

# Co-regulation of *Nr1d1* and *Ppara* in age-related changes of lipid metabolism and its modulation by calorie restriction

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**Keywords:** aging, calorie restriction, lipid metabolism, NR1D1, PPAR $\alpha$

**Received:** January 6, 2025

**Accepted:** June 24, 2025

**Published:** July 28, 2025

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## ABSTRACT

Aging is associated with a decline in liver function, which increases the risk of age-related metabolic disorders. Calorie restriction (CR) counteracts age-related changes in the liver; however, the underlying molecular mechanism remains elusive. In this study, we integrated transcriptomic, bioinformatic, and molecular analyses to investigate the effects of aging and CR on age-related gene expression in the rat liver, focusing on the interplay between the circadian rhythm and lipid metabolism. Our results revealed aging-induced upregulation of *Nr1d1*, a key circadian repressor, and downregulation of *Ppara*, accompanied by decreased expression of fatty acid oxidation genes and increased expression of lipogenic genes. CR attenuated these age-related changes and restored circadian rhythm-related gene expression. Furthermore, we demonstrated that *Nr1d1* overexpression inhibited PPAR $\alpha$  binding to peroxisome proliferator response elements (PPRE), resulting in decreased fatty acid oxidation gene expression. Our findings suggest that age-related dysregulation of *Nr1d1* contributes to impaired lipid metabolism in liver aging, and CR may exert its beneficial effects by modulating the interaction between NR1D1 and PPAR $\alpha$ . This study provides novel insights into the molecular mechanisms linking circadian rhythms and lipid metabolism in hepatic aging.

## INTRODUCTION

Aging is a complex process characterized by progressive physical, functional, and physiological declines [1]. The liver, a vital organ responsible for maintaining homeostasis through various metabolic pathways, undergoes significant age-related morphological and functional changes with age [2]. These alterations can lead to the deterioration of liver function, increasing the risk of various diseases and negatively affecting overall health [3–6]. Therefore, efficient strategies to mitigate the effects of aging on the liver are crucial for promoting health and longevity.

Calorie restriction (CR), a well-established anti-aging intervention, has shown promise in modulating the aging process by regulating inflammation and metabolism [7, 8]. Several studies have reported beneficial effects of CR on liver metabolism and inflammation, with significant improvements in gene expression profiles and lipid accumulation associated with hepatic aging [9–15]. CR has been consistently recognized as the golden standard for anti-aging interventions, demonstrating robust effects on lifespan and health span across species. However, the precise mechanisms underlying the effects of CR on age-related changes in the liver remain unclear.

Recent research has highlighted the intricate interplay between metabolism and circadian rhythms. Circadian regulation of enzymes involved in metabolite synthesis, such as fatty acids and cholesterol, demonstrates the profound influence of circadian rhythms on metabolic gene expression [16, 17]. Genetic alterations in core clock genes can disrupt metabolic functions, leading to impaired gluconeogenesis, hepatic steatosis, obesity, and abnormal lipid and glucose metabolism [18–20]. Age-related declines in circadian rhythms have been linked to disruptions in metabolic tissue homeostasis [21–23], particularly in the liver [24–26]. Our previous study reported that aging induces dysregulation of circadian genes such as *Nr1d1*, suggesting a close relationship between the aging process and circadian clock dysfunction [27].

Lipid metabolism plays a central role in the aging process, with lipid accumulation exerting detrimental effects on cells and organs, contributing to age-related diseases and reduced lifespan [28–30]. Age-related changes in lipid composition and accumulation have been implicated in metabolic dysfunction-associated fatty liver disease (MAFLD), which increases in prevalence with age [31]. Lipid metabolism dysfunction accelerates the aging process [32], with several studies reporting increased hepatic lipid accumulation during aging [15, 33, 34]. Therefore, identifying key regulators linking circadian rhythms and lipid metabolism in aging contexts is crucial for developing effective healthy aging strategies.

Bioinformatics and systems biology approaches offer valuable tools for unraveling aging mechanisms and identifying critical aging biomarkers [35, 36]. These tools integrate statistical analyses, mathematical models, and pathway/network construction to provide comprehensive understanding of aging. Various studies using biological big data have shown metabolic changes during aging [37–41]. However, many bioinformatics studies remain at the systemic level and lack detailed exploration of specific mechanisms through which anti-aging interventions modulate aging. There is a pressing need for studies that bridge bioinformatics and systems biology with targeted biological experiments to elucidate the molecular underpinnings of aging and the effects of anti-aging interventions.

Our previous studies extensively characterized the beneficial effects of CR on various organs during aging [7, 14, 42]. Building on these findings, the study aimed to investigate age-related gene expression changes in the liver and identify key gene candidates modulated by CR. We employed an integrated approach combining RNA-seq analysis, bioinformatics tools, and targeted biological experiments. By focusing on metabolic

homeostasis in the liver, we sought novel insights into molecular mechanisms underlying hepatic aging and the potential of CR to mitigate these effects. We hypothesized that CR counteracts age-related dysregulation of key circadian and metabolic regulators, thereby maintaining metabolic homeostasis in the aging liver. Through this investigation, we discovered that circadian and metabolic regulators *Nr1d1* and *Ppara* were modulated by CR during aging.

## RESULTS

### Transcriptomic analysis of gene expression changes during aging and CR

We developed an integrated approach combining bioinformatics, systems biology, and empirical biological analyses to investigate aging and CR effects on gene expression in the liver (Figure 1). Liver tissues from young rats (Young group), aged rats (Old group), and aged rats treated with CR (Old-CR group) were harvested for RNA-Seq analysis. We compared mRNA expression levels between the young and old groups (Old vs. Young) and between the old group and old-CR groups (Old-CR vs. Old) to identify differentially expressed genes (DEGs) (Supplementary Table 1). The Old vs. Young comparison revealed 599 upregulated genes and 578 downregulated genes (Figure 2A). In the Old-CR vs. Old comparison, 459 genes were upregulated, and 663 genes were downregulated (Figure 2B).

To elucidate DEG functions, we analyzed all genes using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) across five categories: biological processes (BP), cellular components (CC), molecular functions (MF), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome. The top 30 terms based on *p*-values are listed, to provide insights into the gene functions affected by aging and CR (Supplementary Table 2).

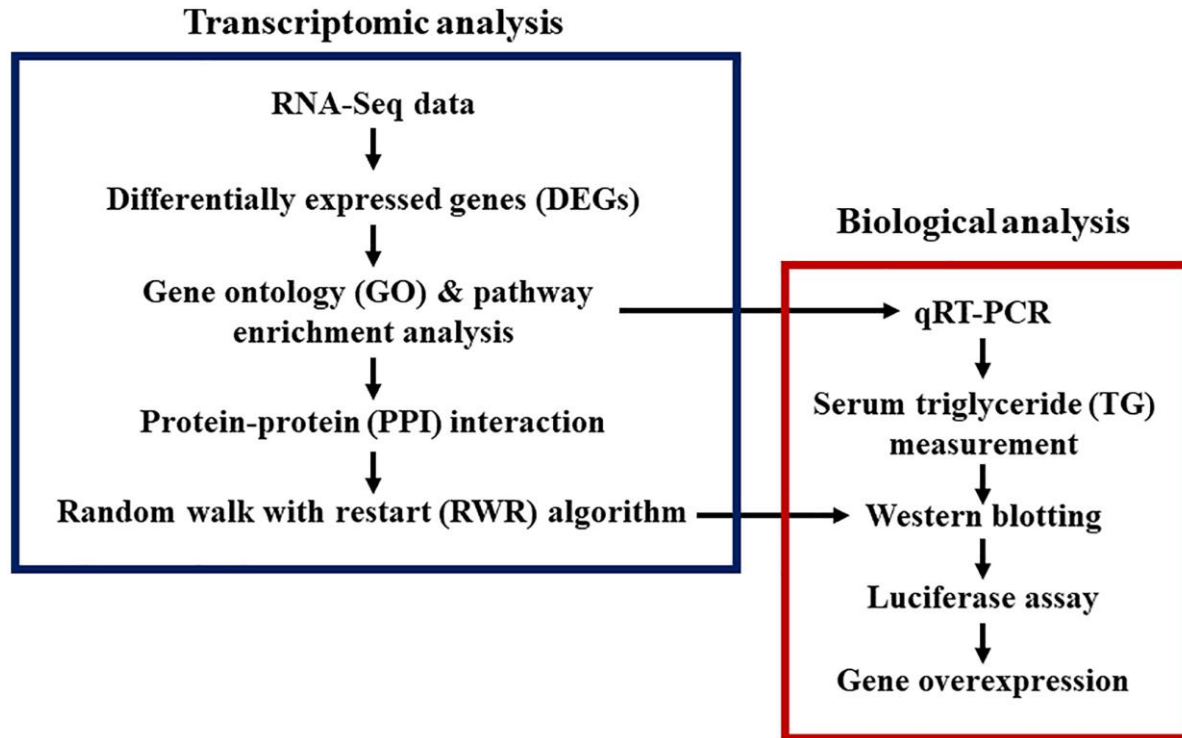
Our results revealed significant changes across various metabolic processes in both Old vs. Young and Old-CR vs. Old datasets. Prevalent BP terms included “aging,” “steroid metabolic process,” “triglyceride metabolic process,” and “xenobiotic metabolic process” (Figure 3A). Key circadian rhythm terms included “circadian rhythm” and “regulation of circadian rhythm,” while immune response terms included “response to lipopolysaccharide”.

CC terms such as “lipid particle,” “collagen trimer,” and “mitochondrion,” were altered in both datasets (Figure 3B). Key MF terms associated with energy metabolism-related “oxidoreductase” and “monooxygenase” and

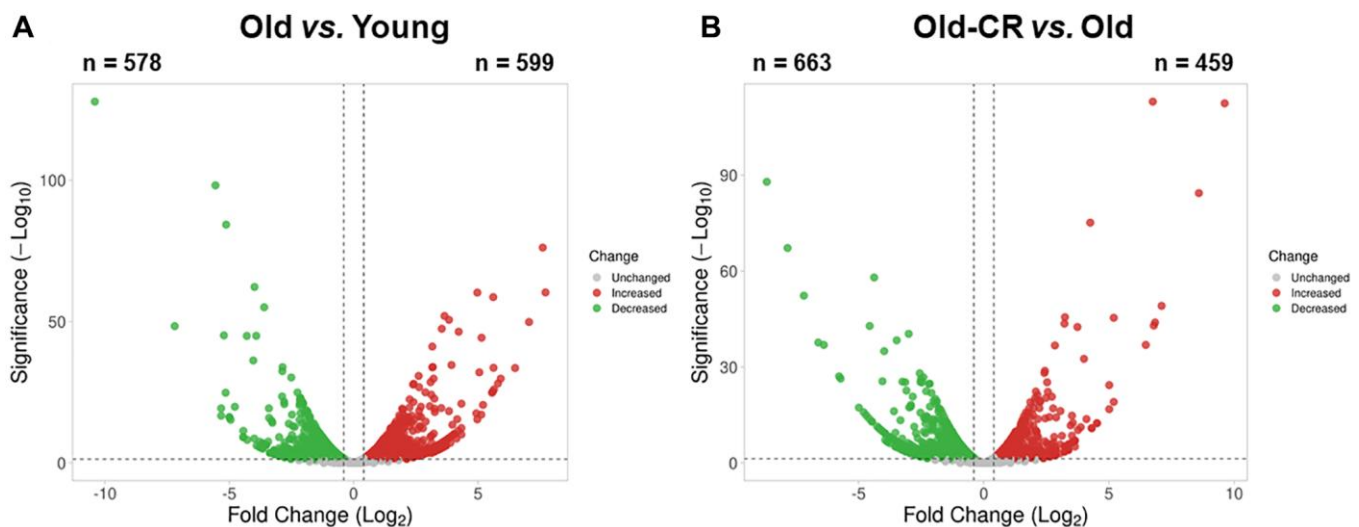
lipid metabolism-related “steroid hydroxylase activity” and “fatty acid binding” (Figure 3C).

KEGG analysis revealed changes in “AMPK signaling pathway,” “PPAR (Peroxisome proliferator-activated receptors) signaling pathway,” and “steroid hormone biosynthesis,” indicating strong associations with lipid

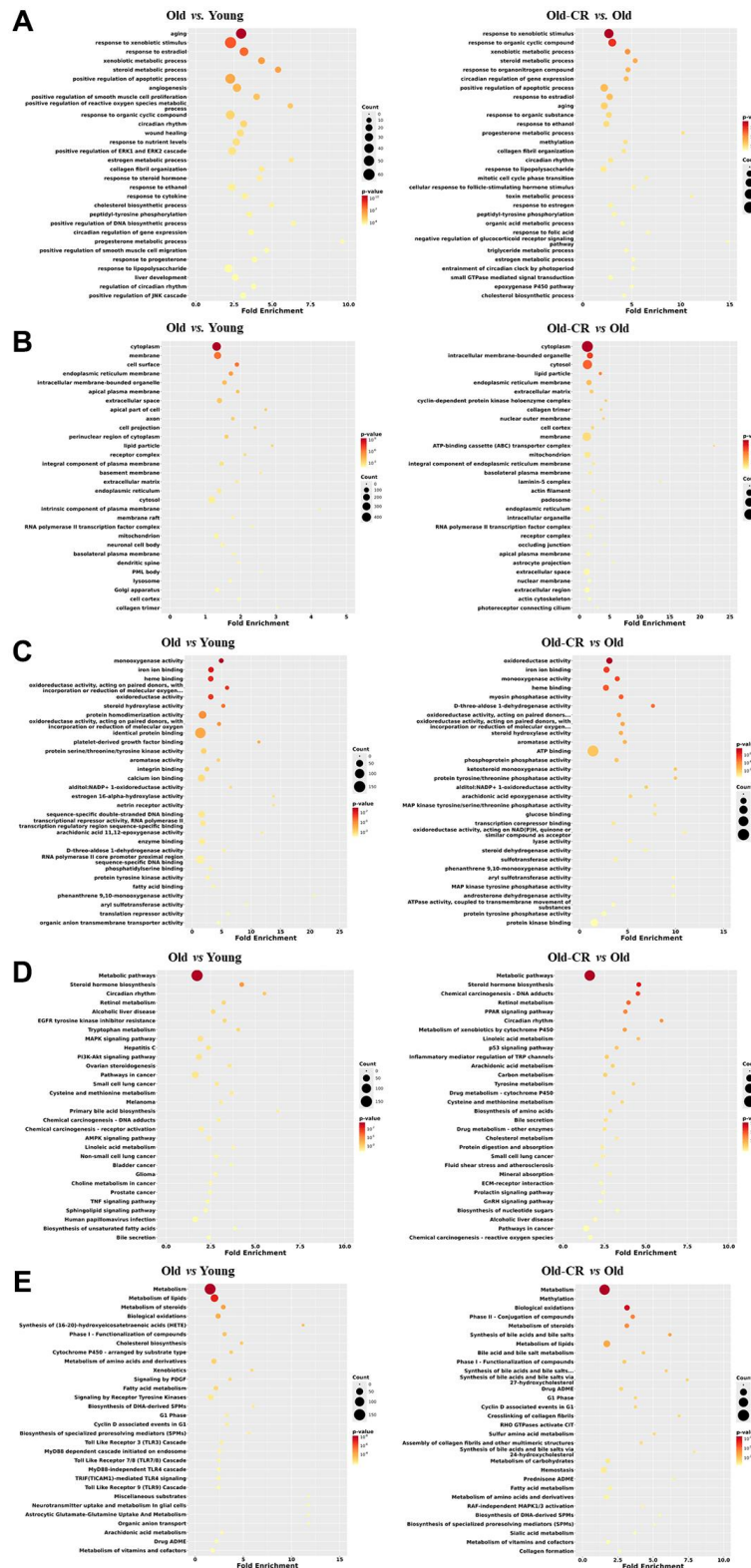
metabolism and circadian rhythm-related terms were also identified (Figure 3D). Reactome terms such as “metabolism,” “metabolism of lipids/steroids,” and “fatty acid metabolism,” were prominent in both datasets. The Old vs. Young dataset showed a higher prevalence of terms related to toll-like receptor signaling, whereas the Old-CR vs. Old dataset was



**Figure 1.** Study designs for transcriptomic and biological analyses of aging and CR. Bioinformatics, systems biology, and biological experiments were combined.



**Figure 2.** Aging-related differentially expressed genes from RNA-Seq data of the (A) Old vs. Young and (B) Old-CR vs. Old datasets from SD rats. Aging upregulated 599 genes and downregulated 578 genes, while CR in aged mice upregulated 459 genes and downregulated 663 genes. Red dots represent upregulated genes between the two groups, whereas green dots represent downregulated genes. Gray dots represent genes with no changes between the two groups. The DEG criteria were  $|FC| > 1.5$  and  $p < 0.05$ . Old-CR, old group under CR.



**Figure 3. Top 30 enriched GO or pathway terms of the DEGs in the Old vs. Young and Old-CR vs. Old datasets from SD rats.** (A) BP, (B) CC, (C) MF, (D) KEGG, and (E) Reactome data. Gene ontology analysis revealed significant alterations in metabolic processes, circadian rhythm, and inflammation (BP); lipid particles, collagen, and mitochondria (CC); and energy/lipid metabolism enzymes (MF). Pathway analysis identified changes in AMPK/PPAR signaling and circadian rhythm (KEGG), with distinct inflammatory signatures in aging and extracellular matrix responses in CR (Reactome). The criterion for a significant term was  $p < 0.05$ . Counts indicate the number of genes related to each term in the analysis. The fold enrichment indicates how much a particular GO term or pathway is overrepresented in the list of genes compared to that expected by chance. Old-CR, old group under CR.

enriched in terms related to the extracellular matrix (ECM) terms, such as “crosslinking of collagen fibrils” and “collagen formation” (Figure 3E).

These results suggest that genes associated with inflammation, metabolism, circadian rhythms, and the ECM exhibited differential expression patterns in aging and CR. Consistent changes in metabolism and circadian rhythm-related GOs and pathways highlight their significant interplay in the aging liver, underscoring their critical roles in liver responses to aging and CR interventions.

### **CR modulation of aging-related changes in lipid metabolism-related gene expression**

To explore CR intervention in age-induced changes, we examined DEG expression patterns from both datasets (Supplementary Table 3). Specifically, we conducted GO and pathway analyses focusing on genes upregulated in the Old vs. Young but downregulated in the Old-CR vs. Old (Table 1). BP terms related to fatty acid, lipid, and cholesterol steroid processes and MF terms, including palmitoyl-CoA 9-desaturase and stearoyl-CoA 9-desaturase activities were identified. Metabolic pathways such as the AMPK and PPAR signaling pathways, lipid metabolism, and fatty acid metabolism were altered.

Examination of lipogenesis and fatty acid oxidation genes revealed that lipogenic genes (*Acl*, *Scd*, *Fasn*) increased with aging and decreased with CR, while fatty acid oxidation genes showed opposite trends (Table 2). qRT-PCR was validated that PPARs and fatty acid oxidation genes (*Ppara*, *Ppard*, *Cpt2*, *Cyp4a1*, *Acaa1a*) were downregulated during aging but upregulated with CR, whereas the lipogenic genes (*Acaca*, *Fasn*) showed the opposite results (Supplementary Table 4 and Figure 4).

Triglyceride (TG) levels were measured to assess the aging and CR effects on lipid metabolism. TG levels were significantly elevated in the Old group compared to Young ( $106.8 \pm 11.23$  vs.  $82.28 \pm 7.111$  mg/dL;  $p < 0.05$ ); but significantly reduced in the Old-CR compared to Old ( $40.73 \pm 3.689$  mg/dL;  $p < 0.0001$ ) (Figure 5). These findings indicate that lipid synthesis genes increased with aging and decreased with CR, affecting lipid accumulation and suggesting that changes in lipogenesis and fatty acid oxidation are crucial in hepatic aging, with CR potentially counteracting these alterations.

### **Expression changes of the circadian gene *Nr1d1* predicted as a key gene linking circadian rhythms and metabolism by CR during aging**

Following lipid metabolism analysis, we examined circadian rhythm genes to identify aging- and CR-

related expression changes (Table 3). Most circadian genes showed significant alterations in both Old vs. Young and Old-CR vs. Old datasets, with *Cry1*, *Cry2*, *Nfil3*, *Nr1d1*, and *Rorc* displaying opposite trends in both datasets. We focused on *Nr1d1*, a circadian repressor that increased with age. Furthermore, qRT-PCR validated circadian gene changes (Figure 6 and Supplementary Table 4), showing that *Nr1d1* and *Nr1d2* increased in the Old group compared to Young and decreased in the Old-CR compared to Old. Conversely, *Bmal1* decreased with aging but increased with CR. *Clock* also decreased with aging. Other genes such as *Rora*, *Rorc*, *Per1*, *Per2*, *Cry1*, and *Cry2* showed with aging and significant increases with CR.

We constructed a protein-protein interaction (PPI) network based on circadian-associated genes, lipid metabolism-related genes, and aging-altered DEGs, trimmed to focus on *Nr1d1* (Supplementary Figure 1 and Figure 7, Supplementary Table 5). Results confirmed that circadian rhythm and lipid metabolism genes were interconnected around *Nr1d1*, with *Ppara* among these connections. These analyses suggest that increased *Nr1d1* expression during aging affects both circadian rhythm and lipid metabolism, which was ameliorated by CR.

### **Co-regulation of *Nr1d1*- and *Ppara*-induced age-related changes in lipid metabolism and its modulation by CR**

To identify the genes closely associated with *Nr1d1*, we applied the RWR algorithm using *Nr1d1* as the seed node (Supplementary Figure 1 and Supplementary Table 6). Among the top 100 genes, several circadian and lipid metabolism genes were identified, such as *Cry1*, *Arntl*, *Nr1d2*, *Fasn*, *Pparg*, and *Acaca*. *Ppara* ranked among top genes, indicating that *Nr1d1* is closely associated with *Ppara*, a major metabolic regulator.

Western blotting detected *Nr1d1* and *Ppara* levels (Figure 8). *Nr1d1* protein levels were higher in the Old group than Young control but significantly downregulated in the Old-CR compared to Old. Conversely, *Ppara* protein levels were lower in Old than Young but increased with CR treatment. Immunohistochemistry assessed the *Nr1d1* expression and localization in rat liver tissues (Figure 9). The Old group showed greater *Nr1d1* intensity than Young, while intensity was significantly diminished in Old-CR compared to Old. These results suggest that the *Nr1d1* and *Ppara* protein expression are affected by aging and CR with opposing trends, highlighting their potential as aging modulators.

**Table 1. GO terms and pathways of genes that were upregulated in the Old vs. Young but downregulated in the Old-CR vs. Old datasets.**

| Category | Term                                                                    | Fold enrichment | p-value     | Genes                                                                                          |
|----------|-------------------------------------------------------------------------|-----------------|-------------|------------------------------------------------------------------------------------------------|
| BP       | positive regulation of apoptotic process                                | 4.8095          | 1.60E-05    | MOAP1, TNFRSF12A, SRC, ZBTB16, PDCD5, PTGS2, PPP2CA, CASP12, UBD, ALDH1A1, HMOX1, TSPO, CYP1B1 |
|          | fatty acid biosynthetic process                                         | 16.37276596     | 2.37E-04    | ACLY, SCD, FASN, SCD2, ACACB                                                                   |
|          | response to organonitrogen compound                                     | 10.68777778     | 0.001206086 | CASP12, UBD, CCNG1, PTGS2, AACS                                                                |
|          | acetyl-CoA metabolic process                                            | 51.30133333     | 0.001440356 | ACLY, FASN, ACACB                                                                              |
|          | peptidyl-tyrosine phosphorylation                                       | 9.271325301     | 0.002040379 | EFEMP1, SPINK1, SRC, PDGFB, EPHA3                                                              |
|          | response to xenobiotic stimulus                                         | 3.129286506     | 0.002650987 | CASP12, SRC, ALDH1A1, PDGFB, HMOX1, TSPO, MVD, CYP4A2, PTGS2, ACACB, AACS                      |
|          | response to fatty acid                                                  | 13.38295652     | 0.003241543 | SCD, SRC, SCD2, PTGS2                                                                          |
|          | regulation of cell-cell adhesion                                        | 28.857          | 0.00466156  | SRC, ADAM8, ZDHHC2                                                                             |
|          | response to organic cyclic compound                                     | 3.800098765     | 0.005063243 | G6PD, LUM, ALDH1A1, PDGFB, CYP1B1, PTGS2, ACACB, AACS                                          |
|          | positive regulation of ERK1 and ERK2 cascade                            | 4.397257143     | 0.005141411 | CCL21, SRC, C5AR2, TRPV4, PDGFB, GAS6, FGF21                                                   |
|          | inflammatory response                                                   | 3.439195531     | 0.008574019 | CXCL9, CCL21, ELF3, C5AR2, FASN, ADAM8, CXCL1, PTGS2                                           |
|          | negative regulation of cell proliferation                               | 3.01772549      | 0.009810343 | ZBTB16, PDCD5, ADORA1, CYP1B1, HMOX1, CD9, CDH13, SLIT3, PTGS2                                 |
|          | lipid biosynthetic process                                              | 19.238          | 0.010367306 | ACLY, SCD, FASN                                                                                |
|          | white fat cell differentiation                                          | 19.238          | 0.010367306 | SCD, PNPLA3, AACS                                                                              |
|          | positive regulation of protein kinase B signaling                       | 5.785864662     | 0.010820854 | CCL21, SRC, GDF15, ADAM8, GAS6                                                                 |
|          | positive regulation of DNA biosynthetic process                         | 17.75815385     | 0.012106014 | SRC, PDGFB, CYP1B1                                                                             |
|          | neutrophil chemotaxis                                                   | 8.208213333     | 0.012599584 | LGALS3, CXCL9, CCL21, CXCL1                                                                    |
|          | positive regulation of phosphatidylinositol 3-kinase activity           | 15.3904         | 0.015934563 | CCL21, SRC, PDGFB                                                                              |
|          | activation of protein kinase B activity                                 | 15.3904         | 0.015934563 | SRC, PDGFB, GAS6                                                                               |
|          | positive regulation of MAP kinase activity                              | 7.417060241     | 0.016521383 | SRC, PDGFB, ADAM8, TPD52L1                                                                     |
|          | response to ethanol                                                     | 4.014886957     | 0.016714469 | G6PD, SPINK1, CASP12, GSN, ALDH1A1, AACS                                                       |
|          | cell chemotaxis                                                         | 7.242541176     | 0.017596824 | CCL21, ARHGEF16, PDGFB, CXCL1                                                                  |
|          | monocyte chemotaxis                                                     | 14.4285         | 0.018017922 | LGALS3, CCL21, PDGFB                                                                           |
|          | endothelial cell-cell adhesion                                          | 102.6026667     | 0.019213353 | CYP1B1, THBS4                                                                                  |
|          | positive regulation of smooth muscle cell proliferation                 | 6.917033708     | 0.019862531 | PDGFB, HMOX1, CDH13, PTGS2                                                                     |
|          | positive regulation of protein tyrosine kinase activity                 | 13.57976471     | 0.020209789 | TRPV4, PDGFB, GAS6                                                                             |
|          | actin filament organization                                             | 4.607904192     | 0.023008755 | GSN, TRPV4, MYO7B, RHOC, TLE6                                                                  |
|          | positive regulation of reactive oxygen species metabolic process        | 12.4787027      | 0.023694306 | PDGFB, CYP1B1, TSPO                                                                            |
|          | aging                                                                   | 3.131767442     | 0.024104355 | CDKN1C, CASP12, GSN, CNP, TSPO, PTGS2, DCN                                                     |
|          | antimicrobial humoral immune response mediated by antimicrobial peptide | 6.346556701     | 0.024854734 | LGALS3, CXCL9, CCL21, CXCL1                                                                    |

|    |                                                            |             |             |                                                                                                                                                                                                                                                                                                                                                             |
|----|------------------------------------------------------------|-------------|-------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| CC | positive regulation of glomerular filtration               | 76.952      | 0.025536074 | PDGFB, GAS6                                                                                                                                                                                                                                                                                                                                                 |
|    | monounsaturated fatty acid biosynthetic process            | 76.952      | 0.025536074 | SCD, SCD2                                                                                                                                                                                                                                                                                                                                                   |
|    | adrenal gland development                                  | 11.5428     | 0.027405876 | CDKN1C, CYP1B1, TSPO                                                                                                                                                                                                                                                                                                                                        |
|    | positive regulation of JNK cascade                         | 6.095207921 | 0.027581281 | CCL21, TRPV4, TPD52L1, ANKRD6                                                                                                                                                                                                                                                                                                                               |
|    | immune system process                                      | 11.26126829 | 0.028691809 | IGHM, IRF7                                                                                                                                                                                                                                                                                                                                                  |
|    | cholesterol biosynthetic process                           | 10.99314286 | 0.030001514 | G6PD, MVD, LSS                                                                                                                                                                                                                                                                                                                                              |
|    | positive regulation of inflammatory response               | 5.863009524 | 0.030460944 | TRPV4, NKG7, ADAM8, IL17RB                                                                                                                                                                                                                                                                                                                                  |
|    | fructose catabolic process                                 | 61.5616     | 0.031818362 | ALDH1A1, ALDH1A7                                                                                                                                                                                                                                                                                                                                            |
|    | positive regulation of smooth muscle cell migration        | 10.26026667 | 0.03406973  | SRC, PDGFB, CYP1B1                                                                                                                                                                                                                                                                                                                                          |
|    | response to nutrient levels                                | 4.071534392 | 0.034078992 | SPINK1, SRC, FASN, ACACB, FGF21                                                                                                                                                                                                                                                                                                                             |
|    | cell adhesion                                              | 2.88055615  | 0.034278073 | TNFRSF12A, SRC, CYP1B1, CD9, CCDC141, MPDZ, THBS4                                                                                                                                                                                                                                                                                                           |
|    | positive regulation of I-kappaB kinase/NF-kappaB signaling | 3.966597938 | 0.036959971 | CCL21, UBD, HMOX1, RHOC, TMEM106A                                                                                                                                                                                                                                                                                                                           |
|    | response to iron (III) ion                                 | 51.30133333 | 0.038060473 | G6PD, CASP12                                                                                                                                                                                                                                                                                                                                                |
|    | kidney development                                         | 3.926122449 | 0.038150589 | CDKN1C, ALDH1A1, RHPN1, CYP4A2, DCN                                                                                                                                                                                                                                                                                                                         |
|    | negative regulation of dendritic cell apoptotic process    | 43.97257143 | 0.044262663 | CCL21, GAS6                                                                                                                                                                                                                                                                                                                                                 |
|    | cellular response to glucose stimulus                      | 4.88584127  | 0.048049626 | PPP2CA, GAS6, AACS, FGF21                                                                                                                                                                                                                                                                                                                                   |
|    | chemokine-mediated signaling pathway                       | 8.394763636 | 0.049034302 | CXCL9, CCL21, CXCL1                                                                                                                                                                                                                                                                                                                                         |
|    | extracellular space                                        | 2.208098626 | 5.51E-04    | WFDC21, CXCL9, SPINK1, GSN, CCL21, CNP, GDF15, LUM, PDGFB, MUP4, CTSW, CXCL1, PLA2G7, THBS4, DCN, LGALS3, PROCR, EFEMP1, PODNL1, CDH13, SLIT3, GAS6, FGF21                                                                                                                                                                                                  |
|    | cytoplasm                                                  | 1.393257601 | 0.00602873  | CDKN1C, LGALS3, EFEMP1, CASP12, ME1, ANXA8, CLIC2, SULT4A1, HTATIP2, ANKRD6, CDKL2, G6PD, SPINK1, MYO7B, DUSP26, IL17RB, TLE6, ACLY, EVC, ALDH1A1, IRF7, CDH13, ADAM8, GAS6, CNP, SRC, PDGFB, DCUN1D2, PTGS2, PLA2G7, PPP2CA, UBD, CCDC141, MPDZ, PDLIM4, FGF21, NEK8, MOAP1, GSN, GDF15, MOK, PRSS36, TPD52L1, KY, SBK1, FASN, CCNG1, PNPLA3, B9D1, PNPLA5 |
|    | caveola                                                    | 6.761153054 | 0.02109186  | SRC, HMOX1, CDH13, PTGS2                                                                                                                                                                                                                                                                                                                                    |
|    | podosome                                                   | 13.2405914  | 0.0212081   | GSN, SRC, ADAM8                                                                                                                                                                                                                                                                                                                                             |
|    | membrane                                                   | 1.41390075  | 0.033419567 | POPDC2, ADAMDEC1, CNP, SRC, C5AR2, PDGFB, CYP4A2, SLC2A5, ZDHHC2, ADGRG2, SCD2, HMOX1, CYP1B1, SLIT3, TAS1R1, CCDC141, CLIC2, CSMD1, GRIA3, ENTPD7, SYT15, RHOC, IL17RB, PROCR, SCD, TRPV4, PNPLA3, CD9, PNPLA5, B9D1, PLP2, TMEM106A                                                                                                                       |
|    | extracellular region                                       | 1.943573049 | 0.043080377 | LGALS3, WFDC21, GSN, GDF15, PDGFB, CD9, PRSS36, GAS6, ZP2, CHRDL1, DCN, THBS4                                                                                                                                                                                                                                                                               |
|    | growth factor activity                                     | 7.79222973  | 2.71E-04    | EFEMP1, GDF15, PDGFB, CXCL1, GAS6, THBS4, FGF21                                                                                                                                                                                                                                                                                                             |
|    | protein homodimerization activity                          | 2.678861789 | 0.004834941 | G6PD, GDF15, ZBTB16, PDGFB, CRYL1, HMOX1, CDH13, MVD, TPD52L1, PTGS2, PDLIM4, ZDHHC2                                                                                                                                                                                                                                                                        |
|    | collagen binding                                           | 9.152777778 | 0.009337216 | LUM, PDGFB, DCN, THBS4                                                                                                                                                                                                                                                                                                                                      |
| MF | chemokine activity                                         | 13.35810811 | 0.020808064 | CXCL9, CCL21, CXCL1                                                                                                                                                                                                                                                                                                                                         |
|    | benzaldehyde dehydrogenase (NAD+) activity                 | 82.375      | 0.023840283 | ALDH1A1, ALDH1A7                                                                                                                                                                                                                                                                                                                                            |
|    | palmitoyl-CoA 9-                                           | 82.375      | 0.023840283 | SCD, SCD2                                                                                                                                                                                                                                                                                                                                                   |

|          |                                                                                |             |             |                                                                                                                                |  |
|----------|--------------------------------------------------------------------------------|-------------|-------------|--------------------------------------------------------------------------------------------------------------------------------|--|
|          | desaturase activity                                                            |             |             |                                                                                                                                |  |
|          | heme binding                                                                   | 4.476902174 | 0.025071838 | SRC, CYP1B1, HMOX1, CYP4A2, PTGS2                                                                                              |  |
|          | stearoyl-CoA 9-desaturase activity                                             | 65.9        | 0.029711838 | SCD, SCD2                                                                                                                      |  |
|          | protein binding                                                                | 1.694571429 | 0.03195988  | CDKN1C, UNC5B, SRC, ZBTB16, PTGS2, PPP2CA, EFEMP1, CASP12, TRPV4, FASN, ADORA1, HMOX1, TSPO, CD9, CCDC141, PDLIM4, MPDZ, GRIA3 |  |
|          | 3-chloroallyl aldehyde dehydrogenase activity                                  | 54.91666667 | 0.035548404 | ALDH1A1, ALDH1A7                                                                                                               |  |
|          | glyceraldehyde-3-phosphate dehydrogenase (NAD+) (non-phosphorylating) activity | 47.07142857 | 0.041350187 | ALDH1A1, ALDH1A7                                                                                                               |  |
|          | AMPK signaling pathway                                                         | 5.919354839 | 0.009421368 | PPP2CA, SCD, FASN, SCD2, ACACB                                                                                                 |  |
|          | Alcoholic liver disease                                                        | 5.48088411  | 0.012249191 | SCD, FASN, SCD2, CXCL1, ACACB                                                                                                  |  |
| KEGG     | Metabolic pathways                                                             | 1.749653733 | 0.014414787 | MT-ND6, G6PD, CYP4A2, PTGS2, ACACB, LSS, PLA2G7, AACS, ACLY, SCD, FASN, ALDH1A1, ME1, SCD2, PNPLA3, CRYL1, HMOX1, MVD, ALDH1A7 |  |
|          | PPAR signaling pathway                                                         | 6.882970743 | 0.019366476 | SCD, SCD2, ME1, CYP4A2                                                                                                         |  |
|          | Cytokine-cytokine receptor interaction                                         | 3.276395667 | 0.03364291  | CXCL9, TNFRSF12A, CCL21, GDF15, CXCL1, IL17RB                                                                                  |  |
|          | Metabolism of lipids                                                           | 2.817831233 | 0.004103554 | ACLY, SCD, FASN, PNPLA3, CYP1B1, TSPO, PNPLA5, MVD, PTGS2, LSS, AACS                                                           |  |
|          | G alpha (i) signalling events                                                  | 3.685106717 | 0.010239663 | PPP2CA, CXCL9, CCL21, SRC, ADORA1, CXCL1, TAS1R1                                                                               |  |
|          | GPCR downstream signalling                                                     | 2.649684044 | 0.016729272 | PPP2CA, CXCL9, CCL21, SRC, ARHGEF16, ADORA1, CXCL1, TAS1R1, RHOC                                                               |  |
|          | Metabolism                                                                     | 1.670611517 | 0.019928616 | MT-ND6, G6PD, LUM, PTGS2, LSS, AACS, DCN, ACLY, SCD, FASN, ALDH1A1, PNPLA3, CRYL1, HMOX1, TSPO, CYP1B1, PNPLA5, MVD, SULT4A1   |  |
| Reactome | Fatty acyl-CoA biosynthesis                                                    | 12.85249042 | 0.021695752 | ACLY, SCD, FASN                                                                                                                |  |
|          | Fatty acid metabolism                                                          | 4.501476114 | 0.023057567 | ACLY, SCD, FASN, CYP1B1, PTGS2                                                                                                 |  |
|          | G1 Phase                                                                       | 9.808479532 | 0.035914728 | PPP2CA, CDKN1C, SRC                                                                                                            |  |
|          | Cyclin D associated events in G1                                               | 9.808479532 | 0.035914728 | PPP2CA, CDKN1C, SRC                                                                                                            |  |
|          | Chemokine receptors bind chemokines                                            | 8.470959596 | 0.046907572 | CXCL9, CCL21, CXCL1                                                                                                            |  |
|          | Signaling by GPCR                                                              | 2.162798195 | 0.048288983 | PPP2CA, CXCL9, CCL21, SRC, ARHGEF16, ADORA1, CXCL1, TAS1R1, RHOC                                                               |  |

**Table 2. Changes in the expression of genes related to lipid metabolism from RNA-Seq data in the Old vs. Young and Old-CR vs. Old datasets.**

| Category             | Gene          | Old vs. Young | p-value  | Old-CR vs. Old | p-value  |
|----------------------|---------------|---------------|----------|----------------|----------|
| Lipogenesis          | <i>Aacs</i>   | 3.972369982   | 3.30E-08 | -2.02791896    | 2.00E-03 |
|                      | <i>Acly</i>   | 1.777685362   | 9.50E-03 | -1.729074463   | 1.10E-02 |
|                      | <i>Acaca</i>  | 1.945309895   | 4.90E-03 | 1.049716684    | 8.30E-01 |
|                      | <i>Acacb</i>  | 1.558329159   | 4.90E-02 | -1.905275996   | 3.00E-03 |
|                      | <i>Fasn</i>   | 2.203810232   | 4.20E-04 | -2.056227653   | 8.90E-04 |
|                      | <i>Pnpla3</i> | 6.105036836   | 4.30E-14 | -2.887858391   | 1.80E-06 |
|                      | <i>Pparg</i>  | -1.197478705  | 6.00E-01 | -1.148698355   | 7.40E-01 |
|                      | <i>Scd</i>    | 1.777685362   | 9.80E-03 | -4.469148552   | 1.70E-11 |
|                      | <i>Scd2</i>   | 3.052518418   | 3.30E-05 | -2.099433367   | 2.90E-03 |
| Fatty acid oxidation | <i>Acox1</i>  | -1.125058485  | 5.90E-01 | 1.494849249    | 6.30E-02 |
|                      | <i>Cpt1a</i>  | -1.021012126  | 9.20E-01 | 1.433955248    | 9.20E-02 |

|               |              |          |              |          |
|---------------|--------------|----------|--------------|----------|
| <i>Cpt2</i>   | 1.205807828  | 4.00E-01 | 1.283425898  | 2.50E-01 |
| <i>Acadvl</i> | -1.079228237 | 7.20E-01 | 1.042465761  | 8.50E-01 |
| <i>Hadhb</i>  | 1.222640278  | 3.70E-01 | 1.248330549  | 3.10E-01 |
| <i>Acadm</i>  | -1.172834949 | 4.70E-01 | -1.117287138 | 6.00E-01 |
| <i>Echs1</i>  | -1.515716567 | 6.30E-02 | 1.292352831  | 2.40E-01 |
| <i>Hadh</i>   | -1.125058485 | 6.00E-01 | -1.189207115 | 4.30E-01 |
| <i>Ppara</i>  | -1.164733586 | 5.70E-01 | 1.394743666  | 1.70E-01 |
| <i>Ppard</i>  | -2.531513188 | 2.40E-04 | 1.310393404  | 2.90E-01 |
| <i>Acox1</i>  | -1.125058485 | 5.90E-01 | 1.494849249  | 6.30E-02 |

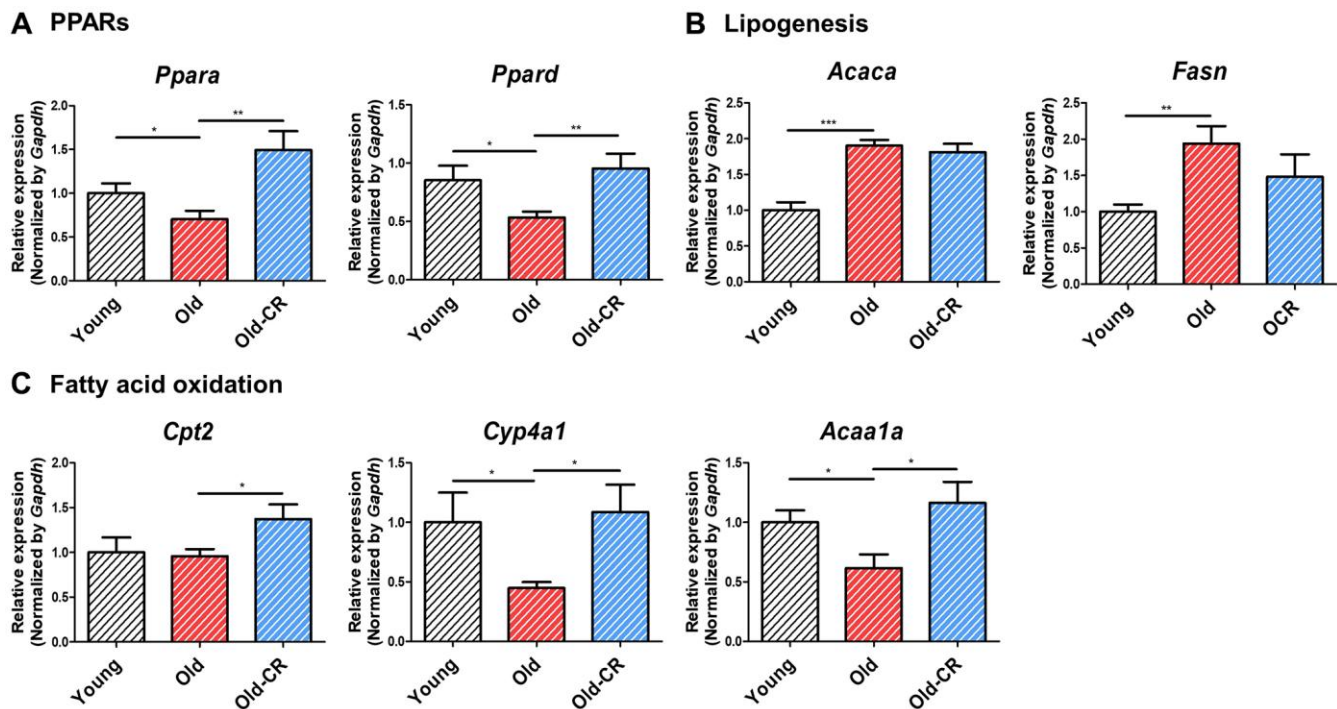
### Effect of NR1D1 overexpression on PPAR $\alpha$ binding to peroxisome proliferator response elements (PPRE) and the expression of circadian- and lipid metabolism-related genes

To investigate the relationship between NR1D1 and PPAR $\alpha$ , we measured PPAR $\alpha$  binding to PPRE in HepG2 cells using luciferase assay (Figure 10A). PPAR $\alpha$  binding to PPRE decreased significantly in a dose-dependent manner with NR1D1 administration. To examine increased NR1D1 effects on gene expression during aging, we measured circadian rhythm and lipid metabolism gene expression in HepG2 cells overexpressing *NR1D1* (Figure 10B). Results showed decreased expression of *BMAL1*, *CLOCK*, *PER1*, *PER2*, *CRY1*, and *CRY2* in *NR1D1*-overexpressing cells and

reduced expression of fatty acid oxidation genes (*ACOX1*, *CPT2*, *PPARA*). These results suggest that NR1D1 competitively binds to promoters, inhibiting PPAR $\alpha$  binding to PPRE and reducing fatty acid oxidation gene expression.

## DISCUSSION

In this study, we identified key regulatory genes in the liver during aging and CR through transcriptomic and biological analyses. Transcriptomic analysis revealed changes in metabolic and circadian-related gene expression during aging and CR, with lipid metabolism showing opposite expression patterns. Notable changes in circadian gene expression were confirmed, particularly *Nr1d1*, which increased during aging and



**Figure 4. Relative mRNA expression of genes related to lipid metabolism that are differentially expressed during aging and CR ( $n = 6$  per group).** (A) *Ppara* and *Ppard* are downregulated in aging but upregulated in CR, (B) while the lipogenic genes *Acaca* and *Fasn* display opposite trends. (C) Fatty acid oxidation-related genes, such as *Cpt2*, *Cyp4a1*, and *Acaa1a*, are downregulated in aging but upregulated in CR. Data are presented as mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  between two groups.

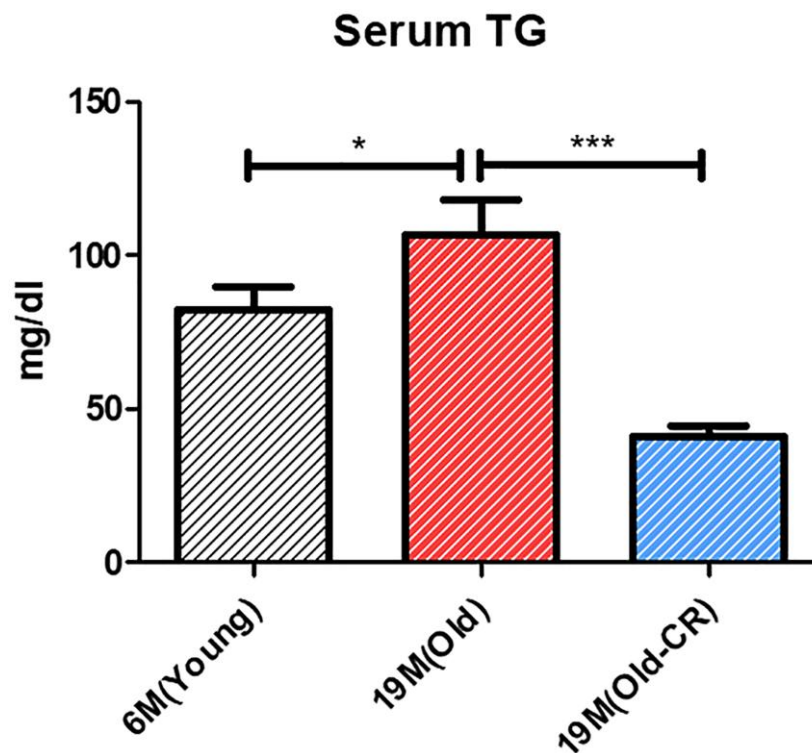
**Table 3. Changes in the expression of circadian genes from RNA-Seq data in the Old vs. Young and Old-CR vs. Old datasets.**

|                      | Old vs. Young | <i>p</i> -value | Old-CR vs. Old | <i>p</i> -value |
|----------------------|---------------|-----------------|----------------|-----------------|
| <i>Bmal1 (Arntl)</i> | −3.116658319  | 4.20E-06        | −9.51365692    | 9.20E-12        |
| <i>Clock</i>         | −1.404444876  | 1.30E-01        | −1.879045498   | 5.40E-03        |
| <i>Cry1</i>          | −1.777685362  | 4.80E-02        | 4.75682846     | 4.90E-09        |
| <i>Cry2</i>          | −1.021012126  | 9.30E-01        | 2.099433367    | 1.00E-03        |
| <i>Dbp</i>           | 12.55334557   | 2.60E-23        | 1.945309895    | 2.10E-03        |
| <i>Nfil3</i>         | −1.647182035  | 3.10E-02        | 1.569168196    | 4.70E-02        |
| <i>Npas2</i>         | −2.848100391  | 1.20E-05        | −23.58830748   | 2.50E-19        |
| <i>Nr1d1</i>         | 2.514026749   | 5.30E-05        | −1.214194884   | 3.80E-01        |
| <i>Nr1d2</i>         | 1.591072968   | 4.00E-02        | 1.484523571    | 7.00E-02        |
| <i>Per1</i>          | 1.021012126   | 9.20E-01        | 2.281527432    | 2.20E-04        |
| <i>Per2</i>          | 1.753211443   | 1.60E-02        | 4.228072162    | 2.00E-10        |
| <i>Rora</i>          | −1.292352831  | 6.70E-01        | 1.2397077      | 7.80E-01        |
| <i>Rorb</i>          | 1             | 1.00E+00        | 1              | 1.00E+00        |
| <i>Rorc</i>          | −1.01395948   | 9.60E-01        | 2.0139111      | 1.50E-03        |

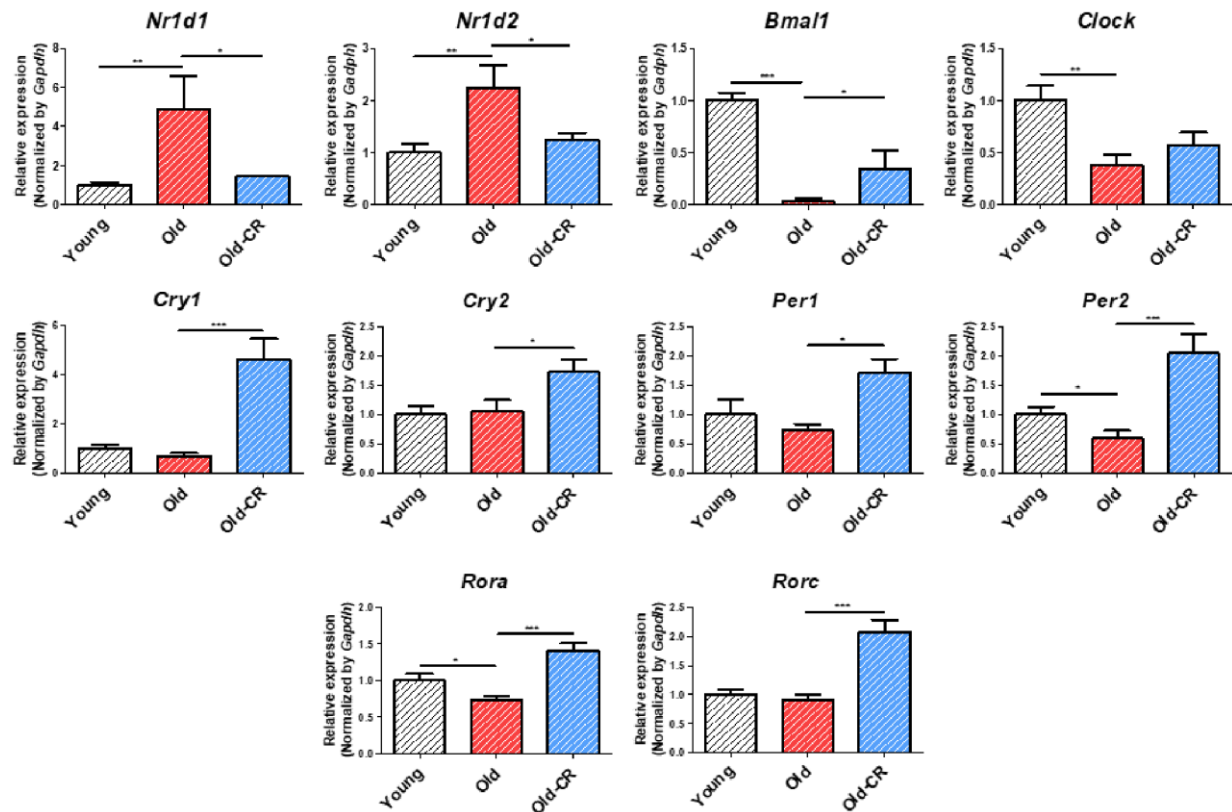
decreased with CR. PPI analysis predicted that *Nr1d1* association with lipid metabolism genes, including *Ppara*. We demonstrated that NR1D1 competitively binds to the PPRE against PPAR $\alpha$  in human cells, reducing fatty acid oxidation gene transcription. These findings suggest that *Nr1d1* overexpression during

aging interferes with Ppara $\alpha$ , leading to hepatic lipid accumulation, which was alleviated by CR.

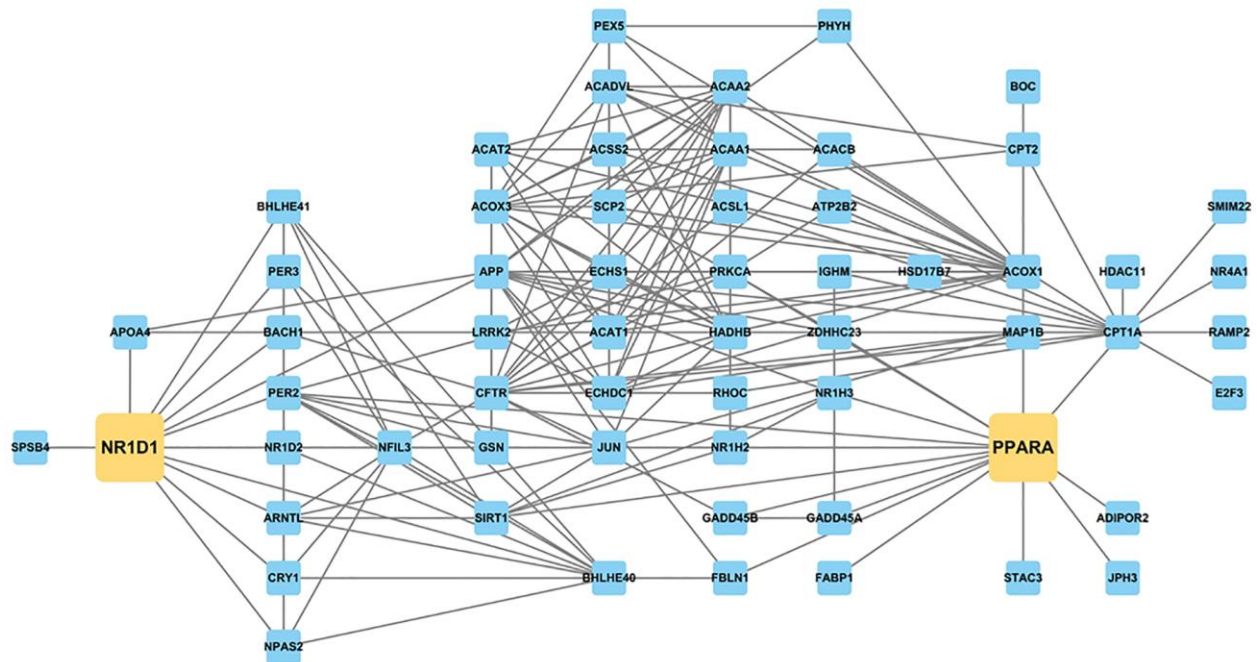
Our study confirmed significant changes in genes associated with lipid metabolism and circadian rhythm during aging and CR. These findings align with prior



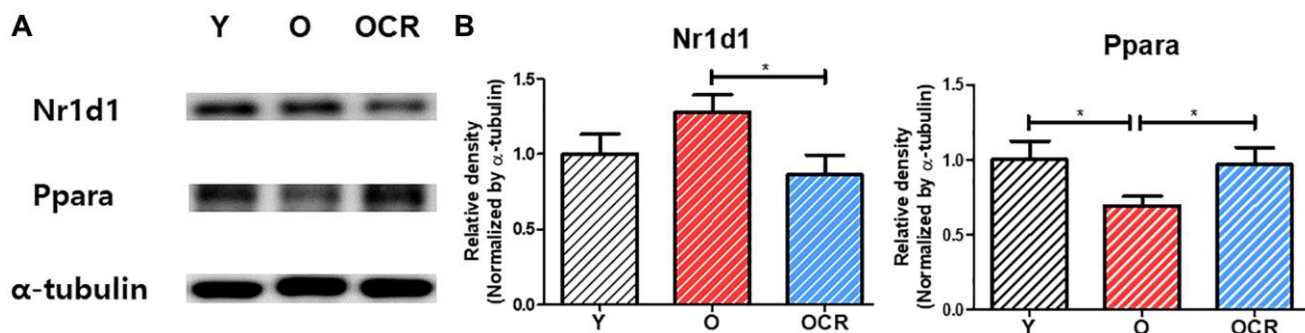
**Figure 5. Effect of aging and CR on serum TG (*n* = 6 per group).** Serum TG levels are increased in Old rats compared to those in Young rats and are decreased in Old-CR rats compared to those in Old rats (Young: 82.28  $\pm$  7.111 mg/dL, Old: 106.8  $\pm$  11.23 mg/dL, and Old-CR: 40.73  $\pm$  3.689 mg/dL). Data are presented as mean  $\pm$  SEM. Old-CR, old group under CR; TG, triglycerides.



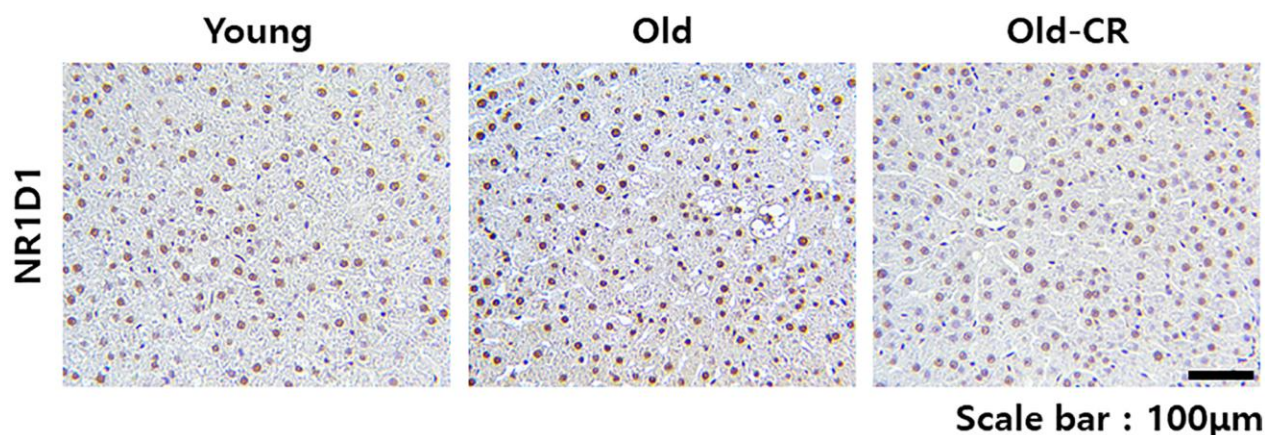
**Figure 6. Relative mRNA expression of circadian genes that are differentially expressed during aging and CR ( $n = 6$  per group).** *Nr1d1* and *Nr1d2* are upregulated in aging but downregulated in CR, while *Bmal1*, *Per2*, and *Rora* display opposite trends. *Clock* is downregulated in aging. *Cry1*, *Cry2*, *Per1*, and *Rorc* are upregulated in CR. Data are presented as mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  between two groups.



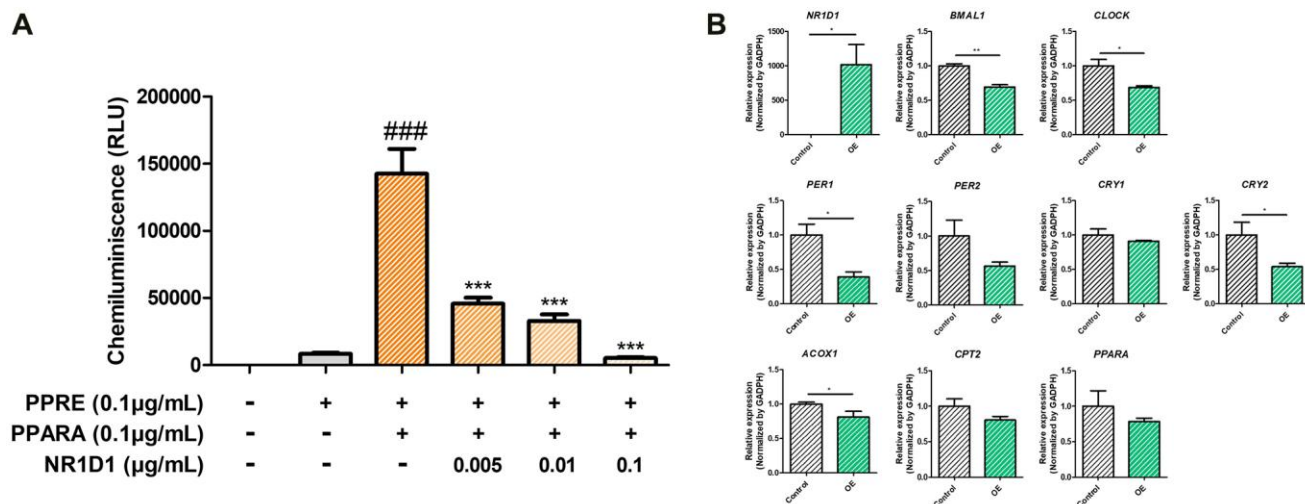
**Figure 7. PPI network constructed with genes related to circadian rhythm and lipid metabolism and aging-altered DEGs, focusing on *Nr1d1* and *Ppara*.** *NR1D1* is highly related to several circadian and metabolic regulators, such as *Bmal1* (*Arntl*), *Nr1d2*, *Cry1*, *Acox1*, *Cpt2*, and *Ppara*.



**Figure 8. Protein expression of NR1D1 and PPAR $\alpha$  during aging and CR ( $n = 6$  per group).** (A) Representative images of NR1D1 and PPAR $\alpha$  bands. (B) Relative expression of NR1D1 and PPAR $\alpha$ . The expression of NR1D1 increases during aging and decreases under CR. In contrast, the expression of PPAR $\alpha$  is downregulated during aging but upregulated by CR. Data are presented as mean  $\pm$  SEM. Abbreviations: Y: Young; O: Old; OCR: Old-CR. \* $p < 0.05$  between two groups.



**Figure 9. Immunohistochemical analysis of Nr1d1 expression in rat liver tissues during aging and CR.** Nr1d1 protein expression is elevated in liver sections from aged mice compared to young controls, while CR intervention significantly attenuated this age-associated increase. Representative images of Nr1d1 staining of liver sections from the Young (left), Old (center), and Old-CR groups (right). Scale bar = 100  $\mu$ m.



**Figure 10. Effects of NR1D1 overexpression on (A) the PPRE binding activity of PPAR $\alpha$  and (B) expression changes of circadian and lipid metabolic genes ( $n = 6$  per group).** HepG2 cells were transfected with pcDNA or the NR1D1 plasmid. The luciferase reporter assay measures the binding of PPAR $\alpha$  to the PPRE element with varying amounts of NR1D1 expression vectors. PPRE binding of PPAR $\alpha$  decreases in a NR1D1 dose-dependent manner. Furthermore, the circadian gene *BMAL1*, *CLOCK*, *PER1/2*, and *CRY1/2* and lipid metabolic genes *ACOX1*, *CPT2*, and *PPARA* are decreased with *Nr1d1* overexpression. Data are presented as mean  $\pm$  SEM. ###, \*\*\* $p < 0.001$  between two groups. OE; overexpression.

literature demonstrating that circadian rhythms and lipid metabolism undergo substantial alterations during hepatic aging, and directly linked to age-related metabolic diseases [29]. The complex interplay between metabolic, hormonal, and cellular mechanisms contributing to age-associated lipid accumulation has been well-documented [43, 44]. Aging modifies the circadian transcriptome of the liver, resulting in tissue-specific alterations in circadian gene expression patterns [25, 45].

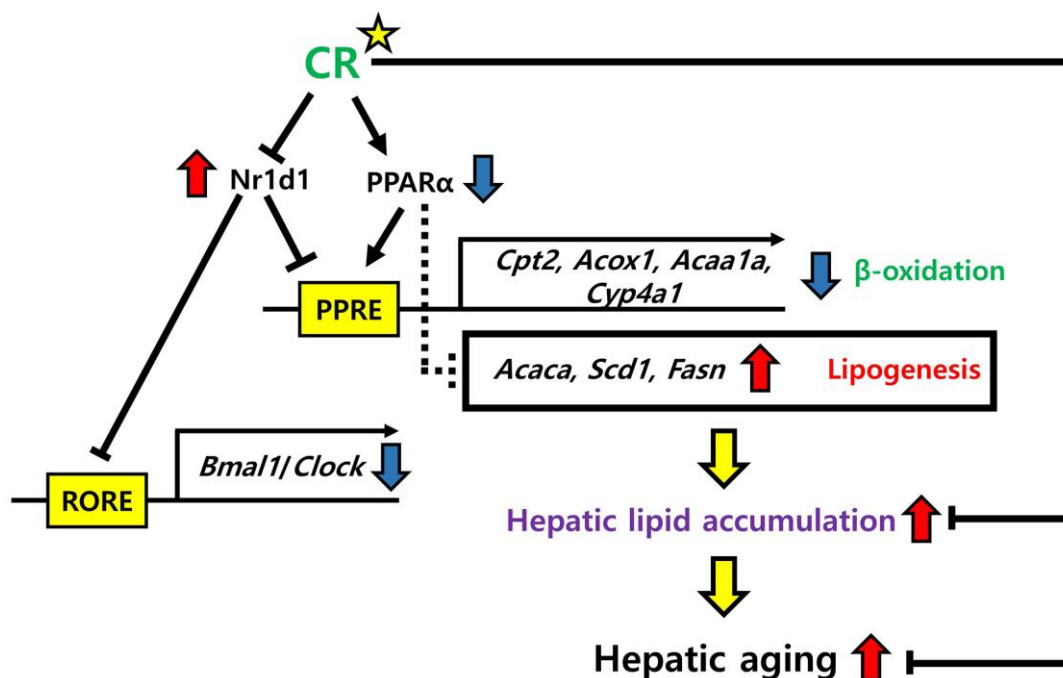
CR, a well-known anti-aging strategy, positively affects circadian rhythm gene expression, helping synchronize circadian rhythms and protect against desynchronization that negatively affects longevity [46]. CR also enhances protein modifications such as acetylation, suggesting a pathway to slow aging by modulating liver metabolism [25]. These findings are consistent with our data indicating significant alterations in circadian rhythm and metabolism-related gene expression during hepatic aging and CR.

We confirmed that *Nr1d1* is upregulated during aging but downregulated by CR. Our previous study demonstrated *Nr1d1* overexpression during hepatic aging [41]. Other studies reported that *Nr1d1* expression is associated with aging and lipid metabolism. For example, reducing *Nr1d1* levels in aged mouse heart

cells promotes growth and reduces cell death, offering a strategy to mitigate heart aging [47]. CR changes transcriptomic levels of longevity and circadian-related genes, including *Nr1d1*, thereby mitigating circadian disruption [48–51].

*Nr1d1* plays a complex, context-dependent role in lipid metabolism regulation. While *Nr1d1* suppression leads to increased hepatic lipid accumulation, particularly with high dietary fat intake [52, 53], other studies reported that NR1D1 positively regulates lipogenic genes such as *SREBP-1c* in hepG2 cells [54]. These seemingly contradictory findings likely reflect the multifaceted nature of *Nr1d1* function, which varies depending on metabolic state, tissue context, and experimental conditions. Our data revealed that in the aging context, *Nr1d1* overexpression disrupts the balance of lipid homeostasis by interfering with PPAR $\alpha$ -mediated fatty acid oxidation, leading to lipid accumulation. This is consistent with studies showing that circadian clock proteins regulated by *Nr1d1*, including *Bmal1*, *Clock*, and cryptochromes, significantly regulate ketogenesis by interacting with PPAR $\alpha$  in mouse models [55].

Our findings suggest that during aging, elevated *Nr1d1* expression shifts this balance toward lipid accumulation by competitively inhibiting PPAR $\alpha$  function, while CR



**Figure 11. Possible mechanisms of NR1D1 and PPAR $\alpha$  modulation of hepatic lipid metabolism in aging and CR.** NR1D1 and PPAR $\alpha$  competitively bind to PPRE. Upregulation of NR1D1 during aging represses the expression of genes involved in  $\beta$ -oxidation, such as *Cpt2*, *Acox1*, *Acaa1a*, and *Cyp4a1* and further induces the expression of lipogenic genes, such as *Acaca*, *Scd1*, and *Fasn*. These transcriptomic changes lead to hepatic lipid accumulation, which aggravates hepatic aging. However, CR may protect against the deterioration of lipid metabolism in the liver by modulating NR1D1 and PPAR $\alpha$ .

restores metabolic balance by normalizing *Nr1d1* levels. Although previous research on *Nr1d1* overexpression has been limited, our study highlights a novel role for *Nr1d1* in significantly influencing lipid metabolism. This expands the understanding of *Nr1d1* functions beyond its established roles, emphasizing the importance of maintaining *Nr1d1* homeostasis as a potential strategy for regulating circadian rhythms and metabolism. Further research is warranted to explore these mechanisms in greater detail.

Our study confirmed that both *Ppara* expression and activity decline during liver aging but are restored by CR. PPARs play crucial roles in lipid metabolism regulation and prevention of obesity and diabetes, which can modulate aging [56–58]. *Ppara*, a member of the PPAR family, plays a protective role against age-related lesions in vital organs, including the liver, kidneys, and heart, thereby mitigating aging progression [59, 60]. These studies indicate that age-related PPAR $\alpha$  decreases can aggravate the aging effects, highlighting its role as an aging modulator. On the other hand, CR leads to metabolic adaptations where the body reduces lipogenesis and increases lipolysis and ketogenesis by activating *Ppara* [9, 61]. Considering the critical role of *Ppara* as a metabolic regulator, comprehensive understanding of its targets and regulatory mechanisms could provide a robust foundation for addressing metabolic decline during aging.

*Ppara* is influenced by circadian genes and reciprocally regulates these genes while modulating lipid metabolism. *Bmal1*, in cooperation with *Clock*, activates *Ppara* expression by binding to E-box elements and acts as an upstream regulator of *Ppara* gene influencing various metabolic processes [62, 63]. Conversely, PPAR $\alpha$  directly regulates core circadian rhythm components, such as *BMAL1* and *NR1D1*, illustrating their significant role in metabolism and daily bodily cycles [64]. These findings support our study's assertion of *Ppara*-*Nr1d1* interaction during aging by demonstrating that *Ppara* functions as a regulator of circadian rhythm components, including *Nr1d1*.

We confirmed that *NR1D1* competitively binds to the PPRE and inhibits the binding of PPAR $\alpha$  to PPRE in hepG2 cells. *NR1D1* modulates gene expression by antagonizing the binding of PPAR $\alpha$  to the PPRE, illustrating a convergence of *NR1D1* and PPAR signaling pathways in the transcriptional regulation affecting lipid metabolism, including  $\beta$ -oxidation [65–67]. In mouse models, *Nr1d1* represses downstream circadian genes like *Bmal1* and *Clock*, while *Ppara* regulates the circadian clock by modulating *Bmal1* [63, 68]. *Bmal1*/*Clock* reciprocally transactivates PPAR target genes via PPRE, indicating bidirectional

influence of circadian and metabolic regulators [69]. These studies correspond with our findings that *Nr1d1* plays a crucial role in modulating lipid metabolism by regulating fatty acid oxidation-related gene expression, which are principal PPAR $\alpha$  targets.

In conclusion, our findings reveal that the co-regulation of *Nr1d1* and PPAR $\alpha$  plays a significant role in modulating hepatic lipid accumulation during aging, while CR may protect against this process, offering new potential strategies to mitigate the age-related metabolic decline with liver aging (Figure 11).

## MATERIALS AND METHODS

### Animals

Young male Sprague Dawley (SD) rats (Young group; 6 months old), old male SD rats (Old group; 19 months old), and old male rats treated with caloric restriction (Old-CR group; 19 months old) were purchased from Samtako (Osan, Gyeonggi-do, Korea). The CR protocol followed established methodologies where old-CR rats received 60% of the average daily food intake measured after 1-week stabilization, maintained for 4 weeks. Young and old rats were provided ad libitum water and a normal chow diet (20% protein, 4.5% fat, 6% fibre, 7% ash, 0.5% calcium, 1% phosphorus) [14, 42]. Rats were kept at  $23 \pm 2^\circ\text{C}$ , with  $60 \pm 5\%$  humidity and a 12-h light/dark cycle. Tissues were frozen in liquid nitrogen for analysis. All experiments were approved by the Pusan National University Institutional Animal Care and Use Committee (approval number PNU-2015-1044).

### RNA-Seq

Total RNA was extracted from liver samples using RiboEx reagent (GeneAll Biotechnology, Seoul, Korea). Equal quantities of RNA from each group ( $n = 5$ ) were pooled for RNA-Seq analysis. cDNA libraries were prepared and sequenced using the MGI-T7 platform (MGI Tech Co., Shenzhen, China) with MGIEasy RNA Directional Library Prep Set. Adapter sequences were removed using Cutadapt (version 2.9) and quality filtered using Trimmomatic (version 0.39) [70, 71]. Reads shorter than 36 bp were excluded from analysis. Reads were aligned to the human reference genome (hg38) and Ensembl (version 102) using STAR (version 2.7.3a). STAR and RSEM (version 1.3.1) were used for alignment and quantification with default parameters [72, 73].

### Differential expression analysis

Gene count data were normalized using the DESeq2 normalization methods. Differential expression

analysis was performed using DESeq2 (version 1.30.1) [74]. Differentially expressed genes (DEGs) were identified using  $p$ -values  $< 0.05$  and absolute fold changes  $\geq 1.5$  (Supplementary Table 1). Volcano plots were generated using VolcanoR web interface [75].

### Gene ontology (GO) and pathway enrichment analyses

GO enrichment analysis examined biological functions, and KEGG pathway analysis investigated DEG-related signaling pathways. Both analyses were conducted using DAVID with statistical threshold of  $p < 0.05$ . Results are listed in Supplementary Table 2.

### qRT-PCR

Primers were synthesized by Bioneer (Daejeon, Korea). Total RNA was isolated from rat liver tissues (20 mg) and *NR1D1*-overexpressing HepG2 cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) ( $n = 6$  per group) and reverse-transcribed using cDNA synthesis kit (GenDEPOT, Baker, TX, USA). qRT-PCR was performed using SYBR Green (Bioneer, Daejeon, Korea) and CFX Connect System (Bio-Rad, Hercules, CA, USA). Unpaired Student's  $t$ -test was used for two-group comparisons (*NR1D1* overexpression vs. Control). When comparing multiple groups simultaneously, one-way analysis of variance (ANOVA) was performed followed by Tukey's multiple comparison post-hoc test to control for family-wise error rate (Young vs. Old vs. Old-CR). Primer sequences are listed in Supplementary Table 4.

### Serum biochemical analysis

Serum samples were prepared by centrifugation ( $4^{\circ}\text{C}$  and  $2,000\times g$  for 15 min) after euthanasia. TG levels were measured using serum kits (Bioassay Systems, Hayward, CA, USA). Serum TG levels were compared between groups using one-way ANOVA followed by Tukey's post-hoc test.

### PPI network analysis

Mixed PPI database was constructed based on the interactions between five databases: HPRD, BioGRID, IntAct, MINT, and STRING [76–80]. Interactions with highest confidence (0.900) were selected from STRING, with all interactions extracted from the other databases. PPI network was constructed to identify hub molecules and examine DEG interactions. Cytoscape (version 3.10.1) visualized networks and calculated topological parameters including degree and betweenness centrality [81].

### Random Walk with Restart (RWR) algorithm on the PPI network

RWR algorithm was applied for node ranking within the PPI network to identify pivotal genes near seed genes [82, 83]. The mathematical underpinning of RWR involves the utilization of a transition matrix ( $M$ ) and probability vectors, where  $P$  signifies the vector denoting node probabilities. The updated equation is expressed, as follows:

$$P(t+1) = (1-\alpha) \cdot M \cdot P(t) + \alpha \cdot P_{\text{init}}$$

Here,  $\alpha$  represents the restart probability (damping factor),  $P(t)$  denotes the probability vector at iteration  $t$ , and  $P_{\text{init}}$  is the initial probability vector. RWR computations used 0.8 restart probability with R package RandomWalkRestartMH [84]. Results are presented in Supplementary Table 6.

### Western blotting

Total protein was extracted from rat liver tissues and boiled for 5 min in loading buffer containing 0.2% bromophenol blue, 125 mM Tris-HCl, 10% 2-mercaptoethanol, and 4% SDS (pH 6.8) ( $n = 6$  per group). Equal amounts of protein (8–10  $\mu\text{g}$ ) were separated by SDS-PAGE using 10% gels and transferred to PVDF membranes at 25 V for 10 minutes using a semidry transfer. Membranes were blocked in 5% nonfat milk for 2 h, then immunoblotted overnight with primary antibodies (1:1,000) at  $4^{\circ}\text{C}$ . After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000) for 1 h at room temperature. Protein detection was performed using enhanced chemiluminescence, and molecular weights were determined using a wide range of protein markers. Immunoblots were visualized using a chemiluminescent HRP substrate (Davinchchemi CAS-400) and analyzed using the ImageJ Software. The detailed procedure has been described previously [41]. Antibodies against NR1D1 (sc-100910), PPAR $\alpha$  (sc-398394), and  $\alpha$ -tubulin (sc-5286) were purchased from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies (GTX213110-01 and GTX213111-01) were purchased from GeneTex (Irvine, CA, USA). Protein levels were compared between groups using one-way ANOVA followed by Tukey's post-hoc test.

### Immunohistochemistry

Paraffin-embedded sections were deparaffinized of using xylene, followed by graded ethanol series (starting at 100% and ending at 70%) and rehydration. Antigen

retrieval was performed using sodium citrate buffer (pH 6.0). Specimens were then incubated with Nr1d1 primary antibodies (Santa Cruz, sc-100910, 1:200) at 4°C overnight in a humidity-controlled environment. After PBS washing, sections were incubated with biotinylated secondary antibodies (1:10,000; VectorLabs) for 30 min at ambient temperature. Diaminobenzidine substrate with hematoxylin counterstaining was used for visualization. Microscopic examinations were performed using a Motic AE30/31 inverted microscope.

## Cell culture

HepG2 cells were purchased from American Type Culture Collection and maintained in Dulbecco's Modified Eagle Medium (Welgene; LM001-11) containing 10% fetal bovine serum (Gibco; S001-01), 100 U/mL penicillin, and 100 µg/mL streptomycin (Hyclone; SV30010) at 37°C in 5% CO<sub>2</sub>. Cells were cultured in sterile plastic plates (SPL, 20100).

## Cell transfection for NR1D1 overexpression

To observe the change in mRNA expression with *NR1D1* overexpression, HepG2 cells were transfected with pcDNA or the NR1D1 plasmid using Lipofectamine 3000 (Invitrogen; L3000015), according to the manufacturer's instructions. The *NR1D1* plasmid was kindly provided by Dr. Mi-Ock Lee (Seoul National University, Seoul, Republic of Korea).

## Luciferase assay

To observe the binding of PPARα or NR1D1 to PPRE, HepG2 cells ( $1.5 \times 10^4$  cells/well) were grown in a 96-well plate in DMEM supplemented with 10% FBS. The PPRE-X3-TK-LUC plasmid (0.1 µg) purchased from BioCat company (Heidelberg, Land Baden-Württemberg, Germany), and *PPARA* and the *NR1D1* plasmid were transfected using Lipofectamine 3000 (0.1 µL) and P3000 (0.2 µL) complexes in Opti-MEM, according to the manufacturer's instructions. An empty pcDNA vector was added to ensure that equal amounts of plasmid DNA were used per transfection. Luciferase activity was measured using a ONE-Glo Luciferase Assay System (Promega, Madison, WI, USA) and a luminescence plate reader (Berthold Technologies GmbH & Co., Bad Wildbad, Germany). Data were analyzed using one-way ANOVA with Tukey's post-hoc test for multiple comparisons.

## Statistical analysis

Sample sizes ( $n = 6$  per group for animal studies,  $n = 3$  for cell culture experiments) were determined based on our previous studies to detect meaningful biological

differences [27, 85]. All statistical analyses were performed using the GraphPad Prism software (version 5; GraphPad Software, La Jolla, CA, USA). Data are presented as mean  $\pm$  standard error of the mean (SEM). Statistical significance was set at  $p < 0.05$  for all analyses.

## Abbreviations

Acaal: Acetyl-CoA Acyltransferase 1A; Acaca: Acetyl-CoA Carboxylase Alpha; Acly: ATP Citrate Lyase; ANOVA: Analysis of Variance; Bmal1: Brain and Muscle ARNT-Like 1; BP: Biological Processes; CC: Cellular Components; Clock: Circadian Locomotor Output Cycles Kaput; Cpt2: Carnitine Palmitoyl-transferase 2; CR: Calorie Restriction; Cry1/2: Cryptochrome 1/2; Cyp4a1: Cytochrome P450 4A1; DAVID: Database for Annotation, Visualization, and Integrated Discovery; DEGs: Differentially Expressed Genes; DMEM: Dulbecco's Modified Eagle Medium; ECM: Extracellular Matrix; Fasn: Fatty Acid Synthase; FBS: Fetal Bovine Serum; GO: Gene Ontology; HRP: Horseradish Peroxidase; KEGG: Kyoto Encyclopedia of Genes and Genomes; MAFLD: Metabolic Dysfunction-Associated Fatty Liver Disease; MF: Molecular Functions; Nfil3: Nuclear Factor, Interleukin 3 Regulated; NR1D1: Nuclear Receptor Subfamily 1 Group D Member 1; OE: Overexpression; PBS: Phosphate-Buffered Saline; Per1/2: Period 1/2; PPI: Protein-Protein Interaction; PPAR: Peroxisome Proliferator-Activated Receptor; PPARα: Peroxisome Proliferator-Activated Receptor alpha; Ppard: Peroxisome Proliferator Activated Receptor Delta; PPRE: Peroxisome Proliferator Response Elements; qRT-PCR: quantitative Real-Time Polymerase Chain Reaction; RNA-seq: RNA sequencing; Rora: RAR Related Orphan Receptor A; Rorc: RAR Related Orphan Receptor C; RWR: Random Walk with Restart; Scd: Stearoyl-CoA Desaturase; SD: Sprague Dawley; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; SEM: Standard Error of the Mean; STRING: Search Tool for the Retrieval of Interacting Genes/Proteins; TG: Triglycerides.

## AUTHOR CONTRIBUTIONS

Noh, S.G. and Chung, H.Y. conceived and designed the experiments. Noh, S.G. analyzed the data and wrote the manuscript. Kim, H.W. and Kim, S. performed all *in vitro* and *in vivo* experiments. Noh, S.G. performed all *in silico* analyses. Yu, B.P., Yoon, J.H., Chung, K.W., Lee, J., and Chung, H.Y. critically revised the manuscript.

## CONFLICTS OF INTEREST

The authors declare no conflicts of interest related to this study.

## ETHICAL STATEMENT

Experimental work with animals was approved by the Pusan National University Institutional Animal Care and Use Committee (Approval Number: PNU-2015-1044) and was performed in compliance with guidelines on animal experimentation at our institution.

## FUNDING

This work was supported by National Research Foundation grants funded by the Korean government (NRF-2023R1A2C2006035, RS-2023-00272618).

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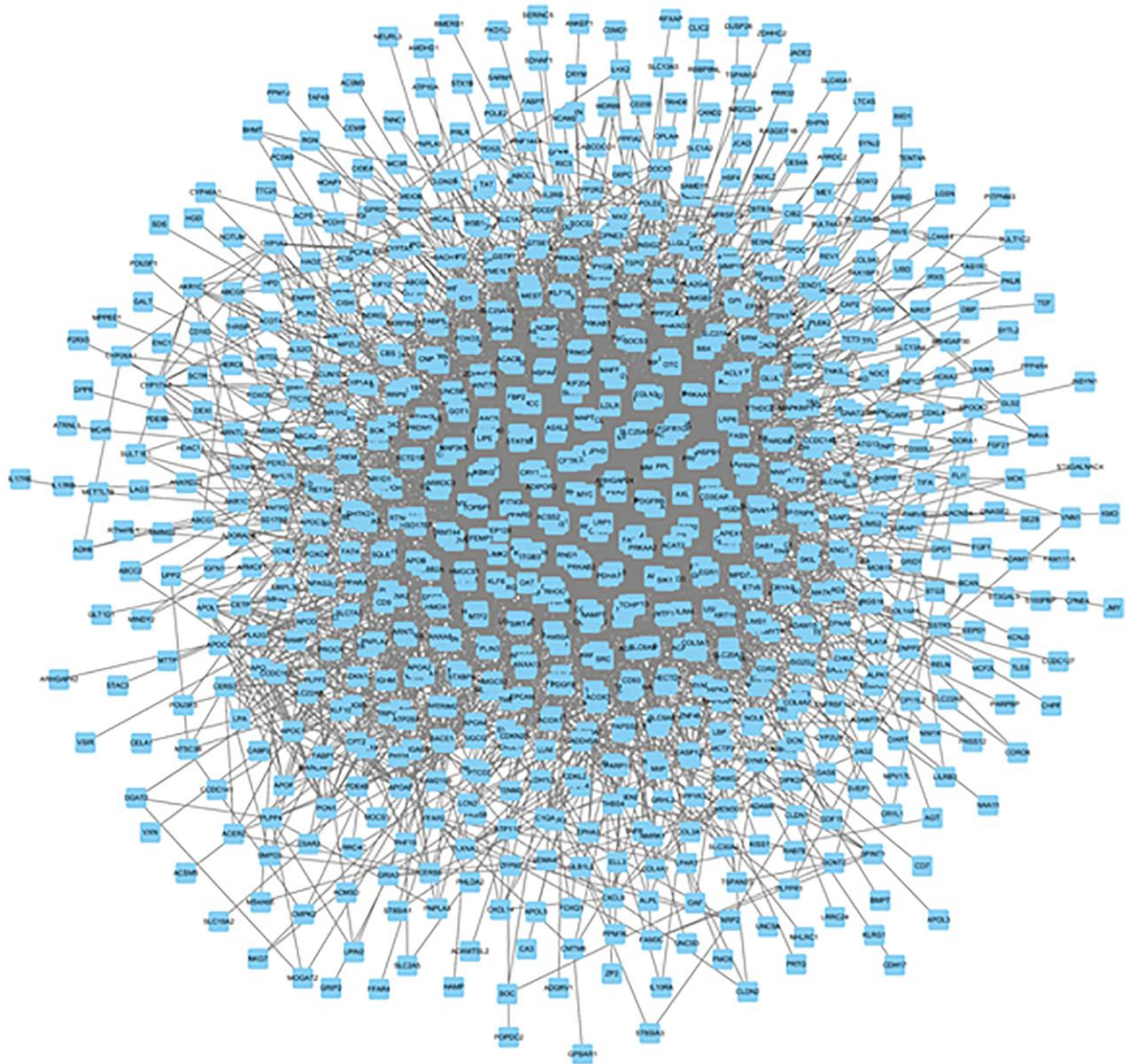
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## SUPPLEMENTARY MATERIALS

### Supplementary Figure



Supplementary Figure 1. PPI network constructed using genes related to the circadian rhythm and lipid metabolism and age-affected DEGs.

## Supplementary Tables

Please browse Full Text version to see the data of Supplementary Tables 1–3 and 5, 6.

**Supplementary Table 1. DEGs from the Old vs. Young and Old-CR vs. Old datasets.**

**Supplementary Table 2. GO terms and pathways of the DEGs from the Old vs. Young and Old-CR vs. Old datasets.**

**Supplementary Table 3. Genes with significant expression changes in the Old vs. Young and Old-CR vs. Old datasets.**

**Supplementary Table 4. Primer sequences for RT-qPCR analysis.**

| Species | Gene          | Forward (5'–3')        | Reverse (3'–5')         |
|---------|---------------|------------------------|-------------------------|
| Rat     | <i>Acaa1a</i> | CCTGATTCCTATGGGGATAACC | GTTGTCACAGGTACGATCTCAG  |
| Rat     | <i>Acaca</i>  | GGCACTCTGATCTGGTCACG   | GCTCCGCACAGATTCTTCAA    |
| Rat     | <i>Bmal1</i>  | TATCACACTACGAAGTCGATGG | CGGCAATCATTTCGACCTATTTT |
| Rat     | <i>Clock</i>  | GAGGTCATCCTTCAGTAGTCAG | TGAAAGCTGAAACTGTGACATG  |
| Rat     | <i>Cpt1a</i>  | AAGCTGTGGCCTTCCAGTTC   | GGATGAAATCACACCCACCA    |
| Rat     | <i>Cry1</i>   | CCTTGATGCCAATCTACGAAAG | AAAGGCTCAGAATCGTACTCAA  |
| Rat     | <i>Cry2</i>   | AAAACCACATGGGGAATAGACT | GTTTCTTTTACCAGTTCAGACC  |
| Rat     | <i>Cyp4a1</i> | CAAGGTGACAAAGAACTACAGC | CAAGGAGCTAGCAATCTGTAGA  |
| Rat     | <i>Fasn</i>   | GCTGGGACACATGTGATGGT   | AGTGAGTGACGGGAGGGCT     |
| Rat     | <i>Nr1d1</i>  | AATGCCAATCATGCATCAGGTA | ATTCAGTGCTTCATTATGACGC  |
| Rat     | <i>Nr1d2</i>  | GAATTCCGTTGTACCACAAGAC | AGGTAAAGACGCTTTATGGACA  |
| Rat     | <i>Per1</i>   | AACATTCTAACACAACCAAGC  | CTGCTGACGACGTATCTTTCTT  |
| Rat     | <i>Per2</i>   | CTCTGACACATCCCAGTCTAG  | GTCCCTGGTGTGGATACTATTC  |
| Rat     | <i>Ppara</i>  | ACGGCGTTGAAAACAAGGAG   | TTGGCAAATTCCTGAGCTC     |
| Rat     | <i>Ppard</i>  | CATTGTCAACAAAGACGGACTG | ATTGAACTTGACAGCAAACCTCG |
| Rat     | <i>Rora</i>   | CAATATACCCAGACATTGTGCG | ACTCCAGATGTTCTAGAAAGTGC |
| Rat     | <i>Rorc</i>   | GTGGAGCATCTGCAATACAATT | CAATGTCAGTGCTGAAGAGTTC  |
| Human   | <i>NR1D1</i>  | TGAATGGCATGGTGTTACTG   | ACTTCTTGAAGCGACATTGC    |
| Human   | <i>BMAL1</i>  | CTGTGCTAAGGATGGCTGTT   | GCTCCTTGACTTTGGCAATA    |
| Human   | <i>CLOCK</i>  | GGCAAAATGTCATGAGCACTTA | AGCCCTAACTTCTGCATAACTT  |
| Human   | <i>PER1</i>   | CAGCTTTTATTGAGTCTCGGG  | CAGTTGATCTGCTGGTAGGAG   |
| Human   | <i>PER2</i>   | CTTATTCACTGCCCGTGTTTC  | GGAAGGAATAACTGGGTAGCAT  |
| Human   | <i>CRY1</i>   | GACGCAGCTATTAAGAACTGG  | TTTGCTGATGAGAGTCTGGAAT  |
| Human   | <i>CRY2</i>   | CTATGAGAGACCCGAATGAAC  | CCGCTTCACCTTTTTTATACAGG |
| Human   | <i>ACOX1</i>  | AGGTCACAGCTGTCCAACCA   | TTACCCAGCCCTGGCTTAAT    |
| Human   | <i>CPT2</i>   | GGTCCAGGTAGAGCTCAGGC   | GTGCTCTGAGGCCTTTGTCA    |
| Human   | <i>PPARA</i>  | TCGGCGAGGATAGTTCTGGAAG | GACCACAGGATAAGTCACCGAG  |

**Supplementary Table 5. Network analysis of the PPI network constructed with genes related to the circadian rhythm and lipid metabolism and age-affected DEGs.**

**Supplementary Table 6. Genes ranked using the Random Walk with Restart (RWR) algorithm on the PPI network with *Nr1d1* as the seed node.**