



# Mitochondrial – nuclear genetic interaction modulates whole body metabolism, adiposity and gene expression in vivo

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## ABSTRACT

We hypothesized that changes in the mitochondrial DNA (mtDNA) would significantly influence whole body metabolism, adiposity and gene expression in response to diet. Because it is not feasible to directly test these predictions in humans we used Mitochondrial-Nuclear eXchange mice, which have reciprocally exchanged nuclear and mitochondrial genomes between different *Mus musculus* strains. Results demonstrate that nuclear-mitochondrial genetic background combination significantly alters metabolic efficiency and body composition. Comparative RNA sequencing analysis in adipose tissues also showed a clear influence of the mtDNA on regulating nuclear gene expression on the same nuclear background (up to a 10-fold change in the number of differentially expressed genes), revealing that neither Mendelian nor mitochondrial genetics unilaterally control gene expression. Additional analyses indicate that nuclear-mitochondrial genome combination modulates gene expression in a manner heretofore not described. These findings provide a new framework for understanding complex genetic disease susceptibility.

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## 1. Introduction

Studies searching for the genetic basis of common disease susceptibility have identified statistical associations between hundreds of loci throughout the nuclear genome, significantly enhancing our appreciation of the number of pathways potentially related to disease development. Investigations seeking a clear genetic etiology for individual susceptibility to common diseases, such as obesity and cardiometabolic syndrome suggest a combination of complex genetic-environmental interactions. However, a well-defined understanding of how these genetic-environmental mechanisms modulate polygenic/complex disease development has not yet been realized.

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Obesity rates have been rising over the past 25 years, and current reports classify more than one-third of US adults as obese [32]. It is a major risk factor for numerous maladies, including diabetes, cancer, and cardiovascular disease [4,5], and although obesity can occur at any age, metabolic and lifestyle changes associated with aging increase obesity risk. While caloric excess and lack of exercise are implicated as the major contributors to the rising incidence of obesity [17,19,20,40], 40–70% of inter-individual variability in body mass index (a common assessment of obesity) is genetic [44]. Multiple single gene mutations within the nuclear genome have been linked to morbid obesity and some features of the metabolic syndrome<sup>reviewed in</sup> [9], but the frequencies of these mutations are low and cannot account for the rising rates of obesity observed in the developed world. Consistent with this notion is the CDC statement in 2012 that “genetic changes in human populations occur too slowly to be responsible for the obesity epidemic” [5].

The origins of eukaryotes are the result of 1.5 billion years of endosymbiosis and co-evolution between the mitochondrial and nuclear genomes. It is therefore reasonable to consider that this collaboration enabled genetic adaptation to environmental challenges through nuclear-mitochondrial genetic interactions. Further, it has been proposed that changes in mitochondrial DNA (mtDNA) sequence were

### Research in context

The search for a genetic cause to obesity and cardiovascular pathologies has spanned decades. Numerous gene targets have been identified, but no single gene mutations are prevalent in large enough populations to adequately explain the surge in obesity and cardiometabolic diseases in humans. Recently, mitochondrial DNA (mtDNA) has emerged as an attractive target to researchers for its potential role in disease susceptibility both by changing mitochondrial function and interacting with the nuclear genome to alter gene expression. To directly test the role mitochondrial genetic background plays in metabolism and nuclear gene expression we have employed genetic manipulate to common mouse strains, performing reciprocal exchange of nuclear and mitochondrial genomes, similar to mitochondrial replacement therapy approved for use in humans. Changing mtDNA background altered the response to high fat diet feeding, changing fat deposition and expression of nuclear genes. Collectively, these results suggest that mtDNA background is playing a pivotal role in nuclear gene expression and influencing individual disease susceptibility. Thus, current mitochondrial replacement therapies may also be changing the nuclear gene profile of the recipient, with as yet unknown consequences.

### eTOC Blurb

Dunham-Snary et al. directly show that different mitochondrial-nuclear genome combinations influence metabolism, adiposity, and gene expression. Different mtDNAs on the same nuclear background can create distinct transcriptional profiles in response to stress, which provides a new framework for the interpretation of genetic linkage studies and understanding complex genetic disease.

influenced by environmental challenges and therefore adaptive during the prehistoric global establishment of human populations [38]. Consequently, we hypothesized that non-pathogenic changes in the mtDNA could play a significant role in metabolism, body composition and gene expression.

Because it is not feasible to directly test the effects of the mtDNA on these factors in humans, we utilized Mitochondrial-Nuclear eXchange (MNX) mouse models [2,11,12,23], which have nuclear and mitochondrial genomes that have been reciprocally exchanged between different *M. musculus* strains to examine the impact of different mtDNA backgrounds on diet-induced changes in body composition, whole body metabolism and nuclear gene expression. For these studies C57BL/6J and C3H/HeN mouse strains were utilized due to their noted differences in susceptibility to diseases associate with metabolism [14–16,22,25,39,43] and distinct mtDNAs [1]. Using C57BL/6J as a reference sequence, C3H/HeN mice have two non-synonymous mutations (nt – nucleotide): nt9461 T/A → C, resulting in substitution of isoleucine for methionine at the first amino acid of *mt-Nd3* (a subunit of electron transport chain Complex I), and nt9348 G → A, resulting in substitution of isoleucine for valine at the 248th amino acid of *mt-Co3*, (a subunit of Complex IV). C3H/HeN mice also have a 'TA' insert at nt9818, which is within tRNA<sup>Arg</sup>. We have previously used C57 and C3H MNX models to show that differences in bioenergetics, ROS production, mitochondrial ETC complex activity and susceptibility to heart failure exist between these two strains of mice and segregate with their mtDNAs [12]. For these studies, we anticipated that the C57BL/6J genetic components (nuclear or mitochondrial genomes) would provoke greater changes in adiposity and gene expression in response to diet, compared to the C3H/HeN background.

## 2. Materials and methods

Additional materials and methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING

### 2.1. Ethics statement

All animal studies carried out were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Alabama at Birmingham.

### 2.2. Experimental model and subject details

#### 2.2.1. Generation of experimental animals

All animals utilized for these studies were generated within the wild type (C57BL6/J – C57n:C57mt and C3H/HeN – C3Hn:C3Hmt) and MNX (C57n:C3Hmt and C3Hn:C57mt) mouse colony maintained at the University of Alabama at Birmingham. All mice had their mtDNA haplotype confirmed by SNP analysis (Supplementary Fig. S3) as previously described [12]. Briefly, these two strains differ in mtDNA mutations at complexes I, IV and tRNA<sup>Arg</sup> as previously described [1], and have differences in complex IV activity in heart that segregate with mtDNA background [12]. Wild type and MNX mouse colonies are maintained by breeding wild type and MNX females to wild type males of corresponding nuclear genetic background. In this manner, all mice within a shared nuclear background are paternally related (e.g. wild type male siblings were used to sire both the wild type and MNX progeny so paternal genetic contribution would be isogenic across nuclear DNA-matched strains). The age at time of study was selected because: 6 weeks is a commonly used for commencing diet experiments, and to minimize the impact of age-related changes in redox balance, inflammation and metabolic dysfunction. Only male mice were selected for this study, as using female MNX mice for experimentation is practically prohibitive (female MNX mice are all used for colony maintenance).

### 2.3. Diets

Three-week-old male wild type and MNX mice were weaned onto a chow diet containing 10% kcal fat (D12450B, Research Diets, New Brunswick, NJ) and provided food and water ad libitum until the age of 6 weeks. At 6 weeks of age, mice either continued on ad libitum chow, or were fed a high-fat (45% kcal), moderate carbohydrate (35% kcal) Western-style diet (D12451, Research Diets, New Brunswick, NJ) ad libitum until sacrifice at 12–13 weeks of age.

### 2.4. Whole body metabolic measures

At 5 weeks of age, animals were acclimated to individual housing in an OxyMax (Columbus Instruments, Columbus OH) Comprehensive Laboratory Animal Monitoring System (CLAMS) at 25 °C as previously described [3]. Metabolic data including oxygen consumption and carbon dioxide production (VO<sub>2</sub> and VCO<sub>2</sub>, respectively), heat (kcal/h), activity and food intake were monitored in real-time, and data were recorded in 15-min intervals for alternating weeks from 6 to 12 weeks of age. All CLAMS data were exported to Comma Separated Value files, which were then converted for analysis in Microsoft Excel 2008 for Mac. Metabolic efficiency was calculated for each individual and corresponding time point as activity/VO<sub>2</sub> (normalized to body weight), and the last 72 h of measurements averaged. Correspondingly, energy expenditure was calculated for each individual and corresponding time point as heat (kcal/h), and the last 72 h of measurements used to

calculate average daily energy expenditure (kcal) normalized by body weight for each animal.

## 2.5. Body weight and body composition

Animal weight was monitored on a weekly basis during cage changes, beginning with a baseline measurement at 5 weeks, prior to acclimation to CLAMS housing. *In vivo* body composition measures of total body fat and lean tissue were obtained at 6, 9 and 12 weeks of age using an EchoMRI 3-in-1 quantitative magnetic resonance (QMR) machine (Echo Medical Systems, Houston, TX).

## 2.6. Euthanasia

Heparinized mice were anesthetized by step-wise 100  $\mu$ L intraperitoneal injections of 0.1 mg/mL chloral hydrate. When mice were unresponsive to stimuli, mice were exsanguinated via removal of the heart as previously described [12]. All animals were harvested between 09:00 and 13:00 h. Comparison of CLAMS parameters for animals within this time range did not show significant differences (data not included).

## 2.7. White adipose tissue deposition

White adipose tissue (WAT) was removed and weighed from inguinal (iWAT, anterior to the upper segment of hind limbs) and epididymal (eWAT, attached to the epididymis and testes) depots, representative of subcutaneous and visceral fats, respectively. Adipose tissues were collected for RNA sequence analysis and stored in RNAlater RNA Stabilization Reagent (Qiagen, Germantown, MD) and flash-frozen in liquid nitrogen (LN<sub>2</sub>) for storage. All tissues were harvested at the end of the study (12–13 weeks of age) between 09:00 and 13:00 h.

## 2.8. RNA sequence analysis

For these studies, the effect of different mtDNA and nuclear background combinations on adipose gene expression was assessed by RNA sequence analysis with RNA isolated from white adipose tissues collected from epididymal (eWAT) and inguinal (iWAT) fat, representing visceral and subcutaneous fat depots, respectively. RNA was isolated with the RNeasy kit (Qiagen, Germantown, MD) per manufacturer instructions. Reverse transcribed cDNA libraries were sequenced with an Illumina HiSeq 2000. Read mapping was conducted with a proprietary algorithm by Expression Analysis ([www.q2labsolutions.com](http://www.q2labsolutions.com)), and read counts were used as input for differential expression analysis.

## 2.9. Quantification and statistical analyses

### 2.9.1. Comparative transcriptome analyses

Transcriptional changes were interrogated for 920 genes previously reported to be associated with fat metabolism and 29,209 genes representing the entire mouse transcriptome. A list of the 920 genes (Supplementary Table S1) reported to be involved in fat metabolism was generated by searching the following terms into the NCBI Gene database (National Center for Biotechnology Information, U.S. National Library of Medicine, Gene, <http://www.ncbi.nlm.nih.gov/gene>): adipose, adipose metabolism, fat metabolism, triglyceride metabolism, and adipogenesis. Comparative transcriptomic analyses were performed by comparing the impact of different nuclear-mitochondrial genome combinations upon dietary response by defining transcriptome differences within the same strain fed high fat diet versus chow diet. For these comparisons, the transcriptome profile for each strain fed the chow diet was defined, and changes in this profile were determined in the same strain fed the high fat diet to identify the number of differentially expressed (DE) genes. All comparative analyses were performed in the R (version

3.2.2) environment using DESeq2 version 1.10.1, with default parameters. This test for differential expression is based on a negative binomial (e.g. Gamma-Poisson) Generalized Linear Model, which utilizes raw, non-transformed read count data as input. Genes were interpreted as differentially expressed if the Benjamini-Hochberg-adjusted p-value was reported as <0.05. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE86826.

Gene lists generated from these comparisons were utilized to determine Biological Process, Molecular Function, and Cellular Component categories using WEB-based Gene Set Analysis (WebGestalt) Toolkit (<http://www.webgestalt.org/option.php>, [46]).

### 2.9.2. Other statistical analyses

Data are expressed as mean + SEM. The significance of differences was analyzed by ANOVA and post hoc analysis for multiple group comparison using GraphPad Prism (GraphPad, La Jolla, CA). The test was considered significant with a p value < 0.05.

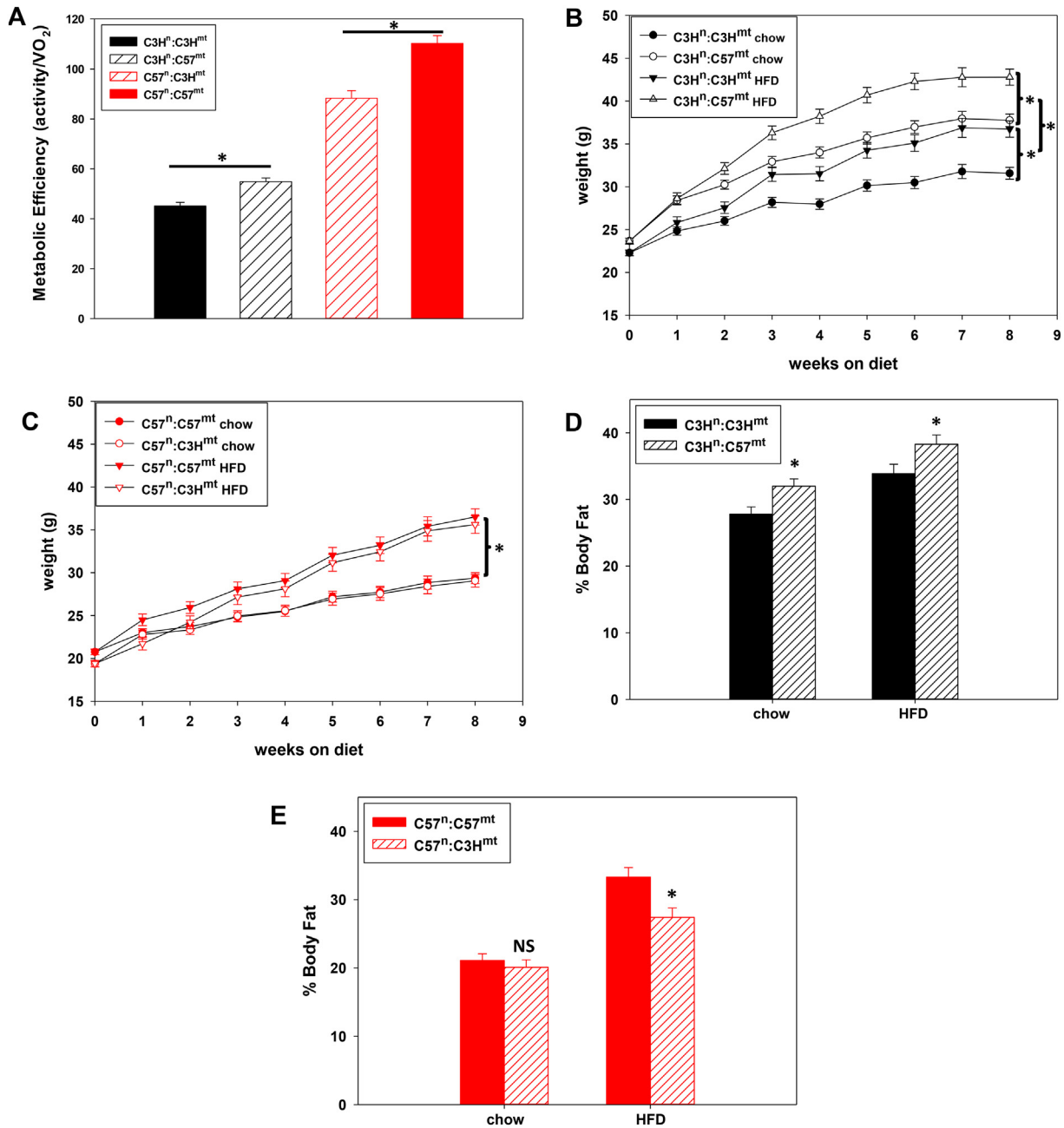
### 2.9.3. Data software and availability

The GEO accession number for the RNA sequence dataset reported in this paper is GEO: GSE86826. All comparative analyses were performed in the R (version 3.2.2) environment using DESeq2 version 1.10.1. Biological Process, Molecular Function, and Cellular Component categories were determined using WEB-based Gene Set Analysis (WebGestalt) Toolkit (<http://www.webgestalt.org/option.php>, [46]).

## 3. Results

### 3.1. Measurement of the impact of nuclear – mitochondrial genetic background on whole body metabolic efficiency and body composition

Wild-type (C57BL/6J – C57<sup>n</sup>:C57<sup>mt</sup> and C3H/HeN – C3H<sup>n</sup>:C3H<sup>mt</sup>) and MNX (C57<sup>n</sup>:C3H<sup>mt</sup> and C3H<sup>n</sup>:C57<sup>mt</sup>) male mice were monitored for changes in activity, metabolic efficiency and food intake using a Comprehensive Laboratory Animal Monitoring System (CLAMS). Mitochondrial genetic background significantly impacted whole animal metabolic efficiency within each nuclear background. More specifically, C57 and C3H mtDNAs were associated with increased and decreased efficiency relative to nuclear background matched controls, respectively (Fig. 1A). As expected, high fat diet significantly increased weight gain in all mouse strains relative to their chow counterparts (Fig. 1B to C) and mtDNA background significantly affected body weight changes in mice harboring the C3H nuclear genetic background on both diets (the C57 mtDNA was associated with greater weight gain; Fig. 1B). No significant differences were observed between mice carrying the C57 nuclear background but having different mtDNAs (Fig. 1C). However, further analysis of body composition revealed that the C57 mtDNA increased adiposity on both C3H and C57 nuclear backgrounds in animals fed high fat diet (Fig. 1D to E) and in C3H nuclear background mice fed chow diet (Fig. 1D). Additional quantitative magnetic resonance (QMR) analyses showed similar effects of mtDNA background upon fat/lean body composition on both C3H and C57 nuclear backgrounds (Supplementary Fig. S1). Mitochondrial genetic background did not have an impact on either activity or food consumption, which instead segregated with nuclear background – C57 nuclear background animals were more active and consumed less food compared to those carrying the C3H nuclear genome (Supplementary Fig. S2A–B). Similarly, energy expenditure (kcal) normalized to lean body mass also segregated with nuclear background (Supplementary Fig. S2C), although energy expenditure was inversely associated with metabolic efficiency (Supplementary Fig. S2D). When metabolic efficiency was used to normalize energy expenditure there were significant differences between both nuclear and mtDNA backgrounds (Supplementary Fig. S2E).



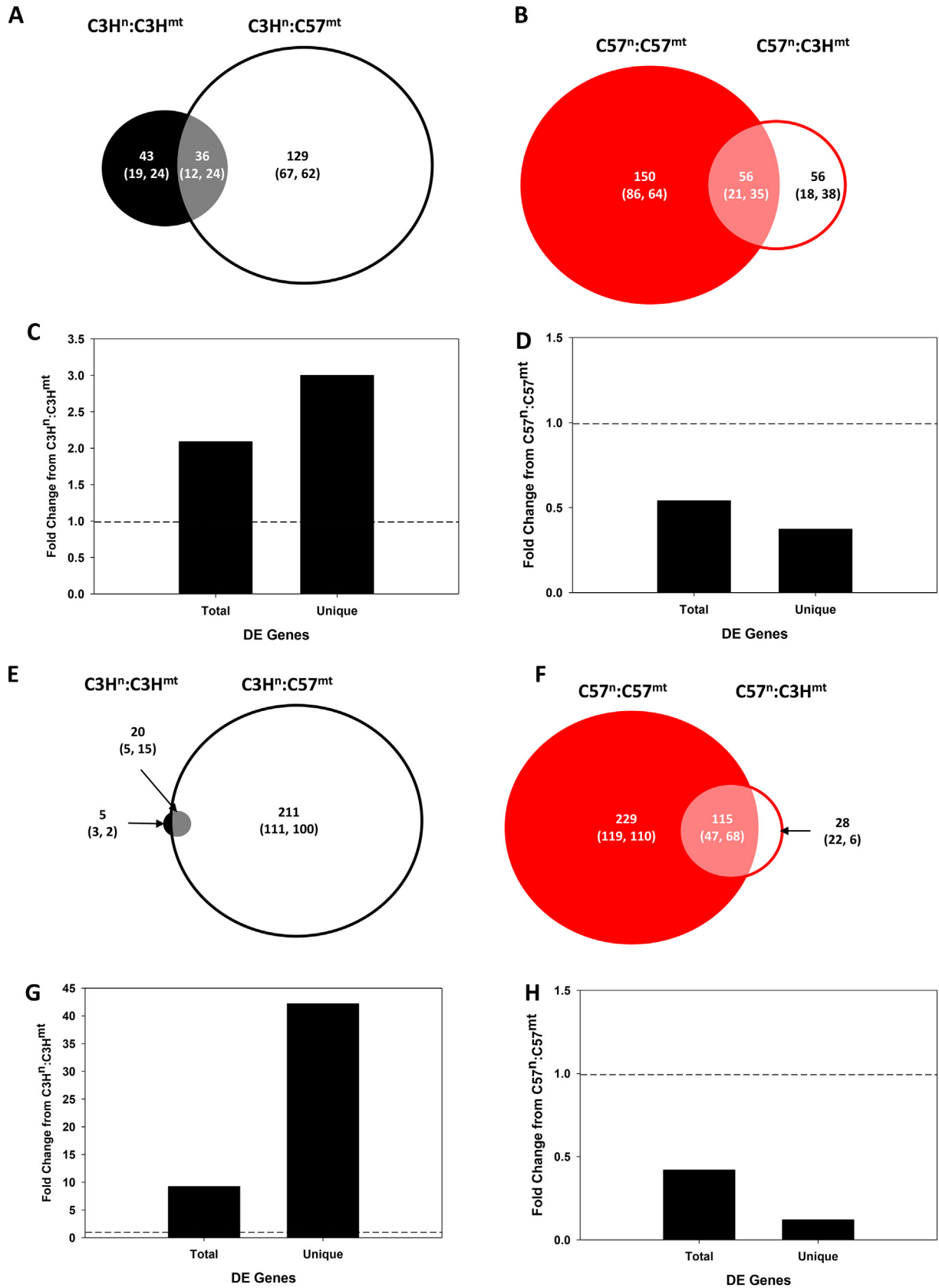
**Fig. 1.** Mice were fed chow or high fat diets from 6 to 12 weeks of age using the Comprehensive Laboratory Animal Monitoring System (CLAMS). (a) Average daily metabolic efficiency (activity/ $VO_2$  normalized to body weight) determined in C3H<sup>n</sup>:C3H<sup>mt</sup>, C3H<sup>n</sup>:C57<sup>mt</sup>, C57<sup>n</sup>:C3H<sup>mt</sup> and C57<sup>n</sup>:C57<sup>mt</sup> mice. (b) Weekly weight measures in all C3H strains fed either chow or high fat (HFD) diets. (c) Weekly weight measures in all C57 strains fed either chow or high fat (HFD) diets. (d) Percent body fat in 12 week old C3H<sup>n</sup>:C3H<sup>mt</sup> and C3H<sup>n</sup>:C57<sup>mt</sup> mice fed chow or HFD. (e) Percent body fat in 12 week old C57<sup>n</sup>:C57<sup>mt</sup> and C57<sup>n</sup>:C3H<sup>mt</sup> mice fed chow or HFD. Asterisks \* indicate a significant difference ( $p < 0.001$ ) exists ( $n = 6-13$ /group).

### 3.2. Comparative transcriptomic analysis to evaluate the role of nuclear – mitochondrial genetic background on fat metabolism gene expression

To determine the impact of different nuclear – mitochondrial genetic combinations on gene expression in response to diet for each individual strain, RNA sequence analysis was performed on epididymal and inguinal white adipose tissues (eWAT and iWAT), representing visceral and subcutaneous fat depots, respectively. For these analyses the transcriptomes of each strain fed the high fat diet and the same strain fed the chow diet (baseline) were evaluated. Initially, transcriptional changes were interrogated for 920 genes previously reported to be associated with fat metabolism (Supplementary Table S1). Fig. 2A shows that for genes reported to be associated with fat metabolism, 36 were

differentially expressed (DE) in both C3H<sup>n</sup>:C3H<sup>mt</sup> and C3H<sup>n</sup>:C57<sup>mt</sup> eWAT (e.g DE genes that were “shared” between strains having the same nuclear background), while 43 and 129 DE genes were identified as specific (“unique”, DE genes only observed with a specific nuclear – mtDNA combination) to the C3H<sup>n</sup>:C3H<sup>mt</sup> and C3H<sup>n</sup>:C57<sup>mt</sup> eWAT, respectively, on the high fat diet relative to the chow-fed baseline. Fig. 2B shows that 56 DE genes were common to both C57<sup>n</sup>:C57<sup>mt</sup> and C57<sup>n</sup>:C3H<sup>mt</sup> eWAT, with the C57<sup>n</sup>:C57<sup>mt</sup> having a greater number of unique DE genes than C57<sup>n</sup>:C3H<sup>mt</sup> eWAT (150 versus 56, respectively). Overall, these results show that the C57 mtDNA increased the number of DE genes responding to high fat diet in mice harboring the C3H nuclear genome (Fig. 2C), whereas the C3H mtDNA decreased response in animals carrying the C57 nucleus (Fig. 2D).





Analysis of iWAT yielded similar results, with increased number of DE genes linked with the C57 mtDNA relative to the C3H mtDNA in the presence of a C3H nucleus (C3H<sup>n</sup>:C3H<sup>mt</sup> versus C3H<sup>n</sup>:C57<sup>mt</sup>) showing 20 DE genes common to both strains and 5 and 211 unique DE genes specific to C3H<sup>n</sup>:C3H<sup>mt</sup> and C3H<sup>n</sup>:C57<sup>mt</sup>, respectively (Fig. 2E). On the C57 nuclear background, the C3H mtDNA decreased the number of DE genes responding to high fat diet (C57<sup>n</sup>:C3H<sup>mt</sup>, 28 unique DE genes), whereas the C57 mtDNA increased the response to 229 unique DE in C57<sup>n</sup>:C57<sup>mt</sup> iWAT, with 115 DE genes common to both strains, (Fig. 2F). As in the case with eWAT, C57 and C3H mtDNA backgrounds (Fig. 2G and H, respectively) modulated the number of DE genes in iWAT responding to high fat diet relative to their wild-type counterparts.

### 3.3. Comparative transcriptomic analysis to evaluate the role of nuclear – mitochondrial genetic background on global gene expression

To ascertain the effects of nuclear – mitochondrial genetic combinations on total gene expression in response to high fat diet relative to chow for each strain, 29,209 genes for eWAT and iWAT were compared. Fig. 3A shows that 472 DE genes were common to C3H<sup>n</sup>:C3H<sup>mt</sup> and C3H<sup>n</sup>:C57<sup>mt</sup> eWAT, whereas a total of 392 and 3313 DE genes were found specific to C3H<sup>n</sup>:C3H<sup>mt</sup> and C3H<sup>n</sup>:C57<sup>mt</sup> eWAT, respectively. A total of 1407 DE genes were found in both C57<sup>n</sup>:C57<sup>mt</sup> and C57<sup>n</sup>:C3H<sup>mt</sup> eWAT; 2875 and 1236 genes were DE on high fat relative to chow diet within C57<sup>n</sup>:C57<sup>mt</sup> and C57<sup>n</sup>:C3H<sup>mt</sup> eWAT, respectively (Fig. 3B). Fig. 3C shows that the presence of the C57 mtDNA on the C3H nuclear genetic background (C3H<sup>n</sup>:C57<sup>mt</sup>) increased the number of nuclear genes in eWAT responding to a high fat diet ~4- to 8-fold, in terms of total and unique DE genes, respectively, compared to C3H<sup>n</sup>:C3H<sup>mt</sup> animals. In contrast, the presence of the C3H mtDNA on the C57 nuclear background (C57<sup>n</sup>:C3H<sup>mt</sup>) was associated with a ~40% to 60% decrease (total and unique DE genes, respectively) in the number of nuclear genes responding to the high fat diet relative to C57<sup>n</sup>:C57<sup>mt</sup> eWAT (Fig. 3D).

For iWAT, 325 DE genes were observed in both C3H<sup>n</sup>:C3H<sup>mt</sup> and C3H<sup>n</sup>:C57<sup>mt</sup>, and 68 and 3757 nuclear genes were DE in C3H<sup>n</sup>:C3H<sup>mt</sup> and C3H<sup>n</sup>:C57<sup>mt</sup> mice fed high fat diet relative to chow diet fed mice, respectively (Fig. 3E). The effect of diet change in C57<sup>n</sup>:C57<sup>mt</sup> and C57<sup>n</sup>:C3H<sup>mt</sup> mice showed that 1781 DE genes were common to C57<sup>n</sup>:C57<sup>mt</sup> and C57<sup>n</sup>:C3H<sup>mt</sup> iWAT, and that 4272 and 419 nuclear genes were DE in C57<sup>n</sup>:C57<sup>mt</sup> and C57<sup>n</sup>:C3H<sup>mt</sup> mice fed high fat diet relative to chow diet, respectively (Fig. 3F). In iWAT, the C3H nuclear genomes harboring a C57 mtDNA (C3H<sup>n</sup>:C57<sup>mt</sup>) had an ~10- to 55-fold increase (total and unique DE genes, respectively) in the number of nuclear genes responding to the high fat diet compared to C3H<sup>n</sup>:C3H<sup>mt</sup> iWAT (Fig. 3G). By contrast, the C57 nuclear – C3H mtDNA combination was associated with decreased transcriptional response to the high-fat diet (36% and 12% of the control for total and unique DE genes, respectively; Fig. 3H) in iWAT compared to those of same nuclear background having a C57 mtDNA (C57<sup>n</sup>:C57<sup>mt</sup>).

Further analysis of the DE genes unique to each strain revealed that changes in number of DE genes were consistent among multiple subgroupings of genes determined via gene ontology analysis ((<http://www.webgestalt.org/option.php>, [46])).

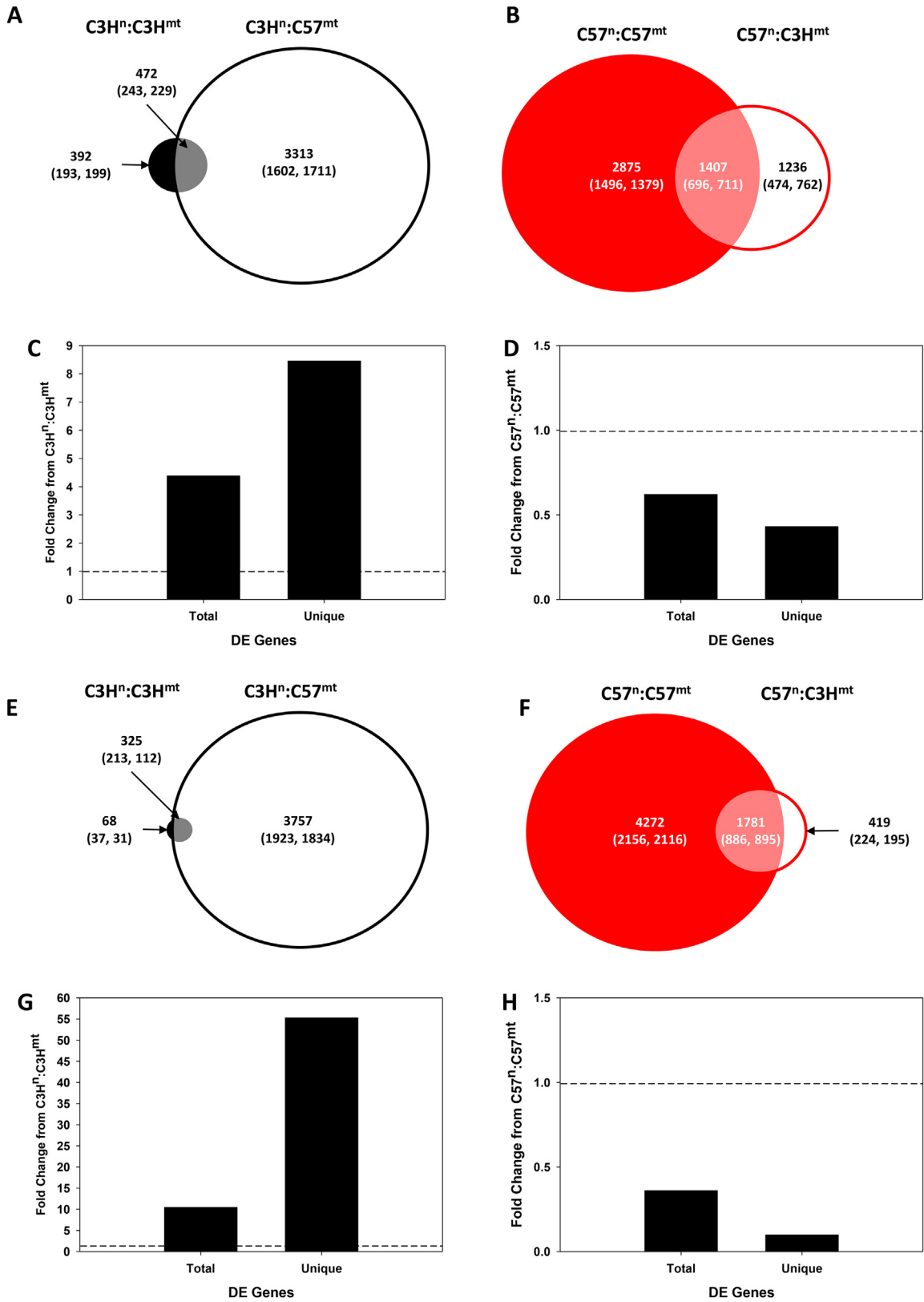
For example, analysis segregating unique DE genes by biological processes in eWAT showed that the C57 mtDNA was associated with increased numbers of DE genes responding to diet relative to the C3H mtDNA, regardless of biological process (Fig. 3I). Parallel analyses in iWAT gave similar results albeit with greater relative changes (Fig. 3J); although both adipose depots showed the same trend with respective mtDNA backgrounds. While the largest numbers of DE genes were consistently associated with metabolic processes, biological regulation, and response to stimulus, the relative differences between subgroupings were consistent between nuclear-mitochondrial genome combinations in that the C57 mtDNA was associated with enhanced DE gene numbers and the C3H mtDNA decreased numbers as shown via MNX versus wild-type ratios for eWAT and iWAT in Fig. 3K and L. These results suggest that the net differences in the number of DE genes between control and MNX mouse strains were not specifically associated with a single category of genes or pathways, but instead, imply that changes in mtDNA background had a global impact on gene expression. Similar associations were also observed using alternative grouping methods including molecular function or cellular component (Supplementary Figs. S3 and S4). Overall, the presence of the C57 mtDNA, irrespective the nuclear background, was associated with a greater number of genes responding to a high fat diet compared to a C3H mtDNA.

### 3.4. KEGG analysis to identify top pathways affected by different nuclear – mitochondrial genome combinations

To further define the potential impact of different nuclear – mitochondrial genome combinations upon cell processes, KEGG analysis was performed to identify the top 10 up- and down- regulated pathways based upon the enrichment of DE genes identified in each strain on high fat diet relative to chow fed baseline in eWAT and iWAT. Comparisons of nuclear-matched wild-type to MNX counterpart in terms of the identified KEGG pathways clearly shows that each nuclear – mitochondrial combination yields different results (Fig. 4). For example, in eWAT, 5 of the 10 upregulated pathways found in C57<sup>n</sup>:C57<sup>mt</sup> were present in C57<sup>n</sup>:C3H<sup>mt</sup> tissue (Fig. 4A). Three of these were common in all strain combinations for eWAT (regulation of actin cytoskeleton, cancer, and MAPK), meaning that of the remaining 7 identified in C57<sup>n</sup>:C57<sup>mt</sup>, only 2 (chemokine signaling and osteoclast differentiation) were specifically shared with C57<sup>n</sup>:C3H<sup>mt</sup>. Interestingly while the metabolic-biosynthesis and insulin signaling pathways were also identified in both strains, they were regulated in opposing directions (Fig. 4A). In mice sharing the C3H nuclear background, only focal adhesion pathways were specifically shared in animals having different mtDNAs (Fig. 4B, the remaining three were found in all strains, see previous), and only two downregulated pathways were common between C3H wild-type and corresponding MNX (Metabolic Pathways and Parkinson's, Fig. 4B).

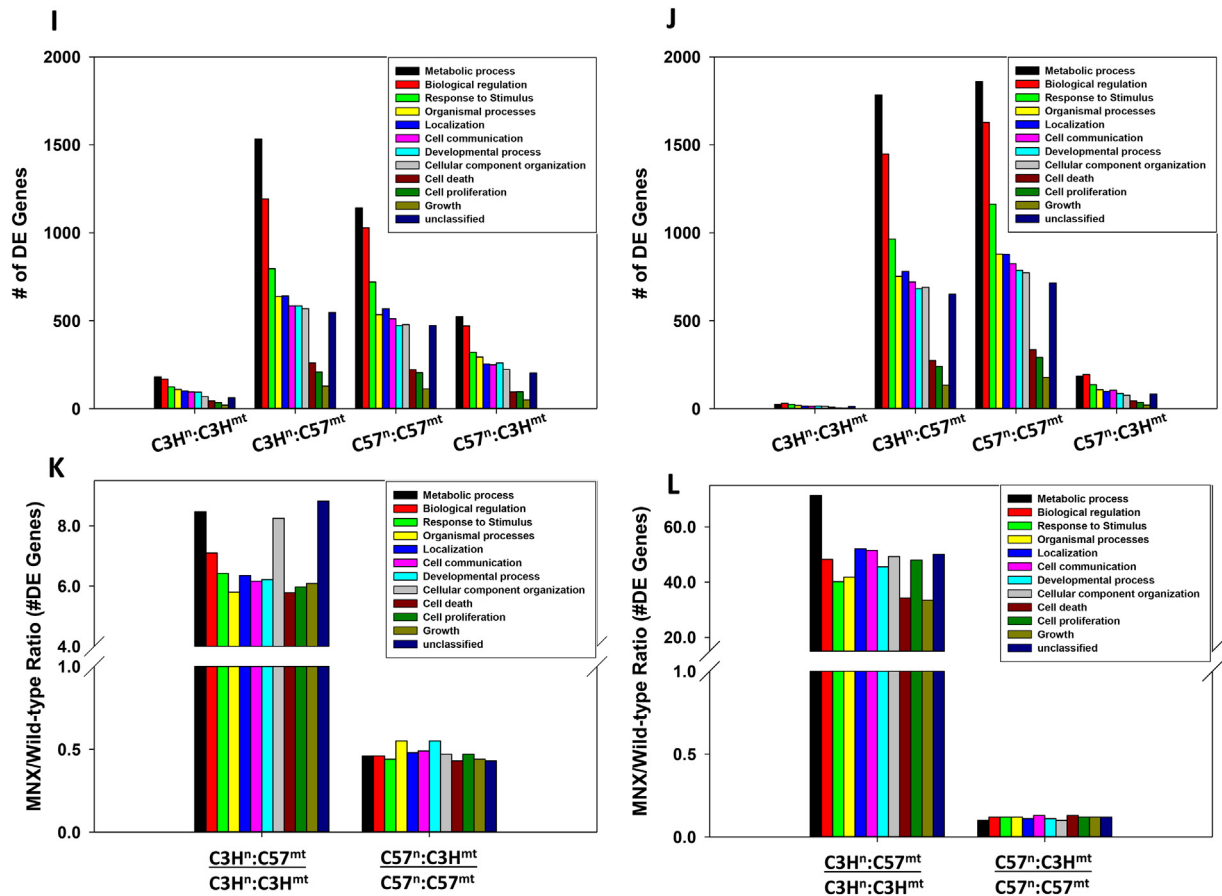
For iWAT, 6 of 10 upregulated pathways were common to both C57<sup>n</sup>:C57<sup>mt</sup> and C57<sup>n</sup>:C3H<sup>mt</sup> (metabolic pathways, focal adhesion, cancer, protein processing in the ER, endocytosis, and regulation of actin cytoskeleton; cancer pathways were common to all strains), whereas only one downregulated pathway was shared (Metabolic Pathways, Fig. 4C).

**Fig. 2.** Number of differentially expressed (DE) genes (high fat relative to chow diet) previously associated with fat metabolism for each strain of mouse (n = 4–5 mice/group). Mice were fed chow or high fat diets from 6 to 12 weeks of age and adipose tissues (eWAT and iWAT) were harvested for RNA sequence analysis. High fat diet results compared to baseline chow results for each strain. (A) Venn diagrams showing the number of DE genes in eWAT specific to nuclear – mitochondrial genome combination (C3H<sup>n</sup>:C3H<sup>mt</sup> – black area; C3H<sup>n</sup>:C57<sup>mt</sup> – white area), and common to both strains (overlapping gray area). (B) Venn diagrams showing the number of DE genes in eWAT specific to nuclear – mitochondrial genome combination (C57<sup>n</sup>:C57<sup>mt</sup> – red area; C57<sup>n</sup>:C3H<sup>mt</sup> – white area), and common to both strains (overlapping pink area). (C) Fold-change in number of total (unique + shared) and unique DE genes associated with fat metabolism in C3H<sup>n</sup>:C57<sup>mt</sup> versus C3H<sup>n</sup>:C3H<sup>mt</sup> eWAT. (D) Fold – change in number of total (unique + shared) and unique DE genes associated with fat metabolism in C57<sup>n</sup>:C3H<sup>mt</sup> versus C57<sup>n</sup>:C57<sup>mt</sup> eWAT. (E) Venn diagrams showing the number of DE genes in iWAT specific to nuclear – mitochondrial genome combination (C3H<sup>n</sup>:C3H<sup>mt</sup> – black area; C3H<sup>n</sup>:C57<sup>mt</sup> – white area), and common to both strains (overlapping gray area). (F) Venn diagrams showing the number of DE genes in iWAT specific to nuclear – mitochondrial genome combination (C57<sup>n</sup>:C57<sup>mt</sup> – red area; C57<sup>n</sup>:C3H<sup>mt</sup> – white area), and common to both strains (overlapping pink area). (G) Fold-change in number of total (unique + shared) and unique DE genes associated with fat metabolism in C3H<sup>n</sup>:C57<sup>mt</sup> versus C3H<sup>n</sup>:C3H<sup>mt</sup> iWAT. (H) Fold-change in number of total (unique + shared) and unique DE genes associated with fat metabolism in C57<sup>n</sup>:C3H<sup>mt</sup> versus C57<sup>n</sup>:C57<sup>mt</sup> iWAT.



In C3H<sup>n</sup>:C3H<sup>mt</sup> and C3H<sup>n</sup>:C57<sup>mt</sup> iWAT, 2 (chemokine and MAPK signaling) of 10 upregulated pathways were found in addition to the commonly shared cancer pathway, and only 1 downregulated pathway (insulin signaling) was identified in both strains C3H nuclear

background iWAT (Fig. 4D). Expression of DE genes associated with metabolic pathways (biosynthetic) were down and upregulated in C3H<sup>n</sup>:C3H<sup>mt</sup> and C3H<sup>n</sup>:C57<sup>mt</sup> iWAT respectively. Overall, these analyses demonstrate that different nuclear – mitochondrial genomic



**Fig. 3.** Number of differentially expressed (DE) genes associated with high fat diet relative to chow baseline within each strain of mouse (total transcriptome,  $n = 4\text{--}5$  mice/group). Mice were fed chow or high fat diets from 6 to 12 weeks of age and adipose tissues (eWAT and iWAT) were harvested for RNA sequence analysis. High fat diet results compared to baseline chow results for each strain. (A) Venn diagrams showing the number of DE genes in eWAT specific to nuclear – mitochondrial genome combination ( $C3H^n:C3H^{mt}$  – black area;  $C3H^n:C57^{mt}$  – white area), and common to both strains (overlapping gray area). (B) Venn diagrams showing the number of DE genes in eWAT specific to nuclear – mitochondrial genome combination ( $C57^n:C57^{mt}$  – red area;  $C57^n:C3H^{mt}$  – white area), and common to both strains (overlapping pink area). (C) Fold-change in number of total (unique + shared) and unique DE genes associated with fat metabolism in  $C3H^n:C57^{mt}$  versus  $C3H^n:C3H^{mt}$  eWAT. (D) Fold-change in number of total (unique + shared) and unique DE genes associated with fat metabolism in  $C57^n:C3H^{mt}$  versus  $C57^n:C57^{mt}$  eWAT. (E) Venn diagrams showing the number of DE genes in iWAT specific to nuclear – mitochondrial genome combination ( $C3H^n:C3H^{mt}$  – black area;  $C3H^n:C57^{mt}$  – white area), and common to both strains (overlapping gray area). (F) Venn diagrams showing the number of DE genes in iWAT specific to nuclear – mitochondrial genome combination ( $C57^n:C57^{mt}$  – red area;  $C57^n:C3H^{mt}$  – white area), and common to both strains (overlapping pink area). (G) Fold-change in number of total (unique + shared) and unique DE genes associated with fat metabolism in  $C3H^n:C57^{mt}$  versus  $C3H^n:C3H^{mt}$  iWAT. (H) Fold-change in number of total (unique + shared) and unique DE genes associated with fat metabolism in  $C57^n:C3H^{mt}$  versus  $C57^n:C57^{mt}$  iWAT. (I–J) Comparison of DE gene numbers from eWAT and iWAT, respectively, in each strain segregated by biological process, determined via gene ontology analysis. (K–L) Ratio of MNX/Wild-type DE gene numbers in eWAT and iWAT, respectively, segregated by biological process.

combinations modulate gene expression patterns that potentially manifest in different, and sometimes opposing biological effects or responses.

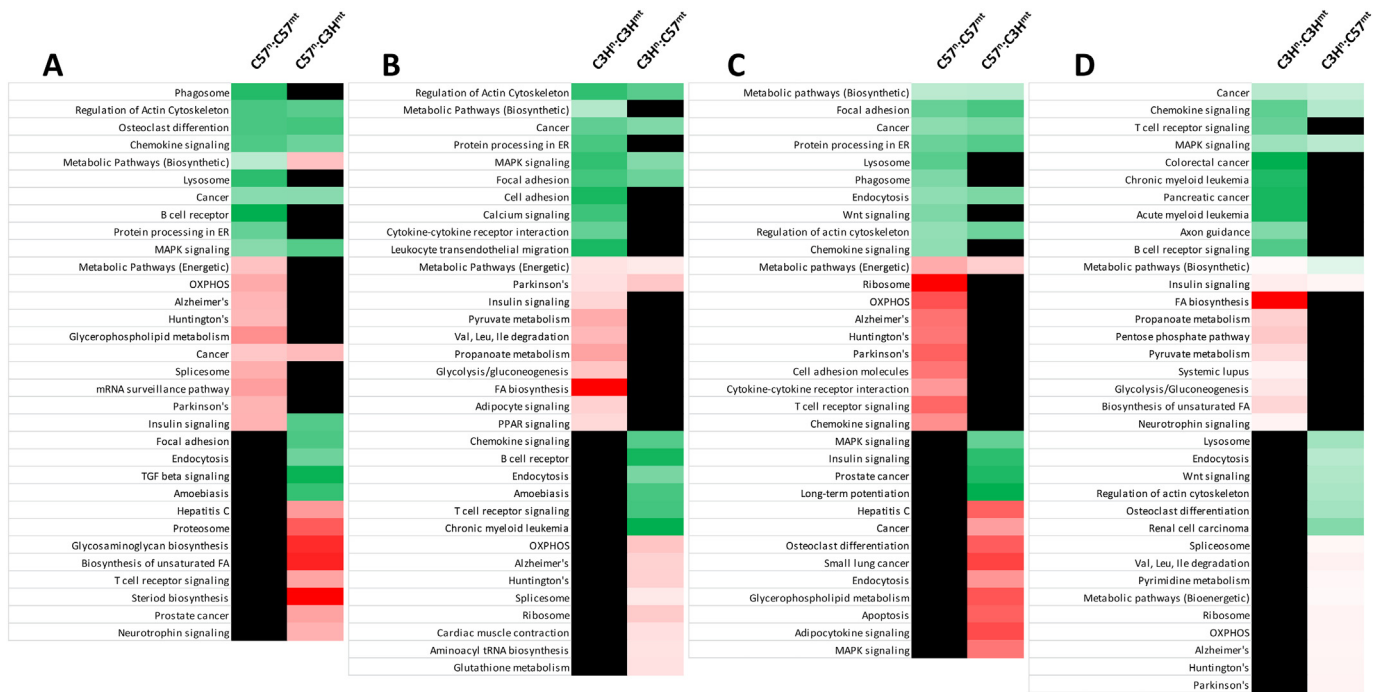
### 3.5. Estimation of genetic background effects upon gene expression

To directly assess the effects of the mtDNA upon gene expression in response to high-fat diet, we performed additional analyses using the data generated for Fig. 3 from wild -type and corresponding MNX strains. We considered DE gene responses observed in Fig. 3 to be due to effects associated with the: (i) nDNA, (ii) mtDNA, (iii) nDNA – mtDNA combination, or, (iv) factors independent of the tested genetic backgrounds (Fig. 5A). To isolate the effects of a mtDNA driven response, comparisons were made to generate respective DE gene lists linked to mtDNA effects by identifying genes common to only strains sharing the same mtDNA (Fig. 5B). Using this approach, 1178 and 1896 genes in eWAT and iWAT were shown to be influenced by mtDNA background, respectively. Fig. 5 presents heat maps for a subset of these DE genes (100 from eWAT and iWAT, Fig. 5C and D, respectively) from each of the nuclear – mitochondrial genome combinations. These results indicate mtDNA background influences

differential gene expression on the same nuclear background. Moreover, Table 1 presents an analysis of all DE genes identified in these comparative studies that examined whether their expression appeared to be defined by nuclear, mitochondrial, nuclear – mitochondrial, or independent of test genetic backgrounds. As seen in the table, for both eWAT and iWAT, the majority (~65% and 56%, respectively) of DE genes appear to be influenced by nuclear-mitochondrial background combination.

Finally, we contemplated these results in context of Mendelian, mitochondrial, or nuclear-mitochondrial genetic expectations. Conceivably, if gene expression is exclusively driven by either the nuclear or the mitochondrial genome, the anticipated outcomes of DE gene changes would segregate by either nuclear or mitochondrial genetic background. Fig. 6 illustrates hypothetical results under a model where gene expression is primarily dictated by nuclear (Fig. 6A) or mitochondrial (Fig. 6B) genomes. Our results (Fig. 6C) while perhaps a degree more similar to those predicted by Fig. 6B, do not clearly represent either extreme, suggesting that neither Mendelian nor mitochondrial genetics act in a unilateral manner, but instead work coordinately to determine the differential gene response to stimuli in adipose tissues (in this instance, a chronic high fat diet).





**Fig. 4.** Ratio of DE gene enrichment for the top 10 upregulated (green) and downregulated (red) pathways determined via KEGG analysis. Intensity represents degree of DE gene enrichment per pathway (# observed DE genes/# expected DE genes) upregulation (green) or downregulation (red) (A) Comparison of eWAT genes in C57<sup>n</sup>:C57<sup>mt</sup> and C57<sup>n</sup>:C3H<sup>mt</sup> tissues. (B) Comparison of eWAT genes in C3H<sup>n</sup>:C3H<sup>mt</sup> and C3H<sup>n</sup>:C57<sup>mt</sup> tissues. (C) Comparison of iWAT genes in C57<sup>n</sup>:C57<sup>mt</sup> and C57<sup>n</sup>:C3H<sup>mt</sup> tissues. (D) Comparison of iWAT genes in C3H<sup>n</sup>:C3H<sup>mt</sup> and C3H<sup>n</sup>:C57<sup>mt</sup> tissues. Black bars indicate pathway was not identified in top 10 pathways by KEGG analysis.

#### 4. Discussion

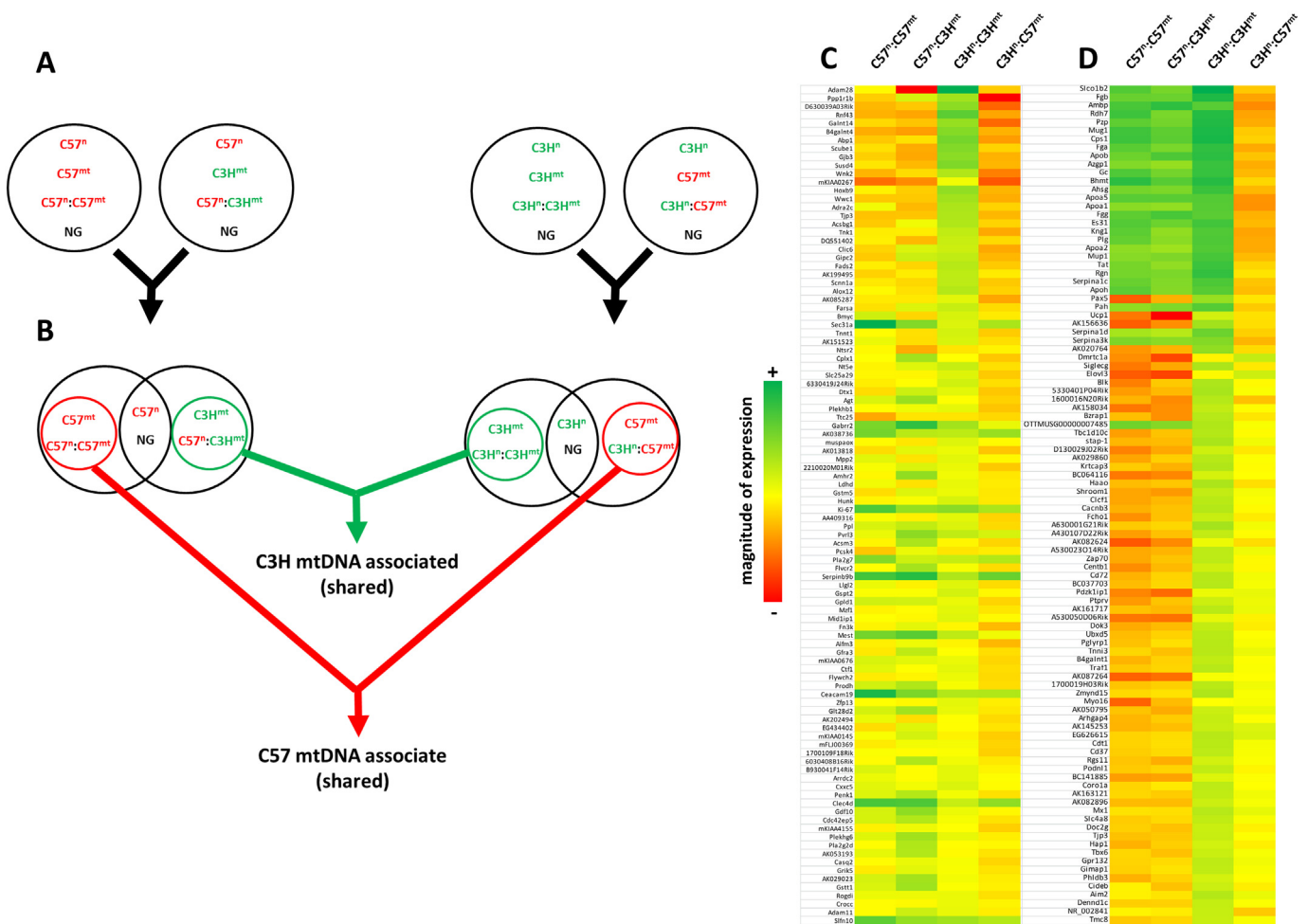
We have previously demonstrated that cellular bioenergetics and mitochondrial economy can be significantly altered by mtDNA background, and in some instances, affect disease susceptibility [11,12]. This study focused upon the effects of nuclear and mitochondrial genetic background on whole body metabolism, body composition and gene expression in adipose tissue. Our results show that animals with C57 mtDNAs are associated with greater metabolic efficiency, adiposity and transcriptional response compared to C3H mtDNA, and that these effects can be more or less pronounced depending on nuclear background. These results are clearly consistent with the notion that different nuclear-mitochondrial genetic combinations influence metabolism, adiposity, and gene expression in different ways.

Our results also show that the degree of transcriptional response influenced by the mtDNA can vary based upon the type of adipose tissue being studied, suggesting that mtDNA background can have varying effects on the number of nuclear genes differentially responding to stimuli in a location- and tissue-dependent manner. For example, the C57 mtDNA consistently associated with increased numbers of DE genes when compared to the C3H mtDNA (Figs. 2–3), irrespective of the nuclear background. The mechanisms underlying these differences are not yet known. Preliminary results suggest that the transcriptional response associated with the C57 mtDNA is associated with genes containing binding sites for transcription factors known to be redox-sensitive (e.g. *NF-κB*, *Trp53*, *SOX*, *Pou5f1*, *Smad*, *STAT*, and *Ets1*). Consequently, it is therefore possible that by changing aspects of mitochondrial function, mtDNA background modulates redox balance and/or metabolites in response to stressors or stimuli (in this case a high fat diet), thereby leading to differential activation of signaling pathways and transcription factors. Further, since most genes contain numerous binding sites for multiple transcription factors, it is possible that a “threshold” of activation exists that is uniquely gene-dependent. Whether this process is a cell autonomous process or a consequence of cell–cell communications resultant from nuclear–mitochondrial interactions is yet to be determined,

however, a combinational effect is likely, in that changes in mtDNA–nuclear genome combinations have been previously shown to alter bioenergetics at both the organelle and cellular level [11,12], and in these studies, at the level of whole body metabolism.

As previously noted, 2 non-synonymous mutations in protein coding subunits exist between the C57 and C3H mtDNA (subunit 3 in complex I and subunit 3 in complex IV) – changes in complex IV activity and overall mitochondrial bioenergetics were found when comparing the two mtDNA's on the same nuclear background, with the C57 mtDNA been linked to greater organelle economy and oxidant production relative to the C3H mtDNA [12]. Because mitochondria are multifunctional organelles, serving as sources of ATP, metabolites, heat and oxidants for a variety of cellular biosynthetic processes, features of these basic functions also enable it to participate in cell signaling (e.g. calcium regulation, apoptosis), immune response, and glucose homeostasis [6,26,27,33,36,37,41,42,47]. The electron transport chain (ETC) and citric acid cycle (CAC) are directly intertwined and at the nexus of these processes, with the former supplying energy (molecular and thermal) and oxidants, and latter providing metabolic intermediates for various anabolic, biosynthetic pathways (Fig. 7). Consequently, it is therefore reasonable to anticipate that a single, nonpathogenic mtDNA mutation (e.g. a complex IV mutation) that changes aspects of mitochondrial bioenergetics also affects both redox and interlinked metabolic signaling capacities by influencing organelle oxidant production and metabolic intermediate flux (via the CAC), which by virtue of its centrality to all cellular biochemical processes, can have far ranging impact on multiple cellular pathways. While beyond the current scope of this study, future investigations into whether mtDNA background can influence transcriptional response via differential activation of transcription factors by changing redox and metabolic intermediate levels in response to stimuli will clarify this possibility.

The etiology underlying individual susceptibility to common diseases including obesity points to a complex interaction of genetic and environmental factors. While the epidemic levels of obesity occurring within the western world are undoubtedly linked to decreased activity



**Fig. 5.** Identification of DE genes influenced by mtDNA background. (A) DE gene responses observed in Fig. 3 were assumed to be the consequence of effects associated with the: i) nDNA (C57<sup>n</sup> or C3H<sup>n</sup>); ii) mtDNA (C57<sup>mt</sup> or C3H<sup>mt</sup>); iii) nDNA – mtDNA combination (C57<sup>n</sup>:C57<sup>mt</sup>, C57<sup>n</sup>:C3H<sup>mt</sup>, C3H<sup>n</sup>:C3H<sup>mt</sup>, or C3H<sup>n</sup>:C57<sup>mt</sup>); or, iv) response independent of the tested genetic backgrounds (NG), as illustrated in the top row of Venn diagrams for each of the 4 strains. To isolate the effects of a mtDNA driven response, comparisons were initially made between each corresponding wild-type and MNX based upon nuclear background (B), to identify partitioning of DE genes based upon assumptions *i-iv*. To generate respective DE gene lists anticipated to be influenced by mtDNA effects, unique DE sets between partitions harboring only a common mtDNA (C57<sup>mt</sup> shown by red circles, C3H<sup>mt</sup> shown by green circles) were compared to generate lists associated with the C3H or C57 mtDNA. Representative heat maps for eWAT and iWAT (C) and (D), respectively, for a subset of 100 DE genes from each of the nuclear – mitochondrial genome combination representing all 4 strains of mice is presented. Each heat map represents the 100 most variable genes selected from a concatenated list of DE genes shared between mtDNA-matched mouse strains. Cell colors represent gene-specific fold-change values for each strain fed high-fat diet, relative to matched chow fed mice. Green cells represent genes upregulated in high-fat fed mice, whereas red cells represent downregulated genes.

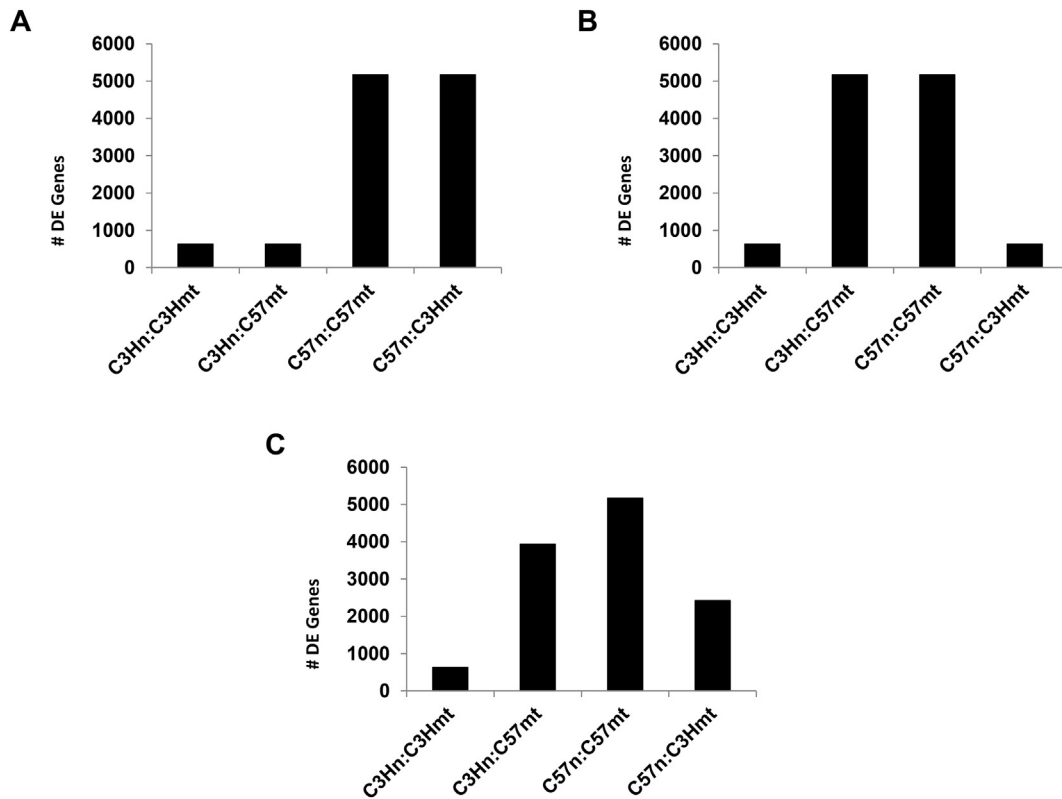
and increased caloric intake, it is clear that some individuals are more prone to adiposity than others. This differential susceptibility likely derives from both polygenic and environmental factors. For example, a large-scale meta-analysis of genome-wide association studies (GWAS) identified over 160 loci linked with adiposity and obesity [28]. While these findings are consistent with a polygenic basis for common disease susceptibility, they do not directly address the genetic mechanisms behind it. Because the genetics of the eukaryote are consequence of endosymbiotic events [29,30], it is logical to consider endosymbiotic or “nuclear-mitochondrial” genetics as a means for governing transcriptional response. Nuclear-mitochondrial interactions clearly exist;

features of anterograde and retrograde signaling have been known for some time [35], and have been investigated and discussed as a means of influencing cell function in response to significant or acute cell stress. However, nuclear-mitochondrial genetic interaction as a means for central control of “normal” function and global gene regulation has not been generally considered.

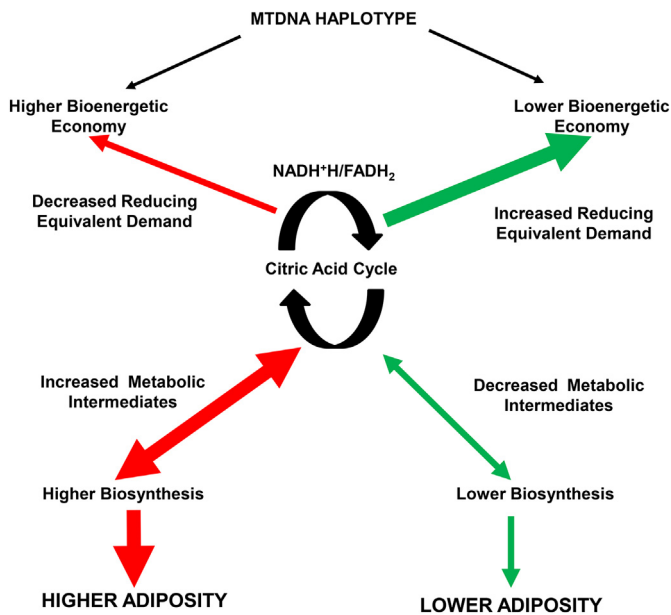
Mitochondrial genetic background-associated changes in nuclear transcriptional response are compatible with predictions from the evolutionary literature. The mutational load of mtDNA exceeds that of nDNA by an order of magnitude, and this difference is thought to have persisted for over a billion years [45]. These observations suggest that the higher mutational load of mtDNA may be compensated by some degree of transcriptional plasticity within the nucleus that involves coadaptation of specific genes that modulate biological processes through pleiotropy. Therefore, the mitochondrial genome may have evolved to modulate key aspects of organelle function to respond and meet environmental challenges that in turn activate processes that regulate nuclear gene expression as a means for adaptation. In this manner, different mtDNA backgrounds can be linked with “hyper-” or “hypo-” responsiveness to stimuli in terms of alteration of organelle function and thus, effects on nuclear gene expression, as observed in this study. In

**Table 1**  
Differentially Expressed (DE) genes in adipose tissue associated with genetic component.

Component	# DE genes (eWAT)	# DE genes (iWAT)
NG	160 (1.9%)	162 (1.9%)
Nuclear	1559 (18.7%)	1732 (20.3%)
Mitochondrial	1178 (14.1%)	1896 (22.3%)
Nuclear – mitochondrial	5460 (65.3%)	4724 (55.5%)
Total	8357 (100%)	8514 (100%)



**Fig. 6.** Bar graphs illustrating anticipated and observed patterns of DE gene response to high fat diet relative to chow baseline based upon (A) Mendelian segregation, (B) mitochondrial segregation, and (C) observed results, representing the average number of DE genes for eWAT and iWAT.



**Fig. 7.** Proposed mechanism of how mtDNA mutations can have broad impact upon multiple cellular systems. MtDNA mutations that change mitochondrial bioenergetic economy (green – high or red – low) place different demands on the citric acid cycle for providing reducing equivalents for the generation of ATP, heat, and oxidants. Consequently, mtDNA mutations that convey higher ETC-OXPHOS economy will decrease demand for reducing equivalents, whereas mutations associated with lower economy will increase reducing equivalent demand (green – decreased demand, red – increased demand) from the citric acid cycle (CAC). Changes in demand for reducing equivalents will accordingly, alter levels of available CAC metabolites/intermediates for other cellular processes (e.g. biosynthesis). By virtue of the centrality of the CAC to all cellular biochemical processes, these changes in flux, initiated by changes in mitochondrial bioenergetics by mtDNA mutation, can have far ranging impact on multiple cellular pathways.

this respect, it is plausible that the same nuclear gene polymorphism can be differentially associated with “risk” due to the coincident mtDNA background that can influence nuclear gene penetrance by affecting transcriptional response. Thus, interpretation of genetic linkage studies should also consider mitochondrial genetic background as a mitigating factor in the interpretation of their results.

These results may also influence current and future investigations into human pronuclear transfer. A recent study reported that mtDNA haplotype could profoundly affect health and longevity in mice [24], although it did not generate founder animals via pronuclear transfer or perform reciprocal nuclear-mitochondrial combinations (as herein). A similar study using a different congenic substrain of mouse observed no significant differences in reactive oxygen species (ROS) levels, body mass or longevity associated with mtDNA variation, and largely attributed this lack of differences to presence or absence of the *Nnt* (nicotinamide nucleotide transhydrogenase), and the relevance of *Nnt* and other “mitonuclear interactions” [18]. *Nnt* has been considered an attractive target of diet-induced obesity research – it was reported in 2010 that C57BL6/NJ mice are significantly more susceptible to diet-induced obesity (DIO) than their *Nnt*-mutant strain-match (the C57BL6/J), but only in response to a 10% fat diet [31]. Nicholson et al. also note that DIO developed under high-fat feeding (60% fat) regardless of *Nnt* status and that *Nnt* deficiency was, counterintuitively, mildly protective during 10% fat feeding. More recent studies have failed to confirm the in vivo relevance of this locus to metabolism and associated pathologies; a repeated direct comparison of *Nnt*-competent (C57BL/6NJ) and *Nnt*-mutant (C57BL/6J) mice confirmed no differences in diet-induced metabolic disease during 45% fat diet feeding [13]. Further, Hull et al. outlined the different effects of *Nnt* status on in vitro and in vivo systems, reporting a significant effect of *Nnt* status on insulin secretion in vitro, but no effect of *Nnt* status in vivo, effectively eliminating a causal role for *Nnt* in differential obesity susceptibility in vivo [13,21]. Regardless, both the C57<sup>n</sup>:C57<sup>mt</sup> and C57<sup>n</sup>:C3H<sup>mt</sup> mice in the study herein share the same nuclear genetic background, and therefore



the observed differences must be due to changes in nuclear-mitochondrial interactions, as reflected by the transcriptome studies. Independent of *Nnt* status, back-crossing strategies, like those conducted by Hirose and Latorre-Pellicer are limited in that they do not entirely eliminate undesired nuclear genes, and it is possible that the retained nuclear genes differ between both crosses and strains [7]. Unlike conventional conplastic approaches which involve the generation of an F<sub>1</sub> hybrid cross strain, followed by subsequent backcrossing on to the desired nuclear background, generation of MNX mice utilizes a pronuclear transfer approach capable of generating maternal founders in a single step, similar to current methods colloquially referred to as “3-parent embryos”. This approach has been already approved in the United Kingdom as a means for mtDNA gene therapy in humans [34], is being considered in the United States [8,10], and reports of the first children resulting from this and similar techniques are surfacing in the media. Evidence provided in this study suggesting that changes in mtDNA background resulting from pronuclear transfer can significantly alter body composition, metabolism and nuclear gene expression brings forward new considerations regarding the long-term health of individuals “conceived” as a 3-parent embryo, particularly female children who will, in turn, establish their own mtDNA lineage. Additional investigation into the impact of pronuclear transfer in other tissues may further elucidate the mechanism of “mito-Mendelian” genetics and the role of the mitochondrial genome in health and disease.

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### Conflicts of interest

SWB holds US patents (#9,040,771 and #9,592,306) for the MNX model.

### Author contributions

Study concept and design, KDS, MWS, MEY and SWB; Acquisition of data, KDS, MWS, RX, and WFR; Analysis and interpretation of data, KDS, MWS, RX, RJM, AP, MEY and SWB; Statistical analysis, KDS, MWS and RX; Technical support, MJS, DGW, WFR; Drafting of manuscript, KDS, MWS, RX, MEY and SWB; manuscript revisions, KDS, MWS and SWB.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2018.08.036>.

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