DOI:
10.1159/000444257

Document Version
Peer reviewed version

SUMOylation of DISC1:

a potential role in neural progenitor proliferation in the developing cortex

Stephanie Tankou[a,g], Kazuhiro Ishii[a], Christina Elliott[b], Krishna C. Yalla[b], Jon P. Day[b], Keiko Furukori[a], Ken-ichiro Kubo[c], Nicholas J. Brandon[d], Gary Hayward[e], Kazunori Nakajima[c], Miles D. Houslay[f], Atsushi Kamiya[a], George Baillie[b], Koko Ishizuka[a], and Akira Sawa[a,h]

Departments of [a]Psychiatry and [e]Pharmacology, The Johns Hopkins University, Baltimore, MD, USA

[b]Molecular Pharmacology Group, CMVLS, University of Glasgow, Glasgow, UK

[c]Department of Anatomy, Keio University School of Medicine, Tokyo, Japan

[d]AstraZeneca Neuroscience iMED, Cambridge, MA, USA

[f]Institute of Pharmaceutical Sciences, King's College London, London, UK

[g]Present address: Center of Neurological diseases, Harvard University, Cambridge, MA, USA

[h]Corresponding author: Akira Sawa, MD, PhD, asawa1@jhmi.edu

Short title: SUMOylation of DISC1

Key words: DISC1, SUMOylation, post-translational modification, cortical development, mental illness

Abstract

DISC1 is a multifunctional, intracellular scaffold protein. At the cellular level, DISC1 plays a pivotal role in neural progenitor proliferation, migration, and synaptic maturation. Perturbation of the biological pathways involving DISC1 is known to lead to behavioral changes in rodents, which supports a clinical report of a Scottish pedigree in which the majority of family members with disruption of the *DISC1* gene manifest depression, schizophrenia, and related mental conditions. The discrepancy of modest evidence in genetics but strong biological support for the role of DISC1 in mental conditions suggests a working hypothesis that regulation of DISC1 at the protein level, such as posttranslational modification, may play a role in the pathology of mental conditions. In this study, we report the SUMOylation of DISC1. This posttranslational modification occurs on lysine residues where small ubiquitin-related modifier (SUMO) and its homologs are conjugated to a large number of cellular proteins, which in turn regulates their subcellular distribution and protein stability. By using *in silico*, biochemical, and cell biological approaches, we now demonstrate that human DISC1 is SUMOylated at one specific lysine 643 (K643). We also show that this residue is crucial for proper neural progenitor proliferation in the developing cortex.

Introduction

Over the past several decades, we have used genetic and neuropathological approaches to explore compelling molecular leads to address the mechanisms of neurological and neuropsychiatric disorders [1-3]. For example, tau and α-synuclein proteins have been underscored as major components of inclusion bodies that occur in the brains of patients with Alzheimer's disease and Parkinson's disease, respectively [4, 5]. The tau and α-synuclein proteins are pathologically phosphorylated [6, 7], and the pathological roles of these posttranslational modifications have been elucidated [8]. Likewise, pathological implications of other posttranslational modifications have also been reported [9].

The *DISC1* locus has received broad attention in the context of psychiatric illness, since a balanced translocation that segregated in a Scottish pedigree with schizophrenia and depression was reported [10]. Since the discovery of the gene, many neurobiologists have studied the biological function of DISC1. At the cellular level, DISC1 plays a pivotal role in neural progenitor proliferation, migration, and synaptic maturation. Perturbation of the biological pathways involving DISC1 is known to lead to behavioral changes in rodents. In contrast to biological results that support the role for DISC1 in mental conditions, genetic evidence to support the contribution of the *DISC1* gene in sporadic schizophrenia has been modest. The discrepancy of modest evidence in genetics but strong biological support for the role of DISC1 in mental conditions led us to propose a working hypothesis that regulation of DISC1 at the protein level, such as posttranslational modification, may play a role in its function in pathology.

DISC1 is located in multiple subcellular domains, including the centrosome, postsynaptic density, and the nucleus [11-13]. Like tau and α-synuclein, specific phosphorylation on DISC1 affects its localization to the centrosome and plays a crucial role in neurodevelopment [14]. Protein targeting to the nucleus is also influenced by posttranslational modifications, such as SUMOylation [15]. SUMOylation occurs on lysine residues whereby small ubiquitin-related modifiers (SUMO) and homologs are conjugated to proteins [16]. SUMOylation has emerged as a major regulator of nuclear

function, including DNA replication, DNA damage response, and transcription [17-19]. Furthermore, an important role for SUMOylation in protein trafficking to the nucleus has also been reported [20].

In the present study, we show that human DISC1 is SUMOylated at lysine 643 (K643) with *in silico*, biochemical, and cell biological approaches. This specific SUMOylation site of DISC1 regulates neural progenitor proliferation in the developing cortex.

Methods

Animals

Pregnant C57BL/6 mice were purchased from Charles River. All animal care and use was in accordance with guidelines for the care and use of laboratory animals issued by the National Institutes of Health and Johns Hopkins University.

Constructs and antibodies

A K643A variant of human DISC1 or a K640A variant of mouse DISC1 (mDISC1) was introduced by PCR-based mutagenesis as mentioned previously [14]. All constructs in the present study were created as described previously [12, 21]. We used short hairpin RNA (shRNA) construct of DISC1 that have been published previously [12]. GFP-tagged SUMO1, FLAG-tagged SUMO1, and Myc-tagged Ubc9 constructs were generous gifts from Dr. Pandolfi (Beth Israel Deaconess Cancer Center, USA), Dr. Hayward (Johns Hopkins University, USA), and Dr. Matunis (Johns Hopkins School of Public Health, USA), respectively. The following antibodies were used: mouse monoclonal anti-HA antibody (Covance); rabbit polyclonal anti-FLAG antibody (Sigma); rabbit polyclonal anti-SUMO1 antibody (Zymed); mouse monoclonal anti-BrdU antibody (BD Biosciences); and a rabbit polyclonal anti-human DISC1 antibody (kindly provided by Dr. Akiyama, University of Tokyo, Japan) [22].

In silico prediction of SUMOylation site(s) in DISC1

Prediction of DISC1 SUMOylation sites was determined by using the SUMOplot™ Analysis Program from ABGENT available at http://www.abgent.com/sumoplot. The score indicates the probability that the targeted lysine is SUMOylated.

Recombinant proteins

To obtain C-terminal DISC1 fragmented glutathione S-transferase (GST) fusion proteins, the C-terminal of DISC1 (amino acids 598-854) was inserted into pGEX vector. The expression plasmids were introduced into BL21 competent E. coli with 0.1 mM IPTG. Recombinant proteins were purified from E. coli with glutathione sepharose or amylase beads.

In vitro SUMOylation assay

In vitro SUMOylation was assayed by use of the SUMOylation assay kit (Boston Biochem). Briefly, reactions contained 10 μg of GST-DISC1-C, 50 μM SUMO proteins, 100 nM SUMO activating enzyme, E1, 5 μM UbcH9, and 1mM Mg-ATP in buffer (50 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM DTT), and were incubated at 37˚C for 2 h. The reactions were also run in the absence of ATP as a negative control. Proteins were run on NuPAGE 4-12% Bis-Tris gel (Invitrogen) and visualized with SimplyBlue SafeStain (Invitrogen).

Ubc9 fusion-directed SUMOylatin (UFDS) assay

UFDS assay was performed as previously described [21] with minor modifications. Briefly, HEK293 cells were transfected with the construct of DISC1-Ubc9 or DISC1-Ubc9(C93S) together with the SUMO1-GFP construct, and lysed 48 h after transfection. Immunoblots were conducted with the anti-DISC1 antibody described above [22].

Cell culture

COS7 cells were used to assay SUMOylation on DISC1. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. The expression constructs were transfected into cells by using FuGENE®6 (Roche Applied Sciences).

Immunoprecipitation

Cells were lysed in a RIPA buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100) containing protease inhibitor mixture (Roche Applied Sciences). Supernatant fractions were obtained from lysates after centrifugation at 13 000 rpm for 15 min. Soluble fractions were used for immunoprecipitation as described previously [14].

In utero electroporation

Pregnant C57BL/6 mice at embryonic (E) day 13 were deeply anesthetized by intraperitoneal administration of 2,2,2-tribromoethanol in tert-amyl alcohol (0.4 mg/g), and intrauterine embryos were surgically manipulated as described previously [12, 23]. Plasmid solutions in volumes of 1-2 μl containing RNAi plasmids (2 μg/μl) together with CAG-driven GFP expression vector (1 μg/μl) were injected into the lateral ventricles, and electronic pulses (35 V, 50 ms, 4 times) were applied using an electroporator (CUY21E, Tokiwa Science) with forceps-type electrode (CUY650-5, Tokiwa Science).

BrdU incorporation assay and brain slice preparation

Incorporation of BrdU was assayed was performed as previously described [14] with minor modifications. 50 mg/kg BrdU (Sigma) was injected intraperitoneally into E13 pregnant mice 48 h after electroporation. Then 2 h after BrdU injection, brains were fixed with 4% paraformaldehyde, and 20 μm coronal sections were obtained with a cryostat (CM 1850, Leica). For BrdU immunostaining, brain slices were incubated in 2 N HCl at 37˚C for 30 min before incubation with mouse anti-BrdU antibody. Images of the slices were acquired with a confocal microscope (Zeiss LSM510 and Olympus FV300).

Statistical analysis

To compare four groups, one-way ANOVA followed by Bonferroni post hoc for multiple comparisons test was used. $p < 0.05$ were considered to be statistically significant ($*p < 0.05$).

Results

SUMOylation of C-terminal DISC1 at lysine residue 643 (K643) in vitro

DISC1 is found at multiple subcellular domains, and its nuclear localization and biological roles have been demonstrated [24]. This subcellular targeting may be determined in part by posttranslational modifications [14]. As a role of SUMOylation for targeting proteins to the nucleus is known [15], we hypothesized that a pool of DISC1 may be SUMOylated. We thus first predicted the SUMOylation site(s) in DISC1 by using a bioinformatics approach with the ABGENT SUMOplot (http://www.abgent.com/sumoplot). By this method, DISC1 at lysine residue 643 (K643) was identified as a strong candidate SUMOylation site (**Fig. 1a**). Since K643 is in the C-terminus of DISC1, we assayed *in vitro* SUMOylation with GST-tagged C-terminal DISC1 protein spanning amino acid residues 598-854 (DISC1-C). In the presence of ATP, which is crucial for SUMO-protein conjugation, DISC1-C was SUMOylated by SUMO1, SUMO2, or SUMO3 (**Fig. 1b**). Next, to confirm whether K643 is essential for SUMOylation in DISC1-C, we performed *in vitro* SUMOylation assays using mutant DISC1-C where lysine residue 643 was substituted into alanine (DISC1-C-K643A). Even in the presence of ATP, DISC1-C-K643A was not SUMOylated by SUMO1 (**Fig 1c**). Thus, these results indicate that C-terminal DISC1 protein is SUMOylated at lysine residue 643 *in vitro*.

SUMOylation of DISC1 at K643 in cells

Next, to test whether the SUMOylation of DISC1 occurs in cells, we exogenously expressed DISC1-C together with SUMO1 in cells and examined whether DISC1-C is conjugated to SUMO. DISC1-C and

SUMO1 were indeed co-immunoprecipitated, and a signal for the immunoprecipitated protein complex was observed at an expected size of SUMOylated DISC1-C, 70 kDa (red arrowhead in IP panel in **Fig. 2a**). Of note, a signal for SUMOylation of DISC1-C was also observed in the lysates (red arrowhead in Input panel in **Fig. 2a**). Then, to determine whether K643 is the SUMOylation site in DISC1-C in cells, we examined whether the mutation in which lysine residue 643 was replaces with alanine (K643A) in C-terminal of DISC1 interfered with the SUMOylation. Immunoblotting with cells transfected with DISC1-C showed a strong signal at 70 kDa that represents SUMOylated DISC1-C, whereas such a signal was abolished in cells expressed with DISC1-C-K643A (**Fig. 2b**). These results suggest that the SUMOylation of DISC1-C occurs at lysine residue 643 in cells.

We next assessed whether the full-length DISC1 could also be SUMOylated in cells. Strong signal that represents SUMOylated DISC1 was detected when DISC1-Ubc9, which enhances the SUMOylation, was exogenously expressed together with SUMO1 in cells (red arrowhead). This SUMOylation on DISC1 was abolished by co-expression of SUMO1 with DISC1-Ubc9(C93S), a dominant negative form of Ubc9 (**Fig. 2c**).

To address the question whether K643 is the main SUMOylation site on full-length DISC1, we compared potential SUMOylation between wild-type full-length DISC1 (DISC1) and full-length DISC1-K643A (DISC1-K643A). Cells were co-expressed with DISC1 or DISC1-K643A together with SUMO1 and Ubc9. DISC1 and SUMO1 were co-immunoprecipitated, and a signal for the immunoprecipitated protein complex was observed at an expected size of SUMOylated DISC1, 125 kDa (red arrowhead in IP panel in **Fig. 2d**), whereas such a signal was abolished in cells expressed with DISC1-K643A. Of note, a signal for SUMOylation of DISC1 was also observed in the lysates (red arrowhead in Input panel in **Fig. 2d**). On the basis of these results, K643 is considered the main SUMOylation site on full-length DISC1.

K640 SUMOylation site in mouse DISC1 (mDISC1) (corresponding to K643 in human DISC1) is crucial for proper proliferation of neural progenitor cells (NPCs) in the developing cortex

Previous studies have shown that DISC1 modulates NPCs proliferation *in vivo* [14, 25]. Thus, we tested how the K640 SUMOylation site in mDISC1 affected the proliferation of NPCs by *in utero* gene transfer combined with BrdU pulse-labeling. Consistent with our previous study [14], the proportion of cells incorporating BrdU was significantly decreased in brains with mDISC1 knockdown (mDISC1 RNAi) compared to control, indicating that NPC proliferation was impaired. These knockdown effects were ameliorated by co-expression with wild type mDISC1, but not by mDISC1-K643A (**Fig. 3**), suggesting that the K640 SUMOylation site in mDISC1 is crucial for proper proliferation of NPCs in the developing cortex.

Discussion

Here we report that DISC1 protein is SUMOylated. By combinig *in silico*, biochemical, and cell biological approaches, we have determined that lysine 643 in human DISC1 (which corresponds to lysine 640 in mouse DISC1) is a major SUMOylation site. We show that mutation in this residue leads to improper neural progenitor proliferation in the developing cortex.

Thus far, several papers have indicated a role for nuclear DISC1 [14, 25]. By examining autopsied brains from patients with neuropsychiatric conditions, Sawamura et al. [26] suggested that nuclear DISC1 in the orbitofrontal cortex may be involved in substance abuse. The Furukubo-Tokunaga lab [27] reported that DISC1 is involved in the regulation of sleep homeostasis, in particular in association with nuclear DISC1. More recently, the Tsai lab [28] has demonstrated that nuclear DISC1 is crucial for the regulation of phosphodiesterase-cyclic AMP signaling that underlies multiple psychiatric conditions. Taken together, although each study is still independent without a cohesive or integrative story of nuclear DISC1 function yet, studying nuclear DISC1 will be important in studying

the pathological mechanisms for mental illnesses. We hope that SUMOylation at K643 in human DISC1 may be a solid molecular probe to lead such mechanistic studies.

Here we observed that the replacement of lysine with alanine at a critical SUMOylation site (mouse K640 that corresponds to human K643) disturbed neural progenitor proliferation in the developing cortex. Although this is not a direct demonstration that SUMOylation of DISC1 is crucial for the neurodevelopmental process, we believe this is very suggestive evidence. To address this question in a more direct manner, although it is beyond the scope of the present study, we may need to develop anti-SUMOylation specific DISC1 antibodies. Equivalent antibodies have been frequently used as tools to study protein phosphorylation [29], and indeed applied to a study in DISC1 in the past [14]. There is thus a successful precedence for using anti-SUMOylation specific antibodies in biomedical studies [7, 30]. Such posttranslational modification-specific antibodies may become useful probes to study the pathology of mental illness.

Acknowledgements

We thank Ms. Y. Lema for preparing figures and organizing the manuscript, Dr. P. Talalay for critical reading of this manuscript. This work was supported by USPHS grants of MH084018 Silvo O. Conte center (A.S.), MH094268 Silvo O. Conte center (A.S. and A.K.), MH069853 (A.S.), MH085226 (A.S.), MH088753 (A.S.), MH092443 (A.S.), MH091230 (A.K.), MH96208 (K.I.), MH105660 (A.S. and K.I.); grants from Stanley, RUSK, S-R, and DANA foundations (A.S.); grants from NARSAD (A.S., A.K., and K.I.); grants from MSCRF (A.S. and K.I.); the Grand Challenge Programme grants from Pfizer (G.B.).

Disclosure Statement

Nicholas Brandon is a full-time employee and a share holder at AstraZeneca.

References

1. Samii A, Nutt JG, Ransom BR: Parkinson's disease. Lancet 2004;363:1783-93.
2. Insel TR: Rethinking schizophrenia. Nature 2010;468:187-93.
3. Ballard C, Gauthier S, Corbett A, Brayne C, Aarsland D, Jones E: Alzheimer's disease. Lancet 2011;377:1019-31.
4. Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI: Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. Proc Natl Acad Sci U S A 1986;83:4913-7.
5. Volpicelli-Daley LA, Luk KC, Patel TP, Tanik SA, Riddle DM, Stieber A, Meaney DF, Trojanowski JQ, Lee VM: Exogenous alpha-synuclein fibrils induce Lewy body pathology leading to synaptic dysfunction and neuron death. Neuron 2011;72:57-71.
6. Grundke-Iqbal I, Iqbal K, Quinlan M, Tung YC, Zaidi MS, Wisniewski HM: Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. J Biol Chem 1986;261:6084-9.
7. Fujiwara H, Hasegawa M, Dohmae N, Kawashima A, Masliah E, Goldberg MS, Shen J, Takio K, Iwatsubo T: alpha-Synuclein is phosphorylated in synucleinopathy lesions. Nat Cell Biol 2002;4:160-4.
8. Iqbal K, Grundke-Iqbal I, Zaidi T, Merz PA, Wen GY, Shaikh SS, Wisniewski HM, Alafuzoff I, Winblad B: Defective brain microtubule assembly in Alzheimer's disease. Lancet 1986;2:421-6.
9. Haj-Yahya M, Fauvet B, Herman-Bachinsky Y, Hejjaoui M, Bavikar SN, Karthikeyan SV, Ciechanover A, Lashuel HA, Brik A: Synthetic polyubiquitinated alpha-Synuclein reveals important insights into the roles of the ubiquitin chain in regulating its pathophysiology. Proc Natl Acad Sci U S A 2013;110:17726-31.

10. St Clair D, Blackwood D, Muir W, Carothers A, Walker M, Spowart G, Gosden C, Evans HJ: Association within a family of a balanced autosomal translocation with major mental illness. Lancet 1990;336:13-6.

11. Camargo LM, Collura V, Rain JC, Mizuguchi K, Hermjakob H, Kerrien S, Bonnert TP, Whiting PJ, Brandon NJ: Disrupted in Schizophrenia 1 Interactome: evidence for the close connectivity of risk genes and a potential synaptic basis for schizophrenia. Mol Psychiatry 2007;12:74-86.

12. Kamiya A, Kubo K, Tomoda T, Takaki M, Youn R, Ozeki Y, Sawamura N, Park U, Kudo C, Okawa M, Ross CA, Hatten ME, Nakajima K, Sawa A: A schizophrenia-associated mutation of DISC1 perturbs cerebral cortex development. Nat Cell Biol 2005;7:1167-78.

13. Hayashi-Takagi A, Takaki M, Graziane N, Seshadri S, Murdoch H, Dunlop AJ, Makino Y, Seshadri AJ, Ishizuka K, Srivastava DP, Xie Z, Baraban JM, Houslay MD, Tomoda T, Brandon NJ, Kamiya A, Yan Z, Penzes P, Sawa A: Disrupted-in-Schizophrenia 1 (DISC1) regulates spines of the glutamate synapse via Rac1. Nat Neurosci 2010;13:327-32.

14. Ishizuka K, Kamiya A, Oh EC, Kanki H, Seshadri S, Robinson JF, Murdoch H, Dunlop AJ, Kubo K, Furukori K, Huang B, Zeledon M, Hayashi-Takagi A, Okano H, Nakajima K, Houslay MD, Katsanis N, Sawa A: DISC1-dependent switch from progenitor proliferation to migration in the developing cortex. Nature 2011;473:92-6.

15. Johnson ES: Protein modification by SUMO. Annu Rev Biochem 2004;73:355-82.

16. Muller S, Hoege C, Pyrowolakis G, Jentsch S: SUMO, ubiquitin's mysterious cousin. Nat Rev Mol Cell Biol 2001;2:202-10.

17. Mao Y, Sun M, Desai SD, Liu LF: SUMO-1 conjugation to topoisomerase I: A possible repair response to topoisomerase-mediated DNA damage. Proc Natl Acad Sci U S A 2000;97:4046-51.

18. Bassi C, Ho J, Srikumar T, Dowling RJ, Gorrini C, Miller SJ, Mak TW, Neel BG, Raught B, Stambolic V: Nuclear PTEN controls DNA repair and sensitivity to genotoxic stress. Science 2013;341:395-9.

19. Yan Q, Gong L, Deng M, Zhang L, Sun S, Liu J, Ma H, Yuan D, Chen PC, Hu X, Liu J, Qin J, Xiao L, Huang XQ, Zhang J, Li DW: Sumoylation activates the transcriptional activity of Pax-6, an important transcription factor for eye and brain development. Proc Natl Acad Sci U S A 2010;107:21034-9.

20. Mahajan R, Delphin C, Guan T, Gerace L, Melchior F: A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. Cell 1997;88:97-107.

21. Jakobs A, Koehnke J, Himstedt F, Funk M, Korn B, Gaestel M, Niedenthal R: Ubc9 fusion-directed SUMOylation (UFDS): a method to analyze function of protein SUMOylation. Nat Methods 2007;4:245-50.

22. Ogawa F, Kasai M, Akiyama T: A functional link between Disrupted-In-Schizophrenia 1 and the eukaryotic translation initiation factor 3. Biochem Biophys Res Commun 2005;338:771-6.

23. Tabata H, Nakajima K: Efficient in utero gene transfer system to the developing mouse brain using electroporation: visualization of neuronal migration in the developing cortex. Neuroscience 2001;103:865-72.

24. Brandon NJ, Sawa A: Linking neurodevelopmental and synaptic theories of mental illness through DISC1. Nat Rev Neurosci 2011;12:707-22.

25. Mao Y, Ge X, Frank CL, Madison JM, Koehler AN, Doud MK, Tassa C, Berry EM, Soda T, Singh KK, Biechele T, Petryshen TL, Moon RT, Haggarty SJ, Tsai LH: Disrupted in schizophrenia 1 regulates neuronal progenitor proliferation via modulation of GSK3beta/beta-catenin signaling. Cell 2009;136:1017-31.

26. Sawamura N, Sawamura-Yamamoto T, Ozeki Y, Ross CA, Sawa A: A form of DISC1 enriched in nucleus: altered subcellular distribution in orbitofrontal cortex in psychosis and substance/alcohol abuse. Proc Natl Acad Sci U S A 2005;102:1187-92.

27. Sawamura N, Ando T, Maruyama Y, Fujimuro M, Mochizuki H, Honjo K, Shimoda M, Toda H, Sawamura-Yamamoto T, Makuch LA, Hayashi A, Ishizuka K, Cascella NG, Kamiya A, Ishida

N, Tomoda T, Hai T, Furukubo-Tokunaga K, Sawa A: Nuclear DISC1 regulates CRE-mediated gene transcription and sleep homeostasis in the fruit fly. Mol Psychiatry 2008;13:1138-48, 1069.

28. Soda T, Frank C, Ishizuka K, Baccarella A, Park YU, Flood Z, Park SK, Sawa A, Tsai LH: DISC1-ATF4 transcriptional repression complex: dual regulation of the cAMP-PDE4 cascade by DISC1. Mol Psychiatry 2013;18:898-908.

29. Xie Z, Sanada K, Samuels BA, Shih H, Tsai LH: Serine 732 phosphorylation of FAK by Cdk5 is important for microtubule organization, nuclear movement, and neuronal migration. Cell 2003;114:469-82.

30. Takahashi-Fujigasaki J, Arai K, Funata N, Fujigasaki H: SUMOylation substrates in neuronal intranuclear inclusion disease. Neuropathol Appl Neurobiol 2006;32:92-100.

Figure legends

Figure 1. SUMOylation of C-terminal DISC1 at lysine residue 643 (K643) *in vitro*

a, DISC1 SUMOylation sites predicted by use of the ABGENT SUMOplot^{TM}.

b, *In vitro* SUMOylation assay with GST-tagged C-terminal DISC1 (DISC1-C). Coomassie blue-staining showed that DISC1-C was covalently conjugated to SUMO1, SUMO2, and SUMO3. Red arrowheads indicate SUMOylated forms of DISC1-C.

c, *In vitro* SUMOylation assay with GST-tagged C-terminal DISC1 (DISC1-C) or mutant C terminal DISC1 where K643 was substituted by alanine (DISC1-C-K643A). Immunoblotting showed that DISC1-C but not DISC1-C-K643A was SUMOylated. Red arrowheads indicate SUMOylated forms of DISC1-C.

Figure 2. SUMOylation of DISC1 at lysine residue 643 (K643) *in cells*

a, Conjugation of HA-DISC1-C with FLAG-SUMO1 (SUMOylation of DISC1) was shown in COS7 cells. COS7 cells were transfected with HA-DISC1-C and/or FLAG-SUMO1. Cell lysates were immunoprecipitated with anti-FLAG antibody and the immunoprecipitates were blotted with anti-HA antibody. Red arrowheads indicate SUMOylated forms of DISC1-C at 70 kDa.

b, COS7 cells were transfected with HA-DISC1-C or HA-DISC1-C-K643A. Cells with HA-DISC1-C showed a strong signal at 70 kDa, which represents a SUMOylated form of DISC1-C, suggesting SUMOylation of DISC1 with endogenous SUMO protein. This signal was significantly abolished in cells transfected with HA-DISC1-C-K643A. Red arrowhead indicates SUMOylated forms of DISC1-C at 70-kDa.

c, SUMOylation of full-length DISC1 was shown in HEK293 cells by Ubc9 fusion-directed SUMOylation (UFDS) assay. SUMO1-GFP fusion was co-transfected with DISC1-Ubc9, an E2 SUMO conjugating enzyme fusion, or DISC1-Ubc9(C93S), a dominant negative form of Ubc9 fusion, in

HEK293 cells. Cell lysates were immunoblotted with anti-DISC1 antibody. Red arrowhead indicates SUMOylated forms of DISC1.

d, COS7 cells were co-transfected with HA-DISC1 or HA-DISC1-K643A together with FLAG-SUMO1 and myc-Ubc9. Cell lysates were immunoprecipitated with anti-HA antibody and the immunoprecipitates were blotted with anti-FLAG antibody. Signals for SUMOylated full-length DISC1 were observed at 125 kDa in cells transfected with HA-DISC1 (red arrowheads), whereas almost no signals were found at 125-kDa in cells transfected with HA-DISC1-K643A.

Figure 3. K640 SUMOylation site in mouse DISC1 (mDISC1), corresponding to K643 in human DISC1, is crucial for proper proliferation of neural progenitor cells (NPCs) *in vivo*

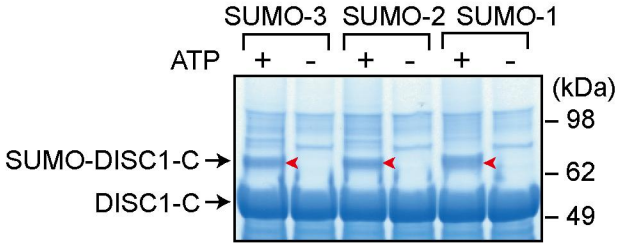
Images show E15 brains that were electroporated with GFP together with control shRNA, mDISC1 shRNA, mDISC1 shRNA + wild-type (wt) mDISC1, or mDISC1 shRNA + mDISC1-K640A at E13. BrdU was injected 2 h before brain extraction. The proportion of BrdU incorporating cells in the ventricular and subventricular zone (VZ/SVZ) in brains with mDISC1 shRNA, was significantly lower than that in brains with control shRNA. This phenotype was rescued by wt mDISC1 but not by mDISC1-K640A. Graph shows the proportion of GFP/BrdU double-positive cells over total GFP positive cells in the VZ/SVZ. Scale bar, 20μm. Error bars indicate s.e.m. **P*<0.05.

Fig. 1

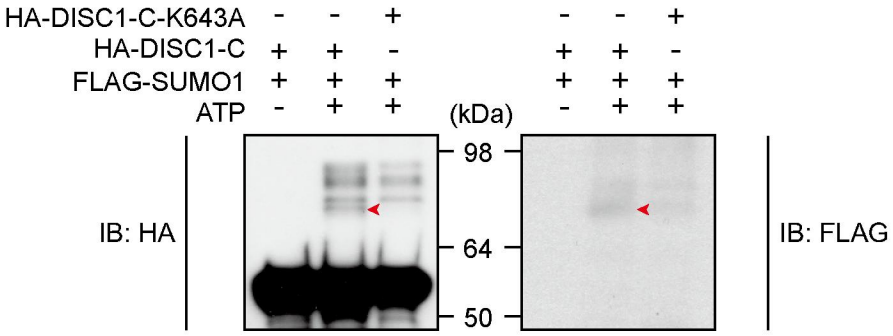
a

No.	Pos.	Group	Score
1	K643	KKLGS~**VKED**~YNRLR	0.93
2	K372	ENDDY~**DKAE**~TLQQR	0.50
3	K768	CAGGE~**QKEE**~SYILS	0.50
4	K781	SAELG~**EKCE**~DIGKK	0.50
5	K332	WDTLL~**RKWE**~PVLRD	0.44
6	K743	HSEDK~**RKTP**~LKVLE	0.34
7	K638	SSRNV~**KKLG**~SVKED	0.31

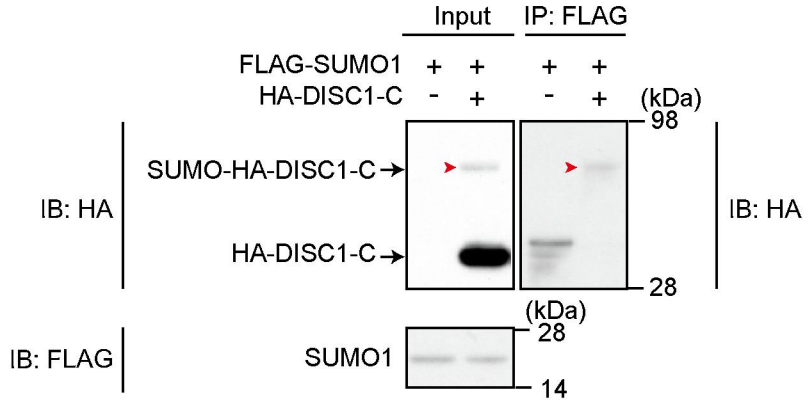
b



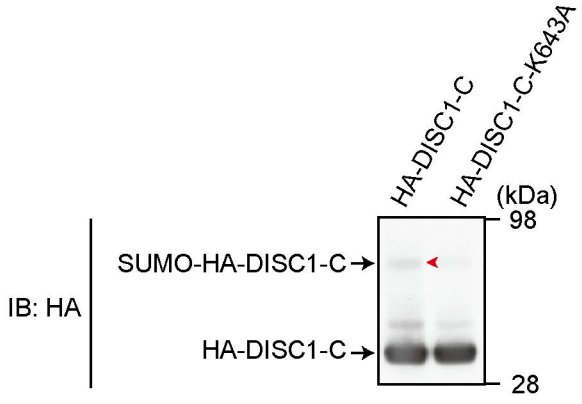
c



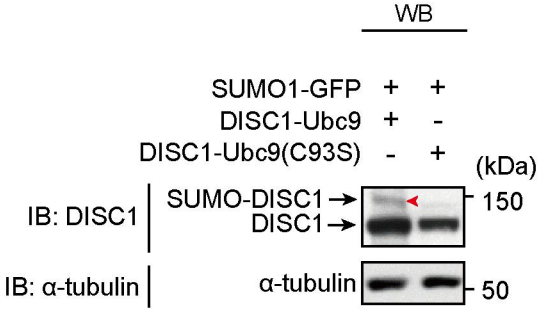
a


Input
IP: FLAG
FLAG-SUMO1 + + + +
HA-DISC1-C - + - +
(kDa)
98
SUMO-HA-DISC1-C
HA-DISC1-C
28
IB: HA
IB: HA
(kDa)
28
IB: FLAG
SUMO1
14

b


HA-DISC1-C
HA-DISC1-C-K643A
(kDa)
98
SUMO-HA-DISC1-C
IB: HA
HA-DISC1-C
28

c


WB
SUMO1-GFP + +
DISC1-Ubc9 + -
DISC1-Ubc9(C93S) - +
(kDa)
IB: DISC1
SUMO-DISC1
DISC1
150
IB: α-tubulin
α-tubulin
50

d

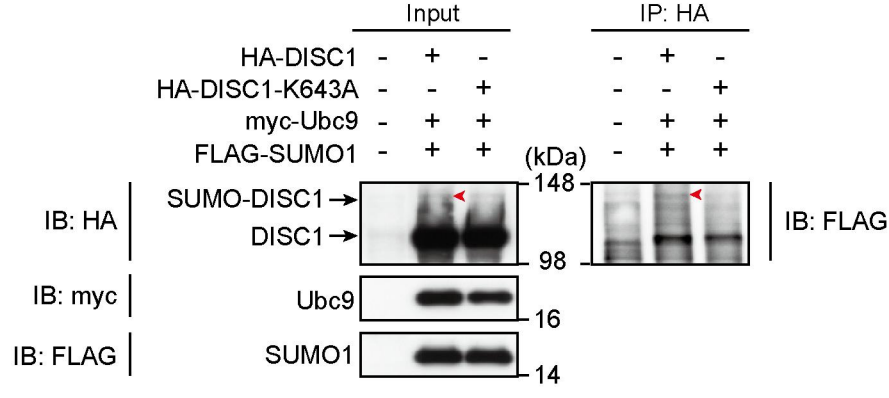
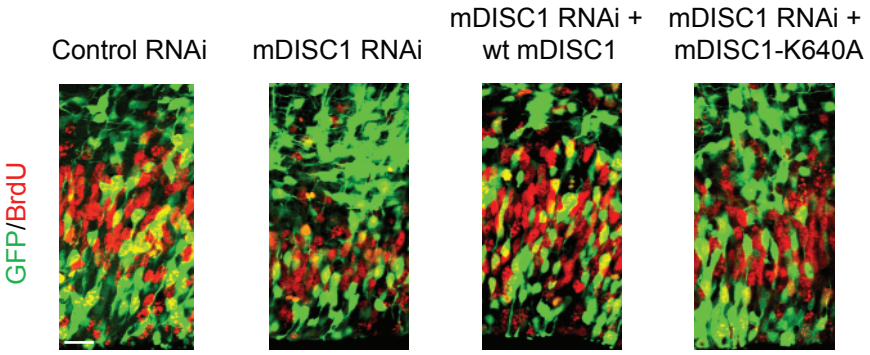
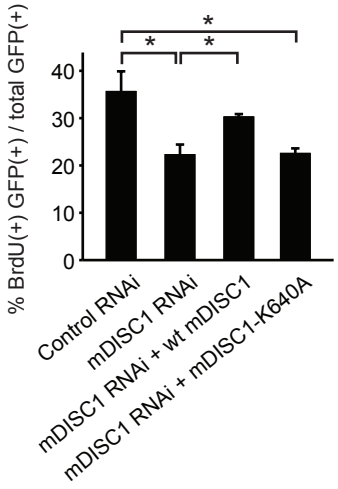

Input
IP: HA
HA-DISC1 - + - - + -
HA-DISC1-K643A - - + - - +
myc-Ubc9 - + + - + +
FLAG-SUMO1 - + + - + +
(kDa)
148
IB: HA
SUMO-DISC1
DISC1
98
IB: FLAG
IB: myc
Ubc9
16
IB: FLAG
SUMO1
14

Fig. 3.


Control RNAi
mDISC1 RNAi
mDISC1 RNAi +
wt mDISC1
mDISC1 RNAi +
mDISC1-K640A
GFP/BrdU


% BrdU(+) GFP(+) / total GFP(+)
40
30
20
10
0
*
*
*
Control RNAi
mDISC1 RNAi
mDISC1 RNAi + wt mDISC1
mDISC1 RNAi + mDISC1-K640A