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Article

Dendritic cell nuclear protein-1 regulates melatonin biosynthesis by binding to BMAL1 and inhibiting the transcription of N-acetyltransferase in C6 cells

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Abstract

Dendritic cell nuclear protein-1 (DCNP1) is a protein associated with major depression. In the brains of depression patients, DCNP1 is up-regulated. However, how DCNP1 participates in the pathogenesis of major depression remains unknown. In this study, we first transfected HEK293 cells with EGFP-DCNP1 and demonstrated that the full-length DCNP1 protein was localized in the nucleus, and RRK (the residues 117-119) composed its nuclear localization signal (NLS). An RRK-deletion form of DCNP1 (DCNP1$^{\Delta RRK}$) and truncated form (DCNP1$^{1-116}$), each lacking the RRK residues, did not show the specific nuclear localization like full-length DCNP1 in the cells. A rat glioma cell line C6 can synthesize melatonin, a hormone that plays important roles in both sleep and depression. We then revealed that transfection of C6 cells with full-length DCNP1 but not DCNP1$^{\Delta RRK}$ or DCNP1$^{1-116}$ significantly decreased the levels of melatonin. Furthermore, overexpression of full-length DCNP1, but not DCNP1$^{\Delta RRK}$ or DCNP1$^{1-116}$, in C6 cells significantly decreased both the mRNA and protein levels of N-acetyltransferase (NAT), a key enzyme in melatonin synthesis. Full-length DCNP1 but not DCNP1$^{\Delta RRK}$ or DCNP1$^{1-116}$ was detected to interact with the *Nat* promoter and inhibited its activity through its E-box motif. Furthermore, full-length DCNP1 but not the mutants interacted with and repressed the transcriptional activity of BMAL1, a transcription factor that transactivates *Nat* through the E-box motif. In conclusion, we have shown that RRK (the residues 117-119) are the NLS responsible for DCNP1 nuclear localization. Nuclear DCNP1 represses NAT expression and melatonin biosynthesis by interacting with BMAL1 and repressing its transcriptional activity. Our study reveals a connection between the major depression candidate protein DCNP1, circadian system and melatonin biosynthesis, which may contribute to the pathogenesis of depression.

Keywords: major depression; DCNP1; N-acetyltransferase; melatonin; circadian system; BMAL1; rat glioma cell line C6

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Introduction

Major depression is a mental disorder that is characterized by a continuous low mood, low self-esteem, insomnia, loss of interest in normally enjoyable activities, and suicide[1, 2]. The circadian system, which controls the sleep-wake cycles, hormonal secretion patterns and mood fluctuations, plays important roles in the pathogenesis and symptoms of depression[3, 4]. Polymorphisms in several core clock genes, such as *PER2*, *BMAL1*, *CLOCK* and *NPAS2*, whose products are necessary components for the generation and regulation of circadian rhythm, appear to be associated with susceptibility to depression[5-7]. However, the molecular mechanisms linking major depression and circadian rhythm are poorly understood.

Melatonin plays important roles in many physiological and pathological conditions, such as circadian rhythm and depression, which may explain the link between them[8-10]. Clinically, 90% of individuals with major depression suffer from insomnia with an abnormal melatonin pathway function[11-14]. Many studies have demonstrated that melatonin decreases with a decreased or phase-shifted peak at night are found in patients with depressive disorders[15-19]. The biosynthetic pathway

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of pineal melatonin has been thoroughly studied and is controlled by the circadian system[9, 10]. It is initially synthesized from L-tryptophan, which is first converted to 5-hydroxy-L-tryptophan (5-HTP) by tryptophan hydroxylase. 5-HTP is then decarboxylated by 5-hydroxytryptophan decarboxylase to produce serotonin[20, 21]. In the darkness, the key enzyme N-acetyltransferase (NAT) is activated and then converts serotonin to N-acetyl-5-hydroxytryptamine, which is subsequently converted to melatonin by hydroxyindole-o-methyltransferase (HIOMT)[22, 23]. NAT is the rate-limiting enzyme in the synthesis of melatonin from L-tryptophan. The *Nat* gene contains an E-box element in its promoter, which is the site of BMAL1/CLOCK heterodimer binding and transactivation[24, 25].

BMAL1 is a gene that encodes a bHLH-PAS domain transcription factor[26]. It plays a key role in generating circadian rhythms[27]. *BMAL1* knock-out mice show a complete loss of circadian rhythm and alterations of locomotion and other behaviors[27]. Furthermore, individuals harboring polymorphisms in *BMAL1* are susceptible to depression[7, 28], implying a possible association between BMAL1 and this disorder. BMAL1 and CLOCK form heterodimers and bind to E-box components in the promoters of *PER*, *CRY*, *REV-ERB* and other clock-controlled genes (CCGs), such as *Nat* to drive their transcription[8, 29].

Dendritic cell nuclear protein-1 (DCNP1) was first discovered in mature or immature dendritic cells, and in humans, DCNP1 is mainly expressed in the brain and skeletal muscle[30]. Recent studies have shown that DCNP1 is closely associated with major depression[31, 32]. The *DCNP1* mRNA levels are dramatically increased in the paraventricular nucleus (PVN) of depressed patients compared with control subjects[33]. Furthermore, a truncated form of DCNP1 ($DCNP1^{1\text{-}116}$) encoded by the T allele of DCNP1 has been reported to lead to an increased risk of major depression[31, 32]. These results indicate that increased DCNP1 expression or DCNP1 mutation-induced dysfunction possibly participate in the pathogenesis of depression. However, the function of DCNP1 as well as its role in depression are largely unknown.

In the present study, we identified a nuclear localization signal (NLS) (RRK, amino acids 117-119) in DCNP1 and demonstrated that full-length DCNP1, but not the truncated form of DCNP1 ($DCNP1^{1\text{-}116}$) or an NLS deletion mutant ($DCNP1^{\Delta RRK}$), represses NAT expression to down-regulate melatonin levels. Full-length DCNP1 binds to and represses BMAL1, a transcription factor that transactivates NAT expression.

Materials and methods

Plasmid constructs

Full-length *DCNP1* cDNA was amplified by PCR using the primers 5′-GAGTCGACACTATGCATTACGGAGCA-3′ and 5′-TTGGATCCAACTCAGGCACGTGGGCTG-3′ with human fetal brain cDNA library as the template (Clontech). The PCR product was then inserted into the pEGFP-N1 (Clontech) vector via its *Sal* I/*Bam*H I sites. pEGFP-$DCNP1^{1\text{-}116}$ and pEGFP-$DCNP1^{\Delta RRK}$ were generated with the primer pairs 5′-GAGTCGACACTATGCATTACGGAGCA-3′ and 5′-CTGGATCCTTGCTGCTATGCAGTTC-3′ or 5′-GCTGCTATGCAGTTCATCCTG-3′ and 5′-ACAGGCCAGACCAGGCGGGAG-3′, respectively, using pEGFP-DCNP1 as the template. Rat *Nat* promoter DNA (-2276 bp to +0 bp) was obtained using PCR with the primers 5′-CCGCTCGAGGAGACCTTCCTGTTCTCCTGGTAC-3′ and 5′-CCCAAGCTTGGGTATCTGGCCACTGACCCCTCC-3′ and C6 cell genomic DNA as the template; the PCR products were then inserted into the pGL3-Basic vector (Promega) via its *Xho* I/*Hin*d III sites. pGL3-$Nat^{\Delta E\text{-box}}$, lacking nucleotides -1192 to -1137, was generated by PCR with the primers 5′-AGAGGACTGCTGTGGGAGTCTGTTCTCTC-3′ and 5′-TTAAATGGGCGATGGCGATGGCTCAAGCAC-3′ using pGL3-*Nat* as the template. The HA-BMAL1 and HA-CLOCK plasmids were kind gifts from Dr Ying XU (Soochow University, China).

Cell culture and transfection

HEK293 cells or C6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) containing 10% fetal bovine serum (Life Technologies). Cells were transfected with expression plasmids using the Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were observed using an inverted system microscope IX71 (Olympus), or harvested for immunoblot or immunoprecipitation analyses.

Immunoblot analysis

Proteins were separated by 12% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Millipore). The following monoclonal primary antibodies were used: anti-BMAL1 (Santa Cruz Biotech), anti-GAPDH (Millipore), anti-GFP (Santa Cruz Biotech), and anti-NAT (Sigma). The secondary sheep anti-mouse IgG-HRP antibody was from Jackson ImmunoResearch. Proteins were visualized using an ECL detection kit (Thermo Fisher Scientific).

To re-probe membranes with another primary antibody, each membrane was first incubated with stripping buffer (50 mmol/L Tris-HCl, pH 6.8, 0.1 mmol/L β-Mercaptoethanol, 20 mmol/L SDS) for 30 min at 50 °C. Then the stripped membrane was subjected to immunoblot analysis following the standard protocol.

Nuclear and cytoplasmic fractionation assay

HEK293 cells transfected with EGFP, EGFP-DCNP1, EGFP-$DCNP1^{1\text{-}116}$ or EGFP-$DCNP1^{\Delta RRK}$ were lysed in fractionation buffer (320 mmol/L sucrose, 3 mmol/L $CaCl_2$, 2 mmol/L MgAc, 0.1 mmol/L EDTA, 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 0.5% NP-40) for 20 min on ice. After centrifugation at 600×*g* for 15 min at 4 °C, the supernatant was collected as the cytoplasmic fraction. The pellet was washed once with fractionation buffer without NP-40 and lysed in cell lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl pH 7.5, 0.5% deoxycholate, 1% NP-40 and protease inhibitor cocktail (Roche)) as the nuclear fraction. Nuclear poly (ADP-ribose) polymerase (PARP) was used as

a nuclear marker, and GAPDH was used as a cytoplasmic marker.

Immunoprecipitation

HEK293 cells co-transfected with HA-BMAL1 along with EGFP, EGFP-DCNP1, EGFP-DCNP1$^{1\text{-}116}$ or EGFP-DCNP1$^{\Delta RRK}$ were harvested 48 h after transfection and sonicated in TSPI buffer containing 50 mmol/L Tris-HCl pH 7.5, 150 mmol/L sodium chloride, 1 mmol/L EDTA, and 1% NP-40 supplemented with cOmplete Mini Protease Inhibitor Cocktail (Roche). Cell lysates were centrifuged at 12 000×*g* for 30 min at 4 °C to remove cellular debris. Supernatants were incubated with a monoclonal anti-GFP antibody (Roche) for 4 h at 4 °C. After incubation, protein G Sepharose beads were used for precipitation. Beads were washed with TSPI buffer six times and then eluted with SDS sample buffer for immunoblot analysis.

RNA isolation, reverse transcription, and real-time quantitative PCR

Total RNA was extracted from C6 cells by Trizol reagent (Life Technologies) as previously described[33] and then reverse-transcribed into cDNA with a TransScript First-Stand cDNA Synthesis Kit (Takara). Real-time quantitative PCR (qRT-PCR) was performed using SYBR Green Master Mix (Applied Biosystems) on a 7500 Real-Time PCR System (Applied Biosystems). The primer pairs were as follows: 5′-TGCTGTGGCGATACCTTCACCA-3′ and 5′-CAGCTCAGTGAAGGTGAGAGAT-3′ for rat *Nat*; 5′-TACGGGGACAGGAAGTTTTG-3′ and 5′-GTGCCACTTCTGGGTTCATT-3′ for rat *Hiomt*; 5′-GCCAGGAACGGAAATTTGTA-3′ and 5′-TCTCAGGTGGAAGCTCTGGT-3′ for rat *Maoa*; 5′-TGGGAAGATTCCAGAGGATG-3′ and 5′-GCTGACAAGATGGTGGTCAA-3′ for rat *Maob*; 5′-TTGCTGACAGGATGCAGAA-3′ and 5′-ACCAATCCACACAGAGTACTT-3′ for rat *β-actin*. The relative target gene mRNA levels were determined using the $2^{-\Delta\Delta CT}$ method.

Chromatin immunoprecipitation and immunoblot analysis

Formaldehyde cross-linking and chromatin immunoprecipitation (ChIP) were performed as previously described[33]. Immunoprecipitation assays were performed with anti-GFP antibodies (Roche) and protein G Sepharose beads (Roche). Immunoprecipitates obtained by ChIP were subjected to PCR with the primers 5′-GGAGACCTTCCTGTTCTCCTG-3′ and 5′-TAGGGAGAGCATGGGCTAAG-3′. The amplification conditions were as follows: 5 min at 95 °C; then 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s.

Dual-luciferase reporter gene assay

HEK293 cells were transfected with a *Nat*- or *Nat*$^{\Delta E\text{-box}}$-luciferase reporter construct along with the EGFP, EGFP-DCNP1, EGFP-DCNP1$^{1\text{-}116}$ or EGFP-DCNP1$^{\Delta RRK}$ plasmids or siRNAs against BMAL1, as indicated. The renilla luciferase expression plasmid pRL-CMV was co-transfected to normalize for variations in the transfection efficiency. Cells were lysed with passive lysis buffer (Promega). The activities of both firefly and Renilla luciferase were measured with a Dual Luciferase Reporter Assay System (Promega) using a Veritas Microplate Luminometer according to the manufacturer's instructions. The absolute values of firefly luminescence were normalized to those of Renilla, and the ratios are presented as the median of three transfection experiments. The activity of pGL3-Basic luciferase was normalized to 1.

Immunocytochemistry

HEK293 cells were washed with PBS and fixed with 4% paraformaldehyde for 5 min at room temperature. Then, cells were treated with 0.25% Triton X-100 for 15 min and blocked with 4% FBS in PBS overnight at 4 °C with anti-BMAL1 antibodies (Santa Cruz). Next, cells were incubated with Alexa Fluor 594 donkey anti-mouse secondary antibodies (Life Technologies). Nuclei were stained with DAPI (Sigma).

Melatonin determination

The melatonin concentrations in the culture medium of C6 cells expressing EGFP, EGFP-DCNP1, EGFP-DCNP1$^{1\text{-}116}$ or EGFP-DCNP1$^{\Delta RRK}$ were measured by ELISA (IBL International) according to the manufacturer's instructions.

RNA interference

Double-stranded oligonucleotides targeting 5′-TAGGCACATCGTGTTATGAAT-3′ of human *BMAL1* mRNA were synthesized by Shanghai GenePharma (Shanghai, China), and an irrelevant oligonucleotide was used as the negative control. Transfection was performed with Lipofectamine RNAiMAX reagent (Life Technologies) according to manufacturer's instructions. Briefly, siRNA and Lipofectamine RNAiMAX reagent (Life Technologies) were mixed in Opti-MEM medium (Invitrogen), incubated for 30 min at room temperature to allow for complex formation, and then added to culture medium. Cells were harvested 72 h after transfection for further analyses.

Statistical analysis

Statistical comparisons between groups and treatments were performed using one-way or two-way analysis of variance (ANOVA) for comparisons among multiple groups, followed by Student's *t*-tests for analyzing significance, as indicated. *P* values <0.05 were considered statistically significant. Data are presented as the mean±SEM.

Results

Identification of the DCNP1 NLS

Our previous study showed that endogenous DCNP1 was probably localized in the nucleus[33]. To confirm the subcellular distribution of DCNP1, we transfected EGFP or EGFP-DCNP1 into HEK293 cells and examined their subcellular localization. Unlike EGFP, which was diffusely distributed throughout whole cells, EGFP-DCNP1 was predominately localized in the nucleus (Figure 1A). By examining the DCNP1 protein sequence, we found that it has a potential NLS (RRK)

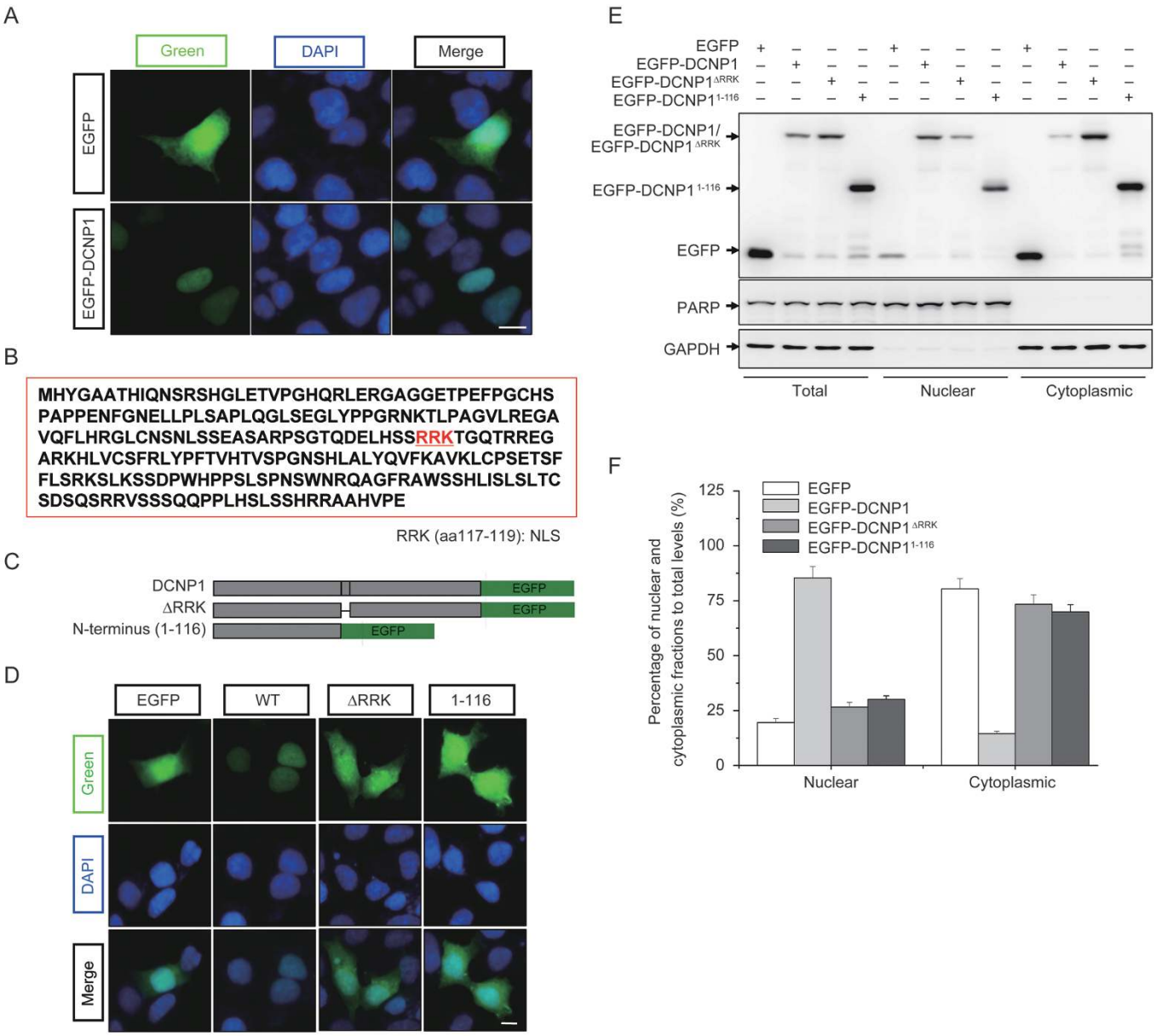

Figure 1. Nuclear localization of DCNP1. (A) Subcellular localization of EGFP-DCNP1 (green). HEK293 cells were transfected with EGFP or EGFP-DCNP1 and visualized using a fluorescence microscope (magnification ×400). Nuclei were stained with DAPI (1 μg/mL) (blue). Scale bars, 5 μm. (B) Schematic illustration of the predicted NLS (aa 117-119) of DCNP1. (C) Schematic illustration of EGFP-tagged expression constructs. (D) RRK is a functional NLS of DCNP1. HEK293 cells transfected with EGFP-DCNP1 or its mutants (1-116 and ΔRRK) were visualized using a fluorescence microscope (magnification ×400). Nuclei were stained with DAPI (1 μg/mL) (blue). Scale bars, 5 μm. (E) HEK293 cells were transfected with EGFP, EGFP-DCNP1 or its mutants (1-116 and ΔRRK). Forty-eight hours later, cells were subjected to nuclear and cytoplasmic fractionation. (F) Percentages of EGFP, EGFP-DCNP1 or its mutants (1-116 and ΔRRK) in nuclear and cytoplasmic fractions relative to their total protein levels in (E) were quantified.

at amino acid positions 117-119 (Figure 1B). We therefore created two deletion mutants of DCNP1, DCNP1$^{\Delta RRK}$, in which the putative NLS (RRK, amino acids 117-119) was deleted, and DCNP1$^{1-116}$, a truncated form of DCNP1 lacking amino acids 117-224, including the RRK motif, which has been reported to increase the risk of major depression[31, 32] (Figure 1C). Full-length DCNP1 showed specific nuclear localization. However, like EGFP, both EGFP-DCNP1$^{\Delta RRK}$ and EGFP-DCNP1$^{1-116}$ showed a diffuse distribution with no clear nuclear pattern (Figure 1D). We also performed nuclear and cytoplasmic fractionation assays to further verify differences in the distribution patterns between full-length DCNP1 and its mutants. As shown in Figure 1E and 1F, full-length DCNP1 was mainly present in the nuclear fraction. However, although EGFP-DCNP1$^{\Delta RRK}$ and EGFP-DCNP1$^{1-116}$ were partially detected in the nuclear fraction, a large proportion of each protein was distributed in the cytoplasm (Figure 1E and 1F). These results suggest that the amino acids RRK at positions 117-119 are

essential for DCNP1 nuclear localization.

Regulation of melatonin levels and *Nat* mRNA levels by full-length DCNP1

As a significant clinical feature of major depression is insomnia, with low levels of melatonin in patients[11-14, 34], we investigated whether DCNP1 or it mutants regulate melatonin production. To address this issue, we transfected EGFP, EGFP-DCNP1, EGFP-DCNP1$^{1\text{-}116}$ or EGFP-DCNP1$^{\Delta RRK}$ into C6 cells, a rat glioma cell line that can synthesize melatonin[35]. Overexpression of EGFP-DCNP1 significantly decreased the melatonin levels, whereas its mutants (1-116, ΔRRK) did not (Figure 2A). To identify which factors are involved in the regulation of melatonin by DCNP1, we examined the expression of two key enzymes in the melatonin synthesis pathway, HIOMT and NAT, and two other key enzymes, monoamine oxidase A and B (MAOA and MAOB), for 5-HT metabolism in the bypass of melatonin synthesis. Significantly decreased levels of *Nat* mRNA were observed in EGFP-DCNP1-transfected C6 cells, but the *Hiomt*, *Maoa* and *Maob* mRNA levels were not changed (Figure 2B). We next examined whether DCNP1 mutants affect NAT expression. In C6 cells overexpressing EGFP, EGFP-DCNP1, EGFP-DCNP1$^{1\text{-}116}$ or EGFP-DCNP1$^{\Delta RRK}$, EGFP-DCNP1 repressed the *Nat* mRNA and protein levels, while EGFP-DCNP1$^{1\text{-}116}$ and EGFP-DCNP1$^{\Delta RRK}$ failed to repress *Nat* mRNA or protein expression (Figure 3A and 3B). These data suggest that full-length DCNP1, unlike its mutants, regulates melatonin synthesis by repressing NAT transcription.

Interactions between DCNP1 and the *Nat* promoter or BMAL1

As full-length DCNP1 localizes to the nucleus and regulates *Nat* mRNA expression, we investigated whether DCNP1 interacts with the *Nat* promoter. We performed a chromatin immunoprecipitation assay to verify this interaction. In cells overexpressing wild-type or mutant DCNP1, the *Nat* promoter co-immunoprecipitated when EGFP-DCNP1, but not its mutants (1-116, ΔRRK), was precipitated using anti-GFP antibodies (Figure 4A), suggesting that DCNP1 interacts with the *Nat* promoter and that this interaction is dependent on DCNP1's nuclear localization. Considering that BMAL1 binds to the E-box in the *Nat* promoter to regulate its transcription[24, 25], we next examined whether regulation of NAT by DCNP1 is mediated by BMAL1. We first tested whether DCNP1 could bind to BMAL1 to regulate the transcription of *Nat*. In HEK293 cells expressing HA-BMAL1 with EGFP or EGFP-DCNP1 or EGFP-DCNP1 mutants (1-116, ΔRRK), HA-BMAL1 co-precipitated with EGFP-DCNP1 using anti-GFP antibodies, but not with EGFP alone or EGFP-DCNP1 mutants (Figure 4B). These immunocytochemical assays also showed that both EGFP-DCNP1 and BMAL1 were nuclear-localized, whereas DCNP1 mutant proteins were diffusely distributed throughout whole cells (Figure 4C).

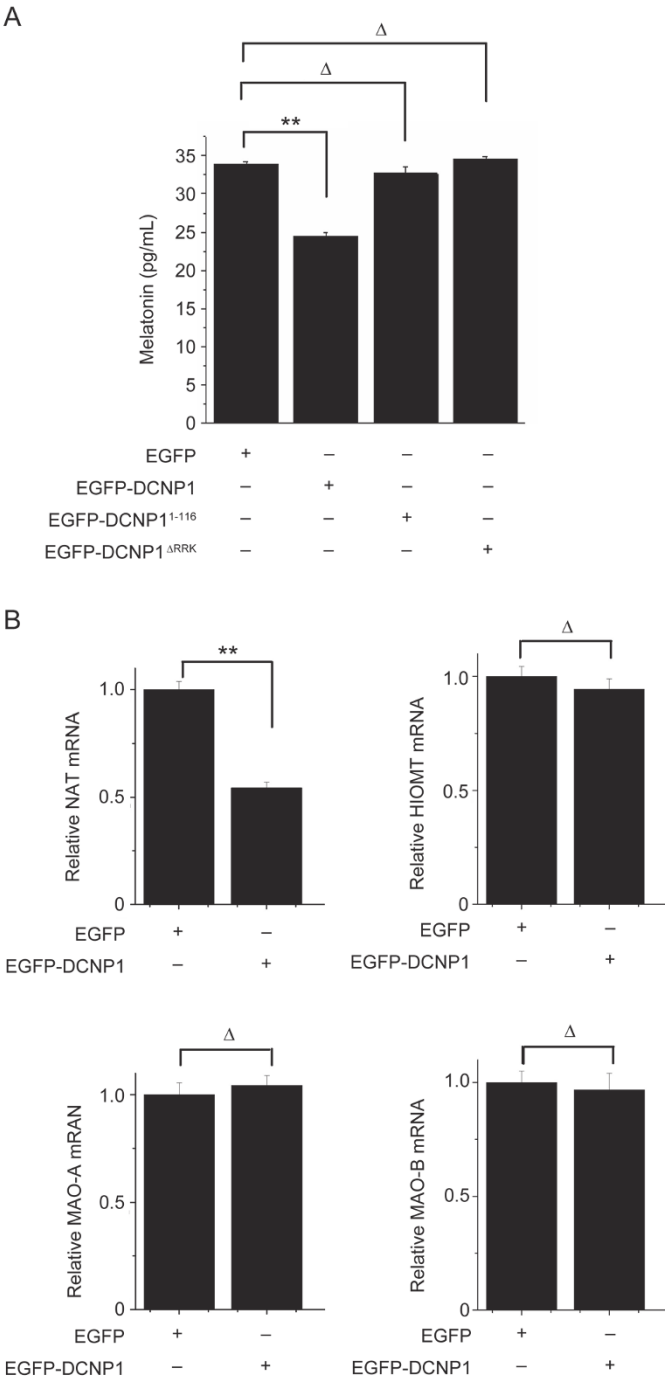

Figure 2. DCNP1 regulates melatonin and *Nat* mRNA levels. (A) Melatonin levels in culture medium of C6 cells transfected with EGFP-DCNP1 or its mutants (1-116 and ΔRRK) were measured by ELISA. The values are the mean±SEM from three independent experiments. $^{**}P<0.01$, $^{\Delta}P>0.05$, one-way ANOVA, followed by the *t*-test. (B) qRT-PCR assays were performed to measure the mRNA levels of *Nat*, *Hiomt*, *Maoa* and *Maob* in C6 cells expressing EGFP or EGFP-DCNP1. The values are the mean±SEM of three independent experiments. $^{**}P<0.01$, $^{\Delta}P>0.05$, *t*-test.

DCNP1 regulates the activity of the *Nat* promoter through BMAL1

As DCNP1 interacts with BMAL1 and suppresses the transcription of *Nat*, we performed a luciferase reporter gene assay to further examine whether DCNP1 regulates *Nat* promoter activity and to determine whether this regulation is BMAL1-dependent. We transfected HEK293 cells with a *Nat*-luciferase

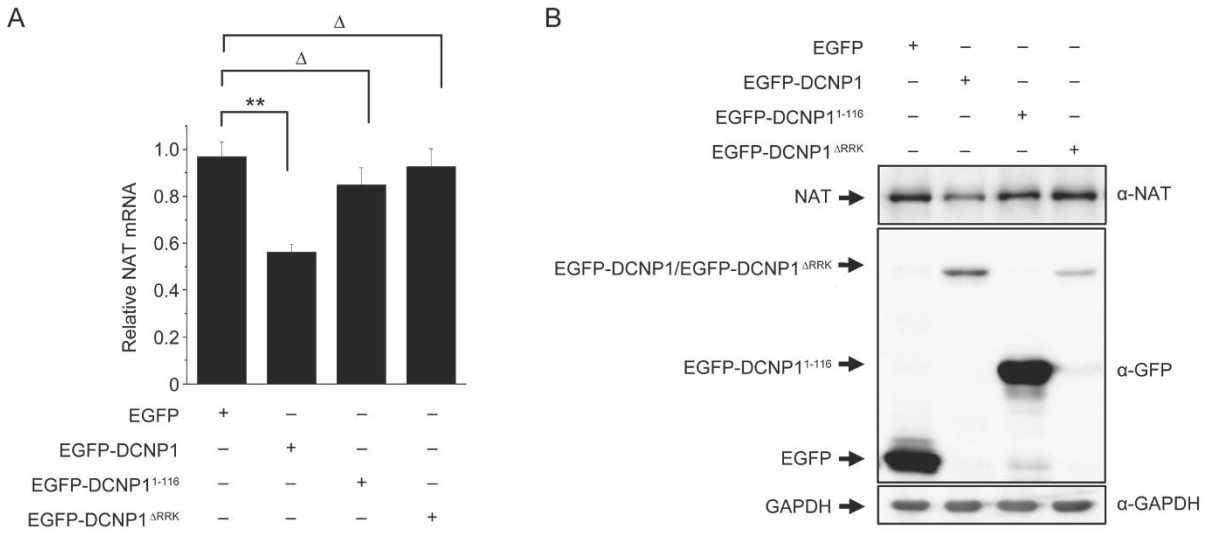

Figure 3. Nuclear DCNP1 regulates *Nat* mRNA and protein levels. qRT-PCR analysis (A) and immunoblot analysis (B) showing the *Nat* mRNA and protein levels in C6 cells expressing EGFP-DCNP1 or its mutants (1-116 and ΔRRK). Antibodies were used as indicated. The values are the mean±SEM of three independent experiments. $^{**}P<0.01$, $^{\Delta}P>0.05$, one-way ANOVA.

construct along with expression plasmids for EGFP, EGFP-DCNP1, and EGFP-DCNP1 mutants (1-116, ΔRRK); HA; or HA-BMAL1/HA-CLOCK. Cells were harvested 48 h after transfection, and the supernatants were used for luciferase activity assays. Overexpression of HA-BMAL/HA-CLOCK heterodimer significantly increased *Nat* promoter activity. EGFP-DCNP1 significantly inhibited the basal *Nat* promoter activity as well as BMAL/CLOCK-induced *Nat* promoter activity (Figure 5A). As BMAL1/CLOCK binds to the E-box in the *Nat* promoter to transactivate *Nat* transcription, we deleted the E-box in the *Nat*-luciferase plasmid (pGL3-*Nat*$^{\Delta E\text{-box}}$ promoter) and used it for co-transfection with the EGFP or EGFP-DCNP1 expression plasmids. Although DCNP1 repressed wild-type *Nat* promoter activity, it failed to influence *Nat* promoter activity when the E-box motif was deleted (Figure 5B), further suggesting that DCNP1 regulation of *Nat* transcription is dependent on BMAL1. To further investigate the dependence of DCNP1-regulated NAT expression on BMAL1, we examined the effects of DCNP1 on *Nat*-luciferase reporter activity in BMAL1-knockdown cells. The knockdown efficiency of *Bmal1* siRNA is shown in Figure 5C. Full-length DCNP1 repressed *Nat* reporter activity in the presence of BMAL1, while it did not affect *Nat* reporter activity when BMAL1 had been knocked down (Figure 5D). We also found that mutants of DCNP1 (1-116, ΔRRK) did not influence *Nat* promoter activity (Figure 5D). These results indicate that inhibition of *Nat* transcription by DCNP1 is dependent on BMAL1. Together, these data suggest that the regulation of melatonin biosynthesis by DCNP1 is dependent on its binding to BMAL1 to repress NAT expression.

In conclusion, we found that the amino acids RRK at residues 117-119 are responsible for nuclear localization of full-length DCNP1. Nuclear-localized DCNP1 binds to BMAL1 to repress its transcriptional activity, thus inhibiting *Nat* transcription and melatonin biosynthesis. However, the NLS-deletion mutants DCNP1$^{\Delta RRK}$ and truncated DCNP1$^{1\text{-}116}$ lack specific nuclear localization and fail to interact with BMAL1 to inhibit BMAL1/NAT-mediated melatonin biosynthesis (Figure 6). Thus, our results suggest that the involvement of nuclear DCNP1 in the pathogenesis of depression may depend on its regulation of the circadian clock and melatonin biosynthesis.

Discussion

In the present study, we determined that the product of *DCNP1*, a candidate gene for major depression, plays an important role in melatonin biosynthesis by regulating NAT expression via BMAL1.

Melatonin is mainly produced in the pineal gland and is also produced by various other tissues and cells[36], such as astrocytes[37], macrophages[38], fibroblasts and skin cells[39]. Melatonin is involved in many biological processes, including sleep-wake timing and blood pressure regulation, mainly through activation of the melatonin receptors MT1 and MT2, which are expressed in many tissues and cells[36, 40]. Melatonin is also closely associated with depression as its levels are dramatically decreased in depression patients and is considered to be a significant marker of depression[15-19]. In addition, a melatonin analog, agomelatine, has been approved for major depression treatment[41]. Agomelatine acts as an agonist of MT1, MT2 and the 5-hydroxytryptamine 2C (5-HT2C) receptor to improve both depressive symptoms and sleep disorders in patients with depression[41]. Our previous study showed that the mRNA levels of DCNP1 were dramatically increased in patients with depression[33]. In the present study, we further characterized the relationship between DCNP1 and the melatonin biosynthesis pathway. We identified a novel NLS, amino acids RRK at residues 117-119, that is critical for

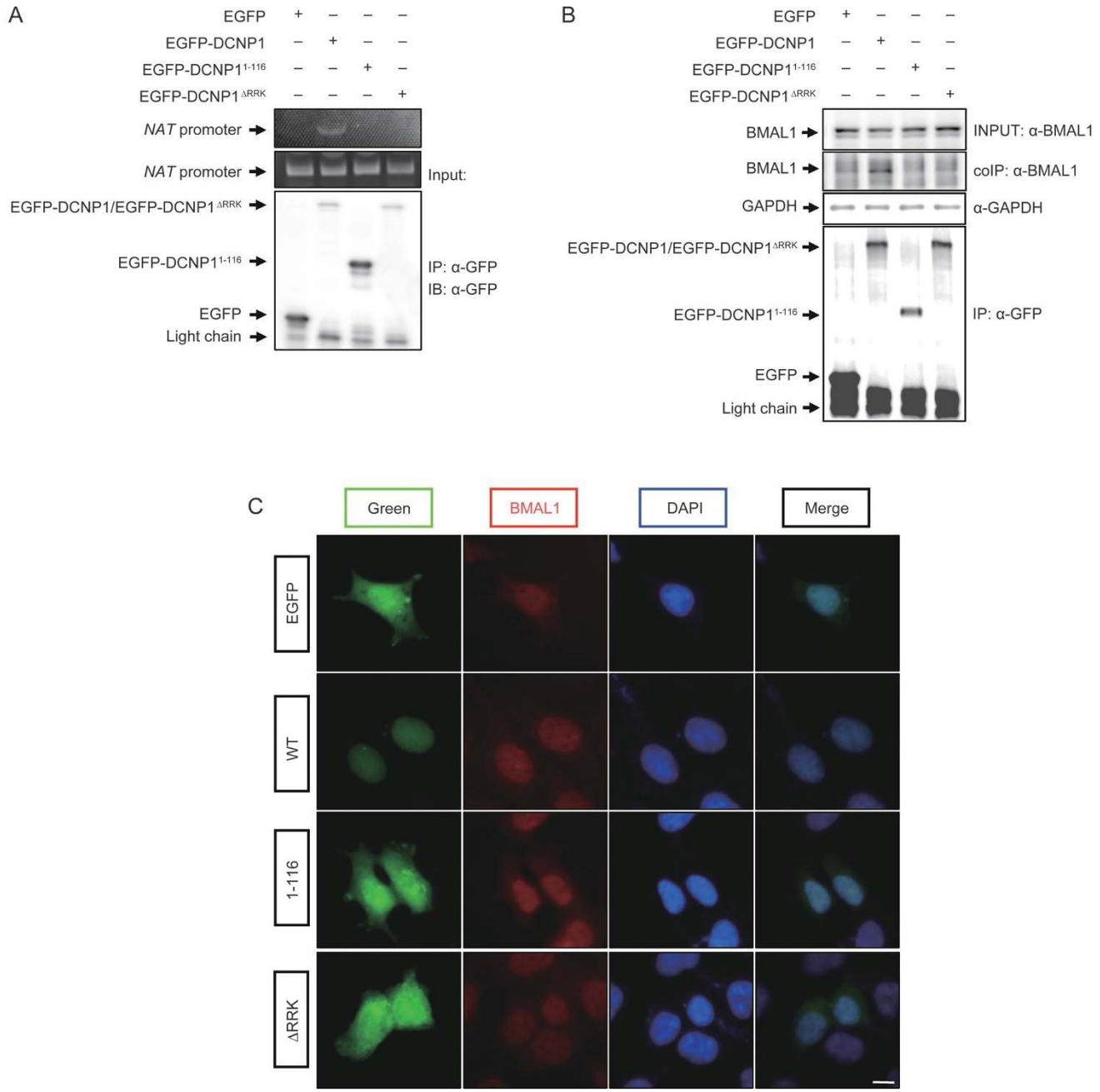

Figure 4. DCNP1 binds to the *Nat* promoter and BMAL1 in the nucleus. (A) HEK293 cells were transfected with EGFP-DCNP1 or its mutants (1-116 and ΔRRK) for chromatin immunoprecipitation assays. Inputs and chromatin immunoprecipitation products were amplified by PCR. (B) HEK293 cells expressing EGFP-DCNP1 or its mutants (1-116 and ΔRRK) were subjected to immunoprecipitation using anti-GFP antibodies. Immunoprecipitates and inputs were detected with the antibodies as indicated. (C) Co-localization of DCNP1 and BMAL1 in the nucleus. HEK293 cells expressing EGFP-DCNP1 or its mutants (1-116 and ΔRRK) were stained with anti-BMAL1 antibodies and visualized using a fluorescence microscope (magnification ×400). Nuclei were stained with DAPI (1 μg/mL) (blue). Scale bars, 5 μm.

DCNP1 nuclear localization. Full-length DCNP1, but not mutants lacking this NLS, represses melatonin biosynthesis. As nuclear DCNP1 is increased in depression patients[33], our present study shows that the negative regulation of melatonin biosynthesis by DCNP1 may contribute to depression pathogenesis.

Although the truncated form DCNP1$^{1\text{-}116}$ has been reported to increase the risk of major depression[31, 32], it did not seem to influence melatonin biosynthesis in our experiments. The premature DCNP1$^{1\text{-}116}$ encoded by the T allele of DCNP1 lacks an NLS and shows a diffuse distribution pattern. We speculate that the truncated DCNP1$^{1\text{-}116}$ may increase the risk for major depression through other signaling pathways rather than by regulating melatonin biosynthesis in the nucleus.

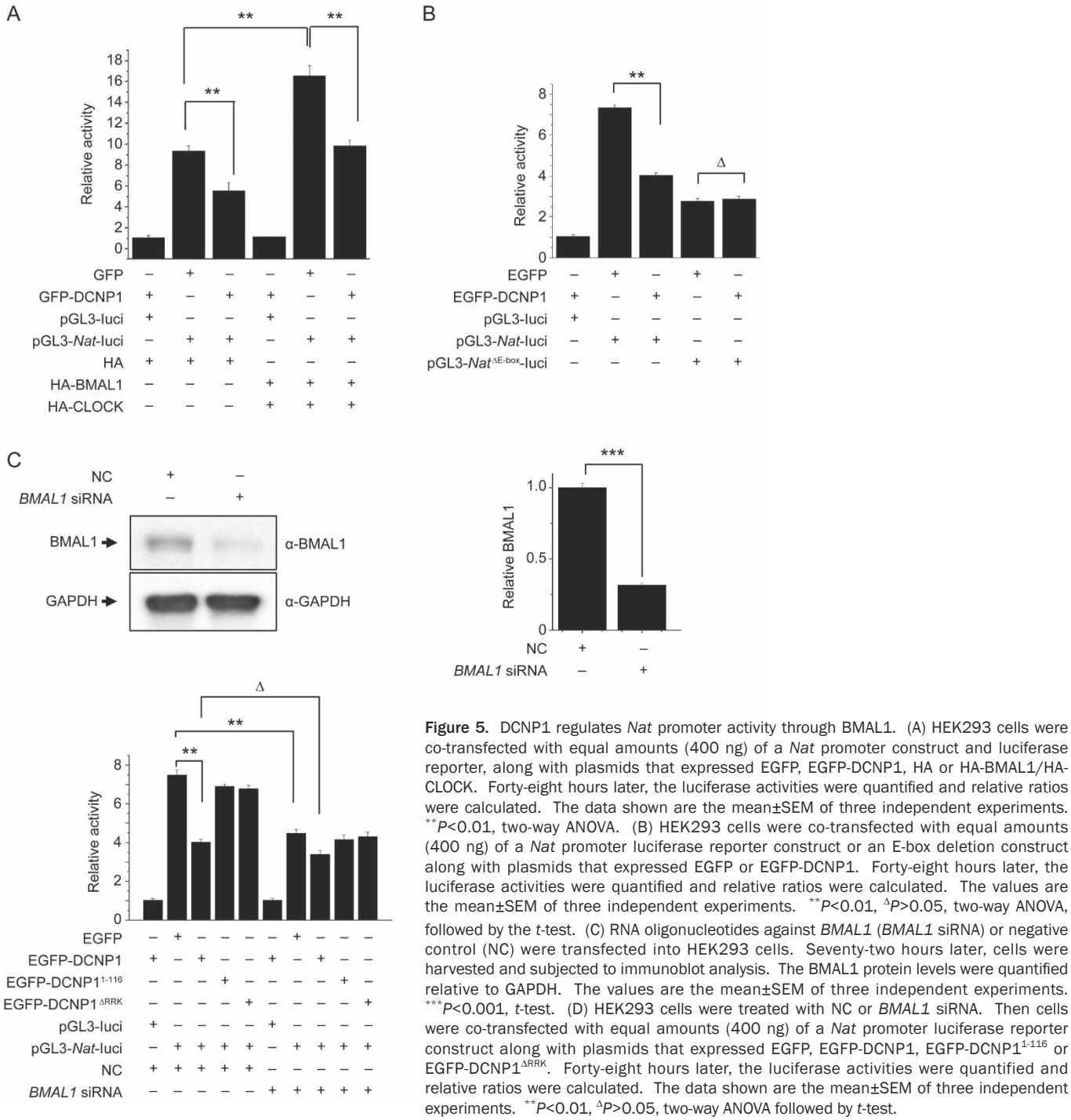

Figure 5. DCNP1 regulates *Nat* promoter activity through BMAL1. (A) HEK293 cells were co-transfected with equal amounts (400 ng) of a *Nat* promoter construct and luciferase reporter, along with plasmids that expressed EGFP, EGFP-DCNP1, HA or HA-BMAL1/HA-CLOCK. Forty-eight hours later, the luciferase activities were quantified and relative ratios were calculated. The data shown are the mean±SEM of three independent experiments. $^{**}P<0.01$, two-way ANOVA. (B) HEK293 cells were co-transfected with equal amounts (400 ng) of a *Nat* promoter luciferase reporter construct or an E-box deletion construct along with plasmids that expressed EGFP or EGFP-DCNP1. Forty-eight hours later, the luciferase activities were quantified and relative ratios were calculated. The values are the mean±SEM of three independent experiments. $^{**}P<0.01$, $^{\Delta}P>0.05$, two-way ANOVA, followed by the *t*-test. (C) RNA oligonucleotides against *BMAL1* (*BMAL1* siRNA) or negative control (NC) were transfected into HEK293 cells. Seventy-two hours later, cells were harvested and subjected to immunoblot analysis. The BMAL1 protein levels were quantified relative to GAPDH. The values are the mean±SEM of three independent experiments. $^{***}P<0.001$, *t*-test. (D) HEK293 cells were treated with NC or *BMAL1* siRNA. Then cells were co-transfected with equal amounts (400 ng) of a *Nat* promoter luciferase reporter construct along with plasmids that expressed EGFP, EGFP-DCNP1, EGFP-DCNP1$^{1\text{-}116}$ or EGFP-DCNP1$^{\Delta RRK}$. Forty-eight hours later, the luciferase activities were quantified and relative ratios were calculated. The data shown are the mean±SEM of three independent experiments. $^{**}P<0.01$, $^{\Delta}P>0.05$, two-way ANOVA followed by *t*-test.

Melatonin biosynthesis is initiated from serotonin, which is first converted to *N*-acetylserotonin by NAT and then further converted to melatonin by HIOMT[22, 23, 36]. Serotonin can also be oxidized by MAOA and MAOB to 5-hydroxyindoleacetic acid (5-HIAA), bypassing melatonin synthesis[10, 20]. In the present study, we found that the levels of melatonin were significantly lower in DCNP1-overexpressing C6 cells. The decrease in melatonin levels induced by DCNP1 are mediated by NAT, but not other enzymes, such as HIOMT, MAOA and MAOB, that are involved in melatonin biosynthesis or breakdown, as only the NAT mRNA and protein levels are significantly down-regulated by overexpression of DCNP1.

The circadian clock protein BMAL1 plays core roles in circadian rhythms and controls the daily rhythms of melatonin[8]. It forms heterodimers with CLOCK to bind to E-box response elements to activate transcription of downstream genes, including circadian clock genes[42, 43]. BMAL1/CLOCK heterodimers also transactivate *Nat* transcription via the E-box in its promoter[24, 25]. In our observations, full-length DCNP1 co-localized and interacted with BMAL1 in the nucleus. In

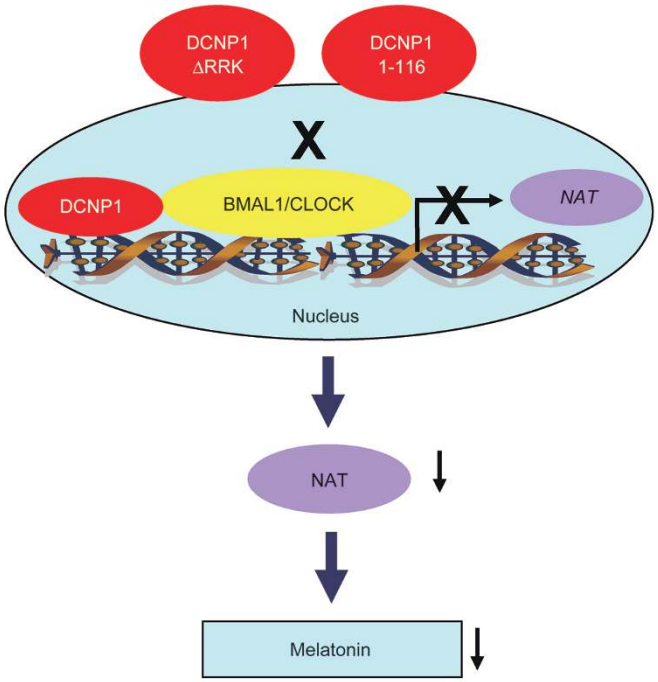

Figure 6. Schematic diagram representing the function of DCNP1 or its mutants (1-116 and ΔRRK) in melatonin biosynthesis. Full-length DCNP1 localizes to the nucleus and binds to BMAL1, thus repressing NAT transcription and subsequently inhibiting melatonin biosynthesis. However, DCNP1 mutants (1-116 and ΔRRK) lack the NLS and cannot inhibit the BMAL1/NAT/melatonin pathway.

addition, full-length DCNP1 interacted with the *Nat* promoter. DCNP1 mutants (1-116 and ΔRRK) lack specific nuclear localization and instead show diffuse distribution throughout the whole cell and fail to interact with BMAL1 or the *Nat* promoter, suggesting that the RRK motif may be important for DCNP1 to bind to BMAL1 and regulate its function. Moreover, luciferase assays showed that full-length DCNP1, but not its mutants, significantly inhibits *Nat* reporter activity, which is dependent on the E-box in the *Nat* promoter, further suggesting that the effects of DCNP1 on NAT expression are mediated by BMAL1.

Interestingly, genetic variants of NAT, the key enzyme in melatonin synthesis, are associated with susceptibility to major depression[44]. In addition, mice harboring *Nat* mutations show depression-like behaviors[45], strongly suggesting an association between the melatonin pathway and depression. Our study also reveals that DCNP1, a major depression candidate gene product, is involved in the regulation of melatonin biosynthesis, implying a role in depression pathogenesis.

In summary, we determined that full-length DCNP1 is involved in regulation of melatonin biosynthesis, dependent on its interaction with BMAL1. Full-length DCNP1 represses BMAL1-mediated *Nat* transcription via an E-box motif and subsequently decreases NAT expression and melatonin biosynthesis. However, mutants of DCNP1 (1-116 and ΔRRK) that lack an NLS fail to interact with BMAL1, leading to a failure to regulate melatonin biosynthesis. Thus, our findings provide linkages between a depression-associated gene product, the circadian system and melatonin metabolism.

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Author contribution

Dong CHEN and Yi-pei LI performed most of the experiments and analyzed the data; Yan-xia YU performed the ELISA assays; Tian ZHOU, Chao LIU, Er-kang FEI, Feng GAO and Chen-chen MU performed some of the immunoblot analyses; Dong CHEN and Hai-gang REN drafted the manuscript and discussed the experiments; Dong CHEN and Guang-hui WANG designed the experiments; Guang-hui WANG and Hai-gang REN interpreted the experiments and revised the manuscript. All of the authors read and approved the final manuscript.

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