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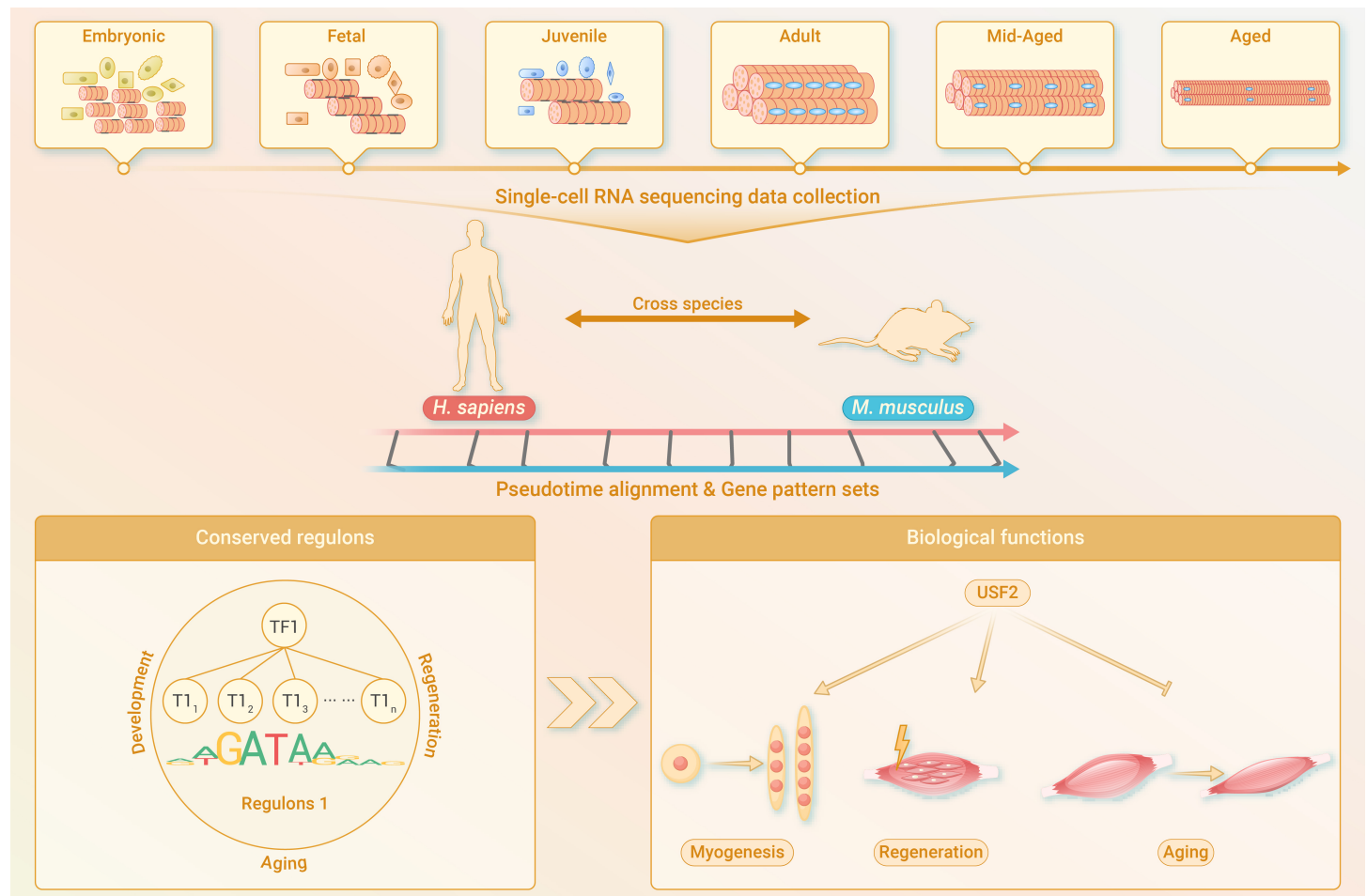
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- A conserved developmental coordinate between human and mouse MuSCs sheds light on their shared biology.
- Conserved regulons enhance the understanding of MuSC behavior during development and aging.
- USF2 is a pivotal conserved regulon essential for muscle cell myogenesis and aging.



Single-cell analysis reveals conserved regulons shaping muscle stem cell behavior during development and aging in mammals

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Muscle stem cells (MuSCs) play a pivotal role in skeletal muscle development, regeneration, and maintenance. Previous studies have extensively investigated the transcriptional changes in MuSCs during various developmental stages in mammals using single-cell RNA sequencing. However, a gap remains in cross-species integrative research examining the factors influencing MuSC activity during development and aging. To address this gap, we constructed a conserved single-cell landscape of MuSCs in mammals, encompassing their developmental and aging stages. Our findings unveil a conserved developmental coordinate between human and mouse MuSCs, shedding light on their shared biology. The single-cell coordinated gene association in pattern sets (scCoGAPS) algorithm and dynamic time warping algorithm were used to investigate the temporal dynamics of skeletal muscle regeneration related gene patterns. Additionally, we scrutinized the conservation of regulons, delineating groups of genes under the influence of common transcription factors, particularly emphasizing the identification of pivotal factors governing MuSC behavior during aging. Notably, we identified USF2, a conserved regulon, as a key regulator influencing muscle cell myogenesis and aging. This research provides critical insights into the conserved aspects that influence MuSC behavior and highlights the significance of USF2 in MuSC regulation. By unraveling the intricate mechanisms underlying MuSC development and aging, our study opens avenues for advancements in regenerative medicine and muscle-related therapeutics.

INTRODUCTION

Muscle stem cells (MuSCs) play a pivotal role in the development, regeneration, and maintenance of skeletal muscle throughout an organism's lifespan.¹⁻⁴ During embryonic development, MuSCs undergo complex molecular and cellular changes, which are tightly regulated to ensure proper muscle formation.⁵⁻⁷ In adulthood, the maintenance of a functional MuSC pool becomes essential for muscle homeostasis and preventing muscle degeneration associated with aging.^{8,9} Thus, understanding the factors that influence MuSC activity during different developmental stages and aging is crucial for developing strategies to enhance muscle regeneration and combat age-related muscle loss, frailty, and functional decline.

Recent advancements in single-cell RNA sequencing (scRNA-seq) technology have enabled detailed exploration of the transcriptome landscapes of skeletal muscle at the single-cell level in mammals.¹⁰⁻¹⁴ These studies provided valuable reference resources to examine MuSC diversity and dysregulation during development and aging. However, there remains a notable gap in cross-species integrative research to fully comprehend the conserved mechanisms that govern MuSC behavior and their implications for muscle health. Cross-species transcriptome comparison at single-cell level helps uncover molecular basis for many conserved and divergent developmental processes among mammalian species.¹⁵⁻¹⁹ Consequently, conducting a comprehensive cross-species study of the conserved biological mechanisms governing MuSC behavior is imperative.

Here, we employed scRNA-seq analysis to examine the transcriptomes of human and mouse MuSCs across different developmental stages and during aging. By comparing the transcriptomes between species, we identified conserved factors responsible for maintaining the state of MuSCs through-

out development and aging. Additionally, we established a developmental coordinate that aligned the progression of MuSCs between human and mouse. Through the analysis of dynamic gene patterns associated with muscle regeneration, we gained insights into the age-related degeneration of muscle structure and function. Our investigation also focused on the conservation of transcription factors (TFs) and their target genes in MuSCs during the aging process. Notably, we highlight the role of upstream transcription factor 2 (USF2), revealing that its overexpression enhanced the regenerative capacity of MuSCs by promoting the process of muscle cell differentiation. These findings contribute to our understanding of muscle health and lay the groundwork for future therapeutic interventions targeting MuSCs in the context of aging and muscle-related disorders.

MATERIALS AND METHODS

Data and materials availability

Previously published single-cell RNA-seq data that were reanalysed here are available in the GEO or ArrayExpress under the accession codes "GSE147457" (human developmental skeletal muscle cells), "GSE143704" (human aging skeletal muscle cells), "GSE149451" (developmental trajectory of human induced pluripotent stem cell (iPSC) derived myogenic precursors), "GSE109774" (mouse developmental skeletal muscle cells), "GSE149590" (mouse aging skeletal muscle cells), "GSE138826" (mouse skeletal muscle cells during regeneration process). Expression level changes of USF2 during C2C12 myoblasts induced to differentiate was obtained under the accession numbers GSE989, during bovine skeletal myoblast myogenic differentiation was obtained under the accession numbers GSE173477, and during porcine skeletal muscle satellite cells differentiation was obtained under the accession numbers GSE112527.

Data Processing analysis

Muscle cells were isolated based on cell type annotations from the literature, and standard procedures for variable gene selection, dimensionality reduction, and clustering were conducted using Seurat package version 3.2.3²⁰. Initial quality checks were performed to ensure sample integrity²¹, measuring parameters such as the number of genes per cell (nFeature_RNA), the number of UMI per cell (nCount_RNA), and the percentage of UMIs originating from mitochondrial genes per cell (percent.mt). Filtering criteria included cells with > 200 nFeature_RNA, > 1000 nCount_RNA, < 5% percent.mt, and genes expressed in at least 3 cells (Figure S1). The Seurat package's 'LogNormalize' method was employed for global scaling normalization of the data. Unsupervised shared nearest neighbor (SNN) clustering was executed with a resolution of 0.4.

Cell cycle states of distinct cell types were assigned based on "S.Score" and "G2M.Score" for each cell, considering the average normalized expression levels of core cell cycle genes, using the "CellCycleScoring" method as per Seurat instructions²². Cells expressing neither G2/M nor S phase markers were categorized as being in the G1 phase. Differential expression genes (DEGs) between individual clusters were identified using the "FindMarkers" function in Seurat, utilizing the non-parametric Wilcoxon rank sum test. The gene set enrichment analysis was performed at the WebGestalt (WEB-based Gene Set Analysis Toolkit) (<http://www.webgestalt.org/>) with "pathway" and "phenotype" functional database.

Integration of muscle cells across species

To jointly define MuSCs across species, Gene-Cell expression matrix of MuSCs from different species and time points were normalized and centered. Variable gene selection was performed, followed by non-negative matrix factorization (NMF), Louvain clustering and uniform manifold approximation and projection (UMAP) projection based on low-dimensional embedding from NMF. parameters for integrated mouse and human MuSCs as follows: (1) $k=20$ (number of latent factors); (2) $knn-K$ (number of nearest neighbors) $=30$; and (3) SLM resolution (number of clusters) $=0.4$.

scCoGAPS gene pattern analysis

To infer phase-specific gene expression programs in the MuSCs during regeneration and aging, scCoGAPS version 3.6.0²³ was used to perform unsupervised identification of patterns of gene set usage that represent common features across cells. The parameters were default single-cell parameters. The identified regeneration patterns were projected into the expression matrix of others using 'ProjectR' (version 1.5.0) to understand each pattern conservation.

Inference of regulons and their activity

Human and mouse gene regulatory network analysis was performed using SCENIC with default parameters. The original implementation of SCENIC is not scalable to large datasets and its results can be significantly affected by sequencing depth. To improve the scalability or robustness, we aggregated data from multiple cells in the same cell cluster to make pseudo-cells as previously described²⁴, and then applied SCENIC using the pseudo-cells. This simple modification effectively increases the data quality as well as reduces the computational burden. To determine the 'on/off' activity of each regulon in each pseudo-cell clusters, we used '0.5*max (AUC scores)' for each regulon as a threshold to binarize the regulon activity scores and created the 'binary regulon activity matrix'. The values of the matrix that corresponded to 'on' regulons in a given pseudo-cell were 1, and 0 for 'off' regulons. From SCENIC analysis, we defined 55 human unique TF regulons, 58 mouse unique TF regulons and 192 orthologous TF regulons (Table S1-S2). Based on 192 orthologous TF regulons, we obtained a merged AUCell score matrix from both human and mouse pseudo-cells. Then the PCC of AUCell score was calculated for each pair of orthologous TF regulons. Regulon modules were identified based on the Connection Specificity Index (CSI)²⁵, using the 'ward.D' clustering method. The distribution of mean AUCell scores across the cell clusters was used to present the relationship between orthologous TF regulon modules and cell clusters.

Pseudotime analysis

Pseudotime analysis was performed using PAGA (threshold=0.05) in Scanpy²⁶. Pseudotemporal orderings were constructed by selecting dividing PAX7+ cells as root. Diffusion pseudotime was calculated for all the remaining cells relative to the root.

RNA-seq Analysis

Raw sequencing data underwent quality control assessment using FastQC version 0.11.8²⁷, followed by adapter removal and low-quality base trimming with Trimmomatic version 0.39²⁸. Reads were aligned to the reference genome (mm10) using Hisat2 version 2.2.1²⁹, and reads count for each gene were quantified using Featurecounts version 2.0.6³⁰. DEGs were identified using DESeq2 version 3.17³¹ with significance thresholds ($P < 0.01$) and multiple testing corrections. The DEGs enrichment analysis was performed at the WebGestalt (WEB-based Gene Set Analysis Toolkit) (<http://www.webgestalt.org/>) with "geneontology_Biological_Process" and "phenotype" functional database.

Isolation and differentiation of mice primary myoblast

Primary myoblasts were isolated from 2- to 5-day-old C57BL/6 mice as described previously³² and cultured in DMEM/F12 -Dulbecco's Modified Eagle Medium (DMEM/F12, Gibco, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (PS, Thermo Scientific, USA) at 37 °C in a 5% CO2 incubator. Differentiation was induced at 50–70% cell density using differentiation medium as described above for 4 days, and the medium was

changed daily.

Cell culture and transfection

C2C12 cells were purchased from China Infrastructure of Cell Line Resource and were cultured in growth medium comprising high-glucose Dulbecco's Modified Eagle medium (DMEM) (H30022.01, HyClone, Connecticut, USA) supplemented with 10% fetal bovine serum (FBS) (Z7186FBS-500, ZETA LIFE, California, USA), 1% penicillin/streptomycin. Transient transfection of cells with siRNA oligos was performed in 12-well plates with Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's instructions and harvested after 48 h.

Western blot analysis

Proteins were extracted by RIPA buffer (Thermo Scientific, Massachusetts, USA) supplemented with a phosphorylase inhibitor (Roche 5892791001, Basel, Switzerland) and a protease inhibitor (Roche 04693132001, Basel, Switzerland). The extracts were boiled with 4× SDS loading buffer (CWBI0; Beijing, China) at 98°C for 5 min, after which western blotting was performed as previously described³³. The antibodies used for Western blot were anti-USF2 (1:500, #AF0291, Affinity, Changzhou, Jiangsu, China) and anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000, Abcam ab9482, Cambridge, UK).

Muscle damage and regeneration evaluation

A muscle injury model was implemented by injecting 100 μ L 10 μ M cardiotoxin (CTX; Sigma-Aldrich) in phosphate-buffered saline (PBS) (Thermo Fisher Am9625, MA, USA) into the left TA muscle of mice. The TA muscle was collected at 0, 1, 3, 5, and 14 days after injury. As a control treatment, PBS (100 μ L) was injected into the right TA muscle of each mouse. For USF2 knockdown in vivo, we obtained AAV9 serotypes of siRNA-USF2 or siRNA-NC from HNCI (Shanghai, China). We similarly injected 100 μ L of AAV expressing siRNA-USF2 or siRNA-NC 28 days before CTX injection. The AAV-treated mice were anesthetized with isoflurane and sacrificed by cervical dislocation, after which muscle tissue samples were collected. Three mice were used at 0, 1, 3, 5, and 14 days after CTX injection for this experiment.

Hematoxylin-eosin (H&E) staining

The TA muscle was fixed with 4% paraformaldehyde for more than 72 hours and then subjected to dehydration embedding. Finally, paraffin sections of muscle were obtained at a thickness of 2 to 4 μ m for HE staining, and whole-slide digital images were collected with a Panoramic DESK Scanner (P-MIDI, P250, 3D HISTECH, Hungary).

5-Ethynyl-2'-deoxyuridine (EDU) assay

Myoblasts were seeded on 12-well plates. At 50% confluence, cells were transfected with overexpression plasmid, siRNA, or miRNA mimics. After transfection for 48 h, myoblasts were exposed to 50 μ M EDU (RiboBio, China) for 2 h at 37°C. Subsequently, cells were fixed in 4% paraformaldehyde for 30 min, neutralized using 2 mg/mL glycine solution, and permeabilized with 0.5% Triton X-100. A solution containing EDU (Apollo Reaction Cocktail; RiboBio, China) was added and the cells were incubated at room temperature for 30 min. The nuclear stain Hoechst 33342 was then added, and incubation was continued for a further 30 min. A fluorescence microscope (DMI8; Leica, German) was used to capture three randomly selected fields to visualize the number of EDU-stained cells.

Primers and oligonucleotides

Primers were designed using Primer Premier5. The siRNAs were obtained from GenePharma (Shanghai, China). All siRNA sequences and primers used in this study are provided in Table S3.

RNA extraction

Total RNA was extracted using TRIzol™ Reagent (Invitrogen, Shanghai, China). The total RNA concentration was determined via spectrophotometry to ensure an OD260/OD280 ratio of 1.8–2.0. The integrity of the RNA was assessed by gel electrophoresis. Total RNA was reverse transcribed using a HiScript III 1st Strand cDNA Synthesis kit (+gDNA wiper) (Vazyme R312-01,

Nanjing, China) according to the manufacturer's instructions.

Real-time quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed on a 7500 FAST Real - Time PCR System (Applied Biosystems) according to the SYBR Premix Ex Taq™ instructions. The cycling conditions for RT-qPCR were as follows: 50°C for 2 min; 95°C for 2 min; and 40 cycles of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Each reaction was performed in a 20 µL mixture containing 10 µL ChamQ SYBR qPCR Master Mix (Vazyme Q311-02, Nanjing, China), 0.5 µL gene - specific primers, 2 µL template cDNA, and 7 µL sterile water. All reactions were repeated three times using cDNA from three independent individuals, and the results were analyzed using the 2- $\Delta\Delta$ CT method. Each experiment was repeated three times. The primers are listed in Table S3.

Statistical analysis

Results are presented as the mean $\pm$ SEM. GraphPad Prism v. 7.0 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. All differences between groups were analyzed by one-way analysis of variance (ANOVA) and the Student's t-test. A significance threshold of $p < 0.05$ was used to determine statistically significant results.

RESULTS

Comparative analysis of gene expression patterns in MuSCs during skeletal muscle development and aging in human and mouse

In this study, we used scRNA-seq data from the NCBI Gene Expression Omnibus to investigate skeletal muscle development and aging in humans and mice. Our dataset included 22 time points for humans (embryonic weeks 5-18 to postnatal years 7-81) and 13 for mice (embryonic days 10.5-15 and postnatal months 1-30). Using cell type annotations from the literature and marker gene expression patterns^{10,34}, we identified a total of 10,374 human and 9,887 mouse cells that were putatively involved in myogenesis (Figure 1A). During early embryonic development, PAX3+ myogenic progenitors (MPs) were predominant, transitioning to MuSCs and differentiating myoblasts-myocytes (MBs-MCs) as development progressed (Figure 1B). Cell cycle phase analysis showed MPs were primarily in the G2M and S phases, indicating active growth, while most MBs-MCs were in the G1 phase, indicating differentiation (Figures 1C & Figure S2).

Notably, our investigation revealed the existence of two discernible populations of MuSCs within the developing human muscle: MuSC1, from embryonic weeks 6-9, and MuSC2, from fetal weeks 12-18 (Figure 1D). Differential gene expression analysis revealed MuSC1 had high levels of embryonic genes like neuronatin (NNAT) and gamma globin genes (HBG1/2), while MuSC2 expressed Delta-like non-canonical Notch ligand 1 (DLK1),³⁵ dystrophin (DMD), and the extracellular matrix gene matrilin 2 (MATN2)³⁶ (Figures 1E-F & Table S4-S5). Similar expression patterns of DLK1 and NNAT were observed in murine MuSCs, suggesting these genes as conserved markers for distinguishing embryonic and fetal MuSC stages (Figure 1G). CDH15 was identified as a marker for distinguishing embryonic and fetal stages in humans but not in murine models due to a lack of significant expression differences (Figure 1H).

Consistent with previous findings, our study also identified two distinct clusters of MuSCs in the aging muscles of both humans and mice. By comparing gene expression profiles between these clusters, we identified 243 differentially expressed genes (DEGs) shared by both species (Figure 1I). Functional analysis indicated these DEGs are enriched in pathways related to skeletal muscle abnormalities and muscle dystrophy (Figure 1J). KEGG analysis highlighted their involvement in muscle development and immune response-regulating signaling pathways (Figure 1K).

Conserved Coordinates in Human-Mouse MuSCs

To investigate the similarities and differences in muscle stem cells (MuSCs) between humans and mice, we conducted an integrative analysis using LIGER to reduce batch effects across individuals, time points, and species (Figure 2A). We categorized the clusters identified by tSNE into six developmental stages: embryonic, fetal, postnatal juvenile, adult, middle-aged, and geriatric based on human age. DEG analysis and functional enrichment

revealed distinct gene expression patterns across these stages. Embryonic and fetal MuSCs showed high levels of cell cycle and oxidative phosphorylation genes, juvenile MuSCs activated genes related to muscle growth and cAMP signaling, and adult MuSCs upregulated genes involved in cell survival, death, and differentiation. Middle-aged and aged MuSCs exhibited high expression of aging-related genes (Figure 2B).

We identified both shared and species-specific latent factors (metagenes) contributing to MuSC activity (Figure S3). Embryonic MuSCs were dominated by the Paired-like homeodomain transcription factor 2 (PITX2)³⁷ and the SRY-related HMG-box 4 (SOX4),³⁸ while fetal MuSCs were characterized by insulin-like growth factor 2 (IGF2).³⁹ Middle-aged and aged MuSCs expressed transcription factor JUNB,⁴⁰ and natriuretic peptide C (NPPC)⁴¹ (Figure 2C).

Given the use of mice as models for human studies, cross-species comparisons of MuSC transcriptomes help in selecting appropriate mouse models. Pseudo-time analysis revealed that MuSCs from different developmental stages form distinct clusters (Figures 2D & Figure S4). Correlation analysis of orthologous gene expression established a developmental coordinate for MuSCs conserved between humans and mice (Figures 2E-F).

Temporal dynamics of gene expression patterns and developmental correlations revealed in skeletal muscle regeneration

Mammalian skeletal muscle has a unique regenerative capacity, primarily driven by MuSCs. However, this regenerative potential decreases with age, along with a decline in MuSC activity. To explore this phenomenon, we used the single-cell coordinated gene association in pattern sets (scCoGAPS) algorithm to identify gene patterns specific to MuSCs at various days post-injury (DPI) (Figure S5). Specifically, patterns 67, 23, 62, 50, and 69 were linked to non-injured MuSCs. Patterns 59, 47, 22, 76, and 55 were specific to 0.5 DPI, a period marked by increased inflammation in muscle tissue. Patterns 5, 8, 9, and 25 were specific to 10 DPI, a stage of substantial myofiber regeneration. Patterns 3, 35, and 72 corresponded to 21 DPI, indicating near-complete regeneration and muscle function recovery (Figure 3A). Gene Ontology (GO) analysis revealed common terms like "Negative regulation of apoptotic process" and specific terms such as "MuSCs maintenance involved in skeletal muscle regeneration" for patterns 56 and 63 (Figure 3B). Notably, the top 20 genes in pattern 81 showed high expression at 0.5 and 2 DPI (Figure 3C), including the long non-coding RNA Malat1, known to regulate muscle fiber development, maturation, apoptosis, and inflammatory response.⁴²

Recognizing that tissue regeneration shares features with embryonic development, we projected all regeneration-associated patterns onto developmental MuSCs in humans and mice. This analysis estimated the correlation of each pattern to MuSCs at various developmental stages in both species (Figure 3D). Using dynamic time warping (DTW), we found that 72% of the patterns had more than 0.5 similarity between human and mouse, indicating conservation (Figure 3E).

To further understand changes in gene activity patterns during regeneration with aging, we projected these patterns onto aging MuSCs in humans (Figure S6). Hierarchical clustering of patterns using pairwise DTW distances classified them into five distinct modules (Figure 3F & Figure S7). Module 2, which included 39 patterns, showed an increasing trend with age, with notable patterns like 81, 9, and 79 associated with inflammatory response and apoptotic processes during regeneration. In contrast, module 3, containing 11 patterns, displayed a decreasing trend with age, featuring patterns like 62, 48, and 27, which were linked to myoblast fusion during regeneration (Figure 3G).

Conserved regulons governing mammalian muscle aging

We conducted further investigations into the genetic network underlying the aging of mammalian muscle. Employing single-cell regulatory network inference and clustering (SCENIC) methodology, we meticulously extracted transcription factor (TF) regulons using aging MuSCs sourced from both human and murine subjects, spanning the adult to geriatric stages. Through this analysis, we identified a total of 192 orthologous TF regulons, which were categorized into 13 major modules representing the aging process in mammalian MuSCs (Figure 4A). For each module, we identified several representative regulators and characterized their functions based on

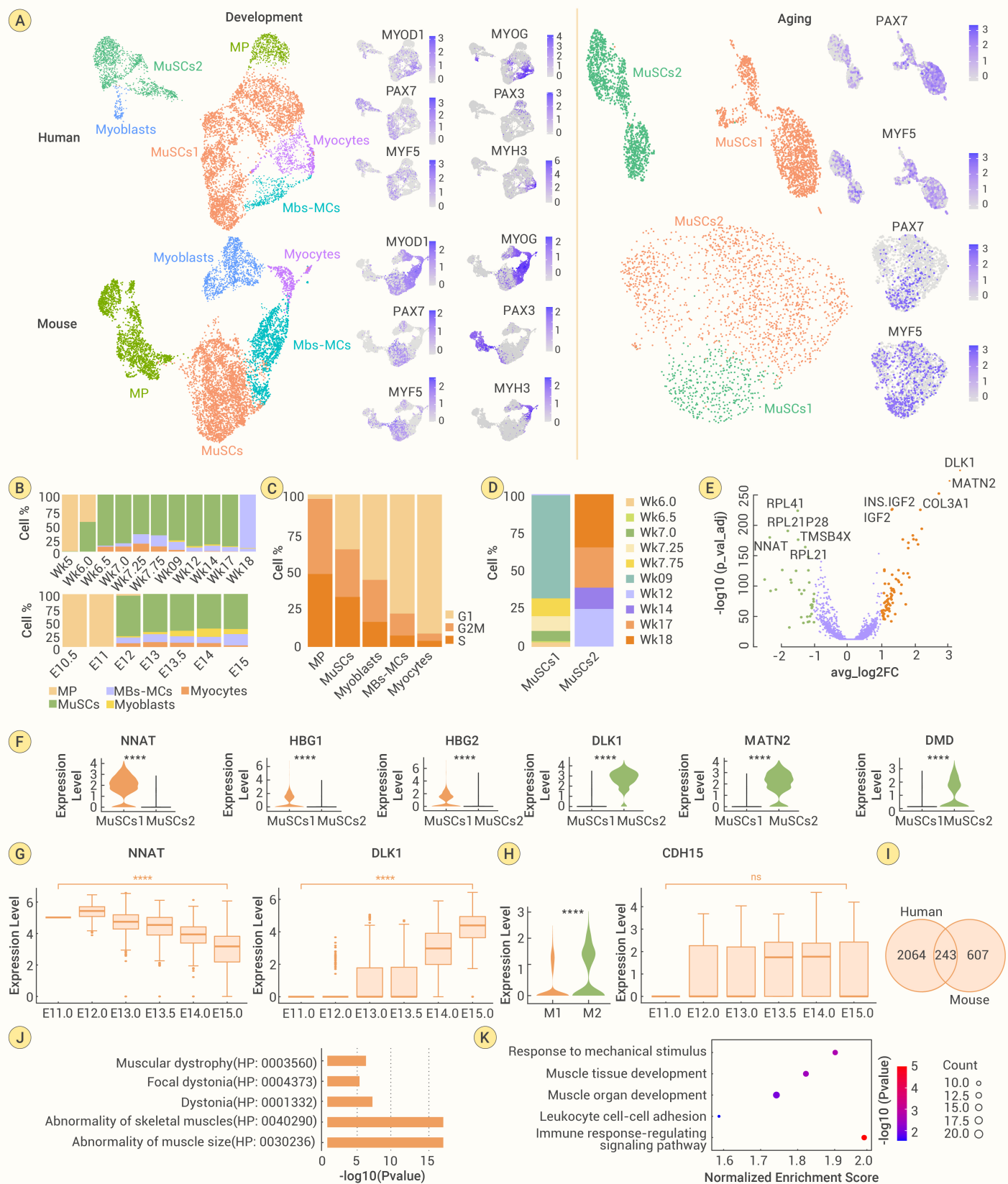


Figure 1. Identification and comparative analysis of MuSCs in the human and murine skeletal muscle (A) UMAP visualization and marker-based annotation of Cells putatively involved in myogenesis during development and aging. Genes indicative of myogenic progenitors (MP) (PAX3+, PAX7-), muscle stem cells (MuSCs) (PAX7+, MYF5+), myoblasts-myocytes (MBs-MCs) (MYOG+, MYOD+) and myocytes (MYH3+, MYOG+) are colored by log-normalized transcript counts. (B) Cell type proportions as annotated in (A) across the developmental stages. (C) Proportion of cell cycle phases in each cell type. (D) Bar plots illustrating the distribution of developmental stages within two distinct subpopulations of human MuSCs. (E) Volcano plot showing DEGs between "MuSC1" and "MuSC2" subpopulations. (F) Violin plots depict the normalized expression values of selected DEGs in human MuSC subpopulations labeled as "MuSC1" and "MuSC2" during development. (G) Box plots illustrate the dynamic expression profiles of NNAT and DLK1 genes in murine MuSCs throughout the developmental stages. (H) Normalized expression values of CDH15 in human developmental MuSC subpopulations "MuSC1" (M1) and "MuSC2" (M2) (Left panel) and in murine MuSCs across various developmental stages (right panel). (I) Venn diagram showing the overlap of DEGs between human and mouse. Enriched phenotype ontologies (J) and KEGG analysis (K) in DEGs between two distinct clusters of MuSCs in the aging muscles of both humans and mice.

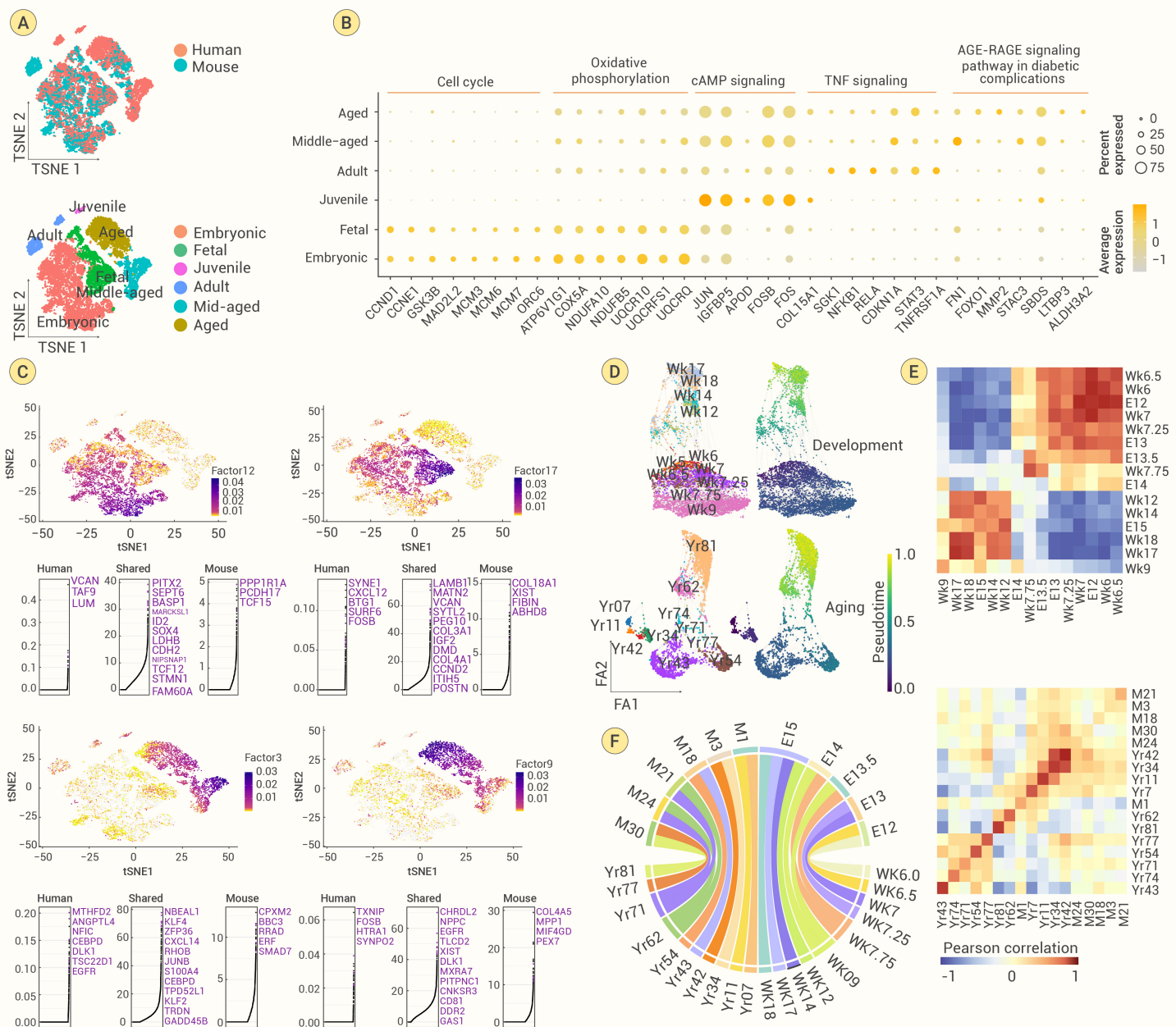


Figure 2. Comparison analysis of MuSCs during development and aging in human and mouse (A) t-SNE visualization of MuSCs analyzed by LIGER, colored by species (up panel) and stages (down panel). (B) Bubble plot showing expression of stage-specific genes. Rows represent genes, while columns represent the stage phases. The size of each circle is proportional to the percentage of cells expressing the gene, and its intensity depicts the normalized average transcript count within expressing cells. (C) Cell factor loading values (top) and gene loading plots (bottom) of top loading species-specific and shared genes (bottom) for factors corresponding to the stage phase. (D) The development trajectory of human and mouse MuSCs based on pseudotime analysis using Scanpy. (E) Heatmap of the correlation coefficients among MuSCs during development and aging. (F) Schematic diagram showing developmental coordinate of the MuSC of human and mouse.

conserved transcription factors and their binding motifs. In our subsequent investigation, we identified a set of regulons that exhibited phase-specific activity patterns in both human and mouse (Figure 4B). Remarkably, our study unveiled two conserved regulons, namely IRF1 and JUND, which displayed dominant activity in the MuSC cell clusters during the geriatric phase across both human and mouse (Figure 4C). JUND, a component of the AP-1 (activator protein-1) family of transcription factors, emerged as a crucial defender against oxidative stress.⁴³ Conversely, the IRF1 gene (Interferon Regulatory Factor 1) has been implicated in the regulation of immune responses, inflammation, and cellular senescence.⁴⁴ The heightened activity of JUND and IRF1 regulons likely represents a response to the oxidative stress and inflammaging typically observed during the aging process.

Conserved role of USF2 as a key driver for myogenesis during muscle development and aging

To further investigate important regulons underlying muscle cell regeneration, development and aging in both mice and humans, we conducted a rigorous regulon identification analysis. This analysis unearthed a distinct set of regulons that exhibit activation during the muscle cell regeneration process and throughout muscle cell development and aging in both human and mouse models (Figure S8). Notably, we identified 30 stage-specific regulons associated with muscle cell regeneration, representing distinct gene regulatory programs at different stages of the regeneration process (Figure 5A). Furthermore, a comprehensive examination of overlapping regulon sets within regenerative MuSCs and the conserved regulons spanning human and murine MuSCs across developmental and aging stages revealed the presence of 13 regulons exhibiting consistent activity profiles across the spectrum of regeneration, development and aging (Figure 5B). Interestingly, among these conserved regulons, we observed the activation of the USF2 regulon specifically at 3.5-5 DPI, which became deactivated after the adult

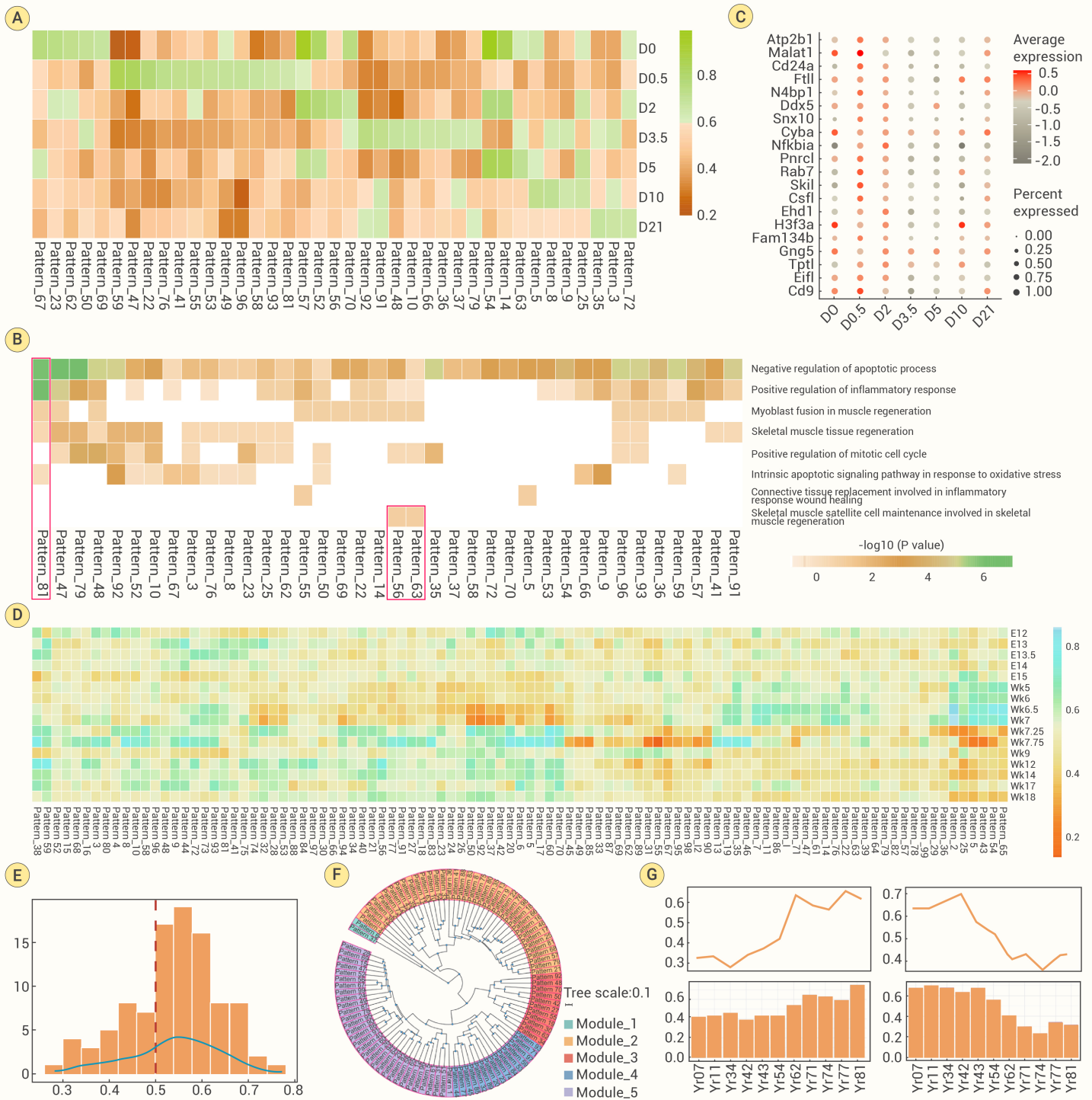


Figure 3. Meta-analysis of MuSCs under regeneration in mammals (A) Specificity of patterns determined by scCoGAPS algorithm in MuSCs of mouse during regeneration. The correlation of stage-specific patterns to the corresponding stage phases was colour-coded. (B) Respective GO of the patterns in Figure 3A. Metascape calculated the statistical significance of each GO term enrichment (p-value) based on the accumulative hypergeometric distribution. The white colour indicated a lack of significance. (C) Bubble plot showing expression levels of the top 20 genes by gene weight of pattern 81. The size of each circle is proportional to the percentage of cells expressing the gene, and its intensity depicts the normalized average transcript count within expressing cells. (D) The patterns of gene expression during regeneration projected onto embryonic MuSCs of human and mouse. The correlation of each pattern to different stages was colour-coded. (E) Density of dynamic time warping (DTW) estimated similarity of the correlation changes during developmental stages between human and mouse. (F) The five distinct modules identified through hierarchical clustering based on the similarity of the gene patterns dynamic changes. (G) Example of gene patterns in module 2 and module 3 that exhibit either increasing or decreasing trends with age, respectively.

stage in both human and mouse (Figure 5C). Consistently, we noted a decrease in USF2 gene expression with aging in both human and murine MuSCs (Figure S9A).

Then, we conducted knockdown (KD) experiments targeting USF2 gene in C2C12 myoblast cells (Figure S10). KD of USF2 altered the expression of several genes (Table S6), with functional enrichment analysis indicating their involvement in muscle tissue development and cell differentiation. Pheno-

type Ontology analysis showed that USF2 KD led to abnormalities in muscle fiber type ratio, impaired muscle contractility, and muscle weakness (Figure 5D). Additionally, KD of USF2 significantly reduced the expression of myog, a key transcriptional activator in muscle differentiation (Figure 5E). These findings underscore USF2's crucial role in maintaining MuSC regenerative capacity, which declines with age, likely through its impact on muscle cell differentiation.

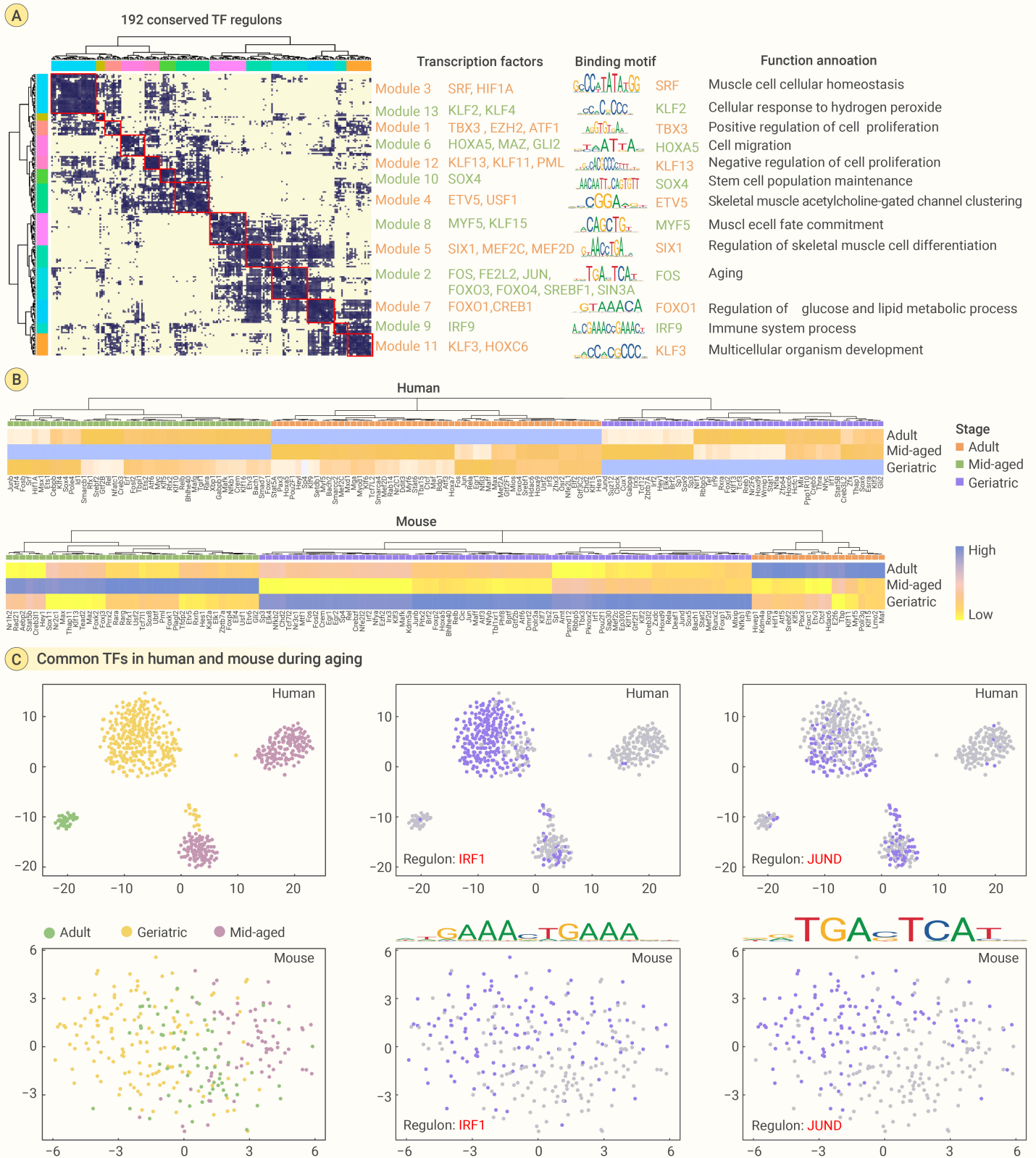


Figure 4. Comparison of aging-related regulons in human and mouse (A) Identification of regulon modules based on the regulon matrix of the human and the mouse. The network shows 192 orthologous TF regulons grouped into 13 major modules, along with representative TF regulons, corresponding binding motifs and functions. (B) Heatmap show stage-specific regulons identified by SCENIC in human and mouse aging MuSCs. The activity values of each regulon at different stage were colour-coded. (C) Binary regulon activity scores (RASS) for regulons IRF1 and JUND in the human and mouse regulon activity t-SNE map; dark blue dots represent one and grey dots represent zero. Other colours are used to mark specific cell types.

In addition, we found a progressive increase in *USF2* expression levels (measured in transcripts per million, TPM) from the proliferative stage to myogenic fusion in C2C12 myoblasts (Figure 5F). In human, *USF2* exhibited high expression in myocytes cells, as marked by the *MYOG*, *MYOD*, and *MYH3* (Figure 5G). Interestingly, protein sequences alignment analysis result showed that the *USF2* protein is highly conserved among mammals (includ-

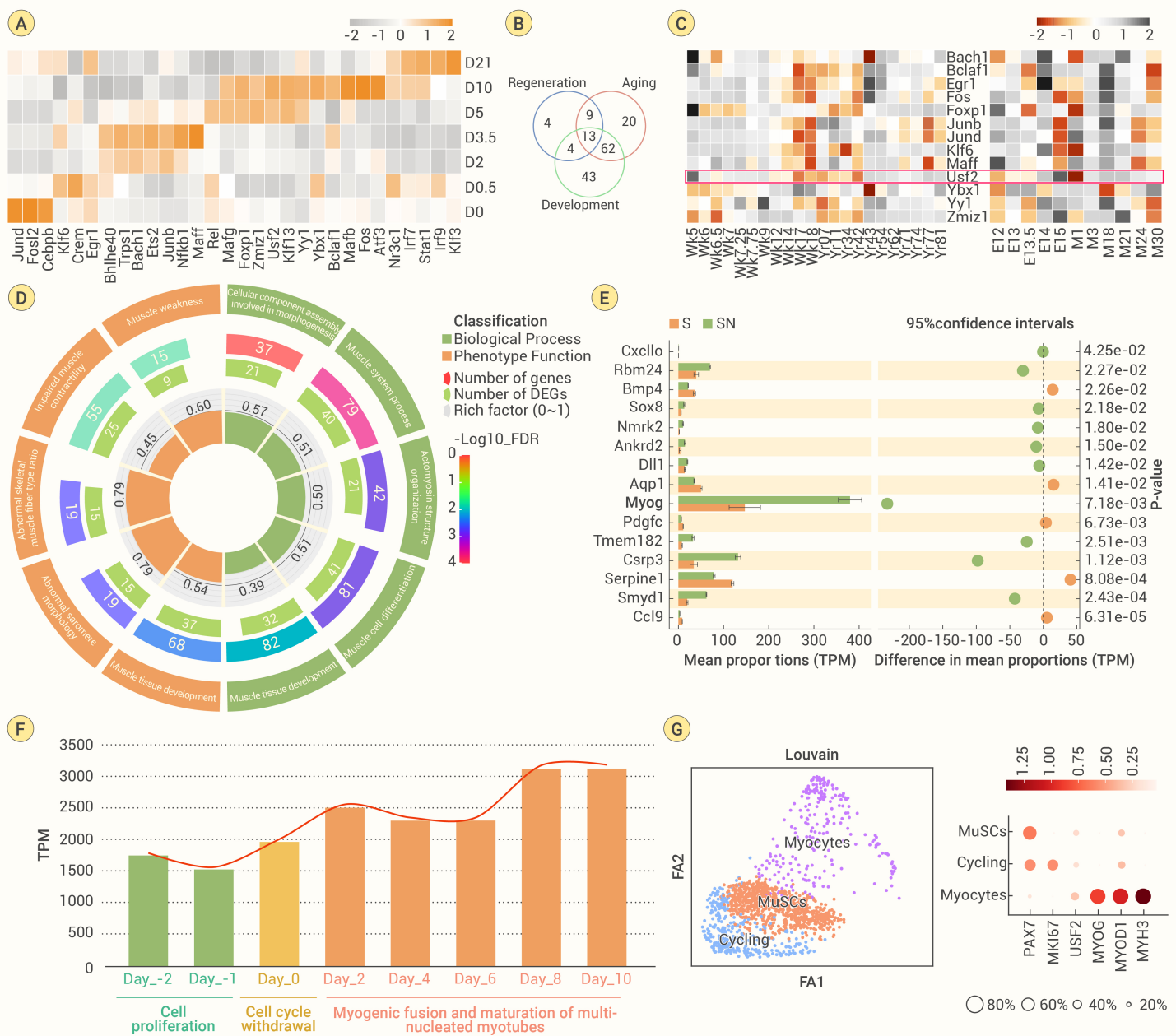


Figure 5. USF2 participates in myogenesis in mammals (A) Specificity of regulons identified in MuSCs of mouse during regeneration. The normalized regulon activity in each stage was colour-coded. (B) Venn diagram showing the overlap between stage-specific regulons identified in mouse MuSCs during regeneration and developmental and aging MuSCs in human and mouse. (C) Dynamic activity changes of 13 regulons in human and mouse developmental and aging MuSCs. (D) Enriched GO and Phenotype Ontology analysis of DEGs between C2C12 myoblast cells transfected with siRNAs for *Usf2* and siRNAs negative control (SN) as control. (E) Top 15 significantly down-regulated gene by KO of *Usf2* gene in C2C12 myoblast cells. (F) Dynamic expression level changes of *Usf2* in C2C12 myoblast cells from proliferation to differentiation. (G) Left panels: Human iPSC cells in the myocyte trajectory, coloured by their expression of selected transcriptional regulators of myogenesis. Right panels: dot plots of selected subtype markers and *USF2*.

ing human, mouse, pig and cattle (Figure S11). Furthermore, we also found a significant increase in *USF2* expression from the proliferative stage to myogenic fusion in both pig and cattle myoblasts (Figure S9B).

USF2 safeguards skeletal muscle from aging and promotes regeneration by promoting myoblast differentiation-mediated myogenesis

To investigate *USF2*'s role in myogenesis, we examined its expression during mouse primary myoblast differentiation, noting an increase (Figure 6A). Overexpression (OE) of *USF2* in myoblasts enhanced cell fusion and multinuclear myotube formation, along with increased MYHC levels (Figures 6B-E & Figures S11A-B). Conversely, knockdown (KD) of *USF2* reduced myoblast differentiation and MYHC levels (Figures S12C-H). In a CTX-induced muscle injury model, *USF2* expression peaked at day 5 of regeneration and declined by day 14 (Figure 6F). Using an AAV vector to deliver siRNA targeting *USF2* (AAV-siUSF2), we reduced *USF2* protein levels

by 74% in the tibialis anterior muscle (Figure S12I). Muscles treated with AAV-siUSF2 showed degenerating myofibers, fibrosis, and inflammation at day 5 post-injury, while control muscles had centrally localized nuclei (Figure 6G). Additionally, AAV-siUSF2 treatment resulted in fewer and smaller regenerated myofibers marked by eMYHC, along with decreased eMYHC protein levels (Figures 6H-I). These findings demonstrate that *USF2* is crucial for skeletal muscle regeneration in mice.

To explore *USF2*'s impact on skeletal muscle aging, we used siRNA to knock down *USF2* in mouse myotubes, leading to accelerated aging, reduced myotube diameter, and increased senescence-associated beta-galactosidase activity (Figures 6J-K). These results suggest that *USF2* is essential for maintaining muscle integrity during aging.

DISCUSSION

Even though recent work has profiled skeletal muscle using scRNA-

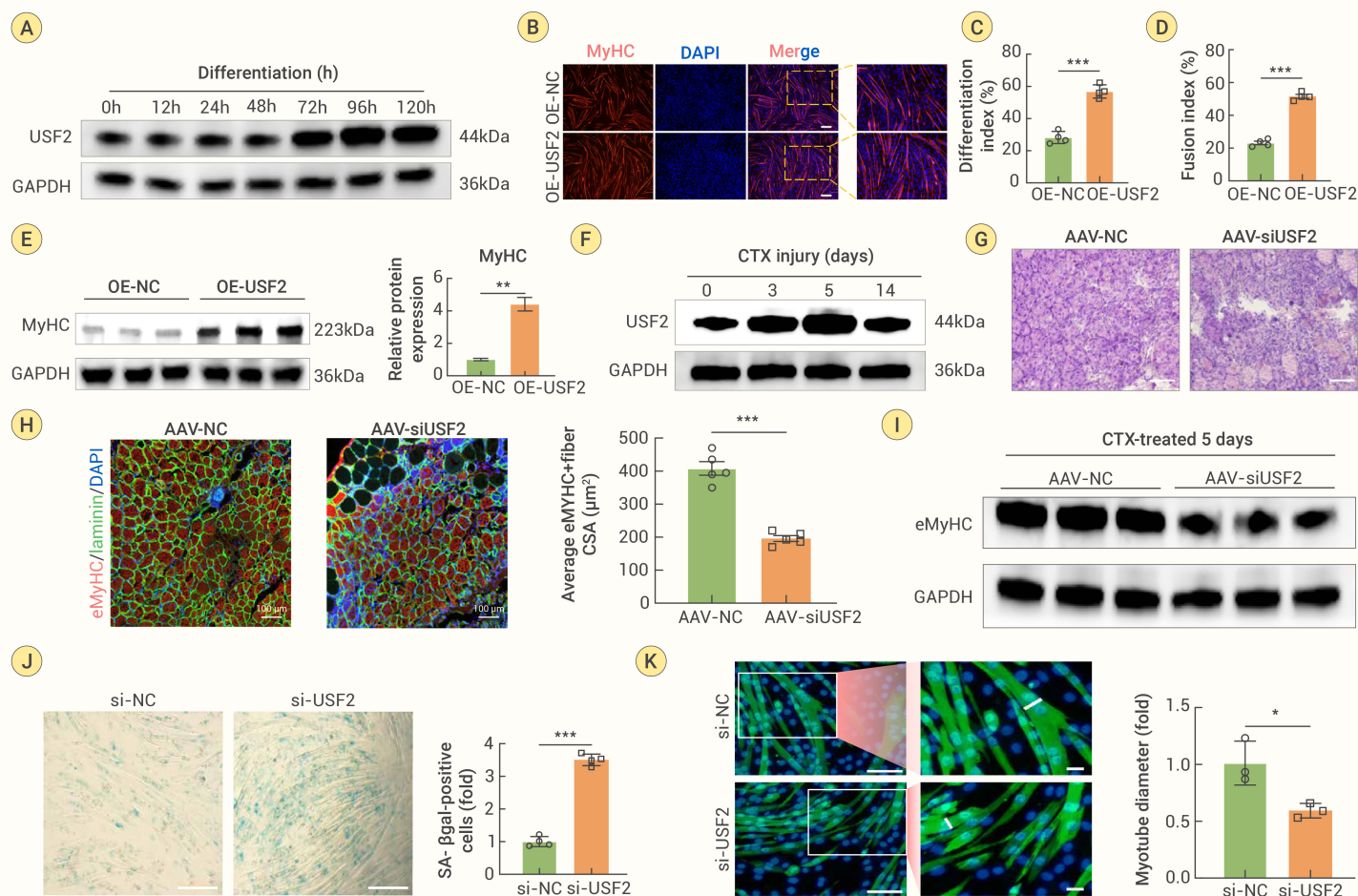


Figure 6. USF2 enhances muscle regeneration and protects mouse myotubes from senescence by promoting myoblast differentiation (A) Immunoblotting analysis depicted USF2 expression changes in mouse primary myoblast cells during differentiation. (B) Immunofluorescence staining revealed MyHC levels in control and USF2-overexpressed cells post-differentiation; Differentiation index (C) and fusion index (D) were calculated. Scale bars: 200 μ m. (E) Immunoblotting analysis of MyHC levels in OE-NC or OE-USF2 mouse primary myoblast cells at 4 days post differentiation, with relative protein expression levels illustrated in the bar chart. (F) Immunoblotting analysis of USF2 in TA muscles of WT mice at 0, 3, 5, and 14 days postinjury (n = 3 mice per group). (G) Hematoxylin and eosin staining of eMYHC+ fibers in TA muscles at 5 days postinjury in scramble siRNA or siRNA-USF2 treated mice, with a scale bar of 200 μ m. (H) Immunofluorescence staining of eMYHC+ fibers in TA muscles of mice treated with either control (AAV-siRNA-NC) or USF2 knockdown (AAV-siRNA-USF2) at 5 days post-injury, with average cross-sectional area quantified (n = 5 mice per group). (I) Immunoblotting analysis of myogenic markers (eMYHC) in AAV-siRNA-NC or AAV-siRNA-USF2 treated TA muscle (n = 3 mice per group) at 5 days postinjury. (J) SA- β -gal staining of mouse primary myotubes transfected with siRNA-NC or siRNA-USF2, with quantification of SA- β -gal-positive cells presented as fold changes (siRNA-USF2 vs. siRNA-NC) (n = 3 biological replicates). (K) Immunofluorescence staining of MYHC in mouse primary myotubes transfected with siRNA-NC or siRNA-USF2, with quantification of myotube diameters presented as fold changes (siRNA-USF2 vs. siRNA-NC) (n = 3 biological replicates). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (statistical significance).

seq,^{11-13,45-48} but our focus was on transcriptional changes in MuSCs during development and aging across species. We created a conserved single-cell landscape of MuSCs in mammals, establishing a developmental coordinate between human and mouse MuSCs. Given the widespread use of mice as mammalian models for studying human diseases and health,⁴⁹⁻⁵² this work provides insights into shared muscle biology and helps in selecting suitable mouse models for studying human muscle development, regeneration, and aging.

Understanding gene regulatory networks in regeneration can lead to targeted reactivation, offering potential treatments for injuries and degenerative diseases.⁵³ Skeletal muscle is ideal for studying regeneration mechanisms and enhancing stem-cell-based therapies. While scRNA-seq studies on muscle repair have identified distinct cell populations involved in regeneration,^{12,47,54,55} comprehensive evaluation of MuSC activity during regeneration has been limited. Using the scCoGAPS algorithm, we identified gene patterns specific to MuSCs at different regeneration stages and confirmed their conservation during development and aging with the DTW algorithm. This highlights key genes and pathways involved in inflammation, myofiber regeneration, and recovery, presenting targets for enhancing muscle regeneration in various contexts.

Age-related muscle dysfunction, like atrophy and sarcopenia, diminishes life quality and increases morbidity and mortality. This is largely due to the decreased ability of MuSCs to generate new muscle cells. Identifying

conserved regulons and transcription factors in aging-related processes in MuSCs is crucial for understanding muscle decline. Key transcription factors such as PITX2, SOX4, IRF1, and JUND play roles in MuSC function during development and aging. The upregulation of JUND and IRF1 during aging suggests their involvement in oxidative stress response and inflammaging, common in age-related muscle dysfunction. These findings offer valuable targets for therapies to mitigate age-related muscle decline and promote healthy aging.

USF2 has emerged as a key player in various cellular processes, including cell cycle regulation,^{56,57} apoptosis,⁵⁸ metabolism,⁵⁹ and response to cellular stress.⁶⁰ The role of USF2 in muscle biology has been unclear, but our findings reveal its dynamic activity, with heightened levels during early muscle regeneration (3.5-5 days post-injury) and deactivation in aging. The downregulation of USF2 in aging MuSCs suggests a link to the age-related decline in MuSC activity. Our study provides strong evidence that USF2 is crucial for myogenesis, muscle regeneration, and protecting against muscle aging by facilitating skeletal muscle differentiation. These insights position USF2 as a key player in muscle regeneration and aging, offering potential targets for therapeutic interventions in muscle-related diseases.

Actually, future investigations incorporating other omics data, such as epigenomic and proteomic profiles, could provide a more comprehensive understanding of the regulatory mechanisms underlying MuSC behavior. Furthermore, additional studies are warranted to explore other transcription factors

and signaling pathways that may contribute to muscle development and aging.

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AUTHOR CONTRIBUTIONS

Z.L.T and Z.S.W conceived and designed the experiments. Z.S.W contributed to the

study design, collected data, conducted bioinformatic analysis, summarized the results and wrote the manuscript. W.C.L performed regulons identification related bioinformatic analysis. W.W, W.W.L and Y.L.Y conducted the molecular, cellular, and animal experiments.

DECLARATION OF INTERESTS

The authors declare no competing interests.

ETHICAL STATEMENT AND PATIENT CONSENT

This study was approved by the life sciences ethics committee of institute of agricultural genomics, Chinese Academy of Agricultural Sciences (AGIS-ER-2021-023).

DATA AND CODE AVAILABILITY

Previously published single-cell RNA-seq data that were reanalysed here are available in the GEO or ArrayExpress under the accession codes "GSE147457" (human developmental skeletal muscle cells), "GSE143704" (human aging skeletal muscle cells), "GSE149451" (developmental trajectory of human induced pluripotent stem cell (iPSC) derived myogenic precursors), "GSE109774" (mouse developmental skeletal muscle cells), "GSE149590" (mouse aging skeletal muscle cells), "GSE138826" (mouse skeletal muscle cells during regeneration process). Expression level changes of USF2 during C2C12 myoblasts induced to differentiate was obtained under the accession numbers GSE989, during bovine skeletal myoblast myogenic differentiation was obtained under the accession numbers GSE173477, and during porcine skeletal muscle satellite cells differentiation was obtained under the accession numbers GSE112527.

SUPPLEMENTAL INFORMATION

It can be found online at <https://doi.org/10.59717/j.xinn-life.2024.100075>