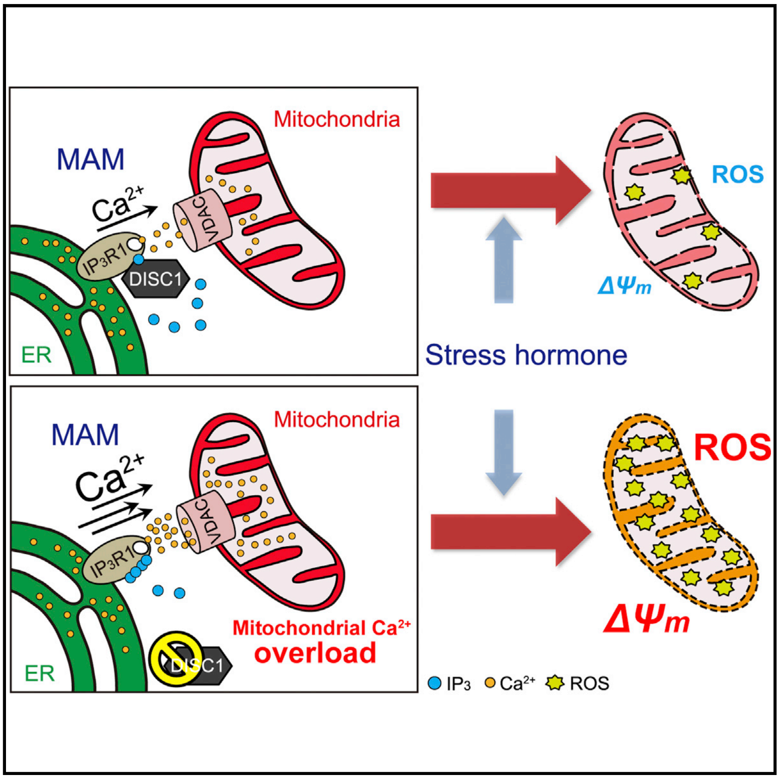
Cell Reports

DISC1 Modulates Neuronal Stress Responses by Gate-Keeping ER-Mitochondria Ca^{2+} Transfer through the MAM

Graphical Abstract



Highlights

- DISC1 is enriched in mitochondria-associated ER membrane (MAM)
- DISC1 interacts with IP_3R1 at MAM and regulates its ligand binding
- DISC1 regulates ER-mitochondria Ca^{2+} transfer through MAM
- In neuronal stress, DISC1 dysfunction impairs mitochondrial function

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In Brief

Park et al. show that DISC1 regulates ER-mitochondria Ca^{2+} transfer through mitochondria-associated ER membrane (MAM). DISC1 dysfunction at MAM increases ER-mitochondria Ca^{2+} transfer during oxidative stress and excessive amounts of corticosterone, which impairs mitochondrial function.

Park et al., 2017, Cell Reports *21*, 2748–2759
December 5, 2017 © 2017 The Author(s).
https://doi.org/10.1016/j.celrep.2017.11.043

DISC1 Modulates Neuronal Stress Responses by Gate-Keeping ER-Mitochondria Ca^{2+} Transfer through the MAM

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https://doi.org/10.1016/j.celrep.2017.11.043

SUMMARY

A wide range of Ca^{2+}-mediated functions are enabled by the dynamic properties of Ca^{2+}, all of which are dependent on the endoplasmic reticulum (ER) and mitochondria. *Disrupted-in-schizophrenia 1* (DISC1) is a scaffold protein that is involved in the function of intracellular organelles and is linked to cognitive and emotional deficits. Here, we demonstrate that DISC1 localizes to the mitochondria-associated ER membrane (MAM). At the MAM, DISC1 interacts with IP_3R1 and downregulates its ligand binding, modulating ER-mitochondria Ca^{2+} transfer through the MAM. The disrupted regulation of Ca^{2+} transfer caused by DISC1 dysfunction leads to abnormal Ca^{2+} accumulation in mitochondria following oxidative stress, which impairs mitochondrial functions. DISC1 dysfunction alters corticosterone-induced mitochondrial Ca^{2+} accumulation in an oxidative stress-dependent manner. Together, these findings link stress-associated neural stimuli with intracellular ER-mitochondria Ca^{2+} crosstalk via DISC1, providing mechanistic insight into how environmental risk factors can be interpreted by intracellular pathways under the control of genetic components in neurons.

INTRODUCTION

The mitochondria-associated endoplasmic reticulum (ER) membrane (MAM) is a specialized subcompartment that makes close contacts between the ER and mitochondria. Electron tomography analyses estimate that a very small distance (10–25 nm) exists between the MAM and the mitochondrial membrane (Giacomello and Pellegrini, 2016), and many chaperones and several key Ca^{2+} channels involved in intracellular Ca^{2+} homeostasis are concentrated at the MAM (Patergnani et al., 2011). Moreover, inositol triphosphate receptors (IP_3Rs) and voltage-dependent anion channels (VDACs) are enriched in the MAM and are physically tethered by glucose-regulated protein 75 (Szabadkai et al., 2006). Consequently, ER-stored Ca^{2+} is rapidly and efficiently transferred into mitochondria through the MAM.

Neurons are extremely polarized to best fit the function for cell-to-cell communication. ER and mitochondria are extensively dispersed throughout the cell body and distal part of neurites, functioning as key components of neuronal local Ca^{2+} signaling (Ramírez and Couve, 2011). ER Ca^{2+} channels control various neuron-specific processes, such as synaptic plasticity and neurotransmitter release (Mattson et al., 2000), and the ER and mitochondria are also very closely associated with the postsynaptic density (PSD), presumably to supply ATP in a Ca^{2+}-responsive manner, in that mitochondrial ATP production appears to be tightly regulated by intracellular Ca^{2+} levels (Berridge et al., 2000), emphasizing the potential importance of the MAM in neurons. Indeed, several lines of evidence suggest that ER-mitochondria connection at the MAM and many related functions are disrupted in neurological diseases such as Alzheimer's disease (Area-Gomez et al., 2012) and amyotrophic lateral sclerosis (Stoica et al., 2014), which display some common features, and mitochondrial dysfunction (Johri and Beal, 2012) and perturbation in intracellular Ca^{2+} homeostasis (Marambaud et al., 2009).

Oxidative stress evokes ER-mitochondria Ca^{2+} transfer at the MAM. Hydrogen peroxide (H_2O_2), superoxide anion (O_2-), and C_2-ceramide, which are generators of oxidative stress, trigger Ca^{2+} release from the ER via IP_3Rs, leading to its transfer into mitochondria (Pinton et al., 2008). Oxidative stress-induced mitochondrial Ca^{2+} accumulation reportedly contributes to mitochondrial depolarization and changes in oxidative phosphorylation, which are blocked following addition of ER Ca^{2+} channel blockers (Gerich et al., 2009). This is interesting because oxidative stress is a key mechanism that underlies various psychological stress-induced cellular and subcellular responses. Short- and long-term treatment with cortisol and other glucocorticoids, which are physiological stress hormones released in response to

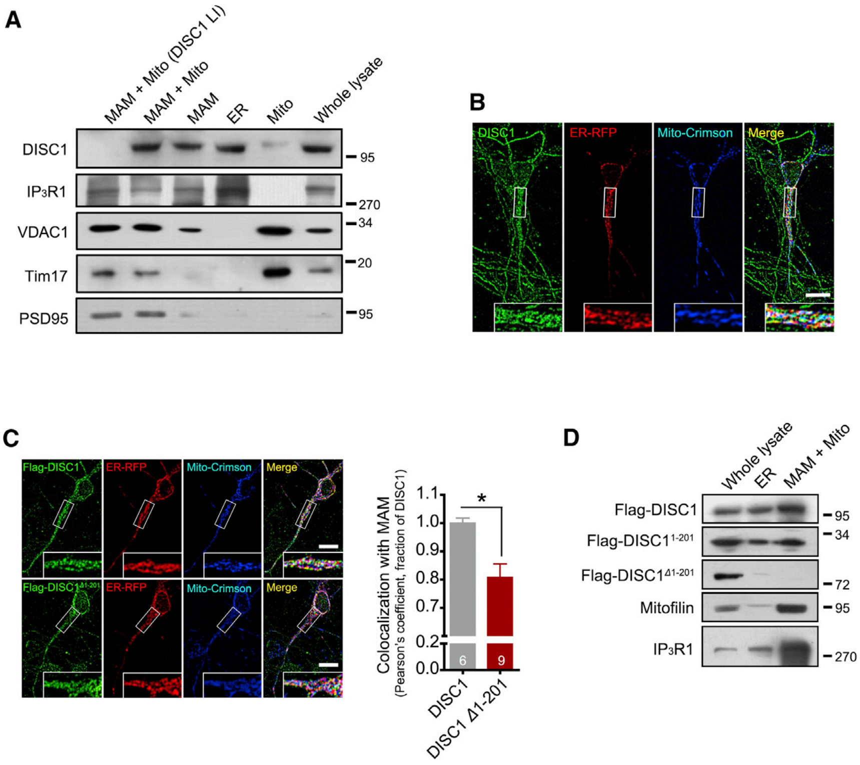

Figure 1. MAM Localization of DISC1

(A) Subcellular localization of DISC1 in organelles biochemically fractionated from adult mouse brain. Endogenous DISC1 appears at 95 kDa. IP_3R1 and VDAC1 were used as an ER and ER-associated mitochondrial marker, respectively. Tim17 and PSD95 were used as a mitochondrial and a synaptosomal marker, respectively.

(B) Staining of endogenous DISC1 in cortical neurons transfected with ER-RFP and Mito-Crimson showing ER and mitochondria, respectively. The scale bar represents 10 μm.

(C) Staining of recombinant DISC1 in cortical neurons cotransfected with ER-RFP and Mito-Crimson. A quantitative analysis for colocalization of recombinant DISC1 with ER-mitochondria merged regions (MAM) was carried out using ImageJ. The scale bars represent 10 μm. Sample size (n) is shown at the bottom of the bars in the graphs.

(D) Subcellular localization of recombinant DISC1. Mitofilin was used as a mitochondrial marker.

Error bars are presented as means ± SEM. $*p < 0.05$, two-tailed t test. All experiments were independently repeated in triplicate.

psychological stress, result in the impairment of oxidative energy metabolism and inhibition of antioxidation pathways, causing mitochondrial energy deficits and drastic elevation of cellular reactive oxygen species (ROS) (Martens et al., 1991; Sato et al., 2010), giving rise to oxidative stress in the brain.

Disrupted-in-schizophrenia 1 (DISC1) was initially underscored from the analysis of a large pedigree that shows aggregation of various major mental illnesses, including schizophrenia, in association with a chromosomal translocation by which the open reading frame for DISC1 was affected (Millar et al., 2000). Subsequent studies have provided evidence that functional perturbation of the DISC1 protein is likely to underlie the pathology of a wide range of major mental illnesses beyond the individual disease category (Niwa et al., 2016). For example, DISC1 mutant animal models display a variety of behavioral phenotypes, including deficits in cognitive memory and social behavioral deficits (Clapcote et al., 2007), that are relevant to endophenotypes of major psychiatric disorders. DISC1 has been implicated in oxidative stress and hypothalamic-pituitary-adrenal (HPA) dysregulation (Johnson et al., 2013; Niwa et al., 2013), suggesting that DISC1 can participate in the interplay between environmental risk factors such as psychological stress and intracellular calcium cascades.

To test this hypothesis, we investigated MAM localization of DISC1 and its influence on Ca^{2+} crosstalk between the ER and mitochondria under physiologically and pathologically relevant conditions. DISC1 deficiency exaggerated IP_3-dependent ER-mitochondria Ca^{2+} transfer through the MAM, leading to Ca^{2+} overload into mitochondria in response to oxidative stress and excessive corticosterone levels. These observations may provide a mechanistic link between the malfunction of DISC1 and neuronal interpretation of stress-associated stimuli with a central view of Ca^{2+}, which is potentially relevant to various psychiatric conditions.

RESULTS

DISC1 Localizes to the MAM

We assessed the intracellular localization of DISC1 in the adult mouse brain and mouse embryonic cortical neurons using biochemical and immunofluorescence techniques. As an initial screen, brains were isolated from adult mice, and serial subcellular fractionation was performed to isolate subcellular organelles. The identity and purity of each fraction was confirmed by immunoblotting with organelle-specific markers such as IP_3R1 (ER/MAM), VDAC1 (ER-associated mitochondria), Tim17 (mitochondria), and PSD95 (synaptosomes) (Figure 1A). Endogenous DISC1 was observed in the crude MAM fraction (MAM + Mito), in which mitochondria are attached to the MAM and synaptosomes are still included before they are separated by further fractionation (Annunziata et al., 2013), that was derived from the brains of adult wild-type mice but not from those of mice that harbor an impaired DISC1 locus (DISC1 LI; Seshadri et al., 2015); this confirms the specificity of the DISC1 antibody (Figure 1A). Endogenous DISC1 was observed in the ER fraction, as reported previously (Park et al., 2015), and in the MAM fraction (Figure 1A). Pure mitochondrial fractions or synaptosomal fractions were effectively removed from the final MAM fractions because Tim17 and PSD95 were not significantly detected (Figure 1A). A parallel immunochemical assay with the same DISC1 antibody revealed a dispersed pattern of endogenous DISC1 in cortical

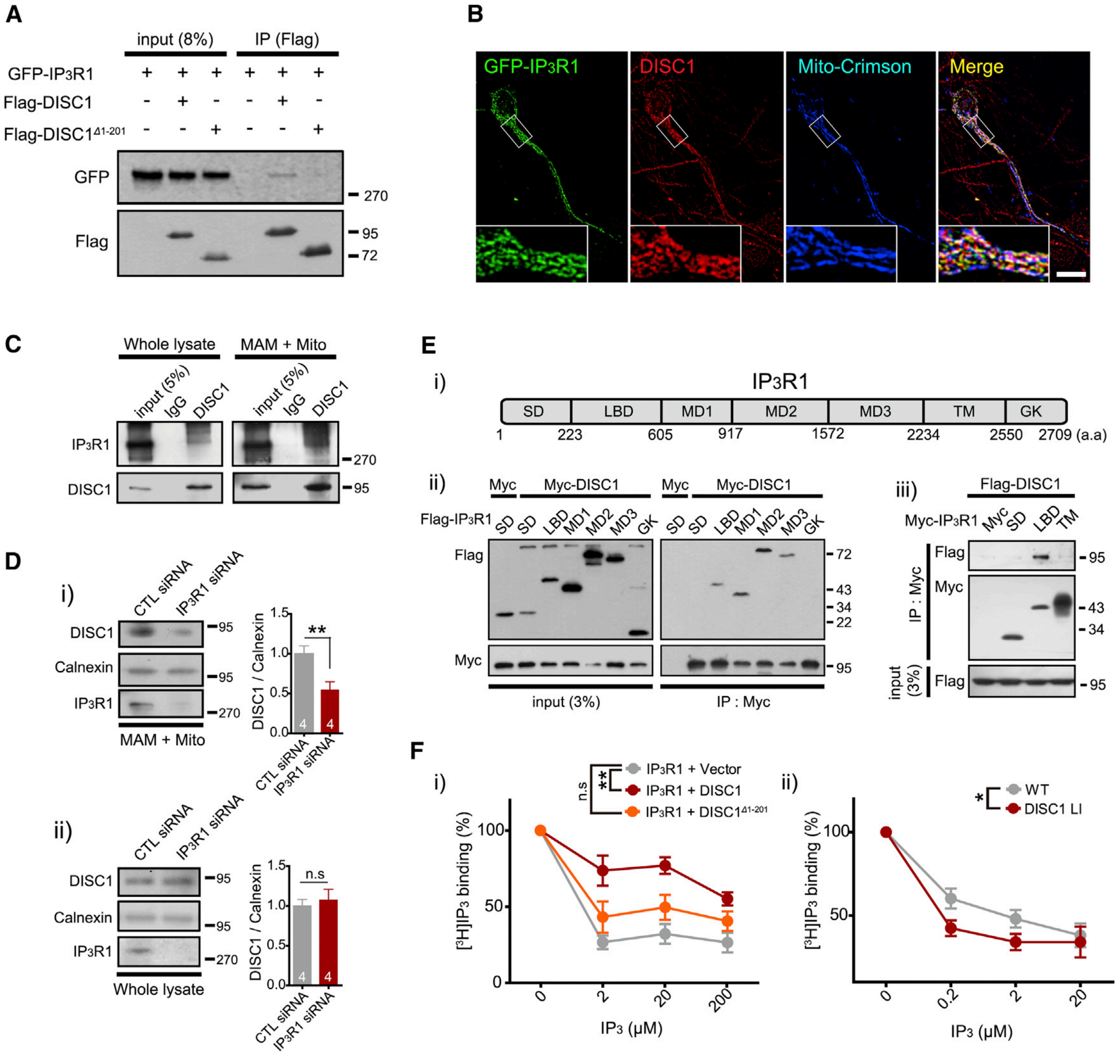

Figure 2. Functional Interaction of DISC1 and IP_3R1 at the MAM

(A) Coimmunoprecipitation (coIP) of GFP-IP_3R1 with recombinant DISC1.

(B) Staining of endogenous DISC1 in cortical neurons transfected with GFP-IP_3R1 and Mito-Crimson. The scale bar represents 10 μm.

(C) CoIP of endogenous DISC1 with IP_3R1 in whole-cell lysates (left) and the crude MAM fraction (right) derived from adult mouse brains.

(D) Alteration in the localization of DISC1 to crude the MAM fraction upon IP_3R1 knockdown (i) without changes in total expression levels (ii) in CAD cells. Densitometric analysis was performed using ImageJ. The DISC1 level was normalized to Calnexin. Sample size (n) is shown at the bottom of the bars in the graphs.

(E) Domain map of IP_3R1 (i) and coIP of IP_3R1 fragments with DISC1 (ii and iii). (SD, suppressor domain; LBD, ligand-binding domain; MD, modulatory domain; TM, transmembrane domain; GK, gate-keeping domain).

(F) Competitive IP_3 binding assay. The influence of increasing concentrations of IP_3 on $[^3H]$IP3 binding to GFP-IP_3R1 in the presence of recombinant DISC1 (i, n = 5) and to endogenous IP_3R1 isolated from adult mouse brains of WT or DISC1 LI (ii, n = 6) was analyzed.

Error bars are presented as means ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, two-tailed t test for (D) and two-way ANOVA for (F). All experiments were independently repeated in triplicate. See also Figure S1.

neurons with partial but prominent colocalization with ER and mitochondria, marked using organelle-specific expression constructs for each (Figure 1B). Furthermore, we determined that the MAM localization of DISC1 is mainly governed by residues 1–201. A statistically lower colocalization with regions where ER and mitochondrial markers intersected in cortical neurons was observed in mutant DISC1 that lacked residues 1–201 ($DISC1^{\Delta1-201}$) compared with wild-type DISC1 (Figure 1C). Moreover, there was less enrichment of $DISC1^{\Delta1-201}$ compared with the wild-type in crude MAM fractions (Figure 1D).

DISC1 Interacts with IP_3R1 at the MAM and Inhibits Ligand Binding

IP_3R1 is predominantly expressed in the brain and is enriched in the MAM. IP_3R1 showed a strong interaction with wild-type DISC1 but not with $DISC1^{\Delta1-201}$ (Figure 2A). Moreover, endogenous DISC1 (Figure 2B) and FLAG-DISC1 (Figure S1A) showed prominent colocalization with GFP-IP_3R1 at contact regions with mitochondria, which were marked by Mito-Crimson, in cortical neurons. Endogenous DISC1 and IP_3R1 were associated in whole lysates and the crude MAM fraction, which were derived from adult mouse brain (Figure 2C; Figure S1B). Next we examined the contribution of IP_3R1 to the MAM localization of DISC1. FLAG-DISC1 was significantly enriched in the crude MAM fraction upon IP_3R1 overexpression (Figure S1C, i). In contrast, IP_3R1 depletion by a specific small interfering RNA (siRNA) decreased FLAG-tagged (Figure S1C, ii) and endogenous (Figure 2D, i) DISC1 levels in the crude MAM fraction without altering total expression levels (Figure 2D, ii). Parallel immunostaining also showed a decrease in MAM localization of DISC1 upon IP_3R1 knockdown (Figure S1D). These results suggest that IP_3R1 contributes to the localization of DISC1 to the MAM.

To identify a particular IP_3R1 domain that could be responsible for binding to DISC1, we generated expression constructs for functional domains of IP_3R1 (Bosanac et al., 2004; Figure 2E, i). DISC1 interacted with multiple domains that encompass the ligand-binding and modulatory domains but not the suppressor, gate-keeping, or transmembrane domains (Figure 2E, ii and iii). Because the ligand-binding and modulatory domains are critical regions for ligand binding of IP_3Rs (Bosanac et al., 2004), we examined the influence of DISC1 expression on the binding of IP_3 to IP_3R1. IP_3R1, DISC1, or DISC1$^{\Delta 1-201}$, isolated from HEK293FT cells by immunoprecipitation with antibodies, were subjected to competitive IP_3 binding assays (Figure 2F, i). DISC1, but not DISC1$^{\Delta 1-201}$, decreased the potency of unlabeled IP_3 to compete for [^{3}H]IP_3 bound to IP_3R1 (Figure 2F, i). Furthermore, when endogenous IP_3R1 and DISC1, isolated from wild-type (WT) or DISC1 LI mouse brain lysates, were subjected to the same assays, unlabeled IP_3 binding to IP_3R1 was significantly increased in DISC1 LI samples (Figure 2F, ii). These results indicate that DISC1 inhibits ligand binding of IP_3R1.

DISC1 Regulates ER-Mitochondria Ca^{2+} Transfer through the MAM

In light of the functional association of IP_3R1 and DISC1 at the MAM, we investigated whether DISC1 regulates ER-mitochondria Ca^{2+} crosstalk. We modified an expression construct for GCaMP6, a genetically encoded Ca^{2+} indicator, by combining the targeting sequences for mitochondria or the ER. Specific localizations of the organelle-specific GCaMP6 constructs were confirmed in cortical neurons (Figures S2A and S2B). In the case of the ER Ca^{2+} measurement, we verified the results using ER-GCaMP3, which has a relatively lower affinity for Ca^{2+} (Henderson et al., 2015; Figures S2A and S2B).

Cortical neurons were preincubated with digitonin and a Ca^{2+}-free form of ionomycin in EGTA- and Ca^{2+}-free buffer for 2 min to allow plasma membrane permeabilization (Fiskum, 1985; Westerink and Vijverberg, 2002) and then were washed to prevent the collapse of the membranes of other organelles. We confirmed that this permeabilization process did not affect basal Ca^{2+} levels (Figure S2C) or general depolarization by the activation of L-type Ca^{2+} channels (Macías et al., 2001; Figure S2D, i and ii) in neurons. After permeabilization, neurons were treated with IP_3, and the IP_3-dependent increase in mitochondrial Ca^{2+} levels was significantly augmented in DISC1 knockdown neurons and rescued by the overexpression of short hairpin RNA (shRNA)-resistant human DISC1 (Figure 3A). Supporting this finding, DISC1 knockdown dramatically decreased ER-stored Ca^{2+} levels upon stimulation with IP_3 in neurons, which was monitored by two different ER Ca^{2+} indicators (Figure S3A). This result suggests that DISC1 modulates Ca^{2+} release via IP_3R1 on the ER side before Ca^{2+} is transferred into mitochondria at the MAM. The augmented Ca^{2+} transfer was consistent with the effects of bradykinin, an IP_3-generating agonist, in non-permeabilized cells (Figure S3C). In contrast, the overexpression of DISC1 reduced the increase in mitochondrial Ca^{2+} levels induced by IP_3 in permeabilized neurons (Figure 3B) and bradykinin in intact cells (Figure S3D).

However, DISC1 knockdown did not significantly change the increase in mitochondrial Ca^{2+} levels induced by 4-chloro-m-cresol (4-cmc), a ryanodine receptor agonist, indicating that DISC1 is relatively specific to IP_3R-mediated Ca^{2+} transfer (Figure S3E). Moreover, DISC1 knockdown did not affect mitochondrial capacity for Ca^{2+} uptake in neurons that were preincubated with 2-aminoethyl diphenylborinate (2-APB), a selective IP_3R blocker (Figure S3F). To further examine the intrinsic capacity of mitochondrial Ca^{2+} uptake, an *in vitro* mitochondrial Ca^{2+} assay was carried out using pure mitochondrial fractions derived from WT or DISC1 LI adult mouse brains. In response to extramitochondrial Ca^{2+} pulses, fluorescence signals of CaGreen-5N, a cell-impermeant Ca^{2+} dye, rose immediately, and after reaching at peaks, signals were sharply decreased, supposedly by mitochondrial Ca^{2+} uptake. The rates of decrease, representing mitochondrial Ca^{2+} uptake rates, were not significantly different between WT and DISC1 LI (Figure S3G). These results suggest that the effects of DISC1 on ER-mitochondria Ca^{2+} crosstalk are not due to the changes in the intrinsic mitochondrial capacity for Ca^{2+} uptake.

To further characterize the association between DISC1 localization to the MAM and the regulation of ER-mitochondria Ca^{2+} transfer, we generated a DISC1 expression construct that was fused with a targeting sequence of yeast UBC6, an ER membrane protein, to target DISC1 on the ER/MAM, as described previously (Yang et al., 1997), and we confirmed its localization to the ER and MAM in neurons (Figure S4A). UBC6-DISC1 significantly reduced IP_3-mediated Ca^{2+} transfer in a manner similar to WT DISC1 (Figure 3B). In contrast, DISC1$^{\Delta 1-201}$ failed to manifest significant changes in Ca^{2+} transfer (Figure 3B). Moreover, DISC1 dominantly expressed at the outer mitochondrial membrane or in the mitochondrial internal space by recombining the anchoring sequence of mouse AKAP1 (Csordás et al., 2010) or the yeast MIA40 leader sequence (Chacinska et al., 2004), respectively (the mitochondrial localization and topologies of those were confirmed as shown in Figures S4B and S4C), failed to change the IP_3-dependent mitochondrial Ca^{2+} response (Figure S4D).

To investigate whether the ER-mitochondria Ca^{2+} transfer regulated by DISC1 is controlled by changes in MAM formation, we used mitofusin 2 (MFN2), a protein tethering the ER to mitochondria at the MAM, and observed that MFN2 knockdown

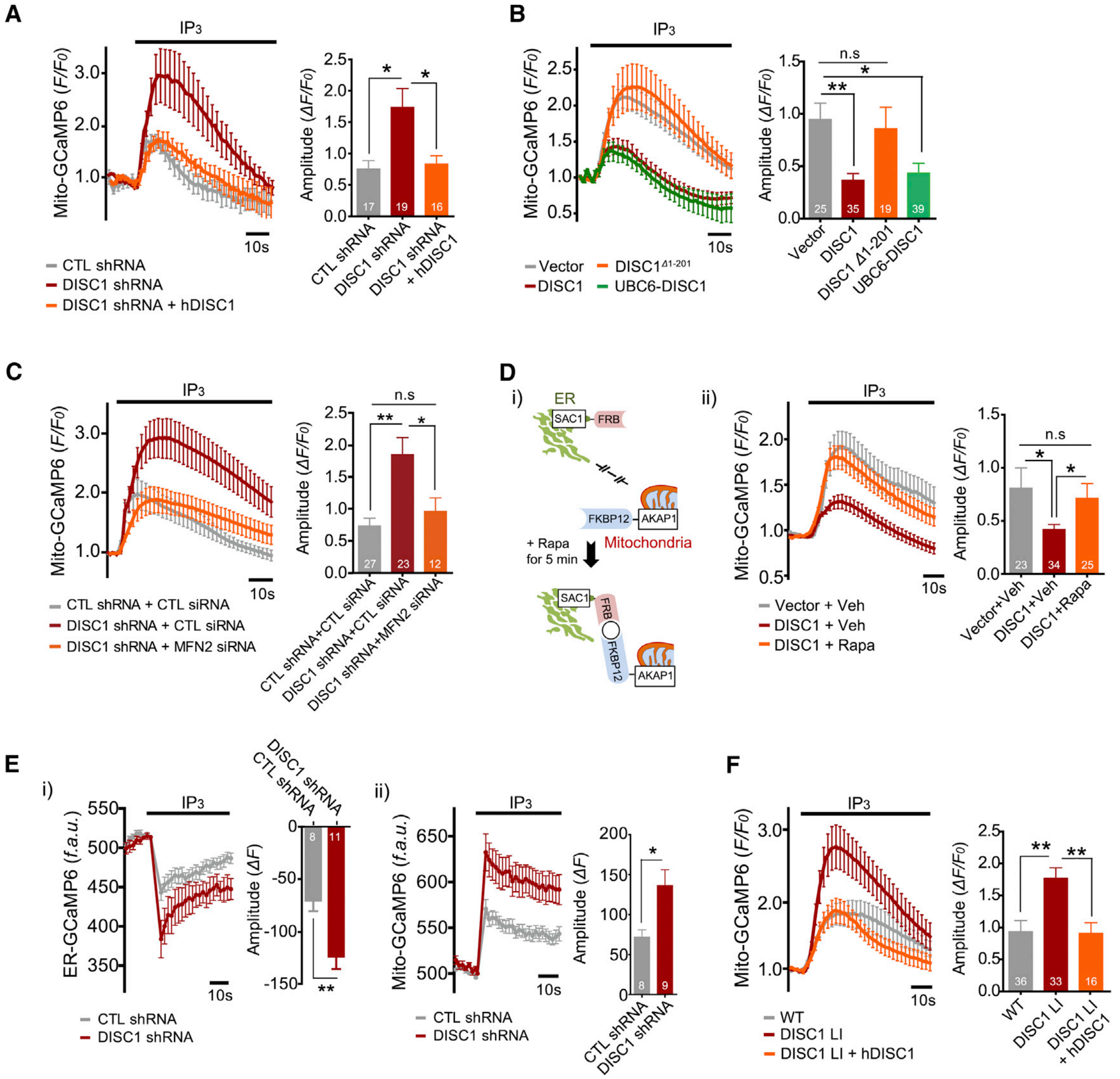

Figure 3. Regulation of IP$_3$-Dependent ER-Mitochondria Ca^{2+} Transfer by DISC1

(A) Increased IP$_3$-dependent ER-mitochondria Ca^{2+} transfer upon DISC1 knockdown and its recovery by hDISC1 overexpression in neurons. Average amplitudes of mitochondrial GCaMP6 in response to 30 μM IP$_3$ were statistically analyzed.

(B) Decreased 30 μM IP$_3$-dependent ER-mitochondria Ca^{2+} transfer upon overexpression of DISC1 and UBC6-DISC1 but not DISC1$^{\Delta 1-201}$.

(C) Reduction of IP$_3$-dependent increase in mitochondrial Ca^{2+} levels upon MFN2 knockdown in DISC1 knockdown cortical neurons.

(D) Schematic illustrating the mechanism of RiBFM (i) and enhancement of IP$_3$-dependent ER-mitochondria Ca^{2+} transfer by RiBFM in DISC1-overexpressed neurons (ii).

(E) Significant reduction of MAM-stored Ca^{2+} (i) and an increase of MAM-mitochondria Ca^{2+} transfer (ii) in response to 30 μM IP$_3$ upon DISC1 knockdown in the crude MAM fraction.

(F) IP$_3$-dependent increase of ER-mitochondria Ca^{2+} transfer in neurons derived from DISC1 LI mouse embryos and its rescue by hDISC1 overexpression.

n is shown at the bottom of bars in the graphs. Error bars are presented as means ± SEM. *$p < 0.05$, **$p < 0.01$, two-tailed t test for (E) and one-way ANOVA for (A), (B), (D), and (F). All experiments were independently repeated in triplicate. See also Figures S2–S7.

reduced ER-mitochondria contacts (Figure S5A, i and ii), as reported previously (de Brito and Scorrano, 2008), and significantly reduced ER-mitochondria Ca^{2+} transfer in DISC1-deficient neurons (Figure 3C). Moreover, we employed the rapamycin-inducible bridge-forming module (RiBFM) (Csordás et al., 2010), which enables the enhancement of ER-mitochondria contact in response to rapamycin treatment (Figure 3D, *i*). The expression patterns of two rapamycin-binding domains that localize to the ER and mitochondria, respectively, dramatically merged after treatment with rapamycin for 5 min (Figures S6A and S6B). Moreover, robust colocalization between the two proteins following rapamycin treatment lasted for 1 hr even after rapamycin was removed (Figures S6B and S6C). The activation of this module by rapamycin increased ER-mitochondria Ca^{2+} transfer in neurons (Figure S6D), as reported previously (Csordás et al., 2010). The dramatic enhancement of ER-mitochondria

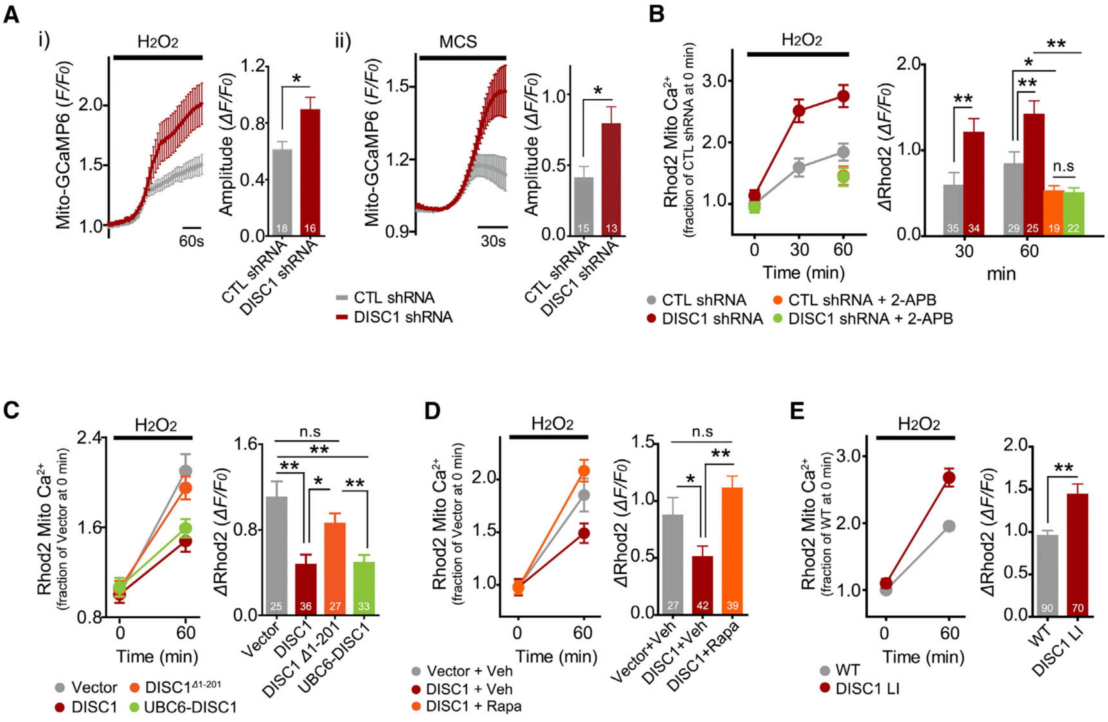

Figure 4. Regulation of Oxidative Stress-Dependent ER-Mitochondria Ca^{2+} Transfer by DISC1

(A) Exaggeration of H_2O_2-induced (i) and MCS-induced (ii) increases in mitochondrial Ca^{2+} levels upon DISC1 knockdown in neurons. Average amplitudes were statistically analyzed based on mitochondrial GCaMP6 Ca^{2+} curves in the response to 5 mM H_2O_2 or 2 mM MCS.

(B) Increase of mitochondrial Ca^{2+} accumulation upon DISC1 knockdown during incubation with H_2O_2 in a time-dependent manner and its significant reduction by 2-APB at 60 min. The intensities of Rhod-2/AM were measured 0, 30, and 60 min after 5 mM H_2O_2 treatment.

(C) Decrease of mitochondrial Ca^{2+} accumulation by H_2O_2 upon overexpression of DISC1 and UBC6-DISC1, but not DISC1$^{\Delta 1-201}$, relative to control vector in cortical neurons.

(D) Significant increase of H_2O_2-dependent mitochondrial Ca^{2+} accumulation by RiBFM in DISC1-overexpressed neurons.

(E) Significant increase of H_2O_2-induced mitochondrial Ca^{2+} accumulation in DISC1 LI cortical neurons.

n is shown at the bottom of the bars in the graphs. Error bars are presented as means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, two-tailed t test for (A) and (E) and one-way ANOVA for (B)–(D). All experiments were independently repeated in triplicate. See also Figures S3 and S7.

contacts by this module also increased Ca^{2+} transfer between the ER and mitochondria upon DISC1 overexpression (Figure 3D, ii). These results collectively support the hypothesis that DISC1-regulated ER-mitochondria Ca^{2+} transfer occurs mainly at the MAM.

To more directly assess Ca^{2+} movement through the MAM under the control of DISC1, *in vitro* Ca^{2+} assays were performed. We isolated crude MAM fractions (mitochondria-attached MAM) from neuroblastoma Cath.a-differentiated (CAD) cells that were transfected with GCaMP6 and shRNAs (Figure 3E, i and ii). We did not observe a significant difference in the basal GFP signal of GCaMP6 between crude MAM fractions derived from control and DISC1 knockdown cells (Figures S5B and S5C). This suggests that DISC1 does not significantly affect tethers between the MAM and mitochondria, which was also validated by colocalization assays that showed no difference in ER-mitochondria contacts between control and DISC1 knockdown neurons (Figure S5D, i and ii). The crude MAM fraction isolated from DISC1 knockdown cells showed a greater reduction in MAM Ca^{2+} in response to IP_3 (Figure 3E, i). Moreover, IP_3-evoked increases in mitochondrial Ca^{2+} levels were high in the fraction derived from DISC1 knockdown cells (Figure 3E, ii).

Cortical neurons cultured from DISC1 LI embryos showed a significant increase in ER-mitochondria Ca^{2+} transfer in response to IP_3, which is similar to DISC1 knockdown, and these exaggerated Ca^{2+} responses were effectively reversed by hDISC1 overexpression (Figure 3F). These results collectively show that loss of function of DISC1 leads to abnormal ER-mitochondria Ca^{2+} crosstalk at the MAM.

DISC1 Regulates Oxidative Stress-Dependent ER-Mitochondria Ca^{2+} Transfer

Recent studies have suggested that intrinsic susceptibility to oxidative stress underlies neuronal environments associated with the pathophysiology of schizophrenia (Emiliani et al., 2014). Intriguingly, oxidative stress triggers progressive Ca^{2+} release from the ER and its transfer into mitochondria in various types of cells, including neurons (Gerich et al., 2009; Pinton et al., 2008). Based on these findings, we tested whether DISC1 affects ER-mitochondria Ca^{2+} transfer that was induced by oxidative stimuli. Under H_2O_2 treatment, cortical neurons displayed slower increases in mitochondrial Ca^{2+} levels compared with increases dependent on IP_3, and DISC1 knockdown exaggerated increases in mitochondrial Ca^{2+} levels (Figure 4A, i). Treatment with mercaptosuccinic acid (MCS), an inhibitor of glutathione peroxidase that endogenously generates H_2O_2 (Gerich et al., 2009), also caused mitochondrial Ca^{2+} uptake that was exaggerated in DISC1 knockdown neurons (Figure 4A, ii). Moreover, ER Ca^{2+} indicators showed that ER-stored Ca^{2+} levels were dramatically decreased in DISC1 knockdown neurons upon stimulation with H_2O_2 (Figure S3B).

In light of the slower increase in mitochondrial Ca^{2+} levels in response to oxidative stress, we measured mitochondrial Ca^{2+} levels under oxidative stress over a longer time period. To measure mitochondrial Ca^{2+} levels at specific time points during incubation with H_2O_2, we used Rhod2/AM, a mitochondrion-specific chemical Ca^{2+} indicator, instead of Mito-GCaMP6 to avoid variations caused by differential expression levels of Mito-GCaMP6 at multiple time points. Mitochondrial Ca^{2+} accumulation observed following H_2O_2 treatment was proportional to the incubation time (Figure 4B). Moreover, DISC1 knockdown neurons exhibited greater mitochondrial Ca^{2+} accumulation after H_2O_2 treatment (Figure 4B), which was abolished by preincubation with 2-APB, indicating that IP_3R is important for DISC1 to regulate oxidative stress-induced ER-mitochondria Ca^{2+} transfer (Figure 4B). Conversely, the overexpression of WT DISC1 and UBC6-DISC1 reduced the mitochondrial Ca^{2+} peak 1 hr following H_2O_2 incubation, but this was not observed with DISC1$^{\Delta 1-201}$ (Figure 4C). The augmentation of ER-mitochondria associations by RiBFM increased mitochondrial Ca^{2+} accumulation in response to oxidative stress, offsetting the effects of DISC1 overexpression on oxidative stress-induced mitochondrial Ca^{2+} accumulation (Figure 4D). Consistent with this finding, cultured cortical neurons derived from DISC1 LI embryonic mice showed significant increases in mitochondrial Ca^{2+} peaks 1 hr after incubation with H_2O_2 compared with WT neurons (Figure 4E).

DISC1 Modulates Oxidative Stress-Induced Functional Abnormalities in Mitochondria

Excessive Ca^{2+} accumulation in mitochondria has been reported to deregulate the activity of the mitochondrial electron transport chain, causing a collapse (depolarization) of mitochondrial membrane potential and promotion of ROS generation (Duchen, 2000; Feissner et al., 2009). Such a mitochondrial dysfunction has been observed in patients with schizophrenia and in animal models that display the phenotypes of schizophrenia, implying that the deterioration of mitochondrial activity may be a component of schizophrenia pathobiology (Clay et al., 2011). Therefore, we examined the influence of DISC1 on oxidative stress-mediated mitochondrial dysfunction in cortical neurons. To measure the changes in mitochondrial membrane potential in response to oxidative stress, neurons were preincubated with tetramethylrhodamine methyl ester perchlorate (TMRM), a chemical indicator of mitochondrial potential, and exposed to H_2O_2. DISC1 knockdown led to an acceleration of H_2O_2-induced collapse of mitochondrial membrane potential in both a time- and dose-dependent manner (Figures 5A and 5B). Consistent with this, exaggerated ROS generation, as measured by dihydrorhodamine-123 (DHR-123), an indicator of mitochondrial ROS, was observed in DISC1 knockdown neurons in a time- and dose-dependent manner (Figures 5C and 5D). The pre-depletion of ER-stored Ca^{2+} reduced H_2O_2-dependent ROS production and eliminated the differences in ROS generation between control and DISC1 knockdown neurons (Figure 5D). Moreover, although UBC6-DISC1 significantly reduced ROS production in response to H_2O_2, DISC1$^{\Delta 1-201}$ failed to elicit a statistical difference compared with the control vector (Figure 5E).

Consistent with these results, neurons derived from DISC1 LI embryos displayed greater changes in mitochondrial membrane potential (Figure 5F) and ROS production (Figure 5G) under incubation with H_2O_2 compared with WT neurons. Altogether, our findings demonstrate that the function of DISC1 at the MAM is closely associated with mitochondrial functionality during oxidative stress via ER-mitochondria Ca^{2+} transfer.

Corticosterone Induces Mitochondrial Ca^{2+} Accumulation in an Oxidative Stress-Dependent Manner

Earlier studies have demonstrated that both acute and chronic treatments with excessive amounts of glucocorticoids result in the impairment of oxidative phosphorylation, causing deficits in mitochondrial ATP production (Martens et al., 1991), and that a drastic elevation of ROS leads to oxidative stress (Sato et al., 2010) in glucocorticoid receptor-rich brain regions, including the hippocampus and cortex. Thus, we hypothesized that excessive glucocorticoids could lead to ER-mitochondria Ca^{2+} transfer by inducing oxidative stress. To test this, we assessed changes in ROS and mitochondrial Ca^{2+} levels in cortical neurons following treatment with corticosterone, a glucocorticoid stress hormone. Following treatment with corticosterone for 1 hr, elevated ROS levels along with significant increases in mitochondrial Ca^{2+} levels were observed (Figures 6A and 6B). To determine whether this glucocorticoid-dependent increase in mitochondrial Ca^{2+} levels relied on the induction of oxidative stress, we used apocynin (APO), an antioxidant and ROS scavenger. Pre-treatment with APO strongly reduced the elevated mitochondrial Ca^{2+} levels induced by corticosterone in neurons (Figure 6B). Moreover, preincubation with 2-APB abolished the corticosterone-dependent increases in mitochondrial Ca^{2+} levels (Figure 6C). Conversely, treatment with corticosterone for 1 hr did not change the capacity of the ER for Ca^{2+} storage (Figure S6G) or IP_3 generation (Figure S6H) in neurons. Collectively, these results indicate that ER-mitochondria Ca^{2+} transfer is controlled by corticosterone interlinked with the induction of oxidative stress.

DISC1 Regulates Corticosterone-Dependent ER-Mitochondria Ca^{2+} Transfer

Having observed that DISC1 modulates ER-mitochondria Ca^{2+} transfer induced by oxidative stress, we addressed whether DISC1 influences mitochondrial Ca^{2+} accumulation in response to corticosterone. Under stimulation with corticosterone for 1 hr, DISC1 knockdown caused an exaggeration of increases in mitochondrial Ca^{2+} levels, and this was significantly reduced by APO treatment (Figure 7A). We further investigated the contribution of MAM localization of DISC1 to corticosterone-induced increases in mitochondrial Ca^{2+} levels. UBC6-DISC1 reduced mitochondrial Ca^{2+} accumulation under incubation with corticosterone, whereas DISC1$^{\Delta 1-201}$ and two different mitochondrion-targeting DISC1s, AKAP1-DISC1 and MIA40-DISC1, did not

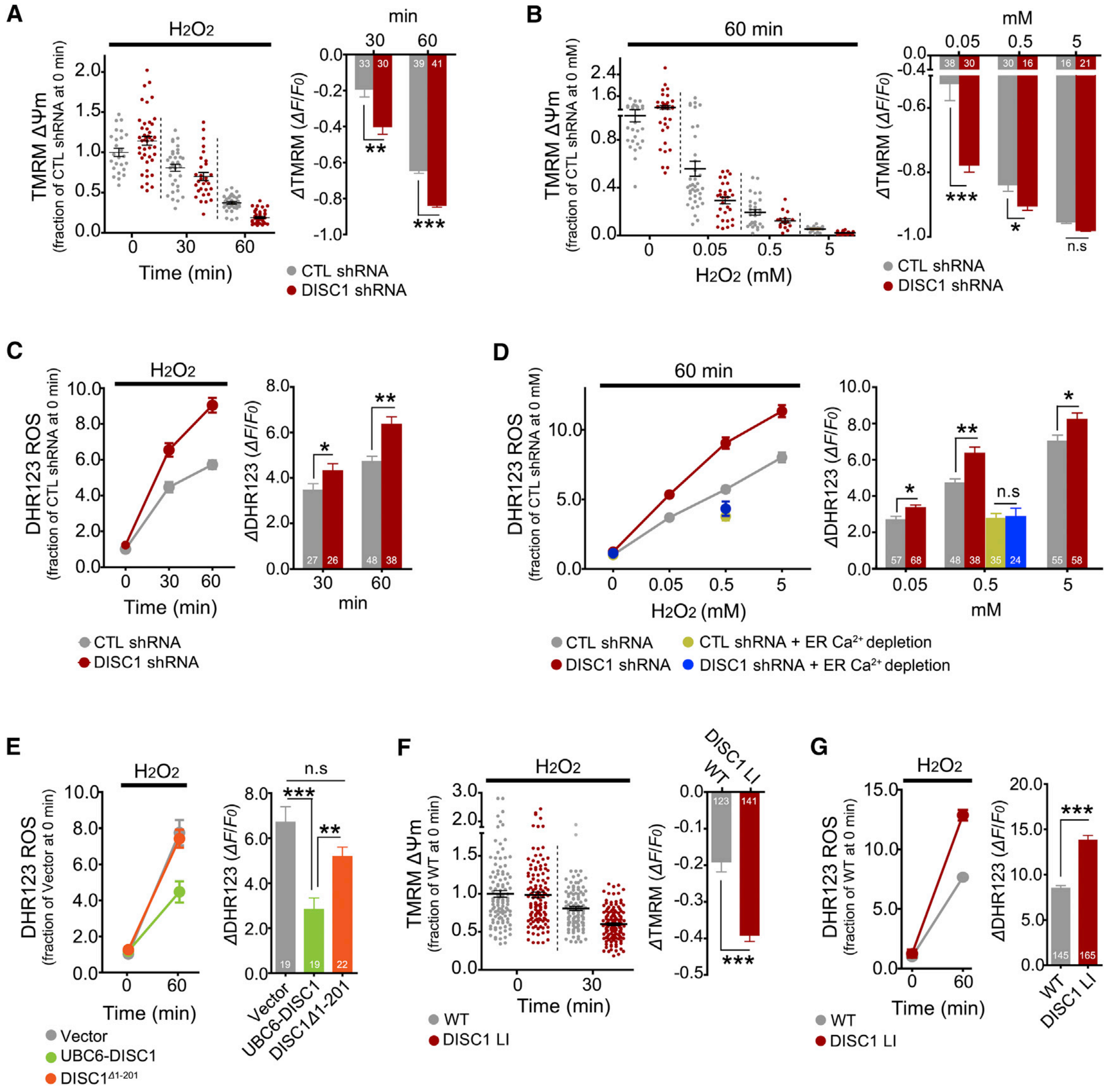

Figure 5. Regulation of Oxidative Stress-Dependent Mitochondrial Depolarization and ROS Generation by DISC1

(A and B) Dramatic reduction of mitochondrial membrane potential in DISC1 knockdown cortical neurons in a time-dependent manner following 0.5 mM H_2O_2 treatment (A) and in a dose-dependent manner for 60 min (B).

(C and D) Exaggerated increase in ROS production in DISC1 knockdown cortical neurons in a time-dependent manner following 0.5 mM H_2O_2 treatment (C) and in a dose-dependent manner for 60 min (D). Depletion of ER-stored Ca^{2+} reduced the difference in the ROS production between control and DISC1 knockdown neurons.

(E) Statistical decrease in H_2O_2-induced ROS production upon overexpression of UBC6-DISC1, but not DISC1$^{\Delta 1-201}$, in cortical neurons.

(F) Dramatic reduction of mitochondrial membrane potential following H_2O_2 (0.5 mM) treatment in DISC1 LI cortical neurons.

(G) Dramatic increase in H_2O_2 (0.5 mM)-induced ROS production in DISC1 LI cortical neurons.

n is shown at the bottom of the bars in the graphs. Error bars are presented as means ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, two-tailed t test for (F) and (G) and one-way ANOVA for (A)–(E). All experiments were independently repeated in triplicate.

(Figure 7B; Figure S4E). Moreover, the influence of DISC1 on corticosterone-induced mitochondrial Ca^{2+} changes was offset by additional MAM formation under the control of RiBFM (Figure 7C). These results indicate that DISC1 at the MAM plays an essential role in glucocorticoid-induced ER-mitochondria Ca^{2+} transfer. Next, we investigated whether excessive Ca^{2+} accumulation in mitochondria in response to corticosterone in DISC1 knockdown neurons leads to ROS overproduction. The augmentation of ROS was significantly high in DISC1 knockdown neurons (Figure 7D), which was not observed under ER Ca^{2+} predepleted conditions (Figure 7D). Moreover, WT DISC1 and UBC6-DISC1 reduced elevated ROS levels following incubation with corticosterone, whereas DISC1$^{\Delta 1-201}$ did not alter elevated ROS levels (Figure 7E). The augmentation of MAM formation by RiBFM enhanced ROS production in DISC1-overexpressing neurons (Figure 7F). In these experimental settings, rapamycin

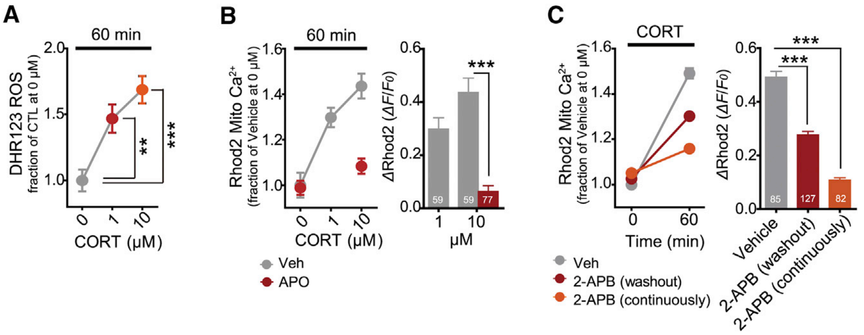

Figure 6. Mitochondrial Ca^{2+} Accumulation by Glucocorticoid in an Oxidative Stress-Dependent Manner

(A) ROS generation following corticosterone (CORT) treatment for 60 min in cortical neurons (n = 38, 21, and 36 for 0, 1, and 10 μM).

(B) Mitochondrial Ca^{2+} accumulation following CORT treatment in cortical neurons and its rescue by co-treatment with 500 μM apocynin (APO).

(C) Reduced CORT-induced mitochondrial Ca^{2+} accumulation by 2-APB (10 μM) treatment.

n for (B) and (C) is shown at the bottom of the bars in the graphs. Error bars are presented as means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA. All experiments were independently repeated in triplicate. See also Figure S6.

itself did not influence the increases in mitochondrial Ca^{2+} levels and the ROS generation evoked by oxidative stress and glucocorticoids (Figures S6E and S6F). Finally, cultured neurons derived from DISC1 LI mouse embryos showed exaggerated ROS generation (Figure 7G) and mitochondrial Ca^{2+} accumulation (Figure 7H) in response to corticosterone. Consistent with the results shown in Figure 7A, APO treatment significantly reduced the difference in mitochondrial Ca^{2+} accumulation between WT and DISC1 LI neurons (Figure 7G).

DISCUSSION

In this study, DISC1 is shown to have an inhibitory effect on IP_3R1 to downregulate the transfer of Ca^{2+} from the ER to mitochondria through the MAM in both physiological processes and potentially pathological conditions. Consequently, DISC1 deficiency abnormally exaggerates ER-mitochondria Ca^{2+} transfer in response to oxidative stress and excessive glucocorticoids, causing abnormal Ca^{2+} accumulation in mitochondria, a dramatic collapse in mitochondrial membrane potential, and the overproduction of ROS (Figure 7I). We provided evidence that the particular N-terminal region (residues 1–201) of DISC1 is critical in this process. Given that the N-terminal region was shown to interact with several interacting partners, such as Miro and TRAKs in mitochondria (Norkett et al., 2016) and PCM1 in centrosomes (Kamiya et al., 2008), further investigations are needed to dissect how these are coordinated in different subcellular contexts utilizing important sequence mutations within this region (Ogawa et al., 2014).

Our findings demonstrate that MAM Ca^{2+} signaling can be a key step in mediating cellular responses to oxidative stimuli and that DISC1 plays a gate-keeping role in this process. With DISC1 deficiency, oxidative stimuli appear to effectively induce the overloading of mitochondria with Ca^{2+} from the MAM, and therefore, DISC1-deficient neurons become more susceptible to injury from oxidative stress and related stimuli, as evidenced by a more rapid collapse of mitochondrial membrane potential and the overproduction of ROS. There is evidence, obtained from analyses conducted in animal models and postmortem brains, that aberrant mitochondrial membrane potential and ROS responses are associated with schizophrenia and related mental disorders (Emiliani et al., 2014). In this regard, the amplification of oxidative stress and consequent mitochondrial dysfunction upon DISC1 deficiency are consistent with the neuronal features that are relevant to the pathobiology of schizophrenia and related mental illnesses. This notion is intriguing because neuronal oxidative stress has been intimately linked to psychological stress. For example, animals subjected to heavy psychosocial stress, induced by physical restraint or early social isolation, exhibited high ROS levels in several brain regions (Jiang et al., 2013). In interneuron-specific *GluN1* knockout mice with N-methyl-D-aspartate (NMDA) receptor hypofunction relevant to schizophrenia (Belforte et al., 2010), postweaning social isolation (PWSI) augmented ROS levels, which was accompanied by remarkable exacerbation of schizophrenia-like phenotypes, and chronic administration of a ROS scavenger during PWSI reduced oxidative stress, with an alleviation of schizophrenia-like behaviors (Jiang et al., 2013). In agreement with these previous results, we observed induction of oxidative stress in cortical neurons following corticosterone treatment, which was causatively linked with a higher increase in mitochondrial Ca^{2+} levels in neurons. When DISC1 expression was altered, corticosterone-induced mitochondrial Ca^{2+} accumulation and ROS production were perturbed, indicating that the physiological stress-mediated oxidative stress pathway is interlinked with MAM Ca^{2+} crosstalk and tightly modulated by DISC1.

After DISC1 was found to localize to mitochondria (James et al., 2004), many studies demonstrated that DISC1 is a crucial regulator of mitochondrial morphology and distribution (Norkett et al., 2016; Piñero-Martos et al., 2016), bioenergetics (Piñero-Martos et al., 2016), and trafficking (Norkett et al., 2016; Ogawa et al., 2014) in collaboration with mitochondrial proteins such as the MICOS complex, syntaphilin, TRAK1/2, and Miro. These results established a role of DISC1 in the physiological function of mitochondria and related cellular processes. Our current findings extend the functions of DISC1 to communication between the organelles, ER, and mitochondria via the MAM. In this regard, the functions of the MAM are quite diverse, beyond the structural tethering of ER and mitochondria, in regulating mitochondrial function. For example, Ca^{2+} signals from the ER spatiotemporally control ATP synthesis in mitochondria (Jouaville et al., 1999) and mitochondrial motility by arresting or releasing mitochondrial movement in microtubules within the physiological range (Yi et al., 2004). Moreover, the MAM modulates mitochondrial fusion and fission by physically constricting mitochondria

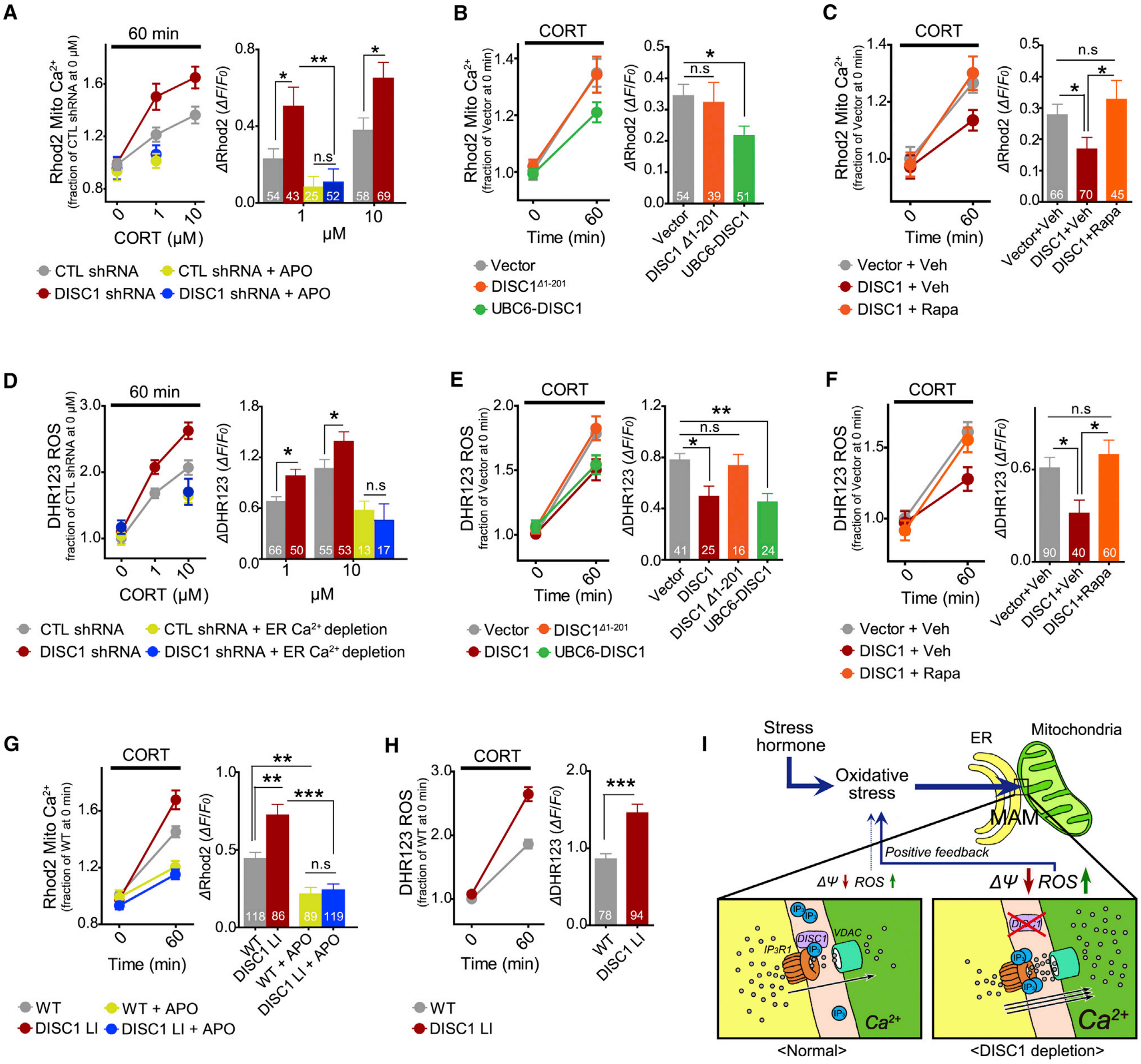

Figure 7. Regulation of Glucocorticoid-Dependent ER-Mitochondria Ca^{2+} Transfer by DISC1

(A) Exaggerated mitochondrial Ca^{2+} accumulation in DISC1 knockdown neurons following treatment with CORT and its dramatic reduction by treatment with APO (500 μM).

(B) Reduction of CORT (1 μM)-induced mitochondrial Ca^{2+} rises upon overexpression of UBC6-DISC1, but not DISC1$^{\Delta 1-201}$, in neurons.

(C) Increase in CORT-dependent mitochondrial Ca^{2+} accumulation by RiBFM in DISC1-overexpressed neurons.

(D) Abnormally increased ROS production in DISC1 knockdown neurons following CORT treatment. ER Ca^{2+}-depletion reduced the difference in ROS production between control and DISC1 knockdown neurons.

(E) Reduction of CORT (1 μM)-induced ROS production upon overexpression of DISC1 and UBC6-DISC1, but not DISC1$^{\Delta 1-201}$, in neurons.

(F) Enhanced CORT-induced ROS generation by RiBFM in DISC1-overexpressing neurons.

(G) Increase in mitochondrial Ca^{2+} accumulation in DISC1 LI cortical neurons following treatment of CORT (1 μM) and its drastic reduction by APO.

(H) Increased CORT (1 μM)-induced ROS production in DISC1 LI cortical neurons.

(I) Postulated model for DISC1's role at the MAM.

n is shown at the bottom of the bars in the graphs. Error bars are presented as means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, two-tailed t test for (H) and one-way ANOVA for (A)–(G). All experiments were independently repeated in triplicate. See also Figures S4 and S6.

(followed by division) using associated tubules, affecting mitochondrial morphology (Friedman et al., 2011). Thus, it would be of interest to investigate the association between DISC1 function in mitochondria and at the MAM in relation to the potential contribution of MAM activity in various aspects of mitochondrial physiology that are governed by DISC1; this refines the implications of mitochondrial dysfunction in psychiatric conditions.

In summary, DISC1 is a factor that can modulate the interpretation of stress into intracellular oxidative stress responses by gate-keeping ER-mitochondria Ca^{2+} crosstalk at the MAM. This finding provides an interesting model of intracellular calcium response to physiological stress, potentially reflecting the molecular basis of sensitivity to environmental insults associated with vulnerability to psychiatric conditions.

EXPERIMENTAL PROCEDURES

Mouse Lines

Pregnant C57BL/6 mice were purchased from Hyochang Science, and cortical neurons were cultured at the stage of embryonic day 15 (E15)–E16. Male WT (C57BL/6) and DISC1 locus impairment mice (DISC1 LI, C57BL/6 background) fed *ad libitum* and kept on a 12-hr light, 12-hr dark cycle for 10–12 weeks were subjected to experiments using brain lysates. All animal procedures were approved by the Pohang University of Science and Technology Institutional Animal Care and Use Committee. All experiments were carried out in accordance with the approved guidelines.

Subcellular Fractionation

Brains isolated from three adult mice were homogenized and centrifuged. A small portion of the supernatant was kept as the whole-lysate fraction, and the rest was centrifuged for 10 min at 13,800 × g at 4°C. The pellet (crude MAM) was collected, and the supernatant was loaded on a sucrose gradient and centrifuged. The white band was collected as the ER fraction. The crude MAM pellet was loaded on a sucrose gradient and centrifuged. The third white band was collected as the synaptosomal fraction, and the resulting pellet was loaded on top of the Percoll gradient and centrifuged. The upper and lower bands were collected as MAM and mitochondria, respectively.

IP_3 Binding Assay

HEK293FT cells transfected with constructs were lysed in NP40 buffer, and the proteins were immunoprecipitated with antibodies. The immunoprecipitated proteins were incubated with 3 nM $[^3H]$-IP_3 and increasing concentrations of cold IP_3 in Ca^{2+}-free cytosol-like medium (CLM) buffer for 1 hr at 4°C. Mouse brains were isolated from adult WT or DISC1 LI mice and lysed in NP40 buffer by sonication. Endogenous IP_3R1 and DISC1 immunoprecipitated with antibodies were incubated with $[^3H]$-IP_3 and increasing concentrations of cold IP_3. Mixture was filtered on a GF/B filter and washed 3 times with CLM buffer. The filters were dried, and radioactivity was measured using a scintillation counter.

Live Ca^{2+} Imaging Using GCaMP6

The neurons (days *in vitro* [DIV] 7–8) transfected with Mito-GCaMP6 together with the appropriate constructs were permeabilized with digitonin and Ca^{2+}-free ionomycin for 2 min at 37°C in modified EGTA- and Ca^{2+}-free buffer and exposed to IP_3. To measure the effects of RiBFM on mitochondrial Ca^{2+} responses, neurons were transfected with Mito-GCaMP6, AKAP1-FKBP12, and FRB-SAC1 together with pFLAG-cmv2 or FLAG-DISC1 on DIV 5–6. Neurons pre-incubated with rapamycin for 5 min were permeabilized and exposed to IP_3.

Statistical Analysis

Data were analyzed by two-tailed independent-sample Student's t test for comparisons between two different groups or one- or two-way ANOVA followed by Bonferroni's *post hoc* test for comparisons among multiple groups and expressed as mean ± SEM. The data met the assumptions of this test, and variances were similar between the groups that are being compared. Differences were considered to be significant when $p < 0.05$. No statistical methods were used to determine sample size, and randomization was not used for analyses.

Data Availability

The authors declare that all relevant data are available from the corresponding author upon request.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.11.043.

AUTHOR CONTRIBUTIONS

S.J.P. conceived the study, performed experiments, analyzed and interpreted data, and wrote the manuscript. S.B.L. and Y.S. performed experiments. S.-J.K., N.L., J.-H.K., and P.-O.B. provided experimental tools. Y.W., K.I., and A.S. provided vital reagents. J.-H.H. and C.P. provided key ideas for experiments. S.K.P. conceived the study, coordinated experiments, interpreted data, and wrote the manuscript.

ACKNOWLEDGMENTS

We thank Ohseop Kwon for designing the images in our figure sets and Dr. Kihean Kim and Viet-Hoan Le for technical support. This work was supported by Samsung Science and Technology Foundation grant SSTF-BA1601-14.

Received: June 2, 2017
Revised: September 21, 2017
Accepted: November 12, 2017
Published: December 5, 2017

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