Archivum Immunologiae et Therapiae Experimentalis (2020) 68:18
https://doi.org/10.1007/s00005-020-00583-5

ORIGINAL ARTICLE

# WNT7A Expression is Downregulated in T Lymphocytes after T-Cell Receptor Activation Due to Histone Modifications and in T-ALL by DNA Methylation

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Received: 8 October 2019 / Accepted: 19 May 2020 / Published online: 26 May 2020


## Abstract

WNT signaling pathway regulates several processes involved in the homeostasis of normal cells. Its dysregulation is associated with pathological outcomes like cancer. We previously demonstrated that downregulation of WNT7A correlates with higher proliferation rates in acute lymphoblastic leukemia. However, the regulation of this gene in pathological and normal conditions remains unexplored. In this work, we aimed to analyze the transcriptional regulation of WNT7A in leukemic cells and in normal T lymphocytes after a proliferative stimulus. WNT7A expression was measured in blood cells and in T lymphocytes after phytohemagglutinin-L (PHA-L) treatment or T-cell receptor (TCR) activation by qPCR and Western blot. Promoter methylation was assessed using methylation-sensitive restriction enzymes, and histone modifications were determined by chromatin immunoprecipitation and qPCR. In T-cell acute lymphoblastic leukemia (T-ALL), WNT7A expression is silenced through DNA methylation of CpG island in the promoter region. In normal peripheral blood cells, WNT7A is mainly expressed by monocytes and T lymphocytes. TCR activation induces the downregulation of WNT7A in normal T lymphocytes by changes in histone methylation marks (H3K4me2/3) and histone deacetylases. A proliferative stimulus mediated by IL-2 keeps WNT7A expression at low levels but in the absence of IL-2, the expression of this gene tends to be restored. Furthermore, after TCR activation and WNT7A downregulation, target genes associated with the WNT canonical pathway were upregulated indicating an independent activity of WNT7A from the WNT canonical pathway. WNT7A expression is silenced by long-term DNA methylation in T-ALL-derived cells and downregulated by histone modifications after TCR activation in normal T lymphocytes.

**Keywords** WNT signaling · Epigenetics · Transcription regulation · T lymphocytes · Leukemia · DNA methylation · Histone modification

## Introduction

The WNT signaling is an evolutionary conserved pathway that regulates several processes like differentiation, self-renewal, polarity and proliferation during development, homeostasis

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and regeneration of many tissues (Staal et al. 2016). This pathway comprises 19 different ligands, at least 10 Frizzled receptors, 2 co-receptors (LRP5/6) and some molecules like the Dickkopf family or the secreted Frizzled-related proteins that might regulate its activity (Niehrs 2012). WNT signaling is often classified as canonical or WNT/β-catenin pathway and non-canonical pathways associated with $Ca^{2+}$ signals or JNK kinases (Planar Cell Polarity) that are independent of β-catenin and are associated with cell movement (Staal et al. 2008).

WNT ligands are glycoproteins with conserved cysteine residues that can induce the canonical or non-canonical pathways after the interaction with Frizzled receptors (Willert and Nusse 2012). Among these, WNT7A is a 39-kDa glycoprotein that has been implicated in female reproductive tract development (Huang et al. 2014; Parr and McMahon 1998), maintenance of the blood–brain barrier, blood–retina barrier (Wang et al. 2018) and corneal epithelium (Kuznetsova et al. 2015). Previous reports have established that downregulation of this protein is associated with higher proliferation rates in several types of cancer, for example, non-small cell lung carcinoma (Stewart 2014; Tennis et al. 2012; Winn et al. 2005), renal cell carcinoma (Kondratov et al. 2012; Xu et al. 2016) and cervical cancer (Ramos-Solano et al. 2015). In a previous report Ochoa-Hernandez et al. (2012) have demonstrated that T-cell acute lymphoblastic leukemia (T-ALL)-derived cells have almost undetectable levels of WNT7A in comparison to normal resting peripheral lymphocytes. Low expression of WNT7A was corroborated in samples of T-ALL patients. In addition, the treatment of these cells with recombinant WNT7A or its ectopic expression using lentiviral vectors have a negative impact on cell proliferation (Ochoa-Hernandez et al. 2012). The negative correlation between WNT7A expression and cell proliferation has been observed in different neoplasias (Kim et al. 2015). These evidences indicate a possible role of WNT7A in the regulation of cell proliferation; moreover, the fact that WNT7A is normally expressed in peripheral $CD3^+$ T cells indicates that this gene could be necessary to maintain a resting phenotype. Therefore, it is important to describe the regulatory mechanisms of gene expression for this gene and the role of WNT7A protein in the biology of normal mature blood cells. As several reports have shown that this ligand is regulated through epigenetic modifications, in this study, we aimed to analyze the transcriptional regulation of WNT7A in T-ALL-derived cells and normal T lymphocytes under resting and activated conditions.

## Materials and Methods

### Isolation of PBMCs, Monocytes, NK, T and B Cells

Peripheral blood mononuclear cells (PBMCs) obtained from healthy volunteers (10 mL of peripheral blood) were isolated by gradient-density centrifugation with Ficoll-Paque™ PLUS (GE Healthcare, Chicago, IL, USA). PBMCs were cultured for 4 h allowing the adhesion of monocytes to the dish (Style Dish, Cat. No. 430167, Corning, New York, USA); by this method, they were separated from lymphocytes. After that, lymphocytes were resuspended in phosphate-buffered saline (PBS) and stained with an anti-CD3 antibody (sc-555332-FITC, BD Pharmingen, San Jose, CA, USA) to select T lymphocytes and with an anti-CD19 antibody (sc-19650-PE, Santa Cruz Biotechnology, Dallas, TX, USA) to select B lymphocytes. After incubation with both antibodies, cells were washed and positive cells for CD3 or CD19 were sorted on a FACSAria (Becton–Dickinson, San Jose, CA, USA). For NK isolation, negative selection was performed from PBMCs using the NK Isolation Kit (Miltenyi Biotec Inc, Bergisch Gladbach, Germany) and cell purity was assessed by flow cytometry staining with antibodies specific for CD3-FITC (Cat. 130-113-128, Miltenyi Biotec Inc, Bergisch Gladbach, Germany), CD16-APC (Miltenyi Biotec Inc, Bergisch Gladbach, Germany) and CD56-PE (Miltenyi Biotec, Bergisch Gladbach, Germany) using the Attune flow cytometer (Thermo Fisher Scientific, Whaltman, MA, USA).

### Cell Culture

PBMCs, lymphocytes and T-ALL-derived cell lines—Jurkat, CEM and MOLT-4—were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100-U/ml penicillin, and 1-mg/ml streptomycin. Cultures were incubated at 37 °C in 5% $CO_2$ atmosphere. All products mentioned before were acquired from Gibco®, Thermo Fisher Scientific, Waltham, MA, USA.

### Activation and Proliferation Induction

PBMCs were obtained from healthy volunteers and the lymphocytes fraction was obtained as previously described. For T-cell receptor (TCR) activation, two methods were used: the Dynabeads Human T-Activator CD3/CD28 (Gibco®, Thermo Fisher Scientific, Waltham, MA, USA) and the T-Cell Activation/Expansion Kit that consists of a system based on the use of Anti-Biotin MACSiBeads Particles and biotinylated antibodies against human CD2, CD3 and CD28 (Miltenyi Biotec Inc, Bergisch Gladbach, Germany). In all cases, $5 \times 10^5$ lymphocytes were used for the experiments. To induce proliferation, recombinant Human IL-2 carrier-free (Biolegend, San Diego, CA, USA) and phytohemagglutinin-L (PHA-L) mitogen (Roche Diagnostic, Basel, Switzerland) were used as described by the manufacturers.

## Quantitative Real-Time PCR Assays

Total RNA was isolated from $5 \times 10^6$ cells using the GeneJet RNA purification Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. 5 µg of total RNA was reverse transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) primed with oligo (dT). Gene expression levels were analyzed by quantitative real-time PCR (qPCR) assays using the LightCycler 2.0 system and the LightCycler FastStart DNA Master PLUS SYBR Green I Kit (Roche Applied Science, Penzberg, Germany). Gene-specific primers were designed with Oligo v.6.0 software (Molecular Biology Insights, Inc., Colorado Springs, CO, USA) using sequences obtained from the National Center of Biotechnology Information. Ribosomal Protein L32 (RPL32) and Ribosomal Protein S18 (RPS18) were utilized as housekeeping genes. All primers and sequences that were used are shown in Table 1.

## Immunoblot Analysis

T lymphocytes ($5 \times 10^6$) were cultured in RPMI medium and activated of either PHA-L or beads against CD2/CD3/CD28 as previously described or treated with 20-mM LiCl (Sigma-Aldrich, St. Louis, MO, USA). Following activation or treatment, cells were lysed in 200-µl RIPA buffer (150-mM NaCl, 1% NP-40, 0.5% SDS, 50-mM Tris pH 8) containing protease inhibitors (Roche, Basel, Switzerland). Then, samples were incubated on ice for 30 min for further sonication (30″ON/30″OFF, 10 cycles) using the Bioruptor® Pico Device (Diagenode, Denville, NJ, USA). Protein extracts were obtained by centrifugation (14000 rpm for 15 min at 4 °C) and quantified using the DC Protein Kit (Bio-Rad, Hercules, CA, USA). For each immunodetection, 50 µg of protein was separated by SDS polyacrylamide electrophoresis and then transferred onto a PVDF membrane (Millipore Corporation, Darmstadt, Germany). The membrane was blocked for 1:30 h with Odyssey Blocking Buffer (Li-COR Biosciences, Lincoln, NE, USA). Then, primary antibodies, Anti-WNT7A goat polyclonal (Cat. Nº sc-26361, Santa Cruz Biotechnology, Dallas, TX, USA), Anti-β-Catenin mouse monoclonal (Cat. Nº sc-59737, Santa Cruz Biotechnology, Dallas, TX, USA), Anti-Tubulin mouse monoclonal (Cat. Nº sc-8035, Santa Cruz Biotechnology, Dallas, TX, USA) and Anti-Actin mouse monoclonal (Cat. Nº sc-8432, Santa Cruz Biotechnology, Dallas, TX, USA), were incubated overnight at 4 °C. After washing with 1 × PBS Tween-20, the membrane was incubated for 2 h with a fluorophore-labeled secondary antibody: Donkey Anti-Mouse IRDye 800 CW (Cat. Nº 926-32212, Li-COR Biosciences, Lincoln, NE, USA) or Donkey Anti-Goat IRDye 680 RD (Cat. Nº 926-68074, Li-COR Biosciences, Lincoln, NE, USA). Finally, the membrane was scanned with the Odyssey Imaging System (Li-COR Biosciences, Lincoln, NE, USA).

## CpG Methylation Detection Assay

To explore the epigenetic regulation of WNT7A, the sequence 1200-bp upstream and 600-bp downstream from the transcription start site was obtained from the Eukaryotic Promoter Database (https://epd.vital-it.ch/) and the CpG islands were identified using the MethPrimer tool (http://www.urogene.org/cgi-bin/methprimer2/MethPrimer.cgi). Genomic DNA were extracted from resting and activated T lymphocytes, and T-ALL-derived cell lines with the DNeasy Blood and Tissue Kit (Qiagen, Germantown, USA), for further digestion with *MspI* and *HpaII* restriction enzymes (New England Biolabs, Inc., MA, USA) at 37 °C for 2 h. Digested DNA was used for PCR amplification with CpG specific primers using the DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and 5% DMSO (Sigma-Aldrich, St. Louis, MO, USA) and the products were visualized on 2% agarose gels.

## Chromatin Immunoprecipitation

For this purpose, $2 \times 10^7$ T lymphocytes were isolated with the PanT Isolation Kit (Miltenyi Biotec Inc, Bergisch Gladbach, Germany) from PBMCs and cell purity was assessed by flow cytometry staining with antibodies specific for CD3-FITC (Miltenyi Biotec Inc, Bergisch Gladbach, Germany) and CD2-APC (Miltenyi Biotec Inc, Bergisch Gladbach, Germany). $1 \times 10^7$ cells were activated using Dynabeads Human T-Activator CD3/CD28 (Gibco®, Thermo Fisher Scientific, Waltham, MA, USA) for 48 h. After activation, resting and activated T lymphocytes were washed with PBS and then crosslinked with 18.5% formaldehyde for 10 min at room temperature followed by quenching with 1.25-M glycine for 5 min. Cells were centrifugated at 2000 rpm for 10 min and washed with cold PBS followed by centrifugation. The pellets were then lysed in 1-mL lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl pH 8.1) and sonication was done to obtain 200–400 bp DNA fragments using the Bioruptor® Pico sonication device (Diagenode, Denville, NJ, USA). Sonicated samples were centrifugated at $14000 \times g$ at 4 °C, and supernatant was collected. Then, the lysate was equally divided for negative control and test samples. Chromatin was subject to immunoprecipitation with 2-µg antibody to H3K4me2 (Cat. 07-030, EMD Millipore, Temecula, CA, USA), H3K4me3 (Cat. 07-473, EMD Millipore, Temecula, CA, USA), H3K9me3 (Cat. 07-442, EMD Millipore, Temecula, CA, USA), H3K27me3 (Cat. 07-449, EMD Millipore, Temecula, CA, USA) and IgG rabbit as negative control using the OneDay ChIP Kit (Diagenode, Denville,

**Table 1** Primers used for PCR amplifications

| Gene | Primer sequence | Product length (bp) |
|---|---|---|
| WNT7A | F: CAA AGA GAA GCA AGG CCA GTA<br>R: GTA GCC CAG CTC CCG AAC TG | 277 |
| CD25 | F: CCT GCT GAT CAG CGT CCT C<br>R: ATG GGA CTG AGC TGG CAT AGA | 273 |
| CD40L | F: AAT CCT CAA ATT GCG GCA CAT<br>R: GTT TGG CGG AAC TGT GGG TAT T | 295 |
| CD69 | F: GGC TACA CAG AGG AAA TGC TA<br>R: CAC TTG TCA GAC CCT GTA ACG | 248 |
| CD71 | F: GGC TGT ATT CTG CTC GTG GAG<br>R: AGC TGG CAG CGT GTG AGA | 212 |
| CD95 | F: CAC CCG GAC CCA GAA TAC<br>R: AAT TAT TGC CAC TGT TTC AGG A | 275 |
| JUN | F: TGG AAA GTA CTC CCC TAA CCT<br>R: CTG AAA CAT CGC ACT ATC CTT | 250 |
| c-MYC | F: GAA CTT ACA ACA CCC GAG CAA<br>R: CAG TAG AAA TAC GGC TGC ACC | 249 |
| CCND1 | F: CGC ACG ATT TCA TTG AAC AC<br>R: CAG CAG GGC TTC GAT CTG | 296 |
| TCF4 | F: TCG GAG GAC AAG AAA TTA GAT<br>R: CGC AGC TTT CGG ATT C | 321 |
| MMP7 | F: TCG AGA CTT ACC GCA TAT T<br>R: CAG CGT TCA TCC TCA TCG AAG | 243 |
| CAPN2 | F: TCG GCT GGA AAC GCT A<br>R: CTT GTC AAG GGT TAA GCG ACT | 341 |
| MMP9 | F: CGC AAG CTG GAC TCG GTC TTT<br>R: TCA CGC GCC AGT AGA A | 355 |
| CELRS1 | F: CGC CTC CTC ACA CTC GTC AGA<br>R: CGG CGG GTA GGT GAC T | 352 |
| CCN2 | F: CGT CCC CAA GAT CAA CC<br>R: ACC TGG CTG GCA CAC TTG TTC | 327 |
| RPL32 | F: CGA TTG ACA ACA GGG TTC GTA G<br>R: ATT TAA ACA GAA AAC GTG CAC A | 320 |
| RPS18 | F: CGA TTG GCG GAA AA<br>R: CAG TCG CTC CAG GTC TTC ACG G | 283 |
| WNT7A CpG region 1 | F: TCA GTC CCT GGA GCC CGA GAG<br>R: GGA CGC GGC GGG TGG T | 321 |
| WNT7A CpG region 2 | F: GCT CCG TGG CTC CCG TGC TC<br>R: ACC GCT CCC ACA CCG CAC C | 396 |
| WNT7A −922 to −754 | F: CGT GCA AAC AGG GAA CAG G<br>R: GGA ACA GAA TGC TTT GAG TGC C | 190 |
| WNT7A −578 to −414 | F: TCG GCT AAC AGC AGG ATC CCA<br>R: CAC ACC CCA GTT CCC CAA CC | 164 |
| WNT7A −518 to −342 | F: TCG AAT AGG TGG GAG TAG GGA A<br>R: TGG AGT GGA CCA GAC TCG G | 195 |
| WNT7A 293 to 483 | F: TTC TCA GCC TGG GCA TGG TCT A<br>R: GCC TTT GTG CGT CCA CCG | 190 |

NJ, USA) as described by the manufacturers. DNA was purified using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, MO, USA) and four different regions of WNT7A promoter were analyzed by qPCR. Primers used for chromatin immunoprecipitation (ChIP)-qPCR are included in Table 1. Data were calculated as relative occupancy of the immunoprecipitated factor at a region using the following equation: $2^{(\mathrm{CpNegCtl}\ -\ \mathrm{CpTarget})}$ where CpNegCtl and CpTarget are mean amplification cycles of PCR done in duplicates on DNA samples from negative control ChIP (IgG rabbit) and targeted ChIP (using specific antibodies).

### Analysis of Histone Deacetylases

PBMCs from healthy volunteers were isolated and the lymphocytes fraction was collected as previously described. Lymphocytes were seeded at a density of $5 \times 10^6$ in 60-mm dishes (Biolite 60 mm Tissue Culture Dish, Cat. Nº 130181, Thermo Scientific, New York, USA) and stimulated with 1-µg/ml PHA-L (Roche Diagnostic, Basel, Switzerland) in the presence of 2-mM NaB (Sigma-Aldrich, St. Louis, MO, USA)—a well-known inhibitor for histone deacetylases (iHDACs)—for 48 h. After that, RNA was isolated and qPCR was carried out to assess CD69, CD25, CD71, CD40L, CD95 and WNT7A expression using RPL32 and RPS18 as reference genes.

### Statistical Methods

The data obtained are shown as mean ± standard error (SME). Mann–Whitney U test was employed to compare means among two different groups. Only $p < 0.05$ were considered significant. Statistical analysis was performed with GraphPad prism v.5 software.

### Ethics Statement

Peripheral blood samples from healthy volunteers were collected at the División de Inmunología, Centro de Investigación Biomédica de Occidente, IMSS. Written informed consent from each volunteer was required prior to blood sample collection. This study was conducted in agreement with the Declaration of Helsinki principles and was approved by the Ethical and Research Committee CLIEIS1305 with the No. R-2013-1305-7.

## Results

### Peripheral Monocytes and T Lymphocytes Express WNT7A

To analyze the expression pattern of WNT7A, RNA was isolated and retrotranscribed into cDNA from total PBMCs, monocytes, NK cells, B and T lymphocytes for further analysis by qPCR using RPL32 and RPS18 as reference genes.

WNT7A expression was detected in all populations analyzed, but interestingly most of the expression of this ligand was found in T lymphocytes and monocytes. In contrast, B lymphocytes and NK cells expressed low levels of WNT7A when compared with total PBMCs expression (Fig. 1a).

### WNT7A Expression is Modulated by Mitogenic Stimuli

As WNT7A expression was detected in resting non-proliferative cells, we wondered if this expression could be modulated by a proliferative stimulus. For this purpose, PBMCs were stimulated with PHA-L—a mitogenic stimulator that preferentially induces T lymphocyte activation and proliferation—for 24 and 48 h. After that, lymphocytes were isolated and the RNA was obtained; subsequently, WNT7A expression was measured by qPCR. As depicted in Fig. 1b, 24 h after the stimulus, WNT7A expression decreases significantly in activated lymphocytes, and a more drastic decrease was observed 48 h post-stimulus when compared with the non-stimulated lymphocytes. All these data reveal that WNT7A gene expression is negatively regulated by a proliferative stimulus in a time dependent manner.

### WNT7A Expression is Similar to Other Molecules Regulated by TCR Activation in T Lymphocytes

With the aim to compare WNT7A expression with the expression of other important molecules of T lymphocytes, some receptors involved in the activation process were analyzed: CD69, CD25, CD71, CD40L and CD95. As depicted in Fig. 1c, the expression levels of WNT7A in resting T lymphocytes were similar to the expression of CD25, which could be related to the non-proliferative state of naïve T lymphocytes. All other molecules have a higher expression when compared with WNT7A.

Furthermore, two different activation processes were carried out to evaluate whether the modulation of WNT7A was a consequence of the TCR activation. The first comprises the binding of an antibody against CD3 and CD28 (α CD3/CD28 stimulation), both molecules implicated in the TCR activation signaling. The second activation process was triggered by antibodies against CD2, CD3 and CD28 (α CD2/CD3/CD28 stimulation). Figure 1d, e shows the effectiveness of both systems to activate T lymphocytes due to the high relative expression of all activation markers, especially with the stimuli triggered by α CD2/CD3/CD28. Then, the WNT7A relative expression was assessed under these conditions and as shown in Fig. 1f, both activation stimuli decrease WNT7A expression. It is worth noticing that even though WNT7A and CD25 had similar expression levels in basal conditions, the expression of both molecules was inversed after activation indicating a negative role of WNT7A over proliferation. Finally, at the protein level, WNT7A expression was diminished after both stimuli used for T lymphocytes activation—PHA-L or CD2/CD3/CD28 beads—for 48 h (Fig. 1g). In addition, WNT7A was slightly detected at the mRNA and protein levels in T-ALL-derived cell lines (Fig. 1h, i); as positive control for WNT7A

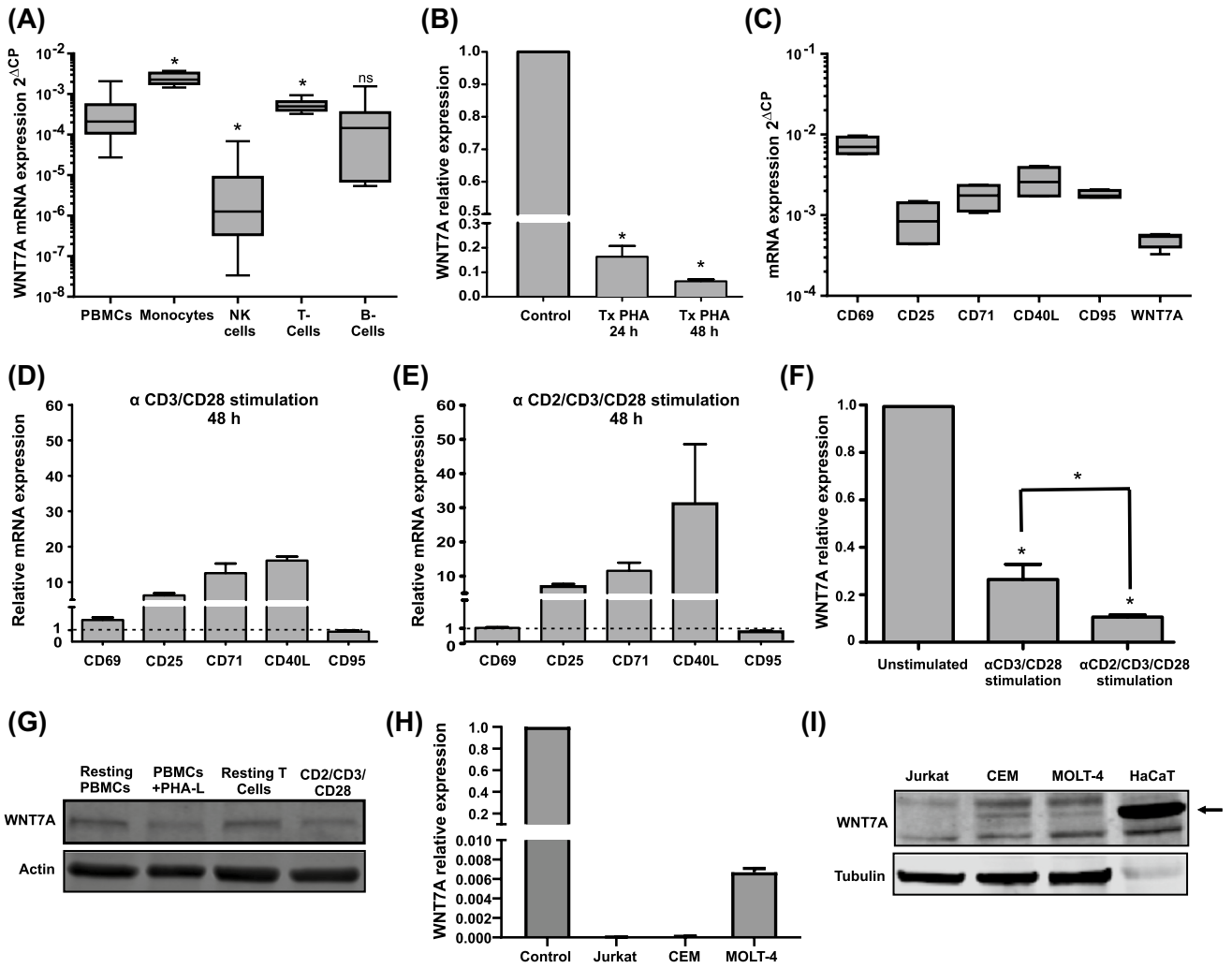


**Fig. 1** Evaluation of WNT7A expression in total PBMCs, blood cell populations, resting and activated T lymphocytes. Comparison of its expression with markers of T-cell activation. **a** Box plot graphic illustrating WNT7A expression depicted as $2^{\Delta CP}$ in total PBMCs and subpopulations. The graphic displays median (dark line), 25–75% (boxes) and interquartile ranges (whiskers). **b** WNT7A relative expression in stimulated lymphocytes with PHA-L for 24 and 48 h. Relative expression was calculated by taking the expression of WNT7A in resting lymphocytes as 1. **c** Comparison between WNT7A expression with the expression of activation markers in resting T lymphocytes depicted as $2^{\Delta CP}$. **d**–**f** Relative expression of activation markers and WNT7A in T lymphocytes after CD3/CD28 or CD2/CD3/CD28 stimulation for 48 h. Relative expression was calculated by setting the expression of all genes in resting T lymphocytes as 1. In all cases quantification was performed using RPL32 and RPS18 as reference genes. $^*p < 0.05$. **g** Immunoblot analysis reveals a reduction of WNT7A protein levels upon T lymphocytes activation with PHA-L or CD2/CD3/CD28 beads for 48 h. Actin was used as protein loading control. **h** WNT7A relative expression in LLA-T-derived cell lines: Jurkat, CEM and MOLT-4. Relative expression was calculated by taking the expression of WNT7A in healthy subjects as 1. RPL32 and RPS18 were used as reference genes. **i** Immunoblot analysis demonstrates a diminished WNT7A expression in LLA-T-derived cell lines—Jurkat, CEM and MOLT-4. HaCaT cells—non-tumorigenic keratinocytes—were used as positive control for WNT7A expression. Tubulin was used as protein loading control

protein expression, we used HaCaT cells—non-tumorigenic keratinocytes.

## WNT7A Expression Decreases in a Time-Dependent Manner and a Proliferative Stimulus Maintains this Expression Low

To study the kinetics of WNT7A regulation during T-cell activation, lymphocytes of healthy volunteers were stimulated with α CD2/CD3/CD28, and the expression of WNT7A and activation markers was measured after 24, 48, 72 and 168 h. In addition, a group of lymphocytes activated for 48 h were induced with a proliferative stimulus by adding IL-2 for 24 h (group 72-h stimulated + IL-2) and 120 h (group 168-h stimulated + IL-2) (Fig. 2). In all groups, early and late activation markers were measured, and relative expression analysis was performed setting to one their expression in basal conditions (resting, non-activated and

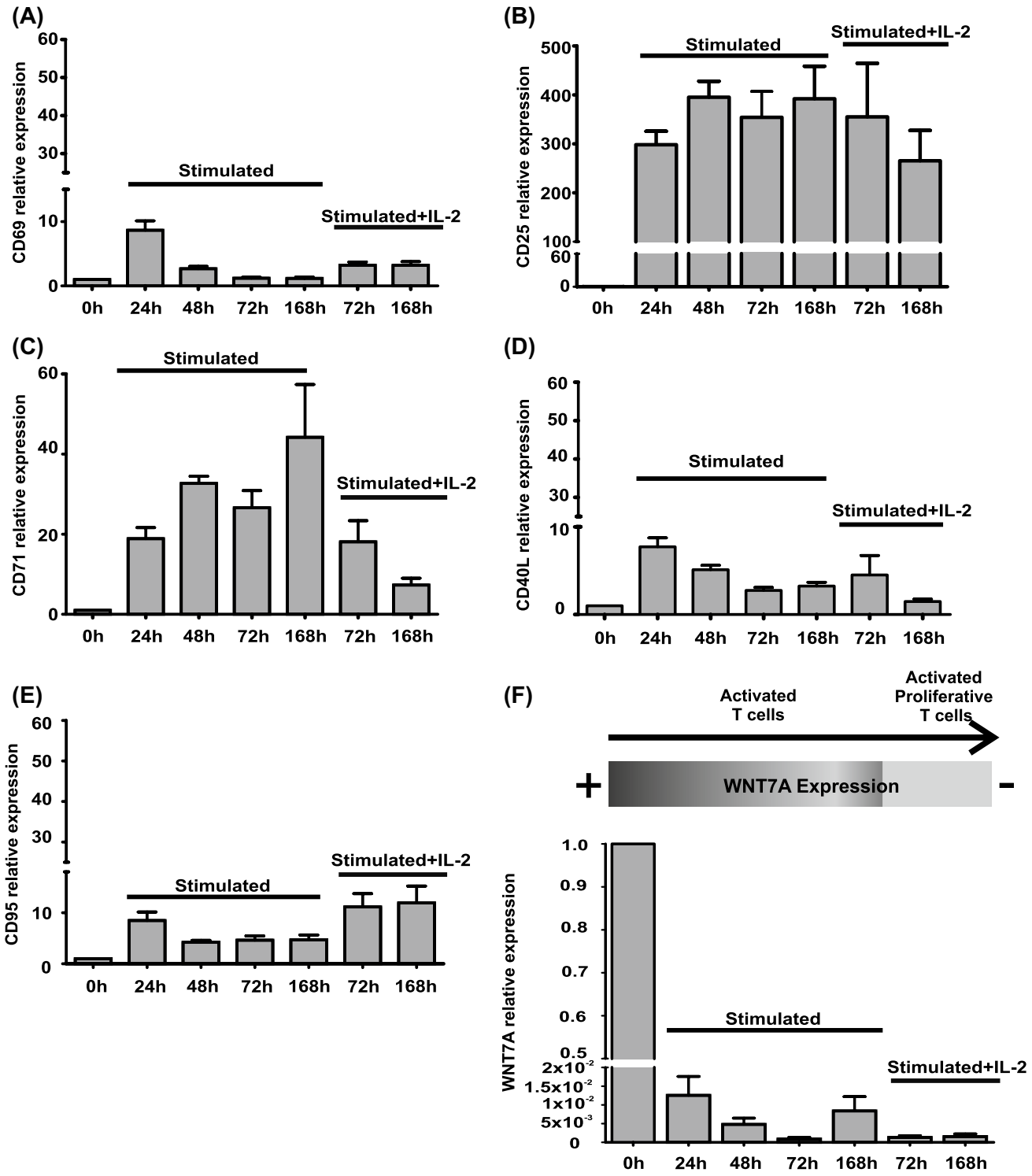


**Fig. 2** Expression of activation markers and WNT7A in activated and proliferative conditions in T lymphocytes. Graphics show relative mRNA expression of activation markers and WNT7A in activated T lymphocytes with CD2/CD3/CD28 system for 24, 48, 72 and 168 h. In addition, lymphocytes activated for 48 h received IL-2 as a proliferative stimulus for 24 and 120 h (Stimulated + IL-2, 72 h and 168 h). Quantification was performed using RPL32 and RPS18 as reference genes and relative expression was calculated by taking the resting T lymphocytes as 1

non-proliferative). As expected, early activation markers were expressed at 24, 48 and 72 h. At 168 h post-activation, the expression of CD69, CD40L and CD95 decreased due to the absence of a proliferative signal. However, in the presence of IL-2, those markers tend to maintain a high expression, especially CD95 (Fig. 2a–e). Once we proved the activated state in T lymphocytes, we measured WNT7A expression, and as expected, it decreased in a time-dependent manner, where the lowest expression was detected 72 h after TCR stimulus. Moreover, 168 h after the stimulus, the activated group without IL-2 shows a restoration of WNT7A expression (Fig. 2f). Interestingly, the proliferation signal mediated by IL-2 maintains the expression of WNT7A at low levels (Fig. 2f stimulated + IL-2 group).

## TCR Activation Induces a Rapid Regulation of WNT7A While Increases WNT Canonical Target Genes

Since we noticed that TCR activation could modulate the expression of WNT7A, we further analyzed the kinetics of WNT7A regulation. For this purpose, T lymphocytes were activated using the CD2/CD3/CD28 system for 1, 2, 4 and 8 h and the expression of WNT7A was measured. To have a comparison parameter of lymphocytes response to activation, we also evaluated the expression of the early marker CD69 (Fig. 3a). The kinetics of WNT7A regulation after TCR activation seems to be complex with an early increase in the mRNA levels of two- to threefold at 1 h and then a drastic diminish to almost undetectable levels after 8 h (Fig. 3b). In the same way, CD69 was upregulated as early as 1 h post activation but its expression starts to decrease after 4 h (Fig. 3a).

WNT7A has been reported as a non-canonical ligand in some cellular models, but there are also reports about its activity as a canonical ligand (Huang et al. 2018; Le Grand et al. 2009). To have an approximation about the status of canonical or non-canonical WNT pathways, the expression pattern of target genes associated with those pathways was evaluated. As depicted in Fig. 3c–e, after TCR activation using the α CD2/CD3/CD28 activation stimulus for 48 h, the expression of WNT canonical target genes—c-MYC, CCND1 and MMP7—was induced; in contrast, the expression of non-canonical target genes—CAPN2, MMP9, CELSR1 and CCN2—was down-modulated.

To further investigate if the activation of the WNT canonical pathway is implicated in the negative regulation of WNT7A after TCR stimulation, we analyze the kinetics of β-catenin and WNT7A protein expression for 1, 2, 4 and 8 h. As expected, β-catenin is stabilized after 1–2 h post stimulation accompanied by the WNT7A downregulation when compared with resting conditions (Fig. 3f). To confirm the role of β-catenin in the regulation of this ligand, T lymphocytes were treated with 20-mM LiCl (an inhibitor of the GSK3β protein) for the same short periods of time. As shown in Fig. 3g, β-catenin is clearly upregulated 2 h in parallel with a downmodulation of WNT7A after the inactivation of GSK3β. These results confirm that the activation of the WNT canonical pathway induces the downregulation of WNT7A expression in activated T lymphocytes.

## Different Epigenetic Mechanisms are Involved in the Negative Regulation of WNT7A in T Lymphocytes and T-ALL-Derived Cells

To better understand the epigenetic mechanisms that might be implicated in the downregulation of WNT7A expression, we analyzed several epigenetic modifications as DNA methylation, histone methylation and histone acetylation. First, the methylation status of CpG islands at WNT7A promoter was analyzed by *MspI/HpaII* restriction enzymes followed by conventional PCR. As shown in Fig. 4a, three CpG islands were found within the WNT7A promoter and gene (−1200 to +600 pb), and these CpG islands were grouped in two regions (CpG1 and CpG2). Both resting and activated T lymphocytes have the same DNA methylation profile at WNT7A promoter, indicating that this mechanism was not implicated in WNT7A expression regulation after TCR activation (Fig. 4a). In addition, since WNT7A expression is very low in T-ALL-derived cells as previously showed, we wondered whether the expression of WNT7A was downregulated through DNA methylation in those tumoral cells. As shown in Fig. 4a, hypermethylation of both CpG regions were clearly observed in Jurkat and CEM cells. Interestingly, MOLT-4 cells showed a similar pattern to normal T lymphocytes, with CpG region 1 methylated but not CpG region 2. These results indicate the possible involvement of DNA methylation in the negative regulation of WNT7A at least in a subset of T-ALL-derived cells. However, DNA methylation was not involved in WNT7A regulation induced by the TCR signaling.

## Changes in Histone Methylation Patterns are Related with Regulation of WNT7A Expression in Activated T Lymphocytes

To assess the role of histone modifications into WNT7A regulation, peripheral T lymphocytes were isolated and purified by negative selection using magnetic beads for further ChIP analysis. The efficiency of the purification was assessed by flow cytometry using CD2-APC and CD3-FITC antibodies (data not shown). Thereafter, four histone methylation marks (H3K4me2 and H3K4me3, related with transcriptional activation, and H3K9me3 and H3K27me3, related with transcriptional repression) were analyzed by ChIP-qPCR in four different WNT7A promoter and

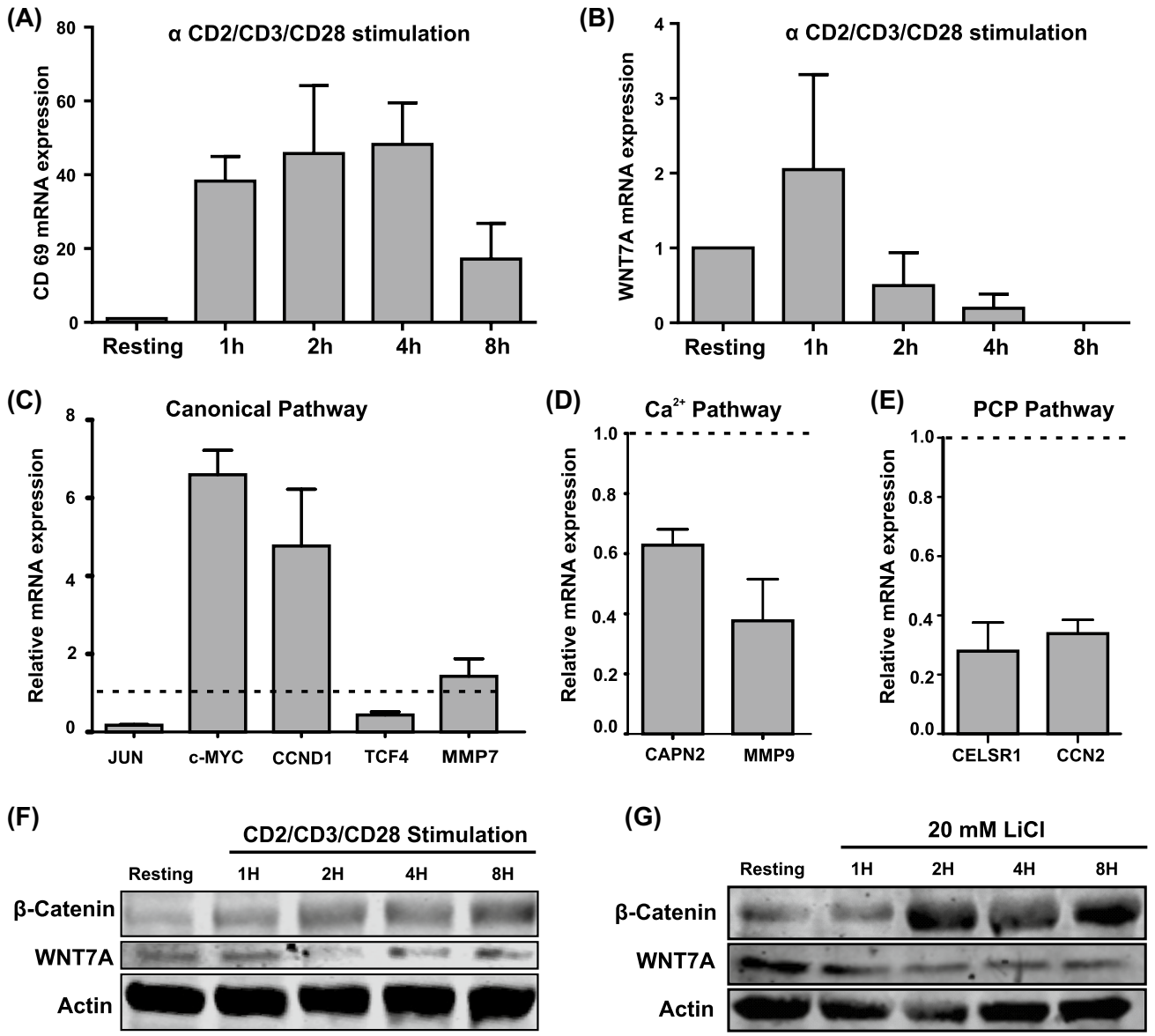


**Fig. 3** TCR signaling induces a rapid modulation of WNT7A expression, the upregulation of WNT canonical target genes and downregulation of non-canonical target genes. CD69 relative expression (**a**) and WNT7A relative expression (**b**) in activated T lymphocytes after CD2/CD3/CD28 stimulation for 1, 2, 4 and 8 h. **c**–**e** Relative expression of target genes related to WNT canonical and WNT non-canonical pathways ($Ca^{2+}$ and PCP) in activated T lymphocytes. Values were calculated taking as 1 the expression of all genes under basal conditions (resting T lymphocytes). In all cases, RPL32 and RPS18 were used as reference genes. **f**–**g** Immunoblot analysis reveals that β-catenin is stabilized after 1–2 h post TCR stimulation in T lymphocytes with a further WNT7A downregulation. Consistent with this, in T lymphocytes treated with 20 mM LiCl, (an inhibitor of GSK3) the stabilization of β-catenin occurs after 2 h accompanied by the WNT7A downregulation. In all cases, immunoblots were normalized to actin protein expression

gene regions to determine the relative occupancy for each mark (Fig. 4b). Interestingly, in all the studied regions, we observed a higher occupancy of H3K4me2/3 in resting T lymphocytes, which is in concordance with the increase in WNT7A expression. However, once the T lymphocytes were activated, the occupancy of these histone methylation marks decreases (Fig. 4b). Contrarily to what was expected, H3K9me3 and H3K27me3 marks—associated with transcriptional repression—were not increased after TCR activation.

## WNT7A Expression in Activated T Lymphocytes is also Regulated by HDACs

To address the role of HDACs in WNT7A regulation, PBMCs from healthy volunteers were activated with PHA-L

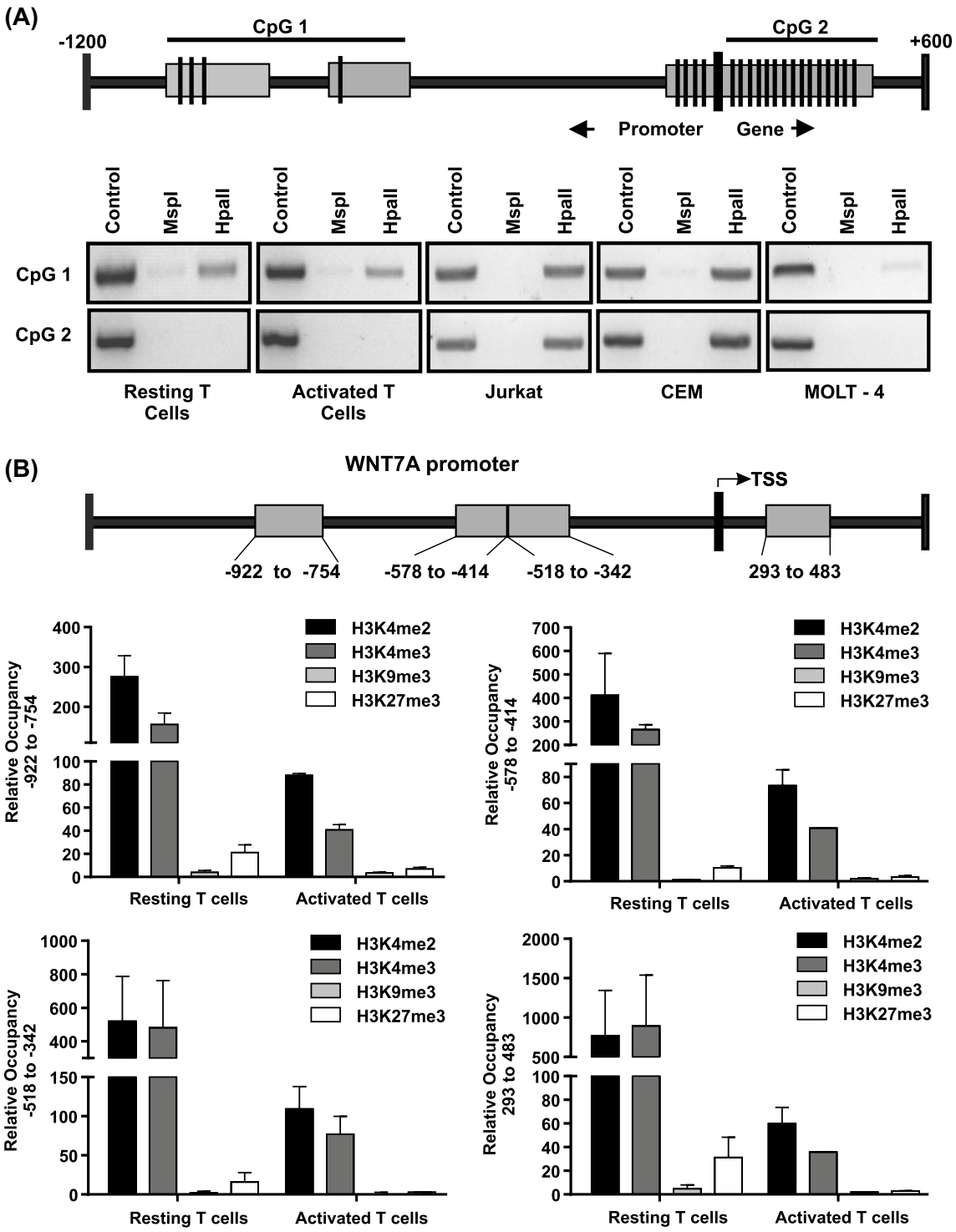


**Fig. 4** Analysis of WNT7A regulation by DNA methylation and histone methylation marks. **a** Representative diagram of sequence –1200 to +600 pb from the transcriptional start site (TSS) of WNT7A promoter. CpG islands and restriction sites for *MspI/HpaII* are depicted as dark boxes and vertical lines, respectively. Resolution in agarose gel electrophoresis of amplification fragments for CpG 1 and CpG 2 after restriction with *MspI*/*HpaII* employing DNA from resting and activated T lymphocytes, Jurkat, CEM and MOLT-4 cell lines. **b** Representative diagram of WNT7A promoter showing the four different regions that were amplified by qPCR after ChIP. Relative occupancy of H3K4me2, H3K4me3, H3K9me3 and H3K27me3 in resting and activated T lymphocytes at the four different regions of WNT7A promoter

in the presence of 2-mM NaB—the iHDACs—thereafter, the lymphocytes fraction were collected and the expression of activation markers and WNT7A expression were assessed by qPCR. As observed in Fig. 5, CD69 expression was similar in resting vs activated lymphocytes, but the expression of CD25, CD71, CD40L and CD95 was highly upregulated after PHA-L treatment, which indicates an activation process. As expected, the expression of WNT7A decreases after activation with PHA-L. Interestingly, lymphocytes stimulated with PHA-L in presence of 2 mM NaB show high expression of CD69 and low expression of CD25, CD71, CD40L and CD95 when compared with stimulated lymphocytes without NaB. In addition, the expression of WNT7A decreases but not so dramatically as without NaB. This finding indicates the possible involvement of histone deacetylation in the regulation of WNT7A expression and its important role after activation of T lymphocytes.

## Discussion

WNT signaling plays an important role in hematopoiesis and its dysregulation is often associated with the malignant transformation of blood cells (Luckey et al. 2014; Tiemessen and Staal 2013). Remarkably, all cells implicated in the hematopoietic system like mesenchymal stem cells and hematopoietic stem cells which have the capacity to differentiate into all blood cell lineages require a specific regulation of WNT signaling at different stages of differentiation (Ahmadzadeh et al. 2016; Bigas et al. 2013; Rattis et al. 2004; Reya et al. 2003); however, the role of WNT ligands in the biology of normal mature blood cells remains poorly understood.

In this study, we report that WNT7A is expressed in PBMCs, mainly in T lymphocytes and monocytes. In agreement with our results, Wallace et al. (2018) found that WNT7A was expressed in monocytes and involved in the phenotype commitment and function of monocyte-derived macrophages. In addition, Sercan et al. (2010) reported that T lymphocytes also express WNT3, WNT5A, WNT9B, WNT10A and WNT16; while B lymphocytes express WNT3, WNT7A, WNT9B and WNT10A. However, in our work, we observed low levels of WNT7A in B lymphocytes. It is important to mention that the analysis of Sercan et al. (2010) was done qualitatively; whereas, we determine WNT7A expression quantitatively. To our knowledge, there are no previous reports regarding WNT7A expression in peripheral NK cells, which was very low when compared with total PBMCs and with other cell blood populations.

An important finding was that WNT7A decreases after mitogenic stimulation using PHA-L or anti-CD3/CD28—anti-CD2/CD3/CD28 in a time-dependent manner, where the lower expression was 48–72 h post-stimulation. Our data indicate that T lymphocytes downregulates WNT7A

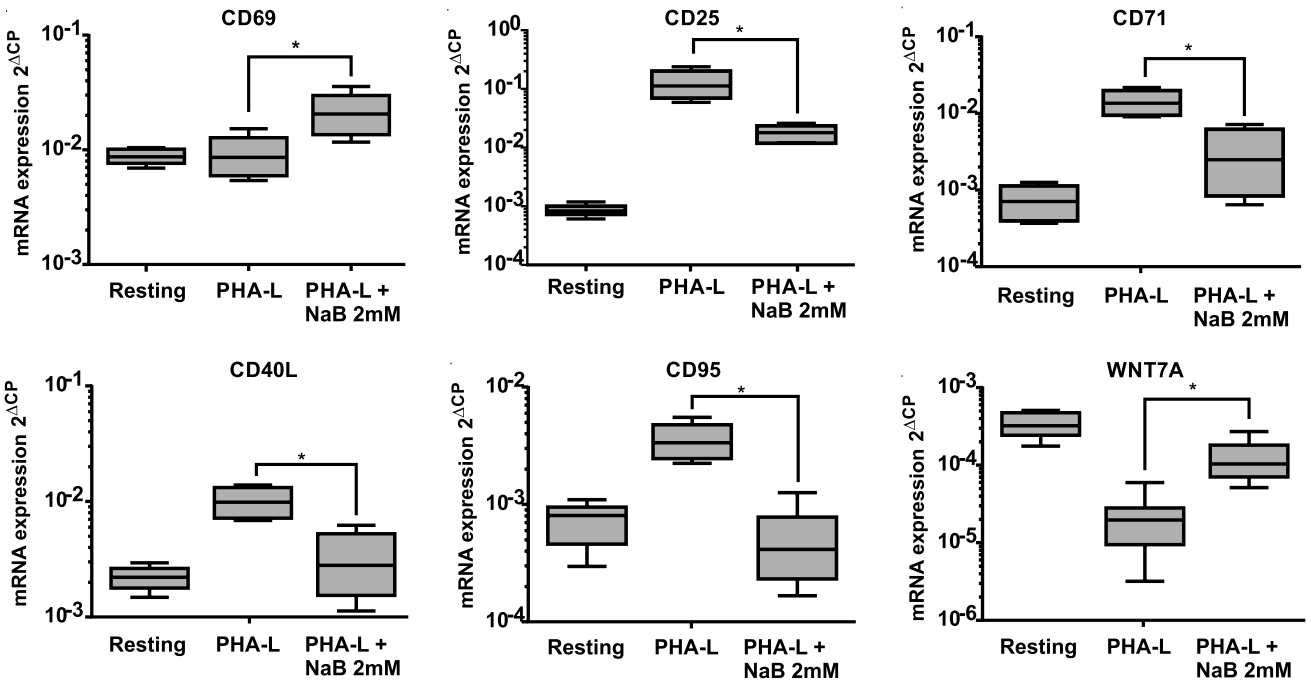


**Fig. 5** Evaluation of WNT7A expression using an inhibitor of histone deacetylases. Box plot graphics illustrating mRNA expression, depicted as $2^{\Delta CP}$ of activation markers and WNT7A in activated T lymphocytes treated with NaB 2 mM for 48 h. Quantification was performed using RPL32 and RPS18 as reference genes. $^{*}p < 0.05$

expression after TCR activation and such regulation depends on TCR signaling. These results are entirely consistent with the fact that WNT7A appears to be a regulator of cell proliferation (Jia et al. 2019; Ma et al. 2017; Ochoa-Hernandez et al. 2012; Qu et al. 2013; Ramos-Solano et al. 2015). Furthermore, it has been established that high concentrations of IL-2 induce a faster cell cycle progression in T lymphocytes (Ross and Cantrell 2018), which agrees with our findings that shows a restoration of WNT7A expression 168 h post-stimulation in the absence of proliferative stimuli. This is relevant since it indicates that T lymphocytes are capable of modulating WNT7A expression according to the stimulus they receive as a regulatory mechanism for cell proliferation.

Previous studies have shown that canonical WNT signaling is relevant in different aspects of T-cell biology, for example, in mature T lymphocytes, TCR and CD28 signaling inhibits GSK3β through PI3K–AKT and PKC which leads to a rapid increase of stabilized β-catenin levels 2 h after the stimulation, allowing its migration to the nucleus (Lovatt and Bijlmakers 2010). In T lymphocytes, TCF-1 and LEF-1 factors are expressed in multiple isoforms due to alternative splicing (Willinger et al. 2006; Xue and Zhao 2012). All isoforms contain a DNA binding domain, but the short TCF-1 isoforms cannot interact with β-catenin. Indeed, TCR signaling induces a shift in the production of TCF-1 isoforms favoring the full-length version of TCF-1, which can bind β-catenin and induce the expression of WNT canonical target genes, including JUN, c-MYC, MMP7, TCF4, MMP9, CCND1. All these genes have been related to important functions of T lymphocytes such as proliferation, migration and differentiation (Tiemessen et al. 2014; Wu et al. 2007; Xue and Zhao 2012). Consistent with this, our data show a major increase in c-MYC, MMP7 and CCND1 expression after TCR activation, while all non-canonical target genes were downregulated. Following this line, we have previously reported that WNT7A could work as a non-canonical ligand (Ochoa-Hernandez et al. 2012); so, it is evident that WNT7A acts in this way in normal T lymphocytes. In addition, our results suggest two regulatory mechanisms for WNT7A expression, one very early that triggers the upregulation and a second, after 2 h, that induces downregulation. A rapid analysis of the promoter sequence of WNT7A (region −700 to 299 relative to the transcription start site) showed the presence of NF-κB and NFAT putative binding sites (data not shown). The activation of NF-κB is an event that follows TCR activation and changes in the intracellular levels of $Ca^{2+}$, also mediated by the TCR signaling, induces the translocation of cytoplasmic NFAT to the nucleus and, therefore, the transcriptional induction of its target genes (Brownlie and Zamoyska 2013). In this sense, both transcription factors could be involved in the very early upregulation of WNT7A. All these hypotheses need to be evaluated at early times in activated normal T lymphocytes, we are working currently on that.

On the other hand, the activation of the TCR has been linked with epigenetic changes that modify genetic expression in activated lymphocytes (Naito and Taniuchi 2013; Rodriguez et al. 2015; Schmidl et al. 2018). We evaluated epigenetic mechanisms to determine its involvement in the downregulation of WNT7A in activated T lymphocytes. We identified three CpG islands in the WNT7A promoter and our results indicate that mainly, the CpG island near to the transcription start site (CpG 2) was of relevance for the WNT7A regulation as this site was found highly methylated in Jurkat and CEM cell lines. It is well known that DNA methylation in promoter CpG sites induces a permanent gene silencing. To our knowledge, this is the first report that associates the methylation status of WNT7A promoter with its expression in T-ALL-derived cell lines. Silencing of WNT7A in tumoral cells suggests a critical role for this gene on the control of cell tumorigenesis. In fact, WNT7A silencing has been associated with poor prognosis in human malignant pleural mesothelioma (Hirata et al. 2015), non-small cell lung cancer (Kim et al. 2015), clear renal cell carcinoma (Kondratov et al. 2012) and cervical cancer (Ramos-Solano et al. 2015). Silencing of WNT7A in tumoral cells needs to be perpetual; therefore, the appropriated mechanism of transcriptional regulation is through DNA methylation. In normal T lymphocytes, the WNT7A expression needs to be regulated temporarily and reversibly; indeed, we observe that after TCR activation and with lack of a proliferative signal, the expression of WNT7A tends to recover. In this sense, methylation and acetylation of histones seem to be an adequate mechanism of genetic regulation. We observed that after TCR activation, the relative occupancy of H3K4me2/3—both associated with actively transcribed genes—decreases; while, the occupancy of H3K9me3 and H3K27me3—both associated with transcriptional repression—remains low when compared with resting T lymphocytes in four different regions of WNT7A promoter. A previous report details the dynamics of genome-wide promoter H3K4me2/3 occupancy over a time course during activation of human-naïve and activated CD4 T cells using ChIP seq and RNA-seq, where they found 611 genes with early promoter H3K4me2/3 changes linked to many important T-cell pathways including PI3K–AKT, JAK/STAT, interferon signaling and WNT signaling (LaMere et al. 2016). Among these genes, H3K4me2/3 marks decreases at WNT7A promoter which is clearly consistent with our results. In addition, using NaB—a well-known iHDACs—we found that the expression of WNT7A was not so drastically inhibited, which strongly suggests the participation of histone deacetylases on the regulation of this gene and other T-cell activation markers. In concordance with our results, there are some reports which have shown that CD69 expression

increases after the treatment with iHDACs in activated T lymphocytes, while CD25 and CD40L decrease (Rasmussen et al. 2013; Skov et al. 2003; Zimmerman et al. 2012). Overall, our results indicate that in normal T lymphocytes, WNT7A is quickly downregulated after TCR activation by epigenetic mechanisms typically through histone modifications such as methylations and deacetylations. In addition, WNT7A might act as a new marker for resting mature T lymphocytes. In tumoral cells, WNT7A silencing is mainly due to DNA methylation. Further studies are necessary to determine the signaling pathways and mechanisms that are induced by WNT7A to regulate cell proliferation in T lymphocytes. Elucidation of those mechanisms will clarify its role in the T-cell biology and in the tumoral counterpart.

**Acknowledgements** This work was supported by the Fondo de Investigación en Salud – IMSS, grant number FIS/IMSS/PROT/G13/1243 and FIS/IMSS/PROT/G14/1316 both to Luis Felipe Jave-Suárez. Christian Barreto-Vargas and Monserrat Alvarez-Zavala are grateful for their scholarship obtained from Consejo Nacional de Ciencia y Tecnología (CONACyT), Mexico. We are grateful to Cristina Artaza Irigaray for proofreading of the manuscript.

**Author Contributions** CB-V and MA-Z performed the experimental work, analyzed results, and contributed with figures and manuscript preparation. MG-C and GH-F provided scientific ideas and analysis of data. AA-L and LFJ-S conceived the study, provided scientific guidance, analyzed results and contributed to manuscript preparation. All authors approved this final version.

## Compliance with Ethical Standards

**Conflict of interest** The authors declare no conflict of interest.

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