RCAN1 Reduces FBXW7 β Transcription by Inhibiting Calcineurin/NFAT Signaling Pathway

Shuai Wang

Jining Medical University

Yu Yang

Jining Medical University

Ruying Song

Jining Medical University

Yiming Gao

Jining Medical University

Yili Wu (✉ yili_wu2004@yahoo.ca)

Wenzhou Medical University

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1 **RCAN1 reduces *FBXW7* transcription by inhibiting**
2 **calcineurin/NFAT signaling pathway**

3 Shuai Wang^{1,2}, Yu Yang^{1,2}, Ruying Song^{1,2}, Yiming Gao^{1,2} and Yili Wu^{3,4} *

4

5 1 Shandong Collaborative Innovation Center for Diagnosis, Treatment and
6 Behavioral Interventions of mental disorders, Institute of Mental Health, Jining
7 Medical University, Jining, China, 272013.

8 2 Shandong Key Laboratory of Behavioral Medicine, School of Mental Health,
9 Jining Medical University, Jining, China, 272013.

10 3 Key Laboratory of Alzheimer's Disease of Zhejiang Province, Institute of
11 Aging, School of Mental Health and The Affiliated Kangning Hospital, Wenzhou
12 Medical University, Wenzhou, China.

13 4 Oujiang Laboratory, Wenzhou, China.

14

15 * Corresponding author at: Institute of Aging, Wenzhou Medical University,
16 University Town, Chashan, Wenzhou, China.

17 E-mail address: yili_wu2004@yahoo.ca (YL. Wu)

18

Abstract

Regulator of calcineurin 1 (RCAN1), a crucial endogenous regulator of calcineurin, is implicated in multiple important physiological and pathological processes. Aberrant expression of RCAN1 is commonly found in brains of patients with Down syndrome (DS) or Alzheimer's disease (AD), accounting for impaired neurodevelopment in DS and neuronal degeneration in AD, respectively. However, the mechanism of RCAN1 in brain development and neurodegeneration remains unclear. FBXW7 functions as vital factor in neurodevelopment and neurodegeneration via mediating proteasomal degradation of its substrates. Deficiency of FBXW7 contributes to impaired neurodevelopment and accelerating neurodegeneration. Here, we show that increased RCAN1 reduces the level of β isoform of FBXW7 (FBXW7 β). RCAN1 inhibits FBXW7 β transcription in a calcineurin dependent manner. Potential NFAT binding sites are identified within the promoter of FBXW7 β , and NFAT is also demonstrated to activate the promoter activity of FBXW7 β . In summary, our work implies that RCAN1 can regulate FBXW7 β expression by inhibiting FBXW7 β transcription via calcineurin/NFAT signaling pathway. It could provide more understanding on the mechanism of FBXW7 regulation and suggest a potential mechanism on functional implication of RCAN1 with impaired brain function in some neurodevelopmental and neurodegenerative diseases.

Keywords

RCAN1, FBXW7, calcineurin/NFAT pathway, transcriptional regulation

43 **Introduction**

44 Down syndrome (DS) patients generally exhibit abnormal morphology and function
45 in the brain [1, 2]. The contribution of triplicated genes in critical region of human
46 chromosome 21 provided valuable insights into the pathogenesis of DS. RCAN1 is
47 one of these factors contributing to these developmental and functional abnormalities
48 in DS brains. *RCAN1* gene, formerly known as *DSCR1* is located q22.1–q22.2 region
49 of human chromosome 21 and is aberrantly up-regulated in DS brains [3, 4]. RCAN1
50 expresses three protein isoforms, named RCAN1.1L, RCAN1.1S and RCAN1.4
51 depending on alternative promoter using, transcripts splicing and translational
52 initiation. RCAN1.1L, hereafter referred as RCAN1, is the dominated isoform which
53 is specially expressed in the brain [3, 5]. RCAN1 with high levels in different brain
54 tissues was demonstrated to function in neural proliferation and differentiation during
55 brain development [6] and in the pathogenesis of Alzheimer's disease [7]. RCAN1
56 transgenic mice show reduced density of dendritic spines hippocampal pyramidal
57 neurons and impaired learning and short- and long-term memory [8]. Plenty of
58 evidence suggests that RCAN1 plays crucial roles in normal brain development and
59 function while the mechanism remains unresolved.

60 F-box and WD repeat domain containing 7 (FBXW7), one of the SCF type of E3
61 ubiquitin ligases, has been widely investigated in the process of tumorigenesis [9, 10].
62 FBXW7 gene expresses three different transcripts via distinct promoters and
63 subsequently encodes three protein isoforms FBXW7 α , FBXW7 β , and FBXW7 γ .
64 FBXW7 α constitutively exists in almost all tissues, while FBXW7 β is specially
65 expressed in the brain [11, 12]. Increasing evidence also shows FBXW7 is implicated
66 in several crucial neurodevelopmental processes, such as neurogenesis, myelination,
67 and cerebral vasculogenesis, and the pathogenesis of some neurodegenerative
68 disorders, such as Alzheimer's disease, Parkinson's disease and Huntington's disease
69 [13]. Dysregulations of FBXW7 is associated with defective brain development and
70 functions while the mechanism of altered FBXW7 expression in these processes is far
71 from resolved [13].

In this study, we report that RCAN1 inhibit FBXW7 expression. RCAN1 overexpression can lead to significant decrease of *FBXW7* β mRNA level in both cortex tissue and different cell lines. Altered calcineurin activity implemented via calcineurin activator and inhibitor can also affect *FBXW7* β mRNA level. Besides, a motif within RCAN1 responsible for interaction with calcineurin is also demonstrated to be essential for transcriptional inhibition of FBXW7 β by RCAN1. Meanwhile, we also show that RCAN1 and NFAT have opposite effect on the promoter activity of FBXW7 β . Taken together, our work indicates that RCAN1 regulates FBXW7 β transcription via calcineurin/NFAT pathway.

Material and methods

Animals

RCAN1 transgenic mice were constructed by insertion of human RCAN1 expression cassette into intergenic region between *Eif4enif1* and *Drg1* on Chromosome 11 in C57BL/6N background. RCAN1 expression cassette consists of *Thy1* promoter followed by RCAN1.1 coding sequence. Original F1 homozygotes were constructed and provided by Cyagen Biosciences (Suzhou) Incorporated and then bred in the Animal Research Unit (ARU) in Jining Medical University in specific pathogen free (SPF) conditions. Homozygotes at the age of 9 months were used in this study.

Cell Culture and Transfection

HEK293, SH-SY5Y and SRNL cells were cultured in high-glucose DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin. SRNL obtained from Dr. Weihong Song's lab refers to SY5Y cell stably expressing RCAN1.1L [5]. Transient transfections were performed by using PEI-based protocol [14]. The plasmids pRCAN1 and pNFAT used in transfection were obtained from Weihong Song's lab as described previously [5, 15].

Plasmids construction

The pRCAN1-Mut plasmid was constructed as follows. The RCAN1 CDS fragment with 631-654bp deletion was amplified by overlap PCR [16] using primers 5'-TATGGTACCATGGAGGACGGCGTGGCC-3'/5'-CTCCTTCTCTTGATCGCTGGGAGTGGTGTCAGTCGCT-3' and 5'-CACCACCTCCCAGCGATCAAGAGAAGGAGGAAGAAGAGGA-3'/5'-TATCTCGAGTCAGCTGAGGTGGATCGGCG-3' and subsequently cloned into pcDNA4 vector after digesting by KpnI and XhoI.

The P_{FBXW7β}-luc plasmid was constructed as follows. The *FBXW7β* promoter fragment was cloned using primers 5'-CTATGGTACCCATGGATCTACAGGACAGTACCCAC-3'/5'-ACCCAAGCTTG CAGCTTGACTGAGAAGAACTCGA-3', followed by cloning into pGL-basic vector after digesting by KpnI and HindIII.

RNA extraction and semi-quantitative PCR

Total RNA was extracted from cortex tissue and cells using Trizol reagent. cDNA was prepared with FastQuant RT Kit (TIANGEN). The primers used in quantitative PCR are 5'-GCTGCCGGTTCCGCTGCCTA-3'/5'-GCCAGCAACTTCTCTGGTCCG-3' and 5'-TTGTTGCCGGTTCTGCTCCCT-3' / 5'-TAGCCATTCCTGGAGGCCTGTAGG-3' for mouse *Fbxw7β* and human *FBXW7β* respectively. *ACTB* and *GAPDH* were used as endogenous reference and the primers were purchased from Sangon Biotech.

Immunoblotting

Cell or tissue lysates were extracted by RIPA buffer and resolved by 12% Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A mouse anti-CDC4 antibody (Santa Cruz, sc-293423) was used to detect FBXW7 expression. β-Actin antibody AC-15 which raised as control were obtained from ZSGB-BIO.

Luciferase assay

Cells were seeded in 24-well plats and grown to near 70% before transfection. Cells

were transfected with 1 ug plasmid DNA per well using PEI reagent. The Renilla luciferase vector (pRL-TK) purchased from Promega was cotransfected to normalize for the transfection efficiency. Cells were harvested at 48 hour after transfection and lysed in 50 μ L passive lysis buffer (Promega) per well. The dual activities of firefly luciferase and Renilla luciferase were detected using the Dual-luciferase reporter assay system (Promega). The relative luciferase activity was calculated by the ratio of firefly luciferase unit to Renilla luciferase unit.

Results

RCAN1 inhibits FBXW7 β expression

Deficits in learning and short- and long-term memory have been reported in RCAN1.1 transgenic mice while involvement of RCAN1 in normal brain development and function is far from resolved. To explore the functional implications and mechanism of RCAN1 in the brain, the RCAN1 transgenic mice were generated. Thy1 promoter drove the expression of human RCAN1.1 cassette specifically in the brain. Western blot shows a high level upregulation of RCAN1 in the cortex of male homozygotes of RCAN1 transgenic mice (TgRCAN1) (Fig. 1A). Interestingly, FBXW7 β protein level decreased in the cortex tissue of TgRCAN1 mice comparing with the wildtype. To verify the functional role of RCAN1 on FBXW7 expression, we also detect the FBXW7 level in SY5Y cell lines. Consistently, FBXW7 β protein level was also reduced in SY5Y cell line with stable overexpression of RCAN1.1 (SRNL) compared to the control (Fig. 1B). These evidence indicate that RCAN1 can regulate FBXW7 β expression.

RCAN1 overexpression downregulates *FBXW7 β* mRNA level

To explore the mechanism of regulatory effect of RCAN1 on FBXW7 β expression, mRNA level of *FBXW7 β* in both mice and cells was also detected. Semi-qPCR revealed that *FBXW7 β* transcript was significantly decreased about 15% in TgRCAN1 comparing with that in wildtype after normalization to the level *Gapdh* (Fig. 2).

Consistently, *FBXW7 β* mRNA level was also reduced around 40% in the SRNL cell line compared to the control of SY5Y (Fig. 2). These evidence indicates the potential transcriptional regulation of *FBXW7 β* by RCAN1.

RCAN1 regulates *FBXW7 β* mRNA level in a calcineurin-dependent pathway

RCAN1 commonly functions as an important endogenous inhibitor of calcineurin in different cellular processes [17]. Hence, it can be suspected whether calcineurin can also influence the mRNA level of *FBXW7 β* . To explore this, calcineurin activator and inhibitor were administered. It showed that Ionomycin, a calcium ionophore which can enhance calcineurin activity, significantly upregulated *FBXW7 β* mRNA level. In contrast, FK506, an inhibitor of calcineurin significantly decreased *FBXW7 β* mRNA level (Fig. 3A). These results showed that *FBXW7 β* mRNA level is also regulated by calcineurin.

Considering both RCAN1 and calcineurin are involved in the regulation of *FBXW7* mRNA level, it can be speculated that RCAN1 regulates *FBXW7 β* expression via inhibiting calcineurin activity. The 211-218th amino acid motif within RCAN1.1 has been demonstrated to be essential for interaction with calcineurin. Deletion of the sequence could deprive of the suppressive activity of RCAN1 on calcineurin [18]. To verify the functional involvement of calcineurin in the regulation of *FBXW7 β* expression by RCAN1, the expression vector contains the mutant RCAN1.1 cassette with deletion of 631-654bp sequence was constructed (pRCAN1-Mut). The plasmid was used to express mutant RCAN1 with 211-218th motif deletion. Consistent with the result in SRNL (Fig. 2), transient expression of full length RCAN1 (pRCAN1) can also decrease the *FBXW7 β* transcript in HEK293, while interestingly, overexpression of mutant RCAN1 cassette cannot downregulate mRNA level of *FBXW7 β* (Fig. 3B), indicating that RCAN1 regulates *FBXW7 β* expression in a calcineurin-dependent pathway.

NFAT increases promoter activity of *FBXW7 β*

Calcineurin stimulates dephosphorylation and translocation of NFAT and subsequently activate the transcription of a variety of target genes [19]. RCAN1 can attenuate NFAT signaling by inhibiting calcineurin activity [20, 21]. Transcription elements screening shows several consensus NFAT recognition sites, (A/T)GGAAA(N)(A/T/C), exist in the upstream region of the first exon of *FBXW7 β* transcript (Fig. 4A). The transcription start site is defined as +1 which has been reported previously [22]. Consistently, *FBXW7 β* mRNA was increased up to 2-fold in response to NFAT overexpression (Fig. 4B). Besides, to verify the functional role of NFAT and RCAN1 in the transcription of *FBXW7 β* , the potential promoter sequence from upstream of translation initiation site of *FBXW7 β* was cloned into the luciferase reporter vector. The dual luciferase assay showed that NFAT can significantly increase the promoter activity of *FBXW7 β* while to the contrary RCAN1 slightly decreased *FBXW7 β* promoter activity (Fig. 4C). These evidence indicate that RCAN1 regulates *FBXW7 β* transcription by inhibiting calcineurin/NFAT activity.

Discussion

RCAN1 is extensively reported to function in brain development. RCAN1 is up-regulated in DS by 1.9-fold in fetal brain and up to 3-fold within the adult hippocampus of DS patients [3, 4]. Aberrant RCAN1 level has been causally associated with defective morphology and function in DS brains. Mice brain with RCAN1 overexpression exhibited shrunken hippocampal volume and decreased number of DG neurons which all resembles the abnormalities in Ts65Dn mice, a DS model [23, 24]. Also, up-regulation of RCAN1 can lead to dendritic spine anomalies similar to those in DS model [25]. However, the mechanism of RCAN1 in brain development and function remains unclear.

In this study, we showed that RCAN1 regulated *FBXW7 β* expression. RCAN1 up-regulation can reduce *FBXW7* level (Fig. 1). Mounting evidence shows that *FBXW7* is functionally implicated in neurodevelopment. *FBXW7* regulates neuronal and glial differentiation via controlling the homeostasis and turnover of Notch and

c-Jun, both of which acts as substrates of FBXW7 [26-29]. FBXW7 deficiency can also lead to structural and functional abnormalities in mouse brain. Deletion of exon 5 of *Fbxw7* leads to substantial changes and morphological abnormalities in brain structure. Neurogenesis was found to be defective [26]. Besides, conditional inactivation of *Fbxw7* in the nervous system was also reported to cause impaired stem cell differentiation and anabolic progenitor cell death [27]. Similarly, conditional knockout of *Fbxw7* in the cerebellar anlage of mouse led to shrunken cerebellar volume and defects in axonal arborization [28]. Moreover, specific knock-out β isoform of *Fbxw7* impairs the neuron survival in response to oxidative stress indicating its crucial effect on neurological function. As RCAN1 overexpression was demonstrated to downregulate FBXW7 β , considering the similar effect of RCAN1 overexpression and FBXW7 deficiency on brain function, it indicates that FBXW7 β may function downstream RCAN1 in some neurological processes.

We also found that RCAN1 inhibits FBXW7 β expression by regulating *FBXW7 β* transcription. The mRNA level of *FBXW7 β* was significantly reduced by RCAN1 overexpression (Fig. 2). Moreover, it also indicates that RCAN1 regulates *FBXW7 β* transcription in a calcineurin-dependent pathway (Fig. 3). Acting as one of the crucial regulators of calcineurin, RCAN1 plays a complex role in regulating calcineurin signaling, depending on both the dosage of RCAN1 and the intracellular levels of angiotensin II (AngII), platelet-derived growth factor (PDGF) and isoproterenol (ISO) [30]. Calcineurin controls transcription of numerous genes via the transcription factor nuclear activator of T-cells (NFAT) [31, 32]. NFAT proteins are primarily inactivated in the cytoplasm until the calcium-dependent signal stimulates the dephosphorylation of NFATs by calcineurin [33]. The dephosphorylated NFATs further activate the transcription of target genes such as *TMP21* [15] and *RCAN1.4* [34]. In this work, we also screened out four potential NFAT binding sites within the promoter region of *FBXW7 β* (Fig. 4). Overexpression of NFAT can also significantly upregulate mRNA level and promoter activity of *FBXW7 β* . Consistently, RCAN1 exhibits an inhibitory effect on the promoter activity of *FBXW7 β* opposite to the role of NFAT (Fig. 4).

In conclusion, our results showed that RCAN1 can regulate FBXW7 β expression by

inhibiting *FBXW7* transcription via calcineurin/NFAT signaling pathway (Fig. 5). It advances our understanding of FBXW7 regulation and provides a potential mechanism that RCAN1 may promote impaired brain function by inhibiting FBXW7 expression.

Abbreviations

RCAN1, Regulator of calcineurin 1; DSCR1, Down syndrome critical region protein 1; FBXW7, F-box and WD repeat domain containing 7; AngII, angiotensin II; PDGF, platelet-derived growth factor; ISO, isoproterenol;

Declarations

Ethics approval and consent to participate

All procedures were compliant with the approved protocol from the Animal Ethics Committee of Jining Medical University and the Chinese animal welfare act and the “Chinese code of practice and use of animals for scientific purposes. There is no data collected from human subjects in this study.

Availability of data and materials

The data of this study are available from the corresponding author upon reasonable request.

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Authors' contributions

S.W. and Y.W. designed the experiments; S.W., Y.Y., R.S. and Y.G. performed the experiments and analyzed the data; S.W. and Y.W. wrote and revised the manuscript.

Competing interests

The authors declared no conflict of interest.

Consent for publication

Not applicable.

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Figure legends

Fig. 1 RCAN1 inhibits FBXW7 β expression. Western blot shows the relative abundance of FBXW7 β protein in mice cortex tissues (A) and cell lines (B). Quantifications are performed by Student's t test. Values are mean $\pm$ SEM; n $\geq$ 3.

Fig. 2 RCAN1 reduces *FBXW7 β* mRNA level. Semi-qPCR reveals RCAN1 overexpression in mice brain (TgRCAN1) and SY5Y cell line (SRNL) downregulate the mRNA level of *FBXW7 β* . Quantifications are performed by Student's t test. Values are mean $\pm$ SEM; n $\geq$ 3.

Fig. 3 RCAN1 regulates *FBXW7 β* expression via calcineurin dependent pathway. (A) The relative abundance of *FBXW7 β* mRNA was detected by semi-qPCR in SY5Y cell line treated with Ionomycin (10 μ M) and FK506 (1 μ M) for 6h and 24h respectively. DMSO was administered as the vehicle control. Quantifications are performed by Student's t test. Values are mean $\pm$ SEM; n $\geq$ 3. (B) HEK293 cells were transfected with pRCAN1 and pRCAN1-Mut respectively for 48h before harvesting. Semi-qPCR and western blot are performed to detect the relative abundance of *FBXW7* mRNA and RCAN1 protein respectively. Quantifications are performed by One-way Anova. Values are mean $\pm$ SEM; n $\geq$ 3.

Fig. 4 NFAT regulate *FBXW7 β* transcription. (A) Sequence features of the human *FBXW7 β* gene promoter. The transcription start site is defined as +1. The potential NFAT binding sites are indicated in bold. The underlined sequences indicate the primers used for clone of *FBXW7 β* promoter. Transcription initiation codon (ATG) is marked with a box. (B) SY5Y cells were transfected with pNFAT1 for 48h before harvesting. Semi-qPCR is performed to detect the relative abundance of *FBXW7* mRNA. Quantifications are performed by Student's t test. Values are mean $\pm$ SEM; n $\geq$ 3. (C) Dual luciferase assay revealed the functional effect of RCAN1 and NAFT

on the *FBXW7 β* promoter activity. Quantifications are performed by One-way Anova.
Values are mean $\pm$ SEM; n $\geq$ 3.

Fig. 5 The schematic illustration of regulatory effect of RCAN1 on *FBXW7 β* expression. NFAT activate the transcription of *FBXW7 β* , while RCAN1 possibly inhibits *FBXW7 β* transcription by blocking NFAT dephosphorylation and translocation in a calcineurin dependent pathway. P within the red circle represents the phosphorylation of NFAT. The arrowhead stands for positive regulation, while T-shaped end stands for inhibition. Dotted line stands for the translocation of NFAT from cytoplasm to nucleus.

Figures

Figure1

Figure 1

RCAN1 inhibits FBXW7β expression. Western blot shows the relative abundance of FBXW7β protein in mice cortex tissues (A) and cell lines (B). Quantifications are performed by Student's t test. Values are mean ± SEM; n ≥ 3.

Figure2

Figure 2

RCAN1 reduces FBXW7 β mRNA level. Semi-qPCR reveals RCAN1 overexpression in mice brain (TgRCAN1) and SY5Y cell line (SRNL) downregulate the mRNA level of FBXW7 β . Quantifications are performed by Student's t test. Values are mean $\pm$ SEM; $n \geq 3$.

Figure3

Figure 3

RCAN1 regulates FBXW7 β expression via calcineurin dependent pathway. (A) The relative abundance of FBXW7 β mRNA was detected by semi-qPCR in SY5Y cell line treated with Ionomycin (10 μ M) and FK506 (1 μ M) for 6h and 24h respectively. DMSO was administered as the vehicle control. Quantifications are performed by Student's t test. Values are mean $\pm$ SEM; $n \geq 3$. (B) HEK293 cells were transfected with pRCAN1 and pRCAN1-Mut respectively for 48h before harvesting. Semi-qPCR and western blot are performed to detect the relative abundance of FBXW7 mRNA and RCAN1 protein respectively. Quantifications are performed by One-way Anova. Values are mean $\pm$ SEM; $n \geq 3$.

Figure4

Figure 4

NFAT regulate FBXW7β transcription. (A) Sequence features of the human FBXW7β gene promoter. The transcription start site is defined as +1. The potential NFAT binding sites are indicated in bold. The underlined sequences indicate the primers used for clone of FBXW7β promoter. Transcription initiation codon (ATG) is marked with a box. (B) SY5Y cells were transfected with pNFAT1 for 48h before harvesting. Semi-qPCR is performed to detect the relative abundance of FBXW7 mRNA. Quantifications are performed by Student's t test. Values are mean ± SEM; n ≥ 3. (C) Dual luciferase assay revealed the functional effect of RCAN1 and NFAT on the FBXW7β promoter activity. Quantifications are performed by One-way Anova. Values are mean ± SEM; n ≥ 3.

Figure5

Figure 5

The schematic illustration of regulatory effect of RCAN1 on FBXW7 β expression. NFAT activate the transcription of FBXW7 β , while RCAN1 possibly inhibits FBXW7 β transcription by blocking NFAT dephosphorylation and translocation in a calcineurin dependent pathway. P within the red circle represents the phosphorylation of NFAT. The arrowhead stands for positive regulation, while T-shaped end stands for inhibition. Dotted line stands for the translocation of NFAT from cytoplasm to nucleus.