



Superenhancer-mediated ferroptosis in age-related hearing loss: cochlear epigenomics

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Abstract

Background Age-related hearing loss (ARHL), also known as presbycusis, is a prevalent condition among older adults and affects a substantial proportion of the global aging population. The underlying mechanisms of ARHL remain unclear, and this study aimed to explore the role of superenhancers (SEs) and the transcription factor Sp1 in regulating hair cell (HC) aging and ferroptosis, a form of regulated cell death associated with iron metabolism.

Methods We utilized a combination of bioinformatics analysis, including transcriptional regulatory element enrichment analysis (TREA) and SE prediction, with SEdb 2.0 to identify key transcriptional regulators and their target genes. Experimental validation was performed using auditory brainstem response (ABR) measurements, immunofluorescence staining, Western blot analysis and quantitative real-time PCR (RT-qPCR) in mouse and cell models. Additionally, we employed CUT&Tag assays to map Sp1 binding sites and performed statistical analyses using SPSS Statistics 25 and GraphPad Prism.

Results Our study revealed that reduced binding of Sp1 to the Fth1 superenhancer triggered HC ferroptosis and the progression of ARHL. We identified Sp1 as a key upstream transcriptional regulator whose binding to the Fth1 SE decreased with aging, leading to reduced Fth1 gene transcription and increased intracellular iron levels. This phenomenon resulted in cellular iron overload, subsequent ferroptosis, and increased reactive oxygen species (ROS) levels, ultimately promoting HC and cochlear aging. In vivo studies with the SE inhibitor JQ-1 confirmed the importance of SE activity in maintaining auditory function.

Conclusions This study provides evidence for the role of Sp1 and Fth1 in the regulation of HC aging and ARHL. These findings suggest that manipulating SE sites and inhibiting ferroptosis may offer novel therapeutic strategies for treating ARHL. Understanding the interplay between SEs, Sp1, Fth1 and ferroptosis reveals novel targets for AAV gene therapy to preserve hearing in aging populations by modulating iron homeostasis during sensory cell senescence.

Keywords Presbycusis · Hair cell degeneration · Sp1 transcription factor · FTH1 gene regulation · Iron homeostasis · Chromatin accessibility · AAV gene therapy · Programmed cell death

Chanyuan Zhang and Ting Yang contributed equally to this work and should be considered as co-first authors.

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Introduction

Age-related hearing loss (ARHL, or presbycusis) affects most older adults, with the prevalence increasing significantly with age [1, 2]. It affects the majority of people as they grow older, with approximately one-third of those aged over 65 years experiencing some degree of hearing impairment, as reported by the World Health Organization. The global aging population has resulted in more than 500 million people suffering from ARHL worldwide [3]. This condition significantly impacts daily communication for older individuals and has been linked to an increased risk of cognitive impairment and dementia. ARHL is characterized by a progressive decline in auditory function associated with aging. Pathologically, this condition is primarily attributed to the loss of hair cells (HCs) and spiral ganglion cells (SGCs) in the cochlea [4]. However, the precise mechanisms underlying ARHL remain largely unclear.

In 2022, Joshua E. Burda and colleagues studied the heterogeneity of differentially expressed genes (DEGs) related to reactive astrocytes across various diseases and identified disruptions in the transcriptional regulators (TRs) of these cells among different diseases [5]. Their investigation of these variations revealed that gene expression diversity results from specific interactions between upstream and downstream TRs, with changes in DEGs requiring multiple critical TR interactions. To identify candidate TRs involved in astrocyte reactivity, they initially used rigorous multi-step upstream transcriptional regulator enrichment analysis (TREA). This method integrates computational and biologically derived data on regulator–target gene interactions from resource databases to predict potential TRs on the basis of DEG profiles. Our study utilized TREA for transcriptomic data obtained from our previous research [6], integrating it with motif analysis (Fig. S1A) performed previously, which pinpointed specificity protein 1 (Sp1) and Klf4 as pivotal upstream TRs driving transcriptomic changes (Fig. 1a). Notably, Sp1, ranked second in binding frequency to motifs in ATAC data, was predicted by an online prediction tool to predominantly regulate downstream genes in cochlear tissues via superenhancers (SEs) (Fig. 1B). Consequently, through comparisons with previously predicted candidate target genes controlled by senescence-associated superenhancers (SASEs), we predicted ferritin heavy chain 1 (Fth1) as the Sp1 downstream gene.

The transcription factor Sp1 is a sequence-specific binding protein that regulates the transcription of nuclear viral genes enriched in GC/GT sequences and participates in various physiological and pathological processes [7]. Its association with aging is well established, with studies indicating its involvement in DNA repair processes. Sp1 levels decrease with age, resulting in reduced activity in

aging animal tissues and senescent cells [8, 9], suggesting a regulatory role in replicative senescence due to accumulated oxidative stress and diminished antioxidant responses [10]. Recent research has indicated that decreased Sp1 expression is correlated with an increased expression of markers of aging, which is likely due to DNA damage, and the loss or mutation of Sp1 results in premature degradation mediated by ataxia telangiectasia mutated kinase (ATM) and increased expression of aging markers [11]. Previous studies have demonstrated that p16 induces G1 cell cycle arrest and mediates aging through the retinoblastoma pathway, with the transcription factors CTCF and Sp1 and members of the Ets family activating p16 transcription [12]. Therefore, although Sp1 is closely linked to aging, its regulatory outcomes in aging vary across tissues and cells, necessitating further exploration into its role, mechanisms, and consequences in cochlear aging.

Fth1 is a major iron storage protein in the body, forming complexes with the ferritin light chain (Ftl) to efficiently bind and store free iron in nontoxic forms within cells, releasing it when needed for cellular and tissue functions [13]. The regulation of iron storage and release processes is crucial for maintaining iron balance and metabolic homeostasis and contributes to understanding iron metabolism, cellular stress responses, and related disease research and treatments. Fth1 is a known marker of ferroptosis, and its downregulation often indicates the occurrence of ferroptosis [14].

Ferroptosis, identified in 2012 [15], is characterized by excessive iron accumulation leading to elevated ROS levels, which trigger iron-dependent regulated necrosis associated with diseases such as cardiovascular diseases, neurodegenerative disorders, and cancer [16]. This pathway involves oxidative-reduction mechanisms related to the Fenton reaction [17], with H_2O_2 catalyzing iron (II/III)-mediated oxidation reactions that induce lipid peroxidation and membrane damage [18], particularly affecting membranes enriched in polyunsaturated fatty acids (PUFAs) or enclosed organelles [19]. The scientists who proposed the concept of ferroptosis have once again published a comprehensive review summarizing recent research findings on ferroptosis, which involve a wide range of biological contexts, including development, aging, immunity, and cancer [20]. In the context of ARHL, the interplay between aging and ferroptosis and the regulatory role of Sp1 remain unclear, highlighting the need for further research to elucidate the roles of these biological processes in aging and disease.

HEI-OC1 cells retain key characteristics resembling those of HCs and have been widely used in previous studies to explore the protective mechanisms of HCs [21, 22]. Therefore, in this study, we used D-galactose (D-gal)-treated HEI-OC1 cells to elucidate the involvement and mechanisms of

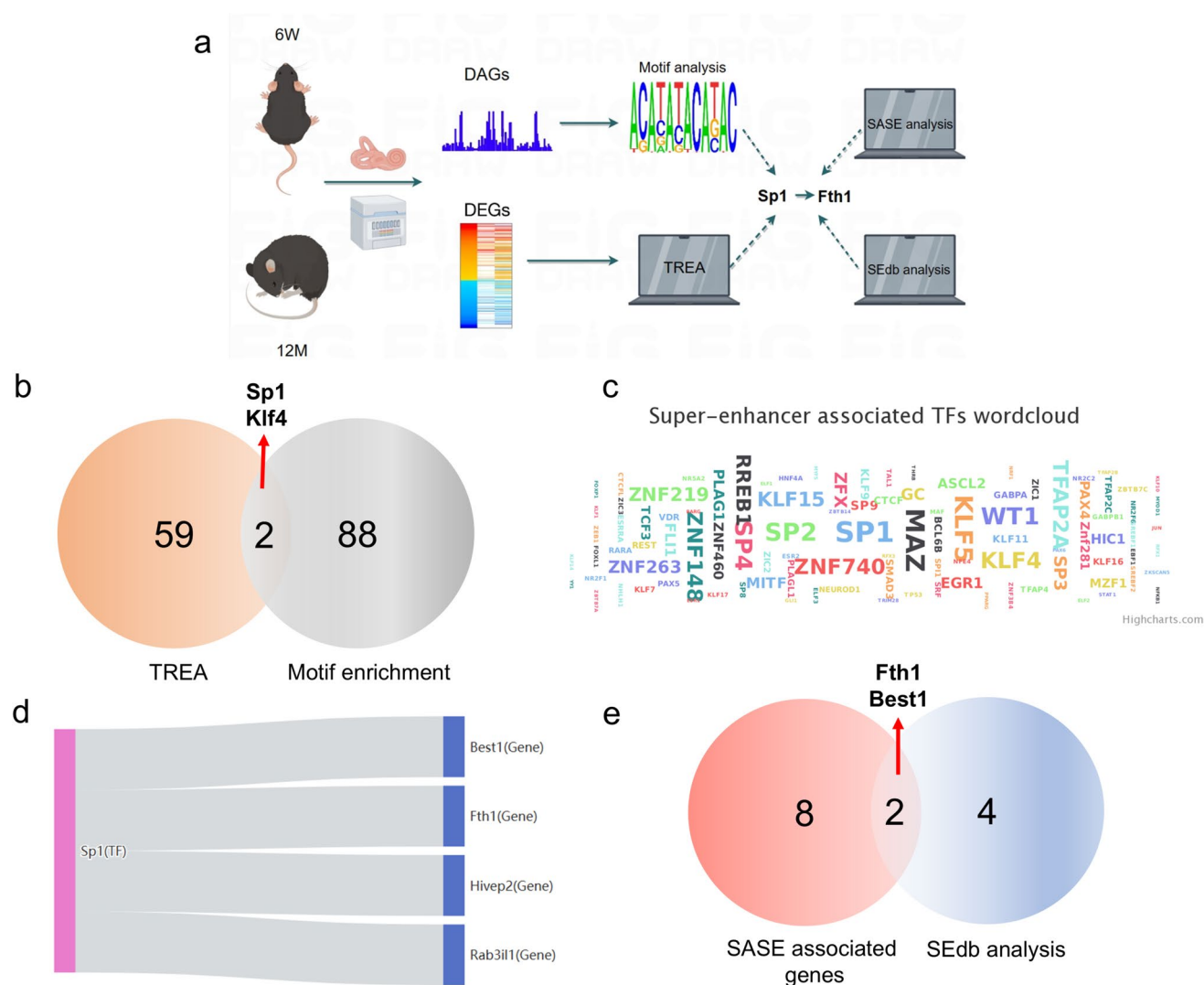


Fig. 1 TREA and SEDb 2.0 coanalysis identifies Sp1 as a key TF that regulates cochlear HC aging via SASEs a) TRs and their target gene prediction schematic. b) TREA analysis and Motif analysis show that Sp1 and Klf4 are core TRs. c) Superenhancer associated TFs word

cloud in cochlea tissue (size of word indicates its frequency or probability of occurrence). d-e) Sp1 downstream gene prediction influenced by super enhancers

Sp1 in the senescence process of HCs, aiming to explore the relationships among aging, ferroptosis, and the potential mechanistic links involving Sp1 and whether intervening in these processes can mitigate ARHL.

Materials and methods

Auditory brainstem response (ABR) measurements

The ABR test was performed with the TDT BioRigRZ system (Tucker-Davis Technologies, USA), as directed by the manufacturer. After the application of anesthesia, subcutaneous needle-like electrodes were inserted under the skin of the mice at specific locations. The mice that were

anesthetized were kept warm throughout the ABR recordings. To evoke auditory brainstem response (ABR) potentials, tone pips with a duration of 5 ms were administered to the eardrum at different frequencies (4 kHz, 8 kHz, 16 kHz, 24 kHz, and 32 kHz). The minimal stimulus levels producing reproducible responses for ABR wave II in various animals were determined as the ABR thresholds. All of the above frequencies were measured.

Animals

The C57BL/6 J mice used in this research were 6 weeks old (6W, $n=10$), 9 months old (9 M, $n=40$), 10 months old (10 M, $n=20$) and 12 months old (12 M, $n=10$). After the ABR tests were performed, the 9 M mice were randomly

divided into two groups. There was no significant difference between the hearing thresholds of the 2 groups. One group of mice was intraperitoneally injected with JQ-1 (30 mg/kg/day, 5% DMSO + 40% PEG300 + 5% Tween 80 + 50% saline; Selleck, USA) for a period of 2 months (from 9–11 months of age), whereas the other group was injected with the same amount of saline as the control. The mice were dosed once a day (qd) during the first 30 days and once every other day (qon) during the second 30 days. The 10 M mice were kept under the same conditions. The 12 M mice were established as an ARHL animal model on the basis of published studies [23, 24] and our previous research [6]. The ABR test was performed on Day 61. The experiments were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of the People's Republic of China, Policy No. 2006398). The experiments involving animals were approved by the Chongqing Medical University Animal Welfare Committee.

Bioinformatics analysis

Transcriptional regulatory element enrichment analysis (TREA)

To identify TRs involved in cochlear aging, this study utilized the method provided by Joshua E. Burda et al. [5] to analyze RNA-seq data. TREA analysis integrates various resource databases, including ChEA, JASPAR and TRANSFAC, to detect multiple forms of gene regulatory interactions targeted by TRs. These interactions encompass experimental findings involving chromatin immunoprecipitation and genetic functional studies, as well as validated DNA binding motif analyses. Consequently, TR-targeted gene interactions identified by TREA include both direct TR-DNA binding mechanisms and indirect forms of gene expression regulation (where TRs may influence downstream target gene expression via different mediators, including chromatin modification and other forms of epigenetic regulation). These resource databases are used to study gene expression datasets, which are enriched for approximately 1350 downstream target genes of TRs. The TREA GitHub repository (<https://github.com/burdalab/TREA>) provides custom Python scripts, protocols, example gene expression datasets, and output files from TREA resource databases.

Transcription factors (TFs) that bind superenhancers (SEs) and prediction of their target genes in the cochlea

SEdb 2.0 (<http://www.licpathway.net/sedb>) is a comprehensive database of human and mouse superenhancers that offers abundant resources on superenhancers in both species. This database annotates the potential functions of

superenhancers in TF gene regulation, along with detailed genetic and epigenetic annotation information about superenhancers. Through its interface for searching for superenhancers on the basis of tissue categories, superenhancers, associated TFs, and target genes could be queried for all samples of a specific tissue type (cochlea).

Whole-mount staining

The cochleae were fixed with 4% PFA at 4 °C overnight and decalcified with 10% EDTA for 72 h at 4 °C. The samples were then divided into basal, middle, and apical segments, exposed to the sensory epithelium, and blocked with a reagent containing 10% normal goat serum in 0.01 M PBS for 30 min. Primary antibodies, including anti-myosin 7a (1:500; Proteus Biosciences, 25–6790), anti-Sp1 (1:200; Novus, USA) and anti-Fth1 (1:500; HUABIO, China) antibodies, were incubated with the samples overnight at 4 °C, followed by incubation with secondary fluorescent antibodies (1:1000) for 1 h at room temperature. Finally, the samples were stained with DAPI (Sigma–Aldrich, USA) and imaged with a Leica SP8 confocal fluorescence microscope (Leica Microsystems). The numbers of HCs and target protein expression in three cochlear turns were analyzed using ImageJ (ImageJ software v1.6.0). At least three samples were examined for each group.

Western blot analysis

The cochleae were isolated carefully, and proteins were extracted with RIPA buffer (Pierce #89,901; Solaribo, Waltham, MA) supplemented with protease and phosphatase inhibitors (Beyotime, Shanghai, China). The protein concentration was measured with a BCA assay (Beyotime, Shanghai, China). Cells (scraped from the plates) and tissue samples were homogenized in buffer, followed by centrifugation at 14,000 g for 10 min at 4 °C. The supernatants were subjected to Western blot analysis by loading the same amount of protein in each line. Proteins were separated by SDS–PAGE, transferred to a PVDF membrane (Millipore, Burlington, MA, USA), and analyzed by immunoblotting with specific antibodies. GAPDH (1:5000; HUABIO, China) was used as an internal control. Protein detection was performed with Super ECL Plus Western Blotting Substrate (BIOGROUND, BG0001, China), and protein intensity was analyzed using ImageJ software. The primary antibodies used in this study were anti-Sp1 (1:2000; Novus, USA) and anti-Fth1 (1:1000; HUABIO, China). The secondary antibodies used included a goat anti-mouse antibody (1:5000; ZSGB-BIO, China) and a goat anti-rabbit antibody (1:1000; CST, China).

Cell culture and treatment

HEI-OC1 cells were cultured in high-glucose DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) at 33 °C in an incubator containing 10% CO₂. The cells were subcultured at 70%–80% confluence with 0.25% trypsin EDTA (Gibco, USA). D-gal (G0750; Sigma, USA) and JQ-1 (HY-13030; MedChemExpress, USA) were applied at concentrations of 30 mg/mL and 1 µM, respectively, to affect the cells.

The cells were pretreated with three types of cell death inhibitors, namely, ferrostatin-1 (Fer-1; Selleck, USA), the autophagy inhibitor 3-methyladenine (3-MA; Selleck, USA), and the necroptosis inhibitor necrostatin-1 (Nec-1; Selleck, USA), before being exposed to the indicated doses of drugs with or without D-gal. The pretreatment methods and working concentrations used were as follows: Fer-1 pretreatment for 2 h at 10 µM (diluted in culture medium after being dissolved in DMSO), 3-MA pretreatment for 6 h at 10 mM (dissolved in culture medium), and Nec-1 pretreatment for 1 h at 10 µM (diluted in culture medium after being dissolved in DMSO).

Cell viability assay

HEI-OC1 cell viability was assessed with a CCK-8 Cell Counting Kit (HY-K0301; MedChemExpress, USA). The cells were cultured in 96-well plates with various concentrations of D-gal for 48 h. The absorbance was measured at 450 nm with a MULTISKAN GO Spectrophotometer (Thermo Scientific, USA) 2 h after the addition of the CCK-8 solution. Viability was normalized to that of the control, and the experiments were repeated at least three times.

ROS detection

The cells were seeded in 96-well plates at 3000 cells/well in 100 µL of media or in 6-well plates at 6×10^6 cells/well in 2 mL. The cells were then exposed to different drugs in DMEM and incubated at 33 °C for 48 h. The cells were loaded with 10 µM DCFH-DA (a fluorescent probe for ROS) (S0033S; Beyotime, China) and assessed using a fluorescence spectrophotometer (Bio-Rad, USA) and FACS analysis (FACScan; BD Biosciences, San Jose, CA, USA) after 30 min of further culture in the dark. The average fluorescence intensity of each group was then standardized against that of the control group.

Mitochondrial membrane potential (MMP) measurement

The estimation of the MMP was conducted through TMRE (Thermo, USA) staining. HEI-OC1 cells were cultured in 96-well plates at a density of 3000 cells per well and

subjected to specific experimental conditions for the indicated duration prior to TMRE staining at a final concentration of 100 nM for 30 min at 37 °C. Imaging was performed with an Olympus BX63 microscope, and the density of fluorescence was detected by a spectrophotometer. The average fluorescence intensity of each group was then standardized against that of the control group.

Fe²⁺ detection measurement

Intracellular Fe²⁺ was detected with FerroOrange (Dojindo, Japan) following the manufacturer's instructions. HEI-OC1 cells and cochleae were exposed to the indicated conditions for the indicated amount of time and subsequently stained with FerroOrange at a concentration of 1 µmol/L for 30 min at 37 °C in the dark. The fluorescence intensity was measured with a fluorescence spectrophotometer. The average fluorescence intensity of each group was then standardized against that of the control group.

Quantitative real-time PCR (RT-qPCR)

Total RNA was isolated from the cells with TRIzol reagent (TaKaRa Biology, Inc., Kusatsu, Shiga, Japan), followed by cDNA synthesis with MIX reverse transcription primers (TaKaRa Biology, Inc.). Real-time quantitative PCR was conducted on a LightCycler 480 instrument with SYBR Green (Roche Diagnostics, Basel, Switzerland). PCRs were conducted with a predenaturation step at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 58.8 °C for 20 s. The relative expression of genes was evaluated via the 2^{−ΔΔCt} method. Two technical replicates were used for each individual experimental replicate. The sequences of the primers used in this study are listed in Table S1.

Immunofluorescence staining

The cochleae were initially fixed in 4% PFA for 24 h at 4 °C, followed by incubation in 8% EDTA at room temperature for 6–8 h. The cochleae were subsequently dehydrated with 10%, 20%, and 30% sucrose solutions at 4 °C for durations of 1 h, 2 h and overnight, respectively. The following day, the cochleae were frozen and sectioned to a thickness of 5 µm. Following rinsing and fixation, the tissue sections were blocked in prehybridization buffer at 37 °C for 1 h and then subjected to overnight incubation with primary antibodies overnight at 4 °C. After being washed with PBS three times the next day, the samples were incubated with secondary fluorescent antibodies for 1 h at 37 °C in the dark and stained with DAPI to label the nuclei. They were then visualized with a Leica SP8 confocal fluorescence microscope.

The primary antibodies used were anti-myosin 7a (1:200; Proteus Biosciences, USA), anti-Sp1 (1:200; Novus, USA) and anti-Fth1 (1:500; HUABIO, China). Each experiment was conducted three times with three turns, as indicated in the figure legends. The fluorescence intensity was measured using ImageJ, and the mean intensity of each group was normalized to that of the control group.

SP1 siRNA transfection

To investigate the effect of Sp1 on ARHL, HEI-OC1 cells were subjected to Sp1 siRNA or negative control (siNC) transfection at a concentration of 100 nM with a riboFECT CP Transfection Kit (RIBOBIO, Guangdong, China), followed by cell harvesting or detection after 48 h. Two sequences of siRNA were designated siRNA-1 and siRNA-2 for initial screening purposes (Table S2).

Sp1 overexpression constructs

Sp1 sequences were inserted into a PCDH-Puro-vector (PCDH-Puro-Sp1) and transfected into 293 T cells along with pMD2.G (5 µg) and pSPAX2 (10 µg) plasmids to produce lentivirus. HEI-OC1 cells were then transduced with lentiviral particles in the presence of polybrene. After 2 days, puromycin was subsequently introduced into the culture medium at a concentration of 3 µg/mL to facilitate the selection of stably transduced cells.

Cleavage under targets and tagmentation (CUT&TAG) assay and data analysis

The cryovial was removed and agitated in a 37 °C water bath to expedite thawing within 1–2 min. Next, the cells were centrifuged for 5 min at 600 × g at room temperature. Cell activity was assessed with a LUNA-FL™ and quantified. The CUT&TAG assay was carried out according to the protocol outlined in a previous study [25], which involved the binding of cells to Concanavalin A-coated magnetic beads, permeabilization of the cell membrane with digitonin, and precise binding of the pA-Tn5 transposase enzyme to the DNA sequence near the target protein under antibody guidance, resulting in factor-targeted tagmentation. The DNA sequence was tagmented, with adapters added simultaneously at both ends, allowing enrichment through PCR to generate sequencing-ready libraries. Following PCR, the libraries were purified with AMPure beads and assessed for quality on an Agilent Bioanalyzer 2100 system. Clustering of the index-coded samples was carried out on a cBot Cluster Generation System with the TruSeq PE Cluster Kit v3-cBot-HS (Illumina) in accordance with the manufacturer's guidelines. The library preparations were subsequently

sequenced on the Illumina NovaSeq platform at Novogene Science and Technology Co., Ltd. (Beijing, China).

Peak calling was conducted with MACS2 (version 2.1.0) with the command 'macs2 -q 0.05 -f AUTO -call-summits-nomodel -shift -100 -extsize 200 -keep-dup all'. The default q value threshold of 0.05 was applied to all the datasets. The proximity of peak summits to transcript start sites of genes can serve as a predictor for protein–gene interaction sites. ChIPseeker was used to identify the genes nearest to the peaks and annotate the genomic regions of the peaks. The association of peak-related genes was confirmed with ChIPseeker [26]. Statistical enrichment analysis of peak-related genes in KEGG pathways was performed with KOBAS software.

Statistical analysis

The data were analyzed by SPSS Statistics 25 software, and GraphPad Prism (version 9.0) was used to perform the statistical analyses. One-way ANOVA, two-way ANOVA, or unpaired Student's t test was performed for comparisons. Values of $P < 0.05$ were considered significant.

Results

TREA and SEdb 2.0 coanalysis identifies Sp1 as a key TF that regulates cochlear HC aging via SASEs

To identify the core TRs that played crucial roles in the process of ARHL, we performed TREA analysis of the DEGs from the RNA-seq data (Table 1, Table S3). By integrating the top motifs obtained from motif analysis of previous research data with the TRs obtained from TREA analysis (Table S4), we discovered that Sp1 and Klf4 were potential specific core TRs within cochlear tissue during the ARHL process (Fig. 1b).

To investigate whether these factors were indeed involved in the regulation of age-related superenhancers, we conducted SE prediction with SEdb 2.0. We found that Sp1 was the top-ranking transcription factor associated with SEs in cochlear tissue (Fig. 1c). Furthermore, the prediction revealed two overlapping genes downstream of these predicted SASEs, namely, Best1 and Fth1 (Fig. 1d–e). Fth1 is a known marker of ferroptosis, and its down-regulation often indicates the occurrence of ferroptosis. Therefore, we hypothesized that Sp1 was a key TF that regulated cochlear HC aging via SEs (Fig. 1a), potentially affecting the expression of Fth1 and thereby influencing ferroptosis. This process would ultimately lead to the aging of HCs and the cochlea and promote the occurrence and progression of ARHL.

Table 1 TR and its downstream genes obtained by TREA

TR	Genes
Arnt	C15ORF38-P3S2; VEGFB; FITM2; ALDH3A1; WIPI2
Atf4	NOCAL; COQ7; WISP1; WIPI1; SKA1; TMEM111; C9ORF46
Brca 1	PPP1R2P9; CLEC4M; LOC439994; TMEM115; SIM2
Cebpb	COQ4; PLCXD3; SPAG11B; C15ORF38-AP3S2
Creb 1	COQ3; NOC4L; CLEC4A; ALDH3A1; ANKS4B; BPIL1
Egr1	RPUSD4; CHFR; CDH2; MAOB; B4GALT1; FARP2
Elk1	RHBDD3; COQ6; NOC4L; NOC3L; ALDH3A1; WISP3
Ets2	NOC2L; PLCXD1; CLEC4D; ALDH3A2; VEGFA
Fev	TRIM14; SERINC1; FAT4; ISCU; PRPF38B
Fos	C15ORF38-AP3S2; FAM75D3; C1ORF227
Foxo3	PLG; Len2; NCRNA00107; LOC339568; Mt1
Gata6	SPAG11A; PLCXD3; SSR3; SPAG11B; NRAS
Gfi 1	COQ4; COQ6; CLEC4C; RLTPR; FITM2; C1ORF227
Hif1a	COQ3; RHBDD2; COQ4; NPPB; NPPA; RLTPR
Hmga 1	SPAG11A; PLCXD3; SSR3; SPAG11B; Insig1; CROT
Hnf4a	COQ5; CLEC4D; CLEC4E; C15ORF38; SLC35F2
Hoxa5	Ptn; NODAL; COQ7; CROT; FITM2; VEGFC
Irf1	Irf1; Irf7; HCG26; BRD2; Irf9; NCRNA00107; FLOT1; Cdkn1a
Irf2	CLEC4M; COQ7; SPAG11A; VCP1P1; NOSIP; SKA3; SF3A3
Irf7	RPAIN; YEATS2; ASH1L; Stat1; SPO11; Oas12
Irf9	CDH9; Irf1; Irf7; CCDC67; SLC11A2; LTB4R; PRG4
Jun	CLEC4M; PLCXD3; SPAG11B; C15ORF38AP3S2
Klf4	NOC2L; NOTUM; PLXND1; C1ORF229; Bmpr1a
Myc	Ptn; COQ4; NOC4L; ERN1; Bmpr1a; WISP1; TMEM116
Mycn	Eef1a1; HOOK1; Hdac2; Zyx; DDX49; Tnfrsf1a
Ncoa1	C10ORF106; ZNF791; GRM6; BTBD6; LDHAL6B
Nfatc2	NCKAP5; MTMR10; IL12RB2; C19ORF24; MVD
Nfe212	COQ6; NCRNA00107; Mt1; Mg11; FOXO4; RIMBP3B
NF-κB1	AP3S2; CLEC4E; CLEC4F; ALDH3A1; C1ORF229
Nfya	COQ4; COQ7; PLCXD2; SSR4; MMAB; FAM75D1
Nkx3-1	RNF144A; NCRNA00107; NACAD; SNORD84
Pbx1	GPC4; PRY2; HCG26; NCRNA00107; BRD2
Pdx1	ING4; CHMP2A; UBE2CBP; DPEP1; DMRT2
Pitx2	COQ3; COQ4; COQ5; COQ6; COQ7; RHBDD2
Prdm1	SPAG11A; SSR3; RLTPR; CROT; NRAP; SLC35F2
Rel	CDH9; NCRNA00107; Fads2; ST3GAL2
Rela	FAM132A; CLEC4M; NOC3L; COQ7; NOC2L
Relb	COQ6; NPPB; MMAB; VEGFB; ALDH3A1
Runx1	COQ6; SPAG11A; SSR1; CLEC4C; ERN2; MMAB
Runx2	NODAL; CLEC4A; PLCXD2; SPAG11B; VEGFB
Smad3	Zyx; Dusp4; BRF1; JMJD8; FOXF1; Mboat2
Smad4	RHBDD2; ALDH3A1; FAM75D3; VEGFA
Sox2	MAGEA11; FAM19A1; Dusp4; SSR3; Amo
Sp1	Ptn; NOC2L; NOTUM; PLXND1; VENTX; PLCXD1
Sp3	NOC2L; NOTUM; PLXND1; VENTX; VEGFB
Spi1	ERCC2; ZFP57; ZFPL1; ZFP91; VEPH1; NOMO3
Srebf1	RHBDD3; COQ3; NOTUM; NPPA; Insig1; ALDH3A1
Srebf2	FAM132A; RHBDD3; RHBDD2; COQ7; NPPB
Stat1	Ptn; SPAG11A; SSR1; PLXND1; CLEC4C; MMAB
Stat3	Ptn; RHBDD1; PLCXD3; CLEC4A; SPAG11B; SSR2
Stat5b	RHBDD1; COQ4; CLEC4G; CROT; SLC35F1; AICDA
Tp53	Ptn; COQ6; SPAG11A; NPPB; PLCXD1; ERN1; FITM2
Usf2	COQ4; NOC4L; NODAL; SSR3; CLEC4C; AP3S2
Xbp1	RPUSD1; SERINC5; COQ7; COQ9; FARS2
Zbtb16	NOC3L; SSR2; C10ORF227; SF3B5; MCF2; ERAL1
Znf148	NOTUM; COQ9; PLXND1; VEGFB; C1ORF229

To determine whether the changes in Sp1 and Fth1 expression in the cochlea with aging were consistent with our hypothesis and to understand their localization in the cochlea, we performed immunofluorescence staining on cochlear sections from the 6W and 12 M mice and selected sections parallel to the modiolus and close to the modiolus for comparison. As shown in Fig. 2a, in both groups of mouse cochleae, Sp1 and Fth1 were predominantly

localized in the basilar membrane region, especially in the inner and outer HCs, indicating their crucial role in maintaining and ensuring the function of the sensory epithelium during the progression of ARHL. Furthermore, compared with those in young mice, the expression of Sp1 and Fth1 in the inner and outer HCs of aged mice (ARHL model mice) was significantly lower in all three turns (Fig. 2b-i), which was consistent with our previous prediction.

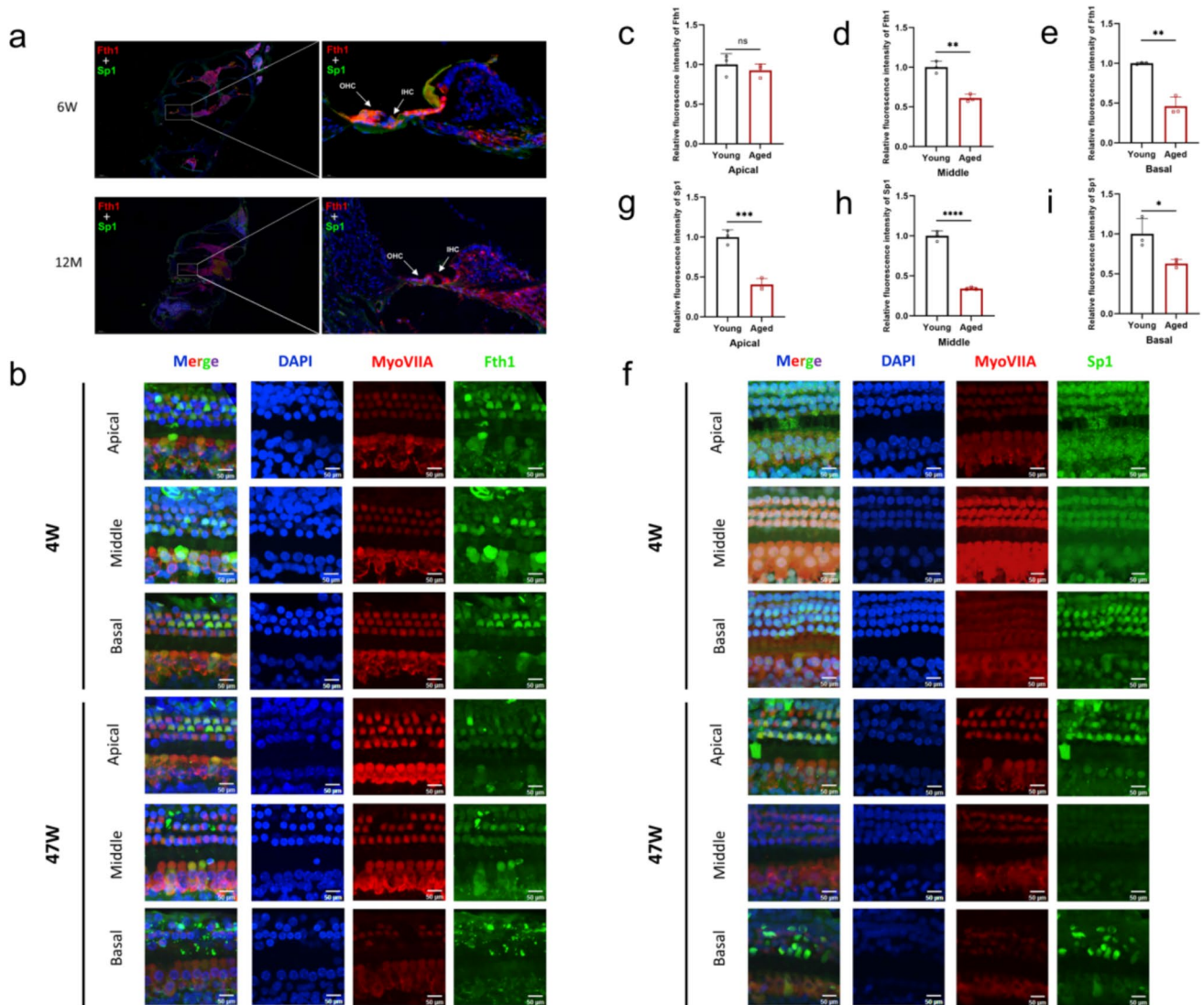


Fig. 2 Changes in Sp1 and Fth1 expression in the cochlea with aging and their localization in the cochlea a) The distribution of Fth1 and Sp1 in cochlear sections from young and aging mice was detected by immunofluorescence double staining (red fluorescence represents the expression of Fth1 in cochlear tissue, green fluorescence represents the expression of Sp1 in cochlear tissue, and blue fluorescence represents the nucleus). OHC: outer hair cell, IHC: inner hair cell. b, f) Representative images of immunofluorescence staining of Fth1 and

Sp1 in HCs from young and old animals (scale bar=50 μ m). c-e) Fluorescence quantitative analysis of Fth1 in the basilar membrane of three turns from young and aged mice. g-i) Fluorescence quantitative analysis of Sp1 in the basilar membrane of three turns from young and aged mice. (c-e, g-i), The two-tailed Student's t-test (t test). Statistical significance: no statistical significance; * p <0.05; ** p <0.01; *** p <0.001; **** p <0.0001

Ferroptosis is the main mechanism by which HCs age

The method used to induce senescence in HEI-OC1 cells with D-gal has been described in our previously published studies. As ROS accumulation is closely related to senescence, we determined the total production of ROS by using the DCFH-DA reagent (Fig. 3a). The staining and flow cytometric analysis results indicated that, after D-gal treatment, the ROS level was significantly greater in HEI-OC1 cells treated with D-gal than in control cells.

To determine the main process of HC aging, we evaluated the major cellular aging pathways induced by D-gal with the necroptosis inhibitor Nec-1, the autophagy inhibitor 3-MA, and the ferroptosis inhibitor Fer-1 (Fig. 3b). First, the CCK-8 results revealed that 3-MA increased cell viability

in the presence of D-gal but decreased it in the absence of D-gal, indicating that autophagy might be one of the biological programs maintaining normal cell function. Nec-1 did not significantly inhibit the decrease in cell viability induced by D-galactose in HEI-OC1 cells (Fig. 3c). However, the ferroptosis inhibitor Fer-1 significantly reversed D-gal-induced cell death and promoted the maintenance of normal cell function. In addition, MMP assessment revealed that 3-MA could not rescue the aging-induced decrease in the MMP, whereas Fer-1 and Nec-1 could not only stabilize the mitochondrial membrane potential in normal cells but also significantly rescue the decrease in the mitochondrial membrane potential caused by aging in HEI-OC1 cells (Fig. 3d-e). The ability of Fer-1 to reverse this decrease was slightly greater than that of Nec-1. DCFA probes were used to detect changes in ROS levels in cells after treatment with

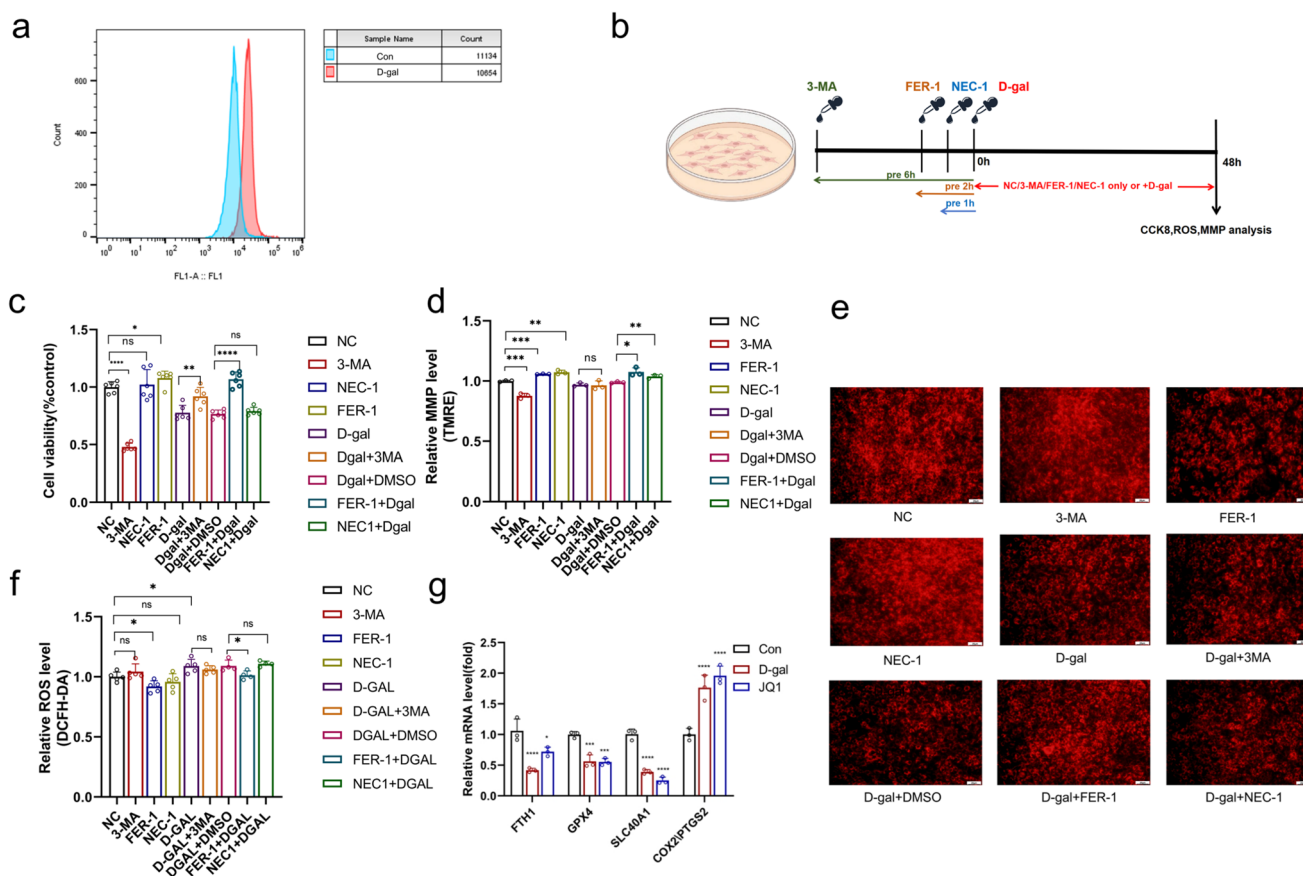


Fig. 3 Ferroptosis is the main mechanism by which HCs age a) Measurement and quantification of intracellular ROS level by flow cytometry using DCFH-DA fluorescent probe. b) Flow chart of HEI-OC1 cells treated by different cell death inhibitors and D-gal. (3-MA 10 mM pre-treated for 6 h, FER-1 10 μ M pre-treated for 2 h, NEC-1 20 μ M pre-treated for 1 h, D-gal concentration was 30 mg/mL) c) Effects of different cell death inhibitors alone or in the presence of D-gal on the viability of HEI-OC1 cells. d-e) Effects of different cell death inhibitors alone or accompanied by D-gal on the mitochondrial membrane potential levels in HEI-OC1 cells. f) Effects of different cell death inhibitors alone or accompanied by D-gal on the cellular ROS level in HEI-OC1 cells. g) Changes in mRNA expression of ferroptosis related protein induced by D-gal or JQ-1 treatment of HEI-OC1 cells. (c-d,f), One-way ANOVA test. (g), Two-way ANOVA test. Statistical significance: no statistical significance; * p <0.05; ** p <0.01; *** p <0.001; **** p <0.0001

tors alone or accompanied by D-gal on the mitochondrial membrane potential levels in HEI-OC1 cells. f) Effects of different cell death inhibitors alone or accompanied by D-gal on the cellular ROS level in HEI-OC1 cells. g) Changes in mRNA expression of ferroptosis related protein induced by D-gal or JQ-1 treatment of HEI-OC1 cells. (c-d,f), One-way ANOVA test. (g), Two-way ANOVA test. Statistical significance: no statistical significance; * p <0.05; ** p <0.01; *** p <0.001; **** p <0.0001

the three inhibitors, and the results revealed that 3-MA and Nec-1 did not significantly reduce the overall ROS level induced by D-gal, whereas Fer-1 reversed the increase in the overall ROS level induced by D-gal (Fig. 3f). These results suggested that ferroptosis might be the main cell death mechanism leading to the aging of HEI-OC1 cells.

To further confirm the occurrence of ferroptosis in aging cells, we used qPCR to detect various markers related to ferroptosis. D-gal treatment significantly increased the transcription levels of prostaglandin endoperoxide synthase 2 (COX2/PTGS2) in HEI-OC1 cells but decreased the transcription levels of Fth1, GPX4, and solute carrier family 40 member 1 (SLC40A1) (Fig. 3g). Interestingly, the changes in ferroptosis marker proteins induced by JQ-1 treatment were similar to those induced by D-gal.

Downregulation of Sp1 in aging cochlear cells leads to decreased Fth1 expression, triggering ferroptosis and aging

To elucidate the changes and interactions between Sp1 and Fth1 during hair cell aging, we first assessed their protein and mRNA expression levels. The PCR results revealed significant downregulation of Sp1 and Fth1 mRNA transcription levels during D-gal-induced aging in HEI-OC1 cells (Fig. 4a). WB revealed a decrease in Sp1 protein expression during the aging of HEI-OC1 cells (Fig. 4b-c) and Fth1 protein was also significantly downregulated (Fig. 4d-e).

To investigate the impact of Sp1 on Fth1 expression and whether Sp1 contributed to ferroptosis-induced aging in HEI-OC1 cells, we designed two siRNAs to knock down

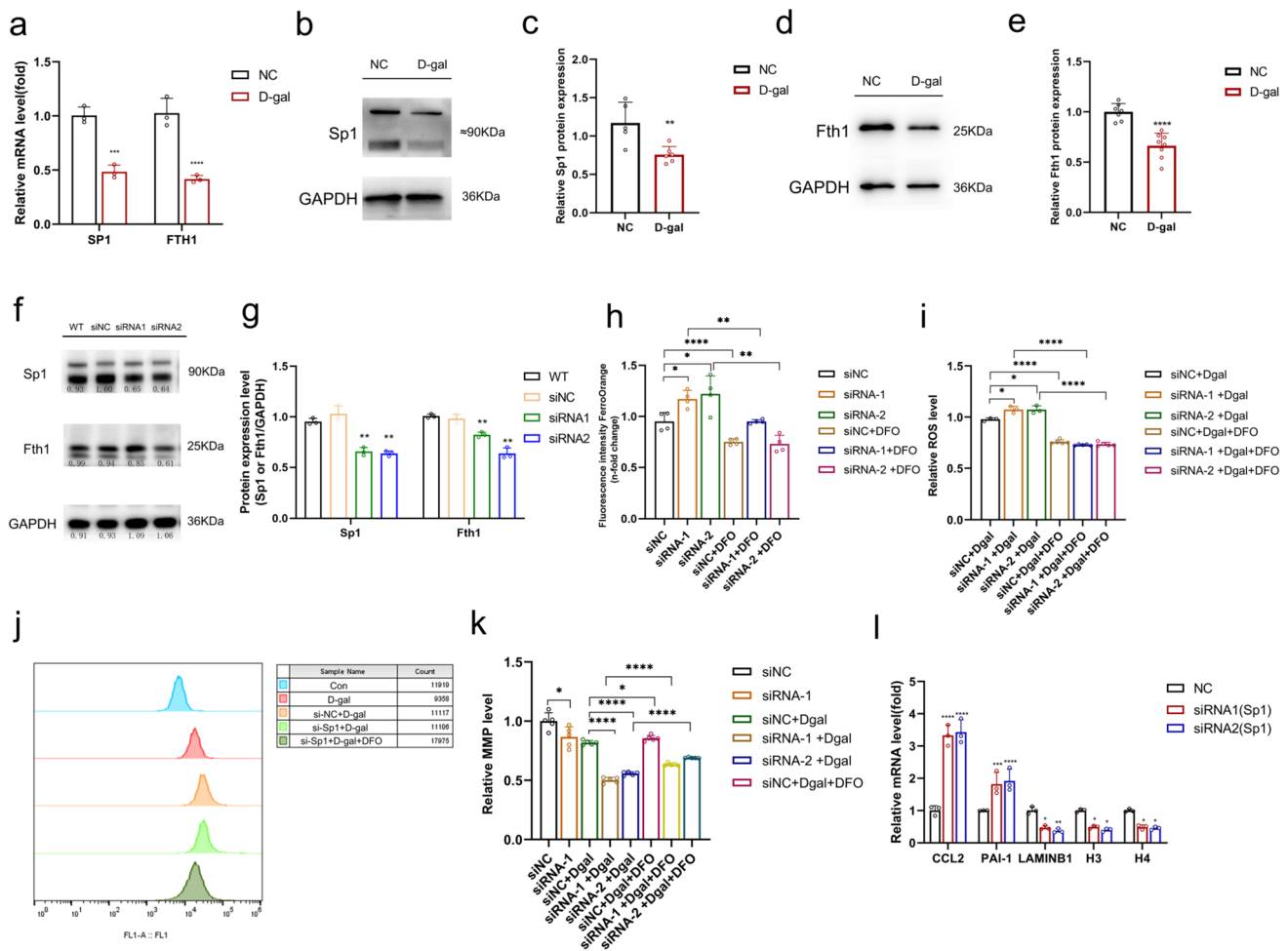


Fig. 4 Downregulation of Sp1 in aging cochlear cells leads to decreased Fth1 expression, triggering ferroptosis and aging a) Transcription expression change of Sp1 in aging HEI-OC1 cells. b-c) Protein expression change of Sp1 in aging HEI-OC1 cells. d-e) Protein expression change of Fth1 in aging HEI-OC1 cells. f-g) Identification of Sp1 knockdown and changes in transcription and protein expression of Fth1 in HEI-OC1 cells. h) Sp1 knockdown resulted in excessive Fe^{2+} content within HEI-OC1 cells and DFO decreased the intracel-

lular iron accumulation induced by Sp1 knockdown. i-k) Downregulating Sp1 with siRNAs led to increased ROS levels and decreased MMP, similar to the effects of D-gal. l) The expression of aging-related markers exhibited changes consistent with senescence. (a,l), Two-way ANOVA test. (c,e), The two-tailed Student's t-test (t test). (h-i,k), One-way ANOVA test. The statistical significance was represented as ns: no statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

Sp1 expression. After transfection, we extracted RNA and protein from the cells and assessed Sp1 mRNA and protein expression levels by PCR and WB. As shown in Fig. 4f–g, both siRNAs effectively reduced Sp1 at the transcriptional and protein expression levels, and Fth1 protein levels decreased in response to Sp1 downregulation induced by both siRNAs, indicating that downregulation of Sp1 led to decreased Fth1 expression.

Fth1/Ftl (ferritin components) play crucial roles in iron transportation by forming iron storage bodies, regulating iron release, and maintaining the balance of iron ions inside and outside the cell, which are essential for cellular function and overall health [26]. Decreased Fth1 expression leads to reduced Fe^{2+} storage, increasing the level of Fe^{2+} within cells and resulting in iron overload. Excess iron generates ROS through the Fenton reaction, leading to ferroptosis. To elucidate whether Sp1 induced ferroptosis in HEI-OC1 cells by causing iron overload, we measured the concentration of Fe^{2+} in cells after Sp1 knockdown with a FerroOrange probe. As shown in Fig. 4h, Sp1 knockdown resulted in excessive Fe^{2+} content in HEI-OC1 cells, resulting in higher FerroOrange signals than those in the normal control group, and deferoxamine mesylate (DFO) decreased the intracellular iron accumulation induced by Sp1 knockdown. These findings indicated that aging induced by Sp1 downregulation in HEI-OC1 cells was associated with excessive intracellular iron content.

To determine whether reducing Sp1 levels could cause ferroptosis and aging in HCs, we measured changes in ROS and MMP levels after siRNA transfection. Downregulating Sp1 with siRNAs led to increased ROS levels and decreased MMP, similar to the effects of D-gal (Fig. 4i–k). We also simultaneously assessed the expression of aging-related markers, as shown in Fig. 4l, which exhibited changes consistent with senescence, including decreased transcription of H3, H4, and lamin B1 and increased transcription of CCL2 and PAI-1. These results collectively demonstrated that the downregulation of Sp1 indeed induced aging in HCs. Moreover, DFO could rescue the changes in the ROS and MMP levels induced by D-gal, which indicated that the ferroptosis that occurred with aging depended mainly on intracellular iron accumulation, with Sp1-mediated downregulation of Fth1 playing a key role.

Sp1 influences Fth1 expression during HC senescence by binding to its SE

To determine whether Sp1 affected Fth1 expression during HC senescence by binding to its SE, we established stable transfectants of Sp1-overexpressing HEI-OC1 cells (control group: NC-Flag; Sp1-overexpressing group: Sp1-Flag). WB analysis of the Sp1 and Fth1 proteins revealed

that Fth1 expression did not significantly change after Sp1 overexpression, indicating that Sp1 overexpression did not affect the expression level of the Fth1 protein (Fig. 5a–b). Additionally, treatment with 1 μM JQ-1 resulted in no significant increase in Fth1 mRNA expression even with Sp1 overexpression; both control and Sp1-overexpressing cells presented a significant decrease in Fth1 mRNA expression after JQ-1 treatment ($p < 0.01$), suggesting that Sp1 overexpression could not rescue the transcriptional downregulation of Fth1 caused by JQ-1 inhibition of SE (Fig. 5c). These findings indicated that JQ-1 effectively blocked the binding of Sp1 to the SE of the Fth1 gene. Moreover, Sp1 overexpression did not decrease the Fe^{2+} concentration or rescue the aging induced by D-gal (Fig. 5d–e). These results supported the hypothesis that the SE of the Fth1 gene must remain active (chromatin-accessible) for Sp1 to bind to it and that the binding of Sp1 to the Fth1 SE could influence Fth1 expression and the aging process of HCs (Fig. 5f).

CUT&TAG assays reveal reduced binding of Sp1 to the Fth1 SE in aging-mimic HCs

To further validate the above hypothesis and identify the binding sites of the Sp1 protein in the Fth1 gene, we performed CUT&TAG assays using anti-Sp1 antibodies in control (Ctrl group) and aging-mimic HEI-OC1 cells (D-gal group). The CUT&TAG technique comprises two main steps: initial CUT&TAG experiments and subsequent sequencing analysis, which constitute a novel method for studying protein–DNA interactions by precisely targeting DNA sequences near the protein of interest under antibody guidance. The detected peaks represent regions where Sp1 may bind, and the gene corresponding to the peak's nearest TSS is considered a peak-related gene. The statistical analysis of the distribution of reads in the genome is shown in Fig. 6a. The horizontal axis represents positions on the chromosome, whereas the vertical axis indicates the number of reads aligned to 1-kb windows. As shown in Fig. 6b, more than half of the peaks were distributed in the promoter region in both groups, which indicated that Sp1 acted mainly by binding to the DNA region near the promoter region. We subsequently conducted KEGG enrichment analysis on the two groups of peak-related genes, where the enrichment factor indicated the ratio of genes in the peak-related genes that were in a specific pathway to the total number of genes annotated in that pathway, and the q value was the p value after multiple hypothesis testing correction (ranging from 0–1), with lower values indicating more significant enrichment. Finally, we selected the top 20 significantly enriched pathways for display. As shown in Fig. 6c–d, in both cell samples, the main functions (e.g., metabolic pathways and signal transduction) of genes associated with Sp1-bound peaks were significantly related to “cellular

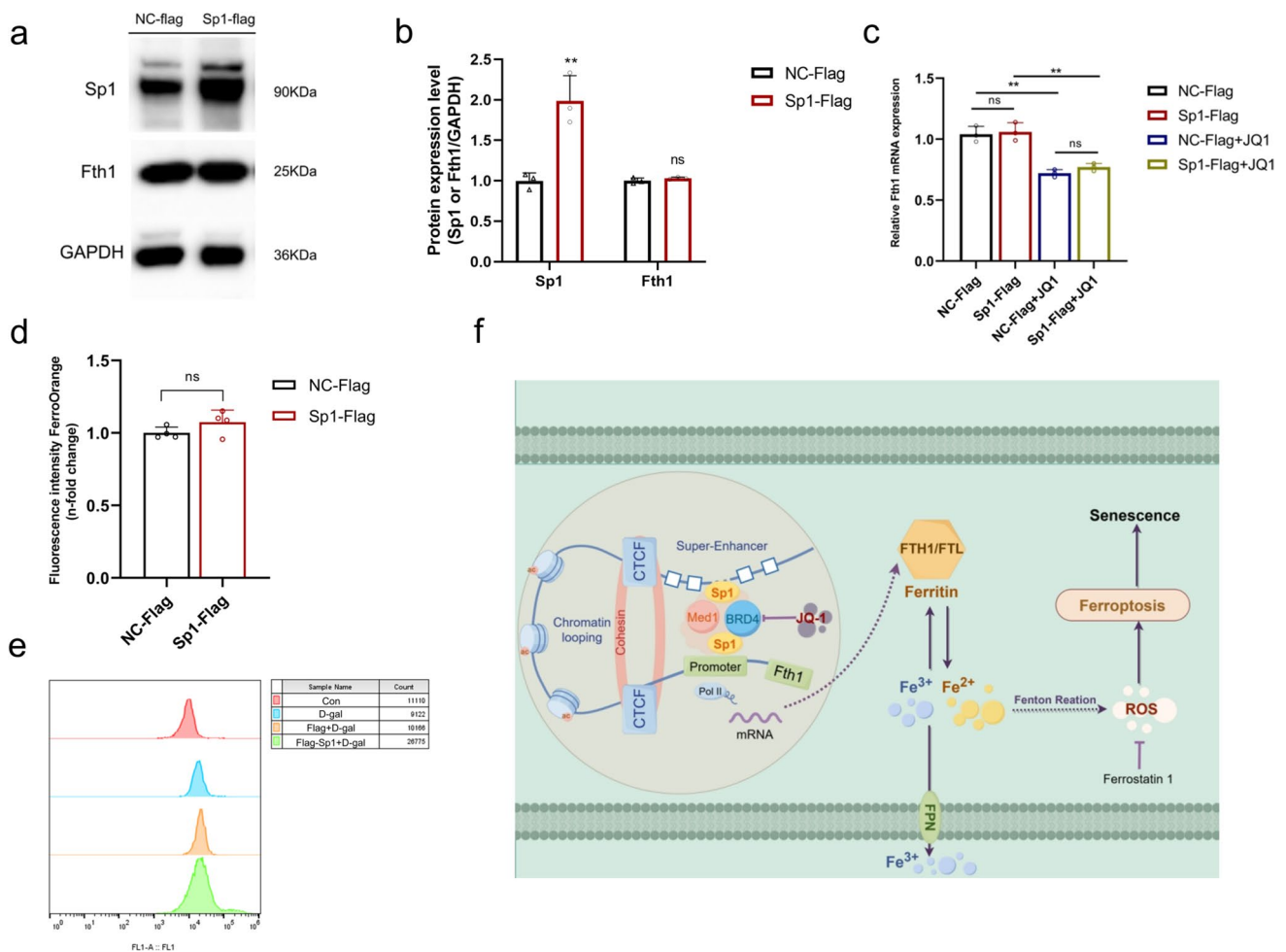


Fig. 5 Sp1 influences Fth1 expression during HC senescence through binding to its SE a-b) WB revealed that Fth1 expression did not significantly change after Sp1 overexpression. c) Treatment with 1 μ M JQ-1 resulted in no significant increase in Fth1 mRNA expression even with Sp1 overexpression; Sp1 overexpression cannot rescue the transcriptional downregulation of Fth1 caused by JQ-1 inhibition of the SE. d) JQ-1 effectively blocks the binding of Sp1 to the SE of the Fth1

gene. e) Sp1 overexpression can not decrease the Fe²⁺ concentration or rescue the aging induced by D-gal. f) Schematic representation of Sp1 and Fth1 possible effects, mechanism(s) of action and biological role of ferroptosis. (b), Two-way ANOVA test. (c), One-way ANOVA test. (d), The two-tailed Student's t-test (t test). The statistical significance was represented as ns: no statistical significance; * p <0.05; ** p <0.01; *** p <0.001; **** p <0.0001

senescence" (Ctrl: q value<0.0001; D-gal: q value<0.0001), which indicated that Sp1 indeed influenced and regulated the senescence process of HEI-OC1 cells by binding to genomic elements. Furthermore, KEGG enrichment analysis of differential peak-related genes bound by Sp1 (Fig. 6e) revealed that "metabolic pathways" ranked second (with 251 overlapping genes in the metabolic pathway) and were significantly enriched (q value<0.0001), revealing that Sp1 protein binding to differential peaks involved mainly metabolic pathways, indicating that the main manner in which the Sp1 protein functioned during HC senescence was by influencing cellular metabolism (such as iron metabolism). We then selected differential peak-related gene-annotated pathways containing the Fth1 gene and pathways of interest for display. As shown in Figure, Fth1 was annotated as a differential peak

gene and participated in pathways related to "ferroptosis" and "necrosis", both of which were significantly enriched (q values<0.05 and 0.0001, respectively); both sets of differential peak-related genes were also shown to participate in "cell senescence", further indicating that Sp1 dynamically regulated the HC senescence process by binding to DNA elements. Notably, "autophagy" was also significantly enriched in this analysis. Ferroptosis appeared to play a particularly significant role in the senescence of HCs among the three modes of cell death (Fig. 6f). However, the primary biological mechanism regulated by the Sp1 protein in HC senescence has not been confirmed. Additionally, the involvement of emerging death pathways, such as iron autophagy, warrants additional investigation and discussion.

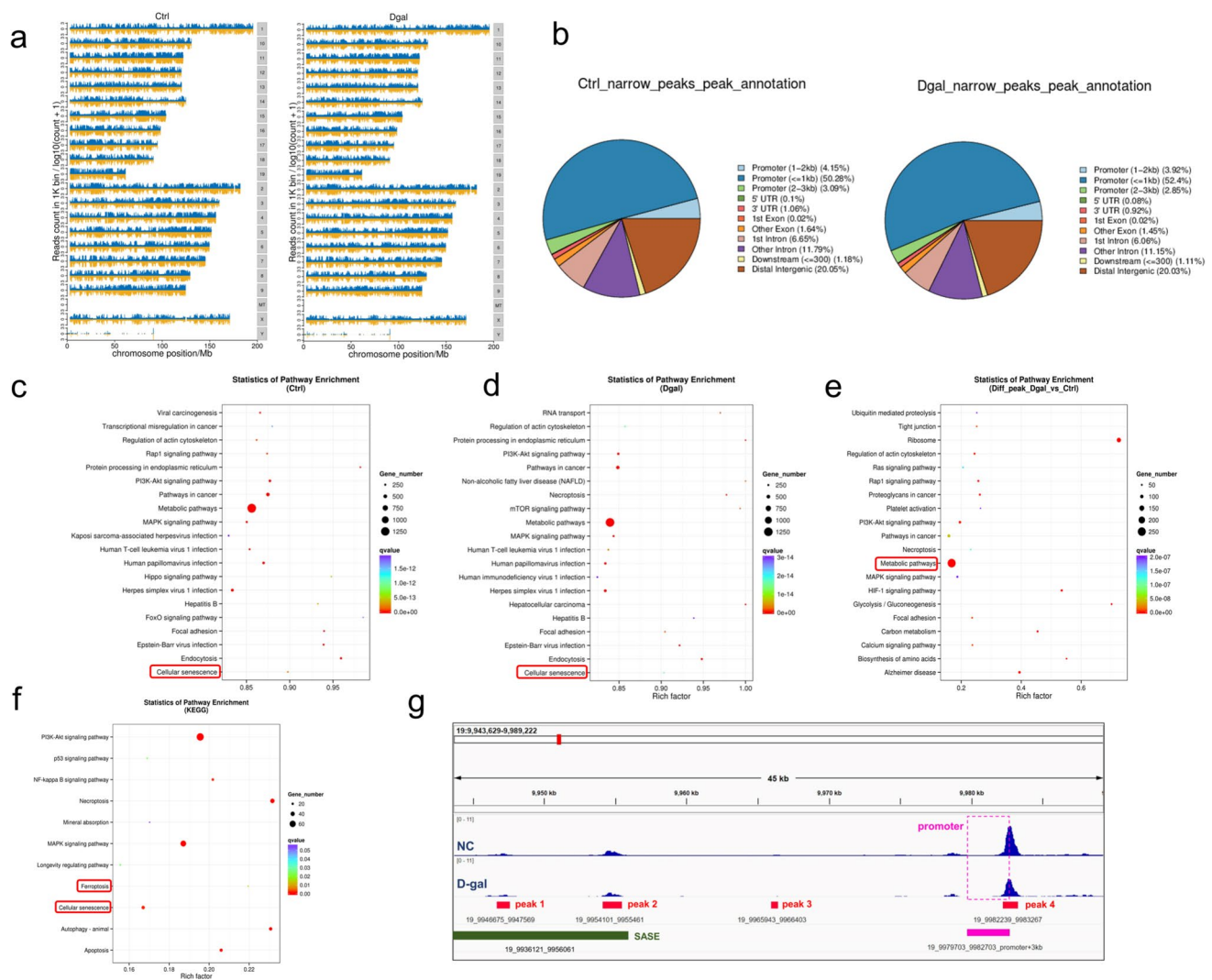


Fig. 6 CUT&TAG assays reveal reduced binding of Sp1 to the *Fth1* SE in aging-mimic HCs **a**) The distribution of the reads in the genome of the two groups (The horizontal axis represents positions on the chromosome, whereas the vertical axis indicates the number of reads aligned to 1 kb windows). **b**) The distribution of peak annotation in two groups, which indicated that Sp1 acts mainly by binding to the DNA region near the promoter region. **c-d**) KEGG enrichment analysis on the two groups of peak-related genes showed the the top 20

significantly enriched pathways (Ctrl: q value < 0.0001 ; D-gal: q value < 0.0001). **e**) KEGG enrichment analysis of differential peak-related genes bound by Sp1. **f**) Ferroptosis appeared to be particularly significant in the senescence of HCs. **g**) Peaks with differential binding of Sp1 to Fth1 were distributed mainly within the promoter region and within 30 kbp upstream, which was consistent with the distribution characteristics of superenhancers

Further analysis revealed that peaks with differential binding of Sp1 to Fth1 were distributed mainly within the promoter region and within the 30-kbp upstream region, which was consistent with the distribution characteristics of superenhancers (Fig. 6g). Red squares represent the positions of differential peaks, green squares represent predicted SASE positions, and the pink dashed box indicates the promoter region of the *Fth1* gene. As shown in the figure, peak 1 and peak 2 were both within the SASE region obtained from conservative prediction based on previous studies (Chr19.9936121–9,956,061), with decreased peak values

($\log_2$ fold change < 0), indicating reduced binding of Sp1 to this site in senescent-like HEI-OC1 cells; peak 4 spanned the transcription start site and was distributed in the promoter and gene regions. On the one hand, these findings indicated that the Sp1 protein might increase *Fth1* expression by binding to the promoter and SE regions through loop formation mediated by CTCF; on the other hand, it did not exclude the possibility that, owing to the large span of the *Fth1* gene's SE, this SE included the promoter region and the vicinity of the transcription start site, which was consistent with the typical span range of 8–30 kb for typical SEs.

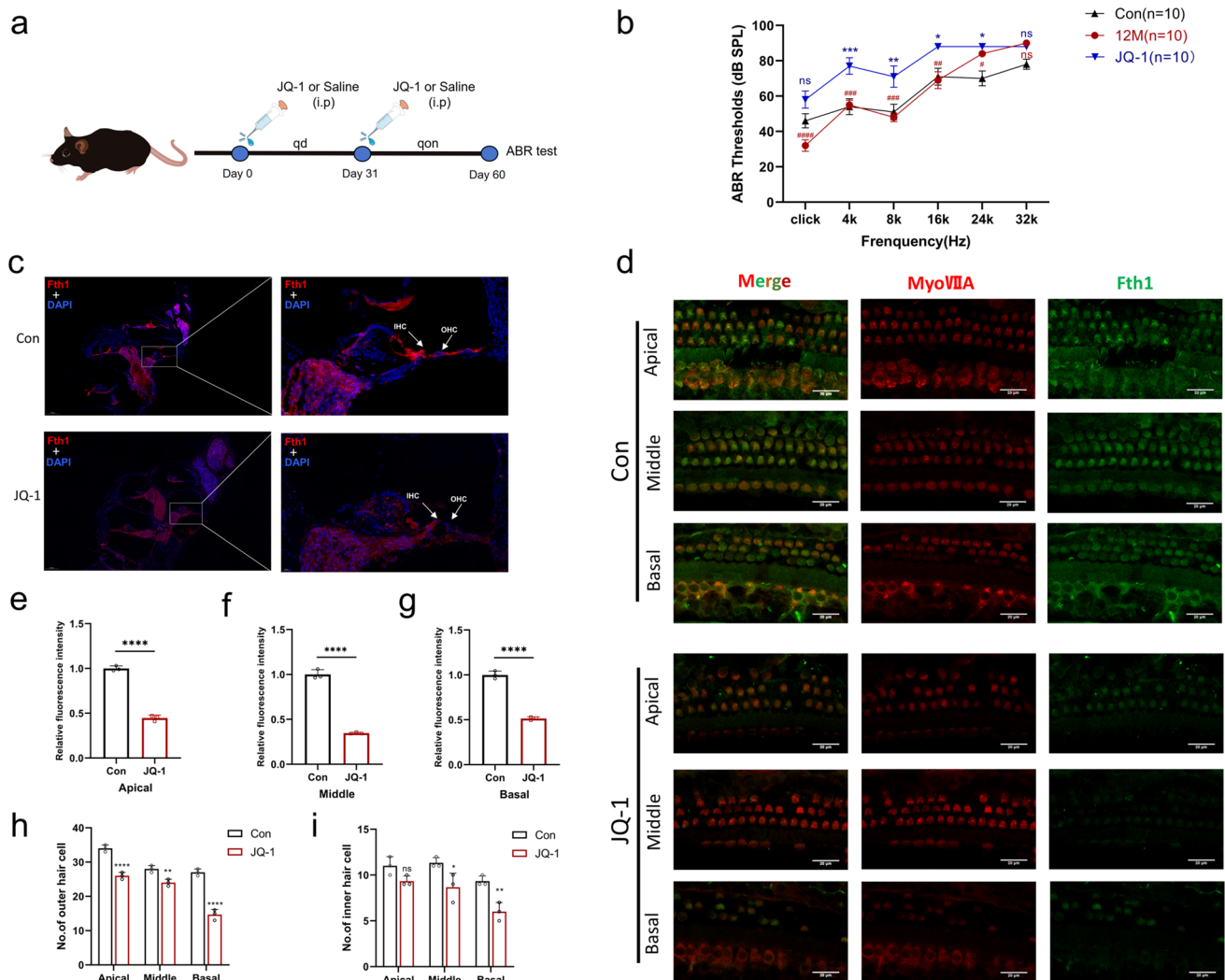


Fig. 7 JQ-1 inhibits Fth1 expression in HCs and accelerates the shift in ABR thresholds in C57BL/6 J mice **a**) The injection procedure of C57BL/6 J mice to investigate the effect of the SE inhibitor JQ-1 on the progression of ARHL. **b**) The change of ABR thresholds after JQ-1 exposure. **c**) Immunofluorescence staining of the cochleae section from control and JQ-1-treated mice revealed significant suppression of Fth1 expression throughout the cochlear layers, which was particularly pronounced in the basilar membrane region. **d–g**) There was a

significant decrease in Fth1 fluorescence in the inner and outer HCs after JQ-1 treatment, indicating significant downregulation of Fth1 expression in the HCs. **h–i**) The number of OHCs and IHCs in different turns also decreased, which might be the result of SE inhibition. (**e–g**), The two-tailed Student's t-test (t test). (**h–i**), Two-way ANOVA test. The statistical significance was represented as ns: no statistical significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

Considering all the above data, the present finding revealed the following mechanism. Sp1 regulates the expression of the Fth1 protein by binding to the SE and promoter of the Fth1 gene. The chromatin accessibility of SEs and promoter regions decreases in senescent HCs, leading to reduced binding of Sp1 to SEs and promoter regions, which results in decreased expression of the Fth1 protein. Consequently, there is a decrease in storage and an increase in the release of Fe^{2+} , followed by iron overload in senescent HCs, stimulation of ROS production, ferroptosis, and ultimately, the progression of ARHL (Fig. 5F).

JQ-1 inhibits Fth1 expression in HCs and accelerates the shift in ABR thresholds in C57BL/6 J mice

To investigate the effect of the SE inhibitor JQ-1 on the progression of ARHL in vivo, we used intraperitoneal injections of JQ-1 in 9 M C57BL/6 J mice for 2 months. The injection procedure is illustrated in Fig. 7a. ABR testing was conducted on the 61st day, which revealed that, compared with the control treatment, JQ-1 treatment resulted in a significant upward shift in ABR thresholds at almost all frequencies in the mice, and the hearing impairment was

more severe in the treated mice than in the ARHL model mice (Fig. 7b). Compared with those in the control group, the mice in the JQ-1 group presented significant increases in auditory thresholds at 4 kHz, 8 kHz, 16 kHz, and 24 kHz, with average increases of 23 dB, 20 dB, 17 dB, and 18 dB, respectively; although the increase in the threshold at the high frequency of 32 kHz was not significant, it still increased by an average of 10 dB. The earlier onset of hearing loss in mice induced by JQ-1 suggested that maintaining SE activity was highly important for overcoming the age-related factors that induce an increase in auditory thresholds in mice. Cochlear sections and immunofluorescence staining of the cochleae of control and JQ-1-treated mice revealed significant suppression of *Fth1* expression throughout the cochlear layers, which was particularly pronounced in the basilar membrane region (Fig. 7c). After JQ-1 treatment, *Fth1* fluorescence significantly decreased in the inner and outer HCs, indicating significant downregulation of *Fth1* expression in the HCs (Fig. 7d–g); the number of OHCs and IHCs in different turns also decreased, potentially resulting from SE inhibition (Fig. 7h–i).

Discussion

Our study aimed to explore the potential molecular switches that are crucial for regulating HC senescence during the development of ARHL. Following the method of Joshua E. Burda et al., the RNA-seq data of the DEGs were subjected to TREA to identify core TRs and their potentially regulated downstream genes. Subsequent hierarchical screening, incorporating network-based predictions and joint analysis with our sequencing data, led to a plausible hypothesis. Sp1 may serve as an upstream TR within cochlear tissue during the development of ARHL, primarily through binding to SE regions, with its downstream target gene being the ferroptosis biological marker *Fth1*. Chromatin accessibility decreases with age, hindering Sp1 from binding to SEs, resulting in the downregulation of *Fth1* expression; moreover, intracellular iron overload elevates ROS levels, which leads to HC senescence and ARHL progression. This hypothesis was progressively validated in these experiments. Sp1 knockdown confirmed the upstream–downstream regulatory relationship between Sp1 and *Fth1*; Sp1 overexpression did not significantly alter *Fth1* expression, indicating that Sp1 regulation of *Fth1* expression relies on the accessibility of SE regions rather than Sp1 protein levels. Even with increased Sp1 protein levels, activity inhibition or chromatin closure of SEs due to epigenetic factors prevented their anti-senescence regulatory function. Additionally, specific SE sites were determined through the CUT&TAG assay, corroborating the results of the SASE prediction in previous

research. However, shortcomings continue to exist in the current research results: H3K27ac signal aggregation is a crucial feature of SE activation, and further assessment is needed to determine whether there is high H3K27ac signal aggregation at these sites for more microscopic observation and identification at the molecular level. Whether chromatin region changes that maintain auditory function can be achieved through interventions in acetylation and other epigenetic modifications to maximally salvage or delay the onset of ARHL from the root cause is also a future research direction and goal.

Our study reveals a cochlear-specific epigenetic–metabolic axis in which Sp1–SE interactions regulate iron homeostasis. Unlike neurodegenerative disorders in which Sp1 modulates protein aggregation [27, 28], cochlear aging centers on SE-controlled iron storage genes (*Fth1*; Fig. 6g). This divergence highlights a tissue-specific vulnerability in age-related degeneration: neurons are predominantly characterized by the accumulation of proteinopathies (e.g., A β and tau in Alzheimer's disease) [29, 30], whereas auditory HCs are particularly susceptible to iron-triggered ferroptosis, a process driven by iron accumulation and lipid peroxidation [31, 32]. Emerging evidence positions the loss of H3K27ac as a contributory epigenetic mechanism to metabolic and inflammatory dysregulation in the aging brain. By analogy, therapeutic strategies aimed at modulating histone acetylation (e.g., via HDAC inhibitors) to restore a youthful epigenetic landscape in the brain might also hold promise for mitigating similar disruptions in the aging cochlea [33, 34].

In this study, three common cell death inhibitors were used to screen the most important pathways involved in HC senescence death, which yielded unexpected results. In terms of various senescence indicators, ferroptosis plays a crucial role in all the cell death pathways leading to the death of HCs, and the inhibition of ferroptosis was demonstrated to effectively rescue D-gal-induced aging. Autophagy has been a hot topic in aging research (including ARHL) in previous studies. Autophagy is considered a cellular protective mechanism against various stressors, helping to remove harmful components in aging or damaged organelles [35]. Recent studies have indicated that autophagy has an inhibitory effect on aging. Researchers have reported that as autophagy levels decrease, ROS levels increase with mitochondrial dysfunction, ultimately leading to cellular senescence, whereas increased autophagy in senescent cells can restore mitochondrial function and cellular regenerative functions [36]. Compared with untreated HEI-OC1 cells, HEI-OC1 cells treated with the autophagy inhibitor 3-MA presented decreased viability, whereas cells treated simultaneously with D-gal presented suppressed autophagy and improved viability. Mitochondrial membrane potential detection revealed an elevated MMP in the

untreated HEI-OC1 cells, whereas no significant change was detected in the cells treated simultaneously with D-gal. The staining and flow cytometric analysis results revealed decreased ROS levels in untreated HEI-OC1 cells, whereas hair cells treated simultaneously with D-gal presented a decrease but not a significant one, and Fer-1 significantly reversed D-gal-induced aging in all the phenotypes. Therefore, we ultimately concluded that ferroptosis was the main pathway involved in HC aging. Our results seemed to conflict with the theory that “autophagy inhibits aging”. In the present study, inhibition of autophagy in HEI-OC1 cells increased cell viability and decreased ROS levels. Recent investigations have shown that cell autophagy is involved in the entire process of ferroptosis and that excessive autophagy promotes ferroptosis through iron accumulation. An authoritative paper in 2023 showed that under specific conditions, cell autophagy can promote ferroptosis; cell autophagy disrupts the redox balance and promotes ROS-dependent ferroptosis, which results in the production of ROS and subsequent induction of autophagy. This process forms a positive feedback loop, amplifying iron death [37]. The relationship between autophagy and ferroptosis is complex and involves vastly different interaction patterns in different contexts, which can explain the phenomenon of aging phenotype recovery after autophagy inhibition observed in this study. Autophagy is inhibited, weakening its promoting effect and amplification effect on ferroptosis, indirectly reflecting that ferroptosis is the main pathway involved in hair cell aging. These findings provide a more in-depth understanding of the complex relationship between autophagy and ferroptosis and a theoretical and experimental basis for future research on the interaction between ferroptosis and autophagy in ARHL.

The connection between autophagy and apoptosis has been widely studied in recent years in the field of hearing research. FOXG1 plays an important role in the process of auditory degeneration by regulating macroautophagy/autophagy, and inhibiting FOXG1 can reduce autophagy activity in normal hair cells, leading to the accumulation of reactive oxygen species and subsequently inducing hair cell apoptosis [38]. Another study revealed that miR-34 in ARHL mice can accelerate hair cell apoptosis by inhibiting autophagy. In addition, KEGG analysis of the differentially expressed peak-annotated genes in this study's CUT&TAG assay results on the Sp1 protein revealed significant enrichment in the apoptosis pathway, indicating the involvement of an inseparable connection between autophagy and apoptosis in Sp1 binding to DNA in the HC aging processes. Additionally, CUT&TAG analysis revealed enrichment of Sp1-bound differential peaks annotated to genes in the mitogen-activated protein kinase (MAPK) pathway. Blockade of the Ras/Raf/MEK/ERK pathway can inhibit erastin-induced ferroptosis

in Ras-mutant cancer cells [39], suggesting that Sp1 may simultaneously regulate HC ferroptosis through the MAPK pathway and participate in complex regulatory or amplification mechanisms, providing clues for future research.

The cochlear basilar membrane is a crucial structure within the inner ear that is situated inside the cochlea and serves to support and protect the sensory organs of the inner ear. It is lined with auditory sensory cells capable of converting sound signals into neural impulses, thus facilitating auditory function. Our study revealed significant upregulation of Sp1 and Fth1 in the cochlear basilar membranes of young mice, whereas both were markedly downregulated in the cochlear basilar membranes of ARHL mice, including both inner and outer HCs. These findings suggested a potential association between abnormal expression changes in Sp1 and Fth1 and the occurrence of ARHL, further confirming the potential roles of these proteins in age-related hearing impairment. Consistent with the findings of *in vitro* studies, systemic administration of the superenhancer JQ-1 in mice can suppress Fth1 expression, particularly in the basilar membrane and hair cells. Sp1 is expressed predominantly in inner and outer hair cells, indicating that hair cells are the target cells for Sp1 regulation within the inner ear. As previously discussed, the downregulation of Fth1 expression in hair cells may lead to iron overload, resulting in iron-induced cell death and thereby promoting hair cell aging and loss. Hearing loss caused by ARHL begins in the high-frequency region of the auditory spectrum and spreads to lower frequency regions with increasing age [40]. The ABR test revealed elevated hearing threshold levels at higher frequencies (24 kHz), mid frequencies (16 kHz), and lower frequencies (4 and 8 kHz), indicating that JQ-1 accelerated the occurrence of ARHL-related hearing deterioration in mice. These findings highlighted the critical role of the HC-specific SE activity of Fth1 in maintaining hearing levels, with basilar membranes from the apex, middle, and base regions all showing low expression of Fth1, which was consistent with the observed upward shift in hearing thresholds across all frequencies following JQ-1 treatment in mice. The low expression of the ferroptosis marker Fth1 in the aging cochlea suggested a potential role for ferroptosis in the progression of ARHL, providing an experimental basis and theoretical evidence for further understanding the relationship between ARHL and SEs. These findings also supported the potential of ferroptosis inhibitors (such as deferoxamine) for delaying the progression of ARHL in mice.

As demonstrated in AAV-OTOF studies in nonhuman primates [41], species-specific efficacy/safety assessments will be essential for clinical translation. Building on this paradigm, future work will validate Sp1/Fth1 gene therapy in aging primate models. Furthermore, inspired by recent successes in auditory gene therapy [41–44], our findings

supported Fth1 as a candidate for AAV-mediated overexpression. Artificial intelligence-driven capsid engineering could optimize cochlear targeting [45], potentially delaying ARHL progression. We acknowledge this limitation and propose future work as follows: Future studies will integrate single-cell transcriptomics to compare Fth1 expression with primate cochlear aging markers [46], leveraging frameworks like the X-Age Project [47].

Conclusion

This study elucidates the mechanisms through which diminished Sp1 binding to the Fth1 SE and promoter promotes HC ferroptosis, thereby accelerating the progression of ARHL. These findings indicate that targeting SE sites and suppressing ferroptosis could represent promising therapeutic approaches for ARHL. Uncovering the relationship among Sp1, Fth1 SE, and ferroptosis opens new avenues for AAV-based gene therapies aimed at maintaining auditory function in the elderly by regulating iron balance during sensory cell aging.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00018-026-06117-0>.

Author contributions C.Y. Zhang, W. Yuan and Y. Sun contributed to the design of the experiments. C.Y. Zhang and T. Yang contributed to performing the experiments and statistical analyses. X.Q. Luo and X.L. Fu were in charge of the animal studies. C.Y. Zhang and T. Yang contributed to writing the manuscript. W. Yuan approved the final submission. All authors have read and agreed to the published version of the manuscript.

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Data availability All data generated in this study are available within the article; Supplementary data will be made available on request.

Declarations

Ethics approval and consent for participate All protocols of the animal study were approved by the Laboratory Animal Welfare and Ethics Committee Of the Army Medical University (Approval number: MU-WEC20226035).

Consent for publication All authors approved the final manuscript and the submission to this journal.

Competing interests The authors declare that they have no competing interests.

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