# Extracellular vesicles as cellular communicators of stress-mediated allostasis

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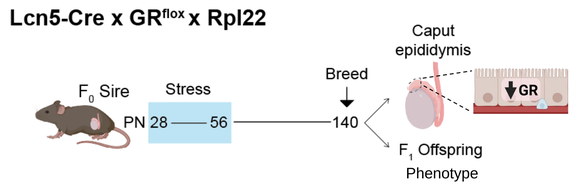
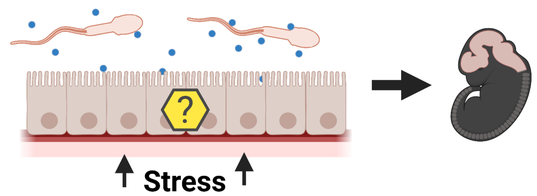
## Mitochondria implicated in the molecular mechanism of intergenerational transmission of stress at the caput epididymis

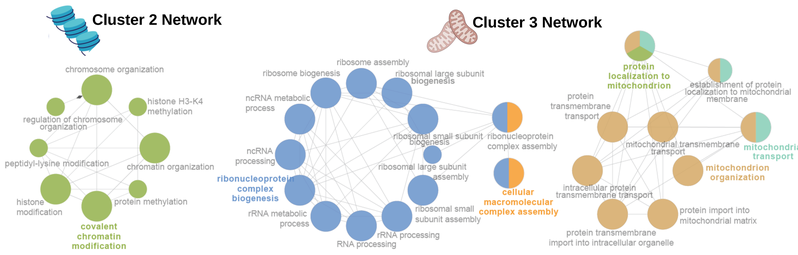
***EEC Glucocorticoid Receptor Knockdown***




**What cellular allostatic mechanism regulates EEC environmental signals to sperm and developing offspring?**

***EEC GR knockdown translatome reveals mitochondrial and nuclear drivers of allostasis***

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## Introduction

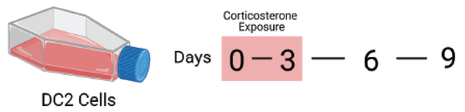
Parental life experiences alter reproductive and embryonic outcomes. In males, sperm maturation depends on signals such as extracellular vesicles (EV) from epididymal epithelial cells (EEC) which are altered by chronic stress to causally influence offspring neurodevelopment. While changes in EV signal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

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

## *Hypothesis*:

We hypothesize changes in cellular allostasis following chronic stress are mediated by lasting changes in cellular energy requirements.

## Model




## Methods

**Animals:** Gr$^{flox}$ (B6.129S6-*Nr3c1*$^{tm2.1Ljm}$/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 Gr$^{flox}$; RiboTag 129 females to generate experimental animals. ***Chronic Variable Stress (CVS) & HPA axis assessment:*** As described in Chan, J., et al. (2020). *Nature Commun.*

**RiboTag immunoprecipitation:** Caput epididymides were dounce homogenized in 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 and sequencing.

**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) to control for the expression of shRNA, and MISSION shRNA Lentiviral Clone Oligo TRCN0000026223 to express shRNA to knockdown GR. Transduced cells were selected with puromycin. Surviving colonies were selected, passaged and cryopreserved until assay.

**DC2 RNA isolation:** RNA was extracted in Trizol according to the manufacturer's protocol. 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:** The Complex I Enzyme Activity Microplate Assay Kit (Abcam) manufacturer's protocol was followed and analyzed using Magellan software (Tecan). Protein concentration was 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 cultures and EVs isolated by ultracentrifugation, pooled by group, and characterized with ZetaView. EVs were added to day 7 vehicle-cultures and assayed 48h later. EVs were incubated with caudal mouse sperm for 0.5 h before assay.

## Stress coordinates nuclear-encoded mitochondrial gene expression and enduring downregulation of gene expression

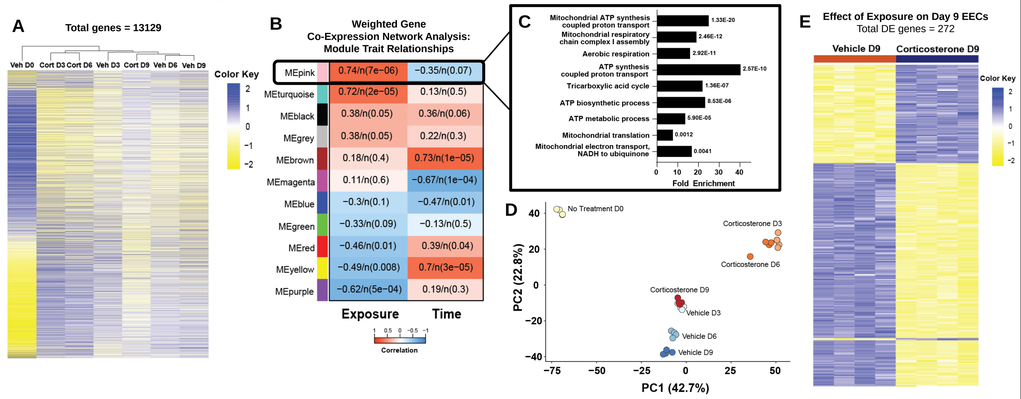



**Figure 1.** ***(A)*** Heatmap of all gene expression over time. Unbiased hierarchical clustering as determined by gene expression visualized by dendrogram. N= 4. ***(B)*** Weighted Gene Co-Expression Network Analysis of all genes expressed across all timepoints. Color modules reflect clusters of highly 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. **(C)** Functional annotation of the top 10 biological processes associated with genes in the MEpink module demonstrating association of genes in this module with mitochondrial bioenergetic processes, FDR < 0.01. ***(D)*** 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. ***(E)*** 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 increased binding of transcriptional repressor H3K27me3 at regions associated with mitochondrial processes following stress

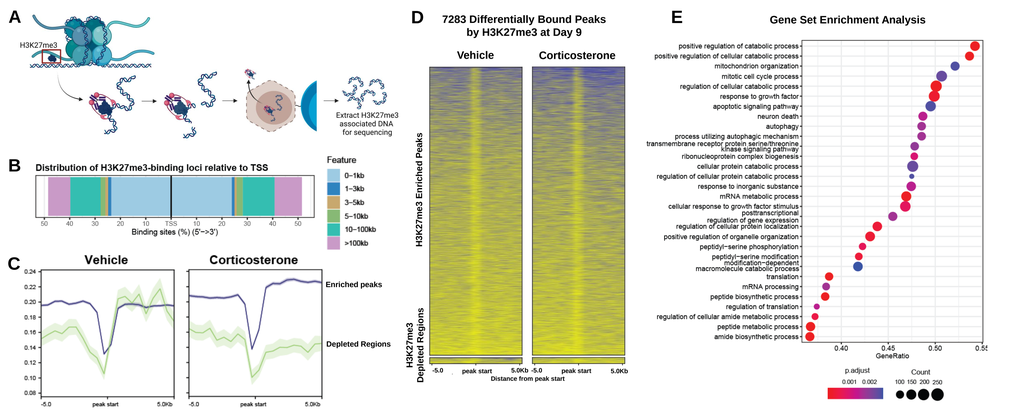



**Figure 2.** ***(A)*** Schematic illustrating the process of Cleavage Under Targets and Release Using Nuclease (CUT&RUN) sequencing. Created with Biorender. ***(B)*** Distribution of the H3K27me3 binding loci relative to transcription start site (TSS) revealing that over 50% of peaks align with regions 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 changes allostatic set point via GR-dependent decreases in mitochondrial respiration maintained by EVs

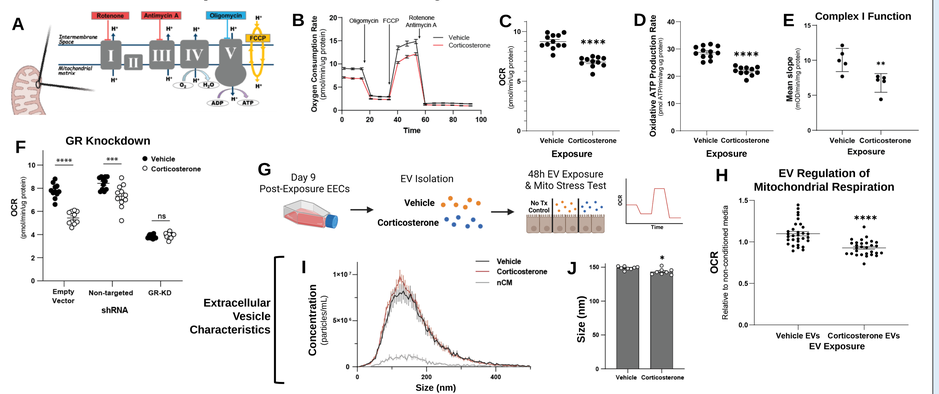
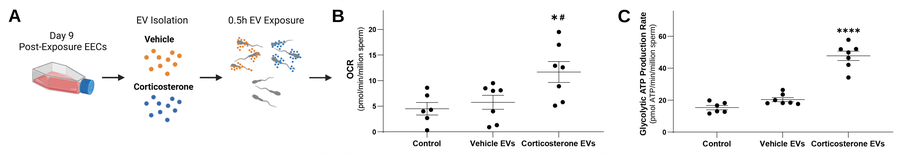



**Figure 3.** ***(A)*** Schematic of the electron transport chain at the inner membrane of the mitochondria. Lines and arrows indicate the site of interference for drugs applied during the Mito Stress Test. Adapted from Agilent Technologies, Inc. Created with Biorender. ***(B)*** Representative plot of oxygen consumption rate (OCR) x time in minutes for Mito Stress Test injections. Arrows indicate when drugs in ***(A)*** are injected into wells. ***(C)*** Two-tailed t-test of exposure x basal mitochondrial OCR demonstrates decrease in OCR in day 9 EECs with prior corticosterone exposure. ***(D)*** 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. ***(C) – (D***) N = 8-9. ***(E)*** Two tailed t-test of exposure x complex I activity demonstrates decrease in complex I function in day 9 EECs with prior corticosterone exposure. N=5. ***(F)*** 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. N=10-12 wells. ***(G)*** Schematic of the collection and isolation of extracellular vesicles (EVs) from day 9 EECs with prior corticosterone exposure. EVs are then incubated with EECs with prior vehicle exposure at day 7 for 48 hours. Following incubation with EVs or control (No Tx Control), day 9 EECs are assayed with the Mito Stress Test by whole cell respirometry to assess whether EVs influence basal mitochondrial respiration. ***(H)*** Two-tailed t-test demonstrates that stress-EVs modulate EEC mitochondrial respiration. ***(I)*** Size distribution of EVs and non-conditioned media control (nCM). ***(J)*** Two-tailed t-test demonstrates that median EV size is reduced with prior EEC corticosterone exposure. N=8. **For all:** Error bars indicate SEM, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns = not significant.

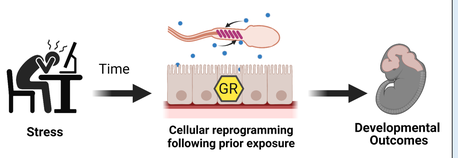
## Stress-dependent EEC EV cargo increase sperm mitochondrial respiration




***(A)*** Schematic of the collection and isolation of day 9 EEC EVs after corticosterone exposure (D0-D3). EVs are incubated with caudal sperm for 0.5h. Sperm are assayed with the Mito Stress Test. ***(B,C)*** One-way analysis of variance demonstrates increase in sperm OCR ***(B)*** and glycolytic ATP production rate ***(C)*** following incubation with EVs from EECs with prior corticosterone exposure. #Comparison to vehicle EVs. N=6-7. Error bars indicate SEM, *P<0.05, #P<0.05, ****P<0.0001

## Summary & Conclusions

- EEC GR regulates two clusters of co-varying genes related to chromatin and mitochondria following chronic stress in male mice
- Paternal stress produced changes in EEC transcriptomics over time. Highly interconnected DEGs and H3K27me3 bound regions are associated with mitochondrial oxidative respiration.
- Changes in the allostatic set point that reduce basal EEC mitochondrial respiration are mediated by GR and EEC EVs.
- Stress-dependent EV cargo causally regulate sperm energy balance and maturation.




Taken together, these data demonstrate the enduring effects of life stress on cellular allostatic set point, reflected by changes cellular energy requirements and intercellular signaling. Future work will investigate the interactions of GR across cell compartments, identify the EV cargo necessary for sperm mitochondrial regulation and the impact of stress-EVs on sperm motility and embryo and brain development.

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