DISC1 (Disrupted-In-Schizophrenia 1) is a centrosome-associated protein that interacts with MAP1A, MIPT3, ATF4/5 and NUDEL: regulation and loss of interaction with mutation

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Disrupted-In-Schizophrenia 1 (DISC1) is a novel gene associated with schizophrenia by multiple genetic studies. In order to determine how mutations in DISC1 might cause susceptibility to schizophrenia, we undertook a comprehensive study of the cellular biology of DISC1 in its full-length and disease-associated mutant forms. DISC1 interacts by yeast two-hybrid, mammalian two-hybrid, and co-immunoprecipitation assays with multiple proteins of the centrosome and cytoskeletal system, including MIPT3, MAP1A and NUDEL; proteins which localize receptors to membranes, including α-actinin2 and β4-spectrin; and proteins which transduce signals from membrane receptors, including ATF4 and ATF5. Truncated mutant DISC1 fails to interact with ATF4, ATF5 or NUDEL. Deletion mapping demonstrated that DISC1 has distinct interaction domains: MAP1A interacts via its LC2 domain with the N-terminus of DISC1, whereas MIPT3 and NUDEL bind via their C-terminal domains to the central coiled-coil domain of DISC1, and ATF4/5 bind via their C-terminal domains to the C-terminus of DISC1. In its full-length form, DISC1 protein localizes to predominantly perinuclear punctate structures which extend into neurites in some cells; mutant truncated DISC1, by contrast, is seen in a diffuse pattern throughout the cytoplasm and abundantly in neurites. Both forms co-localize with the centrosomal complex, although truncated less abundantly than full-length DISC1. Although both full-length and mutant DISC1 are found in microtubule fractions, neither form of DISC1 appears to bind directly to microtubules, but rather do so in a MIPT3-dependent fashion that is stabilized by taxol. Based on these data, we propose that DISC1 is a multifunctional protein whose truncation contributes to schizophrenia susceptibility by disrupting intracellular transport, neurite architecture and/or neuronal migration, all of which have been hypothesized to be pathogenic in the schizophrenic brain.

INTRODUCTION

Schizophrenia is a disabling mental illness which is associated with a high rates of morbidity and mortality (1,2). Both positive (hallucinations, delusions) and negative (social withdrawal, cognitive dysfunction) symptoms characterize the disease (3). Compared with the ~1% risk of schizophrenia in the general population, the lifetime risk of developing the disease in relatives of schizophrenics is greatly elevated, ranging from 6% in parents to ~50% in identical twins (4). Schizophrenia susceptibility therefore has a large genetic component, but environmental factors also play a prominent role (2). Through genetic linkage and association studies, multiple chromosomal regions have been implicated as containing schizophrenia susceptibility genes (5,6). Recently, several genes have been identified within these regions which have biology plausibly related to schizophrenia, including COMT (catechol-O-methyltransferase) (7,8), dysbindin (9) and NRG1 (10,11).

DISC1 (Disrupted-In-Schizophrenia 1) was initially implicated in schizophrenia by an alternative strategy, which identified genes at the breakpoints of a balanced [(1:11)(q42.1;q14.3)] chromosomal translocation which segregated with a high incidence of schizophrenia and other...
psychiatric disease in a large family (12). The finding was compelling both because the high incidence of disease suggested that the translocation conferred a high degree of disease risk, and because of the unequivocal nature of the genetic lesion, a translocation which resulted in truncation of the DISC1 protein. This is in contrast with most previous findings in schizophrenia genetics, which have associated polymorphisms of unclear functional significance with small increases in schizophrenia risk. Furthermore, a subsequent study in an unrelated Finnish population demonstrated significant linkage to a microsatellite marker within the DISC1 locus to schizophrenia (13). Finally, Weinberger and colleagues have recently found a highly significant association of a SNP in DISC1 with schizophrenia in a third independent population (Daniel R. Weinberger, personal communication). DISC1 is therefore a compelling candidate schizophrenia susceptibility gene based on genetic evidence. We have undertaken to understand DISC1’s normal biology, and the effects of DISC1 truncation, as an avenue to understanding the heightened disease susceptibility this truncation confers.

As part of this effort, we recently demonstrated that regions of the primate brain which express DISC1 have been implicated in schizophrenia in humans (14), including the hippocampus, lateral septum, amygdala, cerebral cortex, cerebellum and paraventricular hypothalamus (15). In addition, we have isolated the mouse ortholog of DISC1, shown that its genetic structure and bioinformatically identified motifs are conserved with the human, and identified a novel splice form in the mouse which begins at a site orthologous to the truncation site in the human gene (16). Interestingly, Disc1 is expressed in the mouse brain in homologous regions to those that express DISC1 in the primate, including the hippocampus, cerebral cortex, olfactory bulbs and cerebellum (16).

In this paper, we describe the results of multifaceted studies into the cell biology of DISC1. Yeast two-hybrid analysis identified multiple interactors which function in the structure and regulation of the cytoskeleton and of the centrosome, the cell’s microtubule organizing center. Immunocytochemical studies of the intracellular expression of DISC1 showed that the protein is normally punctate and cytoplasmic, concentrated in an asymetrically perinuclear distribution that includes the centrosome, but with punctata extending into neuronal processes. Subcellular fractionation studies confirmed centrosomal and microtubular association of DISC1. Truncation of DISC1 at the schizophrenia-associated breakpoint disrupts the normal perinuclear pattern of DISC1 localization, producing instead a diffuse cytoplasmic distribution, although at least partial centrosomal and microtubular association is maintained. In addition, truncation DISC1 no longer interacts with ATF4, ATF5 or NUDEL. Treatment of DISC1 expressing cells with agents that disrupt the cytoskeleton also disrupt DISC1 localization and the intracellular transport.

RESULTS

Identification of DISC1 interactors by yeast two-hybrid analysis

In order to identify proteins that physically interact with DISC1, cDNA libraries were screened by yeast two-hybrid (YTH) analysis. Libraries expressing fusion proteins with the GAL4 AD (activation domain) were screened for DISC1 interacting proteins using a FL-DISC1-BD (full-length DISC1 GAL4 DNA-binding domain) fusion protein as a bait. When the AD and the BD are brought in close proximity, they initiate transcription through the GAL promoter of four yeast reporter genes: HIS3, ADE2, LacZ and MEL1. Brain and heart libraries were chosen since DISC1 expression had been detected on northern blots of human (12), mouse, and rat (16) tissues. Yeast were cotransformed with the DISC1 bait and the fusion library, and plated at medium stringency (dropout medium minus his, leu, trp) in order to identify low affinity interactors. Forty-nine positive colonies were identified from the brain library, and 69 positive colonies from the heart library. Owing to the large number of positives, colonies were replated in high stringency medium (minus ade, his, leu, trp and X-gal), which reduced the number of positive colonies to 19 from the brain library and 40 from the heart library.

Plasmid DNA from the YTH interactors was isolated and sequenced (Table 1). Many of the clones were identical; for example, from the heart library, eight clones encoded ATF5, four clones encoded SYNE-1 and two clones encoded NUDEL. Two interactors, EIF3 (eukaryotic initiation factor 3) and MIP3, were identified in both the brain and heart libraries. The majority of the cDNAs were partial clones. The only clones isolated from the YTH libraries in full-length form were EIF3, NUDEL and RanBP.

The positive interactors were further verified by multiple assays, including β-galactosidase, cotransformation, yeast mating, and biosensor assays (see summary of results in Table 1). The β-galactosidase filter assay allows visual colorimetric detection of protein–protein interactions. For this assay, yeast cotransformed with the candidate YTH interactor-AD and FL-DISC1-BD were plated on selective medium; positive colonies were filter-lifted and β-galactosidase activity demonstrating transcriptional activation of the GAL promoter determined by colorimetric detection. ATF4, ATF5, EIF3, KIAA1377, MAP1A and NUDEL produced the greatest β-galactosidase activity. For co-transformation assays, each YTH interactor-AD was cotransformed with FL-DISC1-BD, plated on selective medium, and positive colonies quantitated (Table 1). For yeast matings, different yeast strains transformed with YTH interactor-AD and with FL-DISC1-BD were mated, plated on selective medium and positive colonies counted. The final confirmatory assay was a biosensor assay, in which an oxygen-sensing fluorophore detects oxygen consumption (indicative of cell growth) of yeast co-transformed with FL-DISC1-BD and a YTH interactor-AD in selective medium. The amount of fluorescence detected correlates with cell growth, which in turn is dependent on the interaction of FL-DISC1 with the YTH interactor (data summarized in Table 1). Only YTH interactors which were confirmed by β-galactosidase and at least one other confirmatory assay were considered true positives and followed up with further study.
As a next step in determining the physiological relevance of the protein interactions identified in yeast, coimmunoprecipitation experiments were performed in mammalian cells. The YTH interactors were subcloned into the mammalian expression vector pcDNA3.1/His containing a Xpress epitope tag. FL-DISC1 was subcloned into a mammalian expression construct, pcDNA3.1/V5-HisTOPO, placing a V5 epitope tag at the carboxy terminal end of DISC1. In addition, a truncated DISC1 (TRUN-DISC1) was generated by PCR placing a stop codon at the translocation breakpoint site (12), and was subcloned into pcDNA3.1/V5-HisTOPO.

The YTH interactors were cotransfected with FL-DISC1-V5 into HEK 293 cells. YTH interactors were immunoprecipitated from whole cell extracts using an anti-Xpress antibody, and coimmunoprecipitation with DISC1 assayed for by western blot. Eleven proteins in multiple transfections were identified as interacting with FL-DISC1 by coimmunoprecipitation (Table 2). Many of the proteins identified were associated with the centrosome, the cytoskeleton, elaboration or maintenance of neuronal processes, and/or signal transduction. Interestingly, four of the proteins confirmed as interacting with DISC1 in mammalian cells (\(\alpha\)-actinin2, MAP1A, RanBPM, and SYNE-1) map to chromosomal loci linked to schizophrenia (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM).

Since the translocation in the schizophrenia patients produces a truncated DISC1 protein, we next tested whether the 11 mammalian interactors in Table 2 maintain their interaction with DISC1 upon truncation. Expression vectors encoding the 10 interactors were each cotransfected with TRUN-DISC1-V5 in HEK 293 cells, and coimmunoprecipitation experiments were performed. Most of the proteins interacted with both FL and TRUN-DISC1, but AT5 and NUDEL did not interact with TRUN-DISC1 (Table 2). The loss of these interactions when DISC1 is truncated suggests mechanisms by which DISC1 truncation may affect cellular functions.

Subcellular localization of full-length and truncated DISC1

Molecular interaction analysis by two-hybrid and subsequent mammalian cell interaction analysis suggested that DISC1 associates with the cytoskeleton and proteins that regulate it. Bioinformatic analysis of DISC1 suggested the presence of coiled-coil domains which often subserve structural functions (17), and leucine zipper repeats often seen in transcription factors. To determine whether the subcellular distribution of DISC1 was consistent with any of these hypothesized functions, we studied the localization of DISC1 in neuronal cells by immunocytochemistry. Sindbis virus vectors expressing Xpress epitope-tagged FL-DISC1, Xpress epitope-tagged TRUN-DISC1, or \(\beta\)-galactosidase, were constructed. Human NT2N neurons were infected with each of the viruses 24 h after plating. The viral vectors used in these experiments produced a lower (and presumably more physiologic) level of expression than was obtained by transient or stable transfection, and allowed the use of differentiated neuronal cells not amenable to transfection. These experimental design features allowed us to maximize the likelihood that localization of epitope-tagged DISC1 would accurately reflect the endogenous distribution in brain cells, which could not be examined directly since our native peptide antibodies are not yet reliable for this purpose (data not shown).

All DISC1 immunoreactivity was cytoplasmic; no immunoreactivity was seen in the plasma membrane or nucleus (Fig. 1A). However, the distributions of FL-DISC1 and TRUN-DISC1 were dramatically different. In most cells expressing FL-DISC1, immunoreactivity was predominantly perinuclear and asymmetric. The majority of the immunoreactivity was seen as fine discrete punctate structures, which in some cells extended into the cellular processes (Fig. 1B). In contrast, in cells expressing TRUN-DISC1, the majority of the immunoreactivity was spread diffusely throughout the cytoplasm and cellular processes (Fig. 1A). Similar patterns of immunoreactivity were seen in other cell lines.

### Table 1. DISC1 interactors identified by YTH assay

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nd: not determined.
Human SH-SY5Y neuroblastoma cells were transiently transfected with FL- or TRUN-DISC1 fused to green fluorescent protein (GFP), or a GFP-only control. The majority of cells expressing FL-DISC1-GFP demonstrated fluorescence in fine punctate structures (Fig. 1C1). A minority of cells demonstrated FL-DISC1-GFP fluorescence in larger punctate structures (Fig. 1C2). Percentages of cells showing fine punctate, coarse punctate and diffuse staining patterns in SH-SY5Y cells transiently transfected with FL-DISC1-GFP were 84, 16 and 0%, respectively. By contrast, in SH-SY5Y cells transiently transfected with TRUN-DISC1-GFP, only 28% of the cells showed a fine punctate staining pattern.
DISC1 interacts with NUDEL

The yeast two-hybrid and mammalian coimmunoprecipitation studies demonstrated that DISC1 interacts with NUDEL, a LIS1-interacting protein which is enriched at centrosomes and neuronal growth cones, interacts with cytoplasmic dynein, and is implicated in neurite outgrowth, neuronal migration and anterograde and/or retrograde transport (20,21). Coimmunoprecipitation studies in mammalian cells indicated that NUDEL interacts with FL-DISC1, but not with TRUN-DISC1 (Table 2). Since NUDEL has a number of important functions potentially relevant to schizophrenia, loss of the DISC1-NUDEL interaction upon DISC1 truncation might have important physiological ramifications. We therefore undertook to map the domains of DISC1 with NUDEL required for interaction in further detail, using a mammalian two-hybrid system and coimmunoprecipitation assays with deletion constructs of both DISC1 and NUDEL in HEK293T cells.

To quantify protein–protein interaction in mammalian cells, we employed the mammalian two-hybrid system, which is similar in its experimental design to that of the yeast two-hybrid system (22). In this system, one protein is fused to the GAL4 DNA-BD and the other to the VP16 AD. Transcription from a GAL4 promoter upstream from a firefly luciferase reporter gene is activated when the BD and AD containing proteins are brought into close proximity, with interaction quantified by a luciferase activity assay.

Full-length and two C-terminal deletion constructs of DISC1 were sub-cloned into pBIND, resulting in the production of fusion proteins of DISC1 and the GAL4 BD (Fig. 3A). The deletion constructs produced the two parts of the DISC1 protein on either side of the translocation breakpoint, i.e. TRUN-DISC1 (a C-terminal deletion containing amino acids 1-597) and NT-del1-DISC1, an N-terminal deletion containing amino acids 598-854. Full-length NUDEL, and two C-terminal NUDEL deletion constructs, were generated in the pACT vector resulting in a fusion protein with the VP16 transactivation domain (Fig. 3B). The deletion constructs remove the carboxy-terminal domain of the NUDEL, which contains the cytoplasmic dynein binding domain from amino acids 256-291 (23), as well as variable amounts of the conserved NUDEL domain (24).

As expected from the yeast two-hybrid and coimmunoprecipitation results, cotransfection of full-length DISC1 and NUDEL with a luciferase reporter gene produced robust transactivation, whereas cotransfection of TRUN-DISC1 and NUDEL produced no luciferase activity (Fig. 3C), indicating that NUDEL interacts with the third of DISC1 that is deleted by the truncation. Interestingly, cotransfection of FL-NUDEL and the DISC1 C-terminal domain (NT-del1-DISC1) produced over twice the level of luciferase activity as cotransfection with FL-DISC1,
consistent with the DISC1-NUDEL interaction domain being in the carboxy terminal domain of DISC1. Analogous experiments with NUDEL deletion constructs showed that either C-terminal NUDEL deletion eliminated all luciferase activity (Fig. 3C). DISC1 is therefore binding to the carboxy terminus of NUDEL, which contains the cytoplasmic dynein binding domain and part of the conserved NUDE motif (23,24).

Coimmunoprecipitation experiments in HEK293 cells were consistent with these results. Multiple DISC1 deletion constructs were generated containing the amino-terminal portion of DISC1 (TRUN-DISC1) or the carboxy-terminal portion (NT-del2-DISC1 and NT-del3-DISC1, Fig. 3A). Using these constructs, we were able to coimmunoprecipitate NUDEL with FL-DISC1, but not TRUN-DISC1 (Fig. 3D). NT-del2-DISC1, which deletes the first 292 amino acids of DISC1 but contains all three leucine zipper and coiled-coil domains, also coimmunoprecipitated with NUDEL. When the leucine zippers and coiled-coil domains were removed to create NT-del3-DISC1, however, DISC1’s binding to NUDEL was abolished. Since the mammalian two-hybrid studies showed binding of full-length NUDEL to amino acids 598–854 of DISC1, and the coimmunoprecipitation studies showed lack of binding of full-length NUDEL to amino acids 697–854 of DISC1, it can be concluded that NUDEL is binding amino acids 598–696 of DISC1, which contains both the last leucine zipper and coiled-coil domain of DISC1.

Double immunofluorescence experiments provided further support for this conclusion. NUDEL and FL-DISC1 or TRUN-DISC1 were transiently cotransfected into SH-SY5Y cells to examine colocalization (Fig. 3E–K). NUDEL transfected alone gave a granular cytoplasmic staining pattern (Fig. 3E), consistent with previous studies (20). When cotransfected with FL-DISC1, the localization pattern of NUDEL was altered dramatically, to produce a punctate staining pattern which corresponded to the pattern of DISC1 expression (colocalization seen as yellow in Fig. 3H). By contrast, cotransfection of NUDEL with TRUN-DISC1 did not change the subcellular distribution of either protein (Fig. 3I–K). These results support the notion that FL-DISC1, but not TRUN-DISC1, associates with NUDEL in cells.
DISC1’s interaction with microtubules

The centrosome is the cell’s principal microtubule organizing center, regulating the assembly of α and β tubulin into microtubules (25). Since DISC1 is localized at the centrosome, we examined whether DISC1 is also localized to, or associates with, microtubules. HeLa cells were transiently transfected with FL-DISC1-GFP, TRUN-DISC1-GFP or GFP alone. DISC1 localization was detected by GFP fluorescence, and microtubule localization was detected by α-tubulin immunocytochemistry. FL-DISC1-GFP did not colocalize with α-tubulin, whereas TRUN-DISC1-GFP demonstrated partial colocalization (data not shown). To further test for any association, transiently transfected cells were treated with nocodazole, which disrupts microtubules, or taxol, which stabilizes them (26). If DISC1 was binding to microtubules,
its subcellular distribution should change in concert with the subcellular distribution of \( \alpha \)-tubulin (27). Although, as expected, microtubules were disrupted by nocodazole and stabilized by taxol, the subcellular distribution of FL-DISC1-GFP or TRUN-DISC1-GFP was not affected by either drug treatment (data not shown). These results suggested that the majority of DISC1 is not stably associated with microtubules. However, it remained possible that a lower-abundance, more transient, or more indirect association existed between DISC1 and microtubules.

We therefore used a more sensitive method, the MAP (microtubule associated protein) spin-down assay, to test for microtubule association of DISC1 (28,29). Whole cell extracts from stably transfected FL-DISC1-V5 and TRUN-DISC1-V5 expressing cell lines were mixed with pre-assembled, taxol-stabilized microtubules, and centrifuged. Pellets (which contain the microtubules and their associated proteins) and supernatants were analyzed by SDS-PAGE and epitope-tagged DISC1 detected by anti-V5 immunoblot. Both FL-DISC1 and TRUN-DISC1 were present in the pellets (Fig. 4), indicating that DISC1 does in fact associate with microtubules. Taken together, the immunocytochemical and biochemical results are consistent with a model in which only a subset of DISC1 protein is associated with microtubules at any one time. These results do not distinguish between direct and indirect binding of DISC1 to microtubules, but the finding that DISC1 interacts with the microtubule associated proteins MAP1A and MIPT3 (Table 2) suggests that at least part of the association may be indirect, mediated by these proteins.

**MIPT3 interacts with the coiled-coil domain of DISC1**

MIPT3, or MIP-TRAF3 (Microtubule-Interacting Protein associated with TNF Receptor Associated Factor-3), was identified as interacting with DISC1 by yeast two-hybrid assay (Table 1). MIPT3 interacts with microtubules and recruits other proteins to the cytoskeleton, so represents a potential molecular mechanism by which DISC1 associates with microtubules (27). To examine the association directly, communoprecipitation studies with deletion constructs of both proteins were performed.

Full-length MIPT3 and an N-terminal deletion containing amino acids 223-625 of MIPT3 were sub-cloned into a mammalian expression vector containing an Xpress epitope tag (Fig. 5A). Communoprecipitation assays were performed in HEK293T cells with FL-MIPT3 or amino acids 223-625-MIPT3, and the previously described deletion constructs of DISC1 (Fig. 3A). Both FL (amino acids 1-625) and TRUN (amino acids 223-625) MIPT3 communoprecipitated with FL-DISC1, TRUN-DISC1 and NT-del2-DISC1, but neither FL- nor TRUN-MIPT3 communoprecipitated with NT-del3-DISC1 (Fig. 5A and B). These results indicate that the interaction domains of MIPT3 and DISC1 are in the C-terminal 400 amino acids (223-625) of MIPT3 and the central 400 amino acids (293-696) of DISC1. These interaction domains contain coiled-coils in both proteins, and the interaction domain of DISC1 further includes several leucine zipper domains; both of these domains frequently mediate protein–protein interactions. Interestingly, the interaction domain of MIPT3 does not include the N-terminal domain that is required for its interaction with microtubules (27).

**MIPT3 recruits DISC1 to microtubules**

The interaction of DISC1 and MIPT3 was next examined in cells by co-immunocytochemistry in transiently cotransfected SH-SY5Y human neuroblastoma cells. MIPT3 transfected alone produced a fibrillar-staining pattern suggestive of cytoskeletal distribution (Fig. 5I). Consistent with this, MIPT3 (detected by Texas Red immunofluorescence, Fig. 5J) colocalized with \( \alpha \)-tubulin (detected by blue Cy5 immunofluorescence, Fig. 5J), with colocalization seen as magenta in Fig. 5K). FL-DISC1-GFP and TRUN-DISC1-GFP transfections produced the usual punctate and diffuse fluorescence patterns respectively (Fig. 5C and F). Strikingly, however, when either FL-DISC1-GFP (Fig. 5L) or TRUN-DISC1-GFP (Fig. 5P) was cotransfected with MIPT3, the distribution of DISC1 changed to a fibrillar, cytoskeletal-type pattern which colocalized with both MIPT3 and \( \alpha \)-tubulin (Fig. 5L-S). These results suggest that MIPT3 recruits DISC1 to microtubules.

In order to further examine this apparent recruitment, HeLa cells were transiently cotransfected with MIPT3 and FL- or TRUN-DISC1, and treated with either nocodazole to disrupt microtubules or taxol to stabilize microtubules (26). In cells transfected with MIPT3 alone, nocodazole disrupted the subcellular distribution of MIPT3 (Fig. 6D) and \( \alpha \)-tubulin (Fig. 6E), whereas taxol condensed MIPT3 (Fig. 6G) and \( \alpha \)-tubulin (Fig. 6H) immunoreactivity into bundles around the nucleus. These results demonstrate that MIPT3 is associated with microtubules, which has been previously determined (27).

When either FL- (Fig. 6J) or TRUN-DISC1 (Fig. 6V) was cotransfected with MIPT3 (Fig. 6K and W) into HeLa cells, the subcellular distribution of DISC1 was altered to mirror MIPT3 alone (Fig. 6A), similar to what had been seen in SH-SY5Y cells (Fig. 5C–S). When the cotransfected HeLa cells were treated with nocodazole, the fibrillar colocalization of MIPT3 (Fig. 6O and AA) and DISC1 (Fig. 6N and Z) was lost; by contrast, in the presence of taxol, the fibrillar pattern of both FL- (Fig. 6R) and TRUN-DISC1 (Fig. 6DD) along with MIPT3 (Fig. 6S and EE) was accentuated and fibrillar bundles were localized around the nucleus. Treatment with nocodazole also caused a loss of DISC1-MIPT3 colocalization, but MIPT3-tubulin colocalization was maintained [see predominance of magenta (red + blue) over yellow (red + green) fluorescence in Fig. 6O and CC]. Conversely, stabilization of microtubules caused an increase in DISC1-MIPT3 colocalization (Fig. 6U and GG). These results suggest that DISC1 and MIPT3 associate in a polymerized-microtubule dependent manner, and
Figure 5. (A) MIP-T3 deletion constructs used in coimmunoprecipitation assays, and summary of their binding to DISC1 deletion constructs (described in Fig. 3A). (B) Results of coimmunoprecipitations in HEK293 cells. (C–S) DISC1's subcellular distribution is altered in the presence of MIP-TRAF3. SH-SY5Y human neuroblastoma cells were transiently transfected with DISC1-GFP and MIP-TRAF3-Xpress. DISC1-GFP protein is visualized in green, MIP-T3 in red and α-tubulin in blue.
Figure 6. Both FL-DISC1 and TRUN-DISC1 bind to microtubules in the presence of MIPT3. (A–I) HeLa cells transiently transfected with MIPT3-Xpress alone were either untreated (A–C), treated with nocodazole (D–F) or treated with Taxol (G–I). (J–U) HeLa cells transiently cotransfected with FL-DISC1-GFP and MIPT3-Xpress were either untreated (J–M), treated with nocodazole (N–Q) or treated with taxol (R–U). (V–GG) HeLa cells transiently cotransfected with TRUN-DISC1-GFP and MIPT3-Xpress were either untreated (V–Y), treated with nocodazole (Z–CC) or treated with Taxol (DD–GG). DISC1-GFP protein is visualized in green, MIPT3 in red and α-tubulin in blue.
that dissociating or stabilizing microtubules dissociates or stabilizes the MIPT3-DISC1 association as well. These experiments were replicated in HEK293 cells with the same results (data not shown). Importantly, DISC1 does not associate with microtubules in cells cotransfected with the N-terminal deletion of MIPT3 (amino acids 223–625; data not shown), consistent with previous data that the microtubule binding site in MIPT3 is in this N-terminal domain (27). These results confirm that MIPT3 recruits both FL-DISC1 and TRUN-DISC1 to microtubules.

Microtubule associated protein 1A interacts with the N-terminus of DISC1

Our YTH assay also identified MAP1A as interacting with DISC1 (Table 1). MAP1A is a microtubule-associated protein which stabilizes microtubules and is expressed predominantly in mature neurons, whereas the related protein MAP1B is expressed mainly during development (30). MAP1A is composed of a heavy and light chain, the light chain being responsible for binding of MAP1A to microtubules and actin filaments (31). Our YTH clone encoded specifically the LC2 light chain of MAP1A. In order to map the domain of DISC1 required for the MAP1A LC2-DISC1 interaction, coimmunoprecipitation experiments were performed with the DISC1 deletion constructs (see Fig. 3A) and MAP1A LC2 (Fig. 7A). FL-DISC1 and TRUN-DISC1 both coimmunoprecipitated with MAP1A LC2. However, neither of the N-terminal deletions, NT_del1-DISC1 or NT_del3-DISC1, coimmunoprecipitated with MAP1A LC2 (Fig. 7B and data not shown). Therefore, the interaction domain of DISC1 with MAP1A LC2 is in the N-terminal 292 amino acids of DISC1.

DISC1 interacts with members of ATF/CREB family via their respective carboxy-terminal domains

The final DISC1 interactors identified by YTH screen that were examined in detail were members of the ATF (activating transcription factor) family: ATF4 (also known as CREB2), ATF5 and ATF7ip (Table 1). ATF4 and ATF5 are closely related members of the ATF/CREB family of transcription factors (32), and have been implicated in a number of cell transcription factor family: ATF4 (also known as CREB2), ATF5 and ATF7ip (Table 1). In addition, ATF4 maps to a schizophrenia locus on 22q13 (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM).

In order to confirm this interaction in mammalian cells, we assayed for morphological colocalization in SH-SY5Y human neuroblastoma cells. Cells were transiently transfected with both DISC1-GFP and ATF5, with DISC1 protein detected as green fluorescence and ATF5 detected by Texas red-anti-Xpress immunoreactivity. When transfected alone, ATF5 immunoreactivity is concentrated in the nucleus as expected for a transcription factor, but also shows some finely punctate immunoreactivity in the cytoplasm (Fig. 8A). When cotransfected with FL-DISC1, ATF5 immunoreactivity is diminished in the nucleus, and takes on a coarsely punctate pattern in the cytoplasm, mirroring (Fig. 8B and C), and colocalizing with FL-DISC1 (yellow in Fig. 8D). When co-transfected with TRUN-DISC1, ATF5 immunoreactivity is not changed, remaining finely granular (Fig. 8F). These results suggest that, consistent with the YTH data, ATF5 interacts with FL-DISC1, but not TRUN-DISC1, in mammalian cells.

To quantify and map the domains of this interaction, we employed the mammalian two-hybrid system. Full-length ATF4 and ATF5 were PCR amplified and subcloned, along with TRUN (amino acids 69–351) ATF4 and TRUN (amino acids 136–282) ATF5, into pACT resulting in fusion proteins with the VP16 transactivation domain (Fig. 8H). The ATF4 and ATF5 deletion constructs were cotransfected with the DISC1 deletion constructs (see Fig. 3A). Cotransfection of FL-ATF4 or TRUN-ATF4 with FL-DISC1 gave a robust transcriptional signal (Fig. 8H–I). In addition, both FL- and TRUN-ATF interacted with NT-del1-DISC1. However, neither FL- nor TRUN-ATF4 interacted with TRUN-DISC1. Similar results were seen with ATF5: both FL- and TRUN-ATF5 interacted with FL-DISC1 and NT-del1-DISC1, but this interaction was lost with truncation of the DISC1 protein (Fig. 8J). As had been seen with the NUDEL deletions (see Fig. 3C), cotransfection of ATF4 or ATF5 with the carboxyl end of DISC1 alone resulted in a larger luciferase signal than with FL-DISC1 (Fig. 8I–J). It was therefore clear that the ATFs interact with the C-terminal end of DISC1, which is lost with truncation.

To map the binding sites of DISC1 and ATF5 more precisely, coimmunoprecipitation studies were performed with deletion constructs of DISC1 and ATF5 in HEK293T cells. Both FL- and TRUN-ATF5 coimmunoprecipitated with FL-DISC1, indicating that the ATF5 C-terminal domain mediates its interaction with DISC1 (Fig. 8K–L). Conversely, neither FL- nor TRUN-ATF5 coimmunoprecipitated with TRUN-DISC1, indicating that the DISC1 C-terminal domain mediates its interaction with ATF5. Consistent with this, both ATF5 proteins coimmunoprecipitated with the N-terminal deletion of DISC1, NT-del2-DISC1. Interestingly, however, the isolated C-terminal 157 amino acids of DISC1 (NT-del3-DISC1) coimmunoprecipitated TRUN-ATF5 only partially, and did not coimmunoprecipitate FL-ATF5 at all (Fig. 8K–L). The entire 256 C-terminal amino acids of DISC1 therefore appear to be required for full binding to ATF4/5.

DISCUSSION

The results presented here demonstrate that DISC1 interacts with a group of proteins known to affect centrosomal and...
Figure 8. DISC1 interactions with ATF4 and ATF5. (A–G) FL-DISC1 or TRUN-DISC1 co-localizations with ATF5 in SH-SY5Y human neuroblastoma cells transiently transfected with DISC1-GFP and ATF5-Xpress. DISC1 protein is visualized in green, ATF5 in red, nuclei in blue. (H) ATF4 and ATF5 constructs used in luciferase assay, and summary of luciferase assay results with each construct. (I) ATF4 Luciferase assay results. (J) ATF5 Luciferase assay results. (K) ATF5 deletion expression constructs used in co-immunoprecipitation assays and a summary of their binding to DISC1 deletion constructs diagrammed in Figure 6A. (L) Results of coimmunoprecipitations in HEK293 cells.
microtubule function, and that truncation of DISC1 to mimic the protein produced in humans with mental illness results in the loss of normal subcellular localization of the protein and loss of association with ATF4, ATF5 and NUDEL. These findings suggest that DISC1 normally functions in the regulation of microtubule dynamics, and that mutation of DISC1 may lead to schizophrenia and other mental illness by disruption of the many neuronal functions orchestrated by the centrosome-microtubule system.

DISC1's subcellular localization is disrupted by truncation

Subcellular localization studies using epitope-tagged DISC1 showed that the full-length protein is localized to punctate cytoplasmic structures, whereas the truncated protein is localized diffusely throughout the cell. Although these studies were performed with heterologously expressed proteins, the subcellular localizations observed are likely to reflect the endogenous distributions for several reasons. First, we expressed DISC1 in four different cell lines (NT2N, SHSY5Y, HEK-293T and HELa), tagged with three different epitopes (V5, Xpress and GFP) by three different methods (stable transfection, transient transfection and Sindbis virus infection) and detected the proteins by both immunocytochemistry and GFP fluorescence. Comparable subcellular localization was seen in all cells by all methods. Second, the two DISC1 proteins and the multiple interactors studied with these methods all gave distinct subcellular localization patterns, suggesting that the patterns are specific and physiological. It will, however, still be important to confirm these localizations with anti-native protein antibodies when these become available.

FL-DISC1 was seen to be expressed in cytoplasmic punctata which varied in quantity and location among cells of all types and expression methods. In most cells, these punctata were abundant and asymmetically perinuclear (Fig. 1A); in some cells, the punctate immunoreactivity extended far into the neuronal processes (Fig. 1B), while in others the punctata were more diffusely distributed (Fig. 1C). The only organelle markers which demonstrated consistent immunocytochemical co-localization with DISC1 were γ-tubulin and pericentrin, which are markers of the centrosome; this colocalization was confirmed by subcellular fractionation (Fig. 2). Importantly, only a fraction of DISC1 punctata was centrosomally localized. The more peripherally located DISC1 punctata are localized to a yet-unidentified vesicle type; some investigators have seen co-localization with mitochondrial markers (I. Schurov, N. Brandon, P. Whiting, personal communication).

Since the punctate structures containing DISC1 were also located in the cytoplasmic periphery and neuronal processes, and DISC1 associates with proteins which function in intracellular transport, it appears that DISC1 itself is trafficked, perhaps in association with microtubule-binding proteins and proteins which localize membrane receptors to their correct synaptic locations such as α-actinin2 [required for NMDAR localization (41)] and β4-spectrin [required for GABAAR localization (43)], which all bind DISC1 robustly (Table 2). Since truncation of DISC1 disrupts its subcellular distribution, this putative trafficking function may also be disrupted by truncation, and contribute to neural systems dysfunction by disrupting correct receptor localization. Systems dysfunction in glutameric and GABAergic circuits are well characterized in schizophrenia (1).

DISC1 associates with centrosomes and the centrosomal protein NUDEL

Centrosomal association of DISC1 was seen by both immunocytochemistry and subcellular fractionation. Although this association was seen in both FL- and TRUN-DISC1 expressing cells, TRUN-DISC1 localized less robustly to centrosomes by immunocytochemistry. Consistent with these data, FL-DISC1 showed strong interaction with the centrosomal protein NUDEL by YTH, mammalian two-hybrid, and co-immunoprecipitation assays (Fig. 3). By contrast, no interaction with NUDEL was detected in these latter assays with TRUN-DISC1. Together, these findings suggest that DISC1 localization to the centrosome normally occurs at least in part via interaction with NUDEL, whereas the residual centrosomal localization of TRUN-DISC1 may occur via other centrosome- or microtubule-associated proteins. Domain mapping experiments showed that a 100 amino acid domain of DISC1 (amino acids 598-697) just distal to the translocation breakpoint, containing a coiled coil domain and a leucine zipper, is necessary and sufficient for NUDEL binding. Conversely, the domain of NUDEL that binds DISC1 is the carboxy terminal 100 amino acids of that protein (amino acids 241-345), which does not include the coiled-coil domain of NUDEL but does include its cytoplasmic dynein binding site and much of its conserved NUDE domain (20). These results are generally consistent with those recently reported by Ozeki et al. (34), who also found that NUDEL interacts with FL but not TRUN-DISC1. However, our data on the domains of interaction of the two proteins differ somewhat from that reported by Ozeki et al. We found by mammalian two-hybrid and co-immunoprecipitation that amino acids 598-697 of DISC1 are necessary and sufficient for binding to the full-length NUDEL protein, whereas Ozeki et al. found by YTH that amino acids 795-854 are necessary and sufficient for binding to an N-terminal deletion of NUDEL. It is possible that the N-terminal NUDEL deletion used by Ozeki et al. produced a differently folded protein than the full-length protein we used, or that the interaction domains differ between yeast and mammalian cells. Further experiments will be required to resolve this difference. By contrast, the results on the domain of NUDEL required for DISC1 binding is consistent between the two papers; taken together, the minimal domain apparently required for DISC1 binding is amino acids 240-280 of NUDEL.

The centrosome is the primary microtubule-organizing center of the cell; when dissociated, microtubules rapidly reassemble from this organelle (19). Long known to be required for spindle pole body formation during mitosis, the centrosome also functions as a microtubule-organizing center in interphase cells, and is responsible for establishment and maintenance of cytoplasmic architecture (19,25,35). Centrosome, and specifically NUDEL, function, have been implicated in neuronal migration during brain development, because of the centrosomal regulation of microtubules, and NUDEL binding to LIS1, which is required for normal cerebral cortical histogenesis (20,21). Disruption of these centrosomal/NUDEL functions by...
loss of DISC1 association may produce developmental and neurite architecture abnormalities, such as have been reported in schizophrenia (14). Consistent with this notion, Ozeki et al. (34) have reported decreased neurite outgrowth in PC12 cells expressing TRUN-DISC1; we have observed similar effects in SHSY5Y and NT2N neurons (data not shown). Finally, DISC1’s association with centrosomes implies a possible role in cytokinesis, and it is possible that the observed variability in the punctate FL-DISC1 pattern among cells may be related to the cell cycle. Although study of a possible correlation between DISC1 localization and cell cycle stage was beyond the scope of this current work, and will be a focus of future studies.

DISC1 and microtubules

DISC1 associates not only with the centrosome, which is the cell’s microtubule organizing center, but also associates with microtubules themselves, as demonstrated by microtubule pull-down assays. DISC1 therefore appears to act as a molecular ‘bridge’ between centrosomes and microtubules. Our observations suggest that DISC1’s interaction with microtubules is indirect; however, since no consistent immunocytochemical colocalization of DISC1 and microtubule markers was seen, DISC1 interacting proteins identified by two-hybrid studies provide the identities of at least some of the proteins that may link DISC1 to microtubules. We observed robust physical interaction between DISC1 and the microtubule-associated proteins MIPT3 and MAP1A, and both proteins interacted equally strongly with both full-length and truncated DISC1. The binding domains of the two proteins onto DISC1 are quite distinct, however, with MAP1A binding to the far N terminus (amino acids 1–292) of DISC1, and MIPT3 binding to the central domain (amino acids 293–696) of DISC1.

MIPT3, also known as M1P-TRAF3, was identified by YTH assay using TRAF3 as a bait (27); TRAF3 was itself identified by YTH assay using the cytoplasmic domain of the tumor necrosis factor receptor (TNFR) family CD40 as bait (36). MIPT3 is a microtubule-associated protein that binds to taxol-stabilized microtubules through its N-terminal 250 amino acids; binding of CD40 ligand causes dissociation of the TRAF3-MIPT3 complex and this is thought to play a role in ligand-induced changes in cell growth and differentiation (27). The C-terminal portion of MIPT3 (amino acids 223–625) is required for its interaction with DISC1, while the N-terminal portion of MIPT3 (amino acids 51–250) is required for its interaction with microtubules (27). This suggests that MIPT3 functions to recruit DISC1 to the microtubule network. Consistent with this notion, co-expression of DISC1 with MIPT3 dramatically altered DISC1 subcellular localization and brought both into colocalization with microtubule α-tubulin (Fig. 5). Nocodazole, which dissociates microtubules, diminished the co-localization of MIPT3 and DISC1, while taxol, which stabilizes microtubules, increased the co-localization of MIPT3 and DISC1; these findings were seen with both FL- and TRUN-DISC1 (Fig. 6). Thus MIPT3 and DISC1 appear to associate with each other and with microtubules in a polymerization-dependent manner. This suggests that DISC1 may influence microtubule function in a regulated fashion that is dependent on the balance between microtubule polymerization and dissociation (37).

DISC1 also demonstrates robust interaction with the light chain LC2 of microtubule-associated protein (MAP)1A. MAP1A is related to family members MAP1B and MAP2, and is expressed predominantly in mature neurons, whereas MAP1B is expressed predominantly during development (30). These MAPs control the polymerization and stabilization of microtubule networks in neurons, and thereby influence cell shape and intracellular transport of vesicles and organelles. The MAP1A heavy chain and its major light chain, LC2, are produced by proteolytic cleavage from a single polypeptide precursor (38). The LC2 subunit contains an actin-binding domain and is necessary and sufficient for microtubule binding and polymerization; it is thus thought to be the active subunit while the heavy chain is thought to be regulatory. Interestingly, in addition to interacting with DISC1, MAP1A physically interacts with PSD95 (39), a scaffolding protein essential for the correct membrane localization of multiple neurotransmitter receptors and other synaptic proteins, including NMDA receptors (40). The amino terminus of DISC1 binds to the LC2 subunit of MAP1A, and therefore was well positioned to affect the ability of MAP1A to polymerize and stabilize microtubules, and traffic proteins, including NMDA receptors, to their correct localization in the synaptic architecture.

Other proteins that interact with DISC1 were not investigated in detail for this report, but are notable in their similarity of function to those binding partners already described. α-actinin2 is an actin-binding protein which links the NR1 and NR2B subunits of the NMDA receptor to their appropriate locations in the postsynaptic density, and is required for appropriate Ca2+-dependent inactivation of the NMDAR (41). FLJ13386 has homology to the Rho-interacting protein Citron, a brain-specific isoform of which associates with PSD95 and appears to couple NMDAR signaling to the Rho pathway (42). Finally, the DISC1 interactor β4-spectrin is required for proper anchoring of voltage-gated sodium channels at the axon initial segment and nodes of Ranvier (43), and its mutation results in tremor, deafness and paralysis in quiverer mice (44). All three proteins are required for proper localization of membrane receptors, and in their absence signaling through these receptors is defective. All of these binding partners maintain their interactions with TRUN-DISC1, but it will be important to determine whether DISC1 truncation affects the function of any of these proteins or the receptors they localize through mechanisms other than loss of binding. Importantly, Ozeki et al. (34) also reported pulling out Citron and spectrin (isoform not specified) using DISC1 in their two-hybrid screen.

DISC1 and receptor signaling

In addition to its association with centrosome- and microtubule-related proteins, DISC1 also interacts with several proteins that are involved in transduction of signals relevant to schizophrenia from the cell membrane to the nucleus, including ATF4 and ATF5. These proteins are closely related members of the leucine zipper Activating Transcription Factor/CREB family; ATF5, also known as ATFx, is 57% identical to ATF4 and is usually considered a member of the ATF4 subfamily (32). Both proteins were both isolated in our initial two-hybrid screen, and were confirmed in the mammalian
two-hybrid and subsequent immunoprecipitation assays. Both proteins interact with DISC1 via their second C-terminal leucine zippers, and importantly, binding of both proteins to DISC1 is lost upon DISC1 truncation. ATF4, also known as CREB2, is a transcription factor that can both induce and repress a variety of genes via heterodimerization with other transcription factors (45), and has been shown in Aplysia to regulate long-term potentiation (46,47). ATF4 and ATF5 have been found, initially surprisingly for transcription factors, to interact directly with GABA_B receptors using two-hybrid screens by multiple groups (48-50), and this interaction has been confirmed by pull-down, co-IP, and immunocytochemistry in neurons in vitro and in vivo. Interestingly, ATF and GABA_B receptors have been found to be colocalized in the somatodendritic compartment of cultured neurons, and activation of GABA_B receptors with the specific agonist baclofen caused ATF4-dependent transcription and translocation of ATF4 either into (49) or out of (50) the nucleus. Although the physiological consequences of these interactions remain unclear, it is likely that the interaction with ATF4 and ATF5 regulates GABA_B receptor responses, signal transduction through the ATFs, or both. The second leucine zipper is the domain in both ATF4 and ATF5 which interacts with DISC1 and GABA_B receptors alike, so it is possible that these binding partners compete for the same site on the ATFs, leading to a potential role for DISC1 in ATF regulation of GABA_B function. Furthermore, since interaction of DISC1 with both ATF4 and ATF5 is lost upon DISC1 truncation, ATF4/5 regulation of GABA_B function may also be lost with DISC1 truncation, and may be responsible for some of the physiological consequences of DISC1 truncation.

SUMMARY AND CONCLUSIONS

Taken together, the protein interaction, domain mapping, immunocytochemistry and subcellular localization results show that DISC1 is a multifunctional protein, which interacts via distinct domains with different components of the intracellular machinery. With these data, it is possible to generate a working model of DISC1’s cellular functions, and how DISC1 dysfunction may be related to schizophrenia (Fig. 9).

The amino terminus of DISC1 interacts with MAP1A, and the central domain of DISC1 containing its three coiled-coil domains interacts with MIPT3; both proteins bind microtubules and probably contribute to the observed microtubule association of DISC1. Disruption of microtubule function may adversely affect neuronal architecture and receptor localization, which have been shown to be abnormal in schizophrenic brain (51). Consistent with this concept, the recently reported mouse knockout phenotype of the microtubule-associated protein STOP includes defects in synaptic plasticity and schizophreniform behavioral deficits that are ameliorated by neuroleptics (52).

A 100 amino acid domain of DISC1 just C-terminal to the truncation site interacts with NUDEL, and is appears to be responsible for the majority of DISC1’s association with centrosomes. Given NUDEL’s association with the molecular motor protein cytoplasmic dynein (20), this domain of DISC1, and its loss with truncation, may affect the anterograde and retrograde transport which depend on this molecular motor (21). DISC1’s association with α-actinin2, β4-spectrin and ATF4/5 suggests that DISC1 may link this molecular motor machinery to specific receptor cargo. NUDEL’s interaction with LIS1 (20) and the involvement of the NUDEL-LIS1-Dynein complex in neuronal migration during cerebral cortical development (53,54) suggests that loss of NUDEL binding in the setting of DISC1 truncation may also lead to abnormal cortical histogenesis, a frequent finding in schizophrenic brain (55).

Finally, the carboxy terminal 157 amino acids of DISC1 interacts with the ATF4 and ATF5. This domain is likely to mediate DISC1’s receptor transport, localization and signal transduction functions through GABA_B and perhaps other neurotransmitter receptors. The loss of ATF4/5 binding to truncated DISC1 may disrupt these processes and contribute to the neurochemical system abnormalities seen in schizophrenia (1).

Given its multiple protein interactions, it is likely that DISC1 exists in a dynamic macromolecular complex including centrosomal proteins, NUDEL, MIPT3, MAP1A and microtubules. Since DISC1 is a phosphoprotein, it is possible that its phosphorylation state regulates its binding and dissociation from its partners, including those that associate with microtubules. Association of microtubules with many MAPs is regulated by phosphorylation (56), and similar effects of NUDEL phosphorylation on microtubule dynamics have been demonstrated (20). Finally, given DISC1’s apparent multifunctionality, it is likely that its truncation has similarly pleiotropic consequences. Defects in ‘integrator’ genes as...
DISC1 and RG54 (57) could be responsible for the multiple subtle physiological deficits that are seen in schizophrenia.

**MATERIALS AND METHODS**

**Cloning of DISC1 and generation of expression constructs**

Primers were designed based on human DISC1 sequence (GenBank no. AF22980). FL-DISC1 was PCR (polymerase chain reaction) amplified from a placenta Marathon-ready cDNA library (Clontech) using primer DISC1-1F (GGAA-GGAGCAGGAGCGACCGACCGCCAGGG) and primer DISC1-2R (TCACTGGGAGGTGGTGCTTACCCG) resulting in a 3222 bp product. A 500 µl reaction contained 2.5 U of TaqGold enzyme (Perkin Elmer), 0.2 µM of each primer, 0.2 mM of each dNTP, 1× and 5% DMSO in 1× reaction buffer (Perkin Elmer). The reaction consisted of an initial denaturation at 94°C for 9 min followed by 35 cycles of denaturation at 94°C for 20 s, annealing of 68°C for 1 min, and an elongation at 72°C for 3 min. Primers were also designed to amplify the truncated form of DISC1 inserting a stop codon at amino acid 598. TRUN-DISC1 was amplified from a placenta Marathon-ready cDNA library (Clontech) using primer DISC1-1F and DISC1-1R (TTTATTATAGATATGCGATCTTTTGGCTTCAAGC) resulting in an 1850 bp product. Reaction conditions were the same as described above. Both FL-DISC1 and TRUN-DISC1 were sub-cloned into pcdNA3.1/V5-HisTOPO (Invitrogen) according to standard protocols to screen for SH-YT candidates, subcloned into a mammalian expression vector pcDNA3.1/His (Invitrogen) which contains an Xpress epitope tag. Immunoprecipitations were performed according to a protocol described by Niethammer and colleagues (20). Briefly, transiently transfected HEK 293 cells were lysed in 100 mM NaCl, 50 mM Tris (pH 7.4), 0.5% NP40, 10 µl/ml protease inhibitor cocktail (Sigma), 1 mM PM5F, 25 mM NaF, and 10 mM Na3VO4. After lysis, extracts were incubated for 1 h at 4°C, after which, cellular debris was removed by centrifugation at 13 000 × g and 30 min. Anti-Xpress antibody (Invitrogen) was added to extracts and incubated at 4°C overnight. Protein A Sepharose beads (Pierce) were added and incubated an additional 2 h at 4°C. Immunoprecipitations were washed and beads were resuspended in SDS-PAGE sample buffer. Samples were analysed by SDS-PAGE followed by western blot analysis with an anti-DISC1 polyclonal antibody. An anti-peptide antibody (1864) generated against the amino-terminus of DISC1 was used to detect FL- and TRUN-DISC1. An anti-peptide antibody (1864) generated against the carboxy-terminus of DISC1 was used to detect NT-del2-DISC1 and NT-del3-DISC1.

**Sindbis virus.** FL-DISC1 and TRUN-DISC1 was subcloned into pSinHis expression vector (Invitrogen) placing the anti-Xpress epitope tag at the amino-terminal end of the protein. RNA Sindbis viruses containing DISC1-FL or DISC1-TRUN was produced using the Sindbis Expression System (Invitrogen). Human NT2 primary neurons (Stratagene) were plated on culture slides and subsequently infected with either a full-length or truncated DISC1 Sindbis virus according to manufacturer's instructions.

**Immunocytochemistry.** SH-SY5Y, HeLa and HEK 293T cells were transiently transfected using Lipofectamine 2000 (Invitrogen). For all immunocytochemistry, cells were fixed with 4% paraformaldehyde in PBS (phosphate buffered saline) for 20 min and subsequently washed in PBS. Cells were blocked with 10% donkey serum in PBS for 30 min and then primary antibody was added at an appropriate dilution in PBS from 2 h to overnight. After washing in PBS, a fluorophore-conjugated
secondary antibody was added for 1 h and washed out with PBS. Cells were cover slipped and examined microscopically using a Nikon Eclipse E1000 microscope.

Antibodies used for DISC1’s co-localization with organelle structures were anti-β1 (StressGen), anti-calnexin (StressGen), anti-SK (Sigma), anti-GIANTIN (Covance), anti-Rab4 (StressGen), anti-LAMP-1 (Santa Cruz), anti-RA1P1 (Santa Cruz), anti-KDEL receptor (StressGen), and anti-catalase (RDI). Antibodies used for co-localization with the tubulins were anti-β-tubulin were from Sigma and anti-α-tubulin from Cytoskeleton.

Stable cell lines. Stable cell lines expressing FL-DISC1-V5, TRUN-DISC1-V5 and a vector control were produced in SH-SY5Y cells. Multiple clonal cell lines from several independent transfections were generated to ensure unique integration sites. Cell lines were characterized for DISC1 expression by Taqman quantitative RT-PCR and western blot analysis.

Centrosome isolation. Centrosomes were isolated from FL-DISC1 and TRUN-DISC1 overexpressing SH-SY5Y stable cell lines according to a protocol by Moudjou and Bornens (18). Centrosome fractions were analyzed by SDS–PAGE and western blot analysis.

Protein processing. In order to test the possibility that DISC1 is normally processed, [35S]methionine pulse/chase experiments were performed on SH-SY5Y cell lines stably expressing FL-DISC1-V5, TRUN-DISC1-V5 or a vector control. Cells were labeled with [32P]inorganic phosphate. Both forms of the protein appear to be phosphorylated.

MAP spin down assay. Microtubule associated protein spin down assays were performed using the Microtubule A associated protein Spin-Down Assay Biochem Kit (Cytoskeleton) according to manufacturer’s instructions. Extracts used in the assay were from FL-DISC1-V5, TRUN-DISC1-V5 and vector control overexpressing SH-SY5Y stable cell lines.

Treatment of cells with nocodazole and taxol. HeLa cells were transiently transfected with expression constructs using Lipofectamine 2000 (Invitrogen). The protocol for treatment with nocodazole and taxol was done as described by Ling and colleagues (27). Briefly, six hours post-transfection, cells were treated with 400 ng/ml nocodazole (Sigma) or 5 mg/ml Taxol (Sigma) overnight. Twenty-four hours post-transfection, cells were fixed and immunocytochemistry performed as described above.

Full-length YTH Interactors. In order to clone full-length sequences for ATF4, ATF5 and MIPT3, primers were generated using the GenBank sequences (Table 1). Full-length MIPT3, ATF4 and ATF5 were generated by PCR amplification as described above from Universal Quick Clone cDNA (Clontech).

Mammalian two-hybrid. FL-DISC1 and TRUN-DISC1 was subcloned into pBIND vector (Promega). NT-del1-DISC1 was generated by PCR amplification as described above using DISC1 specific primers and subsequently subcloned into pBIND. ATF4, ATF5 and NUDEL were subcloned into pACT (Promega). Luciferase assays to test for protein–protein interactions were performed using the Luciferase Assay Kit (Stratagene) according to the manufacturer’s protocol.

REFERENCES