DISC1 regulates trafficking and processing of APP and Aβ generation

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Abstract

We report the novel regulation of proteolytic processing of amyloid precursor protein (APP) by DISC1, a major risk factor for psychiatric illnesses, such as depression and schizophrenia. RNAi-knockdown of DISC1 in mature primary cortical neurons led to a significant increase in the levels of intracellular α-C-terminal fragment of APP (APP-CTFα) and the corresponding N-terminal secreted ectodomain product sAPPα. DISC1 knockdown also elicited a significant decrease in the levels of Aβ42 and Aβ40. These aberrant proteolytic events were successfully rescued by co-expression of wild-type DISC1, but not by mutant DISC1 lacking the amino acids required for the interaction with APP, suggesting that APP-DISC1 protein interactions are crucial for the regulation of the C-terminal proteolysis. In a genetically-engineered model in which a major full-length DISC1 isoform is depleted, consistent changes in APP processing were seen: an increase in APP-CTFα and decrease in Aβ42 and Aβ40 levels. Finally we found that knockdown of DISC1 increased the expression of APP at the cell surface and decreased its internalization. The presented DISC1 mechanism of APP proteolytic processing and Aβ peptide generation, which is central to Alzheimer's disease pathology, suggests a novel interface between neurological and psychiatric conditions.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

Supplementary information is available at Molecular Psychiatry's website.

Keywords

Alzheimer's disease; Depression; APP; Proteolytic processing; Trafficking; DISC1

INTRODUCTION

The cloning of the *amyloid precursor protein* (*APP*) gene was a landmark in research of Alzheimer's disease (AD), with its identification as the precursor for Aβ (amyloid beta). Aβ is a key component of the senile plaque, one of the key pathological hallmarks of AD.[1] A variety of Aβ peptides are generated through C-terminal processing of the APP protein.[2] Following its cloning, several groups identified rare genetic variants of the *APP* gene, including those associated with familial cases of early onset AD.[3] These reports included an interesting case with schizophrenia (SZ), carrying a mutation of the *APP* gene at codon 713.[4] For the past two decades, the significance of Aβ peptides, APP C-terminal processing, and APP itself has been extensively examined. These studies have expanded the concept of Aβ from amyloidogenic peptides that drive pathologic cascades, which includes an aberrant synaptic plasticity.[1,2,5] Involvement of this Aβ mechanism may not be limited just to AD, but also to other neurological diseases, such as Down syndrome and Parkinson's disease.[6]

DISC1 is a risk factor for a wide variety of psychiatric disorders.[7,8] We previously published binding of APP to DISC1, through the C-terminal intracellular domain of APP.[9] A major unanswered question is whether this interaction modulates the proteolytic processing of APP and the generation of Aβ peptides. In the present exploratory study, we address this key question and discuss its possible significance for neuropsychiatric disorders.

MATERIALS AND METHODS

Mice

We used a representative model for Alzheimer's disease (AD), 3xTg-AD mice.[10] *Disc1* locus impairment model was recently generated, and the basic characterization, such as Southern blotting, was described (under review). We used these genetically-engineered models with age-and gender-matched wild-type mice.

Cell culture and virus production

From embryonic day 17–18 (E17–18) Sprague-Dawley rats or E14 C57BL/6J mice, cortical primary neuron cultures were prepared, as described previously.[11] Lentiviral constructs expressing DISC1 RNAi were generated by subcloning shRNA sequences[12,13] and H1 promoter into the Pac1 site of the FUGW lentiviral backbone.[14] Lentiviral constructs expressing HA-tagged various DISC1 constructs were generated by replacing EGFP in the FUGW lentiviral vector.

Immunodetection

Immunoprecipitation and Western blotting were performed as described previously.[11] The following antibodies were used: Rabbit polyclonal antibodies to APP (C-terminal specific, Sigma), rabbit polyclonal antibodies to DISC1 (mE3 antibody and 584-2 antibody),[15,16]

guinea pig polyclonal antibody to DISC1 (578-1),[16] mouse monoclonal antibodies to DISC1 raised against 594–852 amino acids of mouse DISC1 (2B3), ADAM Metallopeptidase Domain 10 (ADAM10) (Millipore), Flag (M2, Sigma), HA (Covance), Tubulin (Sigma), and Actin (Santa Cruz).

Quantification of sAPPα

For sAPPα in the conditioned medium of rat primary neurons or mouse brains, Mouse/Rat sAPPalpha ELISA kit (Immuno-Biological Laboratories, Japan) was used for quantification according to the manufacturer's protocol. Cortex samples were homogenized in tissue homogenization buffer (THB; 0.25 M sucrose, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA) containing complete protease inhibitor cocktail (Roche). After centrifugation at $1,600 \times g$ for 5 min at 4°C, the post-nuclear supernatant was further centrifuged at $100,000 \times g$ for 60 min at 4°C.[17] The resulting supernatant was collected as the "soluble fraction" and used to detect sAPPα using ELISA analysis.

Quantification of Aβ

For Aβ in the conditioned medium of rat primary neurons or mouse brains, Human/Rat beta-amyloid (42 or 40) ELISA kit (Wako, Japan) was used for quantification according to the manufacturer's protocol. Endogenous soluble Aβ (Aβ42 and Aβ40) in the cortex was measured using diethylamine-extraction method.[18] Briefly, the above described brain homogenate in THB was further homogenized with an equal volume of cold 0.4% diethylamine (DEA) and 100 mM NaCl on ice. After centrifugation at $100,000 \times g$ for 1 h at 4°C, the supernatant was neutralized with 1:10 volume of 0.5 M Tris base (pH 6.8) and used to measure Aβ42 and Aβ40 by ELISA. For insoluble Aβ (42 and 40), the remaining pellet following DEA fractionation was resuspended in one volume of 70% formic acid, sonicated, and spun at $13,000 \times g$ for 15 min at 4°C. The supernatant was collected (formic acid fraction containing insoluble proteins) and neutralized in 20 volumes of neutralization buffer (1 M Tris-HCl, 0.5 M Na_2PO_4).

Biotinylation of cell surface proteins

Biotinylation of cell surface proteins was performed as previously described [19,20] in primary neuron cultures using cell surface protein isolation kit (Pierce) according to the manufacturer's protocol. Briefly, cells were incubated with ice-cold PBS containing Sulfo-NHS-SS-Biotin (Pierce) for 30 min with gentle rocking at 4°C. Cells were then lysed and immunoprecipitated with NeutrAvidin beads (Pierce). Precipitated proteins were eluted from the NeutrAvidin beads with loading buffer containing dithiothreitol and heated for 5 min at 95°C and then analyzed by Western blotting.

Live cell surface staining

Surface immunostaining in cortical neurons was performed as described previously with some modification.[21] Primary cortical neurons prepared from timed-pregnant mice (14.5 days post-coitum) were cultured for 10 to 12 days and transfected with N-terminally GFP-tagged APP by lipofectamine 2000 (Invitrogen). GFP tag was inserted immediately downstream of signal sequence of APP to allow for proper processing and membrane

localization of the protein.[21] Two days post-transfection, live cultures were incubated in media containing APP N-terminal antibody (22C11, Millipore, 10ug/ml) for 10 min to label surface APP and fixed for 5 min with prewarmed 4% paraformaldehyde in PBS without permeabilization. The cultures were then washed twice with PBS and the surface APP-APP antibody conjugate was detected with Alexa 546-linked anti-mouse IgG (1:500, Invitrogen, 1h, room temp.). To label transfected cells, cultures were subsequently washed, permeabilized with cold methanol (−20°C) for 90 sec, and immunostained with anti-GFP (1:2,000, rabbit polyclonal, Invitrogen) followed by Alexa 488-linked anti-rabbit IgG (1:500, Invitrogen).

Internalization assay

The surface APP internalization assay was performed in cortical neurons as described previously with some modification.[21] Primary cortical neurons transfected with GFP-APP as above were incubated with media containing APP antibody (22C11, 10 μg/ml) for 10 min, washed with prewarmed DMEM, and incubated at 37°C for 30 min to allow for internalization of the APP-APP antibody conjugate. After brief fixation in 4% PFA (5 min), the surface-remaining APP-antibody conjugate was masked with HRP-linked anti-mouse antibody (1:500) for 2 h. Cells were subsequently permeabilized with cold methanol (−20°C) for 90 sec, and the internalized APP was labeled with Alexa 546-linked anti-mouse IgG (1:500) for 1 h. Complete masking of the surface APP-antibody conjugate was confirmed by control experiments in which non-permeabilized cells gave no staining with an anti-mouse secondary antibody.

ADAM10 (α-secretase) activity assay

Endogenous ADAM10 activity was measured using a fluorometric assay with a SensoLyte 520 ADAM10 Activity Assay Kit (AnaSpec Inc.) according to the operation manual.

Statistics

For determination of the statistical significance between two groups, a 2-tailed Student's t-test (paired or unpaired as appropriate) was employed. To compare three or more groups, one-way ANOVA followed by Bonferroni post hoc for multiple comparisons were used. A value of $p < 0.05$ was considered significant. In each experiment, all experimental values were normalized to control levels.

Study approval

All protocols were approved by Animal Care and Use Committee of the Johns Hopkins University, as well as of the Beckman Research Institute of City of Hope.

RESULTS AND DISCUSSION

After confirming our previously published data, demonstrating the APP-DISC1 protein interaction at the endogenous level, using a different set of antibodies for co-IP (Supplementary Figure S1), we examined the influence of DISC1 on APP processing by expressing two independent small hairpin RNAs (shRNAs) to DISC1 in lentiviral vectors.[12] The DISC1 shRNA viruses, or a control virus carrying scrambled sequence, were infected

into primary cortical neurons at 19 days *in vitro* (DIV19), and the effects on APP processing and Aβ generation were examined 6 days after infection (DIV25). Both DISC1 shRNAs significantly knocked down endogenous DISC1 in contrast to the control scrambled shRNA, which was shown by more than one antibody (Supplementary Figure S2). We did not observe robust alteration in the level of full-length APP by the change of DISC1 (Figures 1a and b, and Supplementary Figure S3a). In contrast, knockdown of DISC1 led to a significant increase in the levels of the α-C-terminal fragment of APP (APP-CTFα) (Figures 1a and c, and Supplementary Figure S3b). Consistent with this observation, we observed a significant enhancement of the levels of secreted APPα (sAPPα), the other product of α-secretase processing of APP, in the conditioned medium (Figure 1d and Supplementary Figure S3c). We next measured the amounts of Aβ42 and Aβ40 in the conditioned medium and observed a significant decrease in the levels of these peptides after infection of DISC1 shRNAs (Figures 1e and f, and Supplementary Figures S3d and e). The magnitudes of the changes in the proteolytic products is much more robust, compared with the alteration in the level of the full-length protein.

To test the specificity of the effect of the DISC1 knockdown, we conducted rescue experiments in which we examined whether the biochemical changes elicited by DISC1 shRNAs are rescued by co-expression of shRNA-resistant wild-type DISC1 (called R1), in which several nucleotide sequences corresponding to the target sequences for the shRNAs were mutated without altering the amino acid sequence. Co-expression of wild-type DISC1 (R1) rescued the changes elicited by DISC1 shRNAs, in particular those in the levels of APP-CTFα, sAPPα, Aβ42, and Aβ40 (Figure 1).

We further assessed whether the changes in APP processing are dependent on the APP-DISC1 protein-protein interaction. Our previous study suggested that N-terminal region of DISC1 participates in the binding,[9] however this large portion includes several domains critical for interactions with other proteins. Thus, we went on to determine a minimal binding domain through which APP and DISC1 interact. We accomplished this by using several N-terminal deletion mutants of DISC1 in co-immunoprecipitation experiments. This sequential domain mapping indicated that deletion of amino acids 1–24, but not that of 1–20, significantly decreased the interaction of DISC1 with APP (Supplementary Figure S4). As far as we are aware, no other crucial protein reportedly binds to DISC1 at this region.[8] Thus, we generated a mutant DISC1 deficient in this APP binding site in a lentiviral expression system (called NR1) and tested its ability to rescue phenotypes elicited by coinfecting primary cortical neuron cultures with DISC1 shRNA. In contrast to rescue by wild-type DISC1 (R1), mutant DISC1 lacking the APP binding domain (NR1) failed to normalize the biochemical changes in APP and Aβ elicited by DISC1 knockdown (Figure 1).

We recently generated a *Disc1* locus impairment model (Jaaro-Peled et al, under review). By utilizing well-characterized DISC1 antibodies,[16] we confirmed that at least a major full-length 100 kD isoform is fully depleted in the *Disc1* locus impairment (Supplementary Figure S5). We then examined possible alteration of APP processing in adult cerebral cortex of homozygote mutants (−/−) compared with wild-type littermates (+/+). Consistent with our observations in primary neuronal cultures infected with DISC1 shRNAs (Figures 1a–c), we

observed a significant increase in APP CTFα, without any major change in full-length APP, in −/− mice, when compared with those in +/+ mice (Figures 2a–c). The levels of sAPPα were elevated and those of soluble Aβ (both Aβ42 and Aβ40) were decreased in −/− mice (Figures 2d–f), in accordance with the data from the primary neuronal cultures with DISC1 shRNAs (Figures 1d–f). We observed no significant change in the levels of insoluble Aβ (both Aβ42 and Aβ40) (Supplementary Figure S6a).

The changes of APP C-terminal processing we observed for DISC1 knockdown and locus impairment are possibly accounted by ADAM Metallopeptidase Domain 10 (ADAM10), one of the major α-secretases responsible for ectodomain shedding of APP in the mouse brain.[22–24] We examined whether DISC1 knockdown altered the expression or enzymatic activity of ADAM10, and found that there was no apparent difference between +/+ and −/− brains (Supplementary Figures S6b and c). Thus, we hypothesized the cellular trafficking of APP as an alternative candidate mechanism to account for the alteration in the processing. In the same experimental setup in primary neurons, we tested whether DISC1 knockdown modifies the cell-surface presentation of APP by using cell-surface biotinylation. We observed a significant increase in the cell surface levels of endogenous APP upon DISC1 knockdown (Figures 3a and b). This change was partially rescued by co-expression of shRNA-resistant wild-type DISC1 (R1), but not of mutant DISC1 lacking the APP-binding amino acids (NR1), indicating that DISC1 participates in the regulation of APP surface expression (Figures 3a and b). These observations were further confirmed by live-cell immunostaining under an impermeable condition that exclusively label cell-surface APP. Neurons from −/− mice displayed higher levels of surface APP than those from +/+ mice (Figure 3c). Consistent with this observation, DISC1 knockdown (D1) in primary neurons from +/+ mice increased the cell-surface APP immunoreactivity (Figure 3d). Furthermore, exogenous supplement of full-length DISC1 (DISC1) in primary neurons from −/− mice normalized the levels of cell-surface APP (Figure 3e). Altered level of cell surface APP is likely to be affected by its internalization. Thus, we examined the levels of internalized APP after its surface labeling by biotinylation and observed that DISC1 knockdown in neurons from +/+ mice led to a decrease in APP internalization, whereas exogenous expression of full-length DISC1 in neurons from −/− mice increased APP internalization (Figures 3d and e). These results indicate that modifications of APP internalization may, at least in part, underlie altered surface expression of APP upon the down-regulation of DISC1. Although we don't exclude the possibility that other enzymes affect DISC1-modulated APP processing, these data suggests that DISC1 is most likely to regulate APP processing through affecting the cellular trafficking of APP.

Molecular disposition of DISC1 may be changed in the pathophysiology of AD. We conducted a pilot study to address this question by using a representative animal model for AD (3xTg-AD, 24 months old).[10] At least in the conditions we tested, we failed to observe significant difference in the levels of APP-DISC1 binding and DISC1 protein expression between age-matched wild-type and 3xTg-AD mice (Supplementary Figure S7).

The main findings of the present study are that DISC1 influences APP C-terminal processing and Aβ peptide generation. DISC1 also affects surface expression and internalization of APP in neurons. The protein interaction of DISC1 and APP, via a specific

domain of DISC1, is crucial for the protein metabolism and cellular distribution of APP. Two distinct approaches (e.g., knockdown of DISC1 by shRNAs and germline deletion of a major full-length isoform of DISC1) consistently support these observations.

Roles for Aβ peptides in disease have recently been discussed not only in AD but also in a variety of neuropsychiatric disorders.[6] Given that DISC1 may underlie a risk for a wide variety of psychiatric disorders,[7,8] it is an interesting question of how the protein interaction of APP-DISC1 and its regulation of Aβ generation may underlie the pathology of neuropsychiatric disorders. Although AD has been emphasized as a condition of severe memory loss and cognitive impairment, associated with progressive neurodegeneration, intervention with psychiatric manifestations, such as depression, is a very important clinical matter.[25,26] Studies of antidepressants and atypical antipsychotics in patients with AD are inconclusive, with several negative findings reported in recent studies.[27,28] Thus, the present exploratory work may open a window for future studies to aid our mechanistic understanding and drug discovery of psychiatric symptoms in AD. One of the experimental strategies in the future may include testing whether non-synonymous variations of DISC1 (L607F, S704C), which are genetically associated with psychiatric symptoms and cognitive aging,[7,8,29–31] could influence DISC1-mediated APP processing. Although it is a very speculative view, altered APP processing due to these variations might account, at least in part, for psychiatric symptoms in AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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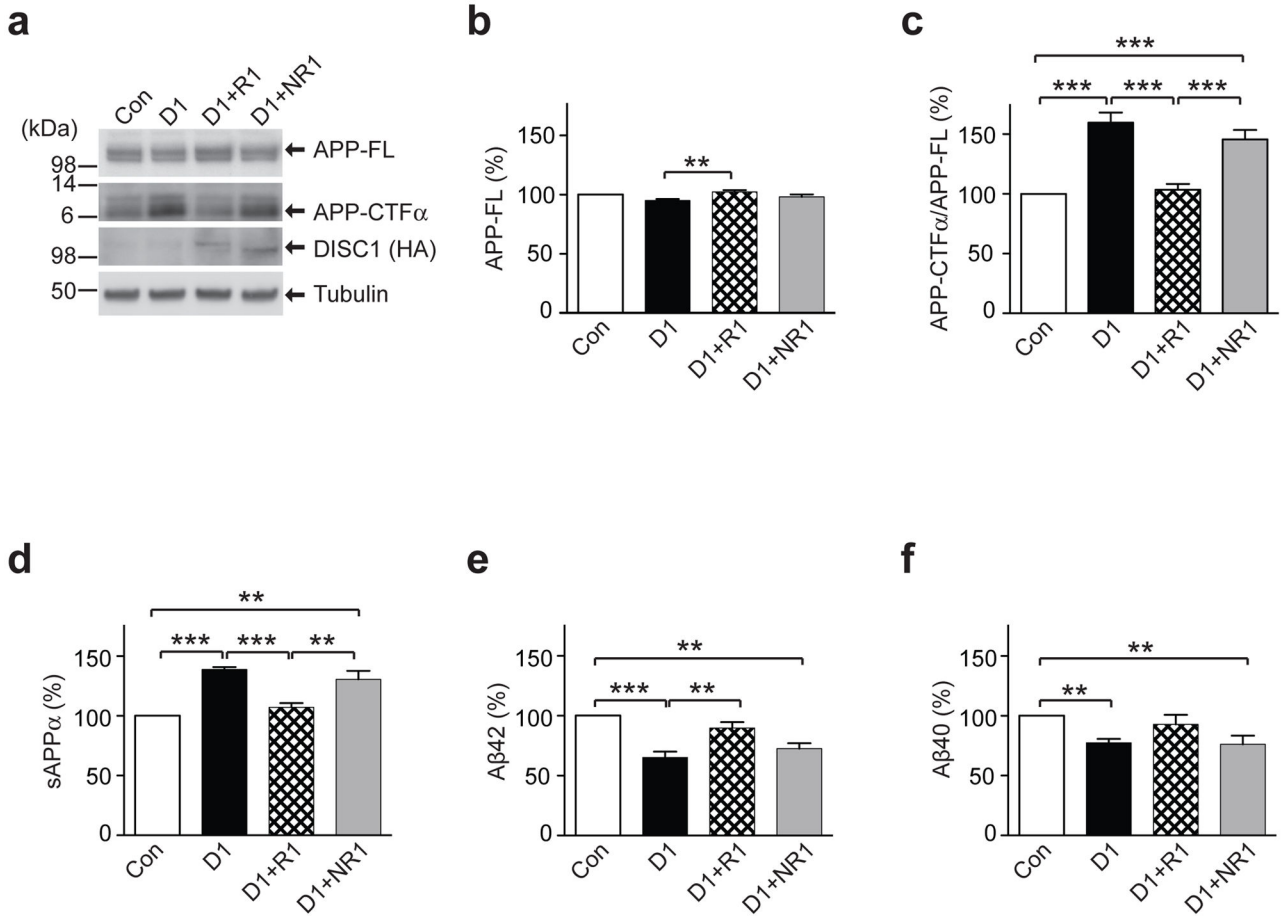
Figure 1. APP processing is regulated by a protein-protein interaction with DISC1
(a) Knockdown of DISC1 by lentiviral-mediated shRNA (D1) alters APP processing, compared to control shRNA (Con). This effect is rescued by co-expression of D1 and an shRNA-resistant wild-type DISC1 construct (R1), but not an shRNA-resistant mutant DISC1 construct lacking the binding domain for APP (NR1). Western blotting for full-length APP (APP-FL) and APP-CTFα was done for each condition using lysates from mature primary cortical neuron cultures. **(b)** Densitometric quantification of APP-FL expression in primary neurons expressing Con, D1, D1+R1, or D1+NR1. **(c)** Densitometric quantification of APP-CTFα expression in primary neurons expressing Con, D1, D1+R1, or D1+NR1. **(d–f)** Analysis of conditioned media by ELISA shows increased sAPPα and decreased Aβ42 and Aβ40 levels following DISC1 knockdown by D1. These effects are rescued by co-expression of R1, but not NR1. Bars represent the mean±SEM for at least five independent experiments. ** $p<0.01$, *** $p<0.001$.
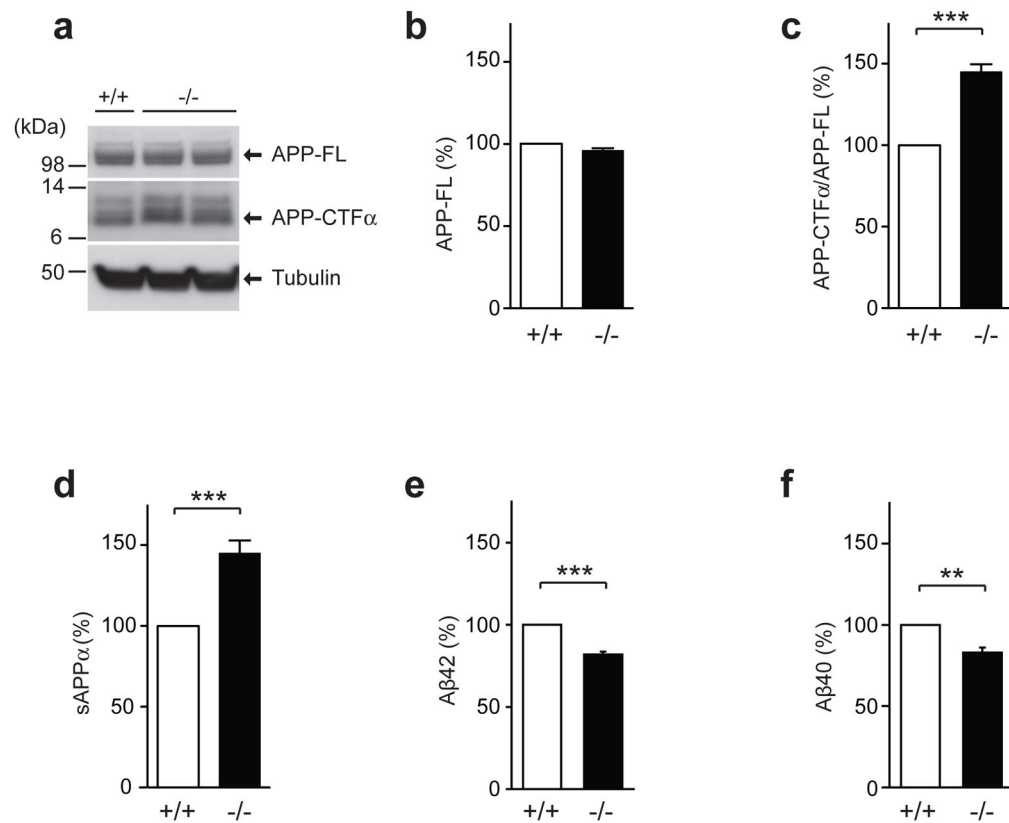
Figure 2. APP processing is dysregulated in DISC1 locus mutant mice
(a) Loss of DISC1 in homozygous DISC1 locus impairment (−/−) mice leads to altered APP processing, compared to wild-type (+/+) controls. Western blotting for APP-FL and APP-CTFα was done using cortical lysates from 3-month-old mice. (b) Densitometric quantification of APP-FL expression in −/− and +/+ mice. (c) Densitometric quantification of APP-CTFα expression in −/− and +/+ mice. (d–f) Analysis of cortical lysates by ELISA shows increased sAPPα and decreased Aβ42 and Aβ40 levels in −/− mice. Bars represent the mean±SEM, n = 5–6 mice per group. ** $p<0.01$, *** $p<0.001$.
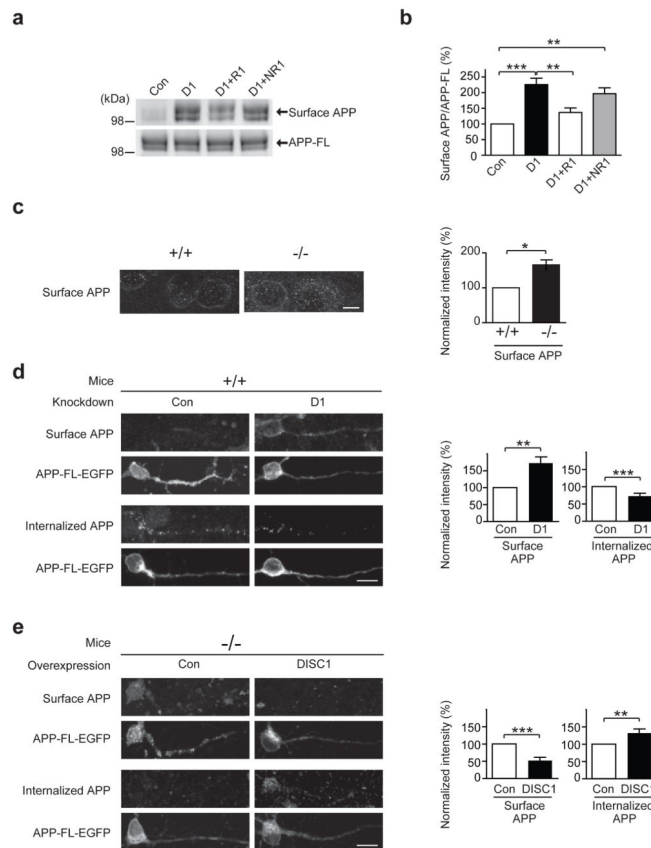
Figure 3. APP trafficking is regulated by DISC1
(a) Knockdown of DISC1 by D1 alters cell surface levels of APP, compared to Con. This effect is rescued by co-expression of R1, but not NR1. Western blotting for APP was done in lysates from mature primary cortical neurons. Surface APP was isolated by biotinylation and precipitation with neutravidin-conjugated beads. **(b)** Densitometric analysis of surface APP in primary neurons expressing Con, D1, D1+R1, or D1+NR1. **(c)** Neurons from −/− mice display higher levels of surface APP than those from +/+ mice. Scale bar, 10 μm. **(d)** Knockdown of DISC1 by D1 increases trafficking of APP to the cell surface. Transfection of mature primary neuron cultures from +/+ mice with D1 increased surface APP (top panels) and decreased internalized APP (3rd from top), compared with Con. Scale bar, 20 μm. **(e)** Expression of exogenous full-length DISC1 rescues the increased trafficking of APP to the cell surface in cells from −/− mice. Transfection of mature primary neuron cultures from −/− mice with a DISC1 construct (DISC1) decreased surface APP (top panels) and increased internalized APP (3rd from top), compared with the empty vector (Con). Scale bar, 20 μm. Bars represent the mean±SEM for at least five independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.