



NSUN2 suppresses ferroptosis via the KLF4/GPX4 axis to preserve stem cells and tissues

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ABSTRACT

RNA 5-methylcytosine (m^5C) methylation has emerged as an important epitranscriptomic regulator of gene expression, yet its role in stem cell maintenance remains not well understood. Here we identify a previously unrecognized function of the m^5C methyltransferase NSUN2 in protecting stem cells from ferroptosis and promoting tissue regeneration. NSUN2 deletion reduced GPX4 expression, decreased intracellular GSH levels, increased Fe^{2+} accumulation, and enhanced lipid peroxidation in stem cells, leading to ferroptotic cell death, loss of tissue stem cells and impaired tissue regeneration across multiple organs. Mechanistically, NSUN2 catalyzed m^5C methylation of *Klf4* mRNA, thereby increasing KLF4 expression and transcriptionally promoting GPX4 to suppress ferroptosis in stem cells. Importantly, KLF4 restoration *in vivo* rescued NSUN2 deficiency-induced stem cell defects and impaired tissue regeneration. Furthermore, NSUN2 gene therapy significantly bolstered tissue stem cells with superior tissue regeneration and repair in multiple tissues. These findings highlight the NSUN2/KLF4/GPX4 axis in safeguarding stem cells against ferroptosis for tissue repair and a promising therapeutic value of NSUN2 in regenerative medicine.

1. Introduction

Stem cell deficiency is a significant issue that underlies a variety of pathologies. For instance, in conditions such as Duchenne muscular dystrophy (DMD), age-associated sarcopenia, traumatic injuries, and amyotrophic lateral sclerosis (ALS) [1–3], insufficient numbers of

muscle stem cells (MuSCs), which are capable of self-renewal to replenish the stem cell pool and generate new myoblasts essential for muscle regeneration, can result in muscle loss and defective tissue repair or regeneration [4]. In the eye, limbal stem cells (LSCs) located at the ocular limbus are vital for the repair and regeneration of the corneal epithelium, which is crucial for maintaining visual acuity [5]. LSCs'

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deficiencies, which occur in many ocular diseases such as infectious keratitis, genetic disorders (e.g., aniridia-associated keratopathy), and thermal or chemical injuries, are one of the leading causes of blindness [6–10]. Given their enormous potential in treating diseases characterized by tissue degeneration or injury [11], it is critical to best preserve stem cells, and there is an urgent need for therapeutic agents capable of effectively preserving stem cells.

Stem cell maintenance requires precise regulation of gene expression, which is dynamically controlled by multiple epigenetic mechanisms [12,13]. In addition to DNA and histone modifications, emerging

evidence highlights the importance of RNA modification in stem cells. To date, over 160 types of RNA modifications have been identified [14]. Among them, RNA 5-methylcytosine (m^5C) modification (methylation of the C5 position of the cytosine residues in RNAs) is a conserved and widely distributed mechanism [15]. It plays a pivotal role in regulating multiple aspects of RNA functions, including RNA stability, transport, and translation [16] to modulate diverse biological processes, such as development, cellular differentiation, and tumorigenesis [17–20]. In mouse embryonic stem cells, transcriptome-wide analyses revealed globally elevated and broadly distributed m^5C modifications, suggesting

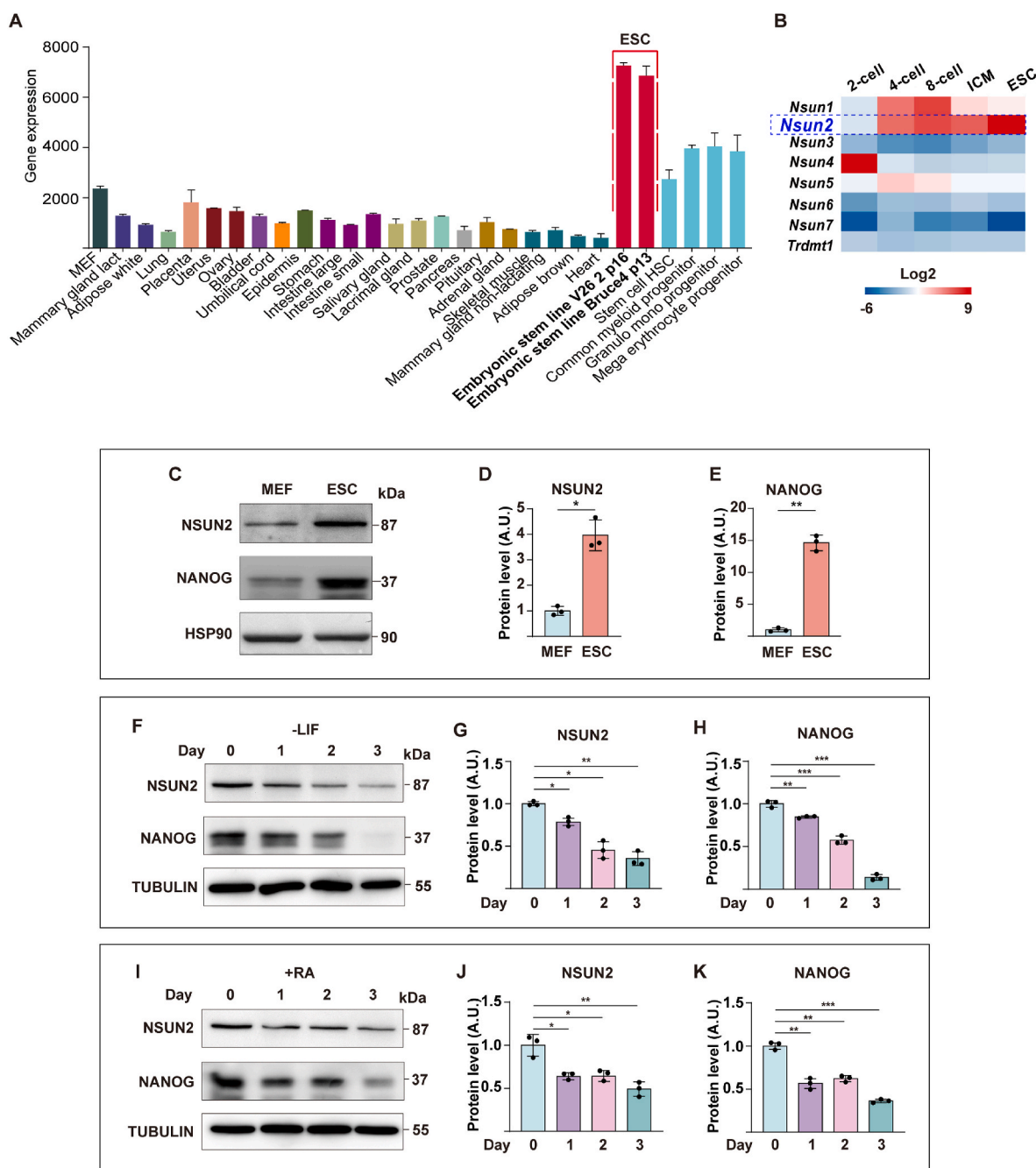


Fig. 1. NSUN2 is most abundantly expressed in stem cells. (A) *Nsun2* is most abundantly expressed in two mouse ESC lines as shown by the BioGPS Gene Expression Atlas analysis. (B) RNA-sequencing (GSE22182) results showing that among the m^5C RNA methyltransferase family, *Nsun2* is the only one enriched in the early stage of mouse embryos and ESCs. (C–E) Western blot analysis showing the expression of NSUN2 and NANOG in mouse embryonic fibroblasts (MEFs) and ESCs. (F–K) Western blot showing decreased NSUN2 and NANOG upon ESC differentiation induced by leukemia inhibitory factor withdrawal (-LIF) (F–H) and retinoic acid treatment (+RA) (I–K). All data are presented as mean $\pm$ SD, $n = 3$ in C to K. Statistical significance was determined using one-sample *t*-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

a complex regulatory landscape in stem cells. Moreover, RNA m⁵C is highly enriched during early embryonic development across multiple species [21]. However, the precise roles and underlying mechanisms of RNA m⁵C modification in preserving stem cells under stress conditions remain poorly defined.

RNA m⁵C modification is catalyzed by the NOP2/Sun domain methyltransferase family, including NSUN1-7 and DNMT2 [22]. Among them, NSUN2 is a key RNA methyltransferase [16,23]. NSUN2 plays important roles in diverse cellular processes, including cell proliferation, migration, and senescence [24–26]. Notably, studies from Michaela Frye and colleagues demonstrated that NSUN2 is essential for maintaining epidermal stem cell homeostasis by balancing their self-renewal and differentiation [27–30]. Moreover, NSUN2 has been implicated in regulating stem cell lineage commitment. For example, NSUN2 depletion impairs the differentiation of germ cells, neuroepithelial stem cells, and tendon stem cells [31,32], whereas NSUN2 has also been reported to promote senescence in dental pulp stem cells [33]. Consistently, *Nsun2* knockout mice display neurodevelopmental defects [34]. Despite these advances, how NSUN2-mediated RNA m⁵C modification regulates stem cell survival and adaptation to stress during tissue damage and regeneration remains not well understood.

In this study, we present our novel discovery highlighting the pivotal role of NSUN2 in protecting the stem cell pool through a newly identified mechanism involving the NSUN2-KLF4-GPX4 axis to mitigate ferroptosis in stem cells. Notably, in models of corneal and muscle injury, NSUN2 gene therapy significantly augmented the population of tissue stem cells and enhanced tissue repair and regeneration. These findings underscore the potential therapeutic significance of NSUN2 in stem cell-based therapies for various degenerative diseases and injuries.

2. Results

2.1. NSUN2 is most abundantly expressed in stem cells

To understand the potential effect of NSUN2 on stem cells, we investigated the expression of NSUN2 across 29 different types of cells and tissues (BioGPS), and the highest abundance of NSUN2 was found in embryonic stem cells (ESCs) compared to other cell types (Fig. 1A). Notably, NSUN2 stands out among the RNA m⁵C methyltransferase family members for its predominant expression in ESCs with a gradual decrease of expression levels during embryonic development (Fig. 1B, GSE22182). Indeed, Western blot analysis confirmed significantly elevated NSUN2 levels in ESCs compared to differentiated cells, such as mouse embryonic fibroblasts (MEFs), mirroring the expression pattern of the master pluripotency marker NANOG (Fig. 1C–E). Furthermore, upon differentiation induction by leukemia inhibitory factor withdrawal (-LIF) or retinoic acid treatment (+RA), NSUN2 levels decreased markedly alongside the loss of NANOG (Fig. 1F–K). These findings strongly suggest a regulatory role of NSUN2 in stem cell dynamics.

2.2. NSUN2 maintains stem cells

We further explored the impact of NSUN2 on stem cells using *Nsun2* knockout mice. At E3.5, a critical stage when ESCs originate from the inner cell mass (ICM), the viability of *Nsun2*^{−/−} embryos was significantly reduced (Fig. 2A), a trend that persisted through postnatal day 21 (Supplemental Fig. 1A). Additionally, the success rate of establishing ESC lines from *Nsun2*^{−/−} embryos was markedly lower compared to their wild-type counterparts (Fig. 2B). ESCs lacking NSUN2 exhibited diminished stemness characteristics evidenced by smaller teratomas (Fig. 2C and D), increased differentiation markers of germ layers (Fig. 2E), fewer alkaline phosphatase positive (AP⁺) colonies (Fig. 2F and G), and more differentiated embryoid bodies (EBs) with reduced expression of the key pluripotency markers NANOG, SOX2, and OCT4 (Fig. 2H–K, Supplemental Fig. 1B) along with elevated levels of germ layer markers (Supplemental Fig. 1C). Moreover, NSUN2 knockdown in

ESCs using three different siRNAs (Fig. 2L and M) recapitulated these phenotypes by showing enhanced ESC differentiation with flattened morphology (Fig. 2N, upper panel), reduced AP staining (Fig. 2N, lower panel), and decreased AP⁺ colonies (Fig. 2O) accompanied by decreased levels of *Nanog*, *Oct4*, *Sox2*, and *Klf7* (Supplemental Fig. 1D–J) and increased expression of the differentiation markers *Sox17*, *Nestin*, and *Brachyury* (Supplemental Fig. 1K). On the other hand, NSUN2 overexpression induced by doxycycline (Dox) enhanced ESC stemness characteristics as evidenced by increased levels of NANOG, OCT4, and SOX2 (Supplemental Fig. 2A–E), and larger numbers and sizes of AP⁺ colonies (Supplemental Fig. 2F–H).

We then investigated the importance of NSUN2's enzymatic activity for its effects on stem cells using an enzymatically inactive mutant of NSUN2 (NSUN2 dead) [35]. Our findings revealed that while overexpression of wild-type NSUN2 (NSUN2 WT) significantly enhanced ESC proliferation (Fig. 2P–R) and increased the size of ESC colonies (Fig. 2S and T), overexpression of the enzymatically dead mutant (NSUN2 dead) failed to do so (Fig. 2P–T), thus highlighting the critical role of NSUN2's enzymatic activity in maintaining stem cell characteristics.

2.3. NSUN2 inhibits stem cell ferroptosis to maintain stem cells

To elucidate the mechanisms underlying NSUN2's effect on stem cells, we conducted RNA sequencing (RNA-seq) on ESCs following NSUN2 knockdown (si*Nsun2*). This analysis revealed 447 differentially expressed genes (DEGs, fold change >1.5, *p* < 0.05) with 275 upregulated and 172 downregulated ones respectively (Fig. 3A). Gene set enrichment analysis (GSEA) showed that the downregulated DEGs in NSUN2 knockdown ESCs were predominantly associated with the glutathione metabolism pathway (Fig. 3B and C) crucial for stem cell maintenance and suppression of stem cell ferroptosis [36,37]. Indeed, *Nsun2*^{−/−} ESCs exhibited significantly reduced levels of glutathione (GSH), a pivotal antioxidant that protects cells against ferroptosis [38] (Fig. 3D). NSUN2 knockdown notably increased ESC susceptibility to ferroptosis as evidenced by enhanced FerroOrange staining (Fig. 3E and F) and elevated lipid peroxidation levels (a hallmark of ferroptosis) [39] upon treatment with Erastin, a ferroptosis inducer [40] (Fig. 3G and H). These findings collectively demonstrate a robust inhibitory role of NSUN2 in ESC ferroptosis.

2.4. GPX4 is a downstream effector of NSUN2 to inhibit stem cell ferroptosis

We then delved into the molecular mechanisms underlying NSUN2's role in inhibiting ESC ferroptosis. RNA-seq analysis following *Nsun2* knockdown revealed a significant decrease in the expression of glutathione peroxidase 4 (GPX4), a critical inhibitor of ferroptosis [41] (Fig. 3I). This finding was corroborated by both qRT-PCR and Western blot analyses (Fig. 3J–L), suggesting that GPX4 may act as a downstream effector of NSUN2. Indeed, like NSUN2, GPX4 knockdown also decreased ESC proliferation (Fig. 3M), and overexpression of GPX4 (Fig. 3N–P) rescued the loss of GSH, consistent with its ability to delay GSH depletion under H₂O₂-induced oxidative stress, reduced ROS levels, and restored impaired ESC proliferation induced by NSUN2 knockdown (Fig. 3Q–T). These results underscore the requirement of GPX4 for NSUN2's inhibitory effect on ESC ferroptosis.

2.5. NSUN2 catalyzes KLF4 m⁵C methylation to promote GPX4 expression

To investigate how NSUN2 modulates GPX4 expression, we utilized m⁵C-RNA immunoprecipitation sequencing (m⁵C-RIP-seq) using ESCs with *Nsun2* knockdown and identified 726 hypo- and 29 hyper-methylated m⁵C sites (Fig. 4A). Gene Ontology (GO) analysis of the hypo-methylated genes revealed enrichment in terms related to transcription regulation across both “molecular function” and “cellular

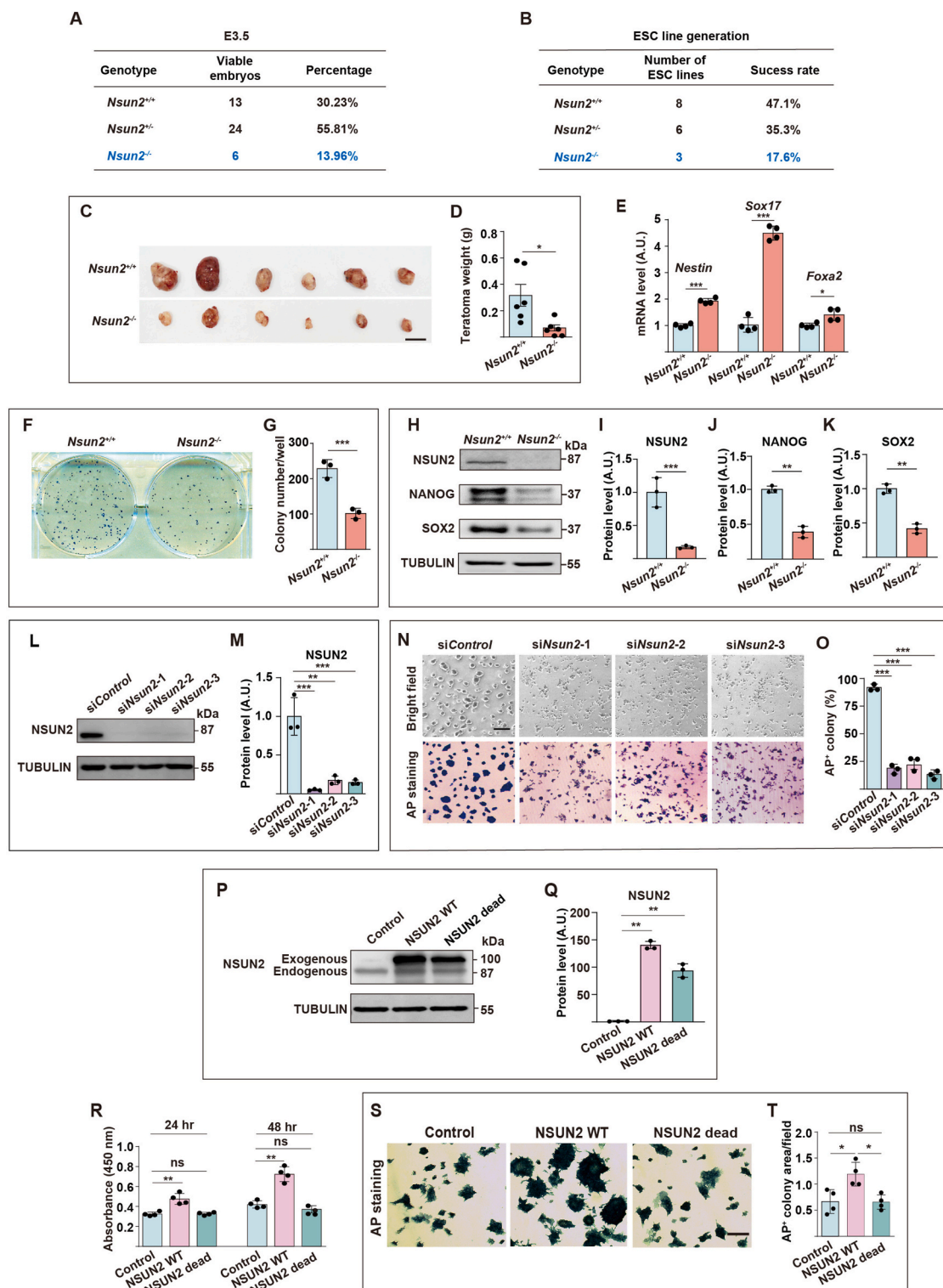


Fig. 2. NSUN2 maintains stem cells. (A) Distribution of genotypes of E3.5 (day post coitum, dpc) embryos from *Nsun2*^{+/-} heterozygous breeding. *Nsun2*^{+/+} n = 13, *Nsun2*^{+/-} n = 24, *Nsun2*^{-/-} n = 6. (B) Distribution of genotypes of ESCs generated from mouse blastocysts from *Nsun2*^{+/-} heterozygous breeding. *Nsun2*^{+/+} n = 8, *Nsun2*^{+/-} n = 6, *Nsun2*^{-/-} n = 3. (C–E) Teratoma formation of *Nsun2*^{+/+} and *Nsun2*^{-/-} ESCs (C). Scale bar: 1 cm. Quantification of the weights of teratoma are shown in (D). qRT-PCR showing the expression levels of differentiation markers (E). (F–G) Colony formation assay using AP staining of *Nsun2*^{+/+} and *Nsun2*^{-/-} ESCs (F). The quantification of AP-positive colonies was shown in (G). (H–K) Western blot showing the expression levels of pluripotency markers in *Nsun2*^{+/+} and *Nsun2*^{-/-} ESCs. (L,M) Western blot showing the knockdown of NSUN2 in ESCs by different siRNA oligos. (N,O) Images showing AP staining of siNsun2 or siControl ESCs (N). Scale bar: 400 μ m. Quantifications of AP-positive colonies are shown in O. (P,Q) Western blot showing the overexpression of NSUN2 WT and NSUN2 dead mutant. (R) CCK8 assay showing higher proliferation of NSUN2 WT-expressing ESCs than the NSUN2 dead-expressing ESCs at different time points. (S,T) NSUN2 WT- but not NSUN2 dead-ESCs formed more round and compact colonies with stronger AP staining (S) and more colonies (T). Scale bar: 200 μ m. All data are presented as mean $\pm$ SD, n = 3 in F–Q, n = 4 in E, R–T, and n = 6 in C, D. Statistical significance was determined using one-sample t-test in H–M and Q, one-way ANOVA in O, R and T, and Student's t-test in D, E, and G. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant.

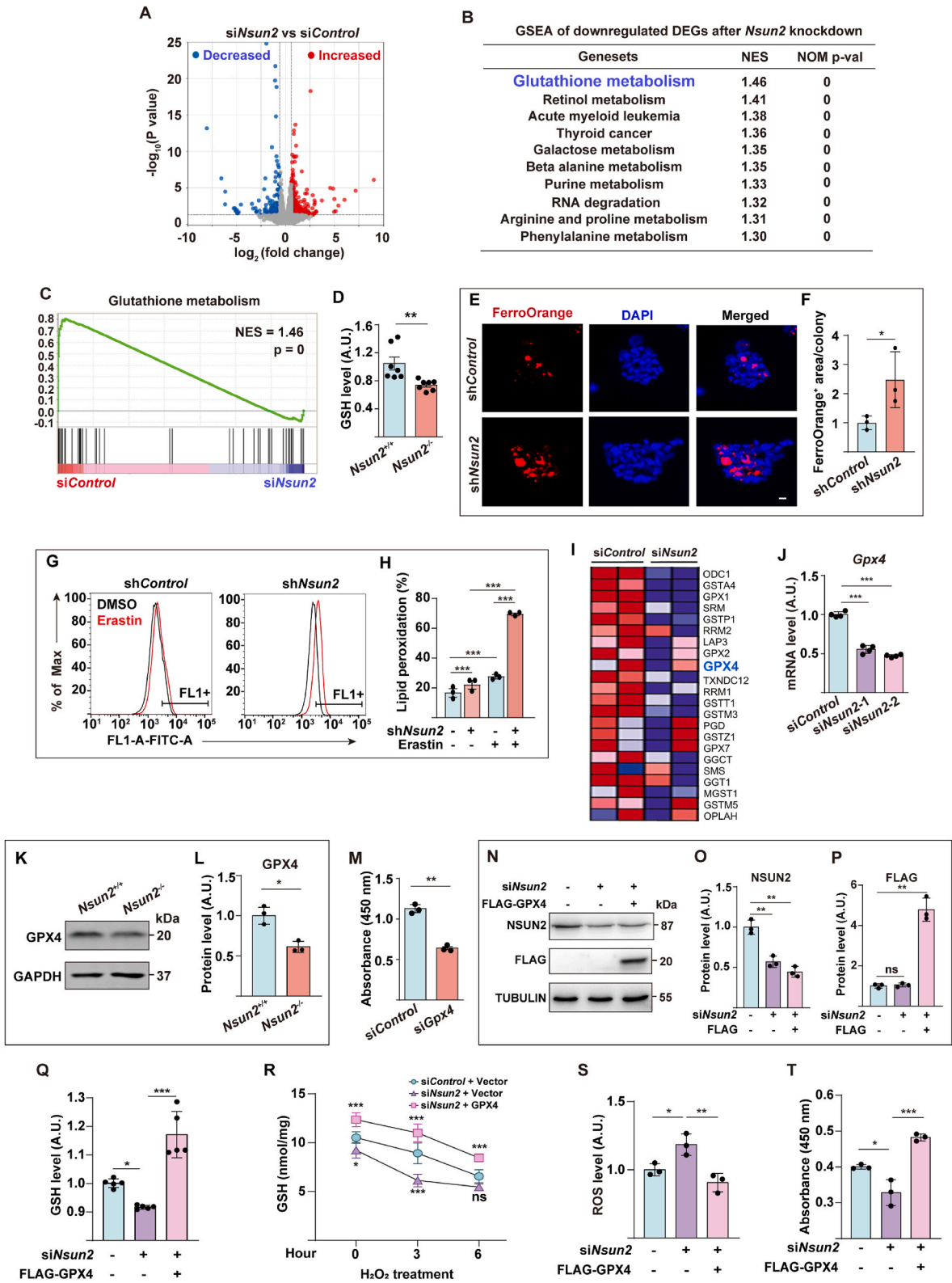


Fig. 3. NSUN2 promotes GPX4 expression to inhibit stem cell ferroptosis. (A) Volcano plot of 447 DEGs (>1.5 -fold, $p < 0.05$) from RNA sequencing results after *Nsun2* knockdown in ESCs. Blue, downregulated DEGs; red, upregulated DEGs; grey, no change. (B) Gene set enrichment analysis (GSEA) showing the top 10 enriched KEGG gene sets from ESCs after *Nsun2* knockdown. NES, nominal enrichment score; NOM p-val, Nominal p-value. (C) GSEA of the gene set “Glutathione metabolism” from siControl and si*Nsun2* ESCs. (D) GSH detection assay showing the levels of GSH in *Nsun2*^{+/+} and *Nsun2*^{-/-} ESCs. (E–F) Intracellular Fe²⁺ detected by FerroOrange in shControl and sh*Nsun2* ESCs (E). Quantification of relative ferroOrange fluorescence (F). Scale bar: 50 μ m. (G,H) Lipid peroxidation assessed by FACS analysis of C11-BODIPY fluorescence in sh*Nsun2* and shControl ESCs after Erastin treatment. (I) Heatmap showing the downregulation of GPX4 in the gene set “Glutathione metabolism” after *Nsun2* knockdown. (J) qRT-PCR showing the mRNA level of *Gpx4* after *Nsun2* knockdown. (K–L) Western blot showing the expression levels of GPX4 in *Nsun2*^{+/+} and *Nsun2*^{-/-} ESCs. (M) CCK8 assay showing decreased proliferation of ESCs with *Gpx4* depletion. (N–P) Western blot showing the expression level of NSUN2 and GPX4 in ESCs with *Nsun2* depletion or GPX4 overexpression. (Q) GSH detection assay showing that GPX4 overexpression rescued GSH levels in *Nsun2*-depleted ESCs. (R) Time-course GSH detection assay showing that GPX4 overexpression maintained higher GSH levels in *Nsun2*-depleted ESCs after H₂O₂ treatment. (S) Cellular ROS assay result demonstrating that GPX4 overexpression reduced ROS levels in *Nsun2*-depleted ESCs. (T) CCK8 assay showing that GPX4 overexpression rescued the impaired proliferation of *Nsun2*-depleted ESCs. All data are presented as mean $\pm$ SD, $n = 3$ in E, G, K–P, S and T. $n = 4$ in J and R. $n = 5$ in Q. $n = 7$ in D. Statistical significance was determined using one-way ANOVA in H, J, Q, S, T and two-way ANOVA in R. One-sample *t*-test was used in L and O–P, Student’s *t*-test was used in D, F and M. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

component” categories (Supplemental Fig. 3A and B). Next, we used the m⁵C-RIP-seq data together with an ESC-related transcription factor (TF) database [42] and identified four common transcription factors (Fig. 4B and Supplemental Table S1). Among them, kruppel-like transcription factor 4 (KLF4) stood out as essential for ESC self-renewal [43] and was also supported by its functional correlation with the key ESC self-renewal genes NANOG, OCT4, and SOX2 (Fig. 4C) as demonstrated by the STRING analysis of the top 10 hypo-methylated genes from the KEGG term “Signaling pathways regulating pluripotency of stem cells” (Supplemental Fig. 3C). m⁵C-Atlas database revealed abundant m⁵C enrichment on *Klf4* mRNA, but nearly none on *Gpx4* mRNA instead (Supplemental Fig. 3D). Indeed, our m⁵C-RIP-seq result showed that *Nsun2* knockdown markedly abolished m⁵C modifications on *Klf4* mRNA (Supplemental Fig. 3E and F).

We then investigated whether KLF4 is a substrate of NSUN2 and found that NSUN2 knockdown significantly reduced both *Klf4* mRNA m⁵C methylation (Fig. 4D) and KLF4 protein levels in ESCs (Fig. 4E–G), while the m⁵C level of *Gpx4* mRNA remained unchanged (Supplemental Fig. 3G), suggesting a direct role of NSUN2 in modulating KLF4 expression given the regulatory effect of NSUN2-mediated RNA m⁵C methylation in gene expression [44,45]. Importantly, overexpression of wild-type NSUN2 (NSUN2 WT), but not the enzymatically inactive mutant (NSUN2 dead), increased KLF4 protein levels (Supplemental Fig. 3H–J), demonstrating that NSUN2 promotes KLF4 expression in an m⁵C-dependent manner.

Next, we investigated whether KLF4 binds to the *Gpx4* promoter using a chromatin immunoprecipitation (ChIP)-qRT-PCR assay and found specific binding of KLF4 to the P1 region (–690 to –500 bp) of the mouse *Gpx4* promoter (Fig. 4H). Importantly, KLF4 knockdown significantly reduced GPX4 expression (Fig. 4I–K), whereas KLF4 overexpression rescued the loss of GPX4 caused by NSUN2 knockdown (Fig. 4L–O). To further determine whether m⁵C modification of KLF4 is required for GPX4 regulation, we generated three individual KLF4 mutants (C611G, C1276T, and C1860T) targeting the top-ranked predicted m⁵C sites identified by the RNA m⁵C finder database (Supplemental Fig. 3K), together with a triple mutant containing simultaneous substitution of all the three m⁵C sites (C611G/C1276T/C1860T). Compared with wild-type KLF4, each single-site mutant displayed reduced ability to restore GPX4 expression in NSUN2-deficient ESCs, whereas the triple mutant showed the least GPX4 rescue (Fig. 4P–R). Consistently, while wild-type KLF4 suppressed intracellular ROS levels, the three m⁵C-site mutants showed diminished protective effects, with the triple mutant displaying the lowest activity (Fig. 4S). Moreover, wild-type KLF4 overexpression mitigated NSUN2 knockdown-induced ferroptosis in ESCs as evidenced by reduced FerroOrange staining (Fig. 4T and U) and decreased lipid peroxidation (Fig. 4V). These findings collectively demonstrate that m⁵C methylation is functionally required for KLF4 to promote GPX4 expression and suppress stem cell ferroptosis downstream of NSUN2.

2.6. NSUN2 maintains muscle stem cell pool to promote muscle regeneration

Stem cell deficiency remains a significant challenge in tissue repair and regeneration [1,13]. NSUN2 is upregulated in activated muscle stem cells (GSE104543) [46], suggesting a potential effect of NSUN2 on muscle stem cells. To investigate this, we employed a cardiotoxin (CTX)-induced muscle injury model [47] (Fig. 5A) and performed AAV-mediated NSUN2 gene therapy (AAV-NSUN2, Fig. 5B and C). We found that AAV-NSUN2 treatment increased the population of muscle stem cells, as indicated by the increased PAX7⁺ (a muscle stem cell marker [48]) and Ki67⁺ cells (PAX7⁺ Ki67⁺, Fig. 5D and E). NSUN2 overexpression also augmented the numbers of activated (PAX7⁺MyoD⁺, Supplemental Fig. 4A and B) and self-renewing muscle stem cells (PAX7⁺MyoD⁺, Supplemental Fig. 4A and C) [49]. Most importantly, NSUN2 gene therapy improved muscle regeneration as evidenced by the larger myofiber areas with better organized muscle structures (Fig. 5F and G) and decreased muscle atrophy by the decreased weight of atrophied muscles (Fig. 5H), demonstrating improved muscle regeneration with increased muscle stem cell population following NSUN2 gene therapy.

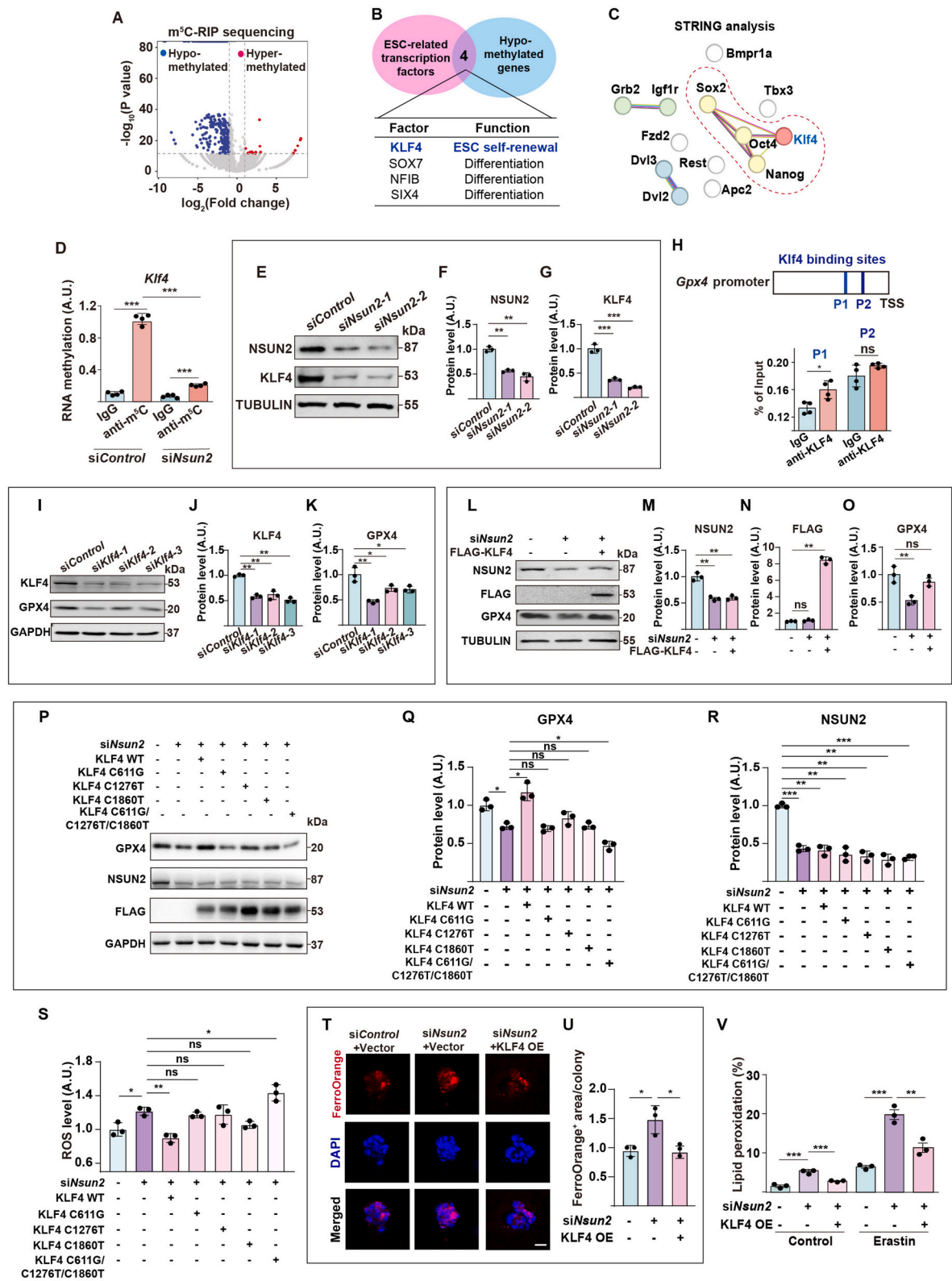
Furthermore, *Nsun2*^{+/-} mice exhibited severely reduced KLF4 and GPX4 levels, alongside impaired myofiber regeneration post-injury (Fig. 5I–N). Notably, AAV-KLF4 treatment significantly restored GPX4 expression and increased the sizes of the regenerating myofibers in *Nsun2*^{+/-} mice (Fig. 5I–N), supporting the importance of the NSUN2/KLF4 axis for tissue regeneration *in vivo*.

2.7. NSUN2 increases the number of putative limbal stem cells and facilitates corneal wound healing

Limbal stem cell deficiency is a debilitating condition resulting from the lack of limbal stem cells [50]. Building on our discovery of NSUN2’s robust role in stem cell maintenance, we investigated its potential to enhance limbal stem cells and facilitate corneal repair/regeneration (Fig. 6A). Using a corneal wound healing model [51], we employed AAV-mediated NSUN2 overexpression (AAV-NSUN2) in mouse corneas (Fig. 6B and C). We found that AAV-NSUN2 treatment increased the population of putative limbal stem cells as evidenced by enhanced P63⁺ (a putative limbal stem cell marker [52]) and Ki67⁺ cells (Fig. 6D and E) with significantly improved corneal wound healing as shown by the reduced wound areas (Fig. 6F and G). These results thus highlight the substantial importance of NSUN2 in augmenting putative limbal stem cell numbers and promoting corneal wound healing. Moreover, *Nsun2*^{+/-} mice displayed markedly fewer P63⁺Ki67⁺ proliferating putative limbal stem cells and impaired epithelial healing post-injury, which was rescued by AAV-KLF4 treatment (Fig. 6H–K).

3. Discussion

In this study, we identify a previously unrecognized role of NSUN2 in



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Fig. 4. NSUN2 catalyzes KLF4 m⁵C methylation to promote GPX4 expression. **(A)** A volcano plot of differentially methylated genes (>1.5-fold, $p < 0.05$) from m⁵C-RIP sequencing after *Nsun2* knockdown in ESCs. Red and blue indicate hyper- and hypo-methylated genes respectively. **(B)** Venn diagram showing the 4 overlapping transcription factors from the hypo-methylated genes from the m⁵C-RIP-seq results and the ESC-related transcription factors from the TF-Marker database. **(C)** STRING analysis showing that *Klf4* may be functionally related to the core pluripotency factors Oct4, Nanog and Sox2. **(D)** m⁵C-RIP qRT-PCR assay validated *Klf4* RNA m⁵C levels in siControl or si*Nsun2* ESCs. IgG was used as a control. **(E–G)** Western blot showing the expression level of KLF4 after *Nsun2* knockdown. **(H)** Upper panel: Putative Klf4 binding sites of mouse *Gpx4* promoter. Lower panel: ChIP-qPCR assay validating the binding of Klf4 to the *Gpx4* promoter. Locations of P1 and P2 are noted. TSS: transcription start site. **(I–K)** Western blot showing the expression level of GPX4 after *Klf4* knockdown in ESCs. **(L–O)** Western blot showing the expression level of GPX4 in *Nsun2*-depleted and KLF4-overexpressing ESCs. **(P–R)** Western blot analysis showing the expression levels of NSUN2, FLAG-KLF4, and GPX4 in *Nsun2*-depleted ESCs overexpressing wild-type KLF4 or KLF4 mutants. Quantification of NSUN2 and GPX4 protein levels is shown in Q, R. **(S)** Cellular ROS assay showing ROS levels in *Nsun2*-depleted ESCs overexpressing wild-type KLF4 or KLF4 mutants. **(T,U)** Intracellular Fe²⁺ detected by FerroOrange in *Nsun2*-depleted and KLF4-overexpressing ESCs. Quantification of the relative FerroOrange⁺ area in (U). Scale bar: 20 μ m. **(V)** Lipid peroxidation analysis using FACS analysis of C11-BODIPY fluorescence in *Nsun2*-depleted and KLF4-overexpressing ESCs after Erastin treatment. All data are presented as mean $\pm$ SD, $n = 3$ in E, I and L–V, $n = 4$ in D and H. Statistical significance was determined using one-way ANOVA in D, T, S, U and V, one-sample t -test in F–G, J–K, M – O and Q–R, and Student's t -test in H. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

protecting stem cells from ferroptosis through the KLF4–GPX4 axis, thereby promoting stem cell preservation and facilitating tissue regeneration following injury (Fig. 7). Mechanistically, the protective function of NSUN2 depends on its methyltransferase activity, which enhances the expression of KLF4 and GPX4 and consequently suppresses ferroptosis in stem cells. Importantly, *in vivo* restoration of KLF4 in NSUN2-deficient mice restored GPX4 expression and improved tissue regeneration, supporting the importance of the NSUN2/KLF4/GPX4 pathway in stem cell-driven tissue repair. Notably, NSUN2 gene therapy markedly increased tissue stem cell numbers and improved regenerative capacity in both corneal and skeletal muscle injury models.

NSUN2 was initially identified as a downstream target of Myc that promotes proliferation and tumorigenesis of human keratinocytes [27, 53]. Subsequent studies established NSUN2 as a key m⁵C “writer” [54] that catalyzes m⁵C modification across multiple RNAs, including tRNA [29,55,56], mRNA [30,32], and non-coding RNA [28], thereby modulating diverse aspects of RNA metabolism and gene expression. NSUN2 has been implicated in the regulation of tumorigenesis [57,58]. Emerging studies have also highlighted its effect on stem cells and progenitor cells, with a major focus on stem cell lineage differentiation in epidermal, germline, and neuroepithelial systems during development [27,31,55], whereas whether NSUN2 regulates stem cell survival or tissue regeneration remains poorly understood. Our current findings, however, mainly focused on the role of NSUN2 in safeguarding stem cells against ferroptosis for tissue regeneration through m⁵C modification of KLF4 RNA. Additionally, NSUN2 may also promote tissue repair through mechanisms including regulation of stem cell proliferation and differentiation, which require further investigation.

Beyond mechanistic insight, our study suggests translational potential for modulating RNA m⁵C signaling in regenerative medicine. NSUN2 gene therapy in adult mice enhanced the self-renewal and proliferation of both muscle and limbal stem cells, leading to better tissue regeneration and repair in both corneal and muscle injuries. Therefore, our study suggests that NSUN2-based gene therapy may offer a novel regenerative strategy to protect stem cells and reinforce tissue repair capacity during tissue repair. Interestingly, analysis of publicly transcriptomic databases indicates that *Nsun2* may not be reduced during aging (GSE229853) [59] and could even be elevated in aged muscle stem cells (GSE152797) [60], possibly reflecting an adaptive response to increased oxidative stress during aging. Noteworthy, in the aged muscle stem cells, a low *Nsun2* expression level is associated with a low GSH state (GSE152797) [60], consistent with our finding that NSUN2 preserves intracellular redox homeostasis. Future studies on tissue injury models using aged animals will be required to determine whether augmenting NSUN2 can enhance tissue regeneration during aging. AAV represents a clinically validated approach for *in vivo* gene delivery and durable transgene expression [61]. NSUN2 is expressed in various cell types, and its downstream targets may vary across tissues, underscoring the need to optimize cell type-specific gene delivery, e.g., tissue-specific promoters and/or engineered capsids to ensure safety and efficacy. This consideration is particularly relevant given the pro-tumorigenic role of NSUN2

reported [57,58], warranting localized delivery, controlled expression levels, and long-term surveillance in future translational studies [62].

The target genes or their modification sites of NSUN2 appear to be cell-type specific. For example, in cardiomyocytes, NSUN2 regulates NCOA4 m⁵C to modulate cellular state [63]. By contrast, in hepatocytes, NSUN2 was shown to regulate GPX4 expression through the m⁵C modification of *Gpx4* mRNA [64]. In this work, we found that NSUN2 knockdown did not significantly alter *Gpx4* mRNA m⁵C modification as shown by m⁵C-RIP-seq, m⁵C-RIP-qPCR, and public dataset analyses, supporting that in ESCs, NSUN2 regulates GPX4 expression mainly through KLF4, possibly due to cell identity-specific remodeling of epitranscriptomic regulation [65,66]. Indeed, as pluripotent cells, ESCs rely on core transcription factors, such as KLF4, OCT4, and SOX2, to coordinate self-renewal and redox homeostasis [67], thereby more likely favoring regulation through upstream transcriptional nodes, such as KLF4. Together, our findings define a NSUN2-KLF4-GPX4 epigenetic-transcriptional axis in the regulation of ESC ferroptosis and show a cell-specific nature of m⁵C-mediated RNA regulation.

KLF4 is a key transcription factor crucial for stem cell self-renewal, functioning in concert with core pluripotency regulators, such as OCT4 and SOX2 [68] [69]. KLF4 expression is tightly controlled by multiple regulatory mechanisms, including transcriptional signaling pathways, epigenetic modifications, and post-translational regulation [70–75]. However, the contribution of RNA post-transcriptional modifications to KLF4 regulation remained largely unexplored. In this work, we show that NSUN2 maintains KLF4 expression through its m⁵C methylation to sustain stem cell pools and tissue regeneration. Furthermore, mutagenesis of the predicted m⁵C sites on *Klf4* impaired its ability to restore GPX4 expression and suppress oxidative stress, supporting the functional importance of KLF4 m⁵C modification. Yet, the m⁵C-RIP-seq approach utilized in our work lacks single-nucleotide resolution [76,77], future studies using higher-resolution mapping techniques will be required to define the complete functional m⁵C landscape of *Klf4* mRNA.

In conclusion, our study unveils a pivotal role for NSUN2 in maintaining stem cells and fostering tissue regeneration. NSUN2 mediates RNA m⁵C modification of KLF4 to induce GPX4 expression, which subsequently shields stem cells from ferroptosis. These findings underscore NSUN2's therapeutic potential for regenerative interventions aimed at treating diverse degenerative diseases and tissue injuries.

4. Materials and methods

4.1. ESC cell line and cell culture

The mouse ESC line E14 (iCell, China) and A172-loxP (a kind gift of Dr. Thomas P. Zwaka, the Icahn School of Medicine at Mount Sinai) were maintained in tissue culture dishes coated with 0.1% gelatin (Sigma) in KnockOut™ DMEM (Gibco) supplemented with 15% (v/v) fetal bovine serum (FBS, Gibco), β -mercaptoethanol (0.1 mM), GlutaMax-I supplement (2 mM), MEM non-essential amino acids (0.1 mM) and leukemia

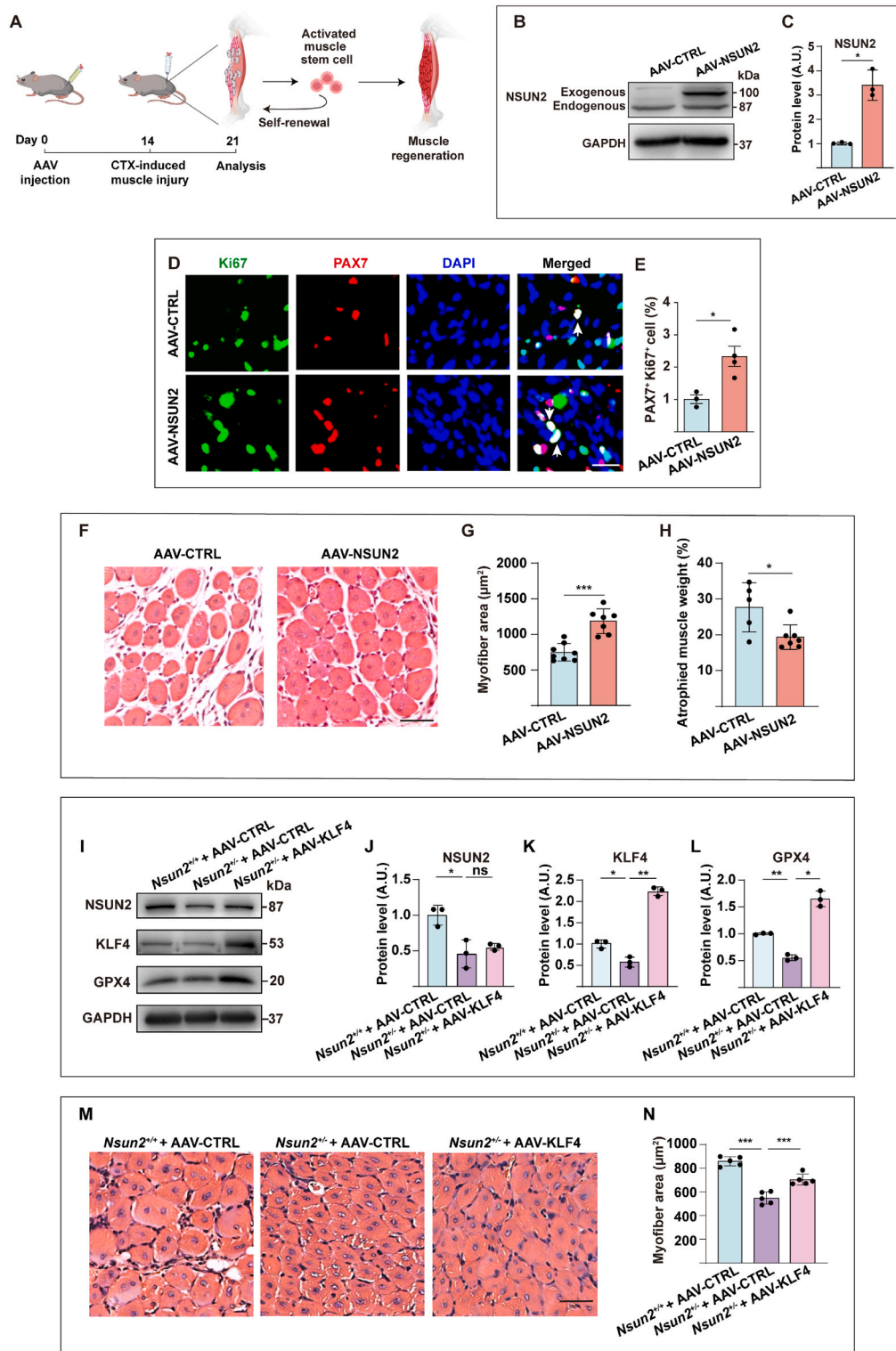


Fig. 5. NSUN2 maintains muscle stem cells and promotes muscle regeneration. **(A)** Schematic representation of the CTX-induced muscle injury model. **(B,C)** Western blot result showing the overexpression of NSUN2 by AAV in tibialis anterior. **(D,E)** Immunofluorescence images of Ki67 (green), PAX7 (red), and DAPI (blue) in the NSUN2-overexpressing muscles in the CTX injury model **(D)**. Scale bar: 100 μ m. The quantification of PAX7⁺Ki67⁺ cells is shown in **E**. **(F–H)** HE staining **(F)** showing that muscles overexpressing NSUN2 formed larger myofiber areas **(G)** and less muscle atrophy **(H)** in the CTX injury model. Scale bar: 100 μ m. **(I–L)** Western blot result showing the protein levels of NSUN2, KLF4, and GPX4 in the tibialis anterior muscles from AAV-KLF4-treated *Nsun2*^{+/-} mice. **(M,N)** HE staining **(M)** demonstrating that AAV-KLF4 treatment increased the sizes of regenerating myofibers **(N)** in *Nsun2*^{+/-} mice in a CTX injury model. Scale bar: 100 μ m. All data are presented as mean $\pm$ SD, $n = 3$ in **B** and **I**, $n = 5$ in **M**, $n = 3$ and 4 for AAV-CTRL and AAV-NSUN2 group respectively in **D**, $n = 8$ and 7 for AAV-CTRL and AAV-NSUN2 group respectively in **F**, $n = 5$ and 7 for AAV-CTRL and AAV-NSUN2 group respectively in **H**. Statistical significance was determined using one-sample *t*-test in **C** and **J–L**, Student's *t*-test in **E**, **G**, **H**, one-way ANOVA in **N**. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

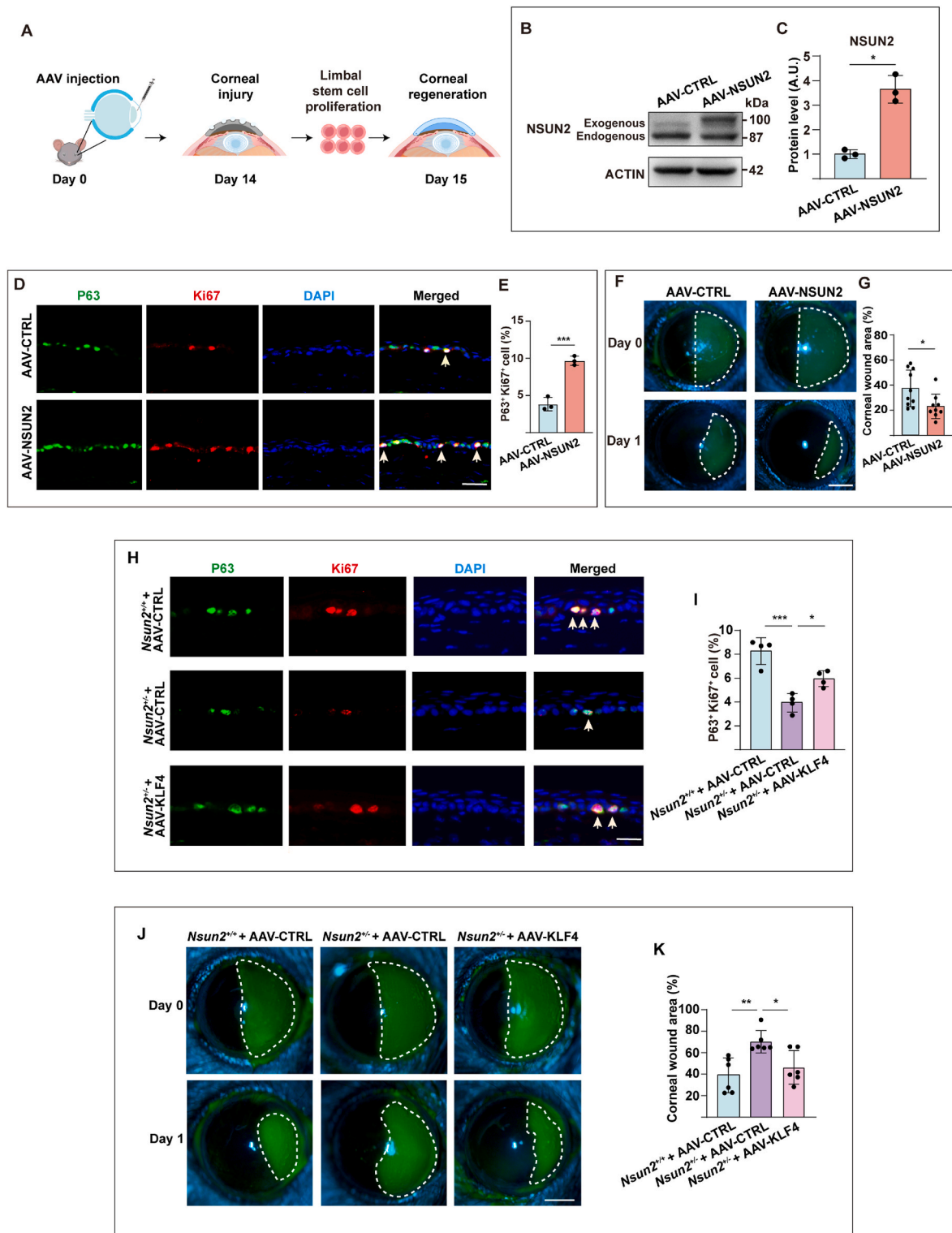


Fig. 6. NSUN2 increases the number of limbal stem cells and facilitates corneal wound healing (A) Schematic representation of the corneal injury mouse model. (B, C) Western blot showing the overexpression of NSUN2 by AAV in cornea. (D, E) Immunofluorescence images of P63 (green), Ki67 (red) and DAPI (blue) in wounded corneas (D). Scale bar: 50 μ m. The quantification of P63⁺Ki67⁺ (white arrows) cells is shown in E. (F, G) Representative fluorescence images showing better wound healing of the NSUN2-overexpressing corneas (F). Scale bar: 2 mm. Quantifications of the corneal wound areas are shown in G. (H, I) Immunofluorescence images of P63 (green), Ki67 (red) and DAPI (blue) of wounded corneas of *Nsun2*^{+/-} mice treated with AAV-KLF4 (H). Scale bar: 50 μ m. The quantification of P63⁺Ki67⁺ (white arrows) cells is shown in I. (J, K) Representative fluorescence images depicting the epithelial wound healing of the *Nsun2*^{+/-} corneas following AAV-KLF4 treatment (J). Scale bar: 2 mm. Quantifications of the corneal wound areas are shown in K. All data are presented as mean $\pm$ SD, n = 3 in B and D, n = 4 in H, n = 6 in J, n = 10 and 9 for AAV-CTRL and AAV-NSUN2 group respectively in F. Statistical significance was determined using one-sample *t*-test in C, Student's *t*-test in E and G, one-way ANOVA in I and K. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

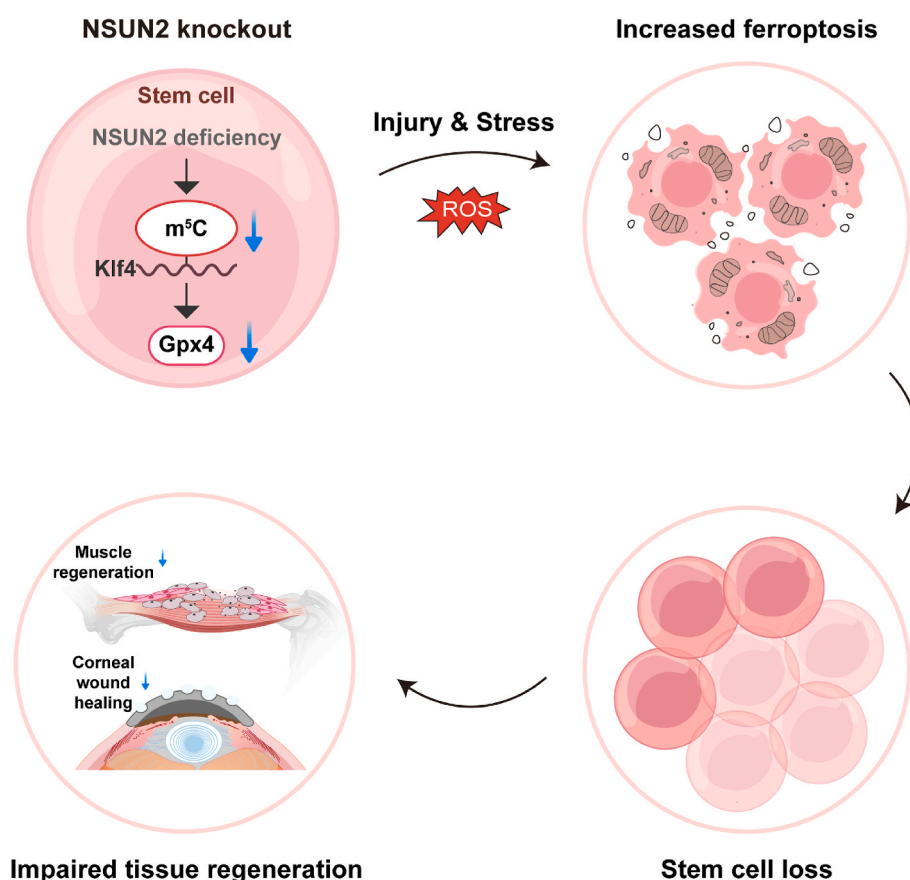


Fig. 7. Critical role of NSUN2 in boosting stem cells and tissue regeneration NSUN2 maintains RNA m⁵C methylation of KLF4, promoting its expression and binding to the GPX4 promoter. This leads to increased GPX4 expression, which subsequently inhibits stem cell ferroptosis, thereby enhancing stem cell survival and tissue regeneration. On the contrary, NSUN2 deficiency decreases KLF4 m⁵C methylation, leading to reduced KLF4 and GPX4 expression and increased stem cell ferroptosis, thus decreased stem cell number and tissue regeneration.

inhibitory factor (LIF, 1000 U/ml, Excell Bio). The HEK293T and MEF cells were cultured in DMEM (Corning) with 10% (v/v) FBS and 1% penicillin-streptomycin (Gibco). For LIF withdrawal-induced ESC differentiation, ESCs were cultured in ESC medium without LIF for 3 days. For retinoic acid (RA)-induced ESC differentiation, ESCs were cultured in ESC medium containing 5 μ M RA (Sigma) with LIF (+LIF) for 3 days.

4.2. *Nsun2* deficient mice

All animal experiments were approved by the Animal Care and Use Committee of Sun Yat-sen University in Guangzhou, People's Republic of China. All animals were housed and maintained on a 12/12 h light/dark cycle at the animal facility of Zhongshan Ophthalmic Center, Sun Yat-sen University. The *Nsun2* deficient mice were generated by GemPharmatech in Nanjing, China, and bred on a C57BL/6J background for more than six generations. Briefly, two sgRNAs targeting *Nsun2* exon3 (5'-GAA-CAAGTACTTTAAGGAGT-3' and 5'-CTTTCTGTCCATCTACTTCC-3') together with Cas9 mRNA were injected into mouse zygotes, which were subsequently transplanted into pseudopregnant mice. The neonatal mutant mice were identified by PCR genotyping and sequencing. The primers used for PCR genotyping are: forward 5'-CAGAA-CAGTGAAGTGGAGGA-3' and reverse 5'-CCTGTAATGCCTTCTGGTGTG-3'.

4.3. ESC generation

Nsun2^{+/+}, *Nsun2*^{+/-} and *Nsun2*^{-/-} ESCs were derived from 3.5 day embryos of blastocyst-stage from *Nsun2*^{+/-} heterozygous breeding. Blastocysts were collected by flushing the oviducts and uteri using M2

media (Sigma). Embryos were collected in four-well plates (Thermo Fisher Scientific) with feeder cells in ESC medium. After 6-12 days, the inner cell mass (ICM) outgrowth was re-plated after trypsinization using 0.25% trypsin (Invitrogen) on four-well plates with feeder cells. When the ESCs were sub-confluent, they were plated into larger gelatin-coated culture dishes. The ESCs were passed every 2-4 days and genotyped by sequencing.

4.4. Teratoma assay

The ESCs (1×10^6 cells/100 μ l PBS) were injected subcutaneously into the dorsal flank regions of six-week-old nude mice (GemPharmatech, China) using a 25-gauge needle. Four weeks after injection, teratomas were dissected from the mice, weighed and collected for qRT-PCR.

4.5. Colony formation assay and alkaline phosphatase (AP) staining

The ESC colony formation assay was performed by plating the ESCs onto 0.1% gelatin-coated 6-well plates at 400 cells/well and culturing for 7 days. Colony formation was analyzed by AP staining before imaging. AP staining was performed using the alkaline phosphatase detection kit (Vector Laboratories) according to the manufacturer's instructions.

4.6. Embryoid body (EB) formation

Nsun2^{+/+} and *Nsun2*^{-/-} ESCs were seeded at a density of 4×10^4 cells/ml using a hanging drop method (25 μ l/drop) and cultured

without LIF (-LIF) in ESC medium for 6 days in 150 mm dishes. At day 6, all the EBs were harvested and transferred into 10 cm culture dishes for qRT-PCR analysis.

4.7. Transfection

To perform an RNAi-mediated gene knockdown assay, ESCs were transfected with siRNA oligos (GenePharma) for the target genes of interest or scrambled siRNA as a control using Lipofectamine 2000 (Invitrogen). Briefly, ESCs were trypsinized, and 6×10^4 cells were seeded in 800 μ l of medium/well in a 12-well plate. For each well, 40 pmol of siRNA was mixed with 100 μ l of Opti-MEM (Thermo), and 2 μ l of Lipofectamine 2000 (Invitrogen) was mixed with another 100 μ l of Opti-MEM. Each mixture was incubated for 5 min. Then, the two different mixtures were combined and incubated for 15 min, and added to each well. After 6 h, the culture medium was changed to new medium, and the ESCs were harvested 48 h after transfection for analysis. The sequences of the siRNA oligos are listed in [Supplementary Table 2](#).

To induce GPX4 or KLF4 overexpression in ESCs, the mouse GPX4 encoding sequences were cloned into the pTSBX-MSCV-Flag plasmid (Transheep, Shanghai, China), whereas the wild-type or mutant KLF4 sequences were cloned into the Lv237 vector (GeneCopoeia, USA). The KLF4 mutant constructs were generated by IGE Biotechnology Co., Ltd. (Guangzhou, China). The indicated plasmids were transfected into ESCs using Lipofectamine 2000. A total of 1×10^6 ESCs were seeded into a 6-cm culture dish. 8 μ g of plasmid was combined with 500 μ l of Opti-MEM, while 10 μ l of Lipofectamine 2000 was mixed with another 500 μ l of Opti-MEM. These mixtures were incubated separately for 5 min before being combined. After a further 15 min incubation, the mixture was added to the cells in each well. The medium was replaced with fresh medium 6 h post-transfection, and the ESCs were harvested 48 h later.

4.8. NSUN2 overexpression in ESCs

To generate stable *Nsun2*-overexpressing ESCs, the human NSUN2 encoding sequences were inserted into the entry plasmid and cloned into the pLent-EF1a-DEST-SFB-IRES-puro-HBV plasmid (a kind gift from Dr. Zhou Songyang at SYSU). The PCR primers used are: forward 5'-ATAGCGGCGCGAATGGGGCGGCGGTGCGTGGTCG-3' and reverse 5'-GGCGCGCCCCGGGGTGGATGGACCCCTGCCGGGTGTCACA-3'. The enzymatically dead *Nsun2* K190 M mutant (NSUN2 dead) was described previously [35]. 1.5 μ g plasmids were transfected into HEK293T cells together with 0.9 μ g pSPAX2, 0.6 μ g pMD2G (Addgene, USA) using Lipofectamine 2000 (Invitrogen). The supernatants, which contained the viral particles, were collected after 24 and 48 h, respectively, and were added to the ESCs in the presence of 5 μ g/mL of polybrene (Santa Cruz). After 6 h, the medium was refreshed. The ESCs were selected using puromycin (2 μ g/mL).

To generate inducible NSUN2 overexpressing ESCs, the inducible gene expression system (pLoxP2 vector, pSalk-Cre vector and A172-LoxP ESCs) was applied as described previously [78,79]. The cDNA encoding mouse NSUN2 was PCR-amplified and cloned into the pLoxP2 targeting vector with a 3Flag-tag inserted at the N terminal. The 3Flag-NSUN2 vector (10 μ g) together with the pal-Cre vector (10 μ g) expressing Cre was electroporated (115 V, 7.5 mess) into the A172-Lux ESCs (1×10^6) using an NEPA21 electroporator (NEPA GENE). NSUN2-expressing ESCs were selected by G418 (300 μ g/mL) and individual clones expanded. Induction of NSUN2 expression was achieved by adding Dox (0, 100, 500 ng/mL, Sigma) into the culture media for 48 h.

4.9. Cell proliferation assay

Cell proliferation was measured using a Cell Counting Kit-8 assay (Do Jindo). Cells were seeded in 96-well culture plates (1×10^4 cells/well) for indicated time points, followed by adding 10 μ l of CCK8 reagent and

incubating for 4 h at 37°C. The absorbance was measured at 450 nm using an ELx800 microplate reader (BioTek).

4.10. Intracameral injection

The intracameral injection was performed as described previously [80,81]. Briefly, after anesthesia with pentobarbital sodium (50 mg/kg), the cornea was punctured with a sterile 30G disposable injection needle approximately 1-2 mm above the temporal corneoscleral limbus. One microliter of AAV2/9-NSUN2 (pAAV-CMV-NSUN2-3 $\times$ FLAG-WPRE, OBiO Technology, China), AAV2/9-KLF4 (ssAAV-CAG-KLF4-3-FLAG-WPRE3, PackGene Biotech, China) or AAV-CONTROL solution (1×10^{10} v.g./eye) was injected into the anterior chamber of each eye from the puncture using a 2.5 μ l syringe with a 33G Small Hub Removable (RN) flat needle tip (Hamilton, USA).

4.11. Corneal wound healing model

The corneal epithelial wound healing model was described previously [51]. Briefly, mice were anesthetized using sodium pentobarbital (50 mg/kg). An Algerbrush II corneal rust ring remover (Alger Co., Lago Vista, TX, USA) was used to debride half of the corneal epithelium and adjoining limbus. Fluorescein sodium-stained corneal wound areas were imaged with a slit-lamp microscope at day 0 and day 1 after injury, and quantified with the ImageJ software. Corneas were collected 24 h after injury for Western blot and immunofluorescence staining.

4.12. Cardiotoxin (CTX)-induced muscle injury model

Eight-week-old C57/BL6J mice were injected with 50 μ l of AAV2/9-NSUN2, AAV2/9-KLF4 or AAV2/9-CONTROL (5×10^{10} v.g./side) into the tibialis anterior muscle of both legs. The muscle injury was performed two weeks later. To induce muscle injury, mice were anesthetized using sodium pentobarbital and administrated with 50 μ l cardiotoxin (50 μ M) via tibialis anterior muscle injection of the right leg. The left legs were injected with an equal volume of saline. 7 days after CTX treatment, tibialis anteriors were collected for weighting, Western blot, HE staining and immunofluorescence staining.

4.13. Immunofluorescence staining

The mouse eyeballs or muscles were fixed with 4% polyformaldehyde (PFA, Sigma) for 2 h at room temperature, and then embedded in 30% sucrose (Sigma) at 4°C overnight. Tissues were then frozen in O.C.T (SAKURA) and cut into 10 μ m sections. Sections were blocked with 5% BSA and 0.5% Triton X-100 in PBS for 1 h at room temperature and incubated with the primary antibodies at 4°C overnight. The secondary antibodies (Life Technologies, Waltham, MA, USA) were incubated for 1 h at room temperature. The sections were imaged using a fluorescence microscope (ZEISS, Oberkochen, Germany). The primary antibodies included Ki67 (AF7649-SP, R&D System), P63 (ET1610-44, HUABIO), PAX7 (AB-528428, DSHB), and MyoD (18943-1-AP, Proteintech). The secondary antibodies included donkey anti-rabbit IgG, Alexa Fluor™ 555 (Life Technologies, A-31572), goat anti-mouse IgG, Alexa Fluor™ 488 (Life Technologies, A-10680) and goat anti-rat IgG, Alexa Fluor™ 488 (Life Technologies, A-11006).

4.14. H&E staining

Mice tibialis anterior were fixed overnight in 4% paraformaldehyde. Dehydrated specimens were fixed in paraffin and sectioned at 5 μ m thickness, and then deparaffinized and rehydrated for H&E staining to observe histological changes.

4.15. RNA sequencing (RNA-seq)

Total RNA was extracted using the Trizol reagent (Invitrogen, Waltham, MA, USA). RNA-seq was performed using an Illumina Nova Seq 6000 sequencer (Illumina, San Diego, CA, USA) by Bohao Bio-tech Inc. (Shanghai, China). The differentially expressed genes (DEGs) were defined at $p < 0.05$ with a fold-change > 1.5 . GO analysis was performed using DAVID (<https://david.ncifcrf.gov/summary.jsp>). Default parameters were used for GSEA analysis using the GSEA software. The RNA sequencing dataset is accessible at the GEO database (GSE220291).

4.16. Lipid peroxidation measurement

To measure lipid peroxidation, 2×10^5 ESCs were plated into a 6-well plate after treatment with $10 \mu\text{M}$ Erastin (Selleck, S7242) for 24 h. Cells were incubated with fresh ESC medium containing $2 \mu\text{M}$ C11-BODIPY 581/591C11 dye (Invitrogen, D3861) for 30 min. The cells were then collected and washed twice with PBS, followed by flow cytometry lipid peroxidation analysis.

4.17. GSH measurement

1×10^6 ESCs were lysed by repeated freezing and thawing. The supernatant was collected after centrifugation according to the manufacturer's instructions (Beyotime, S0053), and the concentration of GSH supernatant was determined at 412 nm using an ELx800 Absorbance microplate reader (BioTek).

For the H_2O_2 -induced oxidative stress assay, ESCs were seeded in 96-well plates and treated with $400 \mu\text{M}$ of H_2O_2 for 0, 3 or 6 h. At each indicated time point, intracellular GSH levels were measured using a glutathione assay kit (Abcam, ab205811) according to the manufacturer's instructions.

4.18. ROS measurement

Intracellular ROS levels in ESCs were determined using the DCFDA/H2DCFDA -Cellular ROS Assay Kit (Abcam, ab113851) according to the manufacturer's instructions. Briefly, live cells were stained with DCFDA solution at 37°C for 30 min in the dark, and the generated fluorescence was subsequently quantified at Ex/Em = 485/535 nm using an Infinite F200 Pro multimode microplate reader (Tecan, Switzerland).

4.19. Detection of intracellular Fe^{2+}

5×10^4 ESCs were seeded in 24-well plates and cultured overnight. Cells were washed 3 times with $1 \times \text{HBSS}$ (Thermo Fisher, 14185052) and then incubated with $1 \mu\text{M}$ of FerroOrange (Dojindo, F374) for 30 min at 37°C . Intracellular FerroOrange fluorescence imaging was taken using an immunofluorescence microscope (Imager.Z2, Zeiss).

4.20. m^5C -RIP-sequencing

Total RNA was isolated using the TRIzol reagent (Thermo Fisher), and m^5C -RIP-seq was performed by Cloudseq Biotech Inc. (Shanghai, China) [82]. Briefly, fragmented RNAs were incubated with anti- m^5C polyclonal antibody (Synaptic Systems, 202003) in IPP buffer for 2 h at 4°C . The mixture was immunoprecipitated by incubation with protein-A beads (Thermo Fisher) at 4°C for 2 h. Bound RNA was eluted with 5-methyladenosine in IPP buffer and then extracted with Trizol reagent (Thermo Fisher). Purified RNAs were used for library generation using the NEBNext® Ultra™ II Directional RNA Library Prep Kit (NEB). Both the input sample without immunoprecipitation and the m^5C IP samples were subjected to 150 bp paired-end sequencing using an Illumina HiSeq sequencer. The m^5C -RIP-seq data is accessible at the GEO database (GSE220292).

4.21. m^5C -RIP qRT-PCR

RNA was extracted as described above. $20 \mu\text{g}$ RNA were incubated with $1 \mu\text{g}$ anti- m^5C antibody (Abcam, ab10805) or mouse IgG (Santa Cruz, sc-2025) in $500 \mu\text{l}$ of immunoprecipitation buffer (150 mM NaCl, 0.1% NP-40, 10 mM Tris-HCl, pH 7.4) with $1 \text{ U}/\mu\text{l}$ RNasin for overnight at 4°C , and 100 ng RNA served as an input control. $30 \mu\text{l}$ of protein A/G beads (Santa Cruz, sc-2003) were pre-blocked with 0.5 mg/ml BSA at 4°C for 2 h and then added to the RNA for immunoprecipitation at 4°C for another 2 h. After washing with immunoprecipitation buffer three times, RNA was eluted from the beads using the TRIzol reagent (TIANGEN) and resuspended in $10 \mu\text{l}$ of water for the m^5C -RIP qRT-PCR assay.

4.22. Chromatin immunoprecipitation (ChIP) assay

ESCs were cultured in 15 cm dishes and crosslinked with 1.42% formaldehyde (Sigma) for 15 min at room temperature, and quenched by 125 mM glycine solution (Sigma) for 10 min. Cells were collected and washed twice with ice-cold PBS, lysed with IP buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA, 0.5% NP-40, 0.1% Triton X-100), and sonicated for DNA fragmentation ($100\text{--}500 \text{ bp}$ on average). 10% (v/v) of the resulting DNA fragments served as an input control and the rest was incubated with a KLF4 antibody (Proteintech, 11880-1-AP) or mouse IgG (Santa Cruz, sc-2025) at 4°C overnight. The immunoprecipitates and input DNA samples were subjected to proteinase K treatment at 65°C to reverse crosslinking, followed by purification using a Tianquick Maxi purification kit (TIANGEN, DP205-02). qRT-PCR was conducted using a QuantStudio™ qRT-PCR device (Thermo Fisher, USA). Klf4 binding sites of the mouse *Gpx4* promoter were predicted using the MATCH algorithm [83]. qRT-PCR primers used to verify the binding sites are as follows:

Gpx4-P1: forward 5'-TCCGGCCGCCGAGATGAGC-3' and reverse 5'-CTGGGAACCTGGAGGATC-3'; *Gpx4*-P2: forward 5'-ATGCGCAGTTGCTGATAAC-3' and reverse 5'-ACTGACGTCTCTTATTGGTC-3'.

4.23. RNA isolation, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using the TRIzol reagent (TIANGEN). cDNA synthesis was performed using a FastKing RT kit with DNase (TIANGEN). qRT-PCR was performed using the SYBR Green reagent (ROCHE) and an ABI QuantStudio 6 Flex device (Life Technologies). Results were normalized against *Gapdh* transcripts. The relative fold changes of gene expression were calculated using a delta-delta Ct method. The sequences of the primers used are listed in Supplementary Table 3.

4.24. Western blot

Proteins were separated on SDS-PAGE gels, followed by protein transfer to a PVDF membrane (BIO-RAD). Primary antibodies used were: anti-NSUN2 (20854-1-AP, Proteintech), anti-GPX4 (CY6959, Abways), anti-KLF4 (11880-1-AP, Proteintech), anti-OCT4 (sc-5279, Santa Cruz), anti-SOX2 (66411-1-Ig, Proteintech), anti-FLAG (F1804, Sigma), anti-NANOG (ab10626, Abcam), anti-GAPDH (RM2002, Beijing Ray Antibody Biotech), anti- β -ACTIN (RM2001, Beijing Ray Antibody Biotech), anti-TUBULIN (RM2007, Beijing Ray Antibody Biotech), and anti-HSP90 (7411, Cell Signaling). Secondary antibodies used included goat anti-mouse, goat anti-rabbit, and rabbit anti-goat antibodies (MUTI SCIENCES). The bands were detected using the Immobilon Western Chemiluminescent HRP substrate (MILLIPORE) and visualized using a GBOX-CHEMI-XX8 device (SYNGENE).

4.25. Statistical analysis

GraphPad Prism (GraphPad Software, Inc.) was used for statistical analyses except for the RNA-seq and m^5C -RIP-seq data. SRplot (<http://www.bioinformatics.com.cn/srplot>) was used for the generation

of volcano plot for DEGs and bubble plots for GO analysis. BioGPS database (<http://ds.biogps.org/?dataset=GSE10246&gene=28114>) was used for the analysis of *Nsun2* expression in different mouse cells/tissues. All data are presented as mean $\pm$ SD. For comparisons between two groups, Student's t-test was used. For comparison among more than two groups, one-way ANOVA analysis was used. The n numbers, p values and other detailed information are provided in the corresponding figure legends.

Ethics approval and consent to participate

This study is not a clinical trial, and no human subjects were involved. All mouse experiments were conducted in accordance with a protocol approved by the Animal Care and Use Committee at the Zhongshan Ophthalmic Center, Sun Yat-Sen University, People's Republic of China (Protocol Number Z2022-078).

Consent for publication

Not applicable.

Availability of data and materials

All data necessary to evaluate the conclusions of the paper are included in the paper and/or in the Supplementary Materials. The m⁵C-RIP-seq data were accessible in the GEO database (GSE220292). The RNA-seq data were accessible in the GEO database (GSE220291).

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CRediT authorship contribution statement

Ying Zhan: Data curation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Boxiong Deng:** Data curation, Formal analysis, Methodology, Writing – original draft. **Xiaoling Zeng:** Data curation, Formal analysis, Methodology, Writing – original draft. **Ying Guo:** Formal analysis, Methodology, Writing – original draft. **Min Xie:** Data curation, Formal analysis, Methodology, Visualization. **Chonghui Tang:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Ying Li:** Methodology, Writing – review & editing. **Yi Tian:** Data curation, Formal analysis, Writing – original draft. **Lijuan Huang:** Formal analysis, Methodology, Visualization. **Ziqing Yang:** Methodology. **Yi Liu:** Funding acquisition, Writing – review & editing. **Jianing Zhang:** Methodology. **Shasha Wang:** Formal analysis, Writing – review & editing. **Haiqing Kuang:** Methodology. **Aijun Deng:** Writing – review & editing. **Xuran Dong:** Writing – review & editing. **Chun Zhang:** Writing – review & editing. **Hong Ouyang:** Writing – review & editing. **Lekun Fang:** Formal analysis, Writing – review & editing. **Weisi Lu:** Data curation, Formal analysis, Funding acquisition, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Xuri Li:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2026.104323>.

Data availability

The RNA sequencing dataset is accessible at the GEO database (GSE220291). The m⁵C-RIP-seq data is accessible at the GEO database (GSE220292).

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