The soul of allostasis is housed in the mitochondria

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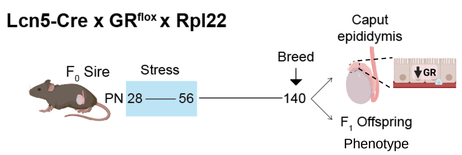
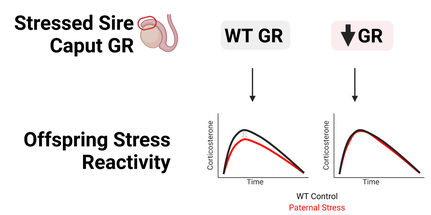
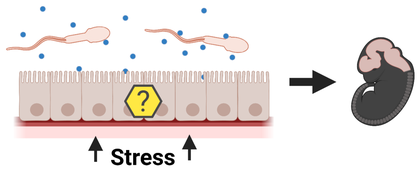
Mitochondria implicated in the molecular mechanism of intergenerational transmission of stress at the caput epididymis

Paternal EEC Glucocorticoid Receptor Knockdown



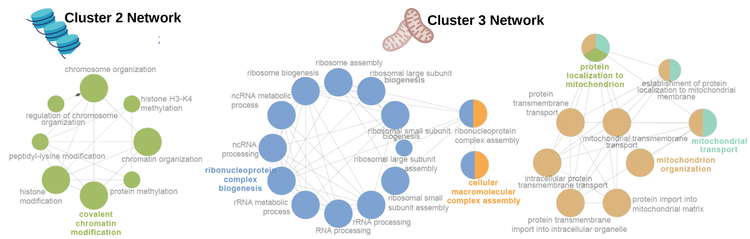
F_1 Offspring Phenotype





What cellular mechanism of allostasis regulates paternal environmental signals to developing offspring?

Paternal EEC translatome reveals mitochondrial and nuclear drivers of allostasis



Background

Paternal life experiences are associated with lasting effects that alter reproductive and embryonic neurodevelopmental outcomes. Sperm maturation depends on signals such as extracellular vesicles (EV) from epididymal epithelial cells (EEC) which are altered by chronic stress to causally influence offspring neurodevelopment. While changes in EV signal are reflective of underlying changes in EEC allostasis, the molecular mechanisms reprogramming EECs in response to stress are unclear. Remarkably, targeted knockdown of the glucocorticoid receptor (GR), a primary regulator of the stress response and allostasis, in paternal EECs inhibits the development of offspring phenotypes following chronic paternal stress and implicates downstream mitochondrial and epigenetic mechanisms as allostatic regulators.

Chan, J.C. et al. *Nature Commun.* 2020

Hypothesis:

We hypothesize changes in cellular allostasis regulating intergenerational transmission of stress are mediated by lasting changes in cellular energy requirements and production.

Methods

Animals: Grflox (B6.129S6-*Nr3c1*$^{tm2.1Lim}$/J) and RiboTag (B6N.129-*Rpl22*$^{tm1.1Psam}$/J) mice were crossed with 129S1/SvImJ females for minimally 3 generations. Lcn5-Cre male mice on a C57Bl/6J background were bred to double heterozygous Grflox; RiboTag 129 females to generate experimental animals. ***Chronic Variable Stress (CVS):*** Postnatal (PN) day 28, pair-housed males were exposed to one stressor each day for 28 days (PN28-56). Order or stressors was randomized each week. Stressors included the following: 36 h constant light, 1 h exposure to predator odor (1:5000 2,4,5 trimethylthiazole (Acros Organics) or 1:2000 phenethylamine (Sigma)), 15 min restraint, novel object overnight, multiple cage changes, 100 dB white noise overnight, and saturated bedding overnight. ***HPA axis assessment:*** Adult mice were tested 2-5 h after lights on by collecting 10 uL tail blood at onset and after completion of a 15 min restraint as well as at 15 and 115 min after the end of restraint (30 and 120 min times). 125I-corticosterone immunoassay (MP Biomedicals) determined corticosterone levels.

RiboTag immunoprecipitation: Whole caput epididymides were dounce homogenized in supplemented homogenization buffer and incubated for 4h with HA antibody. Antibody-protein complexes were isolated with Dynabeads overnight and washed 3x with high salt buffer before processing for RNA-seq library prep.

DC2 corticosterone treatment: Immortalized mouse distal caput epididymal epithelial (DC2) cells cultured to confluency for 72 hours were treated with 500 mg/mL corticosterone (Sigma) or ethanol vehicle. Media was changed after 72 hours to washout treatment. Media was changed every 3 days until time of assay.

DC2 GR knockdown: Confluent DC2 cells were exposed to 18,000 transducing units of viral particles with 8 ug/mL hexadimethrine bromide overnight. Empty vector was used to control for the effects of transduction, non-mammalian shRNA control transduction Particles (Sigma) were used to control for the expression of shRNA, and MISSION shRNA Lentiviral Clone Oligo TRCN0000026223 was used to express shRNA to knockdown GR. Transduced cells were selected with puromycin. Surviving colonies were selected, passaged and cryopreserved until required for downstream assays.

DC2 RNA isolation: DC2 EECs were trypsinized and RNA extracted in Trizol. RNA concentration was measured by Qubit prior to RNA-seq library prep and sequencing on an Illumina NextSeq 500. Network analysis performed with WGCNA package.

Mito Stress Test (MST): DC2 cells were cultured and treated in XFe96 FluxPak plates (Agilent). On the day of the assay, media was changed to base media (Agilent) pH 7.4 supplemented with 1.0 mM sodium pyruvate, 4.0 mM glutamine, and 15.0 mM glucose. A Seahorse XFe96 cartridge was loaded with 1.5 µM oligomycin, 2 µM trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP), and 0.5 µM Rotenone/Antimycin A (Agilent) in base media. Media was replaced prior to the run.

Complex I Enzyme Activity: Activity was measured following the procedures outlined in the Complex I Enzyme Activity Microplate Assay Kit (Abcam) on an Infinite M1000 plate reader (Tecan) and analyzed using Magellan software (Tecan). Protein concentration for normalization quantified by Pierce BCA Protein Assay Kit (Thermo Scientific).

DC2 CUT&RUN sequencing: The protocol was adopted from the Nature Protocols manuscript by Skene, Henikoff & Henikoff. DNA libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB) with the NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set I) (NEB). Individually barcoded libraries were pooled and paired-end sequencing was performed on an Illumina NextSeq 500. Differential binding analysis performed with DiffBind package, and visualized with deeptools.

EV Incubation: Media was collected from treated DC2 cultures at day 9 and EVs isolated by ultracentrifugation, pooled by group, and characterized on a ZetaView. EVs were supplied to vehicle treated cultures in otherwise EV depleted media on day 7 and assayed 48h later.

Stress coordinates nuclear-encoded mitochondrial gene expression and generates lasting changes in EEC transcriptome

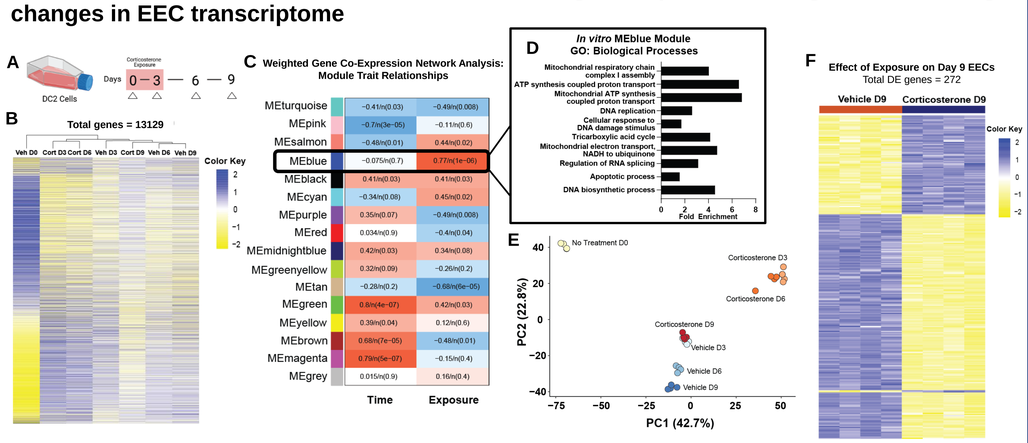


Figure 1. ***(A)*** Schematic illustrating our validated *in vitro* model of paternal stress and RNA-sequencing collection timeline. Created with Biorender. ***(B)*** Heatmap of all gene expression over time. Unbiased hierarchical clustering as determined by gene expression visualized by dendrogram. N= 4. ***(C)*** Weighted Gene Co-Expression Network Analysis of all genes expressed across all timepoints. Color modules reflect clusters of highly interconnected genes. Columns outline the factors considered. Pearson correlation between each module and factor provided as the left value, with significance of the correlation reported in parenthesis. (***D***) Functional annotation of the top 10 biological processes associated with genes in the MEblue module demonstrating association of genes in this module with mitochondrial bioenergetic processes, FDR < 0.01. ***(E)*** Principal component analysis of gene expression across all samples. Each circle represents a sample, while color denotes group membership. These data reveal that component 1 accounts for more than 42.7% of the variance between groups and aligns with treatment group while component 2 accounts for 22.8% of variance and aligns with maturation. *N*=4; adjusted *P*<0.05. ***(F)*** Differential expression analyses reveal 272 DEGs between day 9 corticosterone and vehicle treated samples visualized by heatmap. Unbiased hierarchical clustering of samples is depicted by color blocking above the sample. The largest differentially expressed cluster reveals downregulation of transcription. *N*=4, adjusted *P*<0.5.

CUT&RUN sequencing reveals binding of transcriptional repressor H3K27me3 at regions associated with mitochondrial processes following stress

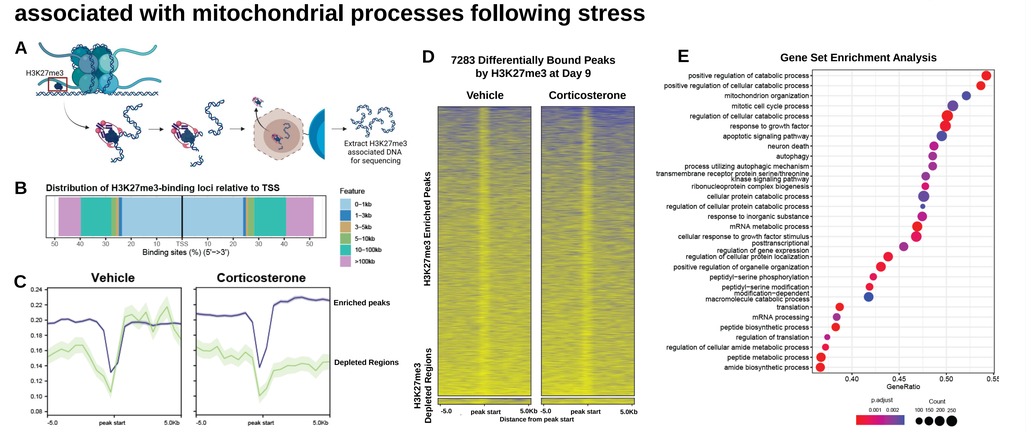
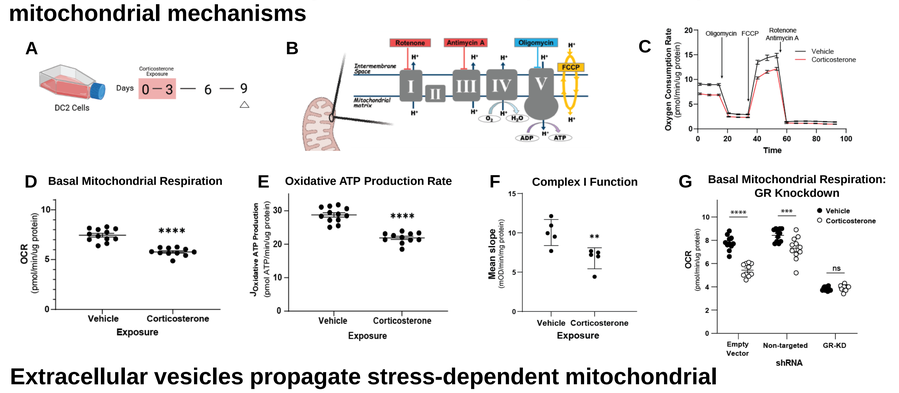


Figure 2. ***(A)*** Schematic illustrating the process of Cleavage Under Targets and Release Using Nuclease (CUT&RUN) sequencing. Created with Biorender. ***(B)*** Distribution of the H3K27me3 binding loci relative to transcription start site (TSS) revealing that over 50% of peaks align with regions within 1 kb of the (TSS). ***(C)*** Signal normalized by bpm for within 5 kb of the peak start for peaks differentially enriched (blue) or depleted (green) H3K27me3 binding as determined by differential binding analysis. Each profile corresponds to a representative sample from each exposure group. Shading indicates SEM. ***(D)*** Heatmap of peaks or regions in the genome that are differentially bound by H3K27me3. Each corresponds with a base pair up to 5kb away from the peak start. Each row corresponds with a peak. Each heatmap reflects data from a representative sample from each group. Differentially bound regions were determined by differential binding analysis (DBA) which revealed H3K27me3 enrichment at nearly 7135 regions and depletion at 147 regions. N = 4-5, FDR <0.05. ***(E)*** Gene set enrichment analysis of all genes aligning with peaks bound by H3K27me3. Each row represents a pathway associated with genes. Color depicts adjusted p-value, size depicts the number of genes represented in the gene set. GeneRatio is calculated by dividing count by the total number of genes in the gene set.

Prior stress promotes changes in allostatic set point via GR-dependent mitochondrial mechanisms



Extracellular vesicles propagate stress-dependent mitochondrial regulation of allostasis

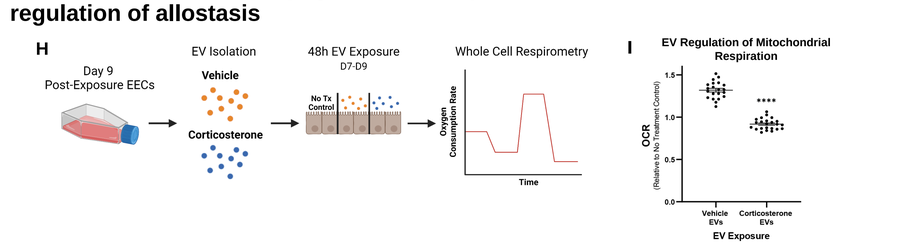


Figure 3. ***(A)*** Schematic illustrating our validated *in vitro* chronic stress model in EECs indicating that assays are performed at day 9. Created with Biorender. ***(B)*** Schematic of the electron transport chain at the inner membrane of the mitochondria. Color coded lines and arrows indicate the site of interference for drugs applied during the Mito Stress Test. Adapted from Agilent Technologies, Inc. (2019), Agilent Seahorse XF Cell Mito Stress Test Kit. Created with Biorender. (***C)*** Representative plot of oxygen consumption rate (OCR) x time in minutes for Mito Stress Test injections. Arrows indicate when drugs identified in ***(B)*** are injected into wells. ***(D)*** Two-tailed t-test of exposure x basal mitochondrial OCR demonstrates decrease in OCR in day 9 EECs with prior corticosterone exposure. ***(E)*** Two tailed t-test of exposure x oxidative ATP production rate (J_{ATPox}) demonstrates significant decrease in J_{ATPox} in day 9 EECs with prior corticosterone exposure. ***(D) – (E)*** Error bars indicate SEM, ****P <0.0001, N = 8-9. ***(F)*** Two tailed t-test of exposure x complex I activity demonstrates decrease in complex I function in day 9 EECs with prior corticosterone exposure. Error bars indicate SEM, **P <0.01, N=5. ***(G)*** Two-way analysis of variance demonstrates decrease in OCR in EECs with prior corticosterone exposure in both empty vector and non-targeted shRNA expressing control groups. No difference between exposure groups in GR-KD cells. Error bars indicate SEM, ***P<0.001, ****P <0.0001, ns = not significant, N=10-12 wells. ***(H)*** Schematic illustrating the collection and isolation of extracellular vesicles (EVs) from day 9 EEC cultures with prior corticosterone or vehicle treatment. These pools are then incubated with EECs with prior vehicle exposure at day 7 for a duration of 48 hours. Following incubation with EVs from treated cultures or no treatment control (No Tx Control), day 9 EEC cultures are assayed with the Mito Stress Test, and whole cell respirometry is used to assess whether treatment with EVs influences basal mitochondrial respiration. ***(I)*** Two-tailed t-test of EV exposure x OCR relative to the average OCR of the no treatment control group demonstrates a decrease in OCR in day 9 EECs following treatment with EVs from EECs with prior corticosterone exposure. Error bars indicate SEM, ****P<0.0001, N=21-23 wells per group.

Summary

- Chronic stress in male mice significantly blunts offspring HPA stress response which was reversed by selectively reducing GR in caput epididymal epithelial cells (EEC). Critically, this validated GR as a target mediating allostasis in the context of paternal intergenerational stress.
- Paternal stress produced marked, enduring and GR-dependent changes in EEC transcriptomics. Interestingly, a vast majority of these GR-responsive genes related to mitochondrial function. Similarly, genes with highly interconnected expression following stress, and H3K27me3 bound regions are associated with metabolic and mitochondrial processes.
- *In vitro* evaluation of mitochondrial function demonstrates decreased basal respiration and ATP production rate following corticosterone treatment. GR knockdown protects against stress-dependent changes in respiration while exposure to EVs from stressed cultures initiate the mitochondrial signature of stress dependent allostatic changes.

Taken together, these data demonstrate the establishment of a new, enduring cellular allostatic set point in response to stress, reflected by cellular energy requirements and mitochondrial energy production, and encourages further investigation regarding the related roles of GR and EVs in the regulation of allostasis and its influence on the mitochondria and cellular baseline.

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