THESIS

AUTISM-ASSOCIATED δ-CATENIN G34S MUTATION PROMOTES GSK3β-MEDIATED PREMATURE δ-CATENIN DEGRADATION INDUCING NEURONAL DYSFUNCTION

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ABSTRACT

AUTISM-ASSOCIATED δ-CATENIN G34S MUTATION PROMOTES GSK3β-MEDIATED PREMATURE δ-CATENIN DEGRADATION INDUCING NEURONAL DYSFUNCTION

δ-catenin is a crucial component of a synaptic scaffolding complex, which regulates synaptic structure and function in neurons. Loss of δ-catenin function is strongly associated with severe autism spectrum disorder (ASD) in female-enriched multiple families. In particular, a G34S (Glycine 34 to Serine) mutation in the *δ-catenin* gene has been identified in ASD patients and suggested to exhibit loss-of-function. The G34S mutation is located in the amino terminal region of δ-catenin, where there are no known protein interaction domains and post-translational modifications. Notably, the Group-based Prediction System predicts that the G34S mutation is an additional target for GSK3β-mediated phosphorylation, which may result in protein degradation. Therefore, we hypothesize the G34S mutation accelerates δ-catenin degradation, resulting in loss of δ-catenin function in ASD. Indeed, we found significantly lower G34S δ-catenin levels compared to wild-type (WT) δ-catenin when expressed in cells lacking endogenous δ-catenin, which is rescued by genetic inhibition of GSK3β. By using Ca^{2+} imaging in cultured mouse hippocampal neurons, we further revealed overexpression of WT δ-catenin is able to significantly increase neuronal Ca^{2+} activity. Conversely, Ca^{2+} activity remains unaffected in G34S δ-catenin overexpression, which is reversed by pharmacological inhibition of GSK3β using lithium. This suggests the G34S mutation of δ-catenin provides an additional GSK3β-mediated phosphorylation site, which could promote δ-catenin premature degradation, resulting in loss-of-function effects on neuronal Ca^{2+} activity in ASD. In addition, inhibition of GSK3β activity is able to reverse G34S-induced loss of δ-catenin function. Thus, inhibition of GSK3β may be a potential therapeutic treatment for δ-catenin-associated ASD patients.

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Next, I would like to acknowledge the Kim Lab, which includes Jiayi for taking care of our mice, Matt helped me with many various aspects of the δ-catenin project, Julie helped me troubleshoot to figure out why protocols weren't working for me or technical problems on GraphPad Prism, and last but not least, my advisor Dr. Seonil Kim who has taught me that you need to be a high functioning sociopath to be a great scientist and has helped me in many aspects with my journey here at Colorado State University along with some future endeavors. I am also very grateful for my committee members, Dr. James Bamburg and Dr. Susan Tsunoda, who have provided very helpful critiques and advice to develop my thesis project to where it is today. In addition, I would like to thank Dr. Mike Tamkun for replenishing our HEK293 cells and Dr. Edward Ziff for the δ-catenin plasmids that really got this project started.

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INTRODUCTION

Autism spectrum disorder (ASD) is a multifactorial neurodevelopmental disorder that begins early in life and is highly heterogeneous in symptom presentation and etiology (1, 2). Restricted and repetitive behavior and impairment in sociability and communication are the two characteristics present to be diagnosed as ASD, but the broad range of the symptom severity may be minor to intense (1). Thus, the characterization of ASD is difficult due to the high comorbidity, as presentation of other psychiatric diagnoses is common in ASD such as Attention Deficit and Hyperactivity Disorder (ADHD), anxiety, depression, and intellectual disability (1). Importantly, there are a large number of the ASD-risk genes identified from various genetic studies that are involved with synaptic activity and plasticity (3-8). This suggests that ASD may result from deficits occurring in the synapse. Significantly, the *δ-catenin* gene is associated with severe autism in female-enriched multiple families (2-4). It has also been found to have more deleterious variants, copy number variations (CNVs), and *de novo* mutations associated with ASD (2, 5). Some of the ASD-associated δ-catenin missense mutations are unable to rescue loss of excitatory synapse density in hippocampal neurons lacking the *δ-catenin* gene, suggesting these δ-catenin mutations induce loss-of-function (2). However, the cellular mechanisms of how ASD mutations cause loss of δ-catenin function in the synapse remain unclear.

δ-catenin is an armadillo repeat protein that is highly prevalent in the brain (6). It is able to adhere to the intracellular side of the postsynaptic membrane by its armadillo-repeat interaction domain with N-cadherin, a synaptic cell-adhesion protein (7, 8) (**Fig. 1**). In addition, the PDZ-binding domain located in the δ-catenin carboxyl-terminal end is able to connect to a glutamate receptor interacting protein (GRIP) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-binding protein (ABP) (8-11) (**Fig. 1**). The resulting N-cadherin-δ-catenin-ABP/GRIP complex functions as an anchorage for a glutamate AMPAR

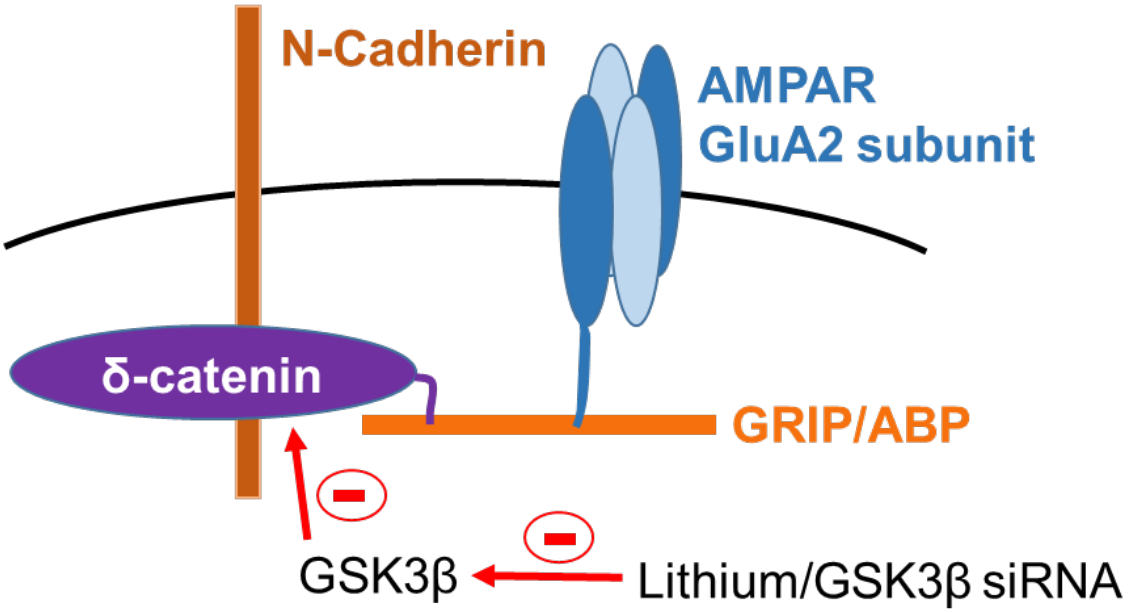

Figure 1. A schematic of N-cadherin-δ-catenin-ABP/GRIP-GluA2 synaptic complex and GSK3β regulation of δ-catenin. The AMPAR GluA2 subunit is tethered to the synaptic membrane by its carboxyl-tail interaction with the GRIP/ABP's PDZ domain. δ-catenin scaffolds to N-cadherin and GRIP/ABP by binding to another of GRIP/ABP's PDZ domain located in amino-terminal region. GSK3β phosphorylates δ-catenin, which leads to δ-catenin degradation. Pharmacological or genetic reduction of GSK3β activity by lithium or siRNA stabilizes N-cadherin-δ-catenin-ABP/GRIP-GluA2 complex.

subunit GluA2 (10) (**Fig. 1**), suggesting δ-catenin plays crucial roles in synaptic structure and function. In fact, δ-catenin homozygous knockout (KO) mice show deficits of hippocampus-dependent learning and memory with disrupted short and long-term synaptic plasticity in the hippocampus (12). δ-catenin KO mice also exhibit a decrease in dendritic arbor size, segment number, tip number, and branching complexity with reduced levels of N-cadherin and GluA2 (12-14). Knockdown of δ-catenin by shRNA in cultured rat pyramidal neurons is accompanied by reduction of spine head width and length (11), which further supports that δ-catenin is important in spine architecture and synaptic plasticity.

Notably, there are genetic links between δ-catenin and other neurological disorders. For example, a yeast-two-hybrid experiment reveals δ-catenin's interaction with the Alzheimer's disease-related protein presenilin 1, affecting senile plaque formation, a pathological hallmark in the disease (15, 16). Epilepsy is commonly associated with ASD occurring in about one third of

ASD individuals (17), and loss of δ-catenin function is also linked to familial cortical myoclonic tremor and epilepsy (FCMTE) (18). Furthermore, Cri-du-chat syndrome is a neurodevelopmental disorder associated with variable hemizygous deletions in the short arm of human chromosome 5p where the *δ-catenin* gene is located (19). Symptoms of Cri-du-chat syndrome include delayed development, severe mental retardation, and verbal skill impairment (20). About 40% of individuals with the syndrome exhibit autistic-like behaviors (21). Importantly, genetics studies of Cri-du-chat syndrome and ASD both identify loss of δ-catenin function (3, 22). Moreover, δ-catenin-associated proteins have been found to be associated with ASD (23, 24), including N-cadherin (25), GRIP (26), and AMPARs (27), which further implicates crucial roles of δ-catenin in the etiology of ASD. This suggests loss of δ-catenin function may be a strong candidate for ASD pathophysiology.

Glycogen synthase kinase 3 beta (GSK3β) is a serine/threonine kinase that plays an important role in many cellular processes (28). For instance, GSK3β is a key regulator of synaptic plasticity with involvement in N-methyl-D-aspartate receptor (NMDAR)-dependent long-term depression (LTD) and long-term potentiation (LTP) (29). Notably, GSK3β regulates the stability of the armadillo-repeat protein family members, including β-catenin, p120-catenin and δ-catenin (30-32). In particular, regulation of β-catenin has been extensively studied. β-catenin turnover is initiated through a multiple protein complex called the β-catenin destruction complex, which consists of the scaffolding protein Axin, adenomatous polyposis coli (APC), casein kinase I-alpha (CKIα), and GSK3β (33, 34). CKIα performs the first priming phosphorylation on β-catenin residue serine 45 (S45), which triggers subsequent β-catenin phosphorylation by GSK3β on β-catenin residues serine 33 and 37 (S33 and S37) and threonine 41 (T41) to target β-catenin for ubiquitination and subsequent proteasomal degradation (30, 35). Importantly, recent studies suggest that changes in GSK3β activity may be an important aspect of ASD pathogenesis (36-43). However, extensive studies have yielded inconsistent data on the role of GSK3β activity in the molecular and behavioral effects. For example, elevated GSK3β activity is

responsible for the ASD-related phenotypes in the mouse model of fragile X mental retardation (FMR) (36-39). Conversely, inactivation of GSK3β is associated with the ASD-related phenotypes found in deletion of the *phosphatase and tensin homolog on chromosome ten (PTEN)* gene in mice (40). Therefore, further studies are needed in order to better understand the roles of GSK3β in ASD pathogenesis. Nonetheless, GSK3β inhibitors may be useful therapeutic interventions for brain disorders because GSK3β has been linked to cognitive processes (44). Pharmacological inhibition of GSK3β can occur by lithium treatment, which can reduce GSK3β activity by increasing the Akt-dependent phosphorylation of the autoinhibitory serine 9 on GSK3β (45) or by being a competitive inhibitor with respect to magnesium binding to GSK3β (44). Reduction of GSK3β activity by lithium in hippocampal neurons *in vitro* and *in vivo* is able to increase levels of β-catenin, δ-catenin, and δ-catenin-associated synaptic complex proteins including GRIP and AMPARs, which supports the findings of increased amplitude of miniature excitatory postsynaptic currents (EPSCs) when lithium treatment was applied to neurons (46). Therefore, an increase in δ-catenin at synapses by pharmacological inhibition of GSK3β activity may provide a cellular mechanism of novel therapeutic effects for loss of δ-catenin function in ASD.

One *δ-catenin* gene missense mutation associated with ASD is glycine 34 to serine (G34S). G34S δ-catenin is unable to rescue the number of excitatory synapses in cultured hippocampal neurons lacking δ-catenin, thus exhibiting loss of δ-catenin function (2). The cellular mechanisms of how G34S δ-catenin induces loss-of-function are unknown. Intriguingly, the amino-terminal region of δ-catenin around G34 has not been reported to contain major post-translational modification sites or protein interaction domains (47). GSK3β-mediated phosphorylation of threonine 1078 (T1078) toward the carboxyl end in δ-catenin targets δ-catenin for ubiquitination then proteasome-mediated degradation (35, 48) (**Fig. 2**). Conversely, GSK3β phosphorylates residues S33, S37 and T41 on the amino terminus side in β-catenin (49) (**Fig. 2**). Similar to β-catenin, p120-catenin has GSK3β phosphorylation sites in the amino

terminus, which induces ubiquitination and destabilization of p120-catenin (31). Importantly, the Group-Prediction System (http://gps.biocuckoo.org) (50) predicts that the G34S mutation can be a target for GSK3β, allowing δ-catenin to have multiple serine residues in the amino-terminal end that mimics GSK3β-mediated phosphorylation of β-catenin (**Fig. 2**). In addition to T1078 in the carboxyl-terminus, these potential sites for GSK3β-mediated phosphorylation in the amino-terminal region may accelerate δ-catenin degradation, inducing loss of δ-catenin function.

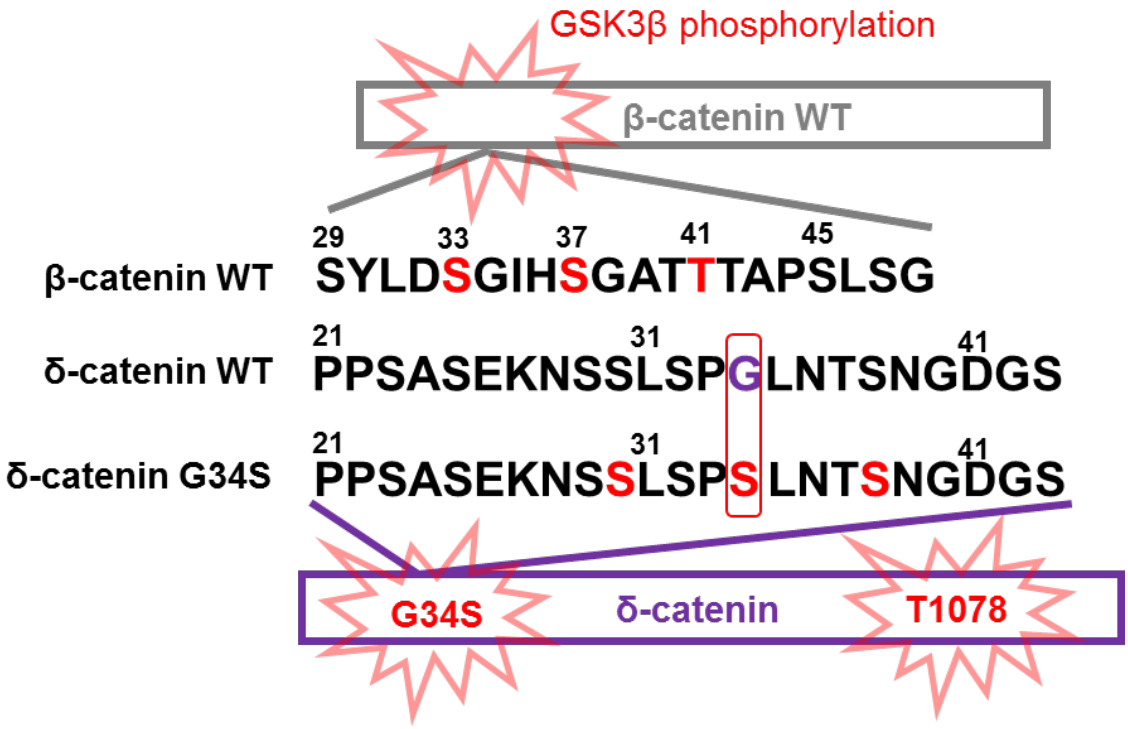

Figure 2. The δ-catenin G34S mutation may add an additional GSK3β-mediated phosphorylation site to induce degradation. GSK3β phosphorylation sites of β-catenin in the amino-terminal region known to induce proteasomal degradation highlighted in red (S33, S37, and T41) are comparable to possible GSK3β phosphorylation sites in the amino-terminus of G34S mutant δ-catenin also highlighted in red (S30, S34, and S38). In addition to GSK3β WT δ-catenin T1078 phosphorylation site in the carboxyl-terminus, these potential amino-terminal GSK3β-mediated phosphorylation sites may accelerate δ-catenin degradation.

Therefore, we hypothesize that the ASD-associated G34S δ-catenin mutation promotes premature degradation via GSK3β-mediated additional phosphorylation, thus causing disruption of synaptic structure and function in ASD. Inhibition of GSK3β may reverse loss of δ-catenin-induced synaptic dysfunction in ASD. In this study, we have found significantly lower δ-catenin

protein levels in cells expressing G34S δ-catenin compared to wild-type (WT) δ-catenin, which is rescued by genetic knockdown of GSK3β. By using Ca^{2+} imaging in cultured mouse primary hippocampal neurons, we further revealed the overexpression of WT δ-catenin is able to significantly increase neuronal Ca^{2+} activity, but Ca^{2+} activity remains unaffected in G34S δ-catenin overexpression, indicating loss of δ-catenin function. More importantly, G34S δ-catenin-induced loss-of-function effects on Ca^{2+} activity is reversed by pharmacological inhibition of GSK3β using lithium. Together, this data provides a novel cellular mechanism of ASD and identifies a potential therapeutic target for δ-catenin-associated ASD patients.

MATERIALS AND METHODS

Cloning

pSinRep5-WT δ-catenin and pSinRep5-G34S δ-catenin plasmids were gifts from Dr. Edward Ziff (New York University Langone Medical Center). HA-tagged WT δ-catenin and G34S δ-catenin were cloned into the mammalian expression vector, pcDNA3.1. The QuikChange XL Site-Directed Mutagenesis Kit (Agilent) was used to generate G34A and G34D mutations from the WT δ-catenin plasmid. The following primers were used with the bolded regions being where the mutations were made to exchange the glycine (GGC): G34A primers 5'-gctccttgagccca***gcc***ttaaacacctccaa-3' and 5'-ttggaggtgtttaaggctgggctcaaggagc-3', and the G34D primers 5'-cagctccttgagccca***gac***ttaaacacctccaatg-3' and 5'-cattggaggtgtttaagtctgggctcaaggagctg-3'.

Cell Culture and Transfection

Human embryonic kidney cells (HEK293) were cultured in DMEM L-Glutamine medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/Streptomycin (Life Technologies) with 37^{o}C humidified 5% CO_2. 500,000 cells were plated in 6-well dishes and 1μg of DNA was transfected when cells reached 75-85% confluency with Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. 25nM control siRNA ON-TARGETplus Non-targeting pool (Dharmacon) and SMARTpool ON-TARGETplus human GSK3β siRNA (Dharmacon) were transfected with 1μg of DNA when cells reached 50% confluency and cell lysates were collected 72 hrs later.

Reagents

Proteasome inhibitor MG132 (Allfa Aesar) was used at 10μM to treat HEK293 cells 1 hr after DNA transfection and incubated for 16 hrs before cell lysis. GSK3β pharmacological inhibitor lithium chloride (LiCl) was used at 2mM to treat cultured hippocampal neurons 16-19 hrs before live Ca^{2+} imaging was performed.

Immunoblotting

Whole cell lysates (20μl /100μl total cell lysate) were loaded onto 10% SDS-PAGE gel and subjected to electrophoresis at a constant 125V. Proteins were then transferred to nitrocellulose membrane at 20V for overnight. Membranes were blocked with 5% non-fat milk and blotted with anti-δ-catenin (BD Biosciences, 1:1000), anti-GSK3β (Cell Signaling Technology, 1:1000), and anti-actin (Abcam, 1:2000). Secondary antibodies used were goat polyclonal anti-mouse antibody (Abcam, 1:4000) and goat polyclonal anti-rabbit antibody (Abcam, 1:4000). Immunoblots were developed with Enhanced Chemiluminescence (ECL) (ThermoScientific). Blots were chosen in the middle of a linear time of exposure and quantified by using NIH ImageJ.

Hippocampal Mouse Neuron Culture

The mouse hippocampal neuron cultures were prepared as previously described (51-54). C57BI6J mouse (Jackson laboratory) hippocampal neurons were dissected from postnatal day 0 (P0) pups. The tissue isolated was digested with 10U/mL papain (Worthington Biochemical Corp) for 15 mins and resuspended in DMEM/F12 Medium (Life Technologies) containing 5% Horse Serum, 5% FBS, 1.5% HEPES, and 1% penicillin/streptomycin (Life Technologies). 500,000 cells were plated on poly lysine-coated glass bottom dishes (Matsunami) for 1.5 hrs in the DMEM/F12 medium in a 37°C humidified 5% CO_2 incubator. The medium was then exchanged with Neurobasal Medium (Life Technologies), 0.5mM Glutamax

(Life Technologies), B27 supplement (Life Technologies), and 1% penicillin/streptomycin (Life Technologies) and cultures were grown until live Ca^{2+} imaging was performed on *in vitro* (DIV) 12-14. The animal care and protocol (16-6779A) was approved by Colorado State University's Institutional Animal Care and Use Committee.

GCaMP Calcium Imaging

DIV4 neurons plated on glass bottom dishes were transfected with either pGP-CMV-GCaMP6f (a gift from Douglas Kim, Addgene plasmid #40755; http://n2t.net/addgene:40755;RRID:Addgene_40755) (55) for imaging of hippocampal pyramidal excitatory neurons or pAAV-mDLX-GCaMP6f-Fishell-2 (a gift from Gordon Fishell, Addgene plasmid # 83899; http://n2t.net/addgene:83899; RRID:Addgene_83899) (56) for imaging of interneurons using Lipofectamine 2000 (Life Technologies) and following the manufacturer's instructions. After transfection, the neurons were grown in Neurobasal Medium without phenol red (Life Technologies) with B27 supplement (Life Technologies), 0.5mM Glutamax (Life Technologies) and 1% penicillin/streptomycin (Life Technologies) for 8-10 days and during imaging. Transfection efficiency was about 2% and near low to no signs of toxicity including swollen neurites and cell body shrinkage. Imaging on an Olympus IX73 microscope was performed when glass bottom dishes were mounted on a temperature-controlled stage at 37^{o}C with 5% CO_2 in a Tokai-Hit heating stage with digital temperature and humidity controller. Ca^{2+} activity in the cell body (excluding dendrites) was measured. A total of 100 images per neuron were captured with a 10 ms exposure time and a 500 ms interval using a 60x immersive objective (NA=1.42). 15 to 30 neurons per glass bottom were imaged. F_{min} was determined as the minimum fluorescence value, and total calcium activity was determined for each neuron by the 100 values of $\Delta F/F_{min} = (F_t - F_{min})/F_{min}$ obtained, and $\Delta F/F_{min} < 0.1$ were excluded due to bleaching.

Immunocytochemistry

Cultured hippocampal neurons transfected with GCaMP6f and HA-tagged WT δ-catenin were fixed in 4% paraformaldehyde in PBS for 30 min after live Ca^{2+} imaging, blocked in 5% normal goat serum (NGS) and 0.3% Triton-X for 30 min, and then incubated overnight with an anti-HA antibody (Santa Cruz Biotechnology, 1:1000) to identify δ-catenin transfected neurons. After 3 washes with in PBS for 10 min each, cells were incubated with Alexa-Flour-647 conjugated secondary antibody (Life Technologies 1:500) in 0.3% Triton-X for 2 hrs, washed, and mounted in FluoroGel Para Phenylenediamine Anti Fading Mounting Medium (Electron Microscopy Sciences). Neurons were imaged with a 60x immersive objective (NA=1.42) using an Olympus IX73 microscope.

Statistics

The statistical comparisons were performed using GraphPad Prism6 software. For single comparisons, unpaired two-tailed Student t-tests were used. For multiple comparisons, one-way analysis of variance (ANOVA) with Fisher's Least Significance Difference (LSD) test and two-way ANOVA with Fisher's LSD test were used in order to determine statistical significance. Results were represented as ± standard mean error (SEM) and p value <0.05 was defined as statistically significant.

RESULTS

G34S δ-catenin mutation promotes GSK3β-mediated premature degradation

We examined whether the G34S δ-catenin mutation promoted GSK3β-mediated premature δ-catenin degradation. To compare WT δ-catenin to G34S δ-catenin protein levels, equal amounts of plasmids expressing WT δ-catenin or G34S δ-catenin were transfected into HEK293 cells lacking endogenous δ-catenin expression with either 25nM control (CTRL) siRNA or 25nM GSK3β siRNA, and δ-catenin expression levels were measured by immunoblots. There was significantly lower δ-catenin expression found in G34S δ-catenin transfected cells compared to WT δ-catenin transfected cells (**Fig. 3a and 3b**). When cells were treated with GSK3β siRNA, we found significantly lower GSK3β protein levels when compared to CTRL siRNA treatment, confirming that GSK3β siRNA treatment was sufficient to reduce GSK3β activity (**Fig. 3a and 3c**). Importantly, lower δ-catenin expression levels in G34S δ-catenin transfected cells were reversed with GSK3β siRNA treatment, but no change in δ-catenin protein levels found in WT δ-catenin transfected cells treated with GSK3β siRNA compared to CTRL siRNA treatment (**Fig. 3a and 3b**), possibly due to a ceiling effect of WT δ-catenin overexpression in HEK293 cells. Thus, inhibition of GSK3β activity was sufficient to rescue the reduction of δ-catenin protein levels in G34S δ-catenin-expressed cells, suggesting G34S δ-catenin is a loss-of-function mutation.

As GSK3β-mediated phosphorylation of G34S δ-catenin could induce proteasome-mediated δ-catenin degradation, we determined if G34S δ-catenin underwent premature proteasomal degradation by inhibiting the proteasome. The proteasome inhibitor MG132 (10μM) was incubated in WT δ-catenin or G34S δ-catenin transfected HEK293 cells for 16 hrs. A significant increase in δ-catenin expression levels were found in G34S transfected cells with MG132 treatment compared to WT δ-catenin transfected cells with MG132 treatment (**Fig. 4a**

and 4b). This finding suggests proteosomal degradation is important for reduction of G34S δ-catenin expression, which may result in loss of δ-catenin function in ASD.

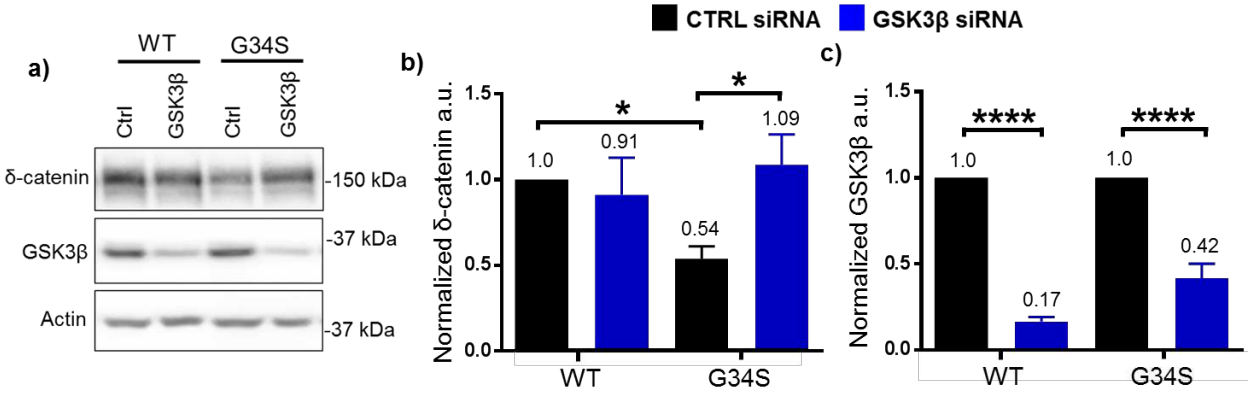

Figure 3. G34S δ-catenin induces GSK3β-mediated premature degradation. a) Representative immunoblots of HEK293 cell lysates transfected with 1μg of WT or G34S δ-catenin and treated with CTRL siRNA or GSK3β siRNA. **b)** Summary graph of average normalized δ-catenin levels showing significantly lower G34S δ-catenin levels are rescued by siRNA-mediated GSK3β knockdown (n=8 experiments, *p<0.05, Two-way ANOVA, Fisher's LSD Test). **c)** Summary graph of normalized GSK3β intensity showing GSK3β siRNA is sufficient in reducing GSK3β levels (n=8 experiments, ****p<0.0001, Two-way-ANOVA, uncorrected Fisher's LSD). Summary graphs are represented by mean ± s.e.m.

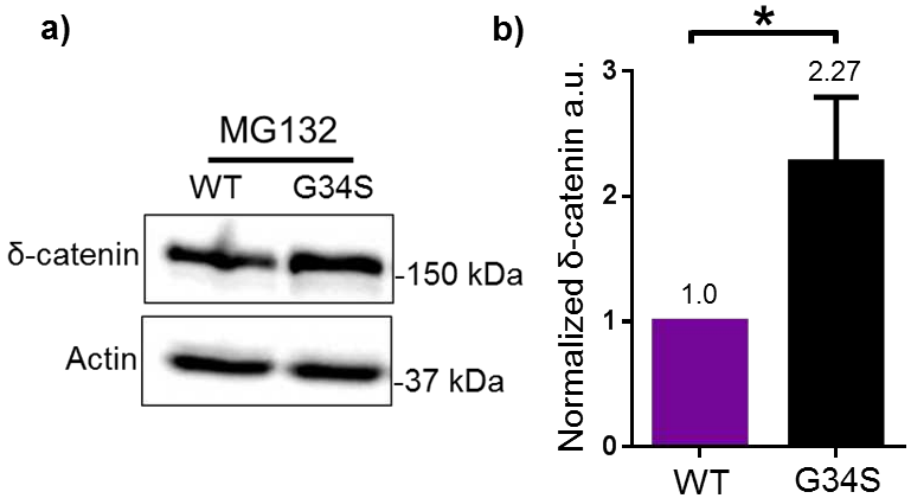

Figure 4. G34S δ-catenin undergoes degradation via the proteasome. a) Representative immunoblots and **b)** summary graph of average normalized δ-catenin levels in HEK293 cells treated with proteasome inhibitor 10μM MG132 for 16-18 hrs showing blocking the proteasome significantly increases G34S δ-catenin levels (n=6 experiments, *p<0.05, unpaired two-tailed Student's t-tests). Summary graphs are represented by mean ± s.e.m.

GSK3β-mediated phosphorylation is important for G34S δ-catenin premature degradation

Additional mutations of δ-catenin at position 34 were made to elucidate if G34S mutant was phosphorylated by GSK3β. First, we substituted glycine 34 for alanine to make G34A δ-catenin mutant that was unable to be phosphorylated by GSK3β. G34A δ-catenin allowed us to determine whether phosphorylation at position 34 was important for inducing premature degradation. Given that G34A δ-catenin was degraded normally by GSK3β phosphorylation of T1078, there was no difference in δ-catenin levels in cells expressing WT δ-catenin and G34A δ-catenin regardless of siRNA treatment (**Fig. 5a and 5b**). This suggests absence of additional GSK3β-mediated phosphorylation is incapable of reducing δ-catenin levels. Next, we generated a G34D δ-catenin mutant in which glycine was substituted with a phospho-mimetic aspartate (D) at position 34. A significant reduction of δ-catenin levels in G34D δ-catenin transfected cells was found compared to WT δ-catenin transfected cells when CTRL siRNA was treated (WT δ-catenin + CTRL siRNA, 1.0 and G34D δ-catenin + CTRL siRNA, 0.7602$\pm$0.081, $p<0.05$) (**Fig. 5a and 5b**) as seen in cells expressing G34S δ-catenin (**Fig. 3a and 3b**). However, GSK3β knockdown with siRNA was unable to increase δ-catenin expression levels in G34D δ-catenin transfected cells (**Fig. 5a and 5b**), unlike GSK3β siRNA treatment in G34S δ-catenin transfected cells (**Fig. 3a and 3b**). This suggests the ASD-associated G34S mutation provides additional GSK3β-mediated phosphorylation of δ-catenin, which may induce premature δ-catenin degradation.

G34S δ-catenin exhibits loss of δ-catenin function in neuronal activity

An analysis of synapse density in hippocampal excitatory synapses revealed that the G34S δ-catenin mutation exhibits loss-of-function, presented by inability to rescue δ-catenin KO-induced loss of synapse density (2). Also, a previous study showed that an increase in δ-catenin protein levels was sufficient to elevate AMPAR surface expression and synaptic activity

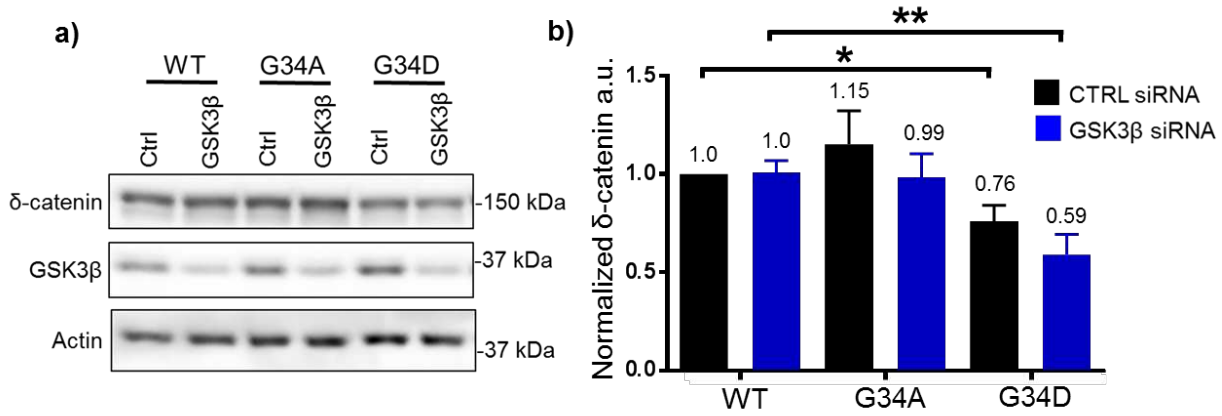

Figure 5. GSK3β-mediated phosphorylation of G34S δ-catenin is important for premature degradation. a) Representative immunoblots and **b)** summary graph of average normalized δ-catenin levels in HEK293 cells showing phospho-mimetic G34D δ-catenin levels are significantly lower than WT δ-catenin, and GSK3β knockdown by siRNA is unable to affect G34D δ-catenin levels. G34A mutation has no effect on δ-catenin levels in the presence or absence of GSK3β siRNA. (n=8 experiments, *p<0.05 and **p<0.01, Two-way ANOVA, uncorrected Fisher's LSD). Summary graphs are represented by mean ± s.e.m.

in cultured neurons (46). Therefore, we examined whether reduction of δ-catenin levels by the G34S mutation affected neuronal activity in hippocampal excitatory cells. As Ca^{2+} is an important second messenger that is involved in neuronal activity (57), a genetically encoded Ca^{2+} indicator GCaMP6f was transfected into cultured primary hippocampal neurons to measure total spontaneous Ca^{2+} activity in the cell body (55). Overexpression of WT δ-catenin in hippocampal neurons (**Fig. 6**) was able to significantly increase Ca^{2+} activity compared to control neurons (CTRL) (**Fig. 7a, 7b, 7k and Table 1**), consistent with a gain-of-function effect on excitatory synapses shown previously (2). Notably, G34S δ-catenin overexpression was unable to increase Ca^{2+} activity (**Fig. 7c, 7k and Table 1**), which was also consistent with the loss-of-function effects on excitatory synapses reported previously (2). Therefore, the G34S δ-catenin mutation did not show the same gain-of-function effects on neuronal activity as WT δ-catenin, confirming a loss-of-function mutation. We also examined overexpression of G34A δ-catenin and G34D δ-catenin effects on neuronal activity to determine if phosphorylation of the δ-catenin amino acid at position 34 affected Ca^{2+} activity. We found overexpression of G34A δ-

catenin was able to significantly increase Ca^{2+} activity (**Fig. 7d, 7k and Table 1**), as seen in WT δ-catenin overexpression. Overexpression of phospho-mimetic G34D δ-catenin did not significantly alter Ca^{2+} activity compared to CTRL, signifying loss of δ-catenin function (**Fig. 7e, 7k and Table 1**). This data suggests the phosphorylation of the G34S δ-catenin ASD-associated mutation could play a critical role in neuronal activity.

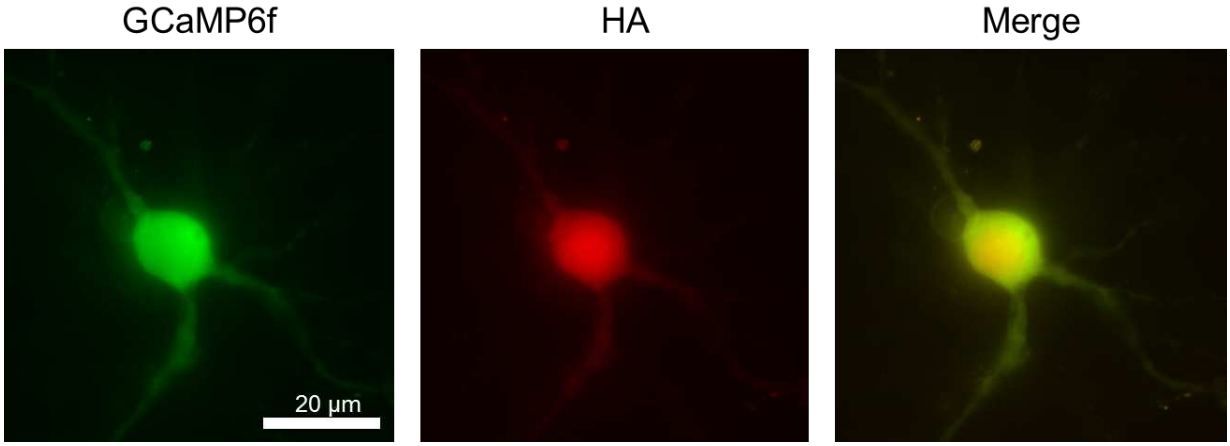

Figure 6. HA-tagged WT δ-catenin and Ca^{2+} indicator GCaMP6f are co-expressed in neurons. Representative images of cultured hippocampal neurons transfected with GCaMP6f and HA-tagged WT δ-catenin, showing that a neuron used for measuring Ca^{2+} activity express both δ-catenin (Red) and GCaMP6f (Green). A bar indicates 20 μm.

G34S δ-catenin-induced loss-of-function effects on neuronal activity is reversed by pharmacological inhibition of GSK3β

As our previous data showed genetic inhibition of GSK3β was able to rescue G34S-induced loss of δ-catenin function effects on protein levels in HEK293 cells (**Fig. 3a and 3b**), we measured whether GSK3β inhibition was also able to reverse G34S δ-catenin-induced loss-of-function in neuronal activity. As chronic changes of GSK3β affect synaptic plasticity (58), we pharmacologically inhibited GSK3β activity in cultured hippocampal neurons by acute 2mM lithium chloride (LiCl) treatment for 16-19 hrs instead of genetic GSK3β siRNA knockdown. LiCl was able to significantly increase in Ca^{2+} activity in CTRL neurons (**Fig. 7f, 7k and Table 1**), suggesting that GSK3β inhibition was able to elevate endogenous δ-catenin levels, increasing

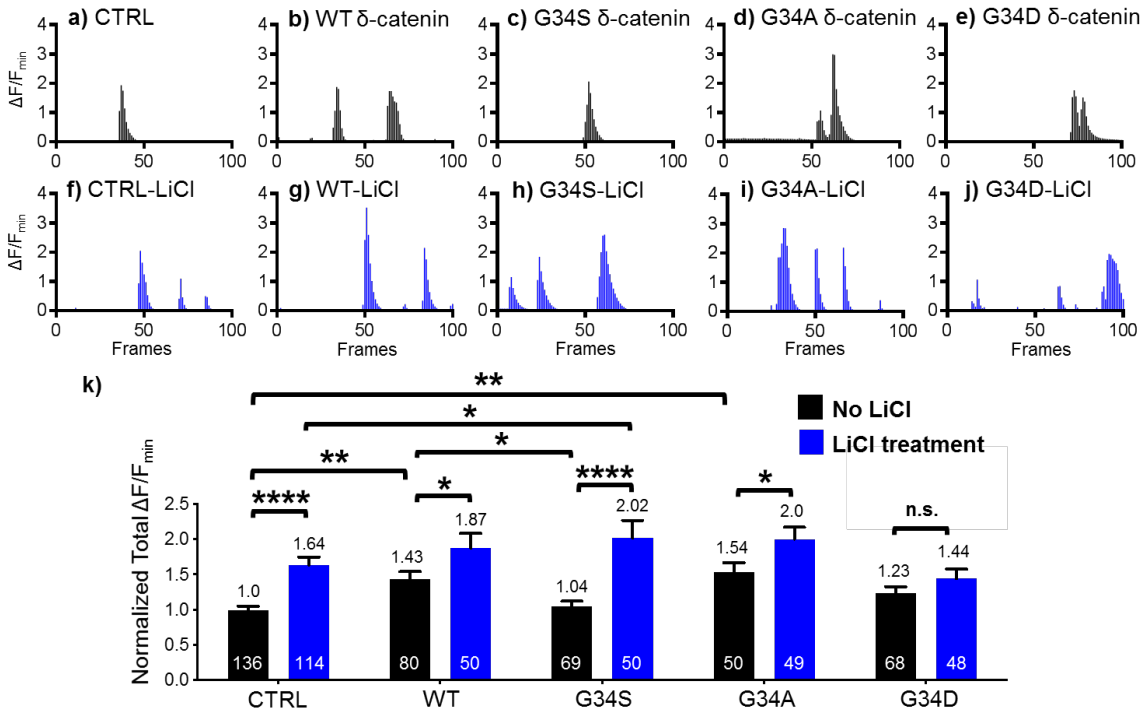

Figure 7. G34S δ-catenin's loss-of-function effects on neuronal activity is reversed by pharmacological inhibition of GSK3β. a-j) Representative traces of GCaMP6f fluorescence intensity and **k)** normalized average total spontaneous Ca^{2+} activity summary graph in cultured mouse hippocampal neurons transfected with **a)** GCaMP6f, **b)** WT δ-catenin and GCaMP6f, **c)** G34S δ-catenin and GCaMP6f, **d)** G34A δ-catenin and GCaMP6f, **e)** G34D δ-catenin and GCaMP6f, and **f-j)** treated with GSK3β inhibitor LiCl (2mM) for 16-19 hrs in subsequent order (n=number of neurons, *p<0.05, **p<0.01 and ****p<0.0001, Two-way ANOVA, uncorrected Fisher's LSD). G34S δ-catenin exhibits loss-of-function effects on neuronal Ca^{2+} activity, which is mediated by GSK3β activity. Summary graphs are represented by mean ± s.e.m.

surface AMPAR levels to enhance neuronal activity as shown in a previous study (46). We found LiCl treatment in neurons overexpressing WT δ-catenin overexpression also had significantly higher Ca^{2+} activity (**Fig. 7g, 7k and Table 1**). Importantly, overexpression of G34S δ-catenin with LiCl treatment significantly increased Ca^{2+} activity compared with LiCl treatment on CTRL neurons (**Fig. 7h, 7k and Table 1**). This suggests lowering GSK3β activity had significant effects on ASD-associated G34S δ-catenin. In addition, Ca^{2+} activity was significantly increased in LiCl-treated neurons overexpressing G34A δ-catenin than LiCl- treated CTRL neurons (**Fig. 7f, 7i, 7k and Table 1**). More importantly, LiCl treatment had no significant effect on Ca^{2+} activity in neurons overexpressing G34D δ-catenin (**Fig. 7j, 7k and Table 1**),

Table 1: Comparison of Ca^{2+} activity in the presence or absence of lithium treatment

Treatment	Average Ca^{2+} activity ($\Delta F/F_{min}$)	Statistics
CTRL – No LiCl	0.99±0.06	**p<0.0001**
CTRL – LiCl treatment	1.64±0.11	
WT – No LiCl	1.43±0.11	**p=0.02**
WT – LiCl treatment	1.87±0.21	
G34S – No LiCl	1.04±0.07	**p<0.0001**
G34S – LiCl treatment	2.02±0.25	
G34A – No LiCl	1.54±0.13	**p=0.02**
G34A – LiCl treatment	2.0±0.17	
G34D – No LiCl	1.23±0.10	n.s. p=0.29
G34D – LiCl treatment	1.44±0.14	

suggesting the mechanism in which G34S δ-catenin induces loss of δ-catenin function can be mediated by GSK3β-induced phosphorylation. Overall, the G34S mutation of δ-catenin provides an additional GSK3β-mediated phosphorylation site, which could promote proteasome-mediated δ-catenin premature degradation, resulting in loss-of-function effects on neuronal activity in ASD. In addition, inhibition of GSK3β activity is able to reverse G34S-induced loss of δ-catenin function

The δ-catenin G34S mutation has no effects on neuronal activity in inhibitory neurons

Notably, ASD has been associated with a disrupted balance of synaptic excitation and inhibition (E/I balance) (59), in which altered synaptic development and activity may contribute to ASD pathophysiology. Therefore, we measured Ca^{2+} activity in inhibitory neurons to determine if ASD-associated G34S δ-catenin affected inhibitory neuronal activity and ultimately altered the E/I balance. To measure inhibitory neuronal activity, we used GCaMP6f under the

control of the GABAergic neuron-specific enhancer of the mouse *Dlx* gene (56), and found that both WT and G34S δ-catenin overexpression were unable to affect Ca^{2+} activity in interneurons (**Fig. 8a-c**). Although this suggests that δ-catenin is likely to affect mainly excitatory neurons, but not interneurons, further studies are needed to understand roles of δ-catenin in interneurons. Furthermore, there were no significant changes in Ca^{2+} activity when neurons overexpressing WT or G34S δ-catenin were treated with LiCl (**Fig. 8d-f**), suggesting that GSK3β-mediated degradation of δ-catenin plays critical roles in excitatory neurons rather than inhibitory neurons. Thus, G34S δ-catenin may cause synaptic dysfunction by altering neuronal activity in excitatory neurons but not inhibitory neurons, supporting the idea that the E/I imbalance is associated with ASD-linked pathophysiology.

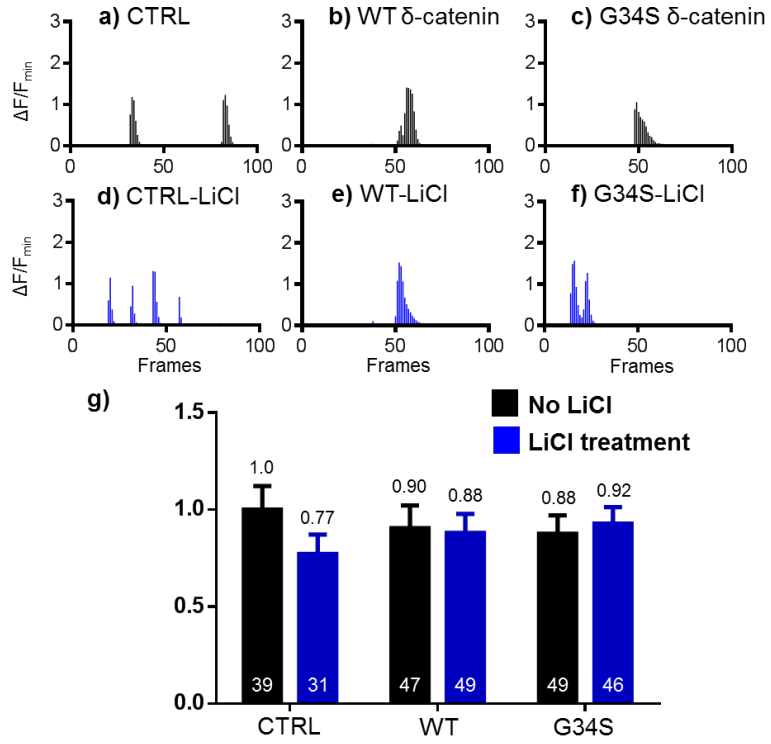

Figure 8. Neuronal Ca^{2+} activity in inhibitory neurons is unaffected by G34S δ-catenin mutant. Representative traces of GCaMP6f under control of mouse GABAergic neuron-specific enhancer and normalized average of total spontaneous Ca^{2+} activity summary graph in cultured mouse hippocampal neurons transfected with **a)** mDlx-GCaMP6f, **b)** WT δ-catenin and mDlx-GCaMP6f, **c)** G34S δ-catenin and mDlx-GCaMP6f, and **d-f)** treated with GSK3β inhibitor LiCl (2mM) for 16-19 hrs in subsequent order (n=number of neurons). Overexpression of WT δ-catenin and G34S δ-catenin has no effect in inhibitory neuronal Ca^{2+} activity in the presence or absence of LiCl. Summary graphs are represented by mean ± s.e.m.

DISCUSSION

Although ASD-associated G34S mutant δ-catenin was previously shown to have the inability to rescue loss of excitatory synapse density in hippocampal neurons lacking the *δ-catenin* gene indicating loss of δ-catenin function (2), the cellular mechanisms of how the G34S mutation causes loss-of-function in the synapse was unclear. In this study, we demonstrated that G34S δ-catenin was degraded prematurely by a GSK3β-mediated additional phosphorylation of δ-catenin to induce loss-of-function, thus causing aberrant neuronal function in ASD. Indeed, we found significantly lower G34S δ-catenin levels compared to WT when expressed in cells lacking endogenous δ-catenin, which was rescued with either genetic GSK3β knockdown (**Fig. 3**) or proteasome inhibition (**Fig. 4**). Our analysis on neuronal activity in cultured mouse primary hippocampal neurons revealed overexpression of G34S δ-catenin was unable to show the same increase in neuronal activity as overexpression of WT, confirming loss of δ-catenin function, which was reversed with pharmacological inhibition of GSK3β by lithium (**Fig. 7 and Table 1**). In addition, we found that δ-catenin may alter the E/I balance, as WT and mutant δ-catenin were unable to affect neuronal activity in inhibitory neurons (**Fig. 8**). Thus, we have provided support that loss of δ-catenin function associated with severe ASD is induced by GSK3β-mediated premature degradation which can be reversed by GSK3β inhibition.

Given that we used primary neurons containing endogenous δ-catenin, lack of gain-of-function by G34S δ-catenin was interpreted as loss-of-function. Therefore, further studies are needed to understand the exact effects of G34S δ-catenin on the synapse by using acute genetic shRNA knockdown of δ-catenin and re-expression of δ-catenin encoding shRNA-resistant protein as shown previously (11) or δ-catenin KO neurons (12). Given that heterozygous loss of the *GSK3β* gene is sufficient to inhibit *in vivo* GSK3β activity and mimic the behavioral and molecular effects of lithium (60), we will breed GSK3β heterozygous KO mice (61) and δ-catenin KO mice, which would further clarify whether genetic inhibition of

GSK3β will improve synaptic efficacy for δ-catenin-linked ASD. In addition, an increase in neuronal activity by reduction of GSK3β activity is possibly due to increased levels of δ-catenin and subsequent higher levels of δ-catenin-associated synaptic complex proteins including GRIP and AMPARs. Hence, AMPAR currents can be measured to confirm this idea.

We have also proposed that the G34S mutation adds an additional GSK3β-mediated phosphorylation site, but have not provided direct evidence that the serine residue at position 34 (S34) is phosphorylated (**Fig. 2**). Thus, further studies are needed to determine if phosphorylation occurs on the ASD-linked G34S mutation by using mass spectrometry with a peptide that contains the amino-terminal region of S34 in G34S δ-catenin. In addition, given that inhibition of the proteasome is able to significantly increase G34S δ-catenin levels, we expect to see significantly higher ubiquitination levels in the G34S δ-catenin compared to the WT δ-catenin if degradation occurs by GSK3β phosphorylation and subsequent ubiquitination. Therefore, immunoprecipitation of WT and G34S δ-catenin to measure ubiquitination levels could provide support for this idea.

Loss of δ-catenin function has also been linked to Cri-du-chat syndrome (19) with autistic-like behaviors exhibited by 40% of individuals with the syndrome (21). More specifically, the syndrome is caused by a hemizygous loss of the tip of human chromosome 5, specifically 5p15.2 where δ-catenin is localized to (19). Our results confirm that G34S δ-catenin is a loss-of-function mutation, which affects neuronal activity, suggesting there may be the same neurobiological mechanisms underlying loss of δ-catenin function-induced autistic-like behaviors in Cri-du-chat syndrome. Interestingly, the missing chromosome regions in Cri-du-chat syndrome also contains the *telomerase reverse transcriptase* (*TERT*) gene, which plays a role in maintaining telomere length (62). Notably, mice that overexpress TERT show ASD-associated behavior, including impaired sociability (63). Therefore, other genes involved in Cri-du-chat syndrome are likely to contribute to ASD-like changes.

Our results have revealed the reduction of GSK3β is able to reverse mutant δ-catenin-induced loss-of-function effects on protein levels and neuronal activity, but there may be other kinases regulating δ-catenin besides GSK3β. For example, a mass spectrometry study has identified δ-catenin as one of possible substrates of c-Jun N-terminal kinases (JNK), a serine/threonine kinase, in the rat brain PSD (64). A JNK-mediated phosphorylation of serine 447 (S477) also targets δ-catenin for proteasomal degradation, leading to reduction of δ-catenin levels and dendritic branching (64). JNK phosphorylates the armadillo protein family member β-catenin on residues S37 and T41 in the amino terminal region, which are also phosphorylated by GSK3β (65). Thus, JNK may provide additional phosphorylation of the ASD-linked G34S δ-catenin mutation, resulting in premature degradation and loss of δ-catenin function. In addition, CKIα is responsible for the priming phosphorylation on β-catenin residue S45, which triggers subsequent β-catenin phosphorylation by GSK3β (30), and it is unknown whether CK1α plays a role in G34S δ-catenin degradation. Consequently, further experiments are needed to determine whether JNK inhibition or CK1α inhibition can reverse mutant δ-catenin-induced loss-of-function.

Importantly, the brain areas responsible for G34S mutation-induced ASD have not yet been explored. According to the Alan Brian Atlas (http://portal.brain-map.org/), δ-catenin is expressed mainly in the cerebral cortex, hippocampus, olfactory bulb, and has lower expression in the thalamus and cerebellum (13). δ-catenin mRNA is present at high levels in the proliferative ventricular zone and developing cortical plate in the developing neocortex (6). δ-catenin also plays a role in the regulation in mature cortical neuronal dendritic complexity which includes arbor size, segment number, tip number (13). Additionally, δ-catenin KO mice exhibited impairment in Pavlovian fear conditioning, which suggests hippocampal and amygdala defects, along with other hippocampal-dependent learning and memory deficiencies (12). The frontotemporal lobe, frontoparietal cortex, amygdala, basal ganglia and anterior cingulate cortex

are believed to be the brain regions that mediate ASD-associated behaviors (66, 67). Thus, further studies on brain regions affected by ASD-linked G34S mutation are a future need for understanding δ-catenin-linked ASD pathophysiology. We have also found the ASD-associated G34S δ-catenin does not affect inhibitory neuronal activity, suggesting that E/I imbalance is likely to contribute to ASD-linked changes. Currently, it is not known whether δ-catenin is present in inhibitory neurons, thus further investigation on our findings that show G34S δ-catenin has no effect on inhibitory neurons is needed.

In conclusion, our data provides a novel cellular mechanism for ASD in which loss of δ-catenin function is triggered by GSK3β-mediated premature degradation, inducing neuronal dysfunction. Inhibition of GSK3β activity may be potential therapeutic treatment for δ-catenin-associated ASD patients.

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