The DISC1 promoter: characterization and regulation by FOXP2

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Received January 26, 2012; Revised and Accepted March 15, 2012

Disrupted in schizophrenia 1 (DISC1) is a leading candidate susceptibility gene for schizophrenia, bipolar disorder and recurrent major depression, which has been implicated in other psychiatric illnesses of neurodevelopmental origin, including autism. DISC1 was initially identified at the breakpoint of a balanced chromosomal translocation, t(1;11) (q42.1;14.3), in a family with a high incidence of psychiatric illness. Carriers of the translocation show a 50% reduction in DISC1 protein levels, suggesting altered DISC1 expression as a pathogenic mechanism in psychiatric illness. Altered DISC1 expression in the post-mortem brains of individuals with psychiatric illness and the frequent implication of non-coding regions of the gene by association analysis further support this assertion. Here, we provide the first characterization of the DISC1 promoter region. Using dual luciferase assays, we demonstrate that a region −2300 to −177 bp relative to the transcription start site (TSS) contributes positively to DISC1 promoter activity, while a region −982 to −301 bp relative to the TSS confers a repressive effect. We further demonstrate inhibition of DISC1 promoter activity and protein expression by forkhead-box P2 (FOXP2), a transcription factor implicated in speech and language function. This inhibition is diminished by two distinct FOXP2 point mutations, R553H and R328X, which were previously found in families affected by developmental verbal dyspraxia. Our work identifies an intriguing mechanistic link between neurodevelopmental disorders that have traditionally been viewed as diagnostically distinct but which do share varying degrees of phenotypic overlap.

INTRODUCTION

Disrupted in Schizophrenia 1 (DISC1) is among the best-supported susceptibility genes for psychiatric illness. The gene was first identified in a large Scottish family in which a balanced chromosomal translocation t(1;11) (q42.1;14.3) disrupting the DISC1 locus segregates with schizophrenia, bipolar disorder and recurrent major depression (1,2). Subsequent studies have supported DISC1’s candidacy as a risk factor for these conditions, and also demonstrated associations of DISC1 gene polymorphisms in autism and Asperger syndrome (3–7).
Nevertheless, with the exception of the t(1;11) translocation, no DISC1 allele or variant has been irrefutably identified as a causative mutation (8,9). Furthermore, several associated variants and haplotypes coincide with non-coding regions of the gene, suggesting that DISC1's involvement in psychiatric illness may, at least in part, be mediated via altered regulation of its expression (5,10–14). In support of this hypothesis, a 50% reduction in DISC1 expression has been detected in lymphoblastoid cell lines derived from carriers of the t(1;11) translocation (15). Furthermore, altered DISC1 expression has been found in the post-mortem brains of patients with psychiatric illnesses in some (16–18), but not all (19–21) studies. Finally, there is evidence that common non-coding variants located upstream of DISC1 alter DISC1 expression in lymphoblastoid cell lines, and affect the expression of genes involved in cytoskeletal function and sensory perception (22).

The aim of this study was to further understanding of DISC1 regulation by investigating its currently uncharacterized promoter region. Of particular interest was a potential role for the transcription factor forkhead-box P2 (FOXP2), which has been implicated in speech and language function (23), in regulating DISC1 expression. The FOXP2 gene was originally identified through mapping studies of a large multi-generational family (the KE family) with a monogenic disorder primarily affecting speech (developmental verbal dyspraxia; DVD) (24). All affected members of the family have a heterozygous non-synonymous FOXP2 mutation, yielding a substitution (R553H) within the highly conserved DNA-binding domain of the encoded protein (25). Affected individuals are profoundly impaired in the selection and sequencing of coordinated orofacial movements required for speech (26–29), and show further deficits in linguistic and grammatical processing, as well as a decrease in IQ in both verbal and, to a lesser extent, non-verbal domains (27,29). Subsequently, another heterozygous FOXP2 point mutation, R328X, which results in a prematurely truncated protein lacking the DNA-binding domain, was identified in a second family segregating DVD (30). Functional characterization has revealed that both mutations can disturb nuclear localization of FOXP2, as well as interfere with its capacity to act as a transcription factor (31). Moreover, chromosomal rearrangements (including translocations and deletions) that disrupt FOXP2 have been reported in other individuals and families with speech and language impairments (32–36).

The presence of FOXP2-binding sites in the DISC1 promoter region has been suggested by two genome-wide chromatin immunoprecipitation (ChIP) screens (37,38) (Fig. 1). ChIP screens identify putative transcription factor-binding sites and suggest that the neighbouring gene may be a candidate for functional regulation by the transcription factor. Based on these observations, we hypothesized that FOXP2 might functionally regulate DISC1 expression. Here, we test this hypothesis and demonstrate, for the first time, that DISC1 expression is repressed by FOXP2. We assess the effect of the FOXP2 point mutations, R553H and R328X, and show that these mutations diminish the ability of FOXP2 to regulate DISC1 expression.

RESULTS
Definition and sequence analysis of the DISC1 candidate promoter region
We initially identified our candidate promoter region for DISC1 as the region spanning from 1000 bp upstream of the transcription start site (TSS) to the translation start site.
A series of DISC1 promoter constructs for assessment in the dual luciferase reporter assay

A series of three nested promoter constructs (pGL4.10-short, -medium and -long) was designed to characterize the candidate promoter region. The long construct extends from −982 to +47 bp relative to the TSS. This construct includes the region identified by Cooper et al. (39), as typically repressing promoter activity in the 45 promoters studied as part of the ENCODE Pilot Project. The medium construct spans the region from −300 to +47 bp relative to the TSS, thus corresponding to the region identified by Cooper et al. (39) as usually contributing positively to core promoter activity. This construct contains the complex dinucleotide repeat region, and an additional 124 bp upstream. The short construct includes the region −129 to +47 bp relative to the TSS, and covers the region up until the 3’ end of the complex dinucleotide repeat region (Fig. 1).

Assessment of DISC1 promoter constructs in the dual luciferase reporter assay

Promoter activity of the long, medium and short DISC1 promoter constructs was assessed in two cell lines: the neuroblastoma cell line, SH-SY5Y, and the human embryonic kidney cell line, HEK293, both of which endogenously express the 100 kDa full-length isoform of DISC1 [SH-SY5Y (43); HEK293: Supplementary Material, Fig. S1a]. One-way analysis of variance (ANOVA) revealed significant differences between the promoter activity of the three constructs in both cell lines (SH-SY5Y: $P < 0.001$, Fig. 2A; HEK293: $P = 0.006$, Fig. 2B). In both cases, Tukey’s honestly significant difference (HSD) post hoc test revealed that the promoter activity of the medium construct was significantly greater than that of the long (SH-SY5Y: $P < 0.001$; HEK293: $P = 0.016$), and the short (SH-SY5Y: $P < 0.001$; HEK293: $P = 0.006$) constructs.

Assessment of the role of the complex dinucleotide repeat region

To assess whether the complex dinucleotide repeat region underlies the enhanced promoter activity of the medium construct, we designed a further construct (pGL4.10-medium 1), which spans the region −176 to +47 bp relative to the TSS, thus comprising the short construct plus the dinucleotide repeat region. This construct does not contain the 124 bp 5’ of the dinucleotide repeat region included in the medium construct, thus permitting a more accurate assessment of the contribution of the dinucleotide repeat region (Fig. 2C). Having established similar promoter activity profiles in HEK293 and SH-SY5Y cells for the long, medium and short constructs, assessment of the medium 1 construct was carried out only in SH-SY5Y cells. Tukey’s HSD revealed that the medium 1 construct had significantly lower promoter activity than the medium construct ($P = 0.001$) and did not differ significantly from either the short, or the long construct (both $P > 0.4$, Fig. 2D).

Assessment of the effect of transcription factor FOXP2 on DISC1 protein expression and transcriptional activity

A previous study has shown HEK293, but not SH-SY5Y, cells to express FOXP2 endogenously (31). Following confirmation by western blot that HEK293 cells do express FOXP2 endogenously (Supplementary Material, Fig. S1b), we used HEK293 cells for the following experiments, in order to assess the effect of FOXP2 in an appropriate cellular context.

First, the effect of FOXP2 on endogenous DISC1 protein expression was assessed by western blotting (Fig. 3A). HEK293 cells were transfected with either a pcDNA4/HisMax vector containing full-length FOXP2 or the pcDNA3.1/HA empty vector, henceforth referred to as the ‘control plasmid’. HEK293 cells transfected with the FOXP2-expressing construct showed a mean decrease in the expression of the 100 kDa full-length isoform of DISC1 of 35.3%, which was statistically significant ($P = 0.00627$, two-tailed independent samples t-test, Fig. 3B).

Next, to determine whether the decrease in DISC1 protein expression could be attributed to repression of transcriptional activity by FOXP2, we carried out dual luciferase reporter assays. HEK293 cells were co-transfected with: (i) a DISC1 promoter construct (pGL4.10-long, -medium or -short); (ii) a FOXP2-containing vector (pcDNA4/HisMax-FOXP2) or the control plasmid; (iii) and the transfection efficiency control, pRL-TK (Fig. 3C). Two-way ANOVA with the independent variables ‘FOXP2 condition’ (control plasmid and wild-type FOXP2) and ‘promoter construct’ (long, medium and short) revealed a significant reduction in DISC1 promoter activity in cells transfected with the FOXP2-expressing construct compared with the control plasmid ($P < 0.001$). As observed previously (Fig. 2B), the effect of promoter construct was also significant ($P < 0.001$), and, in addition, the interaction between promoter construct and FOXP2 condition was also significant ($P = 0.00388$).
To investigate the interaction between promoter construct and FOXP2 condition, simple main effects were analysed. This was achieved by performing a series of one-way ANOVAs followed by Tukey’s HSD post hoc tests. First, the effect of FOXP2 on promoter activity was assessed independently for each promoter construct. Tukey’s HSD revealed a decrease in promoter activity for all constructs in FOXP2-transfected cells compared with control plasmid-transfected cells, with this decrease attaining statistical significance for both the long ($P = 0.0101$) and the medium ($P = 0.00923$) constructs, but not the short ($P = 0.114$) construct. Secondly, the effect of promoter construct was assessed independently for each FOXP2 condition. In

Figure 2. Characterization of the DISC1 candidate promoter region using the dual luciferase reporter assay. (A and B) Dual luciferase reporter assays comparing the promoter activity of the short, medium and long DISC1 promoter constructs reveal that the medium construct yields the highest level of promoter activity in SH-SY5Y (A) and HEK293 (B) cells [$n = 3$, for (A) and (B)]. (C) Design of the pGL4.10-medium 1 (M1) construct to assess the contribution of the complex dinucleotide repeat region. The genomic locations of the primers used to amplify the M1 construct (forward: M1, and reverse: R) are shown in relation to the primers used to amplify the medium (forward: M) and short (forward: S) constructs, which were amplified using a common reverse primer (R). (D) Dual luciferase reporter assays suggest that the complex dinucleotide repeat region does not underlie the enhanced promoter activity of the medium promoter construct ($n = 3$). Statistical significance was assessed by one-way ANOVA followed by Tukey’s HSD post hoc test. $* P \leq 0.05$, $** P \leq 0.01$, $*** P \leq 0.001$. Error bars represent standard error of the mean.
keeping with our initial characterization of the long, medium and short promoter constructs, Tukey’s HSD revealed that in both FOXP2-transfected and control plasmid-transfected cells, the activity of the medium construct was significantly greater than the activity of the short (control plasmid: \( P \leq 0.001 \); FOXP2: \( P \leq 0.001 \)) and the long (control plasmid: \( P = 0.00369 \); FOXP2: \( P = 0.00369 \)) constructs. Additionally, in cells transfected with FOXP2, the activity of the long construct was significantly greater than the activity of the short construct (\( P = 0.0310 \)), while in the control plasmid-transfected cells the same pattern of activity was observed but only approached statistical significance (\( P = 0.0577 \)).

Assessment of the effect of FOXP2 mutations implicated in speech and language disorder on FOXP2-mediated transcriptional repression of DISC1

Two FOXP2-expressing constructs each containing one of the rare coding mutations, R553H or R328X, which are found only in individuals affected with DVD (25,30) were assessed for their ability to repress transcription of DISC1 using the dual luciferase reporter assay (Fig. 4). HEK293 cells were co-transfected with: (i) a DISC1 promoter construct (pGL4.10-long, -medium or -short); (ii) a FOXP2-containing vector (pcDNA4/HisMax-FOXP2, -FOXP2.R553H or-FOXP2.R328X) or the control plasmid; (iii) and the transfection efficiency control, pRL-TK (Fig. 4). Separate two-way ANOVAs were carried out for the two FOXP2 mutations with the independent variables ‘FOXP2 condition’ (control plasmid, wild-type FOXP2 and FOXP2.R553H or FOXP2.R328X) and ‘promoter construct’ (long, medium and short). For both mutations, ANOVA revealed significant main effects of FOXP2 condition (\( \ast\ast\ast P \leq 0.001 \)), determined by two-way ANOVA, and the significant effect of FOXP2 on the long (\( \ast P \leq 0.05 \)) and the medium (\( \ast\ast P \leq 0.01 \)) constructs, determined by two-tailed independent samples Student’s \( t \)-tests are indicated on the graph (\( n = 3 \)). Error bars represent standard error of the mean.

**Figure 3.** DISC1 protein expression and promoter activity is regulated by wild-type FOXP2. (A) Representative image of western blot analysis of DISC1, FOXP2 and GAPDH protein expression in HEK293 cells transfected with either pcDNA4/HisMax-FOXP2 or the control plasmid (pcDNA3.1/HA). (B) Quantification of DISC1 protein expression normalized to GAPDH. DISC1 expression was reduced in cells transfected with the FOXP2-expressing construct compared with the control plasmid (\( n = 5 \)). Statistical significance was assessed by a two-tailed independent samples Student’s \( t \)-test, \( \ast\ast P \leq 0.01 \). (C) Dual luciferase reporter assays show down-regulation of DISC1 promoter activity in cells transfected with the FOXP2-expressing construct compared with the control plasmid. The significant main effect of FOXP2 (\( \ast\ast\ast P \leq 0.001 \)), determined by two-way ANOVA, and the significant effect of FOXP2 on the long (\( \ast P \leq 0.05 \)) and the medium (\( \ast\ast P \leq 0.01 \)) constructs, determined by two-tailed independent samples Student’s \( t \)-tests are indicated on the graph (\( n = 3 \)). Error bars represent standard error of the mean.
In this study, we have performed the first characterization of the promoter of DISC1, a leading candidate gene for schizophrenia and bipolar disorder, which has also been implicated in autism. We have also provided the first functional demonstration that DISC1 is transcriptionally repressed by FOXP2, a transcription factor that is mutated in individuals with a rare autosomal dominant form of DVD (25,30), and which has been shown to regulate targets involved in common language impairments and autism (44). Furthermore, we have shown that two rare FOXP2-coding mutations found only in individuals with DVD diminish the ability of FOXP2 to regulate DISC1 expression, thus furthering understanding of the functional effects of these mutations. These findings highlight a point of molecular convergence between neurodevelopmental disorders that have been traditionally viewed as diagnostically distinct.

Our analysis of the DISC1 promoter region revealed a lack of canonical core promoter elements in the expected location relative to the previously identified TSS (17). The region was found to contain a CpG island, a feature of many constitutively expressed genes, which is in keeping with DISC1’s ubiquitous pattern of expression (1). The highest level of transcription was obtained from the medium promoter construct, which spans the region from −300 to +47 bp relative to the TSS. This region contains a complex dinucleotide repeat region, a feature previously identified as contributing to the transcriptional activity of some promoters (41,42). In the case of the DISC1 promoter, however, the complex dinucleotide repeat region did not appear to underlie the enhanced transcriptional activity of the medium construct (although, it should be noted that our analysis would not have detected any contribution of the complex dinucleotide repeat region that was dependent on the genomic context of upstream sequence). Diminished gene expression from the long compared with the medium construct suggests the presence of repressive elements in the region −982 to −301 bp relative to the TSS. As such, our findings are in keeping with those of the ENCODE project (39).

We observed significant downregulation of the activity of the long and medium promoter constructs in cells transfected with FOXP2, with the activity of the short construct showing the same trend. These observations are compatible with the potential FOXP2-binding sites identified by ChIP screens (37,38). Both ChIP studies identified FOXP2-bound regions that span all three promoter constructs. Consistent with the initial characterization of the R553H and R328X FOXP2 point mutations (31), we found that both mutations reduced the ability of FOXP2 to exert transcriptional repression on DISC1 promoter activity. In contrast to carriers of the t(1;11) translocation, who show a 50% reduction in DISC1 expression, individuals carrying the R553H and R328X FOXP2 point mutations would be expected to show increased expression of DISC1. Future studies should assess whether any deviation from normal DISC1 expression level is pathogenic, and, if so, whether increases and decreases in DISC1 expression contribute to overlapping or distinct phenotypes.

Accumulating evidence supports the view that psychiatric conditions that have traditionally been considered clinically distinct might share overlapping aetiologies (45,46). This possibility was initially suggested by the shared behavioural characteristics and cognitive deficits observed in schizophrenia, bipolar disorder and autism (45). Further support came from the finding of overlap in the genetic variants that predispose to
these disorders, suggesting that variation in certain genes alters neurological processes whose abnormal functioning results in phenotypes common to multiple psychiatric conditions (46).

Our demonstration that DISC1 is a transcriptional target of FOXP2 suggests that there may be mechanistic overlap between the neurodevelopmental conditions associated with variation in the two genes. When viewed as a collection of observable phenotypes, the condition resulting from FOXP2 point mutations (25,30) and those conditions associated with variation in the DISC1 (schizophrenia, bipolar disorder and autism) gene are quite distinct; however, there are areas of overlap. Speech and language dysfunction is the core phenotype associated with point mutations in the FOXP2 gene. In addition to speech and language dysfunction, some affected members of the KE family also show lowering of non-verbal IQ, although the effects on verbal cognition appear more severe and wide-ranging. To varying extents, cognitive dysfunction and abnormalities in some aspects of language function are observed in autism, schizophrenia and bipolar disorder (45,47–60). Determining whether or not these phenotypes result, at least in part, from common neurophysiological abnormalities stemming from altered FOXP2 and/or DISC1 function should be the focus of future research. A possible neurophysiological basis for the convergence of FOXP2 and DISC1 function is the synapse where both genes are known to play an important role (38,61–70). Cognition relies on normal synaptic function (71), and synaptic dysfunction has been identified in schizophrenia (72,73), autism (74) and bipolar disorder (75).

In the context of synaptic function, FOXP2′s role in the regulation of CNTNAP2, the gene that encodes CASPR2, a member of the neurexin (NRXN) family (44) is of particular interest. NRXNs are cell adhesion molecules with key roles in synaptic function (76). Mutations and polymorphisms in CNTNAP2 have been independently associated with specific language impairments (44) and with autism (77–80). Two other NRXN family members, NRXN1 (81–86) and NRXN2 (87), have been implicated in autism, and variation in NRXN1 has been linked to schizophrenia (87–90). Recently, our group identified a role for DISC1 in the regulation of NRXN expression: in a mouse model of schizophrenia in which Disc1 carries a non-synonymous point mutation, L100P (91), we observed disturbed developmental expression of Nrnx1 and Nrnx3 (92).

In conclusion, our data link FOXP2, DISC1 and the NRXN family in a molecular network that, when altered, confers risk for neurodevelopmental conditions in which various aspects of linguistic and cognitive function are disturbed, possibly via aberrant synaptic function. Future work should focus on investigating the physiological consequences of variation in these genes to further understanding of how their disturbance contributes to the shared and distinct aetiologies of these conditions.

MATERIALS AND METHODS

In silico analysis of the DISC1 candidate promoter region

DISC1 has previously been shown by 5′ rapid amplification of cDNA ends (5′RACE) to have one TSS, excluding that which arises from splicing of DISC1 to the downstream gene

<table>
<thead>
<tr>
<th>Core promoter element</th>
<th>Location searched (base pair relative to TSS)</th>
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<tbody>
<tr>
<td>Transcription factor IIB recognition element</td>
<td>–43 –31</td>
</tr>
<tr>
<td>TATA box</td>
<td>–35 –24</td>
</tr>
<tr>
<td>Initiator</td>
<td>–3 –1</td>
</tr>
<tr>
<td>Motif ten element</td>
<td>&gt;16 &gt;18</td>
</tr>
<tr>
<td>Downstream promoter element</td>
<td>&gt;27 &gt;29</td>
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Core promoter elements were searched for using the YAPP Eukaryotic Core Promoter Predictor program (http://www.bioinformatics.org/yapp/cgi-bin/yapp.cgi). This program permits the user to specify the location of the TSS and then searches for core promoter elements in their expected location relative to the TSS, as specified above. The inputted sequence is compared, using a sliding windows algorithm, to position weight matrix representations (Supplementary Material, Table S1) of the core promoter elements and a matrix similarity score calculated. Core promoter elements are identified when the matrix similarity score exceeds a pre-defined threshold. The DISC1 candidate promoter region (chr1: 231761561–231762613) was searched using three thresholds: the default threshold of 0.8, 0.7 and 0.6.

Translin-associated factor X (TSNAX) (17). We therefore considered this TSS located at chr1: 231762561 (February 2009, GRCh build 37; http://genome.ucsc.edu, last accessed 20 October 2011) as the DISC1 TSS for subsequent definition of the promoter region. An initial candidate promoter region extending from 1000 bp upstream of the TSS to the translation start site (chr1: 231761561–231762613) was downloaded from the UCSC genome browser (http://genome.ucsc.edu/) and analysed using the eukaryotic core promoter predictor program YAPP (http://www.bioinformatics.org/yapp/cgi-bin/yapp.cgi, last accessed 15 September 2011), which permits the user to search for core promoter elements, and synergistic combinations of these elements, in their expected location relative to a known TSS (Table 1). Following a previously described method (93), the test sequence is compared against positional weight matrix representations (Supplementary Material, Table S1) of core promoter elements to calculate a matrix similarity score, which only reaches one when the test sequence corresponds to the most conserved nucleotide at every position of the matrix. Searches were carried out using similarity score thresholds of 0.8, 0.7 and 0.6. CpG islands were identified using the ‘CpG Islands’ track of the UCSC genome browser.

Luciferase reporter vectors

Three luciferase reporter vectors purchased from Promega were used in this study: the promoterless vector, pGL4.10, which encodes firefly luciferase reporter gene luc2; pGL4.13, which expresses luc2 under the control of an SV40 promoter; and pRL-TK, which expresses Renilla luciferase (Rluc) under the control of a thymidine kinase promoter.

Cloning of DISC1 promoter fragments

A series of four nested fragments covering different extents of the DISC1 candidate promoter region were polymerase chain
reaction (PCR) amplified from a bacterial artificial chromosome (RP11-17H4; BACPAC) using the Expand High Fidelity PCR System (Roche), which contains a thermostable DNA polymerase with proofreading ability. Primer sequences used were as follows: DISC1_long F: 5′-TCAAGCTAGCACCATCCCTGCGGCTAGGCGC-3′; DISC1_short F: 5′-TCAAGCTAGCAAGCTTCTGCATCTGACCTGCCTCCTC-3′; DISC1_medium 1 F: 5′-TCAAGCTAGCTGTGTGTGTATGTC-3′; DISC1_short F: 5′-TCAAGCTAGCCAGGGGGCATTGCTGGAA-3′; all fragments were amplified using a common reverse primer: DISC1_promoter R: 5′-TCAAAAGCTTCCGCTGCGAGCTCCTC-3′. Following confirmation by sequencing, fragments were digested using NheI and HindIII restriction enzymes (Roche), and ligated using the Rapid DNA Ligation Kit (Roche) upstream of the coding sequence of luc2, in the promoterless vector pGL4.10. Prior to use, the promoter constructs were sequenced to confirm integrity.

**FOXP2 constructs and the pcDNA3.1/HA control plasmid**

The generation of the three FOXP2 constructs used in this study, pcDNA4/HisMax-FOXP2, pcDNA4/HisMax-FOXP2.R553H and pcDNA4/HisMax-FOXP2.R328X has been described previously (31). Briefly, the full-length coding region of the major transcript of FOXP2, isoform 1, was PCR-amplified from a commercial Human Foetal Brain Total RNA (Clontech) following reverse transcription, and cloned into the expression vector pcDNA4/HisMax (Invitrogen). The R553H and R328X mutant constructs were generated using the Quick-Change Site-Directed Mutagenesis kit (Stratagene), according to the manufacturer’s instructions.

For the assessment of the effect of the FOXP2 constructs on DISC1 protein expression and promoter activity, the control plasmid, pcDNA3.1/HA (Invitrogen), which is highly similar in size to pcDNA4/HisMax, was used as a control for the molar quantity of foreign DNA transfected into the cell. Western blotting revealed that DISC1 protein levels in untransfected HEK293 cells and cells transfected with the control plasmid did not differ significantly ($P = 0.979$, two-tailed independent samples t-test; Supplementary Material, Fig. S2).

**Cell culture and transient transfection**

The human neuroblastoma cell line, SH-SY5Y, and the human embryonic kidney cell line, HEK293, were maintained in Dulbecco’s Minimal Essential Medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO$_2$ atmosphere. Cells were grown to ∼80% confluency in tissue culture flasks before seeding onto either 96-well plates at 5 × 10$^4$ cells per well (dual luciferase reporter assays), or 6-well plates at 5 × 10$^5$ cells per well (western blots), in order to reach ∼80% confluency following overnight incubation. Transient transfections were carried out using Fugene HD (Roche) or Nucleofector (Lonza Biologics), according to the manufacturers’ instructions.

**Dual luciferase reporter assay**

For the initial analysis of the DISC1 promoter region, SH-SY5Y and HEK293 cells were co-transfected with: (i) 163.7 µg of the appropriate reporter construct [promoterless pGL4.10 for the negative control, pGL4.13 vector for the positive control or pGL4.10-DISC1 promoter construct (long, medium, medium 1 or short)], and (ii) 3.3 µg of the transfection efficiency control pRL-TK.

For the assessment of the effect of FOXP2 and the mutated forms of FOXP2, FOXP2.R553H and FOXP2.R328X, on DISC1 promoter activity, SH-SY5Y cells were co-transfected with three constructs: (i) 163.7 µg of the appropriate reporter construct [promoterless pGL4.10, pGL4.13 or pGL4.10-DISC1 promoter construct (long, medium or short)], (ii) 3.3 µg of the transfection efficiency control pRL-TK, and (iii) 166.7 µg of a FOXP2 expression construct (pcDNA4/HisMax-FOXP2/-FOXP2.R553H/-FOXP2.R328X), or the control plasmid, pcDNA3.1/HA (Invitrogen). Twenty-four hours post-transfection, each well was supplemented with 100 µl fresh DMEM with 10% FBS.

Cells were rinsed with phosphate buffered saline (PBS) and lysed either 24 h (initial analysis of the DISC1 promoter region) or 48 h (assessment of the effect of FOXP2 post-transfection with 20 µl passive lysis buffer (PLB; Promega). Luciferase activity was quantified using the Dual-Luciferase Reporter Assay System (Promega), using an Omega FLUOstar luminometer (BMG Labtech).

To control for transfection efficiency, firefly luciferase (pGL4.10 and pGL4.13) values were divided by Renilla luciferase values to obtain relative luciferase values. Background luciferase activity (the mean relative luciferase value obtained from cells transfected with the promoterless vector pGL4.10) was subtracted from all relative luciferase values. For the initial characterization of the DISC1 promoter, these values were then divided by the mean relative luciferase value obtained from the six wells transfected with pGL4.13 on each plate, in order to control for between plate variability.

Transfections were performed in sextuplicate (initial analysis of the DISC1 promoter region) or triplicate (assessment of the effect of FOXP2) and repeated in three independent experiments.

**Western blotting**

HEK293 cells were grown to ∼80% confluency in tissue culture flasks before seeding onto six-well plates at 5 × 10$^5$ cells per well, in order to reach ∼80% confluency following overnight incubation. Cells were then transfected with 2 µg of pcDNA4/HisMax-FOXP2 or the control plasmid pcDNA3.1/HA in five biological replicates. Twenty-four hours post-transfection, each well was supplemented with 2000 µl fresh DMEM with 10% FBS. Forty-eight hours post-transfection, cells were rinsed with PBS and lysed with 400 µl PBS-1% Triton X-100-10% glycerol protein extraction buffer, containing Complete Protease Inhibitor Cocktail (Roche). Protein lysates were gently rotated for 30 min at 4°C, before being centrifuged at 16 000g for 20 min at 4°C to remove cell debris. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories).

Immediately before electrophoresis, protein samples (40 µg) were mixed with 5 × loading buffer [final concentration 0.16 × Tris-chloride (pH 6.8), 5% sodium dodecyl sulphate (SDS), 25% glycerol, 6% β-mercaptoethanol, 0.02%
Bromophenol blue] and heated at 100°C for 2 min. Protein samples and the Precision Plus Protein All Blue Standards molecular weight marker (Bio-Rad Laboratories) were fractionated on a 7% SDS-polyacrylamide-tris-aceate gel (Invitrogen) and electrophoretically transferred onto a polyvinyl difluoride membrane (Invitrogen). Gel electrophoresis and transfer efficiency were assessed by Ponceau S staining.

The membrane was immersed in blocking buffer [PBS containing 0.2% Tween (PBS-T) and 5% non-fat milk] for 1 h, followed by incubation (overnight at 4°C) followed by either 1 h (GAPDH) or 4 h (DISC1 and FOXP2) at room temperature with the primary antibody [DISC1, 1:1000, a kind gift from Dr Tetsu Akiyama, University of Tokyo (94); FOXP2, 1:500, Abcam; GAPDH, 1:100 000, Millipore] in blocking buffer. Membranes were then washed in PBS-T, and incubated with a horseradish peroxidase-conjugated secondary antibody (for both DISC1 and FOXP2: swine-anti-rabbit, 1:2000, Dako; for GAPDH: goat-anti-mouse, 1:1000, Dako), in blocking buffer for 1 h. Membranes were washed in PBS-T as before. Immunoreactive bands were visualized using the ‘ECL’ or ‘ECL Plus’ kit (GE Healthcare) on light-sensitive film.

Bands were quantified by densitometry using GeneTools image analysis software (Syngene) and optical density values for DISC1 immunoreactive bands were normalized to the optical density values for GAPDH. Between blot differences were normalized by standardization to a calibrator value.

Statistical analysis
Statistical analysis was carried out using SPSS (Statistical Package for the Social Sciences) 17.0 (Apache Software Foundation). Dual luciferase reporter assay data were assessed using either one-way or two-way ANOVA, followed by Tukey’s post hoc test. Significant interaction terms were investigated by splitting the data file by each independent variable and performing either two-tailed independent samples Student’s t-tests or one-way ANOVAs followed by Tukey’s HSD post hoc test. Western blot data were analysed using a two-tailed independent samples Student’s t-test.

Differences were considered statistically significant when \( P \leq 0.05 \).

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS
K.L.E., P.A.T. and D.J.P. are members of The University of Edinburgh Centre for Cognitive Ageing and Cognitive Epidemiology, part of the cross council Lifelong Health and Wellbeing Initiative (G0700704/84698). Funding of the CCACE from the BBSRC, EPSRC, ESRC and MRC is gratefully acknowledged. We thank Dr Kirsty Millar for helpful comments on a draft of this manuscript.

Conflict of Interest statement: The authors declare no conflict of interest.

FUNDING
This work was supported in part by the Scottish Funding Council Strategic Awards Grant ‘Biomarkers for Battling Chronic Disease’ to D.J.P., and an RCUK fellowship to P.A.T. S.E.F. received support from the Simons Foundation Autism Research Initiative (SFARI, grant number 137593) and the Max Planck Society.

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