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Opposing Actions of the Synapse Associated Protein of 97 kDa Molecular Weight (SAP97) and Disrupted in Schizophrenia 1 (DISC1) on Wnt/ β -catenin Signaling

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Abstract

It has been suggested that synapse-associated protein of 97kDa molecular weight (SAP97) is a susceptibility factor for childhood and adult neuropsychiatric disorders. SAP97 is a scaffolding protein that shares direct and indirect binding partners with the *DISC1* gene product, a gene with strong association with neuropsychiatric disorders. Here we investigated the possibility that these two proteins converge upon a common molecular pathway. Since DISC1 modifies Wnt/ β -catenin signaling via changes in GSK3 β phosphorylation, we asked if SAP97 impacts Wnt/ β -catenin signaling and GSK3 β phosphorylation. We find that SAP97 acts as inhibitor of Wnt signaling activity and can suppress the stimulatory effects of DISC1 on β -catenin transcriptional activity. Reductions in SAP97 abundance also decrease GSK3 β phosphorylation. In addition, we find that over expression of DISC1 leads to an increase in the abundance of SAP97, by inhibiting its proteasomal degradation. Our findings suggest that SAP97 and DISC1 contribute to maintaining

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Wnt/ β -catenin signaling activity within a homeostatic range by regulating GSK3 β phosphorylation.

Graphical abstract

Keywords

SAP97; DISC1; Wnt; β -catenin; autism; schizophrenia

Introduction

The postsynaptic density (PSD) is composed of hundreds of proteins that function in the dynamic regulation of synaptic efficacy and plasticity [1]. SAP97 (also known as discs large 1, DLG1) is a PSD protein that binds to the extreme C-terminus of the GluA1 subunit of AMPA-type glutamate receptors [2]. While early *in vitro* work implicated SAP97 in trafficking of GluA1 into synapses [3, 4], subsequent studies (both *in vitro* and *in vivo*) do not support this view [5, 6]. Nonetheless, SAP97 plays an important role in organizing brain circuitry [5].

Several observations suggest that SAP97 could contribute to neuropsychiatric disorders. The 3q29 microdeletion syndrome results from a ~1.6 Mb deletion on the long arm of chromosome 3 spanning 20 genes, including SAP97. Individuals with 3q29 microdeletion disorder display autism and intellectual disability [7], and loss of SAP97 is thought to be at least partially responsible. Genome wide association studies have also identified 3q29 microdeletion as a significant risk factor for schizophrenia, imparting a 17-fold increase in risk [8]. A study of PSD protein expression (e.g., SAP97, PSD95, chapsyn-110, GRIP1 and SAP102) in post-mortem brain tissue from schizophrenic patients revealed a selective decrease in SAP97 abundance in the prefrontal cortex [9]. Single nucleotide polymorphisms in SAP97 have also been linked to an increased risk of schizophrenia in males [10, 11]. *De novo* pathogenic mutations have been found in rare individuals with schizophrenia [12, 13].

The potential mechanism(s) by which SAP97 could contribute to neuropsychiatric disorders is unknown.

The *Disrupted in Schizophrenia-1 (DISC1)* gene has been repeatedly associated with mental disorders (for review see [14–16]). The Tsai lab has shown that *DISC1* modulates Wnt/ β -catenin signaling. Wild-type *DISC1* activates the Wnt/ β -catenin signaling pathway by specifically inhibiting the kinase activity of GSK3 β ; this results in increased abundance of the β -catenin protein and its transcriptional activity [17]. Many mutant versions of *DISC1* associated with cognitive or psychiatric disorders are impaired in their ability to activate Wnt/ β -catenin signaling. In addition, knock down of *DISC1* or expression of mutant *DISC1* can lead to changes in brain development in mouse and zebrafish [18].

For four reasons we considered the possibility that SAP97 may operate in the *DISC1*/Wnt/ β -catenin signaling cascade. First, SAP97 directly interacts with Adenomatous Polyposis Coli protein (APC) [19], which is a component of the β -catenin destruction complex. Second, we identified several two-step connections between SAP97 and *DISC1* using the Schizophrenia gene resource interaction network [20]. Third, the clinical spectrum of cognitive or psychiatric disorders associated with genetic lesions of *DISC1* and SAP97 overlap. Fourth, SAP97 has been shown to modify NMDAR function [21], and NMDAR activation has been implicated in modifying gene expression by β -catenin [22]. Here we show that SAP97 acts as a negative regulator of Wnt/ β -catenin signaling and has an epistatic relationship to *DISC1*. The integration of these two proteins into a single molecular pathway may explain the intriguing overlap in pathologies associated with abnormalities in these genes.

EXPERIMENTAL PROCEDURES

Cell Culture

HEK293 cells were cultured in DMEM containing 10% FBS and penicillin/streptomycin under standard culture conditions. Lipofectamine 2000 (Invitrogen) was used for transfection following the manufacturer's guidelines for DNA transfection and RNA transfection in cases where RNA was present.

Dissociated rat hippocampal neurons were cultured using standard culture techniques as described in [23]. Hippocampi and cortices from E 17–19 rat embryos were removed from anesthetized Sprague-Dawley rats and trypsinized in Dulbecco's minimum essentials medium (DMEM: Lonzo, Whittaker Bioproducts) containing 0.027% trypsin at 37°C, water bath for 15 minutes. Clumps of cells were reduced to single cells by triturations, in media consisting of DMEM (Lonzo) supplemented with 10 % fetal calf serum (Hyclone Labs). The single cell suspension was plated on plastic (Nunc) dishes and maintained in a 37°C humidified 5% CO₂ incubator. For serum-free cultures that inhibit glia growth, the dissociated cells were plated at a density of 100,000 cells/ml in Neurobasal media (Gibco) supplemented with B27 (Gibco). Mitotic inhibitors and antibiotics were not used in the Neurobasal media.

Plasmids

All GFP tagged proteins were expressed in the pEGFP (Clontech) plasmid, myc tagged proteins were expressed in the pGW1 vector. M50 Super 8× TOPflash (TCF reporter) was purchased from Addgene. The sequence for SAP97 shRNA knockdown was 5'-CCCAAATCCATGGAAAATAT -3'. DISC1 siRNA was purchased from Santa Cruz

Luciferase Assay

HEK293 cells growing in 60mm dishes were transfected overnight with 50ng of pRL-SV40, 1ug of M50 Super 8× TOPflash, and 4ug of the plasmid of interest (or 2ug + 2ug for epistasis experiments) using Lipofectamine 2000 overnight. The following day 60mm dishes were trypsinized and replated into 96 well format (Nunc black microwell SI) in media containing 450ng/ml Wnt3a (R&D Systems) or vehicle. The following day firefly and renilla luciferase activity was quantified using the Dual Luciferase Reporter Assay (Promega) and read on a Veritas Microplate Luminometer (Promega). Firefly luciferase activity was normalized to renilla luciferase activity and all values were normalized to the unstimulated control group in each data set.

Quantitative RT PCR

RNA was isolated from hippocampal neuron cultures with the RNeasy plus mini kit (Qiagen). cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR reactions were carried out using Power SYBR Green Master Mix (Applied Biosystems) on a StepOne Plus Real Time PCR System. Relative transcript abundance was determined using the delta delta CT method [24]. All transcript abundances were reported relative to expression of ribosomal S12 (Rps12). See Table 1 for primers used for quantitative RT PCR.

Western blots

For quantitative western blots, cells were lysed in RIPA buffer (150mM NaCl, 50mM Tris pH 8.0, 1mM EGTA, 5mM EDTA, 1% NP40, 0.5% Sodium Deoxycholate, 0.1% SDS) with complete protease and phosphatase inhibitor cocktails. Lysates were separated by denaturing SDS-PAGE and transferred to a nitrocellulose membrane. Western blots were imaged using the Licor Odyssey system and band intensity was quantified using ImageJ. All band intensities were normalized to actin. The following primary antibodies were used for protein detection: SAP97 (UC Davis Neuro Mab clone K64/15 or Abcam ab3437), DISC1 (abcam ab59017), GSK3β (abcam ab93926), pY216- GSK3β (BD transduction 612313), actin (Sigma A2066 or Abcam ab3280), APC (N-APC [25]).

Recombinant Herpes Simplex Virus

Recombinant herpes simplex virus was generated and utilized for neuronal expression as previously described in [26] using the Viral Core Facility at the McGovern Institute for Brain Research at MIT. The miRNA sequence used to target SAP97 was 5'-CAGTGACTGCCT TAAAGAATA-3'.

Recombinant Lentivirus production

DISC1 shRNA that recognizes both rat and mouse, and DISC1 cDNA was cloned into the pLentiLox 3.7 vector as described previously [17]. HEK293 cells were cultured in 10-cm dishes and transfected after reaching 70% confluency. 1 hour prior to transfection, cells were fed with fresh culture medium. For each dish, we mixed plasmids (20 µg lentiviral plasmid together with 5 µg pCMV-VSVG and 5 µg psPax2) and 60 µg Polyethylenimine (PEI) separately in DMEM with a 5-min incubation at room temperature. Then, PEI solution was mixed with the plasmids. After 15 min incubation, this solution was added dropwise to each dish. Six dishes were used for each lentivirus packaging. Transfected cells were incubated for 8 hours in a CO₂ incubator and cultured for additional 48 hours with fresh culture medium. Collected medium were filtered with 0.45 µm filter and centrifuged for 2 hours at 4 °C and 100,000 g. Lentiviral pellet was resuspended in PBS with mild shaking for 8 hours at 4 °C. The viral titer was determined to be 2TU/ml following an established protocol [27].

Inhibition of transcription, translation and proteasomal degradation

24 hours after transfection HEK293 cells were given fresh media containing either 5 µg/ml Actinomycin D, 10 µg/ml cycloheximide, or 10 µM MG132. Cells were grown for 6 hours in the presence of these compounds and then cell lysates were prepared for western blotting, as described previously.

RESULTS

SAP97 Inhibits Wnt/β-Catenin Transcriptional Activity

We began by asking whether manipulation of SAP97 abundance modifies Wnt3a evoked β-catenin transcriptional activity. Changes in SAP97 abundance are documented in Figures 2 and 3 and discussed below. HEK293 cells were cotransfected with TCF/LEF-luciferase (a reporter of β-catenin transcriptional activity), a control luciferase, and either a SAP97 overexpression construct (SAP97-GFP), or a SAP97 knockdown (KD) construct (e.g., shRNA). They were then treated with recombinant Wnt3a or vehicle, and β-catenin transcriptional activity was assessed via the luciferase reporter. We found a significant effect of SAP97 abundance on Wnt3a evoked β-catenin transcriptional activity (Significant interaction between groups by two way ANOVA, $F_{(3,40)}=314.63$ $p<0.001$). Overexpression of SAP97-GFP resulted in a ~50% reduction in Wnt3a-induced β-catenin transcriptional activity compared to cells expressing GFP, while no statistically significant effect of SAP97 was observed in vehicle treated cells (Figure 1 A) (Scheffe's posthoc $p<0.05$ for Wnt3a stimulated cells and $p>0.05$ for unstimulated cells). Conversely, SAP97 KD resulted in a ~50% increase in Wnt3a-induced β-catenin transcriptional activity compared to cells expressing a scrambled shRNA. No statistically significant effect of SAP97 was observed in vehicle treated cells (Figure 1 A) (Scheffe's posthoc $p<0.05$ for Wnt3a stimulated cells and $p>0.05$ for unstimulated cells). These results suggest that SAP97 acts as a negative regulator of Wnt signaling.

Tsai and colleagues reported that DISC1 enhances Wnt3a evoked β-catenin transcriptional activity [17] and we wanted to confirm this. HEK293 cells were cotransfected with TCF/LEF-luciferase, a control luciferase, and either a DISC1 overexpression construct (DISC1-

GFP), or a DISC1 KD construct. They were then treated with recombinant Wnt3a or vehicle, and β -catenin transcriptional activity was assessed via the luciferase reporter. We found a significant effect of DISC1 abundance on Wnt3a evoked β -catenin transcriptional activity (Significant interaction between groups by two way ANOVA, $F_{(3,40)}=75.15$ $p<0.001$). Overexpression of DISC1-GFP resulted in a ~20% increase in Wnt3a-induced β -catenin transcriptional activity compared to cells expressing GFP alone. No statistically significant effect of DISC1 abundance was seen in vehicle treated cells (Figure 1 B) (Scheffe's posthoc $p<0.05$ for Wnt3a stimulated cells and $p>0.05$ for unstimulated cells). Conversely, DISC1 KD resulted in a ~50% reduction in Wnt3a-induced β -catenin transcriptional activity compared to control cells. No significant effect of DISC1 was observed in vehicle-treated cells (Figure 1 B) (Scheffe's posthoc $p<0.05$ for Wnt3a stimulated cells and $p>0.05$ for unstimulated cells). The magnitude of effects of DISC1 OE and KD precisely match those reported by the Tsai lab [18] and further support a role for DISC1 in enhancing Wnt evoked β -catenin transcriptional activity.

Since SAP97 and DISC1 modulate Wnt signaling in HEK293 cells, but in opposite directions, we wondered whether there is an epistatic relationship between them. To this end we investigated Wnt3a induced β -catenin transcriptional activity in HEK293 cells overexpressing both SAP97 and DISC1 simultaneously. HEK293 cells were cotransfected with TCF/LEF-luciferase, a control luciferase, and one of the following plasmid combinations: SAP97-GFP + DISC1-GFP, SAP97-GFP + GFP, or DISC1-GFP + GFP. They were then treated with recombinant Wnt3a or vehicle, and β -catenin transcriptional activity was assessed via the luciferase reporter. Comparing Wnt3a evoked β -catenin transcriptional activity in cells overexpressing both DISC1 and SAP97 to cells overexpressing either DISC1 or SAP97 alone (DISC1-GFP + GFP or SAP97-GFP + GFP), allowed us to determine if the effect of one factor on β -catenin transcriptional activity was dominant over the other. We found a significant reduction in Wnt3a-evoked luciferase activity in DISC1 + SAP97 overexpressing cells in comparison with DISC1 + GFP overexpressing cells (Figure 1 C) (39% reduction, Two factor ANOVA $F_{(2,30)}=9.97$ $p<0.001$, Scheffe's posthoc $p<0.05$). In addition, there was no difference between the Wnt3a-evoked luciferase response in DISC1 + SAP97 overexpressing cells in comparison with SAP97 + GFP overexpressing cells (figure 1 C) (Scheffe's posthoc $p>0.05$). These results indicate a dominant effect of SAP97 on β -catenin transcriptional activity, achieving a similar level of inhibition of β -catenin transcriptional activity in the presence or absence of DISC1. This suggests that SAP97 acts downstream of DISC1 in a single pathway.

In a complementary set of experiments, we investigated whether knockdown of SAP97 had a dominant effect over DISC1 overexpression on β -catenin transcriptional activity. To this end we studied Wnt3a induced β -catenin transcriptional activity in cells overexpressing DISC1 and compared this to cells with knockdown of SAP97 $\pm$ DISC1 overexpression. HEK293 cells were cotransfected with TCF/LEF-luciferase, a control luciferase, and one of the following plasmid combinations: 1) DISC1 + scrambled shRNA, 2) DISC1 + SAP97 shRNA or 3) SAP97 shRNA + scrambled shRNA. They were then treated with recombinant Wnt3a or vehicle, and β -catenin transcriptional activity was assessed via the luciferase reporter. If the effects of SAP97 were dominant to DISC1 then knockdown of SAP97 should yield the maximum possible increase in Wnt3a evoked β -catenin transcriptional activity. In support of

this hypothesis, we found a significantly higher Wnt3a-evoked luciferase activity in DISC1 + SAP97- KD cells than in DISC1 overexpressing cells (Figure 1 D) (41% increase, Two factor ANOVA $F_{(2,30)}=9.65$ $p<0.001$, Scheffe's posthoc $p<0.05$). However, contrary to the idea that SAP97 yields the maximum possible increase in β -catenin transcriptional activity, we also found a greater increase in Wnt3a-evoked β -catenin transcriptional activity in DISC1 + SAP97-KD knockdown cells compared with SAP97 KD cells (Figure 1 D) (83% increase, Scheffe's posthoc $p<0.05$). These results show that the effects of DISC1 overexpression can be additive to the effects of SAP97 knockdown. This suggests that knockdown of SAP97 may be semi-dominant over DISC1, with the greatest increase in Wnt signaling occurring in cells with a reduced level of SAP97 and an increased level of DISC1. These findings are still consistent with SAP97 being downstream of DISC1 in a shared pathway, but they do not exclude the possibility that SAP97 and DISC1 can act in parallel pathways to exert an additive effect on β -catenin transcriptional activity. If they function in the same pathway, the effect of SAP97 knockdown may not be dominant because incomplete knockdown of SAP97 might allow for overexpression of DISC1 to further increase β -catenin transcriptional activity.

One caveat of all luciferase assays is that the expression levels of the luciferase constructs being assayed are influenced not only by experimental manipulation of cellular proteins (i.e. increased expression of SAP97 or knockdown of SAP97) but also by the presence of strong promoters (i.e. CMV, U6, SV40) in the expression vectors used to manipulate cellular protein levels. The presence of multiple copies of these promoters per cell can result in depletion or partitioning of transcriptional factors necessary for efficient expression of the luciferase constructs of interest. For this reason, we took special care to control for matching backbone vectors and total amount of transfected material in the comparison groups. These are the best controlled and most accurate comparisons to make, and between-panel comparisons of absolute values are likely to be flawed. For this reason, it is important to focus on the relative changes in Wnt3a-induced luciferase reporter activity as opposed to the absolute level of TCF/LEF induction in any set of experiments.

SAP97 Alters Expression of β -catenin Transcriptional Targets in Hippocampal Neuron Cultures

While our results using the TCF/LEF luciferase reporter in HEK293 cells provide evidence that SAP97 is an inhibitor of the Wnt/ β -catenin signaling pathway, use of a reporter construct inherently suffers from the drawbacks noted above, and does not reveal the relevance of this phenomena to neurons. To determine if SAP97 acts as an endogenous inhibitor of Wnt/ β -catenin signaling, we asked if overexpression or knockdown of SAP97 in neurons affects endogenous β -catenin transcriptional targets. We examined a group of potential β -catenin targets by RT-qPCR in rat primary hippocampal neurons after overnight stimulation with Wnt3a. Based on the literature, we chose to screen Axin2, c-Myc, CyclinD1, Lef1, and Sox4 [28–32] (Figure 2A). A two-factor ANOVA revealed a significant interaction between Wnt3a treatment and gene expression ($F_{(4,30)}=61.83$ $p<0.001$). We found that overnight stimulation with Wnt3a led to a significant upregulation of Axin2, cMyc, and CyclinD1 mRNA compared to vehicle treated cells (Scheffe's posthoc $p<0.05$).

The levels of Lef1 and Sox4 mRNA were not affected by overnight Wnt3a treatment in these cells (Scheffe's posthoc $p>0.05$).

To determine if SAP97 can modify transcription of these β -catenin target genes, we engineered recombinant herpes simplex viruses (HSV) to express LACZ, SAP97, a SAP97 targeting miRNA, and a scrambled control miRNA. We confirmed that infection with these HSVs resulted in the expected changes in SAP97 abundance by western blotting lysates of hippocampal cultures 48 hours post infection. Infection with HSV-SAP97 resulted in a significant increase in SAP97 expression compared to HSV-LACZ, while infection with HSV-SAP97 miRNA resulted in a significant decrease in SAP97 abundance compared to HSV-control miRNA (Figure 2B).

Next we coupled HSV infection with Wnt3a treatment to determine if modifying SAP97 abundance could alter Wnt3a induced β -catenin transcription of these genes. Hippocampal neurons were infected with HSV-control miRNA or HSV-SAP97 miRNA for 48 hours and then treated with Wnt3a overnight. A two-factor ANOVA revealed a significant interaction between SAP97 abundance and gene expression ($F_{(4,30)}=28.48$ $p<0.001$). For those transcripts that showed increases in abundance in uninfected neurons after Wnt3a treatment (Axin2, cMyc, and CyclinD1), we found enhanced transcription after Wnt3a treatment in neurons where SAP97 had been knocked down (Scheffe's posthoc $p<0.05$) (Figure 2C). SAP97 knockdown did not alter the transcription of Lef1 or Sox4, transcripts which did not show enhanced expression after Wnt3a treatment in uninfected neurons (Scheffe's posthoc $p>0.05$). These results suggest that endogenous SAP97 suppressed Wnt3a-dependent transcription of specific β -catenin targets, consistent with our data from the TCF/LEF luciferase reporter in HEK293 cells.

Next, hippocampal neuron cultures were infected with HSV-LACZ or HSV-SAP97 for 48 hours and then treated with Wnt3a overnight. A two-factor ANOVA revealed a significant effect of SAP97 on gene expression ($F_{(4,30)}=4.08$ $p<0.05$). All five genes assayed showed decreased expression when SAP97 was overexpressed (Scheffe's posthoc $p<0.05$) (Figure 2D), indicating that Wnt-dependent transcription of β -catenin targets can be blocked by raising the level of SAP97. While decreased expression of Axin2, cMyc and CyclinD1 complements our observations with SAP97 knockdown, the effects on Lef1 and Sox4 transcription were unexpected. The transcription of these genes was not enhanced by Wnt3a treatment in uninfected neurons, but was inhibited after Wnt3a treatment by SAP97 overexpression. One possibility is that Lef1 and Sox4 may be maximally transcribed in the absence of exogenously provided Wnt3a. Alternatively, transcription of Lef1 and Sox4 may require other transcription factors in addition to β -catenin in order to be transcribed. If this were the case, inhibition of β -catenin by SAP97 might inhibit transcription of these genes, while activation of β -catenin by SAP97 knockdown might not be sufficient to enhance their transcription alone. Overall, these results are consistent with our results using the TCF/LEF luciferase reporter in HEK293 cells, and suggest that SAP97 is an inhibitor of β -catenin mediated transcription.

Potential molecular mechanism for SAP97 regulation of DISC

The β -catenin destruction complex is composed of Axin, APC, GSK3 β and casein kinase-1 (CKI). Given the physical association of SAP97 with APC [19] and the involvement of APC in the β -catenin destruction complex, we asked if SAP97 might be modifying β -catenin signaling through changes to APC. Hippocampal neurons were infected with HSV-LACZ, HSV-SAP97, HSV-control miRNA or HSV-SAP97 miRNA. 72 hours after infection cell lysates were generated and western blots were performed to assess APC levels. We found a decrease in APC abundance after overexpression of SAP97 (~40% reduction, Students t-test $p < 0.05$) and an increase in APC abundance with KD of SAP97 (~30% increase, Students t-test $p < 0.01$) (figure 3 A & B). When complexed with Axin and GSK3 β , APC acts to destabilize β -catenin and inhibit β -catenin transcription. The changes that we observe in APC abundance after overexpression or knockdown of SAP97 are therefore unlikely to mediate the effects of SAP97 on β -catenin transcription, as they are opposite to the changes one would predict if APC were responsible for mediating the observed effect of SAP97 on β -catenin activity.

A critical regulatory step in the targeting of β -catenin for degradation is its phosphorylation on serine 45 (S45) by CKI and serines 33 and 37 (S33/37) and threonine 42 (T42) by GSK3 β . Phosphorylation at these sites targets β -catenin for degradation [33]. DISC1 is thought to increase Wnt signaling by specifically inhibiting GSK3 β at tyrosine 216 (Y216) [17]. In light of this, we asked whether SAP97 influences the activity of GSK3 β (monitored by its state of phosphorylation on Y216). We biochemically interrogated components of this signaling pathway with or without SAP97 knockdown in HEK293 cells (figure 3C). First, we were able to confirm the efficacy of the SAP97 shRNA (~40% reduction in SAP97, Students t-test $p < 0.05$) and this was associated with no change in DISC1 abundance. Second, we found a significant reduction of phosphorylated Y216 of GSK3 β in cells with SAP97 knockdown, in comparison with controls, but no change in the abundance of GSK3 β itself (~35% reduction in pY216 GSK3 β , Students t-test $p < 0.05$). These observations suggest that SAP97, like DISC1, may mediate its effects on Wnt signaling by modifying the phosphorylation state of GSK3 β .

Increased Expression of DISC1 Increases SAP97 Abundance

Our epistasis experiments indicate that SAP97 could act downstream of DISC1 in a signaling cascade. Given our observation that knockdown of SAP97 increases GSK3 β phosphorylation, this raised the possibility that DISC1 might alter GSK3 β phosphorylation by influencing the abundance of SAP97. If true, it leads to the prediction that increasing DISC1 abundance would reduce SAP97 abundance. Contrary to this formulation, we found that overexpression of DISC1 in HEK293 cells robustly increased the abundance of both endogenous SAP97 and overexpressed myc-SAP97 (figure 4 A & B, respectfully). This effect was specific since DISC1 overexpression had no effect on the expression of a different PSD protein, Protein Interacting with PRKCA 1 (PICK1 figure 4 C). Consistent with the lack of effect of SAP97 knockdown on DISC1 levels (figure 3 C), overexpression of SAP97 had no effect on DISC1 abundance (Figure 4 B). Overall these observations suggest that DISC1 does not regulate Wnt signaling activity through modification of SAP97 abundance. This is consistent with previous results that DISC1 directly interacts with GSK3 β in order to

modulates β -catenin levels [17]. Instead, taken with our previous data, our findings suggest another level of regulation of DISC1-mediated Wnt signaling. When levels of the GSK3 β inhibitor DISC1 increase, so does the abundance of the GSK3 β activator SAP97. This may act as a brake on DISC1 induced increases in Wnt activity in order to keep GSK3 β phosphorylation and Wnt signaling within a discrete homeostatic range.

To extend this observation, we attempted to determine if this effect was detectable in neurons. Hippocampal neurons were infected with HSV-LACZ, HSV-SAP97, HSV-control miRNA, or HSV-SAP97 miRNA. Consistent with our findings in HEK293 cells, we found no effect of SAP97 overexpression or knockdown on DISC1 abundance (Figure 4 D). Next we engineered a lentivirus to overexpress DISC1. Hippocampal neurons infected with lenti-DISC1 showed elevated levels of DISC1, as well as increased abundance of SAP97 (Figure 4 E). Thus, as seen in HEK293 cells, overexpression of DISC1 increased the abundance of SAP97, while modifying SAP97 levels had no effect on DISC1 abundance.

In principal, DISC1 could increase the abundance of SAP97 by increasing transcription of SAP97, increasing translation of SAP97 mRNA, or decreasing degradation of SAP97. To determine the mechanism by which DISC1 overexpression increased the abundance of SAP97 we compared cells transfected with SAP97 + GFP versus cells transfected with SAP97 + DISC1 under one of four different treatment conditions 1) vehicle, 2) Actinomycin D (ActD), to suppress RNA transcription, 3) cycloheximide (CHX), to inhibit translation, or 4) MG-132, to inhibit proteasomal protein degradation (figure 4 F). HEK293 cells were transfected with these constructs, allowed to recover for 24 hours and then treated for six hours with active agent or vehicle. As shown above (Figure 4, B), co-expression of GFP-DISC1 with myc-SAP97 leads to a robust increase in the abundance of myc-SAP97 (Figure 4, panel F, lanes 1 versus 2).

If DISC1 were acting to increase the abundance of SAP97 by enhancing transcription of SAP97, ActD should suppress the effect of DISC on SAP97 abundance by blocking transcription. If true, this would decrease the difference in SAP97 abundance between the myc-SAP97 + GFP and myc-SAP97 + DISC1 expressing cells. Inconsistent with this prediction, cells treated with ActD show an increased difference in SAP97 abundance between groups when compared to vehicle treated cells (Cf. Figure 4, panel F, lanes 4 versus 3 compared with lanes 2 versus 1). These observations suggest that the increased abundance of SAP97 when co-expressed with DISC1 is not due to DISC1-dependent enhancement of SAP97 transcription.

If DISC1 were acting to increase the abundance of SAP97 by enhancing SAP97 translation, CHX should suppress the effect of DISC on SAP97 abundance by blocking translation. If true, this would decrease the difference in SAP97 abundance between the myc-SAP97 + GFP and myc-SAP97 + DISC1 expressing cells. Inconsistent with this prediction, cells treated with CHX show an increased difference in SAP97 abundance between groups when compared to vehicle treated cells (Cf., Figure 4, panel F, lanes 6 versus 5 compared with lanes 2 versus 1). These observations suggest that the increased abundance of SAP97 when co-expressed with DISC1 is not due to DISC1-dependent enhancement of SAP97 translation.

If DISC1 were acting to increase the abundance of SAP97 by inhibiting SAP97 protein degradation, MG132 should mimic the effect of DISC1 on SAP97 by blocking proteasomal protein degradation. If true, one would expect the difference in SAP97 abundance between myc-SAP97 + GFP and myc-SAP97 + DISC1 expressing cells to decrease after treatment with MG-132. Consistent with this prediction, cells treated with MG132 have a decreased difference in SAP97 abundance between groups when compared to vehicle treated cells (Cf., Figure 4, panel F, lanes 8 versus 7 compared lanes 2 versus 1). This suggests that DISC1 increases the abundance of SAP97 by inhibiting its proteasomal degradation.

DISCUSSION

We find that SAP97 is a negative regulator of the Wnt/ β -catenin signaling pathway. The biochemical actions of SAP97 on Wnt/ β -catenin signaling are opposite those of DISC1, with both proteins modifying the phosphorylation state of GSK3 β , a critical component of the β -catenin destruction complex. DISC1 also stimulates the accumulation of SAP97, through inhibition of its proteasomal degradation, and this likely counteracts some of the effects of DISC1 on GSK3 β phosphorylation and Wnt/ β -catenin signaling. The data suggest that SAP97 acts as a brake on DISC1-induced increases in β -catenin transcriptional activity in order to maintain Wnt/ β -catenin signaling within a narrow range.

GSK3 β appears to be a common target through which DISC1 and SAP97 modify Wnt signaling activity. DISC1 has previously been described as a specific inhibitor of GSK3 β . Since knockdown of SAP97 leads to decreased GSK3 β phosphorylation, endogenous SAP97 probably acts as an activator of GSK3 β . Whether DISC1 inhibition of GSK3 β activity depends on SAP97 (e.g. antagonizing its effect on GSK3 β activity) is not known. It is worth noting that SAP97 physically interacts with APC and APC is a key scaffolding protein of the β -catenin destruction complex. This suggests that the regulation of GSK3 β phosphorylation by SAP97 may be occurring at the level of the destruction complex.

In summary, we identify a novel role for SAP97 in modulating Wnt/ β -catenin signaling, we identify GSK3 β as the target through which SAP97 influences Wnt/ β -catenin signaling, and demonstrate that increased abundance of DISC1 leads to increased abundance of SAP97. These findings are of significant importance because they establish two proteins associated with a shared set of neuropsychiatric disorders as members of a signaling pathway. They also suggest a possible feedback mechanism between DISC1 and SAP97 designed to maintain GSK3 β phosphorylation and Wnt/ β -catenin signaling activity within a homeostatic range.

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Abbreviations

SAP97	synapse associated protein of 97 kDa molecular weight
DISC1	disrupted in Schizophrenia 1
PSD	postsynaptic density
DLG1	discs large 1
GSK3β	glycogen synthase kinase 3 beta
APC	adenomatous polyposis coli protein
CKI	casein kinase-1
HEK	human embryonic kidney
TCF	T-cell factor
LEF	lymphocyte enhancer-binding factor

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Highlights

- SAP97 is a novel inhibitor of Wnt induced β -catenin transcriptional activity.
- SAP97 may mediate this activity through phosphorylation of GSK3 β .
- Increased DISC1 expression stabilizes SAP97.

Figure 1. SAP97 inhibits Wnt3a induced β -catenin transcriptional activity in HEK cells
 Values represent the mean of TCF/LEF firefly luciferase intensity normalized to renilla SV40 luciferase intensity $\pm$ SEM (n=6, * = $p < 0.05$ by scheffe's post hoc).

Figure 2. SAP97 inhibits Wnt3a induced β -catenin transcriptional activity in hippocampal neurons

A) Relative quantitation of transcript abundance by qPCR in uninfected hippocampal neuron cultures treated overnight with 100 ng/ml Wnt3a or vehicle (n=4, $\ast$ =p<0.05 by scheffe's post hoc). B) Representative western blots from hippocampal neuron cultures infected with HSV-control miRNA, HSV-SAP97 miRNA, HSV-LACZ, or HSV-SAP97, blotted for SAP97 and Actin. C) Relative quantitation of transcript abundance by qPCR in HSV-control miRNA and HSV-SAP97 miRNA infected hippocampal neuron cultures treated overnight with 100 ng/ml Wnt3a or vehicle (n=4, $\ast$ =p<0.05 by scheffe's post hoc). D) Relative quantitation of transcript abundance by qPCR in HSV-LACZ and HSV-SAP97 infected hippocampal neuron cultures treated overnight with 100 ng/ml Wnt3a or vehicle (n=4, $\ast$ =p<0.05 by scheffe's post hoc).

Figure 3. Changes in SAP97 abundance lead to alterations in APC levels and GSK3 β phosphorylation at tyrosine-216

A) Representative western blots of APC and actin from HSV infected neurons where SAP97 is either overexpressed or knocked down. B) Quantification of normalized band intensities (n=3). C) Representative western blots from HEK cells transfected with scrambled shRNA or SAP97 shRNA. Band intensities were normalized to actin and quantified (n=3).

Figure 4. DISC1 stabilizes SAP97 in HEK cells

A) Overexpression of GFP-DISC1 increases the abundance of endogenous SAP97 as compared to overexpression of GFP. B) Overexpression of GFP-DISC1 increases the abundance of myc-SAP97 but expression of myc-SAP97 has no effect on the abundance of GFP-DISC1. C) Overexpression of GFP-DISC1 has no effect on the abundance of myc-PICK1. D) Hippocampal neurons were infected with HSV-SAP97 or HSV-miRNA SAP97 and appropriate controls. Representative western blots showing no alteration to DISC1 abundance after overexpression or knockdown of SAP97 (n=3). E) Representative western blot showing Hippocampal neurons infected with lenti-DISC1 effectively overexpress DISC1, which leads to an increase in the abundance of SAP97 (n=3). F) Cells were transfected with either myc-SAP97 + GFP or myc-SAP97 + GFP-DISC1 and then treated with vehicle, Actinomycin D (Act D), Cycloheximide (CHX), or MG-132. Proteasomal inhibition via MG-132 treatment results in the smallest difference in myc-SAP97 abundance, suggesting that DISC1 enhances SAP97 abundance by inhibiting its proteasomal degradation.

Table 1

Transcript

Name	Primers
Axin2	5' TGACTCTCCTTCCAGATCCAA 3' 5' TGCCCACGCTAGGCTGACA 3'
cMyc	5' TGGAGTGAGAAGGGCTTTGCCT3' 5' TGTGGGTCAGCGGGAGGAT 3'
CyclinD1	5' TGCAAATGGAACTGCTTCTG 3' 5' CGGATGGTCTGCTTGTCTC 3'
Lef1	5' CCCCGAAGAGGAGGGCGACT 3' 5' TCCGACCACCTCATGCCCGTT 3'
Sox4	5' GACCTGCTCGACCTGAACC 3' 5' ACTCCAGCCAATCTCCCGA 3'
Rps12	5'- AAATCGATCGAGAGGGGAAG -3' 5'- CTTGGCCTGAGATTCTTTGC -3'