Age-related decrease in mitochondrial IncMtDloop expression potentiates Alzheimer's pathogenesis

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Abstract

Loss of mitochondrial homeostasis are evident in Alzheimer's disease (AD)¹. However, the underlying mechanisms remain largely elusive. In this study, we report that *IncMtDloop*, an age-related and conserved long noncoding RNA derived from mitochondrial DNA (mtDNA) D-loop, is crucial for sustaining mitochondrial transcription and homeostasis, and associates with Alzheimer's pathogenesis. Notably, the level of *IncMtDloop* expression is diminished in the brains of human AD patients and the 3xTg mouse model, whereas the failure to recruit mitochondrial transcription factor A (TFAM) leads to the deregulated mitochondrial transcription. Restoring *IncMtDloop* expression not only significantly improves mitochondrial transcription, morphology and function, but also enhances mitophagy, ameliorates AD-like pathology, which these subcellular effects extend to a dramatic reversal of deficits in synaptic and cognitive behaviors of the 3xTg mouse model. Collectively, our findings show the decreased *IncMtDloop* expression potentiates AD risk, thereby revealing an unexpected mitochondrial mechanism contributing to AD pathogenesis and opens a new door for therapeutic intervention.

Background

Alzheimer's disease (AD) is by far the most common form of dementia in our aging societies^{2,3}. Currently, it remains no cure or effective treatment to manage the progression of the disease⁴⁻⁶. Mutations on genes regulating the production of β -amyloid (A β) plaques and tau tangles were identified in early-onset familial cases, but these could hardly explain the etiologies behind the more common but late-onset sporadic cases, which are multifactorial in nature.

Mitochondria are the major metabolic powerhouse in eukaryotic cells, generating more than 90% of cellular ATP which powers majority of cellular functions^{7,8}. Declines in total mitochondrial quality and activities have been associated with normal aging but also the development of a wide range of age-related disease⁹⁻¹¹. While the relationship between nuclear genome integrity and brain aging is well recognized, the potential contribution of mitochondrial DNA (mtDNA) is also questioned^{12,13}. The mitochondrial genome encodes 13 proteins of oxidative phosphorylation (OXPHOS) chain, which are crucial to ATP production and respiratory capacity, 2 rRNAs and 22 tRNAs, all of which are involved in the mitochondrial transcription, replication and translation in situ. The mtDNA exhibits profound influence on cellular function, including biogenesis, dynamics, metabolism and degradation¹⁴⁻¹⁶. Growing out from gene mutations to alternations at functional levels, dysregulated mitochondrial trafficking, biogenesis and mitophagy are now found to be associated with accelerated brain aging, neurodegeneration, and notably the pathogenesis of late-onset sporadic AD^{17,18}.

Long non-coding RNAs (lncRNAs) are non-protein coding transcripts with length longer than 200 nucleotides¹⁹. They represent a class of transcriptional regulatory machinery which is also one of the fastest evolving parts of the primate genome²⁰⁻²². lncRNAs may regulate gene transcription or epigenetic regulators by acting as RNA scaffolds for the assembly of chromatin- and gene-regulating higher-order complexes and to guide them to specific loci within the genome^{19,23,24}. On the other hand, lncRNAs may serve as cis- and trans-acting modulators of protein-coding gene expression, regulators of mRNA processing, modulators of post-transcriptional control by altering mRNA stability or serving as molecular sponges or even cell signaling molecules²⁵. In the human genome, around 40% of the known lncRNAs are uniquely expressed in the brain, which are essential part of neurogenesis, including differentiation, migration and patterning of neural progenitors; so as being crucial for effective action potential and synaptic plasticity in mature neurons²⁶⁻²⁸.

Although most of our current knowledge on lncRNAs are still those encoded from the nuclear genome, mammalian cells do express a family of long noncoding mitochondrial RNAs (lncmtRNAs). On mtDNA sequences that are complementary to genes encoding MT-ND5, MT-ND6, and Cytochrome b, three mitochondrial lncRNAs were identified where these antisense noncoding mitochondrial RNA (ASncmtRNA) expressions are being regulated by nuclear-encoded mitochondrial processing proteins^{29,30}. On the other hand, sense noncoding mitochondrial RNA (sncmtRNA) is also reported and characterized as a 2,374 nucleotide (nt) long transcript regulating the replicative state of a cell by forming a stem-loop structure complementing with the inverted repeat of 121 nt sequencing located at the 5' end of the mitochondrial 16S rRNA to regulate its stability^{31,32}. Apart from physiological processes, lncmtRNAs are also associated with disease pathogenesis. For example, the mitochondrial long noncoding RNA uc002bqs.1, also known as Long Intergenic noncoding RNA Predicted Cardiac Remodeling (LIPCAR), is a circulating lncmtRNA serving as a biomarker for cardiac remodeling and predicts the survival of patients with cardiac failure^{33,34}.

With deep sequencing approach, we have previously characterized the dynamic changes of global lncRNA expression in brain tissues harvested from rhesus macaque across the development and aging time span²². From there, *AC027613.1* is identified as an age-related and well-conserved lncRNA derived from mtDNA D-loop. In this study, we termed it *lncMtDloop*, and investigated its function in the regulation of mitochondrial transcription and homeostasis, as well as the contribution of its decreased expression to Alzheimer's pathogenesis.

Results

Levels of *lncMtDloop* expression decrease in the brains of human AD patients and the mouse model

In our previous report, cluster analysis revealed that *AC027613.1* was one of the newly identified lncRNAs in rhesus macaque brain, with important relevance in brain development and aging²². In this study, extended characterization on *AC027613.1* gene sequence showed that it is indeed located within the D-loop regions of mitochondrial genome (**Fig. 1a**), therefore we renamed it as *IncMtDloop*. With RNAscope *in situ* hybridization (RISH) using specific probe set against the mouse *IncMtDloop*; in combination with immunocytochemical (ICC) assays with antibody against ATP synthase F1 subunit alpha (ATP5a) serving as a specific mitochondrial marker revealed that *IncMtDloop* is predominantly located inside mitochondria of primary hippocampal neurons (**Fig. 1b, c**). To demonstrate the significance *IncMtDloop* in mitochondria evolution, gene comparative analysis was performed which revealed that it was a well conserved mitochondrial lncRNA among various mammalian species (**Extended Data Fig. 1a**). Despite size differences were identified in human (572bp), macaque (695bp) and mouse (939bp), key block sequences on *IncMtDloop* were evolutionally conserved, with all of them were expressed and localized in mitochondria of respective cell lines (i.e. Human SH-SY5Y, monkey Cos-7 and mouse N2A cells) of these different species (**Supplementary Fig. 1a-d**), as revealed by the same RISH-ICC assays mentioned above.

To confirm whether *IncMtDloop* is a true, novel mitochondrial lncRNA species without any protein-coding potential, we performed Phylo Codon Substitution Frequencies (PhyloCSF) analysis on the UCSC browser (**Extended Data Fig. 1b**). The corresponding CPC score (**Extended Data Fig. 1c**) and CPAT coding probability (**Extended Data Fig. 1d**) were almost reaching the minimum as compared to a positive control of reference coding transcripts, therefore supporting *IncMtDloop* has no protein-coding potentials. The secondary structure of *IncMtDloop* was predicted as well based on minimum free energy (MFE) (**Extended Data Fig. 1e**). These data revealed that *IncMtDloop* likely assembles a complex double-stranded stem-loop, which is frequently found in other known lncRNAs. With RISH-ICC system, it also indicated that *IncMtDloop* is abundantly expressed in mouse primary neurons, microglia and astrocytes, hinting that it is of great importance in supporting the normal mitochondrial physiology and function in the brain (**Supplementary Fig. 1e**).

With the RISH assay followed by immunohistochemistry analyses (RISH-IHC), significant reductions in *IncMtDloop* levels were observed in the prefrontal cortex (PFC), hippocampal CA1 as well as the dentate gyrus (DG) regions of human AD samples as compared to age-and sex-matched non-demented controls (**Fig. 1d, e**). Similar observations were found by Northern blotting and ssRT-qPCR analyses in the same set of human brain tissue samples (**Fig. 1f, j and k**). These phenomena are also conserved in aged (12 months old) 3xTg mice—a triple transgenic mouse model of AD exhibits both classic A β pathology and tauopathies with the same set of analyses (**Fig. 1g-i, l and m**). Of note, decreases in *IncMtDloop* expression were differentially found in 3xTg mice multiple organs which its function linkage to mitochondrial organelle (**Supplementary Fig. 1f**).

A β cascading hypothesis is one of dominating hypotheses addressed for explaining the onset and pathogenesis of AD ³⁵. With RISH, it revealed that levels of *IncMtDloop* were significantly reduced in 3xTg hippocampal neuronal culture as compared to wild type controls, so as when wild type neurons were exposed to exogenous A β_{42} treatment (**Extended Data Fig. 2a, b**). The loss in *IncMtDloop* was further confirmed by ssRT-qPCR and northern blot analyses (**Extended Data Fig. 2c-f**). Mutually, restoration of *IncMtDloop* in 3xTg hippocampal neuronal culture reduced the accumulation of A β (**Extended Data Fig. 2g, h**). As *IncMtDloop* is widely expressed in other cell types in the brain, as demonstrated earlier (**Extended Data Fig. 2a**), we then asked if their *IncMtDloop* expression may also respond to A β cascade signaling stimulus. By the RISH-ICC strategy, significant reductions in *IncMtDloop* signals were again observed in 3xTg cultured cortical neurons (**Supplementary Fig. 2a, b**) but not in primary microglia (**Supplementary Fig. 2d, e**) and astrocyte cultures (**Supplementary Fig. 2g, h**), as when the comparisons were made to wild type controls. Consistently, ssRT-qPCR results revealed similar patterns of observations (**Supplementary Fig. 2c, f and i**). Collectively, these data indicated that decreased *IncMtDloop* expression is unique to neuronal populations and associated with A β cascade signaling and pathology.

p32 promotes the mitochondrial localization of *IncMtDloop*

Previous studies indicated that lncRNA may serve as scaffolds or guides for multiple proteins and even facilitate the assembly of multiprotein complexes ³⁶. We speculated that *IncMtDloop* may also serve similar roles to mitochondrial proteins, therefore its potential binding partners were unbiasedly evaluated by RNA pull-down assay followed by silver staining and mass spectrometry (SS-MS) analyses (**Fig. 2a**). Despite our earlier data indicated that *IncMtDloop* is predominantly located in mitochondria, chances are some of which may also present in the cytosol region, we therefore performed analyses in both cytosolic and mitochondrial fractions enriched from hippocampal tissue lysates of wild type mice. Full-length *IncMtDloop* sequence conjugated with biotin probes was used as bait, upon incubation with different fractions of lysates, streptavidin-conjugated beads were used to capture the biotin-containing complexes, followed by SS-MS global analyses and western blotting as validation. Using the anti-sense *IncMtDloop* as negative control, the full-length sense *IncMtDloop* uniquely pulled down a group of interacting proteins (**Supplementary Fig. 3a-c**). With these candidates, large-scale prediction on protein-RNA associations and the interaction propensities between polypeptide and nucleotide chains were conducted using the catPAPID algorithm on the basis of the physical-chemical properties between the binding partners³⁷⁻³⁹. Among all uniquely pulled down proteins by the *IncMtDloop*, Syntaxin1B (Stx1b), ATP5d and p32 were among the ones with the highly potential interaction scores and discriminative power with *IncMtDloop* in mitochondrial fraction (**Fig. 2b**). For that in the cytosolic fraction, Aly/REF export factor (Alyref), tubulin polymerization promoting protein (Tppp) and p32 were those with the highest interaction scores and discriminative powers instead (**Fig. 2c**). With the mitochondrial extracts, interactions between *IncMtDloop* and ATP5d, as well as that with p32 but not Stx1b were confirmed by Western blotting analyses (**Fig. 2d**).

Similarly, *IncMtDloop* interactions with non-mitochondrial proteins Alyref and Tppp were also confirmed from cytosolic extracts (**Fig. 2e**).

p32 is also known as SF2-associated p32, p32/TAP and gC1qR. It is a conserved eukaryotic protein that localizes in the mitochondria, despite it is also present in other subcellular compartments^{40,41}. It is thought to be involved in mitochondrial oxidative phosphorylation regulation and nucleus-mitochondrial communication^{42,43}. As interaction between in vitro transcribed-*IncMtDloop* and p32 was detected in both cytosolic and mitochondrial extracts (**Fig. 2f**), thus indicating high confidence of interaction. Together with the distinct discriminative power and interactive score between p32 and *IncMtDloop* (**Fig. 2g**) as revealed by the catRAPID algorithm, as well as their colocalization pattern revealed by the RISH-ICC in primary neurons (**Fig. 2h, i**), we therefore use this protein as a study model for identifying the structural motifs on *IncMtDloop* that are critical for mediating RNA-protein interactions. Using RNA immunoprecipitation (RIP) assay followed by Northern blotting or qPCR analyses, *IncMtDloop* could be efficiently precipitated by p32 proteins in extracts of hippocampus and prefrontal cortex tissues freshly harvested from 12-month-old wild type mice (**Fig. 2j, k**). With computational prediction analysis by catRAIPD, a number of regions located at the 5' and 3' terminal domains of the *IncMtDloop* were predicted being essential for protein-RNA interactions (**Supplementary Fig. 3d, e and f**). Based on this information, a series of truncated *IncMtDloop* were constructed and subjected to the same RIP assay (**Fig. 2l, m**). Among various constructs, only the one that constitutes the 3'-terminal 637-939 nt fragment revealed significant interactions with p32. While the levels of RNA transcripts of neither p32 nor *IncMtDloop* showed direct relationship with one another (**Extended Data Fig. 3a-d**), protein levels of p32 were diminished in the hippocampal tissues of 3xTg mice with significance found only in aged ones (i.e. 12 months old) as compared to age-matched wild type (**Extended Data Fig. 3e, f**); hinting the reduced *IncMtDloop* level as a result of aberrant Ab cascade signaling might affect the p32 protein abundance potentially its coordinating roles on mitochondrial translation⁴⁴, mitophagy and oxidative fuel metabolism⁴¹ in neurons. Morphological alterations of mitochondria are reported to be associated to metabolic and energy deficiency in neurons in AD and other neurodegenerative disorders. Notably, mitochondrial dysfunction is also a hallmark of Ab-induced neuronal toxicity in AD^{45,46} and similar changes were well-documented previously in the AD-mouse model of our choice⁴⁷. Given that strong binding of *IncMtDloop* with p32 protein which processes a mitochondrial-targeting signal at its N-terminus⁴⁸, it is therefore possible that endogenous p32 may facilitate the transport of ectopically expressed *IncMtDloop* into the mitochondria by protein-RNA interaction. Using primary neurons where ectopic *IncMtDloop* was expressed, lentiviral particles of either carrying scrambled or p32 specific siRNA constructs were infected to these cells. Upon p32 knockdown in N2A cell line, using RNAscope and qPCR assays significant reductions in mitochondrial located ectopic *IncMtDloop* RNA were observed as compared to control (**Extended Data Fig. 3g, h**). Accordingly, p32 can serve as an ideal binding partner of ectopically expressed *IncMtDloop*, targeting them to mitochondrial region.

Recruitment of TFAM by IncMtDloop is indispensable for the mitochondrial transcription initiation

Damages to mtDNA accumulate over the aging process, as also the neural mtDNA mutations can contribute to aging-associated neurodegenerative disease like AD⁴⁹. As we found reduced levels of mtDNA copy number in 12-month-old 3xTg mice (**Fig. 3a**), we further explore the connection between *IncMtDloop* and mtDNA. In mitochondrial fraction, *IncMtDloop* can also interact with transcription factor complex in SS-MS results, suggesting that *IncMtDloop* may interact with mitochondrial transcription factor as a guide to regulate mitochondrial genome integrity. mtDNA is packaged in DNA-protein complexes named nucleoids, which contain the proteins required for mtDNA transcription, replication, translation and degradation⁵⁰. Within these mtDNA transcription factors, we performed a collection of prediction on protein-RNA associations using the catPAPID algorithm. Among these proteins, TFAM, a promoter-binding protein and a key role in mitochondrial transcription initiation^{51,52}, attracted our attention for its established role in interaction scores (**Fig. 3b**). Consistently, we performed western blot and RIP and reconfirmed the interaction between *IncMtDloop* and TFAM (**Fig. 3c, d**), and found 1-626 nt fragment of *IncMtDloop* revealed significant interactions with TFAM (**Fig. 3e, h**). We found the mRNA and protein expression levels of TFAM in 12-month-old 3xTg mice without significant reduction compared with WT (**Extended Data Fig.4a-c**) and there was little regulatory influence of *IncMtDloop* in TFAM protein expression (**Extended Data Fig.4e, f**). Given the TFAM initiates mitochondrial transcription by binding to the HSP and LSP upstream of the transcription start site (TSS) in a reversed orientation^{53,54}, we then tested whether *IncMtDloop* regulated the TFAM occupation of the promoter locus. We analyzed +200~-1000 bp locus within mitochondrial D-loop region, and found +200~-400 bp segment within the mitochondrial D-loop region is the sufficient binding site for TFAM through the luciferase reporter assays (**Fig. 3g and Extended Data Fig. 4d**). With chromatin immunoprecipitation (ChIP) results, we found that overexpression of *IncMtDloop* increase the binding ability of TFAM with promoters within the mitochondrial D-loop region (**Fig. 3f**). The apparent increases in mitochondrial mRNA levels were the broad effect of regulatory function of *IncMtDloop* (**Extended Data Fig. 4g**). These data suggested that TFAM could be recruited to the promoters within the mitochondrial D-loop region by *IncMtDloop* to assemble the initiation complex, and trigger mtDNA transcription (**Fig. 3i**).

Restoring *LncMtDloop* alleviates AD-related changes in mitochondrial morphology and oxidative fuel metabolism

mtDNA encodes the core component of OXPHOS complexes that is closely related with the synthesis of cellular ATP. As we found decreased levels of mtDNA copy number in AD, these accumulated damages to mtDNA integrity can have pleiotropic effects on mitochondrial function. To test whether diminished level of *IncMtDloop* contributes to mitochondrial abnormalities in AD-mouse model, we adopted a reverse approach and see if replenishment of neural *IncMtDloop* could reverse that phenomenon. With the aged

11-month-old 3xTg mice and their age-matched controls, adeno-associated viral particle (AAV) carrying a construct that expresses GFP-tagged *IncMtDloop* under the regulation of Synapsin-1 promoter (i.e. SYN: AAV9- *IncMtDloop*) was stereotactically microinjected into the hippocampus region. At six weeks after the initial injection, brain tissues were harvested, sliced and observed under the microscope to validate GFP-tagged *IncMtDloop* expression (**Extended Data Fig. 4e**). Next, brain tissues were then subjected to transmission electron microscopy analysis for mitochondrial ultrastructure analyses (**Fig. 4a**). With the images, mitochondrial ultrastructures were classified based on their number of cristae, these included Class I (>3 cristae); II (2-3 cristae) and III (no or 1 crista) (**Fig. 4c, d**); as well as the matrix densities including Class A (Dense matrix) and B (swollen and hypodense matrix) (**Fig. 4e, f**). In 3xTg mice, approximately 30% of the mitochondria belong to Class III and around 40% in Class B, which means that they are mainly small, short and fragmented in morphologies. These mitochondrial cristae and matrix conditions were however improved when *IncMtDloop* was ectopically expressed in 3xTg neurons. The mitochondrial cristae remodeling and consequently the mitochondrial morphologies were significantly improved (**Fig. 4b, d, f and g**).

Mitochondria indeed exist as dynamic networks that often change shape and subcellular distribution. The number and morphology of mitochondria within a cell are controlled by precisely regulated rates of organelle fusion and fission⁵⁵. We therefore speculated that ectopic re-expression of *IncMtDloop* might have also altered the dynamics of mitochondria. To visualize that, we performed live imaging of a new MitoTracker probe (MitoESq-635), which is an enhanced squaraine dye that could be excited at 635 nm and 775 nm the stimulated emission depletion (STED) super-resolution microscopy^{56,57}. In 3xTg neurons, mitochondrial dynamics analyses revealed abnormal patterns of fission and fusion, resulting in more abundant amounts of fragmented mitochondria than wild type neurons (**Fig. 4h, Video S1 and S2**). With ectopic re-expression of *IncMtDLoop*, this significantly improved such dynamics and mitochondrial network in 3xTg neurons (**Fig. 4h, Video S3 and S4**). Recent studies suggest that modelling of mitochondrial architecture by changing its dynamics is crucial for bioenergetic adaption to metabolic demands⁵⁸. In line with this, mitochondrial cytochrome c oxidase activities, an enzyme that is responsible for oxygen consumption in the process of mitochondrial oxidative phosphorylation⁵⁹, were found to be significantly compromised in hippocampal tissues of 3xTg mice as compared to age-matched wild type controls (**Fig. 4i**). With ectopic expression of *IncMtDloop* in hippocampal tissues of 3xTg mice, this treatment restored the mitochondrial cytochrome c oxidase activities to the level that is comparable to the control group. Further analyses on cellular oxygen consumption rate by the Seahorse Mito Stress Test revealed that ectopic expression of *IncMtDloop* in 3xTg primary cortical enhanced both basal and reserved respiratory capacities (**Fig. 4j, k and l**), confirming its effect in facilitating mitochondrial oxidative phosphorylation. Collectively, our results revealed that *IncMtDloop* played an important role in maintaining mitochondrial ultrastructure, network morphologies and metabolic functions, and that loss of which, as in part triggered by Ab cascade signaling, contributed to mitochondrial impairment found in AD.

Restoring *IncMtDloop* expression alleviated AD-like pathology in the brain of 3xTg mouse model

Previous studies have proposed the presence of a feed-forward relationship between the severity of AD-related pathologies and the degree of mitochondrial dysfunction exists^{60,61}. With the effects found on global mitochondrial function and physiology, we speculated that these changes may well-extend to neuropathology levels. Utilizing the RISH-ICC technology, we found that after inoculation of AAV viral particle carrying vehicle construct or one that expresses *IncMtDloop* into hippocampal tissues, ectopic re-expression of *IncMtDloop* in 3xTg also resulted in significant reduction in the intensity and sizes of Ab deposits in CA1, CA3 and DG (**Fig. 5a, b**). ELISA assays were also performed in freshly harvested hippocampal tissues which also confirmed similar findings on Ab content (**Fig. 5c, d**). In addition to Ab plaques, tangles that made up of hyperphosphorylation of tau is also a hallmark of AD. Among various different phosphorylation sites, phosphorylation at Thr231, Ser396/404, Ser202, Ser202/Thr205 and Ser212/Thr214 are considered as the ones mostly implicated in AD⁶². In the hippocampus of 3xTg mice, while total level of mouse Tau (TAU-5) remained unchanged among all the groups, western blot results revealed that levels of phosphorylated tau (pSer202/Thr205, pT231 and human tau) were significantly elevated in those harvested from 3xTg mice (**Fig. 5e, f**). Moreover, the same phenomena were observed in primary neurons and the brain of 3xTg mouse model (**Supplementary Fig. 5**).

Dysregulated mitochondrial trafficking, biogenesis and mitophagy are now found to be associated with accelerated brain aging, neurodegeneration, and notably the pathogenesis of late-onset sporadic AD^{17,18}. Dysregulated mitophagy can be another conserved phenotype of AD⁶³. To differentiate if the functional enhancing effects induced by *IncMtDloop* is from enhancing mitophagy or other possibilities, we first analyzed the levels of various mitophagy-associated proteins. Immunoblotting revealed that key players in mitophagy, including Parkin, PINK1, LC3B-II and ULK1 were reduced in hippocampus tissues harvested from 3xTg as compared to age-matched controls and these reductions were restored upon ectopic expression of *IncMtDLoop* (**Fig. 5g-k**). Similarly, immunofluorescence studies also revealed that signal intensities of LC3B, Parkin and PINK1 in hippocampal CA1 and CA3 regions were enhanced upon ectopic expression of *IncMtDloop* (**Extended Data Fig. 5**).

Restoring *IncMtDloop* improves synaptic functions and reverses behavior deficits of 3xTg mouse model

Mitochondrial dysfunction has been identified as a mechanism responsible for cognitive and memory impairment^{64,65}. Deficits in synaptic function and cognitive impairments are also being well documented in AD-mouse model including 3xTg mice. To investigate if the beneficial effect on mitochondria as a result of re-expression *IncMtDloop* also lead to improvements in synaptic plasticity and cognitive decline in 3xTg mice, viral particles carrying relevant constructs were microinjected into the

hippocampus of 11 months old of 3xTg mice. At 6 weeks after microinjection, an array of electrophysiological and behavioral tests was subsequently performed (**Fig. 6a**). At electrophysiological level, field excitatory postsynaptic potentials (fEPSPs) of the Schaffer collateral pathway (**Fig. 6b**) as well as long-term potentiation (LTP) were evaluated which revealed that the impairments found in 3xTg mice could be restored upon ectopic re-expression of *IncMtDloop* (**Fig. 6c, d**). Further evaluation of on animal social, cognitive and memory-associated behavior was also performed. In a nest-building paradigm (**Extended Data Fig. 6a, b**) and open field paradigm (**Extended Data Fig. 6c, d**), 3xTg mice revealed sub-optimal performance than wild type controls, but that could be rescued upon ectopic re-expression of *IncMtDloop*. Similarly, in a novel object recognition paradigm, 3xTg was less responsive to an introduction of a novel object than wild type controls, but this could be improved upon re-expression of *IncMtDloop* (**Extended Data Fig. 6e**). Subsequently contextual and cue fear conditioning test was also performed as it is composed hippocampus-dependent learning and memory task. Again, *IncMtDloop* re-expression significant improved cued freezing time compared of 3xTg mice (**Fig. 6e, f, and Extended Data Fig. 6g**). Finally, to assess the effect of the *IncMtDloop* re-expression on the animal spatial memory, Morris water maze (MWM) test was performed. While 3xTg mice were deficient in their performance, restoration of *IncMtDloop* in hippocampus significantly reversed all the parameters in the MWM as compared to that of AAV9-control mice (**Fig. 6g-j and Extended Data Fig. 6f**). Collectively, these data confirmed that restoring *IncMtDloop* expression improved synaptic function and resulted in a dramatic reversal of behavioral deficits of the mouse model.

Discussion

In this study, we investigated that the loss of a well conserved, novel mitochondrial DNA D-loop derived lncRNA—*IncMtDloop*—is found in brain tissues of AD patients and those harvested from the 3xTg classic model of AD. With the 3xTg mouse model, we have evaluated the effect of *IncMtDloop* loss on neuronal mitochondrial homeostasis, and their linkages to altered cellular fuel metabolism, mitophagy, neuronal firing and behavior related to cognition and memory (**Supplementary Fig. 5**). lncRNAs are abundant in mammalian brains^{20,26}, yet detailed understanding on their biological functions, particularly those uniquely located in mitochondria, remain incomplete. So far, several mtDNA derived lncRNAs have been identified in mammalian cells, these included LNCND5, LNCND6 and LNCCYTB, and they may play important roles in regulating mitochondrial gene expression and mitochondrial homeostasis²⁹. These, unlike the more well-defined nuclear-encoded lncRNAs, the biosynthesis and functions of mitochondria-derived lncRNAs remain unclear. While we may still lack much of the mechanistic understandings regarding to how lncRNAs are being regulated, their importance on normal brain biology and physiology, and implications on neurodegenerative disorders were reflected by the altered expressions in these scenarios⁶⁶⁻⁶⁸.

Our bioinformatics analyses and other experiments revealed that, *IncMtDloop*, as a mitochondria-located lncRNA, can mechanistically interacts with p32, which increase the mitochondrial location of exogenous

IncMtDloop. Using p32 as a study model of protein-RNA interaction due to the highest interaction score predicted between this protein and *IncMtDloop*, we identified the 3' terminal region located between 627-939 nt of *IncMtDloop* as the likely protein-binding motif that is sufficient and necessary for its interaction with p32. While p32 is located in multiple subcellular compartments, it appears to have a role in facilitating the mitochondrial localization of ectopically expressed *IncMtDloop*. This property could be utilized as a treatment strategy to re-introduce exogenous *IncMtDloop* into the mitochondria when the endogenous level became diminished. This also renders a good strategy to investigate its physiological roles related to mitochondria. Classically, lncRNAs are believed to be involved in transcriptional, translational and post-translational regulations. In the mitochondria where histone proteins are absent, mutations in mtDNA appear more frequently than in nucleus. Accumulation of mtDNA mutations in nucleotides would lead to mtDNA loss, which impact cellular metabolism and extensively affect the risk of complicated late-onset brain diseases, including AD, Parkinson's disease and metabolic disease. Indeed, we found the apparent mtDNA loss in 3xTg mouse model and further identified *IncMtDloop* can interact with TFAM and activate mtDNA expression in *cis* by recruitment of TFAM to the promoters of mitochondrial D-loop region. Of note, the functional analyses of *IncMtDloop* showed the rescued level of mtDNA copy number,

This newly identified lncRNA is also likely to have important clinical relevance as the levels of *IncMtDloop* are reduced in the brains of patients with AD, so as in the amyloid, tau and presenilin-based 3xTg mouse model. We found significant neural cell type variations, with the age- and disease-related loss of *IncMtDloop* being most notable in neurons rather than other cell types. In recent decades, the "mitochondrial cascade hypothesis" of AD pathogenesis has seen increasing attention. Part of the appeal of this view of AD is that the nervous system is an energy-intensive organ with a high demand for the synthesis of ATP. This makes the brain particularly vulnerable to mitochondrial dysfunction. As we found *IncMtDloop* can rescue mtDNA loss in 3xTg mice, the mitochondrial function of *IncMtDloop* in AD appears relatively underexplored. To bridge this gap, we used the newly developed MitoESq-635 combined with STED imaging to document the morphological consequences of *IncMtDloop* loss on the morphological integrity of the mitochondria in 3xTg neurons. When combined with electron microscopy, this further revealed extensive mitochondrial abnormalities in cells with suboptimal level of *IncMtDloop*. At a more global cellular level, mitochondria network in the 3xTg neurons were found to be more fragmented, with swollen cristae and a hypodense matrix than those in wild type cells. Not surprisingly, these abnormal mitochondria are defective in fusion and fission. In AD, the damaged fusion and fission in mitochondrial dynamics would affect mitochondrial genome and nucleoid organization, leading to mtDNA loss and mitochondrial dysfunction¹⁶.

Unexpectedly, *IncMtDloop* has a substantial impact on the clearance of A β accumulation, tauopathies and AD related mitophagy in neurons. It is quite significant, therefore, that restoration of *IncMtDloop*

levels alleviated virtually every behavioral and electrophysiological deficit we tested in 3xTg mice. These mitigations ranged from the recovery of hippocampal LTP to restoration of deficits in hippocampal-dependent learning and memory performance. Importantly, since AAV9-*IncMtDloop* injections were done after the symptoms of the 3xTg mice, which were already established both neuropath and behavior. This is a really important suggestion that restoration of *IncMtDloop* can actually reverse the course of the disease, not just prevent it.

In addition, although the causal nature of the lncRNA is clear, the exact mechanism behind the improvements needs to be worked out in more detail. It is not unexpected that deficits in energy production would degrade neurophysiological function, yet it is not immediately obvious how changes in *IncMtDloop* expression, either directly or indirectly would serve to regulate A β cascade signaling. In summary, we show how an unappreciated aspect of the decreased mitochondrial *IncMtDloop*, the inability of it decrease in neurons to regulate mitochondrial transcription and homeostasis, contributes to Alzheimer's pathogenesis. In future, a search for small RNA molecule mimics against *IncMtDloop* with appropriate pharmacokinetic properties may facilitate the translation of our findings into practical clinical interventions.

Methods

Constructs, oligonucleotide and plasmids. pcDNA3.1-T7-*IncMtDloop* (1-939) was ordered from Genecreate company. pcDNA3.1-T7-*IncMtDloop* #1 (Δ 314-939), pcDNA3.1-T7-*IncMtDloop* #2 (Δ 1-313,627-939), pcDNA3.1-T7-*IncMtDloop* #3 (Δ 1-626), pcDNA3.1-T7-*IncMtDloop* #4 (Δ 627-939), pcDNA3.1-T7-*IncMtDloop* #5 (Δ 1-313), pcDNA3.1-T7-*IncMtDloop* #6 (314-626) were mutant from pcDNA3.1-T7-*IncMtDloop* (1-939). siRNA for p32 and TFAM were designed and synthesized with standard protocols. For overexpression of *IncMtloop* in cell lines, pSLenti-CMV-*IncMtloop* and pSLenti-CMV-GFP were ordered from OBiO company. For overexpression of *IncMtloop* in mice, pAAV9-SYN- *IncMtloop* and pAAV9-SYN-GFP were ordered from OBiO company. For luciferase reporter assay, the fragments of different locus of mitochondrial D-loop (+200-0, +200-200, +200-400, +200-600, +200-800, +200-1000) were cloned into pGL4.10-basic vector (Promega).

Human brain samples—Human autopsy paraffin-embedded 10 μ m brain sections were from the following sources with approval from the appropriate local regulatory authorities. We examined 16 case-patients graciously provided by the University of Pittsburgh Alzheimer's Disease Research Center (ADRC) brain bank with approval from the Committee for Oversight of Research and Clinical Training Involving Decedents (CORID). Each case had been diagnosed neuropathologically and ranked by Braak stage (**Supplementary Table 1**). Additional frozen tissue was a generous gift of the ADRC at Washington University in St. Louis (Grant P50-AG-05681) with approval from the Neuropathology Core (protocol #T1016) at Hongkong University of Science and Technology.

Mouse line

All mouse lines including the 3xTg line (B6. Cg-Tg (APPSwe, tauP301L)1Lfa PSEN1tm1Mpm/J; The Jackson Laboratory) and age- and gender- matched wild-type mice controls were maintained on C57/BL6 background. Mice were group-housed in cohorts of five, separated by sex in individually home cages under specific-pathogen-free conditions with free access to food and water. The light and dark cycle was 12 hour / 12 hour and the temperature was kept constant at 23 ± 2 °C and humidity ($50 \pm 5\%$). Mice were randomly assigned to the experimental groups. Behavioral experiments were performed at the dark cycle. Ages of mice used for each experiment were 11-13 months. All animal experiments were conducted with the licenses from Kunming Institute of Zoology, and were approved by the Animal Care and Use Committee of Kunming Institute of Zoology CAS. All animal experiments were also complied with the Biomedical Ethics Committee for animal use and protection of Peking University.

Primary cells

For primary microglia cultures, wildtype female mice or 3xTg female mice were mated with the wildtype male mice or 3xTg male from an unrelated colony. The hippocampus was isolated from newborn mice (P0-P4), and cut into small pieces. The hippocampal tissue was digested with Neurosphere Dissociation Kit (Cat# 130-095-943) and centrifuged for 5 min at 1500rpm. The purified microglia were resuspended in DMEM/F-12 (Thermo Fisher Scientific) medium with 10% FBS and penicillin-streptomycin (Invitrogen), and then seeded on 6- or 24-well plates with 14-21 days^{69,70}. Cultures were kept in a 37 °C incubator with 5% CO₂. 1/2rd of the medium was replaced with fresh medium every 3 days. For primary astrocyte cultures, wildtype female mice or 3xTg female mice were mated with the wildtype male mice or 3xTg male from an unrelated colony. The hippocampus was isolated from newborn mice (P0-P4), and cut into small pieces. The hippocampal tissue was digested with Neurosphere Dissociation Kit (Cat# 130-095-943). After mechanical dissociation and centrifugation, the cells were resuspended in DMEM/F-12 (Thermo Fisher Scientific) medium with 10% FBS, penicillin-streptomycin (Invitrogen), 15 mM HEPES, 14.3 mM NaHCO₃, 1% Fungizone[®] and 0.04% gentamicin, and then seeded on 6- or 24-well plates pre-coated with poly-L-lysine with 14-21 days⁷¹. Cultures were kept in a 37 °C incubator with 5% CO₂. 1/2rd of the medium was replaced with fresh medium every 3 days. For primary mouse hippocampal neuronal cultures, wildtype female mice or 3xTg-AD female mice were mated with the wildtype male mice or 3xTg-AD male from an unrelated colony. The hippocampus was isolated from embryos of day E17, and cut into small pieces. The hippocampal tissue was digested with Neurosphere Dissociation Kit (Cat# 130-095-943). The primary mice hippocampal neurons were resuspended in Neurobasal plus medium supplemented with B27-P (Thermo Fisher Scientific), Glutamax (Thermo Fisher Scientific) and penicillin-streptomycin (Invitrogen) and then seeded on 6- or 24-well plates pre-coated with poly-L-lysine with 14-21 days⁷². Cultures were kept in a 37 °C incubator with 5% CO₂. 1/2rd of the medium was replaced with fresh

medium every 3 days. For the A β ₁₋₄₂ fibrils, A β ₁₋₄₂ peptide (sigma) was dissolved in sterile PBS, and diluted to a final concentration of 250 μ M and incubated on a shaker at 37 °C for 84 h. In DIV14 or DIV21, cultures were treated with A β ₁₋₄₂ fibrils in indicated concentrations for 18 h and cells were collected for subsequent experiments⁷³.

Cell line

N2A cells, cos-7 cells and SH-SY5Y cells were cultured in DMEM (Thermo Fisher Scientific) medium with 10% FBS and penicillin-streptomycin (Invitrogen). Cultures were kept in a 37 °C incubator with 5% CO₂. 1/2rd of the medium was replaced with fresh medium every 3 days.

AAV microinjections in mice

For bilateral injection of AAVs, sex-matched 11-month-old wild type and 3xTg mice were anesthetized with isoflurane. Wiping 70% ethanol were used to sterilize the surgical area, removing the skin above the skull by using aseptic techniques, and drilling small holes for the sites of injection. A volume of 0.5 μ l virus per hemisphere was injected at a speed of 0.075 μ l min⁻¹. The target sites for AAV injection were marked on the skull (AP -2.0 mm, ML $\pm$ 1.5 mm, DV -2.0mm). After surgery, mice were housed under standardized conditions for 4-6 weeks.

RNA extraction and real-time qRT-PCR

Total RNA was extracted from cultures or mice tissues using RNAiso plus reagent following the manufacturer's instructions. RNA quantity and quality were evaluated by Nano Drop ND-1000 spectrophotometer. The real-time quantitative reverse transcription PCR (qRT-PCR) was using Reverse Transcription kit (vazyme) and SYBR Green I Master (vazyme). qPCR was performed in 10 μ l reaction system including 1 μ l cDNA, 5 μ l 2 $\times$ Master Mix, 0.1 μ l of forward primer (10 μ M), 0.1 μ l of reverse primer (10 μ M), and 3.8 μ l of double distilled water. β -Actin was used as an endogenous reference for data normalization. Primer sequences are described in Supplementary Table. Cycle threshold values were used to calculate fold changes in gene expression using the 2^{- $\Delta\Delta$ Ct} method. The primer sequences of probes were seen in **Supplementary Table 2**.

Tissue preparation and western blot

Samples from mouse brain and primary cell culture were collected and homogenized in RIPA buffer containing protease inhibitors and phosphatase inhibitors. After centrifugation, supernatants were heated to 95°C for 10 min, and resolved on 6-12% polyacrylamide precast gels. The gel was transferred to PVDF membrane at 250 mA for 90 min at 4°C and blocked for 1 hr at room temperature in 5% BSA in Tris buffered saline with 0.05% Tween 20. Membranes were incubated in primary antibody overnight at 4°C. Antibodies used for western blot were: anti-6E10 (803001, BioLegend), anti-p32 (ab24733, Abcam), anti-ATP5a (ab14748, Abcam), anti-LC3B (ab51520, Abcam), anti-PINK1 (ab23707, Abcam), anti-parkin (#2132, Cell Signaling), anti-AT8 (MN1020, Thermo Fisher Scientific), anti-AT180 (MN1040, Thermo Fisher Scientific), anti-HT7 (MN1000, Thermo Fisher Scientific), anti-TAU-5 (AHB0042, Thermo Fisher Scientific), anti- β -actin (ab8227, Abcam), anti-COX IV (ab33985, Abcam), anti-Stxb1 (ab183722, Abcam), anti-Tppp (ab92305, Abcam), anti-Aly/Ref (ab202894, Abcam), anti-ATP5D (ab174438, Abcam), anti-TFAM (ab252432, Abcam). All blots were imaged using HRP-conjugated secondary antibodies. After three washes for 10 min each, the fluorescence signals were quantified using Image J software.

Northern blot

Total RNA (10 μ g for human postmortem PFC and hippocampal frozen tissues of AD and control individuals as well as wild type and 3xTg mouse brain fresh tissues, 2 μ g for mouse primary neurons) was denatured using NorthernMax[®]-Gly sample loading dye (Ambion) and resolved on 1.2% agarose gel in MOPS buffer. The gel was soaked in 1×TBE for 20 min and transferred to a Hybond-N+ membrane (GE Healthcare) for 1 h (15 V) using a semi-dry blotting system (Bio-Rad). Membranes were dried and UV-crosslinked with 150 mJ/ cm² at 254 nm. Pre-hybridization was done at 68 °C for 1 h, and using DIG Northern Blot Starter KIT (12039672910, Roche). DIG-labeled *in vitro* transcribed *IncMtDLoop*, and control probes were hybridized overnight. The membranes were washed three times in 2× SSC, 0.1% SDS at 68 °C for 30 min, followed by three 30 min washes in 0.2× SSC, 0.1% SDS at 68 °C. The immunodetection was performed with anti-DIG AP-conjugated antibodies. Immunoreactive bands were visualized using CDP star reagent (Roche) and a LAS-4000 detection system (GE Healthcare). The primer sequences of probes were seen in the **Supplementary Table 2**.

Immunofluorescence staining

Mice were perfused with PBS and the brains were fix with 4% paraformaldehyde (PFA). The brains were sliced into 30 μ m thick with a Leica CM1950 freezing microtome. For immunofluorescence staining, brain sections were permeabilized and blocked in PBS with 0.3% Triton X-100 and 10% goat serum at room temperature for 1 hour, followed by incubation with the primary antibody: anti-6E10 (803001, BioLegend), anti-p32 (ab24733, Abcam), anti-ATP5a (ab14748, Abcam), anti-LC3B (ab51520, Abcam), anti-PINK1 (ab23707, Abcam), anti-parkin (#2132, Cell Signaling), anti-AT8 (MN1020, Thermo Fisher Scientific), anti-AT180 (MN1040, Thermo Fisher Scientific), anti-HT7 (MN1000, Thermo Fisher Scientific), anti-TAU-5

(AHB0042, Thermo Fisher Scientific). For the confocal microscopic analysis (TCS SP8, Leica), the primary antibodies were visualized with Alexa-Fluor488, Alex-Fluor594 and Alexa-Fluor647 secondary antibodies (Invitrogen). DAPI (ab104139, Abcam) was used for nuclear counter staining. For primary cultures, mice hippocampal neurons, microglia and astrocyte were fix with 4% PFA, then permeabilized and blocked in PBS with 0.3% Triton X-100 and 10% goat serum at room temperature for 1 hour. Primary antibodies were: anti-LC3B (ab51520, Abcam), anti-PINK1 (ab23707, Abcam), anti-parkin (#2132, Cell Signaling), anti-ATP5a (ab14748, Abcam), anti-Iba1 (019-19741, Wako), anti-GFAP (837201, BioLegend), anti-p32 (ab24733, Abcam), anti-MAP2 (ab32454, Abcam), anti-AT8 (MN1020, Thermo Fisher Scientific), anti-HT7 (MN1000, Thermo Fisher Scientific), anti-TAU-5 (AHB0042, Thermo Fisher Scientific). For the confocal microscopic analysis (TCS SP8, Leica), the primary antibodies were visualized with Alexa-Fluor488, Alex-Fluor594 and Alexa-Fluor647 secondary antibodies (Invitrogen). DAPI (ab104139, Abcam) was used for nuclear counter staining.

RNAscope assay for lncRNA detection

Detection of *IncMtDloop* was performed on cryostat brain slices of mice and primary cultured cells. At first, we prepared and pretreated the samples with RNAscope[®] H₂O₂ and proteas reagents (Cat# 322381). Next, we cover each slide or cells cultured on cover slips with the probe sets against human and mouse *IncMtDloop* (Cat# 518381 and 518361). A positive-control probe, *Homo sapiens peptidylprolyl isomerase B (PPIB)*, and a negative-control probe for *bacterial gene dapB* were include for each group. The probe hybridization was performed in the HybEZ[™] for 2h at 40°C. The signal amplification was conducted according to the manufacturer's instruction using RNAscope[®] Multiplex Fluorescent Detection Reagents (Cat# 323110). The fluorescence signals of *IncMtDloop* were visualized with TSA[®] plus fluorophore. The brain slices and cells cultured on cover slips were imaged on the confocal microscopy (TCS SP8, Leica).

STED superresolution for live cells

Primary neuronal cultures were cultured at a suitable density (moderate) and seeded on glass-bottomed dishes. At DIV3, neurons were transfected with lentiviral vector to evaluate the gain-of-function of *IncMtDloop*. At DIV14, primary neuronal cultures were incubated with MitoESq-635 at a concentration of 1μM for 5 minutes in a humid atmosphere with 5% (v/v) CO₂ at 37°C to label the mitochondrial membrane, then imaged with STED nanoscopy. STED imaging was performed with Leica TCS SP8 STED 3X system equipped with a white light laser for excitation and a 775-nm pulsed laser for STED depletion. A ×100 oil-immersion objective (Leica, N.A. 1.4) was employed.

Electron microscopy

The mice were perfused with paraformaldehyde-glutaraldehyde mixed fixative (2%/2.5%), and the hippocampus was removed and post-fixed at 4°C using paraformaldehyde-glutaraldehyde mixed fixative (2%/2.5%) in 0.1M PB (PH7.4). The hippocampal samples from each group were washed with 0.1M PB (PH7.4) three times for 15 min, and then post-fixed with 1% OsO₄ for 2h at 4°C, followed by serial ethanol dehydration and acetone transition for 5 min, embedding in Epon 812 resin, polymerization and polymerization at 60°C for 48h. The hippocampal samples were sliced into 60nm for ultrathin sections of transmission electron microscopy with a Leica UC7 ultramicrotome. Ultrathin sections were loaded onto 100-mesh Cu grids and double stained with 2% uranyl acetate and lead citrate before observations employing a JEM 1400 plus transmission electron microscopy.

RNA pulldown and mass spectrometry (MS)

IncMtDloop was in vitro transcribed using the mMESSAGE mMACHINE T7 ULTRA Kit (Ambion, AMB1345), followed by biotinylation with the Pierce RNA 3' End Desthiobiotinylation Kit (Thermo Fisher Scientific, 20163) RNA pull-down was performed using a Magnetic RNA-protein Pull-Down Kit (Thermo Fisher Scientific, 20164) with the manufacturer's guidelines. The retrieved protein was detected by silver staining (Thermo Fisher Scientific, 24612). The specific bands were identified by MS and western blot. For MS, specific bands and retrieved protein pull-downed by *IncMtDloop* or antisense probes were collected, and digested in 50mM triethylammonium bicarbonate with chymotrypsin and modified trypsin at 30°C overnight. After digestion, each peptide samples were extracted and speed vacuum-dried. Dried proteins were resuspended in 200µl volume of buffer (0.1% formic acid and 2% acetonitrile) and loaded on a Bruker Autoflex speed TOF/TOF MALDI-TOF system for LC/MS/MS. The acquired peptide-sequencing data were loaded in Proteome Discoverer (version 1.3.0.339, Thermo Scientific) and analyzed with Mascot software (version 2.3.01, Matrix Science) against a *Mus musculus* protein database to identify proteins.

RNA immunoprecipitation (RIP)

RIP was performed using a Magnetic RIP RNA-Binding Protein Immunoprecipitation (Millipore, 17-700) in accordance with the manufacturer's guidelines. The primary cultured neurons were treated with ice-cold PBS and IP lysis buffer (Thermo Fisher Scientific, 87787) supplemented with RNase inhibitors and protease inhibitor cocktail. The supernatants were incubated with the anti-p32 (ab24733, Abcam) antibody or control mouse IgG with rotation at 4°C overnight, and then protein A/G beads were added to each group. After the incubation at 4°C overnight, we collected the precipitates and extracted RNA by proteinase K-chloroform method. The enrichment of *IncMtDloop* was detected using qRT-PCR.

Luciferase reporter gene assays

Luciferase assays were performed using the Dual-Luciferase[®] Reporter Assay Kit (Promega, E1960). N2a cells were planted in 96-well plates at 2.0×10^4 cells/well. After culturing for 2 days, si-RNA for TFAM was transfected into the cell lines by using Lipofectamine 2000 (Invitrogen, 11669019). After transfection for 2 days, cells were cotransfected with the luciferase reporter constructs (150ng/well) and internal control plasmid pRL-CMV (30ng/well). 2 days after transfection, the cells were collected to measure the luciferase activity using a luminometer according to the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP) assays

N2A cells were collected at ~90% confluency from 15cm dishes, then performed CHIP assay using the SimpleChIP[®] Enzymatic Chromatin IP Kit (Cell Signaling, 9003). Firstly, cells were cross-linked with 37% formaldehyde for 10 min, quenched, and digested with micrococcal nuclease for 20 min at 37°C. The digested chromatin was sonicated in 500µl ChIP buffer on ice using a sonicator (three times, 20s each). Chromatin was Sheared chromatin was centrifugated at 9400 x g for 10 min at 4°C. The supernatant was collected and incubated with specific antibodies against TFAM (ab252432, Abcam) or IgG antibody at 4°C overnight with rotation, then incubated with protein G magnetic beads at 4°C for 2 hours with rotation. After purification, immunoprecipitated DNA was analysed by quantitative real-time PCR (40 cycles). Primer pairs must range from 150bp to 22bp long. The primer sequences of the promoter of mtDNA containing the TFAM binding domain were shown in the **Supplementary Table 2**.

The lncRNA-protein interaction prediction

The coding probability of *lncMtDloop* was examined with the PhyloCSF analysis in UCSC browser, Coding Potential Assessment Tool (CPAT, <http://lilab.research.bcm.edu/cpat/index.php>) and Coding Potential Calculator (CPC, <http://cpc.cbi.pku.edu.cn/>). The secondary structure of *lncMtDloop* was predicted on RNAfold WebServer (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>) based on partition function and minimum free energy (MFE). The CatRAPID was used for the prediction of protein-RNA binding propensity between *lncMtDloop* fragments and possible proteins (<http://service.tartagliolab.com/page/catrapid.group>).

Measure of extracellular fluxes using Seahorse XF96

20,000 primary neurons were seeded in each well of a XF 96-well cell culture microplate in 80 ml of culture media, and incubated overnight at 37°C in 5% CO₂. The four corners were left only with medium for background correction. Culture medium is replaced with 180 ml of bicarbonate-free DMEM and cells

are incubated at 37°C for 30 min before measurement. Oxygen consumption rates (OCR) were measured using a XF96 Extracellular Flux Analyzer⁷⁴. OCR was determined at four levels: with no additions, and after adding: oligomycin (1 μM); carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 0.4 μM); and rotenone (2 μM) (additives purchased from Sigma at highest quality). After each assay, manual inspection was performed on all wells using a conventionally light microscope. Wells for which the median OCR level did not follow the expected order, namely, median (OCR(Int₃)) > median (OCR(Int₁)) > median (OCR(Int₂)) > median (OCR(Int₄)), were discarded (977 wells, 10.47%). Of note, we excluded from the analysis contaminated wells and wells in which the cells got detached (461 wells, 4.94%).

Aβ ELISA

Hippocampus was isolated from 12-month-old 3×Tg mice. Hippocampal tissues were rinsed with PBS, and homogenized with a tissue homogenizer. The tissues were treated with an equal volume of RIPA buffer supplemented with protease inhibitor cocktail and lysed on ice for 30 minutes with gentle agitation. After centrifugation, aliquots of the lysates were removed and subjected to Aβ measurement using Aβ₁₋₄₀ and Aβ₁₋₄₂ ELISA kits following the manufacturer's instructions.

Cytochrome c Oxidase Assay

Hippocampus was isolated from 12-month-old 3×Tg mice and subjected to Cytochrome c Oxidase measurement using Cytochrome c Oxidase Assay kits following the manufacturer's instructions. The colorimetric assay of the samples is based on observation of the decrease in absorbance at 550nm of ferrocytochrome c caused by its oxidation to ferricytochrome c by cytochrome c oxidase.

Electrophysiology

LTP studies were performed on hippocampal slices from 12-month mice. Mice were anaesthetized with isoflurane, decapitated, and brains were rapidly removed in pre-ice cold ACSF (120 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 2 mM MgSO₄, 10 mM D-glucose, 2 mM CaCl₂ with 95% O₂, 5% CO₂ [pH 7.4]). Transverse hippocampal slices were cut at a thickness of 350 μm, and placed on infusion chambers in ACSF. The fEPSPs were recorded from the CA1 stratum radiatum region by an extracellular borosilicate glass capillary pipette (resistances of 3-5 MΩ) filled with ACSF. Stimulation of Schaffer collaterals from the CA3 region by a bipolar electrode. Signals were amplified using a MultiClamp 700 B amplifier (Axon), digitized using a Digidata 1440A Data Acquisition System (Axon) with 1kHz low pass filter and 2 kHz high pass filter and analyzed using Clampex 10.7 software. For each experiment 8-9 sections from 3-4 animals/genotype were used.

Behavioral testing

For nest building behavior, mice were individually housed with nesting materials of facial tissue strips. Mice were given the nesting material on day 1. Pictures were taken and nests were scored 24 h later. For open field test, mice were placed into the open field arena (made of opaque white plastic material, 35 cm × 35 cm separated into 5×5 grids) by a blinded experimenter and allowed to explore the arena for 15 min. The number of crossing grids and percentage of time spent in the center (22 cm × 22 cm) were quantified using video tracking software. For novel object recognition test, the test was performed on three consecutive days. On the first day, mice were placed in the center of an empty open box and allowed to explore for 10 min. On the second day, the mice were placed in the open box with two identical objects in the two corners and allowed to explore for 10 min. On the third day, the mice were placed in the same box but with an object replaced by a novel one and allowed to explore for 10 min. The discrimination index was referred to the time spent exploring the novel object relative to the time spent exploring both objects. For fear conditioning test, fear memory was measured by pairing a conditioned stimulus with an unconditioned stimulus. We used the Video Freeze Conditioning 'Video Freeze' software to record freezing behavior. On day one, mice were placed inside the chambers; after 120-s baseline, a 30-s CS tone followed, and a US foot shock was given during 120-150s, 180-210 s and 240-270 s. On day two, both contextual and cued phases were done. During phase one, mice were placed in the same testing chambers used on day one for 5 min. During phase two, 3 h later, mice were placed into modified chambers with plastic inserts, and after 300 s the CS tone was played for 30 s at 1 min intervals (five CS tones in total). After 10 min, mice were returned to their housing cages^{75,76}. For morris water maze, the pool (150 cm diameter) filled with water that is maintained at room temperature. A 12 cm diameter transparent platform was placed 1 cm below the water surface at first quadrant. Mice were trained for 5 consecutive days, with 4 trials per day. Each training lasted 60 s until the mouse arrived on platform. After 24 h from the last training, the test was conducted without a platform for 60s to test their memory performance. The latency time to the area of removed platform, the time spent in the first quadrant and number of crossing the area of removed platform was recorded.

Quantification and Statistical analysis

All statistical analysis was performed using GraphPad Prism 7 software. Results are showed as mean ± SEM of three independent experiments. Significance between two groups was analyzed by two tailed Student's test. Significance between three groups or more was analyzed by one-way ANOVA or two-way ANOVA by Tukey's or Dunnett's multiple comparisons test. In all figures, *P <0.05, **P<0.01, ***P <0.001, ****P<0.0001, p value of more than 0.05 was considered non-significant.

Declarations

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Contributions

W.X. and J.L. conceived and designed all experiments and interpreted results. W. X., J.S., K.X., S.L., Y.Z., Z.Z., Z.W., L. F. and Zheng. Y. performed the experiments and analyzed the data. W.X. and J.L. designed

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Ethics declarations

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

Decrease in *LncMtDloop* expression found in the brains of human AD patients and the mouse model. (a) Schematic illustrating the detailed structure of mitochondrial non-coding region, D-loop, and transcripts of *LncMtDloop* in human, macaque and mouse. The two strands of mtDNA are indicated as the heavy and light strands. Abbreviations: CBS, conserved sequence block; D-loop, displacement loop; HSP, heavy strand promoter; HVS, hypervariable segment; LSP, light strand promoter; TAS, termination associated

sequence. (b) Representative images of RNAscope ISH together with immunofluorescence showing the location of *IncMtDloop* and mitochondria in hippocampal neurons of wild type mouse. (c) Relative intensities of *IncMtDloop* and mito-ATP5a fluorescence signals illustrated in white dotted line in b) were quantified by Image J. (d) Representative images of RNAscope ISH showing *IncMtDloop* expression. 15 μ m paraffin sections of prefrontal cortex (PFC) and hippocampus (CA1 and DG) from age- and sex-matched control and AD individuals were performed RNAscope with human- specific probe sets against *IncMtDloop*. Scale bar, 10 μ m. (e) Relative intensity of RNAscope fluorescence illustrated in A) were quantified by Image J. Bars indicate mean $\pm$ SEM. n = 8 patients per group. *P <0.05, by unpaired t- test. (f) ssRT-qPCR was performed for validation of *IncMtDloop* expression. Total RNAs were extracted from postmortem frozen PFC and hippocampal tissues of age- and sex- matched control and AD individuals. Bars indicate mean $\pm$ SEM. n = 8 patients per group. *P <0.05, by unpaired t-test. (g) Representative images of RNAscope ISH showing *IncMtDloop* expression. 10 μ m cryostat brain sections from 12-month-old wild type and 3xTg mice were performed RNAscope with mouse-specific probe sets against *IncMtDloop*. Scale bar, 15 μ m. (h) Relative intensity of RNAscope ISH fluorescence illustrated in D) are quantified by Image J. Bars indicate mean $\pm$ SEM. n = 6 mice per genotype. *P <0.05, by unpaired t-test. (i) ssRT-qPCR was performed for validation of *IncMtDloop* expression. Total RNAs were extracted from fresh PFC and hippocampal tissues of 12-month-old wild type and 3xTg mice. Bars indicate mean $\pm$ SEM. n = 6 mice per group. *P <0.05, by unpaired t-test. (j) Representative northern blots of *IncMtDloop* transcript in postmortem frozen PFC and hippocampal tissues of control and AD individuals. (k) Relative intensities of northern blot signals illustrated in panel i) were quantified by use of Image J. Bars indicate mean $\pm$ SEM. n = 8. *P <0.05, by unpaired t-test. (l) Representative northern blots of *IncMtDloop* transcript in fresh PFC and hippocampal tissues of wild type and 3xTg mice. Bars indicate mean $\pm$ SEM. n = 6. *P <0.05, by unpaired t-test. (m) Relative intensities of northern blot signals illustrated in panel l) were quantified by use of Image J. Bars indicate mean $\pm$ SEM. n = 6 mice per genotype. *P <0.05, by unpaired t-test.

Fig 2

Figure 2

***LncMtDloop* binds to mitochondria-related proteins.** (a) Schematic illustrating the procedure of *IncMtDloop* RNA pull-down and mass spectrometry (MS). (b) The interaction propensity of *IncMtDloop* with binding partners in mitochondrial preparations from hippocampal tissues of 6-month-old wild type mice, predicted by CatRAPID, referring the results of mass spectrometry. The left row indicates the scores of interaction propensity of the proteins. The right row indicates the discriminative power of the proteins,

representing as centesimal system. (c) The interaction propensity of *IncMtDloop* with binding partners in cytosolic preparations from hippocampal tissues of 6-month-old wild type mice, predicted by CatRAPID, referring the results of mass spectrum. The left row indicates the scores of interaction propensity of the proteins. The right row indicates the discriminative power of the proteins, representing as centesimal system. (d) RNA pull-down using biotinylated *IncMtDloop* and antisense-*IncMtDloop* in mitochondrial preparations of hippocampal tissues of 6-month-old wildtype mice, following by western blot of Stx1b, ATP5d and p32. +, *IncMtDloop*; –, anti-*IncMtDloop*. (e) RNA pull-down using biotinylated *IncMtDloop* and antisense-*IncMtDloop* in cytosolic preparations of hippocampal tissues of 6-month-old wildtype mice, following by western blot of Stx1b, ATP5d and p32. +, *IncMtDloop*; –, anti-*IncMtDloop*. (f) RNA pull-down using biotinylated *IncMtDloop* and antisense-*IncMtDloop* in mitochondrial and cytosolic preparations, respectively, from hippocampal tissues of 6-month-old wildtype mice, following by western blot of p32. +, *IncMtDloop*; –, anti-*IncMtDloop*. (g) Heatmap illustrating the interaction propensity of the *IncMtDloop* binding to p32, as predicted by CatRAPID. The X axis represents RNA nucleotide sequence location, and the Y axis represents the protein residue location. Colors scale bar with red shades indicate the predictions with high interaction propensity. (h) Representative images of RNAscope ISH and immunostaining showing co-localization of *IncMtDloop* (green) and p32 (red) in wild type hippocampal neurons. (i) Relative intensities of fluorescence signals of *IncMtDloop* and p32 illustrated in panel H) were quantified by use of Image J. (j) Representative northern blots of *IncMtDloop* transcript in the RIP assay by p32 in extract preparations from fresh brain tissues of 12-month-old wild type mice. (k) qPCR for detectable *IncMtDloop* from RIP analysis of p32 in preparations of fresh hippocampal tissues of 12-month-old wild type mice. Results were normalized to the control IgG RIP group. Bars indicate mean $\pm$ SEM. n = 6 animals per group. ***P<0.001, by unpaired t-test. (l) Schematic map for constructing six truncated mutants of *IncMtDloop* to identify its protein-binding motifs. (m) RNA pull-down using biotinylated distinct fragments of *IncMtDloop* and antisense-*IncMtDloop* in mitochondrial preparations from hippocampal tissues as described above, following by western blot of p32. The indicated six truncates of *IncMtDloop* were transcribed in vitro and tagged-RNA streptavidin to perform RNA pull-down assays.

Fig 3

Figure 3

***IncMtDloop* recruits TFAM to promote the mitochondrial transcription initiation.** (a) qPCR was performed for validation of ratio of mtDNA/nDNA expression. Total RNAs were extracted from fresh hippocampal and PFC tissues of 12-month-old wild type and 3xTg mice. Bars indicate mean $\pm$ SEM. $n = 6$ mice per group. * $P < 0.05$, by unpaired t-test. (b) The interaction propensity of *IncMtDloop* with potential binding proteins in mitochondrial nucleoid predicted by CatRAPID, The left row indicates the scores of interaction

propensity of the proteins. The right row indicates the discriminative power of the proteins, representing as centesimal system. SSBP, mitochondrial single stranded DNA-binding protein; TEFM, mitochondrial elongator factor; TERF1-4, mitochondrial transcription termination factors 1-4; TFAM, mitochondrial transcription factor A; TFB1M/TFB2M, mitochondrial transcription factors B1/B2. (c) RNA pull-down using biotinylated *IncMtDloop* and antisense-*IncMtDLoop* in mitochondrial preparations from hippocampal tissues of 6-month-old wildtype mice, following by western blot of TFAM. +, *IncMtDloop*; –, anti-*IncMtDloop*. (d) qPCR for detectable *IncMtDloop* from RIP analysis of TFAM in preparations of fresh hippocampal tissues of 12-month-old wild type mice. Results were normalized to the control IgG RIP group. Bars indicate mean $\pm$ SEM. n = 6 animals per group. **P<0.01, by unpaired t-test. (e) Heatmap illustrating the interaction propensity of the *IncMtDloop* binding to TFAM, as predicted by CatRAPID. The X axis represents RNA nucleotide sequence location, and the Y axis represents the protein residue location. Colors scale bar with red shades indicate the predictions with high interaction propensity. (f) Mitochondrial promoter within D-loop region is sufficient for the binding of TFAM by ChIP-RNA analysis. Bars indicate mean $\pm$ SEM. n = 3 per group. *P <0.05, ****P <0.0001, by unpaired t- test. (g) Six different loci of mitochondrial promoter in D-loop regions were constructed into pGL4.10 vector and performed luciferase reporter assays in TFAM-silenced N2a cells. Bars indicate mean $\pm$ SEM. n = 8-10 per group. *P <0.05, by unpaired t- test. (i) Schematic illustrating the mechanism of *IncMtDloop* and TFAM.

Fig 4

Figure 4

Restoring *IncMtDloop* expression alleviates AD-related alterations in mitochondrial morphology and function. (a) Representative electron micrographs showing mitochondrial ultrastructure of hippocampal neurons of 12-month-old wild type and 3xTg mice w/o overexpressing *IncMtDloop*. n = 10 random fields from hippocampal tissue samples of 6 animals per group. (b) Percentage of changes in mitochondrial morphology (tubular, short tubular, fragmented and large spherical) was quantified in hippocampal

neurons as described above. Bars indicate mean $\pm$ SEM. $n = 6$ per group. $*P < 0.05$, $**P < 0.01$, by one-way ANOVA with Tukey's multiple comparisons test. (c and d) Representative electron micrographs of mitochondrial cristae number (top), and percentage of cells with different categories: Class I (more than three cristae), Class II (two to three cristae) and Class III (no or one cristae), in hippocampal neurons as described above. Bars indicate mean $\pm$ SEM. $*P < 0.05$, $****P < 0.0001$, by one-way ANOVA with Tukey's multiple comparisons test. (d and f) Representative electron micrographs of mitochondrial matrix density (bottom), and percentage of cells with different categories: Class A (dense matrix), Class B (swollen mitochondria with hypodense matrix), in hippocampal neurons as described above. Bars indicate mean $\pm$ SEM. $***P < 0.001$, $****P < 0.0001$, by one-way ANOVA with Tukey's multiple comparisons test. (g) Quantification of changes in mitochondrial length in hippocampal neurons as described above. Bars indicate mean $\pm$ SEM. $n = 71-89$ mitochondria from 6 animals per group. $**P < 0.01$, $****P < 0.0001$, by one-way ANOVA with Dunnett's multiple comparisons test. (h) Representative dynamical images of mitochondrial fission and fusion using STED nanoscope. Wild type and 3xTg primary hippocampal neurons were infected with Lv- *IncMtDloop* at DIV5, cells were labeled with MitoSq-635 (0.1 μ M) and prepared for tests at DIV14. (i) The effect of restoration of *IncMtDloop* on cytochrome c oxidase activity using isolated mitochondria of hippocampal neurons as described above. Bars indicate mean $\pm$ SEM. $n = 8$ per group. $****P < 0.0001$, by one-way ANOVA with Dunnett's multiple comparisons test. (j) The mitochondrial oxygen consumption rate (OCR) was assessed in primary hippocampal neurons. 3xTg primary hippocampal neurons were infected with Lv- *IncMtDloop* or Lv-control at DIV5, cells were prepared for seahorse assay at DIV14. (k and l) Restoration of *IncMtDloop* displayed increased reserved capacity and basal levels of OCR in primary hippocampal neurons as described above. Bars indicate mean $\pm$ SEM. $*P < 0.05$, $**P < 0.01$, by unpaired *t*-test.

Fig 5

Figure 5

Restoring *IncMtDloop* expression reverses AD-related pathologies in the brain of the mouse model. (a) Representative IF images showing 6E10 in hippocampus of wild type and 3xTg mice. The viral particles of AAV9-*IncMtDloop* were microinjected into hippocampus of 11-month-old mice, and 10μm cryostat slices were prepared and IF with 6E10 antibody at the age of 13 months. (b) Quantification of average Aβ plaque area and fluorescence intensity of 6E10 in CA1, CA3 and DG of wild type and 3xTg mice as described above. Bars indicate mean ± SEM. n = 6 mice per group. ***P* < 0.01, ****P* < 0.001, by one-way ANOVA with Tukey's multiple comparisons test. (c and d) ELISA analysis showing changes in levels of Aβ₄₂ and Aβ₄₀ in hippocampal tissues of wild type and 3xTg mice as described above. Bars indicate mean ± SEM. n = 6 mice per group. ***P* < 0.01, ****P* < 0.001 by one-way ANOVA with Dunnett's multiple

comparisons test. (e) Western blot results of AT8, AT180, human tau and total tau in hippocampus of 12-month-old wild type and 3xTg mice w/o overexpressing *IncMtDloop*. (f) Relative intensities of AT8, AT180, human tau and total tau signals illustrated in e). Bars indicate mean $\pm$ SEM. n = 6 mice per group.

* $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, by one-way ANOVA with Dunnett's multiple comparisons test. (g)

Western blot results of parkin, PINK1, LC3B-II and ULK1 showing changes in the event of mitophagy.

Proteins were extracted from hippocampus of 12-month-old wild type and 3xTg mice w/o overexpressing *IncMtDloop*. (h-k) Relative levels of parkin, PINK1, LC3B-II and ULK1 signals illustrated in A). Bars indicate mean $\pm$ SEM. n = 6 animals per group. * $P < 0.05$, by one-way ANOVA with Dunnett's multiple comparisons test.

Fig 6

Figure 6

Restoring *IncMtDloop* reverses deficits in synaptic plasticity and cognitive behaviors of the mouse model.

(a) Schematic illustrating a timeline of performing synaptic function and behavior tests. (b) Schematic illustrating the location of recording and stimulating electrodes of synaptic transmission with the Schaffer collateral pathway. (c) Field excitatory postsynaptic potentials (fEPSPs) evoked by stimulation of Schaffer collateral pathway during LTP experiment from acute hippocampal slices from wild type and

3xTg mice w/o overexpressing *IncMtDloop*. Bars indicate mean $\pm$ SEM. n (brain slice) = 8-10 per group from 7 animals per genotype. (d) Representative traces and graphs for electrophysiological recordings with acute hippocampal slices from each group (top). Quantification of average fEPSP from the last 10min of the time course from (C). Bars indicate mean $\pm$ SEM. n (brain slice) = 8-10 per group from 7 animals per genotype. * P <0.05, ** P <0.01, by one-way ANOVA with Dunnett's multiple comparisons test (bottom). (e-f) Contextual fear conditioning test and cued fear conditioning test were performed in mice as described above. Bars indicate mean $\pm$ SEM. n = 9-11 animals per group. * P <0.05, by one-way ANOVA with Dunnett's multiple comparisons test. (g) Escape latencies of each group in the MWM during a 5-day training period. Bars indicate mean $\pm$ SEM. n = 7 animals per group. * P <0.05, ** P <0.01, by two-way ANOVA with Tukey's multiple comparisons test. (h) Escape traces to the hidden platform of each group in the MWM at day 6. (i) Latencies to the target platform of each group in the MWM at day 6. Bars indicate mean $\pm$ SEM. n = 7 animals per group. * P <0.05, by one-way ANOVA with Dunnett's multiple comparisons test. (j) The time exploring in the target quadrant in the MWM at day 6. Bars indicate mean $\pm$ SEM. n = 7 animals per group. * P <0.05, by one-way ANOVA with Dunnett's multiple comparisons test.

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