Epigenetic repression of Wnt receptors in AD: a role for Sirtuin2-induced H4K16ac deacetylation of Frizzled1 and Frizzled7 promoters

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INTRODUCTION

Alzheimer’s disease (AD) is the most common form of dementia, clinically characterised by progressive cognitive impairment and memory loss. One of the early events in AD is the loss of synapses, a process strongly correlated with cognitive decline [1, 2]. Interestingly, several signalling pathways required for synapse function and integrity are dysregulated in AD [3, 4]. Of particular interest is the Wnt signalling pathway(s). First, the secreted Wnt antagonist DKK1 is elevated both in the brain of AD patients and models [5]. DKK1 is a key component of the Wnt signalling pathway(s). First, the secreted Wnt antagonist DKK1 is elevated both in the brain of AD patients and models [5]. DKK1 is a key component of the Wnt signalling pathway(s). Second, LRP6 Wnt co-receptor is required for synapse integrity and three variants of this receptor are linked to late-onset AD [6]. Second, LRP6 Wnt co-receptor is required for synapse integrity and three variants of this receptor are linked to late-onset AD [6]. Of particular interest is the Wnt signalling pathway(s). First, the Wnt antagonist DKK1 is elevated both in the brain of AD patients and models [5]. DKK1 is a key component of the Wnt signalling pathway(s). Second, LRP6 Wnt co-receptor is required for synapse integrity and three variants of this receptor are linked to late-onset AD [6]. However, little is known about how these Fzd receptors are regulated in AD.

Growing evidence supports a role for deficient Wnt signalling in Alzheimer’s disease (AD). First, the Wnt antagonist DKK1 is elevated in AD brains and is required for amyloid-β-induced synapse loss. Second, LRP6 Wnt co-receptor is required for synapse integrity and three variants of this receptor are linked to late-onset AD. However, the expression/role of other Wnt signalling components remain poorly explored in AD. Wnt receptors Frizzled1 (Fzd1), Fzd5, Fzd7 and Fzd9 are of interest due to their role in synapse formation/plasticity. Our analyses showed reduced FZD1 and FZD7 mRNA levels in the hippocampus of human early AD stages and in the hAPPNLGF/NLGF mouse model. This transcriptional downregulation was accompanied by reduced levels of the pro-transcriptional histone mark H4K16ac and a concomitant increase of its deacetylase Sirt2 at Fzd1 and Fzd7 promoters in AD. In vitro and in vivo inhibition of Sirt2 rescued Fzd1 and Fzd7 mRNA expression and H4K16ac levels at their promoters. In addition, we showed that Sirt2 recruitment to Fzd1 and Fzd7 promoters is dependent on FoxO1 activity in AD, thus acting as a co-repressor. Finally, we found reduced levels of SIRT2 inhibitory phosphorylation in nuclear samples from human early AD stages with a concomitant increase in the SIRT2 phosphatase PP2C. This results in hyperactive nuclear Sirt2 and favours Fzd1 and Fzd7 repression in AD. Collectively, our findings define a novel role for nuclear hyperactivated SIRT2 in repressing Fzd1 and Fzd7 expression via H4K16ac deacetylation in AD. We propose SIRT2 as an attractive target to ameliorate AD pathology.

MATERIAL AND METHODS

Human tissue

Anonymised human samples were obtained from the Cambridge Brain Bank (CBB) and the Queen Square Brain Bank (QSBB), with informed consent from donors and approval from appropriate local ethics committees. Anonymised Human Tissue (AHT) approval from the Hammersmith Hospital Research Ethics Committee (15/H0715/01) was granted to perform the transcriptional and epigenetic analyses. AD patients were recruited at the University of Cambridge Neurodegenerative Disease Research Group (CamNDRG) AD centres. AHT approval was also obtained to perform DNA methylation analysis of post-mortem brain samples from the Cambridge Biomedical Research Centre (CBRC) dementia cohort. AD and control diagnoses were confirmed and validated by a neuropathologist using National Institute on Aging and Alzheimer’s Association criteria for AD and non-demented controls. AD patients were matched for age and sex, and all control brains were confirmed to have no evidence of neurodegeneration.

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Animals

All procedures involving animals were conducted according to the Animals Scientific Procedures Act UK (1986) and in compliance with the ethical standards at University College London. Further information can be found in supplementary methods.

Fig. 1  *Frizzled 1* and *Frizzled 7* are downregulated in early AD. A Scheme representing a synapse showing the localisation at pre- and/or post-synaptic sides for Fzd1, Fzd5, Fzd7 and Fzd9. B qPCR analyses showing reduced mRNA levels of FZD1 and FZD7 in human hippocampal samples from Braak stages I-III (BI-III) subjects compared to controls. No changes are observed for FZD5 and FZD9 in early AD. C qPCR analysis shows the reduced mRNA levels of Fzd1 and Fzd7 in 2-month-old hAPPNLGF/NLGF hippocampus (NLGF). No differences in Fzd5 and Fzd9 levels are observed in NLGF hippocampal mRNA. D Representative smFISH images of WT and NLGF CA1 hippocampal region. First column shows merged images with DAPI (blue), Fzd1 (green), Fzd7 (yellow) and Rbfox3 (magenta) mRNAs. Fzd1 in black (second column) and its representative neuronal Rbfox3+ cells corresponding to 3 and >3 Fzd1 copies (third column). Fzd7 in black (fourth column) and its representative cells corresponding to one Fzd7 copy (fifth column). Scale bars represent 50 µm and 12.5 µm in the zoomed inserts. Single-cell analyses expressed as H-score for Fzd1 (E) and Fzd7 (F) in neuronal (Rbfox3+) and non-neuronal (Rbfox3-) cells. Single-cell distribution of neurons (Rbfox3+) containing 1, 2, 3 or >3 transcripts for Fzd1 (G) or Fzd7 (H). Data are represented as mean ± SEM. Statistical analysis by t Test in B for FZD1, FZD7 and FZD9 and by Mann-Whitney for FZD5; in C t-Test for all genes analysed; in E and F t-Test for neuronal and non-neuronal; in G t-Test for 1, 2 and 3 copies and by Mann-Whitney for >3 copies; in H t-Test for 1 and 2 copies and by Mann-Whitney for 3 and >3 copies. N is indicated in each bar by the number of symbols. Asterisks indicate *p < 0.05; **p < 0.01, ***p < 0.005.

Statistical analysis

All values are presented as mean ± SEM. Statistical analyses were performed using SPSS v25 (IBM). Outliers were determined with the explore tool (Tukey’s method). Data normality and homogeneity of variances were tested by the Shapiro-Wilk and Levene tests, respectively. Mann–Whitney U test (two groups) or Kruskal–Wallis followed by Dunn’s multiple comparison (more than two groups) tests were used for non-normally distributed datasets. For normally distributed data; one-sample t test (two groups with control values equal one), Student’s t test (two...
RESULTS

Frazzled1 and Frazzled7 expression is downregulated in AD

Deficient Wnt signalling has been linked to AD by studies on the Wnt antagonist DKK1 and LRPs6 genetic variants [21]. In addition, Wnt ligands have been shown to be protective against Aβ insult [21]. However, very little is known about the regulation of Frazzled receptors (Fzd) in AD. Four Fzd receptors (Fzd1, Fzd5, Fzd7 and Fzd9) have been shown to regulate synapse formation and/or function [17-20] (Fig. 1A). We, therefore, evaluated the expression levels of these receptors. We performed RT-qPCR on human hippocampal samples from control and from subjects with early Braak stages but no cognitive deficits (BI-III; Table S1). We found reduced Fzd1 and Fzd7 mRNA levels in BI-II samples (Fig. 1B). In contrast, Fzd5 and Fzd9 mRNA levels were unchanged (Fig. 1B). These results suggest that two Fzds with synaptic function are downregulated in early stages of AD.

Next, we investigated whether the mRNA levels of these Fzds were also affected in an AD model. We used the knock-in AD line hAPP/NGF-NLGF (NLGF), which carries the humanised form of APP with the Swedish, Iberian and Arctic mutations, leading to Aβ overproduction [22]. We analysed Fzd expression in hippocampal samples of NLGF animals at 2-months-old, an age when Aβ plaques start to appear [22]. Our results showed reduced levels of Fzd1 and Fzd7 expression in NLGF samples, whereas Fzd5 and Fzd9 remained unchanged (Fig. 1C). Together, these results demonstrate that Fzd1 and Fzd7 expression were reduced in both human BI-III subjects and the AD mouse model at an early disease stage.

Fzds are expressed by different brain cells, including neurons, astrocytes and microglia [23, 24] (Fig. S1A). We therefore asked whether reduced mRNA levels of Fzd1 and Fzd7 were neuronal specific. We performed single molecule RNA fluorescent in-situ hybridisation (smFISH) for Fzd1 and Fzd7 in the CA1 area of the hippocampus (Fig. 1D). Single-cell analyses revealed reduced Fzd1 levels in NLGF neuronal cells (Rbfox3-), without changes in non-neuronal cells (Rbfox3+: Fig. 1E) when compared to control animals. However, no changes in the overall levels of Fzd7 were observed (Fig. 1F). Next, we analysed the distribution of transcript copy number in neuronal cells. We found that neurons containing ≥3 Fzd1 transcripts were reduced in AD (Fig. 1G). Interestingly, we observed a reduced number of neurons containing one Fzd7 transcript in the NLGF (Fig. 1H). The lack of difference in H-score for Fzd7 could be explained by the lower weighting for percentage of cells with 1 copy (see methods). Together, our results demonstrate that Fzd1 and Fzd7 RNA levels are reduced in both human BI-III and AD mouse model, with a clear downregulation of neuronal Fzd1 expression and reduced number of neurons containing one Fzd7 transcript in the AD mouse hippocampus.

Fzd1 and Fzd7 promoters present reduced H4K16ac levels with concomitant increase of Sirt2 in AD

The reduced levels of Fzd1 and Fzd7 expression in the human brain at early AD stages led us to hypothesise that a shared epigenetic regulation could contribute to their dysregulation. A previous study showed that the pro-transcriptional histone mark acetylated Histone H4 Lysine 16 (H4K16ac) is enriched at promoters of several Wnt signalling pathway components [25]. Chromatin immunoprecipitation (ChIP)-qPCR showed high levels of H4K16ac, and concomitant low levels of total H4, at actively transcribed genes Actb and Eif5 (Fig. S1B, C), which have high levels of H4K16ac in the human brain [26]. In contrast, the repressed genes Hoxa1 and Krt16 exhibited low levels of H4K16ac and high levels of H4 (Fig. S1B, C) [26]. Higher levels of H4K16ac were found at Fzd1 and Fzd7 promoters than at Fzd5 and Fzd9 promoters (Fig. S1B, C), suggesting that H4K16ac is enriched at the Fzd1 and Fzd7 promoters and might contribute to their regulation.

Next, we analysed H4K16ac levels in human hippocampal samples. First, we found that H4K16ac levels were not altered by the post-mortem interval time (Fig. S1D). ChIP-qPCR experiments showed that H4K16ac was reduced at Fzd1 and Fzd7 promoters in BI-III (Fig. 2A, B), whereas no changes were observed at the promoters of our internal controls Fzd5 and Fzd9 (Fig. 2A) or external controls genes Actb, Eif5, Hoxa1 or Krt16 (Fig. S1E), collectively referred here as control genes. Reduced H4K16ac levels at Fzd1 and Fzd7 promoters were also observed in the NLGF hippocampus (Figs. 2C, S1F). Changes in H4K16ac levels could arise from nucleosome remodelling or from differential levels of H4K16ac per se. We found no changes in nucleosome remodelling when analysed by H4 total levels, thus changes of H4K16ac at Fzd1 and Fzd7 promoters were likely due to reduced H4K16ac levels (Fig. S1G, H). Together, these results show reduced H4K16ac levels, which could contribute to Fzd1 and Fzd7 repression in early AD.

Finally, we interrogated which of the three H4K16ac deacetylases (Histone Deacetylases 2 (HDAC2), Sirt1 or Sirt2 [27-29]) could be involved in regulating Fzd1 and Fzd7 in AD. Interestingly, HDAC2 and Sirt2 play a neurodegenerative role, whereas Sirt1 is neuroprotective [30, 31]. Therefore, we analysed Hdac2 and Sirt2 occupancy at Fzd promoters. First, we found that Hdac2 or Sirt2 were not enriched at Fzd promoters in WT (Fig. S1J). Interestingly, ChIP-qPCR experiments showed increased Sirt2 occupancy only at Fzd1 and Fzd7 promoters in the hippocampus of AD mice (Figs. 2D, S1K). In contrast, no changes were found for Hdac2 levels across all the genes analysed (Fig. S1L). These results show that reduced expression of Fzd1 and Fzd7 correlates with reduced levels of H4K16ac and with a concomitant increase of its histone deacetylase Sirt2 at their promoters.

Nuclear Sirt2 is sufficient to downregulate expression of Fzds

To study the possible role of Sirt2 in regulating Fzds, we overexpressed human Sirt2 in primary neuronal cultures and evaluated Fzd mRNA levels. Our results showed that increased Sirt2 expression downregulated Fzd1 and Fzd7 expression in neurons, without affecting Fzd5 and leading to increased Fzd9 expression (Fig. 2E). Intriguingly, Sirt2 is known to be cytosolic in HEK cells [29] (Fig. S2A), whereas we observed a nuclear effect of Sirt2. Interestingly, immunostaining experiments showed that 34.51% of Sirt2 is found in the nucleus in neurons (Fig. 2F, G). In addition, 30-42% of Sirt2 is found in nuclear fractions of human hippocampal samples (Figs. 2H, S2D), suggesting that Sirt2 nuclear localisation could be different in postmitotic cells compared to HEK. To drive Sirt2 nuclear translocation, we incorporated a nuclear localisation signal to the Sirt2 N-terminus (NL-Sirt2; Fig. S2B, C) and studied its impact on Fzds expression. We found that NL5-Sirt2 downregulated Fzd1 and Fzd7 expression to the same levels of WT Sirt2 in neuronal cultures (Fig. 2E). These results suggest that nuclear Sirt2 is sufficient to downregulate Fzd1 and Fzd7 and that Sirt2 nuclear localisation is cell-type dependent.

Sirt2 inhibition prevents synapse loss and rescues Fzds epigenome and transcription in AD

To test if Sirt2 is required for Fzd1 and Fzd7 downregulation in AD, we established an AD cellular model: hippocampal primary neuronal cultures were cultured for 15DIV and treated overnight with Aβ0 (Figs. 3A, S2E), leading to Fzd1 and Fzd7 reduced expression, reduced H4K16ac and increased Sirt2 levels at their promoters and also synapse loss, without modulating total Sirt2 mRNA or protein levels (Fig. S2F-K). Next, we studied whether Sirt2 inhibition could prevent Fzd downregulation and synapse loss. We used a non-toxic concentration of the specific Sirt2 inhibitor AGK2 (Fig. S2L)
that leads to increased acetylation of the Sirt2 substrate H3K18ac (Fig. S2M). We found that Sirt2 inhibition indeed prevented Fzd1 and Fzd7 downregulation and Aβ-induced synapse loss in our AD cellular model (Fig. 3B, C). These results suggest that Sirt2 is required for Fzd1 and Fzd7 downregulation and synapse loss upon Aβ insult in neurons.

To further study the role of Sirt2 in regulating Fzds expression in the context of AD, we prepared hippocampal organotypic cultures (HOC) from WT and NLGF animals. Consistent with our results in BI–III and NLGF mice, we found reduced H4K16ac and increased Sirt2 levels at Fzd1 and Fzd7 promoters and a concomitant reduction in their transcription in NLGF-HOC (Fig. S3A–D). Next, we studied whether Sirt2 inhibition could rescue Fzds expression in our HOC AD model. First, the Sirt2 specific inhibitor AGK2 showed no toxicity (Figs. 3D, S3E, Table S2) and effectively suppressed Sirt2 activity as shown by increased acetylation of the Sirt2 substrate H3K56ac (Fig. S3F). Indeed, Sirt2 inhibition by AGK2 rescued Fzd1 and Fzd7 expression in the NLGF-HOC, without affecting control genes (Fig.3E, F). Second, we treated our HOC model with a second specific and structurally distinct Sirt2 inhibitor; AK7 (Table S2) (32). AK7 treatment was not toxic and effectively suppressed Sirt2 activity (Figs. 3D, S3E, S3G). Importantly, Sirt2 inhibition by AK7 rescued Fzd1 and Fzd7 mRNA levels in the NLGF-HOC model, without modulating control genes (Fig. 3G). Thus, Sirt2 inhibition by two distinct small molecules suggests that Sirt2 represses Fzd1 and Fzd7 in the context of AD. Finally, we analysed whether AK7 treatment also rescued...

Fig. 2 Downregulation of Fzds in AD correlates with reduced levels of H4K16ac and the concomitant increase in Sirt2 at their promoters. A ChIP-qPCR analyses of H4K16ac at the promoters of FZD1, FZD7, FZD5 and FZD9 in Human Control and Braak I-III subjects (BI–III) showing reduced acetylation levels at FZD1 and FZD7 promoters in AD. H4K16ac levels remain unchanged at FZD5 and FZD9 promoters. B Scheme representing the epigenetic changes observed in AD, where FZD1 and FZD7 promoters present reduced levels of H4K16ac and increased levels of the histone deacetylase SIRT2. C ChIP-qPCR experiments showed reduced H4K16ac at Fzd1 and Fzd7 promoters in NLGF hippocampal samples. No changes are observed for Fzd5 or Fzd9. D ChIP-qPCR analyses of Sirt2 at the promoters of Fzd1, Fzd7, Fzd5 and Fzd9 in WT and NLGF hippocampal samples showing increased Sirt2 levels at Fzd1 and Fzd7 promoters in AD. No differences are observed at Fzd5 or Fzd9 promoters. E qPCR analysis showing reduced mRNA levels of Fzd1 and Fzd7 in neuronal cultures overexpressing WT SIRT2 or NLS-SIRT2. No changes are observed for Fzd5. However, WT SIRT2 induced Fzd9 transcription. Quantification (F) and representative images (G) showing Sirt2 is found in the nucleus of postmitotic neurons. In G the first column shows merged images with DAPI (blue), GFP (green) and Sirt2 (white), second column shows GFP, third column shows Sirt2 (white) and last column shows DAPI (blue). H Quantification of cytosolic and nuclear SIRT2 and representative WB showing that 30-42% of SIRT2 is found in the nucleus in human brain. Data are represented as mean ± SEM. Statistical analyses by t-Test in A for FZD1, FZD7 and by Mann-Whitney for FZD5 and FZD9; in C t-Test for Fzd1, Fzd5 and Fzd9 and by Mann-Whitney for Fzd7; in D t-Test for all genes; in E one-way ANOVA followed by Games–Howell multiple comparison for all genes. N is indicated in each bar by the number of symbols. Asterisks indicate *p < 0.05; **p < 0.01, ***p < 0.005.
Indeed, we found that the levels of this pro-
transcriptional histone mark were restored at 
$Fzd1$ and $Fzd7$ promoters in the NLGF-HOC treated cultures (Figs. 3H, S3H).

Interestingly, Sirt2 has other histone substrates, including H3K18ac and H3K56ac [33]. However, we found that these two marks were not enriched at $Fzd1$ or $Fzd7$ promoters in WT (Fig. S3I, J), and no

differences were observed in hippocampus of the NLGF model compared to control (Fig. S3K, L). These results suggest that Sirt2 impedes $Fzd1$ and $Fzd7$ transcription by specifically reducing H4K16ac levels in their promoters in the AD context.

Finally, we tested the role of Sirt2 in regulating $Fzd1$ and $Fzd7$ transcription in vivo by using the Sirt2 inhibitor AK7, which crosses...
the blood-brain barrier [34]. Mice were injected intraperitoneally with 20 mg/kg twice a day for 15 days (Fig. 3I), as previously reported [35]. AK7 administration effectively inhibited Sirt2 in the brain (Fig. S3M), rescuing Fzd1 and Fzd7 expression (Fig. 3J) and H4K16ac levels at their promoters (Fig. 3H) in NLGF animals. Similar to our in vitro studies, AK7 did not modulate the mRNA levels of control genes or the levels of H4K16ac at their promoters (Figs. 3K, S3N). Interestingly, we found no changes in H3B2 levels (Fig. S3O), as previously reported with the same AK7 dosage in AD. To test this hypothesis, we treated HOC with the specific AK7, Fzd7, and Fzd1 FoxO1 levels at Fzd7 and Fzd7 promoters in AD while not changes are observed in WT or Fzd5 and Fzd9 promoters. Data are represented as mean ± SEM. Statistical analyses by Two-way ANOVA followed by Games-Howell post hoc in B for all genes analysed; in C by Kruskal-Wallis followed by Dunn’s multiple comparison; in E by Two-way ANOVA followed by Tukey’s post hoc for Fzd1, Fzd5 and Fzd7 and by Kruskal-Wallis followed by Dunn’s multiple comparison for Fzd9; in G Two-way ANOVA followed by Tukey’s post hoc for Fzd1, Fzd7 and Fzd9 and by Kruskal-Wallis followed by Dunn’s multiple comparison for Fzd9; in H Kruskal-Wallis followed by Dunn’s multiple comparison for all Fzd1, Fzd5 and Fzd7 and by Two-way ANOVA followed by Tukey’s post hoc for Fzd9; in G Two-way ANOVA followed by Tukey’s post hoc for Fzd1 and Fzd9 and Kruskal-Wallis followed by Dunn’s multiple comparison for Fzd9; in J Two-way ANOVA followed by Tukey’s post hoc for Fzd1, Fzd7 and Fzd9; and in K Two-way ANOVA followed by Tukey’s post hoc for Fzd1, Fzd7 and Fzd9. N is indicated in each bar by the number of symbols. Asterisks indicate *p < 0.05; **p < 0.01; ***p < 0.005.

FoxO1 recruits Sirt2 to Fzd1 and Fzd7 promoters in AD

Increased Sirt2 occupancy at Fzd1 and Fzd7 promoters suggest that Sirt2 levels might be upregulated in AD. To test this hypothesis, we analysed Sirt2 mRNA and protein levels in BI-III human hippocampal samples and found no changes (Fig. S4A, B). Similarly, no changes in Sirt2 protein levels were observed in NLGF-HOC model (Fig. S4C), but we observed reduced Sirt2 mRNA levels in NLGF-HOC (Fig. S4D). In addition, no differences in Sirt2 nuclear levels were observed in BI-III, but we found increased nuclear Sirt2 in NLGF-HOC (Figs. 4A, 4E, F). These results indicate that increased Sirt2 occupancy at Fzd1 and Fzd7 promoters do not correlate with increased total/nuclear levels of Sirt2 in the human BI-III, suggesting that Sirt2 might be recruited to Fzd promoters by co-factors.

Sirt2 interacts with FoxO1 and FoxO3a transcription factors [36, 37], which could recruit Sirt2 to specific loci. Using ChIP-DEER [38], we found putative FoxO1, but not FoxO3a, binding sites at Fzd1 and Fzd7 promoters (Fig. S4G). Next, we analysed FoxO1 occupancy at Fzd1 and Fzd7 promoters in AD. We found increased FoxO1 levels at Fzd1 and Fzd7 promoters in NLGF hippocampal samples (Figs. 4B, S4H), suggesting that FoxO1 could contribute to the recruitment of Sirt2 to Fzd1 and Fzd7 promoters in the context of AD. To test this hypothesis, we treated HOC with the specific FoxO1 activity inhibitor AS1842856 (FoxO1I, Fig. 4C, Table S2) [39], and found no cytotoxicity (Fig. S4I). We next analysed FoxO1 occupancy upon FoxO1 inhibition and found reduced Sirt2 levels at Fzd1 and Fzd7 promoters in the context of AD (Fig. 4D, E). No changes were observed at control gene promoters (Figs. 4E, S4J). However, we found reduced Sirt2 levels at Hoxa1 promoter, which has three putative FoxO1 binding sites (Fig. S4G, S4I). These results suggest that Sirt2 recruitment to Fzd1 and Fzd7 promoters in AD depends on FoxO1 binding activity.

To further test the role of FoxO1 in repressing Fzd1 and Fzd7 in the context AD, we treated our cellular AD model with a non-toxic concentration of FoxO1 inhibitor (Fig. S4K), which indeed prevented Aβ-induced Fzd1 downregulation (Fig. 4F). No changes were observed for control genes (Fig. 4F). However, FoxO1 inhibition downregulated Fzd7 expression in WT samples and consequently failed to prevent Fzd7 downregulation in the context of AD (Fig. 4F).

Interestingly, Fzd7 was the only Fzd receptor that displayed high FoxO1 occupancy in WT (Fig. S4L). Furthermore, FoxO1 inhibition in WT-HOC led to reduced H4K16ac levels at Fzd7 promoter (Fig. S4M). Together these results suggest that FoxO1 activity is required for Fzd7 basal expression.

In the context AD, we treated neurons with the Sirt2 inhibitor AGK2 together with the FoxO1 inhibitor. Our results showed that inhibition of these two proteins prevented Fzd1 downregulation in the context of AD (Fig. S4N) with no changes in control genes (Fig. S4N). In contrast, this double inhibition led to Fzd7 downregulation as we observed with inhibition of FoxO1 alone (Fig. S4N). Altogether, these results suggest that FoxO1 recruits Sirt2 to Fzd1 and Fzd7 promoters leading to their downregulation in the context of AD. The difference in the response to the FoxO1 inhibition between Fzd1 and Fzd7 might reflect difference in the regulation under basal conditions.

Increased nuclear Sirt2 activity represses Fzd1 and Fzd7 in AD

Sirt2 activity can be modulated by phosphorylation. We, therefore, analysed the phosphorylation of Sirt2 at its Serine 331 (pSirt2), which inhibits its activity [40, 41]. We found reduced pSirt2 levels in nuclear fractions of BI-III hippocampal samples (Fig. 4A), without changes in total pSirt2 levels (Fig. S4A). Similarly, lower levels of nuclear and total pSirt2 were observed in NLGF-HOC (Fig. S4C, S4F). Decreased levels of Sirt2 inhibitory phosphorylation could be regulated by specific phosphatases, such as the Sirt2 phosphatase PP2Ca [41], which is upregulated at the RNA level in an AD model [42]. We, therefore, analysed the expression of the Sirt2 phosphatases PP2Ca/β [41] and found no changes in the mRNA or total protein levels in hippocampal samples from human BI-III or NLGF-HOC (Fig. S5A–F). Next, we analysed nuclear localisation and found increased levels of PP2Ca, but not PP2CB, in BI-III subjects (Figs. 4G, S5G). Importantly, Pp2ca...
was also upregulated in nuclear samples of NLGF-HOC (Fig. S5H). These results suggest that increased nuclear levels of PP2Cα could lead to SIRT2 nuclear hyperactivity, favouring the repression of Fzd1 and Fzd7 in AD.

To establish the role of PP2C in Fzd1 and Fzd7 expression in AD, HOC were treated with a non-toxic concentration of sanguinarine (SAN), a PP2C specific inhibitor [43] (Fig. 4D, S4I, Table S2) and we analysed the impact of SAN on the expression of Fzd1 and Fzd7. Our results showed that Pp2c inhibition rescued Fzd1 and Fzd7 expression and H4K16ac levels in the NLGF-HOCs, without affecting the expression of control genes (Figs. 4H–J, S5I). Consistently, increased pSirt2 levels were only found in NLGF-HOC SAN-treated...
Fig. 4 Increased Sirt2 activity in AD impairs Fzd5s transcription. A WB analyses of total and pSIRT2 levels in hippocampal nuclear extracts of human control/BIII subjects showing decreased levels of pSIRT2.2 at early AD. B ChiP-qPCR analyses of FoxO1 in WT and NLGF hippocampal samples showing increased FoxO1 levels at Fzd1 and Fzd7 promoters in AD. No differences are observed at Fzd5 or Fzd9 promoters. C Scheme representing Sanguinarine (SAN) and AS1842856 FoxO1 inhibitor (Fox1Oi) treatment in the in vitro AD organotypic model for 7 days and 72 h respectively. D Scheme representing the levels of Sirt2 at Fzd1 and Fzd7 and upon FoxO1 inhibition in AD. E ChiP-qPCR showing that FoxO1 inhibition reduces Sirt2 levels at Fzd1 and Fzd7 promoters in hippocampal organotypic cultures of NLGF while not changing the levels of Sirt2 in WT or at Fzd5 or Fzd9 promoter. F FoxO1 recruits Sirt2 to Fzd1 and Fzd7 promoters in AD. G qPCR analyses of Fzds expression upon FoxO1 inhibition in vehicle (Veh) and Ajo treated neurons, showing that FoxO1i prevents Fzd1 downregulation without modulating Fzd5 or Fzd9 mRNA levels. FoxO1 inhibition downregulates Fzd7 expression per se and fails to prevent its downregulation in Ajo treated neurons. H WB analyses of PP2C2 in hippocampal nuclear extracts of human control/BIII subjects, sowing increased levels of PP2C2 in human BI–III group. I qPCR analyses of total mRNA levels from WT and NLGF hippocampal organotypic cultures treated with vehicle or SAN. Our results show that SAN treatment rescues Fzd1 and Fzd7 mRNA levels and does not show any effect on Fzd5 or Fzd9 mRNA levels. J Scheme representing increased nuclear levels of control/BIII subjects, sowing increased levels of PP2C2 in human BI–III group. K qPCR analyses of total mRNA levels from WT and NLGF hippocampal organotypic cultures treated with vehicle or SAN. Our results show that SAN treatment rescues Fzd1 and Fzd7 mRNA levels and does not show any effect on Fzd5 or Fzd9 mRNA levels.
Our results are in line with a previous report showing no changes in Aβ levels in two AD models treated with the same AK7 regime [35]. These apparently contradictory results suggest that shorter Sirt2 levels at Fzd1 and Fzd7 promoters suggest that Sirt2 is specifically recruited by a co-factor with DNA binding capacity. Interestingly, Sirt2 interacts with the transcription factor FoxO1 [36], which has predicted binding sites at Fzd1 and Fzd7 promoter region. FoxO1 can positively or negatively regulate gene transcription in different biological conditions [60]. We found that FoxO1 is enriched at Fzd7 and is required for its basal transcription in neurons as FoxO1 inhibition downregulated Fzd7 expression and reduced the levels of H4K16ac at its promoter under basal conditions. But, FoxO1 did not modulate Fzd1 expression under basal conditions. In contrast, our results showed that FoxO1 inhibition prevents Sirt2 recruitment to Fzd1 and Fzd7 promoters and prevents Fzd1 downregulation in the context of AD, suggesting that FoxO1 acts as a co-repressor. Together, these results suggest that FoxO1 acts as a repressor for Fzd1 in AD and that this transcription factor has a dual role for Fzd7: from positive regulation of Fzd7 transcription in basal conditions to negative regulation of Fzd7 transcription in AD context. Interestingly, this transcriptional repression could also regulate other genes with synaptogenic or neuroprotective attributes such as the Wnt ligands Wnt3a, Wnt5a/b or the neurotrophic factors Ngf or Ntf3 [13, 50, 61, 62], as all of them present putative FoxO1 binding sites in their promoters when analysed by CiiDER (Fig. S50). This mechanism could also regulate other genes implicated in AD. This epigenetic regulation of Wnt receptors by the Sirt2-H4K16ac could also modulate the expression of these genes in other cellular contexts and diseases.

In summary, we report a novel role for nuclear SIRT2 in regulating Fzd receptors in AD. We propose that nuclear SIRT2 is hyperactivated in AD, and that FoxO1 recruits Sirt2 to Fzd1 and Fzd7 promoters leading to reduced H4K16ac, which in turn impairs their transcription. Thus, SIRT2 is a promising target for developing new AD therapies to restore the expression of key Wnt receptors.

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