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

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ABSTRACT:
Growing evidence supports a role for deficient Wnt signalling in synapse degeneration in Alzheimer´s disease (AD). First, the Wnt antagonist DKK1 is elevated in the AD brain 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 particular interest due to their role in synapse formation and plasticity. Our analyses showed that *FZD1* and *FZD7* mRNA levels were reduced in the hippocampus of human preclinical AD (PAD) cases and in the hAPP$^{NLGF/NLGF}$ 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 inhibitory phosphorylation on Sirt2 in nuclear PAD samples and increased levels of the Sirt2 phosphatase PP2C, leading to hyperactive nuclear Sirt2 and favouring *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.

INTRODUCTION:

Alzheimer's disease (AD) is the most common form of dementia, clinically characterised by progressive cognitive impairment leading to memory loss and difficulties in daily functioning. The AD brain is characterised by the presence of intracellular neurofibrillary tangles, composed of hyperphosphorylated tau, and extracellular Amyloid-β (Aβ) depositions forming Aβ-plaques *(1)*. One of the early events in AD is the loss of synapses, a process strongly correlated with cognitive decline *(2, 3)*. Over the last decades extensive research efforts have been made to elucidate the molecular mechanisms leading to synapse loss in AD *(4, 5)*. However, our understanding of what triggers synapse dysfunction and loss in AD remains poorly understood.

Several signalling pathways regulating synapse function and integrity are dysregulated in AD, eventually leading to synapse loss *(6, 7)*. Of particular interest is the Wnt signalling pathway(s). First, the secreted Wnt antagonist DKK1 is elevated in the brain of AD patients and models *(8–10)* and by exposure to Aβ oligomers *(11)*. Importantly, blockade or knockdown of Dkk1 protects synapses against Aβ *(11, 12)*. Second, three genetic variants of the Wnt co-receptor Low-Density Lipoprotein Receptor-Related Protein-6 (LRP6) have been linked to late-onset AD (LOAD) *(13, 14)*. Moreover, Wnt3a and Wnt5a are protective against Aβ insult *(15, 16)*. Further evidence comes from the finding that induced expression of Dkk1 in the adult mouse hippocampus leads to synapse loss, plasticity defects and cognitive impairment, features observed in AD mouse models *(17, 18)*. Importantly, these synaptic defects can be reversed by ceasing Dkk1 expression *(17)*. Together, these findings demonstrate that Aβ deregulates Wnt signalling, a cascade that is crucial for synapse integrity, and that boosting Wnt signalling could be protective in AD.

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Deregulation of Wnt signalling in AD could be mediated by different mechanisms; elevation of Wnt antagonists, such as Dkk1, or down-regulation of key Wnt components such as Wnt proteins or their receptor Frizzled (Fzd) *(19)*. Interestingly, several Fzd receptors are sufficient and/or required for synaptic assembly. For example, Fzd1 and Fzd9 are sufficient to promote pre- and postsynaptic assembly respectively whereas Fzd5 and Fz7 are required for the assembly of presynaptic and postsynaptic sites respectively *(20–23)* (Fig. 1A). Fzd7 is also required for N-methyl-D-aspartate receptor (NMDAR)-mediated synaptic plasticity *(20)*. However, little is known about the regulation and/or function of these synaptic Fzds in AD.

Here, we investigated whether Fzd receptors are deregulated in AD and the mechanisms involved. We found that *Fzd1* and *Fzd7* are downregulated in early AD by a shared epigenetic mechanism dependent on the hyperactivity of the nuclear Silent Mating Type Information Regulation-2 or Sirtuin2 (Sirt2). Using pharmacological approaches in hippocampal organotypic cultures from WT and hAPP$^{NLGF/NLGF}$ mice, we demonstrated that the Protein Phosphatase 2C (PP2C) is responsible for nuclear Sirt2 hyperactivity. In addition, we found that the transcription factor FoxO1 recruits Sirt2 to *Fzd1* and *Fzd7* promoters, repressing their expression by reducing the levels of the active histone mark H4K16ac at their promoters. Importantly, *in vivo* inhibition of Sirt2 also rescues H4K16 levels at *Fzd1* and *Fzd7* promoters as well as their transcription in the hAPP$^{NLGF/NLGF}$ mouse model. In summary, our results demonstrate a novel role for nuclear Sirt2 in AD which downregulates *Fzd1* and *Fzd7* expression through the deacetylation of the pro-transcription histone mark H4K16ac.

RESULTS:

The expression of *Frizzled1* and *Frizzled7* receptors is downregulated in AD

Wnt signalling has been linked to AD by studies on the Wnt antagonist DKK1 and the genetic variants of the Wnt coreceptor LRP6 *(24)*. However, very little is known about the regulation and/or function of Frizzled receptors (Fzd) in the context of AD. Four receptors of the 10 members of the Fzd family (Fzd1, Fzd5, Fzd7 and Fzd9) have been shown to regulate synapse formation and/or function *(20–23)* (Fig. 1A). We therefore evaluated the expression levels of these Fzd receptors at early human AD stages, a time when synapse loss begins *(2)*. We performed RT-qPCR on human hippocampal RNA samples from control and subjects with early AD, which have AD histopathological features but no cognitive deficits (preclinical AD; PAD; Supp. Table 1). Our results showed that at early AD stages, *FZD1* and *FZD7* mRNA levels were significantly downregulated (Fig. 1B). In contrast, *FZD5* and *FZD9* mRNA levels were unchanged in the human PAD group (Fig. 1B). These results suggest that two *Fzds* with a synaptic function are downregulated in early stages of AD.

Next, we investigated whether the mRNA levels of these *Fzds* were also affected in a mouse model of AD. We used the knock-in AD mouse model hAPP$^{NLGF/NLGF}$ (NLGF) as it avoids potential artefacts introduced by Amyloid-β Precursor Protein (APP) overexpression. This model carries the humanized form of the APP with the Swedish, Iberian and Arctic mutations, leading to Aβ overproduction *(25)*. We analysed the expression of *Fzd1*, *Fzd5*, *Fzd7* and *Fzd9* in the hippocampus of WT and NLGF animals at 2-months-old, a time when Aβ plaques start to appear *(25)*. Our results showed reduced levels of *Fzd1* and *Fzd7* mRNA levels in NLGF samples, whereas *Fzd5* and *Fzd9* mRNA levels remained unchanged (Fig. 1C), similar to the results obtained in human PAD hippocampal samples (Fig 1B). Together, these results demonstrate that expression levels of *Fzd1* and *Fzd7* receptors were reduced in both early AD subjects and AD mouse model at an early disease stage.

Finally, we asked whether the reduced mRNA levels of *Fzd1* and *Fzd7* were neuronal specific, as these receptors are also expressed in other cell-types. We performed single molecule RNA fluorescent in-situ hybridisation (smFISH) for *Fzd1* and *Fzd7* in the CA1 area of the hippocampus of WT and NLGF (Fig. 1D). Our single-cell analyses showed reduced *Fzd1* levels in NLGF neuronal cells (Rbfox3^{+}), without changes in non-neuronal cells (Rbfox3^{-}; Fig. 1E). However, no overall changes in *Fzd7* levels were observed in neuronal or non-neuronal cells (Fig. 1F). Next, we analysed the distribution of transcript copy number in neuronal cells. We found that neurons containing 3 or more *Fzd1* transcripts are reduced in AD (Fig. 1G). Interestingly, we observed a reduced number of neurons containing one *Fzd7* transcript in the AD mouse model (Fig. 1H). The lack of difference in H-score for *Fzd7* can be explained by the lower weighting for percentage of cells with 1 copy (see methods). Altogether, our results show that *Fzd1* and *Fzd7* levels are reduced in both human PAD and AD mouse model, with a clear downregulation

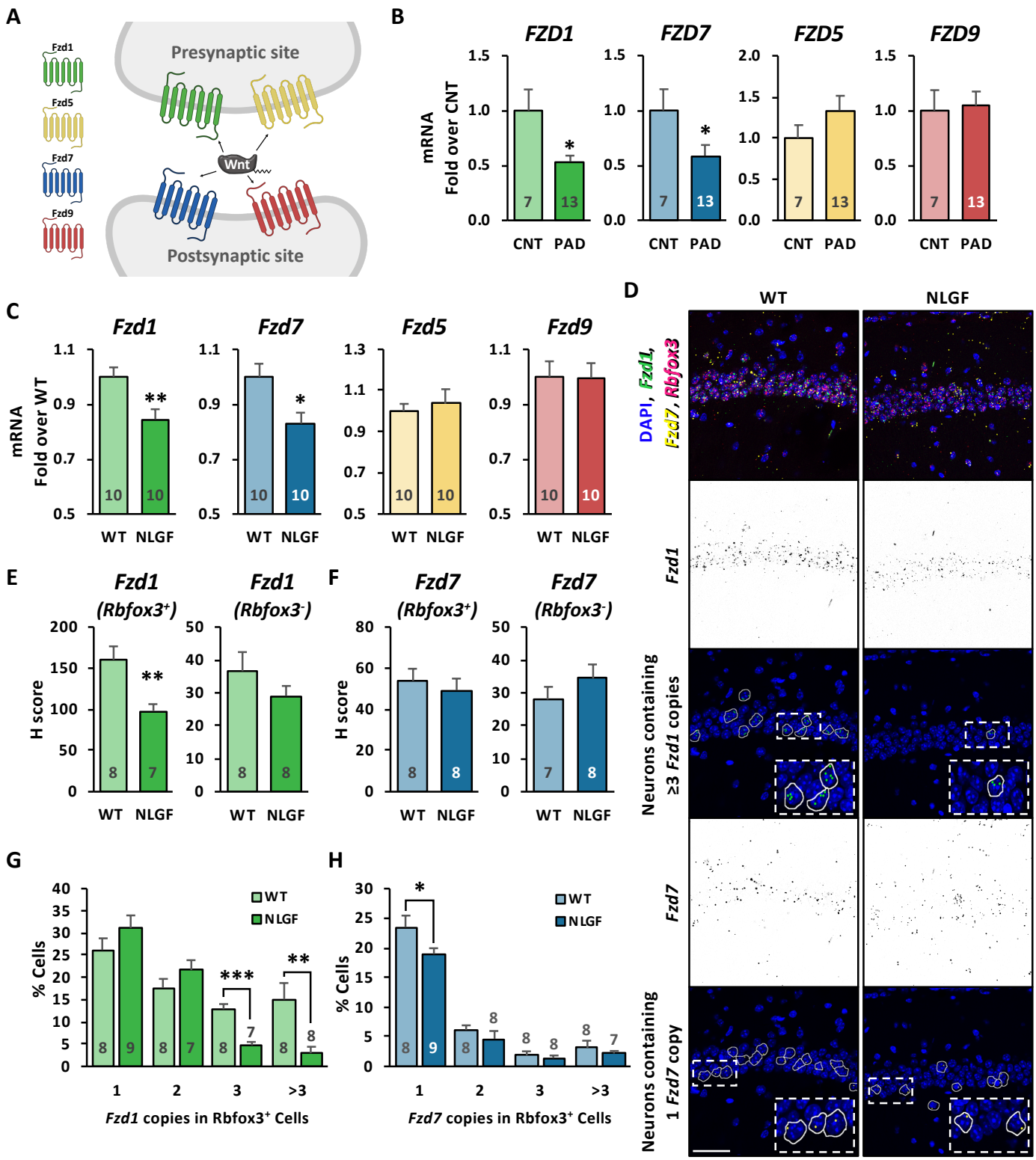

Figure 1: *Frizzled1* and *Frizzled7* expression is downregulated in early AD
A) Schematic showing the localization of Fzd1, Fzd5, Fzd7 and Fzd9 at the pre- and/or post-synaptic sides and the action of Wnts on both sites of the synapse. B) qPCR analyses showed reduced mRNA levels of *FZD1* and *FZD7* in human hippocampal samples from preclinical AD (PAD) subjects compared to controls (CNT). No changes were observed in the levels of *FZD5* and *FZD9* mRNAs at early AD. C) qPCR analyses showed reduced mRNA levels of *Fzd1* and *Fzd7* in 2-month-old NLGF hippocampus. No differences in *Fzd5* and *Fzd9* levels were observed in NLGF mice. D) Representative smFISH images of WT and NLGF CA1 hippocampal region. Top row shows merged images with DAPI (blue), *Fzd1* (green), *Fzd7* (yellow) and *Rbfox3* (magenta) mRNAs. *Fzd1* in black (second row) and its representative neuronal *Rbfox3*$^+$ cells corresponding to 3 and >3 *Fzd1* copies (third row). *Fzd7* in black (fourth row) and its representative cells corresponding to one *Fzd7* copy (fifth row). Scale bars represent 50 µm and 12.5 µm in the zoomed in inserts. E-F) Single-cell analyses expressed as H-score for *Fzd1* (E) and *Fzd7* (F) in neuronal (*Rbfox3*$^+$) and non-neuronal (*Rbfox3*$^-$) cells. G-H) 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. Values inside bars indicate number of samples; asterisks indicate *$p<0.05$; **$p<0.01$.

of neuronal *Fzd1* mRNA levels and reduced levels of neurons containing one *Fzd7* transcript in the AD mouse hippocampus.

Fzd1 and _Fzd7_ promoters present reduced levels of H4K16ac with concomitant increase of Sirt2 in early AD

Given that *FZD1* and *FZD7* exhibited reduced mRNA levels in the hippocampus at early AD stages, we hypothesised that a shared epigenetic regulation could contribute to their dysregulation. A previous study showed that transcriptional prone acetylated histone mark Histone H4 Lysine 16 (H4K16ac) is enriched at promoter regions of several components of the Wnt signalling pathway *(26)*. We therefore analysed whether the levels of H4K16ac were enriched at *Fzd1* and *Fzd7* promoters in the hippocampus by performing chromatin immunoprecipitation (ChIP)-qPCR. Indeed, high levels of H4K16ac are present at *Fzd1* and *Fzd7* promoters, similar to those at the actively transcribed gene *Eif5* (Supp. Fig 1A), a gene reported to have high levels of H4K16ac in the human brain *(27)*. In contrast, *Fzd5* promoter region exhibited low levels of H4K16ac (Supp. Fig. 1A), similar to those observed at the repressed gene *Krt16*, which has low levels of H4K16ac *(27)*. These results suggest that this histone mark is highly present at the promoters of *Fzd1* and *Fzd7* genes and therefore could contribute to the transcriptional regulation of these Wnt receptors.

Next, we compared the abundance of this histone mark in human hippocampal samples from control and PAD. First, we determined that the total levels of H4K16ac were not altered by the post-mortem interval (PMI) time in our human hippocampal samples (Supp. Fig. 1B). ChIP-qPCR experiments showed that H4K16ac was reduced at *FZD1* and *FZD7* promoters in PAD (Fig. 2A-B). In contrast, this signature was unchanged at the promoter of our internal control *FZD5* (Fig. 2A) or at promoters of external controls *ACTB* (an active gene) and *HOXA1* (a repressed gene; Supp. Fig. 1C). Changes in H4K16ac levels at a locus could arise from nucleosome remodelling, changes in chromatin compaction state, 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 due to the reduced H4K16ac levels at these promoters (Supp. Fig 1D). We next interrogated whether these epigenetic changes also occurred in the NLGF hippocampus. Our results showed reduced H4K16ac levels at the promoters of *Fzd1* and *Fzd7* in this AD model, with no changes at the promoter of control genes *Fzd5*, *Actb* and *Hoxa1* (Fig. 2C, Supp. Fig. 1E). No changes in H4 levels were found at the promoters analysed, suggesting no changes in nucleosome remodelling in the AD mouse model (Supp. Fig. 1F). Altogether, these results demonstrate that reduced levels of H4K16ac could contribute to the decreased expression of *FZD1* and *FZD7* in early AD.

Next, we investigated the mechanism leading to reduced levels of H4K16ac at the promoters of *Fzd1* and *Fzd7* in early AD. A possible mechanism is through the action of H4K16ac deacetylases such as Histone Deacetylases 2 (HDAC) *(28)*, Sirtuin1 (Sirt1) *(29)* or Sirt2 *(30)*. Importantly, these three histone deacetylases have been linked to neurodegeneration: HDAC2 and Sirt2 play a neurodegenerative role, whereas Sirt1 is neuroprotective *(31, 32)*. Therefore, we analysed the abundance of HDAC2 and Sirt2 at the promoters of the *Fzds* affected in early AD. Our results showed that HDAC2 levels remained unchanged across all the genes analysed in the hippocampus of NLGF mice (Supp. Fig. 1G). We next analysed the occupancy of Sirt2 on the regulatory regions of our genes of interest. ChIP-qPCR experiments showed that the occupancy of Sirt2 was increased only at *Fzd1* and *Fzd7* promoters in the hippocampus of the AD mouse (Fig. 2D, Supp. Fig. 1H). Therefore, our results demonstrate 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.

Sirt2 inhibition rescues _Fzds_ epigenome and transcription in AD

To test if reduced levels of H4K16ac observed at *Fzd1* and *Fzd7* promoters in early AD depend on Sirt2 activity, we prepared hippocampal organotypic cultures (HOC) from WT and NLGF animals. Consistent with our results in PAD and NLGF mice, we found that 15DIV HOC derived from NLGF showed reduced mRNA levels of *Fzd1* and *Fzd7* but no changes in Fzd5, which was accompanied by reduced levels of H4K16ac and increased levels of Sirt2 at their promoters (Supp. Fig. 2A-D). Next, we studied whether Sirt2 regulates *Fzd* transcription using the specific Sirt2 inhibitor AGK2 *(33)* in our HOC model (Fig. 3A). First, we tested cell viability by Fluorescent Activated Cell Sorting (FACS) and found that AGK2 was not toxic under these conditions (Supp. Fig. 3A, Supp. Table 2). Next, we tested Sirt2 inhibition by AGK2 in the HOC model by analysing levels of H3K56ac, a known Sirt2 substrate *(34)*. Our experiments showed increased H3K56ac

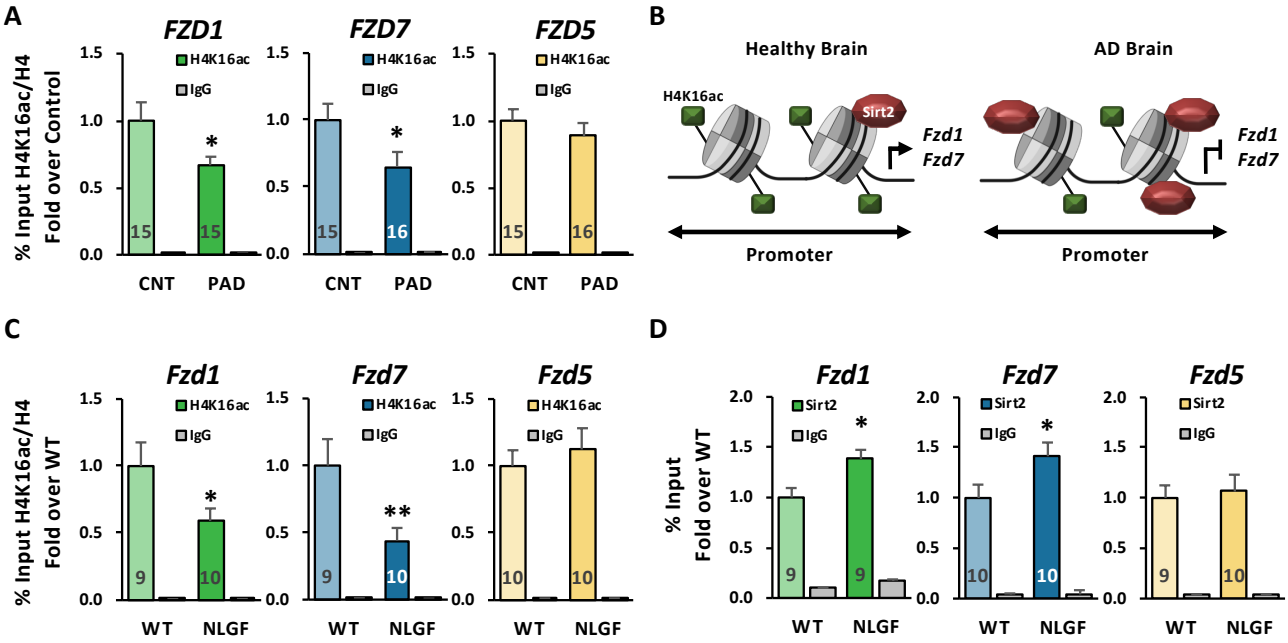

Figure 2: Downregulation of *Fzds* in AD correlates with reduced levels of H4K16ac and the concomitant increase in Sirt2 at *Fzds* promoters

A) ChIP-qPCR analyses showed reduced H4K16ac levels at *FZD1* and *FZD7* promoters in preclinical AD subjects (PAD). H4K16ac levels remained unchanged at *FZD5* promoter. 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 were observed for *Fzd5*. D) ChIP-qPCR analyses demonstrated increased levels of Sirt2 at the promoters of *Fzd1* and *Fzd7* in NLGF hippocampal samples when compared to controls. No differences were observed at *Fzd5* promoter. Data are represented as mean + SEM. Statistical analyses by *t*-test in A for *FZD1*, *FZD7* and by Mann-Whitney for *FZD5*; in C *t-t*est for *Fzd1* and *Fzd5* and by Mann-Whitney for *Fzd7*; in D *t*-test for *Fzd1*, *Fzd7*, *Fzd5*. Values inside bars indicate number of samples; asterisks indicate *$p<0.05$; **$p<0.01$.

levels, consistent with the role of AGK2 as a pharmacological inhibitor of Sirt2 (Supp. Fig. 3B). Finally, we asked whether AGK2 could rescue the reduced expression of *Fzd1* and *Fzd7* in the AD model. Indeed, our RT-qPCR experiments showed that AGK2 rescued *Fzd1* and *Fzd7* mRNA expression to control levels in the NLGF-HOC (Fig. 3B-C). In contrast, the levels of Fzd5 mRNAs remained unchanged across groups (Fig. 3B). To further demonstrate that Sirt2 inhibition rescues *Fzd1* and *Fzd7* mRNA levels, we treated our HOC model with a second specific and structurally distinct Sirt2 inhibitor; AK7 (Supp. Table 2) *(33)*. AK7 treatment was not cytotoxic when analysed by FACS and effectively supressed Sirt2 activity as shown by increased levels of H3K56ac in our HOC model (Fig. 3A, Supp. Fig. 3A, Supp. Fig. 3C). Importantly, Sirt2 inhibition by AK7 rescued *Fzd1* and Fzd7 *mRNA* levels in the NLGF-HOC model, without affecting Fzd5 mRNAs levels (Fig. 3C-D). Together, Sirt2 inhibition by two distinct small molecules demonstrate that Sirt2 contributes to the downregulation of expression *Fzd1* and *Fzd7* in AD (Fig. 3C).

Based on these results, we predicted that the levels of H4K16ac at the promoters of *Fzd1* and *Fzd7* genes could also be rescued by Sirt2 inhibition. We therefore analysed H4K16ac levels after AK7 treatment in WT and NLGF-HOC model and found that the levels of this pro-transcriptional histone mark were restored at *Fzd1* and *Fzd7* promoters in the NLGF-HOC treated cultures (Fig. 3E). AK7 treatment had no effect on H4K16ac levels at promoters of control genes *Fzd5, Actb* and Hoxa1 across genotypes (Fig. 3E, Supp. Fig. 3D). These results demonstrate that Sirt2 impairs *Fzd1* and *Fzd7* transcription by reducing H4K16ac levels in the AD organotypic cultures.

To further examine the role of Sirt2 in regulating *Fzd1* and *Fzd7* transcription we utilised the Sirt2 inhibitor AK7 *in vivo*. This inhibitor has been shown to cross the blood brain barrier and to be safe for animal dosage *(35)*. Therefore, we tested the impact of AK7 on the expression of *Fzd* receptors in the hippocampus of NLGF mouse AD model. Given the limited brain availability of AK7 *(36)*, mice were injected intraperitoneally with 20 mg/Kg twice a day for 15 days (Fig. 3F), as previously reported *(37)*. First, we tested the *in vivo* effect of AK7 as a Sirt2 inhibitor by evaluating H3K56ac levels. We found that this histone mark was elevated upon AK7 administration,

suggesting an effective Sirt2 inhibition in the brain (Supp. Fig. 3E). Next, we examined whether AK7 rescues *Fzds* mRNA levels. We found that AK7 treatment rescued *Fzd1* and *Fzd7* mRNA levels (Fig. 3G) as well as the abundance of H4K16ac at their promoters (Fig. 3H) in NLGF animals. In contrast, AK7 had no effect on the expression of these *Fzds* in WT animals (Fig. 3H). Similar to our *in vitro* studies, AK7 did not change the mRNA or epigenetic levels of our control genes *Fzd5*, *Actb* and *Hoxa1* (Fig. 3H, Supp. Fig. 3F). Previous studies using a higher and longer dosage of AK7 have linked Sirt2 inhibition with reduced levels of Aβ in AD models *(38)*. However, we did not find changes in the *in vivo* levels of $A\beta_{42}$ (Supp. Fig. 3G), as previously reported with the same AK7

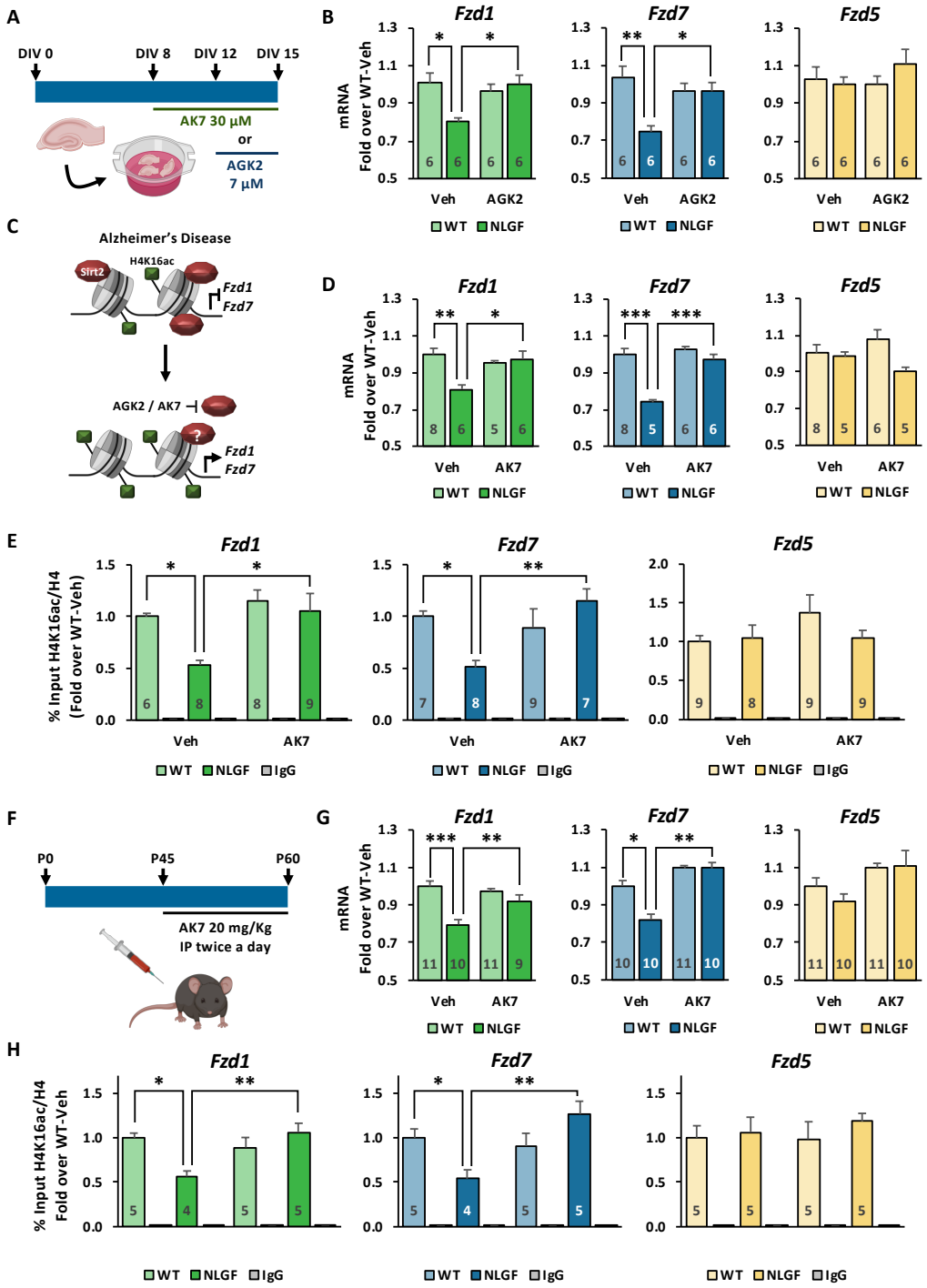

Figure 3: Sirt2 inhibition rescues *Fzds* epigenome and transcription in AD

A) Scheme representing AGK2 and AK7 treatments of HOC. Hippocampal slices were cultured for 15 DIV and treated with 7 µM of AGK2 for 72 hours or with 30 µM of AK7 for 7 days. B) qPCR analyses of mRNA levels from WT and NLGF hippocampal cultures treated with vehicle or AGK2. Our results showed that AGK2 treatment rescues *Fzd1* and *Fzd7* mRNA levels without modulating *Fzd5* mRNA levels. C) Scheme representing the epigenetic state of *Fzd1* and *Fzd7* promoters in AD and how Sirt2 inhibition rescues H4K16ac levels and their mRNA expression. D) qPCR results show that Sirt2 inhibition by AK7 rescued *Fzd1* and *Fzd7* mRNA levels in AD treated cultures, without modulating *Fzd5* mRNA levels. E) ChIP-qPCR showing that AK7 treatment rescued the levels of H4K16ac at *Fzd1* and *Fzd7* promoters in NLGF-HOC while not changing the levels of H4K16ac in WT or at *Fz5* promoter. F) Scheme showing the dosage regime for *in vivo* inhibition of Sirt2 by intraperitoneal injections of 20 mg/Kg of AK7 twice a day for 15 days, from 1.5 to 2-months-old WT and NLGF animals. G) qPCR analyses of mRNA levels from WT and NLGF hippocampal samples treated with vehicle or AK7. Our results showed that AK7 rescued *Fzd1* and *Fzd7* mRNA levels back to WT in NLGF treated animals and did not show any effect on *Fzd5* mRNA levels. H) ChIP-qPCR showed that Sirt2 inhibition by AK7 rescued the levels of H4K16ac at *Fzd1* and *Fzd7* promoters in AD while no changes were observed in WT or at *Fzd5* promoter. Data are represented as mean + SEM. Statistical analyses by Two-way ANOVA followed by Tukey's post hoc in B for all genes analysed; in D Two-way ANOVA followed by Tukey's post hoc for *Fzd1* and *Fzd7* and Kruskal-Wallis followed by Dunn's multiple comparison for *Fzd5*; in E Kruskal-Wallis followed by Dunn's multiple comparison for all genes analysed; in G Two-way ANOVA followed by Tukey's post hoc for *Fzd1* and *Fzd7* and Kruskal-Wallis followed by Dunn's multiple comparison for *Fzd5*; in H Two-way ANOVA followed by Tukey's post hoc for *Fzd1* and *Fzd5* and Two-way ANOVA followed by Games-Howell post hoc for *Fzd7*. Values inside bars indicate number of samples; asterisks indicate *p<0.05; **p<0.01; ***p<0.005.

dosage in two different AD models *(37)*. Collectively, these results show that *Fzd1* and *Fzd7* mRNA levels are downregulated through Sirt2 deacetylation of H4K16ac in the adult hippocampus of an AD model (Fig. 3C).

FoxO1 recruits Sirt2 to *Fzd1* and *Fzd7* promoters in AD

Our results demonstrated that increased Sirt2 occupancy at *Fzd1* and *Fzd7* promoters results in the epigenetic repression of the transcription of these Wnt receptors at early AD stages. These results suggest the possibility that Sirt2 levels are upregulated in AD. To test this hypothesis, we analysed total SIRT2 protein levels in the hippocampus of PAD human samples (Supp. Fig. 4A). However, we found no significant changes. Given that Sirt2 is also present in the nucleus *(39)*, we next examined if nuclear Sirt2 levels are upregulated in early AD. We therefore prepared nuclear enriched fractions from human control and PAD samples (Supp. Fig. 4B). However, no differences in SIRT2 nuclear localisation were found in human PAD hippocampal samples (Fig. 4A). These results indicate that increased occupancy of Sirt2 at *Fzd1* and *Fzd7* promoters does not correlate with increased total or nuclear levels of Sirt2, suggesting that Sirt2 might be recruited to Fzds promoters by co-factors.

Sirt2 interacts with different transcription factors, including FoxO1 and FoxO3a *(40, 41)*, which could recruit Sirt2 to specific loci. We used CiiiDER *(42)* to evaluate putative FoxO1 and FoxO3a binding sites and found that *Fzd1* and *Fzd7* promoters contain FoxO1 binding sites (Supp. Fig. 4D). We therefore tested whether FoxO1 activity is required for Sirt2 recruitment to *Fzds* promoters in AD. We treated our HOC model with the specific FoxO1 activity Inhibitor AS1842856 (FoxO1i, Fig. 4B, Supp. Table 2) *(43)*. No cytotoxicity was observed by FACS upon FoxO1i treatment (Supp. Fig. 4E). Next, we analysed Sirt2 occupancy upon FoxO1 inhibition and found that FoxO1 inhibition reduced the levels of Sirt2 at *Fzd1* and *Fzd7* promoters in the NLGF-HOC model (Fig 4C-D). In contrast, no changes in Sirt2 levels were observed at *Fzd5* and *Actb* promoters upon FoxO1 inhibition (Fig. 4C, Supp. Fig. 4F). However, reduced levels of Sirt2 were found at the promoter of the control gene *Hoxa1* in both WT and NLGF HOCs (Supp. Fig. 4F). Interestingly, *Hoxa1* has three putative FoxO1 binding sites in the region analysed by CiiiDER (Supp. Fig. 4D). Altogether, these results suggest that Sirt2 recruitment to *Fzd1* and *Fzd7* promoters in AD depends on FoxO1 binding activity.

Increased nuclear Sirt2 activity impairs *Fzd1* and *Fzd7* transcription in AD

Sirt2 activity can be modulated by phosphorylation *(44, 45)*. We therefore analysed the phosphorylation state of Sirt2 at its Serine 331 (pSirt2), which has been shown to inactivate Sirt2 *(44)*. First, we evaluated the levels of pSIRT2 in human control and PAD hippocampal samples by western blot analyses and found no differences (Supp. Fig. 4A). Next, we explored whether changes in pSirt2 might take place in the nucleus. Our results showed that pSIRT2 levels were reduced in nuclear enriched fractions from human PAD hippocampal samples (Fig. 4A). Similarly, lower levels of nuclear pSirt2 were observed in NLGF-HOC model (Supp. Fig. 4G). These findings strongly suggest that Sirt2 activity increases in the nucleus at early AD stages and supports the idea that increased levels of active Sirt2 at *Fzd1* and *Fzd7* promoters lead to reduced H4K16ac levels, resulting in decreased transcription of these Wnt receptors.

Our findings are consistent with the hypothesis that nuclear Sirt2 is more active in AD as per decreased levels of its inhibitory phosphorylation. This could be regulated by specific phosphatases. We therefore investigated if lower levels of pSirt2 correlate with changes of the Sirt2 phosphatases PP2Cα/β *(45)*, as Pp2cα has been reported to be upregulated at the RNA level in an AD mouse model *(46)*. First, we found no differences of PP2Cα/β total levels in hippocampal samples from human PAD when compared to controls (Supp. Fig. 4H). Next, we analysed if these phosphatases were differentially localised in the nucleus. Indeed, the levels of PP2Cα, but not PP2Cβ, were upregulated in nuclear samples from PAD subjects (Fig. 4E, Supp. Fig 4I). In addition, Pp2cα was also upregulated in nuclear samples of NLGF-HOC (Supp. Fig. 4J). Altogether, these results suggest that increased nuclear levels of PP2C could lead to Sirt2 hyperactivity in the nucleus, thus favouring the repression of *Fzd1* and *Fzd7* in AD.

To establish the role of PP2C in *Fzd1* and *Fzd7* expression in AD, we treated our HOC with sanguinarine (SAN), a PP2C specific inhibitor (Supp. Table. 2) *(47)*. First, we tested cell viability by FACS in HOCs treated with SAN and found no toxicity (Fig. 4B, Supp. Fig 4E). Next, we analysed if SAN rescues the expression of *Fzd1* and *Fzd7*. Our results showed that Pp2c inhibition had no effect on the levels of *Fzd1* and *Fzd7* expression in WT-HOCs (Fig. 4F). However, Pp2c inhibition rescued *Fzd1* and *Fzd7* expression in the NLGF-

HOCs, without affecting the levels *Fzd5* levels (Fig. 4F). Finally, we analysed if this enhanced *Fzd1* and *Fzd7* expression correlates with changes in H4K16ac levels at their promoters. Indeed, our ChIP-qPCR experiments showed that SAN rescued the levels of H4K16ac at *Fzd1* and *Fzd7* promoters in NLGF-HOCs treated with SAN (Fig. 4G-H). Notably, H4K16ac levels remained unchanged at the promoters of the control genes *Fzd5, Actb* and *Hoxa1* (Fig. 4G, Supp. Fig. 4K).

To assess whether the epigenetic and transcriptional rescue upon Pp2c inhibition was due to changes in Sirt2 activity, we analysed nuclear extracts from WT and NLGF-HOCs treated with SAN. Our results showed higher levels of pSirt2 in AD treated samples (Supp. Fig. 4L), supporting the idea that increased nuclear levels of Pp2c are responsible for the increased Sirt2 activity leading to *Fzd1* and *Fzd7* epigenetic and transcriptional repression in early AD.

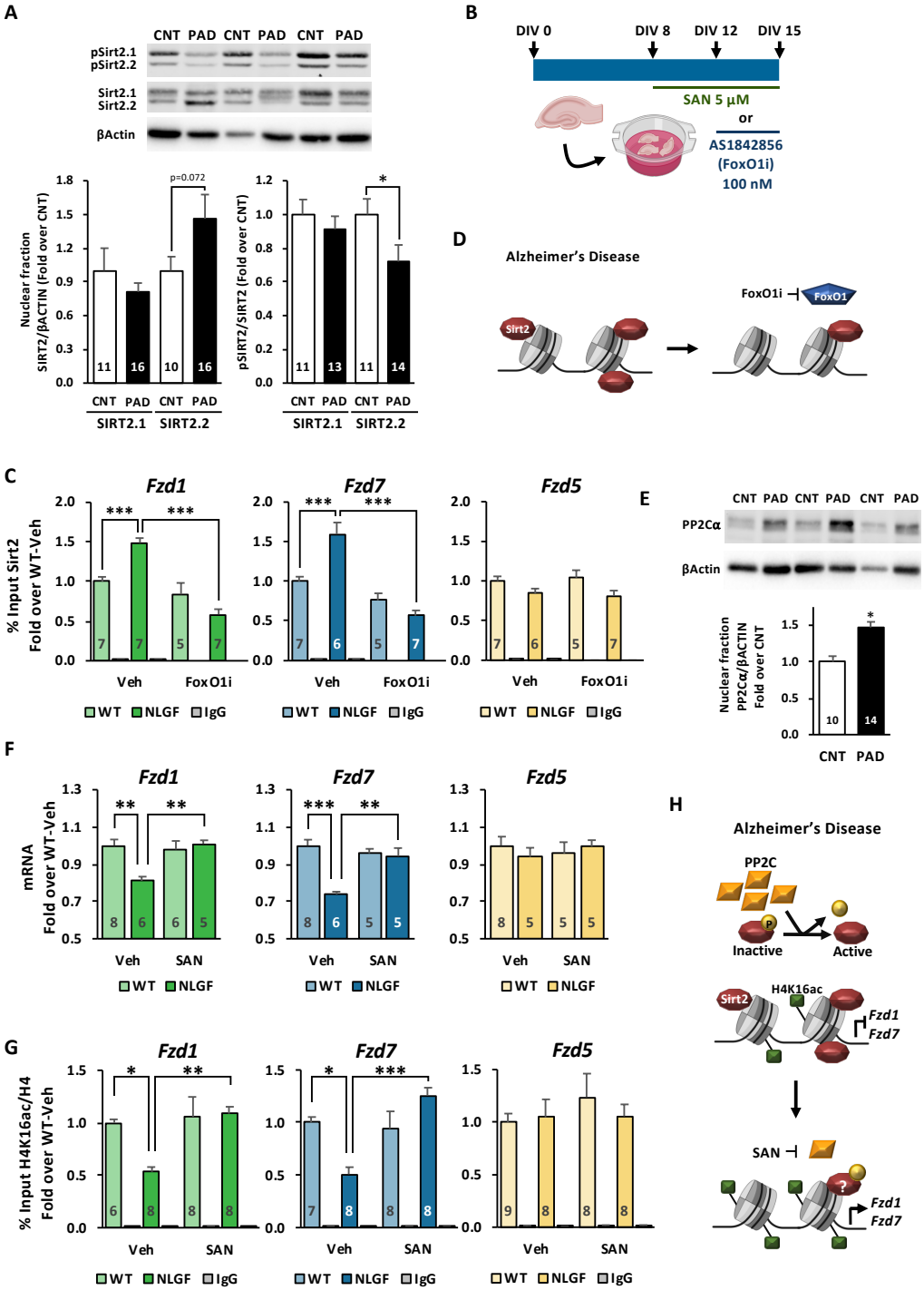

Figure 4: Increased Sirt2 activity in AD impairs *Fzds* transcription

A) WB analyses of total and pSIRT2 levels in hippocampal nuclear extracts of human CNT/PAD subjects showed decreased levels of pSirt2.2 at early AD. B) Scheme illustrating the experimental design where HOC were treated with 5 µM of Sanguinarine (SAN) or 100 nM of AS1842856 FoxO1 inhibitor (Fox1OI) for 7 days and 72h respectively. C) ChIP-qPCR showed that FoxO1i reduced Sirt2 occupancy at *Fzd1* and *Fzd7* promoters in NLGF-HOC whereas not changes were observed in the levels of Sirt2 in WT or at *Fz5* promoter, suggesting Sirt2 recruitment to *Fzd1* and *Fzd7* promoters depends on FoxO1 activity in AD. D) Scheme representing the levels of Sirt2 at *Fzd1* and *Fzd7* in AD and upon FoxO1 inhibition in AD. E) WB analyses of PP2Cα in hippocampal nuclear extracts of human control/PAD subjects showed increased levels of PP2Cα in human PAD group. E) qPCR analyses of total mRNA levels from WT and NLGF-HOC treated with vehicle or SAN. Our results showed that SAN treatment rescued *Fzd1* and *Fzd7* mRNA levels and did not show any effect on *Fzd5* mRNA levels. F) ChIP-qPCR showed that SAN treatment rescued the levels of H4K16ac at *Fzd1* and *Fzd7* promoters in AD while no changes were observed in WT or at *Fzd5* promoter. H) Scheme showing increased nuclear levels of phosphatase PP2C in AD and how PP2C inhibition in AD rescues H4K16ac levels at *Fzd1* and *Fzd7* promoters and their transcription. Data are represented as mean + SEM. Statistical analyses by *t*-test in A for total Sirt2.2 and nuclear Sirt2.1 and Srit2.2, and by Mann-Whitney for total Sirt2.1; in C Two-way ANOVA followed by Tukey's post hoc for all genes analysed; in E by *t*-test; in F Two-way ANOVA followed by Tukey's post hoc for *Fzd1* and *Fzd7* and Kruskal-Wallis followed by Dunn's multiple comparison for *Fzd5*; in G Two-way ANOVA followed by Tukey's post hoc for *Fzd7* and Kruskal-Wallis followed by Dunn's multiple comparison for *Fzd1* and *Fzd5*. Values inside bars indicate number of samples; asterisks indicate *$p<0.05$; **$p<0.01$; ***$p<0.005$.

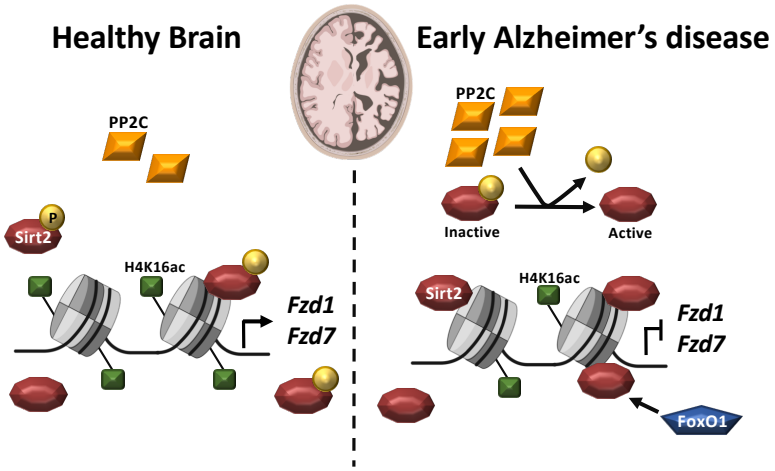

Figure 5: Schematic model of Fzd1 and Fzd7 regulation by Sirt2 in AD
Scheme representing the nuclear localization of the histone deacetylase Sirt2, its phosphatase PP2C and the epigenetic regulation of *Fzd1* and *Fzd7* in healthy and in the early AD brain. In the healthy brain, high levels of H4K16ac and low levels of Sirt2 coexist at *Fzd1* and *Fzd7* promoters, leading to *Fzd1* and *Fzd7* transcription. At early AD stages, increased nuclear levels of the phosphatase PP2C activates Sirt2 by removing its inhibitory phosphorylation. In turn, FoxO1 activity is required for Sirt2 recruitment to *Fzd1* and *Fzd7* promoters leading to reduced levels of H4K16ac and impairing *Fzd1* and *Fzd7* transcription.

Altogether these results demonstrate that Pp2c is upstream of Sirt2 in regulating *Fzd1* and *Fzd7* expression in early AD (Fig. 4H).

DISCUSSION:

In this study, we present novel findings demonstrating that synaptic Wnt signalling receptors *Fzd1* and *Fzd7* are epigenetically downregulated in AD by the hyperactivation of nuclear Sirt2 in AD. In addition, nuclear Sirt2 recruitment to *Fzd1* and *Fzd7* promoters depends on FoxO1 DNA binding activity, leading to increased Sirt2 levels and a concomitant H4K16ac deacetylation at *Fzd1* and *Fzd7* promoters, therefore favouring *Fzd1* and *Fzd7* transcriptional repression (Fig. 5).

Wnt signalling has been linked to LOAD by three LRP6 genetic variants and by a large body of research demonstrating the synaptotoxic role of the Wnt antagonist Dkk1 in AD *(11–14)*. However, the role of other Wnt signalling components in AD have not been extensively studied. Here, we report for the first time that two synaptic Fzds, *Fzd1* and *Fzd7*, display reduced expression levels at the hippocampus of early AD patients and 2-month-old NLGF model. Our results showed a reduction in neuronal *Fzd1* and *Fzd7* levels in AD (Fig. 1D-H). Interestingly, Fzd1 and Fzd7 are localised at the pre- and post-synaptic side respectively *(20, 21)*, thus their reduced levels could impact both sites of the synapse. Silencing *Fzd7* leads to reduced spine number, and their innervation, in hippocampal cultures and impairs LTP in HOC *(20)*. In addition, *in vivo Fzd7* knockdown leads to reduced dendritic arborization during postnatal development *(48)*. At the presynaptic site, Fzd1 overexpression leads to increased Bassoon clusters, whereas blockade of this receptor with the extracellular Fzd1 cysteine rich domain (CRD), which sequesters endogenous Wnts, leads to reduced Bassoon cluster number in hippocampal cultures *(21)*. Interestingly, Wnt3a prevents Aβ-induced synapse loss in a Fzd1 dependent manner *(49)*. Thus, reduced Fzd1 would also fail to protect synapses in AD. Together, these results suggest that reduced levels of *Fzd1* and *Fzd7* could contribute to the impaired synaptic plasticity and synapse loss observed in early AD.

Fzd1 and *Fzd7* expression are regulated by epigenetic mechanisms such as noncoding RNAs and DNA methylation in different biological processes and diseases *(50–53)*. In this study, we focused our attention on the transcriptional regulation of Fzd receptors in AD and how these changes relate to pro-transcriptional histone mark H4K16ac, which is enriched in several genes encoding Wnt signalling pathway components *(26)* (Supp. Fig.1A). Our results identify a novel role for Sirt2 in deacetylating H4K16ac at *Fzd1* and *Fzd7* promoters in AD. Using *in vitro* and *in vivo* approaches, we demonstrated that Sirt2 inhibition prevents H4K16ac deacetylation in AD (Fig. 5). In addition, Sirt2 has other histone substrates, including H3K18ac and H3K56ac *(34)*. However, we found no differences in these histone modifications (Supp. Fig. 5A), suggesting that Sirt2 deacetylation of H4K16 is a specific event at *Fzd1* and *Fzd7* promoters in AD. Interestingly, H4K16ac has been linked to Fzds regulation in brain development as H4K16ac deacetylation by Sirt1 regulates *Fzd5* and *Fzd7* transcription during cortical neurogenesis *(54)*. Taken together with our results, the data strongly suggest that H4K16ac deacetylation regulates different *Fzds* transcription during development and in neurodegeneration *via* Sirt1 and Sirt2 respectively. These studies also show different roles for Sirt1 and Sirt2, consistent with the idea that Sirt1 is neuroprotective whereas Sirt2 plays a role in neurodegeneration *(31, 32)*.

Increasing evidence suggests a neurodegenerative role for Sirt2. First, a genetic variant of *SIRT2* has been linked to LOAD in *APOEε4*-negative population *(55)*. Second, increased SIRT2 levels, and a concomitant reduction in the SIRT2 substrate acetylated α-tubulin, are observed in an *in vitro* cellular AD model *(56, 57)*. Third, *in vivo* Sirt2 inhibition with AK7 ameliorates cognitive deficits in three different AD models *(37, 38)*. These studies led to propose that the neuroprotective effect of Sirt2 inhibition is through increased levels of acetylated α-tubulin, which promote microtubule stability *(57)*. However, this hypothesis is contradicted by the finding of increased acetylated α-tubulin levels in human hippocampal AD samples *(58)*. Thus, the beneficial effect of Sirt2 inhibition in AD could arise from substrates other than acetyl-α-tubulin. Supporting this view, Sirt2 inhibition by AK7 reduces Aβ burden in APP/PS1 mice by deacetylating Reticulon 4B (RTN4B) and leading to reduced BACE1 expression *(38)*. Nonetheless, *in vivo* Sirt2 inhibition with lower AK7 concentration and for a shorter time does not modulate Aβ burden but rescues cognitive defects in AD mouse models (Supp. Fig. 3G) *(37)*, suggesting that other mechanisms apart from reduced Aβ production might be protective. Therefore, it is likely that the neuroprotective effect of Sirt2 inhibition involves several cytosolic and nuclear Sirt2 substrates.

Although we observed no changes in total or nuclear SIRT2 levels in AD (Fig. 4A, Supp. Fig. 4A), we observed a specific reduction of Sirt2 phosphorylation in nuclear factions of presymptomatic AD patients, a post-translational modification known to inhibit Sirt2 deacetylase activity *(44, 45)*. These results suggest the presence of hyperactive nuclear Sirt2 in AD. We also showed that the levels of the Sirt2 phosphatase PP2Cα are increased in nuclear preparations from human PAD subjects (Fig. 4A), in line with previous results showing increased mRNA levels of PP2Cα in an AD mouse model *(46)*. Consistently, we found that PP2C-induced nuclear Sirt2 hyperactivity is upstream of H4K16 deacetylation by Sirt2 at *Fzd1* and *Fzd7* promoter in AD (Fig. 5), demonstrating for the first time a role for PP2C in AD.

Increased 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 *(40)*, which has predicted binding sites at *Fzd1* and *Fzd7* promoter region, but not at *Fzd5* (Supp. Fig. 4D). FoxO1 can positively or negatively regulate gene transcription in different biological conditions *(59)*. Indeed, our results showed that FoxO1 inhibition prevents Sirt2 recruitment at Fzd promoters in AD (Fig. 4D), suggesting that FoxO1 acts as a co-repressor of *Fzd1* and *Fzd7* expression in the context of AD. Interestingly, this transcriptional repression could also regulate other genes with synaptogenic or neuroprotective attributes such as the Wnt ligands *Wnt3a*, *Wnt5a/b* or the neurotropic factors *Ngf* or *Ntf3 (16, 49, 60, 61)*, as all of them present putative FoxO1 binding sites in their promoters when analysed by CiiiDER (Supp. Fig. 5B). Taken together, our results demonstrate a novel role for nuclear hyperactive Sirt2 and that Sirt2 recruitment to *Fzd1* and *Fzd7* promoters depends on FoxO1 activity in AD, leading to their epigenetic repression. This mechanism could also regulate other genes implicated in AD. Moreover, the epigenetic regulation of Wnt receptors by the Sirt2-H4K16ac could also modulate these genes in other cellular contexts.

In summary, we report a novel role for nuclear Sirt2 in regulating *Fzd* receptor 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 the transcription of these Wnt receptors. Thus, Sirt2 is a promising target for developing new AD therapies by restoring the expression of key Wnt receptors.

MATERIAL and METHODS:

Human Tissue

Anonymised human hippocampal samples from control and presymptomatic AD subjects were obtained from Cambridge Brain Bank (CBB), Division of the Human Research Tissue Bank, Addenbrooke's Hospital, Cambridge UK. All samples were obtained with informed consent under CBB license (NRES 10/HO308/56) approved by the NHS Research Ethics Services. Tissues were stored at -80°C. Demographic data and Braak stages for each sample set are shown in Supp. Table 1.

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 (UCL). Human $APP^{NLGF/NLGF}$ AD knock-in (NLGF) line and WT littermates were used in this study. Genotyping was performed using DNA from ear biopsies using the following cocktail of primers: 5′-ATCTCGGAAGTGAAGATG-3′, 5′-ATCTCGGAAGTGAATCTA-3′, 5′-

TGTAGATGAGAACTTAAC-3′ and 5′-CGTATAATGTATGCTATACGAAG-3′ *(25)*. Animals were kept in ventilated racks at 22±2°C and 55±10% humidity with a 12h/12h light cycle and free access to food and water at all times.

Statistical analysis

All values are presented as mean + SEM. Statistical analyses were performed using SPSS v25 (IBM). Outliers were determined with the explore tool with normality plot. Normal distribution of the data and homogeneity of variances were tested by the Shapiro-Wilk test and Levene test, respectively. For non-normally distributed variables, comparisons between two groups of data were analysed by Mann-Whitney U-test, comparisons between more than two groups were analysed by Kruskal-Wallis followed by Dunn's multiple comparison test. For normally distributed data, comparisons between two groups were analysed by Student's t-test. If no variances were found in one of the groups, the statistical test applied was one-sample *t*-test. Two-way ANOVA was used for comparisons between groups in datasets with two factors and followed by post hoc pair-wise comparisons assuming (Tukey's) or not assuming equal variances (Games-Howell). In the figures, asterisks indicate p values as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

SUPPLEMENTARY INFORMATION:

For further information see Supplementary Figures, Supplementary Material & Methods and Supplementary Tables.

AUTHOR'S CONTRIBUTION:

E.P. and P.C.S. conceived this study. E.P. N.MF, M.P. and K.V. performed *in vivo* experiments. E.P. S.J. and P.W. performed RNAscope and experiment in human samples. E.P., P.PV. and S.B. performed *in vitro* experiments. T.S. and T.C.S. provided the mouse model hAPP$^{NLGF/NLGF}$. E.P. and P.C.S. wrote the manuscript with input from all the authors.

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CONFLICTS OF INTEREST:

The authors declare no conflicts of interest.

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