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RCAN 1 and 3 proteins regulate thymic positive selection

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ABSTRACT

Cooperation between calcineurin (CN)-NFATc and RAF-MEK-ERK signaling pathways is essential in thymocyte positive selection. It is known that the Regulators of Calcineurin (RCAN) proteins can act either facilitating or suppressing CN-dependent signaling events. Here, we show that *RCAN* genes are expressed in lymphoid tissues, and address the role of RCAN proteins in T cell development. Overexpression of human *RCAN3* and *RCAN1* can modulate T cell development by increasing positive selection-related surface markers, as well as the "Erk^{hi} competence state" in double positive thymocytes, a characteristic molecular signature of positive selection, without affecting CN activity. We also found that RCAN1/3 interact with RAF kinases and CN in a non-exclusive manner. Our data suggests that the balance of RCAN interactions with CN and/or RAF kinases may influence T cell positive selection.

1. Introduction

$CD4^+$ T and $CD8^+$ T lymphocytes play a crucial role in cell-mediated adaptive immunity against infections as well as in anti-tumoral immunity. In the thymus, T cell development requires the recognition of self-peptide-MHC complexes by immature thymocytes leading to the selection of a self-restricted and autotolerant T cell repertoire [1]. TCR signaling sets the avidity/affinity threshold that dictates thymocyte fate; driving cells to death by neglect, rescue from apoptosis by positive selection or cell deletion by negative selection [2]. Distinct signaling signature occur in thymocytes depending on whether they are being positively or negatively selected, including the CN-NFATc and the RAF-MEK-ERK MAPK signaling pathways, both essential to positive selection [1,2].

Calcium mediated activation of CN leads to CN-dependent dephosphorylation of their substrates, including the cytosolic NFATc transcription factors 1 to 4 (NFATc1-4), key regulators of the immune response. These are then translocated to the nucleus leading to cytokine gene expression via cooperation with other transcription factors [3]. In the thymus, the CN subunits CNAβ and CNB, in cooperation with NFATc3, are essential for positive selection [4–6].

As RAF/ERK and CN/NFATc regulate thymocytes positive selection and mature T cell responses, both signaling pathways need to be fine-tuned in the immune system. Indeed, CN is the target of

Abbreviations: BM, bone marrow; CIC, calcipressin (renamed as RCAN) inhibitor of calcineurin; CN, calcineurin; DP, double positive; DN, double negative; mAb, monoclonal antibody; NFATc, cytosolic Nuclear Factor of Activated T-cells; RCAN, Regulator of Calcineurin; SP, single positive; WT, wild type.

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the immunosuppressive drugs cyclosporine A and FK506 (Tacrolimus). However, prolonged treatment with these drugs is associated with severe side effects [7]. As an emergent alternative, several endogenous inhibitors of CN have been described, including the family of Regulators of Calcineurin (RCAN) proteins [8]. RCAN proteins, which are highly conserved from yeast to human (RCAN1, RCAN2 and RCAN3 in human) [9,10], bind to CN and inhibit NFATc-mediated gene expression in human T cells [9,11]. Nevertheless, *in vivo* functional studies have emphasized the dual role of RCAN proteins, either promoting or suppressing CN signaling, depending on the different RCAN motifs and the cellular context [12–16].

Recent data indicates that RCAN1-1 isoform affects T cell development in a CN pathway-independent manner, suggesting that additional interactions with other signaling pathways can occur [17]. In this context, several proteins involved in TCR signaling are known to interact with members of RCAN family, including IRAK-1 inhibitor Tollip [18], the NF-κB-inducing kinase (NIK) [19] and the kinase CRAF [20].

In order to gain information about the function of RCAN proteins in the immune system, we determined the gene expression of *mRcan* family members in mouse adult and embryonic lymphoid tissues. Moreover, overexpression of human *RCAN3* (hRCAN3) and of human *RCAN1* (hRCAN1) in two different mice models show that *hRCAN1/3* influences positive selection-related signals.

2. Material and methods

2.1. Mice

Ten to twelve week-old C57BL/6 female mice purchased from Harlan Laboratories were used for BM reconstitution experiments and for *Rcan* gene expression analysis. PAC77 *RCAN1* transgenic mice (TghRCAN1), was generated by introducing a 135 kb PAC genomic clone into mixed genetic background C57BL/6 x CBA mice (Supporting Methods). Seven to twelve week-old female TghRCAN1 mice were used. Mice were maintained at the IDIBELL facility in accordance with Institutional guidelines. Animal protocol procedure was approved by Ethics committee of the institution (EC-IDIBELL) and for CEA-Generalitat de Catalunya (Catalonian Government Ethical Committee) (protocol 5769). The procedure meets local and national legislation, which is a transposition of the 2010 63 EU directive.

2.2. Genotyping and characterization of hRCAN1 expression in TghRCAN1 mice

TghRCAN1 mice genotyping was carried out by DNA amplification of the 3′ UTR of the *hRCAN1* gene from animal tail (Supporting methods). Mouse endogenous IL2 precursor locus DNA amplification was used as loading DNA control (Table S1). *hRCAN1-1* and

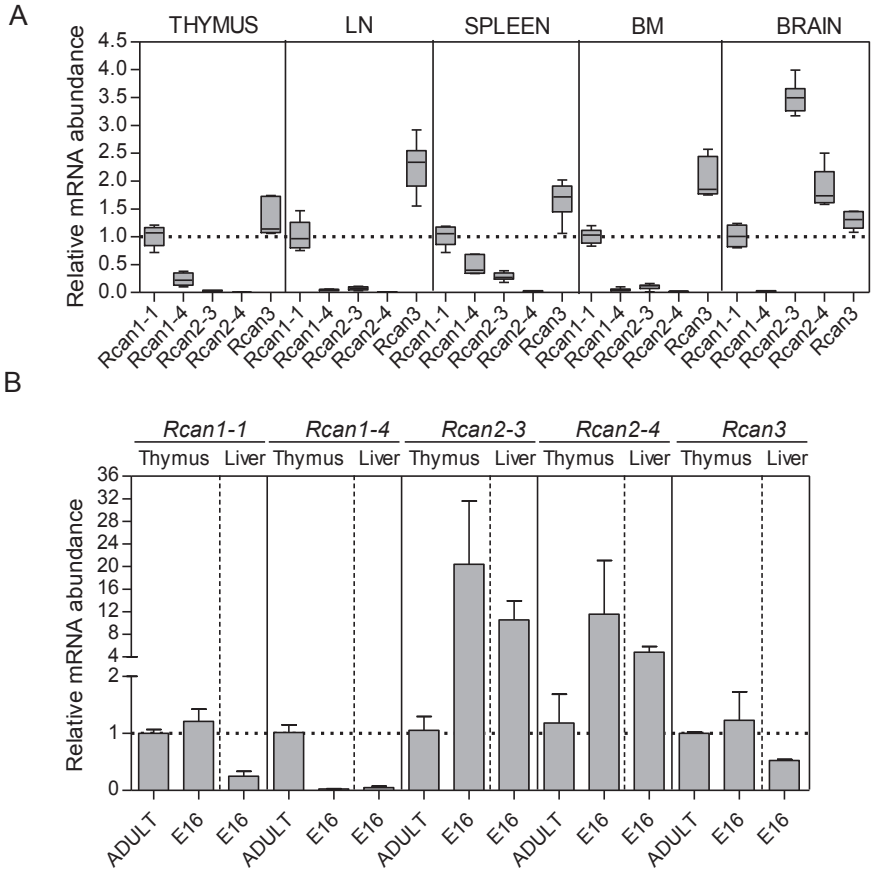

Fig. 1. *Rcan* mRNAs are expressed in adult and embryonic mouse lymphoid tissues. Quantification of mouse *Rcan* transcript variants was performed by q-PCR analysis of cDNA samples from adult and E16 embryonic tissues. (A) Relative abundance of *Rcan* transcripts in adult lymphoid tissues. As a positive control of *Rcan* gene expression [25], brain tissue is shown. Values were normalized to *Hprt* and the abundance of each mRNA form is shown relative to the expression levels of *Rcan1-1* in each specific tissue; $n \geq 5$. (B) Relative abundance of *Rcan* mRNA forms in E16 fetal thymic lobes and liver. Bars represent the mean value ± SEM of two pools of fetuses from different litters collected at the same developmental stage analyzed independently. Values were normalized to *Hprt* gene expression and then relative to its own expression level in adult thymus. (A and B) Horizontal dashed line corresponds to the expression value of 1.

hRCAN1-4 overexpression was confirmed at mRNA level by semi-quantitative PCR using specific primers (Table S1) and normalized to mouse *Gapdh* gene expression values. mRNA samples were resolved in 8% acrylamide continuous gel and stained with EtBr. TghRCAN1 tissues were lysed in RIPA buffer (Supporting methods), and 80 μg of soluble extract were used to western blot analysis using anti-hRCAN1 antibody.

2.3. Real time quantitative PCR (qPCR) analysis of mouse Rcan transcripts

Total RNA was isolated from fetal liver and thymi (E16 day of gestation) and adult (10–12 weeks) lymphoid organs (spleen, thymus, BM and maxillary, axillar and inguinal lymph nodes) and adult brain using TRIzol® Reagent (Invitrogen Corporation, Carlsbad, USA) according to the manufacturer's protocol. Total RNA (1.7 μg) previously treated with DNAse™ (Applied Biosciences, USA), was reverse transcribed using the Superscript II enzyme (Invitrogen Corporation, Carlsbad, USA) and analyzed by qPCR using specific primers and UPL probes (Table S2) using the $2^{-\Delta\Delta CT}$ method [21] as previously described [11]. *Hprt1* and *Gusb* genes were used as internal controls of cDNA amplification, and results are shown normalized to *Hprt1* values.

2.4. Anti-RCAN1 and anti-RCAN3 antibody generation

Monoclonal antisera against hRCAN1 protein and hRCAN3 protein were generated at the Technological Center LEITAT, Biomed Division (Barcelona, Spain), by immunizing mice with the synthetic peptide RPEYTPIHLS at the C-terminus of human RCAN1 protein (from Peptide 2.0, Chantilly, VA, USA) or with amino acids 2 to 65 of human RCAN3 protein grown in bacteria, respectively (depicted in Fig. S2). IgG fractions were purified using protein G affinity chromatography (GE Healthcare, USA) following the manufacturer's protocol.

2.5. Reconstitution of mice with human RCAN3-transduced BM cells

The human full length HA-tagged *RCAN3* sequence was subcloned in the retroviral vector pMIG (Addgene plasmid 9044; [22]). The protocol from Shah and colleagues [23] was used to reconstitute animals with retrovirally transduced BM cells with slight modifications.

2.6. Flow cytometry

Thymocytes where isolated from fresh thymus by forcing the tissue through a 70 μm nylon mesh and maintained in 10% FBS RPMI media on ice. 3×10^6 cells were stained with fluorescence-conjugated antibodies against surface markers (Table S3). For intracellular staining of phosphoproteins, thymocytes were stimulated with 10 μg/ml anti-CD3 (Immunotools, Germany) plus anti-CD28 antibody (BD Pharmigen, USA) and crosslinking with 10 μg/ml of anti-hamster IgG (ThermoScientific Pierce, USA) for 3 min (for pERK, pAKT and pP38 detection) or for 15 min (for pSAP/JNK detection) at 37 °C. Afterwards, cells were fixed and permeabilized with Lyse/Fix and Perm Buffer II (BD Biosciences, USA) and stained with specific anti-phosphoprotein primary antibodies (Table S3),

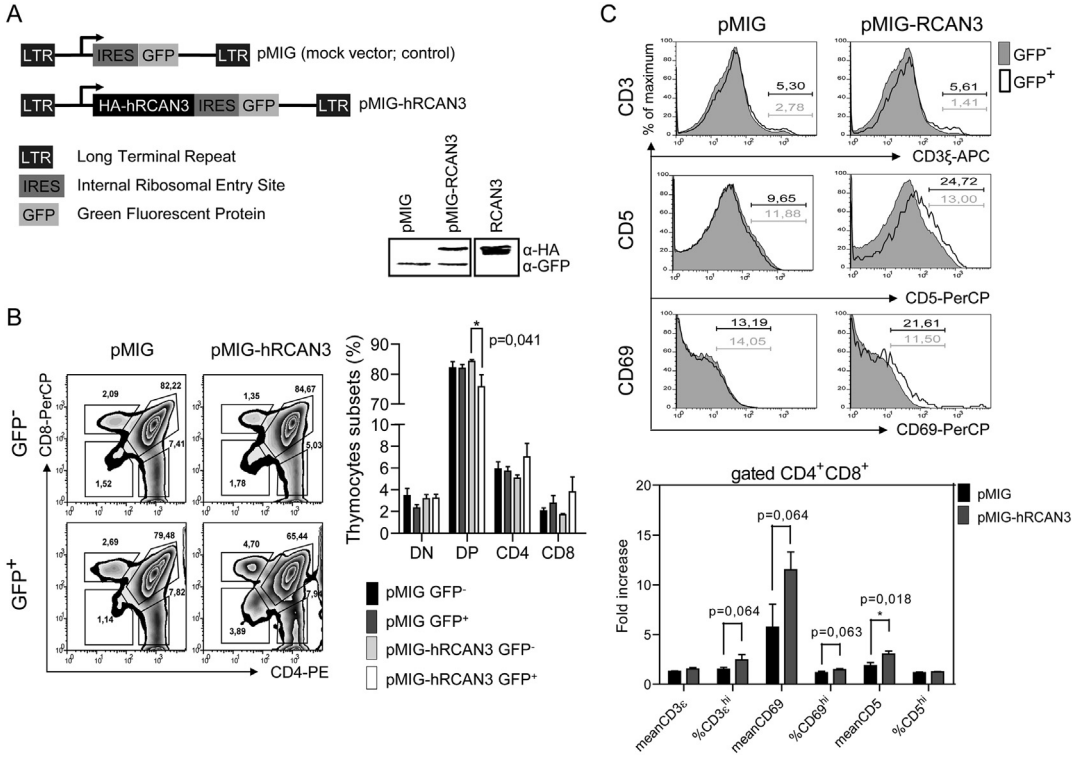

Fig. 2. *hRCAN3* overexpression increases positive selection of thymocytes. (A) Representation of retroviral constructs used to transduce BM progenitors and estimation of the transduction efficiency of the retrovirus in NIH/3T3 cells. HEK293T cells overexpressing HA-hRCAN3 were used as a positive control. (B) Representative dot plots (left panel) and global analysis (right panel) of CD4 versus CD8 staining of thymus in both GFP^- (endogenous) and GFP^+ (transduced) gated subpopulations from pMIG and pMIG-hRCAN3 reconstituted animals. (C) Upper panel, representative histograms showing CD3ξ, CD69 and CD5 staining in gated DP thymocytes from pMIG and pMIG-hRCAN3 mice. Lower panel, global analysis of MFI in CD3, CD5 and CD69, and $CD3\xi^{hi}$, $CD5^{hi}$ and $CD69^{hi}$ populations in gated DP subset. Values show the value in gated GFP^+ cells in each mouse normalized to the values of their GFP^- cells. (B and C) Diagram bars represent mean ± SEM of four independent experiments, $n \geq 7$.

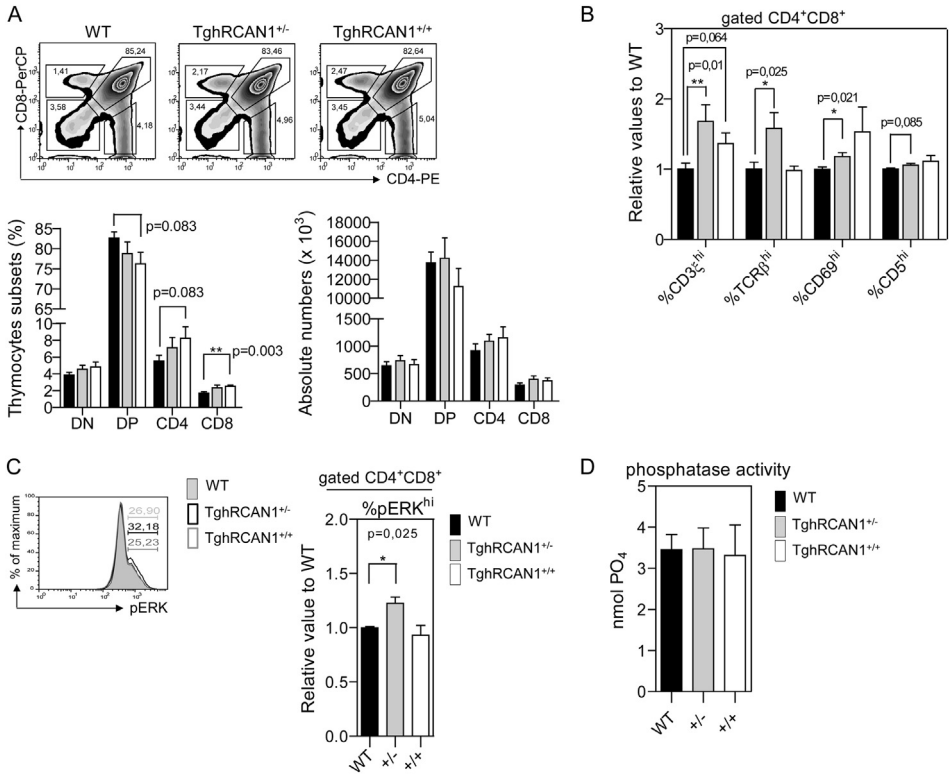

Fig. 3. Overexpression of the *hRCAN1* gene increases positive selection of thymocytes. (A) Upper panel, representative CD4 versus CD8 staining dot plots from adult WT, TghRCAN1$^{+/-}$ and TghRCAN1$^{+/+}$ thymi. Lower panel, percentage (left panel) and total cells (right panel) of each thymocyte subset from each genotype. (B) Global analysis of CD3ξ^{hi}, TCRβ^{hi}, CD69^{hi} and CD5^{hi} subpopulations in gated DP thymocytes from each genotype. (C) Left panel, representative histogram of pERK levels in gated DP thymocytes after anti-CD3 and anti-CD28 stimulation. Right panel, analysis of the percentage of pERK^{hi} subpopulation in gated DP thymocytes from each genotype. (A, B and C) Data represents mean ± SEM of at least two independent experiments, n ≥ 6. (B and C) Values in bar diagrams are shown relative to the mean value for WT mice within each experiment. (D) *In vitro* phosphatase activity of CN in thymocytes from the different genotypes. Mean values ± SEM are depicted, (WT and TghRCAN1$^{+/-}$ n = 3; TghRCAN$^{+/+}$ n = 2).

and subsequently stained with anti-rabbit AlexaFluor 467 (Invitrogen/Life Technologies) and fluorochrome-conjugated antibodies against surface markers (see Table S3). In all cases, dead cells were gated out based on the forward scattering (FSC) and side scattering (SSC) profile. Samples were captured in a FACSCalibur (BD Biosciences) cytometer and data was analyzed with FlowJo Tree Star software.

2.7. Calcineurin phosphatase activity

CN phosphatase activity from thymocytes towards phosphorylated RII substrate was analyzed using the calcineurin cellular assay kit (ENZO Life Sciences) as previously described [24]. Each reaction was performed in the presence of 40 units of human recombinant CN.

2.8. Pull-down and co-immunoprecipitation assays

Jurkat T cells (2.5×10^7) were used for pull-down experiments as described in Ref. [11]. For coimmunoprecipitation (coIP) assays, G protein Sepharose beads (GE Healthcare) were incubated with 2 μg of anti-BRAF (Santa Cruz, CA USA), or unrelated IgG, for 3 h at 4 °C, washed and incubated with soluble extracts from Jurkat T cells and analyzed by western blot using anti-CRAF (1:1000), anti-BRAF (1:500) and anti-CN A (1:500) antibodies (BD Biosciences).

2.9. Statistical analysis

Non-parametric Mann–Whitney test (two-tailed; unpaired) was used to calculate statistical significance using the SPSS 15.0 software (SPSS, USA). Asterisks indicate $^*p$ value ≤ 0.05, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. p-values below 0.1 are also indicated.

3. Results and discussion

3.1. Mouse Rcan genes are differentially expressed in adult and embryonic lymphoid tissues

RCAN1 gene encodes two major protein products, RCAN1-1 and RCAN1-4. In turn, *RCAN2* gene encodes RCAN2-3 and RCAN2-4 isoforms. In contrast, one unique protein product has been detected for human and mouse *RCAN3* [9,10,25]. Since a precise description of *RCAN* gene expression in lymphoid tissues has not previously been performed, we first investigated the expression pattern of mouse *Rcan* genes. As shown in Fig. 1A, all *Rcan* mRNA forms are detected in adult lymphoid tissues. Notably, in lymphoid tissues, both *Rcan1-1* and *Rcan3* are the major forms, with *Rcan3* being the most abundant. In contrast, *Rcan1-4* and *Rcan2-3* were expressed at lower levels, while *Rcan2-4* mRNA was almost undetectable.

Since early T cell development initiates with the recruitment of lymphoid progenitors to the thymic anlage from 13.5 days of gestation, we also analyzed *Rcan* gene expression in E16 fetal thymi and compare it to that in fetal liver, an organ with a hematopoietic function during the fetal stage. Expression of *lymphocyte antigen 75*/CD205 (*Ly75/DEC-205*) gene was analyzed to corroborate the purity of embryonic thymi [26] (Fig. S1). Interestingly, both *Rcan1-1* and *Rcan3* mRNAs are similarly abundant in fetal and adult thymi, being lower in embryonic livers (Fig. 1B). These results suggest that both transcripts may play a role in thymocytes in both the adult and

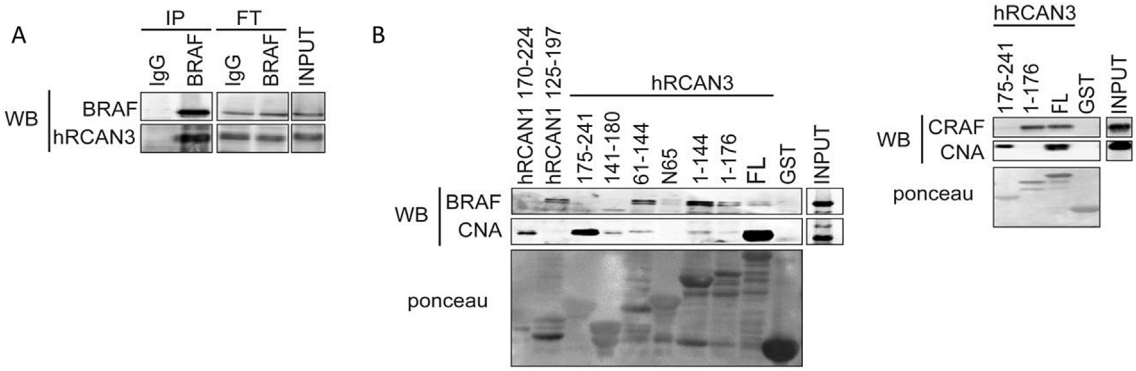

Fig. 4. RCAN1-1 and RCAN3 proteins interact with RAF protein kinases and CN in a non-exclusive manner. (A) Endogenous BRAF protein was immunoprecipitated from Jurkat T cells with anti-BRAF mAb, and mouse IgG as a control, and analyzed for its binding to RCAN3 by WB with anti-hRCAN3 antibody. (B) Pull down experiments using recombinant GST-RCAN1-1 and GST-RCAN3 fusion proteins and deletion mutants as a bait and soluble protein extract from Jurkat T cells as source of endogenous CN and RAF proteins. Samples were analyzed by WB and analyzed with anti-BRAF, anti-CRAF and anti-CNA mAb.

early developmental stages. In addition, *Rcan1-4* is expressed preferentially in adult thymus whereas *Rcan2* transcripts are increased in both fetal tissues compared to adult thymus (Fig. 1B). This switch in *Rcan* gene expression from fetal to adult thymi might be associated with the distinct nature of signals occurring in the thymic niche at these ages.

All these data indicate that *Rcan* transcripts may have specific roles instead of redundant roles in the immune system.

3.2. *hRCAN3 overexpression promotes the expression of positive selection-related markers*

Since *Rcan1* and *Rcan3* were preferentially expressed in adult thymi (Fig. 1A), we next evaluated their putative role in thymocytes development. Since *Rcan3* transgenic mouse is not available, we performed reconstitution experiments with retrovirally transduced BM cells to overexpress *hRCAN3*. As a control, pMIG vector (mock vector) was used (Fig. 2A).

In order to explore the functional role of RCAN3 in T cell development, we first analyzed thymocyte populations, comparing GFP^+ versus GFP^- populations from pMIG and pMIG-hRCAN3 reconstituted mice. Our data revealed that mice that received BM cells overexpressing *hRCAN3* showed a significant decrease in double positive (DP; $CD4^+CD8^+$) cells with a trend towards an increase of single positive (SP; $CD4^+$ or $CD8^+$) populations (Fig. 2B).

Subsequently, we analyzed the impact of *hRCAN3* overexpression on the acquisition of positive selection-related markers such as CD3ε, CD5 and CD69 on the surface of DP cells [27–29] (Fig. 2C). *hRCAN3* overexpression increases the percentage of $CD3\xi^{hi}$, $CD69^{hi}$ cells, as well as the MFI (mean fluorescence intensity) of CD69 and CD5 within gated DP thymocytes, suggesting a functional association between hRCAN3 upregulation and the increase of positive selection-related signals.

3.3. *hRCAN1 overexpression promotes the expression of positive selection-related markers*

Considering that *mRcan1-1* and *mRcan3* mRNA forms are the most abundant in thymocytes (Fig. 1) and the high amino acid sequence identity between them (Fig. S2), it would be expected that some RCAN3 and RCAN1 functions could overlap. Hence, we next analyzed a transgenic mouse containing the complete *hRCAN1* gene including its 5′ flanking region containing both promoters regions that regulate *hRCAN1-1* and *hRCAN1-4* expression (TghRCAN1). All TghRCAN1 animals were viable, fertile and displayed no macroscopic general abnormalities, but a reduced body weight compared to wild type (WT) animals (Fig. S3A). Overexpression of both *hRCAN1-1* and *hRCAN1-4* in lymphoid tissues was confirmed at the mRNA (Fig. S3B) and protein (Fig. S3C) level.

We next tested whether *hRCAN1* overexpression influenced T cell development. As shown in Fig. 3A, *hRCAN1* overexpression decreased the percentage of DP thymocytes and significantly increased SP $CD8^+$, and to a lesser extent $CD4^+$ cells. When analyzing the impact of *hRCAN1* overexpression on the acquisition of positive selection related markers within DP cells, we observed a significant increase of $CD3\xi^{hi}$, $TCR\beta^{hi}$ and $CD69^{hi}$ subpopulations and, to a lesser extent, of $CD5^{hi}$ subpopulation in $TghRCAN1^{+/-}$ mice compared to the other genotypes (Fig. 3B). $TghRCAN1^{+/+}$ mice showed more variability with a tendency towards an increase in $CD69^{hi}$ and $CD3\xi^{hi}$ populations (Fig. 3B).

It has been previously described that positive selection of DP thymocytes depends on their ability to achieve an "ERK^{hi} competence" state, where cells are able to phosphorylate and activate ERK in response to low-avidity ligands [6]. It is noteworthy that only a small population among DP is able to achieve this high phosphorylation status of ERK [6]. Therefore, we analyzed ERK activation in DP thymocytes from TghRCAN1 mice. In accordance with the increase of positive selection markers in DP cells, we observed a significant increase in the percentage of $pERK^{hi}$ in DP cells from $TghRCAN1^{+/-}$ mice (Fig. 3C), but not in other downstream TCR signaling kinases such as pAKT, pP38 and pJNK in DP cells (data not shown).

Therefore, using two different *in vivo* models, our results indicate that overexpression of both *hRCAN1* and *hRCAN3* proteins increase positive selection related signals in the thymus, suggesting, to some extent, a functional overlapping role of these proteins during T cell development.

Interestingly, a different transgenic model specific for the hRCAN1-1 isoform, not including the hRCAN1-4 isoform, showed an impairment of positive selection, reducing the thymic production of both $CD4^+$ and $CD8^+$ T cells in heterozygous animals [17]. These dissimilar results suggest that differential expression of hRCAN1 isoforms differentially affects thymocyte development. Indeed, in our results, the effects on positive selection markers in TghRCAN1 were more evident in heterozygous animals. This could be explained by the reduced protein levels of RCAN1-4 in homozygous compared to heterozygous animals (Fig. S3C), likely due to the higher expression of RCAN1-1 isoform, which could inhibit NFATc-dependent RCAN1-4 gene expression.

It is known that the acquisition of "ERK^{hi} competence" state among DP undergo positive selection requires the CN-NFATc signaling pathway [6]. Due to the described role of RCAN proteins as modulators of CN activity towards NFATc, we next analyzed the CN enzymatic activity of thymocytes from our TghRCAN1 model. Surprisingly, we did not find differences in CN activity (Fig. 3D), similarly to that reported in transgenic mice overexpressing exclusively the hRCAN1-1 isoform [17]. This suggests that hRCAN1 may be exerting its effects on thymocyte selection through the interaction with other proteins different to CN.

3.4. RCAN1-1 and RCAN3 interact with RAF kinases

It is well established that RCAN proteins interact with other proteins other than CN, thereby modulating other signaling pathways [18,19,30,31]. Indeed, RCAN1-4 isoform is able to interact with CRAF kinase [20]. However, no studies have yet addressed the possible interaction between RCAN1-1 and RCAN3 with RAF proteins. By coimmunoprecipitation assays, we were able to determine that endogenous BRAF coimmunoprecipitates with endogenous hRCAN3 (Fig. 4A). In addition, RCAN1 and RCAN3 are able to interact with both BRAF and CRAF kinases. Furthermore, this interaction of RAF kinases on RCAN takes place in a different site from that previously described for the CNA interaction [9], and in a non-mutually exclusive manner, reinforcing the idea that CN-NFATc, RAF/ERK signaling pathways and RCAN proteins may be closely regulated.

In conclusion, we evidence that *mRcan* genes are expressed in lymphoid tissue and that overexpression of *hRCAN3* and *hRCAN1* in two mice models influence positive selection-related signals and thymocyte development.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.029.

Transparency document

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