Restoring nuclear entry of Sirtuin 2 in oligodendrocyte progenitor cells promotes remyelination during ageing

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The age-dependent decline in remyelination potential of the central nervous system during ageing is associated with a declined differentiation capacity of oligodendrocyte progenitor cells (OPCs). The molecular players that can enhance OPC differentiation or rejuvenate OPCs are unclear. Here we show that, in mouse OPCs, nuclear entry of SIRT2 is impaired and NAD⁺ levels are reduced during ageing. When we supplement β-nicotinamide mononucleotide (β-NMN), an NAD⁺ precursor, nuclear entry of SIRT2 in OPCs, OPC differentiation, and remyelination were rescued in aged animals. We show that the effects on myelination are mediated via the NAD⁺-SIRT2-H3K18Ac-ID4 axis, and SIRT2 is required for rejuvenating OPCs. Our results show that SIRT2 and NAD⁺ levels rescue the aged OPC differentiation potential to levels comparable to young age, providing potential targets to enhance remyelination during ageing.
The increasingly ageing global population has ignited great interest in identifying rejuvenating molecules that may delay ageing and enhance the regeneration of the aged central nervous system (CNS). The ensheathment of axons by myelin gives structure to saltatory conduction and provides metabolic support to maintain both axonal functional integrity and long-term survival. Therefore, proper myelination is not only a prerequisite for, but also a consequence of, normal CNS activity. As the human brain ages, white matter volume shrinks more prominently than grey matter and some myelin sheaths exhibit myelin ageing, which in turn drives CNS ageing. The reasons for myelin ageing remain unclear. However, the decreased remyelination capacity of oligodendrocyte progenitor cell (OPC) is one of the popularly proposed mechanisms.

Demyelination is the key pathological feature of the autoimmune inflammatory diseases of CNS such as multiple sclerosis (MS) and an early pathological hallmark of neurodegenerative diseases. Consequently, demyelination may cause devastating irreversible axonal degeneration. Remyelination by oligodendrocytes differentiated from OPCs occurs throughout life. Unfortunately, with ageing, the efficiency of remyelination declines mainly due to the reduced capacity of OPC differentiation. Such decline leads to disability in MS, whose course usually spans several decades and progresses with ageing. It is essentially untreated when MS develops into a progressive phase. Identifying new molecular targets in OPC to rejuvenate the aged OPC therefore holds much promise for this unmet medical need.

As the central player for myelination and remyelination, OPCs are distributed throughout the CNS and are orchestrated by epigenetic mechanisms likely involving sirtuins, the nicotinamide adenine dinucleotide (NAD⁺)-dependent histone deacetylases. Among the seven members of sirtuins, it remains elusive which members of the sirtuin family are expressed in the OPC. Sir2 (SIRT2) is the only member which is predominantly localised in the cytoplasm, and its role in the CNS remains largely unclear. So far, although mature oligodendrocytes express SIRT2, it is not clear whether OPCs in the CNS express SIRT2, or what role SIRT2 plays in remyelination in the aged CNS.

Enhancing remyelination in the aged CNS of animal models has proven to be an effective strategy to rejuvenate aged OPCs, and recent studies have demonstrated that a youthful systemic environment, fasting, or metformin can restore remyelination in the aged CNS, indicating that the aged OPC can be rejuvenated. Emerging evidence suggests that the metabolic profile defines an OPC’s cell fate and shapes its ability to differentiate. However, so far it is not clear how the endogenous metabolic fingerprints of OPCs are affected by ageing.

Herein, we report that the depletion of SIRT2 and its nuclear localisation together with declined NAD⁺ features of aged OPCs. Importantly, supplementation of NAD⁺ by β-nicotinamide mononucleotide (β-NMN) induces re-expression and restores nuclear entry of SIRT2 in OPCs, and rejuvenates aged OPCs by promoting them differentiating into mature oligodendrocytes and eventually enhances new myelin generation in the aged CNS. Mechanistically, we reveal that SIRT2 is necessary for the effect of NAD⁺ on OPCs. This study identifies SIRT2 as a molecular target for OPCs, and by restoring the nuclear entry of SIRT2 in OPCs, β-NMN delays myelin ageing in the normal CNS and enhances remyelination in the demyelinated aged CNS.

**Results**

**Nuclear entry of SIRT2 in OPCs during remyelination is impaired in the aged mice in vivo.** For myelination and remyelination, the OPC is the central player. However, it remains unclear which members of the seven sirtuins are expressed in OPCs. Using primary cultured rat OPCs (Fig. 1a), the mRNA levels of sirt1 to sirt7 were compared by qRT-PCR. Surprisingly, we found that the abundance of sirt2 mRNA was dominant over any other member of sirtuin family for over 50 folds in the OPC (Fig. 1b), and similar pattern was seen in the mature oligodendrocyte (Fig. 1b). The mRNA level of sirtuins in OPCs and mature oligodendrocytes were almost identical between rat and mouse (Supplementary Fig. 1a). Among different cell types in the rat brain, sirt2 mRNA expression was the highest in mature oligodendrocyte, and the second highest in OPC, whereas very little in neuron, microglia, and astrocyte (Fig. 1c).

Further, we investigated whether OPCs express the SIRT2 protein during myelin development in vivo. The SIRT2 expression profile in both white matter such as corpus callosum and optic nerve and the grey matter were detected in the postnatal mouse brain. SIRT2 was indeed expressed in OPCs in the brain from postnatal day 0 (P0) to P21 (Fig. 1d), the crucial time window for postnatal development of CNS myelin, and the expression declined from P0 in a time-dependent manner. At P0, when OPCs start differentiating, SIRT2 was expressed in a significant number of OPCs, consistent with our in vitro data (Fig. 1b). While at P21, the differentiation of OPCs slows down, the number of OPCs expressing SIRT2 reduced dramatically. Interestingly, about 2/3 of the SIRT2+ OPCs had SIRT2 in their nuclei (Fig. 1d–e). At P90, when myelin development has been completed, SIRT2 was depleted in OPCs and was exclusively expressed in the cytoplasm of mature oligodendrocytes (Fig. 1d–f; Supplementary Fig. 1b, c and Supplementary Fig. 2a). To investigate whether OPCs in the primate brain express SIRT2, with the brain of the marmoset, we showed that the nuclear expression of SIRT2 in OPCs was indeed observed in the cortex at P3 (Fig. 1g and Supplementary Fig. 1d–e), whereas in the adult (aged 8 years) SIRT2 was not observed in OPCs but exclusively in the cytoplasm of mature oligodendrocytes (Fig. 1h). However, in cell types other than OPCs and mature oligodendrocytes, such as the astrocyte, neuron, microglia, and endothelial cell, the expression of SIRT2 was not detectable in the brain of either mouse or marmoset (Supplementary Fig. 2b, c). In the adult human cerebral cortex, SIRT2 was also expressed in the cytoplasm of mature oligodendrocyte-like cells and on myelin-like structures (Fig. 1i).

Our results indicate that SIRT2 is expressed in postnatal OPCs, and its expression pattern switches from nucleus-containing to cytoplasm-containing in the major OPCs during myelin development to exclusively cytoplasmic in mature oligodendrocytes. This is an evolutionary reserved phenomenon spanning from mouse, monkey to human.

Further, we explored how the SIRT2 protein level in the brain changes with progressive ageing, and found that SIRT2 declined in old mice aged 18 months (M) when compared to young mice aged 6 M (Fig. 1j, k). It is currently unclear how SIRT2 expression is regulated by remyelination during ageing. To this end, we induced a focal demyelination by injecting lysolecithin (LPC) into the corpus callosum of the mouse brain, and remyelination occurs. Interestingly, at 5 days post-lesion (dpl), when OPC proliferation reaches its peak, SIRT2 re-expression occurred in about 2/3 of the SIRT2+ OPCs, nuclear localisation of SIRT2 was seen in about half of SIRT2+ OPCs (Fig. 1m). In contrast, in the old mice aged 21 M, demyelination upregulated the level of SIRT2+ OPCs to a much lower level than that of the young mice (Fig. 1l, Supplementary Fig. 2d), equivalent to 1/3 in the proportion (Fig. 1m) and about half in the density of SIRT2+ OPCs of the young mice (Fig. 1n). Among SIRT2+ OPCs, nuclear SIRT2+...
OPCs in the aged was only 1/3 of the young (Fig. 1l–o, Supplementary Fig. 2d). Our results reveal that the nuclear localisation of SIRT2 in OPCs during remyelination declines during ageing. Interestingly, our results show that the depleted nuclear entry of SIRT2 in aged OPCs within the demyelination lesion correlates well with the decline of remyelination capacity during ageing12,18,20.

SIRT2 is critical for remyelination of the mice in vivo. So far, the function of SIRT2 in OPCs remains unclear. To explore this, we used SIRT2−/− mice whose knockout efficiency was confirmed by Western blot (Fig. 2a). In the primary OPC culture, SIRT2 knockout impaired both the proliferation (Fig. 2b, c) and the differentiation (Fig. 2d, e) of OPCs. We further induced demyelination in the mice by injecting LPC. Following
demyelination, compared with the WT mice, SIRT2−/− mice showed significantly decreased numbers of both proliferating OPCs (K67+/Olig2+ cells) (Fig. 2f–i) and differentiated oligodendrocytes (CC1+/Olig2− cells), and the differentiation of OPCs is more severely impaired (Fig. 2k–o).

To accurately evaluate the remyelination efficiency, we used the transmission electron microscopy (TEM) technique to distinguish unambiguously the normal myelinated, demyelinated (no myelin) or remyelinated (newly formed myelin, thinner) axons within the lesion (Fig. 2p, q). Taking the published studies8–10 into account, together with our own careful observation, in this work we established a set of graded evaluation criteria from grade 0 (normal) to grade 4 on the ultrastructure of myelin in the corpus callosum (Supplementary Data 1). Since myelin can become lose via demyelination in the old, the G-Ratio cannot entirely represent the myelin thickness. Therefore, combining G-Ratio with the distance between the dense line (DL) becomes a more reliable way to evaluate myelin thickness and compaction.

According to this set of criteria, at 21 dpl, when remyelination is completed, we showed that the young SIRT2−/− mice (aged 6 M) showed more impaired remyelination efficiency not only than WT young but also than WT old (aged 18 M) (Fig. 2r, s). Graded myelin analysis further revealed this was mainly due to SIRT2−/− mice having more grade 2 myelin pathology than WT old (Fig. 2t), indicating that the general myelin structure in SIRT2−/− mice is worse than that of WT old mice. Moreover, SIRT2−/− mice exhibited thinner and looser newly-formed myelin than WT young mice: a result no different to the WT young but also than WT old (aged 18 M) (Fig. 2r, s). The genetic deletion of SIRT2 in OPCs was confirmed by immunofluorescence and quantification of SIRT2+ OPCs and nuclear SIRT2+ OPCs in corpus callosum of WT young and old mice (n = 3). NL, non-lesion, L, demyelination lesion induced by LPC at 5 dpl. Scale bar, 10 μm. All data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 by two-tailed t-test (k) or one-way ANOVA followed by Tukey’s post hoc test (m–o). In all instances **p < 0.001, n.s. no significance. In (k), **p = 0.004; in (m), *p = 0.002 (L-Young vs. L-Old); in (n), *p = 0.007 (L-Young vs. L-Old).

Elevating NAD+ by β-NMN enhances SIRT2 nuclear entry in OPCs of the aged mice within demyelination lesion in vivo.

Age-related changes and defects in the myelin structure are key features of CNS ageing9–11,37. Progressive telomere attrition is a primary cause of organisinal ageing38. Critical telomere shortening caused by telomerase deficiency in the third generation of telomerase RNA component knockout mice (G3 Terc−/− mice) leads to premature ageing38. After confirming that G3 Terc−/− mice had shorter telomeres than the age-matched WT mice (Supplementary Fig. 6a, b), using TEM technique and samples from corpus callosum, we confirmed that G3 Terc−/− mice exhibited a premature ageing phenotype of myelin ultrastructure in the brain (Supplementary Fig. 6e–k). Using primary cultured OPCs and LPC-induced in vivo demyelination, we further revealed that G3 Terc−/− mice exhibited compromised capacity of proliferation (Supplementary Fig. 7a, b; e–i), differentiation (Supplementary Fig. 7c, d; j–n) and remyelination (Supplementary Fig. 7o–v) in comparison with the age-matched WT mice, similar to the old mice three times of its biological age, confirming the premature ageing phenotype of OPC in G3 Terc−/− mice. In parallel, we found that SIRT2 protein level in the brain declined in G3 Terc−/− mice, equivalent to that of the naturally-ageing old mice three times of its biological age (Supplementary Fig. 6c, d).

Endogenous metabolites play a vital role in stem cell ageing25. However, it is not clear how the global metabolome was changed in OPCs during ageing. To address this question, we used premature ageing OPC from G3 Terc−/− mice to avoid the technical difficulties of culturing the aged OPC and compared the global metabolome of OPCs from G3 Terc−/− mice with that of age-matched WT mice. To capture the native metabolism status, OPCs were obtained by immunopanning, which took 4 hours (h) for isolation (Fig. 3a). Importantly, the targeted metabolic profiling results showed that NAD+ was one of the top metabolites that significantly decreased in G3 Terc−/− OPCs (Fig. 3b and Supplementary Data 2). The decreased NAD+ level was further confirmed in the brain of young G3 Terc−/− mice...
aged 6 M, which had decreased to 1/3 of that in age-matched WT mice, and equivalent to that of old WT mice aged 15 M (Fig. 3c).

We demonstrated here that depleted SIRT2 in the aged OPC correlated with the decline of NAD$^+$, and that these two processes correlated with declined remyelination during ageing as previously shown 12,18,20. We further ask whether NAD$^+$ supplementation could restore the age-related decline of SIRT2 in aged OPCs. β-NMN, an immediate biosynthesis precursor of NAD$^+$, was used to supplement NAD$^+$. Using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) (Fig. 3d) we investigated the changes of proteome differentially regulated by NAD$^+$ or DMSO in cultured rat OPCs. The volcano plot shows markedly elevated expression of 399 genes, most of which are involved in metabolism. The metabolic
Supplementary Fig. 11i). These results indicated that NAD+ supplementation delays myelin ageing in vivo by making myelin more compact in both G3 Terc−/− premature ageing and normal aged mice.

Elevating NAD+ by β-NMN enhances remyelination in the aged mice in vivo. To explore whether NAD+ supplementation affects the proliferation and the differentiation of OPCs, we supplied β-NMN to in vitro cultured OPCs and found that β-NMN improved the proportion of proliferating OPCs from G3 Terc−/− mice, but had no effect on OPCs from WT mice (Fig. 4a, b). Importantly, supplementing β-NMN to primary cultured OPCs improved the proportion of differentiated oligodendrocytes in both G3 Terc−/− mice, WT mice and WT rats (Fig. 4c, d and Supplementary Fig. 9), and upregulated myelin basic protein (MBP) at the protein level in WT rats (Fig. 4e, f). This data indicates that NAD+ supplementation enhances both proliferation and differentiation of young OPCs in vitro.

To test whether the long-term NAD+ supplementation (daily injection for 3 M) affects the proliferation and/or differentiation of OPCs in vivo in non-lesion condition, we found in both untreated G3 Terc−/− (6 M) and WT old (21 M) mice, the density of OPCs, oligodendrocytes, the oligodendrocyte lineage cells, and the proportion of the oligodendrocyte lineage cells among all cells declined in comparison with untreated WT young (6 M) mice (Supplementary Fig. 10a–l). We further explored whether β-NMN affected these impaired indexes in G3 Terc−/− and WT old mice. Our results showed that β-NMN increased the density of OPCs, the differentiated oligodendrocytes, the oligodendrocyte lineage cells, and the proportion of the differentiated oligodendrocytes among all cells in both G3 Terc−/− mice and WT old mice (Supplementary Fig. 10a–l).

Importantly, β-NMN fully restored all the impaired indexes described above in both G3 Terc−/− mice and WT old mice to the levels of WT young mice, confirming that β-NMN rejuvenates OPCs in both G3 Terc−/− mice and WT old mice.

Elevating NAD+ by β-NMN delays myelin ageing in vivo, we i.p. injected G3 Terc−/− mice with β-NMN or PBS for 3 months (Fig. 4g). This long-term NAD+ supplementation exerted a similar effect on myelin ultrastructure in both G3 Terc−/− mice (Fig. 4g–n) and WT old mice aged 21 M (Fig. 4o–v). NAD+ supplementation significantly increased the frequency of normal myelin (grade 0), generally decreased the myelin pathological level (grade 1–4, Fig. 4i, q) and decreased the distance between DL (Fig. 4j, k, r, s). These results indicate that long-term NAD+ supplementation delays myelin ageing in vivo by making myelin more compact in both G3 Terc−/− premature ageing and normal aged mice.

pathways consistently ranked as the top, revealed by biological processes analysis (Fig. 3e, f). Among all seven family members of sirtuins, we screened out that SIRT2 was the only one upregulated by NAD+ in cultured rat OPCs at both protein level by LC-MS/MS (Fig. 3c) and at transcriptional level by qRT-PCR (Fig. 3g and Supplementary Data 3). This set of data are consistent with our results of qRT-PCR and immunostaining of SIRT2 on OPC (Fig. 1).

Meanwhile, β-NMN significantly downregulated the mRNA level of several cellular senescence-related genes18,39 in OPCs from G3 Terc−/− mice (Supplementary Fig. 8a), suggesting multiple mechanisms could be involved in the effect of β-NMN on OPCs.

To test whether β-NMN administration restores NAD+ and SIRT2 level in the brain in vivo, we i.p. injected β-NMN or PBS for 3 months (Fig. 3h), and found in the brain tissue of G3 Terc−/− mice treated with β-NMN, the level of both NAD+ and SIRT2 protein was doubled than that of PBS-treated control mice (Fig. 3i–k). We further tested whether NAD+ supplementation could restore age-related decline of nuclear localisation of SIRT2 in OPCs in vivo. In terms of SIRT2 nuclear localisation in OPCs, almost no nuclear SIRT2 OPC was observed in normal adult brain, while NAD+ repletion significantly increased nuclear SIRT2+ OPC density in non-lesion condition (Fig. 3l–n and Supplementary Fig. 8b). Importantly, LPC-induced demyelination further enhanced NAD+ induced SIRT2 nuclear localisation in the aged OPCs over twofolds (Fig. 3l–n and Supplementary Fig. 8b), recapitulating SIRT2 nuclear localisation in OPCs in vivo, a phenomenon that occurred during myelin development (Fig. 1d, g). This set of data indicate that elevating NAD+ level by β-NMN enhances SIRT2 nuclear entry in premature ageing OPCs.

Elevating NAD+ by β-NMN delays myelin ageing in the aged mice in vivo. To explore whether NAD+ supplementation affects the proliferation and the differentiation of OPCs, we supplied β-NMN to in vitro cultured OPCs and found that β-NMN improved the proportion of proliferating OPCs from G3 Terc−/− mice, but had no effect on OPCs from WT mice (Fig. 4a, b). Importantly, supplementing β-NMN to primary cultured OPCs improved the proportion of differentiated oligodendrocytes in both G3 Terc−/− mice, WT mice and WT rats (Fig. 4c, d and Supplementary Fig. 9), and upregulated myelin basic protein (MBP) at the protein level in WT rats (Fig. 4e, f). This data indicates that NAD+ supplementation enhances both proliferation and differentiation of aged OPCs while only promoting differentiation of young OPCs in vitro.

To test whether the long-term NAD+ supplementation (daily injection for 3 M) affects the proliferation and/or differentiation of OPCs in vivo in non-lesion condition, we found in both untreated G3 Terc−/− (6 M) and WT old (21 M) mice, the density of OPCs, oligodendrocytes, the oligodendrocyte lineage cells, and the proportion of the oligodendrocyte lineage cells among all cells declined in comparison with untreated WT young (6 M) mice (Supplementary Fig. 10a–l). We further explored whether β-NMN affected these impaired indexes in G3 Terc−/− and WT old mice. Our results showed that β-NMN increased the density of OPCs, the differentiated oligodendrocytes, the oligodendrocyte lineage cells, and the proportion of the differentiated oligodendrocytes among all cells in both G3 Terc−/− mice and WT old mice (Supplementary Fig. 10a–l).

Importantly, β-NMN fully restored all the impaired indexes described above in both G3 Terc−/− mice and WT old mice to the levels of WT young mice, confirming that β-NMN rejuvenates OPCs in both G3 Terc−/− mice and WT old mice.

To explore whether elevating NAD+ delays myelin ageing in vivo, we i.p. injected G3 Terc−/− mice with β-NMN or PBS for 3 months (Fig. 4g). This long-term NAD+ supplementation exerted a similar effect on myelin ultrastructure in both G3 Terc−/− mice (Fig. 4g–n) and WT old mice aged 21 M (Fig. 4o–v). NAD+ supplementation significantly increased the frequency of normal myelin (grade 0), generally decreased the myelin pathological level (grade 1–4, Fig. 4i, q) and decreased the distance between DL (Fig. 4j, k, r, s). These results indicate that long-term NAD+ supplementation delays myelin ageing in vivo by making myelin more compact in both G3 Terc−/− premature ageing and normal aged mice.
demyelinated region of the corpus callosum under TEM (Fig. 5k, l). We showed that at 21 dpl, NAD$^+$ repletion by β-NMN injection in G3 Terc$^{-/-}$ mice doubled the frequency of remyelinated axons (Fig. 5m), increased the frequency of normal myelin (grade 0) and generally decreased the myelin pathology level in comparison with G3 Terc$^{-/-}$ control mice (grade 1–4, Fig. 5n). These results confirmed that NAD$^+$ repletion enhances remyelination efficiency in G3 Terc$^{-/-}$ mice. Furthermore, NAD$^+$ repletion also decreased both the G-Ratio (Fig. 5o–p) and the distance between DL (Fig. 5q, r), indicating that NAD$^+$ not only increases the thickness of newly-formed myelin but also makes it more compact.
Fig. 3 Elevating NAD+ by β-NMN enhances SIRT2 nuclear entry in OPCs of the aged mice within demyelination lesion in vivo. a Schematic of OPC immunopanning protocol. b Heatmap of top 29 metabolites significantly different between the OPCs of WT and G3 Terc−/− mice (n = 3). c NAD+ levels in brain of WT and G3 Terc−/− mice (n = 4). d, e Volcano plot of 2585 proteins that are differentially expressed between rat OPCs treated with β-NMN or DMSO for 48 h (n = 3). β-NMN, 1 mM. f KEGG enrichment analysis of proteins upregulated by β-NMN in OPCs. g Relative mRNA levels of sirtuins in OPCs treated with β-NMN or DMSO for 48 h (n = 3). h β-NMN was once daily i.p. injected to G3 Terc−/− mice for 3 months. i NAD+ levels in brain of G3 Terc−/− mice (n = 4). j k Relative SIRT2 protein level in brains of G3 Terc−/− mice (n = 5). l Immunofluorescence and quantification of SIRT2+ OPCs in corpus callosum of G3 Terc−/− mice with or without demyelination (n = 5). NL, non lesion, L, demyelination lesion induced by LPC at 5 dpl. Scale bar, 10 μm. All data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 by two-tailed t-test (g, i, k), one-way ANOVA followed by Sidak’s post hoc test (c) or two-way repeated ANOVA followed by Sidak’s post hoc test (n). In all instances, **p < 0.001, n.s. no significance. In (c), *p = 0.008 (WT 6 M vs. WT 15 M), **p = 0.001 (WT 6 M vs. G3 Terc−/−); in (g), *p = 0.03; in (i), **p = 0.0037; in (k), ***p = 0.002; in (n), *p = 0.04 (NL).

To evaluate whether the improved myelin ultrastructure could translate into the recovered signal conduction of the relevant axons in G3 Terc−/− mice, we next tested the effect of NAD+ repletion on nerve signal conduction. Using acute brain slices, we stimulated one side of the corpus callosum and recorded at the other side the compound action potential (CAP) with two clearly separated peaks, corresponding to the activation of myelinated and unmyelinated axons, respectively (Fig. 5s−u). We found that NAD+ supplementation increased the proportion of effective conduction of G3 Terc−/− mice from 40% to 92%, to the level of the WT young mice (Fig. 5v). The CAP amplitude ratio of myelinated axons (Amp 1) to unmyelinated axons (Amp 2) represents the dominant level of myelinated axons40. These results showed that the ratio of Amp 1/Amp 2 of G3 Terc−/− mice was lower than that of WT young mice. Importantly, NAD+ supplementation increased the Amp 1/Amp 2 ratio of G3 Terc−/− mice to the level of WT young mice (Fig. 5w), whereas NAD+ repletion did not change either fast or slow conduction velocity in all groups tested (Fig. 5x−y). These results validated that NAD+ repletion indeed functionally rejuvenates remyelination in G3 Terc−/− mice.

Having proven that long-term pretreatment with NAD+ enhances remyelination efficiency in demyelinated G3 Terc−/− mice, we wondered whether immediate NAD+ supplementation can also enhance proliferation or differentiation of OPCs in G3 Terc−/− mice in vivo. To test this, we started a regime of daily i.p. injection of β-NMN and a focal induction of demyelination simultaneously (referred to as immediate NAD+ supplementation). At 5 dpl (Supplementary Fig. 12a, b), the immediate NAD+ supplementation did not affect the density of the oligodendrocyte lineage cells (Supplementary Fig. 12c), or the proliferating OPCs (Supplementary Fig. 12d) and the proportion of proliferating OPCs (Supplementary Fig. 12e). At 10 dpl (Supplementary Fig. 12f, g), immediate NAD+ supplementation did not change the density of the oligodendrocyte lineage cells (Supplementary Fig. 12h), but increased the density and proportion of differentiated oligodendrocytes by 140% (Supplementary Fig. 12i) and 80% (Supplementary Fig. 12j) respectively.

To test whether immediate or delayed NAD+ supplementation enhances remyelination efficiency in demyelinated G3 Terc−/− mice in vivo, immediate NAD+ administration and lesion started simultaneously while delayed NAD+ administration started at 3 dpl when demyelination had been fully established (Supplementary Fig. 12k). Both immediate and delayed NAD+ supplementation exerted almost identical effects in all indexes tested. NAD+ supplementation significantly increased both the proportion of remyelinated axons (Supplementary Fig. 12l, m) and the frequency of normal myelin (Supplementary Fig. 12n), indicating that NAD+ enhances remyelination and alleviates myelin pathology. Meanwhile, NAD+ increased the thickness of myelin (Supplementary Fig. 12o, p) while simultaneously decreased the distance between DL (Supplementary Fig. 12q, r), indicating that NAD+ makes the newly formed myelin thicker and more compact and therefore higher quality. Together, these results show that NAD+ supplementation by long-term pretreatment, immediate or delayed administration, significantly enhances remyelination efficiency in demyelinated G3 Terc−/− mice in vivo.

The enhancing effect of β-NMN on remyelination of the mice requires SIRT2. As re-expression of SIRT2 and restoring nuclear entry of SIRT2 in the aged OPCs induced by β-NMN is sufficient to rejuvenate the aged OPCs, we further queried whether SIRT2 is required for NAD+ to take effect on OPCs. Using primary cultured mouse OPCs, we found that the effect of NAD+ on OPC differentiation was diminished by a SIRT2 inhibitor, thiomyrystoil (TM), in a dose-dependent manner, and was fully blocked at doses over 5 μM. (Fig. 6a). In primary OPC cultured from SIRT2−/− mice, SIRT2 knockout completely abolished the effect of NAD+ on both the proliferation (Fig. 6b, c) and the differentiation (Fig. 6d, e) of OPCs. The in vitro pharmacological inhibition and genetic deletion data indicated that for NAD+ to enhance the differentiation of OPCs requires SIRT2.

To further validate the role of SIRT2 in the enhancing effect of NAD+ on remyelination in vivo, we applied long-term NAD+ supplementation in SIRT2−/− mice and WT young mice with the same age, both of which received LPC injection-induced demyelination. We found long-term NAD+ supplementation did not affect the proliferation of OPCs in both SIRT2−/− mice and age-matched WT young (6 M) mice (Fig. 6f−j). Interestingly, in WT young mice, long-term NAD+ supplementation promoted the differentiation of OPCs (Fig. 6k−o) and improved the myelin quality (Fig. 6p−w). By contrast, in SIRT2−/− mice, long-term NAD+ supplementation did not affect the impaired capacity of OPCs on proliferation (Fig. 6f−j), differentiation (Fig. 6k−o) or remyelination efficiency (Fig. 6p−w). Loss of SIRT2 in mice completely abolished any changes in all indexes we observed in comparison with age-matched young mice. Collectively, our in vitro and in vivo data confirmed that the effect of NAD+ on the differentiation of OPCs and remyelination requires SIRT2.

β-NMN induces the nuclear entry of SIRT2 and suppresses transcription of ID4, thus promoting OPC differentiation in vitro. To further unveil the molecular mechanism underlying the effect of NAD+ on OPCs, we showed that β-NMN treatment increased the nuclear proportion of SIRT2 in cultured mouse OPCs (Fig. 7a−c). The fact that β-NMN promoted nuclear entry of SIRT2 was validated in OLN93 cells by cellular fractionation and Western blot (Fig. 7d). These in vitro results recapitulate the in vivo phenotype that NAD+ induces SIRT2 nuclear localisation in OPCs. SIRT2 exerts its function through deacetylating several histone sites such as H3K18 and H3K56, which had been identified previously41,42. We detected the acetylation level of these histone sites such as H3K18 and H3K56, which had been identified previously41,42.
In contrast, the acetylation level of H3K56 remained unchanged (Fig. 7e).

ID4 has been identified as a transcription factor which inhibits the differentiation of OPCs by repressing the transcription of MBP\(^{43,44}\). The mRNA level of ID4 was elevated to 6 folds in OPCs of G3 Terc\(^{-/-}\) mice compared to OPCs of WT mice. Importantly, in the OPCs of G3 Terc\(^{-/-}\) mice, β-NMN treatment decreased the elevated mRNA level of ID4 to less than half (Fig. 7f) but did not change the mRNA level of ID2 (Supplementary Fig. 13a). In OPCs of WT mice, β-NMN also downregulated the protein level of ID4 (Fig. 7g–h), which was inversely correlated with β-NMN induced upregulation of MBP (Fig. 4e–f). Since we have established that impaired differentiation of OPCs is the key pathology for SIRT2\(^{-/-}\) mice (Fig. 2),
we asked whether ID4 is elevated in SIRT2−/− mice. Indeed, the mRNA level of ID4 increased sharply in the primary cultured OPCs of SIRT2−/− mice (Fig. 7i). Consistently, the mRNA level of ID4 in the brain of SIRT2−/− mice was higher than that of WT mice (Fig. 7j). Using chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) analysis (Fig. 7k), we found that β-NMN increased SIRT2 binding to the promoter of ID4 (Fig. 7l), but not to ID2 (Supplementary Fig. 13b), whereas β-NMN decreased H3K18Ac binding to the promoter of ID4 (Fig. 7m). These data confirmed that SIRT2 inhibits the transcription of ID4 by deacetylating H3K18.

Based on our results, we propose a working model of NAD+ effecting on OPCs: aged OPCs exhibit depleted SIRT2 nuclear localisation and have lower NAD+ level. Supplementation of β-NMN elevates the NAD+ level, restores nuclear entry of SIRT2 in OPCs and deacetylates H3K18, thus suppressing the transcription of ID4 and further promotes OPC differentiation. NAD+ repletion restores SIRT2 nuclear entry in OPCs, promotes OPCs differentiating into mature oligodendrocytes, and eventually delays myelin ageing in the normal CNS and enhances myelin repair in the demyelinated aged CNS (Fig. 8).

Discussion
The present study identifies SIRT2 as a molecular target for OPC. Werner et al. and Li et al. reported that SIRT2 is expressed throughout the oligodendrocyte lineage cells, and exclusively in mature oligodendrocytes in the adult CNS29,30. The transcriptome database provided by Cahoy et al. and Zhang et al. also revealed that the mRNA of SIRT2 is enriched in oligodendrocytes lineage cells45,46. In this work, we showed that the sirt2 mRNA in cultured OPCs was overwhelmingly dominant over any of the other six members within the sirtuin family, and ranked the second highest among various cell types in the brain, just next to mature oligodendrocytes. Consistent with previous results39–41, we demonstrated that SIRT2 was indeed exclusively localised to the cytoplasm of mature oligodendrocytes. However, surprisingly, we further revealed that the SIRT2 protein was transiently expressed in the nucleus of OPCs during myelin development while depleted in OPCs of the adult CNS, indicating that SIRT2 is a specific target for oligodendrocyte lineage cells. The switch of SIRT2 localisation from OPCs during development to mature oligodendrocytes in the adult CNS is reserved to mammals and, in particular, primates. Interestingly, in the adult CNS, demyelination induced the re-expression of SIRT2 in the nuclei of OPCs, recapitulating a developmental program. However, such recapitulation was impaired in the aged CNS, correlating with impaired remyelination in the aged CNS47.

Given that nuclear SIRT2 expression occurred during myelin postnatal development, and this program was recapitulated within the demyelination lesion in the adult CNS, we next explored the function of SIRT2 in OPCs. To clearly investigate the role of SIRT2 specifically in OPCs in vivo, we used NG2-CreERT; Sirt2flox/flox mice, and i.p. injected tamoxifen to exclusively delete SIRT2 in OPCs in a temporally controllable manner. Our results showed that the conditional knockout of SIRT2 in OPCs reduced the frequency of remyelinated axons, decreased the thickness of newly formed myelin, and reduced the frequency of normal-looking newly formed myelin. These results provide direct evidence that SIRT2 in OPCs indeed plays a critical role in remyelination, and have established a causality between the loss of SIRT2 in OPCs and impaired remyelination. Intriguingly, the autotobody of SIRT2 is prevalent in the cerebrospinal fluid of secondary progressive MS patients48, whose pathological feature is failed remyelination. In addition, SIRT2 protein levels diminished within the lesion in both the EAE model and in MS patients49.

Next, we demonstrate that elevating NAD+ with β-NMN delays myelin ageing and enhances myelin repair in the aged CNS. Using G3 Terc−/− mice, a premature ageing OPC model established in this work, we revealed that NAD+ was one of the top depleted metabolites in the aged OPCs. The present study provides a comparison of the metabolic fingerprints of OPCs between young and old, laying a foundation for future studies concerning the metabolism of aged OPCs. Since NAD+ is a regulator of SIRT2 activity27,50, we supplemented β-NMN, the immediate precursor of NAD+51, in a dose comparable to the clinical trial in which the long-term clinical safety of β-NMN has been proven52, and found that NAD+ repletion restored the nuclear entry of SIRT2 in the aged OPCs and delayed myelin ageing. Furthermore, we demonstrated that NAD+ supplementation enhances remyelination efficiency both ultrastructurally and functionally in the aged CNS in both a preventive and therapeutic way; these results highlight the potential for this work to go towards clinical translational studies, bringing hope to potential treatments for the progressive phase of MS, a so far unmet therapeutic challenge52.

To elucidate the molecular mechanism underlying the effect of NAD+ on OPCs, by using multiple techniques, we unveiled that nuclear SIRT2 deacetylases H3K18, thus making chromatin condensed and repressed. This leads to the decreased transcription of ID4 and further promoting OPC differentiation by disinhibiting the transcription of myelin genes such as MBP. Importantly, SIRT2 is necessary for the effect of NAD+ on OPCs. Interestingly, the localisation switch of SIRT2 is similar to that of ID4, which also switches from the nuclei of OPCs to the cytoplasm of mature oligodendrocytes shown in the previous study53. The current work reveals that the effects of β-NMN are mediated through NAD+−SIRT2−H3K18Ac-ID4 sequentially, and that SIRT2 is necessary for NAD+ to rejuvenate the aged OPCs. Nevertheless, the process by which NAD+ causes nuclear entry of SIRT2 in OPC awaits further study. For translational purposes,
oral administration is desirable in view of the effects of oral administration of β-NMN on OPCs, other neural functions, and other systems need to be systematically examined. Recent studies have shown that in young mice, supplementing unstable NAD$^+$ decreases the EAE score, correlating with beneficial Th1/Th17 immune responses$^{54}$ or helpful CD4$^+$ T cell differentiation$^{55}$, and supplying niacin, an indirect NAD$^+$ precursor, modulates macrophages$^{19}$, suggesting that NAD$^+$ has versatile targets. Our work clarifies that β-NMN rejuvenates aged OPCs, the central player for myelination and remyelination$^{12}$, by restoring the nuclear entry of SIRT2. Together, we report that the nuclear entry of SIRT2 is depleted and NAD$^+$ is substantially reduced in the aged OPCs. Supplementing β-NMN restores the nuclear entry of SIRT2 and...
rejuvenates aged OPCs by promoting their differentiation into mature oligodendrocytes and eventually enhances new myelin generation in the aged CNS. Mechanistically, the effects of β-NMN are mediated through NAD⁺/SIRT2-H3K18ac-ID4 sequentially, and SIRT2 is necessary. The present study identifies SIRT2 as a molecular target for rejuvenating the aged OPCs, and by restoring nuclear entry of SIRT2 in the aged OPCs, β-NMN delays myelin aging in normal and enhances remyelination in demyelinated aged CNS, paving the way for future clinical translation study.

Methods

**Human brain tissue.** Human brain tissue was provided by National Health and Disease Human Brain Tissue Resource Center (http://zjubrainbank.jzlu.edu.cn) and studies were approved by Medical Ethics Review Committee of Zhejiang University School of Medicine (ETHICS number: 2018-109). The sample was anonymously coded in accordance with local ethical guidelines, and written informed consent was obtained. Patient was male aged 53 years.

**Marmoset brain tissue.** The marmoset brain tissue was from Lixia Gao’s lab. One was collected from a P3 healthy male Marmoset and another was collected from an 8 years old healthy male Marmoset. All studies with marmosets were approved by the Experimental Animal Welfare Ethics Review Committee of Zhejiang University (ETHICS number: ZJU20190907).

**Mice and diet.** C57BL/6, G3 Terc⁻/⁻ mice, G3 Terc⁻/⁻ mice, G3 Terc⁻/⁻ mice were used in this study. They are male mice of different ages (3 M 6 M 18 M 21 M) on a C57BL/6 background. The C57BL/6 mice were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The G3 Terc⁻/⁻ mice were from the Zhenyu Ju laboratory and genotyped by PCR reactions with three primers to amplify the WT and Knockout alleles (1, 5′-TTCTGACCACCACCAACTTCAAT-3′, 2, 5′-GGGGGCTG CTAAGAAGGCATGCATCTCAAT-3′, 3, 5′-CTCAAGGCGGCACTCCTCTCAAC-3′). The sizes of the PCR products of WT and Knockout alleles are 220 bp and 180 bp, respectively. The SIRT2⁻/⁻ mice were from the Hozhou Chen laboratory and genotyped by PCR reactions with three primers to amplify the WT and Knockout alleles (1, 5′-GACTTCCACGGCGATGCCCTC-3′, 2, 5′-GCTGAGCTAGCTACAGCTAGC-3′, 3, 5′-TCAAACTGGCCGCAACTCCTGAG-3′). The sizes of the PCR products of WT and Knockout mice are 358 bp and 700 bp, respectively. The Sirt2lox/lox mice were purchased from Shanghai Model Organisms (NM-CKO-190038, Shanghai, China) and genotyped using PCR reactions with two primers to amplify the WT and Sirt2lox/lox alleles (1, 5′-TCTTACAACACCTAATGACCTAGC-3′, 2, 5′-CCAT CATCTTACTCCACACTCT-3′). The sizes of the PCR products of WT and Sirt2lox/lox alleles are 214 bp and 364 bp, respectively. The NG2-CreERT² mice were from the Chong Liu laboratory and genotyped using PCR reactions with three primers to amplify the WT and Knockout alleles (1, 5′-GATGTTGATAAAG GCGGACATCCT-3′, 2, 5′-TCTGAAGCTTAAATTTTCCATATGAGTCCA-3′, 3, 5′-TCACACCATCATATACTC-3′). The sizes of the PCR products of WT and Knockout mice are 261 bp and 220 bp, respectively. NG2-CreERT²; Sirt2lox/lox mice were obtained by crossing the Sirt2lox/lox mice with the NG2-CreERT² mice. All mice were housed in an environment of suitable temperature and humidity with ad libitum access to water and food (25 °C, suitable humidity (typically 50%), 12 h dark/light cycle) at the Zhejiang University Animal Experimental Center. All mice appeared healthy, received regular monitoring from animal care staff and were not involved in prior procedures or testing. For β-NMN treated mice, β-NMN (#BTO5, Bontac) were dissolved in PBS and intraperitoneally injected daily (10 mg/kg bodyweight). The NG2-CreERT²; Sirt2lox/lox mice were intraperitoneally injected with tamoxifen (Sigma, #T7564, 100 mg/kg) for 5 days34 to 36 so that the Cre recombinase-mediated excision creates a nonfunctional allele exclusively in OPCs in a temporally-controlled manner, and the tamoxifen was dissolved in a 9:1 ratio of corn oil to ethanol. Four days post the last administration of tamoxifen, demyelination was induced by focal injections of LPC into the corpus callosum and detected remyelination at 21 dpl with TEM. All mice were randomly allocated to experimental groups. All studies with mice were approved by the Experimental Animal Welfare Ethics Review Committee of Zhejiang University (ETHICS number: 14660).

**Primary OPC culture, microglia and astrocytes.** For the rat OPC culture, two newborn SD rat cerebral cortices were dissected out in ice-cold HBSS (#14025092, Gibco), then the tissues were minced and digested for 12 min (min) at 37 °C in 0.25% trypsin (#25200856, Gibco). The digestion was terminated with DMEM/F12 (10290082, Gibco) containing a 1% foetal bovine serum (FBS, #10099141, Gibco). Then the tissue was washed into a single-cell suspension with a pipette and the cells were collected by centrifuging at 500 g for 5 min at 4 °C. The cells were then plated in a 75 cm² tissue culture flask which was pre-coated with poly-D-lysine (PDL, #P0899, Sigma, 0.1 mg/mL). Cells were maintained in DMEM/F12 containing 10% FBS and incubated at 37 °C with 5% CO₂. The medium was then completely changed at day 2, day 5, day 8. At day 8, the cells were shaken in a shaker at 200 rpm for 2 h at 37 °C and the medium was centrifuged at 300 g for 5 min to collect microglia. Then fresh medium was added into the flask. For OPC purification, the flask was shaken at 250 rpm for 16–18 h and the medium was collected at 1000 g for 5 min after the OPCs were treated with 300 μM cytarabine to inhibit the growth of glial cells. The firmly-attached cells at the bottom of the flask are astrocytes. The astrocytes were collected by 2 min digestion in 0.25% trypsin at 37 °C with a 5 min centrifugation at 300 g. After purification, microglia and microglia were seeded onto dishes and cultured in DMEM/F12 containing 10% FBS. OPCs were seeded onto coverslips or dishes coated with PDL and cultured in DMEM/F12 containing 10% FBS. After 3–4 h culture, the medium of OPCs was changed to Neurobasal™ (#21103049, Gibco) medium containing B27™ Supplement (#17504044, Gibco) and N2 Supplement (#17502001, Gibco). All studies with rats were approved by the Experimental Animal Welfare Ethics Review Committee of Zhejiang University (ETHICS number: 14660).

For mouse OPC culture, four newborn mouse cerebral cortices were dissected out in ice-cold HBSS. Then the tissues were minced into pieces and blown into cell suspension with a 1 mL pipette. The cell suspension was then transferred into a 75 cm² tissue culture flask which was pre-coated with PDL and maintained in DMEM/F12 containing 10% FBS and incubated at 37 °C. The medium was completely changed at day 3, day 5, day 7. The next steps are the same as those for the rat OPC cultures. For β-NMN treatment, OPCs were treated with 1 mM β-NMN, and β-NMN was dissolved in PBS.

**Primary culture of neuron.** For the rat neuron culture, the hippocampi from embryonic day 18 rats were dissected out in ice-cold HBSS and digested for 12 min at 37 °C in 0.125% trypsin. The digestion was terminated with DMEM (#11665092, Gibco) containing 10% F12 (#11765062, Gibco) and 10% FBS (#10099141, Gibco). Then the tissue was gently pipetted into a single-cell suspension with a pipette (1 mL) and left still for 2 min. The single-cell suspension was then transferred to a new 1.5 mL tube and centrifuged at 200 g for 5 min at 4 °C. The cells were resuspended in Neurobasal™ medium containing 2% B27™ Supplement and 1% Glutamax (#35050061, Gibco) and seeded onto dishes that were pre-coated with PDL. 1.25 μM cytarabine was added to inhibit the growth of glial cells.
Cell lines and cell culture. HEK293T (Human embryonic kidney) and OLN93 (Rat oligodendrocyte) cell lines were used in this study. Both of these two cell lines were cultured in DMEM (#11965092, Gibco) with 10% FBS (#04-001-1ACS, Biological Industries) and 1% Penicillin/Streptomycin (#15140163, Gibco) at 37 °C with 5% CO2. HEK293T cells were purchased from the American Type Culture Collection (ATCC). The OLN93 cells were gifted by the Hui Fu laboratory (Wuhan University).

Immunofluorescence for cells. Cultured cells were rinsed with PBS three times and fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature (RT). Then the cells were washed three times with PBS and blocked with 5% normal donkey serum (NDS, #D9663, Sigma), 0.3% Triton X-100 (#T8787, Sigma) in PBS for 2 h at 4 °C. The cells were then incubated with primary antibodies (diluted with 2.5% NDS, 0.3% Triton X-100 in PBS) for 4 h at RT and then washed with PBS for three times. Primary antibodies used in this study include: rat anti-Ki67 (#14-5698-82, Thermo
Fig. 6 The enhancing effect of β-NMN on remyelination of the mice requires SIRT2. a Quantification of differentiated oligodendrocytes from PO WT mice cultured for 48 h (n = 3). TM, Thyminylstrol. b–e Images and quantification of proliferating OPCs (b, c) cultured for 36 h and differentiated oligodendrocytes (d, e) cultured for 48 h from PO SIRT2/−/− mice (n = 3). Scale bars, 50 μm. f Experiment design for testing the impact of β-NMN on OPC proliferation in vivo. g–j Images and quantification of oligodendrocyte lineage cells and proliferating OPCs within the demyelination lesions at 5 dpl (n = 5). Scale bar, 50 μm. k Experiment design for testing the impact of β-NMN on OPC differentiation in vivo. l–o Images and quantification of oligodendrocyte lineage cells and differentiated oligodendrocytes within the demyelination lesions at 10 dpl (n = 5). Scale bar, 50 μm. p Experiment design for testing the impact of β-NMN on remyelination efficiency in vivo at 21 dpl. q TEM micrographs within the lesions at 21 dpl. Scale bar, 1 μm. r–w Quantification of the proportion of myelin pathel level (r), remyelination axes (s), G-Ratio average (t), individual G-Ratio distribution (u), linear regression and distance between DL (v, w) within the lesions at 21 dpl (n = 5). All data are presented as mean ± SEM. The center, upper and lower quartiles represent the median, upper and lower quartiles, respectively. *p < 0.05, **p < 0.01, ***<p < 0.001 by two-tailed t-test (c, e) or one-way ANOVA followed by Tukey’s post hoc test (a, h, j, m–s, t, w) or two-way repeated ANOVA followed by Sidak’s post hoc test (r). In all instances *p < 0.001. n.s. no significance. In (a), **p = 0.004 (DMSO vs. DMSO + β-NMN), **p = 0.002 (DMSO + β-NMN vs. β-NMN + 5 μM TM); In (n), p = 0.02 (WT + PBS vs. WT + β-NMN); In (o), p = 0.05 (WT + PBS vs. WT + β-NMN); In (r), p = 0.02 (grade 2, WT + PBS vs. WT + β-NMN).
as the ratio of the diameter of inner circle over that of the outer circle on the same myelin, which is inversely correlated with myelin thickness. The G-Ratio of unmyelinated and demyelinated axons is 1, which is the maximum value of the G-Ratio. The diameter of axons was calculated by area, which was also measured by tracking the outer surface of axons. Axon diameters were used in our scatter plot of axon diameter and G-Ratio, which represent the linear relationship between the G-Ratio and the axon diameter. The distance between DL was calculated by dividing the distance from the outermost DL to the innermost DL by the number of DL. In the mouse corpus callosum, the mean diameter of unmyelinated axons is more or less constant, with an overall mean diameter of 0.25 ± 0.01 μm. Therefore, only axons with a diameter >0.25 μm were counted as unmyelinated axons. These axons are more likely to be axons without remyelination, rather than axons that were initially unmyelinated.

**Evaluation of axonal pathology.** “Defective axons” was evaluated by: shrunk axons, mitochondria accumulation or swelling, myelin sphere inclusion in axon or in the space between myelin and axon, and axon break. Only the axons with diameters >0.25 μm and were wrapped with myelin were analysed.
NAD<sup>+</sup> enhances SIRT2 nuclear entry and rejuvenates aged OPCs. NAD<sup>+</sup> targets OPCs and restores SIRT2 nuclear localisation in aged OPCs and delays myelin ageing and enhances myelin repair following demyelination in the aged CNS.

**Immunopanning of OPCs.** As previously described<sup>40</sup>, 50 µL BSL-1 (#FL-1000, Vector Laboratories) was diluted in 22 mL DPBS, and four plates were coated with BSL-1 solution at RT for at least 4 h, then replaced by DPBS containing 0.2% BSA. Two 100 mm Petri dishes were incubated with 15 mL Tris buffer containing 72 µg mL<sup>–1</sup> trypsin, 26 µM NaHCO<sub>3</sub>, 25 mM Dextrose, 2 mM MgSO<sub>4</sub>, and 2 mM CaCl<sub>2</sub>; (250 µm thick, four slices before and after bregma dissected and incubated in the papain solution at 37 °C for 45 min overnight. Then, one of the secondary antibody-coated plates was washed three times with DPBS, and incubated with the primary antibody solution, which contained 10 µL rat anti-PDGFRα antibody (#558774, BD Biosciences-Pharmingen, 1:1000), 10 mL DPBS and 0.2% BSA, at RT for at least 2 h.

For tissue dissociation, 7-day-old WT and G3 Terc<sup>−/−</sup> mouse brains were dissected and incubated in the papain solution at 37 °C for 45–60 min. A trypsin inhibitor buffer was gently added to stop digestion. The dissociated cell solution was carefully layered over a 4 mL standard trypsin inhibitor buffer in a new 14 mL polystyrene tube. After centrifuged at 200 g for 15 min, the cell pellet was resuspended with DPBS.

To deplete microglia, the cell suspension was incubated in a BSL-1 coated plate for 15 min at RT. Then, the supernatant was successively incubated in new BSL-1 coated plates for three times. The supernatant was transferred to a new tube, centrifuged at 200 g for 10 min and resuspended with a 6 mL panning buffer (DPBS, 0.02% BSA). To deplete the non-specific binding, cells were transferred to the secondary antibody-coated plate and incubated at RT for 8 min. Then the supernatant was transferred to the primary antibody-coated plate and incubated at RT for 15 min to collect OPCs. The floating cells were washed away with DPBS for 8–10 times. More washing should be performed if there is still significant number of floating cells. The remained cells were collected and stored at −80 °C until the next experiment.

**Corpus callosum compound action potential (CAP) recording.** As described previously, mice were first anesthetised with intraperitoneal injection of sodium pentobarbital (50 mg/kg) and decapitated. Then the brains were dissected out and transferred to an ice-cold slicing solution (2.5 mM KCl, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 26 mM NaHCO<sub>3</sub>, 10 mM Dextrose, 2 mM MgSO<sub>4</sub>, 2 mM CaCl<sub>2</sub>), which was bubbled with mixed gas (95% O<sub>2</sub>, 5% CO<sub>2</sub>). Coronal slices were cut (250 µm thick, four slices before and after bregma ~1.0 mm) using a vibratome (Leica VT1200S). The slices were then transferred to an incubation chamber filled with artificial cerebrospinal fluid (ACSF) (126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 26 mM NaHCO<sub>3</sub>, 25 mM Dextrose, 2 mM MgSO<sub>4</sub>, 2 mM CaCl<sub>2</sub>; 315–325 mMosm, pH = 7.2–7.3) and maintained at 34.5 °C for 45 min. After incubation, slices were kept in the same solution at RT and allowed to equilibrate at least 30 min prior to recording.

For electrophysiological recordings, Slices were transferred to a recording chamber perfused at 2 mL/min rate with aerated ACSF at 20.5–22.7 °C. Referring to a previous report<sup>41</sup>, a tungsten bipolar electrode was used for stimulation in the corpus callosum of one hemisphere and a glass electrode (impedance of 1–3 MΩ) filled with ACSF was placed in the contralateral hemisphere for recording. The stimulating pulses of 0.1 ms duration, 1 mA current and 10 s interval were applied via an isolator (ISO-Flex, A.M.P.I). Evoked CAPs were recorded by a MultiClamp 700B amplifier (Molecular Devices) and sampled by a Micro3 1401 (CED, Cambridge Electronic Design) at 25 kHz using Spike2 software (Bessel filter set to 10 kHz) for offline analysis. For each slice, we recorded two trials with the same stimulation site of two and different recording sites in the current clamp mode.

For data analysis and statistics, 100 repeated responses of each trial were averaged for waveform analysis. The time courses of conduction (T1 and T2) were calculated from the onset of stimulus artifact to negative peaks. Conduction velocity was estimated by the difference of two different distances between the stimulating and recording electrodes (ΔDistance) divided by the difference of corresponding peak latency (ΔT) in the same slice, that is, Fast velocity = ΔDistance/ΔT1; Slow velocity = ΔDistance/ΔT2. CAP amplitude was measured as the vertical distance from the local negative peak of two depolarising phases of the CAPs (Amp.1 and Amp.2) to a tangent joining preceding and following positivities. Due to the serious damage of myelin in some brain slices, the effective conduction was measured to reveal the percentage of slices which lack the fast component even with higher intensity of stimulus.

**Targeted metabolic profiling.** 500 µL 80% Methanol (#67-56-1, Millipore) was added to OPCs (10<sup>5</sup> cells per sample) purified by immunopanning and vortexed fully to extract metabolites. 480 µL supernatant was collected after 15 min centrifugation (13000 × g, 4 °C) and dried at SpeedVac Concentrator (EIS110-230, ThermoFisher) for 4 h. The dried metabolites were then reconstituted in 30 µL of 0.03% formic acid in analytical water. After being vortexed and centrifuged, the supernatant was transferred to an HPLC vial for metabolomics analysis. The targeted metabolite profiling was performed using an LC–MS/MS approach. Separation was achieved on an ACQUITY UPLC HSS T3 column (2.1 × 150 mm, 1.8 µm) using an Ultra High Performance Liquid Chromatograph (UHPLC) system (Nexera×2 LC-30A, Shimadzu) with the following gradient: 0–3 min, 1% mobile phase B; 3–15 min, 1–99% B; 15–17 min, 99% B; 17–17.1 min, 99–1% B; 17.1–20 min, 1% B. The mobile phases employed were 0.03% formic acid in water (A) and 0.03% formic acid in acetonitrile (B). The column was maintained at 35 °C and the samples were kept in the autosampler at 4 °C. The flow rate was 0.25 mL/min, and the injection volume was 20 µL. The mass spectrometer was a triple quadrupole mass spectrometer (QTRAP 6500+, Sciex) with an electrospray ionisation (ESI) source. The sample analysis was performed in multiple reaction monitoring mode. A total of 256 metabolites were monitored with 160 ion transitions in positive mode and 96 ion transitions in negative mode. A chromatogram review and peak area integration were performed using the MultiQuant software v.3.0 (Sciex). The peak area for each detected metabolite was normalised against the...
NAD⁺ quantification. NAD⁺ quantification was performed using NAD⁺/NADH Quantification Colorimetric Kit (#K337, BioVision) according to the instructions. Briefly, 20 mg brain tissue was washed with cold PBS and homogenized in 400 µL of NADH/NAD+ Extraction buffer in a micro-centrifuge tube, then centrifuged at 18000 g for 5 min and the supernatant was transferred into a new tube and diluted 70 times with NADH/NAD+ Extraction buffer. For total NAD detection, 50 µL of diluted samples was transferred into a labeled 96-well plate. For NADH detection, the samples were incubated in a 60 °C water bath for 30 min to decompose the NAD⁺. For a standard curve preparation, 10 µL of 1 mol/mL NAD standard was diluted with 990 µL NADH/NAD+ Extraction buffer to generate 10 pmol/µL standard. The samples were diluted 1000 times from the NADH/NAD+ Extraction buffer to generate a 0, 20, 40, 60, 80, 100 pmol/well standard. 100 µL of reaction mix (98 µL of NAD Cycling Buffer and 2 µL of NAD Cycling Enzyme Mix) was added into each well and incubated at RT for 5 min. Then 10 µL of NADH developer was added into each well and incubated at RT for 1-4 hours. The reactions were stopped by adding 10 µL of stop solution and the plate was read at OD 450 nm. The content of NAD⁺ was calculated by subtracting the content of NADH from total NAD.

Western blot analysis. For tissue lysis, the tissues had been ground in liquid nitrogen and lyzed in lysis buffer [50 mM Tris-HCl-pH 7.5, 5 mM EDTA, 1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 3.69% CHAPS, 1% Triton X-100, 50 mM NaCl, 1 mM DTT (#13483-12-3, Biohours), protease inhibitor (#18519900, Roche), phosphatase inhibitor A (#B15001-A, Biotool) and B (#B15001-B, Biotool), MG132] for 30 min on ice. Then, the mixture was centrifuged at 10000 g for 5 min at 4 °C and the supernatant was stored at −80 °C. For cell lysis, the cells were collected by centrifugation at 300 g for 5 min and lyzed in lysis buffer at 4 °C for 30 min. For cellular fractionation, the cytoplasm and the nucleus of ONL93 cells were separated by using the "PARIS™kit" (ThermoFisher, #AM1921). Protein quantification was implemented using the BCA protein assay kit measured with a microplate reader. SDS running buffer for 60 min for separation. Proteins were then transferred onto a 12% Blot Bis-Tris plus SDS gels (#FD346, Fdbio science) and run at 200 V in an (#FD006, Fdbio science) after boiling to 95 °C for 5 min. Proteins were loaded onto the PVDF membrane (Millipore) and transferred in a Blotting Substrate (#32106, Thermo Fisher Scientific) for 2 min and imaged using the BioSpectrum Imaging System (BioRad). The membrane was stripped using stripping buffer (25 mM Glycine and 1% SDS in ddH₂O, pH 2.0) for 20 min at RT, blocked at RT for 2 h in 5% skim milk blocking buffer in TBS-Tween (TBST). Primary antibodies were incubated overnight at 4 °C on a roller in 0.1% TBST. Primary antibodies used in this study include: rabbit anti-SIRT2 (#S8447, Sigma, 1:1000), mouse anti-beta-actin (#A5441, Sigma, 1:10000), mouse anti-Tubulin (#M1305-2, HUABIO, 1:5000), mouse anti-Ac-Tubulin (4341, #F7451, Sigma, 1:1000), rabbit anti-rH3 (#ab1791, Abcam,1:10000), rabbit anti-H3K18ac (#9694, Active motif, 1:1000), rabbit anti-H3K56ac (#9282, Active motif, 1:1000), rabbit anti-HP1beta (#MC3409, Abcam, 1:5000), rabbit anti-Id4 (#LS-C83409, Bioss, 1:1000). Blots were washed with TBST (10 min, three times) and incubated at RT for 2 h with HRP secondary antibodies in TBST. Secondary antibodies used in this study include: Peroxidase affinitive donkey anti-rabbit IgG (H + L) (#711-055-152, Jackson Immunoresearch) Peroxidase affinitive donkey anti-mouse IgG (H + L) (#715-035-151, Jackson Immunoresearch, 1:10000). Blots were then washed with TBST (10 min, three times), microscans was one for both MS and MS/MS scans and the time of maximum ion injection was 50 and 100 ms, respectively. The dynamic exclusion settings used were 10 s for charge exclusion and on; and exclusion duration, 30 s. MS scan functions and LC solvent gradients were controlled by the Xcalibur data system (Thermo Scientific).

For data analysis, the acquired MS/MS data were analysed using the Uniprot Knowledgebase (https://www.uniprot.org, Swiss-Prot Rattus norvegicus database released on Apr. 1, 2019) using PEAKS Studio 8.5 (Bioinformatics Solutions, Waterloo, Ontario, Canada). The database search parameters were set as the following: MS and MS/MS tolerance of 20 ppm and 0.1 Da, respectively, FDR was set as 1% and the protein identification threshold was set as (10 logP) ≥ 20. The protein was identified when at least one unique peptide was matched. Protein quantification was based on label-free quantitative analysis.

Lentiviral constructs and production. We initially developed the lentiviral transfer plasmid. For SIRT2 overexpression, we inserted the sirt2 gene into the pCDH-CMV-EF1a-FLAG-P2A-copGFP plasmid. For SIRT2 knockdown, we designed shRNA sequences using the BLOCK-iTM RNAi Designer (Thermo Fisher Scientific) and inserted the sequences into the pGCR-puro (psPAX2, #12260, Addgene) plasmids to generate lentiviral particles. Then the lentivirus producer cell line HEK293T was transiently co-transfected with transfer (pCDH-CMV-EF1a-SIRT2-flac-pGFP) or pGreenPuro-shSIRT2 plasmid, envelope (pMD2.G, #12259, Addgene) and packaging (psPAX2, #12260, Addgene) plasmids to generate lentiviral particles. HEK293T cells were transfected using Polyethylenimine (PEI, #23966, Polysciences Inc., Warrington, PA) for mass spectrometry analysis. The full-scan MS spectrum (m/z 300-1800) was acquired followed by top 20 MS/MS events, sequentially generated on the first to the twentieth most intense ions selected from the full MS spectrum at a 28% normalised collision energy. The full-scan resolution was set to 70000 with automated gain control (AGC) target of 3E6. The MS/MS scan resolution was set to 17500 with an ion isolation window of 1.8 m/z and AGC target of 1E5. The number of microscans was one for both MS and MS/MS scans and the time of maximum ion injection was 50 and 100 ms, respectively. The dynamic exclusion settings used were 10 s for charge exclusion and on; and exclusion duration, 30 s. MS scan functions and LC solvent gradients were controlled by the Xcalibur data system (Thermo Scientific).

ChIP-qPCR. For the cross-linking of protein and DNA, 270 µL of 37% formaldehyde was added into a 10 cm dish when the cell confluency reaches 90%, and the dish was shaken slowly for 10 min at RT. Then 500 µL of 2.5 M glycine was added into the medium and the dish was shaken for 5 min to quench cross-linking. The cells were washed three times with cold PBS and digested in 0.25% trypsin for 4
3 min, then collected by centrifugation (800 g for 5 min at 4 °C) and washed twice with cold PBS. Cells were lysed in 500 μL CHiP lysis buffer (50 mM Tris-HCl pH 8.0, 0.5% SDS, 5 mM EDTA, protease inhibitor) for 5 min at 4 °C and centrifuged briefly. The samples were sonicated to shear the chromatin into about 300 bp fragments (typically, 0.5 s pulses followed by 0.5 s rest periods for 15 min at output 10). Then 5–10 μg of antibody or IgG was added into samples and incubated overnight at 4 °C. Antibodies used in this study include: Rabbit anti-SIRT2 (#8447, Sigma, 1:100), mouse anti-flag (M185-3L, MBL, 1:100), rabbit anti-H3K18Ac (#9694, Active motif), normal mouse IgG (#A7028, Beyotime), and normal rabbit IgG (#A7016, Beyotime). A 1% volume of the sample was pipetted out as input and the rest sample was centrifugated and the supernatant was transferred to a fresh micro-centrifuge tube. 50 μL Protein A/G PLUS-Agarose (#A-0383, SANTA CRUZ) was blocked in the block buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA-pH 8.0, 1% BSA) twice for 5 min and centrifuged for 2 min (800 g, 4 °C). The samples were incubated with agarose for 2 h at 4 °C and centrifuged for 2 min (800 g, 4 °C) and then the samples were washed with wash buffer 1 (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton-X-100, 0.1% SDS), wash buffer II (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM EDTA, 1% Triton-X-100, 0.1% SDS), wash buffer III (10 mM Tris-HCl pH 8.0, 0.25 M LiCl, 1 mM EDTA, 1% NP-40), TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA-pH 8.0), TE buffer for 10 min at 4 °C followed by 2 min of centrifugation (800 g, 4 °C), respectively. The agarose was resuspended with 100 μL elution buffer (0.1 M NaHCO3, 1% SDS, 1 mg/ml protease K) twice by using a rotatory shaker for 10 min at RT and the supernatant was collected after centrifugation (800 g, 2 min, RT). The sample tubes were placed in a 65 °C water bath overnight to release cross-linking. The DNA was purified from samples using a Universal DNA Purification Kit (D9214, TIANGEN) as instructed by the manufacturer. For qPCR, DNA, primers and ChamQ Universal SYBR qPCR Master Mix (#Q711, Vazyme) were mixed as instructed by the manufacturer. Fold changes were calculated using the Delta-Delta Ct method in Microsoft Excel. The primer sequences are as follows: CHIIP-1D4-F, 5′-TTGGCACGTGCCTCCTCTGGT-3′, CHIIP-1D4-R, 5′-CCCTCGAAATGACCGACTC-3′.

Statistics and reproducibility. All data analyses were performed using GraphPad Prism 8.0 or R. The data are shown as mean ± SEM. All the images, immunoblots and statistical graphs are representatives of at least three experiments, unless otherwise stated. The “n” numbers for each experiment are specified in the figure legends. For in vivo experiments, each data point represents an individual animal. In vitro experiments, each data point represents a biological replicate. The differences were considered statistically significant if p < 0.05 (*), p < 0.01 (**) or p < 0.001 (***)).

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Author contributions

J.-W.Z. and Z.J. conceived the project; J.-W.Z., Z.J., X.-R.M., and X.Z. designed the experiments; X.-R.M., X.Z., Y.X., H.-M.G., Z.-J.D., D.-X.W., Y.W., Y.Z. and L.G. assisted with the experiments and helped to analyse the data; J.-W.Z. and X.-R.M. wrote the paper; Y.S., H.-Z.C., X.Z. and Li Li edited the paper; revised the paper; J.-W.Z. supervised all phases of the project.

Competing interests

The authors declare no competing interests.

Additional information

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