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Multidimensional informatic deconvolution defines gender-specific roles of hypothalamic GIT2 in aging trajectories

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ABSTRACT

In most species, females live longer than males. An understanding of this female longevity advantage will likely uncover novel anti-aging therapeutic targets. Here we investigated the transcriptomic responses in the hypothalamus – a key organ for somatic aging control – to the introduction of a simple aging-related molecular perturbation, *i.e.* GIT2 heterozygosity. Our previous work has demonstrated that GIT2 acts as a network controller of aging. A similar number of both total (1079-female, 1006-male) and gender-unique (577-female, 527-male) transcripts were significantly altered in response to GIT2 heterozygosity in early life-stage (2 month-old) mice. Despite a similar volume of transcriptomic disruption in females and males, a considerably stronger dataset coherency and functional annotation representation was observed for females. It was also evident that female mice possessed a greater resilience to pro-aging signaling pathways compared to males. Using a highly data-dependent natural language processing informatics pipeline, we identified novel functional data clusters that were connected by a coherent group of multifunctional transcripts. From these it was clear that females prioritized metabolic activity preservation compared to males to mitigate this pro-aging perturbation. These findings were corroborated by somatic metabolism analyses of living animals, demonstrating the efficacy of our new informatics pipeline.

1. Introduction

For most forms of eukaryotic life females live longer than males (Austad and Fischer, 2016). Superior female longevity has been demonstrated for a tremendous array of organisms, *e.g.* prairie-dogs, lions, red deer, short-finned pilot whales, domestic cats, domestic dogs, laboratory based mice and rats, multiple non-human primate species and humans (Allman et al., 1998; Austad, 2011; Austad and Fischer, 2016; Berg and Simms, 1960; Bronikowski et al., 2011; Clutton-Brock and Isvaran, 2007; Egenvall et al., 2000; Kasuya and Marsh, 1984; McCay et al., 1989; O'Neill et al., 2015; Sprott and Austad, 1996; Strong et al., 2013; Swindell, 2012; Turturro et al., 1999). Assessing longevity of wild, or even domesticated animals is fraught with uncontrollable variability in data collection. Monitoring of laboratory-based organisms has provided perhaps the most reliable lifespan data in regards to gender diversity: the most reliable studies to date have been conducted with both rats and mice. The NIH-National Institute on Aging Biomarkers program raised thousands of rats of two inbred strains (F344

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and Brown Norway) and one F1 hybrid in both *ad libitum*-fed and food-restricted conditions, including two different diets and found, in all cases, females lived slightly longer than males (Austad and Fischer, 2016). The best controlled murine longevity data has come from the Interventions Testing Program (ITP) that evaluates compounds that can be administered in food or water for their ability to extend mouse longevity. The ITP employed genetically heterogeneous mice and performed all experiments at three coordinated sites simultaneously. Examining the median longevity of control mice from multiple cohorts at the three sites revealed remarkable data consistency: in all cohorts, at all sites, female mice lived longer than males (Strong et al., 2013).

While laboratory rodents provide excellently controlled data, the most biomedically relevant gender-specific longevity data pertains to humans. Across diverse world populations – including many different cultural and environmental confounders - there is a near unanimity of data supporting female longevity superiority. In 176 of the 178 countries, islands, and principalities for which the United Nations currently keeps records, a female longevity advantage is evident (http://unstats.un.org/unsd/demographic/products/dyb/dyb2014.htm). In addition to this global meta-data, the Human Mortality Database (http://www.mortality.org/), comprising male/female mortality data from 38 countries, demonstrates that female life expectancy at birth exceeds male life expectancy for every year of data stored (Austad and Bartke, 2015). Gender disparities of lifespan greatly facilitate the development of novel theories concerning the complex aging process, however relatively few studies have been performed on this issue (Austad and Fischer, 2016). While multiple theories have been proposed for gender differences, many mechanistic hypotheses have focused on asymmetric inheritance of sex chromosomes, hormonal influences on inflammatory and immunological responses, differential resistance to oxidative damage, modified mitochondrial functionality and energy usage diversity. With regards to the later aspects, it has recently been shown that human female brains tend to demonstrate a more youthful metabolic functionality compared to their male counterparts (Goyal et al., 2019).

Aging is associated with an accumulation of molecular alterations, affecting nearly every tissue in the body. These alterations affect cell survival, genomic stability, gene expression, cellular replication, oxidative damage by reactive oxygen species (ROS) and protein expression/post-translational modification (Rowe and Kahn, 1987; Van Gastel et al., 2018a). The ability of the body to manage this stress (*'molecular resilience'*) degrades at a systems-wide level with increasing age due to the degradation of the efficiency of energy-generating metabolic systems and global loss of signaling complexity (Ingram and Chakrabarti, 2016; Lipsitz and Goldberger, 1992; Picca et al., 2019; Vargas et al., 2015). Disturbances of the primary energy-synthesizing system, *i.e.* mitochondrial oxidative phosphorylation, leads to ATP (adenosine trisphosphate) depletion and an increase in the harmful effects of unregulated hyperglycemia (Barzilai et al., 2012; Daum et al., 2013; Terman, 2006). Disruptions to oxidative phosphorylation have been linked to aging through the generation of glucose resistance and changes in body fat composition (Barzilai et al., 2012). Due to this incremental loss of metabolic integrity the body becomes more prone to pathophysiologies linked to energy insufficiency (Martin et al., 2015). In complex physiological systems (central nervous or endocrine), these progressive changes are likely organized by inter-connected networks of functionally related proteins. Coordination of complex systems often relies on the presence of a small number of crucial factors possessing network-controlling functions as opposed to individual dedicated functions. At a biological level, such network controllers are analogous to the 'loose connectors' proposed by Watts and Strogatz in their seminal work on communication in small-world networks (Watts and Strogatz, 1998). Our previous work has demonstrated that multi-dimensional coordinating proteins such as GIT2 may act as keystone 'master-controllers' of aging networks (Chadwick et al., 2012). Healthy aging relies on coordinated hormonal/neuronal communication between the central nervous system and the periphery (Chadwick et al., 2012; Mielke and Wang, 2011; Van Gastel et al., 2018a). The hypothalamus is one of the most important structures mediating efficient interactions between energy balance and neurological systems. This crucial brain region regulates multiple metabolic pathways by synthesizing and secreting numerous neuro-hormones that regulate the secretion of trophic hormones from the anterior pituitary and helps maintain the fidelity of 'neurometabolic' network communication during the aging process (Chadwick et al., 2012; Conrad and Bimonte-Nelson, 2010; Frolkis et al., 1972).

An individual's aging trajectory is a complex sum of multiple synergistic and interconnected molecular pathologies including mitochondrial dysfunction, alterations in metabolism and nutrient sensation, dysfunctions in DNA repair, chronic inflammation, attenuated stress responses and the accumulation of oxidative damage (Lopez-Otin et al., 2013; van Gastel et al., 2018a, b; Leysen et al., 2018). Given these widespread aging-related activities, it is not surprising that the interaction between these molecular disruptions appear to underpin nearly all forms of major human disease (Colman et al., 2009; Figueira et al., 2016; Franceschi et al., 2018; Perkins et al., 2018; Siddiqui et al., 2017). One of the most penetrant pro-aging perturbations is the increase in oxidative damage linked to metabolic disruption. Prematurely elevated ROS levels lead to increased functional stress on DNA damage repair (DDR) systems resulting in genomic frailty (Van Gastel et al., 2018a). Reinforcing its role in aging, GIT2 has been shown to be highly sensitive to temporal aging and metabolic stressors such as ROS (Chadwick et al., 2012, 2010). GIT2 was first identified as a multifunctional G protein-coupled receptor (GPCR) interacting protein (Premont et al., 1998, 2000), but has subsequently been shown to regulate aging via its ability to coordinate somatic responses to energy deficits, regulate DDR activities, control immunosenescence and manage ROS-related stress (Chadwick et al., 2012; Lu et al., 2015; Martin et al., 2015). Hence, GIT2 forms a link between age-related cellular damage and the maintenance of DNA repair/stability, a process crucial to successful and healthy aging ((Madabhushi et al., 2014; Lu et al., 2015; Martin et al., 2015; Van Gastel et al., 2018a). In the DDR paradigm GIT2 forms functional complexes with DDR proteins such as MRE11, γ-H2AX and ATM and directly promotes DNA repair by facilitating the regulation and stabilization of repair proteins, such as BRCA1, through a poly(ADP-ribose) (PADPR) polymerase (PARP)-linked process (Lu et al., 2015). Several stress-associated GIT2-interacting DDR proteins (ATM, BRCA1, p53) also play important roles in circadian clock regulation (Gery et al., 2006; Kowalska et al., 2013; Miki et al., 2013; Storcelova et al., 2013; Vieira et al., 2014). This cellular clock regulation plays an important role in the aging process, as it regulates chronological aging by conferring daily rhythmicity to physiological functions (Hastings and Goedert, 2013; Reppert and Weaver, 2002; Sancar, 2004; Takahashi et al., 2008). Thus an evolutionary synergy exists between clock genes and DDR proteins (Uchida et al., 2010). Recent data has shown that genomic deletion of GIT2 affects cellular functions related to the circadian rhythm, *i.e.* diurnal/nocturnal metabolic changes (Martin et al., 2015) and immune cell activity (Siddiqui et al., 2017). Hence, cellular clock mechanisms not only control the chronological aging of cellular tissue but also regulate DNA damage, telomeric stability and metabolic activities (Collis and Boulton, 2007; Kagawa, 2012; Khapre et al., 2010). In this context GIT2 is linked to the insulinotropic control of energy metabolism and its age-related dysfunction. Genomic deletion of GIT2 results in a disruption of the beta cell mass, alpha cell involution as well as reduced plasma insulin levels and insulin resistance (Martin et al., 2015). A significantly reduced respiratory exchange ratio (RER) was also observed at a relatively young age (4 months) indicating that a switch occurred, away from glucose towards adipose and then, potentially, protein to generate energy. Hence, the transition from 'healthy' to 'pathological' aging may correspond with a significant downshift in RER as nearly all the processes engendered by energy insufficiency result in pathological loops (Belikov, 2019; Martin et al., 2015). More recently GIT2 has also been

associated with cellular senescence and the creation of pro-inflammatory senescence-associated secretory phenotype (SASP: Siddiqui et al., 2017). This study revealed an inter-related group of GIT2-associated factors connected to senescent/DNA stability functionality (Siddiqui et al., 2017), *e.g.* high mobility group (HMG) proteins (Lu et al., 2015; Siddiqui et al., 2017), which are stress-sensitive DNA-modulatory factors involved in transcription/translation and DNA repair activities. HMG proteins also control GIT2 functions in DDR responses, in addition to PARP activity modulation (Lu et al., 2015; Masaoka et al., 2012). HMGB2 is critically involved in regulatory mechanisms involved in DNA damage (Syed et al., 2015) and senescence control (Biniossek et al., 2013), further strengthening the relationship between GIT2 and cell senescence.

In our current study we have specifically attempted to uncover potential GIT2-associated functions that may underpin superior female longevity. We have identified at a very early life stage (2 months old) distinctions in potential anti-aging protective behavior before the onset of any significant lifestyle-induced aging trajectory variations. This suggests a pre-programmed enhanced 'training-like' resilience in females compared to males. Hence, female mice demonstrate a reactive ability to modify their hypothalamic status to nullify their experience of stressful perturbations, similar to a well-trained athlete compared to a non-trained subject. Our data suggests that there is an innate resilience distinction in females that is related to their ability to sense stress and modify both their metabolic function and circadian rhythms to ensure healthy aging. The comparison between male and female mice in this report is of great potential therapeutic importance as previously a gender-specific hypothalamic response was seen to variations in dietary energy intake such as caloric restriction an intermittent fasting (Martin et al., 2008) that have been demonstrated to significantly attenuate the generation of age-related disease (Colman et al., 2009; Johnson et al., 2007; Martin et al., 2006). We hypothesize that GIT2 may serve as a functional channel between cellular senescence, clock regulation and DNA damage, and thus could have the capability to powerfully modify the accumulation of age-related cellular damage. Our data here suggests that GIT2 likely plays a crucial role in preparing an organism for the aging process in a gender-dependent manner. Our research indicates that the stress sensor functionality of GIT2 is more important in females compared to males and may indeed act as one of the key factors in controlling healthy aging trajectories.

2. Materials and methods

2.1. Experimental animal models

GIT2 gene-trap heterozygous mice (GIT2-HET) were originally obtained from Dr. Richard T. Premont (Duke University Medical Center, Durham, NC, USA) and genotyped by genomic PCRs as previously described (Schmalzigaug et al., 2007). PCR amplification was performed using standard protocols. Primers used to screen the GIT2-HET mice were as follows: forward primer 5′-TCTCCTGGAACTCAGGGATT, reverse primers (wild type, WT) 5′-CATTTCAGAGTCTGCTGCCTTA 1 and (HET) 5′-GGCTACCGGCTAAAACTTGA. WT (C57BL/6) and GIT2-HET mice were group-housed (genotypes were housed separately) in temperature-controlled (22 °C) and humidity-controlled (45%) rooms with a 12 h (hr) light–dark cycle with food and water available *ad libitum*. All experimental mouse models were fed using a standard control animal chow containing 19% protein, 64% carbohydrates, and 17% fat (diet #101845 from Dyets Inc., Bethlehem, PA, USA). Experiments were performed during the light phase of the light–dark cycle in accordance with NIH guidelines. Animal care and experimental procedures followed NIH guidelines and were approved by the National Institute on Aging Animal Care and Use Committee (433-LCI-2015).

2.2. Whole somatic metabolic and behavioral analyses

Murine behavior and metabolic parameters were measured, over a 48 h period, using a Columbus Instruments Comprehensive Lab Animal Monitoring System (CLAMS). The CLAMS system was used to assess X, Z total activity (counts), O_2 consumption (milliliter/kilogram/hr), CO_2 production (milliliter/kilogram/hr), total energy expenditure (TEE: kcal/hr/kg), heat production (kcal/hr) and respiratory exchange ratio (RER = VCO_2/VO_2), as described previously with modifications (Cai et al., 2013b; Kennedy et al., 2007; Shin et al., 2009). Eight mice per group were single housed for 48 h in the system. The mean weight of WT-control mice (male – 32.45 ± 0.68 g; female – 24.36 ± 0.44 g) was slightly less but not significantly different from the GIT2-HET counterparts (male – 37.06 ± 1.17 g; female – 27.44 ± 0.99 g). Statistical analysis was performed using a two-tailed Student's *t*-test, and $p \leq 0.05$ was considered statistically significant.

2.3. Western blotting

Murine hypothalami were extracted through expert micro-dissection. Protein extraction was performed using the Qproteome® extraction kit (Qiagen, Valencia, CA USA) following the manufacturer's instructions. Allowing extraction of the differential cellular compartments (cytoplasm, plasma membrane, nuclear/large organelle, and cytoskeleton). All protein extracts were quantified using BCA reagent (ThermoScientific, Rockford, IL, USA) and then normalized for each specific experiment before resolution with SDS-PAGE and semi-dry electrotransfer (Bio-Rad, Hercules, CA, USA) to PVDF membranes (PerkinElmer Life Sciences; Waltham, MA, USA). Membranes were blocked using a 4% BSA for Western blot as described previously (Chadwick et al., 2011), and primary antibody immune-reactive complexes were identified using alkaline phosphatase-conjugated secondary antisera (Sigma-Aldrich, St. Louis, MO, USA) with enzyme-linked chemifluorescence (GE Healthcare) and visualized with a Typhoon 9410 phosphorimager (GE Healthcare). Proteins were identified using primary antisera at 1:1000 to 1:10 000 dilutions, followed by species-specific alkaline phosphatase-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA) at 1:7000 dilution. Primary antibodies specific for vacuolar protein sorting-associated protein 33A (Vps33a: Abcam ab88254), lysophospholipid acyltransferase 2 (Mboat2: ThermoFisher Scientific PA5-53238) and thioredoxin interacting protein (Txnip: Cell Signaling Technology mAb #14715) were obtained from the identified sources and employed according to the manufacturer's instructions.

2.4. RNA extraction and oligonucleotide microarray hybridization and analysis

RNA isolation from three animals in each experimental genotype and gender group was carried out using the Qiagen RNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA), as described previously (Gesty-Palmer et al., 2013). RNA conversion to cDNA and subsequent hybridization with Sentrix MouseRef-8 Expression BeadChips (Illumina, San Diego, CA, USA) was performed as described previously (Chadwick et al., 2010; Martin et al., 2012). Microarray data were analyzed using DIANE 6.09, a spreadsheet-based microarray analysis program based on the SAS JMP7.0 system10. Raw microarray data were subjected to filtering and z-normalization and tested for significant changes as described previously (Chadwick et al., 2010). Initial filtering identified genes with a z-ratio of ≥ ± 1.50, with the z-ratio being derived from the difference between the averages of the observed gene z-scores divided by the standard deviation of all of the differences for that particular comparison. Genes were then refined by calculating the False Discovery Rate (FDR), which controls for the expected proportion of falsely rejected hypotheses, and including only those genes with FDR < 0.05. These data were further analyzed using analysis of variance

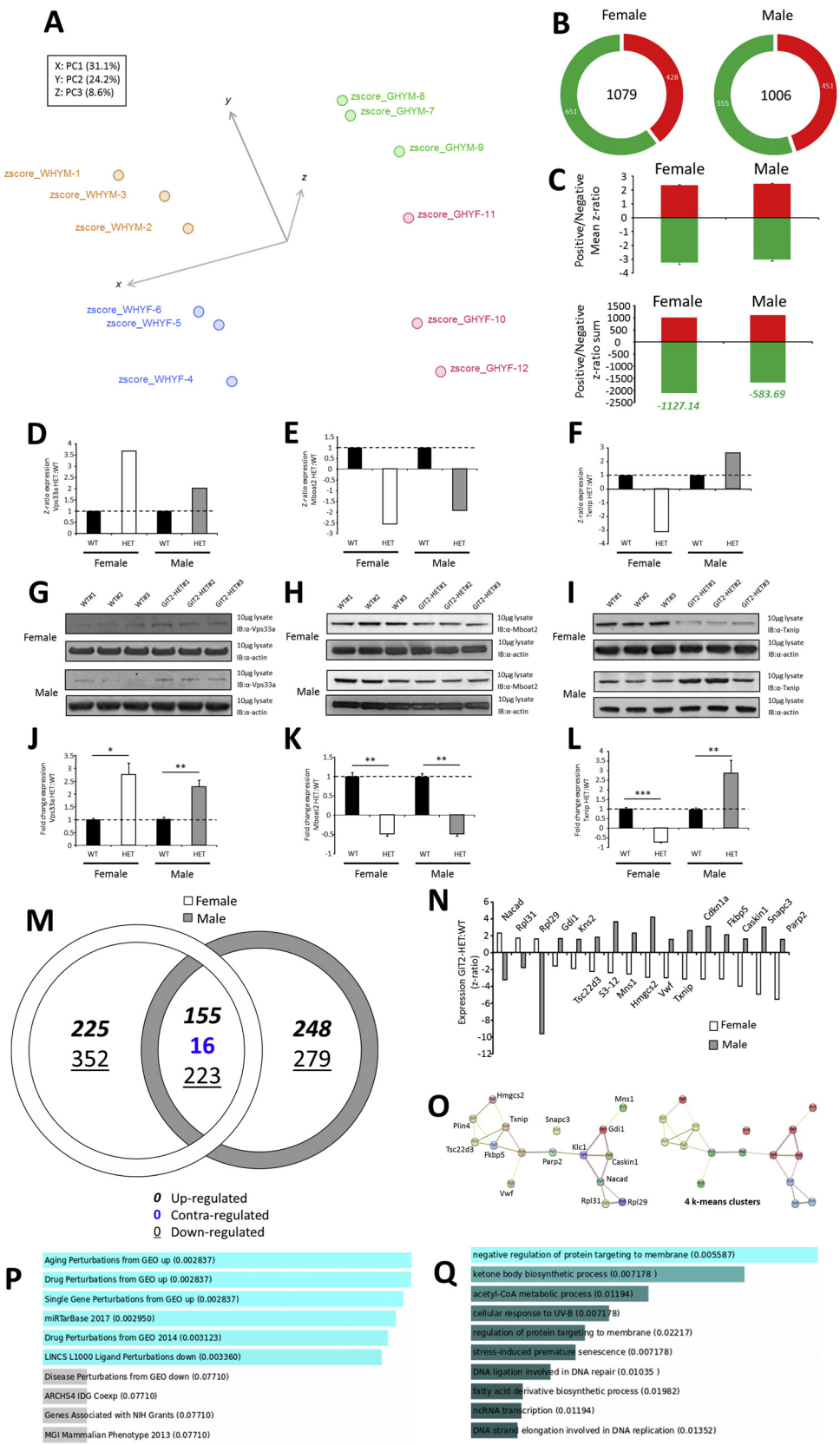


Fig. 1. Transcriptomic microarray analysis of male and female GIT2-HET mice compared to their relative wild type (WT) gender-specific controls. A – 3-dimensional Principal Component Analysis plot for triplicates of WT female hypothalamus (WHYF), WT male hypothalamus (WHYM), GIT2-HET female hypothalamus (GHYF) and GIT2-HET male hypothalamus (GHYM) transcriptomic data. B - Total number of up- and down-regulated transcripts (upregulated denoted in red, downregulated in green) in female and male GIT2-HET mice compared to WT controls. C - Comparison between female and male GIT2-HET transcript z-ratio scores. The upper panel indicates the average of the upregulated or downregulated z-ratios for female and male GIT2-HET animals. Lower panel demonstrates the total sums of upregulated or downregulated transcripts for female or male GIT2-HET mice. Individual z-ratio transcript profiles for Vps33a (D), Mboat2 (E) and Txnip (F) in female/male GIT2-HET. Selective western blot validation for male/female GIT2-HET expression differential to WT controls of Vps33a (G), Mboat2 (H) and Txnip (I). Statistically significant alteration of mean protein expression for female/male GIT2-HET Vps33a (J), Mboat2 (K) and Txnip (L) compared to WT controls. M – Numerical venn-based separation of up- or downregulated transcripts between female (white circle) or male (grey circle) GIT2-HET hypothalamic transcriptomes. Proteins common but regulated in opposite polarities between genders are indicated in blue (contra-regulated). N – Individual proteins significantly-regulated and common between females (white bars) and males (grey bars) that possess gender-specific polarities. O – STRING-based informatic network of 16 differentially-regulated proteins shared between genders. The generated network possesses a protein-protein interaction enrichment p-value of 0.00236. The left panel indicates the individual protein names while the right panel indicates grouping of the interacting proteins based on k-means clustering into four groups. P – Enrichr-based Most-Popular Gene Library analysis of the 16 differential gender-specific proteins common to female and male GIT2-HET hypothalamic transcriptomes. For each bar the enrichment probability is indicated in parentheses. Q – Enrichr-based Gene Ontology annotation of the 16 differential gender-specific proteins common to female and male GIT2-HET hypothalamic transcriptomes. For each bar the enrichment probability is indicated in parentheses.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with significance set at $p < 0.05$. This allowed us to identify transcripts that differed in their intensity across all of the animal replicates and the various experimental conditions of the mice employed in this study.

2.5. Bioinformatic analyses

Microarray-derived gene transcript lists were initially separated into specific expression polarity regulation groups using VennPlex venn

diagram analysis (Cai et al., 2013a) before Gene Ontology (GO; biological process) and signaling pathway/upstream analysis annotation using the ToppGene ToppFun annotation suite (https://toppgene.cchmc.org/) and Ingenuity Pathway Analysis (IPA) respectively. Predicted protein interaction networks were derived using STRING (https://string-db.org/) and were subsequently interrogated for functional ramifications using Enrichr-based GO and Enrichr Library analysis (http://amp.pharm.mssm.edu/Enrichr/). Textrous!-based natural language processing (Chen et al., 2013b), using both latent semantic indexing (LSI)-based Collective and Individual processing modes, was employed to derive biomedically relevant Gene Symbol – noun-phrase associations for significantly-regulated transcriptomic datasets. For both GO and IPA Canonical Signaling Pathway (CSP) analysis, we employed a cutoff of at least two transcripts (from the original filtered/analysis of variance datasets) needing to be present to minimally populate a particular GO term group or canonical IPA pathway, with a probability (p) of enrichment value of ≤ 0.05. LSI-based analysis was performed as described previously using Textrous! (Chen et al., 2013b; Martin et al., 2015; Siddiqui et al., 2017). Textrous! as well as other LSI-based informatics platforms (*e.g.* GeneIndexer), correlate the strength of association, (Chadwick et al., 2012, 2010) between specific transcripts in a dataset with user-defined interrogation terms. Textrous! is able to investigate connections between input transcripts/proteins (using input Gene Symbols) and biomedical words and more syntactic noun-phrases using complete extracted data (in the form of curated gene-word documents) from PubMed Central (https://www.ncbi.nlm.nih.gov/pmc/), OMIM (Online Mendelian Inheritance in Man: https://www.omim.org/), and the Mammalian Phenotypes Database at the Jackson Laboratories Mouse Genomic Informatics portal (http://www.informatics.jax.org/vocab/mp_ontology). For Textrous!-based natural language processing, the possible LSI correlation scores (termed Cosine Similarity Scores) for a gene/protein to be associated with an input interrogation term range from 0 to 1, with the stronger correlation scores approaching 1. A correlation score of ≥ 0.1 indicates at least an implicit correlation, between the specific gene and the user-defined input interrogation term (Xu et al., 2011). To generate more data-specific high-dimensionality data annotation for Textrous!-based text outputs, significant data wordclouds can be created from the Textrous!-based semantic noun and noun-phrase outputs using the web-based JasonDavies wordcloud generator (https://www.jasondavies.com/wordcloud/). The text size within the word clouds is directly proportional to the input word frequency. Numerical analyses of word-phrase frequencies from Textrous! noun-phrase wordcloud outputs were made using WriteWords (http://www.writewords.org.uk/) frequency calculator (Martin et al., 2015). The use of freeform quantitative wordclouds to convey complex non-canonical signaling-activity relationships is becoming more and more recognized as a novel technique to investigate high-dimensionality data (Baroukh et al., 2011; Cheung et al., 2012; Lynch et al., 2015).

2.6. Statistical analyses

In each histogram or figure, data represent the means ± SEM (standard error of the mean). Statistical analyses (Student's *t*-test) were performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). Significance level is indicated in each figure as $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

3. Results

3.1. GIT2 heterozygosity induces profound transcriptomic responses in female and male hypothalami

Compared to WT counterparts both female and male GIT2-HET hypothalamic transcriptome datasets demonstrated a clear spatial separation, as well as a clear female-male principal component analysis-based distinction (Fig. 1A). In both male and female mice at 2 months of age there were numerous significant alterations in hypothalamic transcriptomic expression patterns, hence 1079 and 1006 significantly altered mRNA transcripts were identified in female and male GIT2-HET mice, respectively (Fig. 1B: Tables S1-female and S2-male). Generating an average transcript z-ratio across these transcripts, it was clear that the degrees of transcript upregulation or downregulation (mean z-ratios of all significant transcripts) between females and males were similar (Fig. 1C-upper panel). With respect to the degree of global expression alterations it was evident that despite a relatively similar total number of significantly altered transcripts between females and males (Fig. 1B and C) there was a global z-ratio downregulation bias amongst the female transcriptomes compared to the males (Fig. 1C-lower panel). To verify that our transcriptomic analyses were predictive of protein expression-level responses we chose three different hypothalamic transcriptomic responses (Fig. 1D–F; Vps33a, Mboat2 and Txnip) to correlate to actual protein expression distinctions (Fig. 1G–I). From our protein expression level analyses (Fig. 1J–L) there was a clear correlation between the effects of GIT2 heterozygosity upon both transcriptomic and protein expression data (Vps33a – female/male elevation; Mboat2 female/male diminution; Txnip female down-/male upregulation).

Differential Venn analysis of female and male transcriptomes revealed a predominant gender-based dependence of responses to GIT2 heterozygosity – 53.4% of the female, and 52.8% of the male transcriptomic responses were unique to each gender (Fig. 1M: Table S3 in Supplementary material). Despite this gender divergence in transcriptomic response commonalities were evident, with 155 upregulated and 223 transcripts downregulated coherently (sharing the same expression polarity) in both genders (Fig. 1M). Sixteen transcripts common to both genders possessed distinct expression polarities (Fig. 1N). This small data corpus however was shown to form a tight coherent functional network (Fig. 1O: Average Node Degree = 2.62; protein-protein interaction enrichment p-value = 0.00236) centered upon the DNA repair protein Parp2 (Poly [ADP-ribose] polymerase 2). Employing further Enrichr-based informatic analysis of this small data set we found a firm correlation of this gender-specific 16-transcript data set with GEO (Gene Expression Omnibus)-based Aging Perturbations (Fig. 1P) as well as significant GO correlations with stress-associated metabolic and senescent activities (Fig. 1Q).

3.2. Female-centric functional diversity of the hypothalamic transcriptomic response to GIT2 heterozygosity

3.2.1. Gene ontology clustering

We initially demonstrated at a rudimentary total z-ratio sum level (Fig. 1C), that the female transcriptomic response to GIT2 heterozygosity was distinct to that of the male, *i.e.* showing a bias towards overall transcript downregulation compared to males. Using classical transcriptomic analyses GO annotation and IPA CSP Analysis (Fig. 2) we again found that at a functional level the female hypothalamic responsivity to the heterozygous reduction of GIT2 levels was more profound than that of the males. With respect to GO (biological process) analysis we found that approximately 88% of the significantly populated GO term groups for the female/male data were uniquely-associated with female mice (Fig. 2A: Table S4 in Supplementary material). Given our previous implication of GIT2 in the pathological aging process, it was interesting to note that GO analysis of the male and female hypothalamic transcriptomes in early life revealed a potent gender-specific phenotype (Fig. 2B and C) that mirrors the global female longevity superiority described previously. Hence, significantly-populated GO term biological process groups in females were focused on aging, DNA damage and senescence (*cellular senescence* GO:0090398; *aging* GO:0016280; *intrinsic apoptotic signaling pathway in response to DNA damage* GO:0008630) while significantly-populated male GIT2-HET term groups were associated with neuronal atrophy, apoptosis and

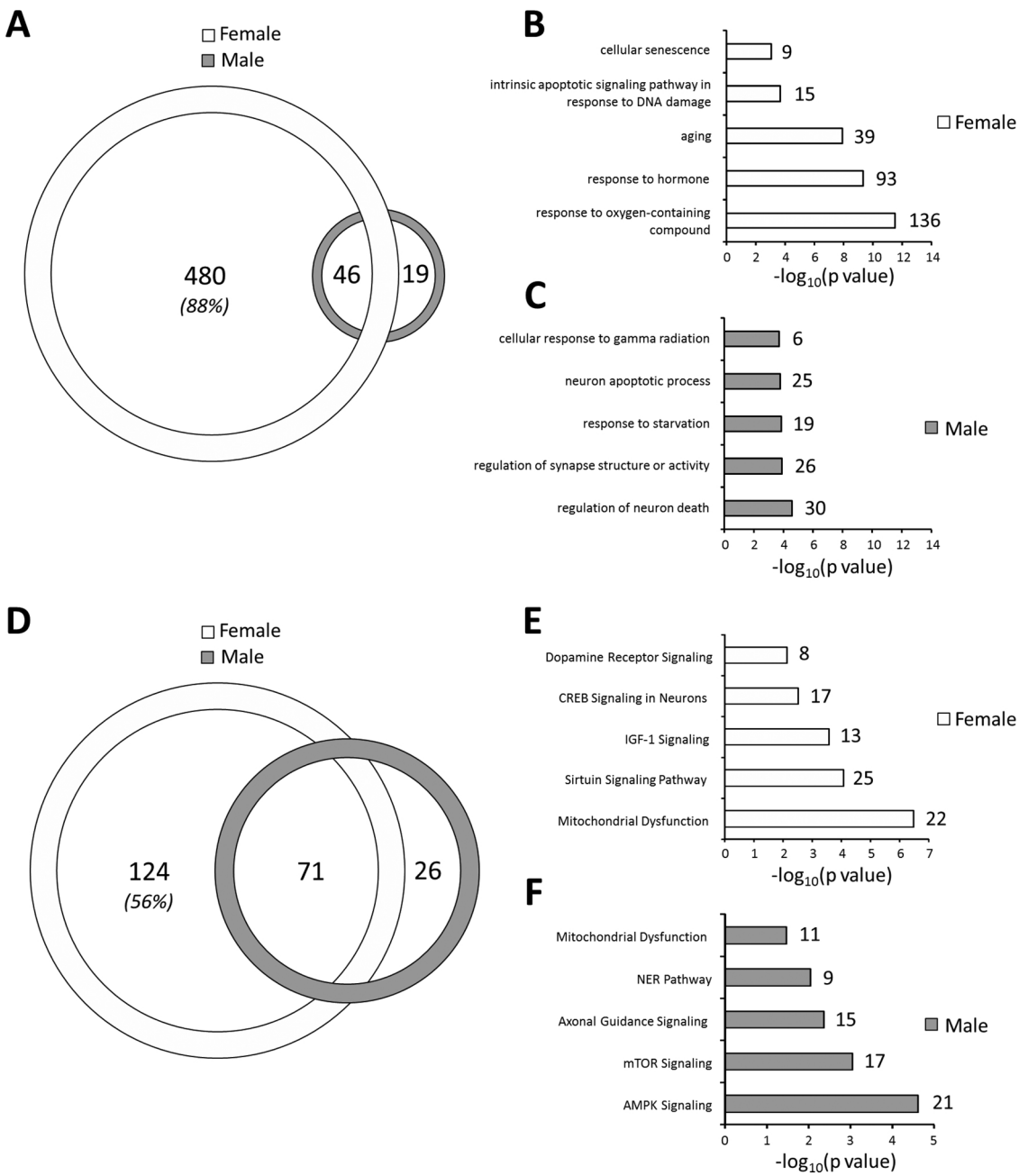


Fig. 2. Gene Ontology and IPA Canonical Signaling pathway annotation of female and male GIT2-HET transcriptomic data. A – Proportional venn diagram separation of female (white circle) and male (grey circle) Gene Ontology terms significantly ($p < 0.05$) populated by female or male GIT2-HET hypothalamic transcriptomic data. B – Selected specific Gene Ontology term groups significantly populated ($p < 0.05$) by female GIT2-HET transcriptomes. C - Selected specific Gene Ontology term groups significantly populated ($p < 0.05$) by male GIT2-HET transcriptomes. D – Proportional venn diagram separation of female (white circle) and male (grey circle) IPA-derived canonical signaling pathways significantly ($p < 0.05$) populated by female or male GIT2-HET hypothalamic transcriptomic data. E – Selected specific canonical signaling pathways significantly populated ($p < 0.05$) by female GIT2-HET transcriptomes. F - Selected specific canonical signaling pathways significantly populated ($p < 0.05$) by female GIT2-HET transcriptomes.

synaptic disruption (*neuron apoptotic process* GO:0051402; *regulation of synapse structure or activity* GO:0050803; *regulation of neuron death* GO:1901214). Thus, it again appears that disruption of GIT2 levels in the female hypothalamus at an early age induces a greater response resonance with respect to aging-related activities than males at the same age.

3.2.2. Signaling pathway analyses

To reinforce our GO-based findings (Fig. 2A–C) we also compared the relative individual CSP annotation outputs for the GIT2-HET hypothalamic transcriptomes (Fig. 2D–F). Similar to the GO-based annotation bias towards the female response to heterozygosity again there was a considerable female pathway annotation bias, *i.e.* 56% of the total significantly populated IPA CSPs were uniquely attributed to female GIT2-HET mice. In comparison, only 11.7% of the hypothalamic transcriptome-derived signaling pathways significantly populated were unique to the male tissues (Fig. 2D: Table S5 in Supplementary material). As with our previous GO annotation we found a robust aging-metabolism phenotype in the female GIT2-HET transcriptome data (‘*IGF-1 Signaling*’, ‘*Sirtuin Signaling Pathway*’, ‘*Mitochondrial Dysfunction*’: Fig. 2E). Male GIT2-HET transcriptome pathway analysis also revealed age-related (‘*mTOR Signaling*’, ‘*AMPK Signaling*’) functions as well as

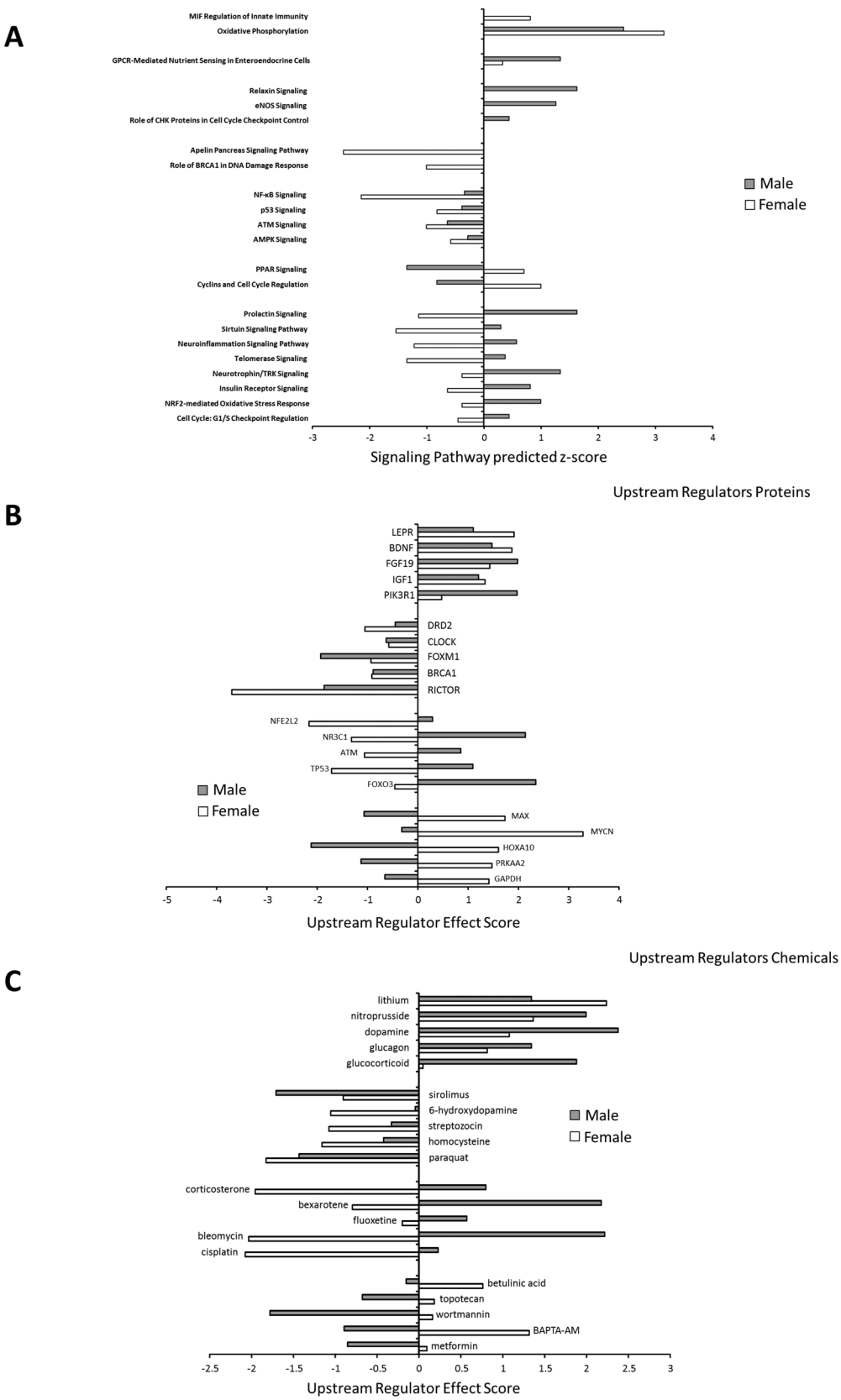


Fig. 3. Comparative numerical analysis of canonical signaling pathway and upstream regulator annotation of female and male GIT2-HET transcriptomic data. A – Clustered signaling pathways significantly populated ($p < 0.05$) by female or male GIT2-HET hypothalamic transcriptome data. Each pathway is represented with an IPA-generated z-score prediction for pathway activation (positive z-score) or inhibition (negative z-score). Clustered upstream protein (B) or chemical (C) transcriptome pattern regulator signatures significantly populated ($p < 0.05$) by female or male GIT2-HET hypothalamic transcriptome data. Each upstream regulator signature is represented with an IPA-generated z-score prediction for stimulatory control (positive z-score) or antagonistic control (negative z-score) of the user-defined input transcriptome dataset.

ones tightly linked to neurophysiological activity (‘*Axonal Guidance Signaling*’). As non-coordinated individual signaling pathway analyses of distinct datasets can often generate a less than optimal mechanism for the comparison of two distinct high-dimensionality datasets, we also performed a direct IPA-based ‘Comparison Analysis’ of the total GIT2-HET transcriptome datasets. Using this analytical platform, we were able to directly compare – within the same analytical workflow – the male and female signaling pathway datasets using predicted z-score

values for added interpretation of signaling activity polarity (Fig. 3A: Table S6). Reinforcing our previous gender-independent transcriptome phenotype observation, a potent impact upon oxidative phosphorylation activity was found in both GIT2-HET genders (Fig. 3A). In contrast to this, a firm male GIT2-HET functional representation of nutrient sensing pathway activity ('*GPCR-Mediated Nutrient Sensing in Enteroendocrine Cells*') was apparent, while for females a much greater functional emphasis was observed for aging, DNA damage repair and inflammaging-associated processes ('*Role of BRCA1 in DNA Damage Response*', '*NF-κB Signaling*', '*ATM Signaling*': Fig. 3A). Given our quantitative z-score predictions for eventual pathway control (as opposed to simple pathway enrichment probabilities), we were also able to identify pathways that possess opposite predicted signaling z-scores. Among these gender-differential pathways it was again evident that male and female hypothalami possess functionally distinct responses to GIT2 heterozygosity. Hence females, in stark contrast to males, demonstrated a distinct positive signaling effect with respect to cell cycle regulation and fatty acid metabolism ('*Cyclins and Cell Cycle Regulation*', '*PPAR Signaling*') while downregulating pathways classically associated with stress and aging ('*Sirtuin Signaling Pathway', 'Neuroinflammation Signaling Pathway' 'Telomerase Signaling Pathway'. 'Insulin Receptor Signaling', 'NRF2-mediated Oxidative Stress Response'*). Therefore, it seems that while experiencing the same functional insult, *i.e.* GIT2 heterozygosity, the female GIT2-HET response is to augment fatty acid metabolism while downregulating stress response/inflammation systems. In this case, it appears that as, the males diametrically oppose these responses, they are more likely experiencing/perceiving a greater pro-aging insult than females. This distinction therefore may represent an extant prevailing superiority of aging-resistance in females compared to males. As such, females may possess a greater level of nuance to their stress responsiveness than males, where the same level of neurometabolic insult clearly inculcates a more typical stress-response process (Fig. 3A). It is likely that the profound divergence in transcriptomic functional activity (Figs. 2 and 3) underpins this resilience superiority in females compared to males.

3.2.3. Upstream protein perturbagen analysis

Using curated perturbagen response datasets to either genetic/proteomic or chemical mediators, it is possible to generate an appreciation of the range of potential mediators/controllers of a specific high-dimensionality dataset. Here, we have employed IPA-based genetic/proteomic- and chemical-based upstream perturbagen analysis to uncover potential instigating factors that may be responsible for the gender differential hypothalamic transcriptome responses in GIT2-HET mice. As with our previous signaling pathway analyses, we applied a direct comparison approach for this form of dataset annotation. With respect to potential upstream protein controllers of the specific gender transcriptome responses to GIT2 heterozygosity, it was clear that neurometabolic responses were prominent in both genders, as upregulation of the leptin receptor (LEPR), brain-derived neurotrophic factor (BDNF) and insulin-like growth factor-1 (IGF-1) were predicted to be controllers of the hypothalamic transcriptomic responses (Fig. 3B: Table S7 in Supplementary material). A further gender-independent inhibitory function of hypothalamic dopaminergic signaling (Dopamine D2 receptor, DRD2), DNA integrity (Breast cancer type 1 susceptibility protein, BRCA1; Forkhead box protein M1, FOXM1) and time perception/energy homeostasis (Circadian locomotor output cycles protein kaput, CLOCK) was evident (Fig. 3B). Reinforcing our posit that at the transcriptome response level female GIT2-HET mice perceive an apparent lack of stress, there was a stark opposition between males and females in the potential transcriptome-based control of stress response systems linked to both oxidative (Nuclear factor erythroid 2-related factor 2, NFE2L2) and psycho-social (Glucocorticoid receptor, NR3C1) insults. For both the potential upstream effects of NFE2L2 and NR3C1 on the hypothalamic transcriptomes the male GIT2-HETs demonstrate a potential response profile commensurate with an active anti-stress response. In this context, it appears that the female GIT2-HET mice do not 'experience' as much of a palpable stress as the males. In addition, in female GIT2-HET there was an apparent reduced level of upstream influence of pro-aging factors such as ATM (ataxia telangiectasia mutated), TP53 (p53 protein) and FOXO3 (Forkhead box protein O3). Underpinning the profound distinction between the stress/aging resilience in female hypothalamic transcriptomes, there was a coherent pattern of potential enhanced transcriptional regulatory activity in females (protein MAX, MAX; *N*-myc proto-oncogene protein, MYCN). Thus, female GIT2-HET mice likely possess a resilience superiority via a more robust hypothalamic protection network activity compared to males experiencing the same GIT2 heterozygosity insult.

3.2.4. Upstream chemical perturbagen analysis

Investigating the potential upstream regulatory activity of small chemical influencers of the GIT2-HET hypothalamic transcriptomes we again found a clear gender-independent influence of dopaminergic signaling pathways, potentially associated with psychoactive signaling paradigms (dopamine, lithium), energy regulation (glucagon) and perceived stress (glucocorticoid: Fig. 3C; Table S8 in Supplementary material). Interestingly a reverse upstream relationship was observed for 6-hydroxydopamine (6 − OHDA), a perturbagen classically linked with the oxidative destruction of dopaminergic neurons. In a similar vein, it was evident that the oxidative toxin paraquat also indicated an anti-stress response (greater in females compared to makes however). Supportive of our posit of an anti-aging status of the female GIT2-HET transcriptome an opposite response signature to DNA-damaging agents such as bexarotene, bleomycin and cisplatin was found. In contrast, the male GIT2-HET transcriptome network suggests the presence of DNA integrity stress as these agents were predicted to be active controllers of the male GIT2-HET transcriptome (Fig. 3C). Interestingly a potent prediction of coordinated apoptosis (betulinic acid, topetecan), mitochondrial disposal (betulinic acid) calcium mobilization control (BAPTA-AM) and anti-aging signaling (metformin) was evident for the females in opposition to that seen in males.

3.3. Gender unique hypothalamic transcriptome response patterns to GIT2 heterozygosity

3.3.1. Classical pathway and perturbagen analysis

Applying signaling pathway and upstream analytical pipelines using either the entire female/male transcriptomic data (female 1079 transcripts; male 1006 transcripts) or using the gender-unique transcriptome data (female 577 transcripts; male 527 transcripts) we assessed the degree of functional divergence when comparing these different input datasets. This process would assess whether the total functional analyses were truly representative of gender-specific activity or if they potentially contained some confounding data. When we compared both the signaling pathway (Table S9 in Supplementary material) and upstream regulator (Table S10 in Supplementary material) annotation between total gender and gender-unique datasets we found a good similarity of functional interpretation of the two different-sized datasets. Hence, despite using approximately half of the input data volume for the gender-unique inputs (versus the total input data sets used previously), we again demonstrated a greater anti-aging/stress response in the females compared to the males (Fig. 4). As such, as with the total gender dataset analysis there was a clear distinction compared to males, at the predicted functional z-score level, of the female unique inhibition of inflammaging ('*Neuroinflammation Signaling*'), stress response *('NRF2-mediated Oxidative Stress Response*'), cellular clock regulation ('*CREB Signaling in Neurons*') and aging mechanisms ('*Telomerase Signaling*' – illustrated as an exemplar heatmap in Fig. 4B). In contrast to these downregulated signaling activities, female GIT2-HET transcriptomes also showed augmentation of cellular differentiation/proliferation ('*Aryl Hydrocarbon Receptor Signaling', Cyclin and Cell Cycle Regulation*') and fatty acid oxidation ('*PPAR Signaling*')

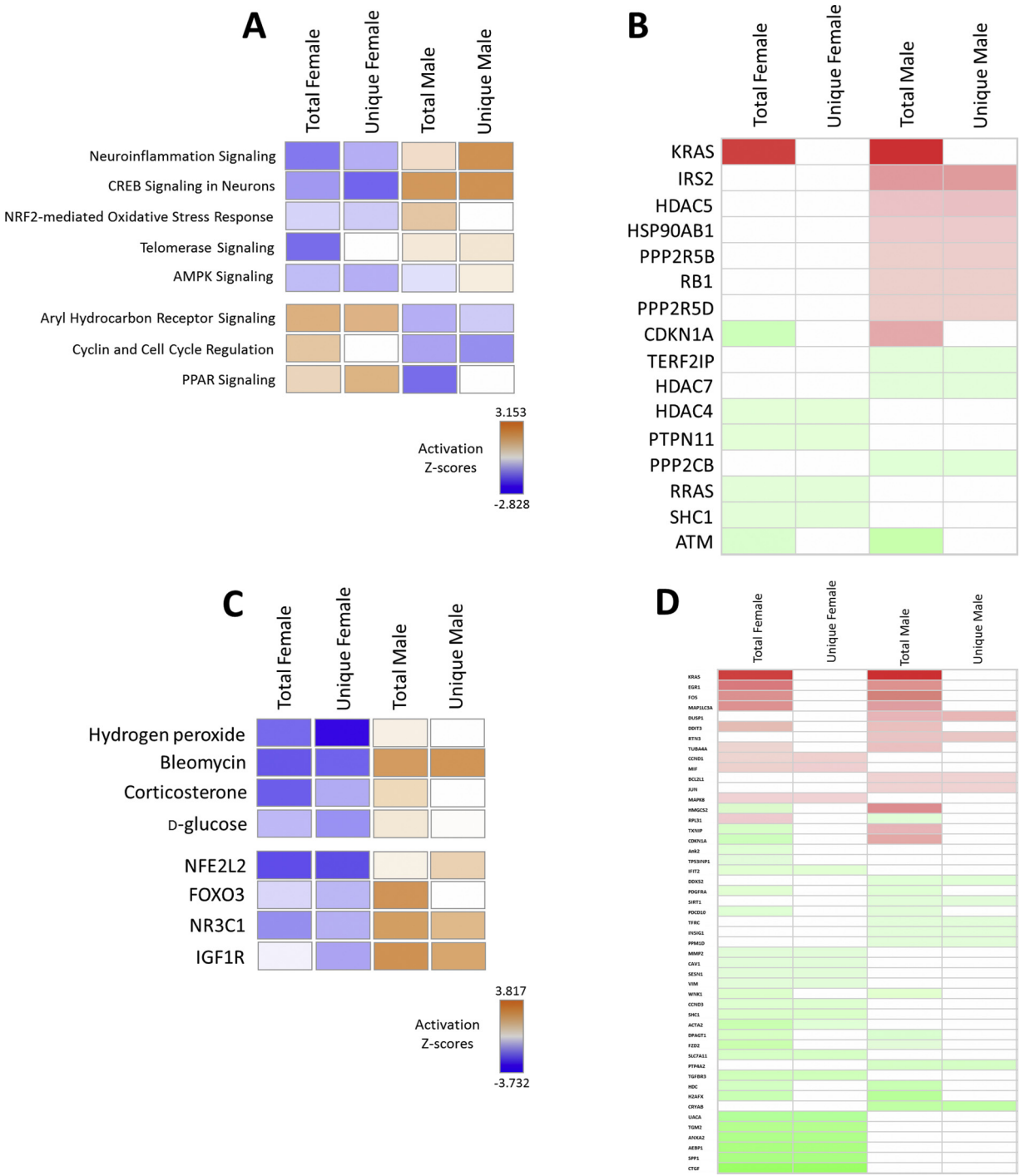


Fig. 4. Comparison of signaling pathway and upstream regulator analytical output generated by total or gender-unique GIT2-HET hypothalamic transcriptome datasets. A – Comparative signaling pathway z-scores generated using total female, unique female, total male or unique male GIT2-HET transcriptome data. Z-score pathway activation (orange) or inhibition (blue) is indicated for each significantly ($p < 0.05$) populated signaling pathway. B – Specific transcriptome data heatmap for the Telomerase Signaling pathway, demonstrating a potent bias for male (total or unique) stimulatory activity. Upregulated transcript are denoted in red, downregulated transcripts in green – color intensity indicates the relative z-ratios. C - Comparative protein or chemical upstream regulator signature z-scores generated using total female, unique female, total male or unique male GIT2-HET transcriptome data. Z-score pathway activation (orange) or inhibition (blue) is indicated for each significantly ($p < 0.05$) populated upstream regulator pathway. D – Specific transcriptome data heatmap for Hydrogen Peroxide as a potential upstream regulator, demonstrating a potent bias for female (total or unique) antagonistic activity. Upregulated transcripts are denoted in red, downregulated transcripts in green – color intensity indicates the relative z-ratios. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activity. With respect to the upstream analysis of the gender-unique datasets again a definitive female bias of stress-responsive upstream activity was seen (Fig. 4C and D). Hence, both the total and unique female transcriptomes were associated with an inhibition of upstream, oxidative stress ('*hydrogen peroxide*' - illustrated as an exemplar heatmap in Fig. 4D; NFE2L2), DNA damage (Bleomycin), psychosocial stress (corticosterone, NR3C1), aging (FOXO3) and neurometabolic (D-glucose, IGF1R) signaling activity. As depicted in Fig. 4, in both the

signaling pathway and upstream regulator processes the male GIT2-HET transcriptomes, both at the total and male-unique level, demonstrated opposite behavior to the analogous female datasets.

3.3.2. Natural language processing analysis

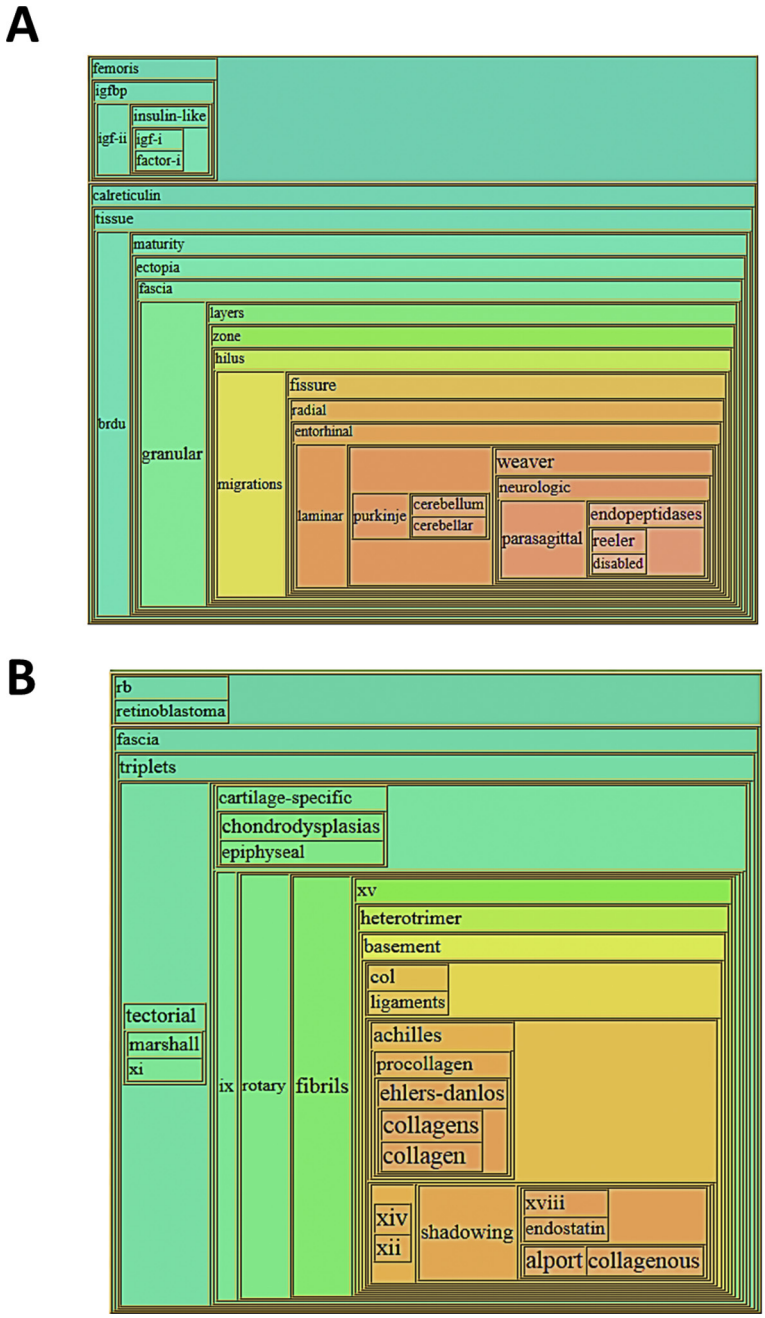
While standard curated database-supported analysis has significantly contributed to high-dimensionality data analyses in the biomedical field, the rise of natural language processing techniques such as LSI has introduced an additional level of nuanced informatics. We have recently demonstrated the importance of LSI for biomedical text mining and the generation of data-driven functional ontologies for high-dimensionality datasets (Maudsley et al., 2018, 2015; Maudsley et al., 2016). Using our novel LSI-based biomedical annotation engine Textrous! (Chen et al., 2013b) we generated complex biomedical textual interpretations of the differential female- and male-unique hypothalamic transcriptomes. Textrous! can generate textual associations in two modes, *i*) Collective Processing where text associations with the whole input data corpus are considered and then arranged into a hierarchical wordcloud and *ii*) Individual Processing where heatmap correlations between specific input Gene Symbol terms are associated with the highest scoring (via Cosine Similarity ranking) biomedical terms. In these processes, Textrous! is able to generate complex and highly-data specific textual descriptions of functional activities. Applying collective processing to the gender-unique transcriptomes from female and male GIT2-HET animals revealed a solid biomedical text-functional divergence resonant with our previous analyses. Hence, for female GIT2-HET unique data (Fig. 5A), Textrous! collective processing generated a coherent hierarchical wordcloud eventually condensing onto two major clades – one associated with IGF-1 signaling and the other linked to pathologies associated with two murine models, *i.e.* Weaver (dopaminergic dysfunction) and Reeler (insulinotropic dysfunction). In contrast to the female hierarchical cloud the male GIT2-HET transcriptome perturbagen response was associated with two condensing clades linked to retinoblastoma (indicating a dysfunctional MAX/MYC activity seen previously – Fig. 3B) and aberrant collagen functionality (Fig. 5B) which has recently been demonstrated in psychosocially stressed murine hypothalamic (Smagin et al., 2018). We next applied the individual processing mechanism to both the female and male unique transcriptome datasets. Despite the general similarity of input data volume (577 female transcripts, 527 male transcripts), a considerably larger biomedical noun-Gene Symbol association matrix was created using the female unique hypothalamic data. The female biomedical noun-Gene Symbol matrix volume was 15050 (50 primary nouns associated implicitly with 301 input Gene Symbols) – the male biomedical noun-Gene Symbol matrix volume however was only 11,450 (50 primary nouns associated with only 229 input genes). This matrix volume difference between female and male data (1.314 ratio of female: male) was greater than the difference in the input Gene Symbol data corpi (1.094 ratio of female: male), suggesting a more robust functional action of the transcriptome response in the female GIT2-HET mice. Using the novel process of noun-phrase chunking (Chen et al., 2013a; Zhang and Elhadad, 2013) we were able to expand the textual association scope from the initial matrices. Using this semantic association process we found 2885 associated noun-phrases (8449 total words) specifically linked to the female GIT2-HET hypothalamic dataset compared to 2480 associated noun-phrases (6924 total words) linked to the male hypothalamic dataset. As with the total Gene Symbol matrix volume differences between female and male datasets, with respect to total associated words (from noun-phrase chunking) a greater female : male word ratio was created (1.22) than based on the input Gene Symbol data bias (1.092 female: male). To generate a gestalt appreciation of these high-dimensionality biomedical text outputs we then condensed, via word frequency representation (JasonDavies Wordcloud Generator: www.jasondavies.com/wordcloud/), these datasets to more comprehensible text clusters. Using this high-intensity data-driven appreciation (using results from 52% and 43.4% of the input female and male Gene Symbols respectively) of transcriptome data we found a robust mitochondrial and cell differentiation bias in the overall female GIT2-HET unique data corpus, while the male GIT2-HET data generated a cloud dominated by terms associated cytoskeletal and neurosynaptic activity (Fig. 6).

3.3.3. High-dimensionality wordcloud deconvolution for GIT2-HET dataset annotation

Given the high volume (female – 15,050; male – 11,450 cells) of the individual processing Textrous! outputs from the unique dataset annotation we next performed noun-Gene Symbol clustering within the primary biomedical noun matrices (Fig. 6). Clustering together closely-related noun groups with their semantically-associated empirically-derived Gene Symbol inputs we generated seven coherent sub-matrices from the female unique (Fig. 7A–G) and five sub-matrices for the male unique dataset (Fig. 8A–E). By extracting the semantically-associated noun-phrases for each of the clustered sub-matrices, we were able to generate more specific high-dimensionality wordcloud outputs (termed mini-clouds (MCs)) for each sub-matrix. For each of these generated MCs we performed word frequency analysis using WriteWords (www.writewords.org.uk/) to create a list of the top 10 highest frequency words found in the noun-phrase outputs from the specific sub-matrices. Based on the nature of these extracted words we clustered these results to demonstrate commonalities and distinctions between the MC interpretations (Fig. 9). In this semantic word association analysis, we found several common sub-features of the female and male GIT2-HET transcriptomes. Hence, mini-cloud number 1 (MC1) for both genders (the largest sub-matrix size for each gender) were strongly representative of cytoskeletal remodeling and coiled-coil domain protein behavior (Fig. 9), with the male sub-matrix demonstrating a greater preponderance of associated transcript upregulation. Female MC4 and the male MCs 2 and 3 also clustered together based on their association with neuroplasticity and synaptic activity – in this respect a male overall clustering bias was seen due to the number of associated MCs. Female MC5 was most closely associated with the male MCs 4 and 5 due to their common transcriptional/DNA management focus – here however there was an evident female bias indicated by a greater preponderance for transcript upregulation. Further confirming our previous analytical platforms, we found that female MC diversity is considerably larger than for males, evidenced by the female-specific MCs 3 (metabolic control), 6 (cellular differentiation control) and 7 (functional signaling and muscle contractility behavior). These additional MCs derived from distinct female sub-matrices was highly indicative of the more profound and diverse phenotypic response of the female hypothalamus to GIT2 heterozygosity. We next investigated whether any significantly regulated transcripts existed that were associated with multiple sub-matrices. We found, for both genders, 11 transcripts that were found in the leading-edge sets of at least two sub-matrices (Fig. 9B and C). Upon cellular compartment (Jensen Compartment Analysis via Enrichr) and GO biological process (Enrichr) analysis (Fig. 9D–G) it was clear that these multidimensional 11 transcripts were differentially focused according to the animals' gender, *i.e.* females were all linked to mitochondrial functionality (Fig. 9D and F) while males were linked to neurosynaptic functionality (Fig. 9E and G).

3.4. Demonstration of functional somatic variance of responses to GIT2 heterozygosity in female and male animals

Our in-depth and nuanced informatics interpretation of the relatively simple hypothalamic transcriptome responses to GIT2 heterozygosity revealed a distinctive molecular phenotype for the female and male GIT2-HETs. As the hypothalamus is perhaps the master organ with respect to somatic control of neuroendocrine behavior (Zhang et al., 2013) we employed a comprehensive behavioral analytical approach to assess the gender-specific effects of GIT2 heterozygosity. Hence, we investigated behavioral and physiological phenotypes of female and



Word	Cosine	Z-score	P-value
weaver	0.4856	2.0417	0.0175
reeler	0.4626	1.9447	0.0228
granular	0.4588	1.9286	0.0238
endopeptidases	0.4474	1.8806	0.0269
parasagittal	0.4418	1.8569	0.0286
fissure	0.4341	1.8243	0.0310
neurologic	0.4173	1.7537	0.0367
tissue	0.4142	1.7403	0.0379
purkinje	0.4051	1.7021	0.0413
insulin-like	0.3912	1.6435	0.0470
cerebellum	0.3900	1.6384	0.0477
zone	0.3861	1.6220	0.0494
radial	0.3849	1.6171	0.0499
calreticulin	0.3845	1.6153	0.0501
hilus	0.3836	1.6113	0.0505
brdu	0.3833	1.6101	0.0506
migrations	0.3825	1.6068	0.0510
disabled	0.3824	1.6063	0.0511
igf-i	0.3807	1.5990	0.0511
femoris	0.3801	1.5968	0.0523

Word	Cosine	Z-score	P-value
collagens	0.6145	2.7504	0.0029
collagen	0.6090	2.7257	0.0032
alport	0.6053	2.7087	0.0033
xiv	0.5953	2.6637	0.0038
xii	0.5895	2.6375	0.0041
collagenous	0.5839	2.6125	0.0044
ehlers-danlos	0.5709	2.5537	0.0053
achilles	0.5623	2.5151	0.0059
fibrils	0.5589	2.4999	0.0062
Chondrodysplasias	0.5337	2.3861	0.0085
shadowing	0.5330	2.3833	0.0085
xviii	0.5284	2.3623	0.0090
tectorial	0.5181	2.3162	0.0102
marshall	0.5161	2.3070	0.0105
col	0.5095	2.2775	0.0113
triplets	0.5022	2.2446	0.0123
basement	0.5017	2.2422	0.0124
procollagen	0.4978	2.2245	0.0130
heterotrimer	0.4945	2.2095	0.0135
xv	0.4884	2.1821	0.0145

Fig. 5. Textrous!-based hierarchical wordcloud analysis of gender-unique GIT2-HET hypothalamic transcriptome data. A – Female unique GIT2-HET agglomerative hierarchical wordcloud indicates words with the greatest semantic association with the input dataset with the largest font in the 'highest' (running from green to red) areas of stepwise wordcloud. The words semantically-associated with the input dataset are indicated in the right of the panel with their specific Cosine Similarity, Z-score and Associations P-values. B - Male unique GIT2-HET agglomerative hierarchical wordcloud indicates words with the largest semantic association with the input dataset with the largest font in the 'highest' (running from green to red) areas of stepwise wordcloud. The words semantically-associated with the input dataset are indicated in the right of the panel with their specific Cosine Similarity, Z-score and Associations P-values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

male GIT2-HET mice using a Columbus Instruments CLAMS (Comprehensive Lab Animal Monitoring System) Oxymax system (Martin et al., 2015). GIT2-HET mice of both genders were assessed over a 48-h period for behavioral responses to differential to WT control mice. For female and male GIT2-HET mice there was a clear distinction between the VO_2 (oxygen consumption: Fig. 10A), VCO_2 (carbon dioxide production: Fig. 10B), TEE (total energy expenditure: Fig. 10C) and thermal heat generation (Fig. 10D), *i.e.* across the circadian cycle female GIT2-HET mice demonstrated a lower level of VO_2, VCO_2, TEE and heat generation compared to WT controls and failed to demonstrate any specific temporal variation across the experimental period. In contrast, male GIT2-HET animals still demonstrated circadian cyclicity of these physiological parameters with also a smaller difference compared to these values found in male WT controls. Thus it appears that female GIT2-HET mice possess a non-circadian cycling diminution of energetic parameters – in contrast to the male GIT2-HET animals. With respect to the observed RER (respiratory exchange ratio) both female and male GIT2-HET mice demonstrated a significant reduction in this parameter indicating a potential shift away from the primary usage of glucose as a metabolic energy source (Fig. 10E), as we have observed before it

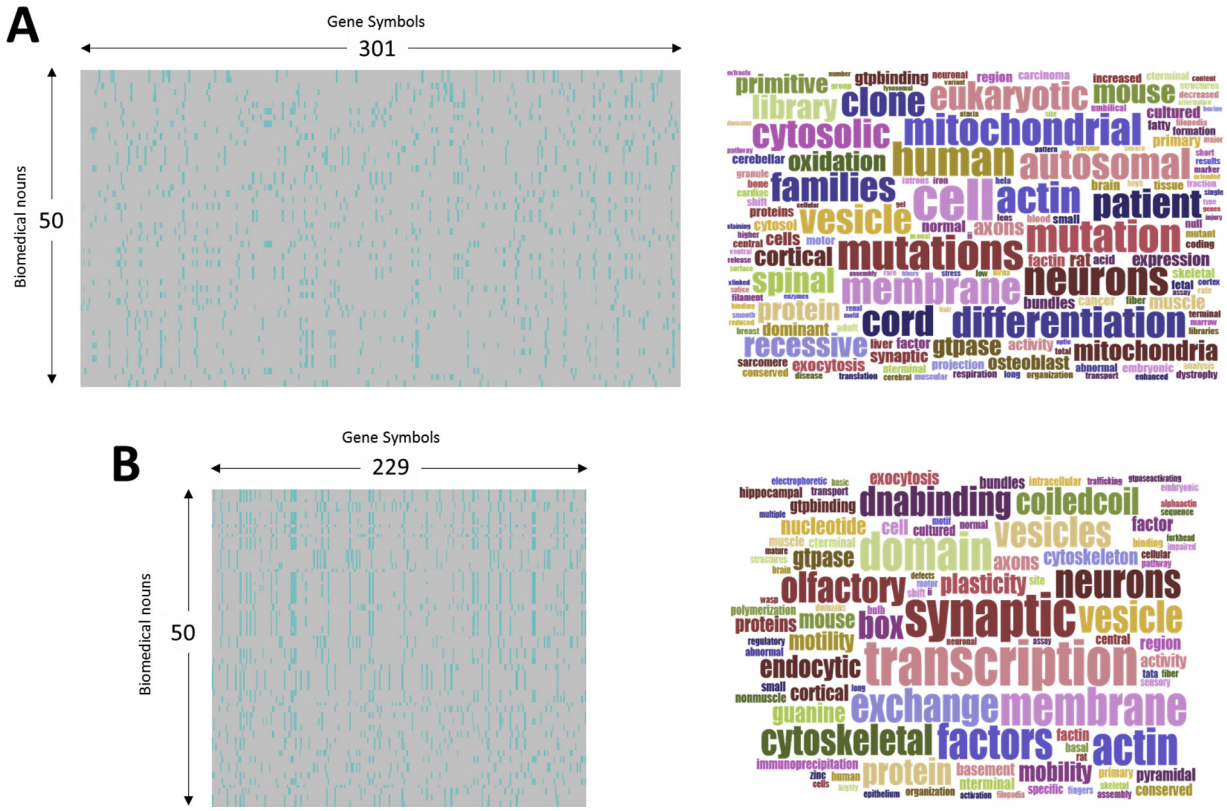


Fig. 6. Textrous!-based individual processing heatmap and high-dimensionality cloud analysis of gender-unique GIT2-HET hypothalamic transcriptome data. A – Female unique transcriptome GIT2-HET Textrous!-based individual processing biomedical noun and transcript heatmap matrix. Taking both the primary biomedical nouns as well as the associated noun-phrases from this list a high-dimensionality wordcloud (right in the panel) can be generated to create a gestalt appreciation of the functional phenotype of input dataset. B - Male unique transcriptome GIT2-HET Textrous!-based individual processing biomedical noun and transcript heatmap matrix. The associated high-dimensionality wordcloud (right in the panel) was generated in an analogous manner to that for panel A.

homozygous GIT2 KO mice (Martin et al., 2015). In accordance with the alterations observed in female GIT2-HET circadian rhythms we also found that with respect to physical activity in the CLAMS cages (x and z axis motion) the female GIT2-HET mice failed to demonstrate any nocturnal activity (Fig. 10F–H) increases, unlike the male GIT2-HETs which demonstrated similar circadian rhythms to male WT controls. Therefore, it is clear from this CLAMS data that indeed despite a similar number of significantly-modulated hypothalamic transcripts (female GIT2-HET 1079, male GIT2-HET 1006) between the two genders there is a significant and multidimensional distinction at the molecular and physiological level between these functional networks in female and male mice, associated with anti-stress and anti-aging functions.

4. Discussion

Here we demonstrate that in an early adult time point, *i.e.* 2 months of age, a partial reduction of GIT2 expression results in a dramatic functional distinction between the molecular and physiological effects in female and male mice. At a *gestalt* level we found a numerical similarity in the total number of significantly altered hypothalamic transcripts found in female and male GIT2-HET mice (Fig. 1). Despite this overall similarity in total numbers there was a definite divergence between the two genders as over half of these significantly altered transcripts were unique to either gender. Among common transcripts there was a small coherent group of 16 factors differentially controlled between the two genders that generated a rationally linked series of factors related to aging, DNA damage and metabolic disruption centered upon Parp2 (Fig. 1). Parp2 was downregulated in females and upregulated in males. Reductions in Parp2 are associated with an increased sensitivity to DNA damaging agents in mice and humans (Gibson and Kraus, 2012; Schreiber et al., 2006). Previously, we have shown that GIT2 forms a dynamic complex with Parp2 in response to DNA-damaging insults (Lu et al., 2015), therefore this attenuation of Parp2 may seem non-beneficial to the female mice. This female idiosyncratic response may however facilitate the inculcation of a secondary protective mechanism, *i.e.* the diversification of energy metabolism away from oxidative phosphorylation to fatty acid beta-oxidation. Intermittent or periodic molecular and/or dietary interventions (*e.g.* ketogenic diets) that divert energy metabolism away from primary oxidative phosphorylation to fatty acid beta-oxidation have been shown to increase longevity (Bruss et al., 2010; Hallbook et al., 2012; Meidenbauer et al., 2014; Roberts et al., 2017). Such a potential activity could be a potential mechanism by which females may help promote their natural longevity superiority. In our study we found that *i*) ketogenic activity and fatty acid biosynthesis were controlled by the divergent polarity female-male transcriptome sub-dataset (Fig. 1Q), *ii*) females upregulated fatty acid-related PPAR signaling activity (Figs. 3A and 4 A) and *iii*) females demonstrate a more profound mitochondrial oxidative phosphorylation dysfunction compared to males (Fig. 2E and F). Diminutions of Parp2 may indeed be an effective longevity strategy as they could facilitate metabolic diversification. Parp2$^{-/-}$ mice demonstrate disrupted insulinotropic functionality and favor fatty acid metabolism (Bai et al., 2011). An enhancement of SIRT1 activity that is directly related to augmented cellular protection systems and longevity, is also seen in these mice (Fig. 2E; (Bai et al., 2011; Geng et al., 2013; Kilic et al., 2015)). In addition to the potential key role of Parp2 in this gender distinction of longevity mechanisms, differential female/male expression was observed for multiple factors associated with pathological aging trajectories (Rpl29 - (Jiang et al., 2011); Gdi1 -(Suszynska-Zajczyk et al., 2014); Plin4 – (Han et al., 2018); Hmgcs2 – (Hirschey

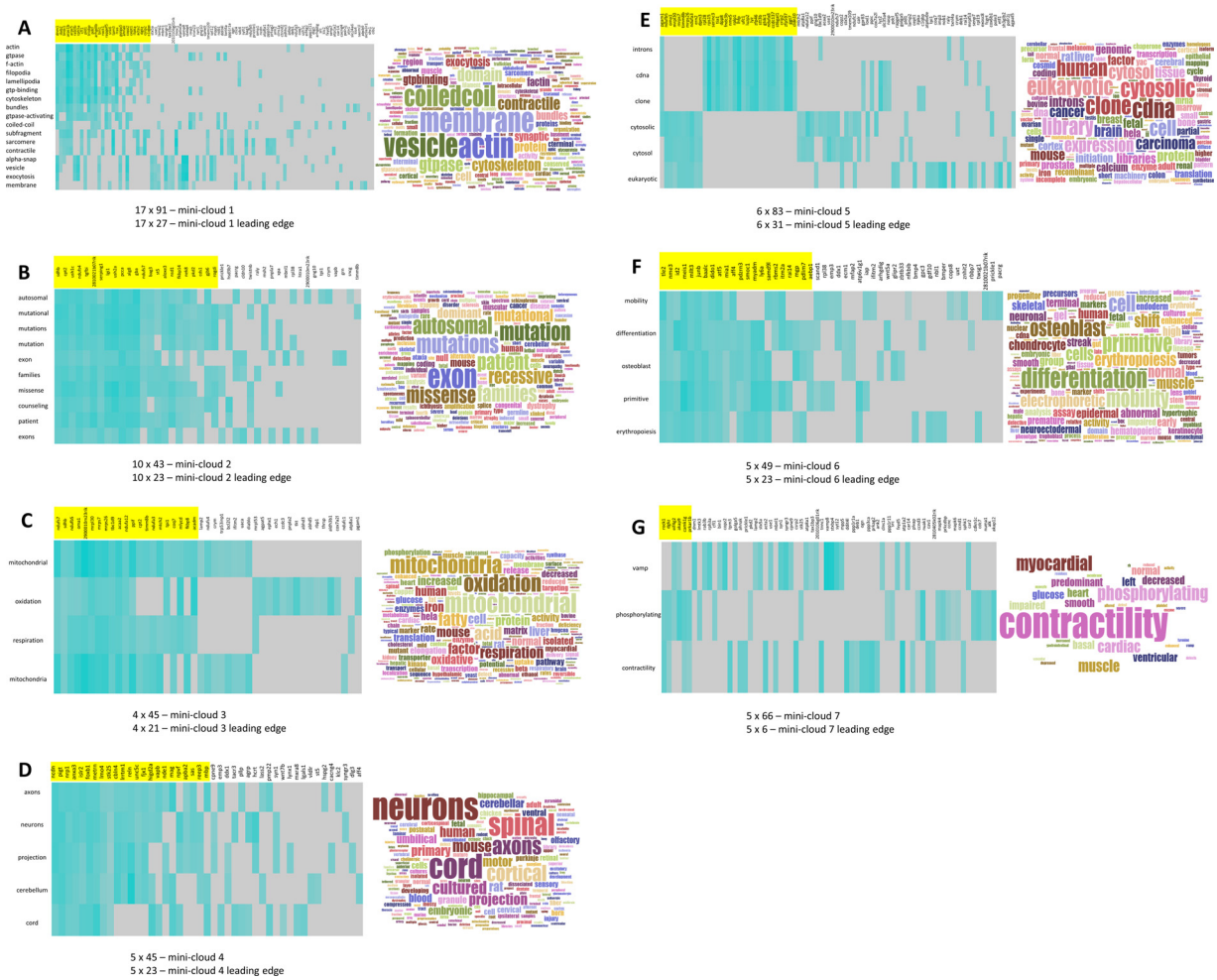


Fig. 7. Textrous!-based sub-matrices and associated mini-cloud dataset representations for deconvoluted female-unique GIT2-HET transcriptomic data. A – G Represent distinctly-populated sub-matrices with their associated noun-phrase mini-cloud (A = 1, B = 2, C = 3, D = 4, E = 5, F = 6, G = 7) representations for female unique GIT2-HET transcriptome data. Within each sub-matrix heatmap the set of transcripts responsible for > 50% of the noun clustering are highlighted in yellow. This specific data subset is denoted the 'leading edge' of the specific sub-matrix. The dimensions of the full, or leading-edge sub-matrices (nouns * transcripts) are given. For each mini-cloud (MC) the word frequencies in the cloud are proportional to the represented font. Specific word colors were varied randomly to accentuate font size differences. All MCs were generated using the JasonDavies Wordcloud Generator (www.jasondavies.com/wordcloud/) using consistent numerical settings. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2011); Vwf – (Seaman and Ragni, 2018); Txnip – (Nasoohi et al., 2018; Oberacker et al., 2018); Cdkn1a – (Khatiwala et al., 2018; Li et al., 2018); Fkbp5 – (Mamdani et al., 2015; Sabbagh et al., 2014); Caskin1 – (Daimon et al., 2015), mitochondrial neurodegeneration (Txnip – (Kim et al., 2019; Nasoohi et al., 2018); Fkbp5 – (Boonying et al., 2019)), cellular senescence (Cdkn1a – (Khatiwala et al., 2018; Li et al., 2018)), metabolic stresses (Kns2 – (Qiu et al., 2010), Tsc22d3 – (Andre et al., 2016); Plin4 – (Gasparin et al., 2018; Richardson et al., 2011); Hmgcs2 – (Zhang et al., 2019); Txnip – (Katsu-Jimenez et al., 2019; Nasoohi et al., 2018)), nutrient selection (Txnip - (Blouet and Schwartz, 2011)), psychosocial stress (Fkbp5 - (Le-Niculescu et al., 2019); Caskin1 – (Katano et al., 2018)), circadian rhythm disruption (Tsc22d3 – (Yurtsever et al., 2016)), ketone metabolism (Hmgcs2 – (d'Avignon et al., 2018d; Geisler et al., 2019)) and inflammaging (Txnip – (Kim et al., 2019; Nasoohi et al., 2018); Cdkn1a – (Khatiwala et al., 2018)).

Thus, a direct comparison of the common but opposite polarity (contra-regulated) transcripts provides a clear indication of the distinctiveness of female versus male responses to GIT2 hypothalamic heterozygosity. Interpretation of the global transcriptome also yielded important insights into the gender-specific control of longevity. Using both Gene Ontology (GO) and IPA CSP analysis it was evident that a greater functional impact (Fig. 2A and D) of heterozygosity was experienced by the females – suggestive of a greater endogenous functionality of GIT2 in females. Hence, despite a similar number of significantly altered hypothalamic transcripts, a huge disparity of GO and CSP annotation was observed, demonstrating a fundamental distinction of transcript inter-relatedness in female hypothalami. With respect to GO biological process annotation a clear female-specific focus upon age-related processes was evident compared to the males that instead demonstrated a degenerative GO biological process phenotype (Fig. 2B and C). A similar phenotypic gender distinction was also seen with the CSP annotation (Fig. 2E and F). At the signaling pathway level the female control of *Sirtuin* and *IGF-1* activity was contrasted by the damage (*NER pathway*) phenotype in male. Both of these findings suggest a distinctive hypothalamic response to GIT2 heterozygosity, *i.e.* females present a capacity to withstand and not respond to the applied perturbation. This can be seen clearly when the predicted z-scores of pathway activation or upstream regulators are cross analyzed (Fig. 4). Female GIT2-HET mice, at the global transcriptome and also the gender-unique transcriptome level potently downregulate pathways normally expected as part of the aging process, *e.g.* neuroinflammation, NRF2 oxidative stress response or Telomerase signaling (Fig. 4A and B) – in contrast their male counterparts actively upregulated these

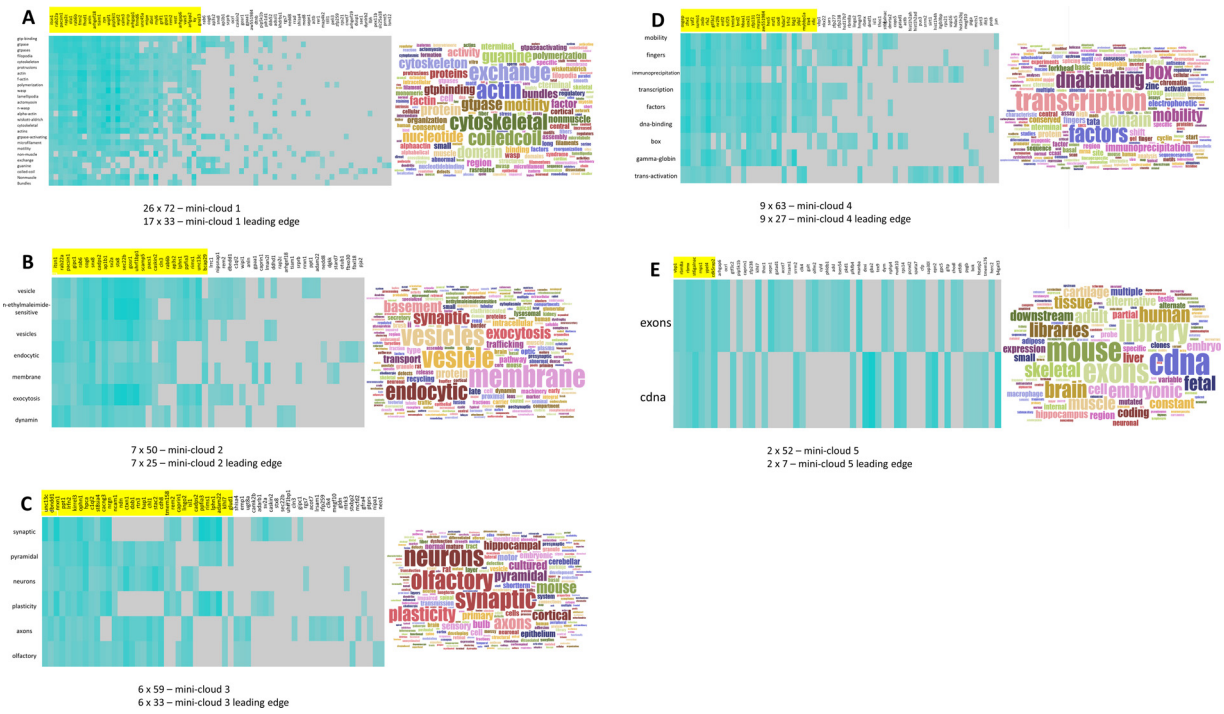


Fig. 8. Textrous!-based sub-matrices and associated mini-cloud dataset representations for deconvoluted male-unique GIT2-HET transcriptomic data. A – E Represent distinctly-populated sub-matrices with their associated noun-phrase mini-cloud (A = 1, B = 2, C = 3, D = 4, E = 5) representations for female unique GIT2-HET transcriptome data. Within each sub-matrix heatmap the set of transcripts responsible for > 50% of the noun clustering are highlighted in yellow. This specific data subset is denoted the 'leading edge' of the specific sub-matrix. The dimensions of the full, or leading-edge sub-matrices (nouns * transcripts) are given. For each mini-cloud (MC) the word frequencies in the cloud are proportional to the represented font. Specific word colors were varied randomly to accentuate font size differences. All MCs were generated using the JasonDavies Wordcloud Generator (www.jasondavies.com/wordcloud/) using consistent numerical settings. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

responses, indicating they indeed 'perceived' the GIT2 heterozygosity perturbation to a greater extent. While downregulating (or could be interpreted as failing to positively engage) classical stress pathways, females demonstrated preparedness in promoting pathways to modify circadian rhythms ('*Aryl Hydrocarbon Receptor Signaling*'; (Jaeger et al., 2017; Ziv-Gal et al., 2013)) and energy metabolism diversification ('*PPAR Signaling*'; (Capri et al., 2006)). Tellingly, the male GIT2-HET significantly attenuated the activity of these pathways. A similar striking divergence of transcriptomic response was observed with the analysis of potential upstream regulators of the GIT2-HET datasets (Fig. 4C and D). Male GIT2-HET hypothalami presented classical signs of stress-induced transcriptomic changes, *i.e.* increases of oxidative (hydrogen peroxide) and psychosocial stresses (corticosterone) as well as DNA damaging agents (bleomycin), appeared to be upstream of the transcriptomic responses in male GIT2-HETs. In regards to these perturbations the females presented the opposite response, *i.e.* a perceived absence of stress. This distinction was also seen with upstream protein regulators, *i.e.* male GIT2-HET transcriptomic responses seemed to be under the control of increased stress pathways (NFE2L2 (Nrf2 pathway: (Sykiotis et al., 2011)), FOXO3 (Martins et al., 2016), NR3C1 (Olczak et al., 2019)). These distinctions of responsiveness raise the possibility that females, at the hypothalamic level, possess an innate aging resilience '*program*' with a differential dependency on the GIT2 aging regulator. In this context, we propose that GIT2 may act as a hypothalamic 'decision maker' with respect to the entrainment of anti-stress/aging somatic programs.

Classical pathway analysis relies heavily upon human-curated data clusters (*e.g.* CSPs) or human-based annotation (GO). Here we also adopted a machine-based annotation process with our application of the LSI-based *Textrous!* platform to generate a more data-dependent appreciation of the GIT2-HET transcriptomes. Using our novel pipeline of mini-cloud generation, we were able to create an unprecedented level of data deconvolution that revealed highly distinct functional signaling clusters that concisely defined the distinctive nature of female versus male GIT2 heterozygosity. Using our mini-cloud data extraction procedure, we were able to demonstrate a profound functional global bias towards female response behavior (Fig. 6) that was underpinned by specific functional clusters regulating cellular differentiation and metabolic diversity (Fig. 9). During temporal stresses, *e.g.* caloric restriction, exercise, dietary deprivation, psychosocial stress it is likely that the body's ability to employ diverse energy substrates rapidly without excessive molecular re-organization will facilitate healthy aging. Controlling a reliable energy production pipeline, despite fluctuations, would prevent time lags in ATP supply or excessive ROS accumulation, both processes that would enhance age-related cellular damage. Reinforcing our informatic analyses we found that female GIT2-HET mice, compared to male GIT2-HETs, presented with larger metabolic shifts in VO_2, VCO_2, RER, greater depression of TEE and heat production (associated with hypothalamic age-control mechanisms: (Tabarean et al., 2010) and a near total deletion of circadian rhythmic activities (Fig. 10). It is therefore unsurprising in this context that maintaining metabolic network flexibility is important for longevity (Hasek et al., 2010; Hong et al., 2014; Kuang and Ebert, 2012; Nunn et al., 2009, 2010; Weir et al., 2017; Westbrook et al., 2009; Zhang and Cao, 2017). As such, our observed phenotypic differences in somatic metabolism therefore support the accuracy of our novel informatic deconvolution workflows.

Many of the current mechanistic theories of aging are focused on the critical role of metabolic diversity (oxidative phosphorylation or β-oxidation) of mitochondrial functionality. It is our contention that GIT2, especially in females, acts as one of the coordinators of metabolic aging and stress sensitivity (Abrahamian et al., 2017; Lanza and Nair, 2010; Martin et al., 2015; Norheim et al., 2019). From our mini-cloud leading-edge analysis a clear molecular bias between male and female

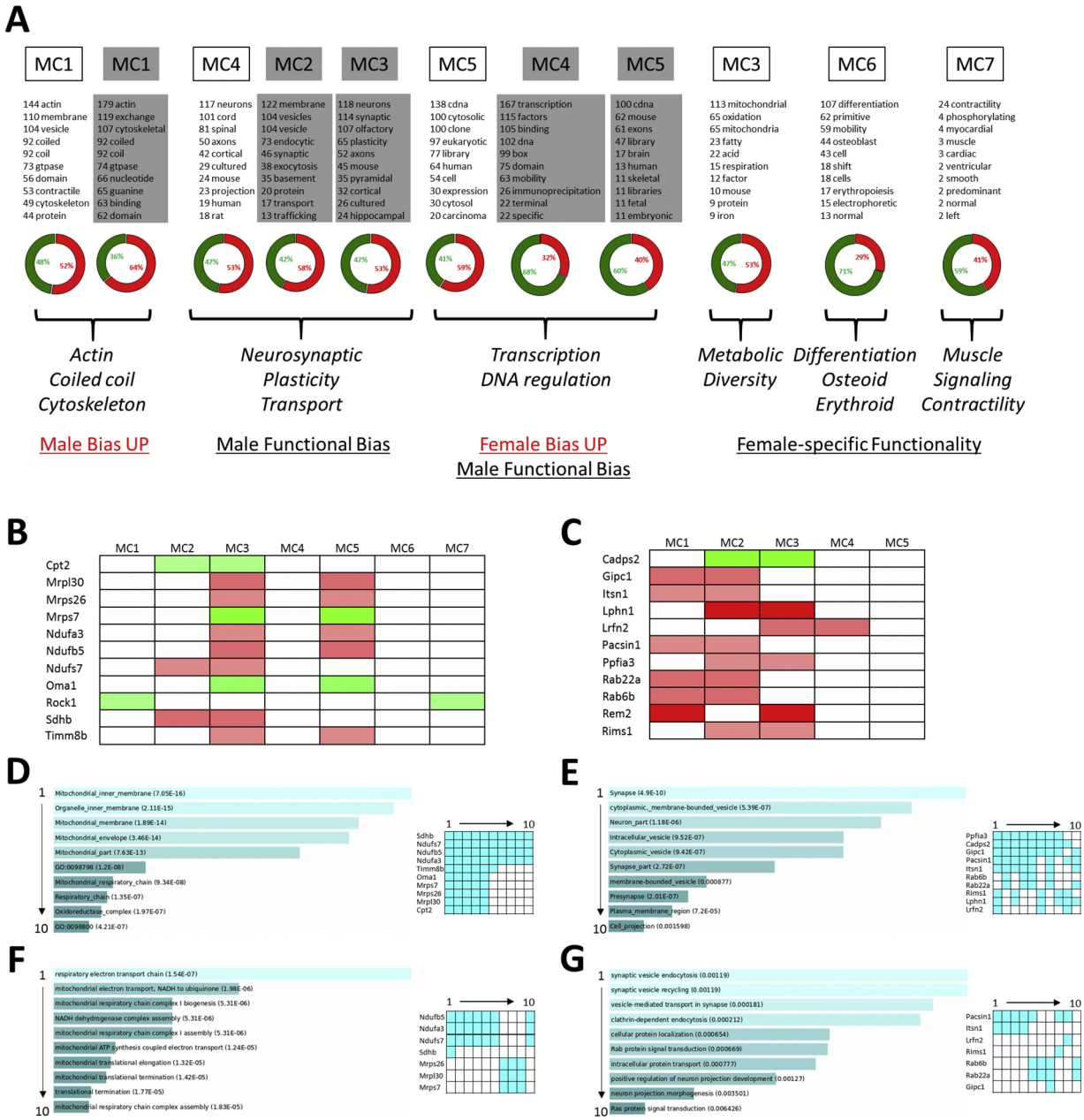


Fig. 9. Functional clustering and word frequency phenotype analysis of female and male unique mini-cloud properties. A – For each specific mini-cloud (MC) created from gender-unique GIT2-HET transcriptomes the top 10 word frequencies (calculated using WriteWords-based analysis: http://www.writewords.org.uk/) are represented. Based on these unbiased MC-based outputs functionally-similar female (white) or male (grey) MCs were clustered. The proportion of up- or downregulated (up – red, down – green) data transcripts within the specific MC is indicated in the associated pie-chart. Identification of multidimensional transcripts present within at least two different sub-matrix leading edge data sets from female (B) or male (C) GIT2-HET mice. The expression polarity of each leading edge transcript and the specific MC it was derived from are denoted in the matrix (upregulation – red, downregulation – green). Enrichr-based Jensen Compartment analysis of female (D) or male (E) multidimensional leading edge transcripts revealed a totally distinct phenotype for each dataset, *i.e.* females = mitochondrial, males = neurosynaptic. The inset heatmaps in each panel indicate the transcript to cellular compartment relationship. Enrichr-based Gene Ontology (biological process) analysis of female (F) or male (G) multidimensional leading edge transcripts revealed a totally distinct phenotype for each dataset, *i.e.* females = mitochondrial activity, males = synaptic activity. The inset heatmaps in each panel indicate the transcript to Gene Ontology term relationship. For each of the panels D–G, the probability values of compartment or Gene Ontology term enrichment are denoted in parentheses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

GIT2-HET transcriptomes was apparent (Fig. 9A). Here we have attempted to create a clearer and more concise mechanism to deconvolute high-dimensionality data for identifying the key distinctive factors between two divergent phenotypes, *i.e.* female versus male GIT2-HET hypothalamic transcriptomes. Using Textrous! heatmap analysis and the construction of mini-clouds we were able to prioritize leading edge sets distinct to each gender. When we investigated the presence of multidimensional factors (Fig. 9B and C) across these gender-specific mini-clouds, we found a simple and clear definition of the fundamental functional distinctions between females and males. Hence, trophic controlling factors in females were overwhelmingly associated with mitochondrial functionality, while analogous male factors instead were associated with synaptic functionality (Fig. 9D–G). Thus, it seems eminently clear that in the face of a consistent perturbation, *i.e.* GIT2 heterozygosity, females concentrate on regulating energy metabolism diversity while males focus on maintaining neurosynaptic architecture

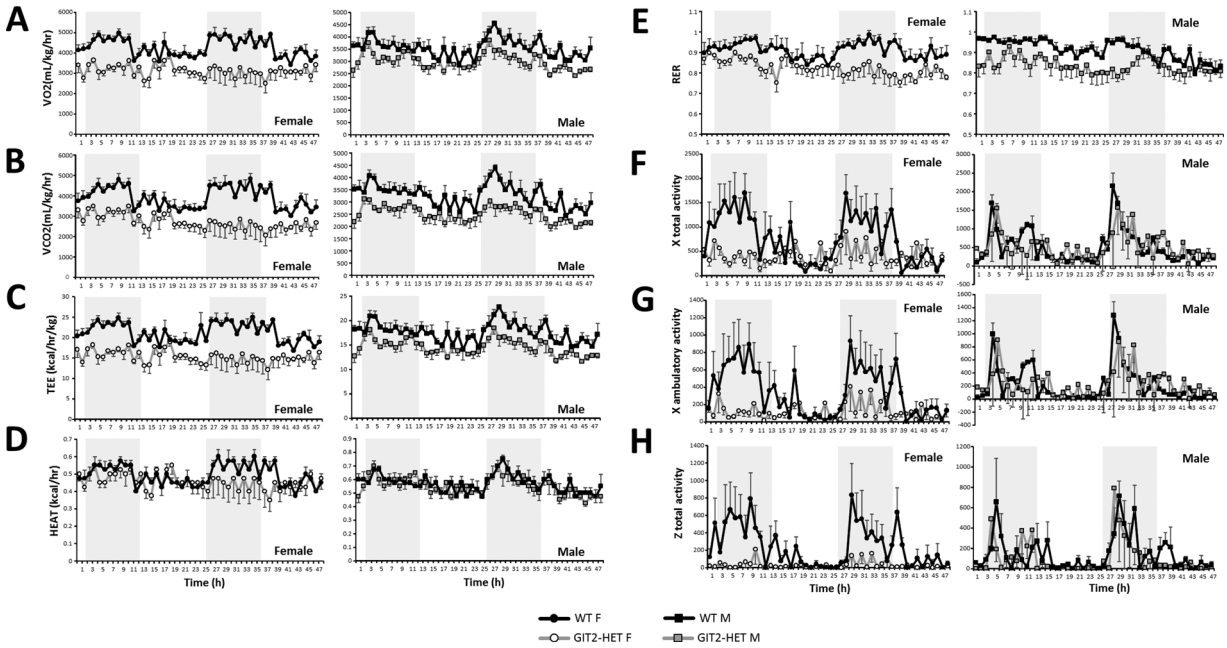


Fig. 10. Whole-body metabolic analysis of WT and GIT2-HET female and male mice. All experimental murine subjects were housed for 48 h in a Columbus Instruments International Comprehensive Lab Animal Monitoring System (CLAMS). VO_2 (A), VCO_2 (B), TEE (total energy expenditure) (C), HEAT (ambient heat generation) (D), RER (respiratory exchange ratio) (E), x-total activity (F), x-ambulatory activity (G) and z-total activity (H) were measured in WT females (black-filled circles), GIT2-HET females (white-filled circles), WT males (black-filled squares) and GIT2-HET males (grey-filled squares). Vertical grey areas in each panel (A–H) indicate the dark period of animal incubation in the CLAMS chamber. Each data point plotted is the mean ± SEM for four independent experimental animals. Statistical significance is indicated in each figure as *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

– this functional bifurcation may have profound effects for aging trajectories in these mice and both genders in general. Given the potential importance of these 11 key factors, future studies examining the temporal variance in expression across both genders have already been instigated. Hopefully, an enhanced understanding of these longevity-regulating networks will reveal crucial mechanisms by which healthspan may be therapeutically regulated.

While there is considerable research investigating the role of dietary stress factors on aging, *e.g.* caloric restriction or intermittent fasting (Colman et al., 2009; Golbidi et al., 2017; Martin et al., 2006; Shintani et al., 2018; Willcox and Willcox, 2014) a smaller body of literature has been developed for the impact of psychosocial/anxiety (Gaffey et al., 2016; Lai and Lee, 2019; Maestripieri and Hoffman, 2011; Prather et al., 2015; Riga et al., 2004; Schwartz et al., 1995; Stein et al., 2018) and psychoactive (Kirkpatrick and Kennedy, 2018; Lindqvist et al., 2015; Tian et al., 2017; Wadhwa et al., 2019) stressor effects on longevity. It is clear however from these studies that a reliable intersection between psychological and psychoactive stress and the rate of aging/onset of age-related disease exists (van Gastel et al., 2019). Further confirming this link, it has been demonstrated that anti-psychotic therapeutics can also entrain longevity-related anti-oxidant pathways such as the telomerase and Nrf2 pathways (Castillo-Quan et al., 2016; Coutts et al., 2019). Thus, it is not surprising that if aging and stress-related activities are functionally linked a physical bridge must also be present. The GIT2 murine model has been demonstrated to possess an excessive stress drive related to greater diurnal corticosterone levels (Schmalzigaug et al., 2009; Van Gastel et al., 2018a), here we found considerable functional overlap between the female GIT2-HET molecular phenotype and physiological processes associated with the stress-aging nexus, *e.g.* altered age-related DNA damage activity (Fig. 2B), modulated dopamine signaling and mitochondrial function (Fig. 2D), modification of upstream dopamine D2 receptor (DRD2) activity (Fig. 3B), altered lithium, dopamine and 6-hydroxy dopamine functional effects (Fig. 3C), transcriptome association with dopaminergic/Parkinsonian Weaver/Reeler murine phenotypes (Fig. 5A: (Cendelin, 2014; Marti et al., 2016; Panagopoulos et al., 1993)) and a potent mitochondrial bias of female GIT2-HET functionality (Fig. 9). With respect to the associations between neurodegenerative conditions (with occasional psychiatric symptoms; (Kashihara, 2017)) such as Parkinson's disease (PD) which has classically been associated with dopaminergic deficits due to damage to nigro-striatal pathways, and the GIT2-HET phenotype, it is interesting to note that some of the most robust risk factors for PD are age-related mitochondrial dysfunction and neuronal demyelination (Reeve et al., 2014; Song et al., 2019; Ubeda, 2007). Further linking female-biased GIT2 signaling to dopaminergic/aging functionalities, we have recently demonstrated in a female model (ovariectomy) of advanced hypothalamus-associated aging linked with mitochondrial dysfunction (Borras et al., 2007), that significant alterations in brain functional connectivity, neuronal demyelination and disrupted dopaminergic receptor signaling were correlated to diminution of female GIT2 expression (Anckaerts et al., 2019). In this study we also found a strong correlation of reduced GIT2 expression with an attenuation of relaxin-3 receptor (Rxfp3) expression. The G protein-coupled Rxfp3 receptor has recently been demonstrated to possess significant potential as a target for psychiatric/psychological stress-related conditions (Kumar et al., 2017; Smith et al., 2014) – given this, it is interesting to note that we also found (Tables S1 and S2) a further correlation between GIT2 expression and Rxfp3 (formerly identified as Gpcr135), *i.e.* GIT2 heterozygosity resulted in a significant reduction of Gpcr135 expression. This repeated commonality of expression regulation suggests that this axis of the Rxfp3 and the G protein-coupled receptor-interacting GIT2 may represent a fundamental regulatory system for aging trajectories of neurometabolic/degenerative conditions.

In our current study, we have deployed novel bioinformatic workflows to deconvolute high-dimensionality transcriptome data to yield an actionable and functionally-relevant data output. We suggest it is likely that female longevity superiority may be associated with the ability of GIT2 to control energy metabolism flexibility. In contrast, males appear to prioritize neurosynaptic functionality to the detriment of metabolic flexibility. These findings are in agreement with our

previous work in which we found that female central nervous system transcriptomic responses are exquisitely sensitive to metabolic perturbations while male counterparts are only sensitive to caloric excess or starvation (Martin et al., 2008, 2009). With respect to the specific locus of female longevity superiority considerable interest has focused upon gender-associated hormonal signaling cascades. It is interesting to note that perhaps the most prevalent – at a worldwide level – energy and age-related disorder, Metabolic Syndrome (Haffner and Taegtmeyer, 2003; Vishram et al., 2014), is tightly linked to gender-controlling hormonal signaling (Kim and Halter, 2014). It has been demonstrated that disruption to androgen-estrogen signaling balances, *e.g.* with menopause or polycystic ovarian syndrome, can augment the susceptibility of females to metabolic syndrome (Janssen et al., 2008; Polotsky et al., 2012; Yang et al., 2016) resulting in negative effects upon healthspan. Therefore, females may possess a longevity superiority likely due to their prioritization of metabolic diversity capacity compared to males.

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Declaration of Competing Interest

There are no conflicts of interest to report.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mad.2019.111150.

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