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RESEARCH ARTICLE

Interaction of DISC1 with the PTB domain of Tensin2

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Abstract The gene for Disrupted-in-Schizophrenia 1 (*DISC1*) is amongst the most significant risk genes for schizophrenia. The DISC1 protein is an intracellular scaffolding molecule thought to act an important hub for protein interactions involved in signalling for neural cell differentiation and function. Tensin2 is an intracellular actin-binding protein that bridges the intracellular portion of transmembrane receptors to the cytoskeleton, thereby regulating signalling for cell shape and motility. In this study, we probed in molecular detail a novel interaction between DISC1 and Tensin2. Western blot and confocal microscopic analyses revealed widespread expression of both DISC1 and Tensin2 proteins throughout the mouse brain. Furthermore, we have developed novel anti-DISC1 antibodies that verified the predominant expression of a 105-kDa isoform of DISC1 in the rodent brain as well as in human cells. In the mouse brain, both proteins showed region-specific expression patterns, including strong expression in the pyramidal cell layer of the hippocampus and dentate gyrus. DISC1–Tensin2 colocalisation was most clearly observed in the Purkinje cells of the mouse cerebellum. Biochemical coimmunoprecipitation experiments revealed an interaction between endogenous DISC1 and Tensin2 proteins in the mouse brain. Further pulldown studies in human cells using Myc-tagged Tensin2 constructs revealed that DISC1 specifically interacts with the C-terminal PTB domain of Tensin2 in a phosphorylation-independent manner. This new knowledge on the DISC1–Tensin2 interaction, as part of the wider DISC1 interactome, should further elucidate the signalling pathways that are perturbed in schizophrenia and other mental disorders.

Keywords Tensin · DISC1 · Schizophrenia · Protein–protein interaction · Protein domain · Cerebellum

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Introduction

Schizophrenia is a widespread psychiatric disorder affecting roughly 1 % of the population worldwide. Results from twin, family and adoption studies highlight the significance of a genetic element amongst the risk factors for the onset of the disease [1]. However, it is most likely that the fullest expression of the disease arises from the combined effects of both genetic and environmental factors that contribute to the neurodevelopmental model of schizophrenia [2]. Amongst the genes most significantly associated with susceptibility to schizophrenia is *DISC1* (Disrupted-In-Schizophrenia 1), which was found to be disrupted directly by a balanced (1;11)(q42.1;q14.3) translocation in a large Scottish pedigree exhibiting familial psychosis [3]. Since that discovery, much research has been done on the structure–function relationships of the DISC1 protein, resulting in the model of DISC1 as a scaffolding protein, acting as a hub for various protein–protein interactions thought to regulate diverse processes including neural progenitor cell differentiation, neurite outgrowth, dendritic spine formation and neurotransmitter receptor expression [4, 5]. Therefore, a wider

knowledge of the array of intracellular signalling networks DISC1 mediates should lead to a greater understanding of the pathogenesis of schizophrenia and other psychiatric disorders involving DISC1-related signalling.

The DISC1 interactome includes proteins that regulate cytoskeletal organisation and process formation. Amongst these binding partners is Tensin2, which can localise to focal adhesions and act as a bridge between the cytoskeleton and the intracellular domains of transmembrane receptors such as receptor tyrosine kinases [6] and β integrins [7]. A C-terminal fragment of Tensin2 was found to interact with DISC1 by yeast two-hybrid screening [8], this region incorporating both a Src homology2 (SH2) and phosphotyrosine binding (PTB) domain in tandem. These domains together provide the potential for multiple protein interactions in a concomitant manner, and both have been shown to bind to diverse protein partners including focal adhesion kinase, p130Cas [9] and DLC-1 [10]. These interactions have been implicated in regulating cytoskeletal dynamics involved in cell motility, and their impairment has been observed in cancer cells, which exhibit a greater tendency to metastasise [11]. However, the expression and function of Tensin2 in the brain, or its involvement in DISC1-regulated signalling networks, have yet to be explored. This is particularly worthy of investigation as Tensin2 expression in the brain appears to be isoform-specific [6] and hence potentially more tailored towards DISC1 interaction. Therefore, the aim of the present study was to verify the physical interaction between DISC1 and Tensin, in whole brain and in cultured cells, and to localise the specific domain in Tensin2 that interfaces with DISC1. Here, we report our findings that Tensin2 binds to DISC1 in the brain, and that the interaction occurs specifically via the Tensin2 PTB domain. These results for the first time implicate Tensin2 as a definitive binding partner involved in DISC1 signalling networks in the brain, and also open up a new area of research into Tensin biology in the brain. Furthermore, we have developed a novel antibody against DISC1 that should help to clarify the uncertain issue of which isoforms(s) of DISC1 exist in the rodent brain.

Materials and methods

Cell culture and brain tissue preparation

Human embryonic kidney 293 (HEK 293), SNB-19 glioma, and DU145 prostate cancer cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % foetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Lonza, Slough, UK). Cells were kept at 37 °C in an incubator with a humidified atmosphere of 5 % CO_2 in air, and passaged once a week.

Brains were freshly obtained from mice of the C57BL/6 strain at ages postnatal day (P)0 and as adults (3 months). All research involving animals was approved by the University of Portsmouth Ethics Committee and by the Home Office Animals Scientific Procedures Act (1986). Animals were killed humanely by cervical dislocation. For western blot analysis, brains were removed rapidly and placed in ice-cold phosphate-buffered saline (PBS) shortly prior to lysis. For immunofluorescence staining analysis, mouse brains were fixed by transcardial perfusion with fixative composed of 1 % paraformaldehyde and 15 % v/v saturated picric acid in PBS, pH 7.4, as described previously [12]. Sagittal sections of the whole brain, at 70 μm thickness, were prepared on a vibratome and stored in PBS containing 0.05 % sodium azide prior to staining.

Cell and tissue extract preparation

For preparation of protein extracts for western blotting, cultured cells were lysed directly in the dish by rinsing in ice-cold PBS followed by addition of ice-cold lysis buffer, composed of: 50 mM Tris-HCl, 150 mM NaCl, 1 % Triton X-100, 0.5 % NP-40, 1 mM EDTA, 10 mM $Na_4P_2O_7$, pH 8.0, supplemented with a protease inhibitor cocktail (Fisher Scientific, Loughborough, UK). For preparation of extracts from mouse brain tissues, brain regions were first isolated by dissection and then finely chopped and minced with sterile surgical scissors. The homogenate was added to the same lysis buffer as described above and kept on ice for 30 min, during which time it was further disrupted by passing through a 20-gauge needle (Fisher Scientific) multiple times, followed by vortexing. Finally, lysates were clarified by centrifugation in a microfuge at 20,800 rcf for 10 min at 4 °C to separate the soluble fraction from the insoluble cell material. The total protein concentration in samples was determined by bicinchoninic acid assay (BCA) assay (Sigma, Gillingham, UK). Samples were the kept frozen at −80 °C until such time as they were used for immunoprecipitation and/or western blot analysis.

SDS-PAGE and western blot

Total protein extracts from C57BL/6 mouse brain and the human cell lines HEK 293, SNB-19 and DU145 were run in equal amounts on 10 % reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were then transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Watford, UK) by the wet transfer method. The membranes were first blocked with blocking buffer, composed of 3 % milk powder in 25 mM Tris, 150 mM NaCl, 0.05 % Tween-20, pH 8.0, for 1 h at room temperature (RT), followed by incubation with specific primary antibodies, either at 4 °C overnight or 2 h at RT.

Antibodies used included a commercial goat polyclonal anti-DISC1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), our in-house rabbit polyclonal anti-DISC1 (raised against amino acids 31–51 of isoform 1; NCBI accession no. NP_777279), and monoclonal anti-beta actin antibody (Pierce, Fisher Scientific). Membranes were washed for 3 × 5 min in wash buffer (25 mM Tris-HCl, 150 mM NaCl, 0.05 % Tween-20, pH 8.0), then incubated with horseradish peroxidase (HRP)- or alkaline phosphatase (AP)-conjugated secondary antibodies (Promega, Southampton, UK) for chemiluminescent and colorimetric detection, respectively.

Immunoprecipitation

Equal amounts (1 mg) of cell and brain tissue extracts were first pre-cleared by mixing with protein A/G-agarose beads (Alpha Diagnostics, San Antonio, TX, USA) together with 2 μL normal rabbit for 1 h at 4 °C. Non-specific immune complexes were removed by gentle centrifugation and separation of the supernatant. The cleared lysate was then incubated overnight at 4 °C with 3 μg immunoprecipitation (IP) antibody, which was anti-Myc (Santa Cruz) for transfected cell lysates and mouse monoclonal anti-DISC1 (gift of A. Sawa, Johns Hopkins, USA) for mouse brain lysates. A non-related antibody, rabbit anti-thioredoxin (Trx), was used as a negative control pulldown (control Ig). The next day, protein A/G-agarose beads were added and incubated for a further 1 h, after which immune complexes were centrifuged and washed once with ice-cold lysis buffer and 4 times with PBS. The proteins complexed to the beads were released by addition of 3× SDS-PAGE loading buffer and brief boiling before loading onto 10 % SDS-PAGE gels for western blot analysis, as described above.

Cloning of Myc-Tensin2 cDNA constructs

The cDNA sequences for individual domains of Tensin2 were cloned into the mammalian expression vector pCMV-Myc (Clontech, Saint-Germain-en-Laye, France), conferring an N-terminal Myc epitope tag on the expressed protein. Tensin2 domain cDNA sequences were mapped from bioinformatic analysis of conserved domains, based on the cDNA sequence for Tensin2 isoform 2 (*TENC1*; NCBI accession no. NM_170754; protein ID NP_736610, 1,409 amino acids). The domain constructs corresponded to amino acids 1,134–1,239 (SH2), 1,370–1,409 (PTB) and 1,134–1,409 (SH2-PTB). The cDNA sequences for full length Tensin2, SH2 domain, PTB domain and SH2-PTB domain pair were amplified by PCR, digested by restriction digestion and ligated directionally into pCMV-Myc vector using specific restriction enzyme sequences (sequences in Online Resource 1). All constructs were verified by DNA sequencing.

Transient transfection of Tensin2 domains

HEK 293 and DU145 cells were transiently transfected with cDNA encoding the various Myc-tagged Tensin2 domains (Fig. 3b). Cells were seeded at sub-confluence in 10-cm dishes or on glass coverslips for subsequent analysis by IP or immunofluorescence staining, respectively. Transfection of cDNAs into cells was performed using a liposome-forming transfection mixture containing transfection and enhancer reagents (Invitrogen Life Technologies, Paisley, UK) mixed with DMEM, according to the manufacturer's instructions. The DNA transfection mix was incubated with the cells for 5 h, after which it was replaced with fresh complete medium, followed by a further 48 h incubation to allow for transfection. Cells were then prepared for analysis by IP/western blot or immunofluorescence staining, as described in other sections.

Immunofluorescence staining and confocal microscopy

Transfected cells were fixed and permeabilised with 4 % paraformaldehyde and 0.2 % Triton X-100 respectively, and immunostained as described previously [13]. Briefly, cells were blocked for 1 h with 10 % porcine serum and then incubated with primary antibody at optimal dilutions in 1.5 % horse serum for 2 h at RT. Antibodies used included anti-Myc (Santa Cruz) and in-house rabbit anti-DISC1 antibody. For staining of mouse brain sections, these were washed in Tris-buffered saline with 0.3 % Triton X-100 (TBS-Tx), and first blocked in TBS-Tx containing 20 % normal horse serum for 1 h at RT. Sections were then washed and incubated with optimal dilutions of in-house rabbit anti-Tensin2 [14] and mouse anti-DISC1 antibodies overnight at 4 °C. After serial washing, fluorescence-conjugated secondary antibodies (Alexa Fluor®-488 and -647; Invitrogen) were applied to cells and tissue sections in 3 % horse serum for 1 h at RT. Samples were then mounted and examined under a confocal laser-scanning microscope (LSM710; Zeiss, Oberkochen, Germany) using using ×63 and ×100 oil objectives. Images were processed with the software Zen2008 Light Edition (Zeiss). Specificity of the antibodies was verified by lack of fluorescence in cells incubated with secondary antibodies alone.

Results

Tensin2 and DISC1 protein expression in the mouse brain

We first carried out western blot analysis of endogenous Tensin2 and DISC1 proteins in brain extracts from both adult (3 months) and postnatal day 0 (P0) C57BL/6 mice. In adult brain, Tensin2 displayed a clear band at the expected

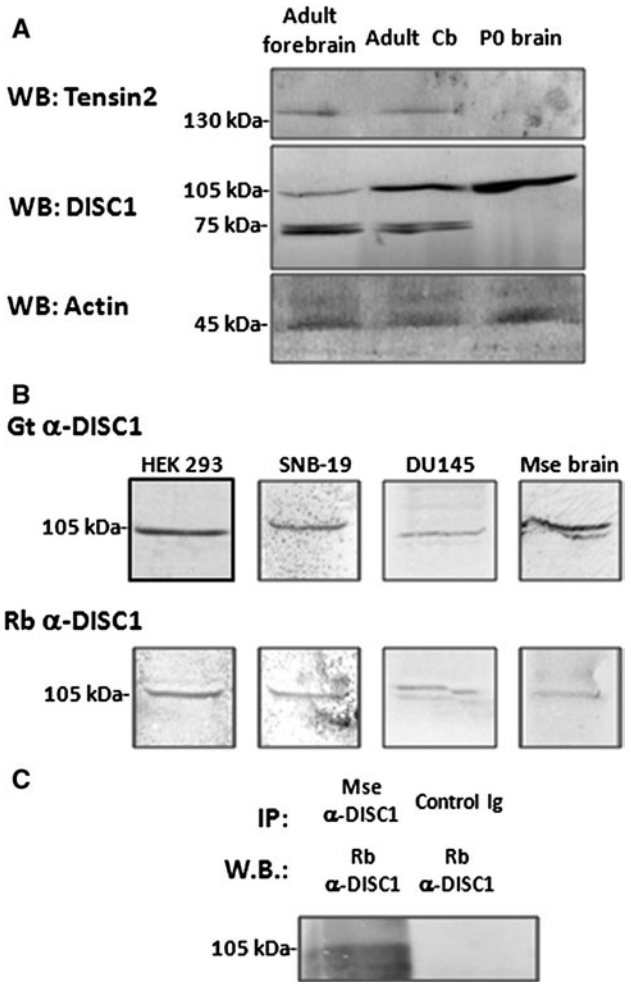

Fig. 1 Detection of endogenous Tensin2 and DISC1 proteins in mouse brain. **a** *Top panel* Tensin2 immunoblot of samples loaded in the following order: adult (3 months) forebrain, adult cerebellum (*Cb*), P0 forebrain. *Middle panel* the corresponding DISC1 immunoblot of the same samples using goat anti-DISC1 antibody. *bottom panel* The corresponding beta actin immunoblot from the same samples as a loading control. **b** Western blot detection of endogenous DISC1 expression in cultured human HEK 293 kidney cells, SNB-19 glioblastoma cells, DU145 prostate cancer cells, and mouse (*mse*) brain. *Upper panel* detection using commercial goat (*Gt*) polyclonal anti-DISC1 antibody, *ower panel* detection using in-house rabbit (*Rb*) polyclonal anti-DISC1 antibody. **c** Recognition of two different epitopes on DISC1 protein using two distinct anti-DISC1 antibodies. IP of endogenous DISC1 from mouse brain was performed using mouse (*Mse*) monoclonal anti-DISC1 antibody, followed by western blot detection using in-house rabbit polyclonal anti-DISC1 antibody. As negative control IP, pulldown was also performed with an unrelated antibody, anti-Trx (control Ig, *lane 2*)

molecular weight of around 140 kDa in extracts from various brain regions, including forebrain and cerebellum (Fig. 1a). In contrast, P0 mouse brain showed barely detectable expression of Tensin2, when loading equal protein amounts on SDS-PAGE and assuming that actin levels are similar. We also developed two novel polyclonal antibodies against DISC1, using as antigen a 21-peptide sequence that had 100, 90 and 80 % sequence identity with the rat (NCBI accession no. Q810H6), mouse and human orthologues, respectively. In western blots, a strong band for DISC1 of around 105 kDa band was consistently detected in samples from neonatal and adult mouse brain regions (Fig. 1a) as well as from a variety of cultured human cell lines (Fig. 1b). The 105-kDa band was a consistent observation when using several DISC1 antibodies from different species and recognising distinct epitopes, including in-house and commercial polyclonal antibodies (Fig. 1b, Online Resource 2). Interestingly, the goat anti-DISC1 antibody also detected an additional band at around 75 kDa, which, however, was present only in adult mouse brain samples, but not P0 samples (Fig. 1a, b). Furthermore, various pairwise combinations of these antibodies were used in IP experiments to verify their specificity and cross-species reactivity. For example, endogenous DISC1 protein that was pulled down from mouse brain using the mouse monoclonal anti-DISC1 antibody, was recognised as DISC1 in western blot by the in-house rabbit antibody (Fig. 1c).

Interaction of endogenous DISC1 and Tensin2 proteins in mouse whole brain extract

Total protein extract of adult mouse forebrain was subject to coimmunoprecipitation (coIP) analysis in order to probe for an interaction between endogenous DISC1 and Tensin2 proteins. Pulldown of DISC1 was carried out using a mouse monoclonal anti-DISC1 antibody, followed by western blot probing for Tensin2. The western blot revealed a band for Tensin2 in the DISC1 pulldown samples, which was absent in control pulldown samples using a non-related antibody (Fig. 2).

The PTB domain of Tensin2 interacts with endogenous DISC1

We next addressed the specific interaction site between Tensin2 and DISC1 by coIP experiments involving different recombinant Tensin2 domains expressed in HEK 293 cells (Fig. 3a). Myc-tagged constructs of the SH2, PTB and SH2-PTB domains of human Tensin2 were transiently expressed in cells, and coIP experiments were performed by pulldown of the Myc-tagged constructs followed by western blot for DISC1. A clear band for DISC1 immunoreactivity was detected in samples containing the pulled down Tensin2 PTB domain as well as the SH2-PTB domain pair (Fig. 3b). The band appears slightly smaller than the band in the "input" lanes of total cell lysates; which is probably due to differences in post-translational modifications and/or protein mobility, as the sample complexities also differ greatly. In contrast, no DISC1 was detected in pulldown

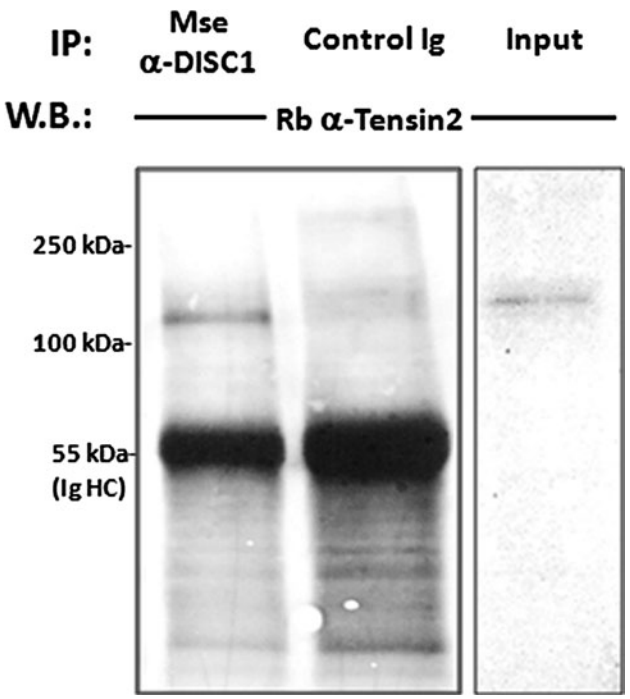

Fig. 2 CoIP of endogenous DISC1 and Tensin2 proteins from adult mouse brain. Pulldown was performed using mouse monoclonal anti-DISC1 antibody (*lane 1*) or the unrelated anti-Trx antibody (control Ig, *lane 2*), followed by western blot probing with an in-house rabbit polyclonal anti-Tensin2 antibody. The strong 55-kDa bands correspond to the heavy chain of the pulldown antibody (Ig HC). Tensin2 detection in total mouse brain extract is shown in the *input lane*. The blot is representative of 3 CoIP experiments with similar results

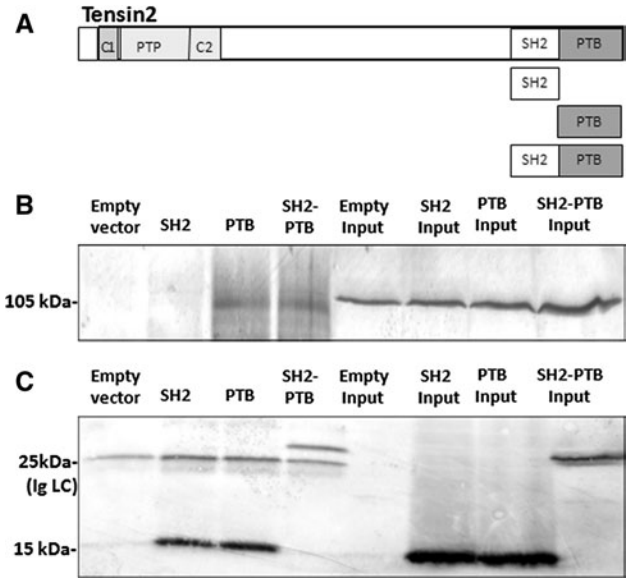

Fig. 3 CoIP experiment of transiently transfected Myc-tagged Tensin2 domains with endogenous DISC1 from HEK 293 cells. **a** Scheme of recombinant Myc-tagged constructs of Tensin2 created. The full-length Tensin2 protein is shown alongside the domain constructs below it that were prepared with N-terminal Myc tags. **b** Cell lysates were subjected to IP pulldown with anti-Myc antibody followed by western blot detection with anti-DISC1 antibody. Samples were loaded in the following order: (1) transfected empty Myc vector, (2) Myc-SH2, (3) Myc-PTB, (4) Myc-SH2-PTB. *Lanes* 5–8 are the total cell lysates of the transfected cells in the same order (*input*), showing equal amounts of endogenous DISC1 protein expression in each sample. **c** The membrane in (**b**) was stripped and reprobed with anti-Myc antibody. Strong bands for Myc-SH2 and -PTB domains (around 15 kDa) and for Myc-SH2-PTB domain (30 kDa) are visible, showing effective transfection. The ~25 kDa bands correspond to the light chain of the pulldown antibody (*Ig LC*)

samples from cells expressing only the SH2 domain or empty Myc plasmid-containing samples. Re-probing the blot with anti-Myc antibody showed that expression of all Myc-tagged Tensin2 domains was strong and corresponded to the expected molecular weights for these constructs, around 15 kDa for Myc-SH2 and Myc-PTB domains and 30 kDa for Myc-SH2-PTB (Fig. 3c).

We also investigated the interaction of specific Tensin2 domains with endogenous DISC1 within cultured human cells by immunofluorescence confocal microscopy. As in the coIP experiments, the same N-terminal Myc-tagged human Tensin2 domain constructs, including the full-length version, were transfected into HEK 293 cells, and were detected by immunofluorescent staining with anti-Myc antibodies (Fig. 3a). Each domain was expressed strongly in a number of transfected cells and showed distinct patterns of fluorescence, as previously reported by us for GFP-tagged Tensin2 domains [13]. The SH2 and PTB domain-containing cells showed a smooth and even fluorescence throughout the cytoplasm and nucleus, whilst full-length Tensin2 showed a discrete punctate pattern of fluorescence throughout the cytoplasm only (Fig. 4). Immunofluorescent localisation of endogenous DISC1 in the HEK 293 cells was strongest in the nucleus, as observed in rodent brain sections (Fig. 5). However, any colocalisation observed in cells showing fluorescence for both DISC1 and Tensin2 PTB and SH2-PTB domains was small and inconsistent.

Detection of regional and subcellular expression of DISC1 in mouse brain using different antibodies

Confocal immunofluorescent localisation of DISC1 in brain sections was carried out using our novel in-house antibodies as well as commercial antibodies. In the mouse brain, DISC1 immunoreactivity was localised to distinct regions of the hippocampus and cerebellum. In the hippocampus, DISC1 staining was strongest in the pyramidal cell layer as well as in the granular cell layer of the dentate gyrus (DG), as previously reported [15] (Fig. 5a). In particular, the strongest fluorescence was in the nuclei of the cells in those layers. The nuclear staining was in particular concentrated in intra-nuclear spots resembling promyelocytic nuclear bodies, as has previously been reported [16]. We also wished to

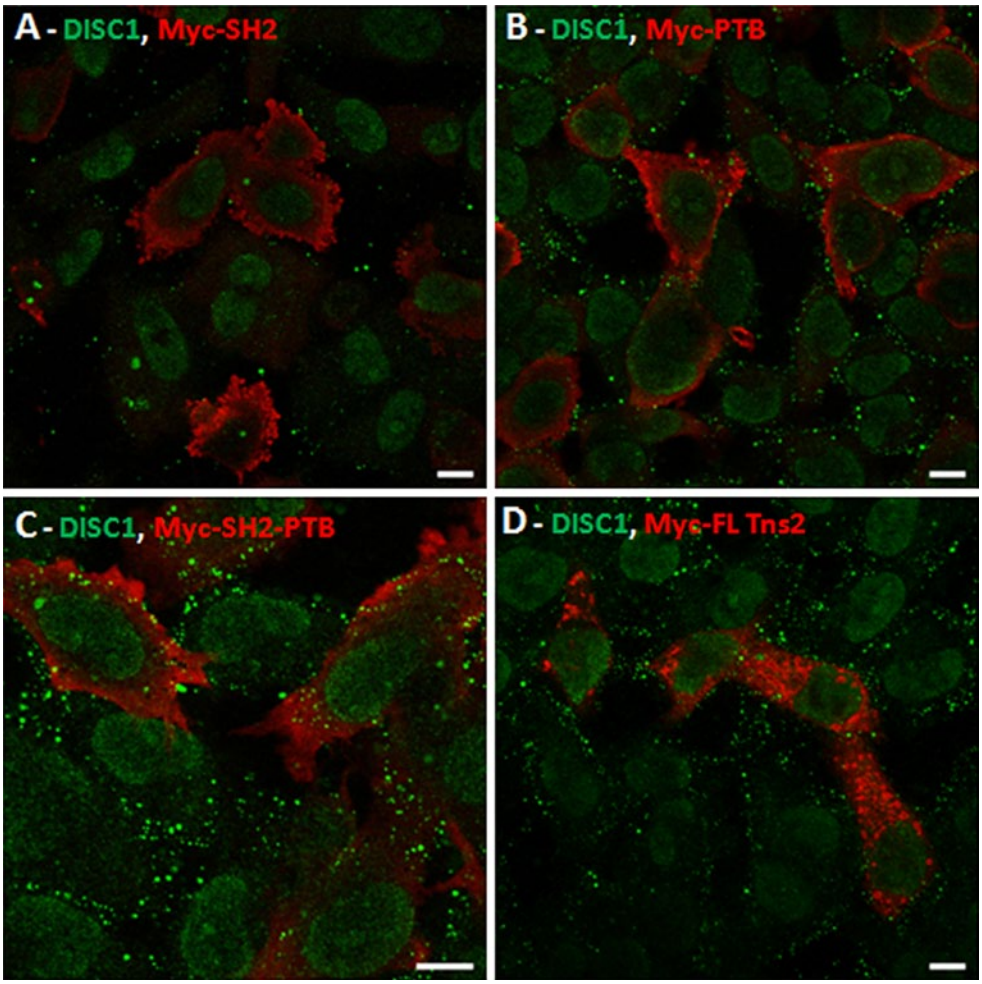

Fig. 4 Fluorescent confocal microscopic analysis of transfected Myc-tagged domains of Tensin2 and endogenous DISC1 in DU145 cells. Cells on coverslips were transiently transfected with cDNAs encoding Myc-tagged Tensin2 domains, following which they were fixed and stained with antibodies against anti-Myc and anti-DISC1 antibodies. Secondary antibodies conjugated to Alexa Fluor®-647 (*red*) and -488 (*green*) were used to detect anti-Myc and anti-DISC1 primary antibodies, respectively. Cells stained were transfected to express the following Myc-tagged Tensin2 domains: **a** SH2, **b** PTB, **c** SH2-PTB and **d** full-length Tensin2 (FL Tns2). *Scale bar* 10 μm

confirm the specificity of the antibodies being used for this application by testing the cross-reactivity potential of two anti-DISC1 antibodies on mouse brain sections. For this, we selected our in-house antibody and a commercial antibody, both of which were raised against distinct sequences within the DISC1 protein. The in-house antibody identified a distinct population of individual neurones within the cortex, with clear punctate fluorescence of the cytoplasm and processes, particularly at the cell periphery (Fig. 5b). The commercial antibody staining was stronger for nuclei as well as punctate for perimembrane regions of the axon, similar to that observed previously in mouse brain. When staining with both antibodies, a significant overlap of the punctate perimembrane fluorescence was observed (Fig. 5b). This further supported the specificity of the DISC1 staining pattern observed using both antibodies in different tissues.

Immunofluorescent colocalisation of DISC1 and Tensin2 in the mouse cerebellum

The expression of DISC1 and Tensin2 together was also investigated in rodent brains by immunofluorescence confocal microscopy. Tensin2 immunoreactivity was localised to distinct cortical regions, hippocampus and cerebellum of the C57BL/6 mouse brain. In the cerebellum, DISC1 immunoreactivity was strongest in Purkinje cells, occurring in large perinuclear clusters as well as a more diffuse signal in dendritic shafts (Fig. 6a). Strong expression of Tensin2 was also observed in Purkinje cells, being concentrated in cell somata and dendrites, with weak-to-no signal being evident in other layers of the cerebellar cortex (Fig. 6b). In an attempt to determine whether Tensin2 and DISC1 immunoreactive patterns in the brain overlap, we performed double-labelling with our in-house antibodies against Tensin2 and the various antibodies against DISC1. Purkinje cells were the only neurons in the cerebellum that showed clear Tensin2 and DISC1 colocalisation (Fig. 6c).

Discussion

Since its identification around 10 years ago, multiple avenues of research on DISC1 have helped to build a picture of it as a major scaffolding protein, acting as an intracellular

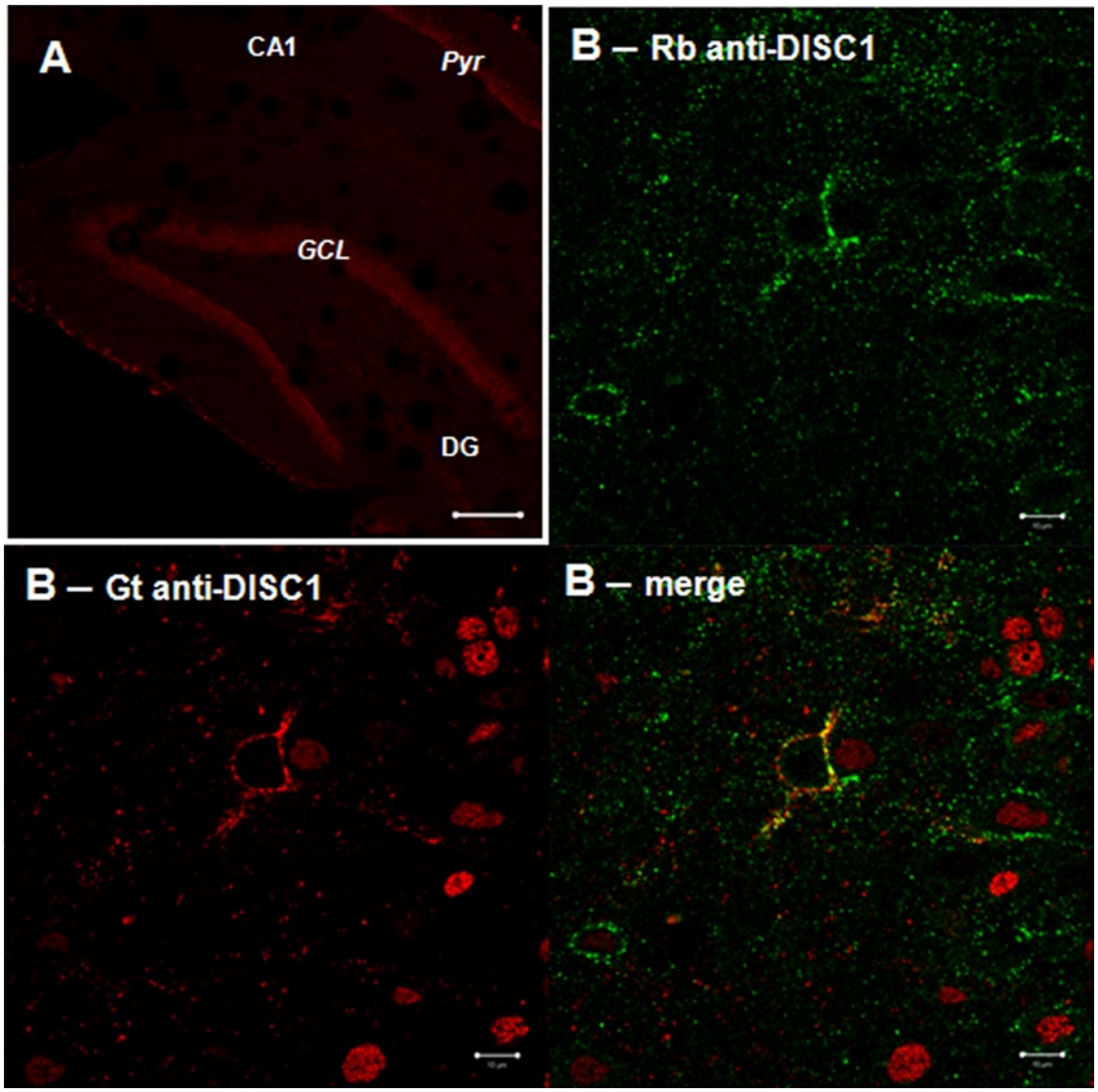

Fig. 5 Immunofluorescent confocal localisation of endogenous DISC1 in mouse brain and verification of different anti-DISC1 antibodies. **a** DISC1 immunoreactivity in sagittal section of hippocampus, showing strong staining in the pyramidal cell layer (*Pyr*) of the CA1 region as well as in the granular cell layer (*GCL*) of the dentate gyrus (*DG*). *Scale bar* 200 μm. **b** Positive colocalisation of DISC1 immunofluorescence in sections of brain cortex, using two distinct anti-DISC1 antibodies recognising different epitopes on the DISC1 protein. Antibodies used were in-house rabbit antibody (*green* fluorescence) and a commercial goat antibody (Santa Cruz, *red* fluorescence). In the third image, *yellow colour* shows sites of overlap of signal from both fluorescent channels. *Scale bars* 10 μm

hub for diverse protein interactions [5]. These protein–protein interactions implicate a significant role for DISC1 in neurodevelopmental pathways, including regulation of neural progenitor cell proliferation and migration, neurite outgrowth and synapse organisation and function [4, 5]. Nevertheless, the observed presence of DISC1 in different organs, cell types and subcellular compartments, as well as the probable existence of multiple isoforms, implicate a wider significance for the protein than has been uncovered up to now. In the present study, we have confirmed by biochemical means an interaction first identified in a yeast two-hybrid screen between full-length DISC1 and a C-terminal fragment of Tensin2 [8]. The DISC1 interactome is large, and many DISC1-binding partners have been identified through such library-scale screens [8, 17, 18], with a subset having been confirmed by further experimentation. The most prominent feature of DISC1 revealed from these studies and others is its localisation to cytoskeleton-rich regions in cells and its interactions with multiple proteins involved in cytoskeletal dynamics [19]. These interactions may be involved in controlling processes such as neurite outgrowth and neuronal migration, axonal elongation and

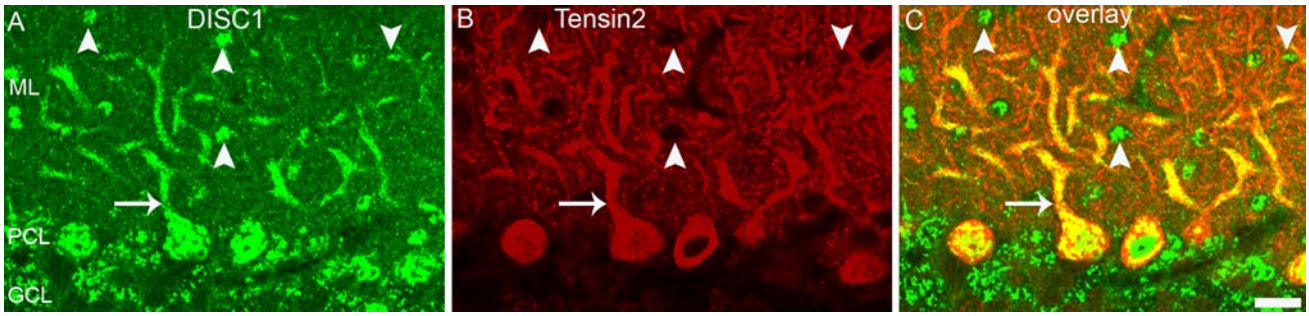

Fig. 6 Colocalisation of Tensin2 and DISC1 immunoreactivity in Purkinje cells (*PCs*) of the mouse cerebellum. **a** DISC1 immunoreactivity is present in all layers of the cerebellar cortex. In the molecular layer, DISC1 immunoreactivity is contained in interneurons (*arrowheads*) and Purkinje cell dendrites (for example, *arrow*). In the PC and granule cell (*GC*) layers, DISC1 immunoreactivity is clustered in PC and GC somata. **b** Tensin2 immunoreactivity in the mouse cerebellar cortex is restricted to Purkinje cell somata and dendrites. Other cell types such as molecular layer interneurons (*arrowheads*) and granule cells are devoid of specific staining. **c** Tensin2 and DISC1 immunoreactivity are colocalised only in PCs and no other cerebellar neurons. *GCL* granule cell layer, *ML* molecular layer, *PCL* Purkinje cell layer. *Scale bar* 20 μm

primary cilia formation. Also, several studies have implicated Tensins as being involved in intracellular signal transduction, including cytoskeletal regulation [14, 20]. Therefore, the characterisation of this new interaction is of potential relevance and significance.

We detected by western blotting expression of both Tensin2 and DISC1 proteins in C57BL/6 mouse brain, as well as endogenous DISC1 in various human cell lines of different origins. In adult mouse brain, Tensin2 displayed a band at the expected molecular weight of around 140 kDa in western blots, whilst it was barely detectable in P0 mouse brain extracts. Nevertheless, P0 brain Tensin2 expression is indeed present but close to the lower detection limit of western blot, as we have observed it in different analyses (data not shown). These observations show that Tensin2 expression is upregulated during development and is particularly switched on after birth. Tensin2 therefore appears not be essential for embryonic development; rather, it is required for organ structure and function in the developing and developed body. The particular isoform of Tensin2 that we have detected in the brain is, however, not known, as the anti-Tensin2 C-terminal antibody we used recognises all the isoforms, which themselves differ due to presence or absence of a C1 domain at the N-terminal.

At present, the issue of DISC1 isoform diversity and expression remains unclear, and has increased in significance and implications in recent years. Many studies to date have relied on the same commercial sources of antibodies, and subsequent studies using different antibodies revealed there to be a degree of variation in the type of DISC1 protein species that is observable by western blotting [5]. Therefore, it was an early priority of our investigation to develop novel in-house antibodies against DISC1 that would be additional specific probes for DISC1, and which would also help verify the specificity of other existing antibodies. The consistent result from western blots of mouse brain and various human cell lines was that DISC1 is in almost all cases apparent as a single, specific band of around 105 kDa. However, only adult mouse brain was distinct in expressing an additional strong band at around 75 kDa, which was completely absent in P0 brain. This additional species may be the same as that which has been detected using other antibodies [5], but further work is required to identify it and the role for its presence in the adult and not the neonate. The western blot results also show a specificity in the cross-species reactivity of the antibody, which was raised against a peptide sequence within the N-terminal of the rat protein, with 90 and 80 % sequence identity with the mouse and human sequences, respectively. In addition, IP experiments showed that pulldown of DISC1 from brain lysates using one antibody yielded positive detection of the protein with the other antibody, and vice versa. Furthermore, positive immunofluorescent colocalisation was observed when staining mouse brain with both the in-house and commercial antibodies.

A yeast two-hybrid screen of DISC1 interactors had detected a C-terminal fragment of the Tensin2 protein as a hit [8]. We therefore first confirmed by biochemical means that a physical interaction takes place between endogenous DISC1 and Tensin2 proteins, which was observed by pulldown experiments from mouse brain extracts. Second, we wished to localise this protein–protein interaction to specific domains or motifs as an important further step in mapping the interactome of both proteins. Therefore, we examined the binding of individual domains of Tensin2 to endogenous DISC1 in human cultured cells. Our coIP analyses revealed a positive interaction of DISC1 only with Tensin2 constructs that housed a PTB domain, namely, the PTB domain alone or in tandem with the SH2 domain. Furthermore, the interaction was phosphorylation-independent, as no difference in the binding was detected with

the inclusion or exclusion of phosphatase inhibitors in the samples (data not shown). This agrees with previous observations of the Tensin2 PTB domain, which interacts with NPXY motifs in the tails of β integrins 1, 3, 5 and 7 [7]. The occurrence of a specific DISC1–Tensin2 PTB domain interaction does not preclude, rather it may promote, a simultaneous or sequential interaction to occur between the SH2 domain and other proteins. This property remains to be investigated, particularly when the Tensin SH2 domain exhibits flexibility in its binding partners depending on its phosphorylation state [21].

Immunofluorescence confocal microscopy analysis revealed both Tensin2 and DISC1 to be widespread in their expression throughout the rodent brain. Tensin2 showed a strong presence within certain brain regions, including discrete layers of the cortex, hippocampus and cerebellum. DISC1 was also discretely expressed within the hippocampus, and was most prominent in the DG, being localised preferentially to the nucleus as well as in a small proportion of structures at or around the axon membrane. In particular, there was a distinct colocalisation of DISC1 and Tensin2 in the cerebellum, occurring mainly in the Purkinje cells. This region and cell-specific coexpression of DISC1 and Tensin2 suggests a greater role for this interaction in the cerebellum and its associated functions. It also may suggest a more significant role than previously thought for the cerebellum and its impairment in some psychiatric disorders.

Our findings of a prominent localisation of DISC1 to the nucleus match previous observations of a pool of DISC1 in the nucleus of mammalian cells, in particular within the promyelocytic nuclear body [16], as well as in rat cortical neurons when using a commercial antibody (Invitrogen; data not shown). The nuclear localisation of DISC1 is possible through the two putative nuclear localisation sequence motifs present within the N-terminal region of the protein. Given the diversity of regional, cellular and subcellular DISC1 localisation within the brain, it will be useful to establish whether specific changes in the anatomical expression of DISC1 are linked to specific mental conditions. For example, the nuclear to cytoplasmic ratio of DISC1 has been found to be altered in brains of schizophrenic patients with an increase in the nuclear pool of DISC1 [22]. Therefore, an increase in nuclear DISC1 as a result of altered signalling upstream may effect changes in gene expression, although this remains to be determined. We have also detected a prevalent punctuate pattern of expression of DISC1 throughout the cytoplasm, as well as in the membrane region of a minority of individual neurones. The latter distribution may indicate the potential for localisation to axon initial segments, which is a potential possibility as DISC1 has previously been reported to strongly interact with beta-spectrin [18]. Previous studies have also reported the association of DISC1 with the actin cytoskeleton, showing that cytoplasmic DISC1 puncta are either randomly dispersed or organised along filament-like structures [23]. These filaments resembled actin-type assemblies, such as lamellipodia, filopodia, stress fibres and arcs. Furthermore, DISC1 has been colocalised with actin or actin-related proteins along stress fibres and peripheral bundles, as well as with microtubules [24]. Therefore, these reported properties of DISC1, together with the fact that Tensins are actin-binding proteins [25], hold open the possibility that the proteins can interact within a certain spatial and/or temporal context in the brain.

In conclusion, we have demonstrated that the schizophrenia-associated protein DISC1 is a binding partner for the cytoskeleton-regulating protein Tensin2, via the latter's PTB domain. Further characterisation of this novel protein–protein interaction should elucidate further the complex and multi-faceted scaffolding role that DISC1 appears to fulfil within neuronal cells, as well as potentially in other cells of the body. Such knowledge can be significant for understanding psychiatric disorders linked to neuronal development, including schizophrenia, with a view to developing more effective targeted therapies in the future.

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