

LETTER

CRISPR-based screening pinpoints H2AZ1 as a driver of senescence in human mesenchymal stem cells

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Dear Editor,

Cellular senescence is characterized by growth arrest and the onset of a senescence-associated secretory phenotype (SASP) (Consortium et al., 2023; Liu et al., 2023; Zhang et al., 2023). Consequently, senescent cells that accumulate within aged organs have the capacity to disseminate pro-senescence and pro-inflammatory signals, promoting structural tissue deterioration and functional decline, culminating in organismal aging (Cai et al., 2022; Consortium et al., 2024). Stem cells, residing within tissues and endowed with remarkable abilities for self-renewal and differentiation, play pivotal roles in the repair and regeneration of injured or aged tissues. As individuals age, stem cells also undergo senescence and exhaustion, leading to a diminished capacity for tissue regeneration and repair. This decline is prominently associated with changes in the inherent flexibility of chromatin structure within stem cells, which instructs their differentiation into specific cell types. Consequently, extensive studies have highlighted the critical role of chromatin modifications in the context of

stem cell senescence (Wu et al., 2024; Zheng et al., 2024). Hence, identifying the key regulators of chromatin structure holds great potential for alleviating stem cell senescence and organ aging (Bi et al., 2024; Liu et al., 2022).

The nucleosome constitutes the fundamental building block of eukaryotic chromatin. Each nucleosome consists of 146 bp of DNA wrapped around a core histone octamer. This octamer comprises two copies of each of the histone proteins H2A, H2B, H3, and H4. Additionally, the histone protein H1, enveloped by 20 bp of DNA, plays a role in connecting adjacent nucleosome cores and stabilizing the higher-order chromatin structure. Consequently, the dynamic remodeling of chromatin structure is governed by different histone proteins, and this regulation occurs primarily through a series of post-translational modifications on different histones. In addition to the canonical histones, a diverse number of histone variants that are expressed independently of DNA replication and persist throughout the cell cycle have been identified. These variants possess the capacity to substitute canonical histones and confer distinct chromatin structural

properties, which in turn impact critical processes such as chromosome segregation, DNA repair, or transcription initiation (Martire and Banaszynski, 2020). However, our current knowledge regarding the interplay between histone variants and stem cell senescence remains limited. In this study, using CRISPR-mediated loss-of-function screening for histone variant-related genes (HVRGs), we identified that depletion of the H2A.Z variant histone 1 (*H2AZ1*) attenuates the senescence of human mesenchymal stem cells (hMSCs). Mechanistically, we demonstrated that *H2AZ1* functions as a transcriptional repressor of pleiotrophin (PTN) by binding to enhancer regions, thereby promoting cellular senescence.

To systematically explore the roles of histone variants in the regulation of hMSCs senescence, we constructed a CRISPR screening library targeting HVRGs. This library, denoted as HVRG library, comprises 96 single guide RNAs (sgRNAs) that target 5 H1 variants, 11 H2A variants, 11 H2B variants, and 5 H3 variants, with 3 sgRNAs assigned to each variant. Additionally, we included 45 non-targeting control sgRNAs (sg-NTCs) serving as controls (Figs. 1A and S1A–C). Subsequently, we conducted the CRISPR screening in three types of stem cell senescence models, including replicative senescent hMSCs (RS hMSCs) (Fig. S1D and S1E), Werner syndrome (WS, *WRN*-deficient) hMSCs (Fig. S1F and S1G), and Hutchinson-Gilford progeria syndrome (HGPS, carrying the heterozygous *LMNA*^{G608G/+} mutation) hMSCs (Fig. S1H and S1I). Notably, the latter two models represent established human stem cell models of premature aging (Wu et al., 2018). To ensure that the majority of cells harbor one sgRNA, we introduced the HVRG library at a low multiplicity of infection (MOI ≈ 0.3). In parallel, cells infected with lentivirus carrying sg-NTCs at the same MOI were utilized as control. After puromycin selection, we performed sequential cell passaging over an 8-week period until the cells infected with sgRNA carrying HVRGs had acquired a relatively rejuvenated phenotype in comparison to the control group, as determined by a reduced presence of senescent-associated β-galactosidase (SA-βgal)-positive cells (Fig. S1J–L). These findings suggested that deficiency of certain histone variants could alleviate senescence in hMSCs. Subsequently, we harvested the rejuvenated cells and ranked the sgRNAs using DNA sequencing. *H2AZ1*, an H2A.Z histone variant, emerged as the only common hit in RS-, WS- and HGPS-based screening, the reduction of which retarded senescence in all three hMSCs senescence models (Fig. 1B–D).

To validate the rejuvenation effects of *H2AZ1* deficiency, we conducted lentivirus-mediated CRISPR knock-out (CRISPRko) in senescent hMSCs (Fig. 1E). Relative to control cells, *H2AZ1* deletion did not impact the differentiation potential of hMSCs into osteoblasts, chondrocytes, and adipocytes (Fig. S1M–O) or genomic integrity (Fig. S1P). As anticipated, the ablation of *H2AZ1* ameliorated

multiple senescent characteristics, as evidenced by a reduction in SA-βgal-positive cells, restoration of compromised proliferation (e.g. enhanced clonal expansion, increased Ki67-positive cells, and EdU-positive cells) (Figs. 1F–H and S1Q), decreased expression of senescence marker p16 and SASP factors (e.g. *IL-6*, *CXCL8*), induction of Lamin B1, HP1α, H3K9me3, and lamina-associated polypeptide 2 (*LAP2*) expression (Figs. 1I, 1J, and S1R–T), decreased reactive oxygen species (ROS) level (Fig. S1U). Transcriptome sequencing (RNA-seq) revealed that *H2AZ1* deletion leads to upregulation of genes related to cell cycle and nuclear chromosome segregation, while genes linked to apoptosis and oxidative stress are down-regulated (Figs. 1K, S1V, and S1W; Table S1). Additionally, in WS and HGPS hMSCs, the *H2AZ1* deficiency also attenuated cellular senescence (Fig. S2A–L), mirroring the effects observed in RS hMSCs. Moreover, in senescent models induced by ultraviolet (UV) irradiation, H₂O₂ treatment, or oncogene (H-Ras^{V12}) transduction, *H2AZ1* depletion reduced the number of SA-βgal-positive cells and improved their proliferation potential (Fig. S3A–L). Next, we assessed the pro-senescence effects of *H2AZ1* by ectopic overexpression in wild-type hMSCs at early passage (young hMSCs) (Fig. 1L). As demonstrated by increased numbers of SA-βgal-positive cells, impaired cell proliferation, and decreased expression of Lamin B1, HP1α, and H3K9me3, *H2AZ1* overexpression accelerated senescence in young hMSCs (Fig. 1M–P and S4A). Moreover, *H2AZ1* overexpression also suppressed the expression of genes associated with proliferation and responsiveness to growth factor, while inducing the expression of genes related to oxygen levels (Figs. 1Q, S4B and S4C; Table S1). In conclusion, these findings suggest that *H2AZ1* plays a driving role in hMSCs senescence and that its depletion retards senescence across diverse biological contexts.

Given that *H2AZ1* as an integrated nucleosome component likely modulates chromatin structure and subsequent gene expression, we performed chromatin immunoprecipitation (ChIP) followed by high-throughput sequencing to explore the potential genomic-binding regions of *H2AZ1*, aiming to uncover the mechanisms through which *H2AZ1* depletion alleviates senescence. Our analysis revealed a global reduction in *H2AZ1* binding signals upon *H2AZ1* deficiency (Figs. 2A and S5A–E). In hMSCs, *H2AZ1* was enriched at both transcriptional start sites (TSSs) and distal regions (Fig. S5F), suggesting that *H2AZ1* may regulate senescence via its *trans*-activity in regulating gene expression. More specifically, the loss of *H2AZ1*-binding events predominantly occurred at distal regions, particularly those located at 3 kb away from TSSs, while its occupancy at TSSs was comparatively less affected (Fig. 2B and 2C). These data led us to speculate that *H2AZ1* regulates gene expression by binding to their distal regulatory regions, which might

be enhancer regions. Hence, we performed an integrated analysis of the ChIP-seq data and RNA-seq data, identifying 47 candidate genes that might be under the regulation of H2AZ1 through its *trans*-activity (Figs. 2D, 2E, and S5G). Among these genes, PTN, encoding pleiotrophin, a secreted growth factor that is essential for hippocampal neurogenesis (González-Castillo et al., 2015), was notably decreased in senescent hMSCs (Fig. S5H).

In the following experiments, we explored the relationship between H2AZ1 and PTN during hMSCs senescence. First, we validated that H2AZ1 occupancy was lost at the PTN enhancer regions in H2AZ1-deficient hMSCs by ChIP-qPCR analysis (Figs. 2F, 2G and S5I). We then found that PTN expression levels are upregulated in H2AZ1-deficient RS hMSCs and downregulated upon H2AZ1 overexpression in young hMSCs (Figs. 2H, 2I and S5J-L), suggesting that PTN is negatively correlated to H2AZ1 in hMSCs. Moreover, we found that H2AZ1 was enriched at five PTN enhancers (PTN E1-5) marked with H3K27ac (Fig. 2F) and that the occupancy of H3K27ac was enriched upon H2AZ1 deficiency (Fig. S5M). In addition, by assessing enhancer sequence activity (Table S2) using a luciferase reporter plasmid (pGL3-promoter vector), we found that all PTN enhancers showed increased luciferase signal. Conversely, H2AZ1 overexpression diminished the enhancer activity of PTN E1 and E5 (Fig. 2J), indicating that H2AZ1 exerts a *trans*-repressive effect on PTN.

To further elucidate the functional role of PTN in hMSCs senescence, we overexpressed PTN in senescent hMSCs and found a decreased percentage of SA- β -gal-positive cells (Fig. 2K-M), reminiscent of the effects observed upon H2AZ1 ablation. Conversely, the absence of PTN promoted hMSCs senescence (Fig. 2N-P), resembling the consequences of H2AZ1 overexpression. Importantly, the H2AZ1-induced senescent traits were ameliorated by PTN overexpression (Fig. 2Q and 2R). Collectively, these findings suggested that the suppression of PTN acts as a

crucial downstream event induced by H2AZ1, reinforcing cellular senescence.

Here, we pioneered studies that identify the role of histone variants in the context of stem cell senescence. Our results revealed that the elimination of H2AZ1 alleviated hMSCs senescence by suppressing PTN expression. As a corroboration of our findings, PTN expression was reduced across a spectrum of aged cells and tissues, supported by data available in the Aging Atlas database. Given the crucial role of histone variants in maintaining chromatin structures, which are implicated in various physiological or pathological processes, the H2AZ1-PTN axis unveiled in this study could have implications beyond cellular senescence, warranting further exploration. It is worth noting that our findings are based on a loss-of-function screening platform, leaving room for the possibility that certain histone variants may play a geroprotective role, as opposed to the pro-senescence function of H2AZ1. Therefore, a gain-of-function screening approach may offer novel insights into the roles of histone variants during aging (Jing et al., 2023).

In alignment with previous reports showing enrichment of nucleosomes incorporating H2AZ1 at promoter regions (Wen et al., 2020), our study revealed enrichment of H2AZ1 at both enhancer and promoter regions across the genome. This study provides the initial evidence that H2AZ1, functioning as a distal regulatory factor, exerts *trans*-repressive control over PTN, consequently promoting senescence in hMSCs. H2AZ1 is well-recognized for its involvement in cell fate determination and cell cycle regulation by replacing canonical H2A within chromosomes, subsequently affecting chromatin structure and gene expression. Hence, beyond its distal *trans*-regulatory role, exploring whether H2AZ1 plays a role in reshaping chromatin high-order structures during cellular senescence represents an exciting topic for further investigation. Furthermore, the upregulation of H2AZ1 and downregulation of PTN

means \pm SEMs. Two-tailed unpaired Student's t-test was performed. The white arrowheads in (H) indicate the Ki67-positive cells. (I) Immunofluorescence staining of H3K9me3 in RS hMSCs after CRISPRko of H2AZ1. Scale bars, 10 μ m. $n = 300$ cells from three biological replicates. Data are presented as the means \pm SEMs. Two-tailed unpaired Student's t-test was performed. (J) Western blot analysis of the indicated proteins in RS hMSCs after CRISPRko of H2AZ1. GAPDH was used as the loading control. $n = 3$ biological replicates. Quantitative data (right) are presented as the means \pm SEMs. Two-tailed unpaired Student's t-test was performed. (K) Bar plot showing Gene Ontology (GO) terms and pathways enrichment analysis for upregulated (red, left) and downregulated (blue, right) differentially expressed genes (DEGs) in RS hMSCs after CRISPRko of H2AZ1. (L) Western blot analysis of H2AZ1 and Flag in young hMSCs transduced with lentiviruses expressing GAL4 or H2AZ1. GAPDH was used as the loading control. $n = 3$ biological replicates. Quantitative data (right) are presented as the means \pm SEMs. Two-tailed unpaired Student's t-test was performed. (M-O) SA- β -gal staining (M), clonal expansion analysis (N), and immunofluorescence staining of Ki67 (O) in young hMSCs transduced with lentiviruses expressing GAL4 or H2AZ1. Scale bars, 100 μ m (M) and 10 μ m (O). $n = 3$ biological replicates. Data are presented as the means \pm SEMs. Two-tailed unpaired Student's t-test was performed. The white arrowheads in (O) indicate the Ki67-positive cells. (P) Immunofluorescence staining of H3K9me3 in young hMSCs transduced with lentiviruses expressing GAL4 or H2AZ1. Data are presented as the means \pm SEMs. Scale bars, 10 μ m. $n = 300$ cells from three biological replicates. Data are presented as the means \pm SEMs. Two-tailed unpaired Student's t-test was performed. (Q) Dot plot showing GO terms and pathways enrichment analysis for upregulated (red, left) and downregulated (blue, right) DEGs in young hMSCs transduced with lentiviruses expressing H2AZ1 compared to GAL4.

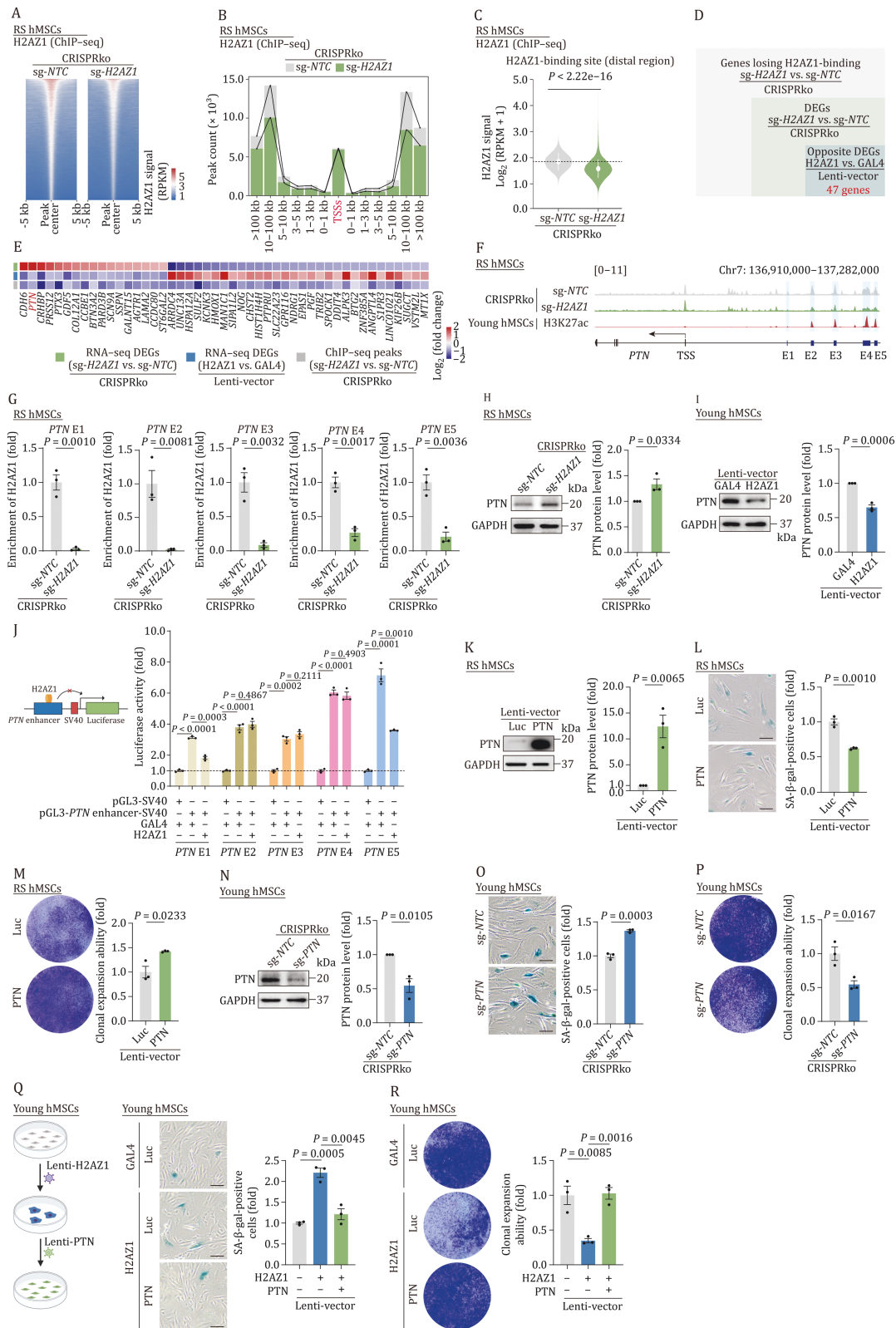


Figure 2. H2AZ1 binds and modulates the PTN enhancer to promote hMSCs senescence. (A) Heatmap showing the H2AZ1 ChIP-seq signals in RS hMSCs after CRISPRko of H2AZ1. The color key from blue to red indicates signals (Reads Per Kilobase Million, RPKM) from low to high. (B) Bar plot showing the distance from H2AZ1-binding sites to TSSs in RS hMSCs after CRISPRko of H2AZ1. (C) Violin plot showing the H2AZ1 ChIP-seq signals at H2AZ1-binding distal regions (3 kb away from TSSs) in RS hMSCs after CRISPRko of H2AZ1. P value was calculated by a two-sided Wilcoxon signed-rank test. (D) Venn diagram showing the number (47) of genes regulated by H2AZ1 via binding at distal regions overlapping with DEGs caused by CRISPRko of H2AZ1 in RS hMSCs but reversed by H2AZ1 overexpression in young hMSCs. (E) Heatmap showing H2AZ1-regulating genes that are differentially expressed

were observed in the muscles of aged monkeys (Jing et al., 2022). These suggested that targeting H2AZ1-PTN axis might offer a promising approach to ameliorating senescence in various cell types and addressing age-related diseases.

In this study, we employed a CRISPR/Cas9-based screening approach to systematically investigate the role of histone variants in human stem cell senescence and uncovered H2AZ1 as a novel driver of hMSCs senescence. Our results not only advance our comprehension of the functions of histone variants in aging but also lay the groundwork for further explorations on H2AZ1 as a potential target for intervention in aging and aging-related diseases.

Supplementary data

Supplementary data is available at <https://doi.org/10.1093/procel/pwae035>.

Footnotes

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J.Q., S.S., W.Z., and G.-H.L. conceptualized the work and supervised overall experiments. M.-H.L. performed the cell culture, CRISPR screening, and phenotypic and mechanistic analyses. X.J. performed bioinformatics analyses. Y.J. performed plasmid construction, RT-qPCR. K.Y. performed luciferase reporter analysis. S.-J.B. performed ChIP-qPCR. S. W. performed immunofluorescence staining. S.M. helped with bioinformatics analyses. M.-H.L., X.J., and Y.J. performed data analysis. J.Q., S.S., W.Z., G.-H.L. M.-H.L., X.J., and Y.J. performed manuscript writing, review, and editing. All authors reviewed the manuscript.

All data associated with this study are present in the paper or the Supplementary Materials. The raw data of screening, WGS data, transcriptomic data, and ChIP-seq data obtained have been deposited in the Genome Sequence Archive in the National Genomics Data Center,

in RS hMSCs by CRISPRko of H2AZ1 but reversed in H2AZ1-transduced young hMSCs. The color key from blue to red indicates the Log_2 (fold change) of DEGs and the differential peak from low to high. (F) Snapshot showing the binding pattern of H2AZ1 on distal regions of PTN in H2AZ1-deficient RS hMSCs and the H3K27ac signals on distal regions of PTN in young hMSCs. H3K27ac ChIP-seq data were obtained from a previous study (Liu et al., 2022). The enhancers are marked with blue squares. (G) ChIP-qPCR analysis of the enrichment of H2AZ1 on the PTN enhancers in RS hMSCs after CRISPRko of H2AZ1. $n = 3$ biological replicates. Data are presented as the means \pm SEMs. Two-tailed unpaired Student's *t*-test was performed. (H and I) Western blot analysis of PTN in RS hMSCs after CRISPRko of H2AZ1 (H) and young hMSCs transduced with lentiviruses expressing GAL4 or H2AZ1 (I). GAPDH was used as the loading control. $n = 3$ biological replicates. Data are presented as the means \pm SEMs. Two-tailed unpaired Student's *t*-test was performed. (J) Luciferase reporter assay measuring the enhancer activity of PTN E (1–5) by transfecting pGL3-PTN E (1–5)-luciferase and Renilla plasmids into H2AZ1-transduced hMSCs. The pGL3-promoter plasmid was used as a negative control. $n = 3$ biological replicates. Data are presented as the means \pm SEMs. Two-tailed unpaired Student's *t*-test was performed. (K, N) Western blot analysis of PTN in RS hMSCs transduced with lentiviruses expressing Luc or PTN (K) and young hMSCs after CRISPRko of PTN (N). GAPDH was used as the loading control. $n = 3$ biological replicates. Data are presented as the means \pm SEMs. Two-tailed unpaired Student's *t*-test was performed. (L and O) SA- β -gal staining of RS hMSCs transduced with lentiviruses expressing Luc or PTN (L), and young hMSCs after CRISPRko of PTN (O). Scale bars, 100 μm . $n = 3$ biological replicates. Data are presented as the means \pm SEMs. Two-tailed unpaired Student's *t*-test was performed. (M and P) Clonal expansion analysis of RS hMSCs (EP, early passage; LP, late passage) transduced with lentiviruses expressing Luc or PTN (M), and young hMSCs after CRISPRko of PTN (P). $n = 3$ biological replicates. Data are presented as the means \pm SEMs. Two-tailed unpaired Student's *t*-test was performed. (Q and R) SA- β -gal staining (Q) and Clonal expansion analysis (R) of H2AZ1-transduced young hMSCs after transduced with lentiviruses expressing PTN. Scale bars, 100 μm (Q). $n = 3$ biological replicates. Data are presented as the means \pm SEMs. Two-tailed unpaired Student's *t*-test was performed.

Beijing Institute of Genomics (China National Center for Bioinformatics) of the Chinese Academy of Sciences, under accession number HRA005568. The raw H3K27ac ChIP-seq data for WT hMSCs: HRA001144 (Liu et al., 2022).

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