

Ciliary dyslexia candidate genes *DYX1C1* and *DCDC2* are regulated by Regulatory Factor X (RFX) transcription factors through X-box promoter motifs

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ABSTRACT: *DYX1C1*, *DCDC2*, and *KIAA0319* are three of the most replicated dyslexia candidate genes (DCGs). Recently, these DCGs were implicated in functions at the cilium. Here, we investigate the regulation of these DCGs by Regulatory Factor X transcription factors (RFX TFs), a gene family known for transcriptionally regulating ciliary genes. We identify conserved X-box motifs in the promoter regions of *DYX1C1*, *DCDC2*, and *KIAA0319* and demonstrate their functionality, as well as the ability to recruit RFX TFs using reporter gene and electrophoretic mobility shift assays. Furthermore, we uncover a complex regulation pattern between *RFX1*, *RFX2*, and *RFX3* and their significant effect on modifying the endogenous expression of *DYX1C1* and *DCDC2* in a human retinal pigmented epithelial cell line immortalized with hTERT (hTERT-RPE1). In addition, induction of ciliogenesis increases the expression of RFX TFs and DCGs. At the protein level, we show that endogenous *DYX1C1* localizes to the base of the cilium, whereas *DCDC2* localizes along the entire axoneme of the cilium, thereby validating earlier localization studies using overexpression models. Our results corroborate the emerging role of DCGs in ciliary function and characterize functional noncoding elements, X-box promoter motifs, in DCG promoter regions, which thus can be targeted for mutation screening in dyslexia and ciliopathies associated with these genes.—Tammimies, K., Bieder, A., Lauter, G., Sugiaman-Trapman, D., Torchet, R., Hokkanen, M.-E., Burghoorn, J., Castrén, E., Kere, J., Tapia-Páez, I., Swoboda, P. Ciliary dyslexia candidate genes *DYX1C1* and *DCDC2* are regulated by Regulatory Factor (RF) X transcription factors through X-box promoter motifs. *FASEB J.* 30, 3578–3587 (2016). www.fasebj.org

KEY WORDS: reading disorder · ciliopathies · gene regulation · hTERT-RPE1 cell line · ciliary proteins

ABBREVIATIONS: ChIP-seq, chromatin immunoprecipitation sequencing; *DCDC2*, doublecortin domain-containing 2 gene; DCG, dyslexia candidate gene; DD, developmental dyslexia; *DYX1C1*, dyslexia 1 candidate 1 gene; hTERT-RPE1, human retinal pigmented epithelial cell line immortalized with hTERT; LR, left/right; PCD, primary ciliary dyskinesia; RFX TF, Regulatory Factor X transcription factor; SH-SY5Y, human neuroblastoma cell line, subline of the neuroblastoma cell line SK-N-SH; siRNA, small interfering RNA; SNP, single nucleotide polymorphism; WT, wild-type

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Developmental dyslexia (DD) is a common neurodevelopmental learning disorder that affects 5–10% of school-age children worldwide (1, 2); a highly heritable trait with a demonstrated biological basis (3). Human genetic studies have implicated multiple loci to be involved in the etiology of DD. Thus far, only a handful of dyslexia candidate genes (DCGs) have been identified from these loci and subsequently characterized. To date, *DYX1C1* (dyslexia 1 candidate 1 gene), *DCDC2* (doublecortin domain-containing 2 gene), and *KIAA0319* are the most replicated and best-studied DCGs (3–7). Functionality of the DD-associated single nucleotide polymorphisms (SNPs) has only been shown in a few instances. For example, we demonstrated that SNPs in the promoter region of *DYX1C1* affect the binding of transcription factor complexes that depend both on the specific allele and on exposure to estrogen (8, 9). Likewise, associated variants in the promoter of *KIAA0319* can affect its expression (10).

By use of cell and rodent models, the 3 DCGs, *DYX1C1*, *DCDC2*, and *KIAA0319*, have been linked strongly to cytoskeletal organization and to neuronal migration in the brain (4, 5, 11–13). Recently, studies have shown an entirely novel connection between DCGs and cilia. Cilia are nearly ubiquitous, hair-like eukaryotic subcellular organelles that project off polarized cell surfaces. In mammals, cilia are present on many different cell types, including most types of neurons (14, 15). Primary cilia house signal reception and transduction components that are critical for development, for example, the Hedgehog and Wnt pathways. Defects in the function of cilia and cilia-associated genes can lead to a class of human genetic disorders broadly called ciliopathies (16, 17). Furthermore, cilia have a demonstrated role in brain development, and dysfunctions in neuronal cilia can lead to brain abnormalities (18, 19).

After our initial discovery that increased expression of *DCDC2* controls the length and signaling of primary cilia (41), more evidence has emerged that links DCGs to cilia. Recent genetic findings support a critical role for these genes in ciliary biology. Knockout mouse and zebrafish morpholino models have further shown that lack of *Dyx1c1* leads to outer dynein arm and inner dynein arm defects, which likely cause impaired ciliary motility (20, 21). Mutations in *DYX1C1* have been found in patients with primary ciliary dyskinesia (PCD) (21, 22) and mutations in *DCDC2* in patients with nephronophthisis-related ciliopathy (23) and heritable deafness (24).

Cilia are composed of hundreds of different protein components. Studies primarily in *Caenorhabditis elegans*, *Drosophila*, and *Danio rerio* have shown that Regulatory Factor X transcription factors (RFX TFs) directly regulate many ciliary genes, including components of the intraflagellar transport complex, axonemal dyneins, and Bardet-Biedl syndrome proteins (25–30), a mechanism that is likely common across many different species (31). In humans, the RFX TF gene family consists of 8 members (*RFX1–8*), which can be divided into groups on the basis of protein domain homologies (26). The RFX TF family of proteins is defined by a highly conserved DNA binding domain that recognizes a sequence motif known as the X-box, typically located in gene promoters (26). RFX TFs are important in brain development and many of the members, namely *RFX1–5* and *RFX7*, are highly expressed in the brain (32, 33), with *RFX1–3* being the most studied.

On the basis of the recently uncovered links between DCGs and cilia, we hypothesized that ciliogenic RFX TFs are involved in the transcriptional regulation of DCGs. This would further strengthen the emerging concept that DCGs play important roles at the cilium and impact the view on the still unknown cell biology that underlies a human brain condition such as DD.

MATERIALS AND METHODS

Identification of X-box promoter motifs

To identify putative X-box motifs in the promoter regions of DCGs, a bioinformatics approach was taken as described by Henriksson *et al.* (34). In short, genomic sequences 3 kb upstream from the start of exon 1 for *DYX1C1*, *DCDC2*, and *KIAA0319* on

the basis of Ensembl protein-coding transcripts (release 75, February 2014) were retrieved by using the UCSC Genome Browser (GRCh37/hg19; University of California, Santa Cruz, Santa Cruz, CA, USA). Thereafter, the nucleic acid pattern search tool EMBOSS fuzznuc (<http://emboss.bioinformatics.nl/cgi-bin/emboss/fuzznuc>) was used to identify putative X-box motif sequences within these promoter regions. The search was based on 7 different X-box motif consensus sequences (Supplemental Table S1) and conducted on both the coding and noncoding strands. Further ranking and prioritization of the identified X-box motifs was done by using the mean PhyloP score on the basis of 100 vertebrates (retrieved from the UCSC Genome Browser) (35). To visualize the conservation of motifs, we aligned the most highly ranked of the identified X-box sequences against 4 other mammalian species—mouse, dog, cow, and cat—and made a consensus sequence logo by using WebLogo (University of California, Berkeley, Berkeley, CA, USA).

Cell culture

The human retinal pigmented epithelial cell line immortalized with hTERT (hTERT-RPE1; CRL-4000; American Type Culture Collection, Manassas, VA, USA) was cultured in DMEM/F12, 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.01 mg/ml hygromycin B at 5% CO₂. To induce ciliogenesis, cells were starved by using Opti-MEM reduced serum medium for 24 h unless indicated otherwise. Human neuroblastoma cell line, subline of the neuroblastoma cell line SK-N-SH (SH-SY5Y) was cultured as described previously (13). In brief, cells were cultured in MEM with Earle's salts and GlutaMax-1 that was supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Luciferase reporter assays

Up to 2 kb of promoter sequence was amplified from human genomic DNA for each of the 3 DCGs, *DYX1C1*, *DCDC2*, and *KIAA0319*. Fragments were then cloned upstream of the luciferase reporter gene in promoterless pGL3basic vector (Promega, Madison, WI, USA; Supplemental Table S2). Genomic regions that were cloned were upstream of DCG exon 1 and contained the identified X-box promoter motifs. To disrupt the X-box motif, we changed up to 8 nucleotides following previously published information and principles (36, 37) using site-directed mutagenesis (Supplemental Table S2). The resulting clones were sequence verified before use. Luciferase assays were performed as described previously (5). In brief, hTERT-RPE1 or SH-SY5Y cells were cotransfected at 80–90% confluence with either the wild-type (WT) or the corresponding X-box mutated pGL3basic construct, together with 10 ng of the pRLTK vector, which constitutively expresses *Renilla* luciferase and functions as an internal normalization control. For hTERT-RPE1 cells that underwent ciliogenesis, standard cultivation medium was replaced by serum-reduced Opti-MEM medium. After 24 h, we lysed and measured luciferase activity by using the Dual-Luciferase Reporter Assay System (Promega) according to manufacturer protocol and a Tecan Luminometer Infinite 200 (Tecan AG, Männedorf, Switzerland). Relative luciferase expression was normalized and results were presented as relative response ratios compared with the indicated WT promoter construct. Experiments were performed in triplicate and were repeated independently 3 times. Statistical significance was analyzed by using 2-sided Student's *t* test.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSA) were performed by using biotin end-labeled probes that contained sequences surrounding the conserved X-boxes of the

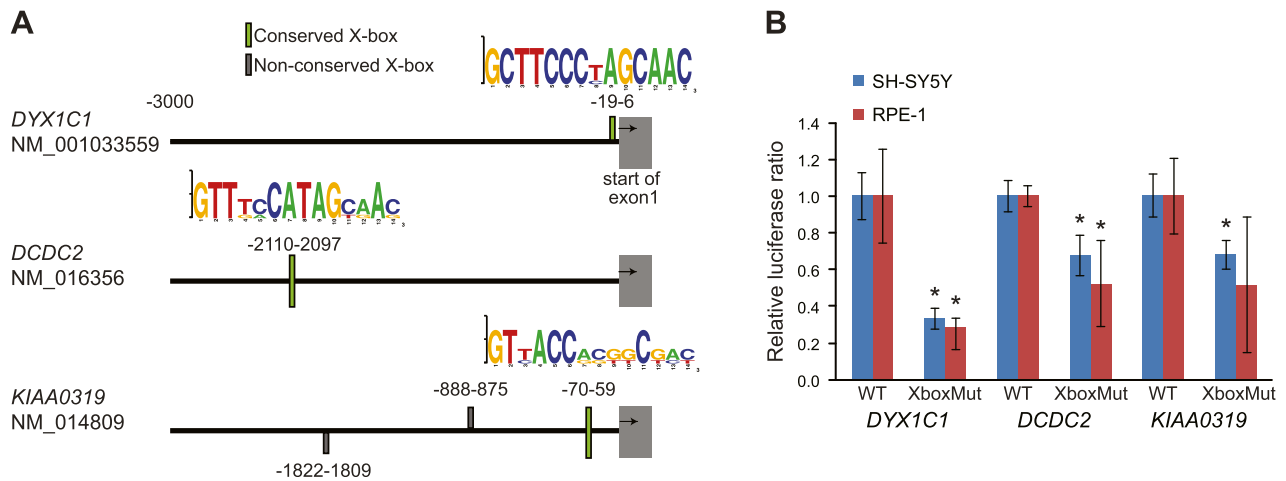


Figure 1. The promoters of *DYX1C1*, *DCDC2*, and *KIAA0319* contain functional X-box promoter motifs. A) Location of putative X-box promoter motifs upstream of *DYX1C1*, *DCDC2*, and *KIAA0319* identified in the bioinformatics screen. X-box sequence logos on the basis of human, mouse, dog, cow, and cat sequence alignments are shown above 3 well-conserved motifs. B) Luciferase expression assays: pGL3 basic vector containing promoter sequences of *DYX1C1*, *DCDC2*, and *KIAA0319* (containing WT or mutated X-box motifs) were transfected into starved hTERT-RPE1 and SH-SY5Y cells under normal growth conditions. Luciferase expression was measured and data are shown as relative ratio to the WT construct. Data are displayed as means \pm SEM. * $P < 0.05$ of 2-sided Student's *t* test.

DCGs *DYX1C1*, *DCDC2*, and *KIAA0319* (Fig. 1A). Probes consisted of double-stranded WT sequences, 29 or 30 bp in length. Sequences are as follows: wt-DYX: forward, 5'-CCCAGCTTCCCTAGCAACCAAGCAGGC-3'; wt-DYX: reverse, 5'-GCCTGCTTGGTTGCTAGGGAAGCTGGGGTT-3'; mut-DYX: forward, 5'-AACCCACATTGACTCTCAAA-CAAGCAGC-3'; mut-DYX: reverse, 5'-GCCTGCTTGTGTT-GAGAGTCAATGTGGGGTT-3'; wt1-DCDC2: forward, 5'-GAGGCTGCGGTTGCTATGGAAACAGCGGGA-3'; wt1-DCDC2: reverse, 5'-TCCCGCTGTTTCCATAGCAACCGCAGCCTC-3'; mut1-DCDC2: forward, 5'-AGGCTGCGACTGGTATGCAAATAGCGGGA-3'; mut1-DCDC2: reverse, 5'-TCCCGCTATTGATACCAAGTCGCAGCCT-3'; wt-KIAA0319: forward, 5'-CGCGCGGTCGCGGTTGGTAACCGCGGCGGC-3'; wt-KIAA0319: reverse, 5'-GCCGCCGCGGTTACCACGGC-GACGCGCGCG-3'; mut-KIAA0319: forward, 5'-CGCGCGCGTCTAGATAACCGCGGCGGC-3'; mut-KIAA0319: reverse, 5'-GCCGCCGCGGTTATCTAGACGACGCGCGCG-3'. Nuclear extracts from serum-starved hTERT-RPE1 cells were obtained by using the NE-PER nuclear and cytoplasmic extraction reagents, according to manufacturer instructions (Thermo Fisher Scientific, Waltham, MA, USA). Each probe was incubated with 10 μ g of nuclear protein extracts for 15 min at room temperature. The remaining steps followed the LightShift Chemiluminescent EMSA Kit protocol (Thermo Fisher Scientific). Samples were electrophoresed on 6% Novex TBE gels (Thermo Fisher Scientific) in 0.5 \times TBE (0.09 M Tris borate, 2 mM EDTA) at 100 V. Gel was blotted on to a nylon membrane (Thermo Fisher Scientific) and the detection was performed according to the manufacturer protocol. For super shift assays, antibodies against RFX1 (H-11, sc-166468), RFX2 (A-18, sc-10659), and RFX3 (T-17, sc-10662; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. RFX2 and RFX3 antibodies were used before in similar experiments (38–40) and their specificity was tested by Western blot (data not shown).

Small interfering RNA knockdowns

hTERT-RPE1 and SH-SY5Y cells were seeded in 6-well plates and grown to 80% confluence. Cells were transfected with 25 μ M of each siGENOME SMARTpool targeting RFX TF genes (RFX1: M-010147-01; RFX2: M-011129-00; RFX3: M-011764-00) at a final

concentration of 75 μ M for any combination of the 3 genes or adjustment of the concentration with a control pool (nontargeting #2: D-001206-14-20; all Thermo Fisher Scientific) using DharmaFECT transfection reagent 1 (T-2001-03; Thermo Fisher Scientific). After 48 h, hTERT-RPE1 cells were serum starved for 24 h and harvested. For SH-SY5Y cells, culture media was changed after 48 h and cells were then harvested after 24 h. RNA from both cell lines was prepared by using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and RNase-free DNase Set (Qiagen) according to manufacturer instructions. All experiments were performed in triplicate and were repeated independently 3 times.

Quantitative real-time PCR

cDNA was synthesized with SuperScript III First-Strand Synthesis SuperMix kit (11752-250; Thermo Fisher Scientific) by using 500 ng RNA. Quantitative real-time PCR (qRT-PCR) was analyzed with cDNA diluted 1:5 using TaqMan expression assays and TaqMan fast Universal PCR Master Mix [4352042; Thermo Fisher Scientific; *DYX1C1*: Hs00370049_m1; *DCDC2*: Hs00393203_m1; *KIAA0319*: Hs00207788_m1; *HPRT1* (hypoxanthine phosphoribosyltransferase 1): Hs02800695_m1; *CDK1* (cyclin-dependent kinase 1): Hs00938777_m1; *TUBA1A* (tubulin α 1a): Hs00362387_m1; *RFX2*: 01100925_m1] or using SYBR green primers for *RFX1*, *RFX3*, and *HPRT1* (10 μ M) and the Fast SYBR Green Master Mix (4385612; Applied Biosystems, Foster City, CA, USA). The following SYBR Green primers were used: *RFX1*: forward, 5'-CAGACGACGCTGCAGGCCAA-3'; *RFX1*: reverse, 5'-TGGCCACCTTTGCTGCCTGG-3'; *RFX3*: forward, 5'-TGGCGATTGACGCTGCAAA-3'; *RFX3*: reverse, 5'-TGGGAAGGCTCACTCTTCTGCT-3'; *HPRT1*: forward, 5'-TCAGGCAGTATAATCCAAAGATGGT-3'; *HPRT1*: reverse, 5'-AGTCTGGCTTATATCCAACACTTCG-3'. *HPRT1* was used as an internal control to normalize expression levels. Reactions were amplified by using the 7500 Fast Real-Time PCR system (Applied Biosystems). The $\Delta\Delta C_t$ method was used to calculate relative expression levels and shown as fold change ($2^{-\Delta\Delta C_t}$). For samples in which the gene was down-regulated, $-2^{\Delta\Delta C_t}$ was calculated. The statistical significance between different conditions of each analyzed gene was analyzed by using ANOVA followed by Tukey's *post hoc* test.

Immunofluorescence

hTERT-RPE1 cells were seeded on coverslips, grown to 80% confluence, and serum starved for 24 h. Cells were incubated on ice for 45 min, then fixed in a 50:50 solution of 4% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and MeOH or in pure MeOH for 15 min at -20°C . Fixed cells were blocked and permeabilized in 5% horse serum (Thermo Fisher Scientific) and 0.05% PBS-Tween for 1 h at room temperature and incubated overnight at 4°C with a primary antibody (rabbit anti-DYX1C1, 1:500; Sigma-Aldrich; rabbit anti-DCDC2, 1:200; Abcam, Cambridge, MA, USA; mouse anti-acetylated tubulin, 1:5000; Sigma-Aldrich; mouse anti- γ -tubulin, 1:500; Abcam). The specificities of anti-DYX1C1 and anti-DCDC2 antibodies have been assessed previously (21, 23). Cells were incubated for 1 h at room temperature with secondary antibodies (donkey anti-rabbit 568; Thermo Fisher Scientific; donkey anti-mouse 568; Thermo Fisher Scientific). Nuclei were stained with DRAQ5 [1,5-bis[2-(di-methylamino)ethyl]amino]s-4,8-dihydroxyanthracene-9,10-dione] (Cell Signaling Technology, Cambridge, United Kingdom) at 1:1000 for 10 min at room temperature and coverslips were mounted in Prolong Gold antifade reagent (Thermo Fisher Scientific). Images were captured on an LSM 510 confocal microscope (Zeiss, Jena, Germany) using Plan-Neofluar $\times 40/\text{NA} 1.3$ oil and Plan-Apochromatic $\times 63/\text{NA} 1.4$ oil objectives and the Zeiss Laser Scanning Microscope LSM 510 software (version 4.2 SP1; Zeiss). Images were captured in multitrack scanning mode in 8-bit format using Z-stack and subsequent maximum intensity projection. Brightness and contrast were adjusted by using LSM 510 software and the far-red channel was pseudo colored in blue. Cilia and nuclei were quantified manually by using ImageJ (National Institutes of Health, Bethesda, MD, USA).

RESULTS

Conserved X-box motifs in the promoters of DCGs

We performed a bioinformatics search to identify candidate X-box motifs within promoter regions (3 kb upstream from exon 1) of the DCGs *DYX1C1*, *DCDC2*, and *KIAA0319*. By using the EMBOSS fuzznuc tool with 7 known X-box motif (consensus) sequences as query patterns (Supplemental Table S1), we identified a total of 7 candidate X-box motifs in the promoter sequences (Fig. 1A, Supplemental Table S3). Conservation estimation using the PhyloP 100 vertebrates mean score revealed that 5 of the candidate X-box motifs (at 3 independent genomic sites) were well conserved across species (PhyloP score >1.0), 1 in each DCG promoter (Fig. 1A, Supplemental Table S3). We considered the 3 well-conserved DCG promoter sites with their X-box motifs as good candidates for being involved in the regulation of DCG gene expression.

X-box motifs in the promoters of *DYX1C1*, *DCDC2*, and *KIAA0319* are functional

We next addressed whether the identified, conserved X-box motifs are functional and required for gene expression. We cloned the X-box-containing promoter regions of *DYX1C1*, *DCDC2*, and *KIAA0319* into a vector with the luciferase reporter gene and produced mutated versions of the X-box motifs by changing multiple nucleotides at essential positions of the motif sequences to abolish any

possible RFX TF binding (Supplemental Table S2). We then transfected WT or mutant constructs into 2 different human cell lines, hTERT-RPE1 (ciliated upon starvation) and SH-SY5Y (nonciliated), that both express high levels of endogenous *RFX1-3*, *RFX5*, and *RFX7* (data not shown). All 3 cloned regions comprised functional promoters that were sufficient to initiate luciferase gene expression well above background. We observed a significant decrease in luciferase activity for X-box mutant constructs for *DYX1C1* and *DCDC2* promoters compared with promoter activity of the corresponding WT motif (Fig. 1B). A similar trend was observed for the *KIAA0319* X-box mutant construct, but a significant change was only observed in SH-SY5Y cells. These results suggest that the identified X-box motifs are functional and required for proper expression of the corresponding downstream gene.

RFX1 and RFX2 bind to conserved X-box motif sequences in the promoters of *DYX1C1* and *DCDC2*

We then performed EMSA experiments with nuclear extracts from ciliated hTERT-RPE1 cells to directly demonstrate *in vitro* the binding of RFX TFs to X-box motif sequences. We observed 2 binding complexes when incubating hTERT-RPE1 nuclear extracts with duplex oligonucleotide probes spanning the X-box promoter motifs from *DYX1C1* or *DCDC2* (Fig. 2A); however, no binding was observed for the *KIAA0319* X-box promoter motif (data not shown). In addition, RFX-to-X-box binding was abolished upon incubation with an excess of unlabeled, biotin-free probes (data not shown). We further demonstrated direct binding of RFX TFs by using EMSA supershift assays by adding anti-RFX1, anti-RFX2, or anti-RFX3 antibodies to the binding reactions (38). We observed only 1 supershifted complex after addition of anti-RFX1 or anti-RFX2 antibodies, which indicated that both RFX1 and RFX2 bind to the X-box motifs in the promoter regions of *DYX1C1* and *DCDC2* (Fig. 2A). Experiments that employed the anti-RFX3 antibody were in parts inconclusive (Fig. 2A and data not shown). All experiments were repeated at least 3 times with similar results. In conclusion, these *in vitro* experiments showing direct binding of RFX TFs to DCG X-boxes underscore that at least the DCGs *DYX1C1* and *DCDC2* are direct RFX target genes.

Knockdown of RFX TFs affects expression of *DYX1C1* and *DCDC2*

Next, we asked whether endogenous RFX TFs drive expression of *DYX1C1*, *DCDC2*, and *KIAA0319*. To address this issue, we performed knockdown of *RFX1*, *RFX2*, and *RFX3* in ciliated hTERT-RPE1 cells and measured expression levels of the DCGs. In addition, we tested for potential combined effects among the RFX TFs by performing double and triple knockdowns of these factors. The level of *DYX1C1* expression was changed significantly only upon knockdown of *RFX1* (Fig. 2B). Expression of *DCDC2* was up-regulated significantly upon silencing of *RFX1* (Fig. 2B). The opposite effect was observed upon

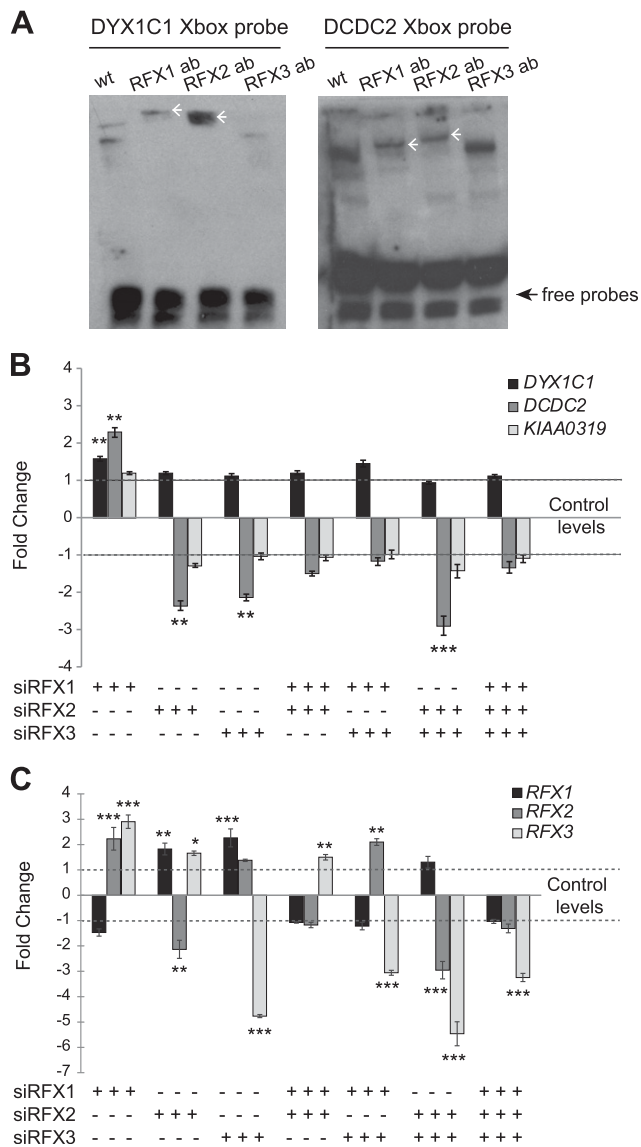


Figure 2. Knockdown of *RFX1*, *RFX2*, and *RFX3* affect the expression of *DYX1C1* and *DCDC2* but not *KIAA0319* in hTERT-RPE1 cells. **A**) Binding of *RFX1* and *RFX2* to the X-box motifs present in *DYX1C1* and *DCDC2* promoters. Biotinylated probes spanning the X-box motifs were incubated with nuclear extracts from serum-starved hTERT-RPE1 cells with or without antibodies (ab) against *RFX1*, *RFX2*, and *RFX3*. Supershifts for both probes were detected for *RFX1* and *RFX2* antibodies (white arrowheads). One representative experiment of 3 is shown. **B**) Fold-change differences in the expression of DCGs upon knockdown of RFX TFs. By using siRNA against *RFX1*, *RFX2*, and *RFX3*, alone or in different combinations, genes were silenced in hTERT-RPE1 cells. Cells were thereafter starved for 24 h to induce ciliogenesis, and expression levels of *DYX1C1*, *DCDC2*, and *KIAA0319* were measured by using qRT-PCR. **C**) Fold-change difference in expression levels of *RFX1*, *RFX2*, and *RFX3* upon siRNA silencing. qRT-PCR data are summarized by using the $\Delta\Delta C_t$ method as displayed as mean fold-change ($2^{-\Delta\Delta C_t}$ or $-2^{\Delta\Delta C_t}$) \pm SEM. Significance of the *post hoc* tests after ANOVA is presented as *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$ after multiple correction of simultaneous comparisons.

reducing the levels of *RFX2* alone or together with *RFX3*. The combination of small interfering RNAs (siRNAs) against *RFX2* and *RFX3* had the most significant effect on *DCDC2* levels, which resulted in a 3-fold down-regulation

(Fig. 2B). Expression of *KIAA0319* was not changed in any of the conditions. Of interest, when we performed similar experiments in the nonciliated neuroblastoma cell line SH-SY5Y, no significant changes were observed for the expression levels of any of the 3 DCGs (Supplemental Fig. S1). These results suggest that in ciliated cells *RFX1* acts as a repressor for *DYX1C1* and *DCDC2* and that *RFX2* and *RFX3* act as an activator complex for *DCDC2*.

It is noteworthy that we observed that in our double or triple siRNA experiments, the effect on the target gene varied on the basis of the combination of silenced genes. To test for the possibility of regulation between these factors, we measured the expression levels of *RFX1*–*3* in all siRNA conditions. Indeed, we observed that knockdown of *RFX1* led to significant increase in the levels of *RFX2* and *RFX3*, which further supported its role as a transcriptional repressor (Fig. 2C). Knockdown of *RFX2* alone and *RFX3* alone increased the expression of *RFX1* (Fig. 2C); however, combined knockdowns of both *RFX2* and *RFX3* did not affect *RFX1* expression. Finally, expression levels of *RFX3* were significantly changed in all conditions. Taken together, these data suggest a complex mutual regulatory mechanism among different RFX TFs, whereby—depending on their relative expression levels in a given cell type—they can up- or down-regulate expression levels of targets, such as *DYX1C1* and *DCDC2*.

Expression of DCGs increases upon induction of ciliogenesis

Given the well-documented role of RFX TFs in ciliary gene regulation (25, 26) and the fact that DCGs *DYX1C1* and *DCDC2* are direct RFX TF targets, we studied the expression of DCGs during ciliogenesis. We performed qRT-PCR at different time points during cilia induction. For this purpose, hTERT-RPE1 cell extracts were isolated at 0, 6, 12, and 24 h after serum starvation. We observed that expression of *DYX1C1*, *DCDC2*, and *KIAA0319* was up-regulated during ciliogenesis, with the highest expression at 24 h (Fig. 3). In parallel, we measured the expression of *RFX1*, *RFX2*, and *RFX3*. Like the 3 DCGs, RFX TF genes also showed a similar increase of expression, but to a lesser extent (Fig. 3). As a control for the growth of cilia, we measured the expression of known cilia-specific genes, *IFT57* (intraflagellar transport 57) and *TUBA1A* (tubulin α 1a). As expected, their expression was up-regulated during ciliogenesis (Fig. 3G, H). At the same time, expression of *CDK1* (cyclin-dependent kinase 1), a cell-cycle gene, was down-regulated as cells enter G_0 and undergo ciliogenesis (Fig. 3I). In parallel and independently, using immunocytochemistry against the ciliary marker acetylated tubulin, we analyzed the process of ciliogenesis at different time points after serum starvation (Supplemental Fig. S2). We observed that after 12 h, >85% of cells had grown cilia. Finally, the same set of experiments was carried out in hTERT-RPE1 cells without serum starvation, and no differential expression of DCGs or of control genes was observed (Supplemental Fig. S3). Taken together, these time course experiments indicate that induction of

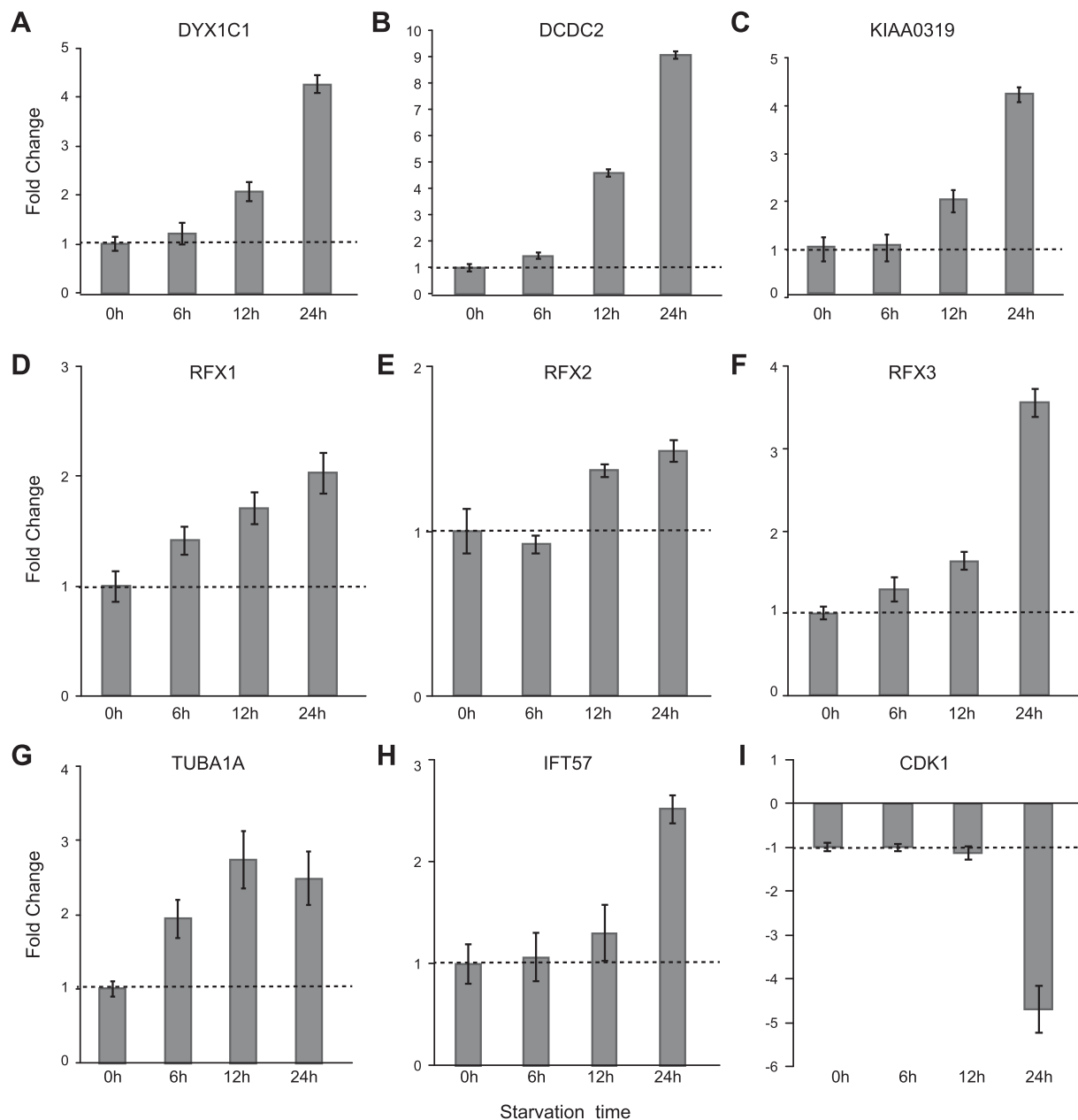


Figure 3. Induction of ciliogenesis in hTERT-RPE1 cells affects the expression levels of DCGs, RFX TF genes, and ciliogenesis/cell-cycle marker genes. hTERT-RPE1 cells were starved for 6, 12, and 24 h to induce ciliogenesis. Fold-change of difference for each time point compared with 0 h is shown for *DYX1C1* (A), *DCDC2* (B), *KIAA0319* (C), *RFX1* (D), *RFX2* (E), *RFX3* (F), *TUBA1A* (tubulin α 1a) (G), *IFT57* (intraflagellar transport 57) (H), and *CDK1* (cyclin-dependent kinase 1) (I). qRT-PCR data are summarized by using the $\Delta\Delta C_t$ method as displayed as mean fold-change ($2^{-\Delta\Delta C_t}$ or $-2^{\Delta\Delta C_t}$) \pm SEM.

RFX TF and DCG expression correlate well with the process of ciliogenesis.

Endogenous *DYX1C1* and *DCDC2* proteins localize to ciliary structures in hTERT-RPE1 cells

On the basis of our and others' previous results that show exogenous, overexpressed *DCDC2* localizes to primary cilia in rat hippocampal neurons and in NIH/3T3 cells (41), and on the basis of results obtained in human ciliated tissues and hTERT-RPE1 cells (23), we tested whether the endogenous proteins

DYX1C1 and *DCDC2* localize to the primary cilium using immunocytochemistry in serum-starved hTERT-RPE1 cells. When costaining for *DYX1C1* together with the cilia-specific marker acetylated α -tubulin or the centrosome-specific marker γ -tubulin, we observed *DYX1C1* accumulation around the basal body of the cilium (Fig. 4A). When we costained for *DCDC2* together with the cilia-specific marker acetylated α -tubulin, specific accumulation of *DCDC2* along the entire ciliary axoneme was observed (Fig. 4B). These results—colocalization experiments of endogenous proteins—strongly support a role for *DYX1C1* and *DCDC2* at the basal body and the cilium, respectively.

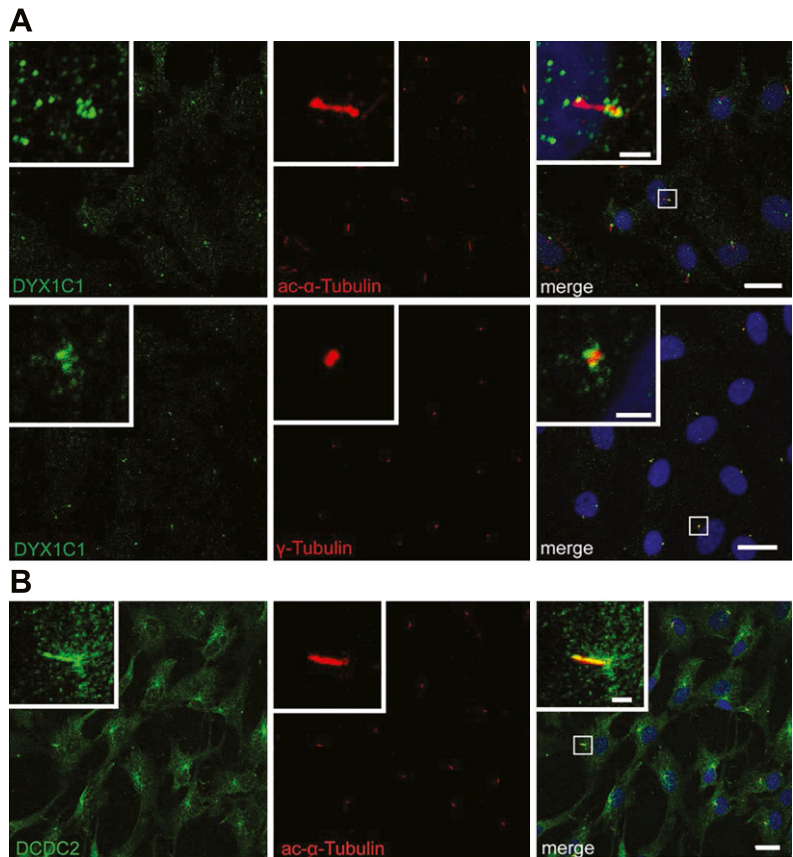


Figure 4. Subcellular localization of endogenous DYX1C1 (A) and DCDC2 (B) in hTERT-RPE1 cells. hTERT-RPE1 cells were grown to 80% confluence, serum starved for 24 h to induce ciliogenesis, then fixed and stained for DYX1C1, DCDC2, acetylated α -tubulin (cilia marker), or γ -tubulin (centrosome marker). Nuclei are stained with DRAQ5. Scale bars, 20 μ m, 2 μ m (insets).

DISCUSSION

Here, we describe for the first time, to our knowledge, that RFX TFs directly regulate 2 of the most replicated and best-characterized DCGs: *DYX1C1* and *DCDC2*. This regulation of DCG expression by RFX TFs occurs *via* evolutionarily well-conserved X-box promoter motifs, which we demonstrate by using a combination of bioinformatics and *in vitro* approaches. In addition, we demonstrate a novel complex regulatory pattern between the RFX TFs, which was previously only suggested by protein-binding assays (28, 43–45). RFX TFs are well known as key regulators of genes involved in cilia structure and function (26). We provide strong evidence that *DYX1C1* and *DCDC2* play a role in human primary cilia and are regulated by RFX TFs in a human cell model.

Our results are in agreement with an emerging body of work that points toward a role for DCGs in ciliary biology. *DYX1C1*, *DCDC2*, and *KIAA0319*—the 3 most replicated DCGs—have been shown to be up-regulated in ciliated tissues (42, 46). *Dyx1c1* and *Dcdc2*, as well as *Rfx2* and *Rfx3*, have been identified in ciliated ependymal cells of the adult mouse brain by using a single-cell RNA-sequencing approach (47). The strongest functional evidence for *DYX1C1* being needed for the proper assembly of cilia was obtained by Chandrasekar *et al.* (20) by morpholino knockdowns of the zebrafish ortholog *dyx1c1*, which resulted in full ciliopathy phenotypes, including *situs inversus*, hydrocephalus, defects in body curvature, shortening of cilia in the Kupffer vesicle, and lack of inner dynein arm and outer dynein arm in motile cilia. Shortly thereafter, rare

homozygous or compound heterozygous deleterious mutations were identified in *DYX1C1* from patients with PCD (21, 22). Similarly, *Dyx1c1*-knockout mice also displayed a PCD phenotype (21). Despite its reported role in cilia, localization of *DYX1C1* to cilia and centrosomes has so far only been achieved in overexpression cell lines (11, 13, 42). Here, we advance by clearly demonstrating that endogenous *DYX1C1* localizes specifically to the base of cilia in human hTERT-RPE1 cells (Fig. 4). For *DCDC2*, further evidence for being a ciliary gene emerged when it was identified as a causative gene for nephronophthisis-related ciliopathy in 2 individuals (23) and for heritable hearing loss (24). These results are consistent with a reported localization to the cilium of overexpressed *DCDC2* (41) and in human ciliated tissues (23). Again, here we advance by clearly demonstrating that endogenous *DCDC2* localizes along the entire axoneme of cilia in human hTERT-RPE1 cells (Fig. 4). In future, experiments should also be conducted to delineate the ciliary role of *DYX1C1* and *DCDC2* in neuronal cells. For *KIAA0319*, there is some evidence from the literature that it could be involved in ciliary processes (42, 46); however, our work offers only indirect clues for such a possible ciliary role for *KIAA0319* as we show that an evolutionarily well-conserved X-box promoter motif is located in its proximal promoter and its expression is up-regulated during the process of ciliogenesis.

Whereas DCGs *DYX1C1*, *DCDC2*, and *KIAA0319* share demonstrated functions in neuronal migration, the role of cilia in neuronal migration and brain development is currently a matter of debate; however, intriguing evidence

has emerged. For instance, a number of ciliary genes are needed for proper brain development (18, 19) and many ciliopathies lead to deficits in cognitive functions, which indicates disrupted brain development under these conditions (16). Another possibility for links between cilia and neuronal development, brain patterning, and morphogenesis is offered by the Shh and Wnt signaling pathways, where crucial pathway components with ciliary localization are involved in key signaling steps of primary cilia (48). Furthermore, both motile and nonmotile cilia are needed to setup the left/right (LR) asymmetry during embryonic development, which could have implications for brain structure and connectivity. Of interest, early studies by Galaburda *et al.* (49, 50) reported reduced *planum temporale* asymmetry in 8 postmortem brain specimens from individuals with DD. Recently, *PCK6* (proprotein convertase subtilisin/kexin type 6), a gene that is involved in LR patterning, was associated with LR relative hand skills in individuals with DD, which further indicated that the cilia-regulated process of determining LR asymmetry and DD might share the same biologic pathways (51). *Rfx2* and *Rfx3* have also been implicated in the regulation of LR asymmetry in the mouse (52, 53).

It is conceivable that RFX TFs exert their impact on neuronal and brain development and possibly on brain-related conditions, such as DD, directly *via* their target genes. Proven targets from the ciliogenic gene battery include genes *TMEM138* (transmembrane protein 138) and *TMEM216* (transmembrane protein 216), which are expressed in human embryonic brain and cause Joubert Syndrome when mutated (54), and the gene *ALMS1* (Alström Syndrome 1), which is important for neurosensory development and causes Alström Syndrome upon mutation (55). Our study tentatively adds DD to this body of work by assigning the ciliary DCGs *DYX1C1* and *DCDC2* as direct targets of RFX TFs. Our work is further supported by independent findings from genome-wide approaches. By using chromatin immunoprecipitation sequencing (ChIP-seq), *RFX3* was found to bind the DCGs *DYX1C1*, *DCDC2*, *KIAA0319*, and *KIAA0319L*, ciliary genes, such as *Arl13b* (ADP-ribosylation factor-like protein 13B), as well as other *RFX* genes (44). In addition, a combination of *rfx2* ChIP-seq and RNA sequencing of *rfx2* morpholino knockdown in *Xenopus laevis* identified *dcdc2* in the group of differentially regulated genes (28). In future, it will be important to study how other RFX TFs not analyzed here affect the expression of DCGs and whether they occupy X-box motifs in different cell systems. Furthermore, alternative possibilities include nonciliogenic RFX TF target gene batteries that impact neuronal and brain development and thus brain-related conditions, such as DD. RFX TF-mediated regulation of target genes and cellular processes other than ciliogenesis and the adaptive immune response (56, 57) are still not well understood and, as such, are a highly speculative topic. Nonciliogenic examples of RFX TF regulation would be transforming growth factor $\beta 2$, which is important for neuronal cell proliferation (58), or RFX TF targets, which are important for neuronal synapse structure and function as originally discovered for the *C. elegans* RFX TF DAF-19 (dauer formation gene 19) (59).

We have successfully focused on a noncoding element of the genome, the X-box promoter motif, and have used it as a tool to uncover the transcriptional regulation of DCGs and how these genes are linked to cilia. The identified X-box in the promoter of *DYX1C1* is located at a transcriptional hub, which contains the DD-associated SNP-3G/A (rs3743205), and where many different TFs bind depending on cell type, state, and environmental stimuli (8, 9). In recent years, increased interest has focused on understanding the functional elements in the noncoding parts of the genome, as exemplified by the Encyclopedia of DNA Elements (60) and Functional Annotation of the Human Genome (61, 62) projects. With advances in whole-genome sequencing, this characterization will become even more important as rare mutations and variants are often detected outside protein coding regions. For example, Bae *et al.* (63) identified a functional X-box in the promoter region of the gene *GPR56* (GPCR 56) by analyzing homozygous small deletions in individuals with brain gyrification defects, including the Broca's area. The authors further demonstrated the binding of *RFX1* and *RFX3* to the X-box, thereby linking these TFs to the regulation of genes that are expressed in language-related areas of the brain. Thus, mutation screening of the noncoding genomic regions characterized here could lead to the identification of causal mutations for DD or for ciliopathies associated with these DCGs. Another approach, looking for X-boxes in already described loci for DD, could facilitate the identification of the respective candidate genes.

In summary, our work with human cells clearly demonstrates that DCGs are regulated by RFX TFs *via* X-box promoter motifs and are linked to the development, structure, or function of cilia. Our work is in agreement with work in other systems that describes the role of DCGs in ciliary biology. Combined, these results lend strong support to an emerging theme that assigns cilia a potentially crucial role in the etiology of DD. Whereas the link between DCGs and cilia is strongly supported by data, the next step—the link between DD and cilia—is much more tenuous and hypothetical. As brain imaging studies suggest overlapping regions of functional importance and involvement of white matter for the 3 DCGs studied here, these regions could serve as starting points for further research to connect the pathways (64). To acquire this more comprehensive understanding, future studies need to clarify which cells of the brain are crucial for the etiology of DD, what the function of cilia is for these cells, and how such results could explain the observed genetic effect on white matter. FJ

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AUTHOR CONTRIBUTIONS

K. Tammimies, A. Bieder, G. Lauter, D. Sugiaman-Trapman, R. Torchet, M.-E. Hokkanen, J. Burghoorn, and I. Tapia-Páez performed experiments; K. Tammimies, E. Castrén, J. Kere, I. Tapia-Páez, and P. Swoboda conceived and designed the study; K. Tammimies, A. Bieder, and P. Swoboda wrote the manuscript; and all authors critically revised the manuscript.

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Ciliary dyslexia candidate genes *DYX1C1* and *DCDC2* are regulated by Regulatory Factor X (RFX) transcription factors through X-box promoter motifs

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Table S3. Detailed description of the identified X-box motifs and their conservation based on PhyloP scores calculated from 100 vertebrates.

Ensembl transcript					RFX consensus (full site only)							X-box position	Conservation based on PhyloP100vert						
Transcript ID	Start of first exon	Strand orientation based on transcript	Location, strand	Sequence	1	2	3	4	5	6	7	Relative to first exon start site	Min	Max	Range	Mean	Variance	Standard deviation	
DYX1C1-002 NM_001033559	chr15:55790575	Forward	chr15:55790581..55790594 -	GCTTCC CT AGCAAC							x	-19 to -6	0,48	4,74	4,27	2,32	1,89	1,37	
DCDC2-001 NM_016356	chr6:24358280	Forward	chr6:24360377..24360390, -	GTTGCT AT GGAAAC			x	x	x		x	-2110 to -2097	0,18	4,51	4,33	2,26	2,31	1,52	
		Reverse	chr6:24360377..24360390, +	GTTTCC AT AGCAAC			x	x	x		x	-2110 to -2097	0,18	4,51	4,33	2,26	2,31	1,52	
KIAA0319-001 NM_014809	chr6:24646383	Forward	chr6:24646440..24646453, -	GTCGCC GT GGTAAC	x	x					x	-70 to -57	-0,05	2,55	2,60	1,11	0,67	0,82	
		Reverse	chr6:24646440..24646453, +	GTTACC AC GGCGAC							x	-70 to -57	-0,05	2,55	2,60	1,11	0,67	0,82	
		Reverse	chr6:24648192..24648205, +	GCCTTT AA AGAAAC			x					x	-1822 to -1809	-1,28	0,85	2,13	-0,03	0,48	0,69
		Forward	chr6:24647258..24647271, -	ATAATT GA GGAAAC								x	-888 to -875	-0,84	1,94	2,78	0,15	0,41	0,64

Table S1. X-box motif sequences used for the bioinformatics search with the EMBOSS fuzznuc tool. All sequence information is shown 5' to 3'.

No.	Consensus	Organism	References
1	GTNRCC (0-3N) RGYAAC	<i>H sapiens</i>	Chen, L., et al. ¹
2	GTNRC (C/N) (0-3N) RGYAAC	<i>H sapiens</i>	Hsu, Y. C., et al. ²
3	RYYNYY WW RRNRAC	<i>C elegans</i>	Efimenko, E., et al. ³
4	RTHNYY WT RRNRAC	<i>C elegans</i>	Efimenko, E., et al. ³
5	GTHNYY AT RRNAAC	<i>C elegans</i>	Efimenko, E., et al. ³
6	GTTNCC NN (0-1N) GGHVAC	<i>H sapiens</i>	Internal
7	RBNNYY NH RGHAAC	<i>H sapiens</i>	Internal

¹ Chen, L., Smith, L., Johnson, M. R., Wang, K., Diasio, R. B., and Smith, J. B. (2000) Activation of protein kinase C induces nuclear translocation of RFX1 and down-regulates c-myc via an intron 1 X box in undifferentiated leukemia HL-60 cells. *The Journal of biological chemistry* 275, 32227-32233

² Hsu, Y. C., Liao, W. C., Kao, C. Y., and Chiu, I. M. (2010) Regulation of FGF1 gene promoter through transcription factor RFX1. *The Journal of biological chemistry* 285, 13885-13895

³ Efimenko, E., Bubb, K., Mak, H. Y., Holzman, T., Leroux, M. R., Ruvkun, G., Thomas, J. H., and Swoboda, P. (2005) Analysis of *xbx* genes in *C. elegans*. *Development* 132, 1923-1934

Table S2. Dyslexia candidate gene promoter construct cloning primers without restriction enzyme sequences and X-box wild type and mutant sequence information, respectively. All sequence information is shown 5' to 3'.

Gene	Forward primers	Reverse primers	X-box wild type ^a	X-box mutant
<i>DYX1C1</i>	TGAGTGTGCCCTAT TTTGGG	CAGTCTTCGTCTG CTGCCAGC	GTCGCC GT GGTAAC	CACGAG GT TCCAAA
<i>DCDC2^b</i>	CTCCCTCTTCCTCC AAACC and GTGTGCGCCAGGCT GGAGTG	CCGTGACTCATGC CTGTAAC and GAAACGAACGAGA AACTGACG	GCTTCC CT AGCAAC	ACTGGT AT GCAAAT
<i>KIAA0319</i>	GGATGTCCACTGAA TGCTGA	ACAGGTGGAGCAA GGTTGCAC	GCTTCC CT AGCAAC	CATTGA CT CTCAA

^a For details of identification see Table S3.

^b The *DCDC2* promoter construct was cloned by PCR sewing two regions together (chr6:24,359,852-24,360,507 and chr6:24,358,078-24,358,893) to avoid a TA-repeat region located at: chr6:24,358,894-24,359,851 in Hg19.