Neoplasma 2022; 69(4): 899–908

doi:10.4149/neo_2022_220106N25

N-type calcium channel v2.2 is a target of TCF21 in adrenocortical carcinomas

Barbara dos Santos PASSAIA[1], Jean Lucas KREMER[1], Maria Candida Villares FRAGOSO[2], Claudimara Ferini Pacicco LOTFI[1,*]

[1]*Department of Anatomy, Institute of Biomedical Science, University of São Paulo, São Paulo, Brazil;* [2]*Adrenal Unit, Hormone and Molecular Genetic Laboratory/LIM42, Hospital of Clinics, School of Medicine; University of São Paulo, São Paulo, Brazil*

**Correspondence: clotfi@usp.br*

Received January 6, 2022 / Accepted May 12, 2022

Transcription factor 21 (TCF21) directly binds and regulates SF1 mRNA expression in tumor and normal adrenocortical cells, and both are involved in the development and steroidogenesis of the adrenal cortex. TCF21 is a tumor suppressor gene and its expression is reduced in malignant tumors. In adrenocortical tumors, it is less expressed in adrenocortical carcinomas (ACC) than in adrenocortical adenomas (ACA) and normal tissues. However, a comprehensive analysis to identify TCF21 targets has not yet been conducted in any type of cancer. In this study, we performed Chromatin Immunoprecipitation and Sequencing (ChIP-Seq) in an adrenocortical carcinoma cell line (NCI-H295R) overexpressing TCF21, with the aim of identifying TCF21 new targets. The five most frequently identified sequences corresponded to the PRDM7, CNTNAP2, CACNA1B, PTPRN2, and KCNE1B genes. Validation experiments showed that, in NCI-H295R cells, TCF21 negatively regulates the expression of the CACNA1B gene. Recently, it was observed that the N-type calcium channel v2.2 (Cav2.2) encoded by the CACNA1B gene is important in Angiotensin II signal transduction for corticosteroid biosynthesis in NCI-H295R adrenocortical carcinoma cells. Indeed, TCF21 inhibits CACNA1B and Cav2.2 expression in NCI-H295R. In addition, in a cohort of 55 adult patients with adrenocortical tumors, CACNA1B expression was higher in ACC than ACA and was related to poor disease-free survival in ACC patients. These results suggest a mechanism of steroidogenesis control by TCF21 in adrenocortical tumor cells, in addition to the control observed through SF1 inhibition. Importantly, steroid production could impair tumor immunogenicity, contributing to the immune resistance described in adrenal cancer.

Key words: TCF21, adrenal cancer, adrenocortical carcinoma, N-type calcium channel, adrenocortical cell cultures

Transcription factor 21 (*TCF21*) is considered a tumor suppressor in several types of cancer because of its low expression in hepatocellular carcinoma, melanoma, lung, and head and neck carcinomas, and kidney cancer [1–4]. In breast cancer cells, *TCF21* reduces migration and invasion capacity, proliferation and angiogenesis, and promotes apoptosis [5]. Similar effects have been observed in colorectal cancer cell lines and gastric cancer cells [6, 7]. In bladder cancer, *TCF21* is more highly expressed in primary cancer samples than in metastatic samples [8]. In adrenocortical tumors, *TCF21* shows lower expression in adrenocortical carcinomas (ACC) than in adrenocortical adenomas (ACA) and normal adrenal glands [9, 10]. *TCF21* gene expression levels are extremely low in ACCs [11]. Adult ACCs are rare but aggressive cancers, whereas ACAs are more common and with favorable outcomes [12, 13]. In children, adrenocortical tumors (ACT) generally show better evolution, especially when diagnosed early [14]. Thus, ACTs have a different prognosis in children and adults. In addition, the combined expression of *TCF21* and *BUB1B* (BUB1 mitotic checkpoint serine/threonine kinase B) was a good prognostic marker for adult patients with ACC, distinguishing two subgroups of patients with different survival times [11]. In pediatric patients with ACT, the combined expression of *TCF21* and *SF1*/*NR5A1* (steroidogenic factor 1/nuclear receptor subfamily 5, group A, member 1) presented diagnostic value for pediatric patients aged <5 years [11]. TCF21 binds directly to the *SF1* promoter, inhibiting its activity and steroidogenic acute regulatory protein (*StAR)* expression in adrenocortical carcinoma cell lines [10]. However, the mechanisms of action of *TCF21* are barely known and its targets are still emerging. In this study, 70 target sequences regulated by TCF21 in H295R cells modified to overexpress *TCF21* (H295R/TCF21) were identified through Chromatin Immunoprecipitation and Sequencing (ChIP-Seq). The five most frequently identified sequences corresponding to *PRDM7* (PR/SET domain 7), *CNTNAP2* (contactin associated protein 2), *CACNA1B* (calcium voltage-gated channel subunit alpha 1B), *PTPRN2* (protein tyrosine phosphatase receptor type N2), and *KCNE1B* (potassium voltage-gated channel

subfamily E regulatory subunit 1B) genes. The *CACNA1B* gene and its product Cav2.2 were analyzed in H295R/TCF21 cells since their expression plays a significant role in the synthesis of aldosterone and cortisol in adrenocortical cells [15]. In H295R/TCF21 cells, we observed the inhibition of *CACNA1B* and Cav2.2 expression and in samples from adult patients with ACT, *CACNA1B* was more highly expressed in ACC than in ACA. In addition, ACC patients expressing high *CACNA1B* levels showed poor disease-free survival. Our results showed that *CACNA1B* is a target of TCF21 in ACC and suggest a mechanism of steroidogenesis control by TCF21 through the inhibition of N-type Cav2.2.

Patients and methods

Cell cultures. The human adrenocortical carcinoma cell line (NCI-H295R) [16] was obtained from ATCC (The ATCC Cell Biology Collection). NCI-H295R cells were cultured in an RPMI medium with 2% fetal bovine serum (FBS) and 1% insulin transferrin selenium (ITS, Gibco, MA, USA). NCI-H295R cells were transiently transfected with pCMVMycPod1, hereafter called H295R/TCF21, or empty vector pCMVMyc, hereafter called H295R empty, as described in Passaia et al. [11]. The human hepatocellular carcinoma cell line (HepG2) [17] was cultured in a DMEM medium with 10% FBS (Gibco, Grand Island, NY, USA). Patient secondary cell cultures from aldosterone-producing adult adenoma, ACA-T23 cells, non-functioning adult carcinoma, ACC-T227 cells, pediatric carcinoma, ACC-PedT218 cells, and a pediatric adenoma, ACA-PedT7 cells were utilized and showed in Supplementary Table S1. Patient cell cultures were obtained as described in Almeida et al. [18]. These cell cultures are called secondary cell cultures because they were utilized until the fourth or sixth passage and they were not immortalized. Mycoplasma contamination was excluded by using the qPCR Mycoplasma test kit (PanReac AppliChem, ITW Reagents, Chicago, IL, USA). The cultures belong to the biorepository approved by the Human Research Ethics Committee of the Institute of Biomedical Sciences, University of São Paulo (USP) (no. 156.12). Patient cell cultures were authenticated by STR DNA profiling analysis. All cultures were kept at 37 °C in a humidified atmosphere of 95% air/5% CO_2.

Patients. Samples of ACT were obtained from 70 individuals, 55 adult (19.0–72.4 years old) and 15 pediatric (0.9–17.7 years old) patients. The clinical and histological characteristics of the patients are summarized in Table 1. These data were obtained from Hospital das Clinicas at School of Medicine of the University of São Paulo, responsible for the evaluation of the patients. The human normal adrenal gland used as an immunoblotting control was obtained from one adult individual without disease related to the adrenal gland, and the adrenal cortex was dissected and lysed as described for immunoblotting. This study was approved by the Human Research Ethics Committees of Hospital das Clinicas and Institute of Biomedical Sciences (no. 822/2016), São Paulo, Brazil. Written informed consent was obtained from all patients and/or their legal guardians. The Cancer Genome Atlas (TCGA) program data were obtained from the cBio Portal for Cancer Genomics (https://www.cbioportal.org/, accessed on November 22, 2020).

Table 1. Clinical characteristics of patients.

Adults	Characteristics	n=55		%
	Mean age (y)		41.4±14.5	–
	Range (y)		19–72	–
	Sex	Female	41	74.5
		Male	14	25.5
	Diagnosis	ACA	29	52.7
		ACC	26	46.3
	Weiss score	<3	29	52.7
		≥3	26	47.3
	Metastasis		25	45.5
	CRD		17	30.9
	Follow-up (mo)		62.7±71.9	–
Pediatric		**n=15**		
	Mean age (y)		4.1±5.2	–
	Range (y)		0.9–17.7	–
		Female	12	80.0
	Sex	Male	3	20.0
	Diagnosis	ACA	9	60.0
		ACC	6	40.0
	Weiss score	<3	4	26.7
		≥3	11	73.3
	Metastasis		5	33.3
	CRD		2	–
	Follow-up (mo)		80.9±63.7	–

Abbreviations: y-years; mo-months; CRD-cancer-related death; ACA-adenomas; ACC-carcinomas

CRISPR/dCas9 activation system. The lentiviruses were produced in HEK-293FT cells using the plasmids sgRNA (MS2) (#61427; Addgene, Cambridge, MA, USA), dCas9-VP64 (#61425; Addgene, Cambridge, MA, USA) and MS2-P65-HSF1 (#61426; Addgene, Cambridge, MA, USA). HepG2 CRISPR/dCas9/TCF21 cells were transduced with the constructs sgRNA TCF21 (sequence AAGGGGTCTA-AGCGCTTTGC) and sgRNA scramble (SCR) (sequence GCACTACCAGAGCTAACTCA), as described in Konermann et al. [19]. Transduction and cell selection were performed as described in Passaia et al. [11].

ChIP-Seq. Approximately 9.0×10^6 cells were plated for 24 h before transfection with plasmid pCMVMycPOD1 and maintained for 48 h after transfection. Chromatin immunoprecipitation was performed with 100 µl of fragmented chromatin, protein G magnetic beads, and 5 µg of anti-TCF21 antibody ChIP Grade (ab32981, Abcam, Cambridge, UK) using the ChIP-IT Express kit (Active Motif, Carlsbad, CA, USA) following the manufacturer's instructions. Chromatin was fragmented by sonication with 6 pulses of 10 s each at 25%

amplitude using a VCX130PB ultrasonic processor (Sonics & Materials, Newtown, CT, USA). Libraries were prepared using the TruSeq® ChIP Sample Preparation kit (Illumina, San Diego, CA, USA), starting with 7 ng of enriched ChIP DNA and selecting 250 bp fragments on average. Sequencing was performed on the NextSeq 500 System (Illumina, San Diego, CA, USA) with 75 bp single-ended reads. Fragmented DNA without immunoprecipitation was used as a positive control, and two replicates were sequenced. Reads were mapped using GRCh38 homo sapiens (human) genome assembly.

The Phred Quality Score (Q>30) of the bases was verified, low-quality and duplicate readings were removed, and Peak calling was performed using MACS2 (v2.1.1) ($p<0.01$) wrapped in the AQUAS ChIP-Seq pipeline (https://github.com/NHLBI-BCB/TF_chipseq_pipeline, accessed on November 22, 2020) [20]. The IDR <0.05 (Irreproducible Discovery Rate) was calculated to identify consistent peaks, which guarantees the reproducibility of the experiment [21]. All readings considered as noise by the blacklist of the Encyclopedia of DNA Elements (ENCODE) were excluded [22]. ChIP-Seq data are available in the database Gene Expression Omnibus (GSE179308).

qPCR and immunoblotting. Total RNA was extracted from 9.0×10^5 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) 48 h after plating. The RNA quality was assessed by agarose gel electrophoresis (2%). RNA quantification and sample contaminants were determined by spectrometry (NanoDrop 2000c, Thermo Fisher Scientific, Waltham, MA, USA). For cDNA synthesis, 4 µg of total RNA and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) were used. qPCR experiments were conducted in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SybrGreen reagents (Invitrogen, Carlsbad, CA, USA); the primers are shown in Supplementary Table S2. A total RNA commercial normal adrenal pool from 5 different donors (BioChain, Newark, CA, USA) was used for qPCR control. The relative expression was calculated by the mean of two endogenous genes: beta-actin (ACTB) and beta-glucuronidase (GUSB). The analysis of gene expression was in triplicate, with the empty vector represented in the figures with the value of 1. For immunoblotting, 9.0×10^5 cells were plated and, after 48 h, the cells were lysed in radioimmunoprecipitation assay buffer (RIPA buffer) containing 50 mM Tris-HCl, 150 mM NaCl, 1% Nodidet (NP40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate at pH 7.5, plus a cocktail of protease and phosphatase inhibitors (Sigma-Aldrich, Saint Louis, MO, USA). After pelleting at 14,000×g for 15 min at 4 °C, the samples were quantified using the Bradford assay. 15 µg of protein were resolved in 15% SDS-PAGE gels or 4–15% SDS-PAGE gradient gels for TCF21 and Cav2.2, respectively. Nitrocellulose membranes were used for protein transfer, monitored by Ponceau staining. The membranes were blocked with 5% fat-free milk and Tris-buffered saline (TBS) solution containing 1% Tween 20 (TBST) for 2 h and then incubated with anti-β actin (1:2000, Santa Cruz Biotechnology, Dallas, TX, USA) or anti-TCF21 (1:1000, sc-377225, Santa Cruz Biotechnology, Dallas, TX, USA), or anti-CACNA1B (1:200, ACC-002, Alomone Labs, Jerusalem, Israel). Antibodies were detected with horseradish peroxidase-conjugated secondary antibody and chemiluminescence using ECL reagents in an Imager 600 (GE Healthcare Life Sciences, Marlborough, MA, USA). Immunoblots were quantified by densitometry using ImageJ software [23] from three different experiments.

Viability assay. $2.0–4.0\times10^4$ cells/well were plated into 96-well plates and, after 24 h, were treated with 100 nM MVIIA ω-Conotoxin, as described by Bleackman et al. [24]. ω-Conotoxin MVIIA is a specific Cav2.2 blocker [25]. A blockade occurs after 2 min of treatment with 100 nM ω-Conotoxin and is not reversible [15, 24, 26]. After 3 min of ω-Conotoxin treatment, 20 µl of tetrazolium compound (Cell Titer MTS reagent) (Promega, Madison, WI, USA) were added to 100 µl of culture medium for 4 h at 37 °C in a humidified atmosphere of 95% air/5% CO_2. The optical density was read at 490 nm using an ELISA plate reader (Bio-Tek Instruments, Winooski, VT, USA).

Statistical analysis. Statistical significance was determined using the GraphPad Prism 9 software. Non-parametric Kruskal-Wallis, Mann-Whitney test, and Spearman's rank correlation coefficient were used to analyze patient samples since these groups do not have a normal distribution. Statistical analyzes of cell culture data that have a normal distribution were used parametric one-way ANOVA and two-sample Student's t-test. One-sample Student's t-test was used to analyze fold-change expression after TCF21 overexpression compared to the empty control set to 1.0. Gene expression of the patients was calculated relative to a commercial normal adrenal pool (BioChain Institute, Newark, CA, USA). Results were considered statistically significant when $p<0.05$.

Results

Seventy TCF21 target sequences were identified through ChIP-Seq. The ChIP-Seq assay was performed using H295R cells overexpressing *TCF21* with a coverage of 25±16% of the entire human genome. Seventy TCF21 target sequences were identified, corresponding to 49 different ensembled genes for which the reproducibility information of the duplicates was checked using IDR <0.05 [21]. The gene sequences identified were ranked by a peak score. The peak score was obtained by the frequency that the peaks were called (Supplementary Table S3). Each peak was identified from the transcription start site (TSS), corresponding to one sequence with its respective Ensembl code. Ten different functions of the TCF21 target genes were identified according to the Gene Ontology Resource (http://geneontology.org/, accessed on November 22, 2020), as shown in Supplementary Table S4.

Validation of the five most frequent targets of TCF21. The five most frequent genes identified as targets of TCF21 were *PRDM7*, *CNTNAP2*, *CACNA1B*, *PTPRN2*, and *KCNE1B* (Supplementary Table S3). The expression of these genes was analyzed in H295R cells overexpressing *TCF21* (H295R/TCF21) in comparison with H295R cells transfected with the empty vector using qRT-PCR. TCF21 cells regulated gene expression positively in H295R cells by 1.89±0.23-fold for *PRDM7* (p=0.0180, T=3.871, DF=4), 5.33±1.5-fold for *CNTNAP2* (p=0.0686, T=2.787, DF=3), and 1.58±0.18-fold for *KCNE1B* (p=0.0511, T=3.155, DF=3), however, only the expression of the PRDM7 gene was statistically significant (Figures 1A–1C). There was no difference in the expression of the *PTPRN2* gene between H295R/TCF21 and control cells (Figure 1D). In contrast, TCF21 transfection significantly inhibited *CACNA1B* gene expression to 0.36±0.04-fold (p=0.0037, T=16.39, DF=2, Figure 1E). The analysis of N-type calcium channel v2.2 protein (Cav2.2) expression, which is coded by the *CACNA1B* gene, was also inhibited in H295R cells overexpressing *TCF21* (Figure 2).

Cav2.2 expression is higher in adult and pediatric adrenocortical carcinoma cell cultures. Since Giordano

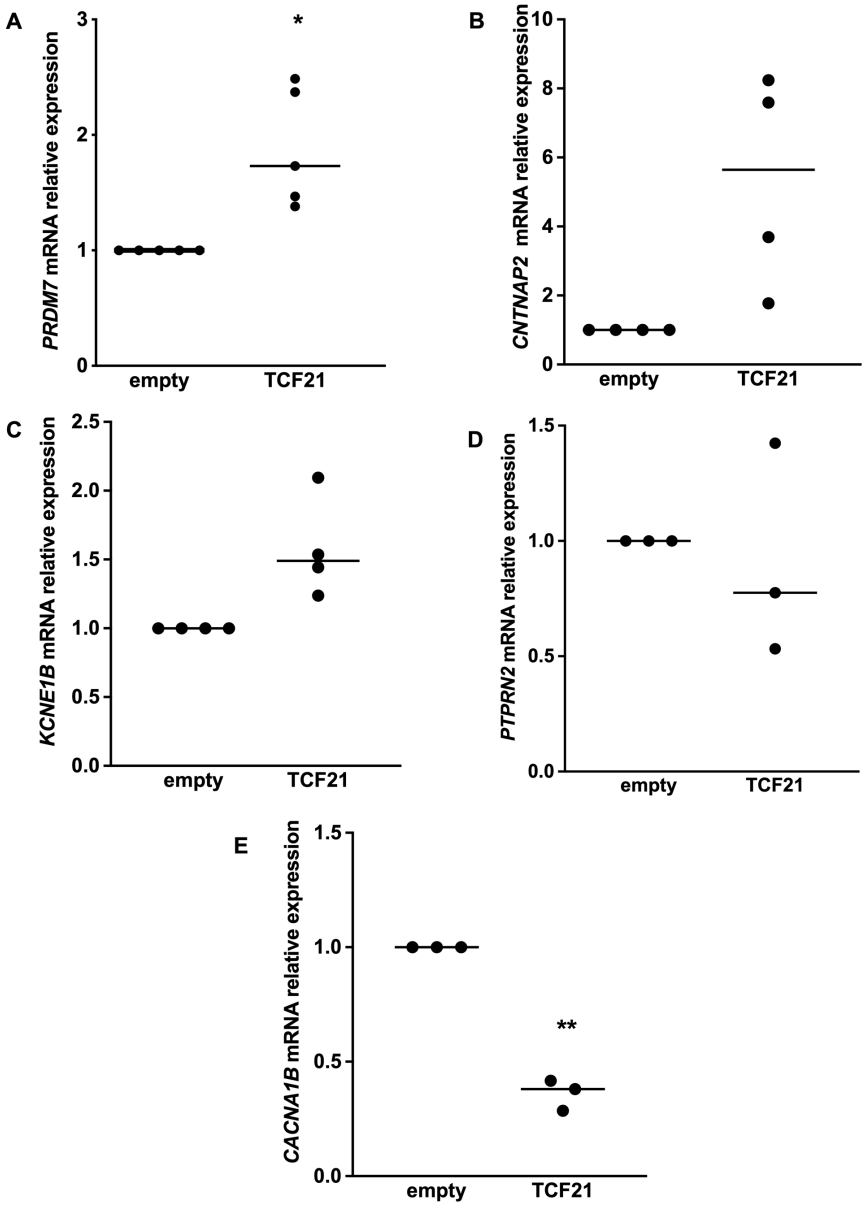

Figure 1. Validation of the five most frequent targets of TCF21 in H295R pCMVMycTCF21 cells. A) mRNA levels of PRDM7 gene, n=5; B) CNTNAP2 gene, n=4; C) KCNE1B gene, n=4; D) PTPRN2 gene, n=3; E) CACNA1B gene, n=3. The results are shown as the mean of 3-5 independent experiments. The p values are indicated in the graph as *p<0.05 and **p<0.01. Statistical significance was assessed by paired t-tests.

et al. [9] described that the *CACNA1B* gene showed lower expression in samples of normal adrenal cortex than in ACC samples, we analyzed the level of Cav2.2 expression by immunoblotting in a normal adrenal cortex and in adrenocortical adenoma carcinoma cell cultures. In relation to the adult normal adrenal cortex, H295R and ACC-T227 cells showed significantly increased Cav2.2 expression. Comparison of Cav2.2 expression of adenoma ACA-T23 cells and normal adrenal cortex showed no statistically significant difference. In pediatric carcinoma cell culture, ACCPed-T218 cells, Cav2.2 was more highly expressed compared to adenoma cell culture, ACAPed-T7 cells (Figure 3, $p<0.0001$, $F(5, 12)=44.90$).

Cav2.2 blockade does not alter the viability of adrenocortical carcinoma cells. H295R and ACC-T227 cells were treated with ω-Conotoxin MVIIA, a specific and irreversible blocker for N-type Cav2.2 [25]. In both H295R and ACC-T227 cells, the blockage of N-type voltage-gated Cav2.2 showed no difference in cell viability of treated cells compared with the control cells (Figures 4A, 4B).

PRDM7 and CACNA1B genes are more expressed in adult ACC than in adult ACA. As *PRDM7* and *CACNA1B* genes are significantly altered in H295R/TCF21 cells, the expression of these genes was analyzed in a cohort of adult and pediatric adrenocortical tumors (Figure 5). Analysis of *PRDM7 and CACNA1B* genes showed significantly higher expression of both genes in adult ACC than in ACA (Figures 5A, 5B, $p=0.0223$ and $p=0.0027$, respectively). In pediatric patients with ACA and ACC, no differences were observed between the expression of *PRDM7* and *CACNA1B* (Figures 5C, 5D). Although pediatric patients with carcinomas present two subgroups of expression of the *PRDM7* and *CACNA1B* genes, no clinical characteristic analyzed defines this division.

CACNA1B expression correlates positively with tumor size in pediatric ACC. In pediatric carcinomas, *CACNA1B* mRNA expression was positively correlated ($r=0.98$; $p=0.0333$) with the tumor size of pediatric carcinomas (Figure 6A), whereas in adult ACC the correlation was negative and moderate ($r=-0.48$; $p=0.0327$) (Figure 6B).

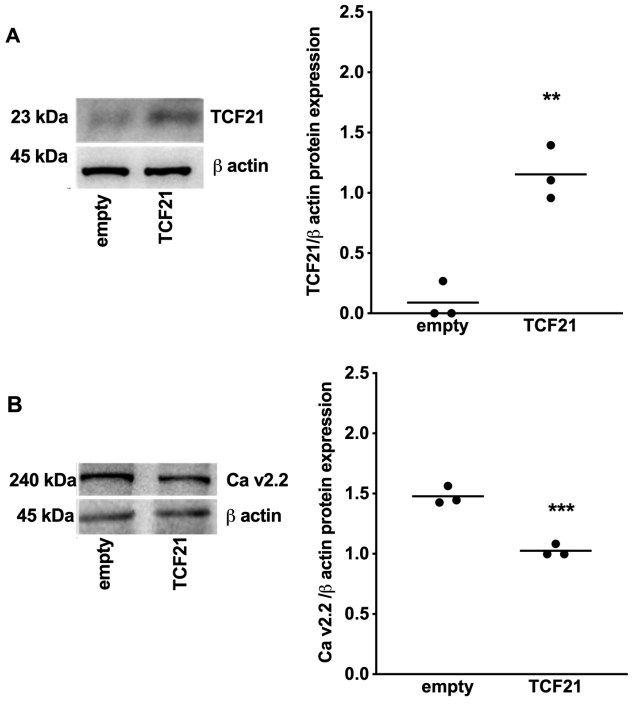

Figure 2. TCF21 expression decreased Cav2.2 expression in H295R pCMVMycTCF21 cells. A) Western blot analysis of TCF21 expression in H295R pCMVMycTCF21 cells (TCF21) and in H295R pCMVMyc control cells (empty). p=0.002, T=6.788, DF=4. Points represent relative TCF21/β-actin expression among samples; B) Western blot analysis of relative Cav2.2 expression in H295R pCMVMycTCF21 cells (TCF21) and in H295R control cells (empty), n=3. p=0.0009, T=8.811, DF=4. Points represent relative Cav2.2/β-actin expression among samples. The results are shown as three independent experiments and their mean. The p values are indicated in the graph as **p<0.01 and ***p<0.001. Statistical significance was assessed by the unpaired t-test.

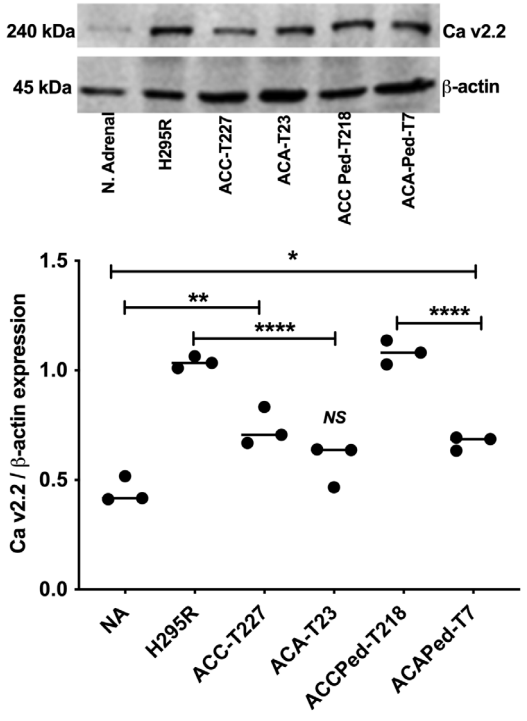

Figure 3. Cav2.2 expression in adrenocortical tumor cell cultures. Western blot analysis for Cav2.2 in total extracts from one adult normal adrenal cortex (Normal Adrenal), the NCI-H295R cell line, adult adrenocortical carcinoma cells (ACC-T227), adult adrenocortical adenoma cells (ACA-T23), pediatric adrenocortical carcinoma cells (ACCPed-T218), and pediatric adrenocortical adenoma cells (ACAPed-T7). Points represent relative Cav2.2/β-actin expression among samples. The results are shown as three independent experiments and their mean. The p-values are indicated in the graph as *p<0.05, **p<0.01, and ***p<0.001. Statistical significance was assessed by Tukey's multiple comparisons test. NS=not significant in relation to the normal adrenal cortex.

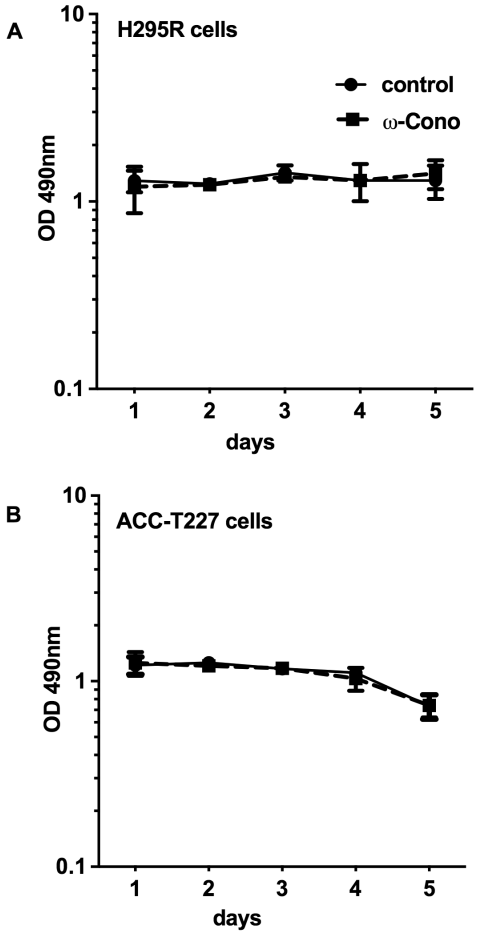

Figure 4. Cav2.2 blockade and viability of adrenocortical carcinoma cells. Dose-response cytotoxicity was analyzed through the methyl thiazoletetrazolium (MTT) assay, measured by optical density (OD) at 490 nm for five days: A) NCI-H295R and B) ACC-T227 cells treated with 100 nM ω-Conotoxin MVIIA (ω-Cono) or untreated (control). The values are expressed as the percentage of viable cells for each condition relative to untreated controls. The results are shown as the mean of three independent experiments. Statistical significance was assessed by analysis of variance (ANOVA).

High CACNA1B expression was related to poor disease-free survival in adult ACC. Analysis of *CACNA1B* expression levels in adult patients with ACC showed that higher *CACNA1B* expression was related to poor disease-free survival compared with patients with ACC with reduced *CACNA1B* expression (Figure 7). Patients with ACC with high *CACNA1B* expression showed metastasis 8.5 months after diagnosis on average. This result suggests that *CACNA1B* may be related to tumor aggressiveness.

Discussion

We identified seventy TCF21 target sequences belonging to 49 different genes using the ChIP-Seq assay in an adrenocortical carcinoma cell line overexpressing *TCF21*. The five most frequently identified sequences corresponded to the *PRDM7*, *CNTNAP2*, *CACNA1B*, *PTPRN2*, and *KCNE1B* genes. Validation experiments showed that *PRDM7* expression was positively regulated in adrenocortical carcinoma cells expressing *TCF21* and was more highly expressed in adult ACC than in ACA. The PRDM family of proteins is defined based on the conserved N-terminal PR domain, which is closely related to the domains of histone methyltransferases [27]. Although PRDM genes play diverse roles in cell cycle regulation, differentiation, and meiotic recombination, these genes present opposite roles in carcinomas [27, 28]. In ACC, the positive relation between *TCF21* and *PRDM7* expression has not been described so far and, to the best of our knowledge, there are no data regarding the role of this gene in the pathogenesis of adrenocortical tumors. Therefore, the understanding of the potential and differential regulation of *PRDM7* by TCF21 in ACA and ACC should be more comprehensively addressed.

The *CACNA1B* gene, the third most frequent target of *TCF21* in the H295R cell line, encodes an N-type voltage-dependent calcium channel, the Cav2.2 protein. *CACNA1B* gene and Cav2.2 protein were negatively regulated by TCF21 in the adrenocortical cell line and more highly expressed in adult ACC than in ACA. These results are in agreement with the findings of Giordano et al. [9], who showed that the *CACNA1B* gene showed lower expression in samples of normal adrenal cortex than in ACC samples. As *TCF21* is expressed to a lesser degree in adult ACC than in ACA and normal adrenals [9–11], these results suggest that TCF21 levels are negatively related to *CACNA1B* in adult ACC. Moreover, high *CACNA1B* gene expression in carcinomas was associated with poor disease-free survival, although Cav2.2 channel blockade alone did not affect cell viability *in vitro*. Even though no difference was observed between pediatric ACA and ACC survival time, *CACNA1B* expression was positively correlated with tumor size in these patients. As small tumor size is one of the characteristics associated with favorable disease prognosis in this age group [29], this observation may be relevant. The *CACNA1B* gene encoding Cav2.2 is located on chromosome 9q34.3 (http://www.ensembl.org/, accessed on November 22, 2020). The Cav2.2 expression was higher in pediatric carcinoma cell culture ACC-Ped218 than in adenoma culture, ACA-T7Ped cells. In contrast, the aldosterone-producing adenoma cell culture, ACA-T23, expressed lower Cav2.2 levels than H295R cells. Juhlin et al. [30] described that in aldosterone-producing adenomas ion channels are frequently mutated, and *CACNA1B* mutations showed a prevalence of 43%. However, according to the Catalog of Somatic Mutations in Cancer (COSMIC), the c.2359C>A (p.L787M) pathogenic mutation in *CACNA1B* was described only in one ACC sample [31]. Interesting, in 90% of pediatric adrenocortical tumors, there is an amplification of chromosome 9q in a region that includes *NOTCH* and *SF1* genes [32]. Since Cav2.2 was more highly expressed in ACCPed-T218 carcinoma cells than in ACAPed-T7

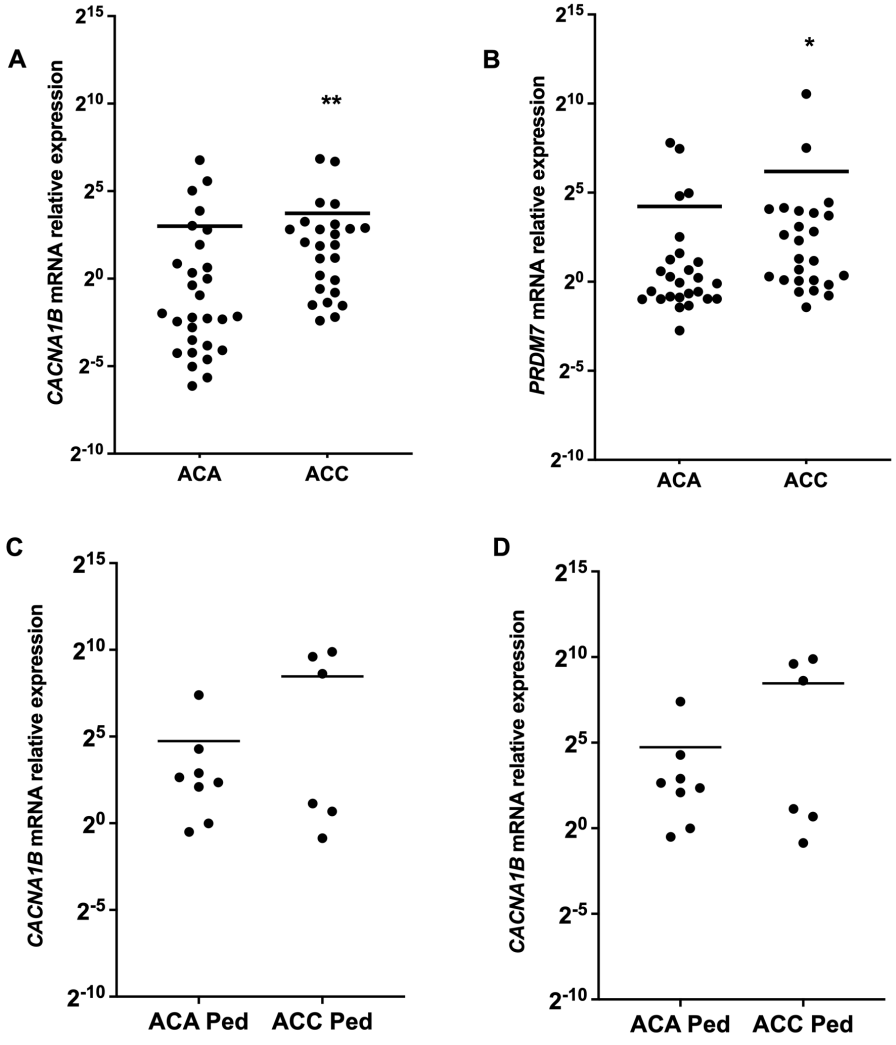

Figure 5. Analysis of CACNA1B and PRDM7 expression in a cohort of adult and pediatric adrenocortical tumors. A) Relative expression of CACNA1B in adult ACA and ACC; B) Relative expression of PRDM7 in adult ACA and ACC; C) Relative expression of CACNA1B in pediatric ACA and ACC and D) Relative expression of PRDM7 in pediatric ACA and ACC, by quantitative real-time PCR. Adult ACA=26; Adult ACC=25; pediatric PedACA=9 and pediatric PedACC=6. The Y-axis shows the fold increase in gene expression relative to the endogenous expression relative to a commercial mRNA pool of normal adrenals. The Mann-Whitney test was used to assess statistical significance ($p<0.05$).

adenoma cells, *CACNA1B* gene expression may be important in pediatric adrenocortical tumors. In addition, it is largely accepted that children have a better outcome than adolescents. In a study by Wieneke and collaborators [33] using the pediatric ACT, biphasic age distribution was described with a poor clinical outcome in the group aged >5 years. In another study by Cecchetto et al. [34], patients aged ≤4 years had a better outcome than older patients. There is epidemiological and molecular evidence suggesting that pediatric adrenocortical tumors represent a distinct disease compared to adult tumors regarding their origin, molecular alterations, clinical characteristics, and prognostic evolution [35]. Therefore, these differences may explain the observed inverse correlation between *CACNA1B* expression and adult and pediatric tumor size.

Although widely expressed in the nervous system, N-type calcium channels are also expressed in the pancreas and adrenal gland [36]. The presence and functional role of N-type Cav2.2 were described in H295R cells by Aritomi et al. [15]. In this study, patch-clamp analysis indicated that the N-type Cav2.2 inhibitor ω-Conotoxin significantly reduced the transient calcium signaling induced by angiotensin II (Ang II) and prevented Ang II-induced aldosterone and cortisol production. Therefore, N-type calcium channels play a significant role in transducing the Ang II signal for steroid biosynthesis. We found that TCF21 binds directly to the *SF1* promoter, inhibiting its activity and *StAR* expression in human adrenocortical carcinoma cells and in rat primary adrenocortical cell cultures, suggesting that *TCF21* regulates adrenocortical steroidogenesis [10,

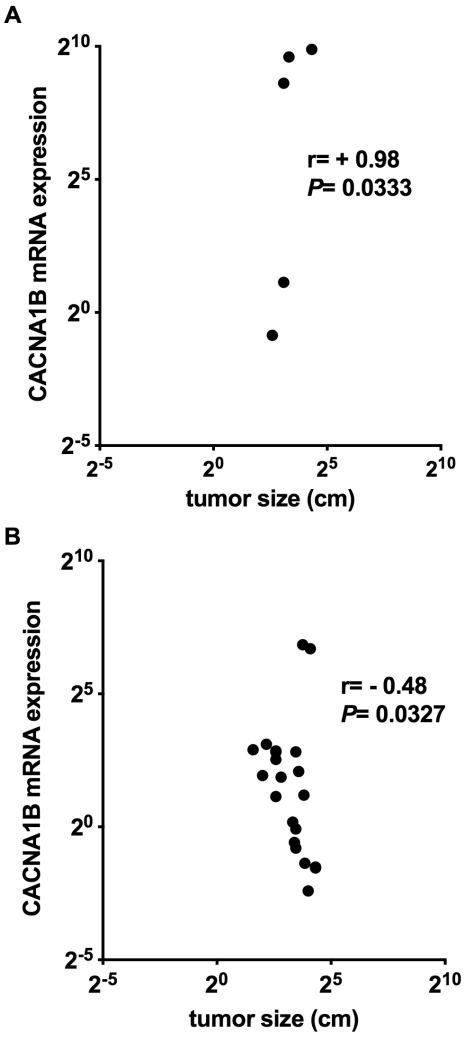

Figure 6. CACNA1B expression correlates with tumor size in pediatric adrenocortical carcinomas. Correlation analysis of CACNA1B expression with tumor size in A) 5 pediatric patients with ACC and B) 21 adult patients with ACC. The r and p-values are indicated – Spearman's rank correlation coefficient.

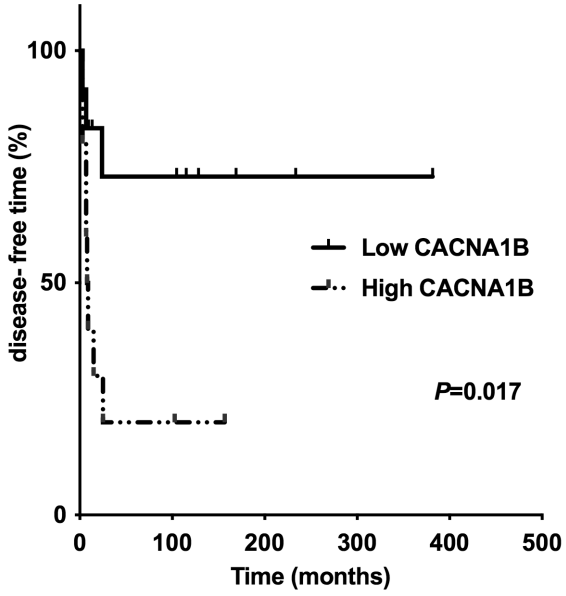

Figure 7. High CACNA1B expression negatively impacts disease-free survival in adrenocortical patients. Kaplan-Meier analysis of disease-free survival according to CACNA1B mRNA levels using an adult ACC cohort. Patients were dichotomized according to median gene expression for survival analysis. Low CACNA1B expression <6.0 (n=12) and high CACNA1B expression >6.0 (n=10). The p-values are indicated by the log-rank test.

37, 38]. Taken together, these results suggest that *TCF21* is part of the control mechanism for the steroidogenic process controlling *SF1* and N-type Cav2.2 expression. Understanding the different mechanisms related to the control of steroid production in adrenocortical tumors may be particularly important since cortisol-secreting ACCs are associated with poor overall survival [39]. In addition, increased steroid production in ACC could impair tumor immunogenicity, contributing to the immune resistance described in this type of cancer [40].

In summary, validation of the most frequent gene sequences identified showed that the regulation of N-type Cav2.2 by TCF21 may be important in cortical hormone-secreting ACC and in the control of steroidogenesis in adrenocortical tumors. Therefore, this mechanism must be taken into consideration in future immunotherapy approaches for adrenocortical carcinomas.

Supplementary information is available in the online version of the paper.

Acknowledgments: We are grateful to Cintia Fridman, Department of Legal Medicine and Medical Ethics, School of Medicine, University of São Paulo, for the STR DNA profiling analysis in cell cultures. The authors would like to acknowledge all research participants contributing to The Cancer Genome Atlas (TCGA) resource for providing high-quality data for analysis. BSP and JLK are the recipient of a scholarship from FAPESP (no. 2016/12381-7; 2016/17285-6), the São Paulo State Research Foundation (FAPESP); CFPL received funding from FAPESP (no. 2015/014199-9; 2018/19035-2) and from CNPq. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

References

[1] LU W, YANG C, DU P, ZHANG JL, ZHANG JC. Expression tendency and prognostic value of TCF21 in hepatocellular carcinoma. Artif Cells Nanomed Biotechnol 2019; 47: 1466–1470. https://doi.org/10.1080/21691401.2019.1601102

[2] ARAB K, SMITH LT, GAST A, WEICHENHAN D, HUANG JP et al. Epigenetic deregulation of TCF21 inhibits metastasis suppressor KISS1 in metastatic melanoma. Carcinogenesis 2011; 32:1467–1473. https://doi.org/10.1093/carcin/bgr138

[3] TESSEMA M, BELINSKY SA. Mining the epigenome for methylated genes in lung cancer. Proc Am Thorac Soc 2008; 5: 806–810. https://doi.org/10.1513/pats.200805-045TH

[4] GOOSKENS SL, KLASSON TD, GREMMELS H, LOGISTER I, PIETERS R et al. TCF21 hypermethylation regulates renal tumor cell clonogenic proliferation and migration. Mol Oncol 2018; 12: 166–179. https://doi.org/10.1002/1878-0261.12149

[5] LI PW, WANG HY, XU LL. [Effect of TCF21 on proliferation, migration and vascular production of breast cancer cells]. Zhonghua Bing Li Xue Za Zhi 2019; 48: 307–311. https://doi.org/10.3760/cma.j.issn.0529-5807.2019.04.009

[6] DAI Y, DUAN H, DUAN C, ZHU H, ZHOU R et al. TCF21 functions as a tumor suppressor in colorectal cancer through inactivation of PI3K/AKT signaling. Onco Targets Ther 2017; 14: 1603–1611. https://doi.org/10.2147/OTT.S118151

[7] YANG Z, JIANG X, LI D, DONG Q, ZHAO H et al. TCF21 inhibits proliferation and chemoresistance through the AKT pathway in human gastric cancer. Gene 2019; 682: 42–49. https://doi.org/10.1016/j.gene.2018.10.011

[8] MOKKAPATI S, PORTEN SP, NARAYAN VM, LIM AH, JAYARATNA IS et al. TCF21 Promotes Luminal-Like Differentiation and Suppresses Metastasis in Bladder Cancer. Mol Cancer Res 2020; 18: 811–821. https://doi.org/10.1158/1541-7786.MCR-19-0766

[9] GIORDANO TJ, KUICK R, ELSE T, GAUGER PG, VINCO M et al. Molecular classification and prognostication of adrenocortical tumors by transcriptome profiling. Clin Cancer Res 2009; 15: 668–676. https://doi.org/10.1158/1078-0432.CCR-08-1067

[10] FRANCA MM, FERRAZ-DE-SOUZA B, SANTOS MG, LERARIO AM, FRAGOSO MC et al. POD-1 binding to the E-box sequence inhibits SF-1 and StAR expression in human adrenocortical tumor cells. Mol Cell Endocrinol 2013; 371: 140–147. https://doi.org/10.1016/j.mce.2012.12.029

[11] PASSAIA BDS, DIAS MH, KREMER JL, ANTONINI SRR, DE ALMEIDA MQ et al. TCF21/POD-1, a Transcritional Regulator of SF-1/NR5A1, as a Potential Prognosis Marker in Adult and Pediatric Adrenocortical Tumors. Front Endocrinol (Lausanne) 2018; 22: 38. https://doi.org/10.3389/fendo.2018.00038

[12] AUFFORTH RD, NILUBOL N. Emerging therapy for adrenocortical carcinoma. Int J Endocr Oncol 2014; 1: 173–182. https://doi.org/10.2217/ije.14.13

[13] TORTI JF, CORREA R (Eds.). Adrenal Cancer. In: StatPearls. Treasure Island (FL): StatPearls Publishing 2020. https://www.ncbi.nlm.nih.gov/books/NBK546580/

[14] MICHALKIEWICZ E, SANDRINI R, FIGUEIREDO B, MIRANDA ECM, CARAN E et al. Clinical and outcome characteristics of children with adrenocortical tumors: a report from the International Pediatric Adrenocortical Tumor Registry. J Clin Oncol 2004; 22: 838–845. https://doi.org/10.1200/JCO.2004.08.085

[15] ARITOMI S, WAGATSUMA H, NUMATA T, URIU Y, NOGI Y et al. Expression of N-type calcium channels in human adrenocortical cells and their contribution to corticosteroid synthesis. Hypertens Res 2011; 34: 193–201. https://doi.org/10.1038/hr.2010.191

[16] GAZDAR AF, OIE HK, SHACKLETON CH, CHEN TR, TRICHE TJ et al. Establishment and characterization of a human adrenocortical carcinoma cell line that expresses multiple pathways of steroid biosynthesis. Cancer Res 1990; 50: 5488–5496.

[17] KNOWLES BB, HOWE CC, ADEN DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 1980; 209: 497–499. https://doi.org/10.1126/science.6248960

[18] ALMEIDA MQ, FRAGOSO MC, LOTFI CF, SANTOS MG, NISHI MY et al. Expression of insulin-like growth factor-II and its receptor in pediatric and adult adrenocortical tumors. J Clin Endocrinol Metab 2008; 93: 3524–3531. https://doi.org/10.1210/jc.2008-0065

[19] KONERMANN S, BRIGHAM MD, TREVINO AE, JOUNG J, ABUDAYYEH OO et al. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. Nature 2015; 517: 583–588. https://doi.org/10.1038/nature14136

[20] ZHANG Y, LIU T, MEYER CA, EECKHOUTE J, JOHNSON DS et al. Model-based analysis of ChIP-Seq (MACS). Genome Biol 2008; 9: R137. https://doi.org/10.1186/gb-2008-9-9-r137

[21] LI Q, BROWN JB, HUANG H, BICKEL PJ. Measuring reproducibility of high-throughput experiments. Annals of Applied Statistics 2011; 5: 1752–1779. https://doi.org/10.1214/11-AOAS466

[22] AMEMIYA HM, KUNDAJE A, BOYLE AP. The ENCODE Blacklist: Identification of Problematic Regions of the Genome. Sci Rep 2019; 9: 9354. https://doi.org/10.1038/s41598-019-45839-z

[23] SCHNEIDER CA, RASBAND WS, ELICEIRI KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012; 9: 671–675. https://doi.org/10.1038/nmeth.2089

[24] BLEAKMAN D, BOWMAN D, BATH CP, BRUST PF, JOHNSON EC et al. Characteristics of a human N-type calcium channel expressed in HEK293 cells. Neuropharmacology 1995; 34: 753–765. https://doi.org/10.1016/0028-3908(95)00078-k

[25] OLIVERA BM, CRUZ LJ, DE SANTOS V, LECHEMINANT GW, GRIFFIN D et al. Neuronal calcium channel antagonists. Discrimination between calcium channel subtypes using omega-conotoxin from Conus magus venom. Biochemistry 1987; 26: 2086–2090. https://doi.org/10.1021/bi00382a004

[26] WILLIAMS ME, FELDMAN DH, MCCUE AF, BRENNER R, VELICELEBI G et al. Structure and functional expression of alpha 1, alpha 2, and beta subunits of a novel human neuronal calcium channel subtype. Neuron 1992; 8: 71–84. https://doi.org/10.1016/0896-6273(92)90109-q

[27] HOHENAUER T, MOORE AW. The Prdm family: expanding roles in stem cells and development. Development 2012; 139: 2267–2282. https://doi.org/10.1242/dev.070110

[28] MZOUGHI S, TAN YX, LOW D, GUCCIONE E. The role of PRDMs in cancer: one family, two sides. Curr Opin Genet Dev 2016; 36: 83–91. https://doi.org/10.1016/j.gde.2016.03.009

[29] PINTO EM, ZAMBETTI GP, RODRIGUEZ-GALINDO C. Pediatric adrenocortical tumours. Best Pract Res Clin Endocrinol Metab 2020; 34: 101448. https://doi.org/10.1016/j.beem.2020.101448

[30] JUHLIN CC, BERTHERAT J, GIORDANO TJ, HAMMER GD, SASANO H et al. What Did We Learn from the Molecular Biology of Adrenal Cortical Neoplasia? From Histopathology to Translational Genomics. Endocr Pathol 2021; 32: 102–133. https://doi.org/10.1007/s12022-021-09667-0

[31] ASSIÉ G, LETOUZÉ E, FASSNACHT M, JOUINOT A, LUSCAP W, BARREAU O et al. Integrated genomic characterization of adrenocortical carcinoma. Nat Genet 2014; 46: 607–612. https://doi.org/10.1038/ng.2953

[32] PINTO EM, CHEN X, EASTON J, FINKELSTEIN D, LIU Z et al. Genomic landscape of paediatric adrenocortical tumours. Nat Commun 2015; 6: 6302. https://doi.org/10.1038/ncomms7302

[33] WIENEKE JA, THOMPSON LD, HEFFESS CS. Adrenal cortical neoplasms in the pediatric population: a clinicopathologic and immunophenotypic analysis of 83 patients. Am J Surg Pathol 2003; 27: 867–881. https://doi.org/10.1097/00000478-200307000-00001

[34] CECCHETTO G, GANARIN A, BIEN E, VORWERK P, BISOGNO G et al. Outcome and prognostic factors in high-risk childhood adrenocortical carcinomas: A report from the European Cooperative Study Group on Pediatric Rare Tumors (EXPeRT). Pediatr Blood Cancer 2017; 64. https://doi.org/10.1002/pbc.26368

[35] LALLI E, FIGUEIREDO BC. Pediatric adrenocortical tumors: what they can tell us on adrenal development and comparison with adult adrenal tumors. Front Endocrinol (Lausanne) 2015; 6: 23. https://doi.org/10.3389/fendo.2015.00023

[36] MILLER RJ. Voltage-sensitive Ca2+ channels. J Biol Chem 1992; 267: 1403–1406.

[37] FRANCA MM, FERRAZ-DE-SOUZA B, LERARIO AM, FRAGOSO MC, LOTFI CF. POD-1/TCF21 Reduces SHP Expression, Affecting LRH-1 Regulation and Cell Cycle Balance in Adrenocortical and Hepatocarcinoma Tumor Cells. Biomed Res Int 2015; 2015: 841784. https://doi.org/10.1155/2015/841784

[38] FRANCA MM, LERARIO AM, FRAGOSO M, LOTFI CFP. New evidences on the regulation of SF-1 expression by POD1/TCF21 in adrenocortical tumor cells. Clinics (Sao Paulo) 2017; 72: 391–394. https://doi.org/10.6061/clinics/2017(06)10

[39] VANBRABANT T, FASSNACHT M, ASSIE G, DEKKERS OM. Influence of hormonal functional status on survival in adrenocortical carcinoma: systematic review and meta-analysis. Eur J Endocrinol 2018; 179: 429–436. https://doi.org/10.1530/EJE-18-0450

[40] COSENTINI D, GRISANTI S, DALLA VOLTA A, LAGANA M, FIORENTINI C et al. Immunotherapy failure in adrenocortical cancer: where next? Endocr Connect 2018; 7: E5–E8. https://doi.org/10.1530/EC-18-0398

https://doi.org/10.4149/neo_2022_220106N25

N-type calcium channel v2.2 is a target of TCF21 in adrenocortical carcinomas

Barbara dos Santos PASSAIA[1], Jean Lucas KREMER[1], Maria Candida Villares FRAGOSO[2], Claudimara Ferini Pacicco LOTFI[1,*]

Supplementary Information

Supplementary Table S1. Secondary cell cultures obtained from tumors of patients.

Cell Culture	Sex	Age (y)	Diagnosis	WS	Classification
ACA-T23	F	43	ACA	1	Hyperaldosteronism
ACC-T227	F	62	ACC	8	-
ACAPed-T7	F	1,6	ACA	5	CS/Virilizing
ACCPed-T218	F	2,75	ACC	8	Virilizing

Abbreviations: y-years; WS-Weiss Score; CS-Cushing Syndrome; ACA-adenoma adrenocortical; ACC-carcinoma adrenocortical; ACAPed- pediatric adenoma adrenocortical; ACCPed-pediatric carcinoma adrenocortical

Supplementary Table S2. Primer sequences used for qPCR.

Gene	Forward	Reverse
ACTB	CCTCGCCTTTGCCGATCC	CGCGGCGATATCATCATCC
GUSB	AGCCAGTTCCTCATCAATGG	GGTAGTGGCTGGTACGAAA
TCF21	GAAAGAAGTGGTGACCGCGA	GTAAAGTGTTCTCGCGGGGT
PRDM7	AGCCACAAGAAGACAAACGTGA	GTGCGGGAAACAACCACAC
CNTNAP2	TTACACTTGGTGGGTTGGCA	CTGTGCAGTTGCGTTCGATG
CACNA1B	CAGTGGTTCCGTGAATGGGA	GTTGGCCGTCTTGTAGGTGA
PTPRN2	AGGTTCCGGCAATGGACTTT	TTCGGGAGGTCTGCAAGTTC
KCNE1B	GCCCTTAGAAGGTGCCGC	GGGATTTTTCCCGGGTCTGA
ATP10A	TACCTGGGGGACCCCTATTG	GTCCAGTAAGGGTTGGACGG

Supplementary Table S3. Target sequences identified in H295RpCMVMycTCF21 cells.

P-Score	Gene	Name	Ensembl	TSS Distance (bp)
670.365	**PRDM7**	**PR/SET Domain 7**	**ENSG00000126856**	**–39401**
657.154	**CNTNAP2**	**Contactin Associated Protein 2**	**ENSG00000174469**	**2;214;725;197;842**
641.278	**CACNA1B**	**Calcium Voltage-Gated Channel Subunit Alpha1 B**	**ENSG00000148408**	**106;735;106;735**
558.899	**PTPRN2**	**Protein Tyrosine Phosphatase Receptor Type N2**	**ENSG00000155093**	**145;881;145;896;145;000**
531.314	**KCNE1B**	**Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B**	**ENSG00000276289**	**–417543**
522.523	ADGRA1	Adhesion G Protein-Coupled Receptor A1	ENSG00000197177	40;777;238;019;460
522.523	TUBA3C	Tubulin Alpha 3c	ENSG00000198033	35358
520.833	SPAAR	Small Regulatory Polypeptide of Amino Acid Response	ENSG00000235387	4294
498.772	FMN2	Formin 2	ENSG00000155816	17;054;293;007
498.772	TPK1	Thiamin Pyrophosphokinase 1	ENSG00000196511	–183668
487.577	PDS5A	PDS5 Cohesin Associated Factor A	ENSG00000121892	8068
472.915	BDH1	3-Hydroxybutyrate Dehydrogenase 1	ENSG00000161267	500;175;001;750;017
471.342	SCGB1C2	Secretoglobin Family 1C Member 2	ENSG00000268320	–19415
469.395	CNTNAP3B	Contactin Associated Protein Family Member 3B	ENSG00000154529	108476
451.316	KCNJ18	Potassium Inwardly Rectifying Channel Subfamily J Member 18	ENSG00000260458	275851
445.001	KCNE1B	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B	ENSG00000276289	–573712
427.519	IRX1	Iroquois Homeobox 1	ENSG00000170549	–271571
426.353	CWH43	Cell Wall Biogenesis 43 C-Terminal Homolog	ENSG00000109182	230092
409.911	OR11H1	Olfactory Receptor Family 11 Subfamily H Member 1	ENSG00000130538	–4462467
408.426	CWH43	Cell Wall Biogenesis 43 C-Terminal Homolog	ENSG00000109182	260700
408.426	IGKV3OR2	Immunoglobulin Kappa Variable 3/OR2	ENSG00000233999	52717

Supplementary Table S3. ***Continued . . .***

P-Score	Gene	Name	Ensembl	TSS Distance (bp)
403.913	RGPD2	RANBP2 Like and GRIP Domain Containing 2	ENSG00000185304	60542
403.768	AC005747.1	AC005747.1	ENSG00000007237	–19726
403.768	DPP10	Dipeptidyl Peptidase Like 10	ENSG00000175497	–165260
403.768	IGHD1OR15	Immunoglobulin Heavy Diversity 1/OR15-1A	ENSG00000271336	–109303
403.768	OR11H1	Olfactory Receptor Family 11 Subfamily H Member 1	ENSG00000130538	–4501498
403.768	USP25	Ubiquitin Specific Peptidase 25	ENSG00000155313	304284
402.778	KCNE1B	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B	ENSG00000276289	–589551
392.973	AC023490.7	AC023490.7	ENSG00000286175	112620
381.474	CWH43	Cell Wall Biogenesis 43 C-Terminal Homolog	ENSG00000109182	224216
377.074	IRX4	Iroquois Homeobox 4	ENSG00000113430	–258955
375.973	NBPF14	NBPF Member 14	ENSG00000270629	590;025;929;656;970;000
366.586	KCNE1B	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B	ENSG00000276289	–634111
365.691	KCNE1B	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B	ENSG00000276289	–407233
365.434	GOLGA8G	Golgin A8 Family Member G	ENSG00000183629	–27094
363.497	KCNE1B	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B	ENSG00000276289	–585069
356.266	ATP10A	ATPase Phospholipid Transporting 10A	ENSG00000206190	10;430;610;469;925;300;000
356.266	GZMB	Granzyme B	ENSG00000100453	–62735
356.266	MTERF1	Mitochondrial Transcription Termination Factor 1	ENSG00000127989	140;107;140;107
356.266	PRR20E	Proline Rich 20E	ENSG00000234278	50041
348.789	KCNE1B	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B	ENSG00000276289	–578252
343.938	GOLGA8F	Golgin A8 Family Member F	ENSG00000153684	–53966
339.332	KCNE1B	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B	ENSG00000276289	–395223
332.515	CNBD1	Cyclic Nucleotide Binding Domain Containing 1	ENSG00000176571	16;424;299;064
332.515	NHSL1	NHS Like 1	ENSG00000135540	5;187;025;041;171;900
332.515	TNS3	Tensin 3	ENSG00000136205	1;071;471;075;946;430;000
332.515	ZMAT4	Zinc Finger Matrin-Type 4	ENSG00000165061	4;397;243;972
329.682	KCNE1B	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B	ENSG00000276289	–625311
308.764	DOCK1	Dedicator Of Cytokinesis 1	ENSG00000150760	4;265;742;610
308.764	LCE5A	Late Cornified Envelope 5A	ENSG00000186207	–19976
308.764	OTOL1	Otolin 1	ENSG00000182447	814551
298.214	KCNE1B	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B	ENSG00000276289	–398181
285.013	CFDP1	Craniofacial Development Protein 1	ENSG00000153774	14;754;459;574;580;300;000
285.013	GTPBP6	GTP Binding Protein 6	ENSG00000178605	2591
285.013	MEIS1	Meis Homeobox 1	ENSG00000143995	–283996
285.013	OR4C45	Olfactory Receptor Family 4 Subfamily C Member 45	ENSG00000260811	33252
285.013	PTPRN2	protein tyrosine phosphatase receptor type N2	ENSG00000155093	103;228;103;243;103;000
285.013	SEPTIN9	Septin 9	ENSG00000184640	53;518;526;505;263;600;000
285.013	TEX51	Testis Expressed 51	ENSG00000237524	–64278
229.412	KCNE1B	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B	ENSG00000276289	–582656
44.375	KCNE1B	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B	ENSG00000276289	–373347
38.125	KCNE1B	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B	ENSG00000276289	–601625
26.875	KCNE1B	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B	ENSG00000276289	–629707
5.875	OR4F16	Olfactory Receptor Family 4 Subfamily F Member 16	ENSG00000284662	56290
3.375	KCNE1B	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B	ENSG00000276289	–398844
3.125	KCNE1B	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B	ENSG00000276289	–405114
525	TCF21	Transcription Factor 21	ENSG00000118526	132
365	KCNE1B	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B	ENSG00000276289	–418590
275	ZNF33B	Zinc Finger Protein 33B	ENSG00000196693	663216
35	KCNE1B	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1B	ENSG00000276289	–600548

Abbreviations: P-Score-Peak-Score; TSS-Transcription Start Site

Supplementary Table S4. Putative biological role of TCF21 target genes identified in ACC cell line.

Functional Activity Categories*	Gene Symbol	Gene Name
Protein Binding	ATP10A	ATPase Phospholipid Transporting 10A
	CNTNAP2	Contactin Associated Protein 2
	DOCK1	Dedicator of Cytokinesis 1
	FMN2	Formin 2
	GOLGA8F	Golgin A8 Family Member F
	GOLGA8G	Golgin A8 Family Member G
	GZMB	Granzyme B
	OTOL1	Otolin 1
	PDS5A	PDS5 Cohesin Associated Factor A
	PRR20E	Proline Rich 20E
	RGPD2	RANBP2 Like-GRIP Domain Containing 2
	SEPTIN9	Septin 9
	TNS3	Tensin 3
	USP25	Ubiquitin Specific Peptidase 25
	ZMAT4	Zinc Finger Matrin-Type 4
	ZNF33B	Zinc Finger Protein 33B
DNA-Binding Transcription Factor	IRX1	Iroquois Homeobox 1
	IRX4	Iroquois Homeobox 4
	MEIS1	Meis Homeobox 1
	PRDM7	PR/SET Domain 7
	TCF21	Transcription Factor 21
G Protein-coupled Receptor	ADGRA1	Adhesion G Protein-Coupled Receptor A1
	OR11H1	Olfactory Receptor Family 11 Subfamily H Member 1
	OR4C45	Olfactory Receptor Family 4 Subfamily C Member 45
	OR4F16	Olfactory Receptor Family 4 Subfamily F Member 16
Voltage-Gated Ion Channel	CACNA1B	Calcium Voltage-Gated Channel Subunit Alpha1 B
	KCNE1B	Potassium Voltage-Gated Channel Subfamily E-1B
	KCNJ18	Potassium Inwardly Rectifying Channel Subfamily J-18
GTP Binding	GTPBP6	GTP Binding Protein 6
	TUBA3C	Tubulin Alpha 3c
Catalytic Activity	BDH1	3-Hydroxybutyrate Dehydrogenase 1
DNA Binding	MTERF1	Mitochondrial Transcription Termination Factor 1
Ion Channel Binding	DPP10	Dipeptidyl Peptidase Like 10
Kinase Activity	TPK1	Thiamin Pyrophosphokinase 1
Protein Tyrosine Phosphatase Activity	PTPRN2	Protein Tyrosine Phosphatase Receptor Type N2

Note: *according to the Gene Ontology Resource; Abbreviation: ACC-adrenocortical carcinoma cell line