbit anti-rabbit, Rockland, RRID: AB_2610848) and X-ray film exposure.

Mass spectrometry of retinal synaptosomes was carried out as previously described (Nakada *et al.* 2017) and as follows; 20 µg of total protein per sample was mixed with 1% SDS, and 8 pmol BSA added as an internal standard. The proteins were precipitated with acetone. The dried protein pellet was reconstituted in 22 µL Laemmli sample buffer and 20µL run as a short (1.5cm) SDS-PAGE gel. The gels were fixed and stained (data not shown). Each sample was cut from the gel as the entire lane and divided into smaller pieces. The gel pieces were washed to remove the Coomassie blue

then reduced, alkylated, and digested overnight with trypsin. The mixture of peptides was extracted from the gel, evaporated to dryness in a SpeedVac and reconstituted in 150µL 1% acetic acid for analysis. The analyses were carried out on a TSQ Vantage triple quadrupole mass spectrometry system (ThermoFisher). The HPLC was an Eksigent splitless nanoflow system (Eksigent) with a 10cm x 75µm i.d. C18 reversed phase capillary column. 7 µL aliquots were injected and the peptide eluted with a 60min gradient of acetonitrile in 0.1% formic acid. The mass spectrometer was operated in the selected reaction monitoring mode. For each protein, the method was developed to measure 2 ideal peptides. Data were analyzed using SkyLine to determine the integrated peak area of the appropriate chromatographic peaks. The response for each protein was calculated as the geometric mean of the two peptide area. These values were normalized to the response for the BSA standard in pmol/100 µg total protein.

Sequencing standard generation

Total genomic DNA isolated from two rat retinas with germline base differences at four known locations on the mitochondrial genome was used. A common forward primer and two reverse primers were used to amplify a short and a long mtDNA fragment (Supplemental Table 1). mtDNA amplicons were ligated into vectors (TOPO TA, Life Technologies) according to manufacturer's instructions. Cloned vectors were transformed into competent *E.coli*. (One Shot TOP10, Life Technologies). Bacteria were plated on agar in the presence of kanamycin (50 µg/mL) and incubated overnight. Visible colonies were picked and transferred to tubes with fresh SOB medium with kanamycin for overnight incubation with shaking. Plasmids were isolated using silica-spin column purification after lysis of the cell pellets according to manufacturer's instructions (Zymo Research).

mtDNA sequencing and analysis

mtDNA was amplified using 1 ng total DNA and long-range PCR (TaKaRa LR DNA Polymerase) with primers specific for the mitochondrial genome (Supplemental Table 1) with the following cold-start PCR reaction conditions; 30 cycles of 98 °C for 10 seconds, 68 °C for 15 minutes, with a final extension step of 72 °C for 10 minutes. DNA amplicons were determined to be of correct size by agarose gel electrophoresis, and quantified by fluorescent assay (PicoGreen, ThermoFisher). 500 pg of mtDNA amplicon per sample were used for sequencing library preparation using Nextera XT reagents according to manufacturer's instructions (Illumina) and as previously described (Masser *et al.* 2013). The average size of libraries was quantified by using the High Sensitivity dsDNA assay reagents and the Bioanalyzer 2100

(Agilent). Molar concentrations of libraries were determined by standard-curve qPCR according to manufacturer's instructions (KAPA Biosystems). Libraries were diluted to 4 nM and pooled for benchtop sequencing (MiSeq, Illumina) using 600 cycle (2x250bp) reagents (Illumina) at a final library concentration of 12 pM.

Paired-end sequencing reads were de-multiplexed and imported for analysis in CLC Genomics Workbench 9.5, RRID: SCR_011853, using the Illumina import workflow. Raw reads were trimmed using adapter removal and for quality with the following settings; ambiguous base limit – 1, minimum quality – 0.001 (Q30). Ten bases were trimmed from each 5' read end and 20 bases trimmed from each 3' read end. The minimum size of reads to be retained was set to 50 bases. The range of trimmed read sizes in every sample was 50-220bp. Trimmed reads were then aligned to the annotated rat mitochondrial genome (NCBI accession # NC_001665.2) using the Large Gap Read Mapping plug-in tool with the following settings; ignore non-specific matches, mismatch score of 1, insertion and deletion scores of 3, 60% read similarity, and 95% read length mapped. Consensus sequences from each animal were extracted from the initial mapping, and trimmed reads were subsequently re-mapped back to the consensus with the same settings but allowing 50% read similarity. Variants were determined from read maps using the Low Frequency Variant Detection tool with the following settings; minimum coverage of 10, minimum count of 2, minimum frequency of 0.1%, required significance of 0.1%. The resulting identified variants were used for tertiary analysis and group comparisons of variant rates (frequency/bp) in a given context of the mitochondrial genome. mtDNA deletion quantitation was determined by the ratio of mapped gapped-reads to total mapped reads relative to ND control average. Average coverage depth per sample was calculated from called variant lists.

Supplemental Tables:

Primers	Sequence (5'-3')	mtDNA location bp	Use
Chen F	CCACGGGACTCAGCAGTGATAAATAT	2233-2261	Whole Retina
Chen R	AATTTCGGTTGGGGTGACCTCGGAG	224-249	Whole Retina
Fillin F	GTGGGACGAGGACTATACTATGG	14427-14449	Whole Retina
Fillin R	TCCCGTACTAATAAACCCAATCACC	3894-3918	Whole Retina
LR set 3 F	CCCACGGGACTCAGCAGTGATAAATA	223-248	Synaptosomes
LR set 3 R	CCCTACACCTGAAACTTCAATGCCAA	16096-16121	Synaptosomes
Standard F1	CTTCCTCCATCATTTCTACTCCTT	5635-5658	Sequencing Standard
Standard R1	ACCTACTGCTGCTTCGC	10075-10091	Sequencing Standard
Standard R2	GGTGATGTGGCGTCTTGT	7032-7049	Sequencing Standard

Supplemental Table 1: mtDNA sequencing primers

	Group	**n/group**	**BG (mg/dL)**	**HbA1c (%)**	**Mass (g)**	**Rat C-peptide (pmol/L)**	**Human insulin (mU/L)**	**Endpoints**
Cohort 1	Non-diabetic	12	97.2 ± 9.5	4.7 ± 0.3	542.0 ± 77.7	2029.2 ± 1187.1	not measured	electroretinography, optical coherence tomography, oxygen consumption rate, mtDNA copy number, mtDNA sequencing
	Diabetic	12	450.1 ± 55.4	11.6 ± 1.2	336.0 ± 55.0	301.3 ± 53.3	not measured	
	Diabetic + Insulin	12	167.8 ± 68.4	8.7 ± 1.9	479.8 ± 29.5	378.1 ± 282.5	not measured	
Cohort 2	Non-diabetic	12	96.9 ± 8.7	4.9 ± 0.2	640.4 ± 46.5	2820.4 ± 1127.2	nd	oxygen consumption rate, mtDNA copy number, mtDNA sequencing, mass spectrometry
	Diabetic	12	451.6 ± 52.9	10.6 ± 1.6	321.3 ± 39.5	386.5 ± 22.4	nd	
	Diabetic + Insulin	12	109.7 ± 67.3	7.4 ± 1.0	460.2 ± 29.0	373.8 ± 51.6	83.3 ± 62.4	

Supplemental Table 2: Animal groups and characterization

Retinal Layer	**ND (mean ± sd) (mm)**	**D (mean ± sd) (mm)**	**D+I (mean ± sd) (mm)**	P
Total	0.201 ± 0.011	0.206 ± 0.010	0.201 ± 0.005	ns
Ganglion Cell Layer	0.017 ± 0.002	0.018 ± 0.003	0.016 ± 0.003	ns
Inner Plexiform Layer	0.045 ± 0.006	0.045 ± 0.003	0.046 ± 0.003	ns
Inner Nuclear Layer	0.024 ± 0.003	0.024 ± 0.002	0.025 ± 0.003	ns
Outer Plexiform Layer	0.010 ± 0.001	0.010 ± 0.010	0.001 ± 0.001	ns
Outer Nuclear Layer	0.052 ± 0.002	0.060 ± 0.003	0.054 ± 0.002	*< 0.001*
Photoreceptor Layer	0.018 ± 0.001	0.017 ± 0.001	0.018 ± 0.001	0.063
Retinal Pigmented Epithelium	0.016 ± 0.002	0.011 ± 0.001	0.011 ± 0.002	*< 0.001*

Supplemental Table 3: Retinal Morphology

	Implicit time (mean ± sd) (ms)				Amplitude (mean ± sd) (µV)			
Scotopic	**ND**	**D**	**D+I**	**P**	**ND**	**D**	**D+I**	**P**
A-wave	28.000 ± 2.000	24.833 ± 6.657	29.000 ± 1.479	0.085	44.362 ± 17.004	27.462 ± 12.989	49.543 ± 14.231	*0.007*
							ND v D	*0.007*
							ND v D+I	0.434
							D v D+I	*0.015*
B-wave	66.909 ± 10.339	76.833 ± 15.356	63.676 ± 4.431	0.165	1147 ± 206.7	1061 ± 179.2	1181 ± 197.4	0.164
Photopic	**ND**	**D**	**D+I**	**P**	**ND**	**D**	**D+I**	**P**
A-wave	11.500 ± 0.612	11.500 ± 0.612	11.833 ± 0.707	0.429	3.903 ± 1.526	4.914 ± 1.355	5.089 ± 1.592	0.213
B-wave	47.042 ± 3.106	46.292 ± 3.275	46.389 ± 46.389	0.461	270.896 ± 60.669	268.757 ± 49.344	336.137 ± 78.485	0.057

Supplemental Table 4: ERG A- and B-wave implicit time and amplitude

Supplemental Table 5: Proteomic data (Excel File)

Standard (%)	gapped	mapped	ratio	Relative to 100%	% gap reads	Site 446 A/G	Site 1472 A/G	Site 1490 C/T	Site 1529 C/T
100	8098	1262840	0.00641253	1	100	99.88	99.93	99.93	99.92
25	3788	2452152	0.001544766	0.24089797	24.09	31.87	32.83	33.8	33.17
5	432	1643789	0.000262807	0.04098342	4.10	5.5	5.58	5.9	6.06
2.5	257	1595316	0.000161097	0.02512216	2.51	3.48	3.31	3.53	3.4
1	69	1462298	4.7186E-05	0.00735841	0.74	1.06	1.24	1.24	1.17
0.5	40	1378841	2.90099E-05	0.00452394	0.45	0.8	0.82	0.81	0.87
0.25	12	694524	1.7278E-05	0.00269442	0.27	0.39	0.52	0.56	0.41
0	9	2546552	3.53419E-06	0.00055114	0.06	0/nd	0/nd	0/nd	0/nd

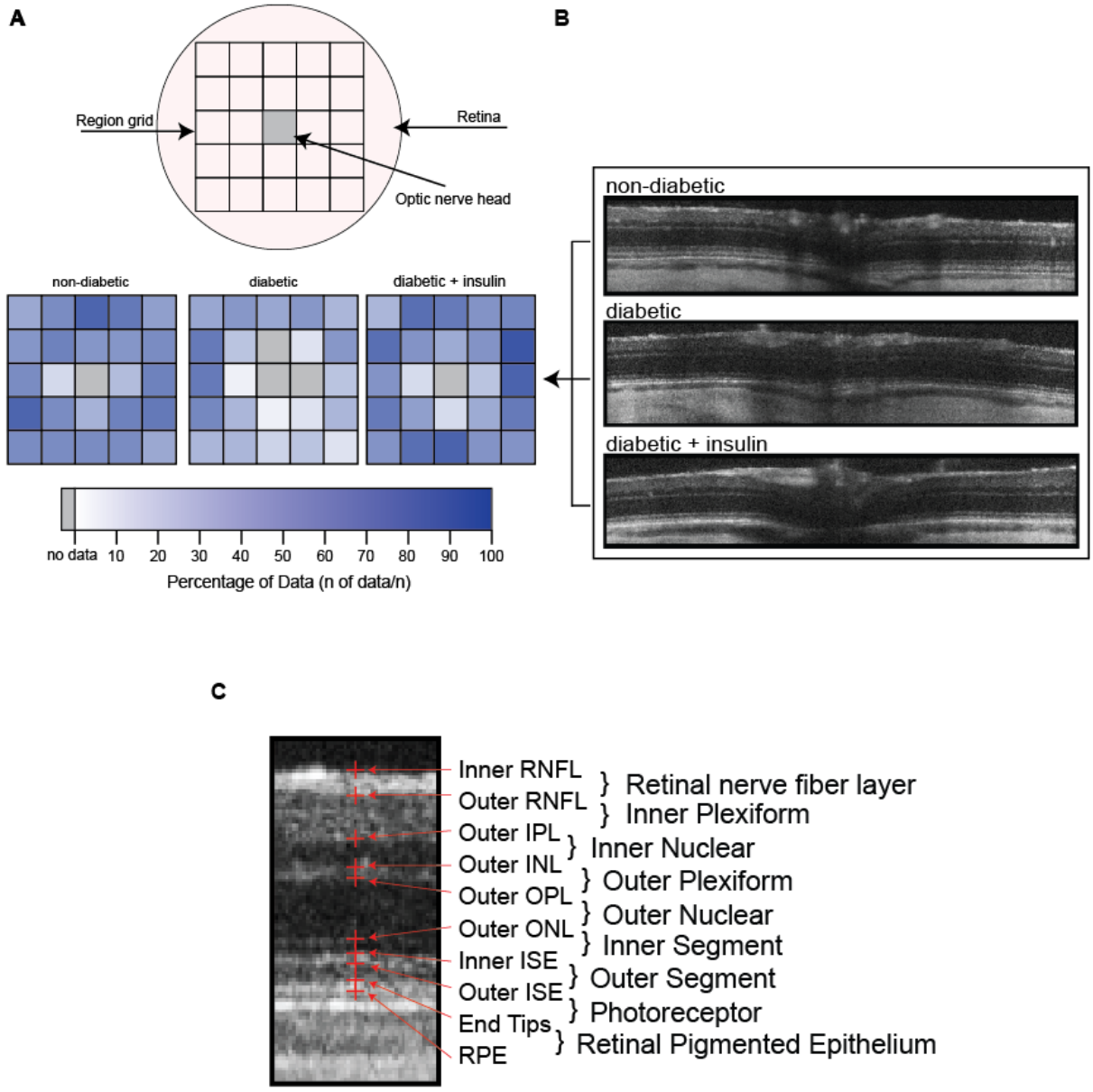
Supplemental Table 6: mtDNA sequencing standards

Supplemental Table 7: Retina Synaptosome mtDNA sequencing variant frequencies (Excel File)

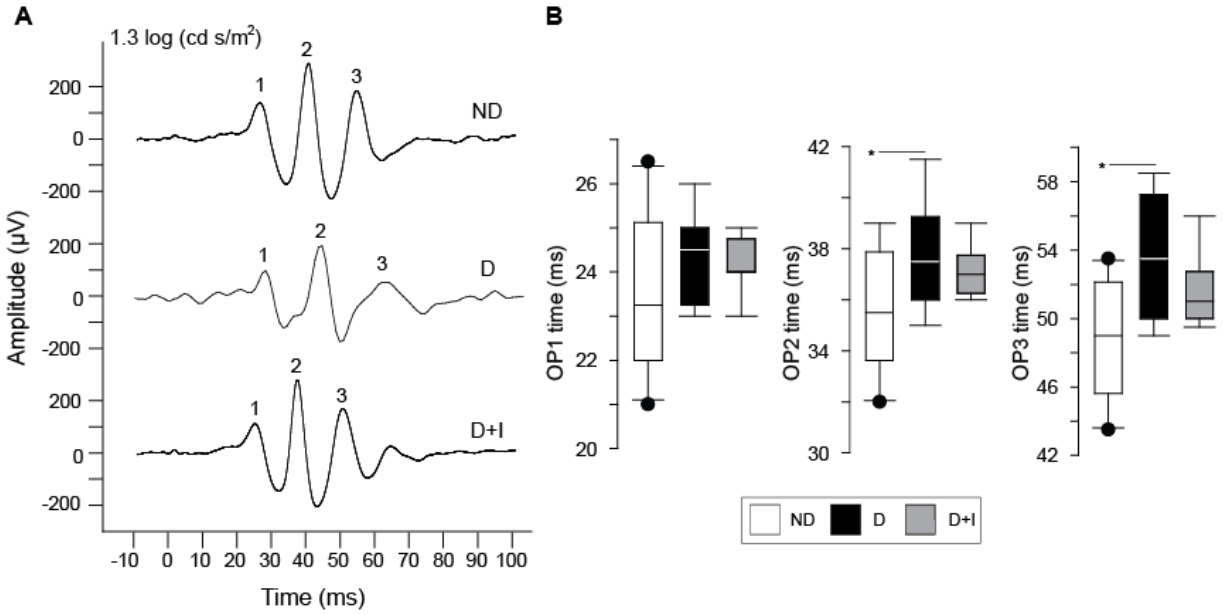
Supplemental Table 8: Re-sequencing Retina Synaptosome mtDNA sequencing variant frequencies (Excel File)

Supplemental Table 9: Whole retina mtDNA sequencing variant frequencies (Excel File)

Supplemental Figures:

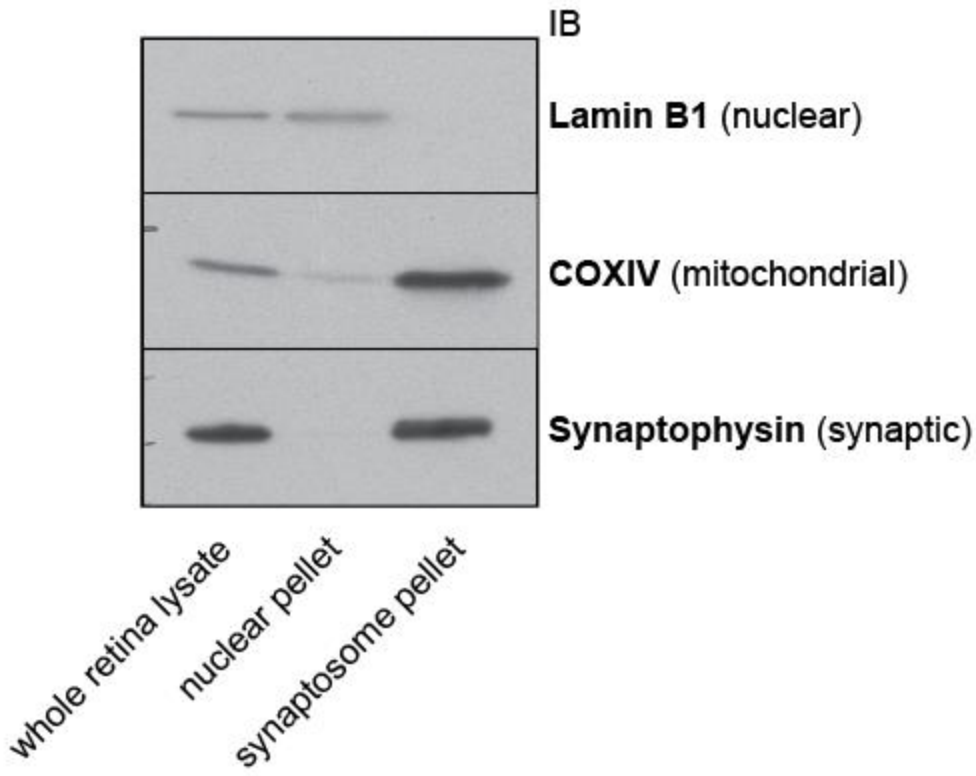


Supplemental Figure 1: Optical coherence tomography data acquisition. **A)** Percentage of data from each group from each data point on the 5x5 grid. **B)** Representative OCT from non-diabetic (top), diabetic (middle), and diabetic with insulin (bottom). Arrow leading to panel A heatmaps, demonstrate where the representative images were taken. **C)** OCT layer definitions. RNFL – retinal nerve fiber layer. IPL – inner plexiform layer. OPL – outer plexiform layer. INL – inner nuclear layer. ONL – outer nuclear layer. ISE – inner segment ellipsoid. RPE – retinal pigmented epithelium.

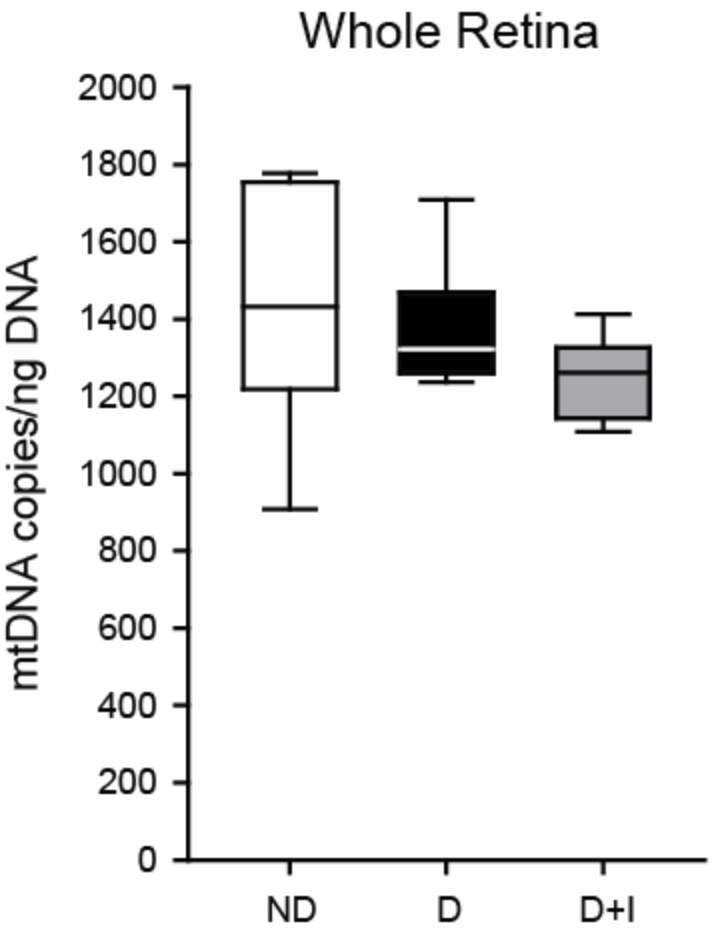


Supplemental Figure 2: Electroretinogram (ERG) photopic oscillatory potentials (OP). **A)** Representative OP traces from non-diabetic (ND – top), diabetic (D – middle), and diabetic with insulin replacement (D+I – bottom) animals showing amplitude (in µV) and time (in ms) after light-adapted light stimulus (1.3 log(cd s/m^2)) at time 0. Numbers (1-3) mark the OPs used for mean implicit time comparison between experimental groups (OP1, OP2, OP3). **B)** Box plots of photopic OP mean implicit time group comparisons for OP1, OP2, and OP3 between ND (white), D (black), and D+I (gray) animals.

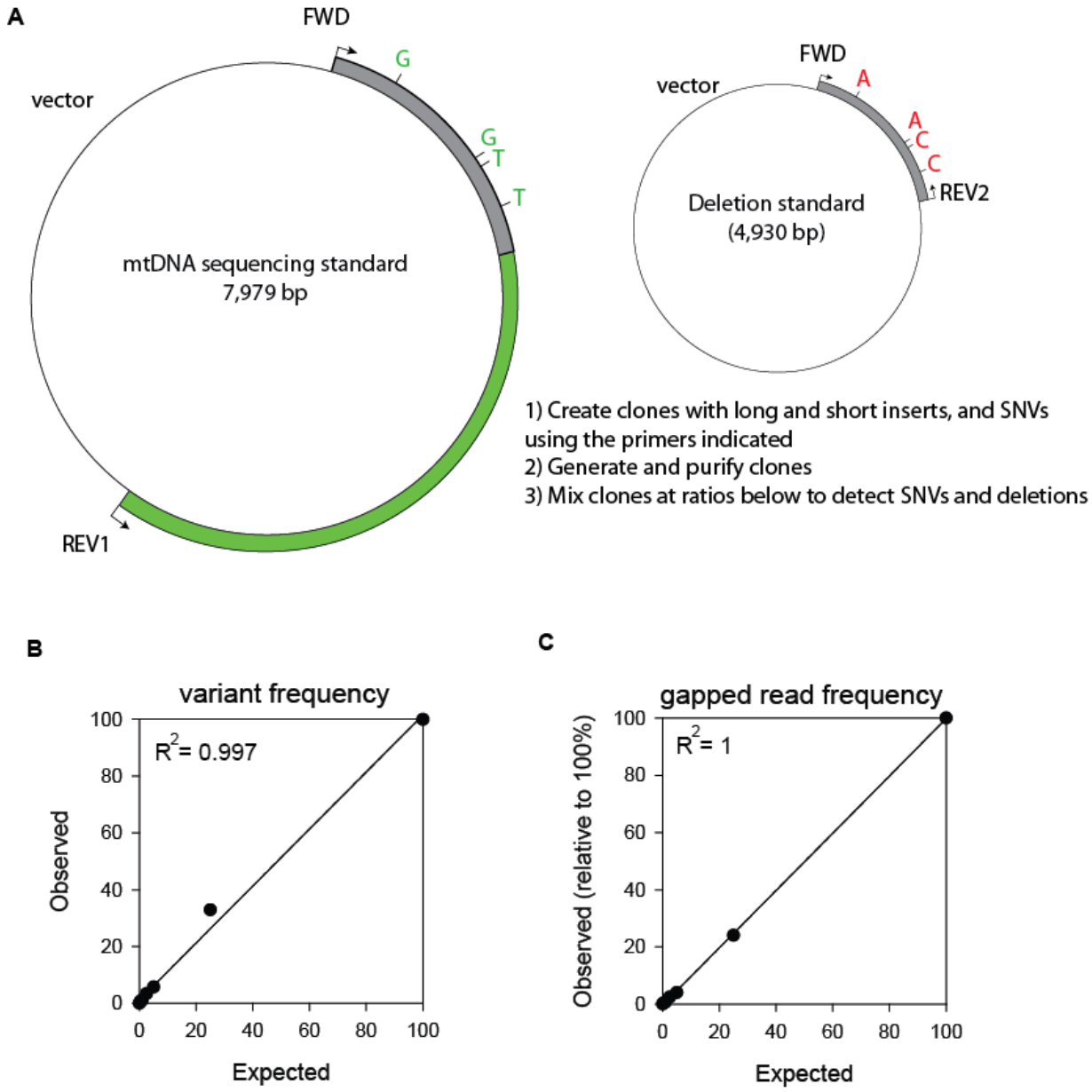
* $p < 0.05$, One-Way ANOVA with Student-Newman-Keuls Method, n=9 animals/group.



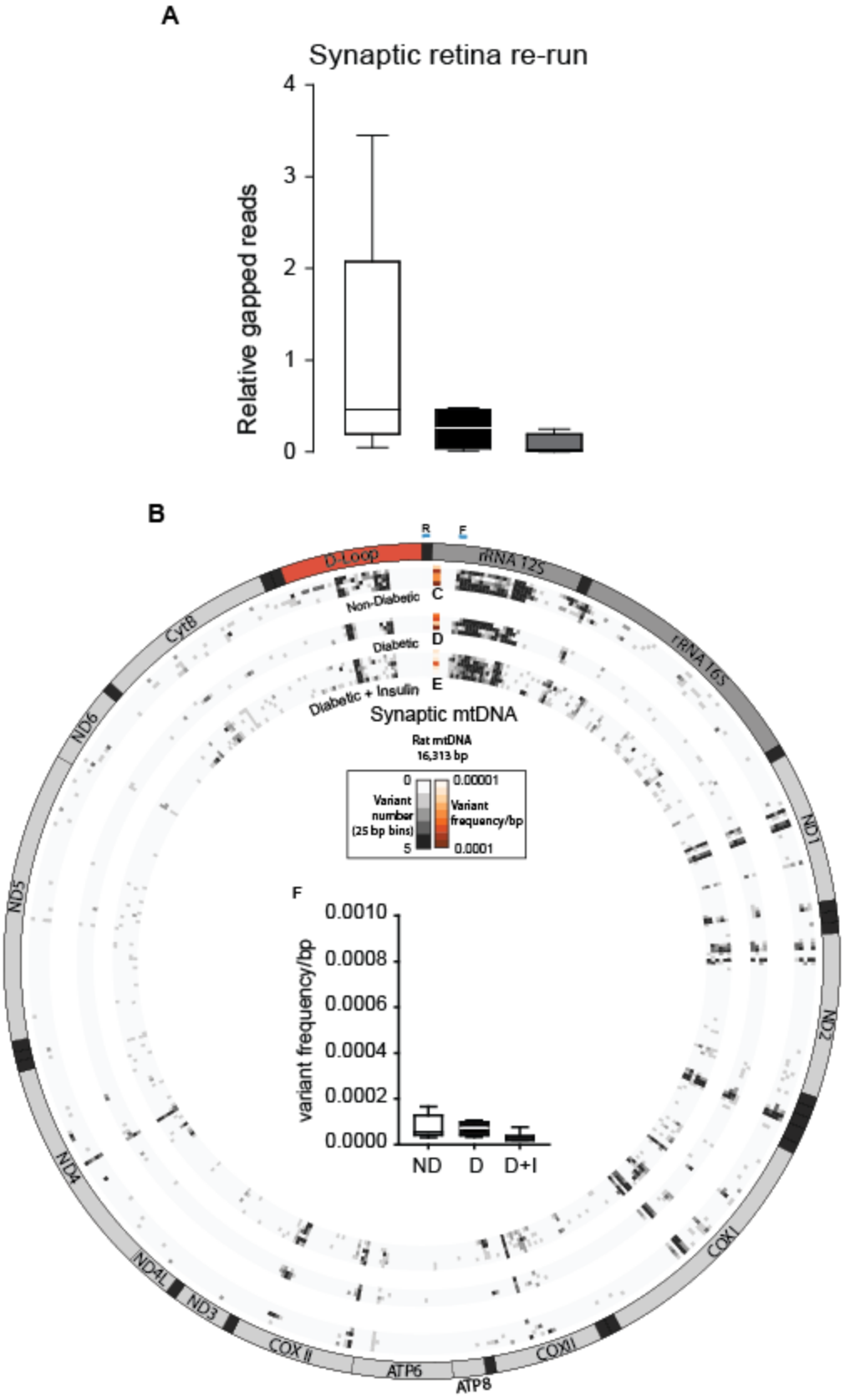
Supplemental Figure 3: Retinal synaptosome preparation western blots. Protein from whole retinal lysate, nuclear and synaptosome pellets after differential centrifugation. Immunoblots (IB) for Lamin B1, 66-70 kDa (nuclear marker), COXIV, 15-16 kDa (mitochondrial marker), and synaptophysin, 38 kDa (synaptic marker). 5 µg soluble protein per well.



Supplemental Figure 4: Mitochondrial DNA (mtDNA) absolute copy number. Box plots of the quantified mtDNA copies from whole retinal DNA from non-diabetic (ND), diabetic (D), and diabetic with insulin replacement (D+I) animals normalized to ng of DNA. n=6 animals/group.

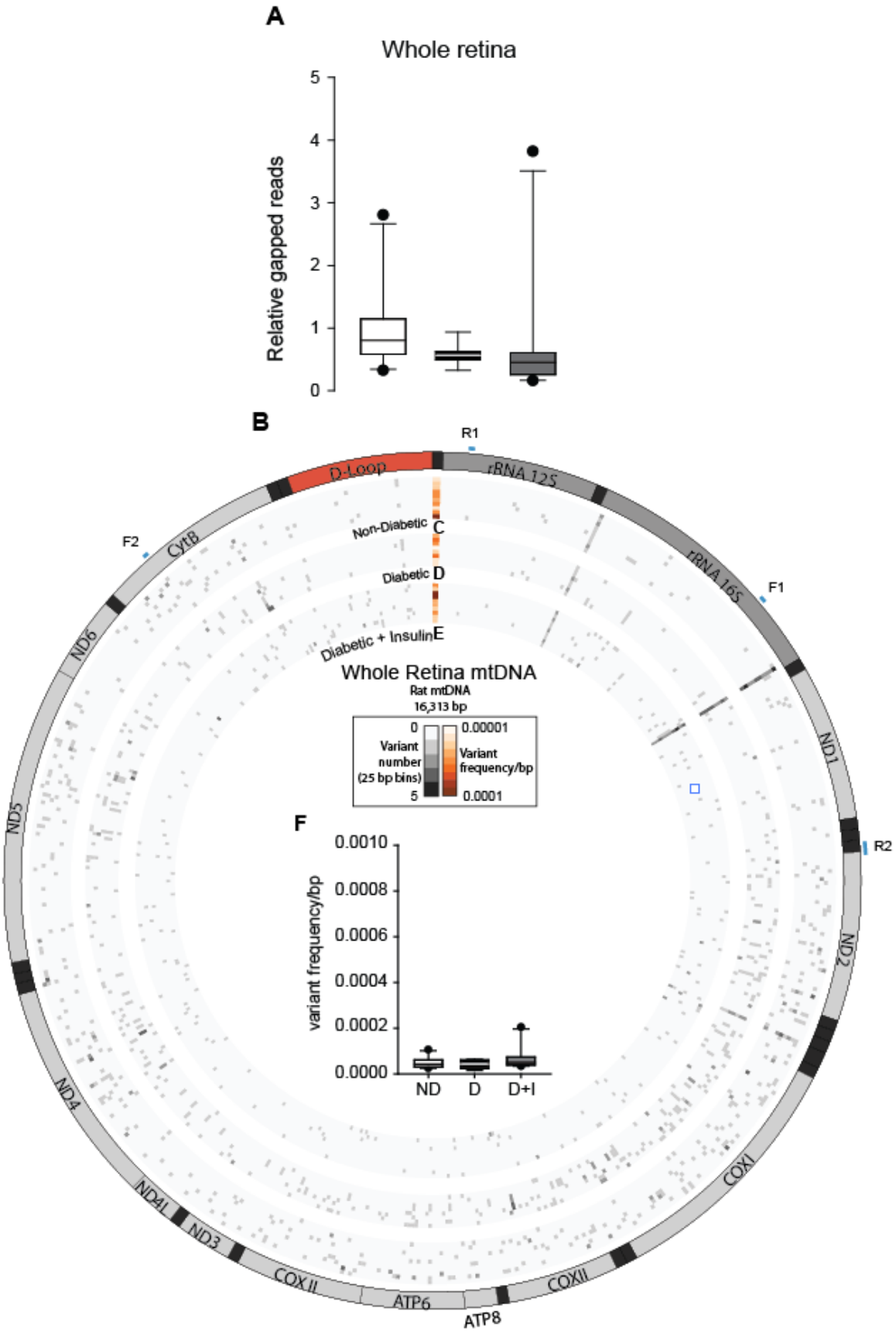


Supplemental Figure 5: Schematic of *de novo* sequencing standards and quantitative validation. **A)** Two artificial 'mitochondrial' genomes were created to serve as sequencing standards. A long and short insert were generated from rat mitochondrial genomes of two different haplotypes. There one standard includes a 7,979 bp insert (left) and the 'deleted' standard is only 4,930bp but the same sequence except for the four bases indicated. **B)** Expected vs Observed quantified variant frequencies. n = 4 sites/standard. **C)** Expected vs Observed (relative to 100% deleted sequencing standard) of gapped reads representing deletions.



Supplemental Figure 6: mtDNA variants from synaptic mtDNA sequencing replication. **A)** Box plots of relative (to non-diabetic, ND, animals) ratio of gapped/total mapped reads from diabetic (D) and diabetic with insulin replacement (D+I) from the mtDNA sequencing from isolated retinal synaptosomes re-run **B)** Ideogram of the 16,313 basepair (bp) mtDNA

genes and annotations; dark gray – ribosomal RNA genes, red – D-Loop region, light gray – protein coding genes, and black – tRNAs. **C)** Number of base variants relative to genomic location of mtDNA per non-diabetic control animal (heatmap rows – gray scale). The single column heatmap represents the mean variant frequency total across the mtDNA for each control animal (total mean variant frequency/bp – orange scale) (n=5 animals). **D)** Number of base variants relative to genomic location of mtDNA per diabetic control animal (heatmap rows – gray scale). The single column heatmap represents the mean variant frequency total across the mtDNA (total mean variant frequency/bp – orange scale) (n=4 animals). **E)** Number of base variants relative to genomic location of mtDNA per diabetic with insulin animal (heatmap rows – gray scale). The single column heatmap represents the mean variant frequency total across the mtDNA (total mean variant frequency/bp – orange scale) (n=6 animals). **F)** Box-plot representation of the mean variant frequency/bp per experimental group. ND1 – NADH dehydrogenase subunit 1. ND2 – NADH dehydrogenase subunit 2. COXI – cytochrome c oxidase subunit 1. COXII – cytochrome c oxidase subunit 2. ATP8 – ATP synthase Fo subunit 8. ATP6 – ATP synthase Fo subunit 6. COX III – cytochrome c oxidase subunit 3. ND3 – NADH dehydrogenase subunit 3. ND4L – NADH dehydrogenase subunit 4L. ND4 – NADH dehydrogenase subunit 4. ND5 – NADH dehydrogenase subunit 5. ND6 – NADH dehydrogenase subunit 6. CytB – cytochrome b.



Supplemental Figure 7: mtDNA variants from whole retina mtDNA sequencing. **A)** Box plots of relative (to non-diabetic, ND, animals) ratio of gapped/total mapped reads from diabetic (D) and diabetic with insulin replacement (D+I) from

mtDNA sequencing of whole retinal samples. **B)** Ideogram of the 16,313 basepair (bp) mtDNA genes and annotations; dark gray – ribosomal RNA genes, red – D-Loop region, light gray – protein coding genes, and black – tRNAs. **C)** Number of base variants relative to genomic location of mtDNA per non-diabetic control animal (heatmap rows – gray scale). The single column heatmap represents the mean variant frequency total across the mtDNA for each control animal (total mean variant frequency/bp – orange scale) (n=10 animals). **D)** Number of base variants relative to genomic location of mtDNA per diabetic control animal (heatmap rows – gray scale). The single column heatmap represents the mean variant frequency total across the mtDNA (total mean variant frequency/bp – orange scale) (n=8 animals). **E)** Number of base variants relative to genomic location of mtDNA per diabetic with insulin animal (heatmap rows – gray scale). The single column heatmap represents the mean variant frequency total across the mtDNA (total mean variant frequency/bp – orange scale) (n=10 animals). **F)** Box-plot representation of the mean variant frequency/bp per experimental group. ND1 – NADH dehydrogenase subunit 1. ND2 – NADH dehydrogenase subunit 2. COXI – cytochrome c oxidase subunit 1. COXII – cytochrome c oxidase subunit 2. ATP8 – ATP synthase Fo subunit 8. ATP6 – ATP synthase Fo subunit 6. COX III – cytochrome c oxidase subunit 3. ND3 – NADH dehydrogenase subunit 3. ND4L – NADH dehydrogenase subunit 4L. ND4 – NADH dehydrogenase subunit 4. ND5 – NADH dehydrogenase subunit 5. ND6 – NADH dehydrogenase subunit 6. CytB – cytochrome b.

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Supplemental File 1:

>mtDNA sequencing standard vector and insert sequence (red+green – 0%, red only- 100%, yellow- variants, white- vector sequence)

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