1 **CircTulp4 functions in Alzheimer's disease pathogenesis by** 2 **regulating its parental gene, Tulp4**

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Abstract

Alzheimer's disease (AD)—one of the most common neurodegenerative diseases worldwide—impairs cognition, memory, and language ability and causes dementia. However, AD pathogenesis remains poorly elucidated. Recently, a potential link between AD and circular RNAs (circRNAs) has been uncovered, but only a few circRNAs that might be involved in AD have been identified. Here, we systematically investigated circRNAs in the APP/PS1 model mouse brain through deep RNA-sequencing. We report that circRNAs are markedly enriched in the brain and that several circRNAs exhibit differential expression between wild-type and APP/PS1 mice. We characterized one abundant circRNA, circTulp4, derived from Intron1 of the gene *Tulp4*. Our results indicate that circTulp4 predominantly localizes in the nucleus and interacts with U1 snRNP and RNA polymerase II to modulate the transcription of its parental gene, *Tulp4*, and thereby regulate the function of the nervous system and might participate in the development of AD.

Keywords: CircTulp4; Alzheimer's disease; Tulp4; Neuronal differentiation; U1 snRNP

Background

Only ~2% of the genes in the human genome encode proteins [1]. Thus, non-protein-coding RNAs such as circular RNAs (circRNAs) and microRNAs (miRNAs/miRs) are now under considerable research scrutiny and generally accepted to influence myriad physiological and pathological processes, including the initiation and progression of neurological diseases such as Alzheimer's disease (AD) [2-6], which is associated with progressive loss of memory, cognition, and behavioral capabilities [7,8]. No effective AD treatments are available, and the genetic basis of AD and the epigenetic control of AD pathogenesis remain largely unelucidated.

CircRNAs are unique among cellular RNAs in that pre-mRNA back-splicing results in covalent linking of the 5'- and 3'-ends [9], which is likely responsible for circRNAs being more stable than linear coding/noncoding transcripts [10]. Given this high stability (lifetimes of hours to days or longer), circRNAs could perform functions distinct from those of linear RNAs and also potentially serve as more reliable biomarkers of health and disease than other RNAs. Intriguingly, in the brain, circRNAs are enriched in neurons and in synaptosomes [11] and their levels are regulated during aging in the fly and the mouse; thus, these circRNAs might play roles in brain aging and aging-associated neurodegenerative diseases [12,13]. For example, CDR1as (CiRS-7: circRNA sponge bound to miR-7) and miR-7 are associated with nervous system development and disease, and CDR1as dysfunction can cause miR-7 upregulation and lead to downregulation of AD-related targets, including ubiquitin-protein ligase A [14-16]. Furthermore, an *SRY*-derived circRNA can act as a natural miRNA sponge to inhibit miR-138 activity [17], which can affect learning and memory by regulating acyl

78 protein thioesterase 1 [18,19]. However, circRNAs and their activities in AD have not been
79 comprehensively analyzed.

80 To identify AD-associated circRNAs, we used deep RNA-sequencing (RNA-seq) for the first
81 comparative profiling of circRNA expression in the brain of APP/PS1 mice (which express
82 APP_{695Swe} and carry PS1-dE9 mutations) and wild-type (WT) mice. We used 6- and
83 9-month-old mice because β -amyloid appears at 6 months and extracellular β -amyloid
84 deposits in the cortex are apparent by 9 months, and synaptic transmission and long-term
85 potentiation are impaired when the mice are 9–12 months old [20]. Our circRNA-profiling
86 data could serve as a useful resource for future development of AD therapeutic targets or
87 novel diagnostics. Moreover, our findings identify circTulp4 as a potential AD biomarker:
88 circTulp4 is enriched in the brain and downregulated in APP/PS1 mice, and circTulp4 mainly
89 localizes in the nucleus, interacts with U1 snRNP and RNA polymerase II to promote the
90 transcription of its parental gene, *Tulp4*, and by affecting *Tulp4* expression, influences
91 neuronal growth and differentiation.

Results

Brain circRNA profiles differ in WT and APP/PS1 mice

We performed RNA-seq analyses on rRNA-/linear-RNA-depleted total RNA isolated from the brain of 6-/9-month-old WT and APP/PS1 mice; from the final clean reads, we identified 15,713 circRNAs (See Supplementary Data). The circRNA candidates were annotated using RefSeq database [22]. Most circRNAs were <5000 nt long (median length, 1000 nt; Fig. 1A), and the majority aligned with protein-coding exons and fewer with introns and intergenic regions (Fig. 1B). We normalized the junction reads (supporting the identification of RNAs as circRNAs) by read length and number of mapped reads (spliced reads per billion mapped reads, denoted as TPM), which permitted quantitative comparisons between junction reads from different RNA-seq data (Fig. 1C). Numerous circRNAs exhibited differential expression across groups, which we quantified using Wilcoxon rank-sum test. In 6-month-old mice, 343 circRNAs were differentially expressed between the two groups: 192 and 151 transcripts were significantly ($p < 0.05$) upregulated and downregulated, respectively, in APP/PS1 mice relative to WT (Fig. 1D); at 9 months, 243 circRNAs were significantly dysregulated: 141 and 102 transcripts were upregulated and downregulated in APP/PS1 mice (Fig. 1E). To generate these volcano maps, cluster analysis was performed on the differentially expressed circRNAs. The brain circRNA profiles of APP/PS1 and WT mice differed (Fig. 1F), and the parental genes of the upregulated/downregulated circRNAs mapped to distinct chromosomes (Fig. 1G).

We next verified that back-splicing events indicated the genuine circular form of the RNA transcripts and further confirmed the differential expression identified using RNA-seq data. For this real-time qPCR analysis, we designed divergent primers against 26 circRNAs

(Supplementary Fig. 1); each primer pair amplified a single product of the expected size. All transcripts were detected in the brain of 2–9-month-old WT and APP/PS1 mice and exhibited significant differential expression. Our qPCR results were highly consistent with the RNA-seq data.

Figure 1. CircRNA profiling in the brain of WT and APP/PS1 mice.

a CircRNA length distribution: x-axis: lengths of circRNAs detected in this study; y-axis:

121 abundance of circRNAs classified by lengths; z-axis: sample information. **b** CircRNAs
 122 derived from distinct genomic regions; circRNA numbers are listed as log2N, where N is the
 123 number of exonic, intronic, or intergenic reads. For example, from the origin, the number of
 124 exonic reads is $\sim 2^{15}$. **c** Density plot of circRNA abundance in WT and APP/PS1 mice. **d, e**
 125 Volcano plots showing circRNAs that exhibited downregulation (green points), upregulation
 126 (red points), or no statistically significant difference (blue points) in WT and APP/PS1 mice;
 127 x-axis: log2 ratio of circRNA expression levels between WT and APP/PS1 mice; y-axis:
 128 false-discovery rate ($-\log_{10}$ transformed) of circRNAs. **f** Heatmap of all circRNAs
 129 differentially expressed between WT and APP/PS1 mouse brains. **g** Numbers of differentially
 130 expressed circRNAs in different chromosomes; yellow, red, and gray bars: upregulated,
 131 downregulated, and total circRNAs, respectively.

132

133 **Supplementary Fig 1. Quantitative PCR validation of circRNA expression.**

134 We selected three sets of circRNAs for verification: 6yes9no: differential expression at 6

135 months, no differential expression at 9 months; 6no9yes: no differential expression at 6

months, differential expression at 9 months; and 6yes9yes: differential expression at both 6 and 9 months. **a** 6yes9no group, **b** 6yes9yes group, **c** 6no9yes group. CircRNA expression was quantified relative to GAPDH expression by using the comparative cycle threshold (Δ CT) method. Data are presented as means $\pm$ SD (n=3, *p<0.05, **p<0.01).

CircTulp4 is a circRNA that mainly localizes in the nucleus and regulates its parental gene

Our RNA-seq screening identified an array of brain circRNAs showing altered expression in APP/PS1 mice relative to WT. We now focus on our investigation on one specific circRNA—circTulp4—as a potential AD biomarker. CircTulp4, derived from *Tulp4* Intron1 (Fig. 2A), was reported to be markedly enriched in the mammalian brain and to feature a sequence well conserved in humans and mice [11]. Our high-throughput sequencing data revealed high circTulp4 levels in the brain, and our qPCR results confirmed significant differential expression of circTulp4 between APP/PS1 and WT mice at both 9 and 12 months (Fig. 5A).

To verify that *Tulp4* encodes an endogenous circRNA, we designed convergent and divergent primers that amplify canonical or back-spliced forms of *Tulp4* RNA (Fig. 2B, upper panel). PCR after reverse-transcription with the divergent primers (black) generated an RNase-R-resistant form of circTulp4, whereas the convergent primers (red) generated an RNase-R-sensitive product from linear *Tulp4* mRNA (Fig. 2B, lower panel). Moreover, Sanger sequencing confirmed the junction sequence in circTulp4 (Fig. 2C).

We next precisely positioned and quantified circTulp4 by using BaseScope (a novel

mutation-specific RNA ISH assay); we used probes designed to target the circTulp4 junction sequence or a negative-control RNA sequence (bacterial *dapB* mRNA). Probe details are listed in Supplementary Data. BaseScope signals were readily distinguishable as punctate red dots within cells, and circTulp4 expression was markedly lower in 9-month-old APP/PS1 mice than WT mice (Fig. 2D); the difference was not notable in 6-month-old mice.

In FISH analysis performed for cellular localization, a junction-specific probe detected abundant nuclear circTulp4 expression in Neuro-2a cells (Fig. 2E); moreover, nuclear mass-separation assays revealed that in primary hippocampal neurons, circTulp4 was mostly localized in the nucleus (Fig. 2F). Because the levels of certain circRNAs (including intron lariats) are positively correlated with those of their parental mRNAs [23], we examined whether circTulp4 exhibits this relationship: siRNA-mediated knockdown of circTulp4 resulted in a reduction in *Tulp4* mRNA and protein levels in primary hippocampal neurons (Fig. 2G); the same effect was produced by three circTulp4-specific siRNAs (Supplementary Fig. 2A-B). Thus, when circTulp4 levels were lowered, *Tulp4* expression was diminished; however, siRNA-mediated knockdown of *Tulp4* mRNA did not affect circTulp4 levels (Supplementary Fig. 2C). Conversely, *Tulp4* mRNA and protein levels were increased when circTulp4 was overexpressed in cells (Fig. 2G). Collectively, these results suggest that circTulp4 can regulate *Tulp4* expression.

Figure 2. CircTulp4 is a circRNA that mainly localizes in the nucleus and regulates its parental gene, *Tulp4*.

a Schematic depicting circTulp4 formation through circularization of Intron1 in *Tulp4*. **b** Upper panel: Convergent (red) and divergent (black) primers designed to amplify linear and back-spliced products. Lower panel: qRT-PCR analysis of abundance of circTulp4 and *Tulp4*

182 mRNA in Neuro-2a cells treated with RNase R; circTulp4 and *Tulp4* mRNA amounts were
183 normalized to the value measured in mock treatment. **c** Sanger sequencing after PCR
184 performed using the indicated divergent flanking primers, confirming “head-to-tail” splicing
185 of circTulp4 in Neuro-2a cells. **d** CircTulp4 expression detected in fresh-frozen brain tissue
186 samples from 6- and 9-month-old WT and APP/PS1 mice, using BaseScope® 2.5 HD visible
187 light staining kit; circTulp4 appears as red dots. Cells were counterstained with hematoxylin.
188 Single-pair probes for circTulp4 targeting a unique junction sequence are specific and
189 sensitive. Original magnification 40×; scale bar: 110 μm. **e** RNA fluorescence in situ
190 hybridization (FISH) for circTulp4; 18S is a cytoplasmic control. Nuclei were stained with
191 4,6-diamidino-2-phenylindole (DAPI); circTulp4 and 18S probes were labeled with Alexa
192 Fluor 555. Original magnification 40×; scale bar: 40 μm. **f** qRT-PCR data indicating circTulp4
193 abundance in cytoplasm and nucleus of primary hippocampal neurons, determined by using
194 nuclear mass-separation assay; GAPDH and U6 are shown for comparison. **g** Tulp4 mRNA
195 and protein levels were decreased after siRNA-mediated knockdown of circTulp4 in primary
196 hippocampal neurons; the specific siRNA targeted circTulp4 at junction sequences. Results
197 for control mismatched-sequence siRNA are shown in black. Parental-gene mRNA and
198 protein levels were increased in primary hippocampal neurons after overexpression of
199 circTulp4 by using a circTulp4 adenovirus vector; adenovirus empty-vector results are shown
200 in black. Results shown are means ± SD (*p<0.05, **p<0.01).

Supplementary Fig 2. CircTulp4 is mainly localized in the nucleus and regulates its parental gene, *Tulp4*.

a Target sequence for siRNAs. **b** Neuro-2a and PC12 cells were transfected with circTulp4 siRNA (100–120 nM) before culturing, and the cells were then cultured for 24 h. The qRT-PCR data indicate that the expression of the parental gene was decreased when circTulp4 was downregulated. **c** Neuro-2a cells were transfected with *Tulp4* siRNA (100 nM) before culturing, and the cells were then cultured for 24 h. The qRT-PCR data indicate that siRNA-mediated knockdown of *Tulp4* mRNA did not affect circTulp4 levels. Results are shown as means $\pm$ SD (* p <0.05, ** p <0.01).

CircTulp4 associates with RNA polymerase II by interacting with U1 snRNP

To investigate how circTulp4 regulates *Tulp4*, we examined circTulp4-protein interactions.

First, we evaluated circTulp4-protein interaction tendency by using CatRAPID, which yields

Interaction Strengths indicating likely occurrence (>50%) or high-confidence prediction

(>75%) of interaction [21] (Section 2.14). Notably, an Interaction Strength of 99% was

obtained for circTulp4 interaction with SNRPA (U1 snRNP) (Fig. 3A).

We next used RAP assays to directly test circTulp4 interaction with U1 snRNA (Fig. 3B).

Because U1 regulatory transcription has been demonstrated [24], U1 snRNP was considered

likely to bind to circTulp4, and thus we designed primers against U1. Because U1 and U2 are

generally in close proximity and frequently function together to form a splicing body, we

checked for U2 capture. We selected 7SK and CDR1as because 7SK has been reported to

function in regulating transcription [25], and CDR1as has been comprehensively investigated

in the nervous system and in degenerative diseases [26]. Lastly, U6 and U7, which are distant

from U1, were used as negative controls. The circTulp4-specific probe captured considerably

more circTulp4 relative to the control probe, and U1 was pulled down to a markedly higher

level than other RNAs (Fig. 3B). Notably, U1 snRNA also colocalized with circTulp4

(detected using a junction probe) in two cell types (Fig. 3C). These results suggest that

circTulp4 can bind to U1 snRNA.

Because U1 interacts with RNA polymerase II to regulate transcription [24], we tested

whether circTulp4 interacts with the U1 protein complex and RNA polymerase II. In RIP

assays performed using antibodies against U1 (U1A and SNRNP70), RNA polymerase II, and

233 U2, RNA polymerase II showed almost no binding to CDR1as but associated with circTulp4
 234 (Fig. 3D). This provides complementary evidence that these molecules interact and thus might
 235 function together.

236 We also investigated potential interactions between circTulp4, U1 snRNA, and *Tulp4*; we
 237 checked for RNA capture by performing ChIRP assays, which revealed that pull-down with
 238 the circTulp4 probe captured U1 snRNA (Fig. 3E). U2 snRNA was also pulled down with
 239 circTulp4, although this association might be indirect. These results verified the RAP results
 240 and demonstrated circTulp4 association with U1 snRNA.

Figure 3. CircTulp4 associates with RNA polymerase II by directly interacting with U1 snRNP.

a Heatmap showing prediction results for U1 and circTulp4 interaction; x-axis and y-axis: indexes of RNA and protein sequences, respectively. Heatmap colors indicate interaction scores, from -3 to 3, of individual amino acid and nucleotide pairs. The total sum represents the overall interaction score. The algorithm CatRAPID identified interaction between U1 and

248 circTulp4 with confidence: Interaction Propensity, 17.14; Interaction Strength, 99%. **b** RNA
 249 antisense purification (RAP) assays were used to pulldown RNAs with circTulp4; U2 snRNA,
 250 CDR1as, U6 snRNA, U7 snRNA, and 7SK are controls. The circTulp4 probe captured
 251 considerably more circTulp4 relative to the control probe, and U1 was pulled down to a
 252 significantly higher level than other RNAs; this suggests that circTulp4 can bind to U1
 253 snRNA. **c** Double FISH for circTulp4 and U1. Nuclei were stained with DAPI; circTulp4 was
 254 labeled with an Alexa Fluor 555-conjugated probe; U1 snRNA was labeled with an
 255 FITC-conjugated probe. Original magnification 40 $\times$; scale bar: 20 μ m. **d** RNA
 256 immunoprecipitation (RIP) assay with antibodies against U1A, SNRNP70, and RNA
 257 polymerase II (Pol II). U2AF65 is shown for comparison. U1A, SNRNP70, and Pol II
 258 co-precipitated a substantial amount of circTulp4 but not CDR1as; pulldown with U2AF65
 259 did not result in marked enrichment of circTulp4. ND: not detected. **e** Pulldown of indicated
 260 RNAs in chromatin isolation by RNA purification (ChIRP) assays for circTulp4. Pulldown
 261 with circTulp4 probes resulted in capture of U1 snRNA. U2 snRNA was also pulled down
 262 with circTulp4, although the association between U2 snRNA and circTulp4 could be indirect.
 263 Results are shown as means $\pm$ SD.

264

265 CircTulp4 recruits U1 snRNP to regulate Tulp4 transcription

266 We divided the 3000-bp *Tulp4* promotor (3000 bp upstream of coding area) into 6 segments
 267 (A–F) and designed a primer for almost every 500-bp stretch. We found that circTulp4
 268 associates with segments E and F, located ~500 bp upstream of the transcriptional start site of
 269 *Tulp4* (Fig. 4A).

287 *Tulp4* promoter, indicating that circTulp4 and U1 snRNP (U1A) occupy a region ~500 bp
 288 upstream of *Tulp4* transcriptional start site; this suggests that circTulp4, U1 snRNP, and Pol II
 289 potentially interact with each other at the promoter region of the parental gene. Results are
 290 shown as means $\pm$ SD.

Ultrasonic condition: 50% energy level; Turn on 5s; Turn off 10s; Ultrasound 20min.

291

292 **Supplementary Fig 3. Chromatin fragmentation for ChIP analyses.**

293 Ultrasound conditions for breaking chromatin into 200–500-bp fragments: 50% energy level;
 294 turn on 5 s, turn off 10 s; ultrasound 20 min.

295

296 **CircTulp4 affects Tulp4 expression and thereby regulates neuronal differentiation, cell** 297 **viability, and neurite growth and branching**

298 To complete our current evaluation of circTulp4 as a potential AD biomarker, we examined
 299 circTulp4 neuronal expression and function. First, qPCR results demonstrated circTulp4
 300 expression in brain tissue from 2–12-month-old APP/PS1 and WT mice, and in accord with
 301 the RNA-seq results, circTulp4 expression in the brain was lower in APP/PS1 than WT mice
 302 at both 9 and 12 months (Fig. 5A). Moreover, in agreement with a previous study [11],

303 circTulp4 was upregulated during neuronal differentiation in different cell types (Fig. 5B,
304 Supplementary Fig. 4A).

305 To investigate circTulp4 function in neuronal cells, circTulp4 expression was silenced using
306 junction-sequence-targeting siRNAs; these siRNAs, but not a control siRNA, markedly
307 lowered circTulp4 levels in two cell types (Supplementary Fig. 2) and potently suppressed
308 cell proliferation (Fig. 5C, Supplementary Fig. 4B). Moreover, circTulp4 downregulation
309 significantly decreased neurite growth and branching in hippocampal neurons and cell lines
310 (Fig. 5D-E, Supplementary Fig. 4C). These findings suggest that circTulp4 functions in
311 neuronal growth and development.

312 Notably, Tulp4 exhibited the same expression pattern as circTulp4 in mouse brain tissue (Fig.
313 5F) and was upregulated during neuronal differentiation (Fig. 5G). The finding that circTulp4
314 knockdown affected *Tulp4* expression suggested that *Tulp4* plays a role in the effects
315 observed following circTulp4 knockdown. Accordingly, siRNA-mediated *Tulp4*
316 downregulation markedly diminished neuronal viability (Fig. 5H, Supplementary Fig. 4D),
317 and Tulp4 knockdown in primary hippocampal neurons also strongly inhibited neurite growth
318 and branching (Fig. 5I). And the subsequent rescue experiment can be found that when
319 knocking down circTulp4 and overexpressing the parent gene Tulp4 at the same time, it has a
320 significant rescue effect on cell viability and neurite damage (Fig. 6A-B). These findings
321 collectively suggest that circTulp4 could influence normal neuronal growth and development
322 by regulating *Tulp4* expression; we propose that this circTulp4 regulatory function underlies
323 the potential link between circTulp4 dysregulation and AD pathogenesis.

Figure 5. CircTulp4 regulates neuronal differentiation, cell viability, and neurite growth and branching through its parental gene, *Tulp4*.

a Relative expression levels of circTulp4 in WT and APP/PS1 mouse cortices were examined

328 using qRT-PCR (9 pairs of mice at each stage). Glyceraldehyde-3-phosphate dehydrogenase
329 gene (*Gapdh*) was used as the reference gene. **b** qRT-PCR analysis of relative expression of
330 circTulp4 in primary hippocampal neurons during development (n=3). **c** Mouse primary
331 hippocampal neurons were transfected with circTulp4 siRNA (100 nM), and 72 h later,
332 CCK-8 assay was performed to evaluate cell viability. **d,e** Mouse primary hippocampal
333 neurons were co-transfected with PT-GFP and circTulp4 siRNA (100 nM) (concentration ratio
334 1:5) before culturing, and 72 h later, neurites were immunostained with anti- β -tubulin III
335 antibody. The length and branch numbers of GFP-expressing neurites were quantified using
336 ImageJ software. Original magnification 40 $\times$; scale bar: 40 μ m. **f** Relative expression of
337 Tulp4 gene and protein in WT and APP/PS1 mouse cortex: qRT-PCR and western blotting
338 analyses (9 pairs of mice at each stage); the results were normalized relative to GAPDH. **g**
339 qRT-PCR analysis of *Tulp4* relative expression in primary hippocampal neurons during
340 development (n=3). **h** Mouse primary hippocampal neurons were transfected with Tulp4
341 siRNA (100 nM), and 72 h later, CCK-8 assay was performed to evaluate cell viability. **i**
342 Mouse primary hippocampal neurons were co-transfected with PT-GFP and Tulp4 siRNA
343 (100 nM) (concentration ratio 1:5) before culturing, and after culturing for 72 h, neurites were
344 immunostained with anti- β -tubulin III antibody. The length and branch numbers of
345 GFP-expressing neurites were quantified using ImageJ software. Results are shown as means
346 $\pm$ SD (*p<0.05, **p<0.01).

Supplementary Fig 4. CircTulp4 regulates neuronal differentiation, cell viability, and neurite growth and branching through *Tulp4*.

a qRT-PCR analysis of circTulp4 relative expression in Neuro-2a and PC12 cells during development in culture. **b** CCK-8 assay was performed to evaluate cell viability. Neuro-2a and PC12 cells were transfected with circTulp4 siRNA (100 nM) and then induced with RA (3 μ g/mL) or NGF (50 μ g/mL) for 48 h before use in CCK-8 assays. **c** PC12 cells were co-transfected with PT-GFP and circTulp4 siRNA (100 nM) (concentration ratio 1:5) before culturing, and after culturing for 48 h, the cells were treated with NGF (50 μ g/mL) for 48 h.

356 Neurites were immunostained with anti- β -tubulin III antibody. The length and branch
 357 numbers of GFP-expressing neurites were quantified using ImageJ software. **d** CCK-8 assay
 358 was used to evaluate cell viability. PC12 cells were transfected with Tulp4 siRNA (100 nM)
 359 and then treated with NGF (50 μ g/mL); after 48-h induction, the cells were used in CCK-8
 360 assays. Results are shown as means $\pm$ SD (* p <0.05, ** p <0.01).

361
 362 **Figure 6. CircTulp4 regulates neuronal differentiation, cell viability, and neurite growth**
 363 **and branching through its parental gene, Tulp4 (rescue experiment)**

364 It can be found that when knocking down circTulp4 and over-expressing the parent gene
 365 Tulp4 at the same time, there is an obvious rescue effect. **a** Mouse primary hippocampal
 366 neurons were co-transfected with pEGFP-Tulp4 and circTulp4 siRNA before culturing, and
 367 after culturing for 72 h, CCK-8 assay was performed to evaluate cell viability. **b** Mouse
 368 primary hippocampal neurons were co-transfected with pEGFP-Tulp4 and circTulp4 siRNA
 369 before culturing, and after culturing for 72 h, neurites were immunostained with
 370 anti- β -tubulin III antibody. The length and branch numbers of GFP-expressing neurites were
 371 quantified using ImageJ software. Results are shown as means $\pm$ SD (* p <0.05, ** p <0.01).

372

373 **Methods**

374 **Animals**

375 WT and APP/PS1 mice (The Jackson Laboratory; strain B6.Cg-Tg(APPswe,
376 PSEN1dE9)85Dbo/Mmjax) were purchased from the Model Animal Research Center of
377 Nanjing University, housed 1/cage under standard specific conditions (25 °C, 50% humidity,
378 12/12-h light/dark cycle, pathogen-free environment), and provided free access to food until
379 6/9 months old. From 3 randomly selected male mice from each group, brain tissues were
380 collected for RNA-seq. All animal experiments were performed in accordance with animal
381 use protocols approved by the Committee for the Ethics of Animal Experiments, Shenzhen
382 Peking University The Hong Kong University of Science and Technology Medical Center
383 (SPHMC) (protocol number 2011-004).

384

385 **RNA-seq**

386 Total RNA was isolated by using TRIzol reagent (Invitrogen) as per manufacturer instructions.
387 After RNA isolation, RNA degradation and contamination were assessed using (1%)
388 agarose-gel electrophoresis, RNA purity was checked using a NanoPhotometer
389 spectrophotometer (IMPLEN, CA, USA), RNA concentration was measured using a Qubit
390 RNA Assay Kit and Qubit 2.0 Fluorometer (Life Technologies, CA, USA), and RNA integrity
391 was evaluated using the RNA Nano 6000 Assay Kit for a Bioanalyzer 2100 System (Agilent
392 Technologies, CA, USA). The complete circRNA study involved the following steps: RNA
393 isolation, quantification, and qualification; library preparation for circRNA sequencing;

sequencing and clustering of reads; quality control; mapping to the reference genome; circRNA identification; quantification of gene-expression level; and differential-expression analysis. Normalized circRNA levels (estimated based on TPM values) were calculated as listed below, and transcripts were considered to be differentially expressed between APP/PS1 and WT mice when their p value was <0.05.

Normalized expression = (mapped reads)/(total reads) × 1,000,000.

Cell culture

Neurons were isolated from the brain of E16.5–E18.5 mice (C57B6), seeded on petri dishes coated with poly-D-lysine (Sigma), and cultured in neurobasal medium (Life Technologies) containing 2% B27 (Life Technologies) and 0.5 mM L-glutamine at 37 °C in a 5% CO₂ incubator. The cell lines Neuro-2a (mouse neuroblastoma N2a cells) and HEK293T were grown in DMEM (GIBCO) supplemented with 10% FBS (HyClone) and 1% antibiotics, and PC12 cells (rat pheochromocytoma cells) were grown in DMEM supplemented with 6% FBS, 6% HS (HyClone), and 1% antibiotics. Differentiation was induced (for 48 h) by maintaining Neuro-2a cells in DMEM containing 0.5% FBS, 10 mM RA (retinoic acid; Sigma), and 1% antibiotics, and PC12 cells in DMEM containing 0.5% FBS, 0.5% HS, 100 ng/mL NGF (nerve growth factor; Sigma), and 1% antibiotics.

Transfection and RNA interference

Neuro-2a cells seeded in 6-well plates (6×10^5 /well) were transfected with 100 nM circTulp4-specific or negative-control siRNA by using Lipofectamine® RNAiMAX

(Invitrogen). At 24 h post-transfection, cells were lysed, RNA was isolated, and siRNA transfection efficiency was tested using quantitative real-time RT-PCR (qRT-PCR). Differentiation was induced for 48 h. Dissected hippocampal neurons seeded in 12- or 24-well plates (1×10^5 /well or 5×10^4 /well) were co-transfected with 50 or 25 nM circTulp4 siRNA and EGFP plasmid (5:1 molar ratio); and immunofluorescence analysis was performed at 72 h post-transfection.

qRT-PCR

Total RNA was extracted using TRIzol reagent (Sigma), according to the manufacturer's protocol, and RNA quantity was measured using a NanoDrop 2000 (Thermo Fisher Scientific). qRT-PCR was performed using the GoScript™ Reverse Transcription System (Promega) and a C1000 Thermal Cycler (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase gene (Gapdh) was used as an internal control. Relative gene-expression levels were quantified using the $2^{-\Delta Ct}$ method.

Cell counting kit-8 (CCK-8) assay

Treated cells were seeded in 24-well plates (1×10^4 /well) and incubated in a 5% CO₂ incubator at 37 °C, and then CCK-8 solution (Cat. no. HY-K0301-100T; MedChem Express, Monmouth Junction, NJ, USA) was added (50 µL/well). After 3/4-h incubation, optical density was measured using a microplate reader (Bio-Rad). Cell viability is presented as a percentage relative to nontreated cells.

Fluorescence in situ hybridization (FISH)

Cy3-labeled circTulp4 probes were designed and synthesized by RiboBio (Guangzhou, China; probe sequences available upon request), and probe signals were detected using a FISH kit (RiboBio), as per manufacturer instructions. Cells were stained with 4'-6-diamidino-2-phenylindole (DAPI; Life Technologies, Carlsbad, CA, USA) before examination under a fluorescence microscope (Zeiss LSM 710).

Immunofluorescence analysis

Neurons were fixed in 4% (w/v) paraformaldehyde (30 min, room temperature) and then incubated with PBS containing 0.4% Triton X-100, 1% bovine serum albumin (Sigma), and 4% goat serum for 30 min to permeabilize the cells and block nonspecific staining. Neurons were incubated overnight at 4 °C with a tubulin primary antibody (mouse monoclonal anti- β -tubulin III, 1:500, Sigma) and for 2 h with a CY3-conjugated anti-mouse secondary antibody (1:500, Jackson Immuno Research), and then the coverslips were mounted on slides with an antifade mounting medium (Beyotime) and examined under a fluorescence microscope.

Western blotting

Protein extracts were prepared in RIPA buffer in the presence of a protease-inhibitor cocktail (Sangon) and 1 mM phenylmethanesulfonylfluoride (Sigma). After determining protein concentrations by using the Bradford protein assay, samples were separated using 10% SDS-PAGE and transferred to nitrocellulose membranes, which were probed with primary antibodies (4 °C, overnight) and then with horseradish peroxidase-conjugated secondary

antibodies (2 h). Signals were developed using a Western Lightning PLUS kit (NEL105001EA, PerkinElmer) and quantified using Quantity One (Bio-Rad).

In situ hybridization (ISH)

We used the single-pair probe ISH approach (BaseScope, Advanced Cell Diagnostics, Newark, CA, USA) based on the multiplex fluorescent ISH RNAscope® method (Advanced Cell Diagnostics [47]). ISH methods are highly specific and sensitive due to the use of a unique probe design employing “ZZ” probe pairs and signal amplification, respectively. Advances in signal amplification over RNAscope® have enabled the use in BaseScope assays of a single-pair probe, comprising a pair of 18–25-bp oligonucleotides, for detecting junction sequences. Table S1 lists the targeted sequences of our customized junction-specific circTulp4 ISH probes. BaseScope ISH assays were performed on 20-µm-thick, fresh-frozen brain-tissue sections from 6-/9-month-old WT and APP/PS1 mice. Sections were deparaffinized in xylene, treated with H₂O₂ (10 min, room temperature) to block endogenous peroxidases, permeabilized by performing antigen retrieval (15 min, 100 °C), and incubated with a protease mixture (30 min, 40 °C). Probes were bound through incubation for 2 h at 40 °C, chemically amplified, and labeled using fluorophores (multiplex ISH) or through alkaline-phosphatase conversion of Fast RED dye (single-pair probe ISH). Table S1 lists the probe sequences.

RNA antisense purification (RAP)

RAP kits (Bersin Bio) were used according to manufacturer instructions. RAP employs specific biotinylated probes that hybridize to target RNAs, which can be pulled down,

reverse-transcribed into cDNA, and identified through qRT-PCR analysis or sequencing. Here, 107 cells were washed with PBS, crosslinked using formaldehyde, lysed with 1 mL of lysis buffer, and homogenized using a 0.4-mm syringe, after which a single 50-bp biotinylated antisense probe (0.2 nmol) targeting the adaptor sequence was added. Probes were denatured (65°C, 10 min) and hybridized (room temperature, 2 h), and then 200 µL of streptavidin-coated magnetic beads were added. After washing to remove nonspecifically bound RNAs, TRIzol reagent was used to recover RNAs that specifically interacted with circTulp4. Table S1 lists the probe sequences.

RNA immunoprecipitation (RIP)

RIP assays were performed using a kit from Millipore (Cat. no. 17-701) and following the manufacturer's instructions. Neuro-2a cells (2×10^7) were lysed in complete RIP lysis buffer and the cell lysates were divided into equal parts and incubated (overnight with rotation, at 4 °C) with anti-Pol II (Cell Signaling Technology (CST), Cat. no. #2629), anti-U1A (Abcam, Cat. no. ab166890), anti-U2AF65 (Abcam, Cat. no. ab37530), IgG (Millipore, Cat. no. pp64B), or anti-SNRNP70 (Millipore, Cat. no. CS203216). Next, magnetic beads (Millipore, Cat. no. CS203178) were added to the cell lysates and the incubation was continued at 4 °C for 1 h, after which the samples were incubated with proteinase K at 55 °C for 1 h. The enriched RNA was obtained using RNA Extraction Reagent (phenol:chloroform:isoamylol = 125:24:1; pH<5.0; Solar Bio), and qRT-PCR was performed on the purified RNA to detect the targets of interest.

Chromatin isolation by RNA purification (ChIRP)

ChIRP was performed using a kit from Millipore (Cat. no. 17-10495), according to the manufacturer's instructions. Briefly, Neuro-2a cells grown in differentiation medium for 24 h were trypsinized (0.25% trypsin) and washed with 1× PBS, and then 2×10⁷ cells were crosslinked with 20 mL of 1% glutaraldehyde/PBS at room temperature (18 °C–25 °C) for 10 min on an end-to-end rotator. The excess glutaraldehyde was quenched by adding 2 mL of 1.25 M glycine and incubating for an additional 5 min. After washing with 20 mL of cold PBS, the crosslinked cells were resuspended in 2 mL of lysis buffer and sonicated (in a cold room) by using a Bioruptor (Diagenode) with the following parameters: H—high setting; pulse interval—30 s ON and 30 s OFF; 10 repeats. After sonication, the fragmented chromatin samples (200–500-bp long) were split into two parts and hybridized with BiotinTEG-labeled tiling probes against circTulp4 (Table S1) or control probes in hybridization buffer at 37°C; after hybridization for 4 h, 100 µL of Pure Proteome Streptavidin magnetic beads (Millipore, Cat. no. CS219080) were added into each reaction and incubated at 37 °C for 30 min, and the beads were then washed 4 times with prewarmed washing buffer at 37 °C for 5 min. The retrieved bead–RNA–protein–DNA complex was split into two parts: 1/10 for RNA isolation, 9/10 for DNA purification. Lastly, the retrieved RNA and DNA were used as templates for real-time quantitative PCR (qPCR), and the obtained data are presented as the percentages of input RNA and DNA, respectively.

Rapid prediction of RNA-protein interaction

CircTulp4-protein interactions were predicted using CatRAPID, an online algorithm that

rapidly predicts RNA-protein interactions based on secondary structure, hydrogen bonding, and molecular interatomic forces. The obtained interaction propensity is a measure of the interaction probability between 1 protein (or region) and 1 RNA (or region). This measure is based on the observed tendency of ribonucleoprotein-complex components to exhibit specific physicochemical properties that can be used to make a prediction. Interaction Strength, a statistical measure for evaluating interaction propensity with respect to CatRAPID training, reflects prediction confidence, ranging from 0% (unpredictability) to 100% (predictability): values of >50% indicate that an interaction is likely to occur, values of >75% represent high-confidence predictions [21].

Chromatin immunoprecipitation (ChIP)

ChIP experiments were performed using a kit from CST (Cat. no. #56383) and following the manufacturer's instructions. Briefly, cells were fixed on plates with 1% formaldehyde for 10 min at room temperature, and after quenching the crosslinking by adding 0.125 M glycine (5 min), the cells were pelleted at $800 \times g$. Cell pellets were resuspended in 1 mL of SDS lysis buffer (1% (w/v) SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) containing Roche complete protease-inhibitor cocktail and incubated for 10 min on ice. Cell extracts were sonicated for 15 min with a Bioruptor (Diagenode) to obtain 500-bp DNA fragments. After saving a 100- μ L sample of the supernatant as input, the remaining supernatant was diluted 1:10 in ChIP dilution buffer (0.01% (w/v) SDS, 1.1% (v/v) Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) containing protease inhibitors. The chromatin solution was precleared and then immunoprecipitated with antibodies against Pol II (CST, Cat. no. #2629),

U1A (Abcam, Cat. no. ab166890), U2AF65 (Abcam, Cat. no. ab37530), LSM10 (Abcam, Cat. no. ab180128), or H3 (CST, Cat. no. #4620), or with (as an antibody control) IgG (CST, Cat. no. #2729). The immune complexes were eluted in 1% (w/v) SDS and 50 mM NaHCO₃, and then the crosslinks were reversed for 6 h at 65 °C. Samples were digested with proteinase K for 1 h at 45 °C, and DNA was extracted using phenol/chloroform/isoamyl alcohol. The eluted DNA was subject to real-time qPCR to detect enriched genomic DNA regions.

Statistical analyses

Distribution normality was assessed using Kolmogorov–Smirnov test, implemented in PRISM statistical software (GraphPad). Multiple normally distributed groups were compared using ANOVA with Tukey post-test, two normally distributed groups were compared using *t* tests, and two non-normally distributed groups were compared using Mann–Whitney U test. High-throughput sequencing-related data were calculated and statistically computed using R software.

Discussion

No effective AD treatment is currently available, and therapies targeting the main AD neuropathological hallmarks (β -amyloid aggregates, neurofibrillary tangles) have shown little success [27]. Thus, epigenetic regulation of AD pathogenesis has recently been widely studied to identify potential new biomarkers/therapeutic targets. Intriguingly, dynamic changes in DNA methylation and long noncoding RNAs in the brain are reported to contribute to AD [28,29]. However, potential circRNA functions in AD development/progression remain largely unknown.

RNA-seq analyses [3,30] have allowed detailed characterization of circRNAs, which present characteristics that could link them to neurodegenerative diseases. Thousands of circRNAs are highly expressed in the mammalian brain, accumulated in neural tissues during aging, conserved between rodents and humans, and more abundant than linear RNAs in neuronal synaptic fractions [11,31]. Thus, circRNAs could perform critical functions in the nervous system, and circRNA dysregulation might underlie neurodegenerative disease development/progression. Accordingly, Lukiw [14] and Zhao et al. [32] reported roles for circRNAs in AD. However, our study represents the first comprehensive circRNA profiling in the brain in the APP/PS1 mouse model of AD. We compared brain circRNA profiles between age-matched APP/PS1 and WT mice and identified several circRNAs showing altered expression in APP/PS1 mice. Moreover, we found marked circTulp4 downregulation in the brain in 9-/12-month-old APP/PS1 mice, which suggests a potential association of circTulp4 dysregulation with AD pathogenesis.

CircRNA functions have been extensively studied but remain incompletely understood.

586 Certain circRNAs could function as miRNA sponges, but only a few circRNAs contain
587 multiple binding sites for trapping specific miRNAs [10,33,34], and circRNAs might not
588 necessarily act as miRNA sponges in human and mouse cells [11]. Interestingly, circRNA
589 knockdown can cause diminished parental-gene expression, which supports the possibility
590 that certain circRNAs upregulate RNA polymerase II-mediated transcription of parental genes
591 [35]. Our findings suggest that circTulp4 is one such circRNA: circTulp4 predominantly
592 localizes in the nucleus and interacts with U1 snRNP and RNA polymerase II to promote
593 *Tulp4* transcription. This ability to control *Tulp4* expression might allow circTulp4 to regulate
594 nervous system functions and thereby influence AD development.

595 We propose (Fig. 7) that circTulp4 and U1 snRNA bind through RNA-RNA interaction, and
596 that the circTulp4-U1 snRNP complex interacts with the RNA polymerase II transcription
597 complex at *Tulp4* promoter to enhance gene expression; once transcription is initiated,
598 circTulp4 generation would further promote the gene's transcription and create a
599 positive-feedback loop.

Figure 7. Working model depicting circTulp4 effects on *Tulp4* expression.

CircTulp4 might form a complex with U1 snRNA through specific RNA-RNA interaction, and the circTulp4–U1 snRNP complex might further interact with the Pol II transcription complex at *Tulp4* promoter to modulate *Tulp4* expression and, thereby, regulate neuronal differentiation and nervous system functions and participate in AD development.

Tulp4 is a protein of the tubby family, which is associated with neuronal differentiation and development, and tubby-protein mutations are implicated in obesity, deafness, and visual impairment [36]: Mice carrying a mutated tubby gene develop delayed-onset obesity, sensorineural hearing loss, and retinal degeneration, and *TULP1* mutations cause retinitis pigmentosa in humans [37,38] and retinal degeneration in mice [39]. Moreover, *Tulp3* mutants show embryonic lethality, with defects in dorsoventral patterning of the spinal cord

[40,41]. However, very few studies have been published on Tulp4. This is the first report that Tulp4 can critically affect nerve-cell differentiation and thus potentially contribute to nervous system growth and development.

In conclusion, several brain-enriched circRNAs are differently expressed between WT and APP/PS1 mice; our RNA-seq database of these circRNAs could serve as a valuable resource for future investigations into circRNA involvement in AD development and suitability as AD pathogenesis biomarkers. Moreover, circTulp4 is downregulated in APP/PS1 mice, and circTulp4 localizes in the nucleus, interacts with U1 snRNP and RNA polymerase II, and promotes *Tulp4* transcription. The circTulp4 ability to control *Tulp4* expression might enable circTulp4 to regulate neuronal differentiation and thereby influence nervous system functions and AD development. Certain circRNAs are stably detected in body fluids (e.g., plasma), which allows their use as biomarkers or therapeutic targets [42-44]. Therefore, we will next test for stable circTulp4 detection in body fluids and determine whether circTulp4 expression correlates with specific clinical stages of AD.

Conclusion

In conclusion, CircTulp4—a circRNA downregulated in APP/PS1 mice—localized in the nucleus and interacted with U1 snRNP and RNA polymerase II to regulate the transcription of its parental gene, Tulp4. CircTulp4 and Tulp4 showed similar expression in brain tissues, and downregulation suppressed neuronal survival/growth and branching. circTulp4 might influence nervous system functions and might participate in the development of AD.

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641 JCYJ20180507182657867, JCYJ20170306161713757).

642

643 **Data availability**

644 Gene-expression data are available at the Gene Expression Omnibus
 645 (<http://www.ncbi.nlm.nih.gov/geo/>; Accession Number GSE-132177). All other data are
 646 available on request.
 647

648 **Ethics approval and consent to participate**

649 All animal experiments were performed in accordance with animal use protocols approved by
 650 the Committee for the Ethics of Animal Experiments, Shenzhen Peking University The Hong
 651 Kong University of Science and Technology Medical Center (SPHMC) (protocol number
 652 2011-004).

653

654 **Conflicts of interest**

655 The authors declare no competing financial interests in relation to this work.

656

657

658 **Author contributions:**

659 JW, WZ, and NM designed the study. NM, JP, QW and BY participated in the animal
 660 experiments including tissue collection and RNA/protein extraction. NM performed the
 661 experiments and collected the data. NM, BY, JW, and WZ analyzed the results and wrote the
 662 manuscript. All authors read and approved the final manuscript.

663

References:

1. Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. ceRNA hypothesis: The Rosetta Stone of a hidden RNA language? CELL 2011;146:353-358
2. Okazaki Y, Furuno M, Kasukawa T, Adachi J, Bono H, Kondo S, Nikaido I, Osato N, Saito R, Suzuki H. Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. NATURE 2002
3. Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, Huarte M, Zuk O, Carey BW, Cassady JP. Chromatin Signature Reveals Over A Thousand Highly Conserved Large Non-coding Rnas In Mammals. NATURE 2009
4. Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. NAT REV GENET 2009;10:155-159
5. Anamaria N, Magali S, Maria W, Angélica L, Tasman D, Ulrich Z, Baker JC, Frank G, Henrik K. The evolution of lncRNA repertoires and expression patterns in tetrapods. NATURE 2014;505:635-640
6. Yvonne T, John R, Pier Paolo P. The multilayered complexity of ceRNA crosstalk and competition. NATURE 2014;505:344-352
7. Destrooper B, Karran E. The Cellular Phase of Alzheimer' s Disease. CELL 2016;164:603-615
8. Canter RG, Penney J, Tsai LH. The road to restoring neural circuits for the treatment of Alzheimer's disease. NATURE 2016;539:187-196
9. Chen LL. The biogenesis and emerging roles of circular RNAs. Nat Rev Mol Cell Biol 2016;17:205-211
10. Jens M. Circular RNAs Are a Large Class of Animal RNAs with Regulatory Potency. NATURE

2013;495:333-338

11. Agnieszka RW, Christin S, Petar GA, Marvin J, Natalia P, Sebastian G, Mor H, Mikaela B, Osnat B, Reut AF. Circular RNAs in the Mammalian Brain Are Highly Abundant, Conserved, and Dynamically Expressed. *MOL CELL* 2015;58:870-885

12. Gruner H, Cort ãS-L ãPez M, Cooper DA, Bauer M, Miura P. CircRNA accumulation in the aging mouse brain. *Sci Rep* 2016;6:38907

13. Westholm JO, Pedro M, Sara O, Sol S, Brian J, Piero S, Celniker SE, Graveley BR, Lai EC. Genome-wide Analysis of Drosophila Circular RNAs Reveals Their Structural and Sequence Properties and Age-Dependent Neural Accumulation. *CELL REP* 2014;9:1966-1980

14. Lukiw WJ. Circular RNA (circRNA) in Alzheimer's disease (AD). *FRONT GENET* 2013;4:307

15. Bingol B, Sheng M. Deconstruction for Reconstruction: The Role of Proteolysis in Neural Plasticity and Disease. *NEURON* 2011;69:22-32

16. Irina L, Shekoyan AR, Hebron ML, Nicole D, Algarzae NK, Charbel E-H M. Diminished parkin solubility and co-localization with intraneuronal amyloid- β are associated with autophagic defects in Alzheimer's disease. *Journal of Alzheimers Disease* 2013;33:231-247

17. Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Bente F, Damgaard CK, J Rgen K. Natural RNA circles function as efficient microRNA sponges. *NATURE* 2013;495:384-388

18. Schröder J, Ansaloni S, Schilling M, Liu T, Radke J, Jaedicke M, Schjeide BM, Mashychev A, Tegeler C, Radbruch H. MicroRNA-138 is a potential regulator of memory performance in humans. *FRONT HUM NEUROSCI* 2014;8:501

19. Tatro ET, Victoria R, Benchawanna S, Jared Y, Stephanie SA, Jeste DV, Achim CL. Short-term recognition memory correlates with regional CNS expression of microRNA-138 in mice. *American*

708 Journal of Geriatric Psychiatry Official Journal of the American Association for Geriatric Psychiatry
709 2013;21:461-473

710 20. Jankowsky JL, Fadale DJ, Jeffrey A, Xu GM, Victoria G, Jenkins NA, Copeland NG, Lee MK,
711 Younkin LH, Wagner SL. Mutant presenilins specifically elevate the levels of the 42 residue
712 beta-amyloid peptide in vivo: evidence for augmentation of a 42-specific gamma secretase. HUM
713 MOL GENET 2004;13:159-170

714 21. Bellucci M, Agostini F, Masin M, Tartaglia GG. Predicting protein associations with long
715 noncoding RNAs. NAT METHODS 2011;8:444-445

716 22. Pruitt KD, Tatiana T, Brown GR, Maglott DR. NCBI Reference Sequences (RefSeq): current
717 status, new features and genome annotation policy. NUCLEIC ACIDS RES 2012;40:D130-D135

718 23. Zhang Y, Zhang XO, Chen T, Xiang JF, Yin QF, Xing YH, Zhu S, Yang L, Chen LL. Circular
719 Intronic Long Noncoding RNAs. MOL CELL 2013;51:792-806

720 24. Kwek KY, Murphy S, Furger A, Thomas B, O'Gorman W, Kimura H, Proudfoot NJ, Akoulitchev
721 A. U1 snRNA associates with TFIIF and regulates transcriptional initiation. Nat Struct Biol
722 2002;9:800-805

723 25. Burenina OY, Oretskaya TS, Kubareva EA. Non-Coding RNAs As Transcriptional Regulators In
724 Eukaryotes. ACTA NATURAE 2017;9:13-25

725 26. Piwecka M, Glazar P, Hernandez-Miranda LR, Memczak S, Wolf SA, Rybak-Wolf A, Filipchuk
726 A, Klironomos F, Cerda JC, Fenske P, Trimbuch T, Zywicka V, Plass M, et al. Loss of a mammalian
727 circular RNA locus causes miRNA deregulation and affects brain function. SCIENCE 2017;357

728 27. Selkoe DJ. Resolving controversies on the path to Alzheimer's therapeutics. NAT MED
729 2011;17:1060-1065

730 28. Zhang S, Qin C, Cao G, Xin W, Feng C, Zhang W. Systematic Analysis of Long Noncoding
731 RNAs in the Senescence-accelerated Mouse Prone 8 Brain Using RNA Sequencing. *Molecular*
732 *Therapy Nucleic Acids* 2016;5:e343

733 29. Zhang S, Qin C, Cao G, Guo L, Feng C, Zhang W. Genome-wide analysis of DNA methylation
734 profiles in a senescence-accelerated mouse prone 8 brain using whole-genome bisulfite sequencing.
735 *BIOINFORMATICS* 2017;33:1591-1595

736 30. Marguerat S, Bähler J. RNA-seq: from technology to biology. *Cellular & Molecular Life Sciences*
737 2010;67:569-579

738 31. Xintian Y, Irena V, Ana B, Tristan W, Irina E, Georgi T, Güney A, Mantian W, Caspar G,
739 Claudia Q. Neural circular RNAs are derived from synaptic genes and regulated by development and
740 plasticity. *NAT NEUROSCI* 2015;18:603-610

741 32. Zhao Y, Alexandrov PN, Jaber V, Lukiw WJ. Deficiency in the Ubiquitin Conjugating Enzyme
742 UBE2A in Alzheimer's Disease (AD) is Linked to Deficits in a Natural Circular miRNA-7 Sponge
743 (circRNA; ciRS-7). *GENES-BASEL* 2016;7:116

744 33. Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Bente F, Damgaard CK, Jørgensen K. Natural
745 RNA circles function as efficient microRNA sponges. *NATURE* 2013;495:384-388

746 34. Guo JU, Vikram A, Huili G, Bartel DP. Expanded identification and characterization of
747 mammalian circular RNAs. *GENOME BIOL* 2014;15:409

748 35. Zhang Y, Zhang XO, Chen T, Xiang JF, Yin QF, Xing YH, Zhu S, Yang L, Chen LL. Circular
749 Intronic Long Noncoding RNAs. *MOL CELL* 2013;51:792-806

750 36. Boggon TJ, Shan WS, Santagata S, Myers SC, Shapiro L. Implication of tubby proteins as
751 transcription factors by structure-based functional analysis. *SCIENCE* 1999;286:2119-2125

37. Banerjee P, Kleyn PW, Knowles JA, Lewis CA, Ross BM, Parano E, Kovats SG, Lee JJ, Penchaszadeh GK, Ott J. TULP1 mutation in two extended Dominican kindreds with autosomal recessive retinitis pigmentosa. NAT GENET 1998;18:177
38. Gu S, Lennon A, Li Y, Lorenz B, Fossarello M, North M, Gal A, Wright A. Tubby-like protein-1 mutations in autosomal recessive retinitis pigmentosa. LANCET 1998;351:1103-1104
39. Ikeda S, Shiva N, Ikeda A, Smith RS, Nusinowitz S, Yan G, Lin TR, Chu S, Heckenlively JR, North MA. Retinal degeneration but not obesity is observed in null mutants of the tubby-like protein 1 gene. HUM MOL GENET 2000;9:155-163
40. Ikeda A, Ikeda S, Gridley T, Nishina PM, Naggert JK. Neural tube defects and neuroepithelial cell death in Tulp3 knockout mice. HUM MOL GENET 2001;10:1325-1334
41. Patterson VL, Christine D, Anju P, Benjamin R, Grimes DT, Stewart ME, Williams DJ, Pam S, Andy G, Murdoch JN. Mouse hitchhiker mutants have spina bifida, dorso-ventral patterning defects and polydactyly: identification of Tulp3 as a novel negative regulator of the Sonic hedgehog pathway. HUM MOL GENET 2009;18:1719-1739
42. Hou J, Lin L, Zhou W, Wang Z, Ding G, Dong Q, Qin L, Wu X, Zheng Y, Yang Y, Tian W, Zhang Q, Wang C, et al. Identification of miRNomes in human liver and hepatocellular carcinoma reveals miR-199a/b-3p as therapeutic target for hepatocellular carcinoma. CANCER CELL 2011;19:232-243
43. Xing Z, Lin A, Li C, Liang K, Wang S, Liu Y, Park PK, Qin L, Wei Y, Hawke DH. lncRNA Directs Cooperative Epigenetic Regulation Downstream of Chemokine Signals. CELL 2014;159:1110-1125
44. Yuan SX, Wang J, Yang F, Tao QF, Zhang J, Wang LL, Yang Y, Liu H, Wang ZG, Xu QG. Long

774 noncoding RNA DANCR increases stemness features of hepatocellular carcinoma by derepression of

775 CTNNB1. HEPATOLOGY 2016;63:499-511

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Table S1. Oligos used in the study

779

Oligo name	Sequence (5' - 3')	purpose
CircTulp4_Div-F	CGCAGAGATAGGAGTACACCA	Divergent primers for Real-time PCR and RT-PCR detection of circTulp4
CircTulp4_Div-R	CCGGTTAATTCAGGAGCCATC	
CircTulp4_Div-F	CGCAGAGATAGGAGTACACCA	RT-PCR detection of circTulp4
CircTulp4_Div-R	TTCTCCACAGACTCCCAACC	Sequencing primer
CircTulp4_con-F	TCCCACTATTAAGCCCCCGA	convergent primers for RT-PCR detection of circEIF3J
CircTulp4_con-R	TGGAGTCTGAGCAGTTGCAG	
mGAPDH_con-F	AACTTTGGCATTGTGGAAGG	convergent primers for RT-PCR and real-time PCR detection of GAPDH
mGAPDH_con-R	GGATGCAGGGATGATGTTCT	
CDR1as-F	AACTACCCAGTCTTCCATCA	Real-time PCR and RT-PCR for CDR1as
CDR1as-R	AGACTTGAAGTCGCTGGAAG	
U1-F	CTCGCTTCGGCAGCACATAT	Real-time PCR and RT-PCR for U1 RNA
U1-R	ACACGCAAATTCGTGAAGCG	
U2-F	CTCGGCCTTTTGGCTAAGAT	Real-time PCR and RT-PCR for U2 RNA
U2-R	TATTCCATCTCCCTGCTCCA	
U4-F	TGGCAGTATCGTAGCCAATG	Real-time PCR and RT-PCR for U4 RNA
U4-R	CTGTCAAAAATTGCCAGTGC	

U5-F	TGGTTTCTCTTCAGATCGCATA	Real-time PCR and RT-PCR for U5 RNA
U5-R	CAAAGCAAGGCCTCAAAAA	
U6-F	ACTCTTACCTCGGCAGGGGAG	Real-time PCR and RT-PCR for U6 RNA
U6-R	GGAAAGCGCGAACGCAGTCC	
U7-F	CAGTGTTACAGCTCTTTTAG	Real-time PCR and RT-PCR for U7 RNA
U7-R	AGGGGCTTTCCGGTAAAAAG	
5s rRNA-F	CCTACAGCACCCGGTATTC	Real-time PCR and RT-PCR for 5s rRNA
5s rRNA-R	CTACGGCCATACCACCCTG	
7SK-F	TGATCGCCAGGGTTGATT	Real-time PCR for 7SK RNA
7SK-R	GGGATGGTCGTCCTCTTC	
promoter-Tulp4-A F	TGACTCAAGGCCACAGAACT	Real-time PCR and RT-PCR for promoter of Tulp4 gene
promoter-Tulp4-A R	ACTGTGCTTGTGAGTCTCCA	
promoter-Tulp4-B F	TGTGAGGCATGCTGTGTTTC	Real-time PCR and RT-PCR for promoter of Tulp4 gene
promoter-Tulp4-B R	TCAGGAGATGGCAAAGGAGC	
promoter-Tulp4-C F	TTTTAGGGTCAGGCAGTGGT	Real-time PCR and RT-PCR for promoter of Tulp4 gene
promoter-Tulp4-C	GGCACAGTGGGACCTAAGTA	

R		
promoter-Tulp4-D F	CCCAATGAGACCCAAGCTGA	Real-time PCR and RT-PCR for promoter of Tulp4 gene
promoter-Tulp4-D R	TTCTATGCCTCACCCCACTC	
promoter-Tulp4-E F	ACTGGTTAGGCATGGTGGTT	Real-time PCR and RT-PCR for promoter of Tulp4 gene
promoter-Tulp4-E R	CGTAAGCTGGCCTCAAACCTC	
promoter-Tulp4-F F	ACCGCCTAAGAAGACACTTGA	Real-time PCR and RT-PCR for promoter of Tulp4 gene
promoter-Tulp4-F R	GTCACACCGTCTCTTACCCA	
CircTulp4 siRNA-1	CCACAACAGTGAGAGTTGT	CircTulp4 siRNA
CircTulp4 siRNA-2	ACAACAGTGAGAGTTGTAA	CircTulp4 siRNA
CircTulp4 siRNA-3	AACAGTGAGAGTTGTAAAGA	CircTulp4 siRNA
CircTulp4-siRNA control	RiboTM product number : siN0000001-1-5	CircTulp4-siRNA control
si-mRNA-Tulp4	CTGCCTTCATTCCCACTAT	Tulp4 siRNA
Tulp4- siRNA control	RiboTM product number : siN0000001-1-5	Tulp4- siRNA control

Bio-circTulp4	AACTTCAACCTGCGAGGCCACAACAG TGAGAGTTGTAAGAGTCCATCCAGGA CCTTCCAG	5'-biotin-labeled oligonucleotide for CircTulp4 pull-down
Bio-circTulp4-control(LacZ, E.coli)	CCAATCCGCGCCGGATGCGGTGTATC GCTCGCCACTTCAACATCAACGGTAA TCGCCATT	As control of circTulp4 pull-down
Bio-circTulp4-control(LacZ, E.coli)	CAAACGGCGGATTGACCGTAATGGGA TAGGTCACGTTGGTGTAGATGGGCGC ATCGTAAC	As control of circTulp4 pull-down
Bio-circTulp4-control(LacZ, E.coli)	CACCACATACAGGCCGTAGCGGTCGC ACAGCGTGTACCACAGCGGATGGTTC GGATAATG	As control of circTulp4 pull-down
CircTulp4_junction probe_FISH	RiboTM Sales order number: S180926133440 Product number: lnc1100177	For circTulp4 FISH probe preparation
18S Probe FISH	RiboTM Sales order number: S180926133723 Product number: lnc110104	For 18S FISH probe preparation
BaseScope™ CircTulp4 Target Probe	product number : 700001-C2	For circTulp4 ISH 1ZZ probe preparation
BaseScope™ Duplex	product number : 700141	For circTulp4 ISH negative Control

Negative Control		Probe
Probe - DapB-1ZZ		

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