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Defining the age-dependent and tissue-specific circadian transcriptome in male mice

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SUPPLEMENTAL INFORMATION

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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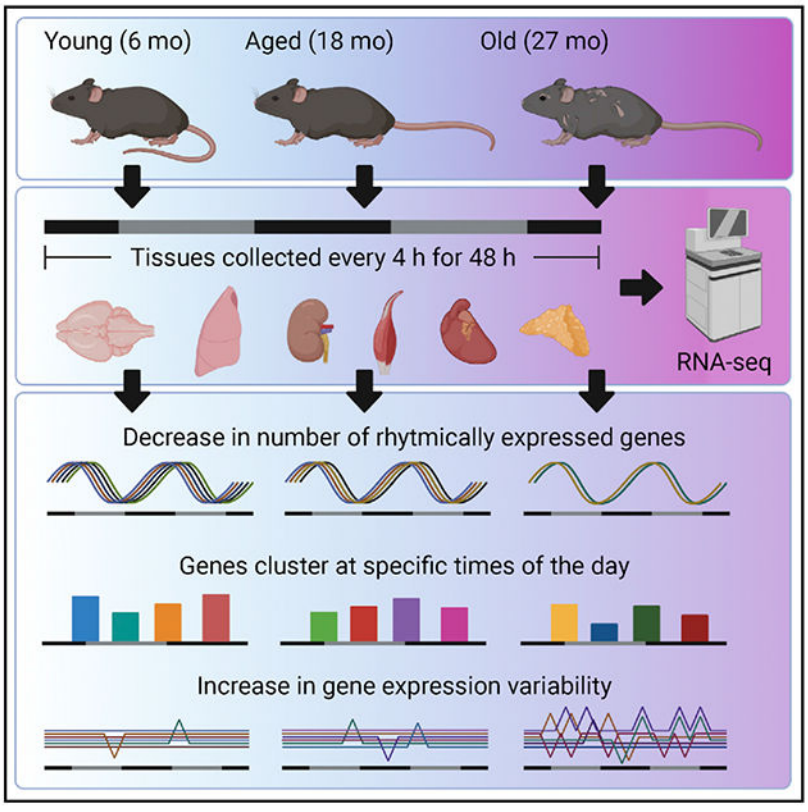
SUMMARY

Cellular circadian clocks direct a daily transcriptional program that supports homeostasis and resilience. Emerging evidence has demonstrated age-associated changes in circadian functions. To define age-dependent changes at the systems level, we profile the circadian transcriptome in the hypothalamus, lung, heart, kidney, skeletal muscle, and adrenal gland in three age groups. We find age-dependent and tissue-specific clock output changes. Aging reduces the number of rhythmically expressed genes (REGs), indicative of weakened circadian control. REGs are enriched for the hallmarks of aging, adding another dimension to our understanding of aging. Analyzing differential gene expression within a tissue at four different times of day identifies distinct clusters of differentially expressed genes (DEGs). Increased variability of gene expression across the day is a common feature of aged tissues. This analysis extends the landscape for understanding aging and highlights the impact of aging on circadian clock function and temporal changes in gene expression.

Graphical Abstract



In brief

Wolff et al. provide an overview of the impact of chronological age on mRNA circadian rhythms in multiple tissues from male mice. Across the organs, there are age-related declines in the number of genes exhibiting circadian expression patterns.

INTRODUCTION

Aging is characterized by a progressive loss of homeostatic control, leading to functional declines and decreased resilience. Over the past 3 decades, there have been numerous studies that used microarray and RNA-seq to examine age-related changes in gene expression across tissues (reviewed in Frenk and Houseley[1]). These studies contributed to the mechanistic understanding of aging biology, leading to defined hallmarks of aging.[2,3] More recent comprehensive RNA-seq studies in rodents captured age-dependent transcriptomic changes across multiple organs and various ages and highlighted age-related increases in inflammation and loss of proteostasis across tissues.[4,5] However, these previous studies did not consider the time of day or the impact of aging on the circadian clock, thus overlooking a critical dimension of aging physiology.

In mammals, virtually every cell in the body has a functional circadian clock. The circadian system consists of a network of central and peripheral oscillators that give rise to various rhythmic outputs largely in a tissue-specific manner.[6,7] The suprachiasmatic nucleus (SCN) of the hypothalamus serve as the central clock that receives the daily light input and regulates the sleep/wake cycle.[8] SCN neurons and peripheral tissue cells share a similar molecular clock mechanism that is based on an autoregulatory transcriptional negative feedback loop. The core feedback loop consists of the transcriptional activators BMAL1 and CLOCK and their negative regulators PER and CRY.[6] The core clock also directs a daily transcriptional program that is cell type specific. It is this circadian transcriptional output that prepares the cell for daily environmental changes and underlies predictive vs. reactive homeostasis.[9,10] Recent studies have established that circadian functions decline over the lifespan. For example, age-related changes in the timing and amplitude of sleep/wake activity, body temperature, and hormone release in rodents and humans have been well documented (reviewed in Hood and Amir[11]). Aging is also associated with a reduced ability to re-entrain to a new light/dark cycle, and increased mortality following repeated “jet lag.”[12–14] It can be posited that circadian attenuation likely contributes to increased damage accumulation,[15] frailty phenotypes,[16] and decreased resilience seen with aging.[17]

Although age-related decline of circadian physiology and behavior has been generally recognized, significantly less is known about age-related changes in the circadian transcriptional output. A few recent aging-related circadian transcriptomic studies show that the core clock genes in aged tissues remain largely intact under light/dark conditions but the genes comprising the transcriptional output are altered (reviewed in Welz and Benitah[18]). For example, the circadian transcriptome is reprogrammed from 3 months to 24 months in the mouse liver,[19] and this was also apparent in stem cells from skin and muscle of 18-month-old mice.[20] Age-dependent circadian transcriptomic reprogramming has also been reported for the human prefrontal cortex[21] as well as in *Drosophila*,[22] demonstrating that altered circadian output is a conserved characteristic of aging. While these initial

investigations brought attention to aging circadian clock output, there has been a lack of systematic design and analyses of the circadian transcriptome across organs and ages. We therefore carried out a 48-h circadian transcriptomic analysis in male mice at three ages, (6, 18, and 27 months) and six organs and tissues (hypothalamus, lung, heart, kidney, skeletal muscle, and adrenal gland). We define age-related changes in the number and identity of the clock output. The temporal resolution of our data also offered an opportunity to examine the genes that were not rhythmic but displayed differential expression patterns at four distinct time domains of the day (e.g., active phase vs. rest phase). We suggest that altered circadian clock output with age should be considered a hallmark of aging that contributes to the changes in cell and tissue homeostasis and likely contributes to frailty and compromised resilience in the old. To disseminate these data, we constructed a "CircaAge" database that provides investigators the ability to query the expression patterns of any gene in any of the organs across circadian time (CT) and age. Mechanistic insights into the interplay between the circadian and aging systems will offer an opportunity to enhance circadian function and promote healthy aging.

RESULTS

Profiling the aging circadian transcriptome across organs

Our study examined the circadian transcriptome in multiple tissues at multiple time points across the lifespan. We obtained male C57B6/J-NIA mice at 4, 16, and 25 months old. The mice were maintained under 12 h:12 h light/dark conditions until 6 (Young), 18 (Aged), or 27 months (Old) of age. Prior to tissue harvest, mice were released into constant darkness (CT or CT0) to study circadian gene expression under free-running conditions. Tissue collections began at CT18 and continued every 4 h for 48 h, with a total of 12 time points, in accordance with the guidelines for analysis of circadian genome data.[23] Our systems-level analysis included the hypothalamus, which contains the central SCN clock, and five other peripheral tissues, lung, kidney, skeletal muscle, heart, and adrenal gland (Figure 1A). We obtained high-quality RNA sequencing (RNA-seq) data from all tissues/organs with the exception of the first 24 h of data from the adrenal gland of aged mice due to a technical issue. These adrenal gland data were included as supplemental data (Figure S1) but excluded in our larger analysis.

Age-dependent decline in the number of circadian clock-regulated genes across tissues

All RNA-seq samples were sequenced to a depth of at least 40 million reads aligned to the mouse genome. To identify the circadian transcriptome, we deployed the cosinor model implemented in the diffCircadian software.[24] Specifically, we defined circadian clock output genes as those with 24-h cosine oscillations in transcript abundance based upon a raw p value <0.01. We provide a table with the number of circadian rhythmically expressed genes (REGs) for each tissue and at each age for different p values and corrected for multiple testing (q values) (Table S1). We first examined the impact of age on the REGs in each organ and tissue (Table S1). Overall, the majority of the REGs in the Young were tissue-specific, consistent with previous multiorgan genomic studies using young mice.[7] Across all tissues, we found an age-associated decline in the number of REGs. The largest REGs change was seen in the kidney with ~75% decline from Young to Old. The heart showed the least change

with ~34% decline. The REGs within each tissue were also age-dependent, with less than 10% of REGs conserved across ages (Figures 1B–1G, S2 and S3). In the hypothalamus, there were 495 REGs in both the Young and Aged groups, but this declined to 196 in Old (Figure 1B and Table S2). We identified 793 REGs in the Young lung, 591 in Aged, and 251 in Old (Figure 1C and Table S2). The kidney had 2,183 REGs in Young, 1,356 in Aged, and 550 in Old (Figure 1D and Table S2). As in the hypothalamus, the kidney circadian transcriptome was clearly age-specific, with only 232 (8%) of the REGs shared across all three ages. In skeletal muscle, the number of REGs decreased from 941 in Young to 699 in Aged and 474 in Old (Figure 1E and Table S2). Again, there was limited overlap of the circadian transcriptome across ages, with just 50 (3%) of the REGs shared across all three ages. We identified 1,186 REGs in Young hearts, 1,068 in Aged, and 785 in Old, with 170 genes shared across all three ages (Figure 1F and Table S2). Finally, in the adrenal gland, we identified 476 REGs in Young, and only 178 in Aged and 216 in Old (Figure 1G). We also plotted the changes in p values for the Young REGs with age and in each tissue (Figure S3). Our analyses demonstrated that the number of REGs declines in all tissues in an age-dependent manner, and that few genes are rhythmically expressed across all ages.

With such large changes in the circadian output with age, we queried the aging effects on the expression patterns of the core clock genes representing the three interlocking loops of the circadian clock (Figures S3 and S5). The expression of *Bmal1* was most robust with age being largely rhythmic across all tissues and ages. The one exception was that the core clock genes in the hypothalamus were either weakly rhythmic or not rhythmic across all ages, likely due to the differences in circadian timing across the cell types and nuclei in this brain region.[7,25] Recent studies had suggested a limited impact of age on the core clock genes.[19,20] However, our statistical analysis revealed a notable decline in the rhythmicity of the repressor components of the clock. We found that *Per1* and *Per2* were dampened from Aged to Old across all peripheral tissues (Figures S3 and S5). The secondary loop genes, *Nr1d1* and *Nr1d2*, also exhibited age-related loss of rhythmicity but only in the skeletal muscle and hypothalamus (Figure S3). Thus, there are common aging effects on selective components of the negative limb of the core clock mechanism across tissues. This observation is consistent with the slowed rate of entrainment of the circadian system with age.[13]

Temporal patterns of age-associated differential gene expression

Our time course collection also provided the opportunity to explore temporal differences in age-related changes in gene expression beyond circadian rhythmicity. We binned the 48-h data over four time domains or phases: the rest phase (light phase in the nocturnal mice), activity onset (rest-active transition), active phase (dark phase), and activity offset (active-rest transition). We used an ordinal analysis strategy to define time domain specific genes that changed in the same direction from Young to Aged to Old. This approach is similar to the linear gene expression changes noted by Shavlakadze and colleagues.[5] To our surprise, we found that a large number of the differentially expressed genes (DEGs) were detected at unique time domains, but this occurred in a tissue-specific manner. These outcomes provide time of day molecular maps of tissue aging with the potential to more

precisely target therapeutic strategies. We describe in each of the sections below the unique clusters of DEGs for the distinct time domains.

Loss of circadian metabolic and immune homeostasis in the aging hypothalamus

The hypothalamus controls essential homeostatic and survival-related functions. It is a small yet highly heterogeneous tissue, with multiple cell types and functionally distinct nuclei, including the SCN. Through projections to other nuclei, the central SCN clock functions to coordinate various circadian rhythms such as endocrine release and sleep/wake behavior.[26,27] The RNA-seq sensitivity enabled the detection of cellular markers specific to cell types and nuclei, for example, *Vip* and *Nms* (SCN), *Agrp, Npy* and *Pomc* (arcuate nucleus), *Hcrt* (lateral hypothalamic area), *Gfap* (astrocytes), and *Aif1* (microglia).[28] The high-quality data provide the first systems-level view of circadian transcripts across ages in the hypothalamus. Circadian rhythmicity analysis uncovered 495 REGs in Young and Aged, but significantly fewer (196) in Old. Overall, there were fewer REGs in the hypothalamus than in the peripheral tissues at the same cutoff, which is due in part to its cellular heterogeneity.[25] While some REGs were present in all three ages, most of them were specific to one age group. Most REGs in Young lost rhythmicity in Old, whereas several genes gained rhythmicity in Old.

We used the Ingenuity Pathway Analysis (IPA) to analyze and integrate the functional pathways enriched for the REGs across ages. This analysis revealed conserved pathways across ages as well as age-specific pathways (Figures 2A and 2B and Table S3). For example, the NRF2 oxidative response was rhythmic across the lifespan, although the genes contributing to the pathway were different across ages. The HIF1, adipogenesis, and neuregulin signaling pathways lost REG enrichment from Young and Aged to Old (Figure 2A). Strikingly, many pathways were enriched only in one of the three ages, with the highest number of the age-specific pathways occurring in Young (n = 80), followed by Aged (n = 37) and Old (n = 27) (Figure 2B). We note that, while changes in enriched pathways can occur when the REG number is reduced, those changes from Young to Aged in the hypothalamus occurred without a change in the REG number. In general, however, we found that the changes in pathway enrichment across all tissues with age are largely driven by changes in specific genes. Interestingly, many of these REG pathways in Old emerged from unique gain-of-rhythm genes. In general, the REG pathways overrepresented in Young were involved in circadian regulation, energy homeostasis, proteostasis, and cell growth and development, whereas those in Old were related to stress and adaptive responses and basic central nervous system functions such as neurogenesis, axonogenesis, and synapse formation.

We next analyzed the REGs based on their CT of peak expression for each age group (Figures 2C–2E, top panels). Strikingly, we observed an age-specific daily distribution of the REGs. While the REGs in the Young hypothalamus had multiple peaks across the circadian cycle, only two peaks were prominent in Aged, with one at activity offset and the other at activity onset. The top enriched functional pathways reflect the time of day-dependent hypothalamic functions (Figures 2C–2E, bottom panels and Table S3). Notably, the REGs in the CRY1-PER2 complex had a peak time between 12 and 16 h in

Young and Aged but lost their rhythms in Old, indicative of compromised active circadian repression and weakened transcriptional outputs. Among the genes that were not highly rhythmic in Young or Aged but gained robust rhythms in Old were vasoactive intestinal polypeptide (*Vip*) and neuromedin S (*Nms*). These two neuropeptides play key roles in photic entrainment, neuronal coupling, and SCN synchronization. Other examples include the corticotropin-releasing hormone (*Crh*) in the hypothalamus-pituitary-adrenocortical axis, and factors involved in neuropeptide signaling (e.g., *Auts2, Drd2, Chrm3,* and *Adora2a*), which became highly rhythmic in Old, likely as an adaptive response to homeostatic stress.

In addition to the REGs, we found a large number of DEGs in the hypothalamus that exhibited unique expression patterns across the four time domains of the day (Figures 2F–2J and Table S3). There were 604 DEGs that were either upregulated (514) or down-regulated (90) across ages at all time domains (Figure 2J). Pathway analysis revealed that the upregulated DEGs had an overrepresentation for the immune and inflammatory responses in Aged and Old tissues (e.g., *Gfap, Aif1, Trem2, Adgre1,* and *Ptgs1*), whereas active transcription-related genes were significantly downregulated. The shift from the metabolically active and proliferative state in Young to an inflammatory state in Old is consistent with findings from genomic studies in both mice[4,29] and rats.[5] The DEGs at activity offset were enriched in neurotransmitter synthesis and neuron differentiation and those at activity onset were in stress and immune responses. Also of note, during the rest phase, genes related to protein transport (e.g., *Vps35, Vps39, Vps45*) and autophagy (e.g., *Atg3*) were significantly upregulated with age (Figure 2G), whereas those involved in vesicular transport (e.g., *Srebf1, Slc2a8, Tbc1d17*) were upregulated during the active phase (Figure 2I). Taken together, our findings from both the REGs and the DEGs highlight the importance of considering the time of day when exploring age-related changes in gene expression and functions.

Inflammation is a major feature of aging in the lung

Broadly speaking the lung functions primarily for gas exchange; however, the organ also serves as a first-line site for defense against pathogenic microbial species.[30,31] Therefore, time of day responses to stressors are critical for maintaining healthy pulmonary and organismal function. The lung REGs were enriched for several stress-related pathways that were maintained across the lifespan, including the unfolded protein response and xenobiotic metabolism (Figure 3A and Table S4). However, immune-related pathways, such as CXCR4 signaling and the hypoxia pathway, HIF1a, lost rhythmicity in the Old (Figure 3B). For example, *Tmem173/Sting1* and *Unc93b1* were rhythmic in the Young but lost rhythmicity with age. The REG pathways that gained rhythmicity in Old were enriched for amino acid metabolism and amino acid hormone synthesis (Figure 3B).

The peak time distribution of the REGs in the lung showed similarities across ages. However, there were significant age-specific changes in the temporal distribution of the REG functions (Figures 3C–3E and Table S4). For example, in the early rest phase (peak time 0–8 h), the REGs in the Young contribute to the immune response and antigen processing pathways but these pathways were lost in the Aged and Old lungs. This would implicate the diminished ability of the lungs to best defend against the immune challenges with age. In the

Old, the pathways overrepresented in the active part of the day (peak time 20–24 h) include response to corticosterone and redox state, suggesting a more reactive set of REG functions related to stress responses.

The lung exhibited large differences in the magnitude of the DEGs across the four time domains. Specifically, activity onset had the largest number of DEGs (1,557) while the rest phase showed the smallest number (100) (Figures 3F–3I and Table S4). Oxidative metabolic pathways were upregulated in the activity onset as well as the rest phase while immune-related DEGs were upregulated in the active phase and activity offset. The upregulation of immune response DEGs in the active phase suggests compensation for the loss of immune REGs in the Young. Overall, this reinforces a decrease in the anticipatory nature of the pulmonary response to pathogens, resulting in a time of day vulnerability to pulmonary disease. Thus, the aging-associated loss of circadian functions likely potentiates the inflammatory response in the lung, potentially contributing to loss of organismal resilience.

Aging blunted the oscillatory expression patterns of ion transporters in the kidney

The kidney is critical for several aspects of systemic homeostasis.[32,33] One of its primary functions is to maintain electrolyte balance through ion transport. Relative to the other tissues, the kidney had the most REGs at Young (2,183) and Aged (1,356), but REG numbers decreased to 550 in Old. Functional cluster analysis of the REGs revealed that many pathways were maintained across age including the unfolded protein response and fibroblast growth factor signaling (Figure 4A and Table S5). However, the aldosterone signaling pathway was enriched in the Young and Aged but lost in the Old (Figure 4A). Notably, the two key REGs unique to kidney function, *Scnn1a* (a subunit of the sodium channel) and *Atp1a1* (a subunit of the Na^+/K^+ -ATPases) were highly rhythmic in the Young but attenuated in the Old. The REG pathways were representative of the hallmarks of aging, including AMPK signaling and ubiquitination, lost oscillations in Old (Figure 4A). Of the pathways enriched solely in Aged, eNOS signaling likely reflects daily maintenance and homeostasis of the epithelium as well as renal hemodynamics[34] (Figure 4B). Finally, NAD^+ salvage pathways and acute immune responses were unique to Old, possibly as an adaptive mechanism to maintain homeostasis.

The overall peak time distribution of the REGs in the kidney did not change dramatically with age. However, pathway analysis highlighted age-specific changes in the temporal distribution of the REG functions (Figures 4C–4E and Table S5). For instance, in the Young and Aged kidneys, genes related to pH homeostasis were oscillating with a peak time between 8 and 12 h, but not in Old. While ion transport processes remained rhythmic in the Old, they peaked at different times compared with Young and Aged. Another notable example was proteostasis-related pathways that were enriched across the circadian cycle in Young and Aged but limited to the late active phase in Old. While pH homeostasis and proteostasis-related genes were rhythmic in the Young and Aged kidney, the daily timing becomes out of phase with age. These age-dependent changes in temporal alignment between key physiological processes may exacerbate aging kidney phenotypes.

There were profound age-related changes in the DEGs, with 2,438 genes differentially regulated with age at all times, in addition to the DEGs that were specific to different time domains (Figures 4F–4J and Table S5). We observed downregulation of mitochondrial genes and an increase in stress fiber assembly at activity onset. Moreover, the upregulated genes at activity onset were enriched for primary focal segmental glomerulosclerosis, highlighting kidney-specific changes in gene expression with age (Figure 4H). As in the lung and hypothalamus, there was a significant upregulation of immune-related pathways with age at all time domains, accompanied by a decrease in proteolysis and lipid metabolism (Figure 4J). In addition, the decreased proteostasis REGs in Old were paralleled by the decreases in the DEGs (Figures 4F–4J; bottom panels).

Skeletal muscle circadian transcriptome changes with age highlight autophagy and myogenic programs

Skeletal muscle is critical for health through its role in regulating movement and metabolism and it is emerging as a source of circulating factors such as myokines.[35,36] In the context of aging, epidemiological studies have shown strong correlations between loss of muscle strength and increased morbidity and mortality.[37] As with other tissues, there was a significant decline in the number of REGs with age. Across the REGs there was a preservation of functional groups including insulin receptor and HIF1 signaling, but pathways such as AMPK, PI3K/AKT, and unfolded protein response lost rhythmicity with age (Figure 5A and Table S6). The functional categories that gained rhythmicity with age include dilated cardiomyopathy and interleukin-1 signaling (Figure 5B).

The distribution of the peak times for the REGs in muscle was similar to that in the hypothalamus. In the Young, the REGs were distributed across the day, but in the Aged the REGs were largely bimodal with clusters at activity onset and offset (Figures 5C–5E and Table S6). Of note, the REGs that contribute to autophagy peaked at activity offset in Young, and *Tfeb*, considered a key upstream regulator of autophagy,[38,39] is one of the REGs in that temporal cluster. In the Aged, *Tfeb* is no longer circadian and the autophagy cluster is centered toward the middle of the rest phase (Figure 5D). Recent studies have implicated changes in the timing of autophagy as a contributor to aging in flies and mice.[40,41] The REGs in the Old muscle were largely unimodal, peaking at activity offset. This cluster includes myofibril assembly and vascular endothelial growth factor pathway suggesting enrichment of muscle tissue maintenance functions in the Old (Figure 5E).

Analysis of the muscle DEGs revealed significant differences across the four different time domains. The largest cluster of DEGs was found in the active phase (833 DEGs) with only 47 DEGs in the rest phase and 136 DEGs were found in all time domains (Figures 5F–5J and Table S6). In the active phase, the downregulated DEGs contributing to RNA processing, transcription, and genome maintenance were overrepresented, whereas autophagy and mitophagy pathways were upregulated DEGs with age. The increase in autophagy DEGs may compensate for the loss of rhythmic control of autophagy with age. Among the DEG pathways that were common across all time domains was the upregulated nuclear factor-κB signaling, which is consistent with inflammation being a common issue that all tissues are responding to with age (Figure 5J). The other upregulated cluster across

all time domains was related to skeletal muscle contraction and sarcoplasmic reticulum, suggesting that maintenance of muscle function is a priority in the Old.

Limited impact of age on circadian and steady-state mRNA expression in mouse hearts

The heart is required for maintenance of gas and nutrient delivery, as well as cellular waste removal through the circulation.[42,43] The heart is unique among all the tissues in that the core clock components exhibit very little change with age and the REG pathways were largely conserved across age. These include many pathways linked to the hallmarks of aging, such as protein homeostasis and senescence (Figure 6A and Table S7). There were some pathways enriched only in the Young and Aged, including some essential processes like p53 and iNOS signaling that were no longer enriched in the Old. In contrast to the other tissues, the heart does not have new REG pathways unique to the Old (Figure 6B).

The peak time distribution of the REGs showed little change, consistent with very little apparent cardiac-specific circadian disruption. Across the three ages, the REGs were evenly distributed throughout the 24-h day (Figures 6C–6E and Table S7), similar to the kidney and lung tissues. In addition to maintaining this daily distribution of genes, pathways related to circulatory maintenance (e.g., angiogenesis and circulatory system development) were consistently peaking at the activity offset across all ages. Despite this pathway conservation with age, there were age-related changes in the heart REGs at the activity onset, where the Young and Aged hearts were enriched for metabolic processes, while the Old were enriched for stress responses.

While aging had a limited impact on the REGs in the heart, it had the highest number of unique DEGs at activity offset (891; Figure 6F). During this transition period, we observed age-related decreases in ribosome biogenesis, RNA processing, and translation processes, suggesting reduced proteostasis in the Old. Relevant to cardiac function, Ca^{2+} signaling pathways were upregulated at activity offset with age. In contrast, DEGs enriched for mitochondria and fatty acid oxidation were decreased at all time domains, supporting the concept of age-related decrements in metabolic capacity in the Aged and Old.[44] In addition, cytokine-related genes were upregulated with age across all time domains in the heart (Figures 6F–6J and Table S7).

Increased daily transcriptional variability is conserved across tissues

Most recently, studies have identified consistent age-associated increases in the variability of gene expression.[45,46] We queried non-rhythmic genes and found that there was an increase in the variability of gene expression with aging in all tissues except the heart (Figures 7A–7F). For example, the hypothalamus and lung had 2,516 and 2,452 genes with high variation, respectively. Surprisingly, we found that even common housekeeping genes, including *Gapdh* and *Rplp0*, were significantly more variable with age across tissues (Table S8). The increase in variability was prevalent from Aged to Old in the hypothalamus, lung, and kidney, but not the heart and skeletal muscle. Skeletal muscle was unique in that most of the variably expressed genes occurred from Young to Aged with fewer changes in Aged to Old. In the heart, the numbers of variably expressed genes were much lower than in other tissues, but the changes were similar from Young to Aged and from Aged

to Old (Figure 7G). Comparison of the variable genes across tissues identified that lung, kidney, and skeletal muscle shared 212 genes, suggesting common age-associated changes in transcriptional regulation (Figure 7H). Functional cluster analysis of these 212 variable genes identified protein transport and ubiquitination, chromatin organization, and immune system processes (Figure 7I and Table S8). The increased variability in gene expression is consistent with the concept that the physiology underlying frailty with age occurs through increased dysregulation of gene expression.[16]

CircaAge: Database of age-dependent changes in circadian and non-circadian gene expression patterns

Genome-wide age-dependent changes in gene expression, circadian or not, are difficult to visualize. To disseminate these data, we developed a web-application database, "CircaAge" (https://circaage.shinyapps.io/circaage/). This publicly accessible resource allows the user to query any gene(s) of their interest and visualize transcript expression patterns across 2 circadian days and three age groups in one central and five peripheral tissues. This database also provides model fitness parameters and significance levels of circadian rhythmicity. Users may specify (1) single or batch entry of genes of interest, (2) one to three ages of interest, and (3) one to six tissue types of interest. Data can be conveniently exported as *.csv files with statistical outputs including peak time, amplitude, basal expression (i.e., Midline Estimating Statistic of Rhythm [MESOR]), phase, R^2, and p values.[24]

DISCUSSION

Aging is characterized by declines in physiological functions and a reduced capacity to maintain homeostasis.[2,3,47] Aging is also characterized by declines in circadian functions, such as sleep/wake cycles.[11,48,49] The circadian clock within each cell directs a daily transcription program that temporally segregates important cell functions to support homeostasis and resilience. This clock function underlies what is known as predictive homeostasis, as the changes within the cell and system occur prior to, and not in reaction to, known changes in the environment linked with the light/dark and rest/activity cycles.[9,50,51] Previous studies of circadian transcriptomes with age have been limited to the liver and stem cells and they were done in only two age groups.[19,20,22] Our goal was to obtain a systems-level understanding of the aging circadian transcriptome through analysis of six tissues and across three ages. In the present work, we identified significant changes in the circadian transcriptomes (i.e., REGs) with age and across all tissues. While there is conservation of some functional pathways, we found that key age-related pathways either lost rhythmicity or showed significant changes in their timing or phase of expression. Our observations identify age-associated changes in circadian gene expression as well as temporal shifts in gene expression. We propose that the age-associated changes in circadian clock output lead to reduced predictive homeostasis and an increased reliance on reactive pathways in response to stressors. This loss of predictive homeostasis pathways is part of a new concept in aging that will contribute to decreased resilience and increased frailty. We suggest that circadian clock output reprogramming be considered as a new hallmark of aging and that the circadian clock and its transcriptional program may serve as a new therapeutic target for improving tissue and systemic health across the lifespan.

Age-related changes in gene expression have been providing significant insights into the understanding of the etiology of aging.[4,5,52–54] Interestingly, clock genes were among the most differentially expressed across tissues with age.[4] We leveraged our time course data to ask whether there are clusters of aging genes and pathways that are unique to specific phases of the day. The discovery that there are tissue-specific and time domain aging clusters includes many aging hallmark pathways. One example is that genes related to autophagy were upregulated with age in the rest phase in the hypothalamus but in the active phase in skeletal muscle. The increase in autophagy-related DEGs occurred as the circadian regulation of autophagy-related genes was lost, consistent with emerging evidence highlighting the importance of the circadian control of autophagy in healthy aging.[40] By considering the time of day, the DEGs provide a new, more precise insight into the complexity of aging.

The age-dependent circadian decline can result from internal and external factors. A better understanding of the role of the circadian clock in aging physiology can offer new strategies for interventions. As environmental factors such as light, food intake, and physical activity are known to influence the circadian system, lifestyles that conform to circadian rhythms may represent an attractive non-pharmacologic regimen to slow down aging and improve healthspan. For example, the time-restricted feeding regimen extends healthspan at least in part by improving the circadian transcriptome.[55,56] It should be noted that our data demonstrated a rapid decline in clock output from Aged to Old. Thus, the middle age period may represent a window of opportunity for early intervention that considers circadian concepts in healthy lifestyle choices. Mechanistically, enhanced circadian function improves cell and systemic homeostasis, which in turn can delay and mitigate damage accumulation and frailty phenotypes, increase resilience, and extend healthspan.

In summary, this aging circadian resource provides a rich dataset highlighting the impact of both age and time of day on the transcriptome in multiple tissues. We suggest that the age-related changes in clock output are linked to a loss of resilience. This dataset will help foster collaborations across multiple disciplines. Future work to determine if the age-related changes in circadian clock output and function can be prevented or reversed by known pro-longevity interventions should be explored.

Limitations of the study

One limitation of our study is that we only analyzed tissues from three ages of male C57B6/J-NIA mice. We recognize the potential for sex differences in the circadian transcriptome, especially given known sex differences in aging,[57,58] warranting additional work in female mice. Also, we found tissue-specific changes in the circadian transcriptome but they were limited to only hypothalamus, lung, heart, kidney, skeletal muscle, and adrenal gland. It is very clear that the circadian clock mechanism regulates very tissue-specific gene expression so how these patterns change with age will also likely be tissue specific. For example, the liver is an important metabolic organ that has been characterized before in the circadian aging field[19] and its inclusion would increase the ability to compare our results with others. Last, we only tested at three ages, 6, 18, and 27 months and in particular, the changes from 18–27 months were very striking. Inclusion of ages from 6–18 months and 18–27 months

will be important to better understand the mechanisms underlying the decline as well as providing an opportunity for testing geroscience-based interventional strategies.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact—Additional information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Karyn Esser (kaesser@ufl.edu).

Materials availability—This study did not generate new, unique reagents.

Data and code availability—Raw RNA-seq data have been deposited under GEO with accession number GSE201207 which is publicly available as of the date of publication. Accession numbers are also listed in the key resources table.

This paper does not report the original code. We implemented a user-friendly interactive website (https://circaage.shinyapps.io/circaage/) for users to query and visualize circadian genes.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal care and use—6 month, 18 month, and 27 month old C57BL/6Nia male mice (n = 24/age) were used to assess circadian robustness and molecular clock transcriptional output. All procedures were approved by the University of Florida Institutional Animal Care and use Committee, in accordance with AAALAC guidelines. Mice were ordered from the National Institute on Aging (NIA) colony 2 months prior to collections. Following a two week quarantine, animals were allowed a two week acclimation period to ensure entrainment to the 12h:12h light/dark cycle (lights on: 6am). Prior to tissue collection, animals were released into constant darkness in light-tight circadian cabinets (Actimetrics, Wilmette, IL, USA). To assess the circadian transcriptome in each age, collections occurred every 4h for 48h, starting at CT18. Mice were euthanized by cervical dislocation under dim red light. 2 animals for each age were collected at each time point.

METHOD DETAILS

RNA isolation and library preparation—RNA was isolated from the hypothalamus, kidney, lung, gastrocnemius, adrenal glands, and heart using a modified TRIzol extraction procedure as previously described.[61,62] Briefly, frozen tissues were lightly ground in a mortar and pestle constantly submerged in liquid nitrogen. Frozen tissue between 10 and 100 mg was placed into RNase-free tubes containing TRIzol and RNase-free stainless-steel beads ranging in diameter from 0.9 to 2.0mm (NextAdvance, Troy, NY, USA). Tissues were homogenized using a bullet blender at 4°C. Chloroform was used to separate the phases, and the RNA-containing aqueous phase was subjected to a modified column purification kit using RNeasy Mini RNA extraction kit for all tissues except the hypothalamus, prepared with RNesay Micro RNA extraction kit (Qiagen, Germantown, MD, USA). RNA was

DNase treated, and purified RNA was checked for integrity using an Agilent Bioanalyzer, all samples had an RNA integrity number above 8.0. Poly-A selected RNAseq libraries were prepared using Illumina mRNA Prep kit (Illumina, San Diego, CA, USA). Libraries were pooled to equal molarity and sequenced on an Illumina NovaSeq (2 × 100bp) to achieve a minimum of 40M reads per sample. FastQ files were downloaded to the University of Florida HiPerGator computing cluster. Raw FastQ files, counts, and normalized counts per million data were uploaded to GEO: GSE201207. A file with the raw counts was also included as Table S9.

QUANTIFICATION AND STATISTICAL ANALYSIS

Bioinformatics and data preprocessing for RNA-Sequencing data—Reads from the RNA-Sequencing data were aligned to the *Mus musculus* genome assembly GRCm38 (mm10) using the HISAT2 software. Duplicated aligned reads were marked and removed using the Picardtools software. The gene expression count data were extracted using the HTseq software. The raw count data were normalized to cpm (counts per million reads), followed by log2 transformation (i.e., log2(cpm+1) where 1 is the pseudo count to avoid log2(0)). For each tissue, we filtered out genes with mean log2(cpm+1) < 1. These non-expressed genes were likely to be false positives, and thus removing them could help reduce the multiple testing burden in the later statistical analysis. All data preprocessing was performed using R software.

Circadian rhythmicity analysis—To identify genes showing circadian rhythmicity patterns for each age group within each tissue type, we deployed the cosinor model implemented in the diffCircadian software. To be specific, the cosinor model assumes the following relationship between the expression level and

$$Y_i = A \times \sin\left(\frac{2\pi}{24}(t_i + \varnothing)\right) M$$

Where i ($1 \leq i \leq n$) is the sample index; n is number of samples in each age group within each tissue type; y_i is the log2 transformed cpm value for sample i; t_i is the circadian time for sample i; A, φ, and M denote the amplitude, phase, and MESOR (Midline Estimating Statistic of Rhythm) of the sinusoidal curve; the period was fixed at 24-h. The goodness of fit of the cosinor model was accessed via the coefficient of determination (R^2), and the p value was determined by the finite-sample-corrected likelihood ratio test.[24] Raw p value<0.01 was used as the statistical significance cutoff to declare rhythmically expressed genes (REGs). A table showing the change in the number of REGs using different statistical thresholds is included (Table S1). We also provide Figure S3 visualizing the change in p value for the Young REGs across age within each tissue. To further compare the phase concordance between age groups, the Watson's Two-Sample Test of Homogeneity was adopted. This comparison was performed for each pair of age groups, and for each tissue, respectively.

Differential expression analysis associated with aging—We first created 4 time domain of day groups: including (i) Active Phase: CT18,22,42,46; (ii) Activity Offset:

CT22,26,46,50; (iii) Rest Phase: CT30,34,54,58; and (iv) Activity Onset: CT34,38,58,62. To detect genes showing increasing/decreasing expression values with respect to the ordinal age groups (Young to Aged to Old), we employed negative binomial models implemented in R software edgeR package, which is specifically designed for RNA-Sequencing count data. In this model, the expression value of a gene was the outcome variable, the ordinal age groups were coded as the continuous predictor (0: Young group; 1: Aged group; 2: Old group). Multiple testing was corrected by the Benjamini-Hochberg method, in which the raw p values were converted to q-values (FDR-adjusted p values), where FDR stands for the false discovery rate. This analysis was performed for each time of day group within each tissue, respectively. Q-value<0.05 was used as the statistical significance cutoff to declare differentially expressed genes (DEGs).

Variability analysis associated with aging—To identify genes showing increasing/decreasing variability in expression values with respect to the ordinal age groups (Young to Aged to Old), we used *DiffVar* function in the *missMethyl* package.[63] The variability for each sample of a gene in one age group was calculated using absolute deviations. A linear model with empirical Bayes shrinkage was then fitted, where the variability was the outcome variable, the ordinal age groups were coded as the continuous predictor (0: Young group; 1: Aged group; 2: Old group). p-value<0.01 was used as the statistical significance to declare variably expressed genes.

Pathway analysis—To examine the function annotation of the putative REGs (raw p value <0.01), DEGs (q-value <0.05), and variably expressed genes (raw p value <0.01), pathway enrichment analyses were performed using Ingenuity Pathway Analysis software (REGs), and both g:Profiler[59] and DAVIDv6.9[60] for the DEGs.

To further compare and integrate the REGs' pathway information (i.e., p values) across all three age groups, an adaptively weighted Fisher's method was adopted, which is a meta-analysis to examine whether a pathway is enriched in at least one age group. Unlike the regular Fisher's method that assigns equal weight for all three age groups, the adaptively weighted method searches for the optimal binary adaptive weight (1 for enriched and 0 for not enriched) for each age group (Young/Aged/Old) given a pathway. These binary adaptive weights can capture the similarities across the three age groups. For instance, an adaptive weight of (1,1,1) indicates a pathway is enriched in all three age groups; and an adaptive weight of (1,0,0) indicates a pathway is enriched only in the young group. A meta-analyzed p value was also reported, followed by multiple comparison correction using the Benjamini-Hochberg method. This analysis was performed for each tissue type, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in their field of research or within their geographical location. One or more authors of this paper self-identifies as a member of the LGBTQIA+ community. One or more of the authors of this paper received support from a program designed to increase minority representation in their field of research.

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Highlights

- The number of rhythmically expressed genes (REGs) decline across all tissues with age
- REGs in young tissues are enriched for the aging hallmarks
- Age-specific differentially expressed genes cluster at specific times of the day
- Increase in gene expression variability is a common feature of aging tissues

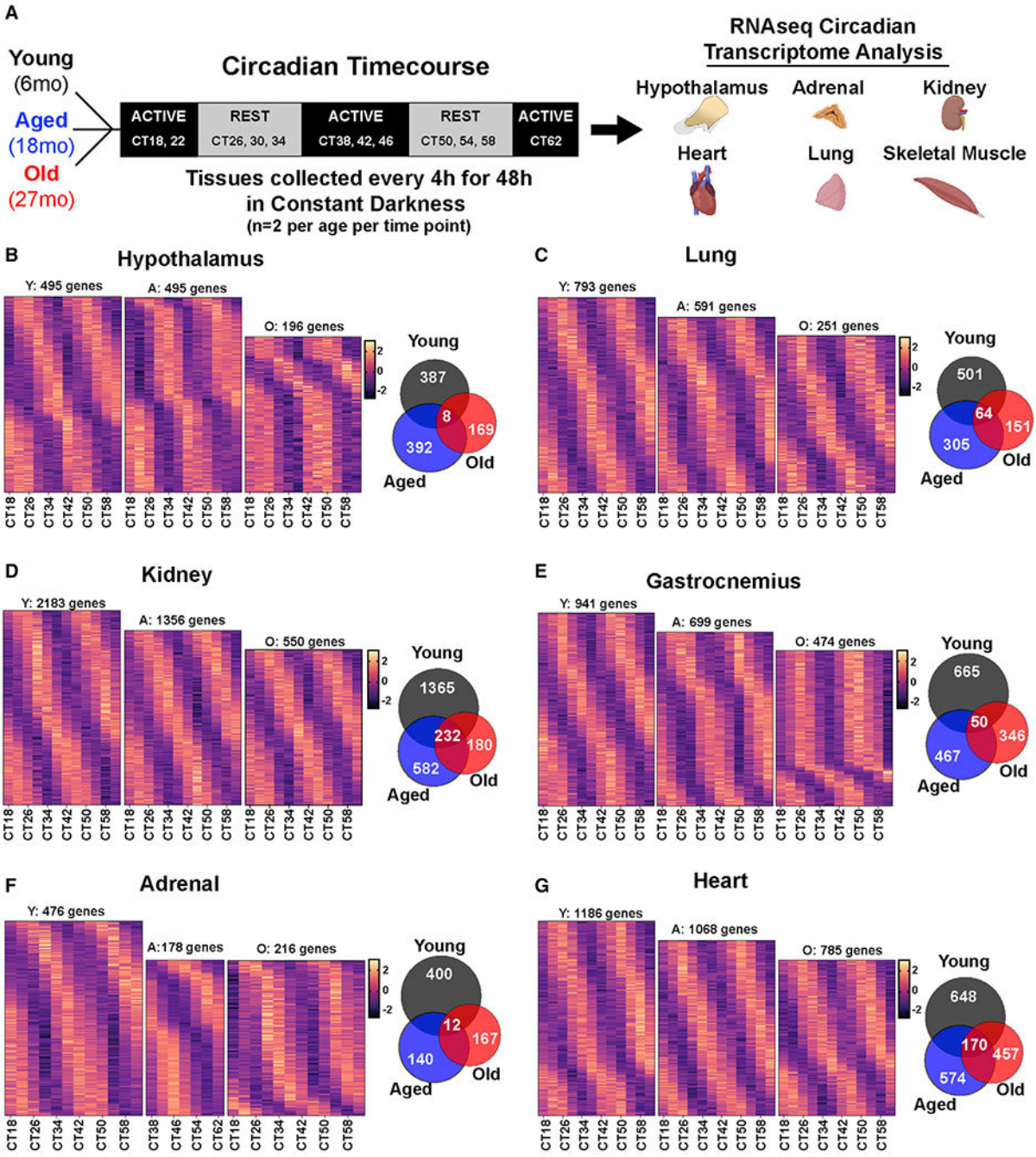

Figure 1. Circadian transcriptome analysis from six tissues and three ages

(A) Simplified study design schematic. Heatmap of z-scored rhythmically expressed genes (REGs) and a Venn diagram of the REGs from each age for the (B) hypothalamus, (C) lung, (D) kidney, (E) skeletal muscle, (F) adrenal gland, and (G) heart. Due to technical difficulties with adrenal samples, only CT38-CT62 from the Aged were included for circadian transcriptome analysis.

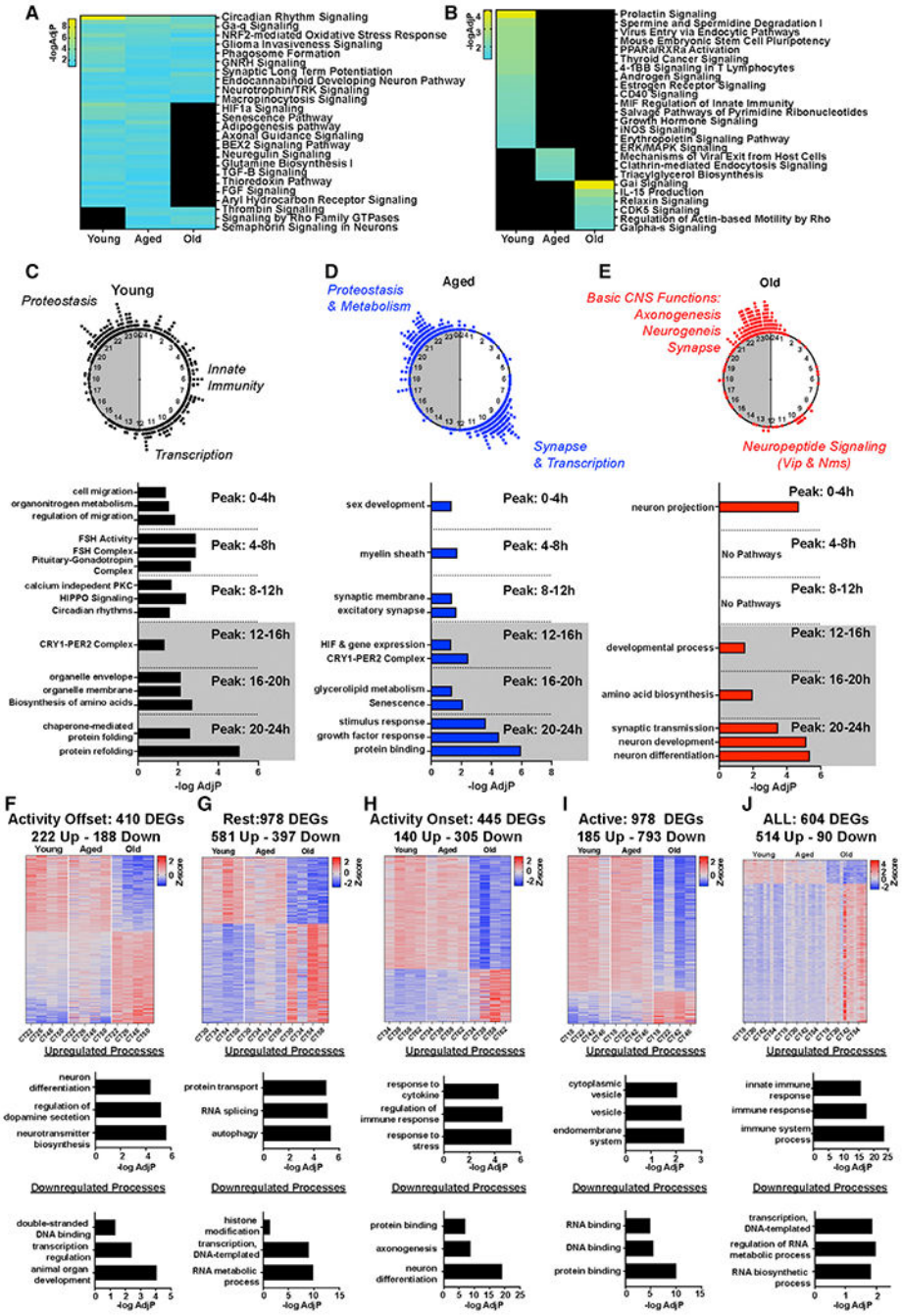

Figure 2. Age-dependent circadian transcriptomic changes in the hypothalamus

(A) Shared IPA pathways enriched by the REGs that cycle in two to three age groups.

(B) Age-specific circadian oscillatory IPA pathways.

(C–E) Peak time maps of all oscillating genes from the hypothalamus of (C) Young, (D) Aged, and (E) Old mice. Each dot represents the peak time of a single significantly circadian gene. Underneath the peak time map is a histogram of top time of day-specific gene expression pathways.

(F–I) Heatmap of z-scored age-related differentially expressed genes (DEGs) and histograms of up- or downregulated pathways from the specific gene sets from the active to rest transition period (F), the rest phase (G), the rest to active transition period (H), and the active period (I).

(J) Heatmap of z-scored age-related DEGs from all time domains and histograms of up- or downregulated pathways from the specific gene sets.

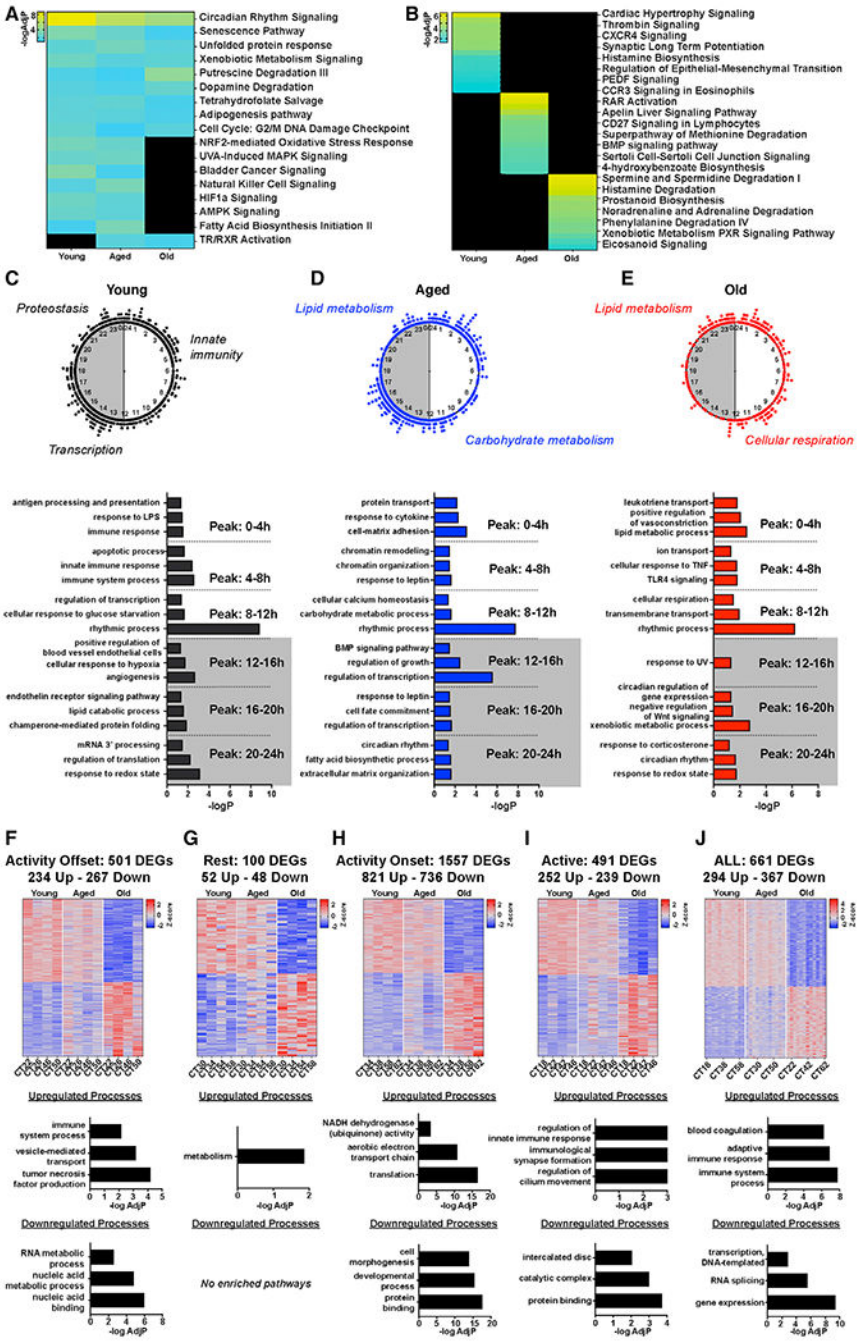

Figure 3. Age-associated changes in the lung circadian transcriptional output and time of day-dependent interpretations of age-related changes in gene expression

(A) Shared IPA pathways enriched by the REGs that cycle in two to three age groups.

(B) Age-specific circadian oscillatory IPA pathways.

(C–E) Peak time maps of all oscillating genes from the lungs of Young (C), Aged (D), and Old mice (E). Each dot represents the peak time of a single significantly circadian gene. Underneath the peak time map is a histogram of top time of day-specific gene expression pathways.

(F–I) Heatmap of z-scored age-related DEGs and histograms of up- or downregulated pathways from the specific gene sets from the active to rest transition period (F), the rest phase (G), the rest to active transition period (H), and the active period (I).
(J) Heatmap of z-scored age-related DEGs from all time domains and histograms of up- or downregulated pathways from the specific gene sets.

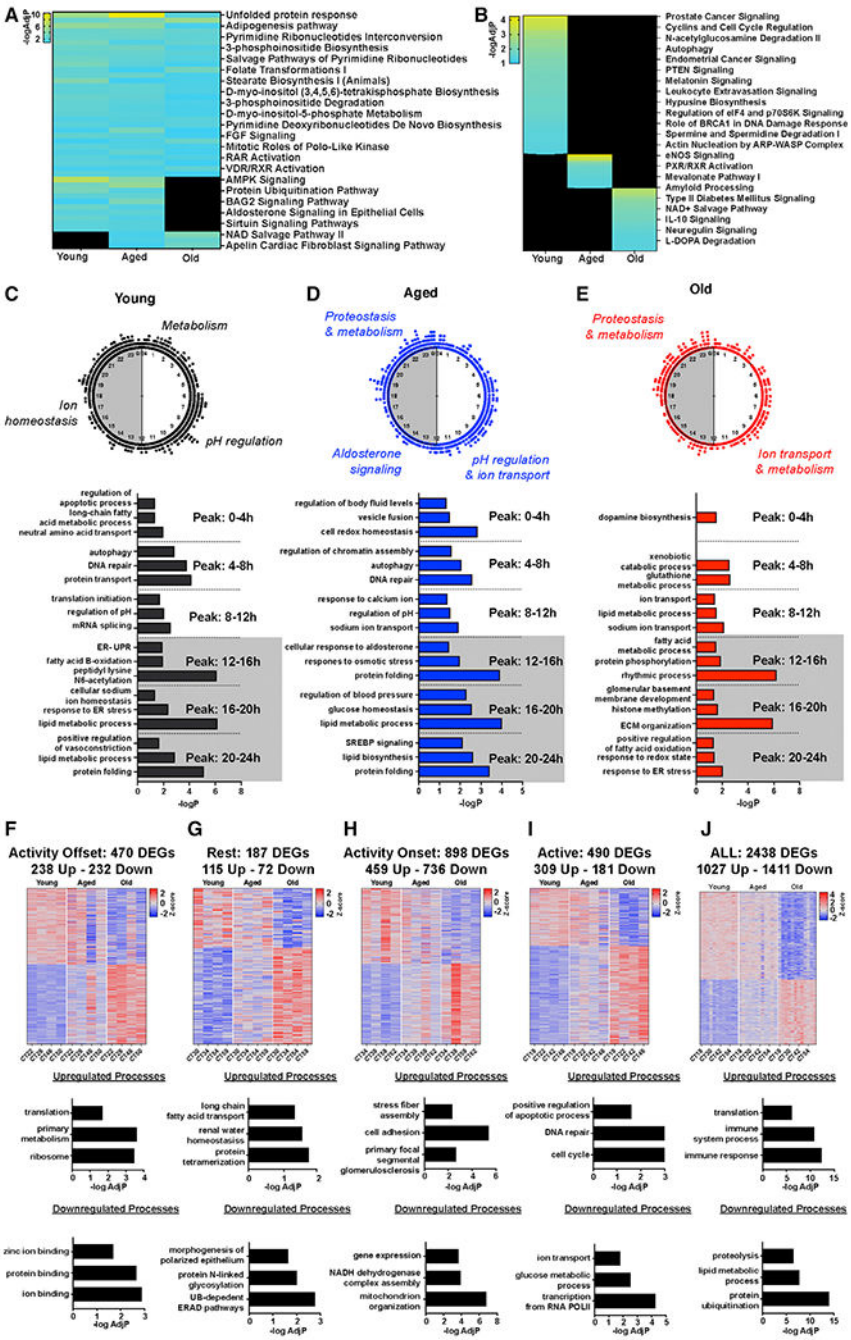

Figure 4. Age-associated changes in the kidney circadian transcriptional output and time of day-dependent interpretations of age-related changes in gene expression

(A) Shared IPA pathways enriched by the REGs that cycle in two to three age groups.

(B) Age-specific circadian oscillatory IPA pathways.

(C–E) Peak time maps of all oscillating genes from the kidneys of Young (C), Aged (D), and Old mice (E). Each dot represents the peak time of a single significantly circadian gene. Underneath the peak time map is a histogram of top time of day-specific gene expression pathways.

(F–I) Heatmap of z-scored age-related DEGs and histograms of up- or downregulated pathways from the specific gene sets from the active to rest transition period (F), the rest phase (G), the rest to active transition period (H), and the active period (I).
(J) Heatmap of z-scored age-related DEGs from all time domains and histograms of up- or downregulated pathways from the specific gene sets.

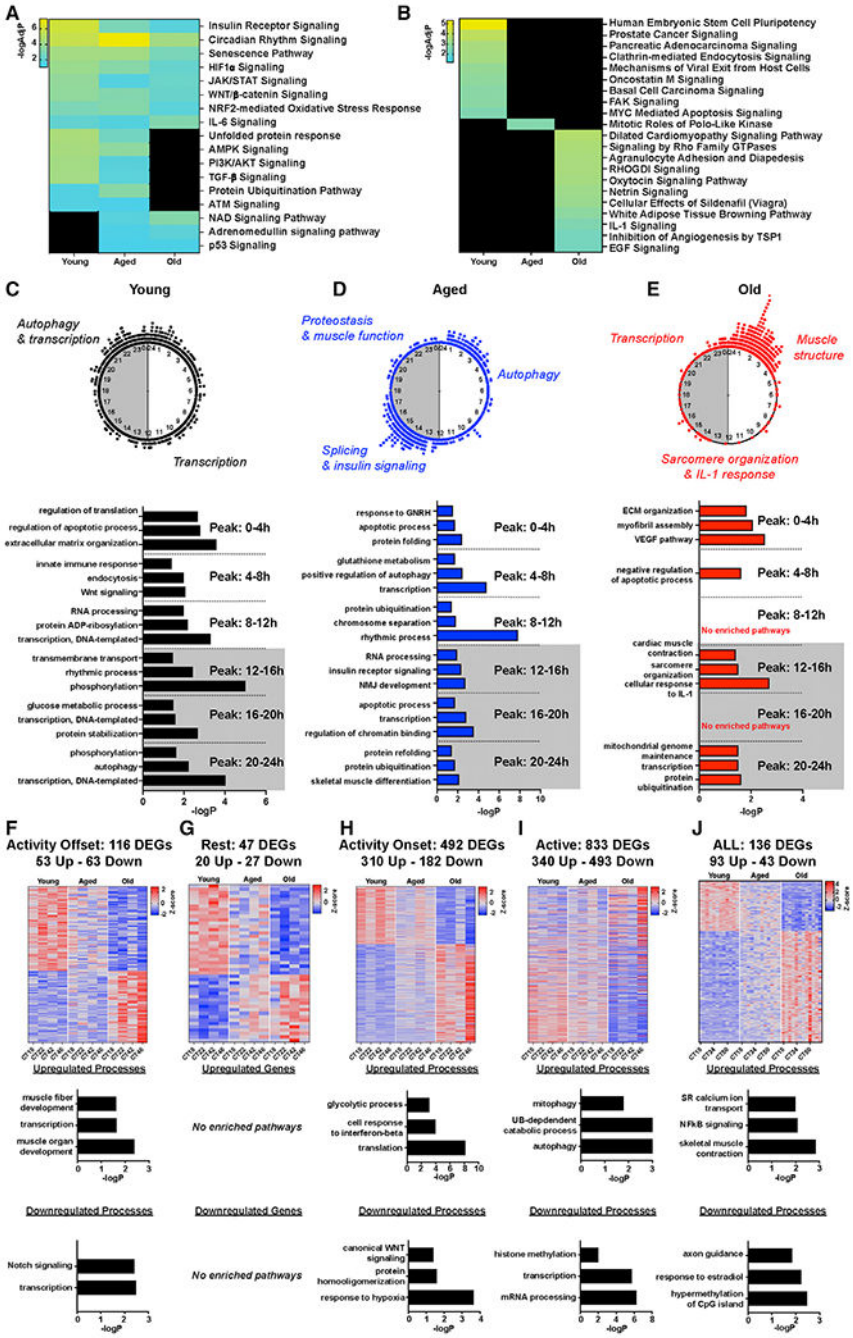

Figure 5. Age-associated changes in the muscle circadian transcriptional output and time of day-dependent interpretations of age-related changes in gene expression

(A) Shared IPA pathways enriched by the REGs that cycle in two to three age groups.

(B) Age-specific circadian oscillatory IPA pathways.

(C–E) Peak time maps of all oscillating genes from the skeletal muscle of Young (C), Aged (D), and Old mice (E). Each dot represents the peak time of a single significantly circadian gene. Underneath the peak time map is a histogram of top time of day-specific gene expression pathways.

(F–I) Heatmap of z-scored age-related DEGs and histograms of up- or downregulated pathways from the specific gene sets from the active to rest transition period (F), the rest phase (G), the rest to active transition period (H), and the active period (I).
(J) Heatmap of z-scored age-related DEGs from all time domains and histograms of up- or downregulated pathways from the specific gene sets.

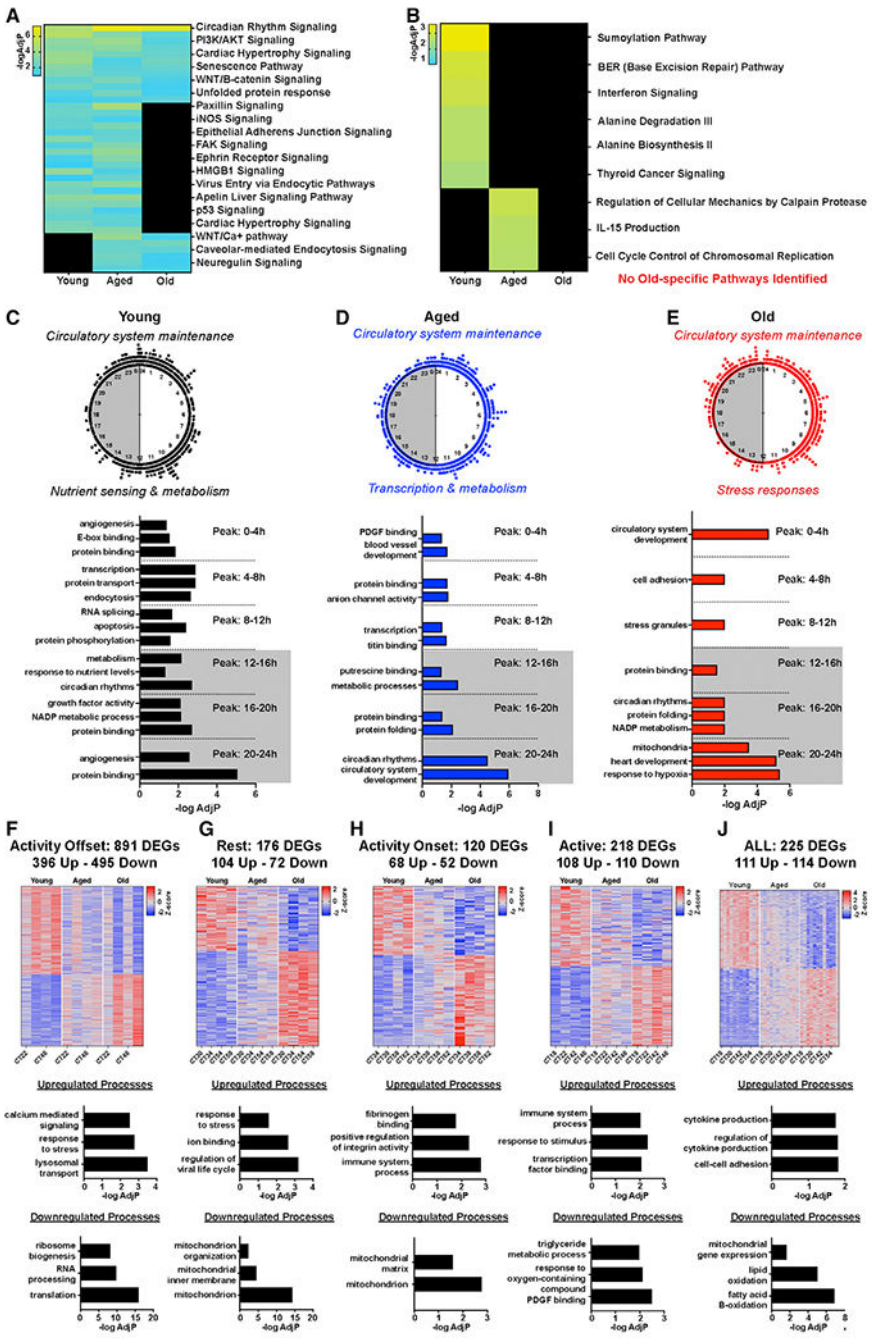

Figure 6. Age-associated changes in the heart circadian transcriptional output and time of day-dependent interpretations of age-related changes in gene expression

(A) Shared IPA pathways enriched by the REGs that cycle in two to three age groups.

(B) Age-specific circadian oscillatory IPA pathways.

(C–E) Peak time maps of all oscillating genes from the heart of Young (C), Aged (D), and Old mice (E). Each dot represents the peak time of a single significantly circadian gene. Underneath the peak time map is a histogram of top time of day-specific gene expression pathways.

(F–I) Heatmap of z-scored age-related DEGs and histograms of up- or downregulated pathways from the specific gene sets from the active to rest transition period (F), the rest phase (G), the rest to active transition period (H), and the active period (I).
(J) Heatmap of z-scored age-related DEGs from all time domains and histograms of up- or downregulated pathways from the specific gene sets.

A

Variably Expressed Genes

	Total	Increased with Age
hypothalamus	2,773	2,516
lung	2,607	2,452
kidney	1,475	1,468
muscle	1,498	1,342
heart	255	92

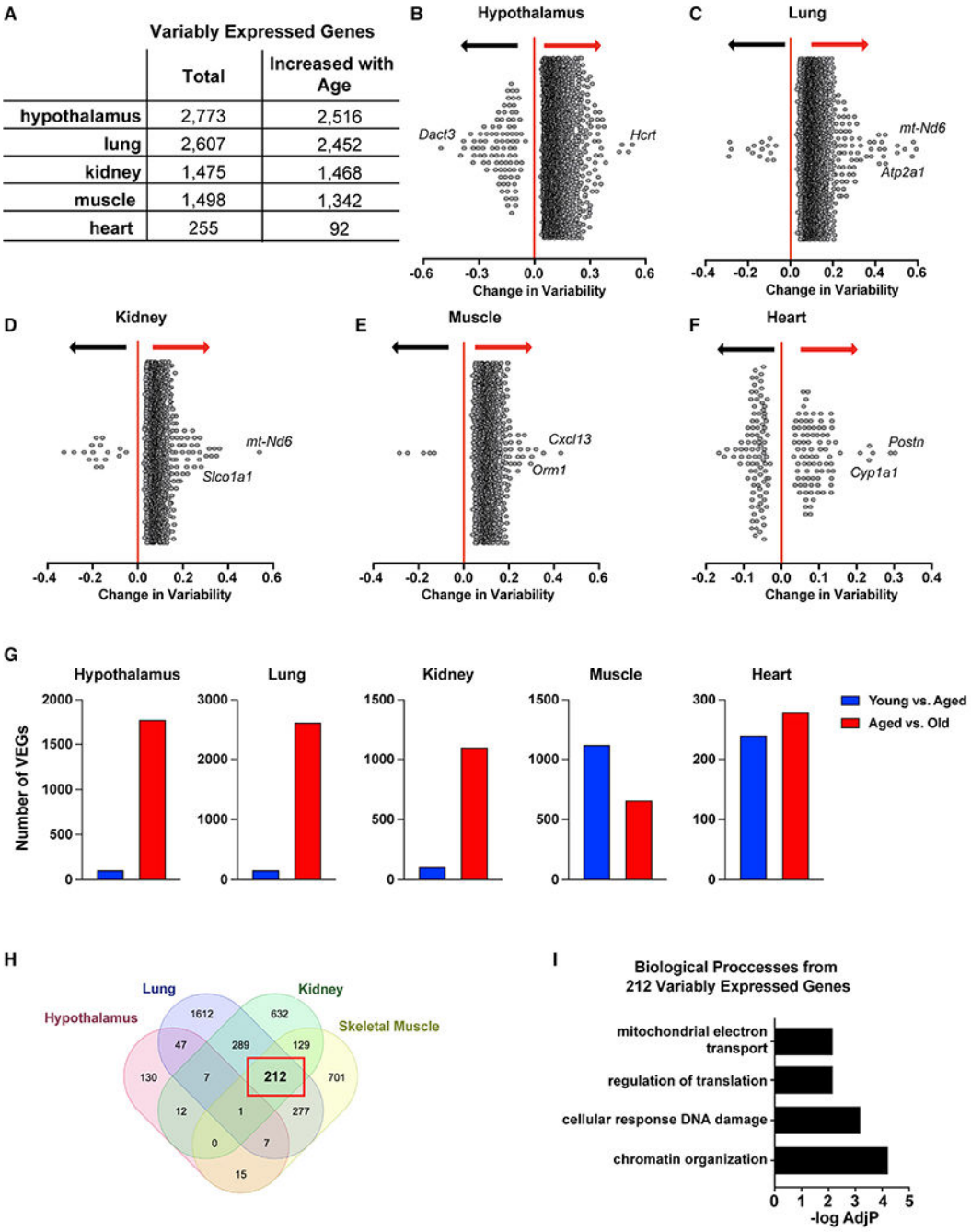

Figure 7. Age-related changes in variably expressed genes across tissues and summary of age-related changes in circadian and non-circadian gene expression

(A) Tissue-specific table of age-related changes in variable gene expression.

(B) Hypothalamic variably expressed genes, “Change in Variability” means “change in variability (measured by absolute deviance) per unit change in age group (young vs. age or age vs. old)”. Change in Variability above 0 indicates genes that are more variably expressed with age, notated by the red arrow.

(C–F) (C) Lung variable gene dispersion plot, (D) kidney variable gene dispersion plot, (E) skeletal muscle variable gene dispersion plot, (F) heart variable dispersion plot.

(G) Number of variably expressed genes changing from Young to Aged and Aged to Old.
(H) Venn diagram of overlapping variable genes across tissues.
(I) Top biological processes enriched by overlapping variable genes in the muscle, kidney, and lung.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
TRIzol reagent	Invitrogen	Cat# 15596018
Critical commercial assays		
RNeasy Mini kit	Qiagen	Cat# 74106
RNeasy Plus Micro kit	Qiagen	Cat# 74034
TURBO DNA-free kit	Invitrogen	Cat# AM1907
Deposited data		
mRNA profiling data of tissues at different ages	This paper	GEO: GSE201207
Experimental models: Organisms/strains		
Mouse: C57BL/6	NIA	C57BL/6Nia
Software and algorithms		
Prism 9	GraphPad	http://www.graphpad.com/
Ingenuity Pathway Analysis	Qiagen	http://www.ingenuity.com/products/pathways_analysis.html
g:Profiler	Raudvere et al.[59]	http://biit.cs.ut.ee/gprofiler/
DAVID	Huang et al.[60]	http://david.ncifcrf.gov/
Other		
Resource website ‘Circaage’	This paper	https://circaage.shinyapps.io/circaage/