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Protein complexes associated with β -catenin differentially influence the differentiation profile of neonatal and adult CD8⁺ T cells

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ABSTRACT

The canonical Wnt Signalling Pathway is a master cell regulator involved in CD8⁺ T cell proliferation and differentiation. In human CD8⁺ T cells, this pathway induces differentiation into memory cells or a “stem cell memory like” population, which is preferentially present in cord blood. To better understand the role of canonical Wnt signals in neonatal or adult blood, we compared the proteins associated with β -catenin, in non-stimulated and Wnt3a stimulated human neonatal and adult naïve CD8⁺ T cells. Differentially recruited proteins established different complexes in adult and neonatal cells. In the former, β -catenin-associated proteins were linked to cell signalling and immunological functions, while those of neonates were linked to proliferation and metabolism. Wnt3a stimulation led to the recruitment and overexpression of Wnt11 in adult cells and Wnt5a in neonatal cells, suggesting a differential connexion with planar polarity and Wnt/Ca²⁺ non-canonical pathways, respectively. ChIP-PCR experiments showed that β -catenin was recruited to a higher level on the promoters of cell renewal genes in neonatal cells and of differentiation genes in those of adults. We found a preferential association of β -catenin with CBP in neonatal cells and with p300 in the adult samples, which could be involved in a higher self-renewal capacity of the neonatal cells and memory commitment in those of adults. Altogether, our results show that different proteins associated with β -catenin during Wnt3a activation mediate a differential response of neonatal and adult human CD8⁺ T cells.

INTRODUCTION

Human neonates are highly susceptible to infections by intracellular pathogens, which are a major cause of infant morbidity and mortality (World Health Organization, 2017). The function of neonatal T cells is considered to be immature, skewed and tolerant. Under very strong stimulation, however, neonatal cells are capable of adult-level T-cell responses, indicating their high activation threshold (Adkins et al., 2004).

The Wnt signalling pathways are master regulators of development and multiple biological functions, including antagonistic roles in undifferentiated cell-renewal and differentiation (Teo and Kahn, 2010). There are 19 mammalian Wnt proteins that can be recognized by 10 frizzled (Fzd) receptors and different co-receptors, the combination of which determine the signalling pathway and outcome of the cell. The canonical pathway can be activated by Wnt1, Wnt3a and Wnt8 and depends on the engagement of Fzd and Low-density lipoprotein receptor-related proteins 5/6 (LRP5/6). It relies on the stabilization of β -catenin, through the disassembly of the β -catenin degradation complex. The degradation complex is formed by the scaffold protein Axin, Adenomatous Polyposis Coli (APC), Glycogen synthase kinase 3 beta (GSK3 β) and Casein Kinase 1 (CK1), which in the absence of Wnt signals keep β -catenin phosphorylated, a mark that provokes its ubiquitination and degradation. Once the Wnt signal is received, β -catenin is stabilized in the cytosol and forms protein complexes that mediate its import to the nucleus and activation of gene expression, which is mediated by the TCF and LEF family of transcription factors (MacDonald et al., 2009). The non-canonical Wnt-dependent signalling pathways are broadly grouped into the Wnt/Ca²⁺ signalling pathway and the planar polarity pathway. The later includes Wnt-RAP1, Wnt-PKA, Wnt-GSK3MT, Wnt-aPKC, Wnt-RYK and Wnt-mTOR signalling pathways (De, 2011; Semenov et al., 2007). Wnt5a, with co-receptors Ror1/2, has been associated with the activation of the Wnt/Ca²⁺ pathway and Wnt11 with the planar polarity pathway. Some controversy exists into the exclusivity of frizzle receptors to recognize Wnt, adding complexity to Wnt signalling pathways (Angers and Moon, 2009; De, 2011; Grumolato et al., 2010; Kühl et al., 2000; Major et al., 2007).

The protein complexes associated with β -catenin translocate to the nucleus and associate with the transcription factors TCF and LEF to displace the GROUCHO family of proteins, in order to allow the transcription of the target genes (Gao et al., 2014). The later are inhibitors of gene transcription that keep the Wnt-responsive-genes silenced. In addition,

the transcription factors TCF and LEF themselves have Histone Deacetylase activities, and also contribute to gene silencing in the absence of activating protein complexes (Cadigan and Waterman, 2012). Gene expression mediated by the canonical pathway is complex, as it depends on the composition of the protein complexes associated with β -catenin and the cell context (Nakamura et al., 2016).

Among the proteins associated with the β -catenin complex in the nucleus, are the co-activator proteins p300 and CBP. These proteins, with a high degree of homology, share a histone acetyl transferase domain, three domains rich in cysteine and a KIX domain for interaction with CREB transcription factor, a steroid binding domain and a bromodomain for association with acetylated histone tails (Ogryzko et al., 1996; Teo and Kahn, 2010). Due to their multiple protein interaction domains, they have a role as scaffold proteins in chromatin opening for transcription of enhancers and genes (Dyson and Wright, 2005; Goodman and Smolik, 2000; Ogryzko et al., 1996; Teo and Kahn, 2010). In association with β -catenin, opposite effects of CBP and p300 have been reported. The inhibition of the association of β -catenin with either CBP or p300 results in different outcomes of the cells. CBP association with β -catenin is essential for the expression of genes involved in self renewal of stem cells, while p300-containing complexes mediate transcription of differentiation related genes (Teo and Kahn, 2010). A dual role for CBP and p300 was also described during myocytes' differentiation. Using mutant forms of CBP and p300, it was shown that p300, but not CBP, is important for cell-differentiation *in vivo* (Roth et al., 2003b). Again, in colorectal cancer, in which a role of Wnt signalling has been clearly established in cell transformation, it was found that CBP promotes proliferation and is a marker of bad prognosis, while p300 is important for differentiation of these cells (Bordonaro and Lazarova, 2015). These reports suggest that p300 and CBP associated with β -catenin act as a switch that promotes either differentiation or cell-renewal, respectively.

The involvement of the Wnt- β -catenin pathway in CD8⁺ T cell differentiation into memory precursor cells (MPEC), but not effector precursor (SLEC) cells, was demonstrated in adult mice models *in vivo*. It was clearly shown that the pathway is active in naïve, MPECs and memory cells, but is silenced in SLECs and effector cells (Boudousquie et al., 2014; Gattinoni et al., 2009). In addition, activation of the canonical Wnt signalling pathway also induces a phenotype that has been called "stem cell memory like (T_{SCM})". These are self-renewing multi-potent CD8⁺ T cells that have a mixed phenotype between memory and

stem cells, which could be useful for bone marrow transplantation purposes and immune cells-mediated cancer treatments (Forget et al., 2012; Li et al., 2014; Scholz et al., 2016). The T_{SCM} phenotype is enriched in neonatal as compared to adult blood (Xu, 2016).

We have previously shown that human neonatal CD8⁺ T cells have a distinctive transcriptomic and epigenetic landscape, characterized by the expression of genes involved in cell cycle and innate immunity. We also found that neonatal cells have an increased homeostatic proliferation, but reduced clonal expansion and effector functions. Several transcription factors were found differentially expressed between adult and neonatal cells, including the β -catenin dependent TCF4 and LEF1 factors (Galindo-Albarrán et al., 2016).

Our aim in this study was to understand the potential dual role of β -catenin mediated signalling in the maintenance of cell-renewal capacity and differentiation of neonatal and adult human CD8⁺ T cells. For this, we made a proteomic identification of the proteins that associate with β -catenin in human neonatal and adult CD8⁺ T cells, before and after stimulation with Wnt3a. We found common and differentially recruited proteins in the neonatal and adult cells that were involved in the formation of complexes with different biological activities. In the adult cells, the complexes formed were associated with the control of immunological function and cell signalling, while in those of neonates with proliferation and metabolism. A notable difference was the Wnt3a-induced association of β -catenin with Wnt11 in the adult cells complexes and Wnt5a in those of neonates, which were accompanied by a differential overexpression of these proteins. This suggests an alternative connexion of Wnt3a activated cells with the cell polarity pathway in adult cells and Wnt/Ca²⁺ in the neonatal samples. Evaluation of β -catenin recruitment to the promoters of target genes, showed a higher enrichment of β -catenin to the promoters of proliferation genes in the neonatal cells. Finally, we found that β -catenin preferentially recruits CBP in neonatal cells and p300 in those of adults. Altogether our results suggest that a cellular switch, mediated by β -catenin associated protein-complexes, could participate in the control self-renewal or differentiation of human CD8⁺ T cells. This clarifies the role of Canonical Wnt signalling Pathway in CD8⁺ T cell populations, which are considered for immunological cancer treatments and cell replenishment in transplantation procedures.

MATERIALS AND METHODS

Cell preparation

Adult CD8⁺ T cells were obtained from leukocyte concentrates of healthy adult donors from Centro Estatal de la Transfusión Sanguínea, in Cuernavaca, Mexico. Neonatal blood was collected from the cord vein of full term vaginal deliveries of healthy babies at Hospital General Parres, with informed mothers consent. Blood was collected just after the deliveries and before placental expulsion. The Ethical Committee of Hospital Parres approved this procedure. Total blood was separated with ficoll-hypaque gradient and incubated overnight for plastic adhesion and monocytes depletion. For CD8⁺ T cell purification, we remixed PBMCs with 2 ml of cultured erythrocytes from the same donor and depleted the unwanted cells with RosetteSep™ CD8⁺ T cell enrichment cocktail (StemCell Technologies, 15063). Cells were further purified by depletion of CD45RO⁺ CD44⁺ and B220⁺ cells with Protein A/G magnetic beads (Pierce, 8803, ThermoFischer), ligated to the corresponding antibodies (CD45RO: UCHL1; CD44: IM7 and B220: RA3-6B2, Biosciences). This method allowed us to have over 94% naïve CD3⁺CD8⁺ T cells (Supplementary figure 1). Cells were maintained in RPMI supplemented with 1% L-Glutamine, antibiotics (100 U/mL Penicillin and 100 µg/mL Streptomycin) and 5% foetal calf serum under 5% CO₂ at 37°C. For all experiments, cells were left untreated or stimulated with Wnt3a recombinant protein (R&D systems, 149122), 50 ng/mL for 12h.

Flow cytometry

Antibodies recognizing the following molecules coupled to the indicated fluorochromes were used to assess the purity of the cell' preparations: PE anti-Human CD8 α (Hit8aOKT8, 50-0089-T100, Tonbo Biosciences), FITC anti-Human CD3 (OKT3, 35-0037-T100, Tonbo Biosciences), APC anti-Human CD45RO (UCHL1, 20-0457-T100, Tonbo Biosciences). Cells were subsequently washed twice in PBS containing 1% FCS and re-suspended for FACS analysis. Flow cytometry was performed on the FACSCanto cytometer using BD FACSDiva software. Data analysis was performed using FlowJo software (FlowJo, LLC).

Immunoprecipitation

Cells were left untreated or incubated in 24 wells plates with Wnt3a for 12h. After washing twice with PBS, cells were lysed in lysis buffer (137 mM NaCl, 20 mM Tris pH 8, 1% NP-40, 10% Glycerol, 50 mM NaF, 2 mM EDTA and 4% protease Inhibitor Cocktail (SIGMA-ALDRICH® P8340)). After clearance by centrifugation at 16,000g for 30 min at 4°C,

lysates were incubated with 2 µg of anti-β-catenin antibody (E-5, sc-7963, Santa Cruz biotechnology) at 4°C for 2h, followed by incubation over-night with protein A/G magnetic beads (Pierce, 8803, ThermoFisher). Beads were washed three times with PBS, using a magnetic separation rack to retain marked cells, after which samples were boiled in SDS sample buffer (SDS 12 %, Tris base 0.6 M pH 6.8, bromophenol blue 1.2 µg/mL and β-mercaptoethanol 5 %).

Mass spectrometry analysis

The β-catenin immunoprecipitates were concentrated in an SDS-PAGE pre-gel. The bands were then cut and the gel fragments were washed sequentially with 50 % (v/v) acetonitrile in water (acetonitrile mixed with 100 mM ammonium bicarbonate (1:1) and 100 % acetonitrile as previously described (Lino et al., 2006)). The proteins in the gel fragments were then reduced, carbamidomethylated, and digested with trypsin, and the resulting peptides were extracted from the gel as previously described (Shevchenko et al., 1996).

These peptides were analysed by mass spectrometry as described (Garcia Montes de Oca et al., 2016) and separated in a nanoAcquity nanoflow LC system (Waters Corp. Milford, MA, USA). The peptides were first trapped in a precolumn (Symmetry® C18, 5 µm, 180 µm × 20 mm, Waters) and then were separated in a 10-cm capillary UPLC column (100-µm ID BEH-C18 1.7 µm particle size) that was run at 35°C with the following gradient: 3–50 % Solvent B (0.1 % formic acid in acetonitrile) in Solvent A (0.1 % formic acid in water) for 30 min, 50–85 % B (1 min), 85 % B (7 min) and 3 % B (22 min). The separated peptides were directly electro sprayed into a linear ion trap LTQ Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) that was equipped with a nano-electrospray ion source and controlled by Xcalibur 2.1 software. This spectrometer was operated in data-dependent acquisition mode, automatically alternating a full-scan (m/z 400–2000) spectrum with a collision-induced dissociation (CID) spectrum of the most abundant peptide ions.

The data was analysed using the X!Tandem search engine on a LabKey or a Trans Proteome Pipeline (Deutsch et al., 2010) platform, and the results were validated with PeptideProphet and ProteinProphet software. The fragmentation spectra were compared with theoretical two H. Sapiens Refseq databases that were obtained from NCBI: one with lymphocyte-related protein sequences and the other with proteins involved in the Wnt Signalling Pathway. The following search parameters were used: constant modifications,

1 carbamidomethylated C, variable modifications, oxidized M. The ion-mass tolerances were
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3 4 Da for precursor ions and 2 Da for fragmentation products. Two separate experiments
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5 with equivalent amount of identified proteins were analysed. In order to include proteins for
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7 further analysis, we selected those in which the ProteinProphet probability was ≥ 0.95 , with
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9 at least four unique identifying peptides per protein and that were identified in the two
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11 experimental samples. All identified proteins were joined in Suppl. Tables 1 and 2.
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13 **Protein analysis**

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15 We performed functional analysis to the differentially β -catenin associated proteins in non-
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17 stimulated or Wnt3a-stimulated conditions from neonate or adult CD8⁺ T cells. For this, we
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19 used the DAVID software (Database for Annotation, Visualization and Integrated Discovery)
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21 in connection with the KEGG PAHTWAY, UP KEYWORDS (Uniprot), GOTERM BP (Gene
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23 Ontology Consortium: Biological Process) and Interprot. We took Benjamini corrected p
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25 values for significant differences between neonatal and adult CD8⁺ T cell samples. For
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27 interactome analysis, we used *String 10.5* to predict protein interactions and visualized
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29 protein complexes with the *Cytoscape 3.5.1* software.
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31 **Chromatin immunoprecipitation**

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33 After stimulation, the cells were washed with PBS and cross-linked in 1% formaldehyde for
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35 20 min. The reaction was stopped with 0.22M Glycine. The nuclei were isolated and
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37 sonicated for 20 min with pulse 10 sec and 60% amplitude to yield fragments of 300 to 500
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39 base pairs. After purification by centrifugation with phenol: chloroform: isoamyl alcohol (25:
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41 24: 1; pH 8), cross-linked protein/DNA complexes were immunoprecipitated with 2 μ g of
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43 anti- β -catenin antibody (E-5, sc-7963, Santa Cruz Biotechnology). Total chromatin was
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45 used as control (input). Both input and immunoprecipitated chromatin was reversed cross-
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47 linked by heating at 50 °C for 12 h and DNA was purified with mini columns (Expin PCR
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49 SV, Genall) and quantitated with a High Sensitivity dsDNA kit in the Qubit fluorometer
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51 (ThermoFischer).
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53 **Real time PCR**

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55 For gene expression evaluation, cells were centrifuged, washed and placed in Trizol
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57 (Invitrogen, 15596-018, ThermoFischer) before freezing. RNA was extracted according to
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59 manufacturer's instructions. The integrity of the RNA was determined by electrophoresis
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and quantified with Qubit RNA HS kit in a Qubit fluorometer. cDNA was synthesized from

total RNA using RevertAid reverse transcriptase (SO131 ThermoFisher). qPCRs were performed using SYBR Green PCR Master Mix (Life Technologies, 4309155, ThermoFisher). The mRNA levels were calculated with standard curves for each gene and were reported relative to those of GAPDH gene as standard.

Specific oligonucleotides were designed to amplify regions corresponding to the mRNA of the genes (Suppl. Table 3). The mRNA sequence of the specific isoforms of the aforementioned genes was obtained from the NCBI database. Subsequently, these sequences were entered into the Primer-BLAST online program to ensure specificity of the amplicon.

For evaluation of the β -catenin associated to specific gene promoters, chromatin immunoprecipitation analysis was performed, followed by qPCR. Specific primers in the gene promoters, containing TCF/LEF binding motifs, were selected using the "Eukaryotic Promoter Database" (EPD; <http://epd.vital-it.ch/>) to find promoters from the position -1000 to +100 of the gene. "MatInspector" analysis in www.genomatix.de allowed us to find the TCF-1/LEF-1 binding motifs. Regions with more than one motif were chosen. Oligos were obtained as described above, from a region -250 +250 of the motifs. Sequences are shown in Suppl. Table 3.

All amplicons produced a single peak after denaturation, indicating a single amplicon.

Immunoblots

Protein extracts or β -catenin immunoprecipitates were boiled with sample buffer for 5 min at 100°C and separated on a PAGE 4-12% mini-gel (mini-PROTEAN®). Proteins were transferred to a PVDF membrane in a wet chamber (Bio-Rad) at 400 mA for 4h. The membrane was blocked with TBST (TBS:Tris base 19 mM, NaCl 137 mM, pH= 7.8 and TBST:TBS+Tween 20), 1% fat free milk for 1h at room temperature and subsequently incubated with anti- β -catenin, anti-CBP, anti-P300 or anti- β -actin antibodies (D2905, sc-1615-R, Santa Cruz Biotechnology). The membrane was washed with TBST three times and subsequently incubated with anti-mouse IgG-HRP (DO80, sc-2302, Santa Cruz Biotechnology) o anti-rabbit IgG-HRP (LO204, sc-2301, Santa Cruz Biotechnology) for 2h at room temperature. After washing the membrane with TBST and TBS, membranes were treated with ECL Western blotting detection reagent (PE191121, 32132, Pierce® ECL Plus) and bands were revealed with radiography films Medical X-ray General Purpose Blue (825

024, Ref 604 0331, Kodak). Densitometry analysis was performed with ImageJ.
(<http://rsbweb.nih.gov/ij/>).

RESULTS

Differences in β -catenin associated proteins in neonatal and adult CD8⁺ T cells

The canonical Wnt signalling pathway controls the antagonistic responses of cell renewal and differentiation. In order to get insights into the proteins involved in neonatal and adult CD8⁺ T cells responses, we purified naïve CD8⁺ T cells (Suppl. figure 1) and immunoprecipitated β -catenin from protein extracts of untreated or Wnt3a stimulated cells. Proteins were identified by mass spectrometry analysis from two independent experiments of each cell population (Suppl. Tables 1 and 2). In the untreated conditions, a total of 101 and 96 proteins were identified in the adult and neonatal samples, respectively, with 40 common proteins (figure 1A). After stimulation with Wnt3a, the number of recruited proteins increased about 4 fold, to 455 and 451 in adult and neonatal cells, respectively, with 402 common proteins (figure 1C).

The functional analysis of the cell-type specific β -catenin recruited proteins showed conserved functions and also differences between neonatal and adult cells (figure 1B). Among the differential functions, we found that in the non-stimulated adult samples, β -catenin associated proteins were involved in Cell Polarity pathway and were rich in Kinase-like domains, Armadillo fold and Shisa family of proteins. Armadillo is a common fold domain present in β -catenin and Wnt-signalling transduction proteins, while the Shisa family refers to proteins that control the maturation and trafficking of frizzled receptors to the cell membrane. In the neonatal cells, the β -catenin associated proteins were enriched in Melanogenesis pathway and in Neural Differentiation and Frizzled binding GOterms. Frizzled receptors associated with β -catenin in the non-stimulated conditions were only found in the neonatal cells. The enrichment in the Melanogenesis pathway could relate to the high ROS production of the neonatal cells (Galindo-Albarran et al., 2016). The neural differentiation GOterm could refer to the recruitment of Presenilin1 and associated proteins in the neonatal immunoprecipitates, which suggests a connection with the Notch pathway in these cells.

The proteins identified after Wnt3a stimulation in adult cells (Fig 1D) were associated with a higher activity of the Wnt Signalling Pathway and were rich in Kringle, Shisa and Kinase proteins. The recruitment of kinases is in agreement with a higher expression of signalling proteins in adult CD8⁺ T cells (Galindo-Albarran et al., 2016). The Proteins with Kringle

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domains are binding mediators and regulators of proteolytic activity. In the neonatal cells the recruited proteins were associated with proliferation, stemness and Hippo signalling pathway that regulates cell proliferation, apoptosis, and stress responses. Altogether, these results suggest a mature, highly controlled, Wnt Signalling Pathway in the adult CD8⁺T cells. In the neonatal cells, however, our results suggest the involvement of this pathway in stemness, cell cycle and developmental processes.

Next, we focused our analysis on the differences of β -catenin immunoprecipitated proteins between neonatal and adult CD8⁺ T cells within the Wnt Signalling Pathway (figure 2). Orange colour refers to common proteins, red to adult-specific proteins and blue to neonatal-specific proteins. In the non-stimulated neonatal cells (figure 2A) we found Wnt5b and Wnt8a, whereas in the adult cells Wnt9b was recruited. Wnt5b is related with cell migration in cancer cells and oestrogenic differentiation (Yang, Yingzi development 2003) (Harada T, Cancer Sci. 2017). Little is known about Wnt8a and Wnt9, except that they participate in development. Only in the adult's samples, association of LEF and TCF with β -catenin complexes was found at basal conditions and only in the neonatal cell samples, frizzled receptors were found associated with β -catenin.

In the Wnt3a-stimulated cells (figure 2B), the number of proteins associated with β -catenin was increased, as expected. In these conditions, recruitment of frizzled receptors was found both in neonatal and adult cells as well as all the proteins of the β -catenin degradation complex and transcription factors TCF and LEF. Only in adult cells MAP3K7 (Tak1), FRAT1 and CXXC4 were recruited, indicating connection with MAPK pathway, ATM-dependent DNA damage control and control of Wnt signalling, respectively. Although in the stimulated cells Axin2 was found in both cell populations, Axin1 was only recruited in the neonatal cells, in untreated and Wnt3a treated samples. A differential role of Axin1 and Axin2 has been reported, the later is more associated with the destruction complex activity (Thorvaldsen et al., 2017). Differences in the recruitment of Wnt proteins were also found in the Wnt3a stimulated cells. Wnt5a Wnt10A and Wnt16 were recruited only in neonatal cells and Wnt11 only in the adult samples, suggesting the connexion with two independent branches of the non-canonical Wnt pathways in the cells from the two different age populations. Wnt11 induces the cell polarity pathway that is more strongly related with cell differentiation (Cohen et al., 2008; Lange et al., 2006; Terami et al., 2004a) and Wnt5a with calcium signalling. Higher calcium waves have been reported in neonatal T cells (Palin et al., 2013).

In neonatal cells we also found Presenilin1 in the β -catenin complexes. This protein is the catalytic subunit of the γ secretase complex (PS-1) and is probably related to a connexion with the Notch Signalling Pathway. On the contrary, only in adult cells the MAPKKK Tak 1 and the nucleic acid binding protein Sbp1 were recruited in the β -catenin complexed proteins, suggesting a link with the MAPK and p53 signalling pathways, respectively. Differences were also found in members of Dkk, DVL and axin family proteins, all of which are controlling proteins of the canonical pathway. Altogether, our results show that the differences in proteins recruited by the β -catenin immunoprecipitates from neonatal and adult CD8⁺ T cells could be biologically significant.

Interactome analysis of the β -catenin immunoprecipitated proteins

In order to identify the protein complexes formed by the specific β -catenin recruited proteins in neonatal and adult cells, we analysed the interactome (figure 3). The protein complexes from neonatal cells were mostly involved in positive regulation of cell cycle, chromatin remodelling, calcium pathways and metabolism. Three proteins involved in several of the neonatal complexes were Wnt5, Axin1 and Presenilin1. Wnt5a was involved in complexes associated with calcium metabolism and proliferation, in association with the replication-dependent histone Hist1H4H. Axin1 was involved in complexes related to proliferation, microtubule organisation, and ubiquitination. Presenilin1 is a protease that cuts the Notch receptor during the Notch Pathway activation. A role of the Notch Signalling Pathway, in association with the Wnt Pathway, has been described during CD8⁺ T cells differentiation into stem cell memory like populations (Kondo 2017).

The preferentially recruited proteins in adult cells were more associated with the regulation of immune system processes, kinases activities and cell signalling. The protein SFRP1, member of the soluble frizzled-related proteins, was very represented in adult cell complexes. This protein is a negative modulator of Wnt signalling, leading to a reduction in β -catenin levels and to an inhibition of proliferation in vascular cells (Ezan et al., 2004). Also central in adult cells complex's formation were MAP3K7 (Tak) and Wnt11.

The specific complexes formation in adult and neonatal cells could be the result of increased gene expression of the population-specific complexes or posttranslational modifications. We thus evaluated the expression of six of the genes involved in the formation of the specific complexes in both cell's populations. As shown in Figure 4, *wnt5A* was only detected in the neonatal cells, both untreated and Wnt3a stimulated. *psen-1* and

axin2 expression did not changed as compared to adult cells or as a result of stimulation. For the adult genes, *wnt11* and *sfpr1* were overexpressed in adult cells, the later only in Wnt3a-stimulated conditions.

Altogether these experiments show that some the differentially recruited proteins could be the result of differential gene expression and corroborate that different protein complexes in neonatal and adult CD8⁺ T cells could be responsible for the enriched presence of T_{SCM} phenotype in cord blood cells.

Association of β -catenin with gene promoters differs in neonatal and adult cells

The differences in protein complexes recruited by β -catenin in neonatal and adult CD8⁺ T cells prompted us to investigate whether there is a differential recruitment of β -catenin to gene promoters in the neonatal and adult cells. For this, we performed experiments of chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) of gene promoters associated with proliferation (*birc5*, *ccnd1*, *pou5f1*, *s100a4*) cell signalling (*cjun*, *fra1*, *fos*, *axin2*) and differentiation (*gzmb*, *pdrn*, *nkd1*). As shown in Figure 5, enrichment of β -catenin was higher for *birc5*, *ccnd1* and *pou5f1* in the neonatal cells. At basal levels, *axin2* promoter was more enriched in β -catenin in the neonatal cells, but after Wnt3a stimulation, adult cells showed enrichment in β -catenin recruitment to the neonatal cell levels.

In the adult cells, a higher enrichment in β -catenin recruitment was observed for *fos* promoter, both in non-stimulated and Wnt3a treated cells. Recruitment of β -catenin to the *jun* promoter only increased in Wnt3a stimulated adult cells. This is in agreement with the bigger involvement of the MAPK pathways in the adult cells. For *gzmb* and *nkd1*, associated with cell differentiation, there was a significant increase in the β -catenin recruitment in the adult cells as compared with those of neonates.

Our gene expression experiments showed that *birc5* was overexpressed in the neonatal cells at basal conditions and was not further stimulated by Wnt3a treatment; *pou5f1* was only overexpressed in response to Wnt3a in the neonatal cells and *axin2* expression was induced by Wnt3a in the adult cells, to the neonatal cells basal levels (figure 5). Only for these three genes there was a correspondence between β -catenin recruitment and gene expression. Although β -catenin could have a permissive role in the expression of the other

genes, their expression is also controlled by other transcription factors, such as the TCR inducible factors, AP-1, NF κ B, NFAT and CREB.

Association of CBP or p300 with β -catenin in neonate and adult cells

Next, we evaluated specifically the association of CBP or p300 with β -catenin in neonatal and adult cells. It has been proposed that the association of β -catenin with either CBP or p300 is a molecular switch that flips the balance towards proliferation or differentiation of the cells, respectively. CD8⁺ T cells from adult or neonate donors differ in their proliferation vs. differentiation profile. Neonatal cells have a higher stemness capacity and adult cells differentiate preferentially into memory cells and its precursors in response to canonical Wnt signals (Boudousquie et al., 2014; Galindo-Albarran et al., 2016; Gattinoni et al., 2009; Xu et al., 2015). In agreement with this notion, our results from the proteomic characterization of the complexes associated with β -catenin in adult cells complexes are balanced towards differentiation and in neonatal cells towards proliferation. To specifically evaluate the association of CBP or p300 with β -catenin in neonatal and adult CD8⁺ T cells, we performed β -catenin immunoprecipitation experiments followed by β -catenin, CBP and p300 immunoblots. As shown in Figure 6, in neonatal but not adult cells, β -catenin was strongly associated with CBP after Wnt3a stimulation. On the contrary, only adult cells increased significantly their association with p300 after Wnt3a stimulation. Altogether, these results suggest that the preferential association of CBP with β -catenin in neonatal CD8⁺ T cells could be contributing to their stemness phenotype after Wnt3a stimulation. On the opposite, the association of p300 with β -catenin in adult CD8⁺ T cells could predispose their differentiation into memory cells and its precursors.

DISCUSSION

In a previous work, we described that neonatal CD8⁺ T cells have a distinct transcriptomic and epigenetic programming, characterized by an inflammatory innate response, a high homeostatic proliferation rate and low signalling and cytotoxic functions (Galindo-Albarran et al., 2016). Among the transcription factors that we found enriched in the neonatal cells were Tcf4 and Lef1, which are the responsive factors of the Wnt-signalling pathway. Both neonatal and adult CD8⁺ T cells responded to Wnt3a treatment with an over 4-fold increase in the amount of proteins that associated to β -catenin. Although 78% of those proteins were shared between adult and neonatal cells (402 proteins), the specifically recruited proteins (102 proteins) could be determinant for the specific neonatal or adult

cells' responses. Adult specific proteins were associated with phosphorylation, cell polarity, MAPK and differentiation, while those specifically recruited in the neonatal cells were involved in proliferation, metabolism and development.

In the adult cells, under basal conditions, association of β -catenin with the transcription factors TCF/LEF and NFAT5 was observed, suggesting the presence of β -catenin already in the nucleus. The pathway regulators APC and FRAT1 were also found in the adult cells immunoprecipitates. This suggests a longer experience with the activity of the pathway in the adult cells, which could be related to the long life of human naïve T cells (Vrisekoop et al., 2008). After stimulation, as expected, a bigger proportion of proteins within the Wnt mediated signalling pathways were found. Among the proteins differentially associated with β -catenin, we retrieved differences in Wnt proteins themselves. In the neonatal cells Wnt5a was found overexpressed as compared to adult cells, suggesting a link of Wnt3a activation with the activation of the Wnt/ Ca^{2+} signalling pathway. Wnt5a is also related to autophagy induction in response to bacterial infections (Jati et al., 2018), which could be part of an Innate immunity like response we previously found in neonatal CD8⁺ T cells. In the adult cells, Wnt11 was overexpressed. This protein induces the Wnt cell polarity pathway and signalling through protein kinase C. It is characterized by cytoskeleton reorganization and changes in cell polarity leading to differentiation in several cell types (Terami et al., 2004b; Uysal-Onganer et al., 2010).

Interactome analysis of the specifically recruited proteins showed that the complexes from neonatal cells were mostly involved in the positive regulation of cell cycle, chromatin remodelling, calcium pathways and metabolism. Wnt5a was found central to calcium metabolism and proliferation, which was also associated with the recruitment of the replication-dependent histone Hist1H4H. Others and us have reported an increased homeostatic proliferation in neonatal CD8⁺ T cells (Galindo-Albarran et al., 2016; Marchant and Goldman, 2005; Schonland et al., 2003). The potential involvement of the β -catenin dependent Wnt-signalling pathway in the neonatal cells proliferation was made evident by the formation of complexes associated with proliferation, the enrichment of β -catenin on the promoters of cell renewal genes and the induction of these same genes transcription by Wnt3a signalling. Oct4 (*pou5f1*) was found overexpressed in the neonatal cells, as well as the apoptosis inhibitor Survivin (*birc5*), which could be involved in self-renewal.

Also important to the neonatal cells complexes formation were Presenilin1, a protease that is involved severing Notch receptor during the pathway activation. A role of Notch

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3 Signalling pathway, complementary to Wnt pathway has been described for differentiation
4 into stem cell memory like populations (Kondo 2017) and also in Alzheimer disease,
5 associated with a PS-1 mutant, in which the blockage of CBP re-established neuronal
6 differentiation (Teo et al., 2005). The preferentially recruited proteins in adult cells were
7 more associated with immune system processes, regulation of kinase activities and cell
8 signalling. The protein SFRP1, member of the soluble frizzled-related complexes was
9 central to several of the adult cell complexes. The *sfrp1* gene was found overexpressed in
10 adult Wnt-3a-stimulated cells. These proteins are negative modulators of Wnt signalling,
11 leading to a reduction in β -catenin levels and to an inhibition of proliferation in vascular
12 cells (Ezan et al., 2004). The product of the gene map3k7 was also only recruited in adult
13 cells. This gene codes for TAK1, an important regulator of cell death, through NF κ B
14 activation and also through NF κ B-independent pathways such as oxidative stress and
15 receptor-interacting protein kinase 1 (RIPK1) dependent pathway (Lui et al., 2011; Mihaly
16 et al., 2014). The nucleic acid binding protein Sbp1 was also recruited to the β -catenin
17 complexed proteins from adult cells, establishing a connexion with the p53 signalling
18 pathway.
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21 To evaluate whether the changes in protein complexes lead to differential recruitment of β -
22 catenin to the promoters of genes associated with self-renewal, signalling and
23 differentiation, we performed ChIP/qPCR experiments. Enrichment of β -catenin over the
24 Input was observed for most of the genes, in both cell populations, however, significant
25 differences between neonatal and adult cells were found. Enrichment of β -catenin was
26 higher in self-renewal associated genes in the neonatal cells. On the opposite,
27 differentiation associated genes recruited a higher proportion of β -catenin to their
28 promoters in adult cells. This remained us of the dual role of the Wnt-signalling pathway
29 reported for other cell types. It has been reported in stem cells, myocytes, neurons and
30 cancer cells that the association of CBP or p300 to β -catenin have opposite roles in the
31 control of self-renewal and differentiation (Bordonaro and Lazarova, 2015; Roth et al.,
32 2003a; Teo and Kahn, 2010; Teo et al., 2005). Because of the high homology of CBP and
33 p300, the distinction between both proteins could be clouded in proteomic analysis, in
34 which we only identified p300 associated with both neonatal and adult cells in Wnt3a
35 treated cells. We thus performed immunoblots to specifically evaluate the interaction of β -
36 catenin with either p300 or CBP, using specific antibodies that distinguish between both
37 proteins. Our results show that in neonatal cells, Wnt3a treatment significantly induced the
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association of CBP with β -catenin, while in adult cells, the same stimulus induced p300 recruitment. The N-terminal 110 amino acids of CBP or p300 are responsible for the association of these proteins with β -catenin. The affinity of this interaction is mediated by MAPK phosphorylation, in the case of CBP in Ser92 and for p300 by Ser12. Using specific inhibitors, it was determined that Erk could be responsible for the Ser92 phosphorylation of CBP and p38 for Ser12 in p300 (Ma et al., 2012; Xia et al., 2011). A differential activation of Erk or p38 MAPK could thus mediate the differential recruitment of CBP or p300 in neonatal and adult CD8⁺ T cells. A different metabolism in neonatal and adult cells could also explain the differences (Scholz et al., 2016). Our results suggest that a cell switch between differentiation and self-renewal, mediated by p300 vs. CBP association with β -catenin, could participate in the higher renewal capacity of neonatal CD8⁺ T, and differentiation of adult cells into memory cells.

Altogether our data shows that both neonatal and adult CD8⁺ T cells respond to Wnt3a with a set of cell population-specific proteins that result in a differential recruitment of β -catenin to the promoters of self-renewal or differentiation genes. These proteins may form complexes that could be responsible for the preferential differentiation of adult cells into memory cells and of neonatal cells into T_{SCM} populations. Our results highlight important differences in protein complexes formed in neonatal and adult cell responses to the canonical Wnt-signalling, which could be important considerations in managing the neonatal cell responses and cord blood transplantation. Additionally, the identification of the complex-forming proteins and potential mechanisms shaping the β -catenin nucleated complexes could be useful for a better control of this important pathway for cancer treatments.

LEGENDS TO FIGURES

Figure 1. Functional analysis of β -catenin immunoprecipitates. **A** and **B**) Proteins from non-stimulated **C** and **D**) or Wnt3a stimulated CD8⁺ T cells. **A** and **C**) Venn diagrams indicating the adult and neonatal cells exclusive and common proteins **B** and **D**) Functional analyses of neonatal (blue) and adult (red) specifically recruited proteins.

Figure 2. β -catenin associated proteins in Wnt-pathways. Proteins from **A**) non-stimulated and **B**) Wnt3a stimulated CD8⁺ T cells. Proteins only found in neonatal cells are depicted in

blue, in adult cells in red and in both cells in orange. The diagram was created in Cell Designer®.

Figure 3. Interactome showing a representation of protein complexes recruited by β -catenin. Complexes formed in **A)** adult or **B)** neonatal CD8⁺ T cells. Red circles represent proteins only recruited in immunoprecipitates from adult cells, blue circles from neonatal cells and orange circles in both samples. Image created using *Cytoscape 3.5.1*.

Figure 4. Expression of genes involved in complexes' formation. Neonatal or adult naïve CD8⁺ T cells were left untreated or stimulated with Wnt3a for 12h. Gene expression was evaluated by RT-qPCR, using GAPDH as control gene. Experiments were performed from at least 3 independent samples of neonatal or adult cells. *p <0.05.

Figure 5. β -catenin bound to gene promoters and gene expression. Primers were designed for a selection of TCF/LEF target genes with self-renewal, signalling or differentiation roles *Upper panels:* Enrichment of β -catenin to the promoters of these genes (ChIP/qPCR). *Lower panels:* gene expression (RT-qPCR). Experiments were performed from at least 3 independent samples of neonatal or adult cells. *p <0.05.

Figure 6. β -catenin, CBP and p300 immunoblots from β -catenin immunoprecipitates. Protein extracts from non-stimulated (NS) or Wnt 3a stimulated neonatal (right panels) and adult (left panels) CD8⁺ T cells were immunoprecipitated with β -catenin antibodies. Representative immunoblots from **A)** adult cells and **B)** neonatal cells. **C-H)** Densitometric analysis of immunoblots, results are reported as means \pm sd of three independent experiments and expressed as optical density value calculated with ImageJ *p <0.05, **B D)** β -catenin blots **E F)** CBP blots **G and H)** p300 Blots. Experiments were performed from at least 3 independent samples of neonatal or adult cells. *p <0.05.

Figure 7. Graphical Abstract

Supplementary Figure 1. Representative flow cytometry evaluation of CD8⁺ T from neonatal and adult cells. **A)** CD3/CD8 dot plots **B)** Depletion of memory cells in adult CD8⁺ T cells. All preparations gave similar results.

Supplementary Table 1. Proteins associated with β -catenin identified by Mass Spectrometry in non-stimulated neonatal and adult CD8⁺ T cells. Only proteins with a probability ≥ 0.95 , with at least 4 independent identified peptides and observed in two independent experiments were considered.

Supplementary Table 2. Proteins associated with β -catenin, identified by Mass Spectrometry in Wnt3a treated neonatal and adult CD8+ T cells. Only proteins with a probability ≥ 0.95 , with at least 4 independent identified peptides and observed in two independent experiments were considered.

Supplementary Table 3. Primers used for ChIP/qPCR or RT-PCR experiments.

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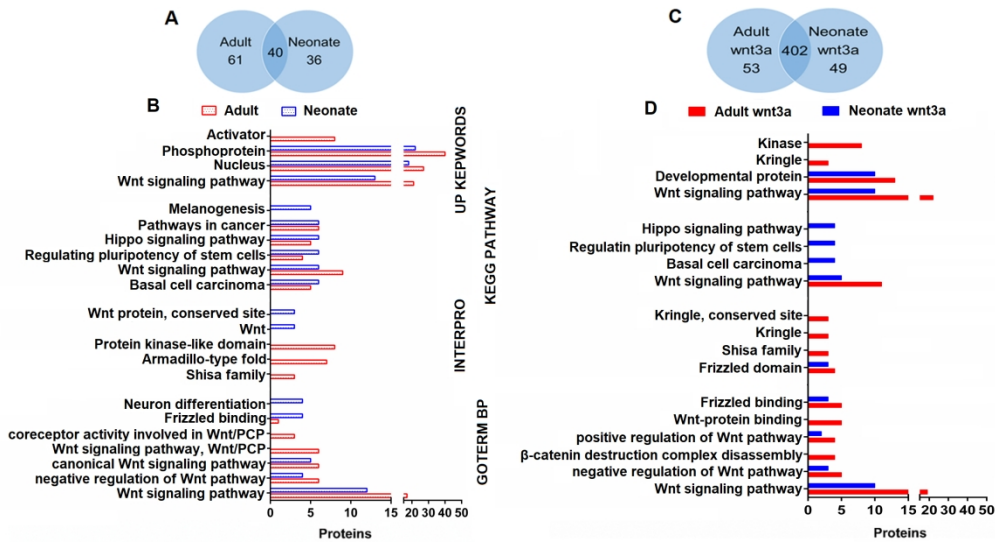


Figure 1. Functional analysis of β -catenin immunoprecipitates. A and B) Proteins from non-stimulated C and D) or Wnt3a stimulated CD8+ T cells. A and C) Venn diagrams indicating the adult and neonatal cells exclusive and common proteins B and D) Functional analyses of neonatal (blue) and adult (red) specifically recruited proteins.

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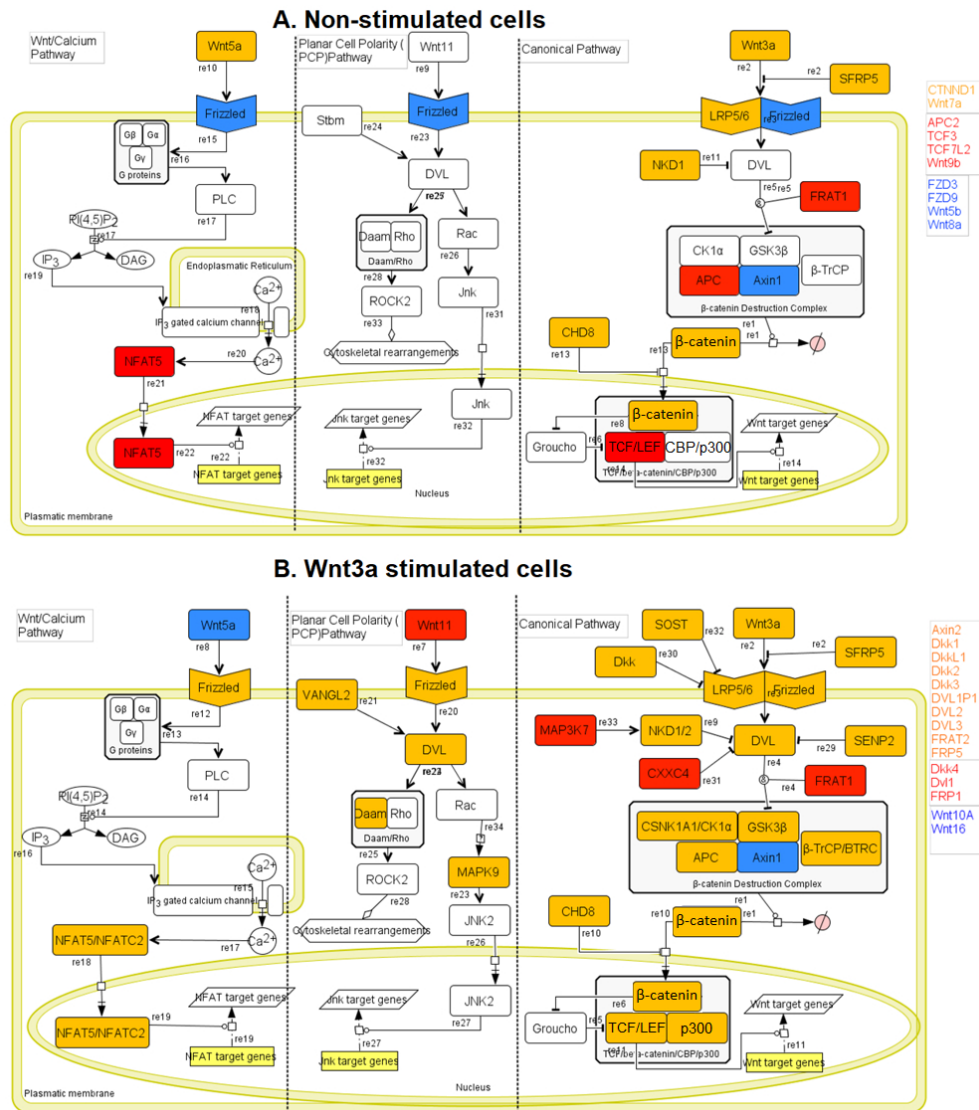


Figure 2. β -catenin associated proteins in Wnt-pathways. Proteins from A) non-stimulated and B) Wnt3a stimulated CD8⁺ T cells. Proteins only found in neonatal cells are depicted in blue, in adult cells in red and in both cells in orange. The diagram was created in Cell Designer®.

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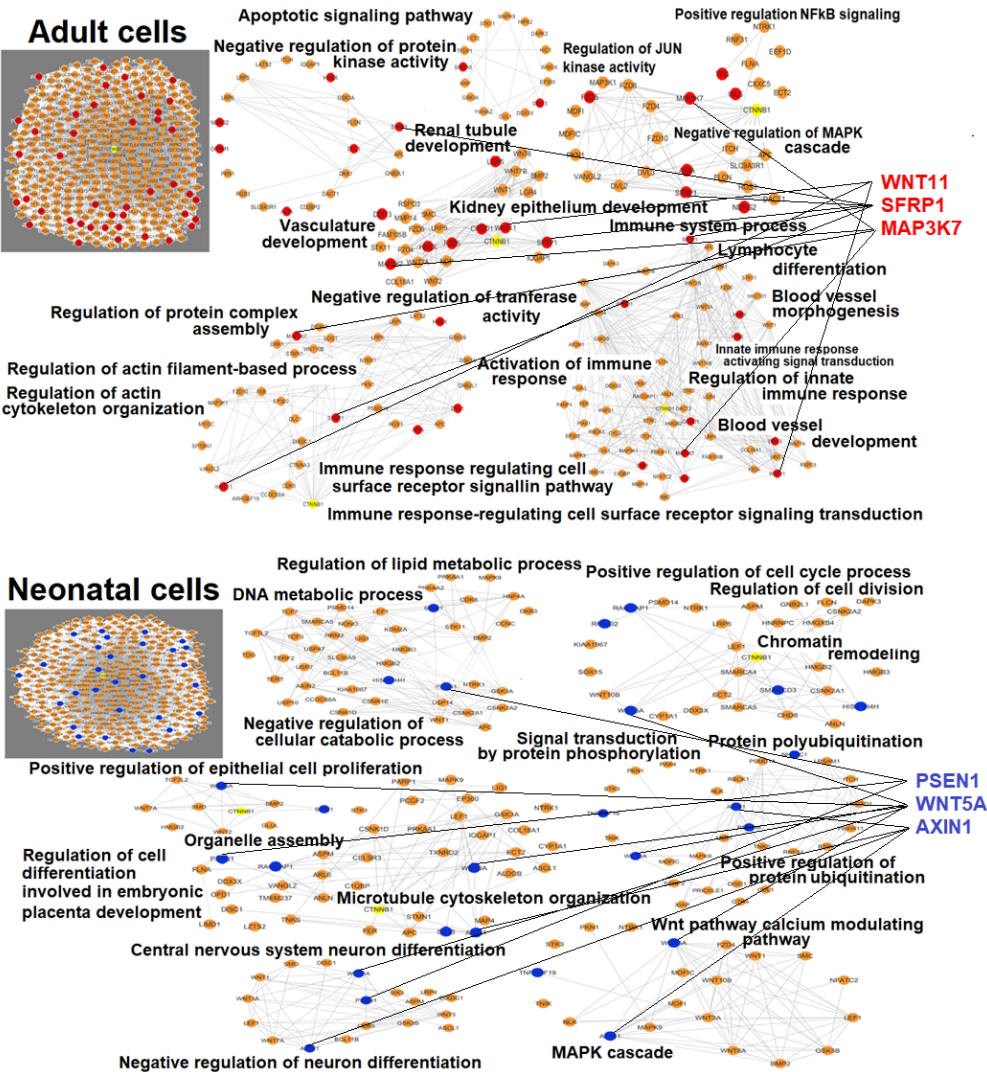


Figure 3. Interactome showing a representation of protein complexes recruited by β -catenin. Complexes formed in A) adult or B) neonatal CD8+ T cells. Red circles represent proteins only recruited in immunoprecipitates from adult cells, blue circles from neonatal cells and orange circles in both samples. Image created using Cytoscape 3.5.1.

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Gene expression

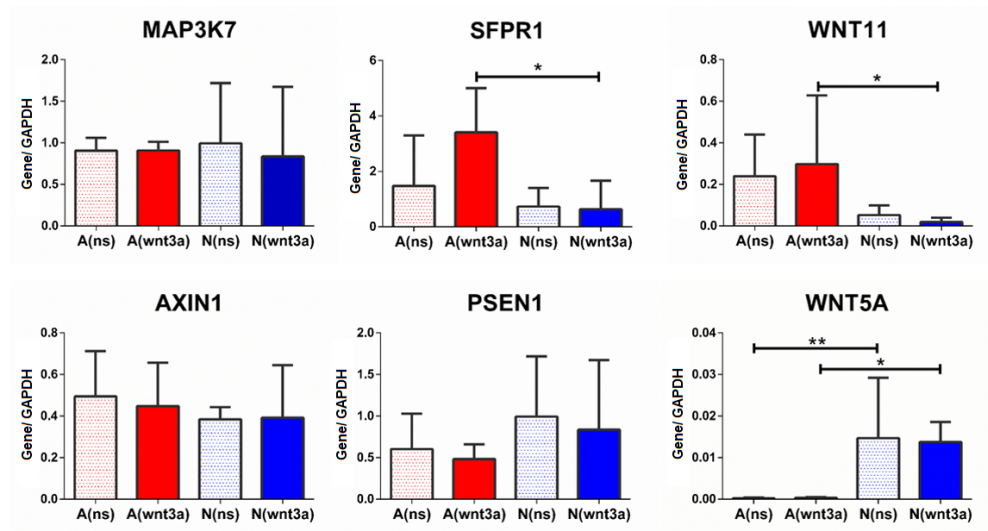


Figure 4. Expression of genes involved in complexes' formation. Neonatal or adult naïve CD8+ T cells were left untreated or stimulated with Wnt3a for 12h. Gene expression was evaluated by RT-qPCR, using GAPDH as control gene. Experiments were performed from at least 3 independent samples of neonatal or adult cells. *p < 0.05.

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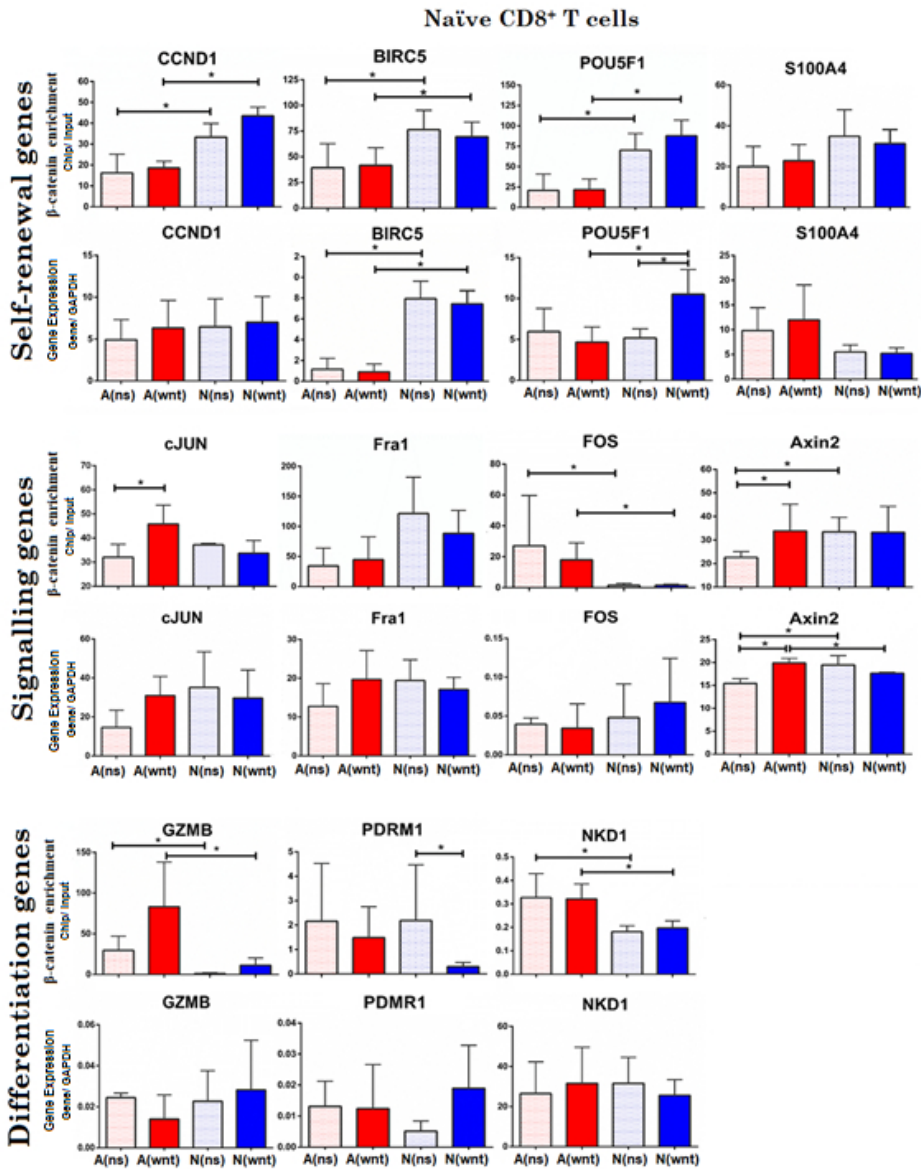


Figure 5. β -catenin bound to gene promoters and gene expression. Primers were designed for a selection of TCF/LEF target genes with self-renewal, signalling or differentiation roles Upper panels: Enrichment of β -catenin to the promoters of these genes (ChIP/qPCR). Lower panels: gene expression (RT-qPCR). Experiments were repeated at least from at least 3 independent samples and evaluated as duplicates. *p < 0.05.

151x192mm (96 x 96 DPI)

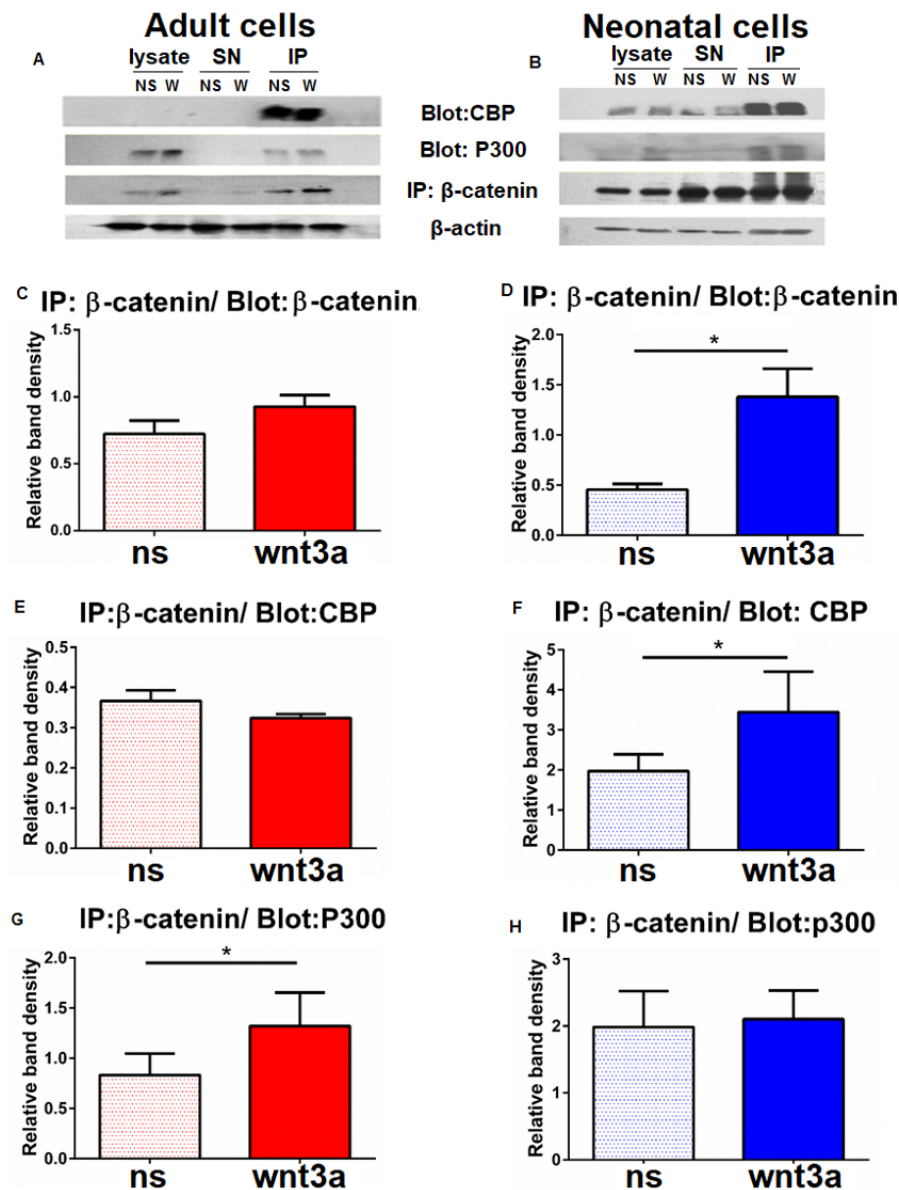


Figure 6. β -catenin, CBP and p300 immunoblots from β -catenin immunoprecipitates. Protein extracts from non-stimulated (NS) of Wnt 3a stimulated neonatal (right panels) and adult (left panels) CD8+ T cells were immunoprecipitated with β -catenin antibodies. Representative immunoblots from A) adult cells and B) neonatal cells. C-H) Densitometric analysis of immunoblots, results are reported as means \pm sd of three independent experiments and expressed as optical density value calculated with ImageJ * $p < 0.05$, B D) β -catenin blots E F) CBP blots G and H) p300 Blots. Experiments were performed from at least 3 independent samples of neonatal or adult cells. * $p < 0.05$.

145x190mm (150 x 150 DPI)

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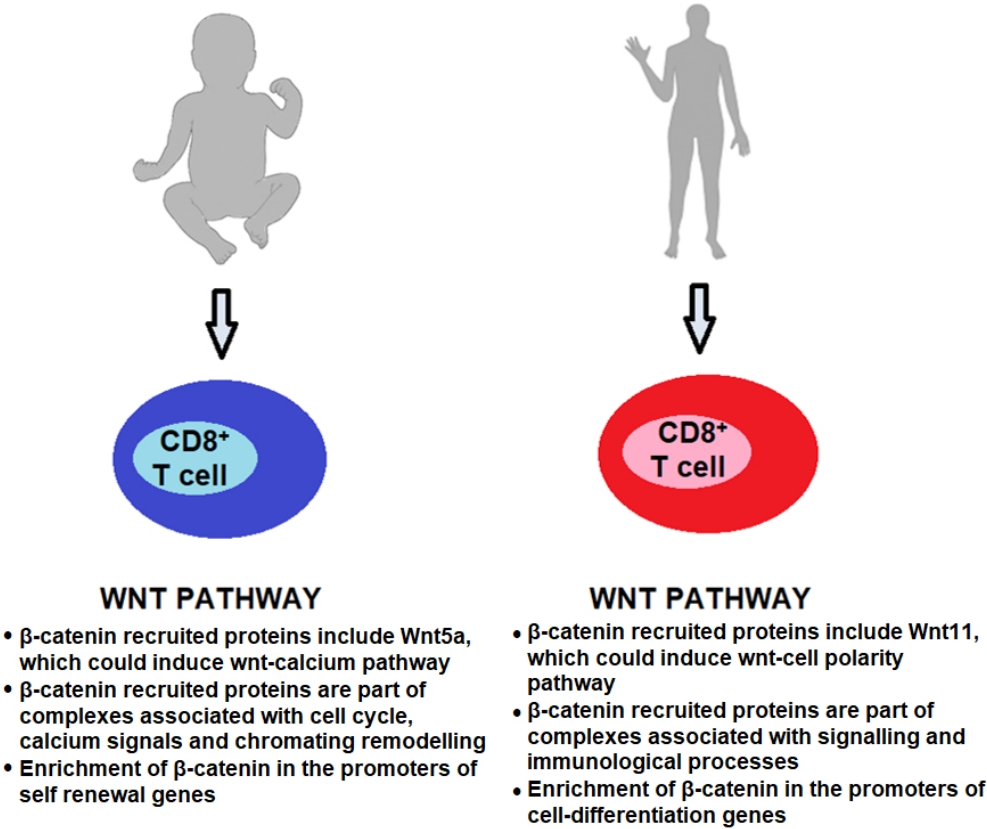
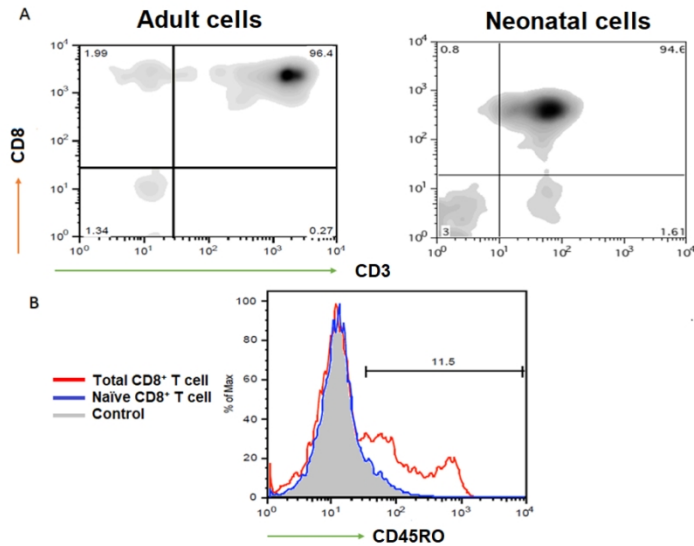


Figure 7. Graphical Abstract

232x198mm (96 x 96 DPI)



Supplementary Figure 1. Representative flow cytometry evaluation of CD8⁺ T from neonatal and adult cells. A) CD3/CD8 dot plots B) Depletion of memory cells in adult CD8⁺ T cells. All preparations gave similar results.

254x139mm (131 x 131 DPI)

Supplementary Table 1. β -catenin immunoprecipitated proteins in unstimulated CD8+ T cells

Adult cells and Neonatal cells (common proteins)														
#	UniProtKB	Gene symbol	#	UniProtKB	Gene symbol	#	UniProtKB	Gene symbol	#	UniProtKB	Gene symbol	#	UniProtKB	Gene symbol
1	Q08117	AES	9	Q9Y5R5	DMRT2	17	Q96N16	JAKMIP1	25	P35227	PCGF2	33	Q15831	STK11
2	Q6IMN6	CAPRIN2	10	O43432	EIF4G3	18	Q53G59	KLHL12	26	Q9UPQ7	PDZRN3	34	O14746	TERT
3	Q9P219	CCDC88C	11	Q7Z7B0	FILIP1	19	O75197	LRP5	27	Q16512	PKN1	35	P56704	WNT3A
4	Q9HCK8	CHD8	12	P34947	GRK5	20	Q9UPN3	MACF1	28	Q96P16	RPRD1A	36	A0A024R316	WNT5A
5	P48730	CSNK1D	13	P49840	GSK3A	21	Q6KC79	NIPBL	29	Q5T4F7	SFRP5	37	O00755	WNT7A
6	P35222	CTNNB1	14	P26583	HMGB2	22	Q969G9	NKD1	30	O60264	SMARCA5	38	O43296	ZNF264
7	O60716	CTNND1	15	Q9H0C8	ILKAP	23	Q5BJF6	ODF2	31	Q8NB90	SPATA5	39	A0A024RC65	
8	Q9NYF0	DACT1	16	P46940	IQGAP1	24	A0A024RC37	P15RS	32	P81877	SSBP2	40	B4DZB8	
Adult cells (exclusive proteins)						Neonatal cells (exclusive proteins)								
#	UniProtKB	Gene symbol	#	UniProtKB	Gene symbol	#	UniProtKB	Gene symbol	#	UniProtKB	Gene symbol	#	UniProtKB	Gene symbol
1	P55196	AFDN	22	Q13233	MAP3K1	43	B8ZZ34	SHISA8	1	Q9NQW6	ANLN	22	Q68DV7	RNF43
2	Q8N7J2	AMER2	23	Q9P0L2	MARK1	44	O95343	SIX3	2	O15169	AXIN1	23	Q9NQG5	RPRD1B
3	P25054	APC	24	P27448	MARK3	45	Q6PML9	SLC30A9	3	Q07021	C1QBP	24	P31350	RRM2
4	O95996	APC2	25	Q71F56	MED13L	46	O14745	SLC9A3R1	4	Q96B18	DACT3	25	Q9NP91	SLC6A20
5	Q8IW93	ARHGEF19	26	Q99972	MYOC	47	Q9NQZ5	STARD7	5	P24534	EEF1B2	26	P51532	SMARCA4
6	Q8IZT6	ASPM	27	O94916	NFAT5	48	O14907	TAX1BP3	6	Q9NPG1	FZD3	27	O95347	SMC2
7	O15234	CASC3	28	O75928	PIAS2	49	P15923	TCF3	7	O00144	FZD9	28	A0A024R158	SMC2L1
8	Q3V6T2	CCDC88A	29	Q96MT3	PRICKLE1	50	P36402	TCF7	8	Q13283	G3BP1	29	Q01082	SPTBN1
9	Q8ND76	CCNY	30	Q7Z3G6	PRICKLE2	51	Q6FWH4	TCF7L2	9	P62805	HIST1H4A	30	Q15583	TGIF1
10	P35638	DDIT3	31	A6NK89	RASSF10	52	Q9UKE5	TNIK	10	B2R4R0	HIST1H4L	31	Q9P2C4	TMEM181
11	P35659	DEK	32	P0DJJD3	RBMY1A1	53	Q13641	TPBG	11	Q9UGU5	HMGXB4	32	P09544	WNT2
12	Q155Q3	DIXDC1	33	Q8NHY2	RFWD2	54	Q70CQ2	USP34	12	P07910	HNRNPC	33	Q9H1J7	WNT5B
13	Q68D97	DKFZp686C13257	34	Q05D03	RHOV	55	Q96K76	USP47	13	Q9BUJ2	HNRNPUL1	34	Q9H1J5	WNT8A
14	Q14517	FAT1	35	Q01973	ROR1	56	Q93008	USP9X	14	O00410	IPO5	35	Q9UGI0	ZRANB1
15	P85037	FOXK1	36	Q01974	ROR2	57	F8W9F9	WNK2	15	A0A024R4R9	LENG8	36	B3KWG6	
16	Q92837	FRAT1	37	Q2MKA7	RSP01	58	O14905	WNT9B	16	O75096	LRP4			
17	Q13322	GRB10	38	P34925	RYK	59	A2A288	ZC3H12D	17	Q9BRK4	LZTS2			
18	Q9ULT8	HECTD1	39	Q8WTS6	SETD7	60	B3KWI2		18	O75161	NPHP4			
19	Q6PKG0	LARP1	40	Q6FHJ7	SFRP4	61	B4DN02		19	P09874	PARP1			
20	Q9HBX8	LGR6	41	Q8N114	SHISA5				20	Q8WY48	PP2150			
21	P18858	LIG1	42	A6NL88	SHISA7				21	Q8NHQ8	RASSF8			

Supplementary Table 2. β -catenin immunoprecipitated proteins in wnt3a treated CD8+ T cells

Adult cells and Neonatal cells stimulated with wnt3a (common proteins)														
#	UniProtKB	Gene symbol	#	UniProtKB	Gene symbol	#	UniProtKB	Gene symbol	#	UniProtKB	Gene symbol	#	UniProtKB	Gene symbol
1	P55196	AFDN	82	Q5SW24	DACT2	163	Q96N16	JAKMIP1	244	Q96KR7	PHACTR3	325	Q14746	TERT
2	P30838	ALDH3A1	83	Q96B18	DACT3	164	Q719H9	KCTD1	245	Q75925	PIAS1	326	Q14186	TFDP1
3	P05062	ALDOB	84	O43293	DAPK3	165	Q9Y2K7	KDM2A	246	Q75928	PIAS2	327	Q15583	TGIF1
4	P09972	ALDOC	85	Q9UHG0	DCDC2	166	Q8NHM5	KDM2B	247	Q8N2W9	PIAS4	328	P10828	THR8
5	Q5JTC6	AMER1	86	O00571	DDX3X	167	A2VDJ0	KIAA0922	248	P14618	PKM	329	Q04724	TLE1
6	Q8N7J2	AMER2	87	P35659	DEK	168	Q53G59	KLHL12	249	Q16512	PKN1	330	Q04725	TLE2
7	Q8N944	AMER3	88	Q8WUY9	DEPDC1B	169	Q1ED39	KNOP1	250	Q9UPG8	PLAGL2	331	Q04726	TLE3
8	Q8IY63	AMOTL1	89	Q9NRI5	DISC1	170	P13646	KRT13	251	Q9NQ55	PPAN	332	Q04727	TLE4
9	Q9Y2J4	AMOTL2	90	Q155Q3	DIXDC1	171	P08729	KRT7	252	Q96MT3	PRICKLE1	333	Q8N4V6	TMEM181
10	Q9Y2G4	ANKRD6	91	Q659G9	DKFZp586H0919	172	Q6PKG0	LARP1	253	Q7Z3G6	PRICKLE2	334	Q66K66	TMEM198
11	Q9NQW6	ANLN	92	Q68D97	DKFZp686C13257	173	Q9NRM7	LATS2	254	Q13131	PRKAA1	335	Q96Q45	TMEM237
12	Q96CW1	AP2M1	93	Q94907	DDX3X	174	Q9UJU2	LEF1	255	P54646	PRKAA2	336	P42167	TMPO
13	P25054	APC	94	Q9UBU2	DDK2	175	A0A024R4R9	LENG8	256	Q86TP1	PRUNE1	337	Q9UKE5	TNIK
14	Q95996	APC2	95	Q9UBP4	DDK3	176	Q8WVC0	LEO1	257	O00487	PSMD14	338	Q95271	TNKS
15	Q8J025	APCDD1	96	Q9UK85	DKKL1	177	Q9BXB1	LGR4	258	Q13308	PTK7	339	Q13641	TPBG
16	Q8IW93	ARHGEF19	97	Q96QB1	DLC1	178	Q9HBX8	LGR6	259	Q5T170	PYGO2	340	P60174	TPI1
17	Q9H0F7	ARL6	98	Q9Y5R5	DMRT2	179	P18858	LIG1	260	Q9H0H5	RACGAP1	341	Q86V40	TRABD2A
18	Q8IUR7	ARMC8	99	Q9NX36	DNAJC28	180	Q9UGP4	LIMD1	261	P63244	RACK1	342	Q6PKC3	TXNDC11
19	Q9P291	ARMCX1	100	A6NLW8	DUXA	181	Q9H9Z2	LIN28A	262	P13631	RARG	343	Q9NNW7	TXNRD2
20	Q9UH62	ARMCX3	101	P54792	DVL1P1	182	Q8NG48	LINS1	263	A6NK89	RASSF10	344	Q86VQ6	TXNRD3
21	O00327	ARNTL	102	O14641	DVL2	183	Q8N448	LNK2	264	Q9BYM8	RBCK1	345	Q94888	UBXN7
22	P50553	ASCL1	103	Q92997	DVL3	184	Q7Z4F1	LRP10	265	P0DJ33	RBMY1A1	346	Q14694	USP10
23	Q8IZT6	ASPM	104	Q9HAK2	EBF2	185	O75096	LRP4	266	P49796	RGS3	347	P54578	USP14
24	L8B567	ATL1-y	105	Q9H8V3	ECT2	186	O75197	LRP5	267	Q7L0Q8	RHOA	348	Q70CQ2	USP34
25	O75787	ATP6AP2	106	P29692	EEF1D	187	O75581	LRP6	268	Q96L33	RHOV	349	Q96K76	USP47
26	Q9Y2T1	AXIN2	107	O43432	EIF4G3	188	Q9Y608	LRRFIP2	269	Q63HN8	RNF213	350	Q93009	USP7
27	Q9HBU1	BARX1	108	Q9BXX0	EMILIN2	189	Q6UWE0	LRSAM1	270	Q5VTB9	RNF220	351	Q93008	USP9X
28	Q9C0K0	BCL11B	109	Q09472	EP300	190	Q86X29	LSR	271	Q96EP0	RNF31	352	Q9ULK5	VANGL2
29	O00512	BCL9	110	Q9Y6I3	EPN1	191	Q86Y78	LYPD6	272	Q68DV7	RNF43	353	Q9UIW0	VAX2
30	Q86UU0	BCL9L	111	Q14517	FAT1	192	Q9BRK4	LZTS2	273	B1APY4	ROR2	354	Q95231	VENTX
31	Q9H694	BICC1	112	Q96IG2	FBXL20	193	Q9UPN3	MACF1	274	Q96P16	RPRD1A	355	O75436	VPS26A
32	Q9H2G9	BLZF1	113	Q9UKB1	FBXW11	194	Q13233	MAP3K1	275	P31350	RRM2	356	Q96QK1	VPS35
33	P12643	BMP2	114	P16591	FER	195	P27816	MAP4	276	Q6UXX9	RSPO2	357	Q9GZS3	WDR61
34	Q9NPI1	BRD7	115	Q96AC1	FERMT2	196	P45984	MAPK9	277	Q9BXY4	RSPO3	358	Q95388	WISP1
35	Q9Y297	BTRC	116	Q7Z7B0	FILIP1	197	Q9P0L2	MARK1	278	Q92541	RTF1	359	O76076	WISP2
36	A0A024R6P6	C14orf129	117	Q8NFG4	FLCN	198	Q7KZ17	MARK2	279	Q9UL12	SARDH	360	Q95389	WISP3
37	Q07021	C1QBP	118	P21333	FLNA	199	P27448	MARK3	280	Q8N9R8	SCAI	361	Q9Y3S1	WNK2
38	Q49A92	C8orf34	119	P85037	FOXK1	200	P23508	MCC	281	Q6P3W7	SCYL2	362	P04628	WNT1
39	Q6IMN6	CAPRIN2	120	O75474	FRAT2	201	Q14676	MDC1	282	Q9HC62	SEN2	363	O00744	WNT10B
40	O15234	CASC3	121	D9ZGF6	FRZB	202	Q99750	MDFI	283	O15047	SETD1A	364	P09544	WNT2
41	Q95931	CBX7	122	Q8N475	FSTL5	203	Q9P1T7	MDFIC	284	Q15047	SETDB1	365	Q93097	WNT2B
42	A6NI87	CBY3	123	Q9UP38	FZD1	204	Q93074	MED12	285	Q5T4F7	SFRP5	366	P56703	WNT3
43	Q8IX12	CCAR1	124	Q9ULW2	FZD10	205	Q71F56	MED13L	286	Q9H788	SH2D4A	367	P56704	WNT3A
44	Q8N163	CCAR2	125	Q86UZ8	FZD2	206	Q9BY79	MFRP	287	Q6ZSJ9	SHISA6	368	Q9Y6F9	WNT6
45	Q3V6T2	CCDC88A	126	Q9NPG1	FZD3	207	A0A024R483	MGC99813	288	A6NL88	SHISA7	369	O00755	WNT7A
46	Q9P219	CCDC88C	127	Q9ULV1	FZD4	208	Q9H2W2	MIXL1	289	B8Z334	SHISA8	370	P56706	WNT7B
47	P24863	CCNC	128	A0A024R9E9	FZD6	209	P50281	MMP14	290	O95343	SIX3	371	Q9H1J5	WNT8A
48	Q8ND76	CCNY	129	Q9H461	FZD8	210	Q99972	MYOC	291	Q6PML9	SLC30A9	372	Q93098	WNT8B

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49	O95400	CD2BP2	130	O00144	FZD9	211	Q00604	NDP	292	O14745	SLC9A3R1	373	D9ZGG3	WNT9A
50	Q6P1J9	CDC73	131	Q13283	G3BP1	212	Q9UQ49	NEU3	293	O00193	SMAP	374	A6NIX2	WTIP
51	O94921	CDK14	132	P08151	GLI1	213	O94916	NFAT5	294	P51532	SMARCA4	375	Q9NZC7	WWOX
52	Q00535	CDK5	133	Q9BZE0	GLIS2	214	Q13469	NFATC2	295	O60264	SMARCA5	376	P98170	XIAP
53	P49336	CDK8	134	P15104	GLUL	215	Q6KCT9	NIPBL	296	O95347	SMC2	377	P63104	YWHAZ
54	Q9NYQ7	CELSR3	135	P30679	GNA15	216	Q969G9	NKD1	297	A0A024R158	SMC2L1	378	Q96IU2	ZBED3
55	Q8WUJ3	CEMIP	136	P35052	GPC1	217	Q969F2	NKD2	298	Q99835	SMO	379	Q9Y330	ZBTB12
56	Q9HCK8	CHD8	137	Q13322	GRB10	218	Q9NVX2	NLE1	299	O60493	SNX3	380	Q86T24	ZBTB33
57	Q9NPF2	CHST11	138	P34947	GRK5	219	Q9UBE8	NLK	300	Q9BQB4	SOST	381	A2A288	ZC3H12D
58	Q9Y471	CMAHP	139	P43250	GRK6	220	Q15233	NONO	301	O15370	SOX12	382	Q8TBF4	ZCRB1
59	P39060	COL18A1	140	P49840	GSK3A	221	Q7Z494	NPHP3	302	Q9UN79	SOX13	383	O43296	ZNF264
60	P48729	CSNK1A1	141	P49841	GSK3B	222	O75161	NPHP4	303	O60248	SOX15	384	Q9ULT6	ZNRF3
61	Q8N752	CSNK1A1L	142	Q9P0R6	GSKIP	223	F1D8N7	NR1A2	304	Q8NB90	SPATA5	385	Q9UGI0	ZRANB1
62	P48730	CSNK1D	143	O60381	HBP1	224	F1D8P1	NR1B3	305	Q496A3	SPATS1	386	B3KW12	
63	P49674	CSNK1E	144	Q9UBI9	HECA	225	F1D8T1	NR2A1	306	A0A024R297	SPIN	387	B3KW36	
64	Q9HCP0	CSNK1G1	145	Q9ULT8	HECTD1	226	O00482	NR5A2	307	Q9Y657	SPIN1	388	B4DZB8	
65	P78368	CSNK1G2	146	V9HVV3	HEL-S-269	227	P04629	NTRK1	308	Q01082	SPTBN1	389	A0A024RC65	
66	Q9Y6M4	CSNK1G3	147	Q14526	HIC1	228	Q9H1E3	NUCKS1	309	Q9BXP5	SRRT	390	B3KWG6	
67	P68400	CSNK2A1	148	Q9H2X6	HIPK2	229	Q6DKJ4	NXN	310	Q13242	SRSF9	391	Q59ER8	
68	P19784	CSNK2A2	149	P26583	HMGB2	230	Q5BJF6	ODF2	311	P81877	SSBP2	392	B7Z3D2	
69	Q9UI47	CTNNA3	150	O15347	HMGB3	231	O75665	OFD1	312	Q15831	STK11	393	A8K4L6	
70	P35222	CTNNB1	151	Q9UGU5	HMGXB4	232	Q99784	OLFM1	313	Q13188	STK3	394	B2RE34	
71	O60716	CTNND1	152	P41235	HNF4A	233	Q96BN8	OTULIN	314	P16949	STMN1	395	A0A140VJV5	
72	Q9UQB3	CTNND2	153	P07910	HNRNPC	234	A0A024RC37	P15RS	315	Q8IWU6	SULF1	396	B4DN02	
73	Q6PD62	CTR9	154	Q9BUJ2	HNRNPUL1	235	Q8N7H5	PAF1	316	O75478	TADA2A	397	B3KX42	
74	Q13616	CUL1	155	P31270	HOXA11	236	O96013	PAK4	317	A0A024R0Y4	TADA2L	398	Q59EF7	
75	Q7LFL8	CXXC5	156	Q9H0C8	ILKAP	237	P09874	PARP1	318	O60907	TBL1X	399	Q53GP3	
76	Q9NQC7	CYLD	157	Q9Y283	INVS	238	Q8N6Y1	PCDH20	319	P15923	TCF3	400	Q8N2N3	
77	P04798	CYP1A1	158	O00410	IPO5	239	P35227	PCGF2	320	P36402	TCF7	401	B3KQX9	
78	Q9Y4D1	DAAM1	159	P46940	IQGAP1	240	P30101	PDIA3	321	Q9HCS4	TCF7L1	402	B3KSZ7	
79	Q86T65	DAAM2	160	Q6DN90	IQSEC1	241	Q9UPQ7	PDZRN3	322	C6ZRJ7	TCF7L2			
80	P98082	DAB2	161	Q96J02	ITCH	242	W0S0X4	Pe1Fe3	323	Q13569	TDG			
81	Q9NYF0	DACT1	162	Q6IE81	JADE1	243	Q99471	PFDN5	324	Q15554	TERF2			
Adult cells stimulated with wnt3a (exclusive proteins)						Neonatal cells stimulated with wnt3a (exclusive proteins)								
#	UniProtKB	Gene symbol	#	UniProtKB	Gene symbol	#	UniProtKB	Gene symbol	#	UniProtKB	Gene symbol	#	UniProtKB	Gene symbol
1	Q96BJ3	AIDA	28	A0A024R2Z8	PTHR1	1	Q9Y4X0	AMMECR1	28	Q9NTX7	RNF146			
2	Q13145	BAMBI	29	P20472	PVALB	2	Q8NEY4	ATP6V1C2	29	Q2MKA7	RSPO1			
3	Q9NR00	C8orf4	30	Q9Y3Y4	PYGO1	3	O15169	AXIN1	30	P29622	SERPINA4			
4	Q99966	CITED1	31	Q8WVD3	RNF138	4	Q9BQE9	BCL7B	31	Q9NPC8	SIX2			
5	Q9H2H0	CXXC4	32	Q01973	ROR1	5	Q53F96	CD2	32	Q6STE5	SMARCD3			
6	P35638	DDIT3	33	Q9NQG5	RPRD1B	6	Q9UI36	DACH1	33	O95863	SNAI1			
7	Q68DS0	DKFZp781N011	34	P34925	RYK	7	Q9NSR3	DKFZp761O2023	34	Q9BT81	SOX7			
8	Q9UBT3	DKK4	35	Q8N474	SFRP1	8	Q9UM22	EPDR1	35	A0A024R9Y9	SPIN-2			
9	O14640	DVL1	36	Q6UW14	SHISA2	9	Q6UWV7	FAM159A	36	Q9BPZ2	SPIN2B			
10	Q92837	FRAT1	37	Q8N114	SHISA5	10	Q9NZH0	GPRC5B	37	Q15532	SS18			
11	P22083	FUT4	38	B4DS77	SHISA9	11	P18283	GPX2	38	Q9NQZ5	STARD7			
12	Q13467	FZD5	39	P63208	SKP1	12	P62805	HIST1H4A	39	Q9Y3Q3	TMED3			
13	V9HWP8	HEL-S-30	40	Q9NP91	SLC6A20	13	B2R4R0	HIST1H4L	40	Q9NS68	TNFRSF19			
14	Q15011	HERPUD1	41	Q6X4U4	SOSTDC1	14	A0A024R5B2	MARK2	41	A6NFA1	TRABD2B			
15	Q03014	HHEX	42	Q8NA61	SPERT	15	Q96J80	MERP1	42	O95859	TSPAN12			
16	D3DWI5	JRK	43	Q2MJR0	SPRED3	16	Q9BRJ9	MESP1	43	Q9GZT5	WNT10A			
17	Q96MU8	KREMEN1	44	Q8N2I9	STK40	17	P50579	METAP2	44	A4D0W7	WNT16			
18	Q8NCW0	KREMEN2	45	Q9BX79	STRA6	18	P17050	NAGA	45	Q9UBV4	WNT16			

19	O75473	LGR5	46	Q06520	SULT2A1	19	Q6VVB1	NHLRC1	46	P41221	WNT5A
20	O43318	MAP3K7	47	Q15750	TAB1	20	Q6P988	NOTUM	47	B3KWB6	
21	Q14696	MESDC2	48	Q92734	TFG	21	Q9P2E7	PCDH10	48	B7Z5Q3	
22	O15146	MUSK	49	O43294	TGFB111	22	Q7Z2D5	PLPPR4	49	Q59G99	
23	Q9UN36	NDRG2	50	I3L2J3	TMEM88	23	P49768	PSEN1			
24	P35372	OPRM1	51	Q5T9L3	WLS	24	A0A024R136	RACGAP1			
25	P11309	PIM1	52	O96014	WNT11	25	Q8NHQ8	RASSF8			
26	P62136	PPP1CA	53	A5Y0M9		26	Q8NHY2	RFWD2			
27	Q03431	PTH1R				27	A0A024R841	RGS3			

For Peer Review

Supplementary Table 3. Primers used for real-time PCR

Genes	Gene transcription primers (5' to 3')	
	Forward	Reverse
CCND1	AGTTGCAAAGTCCTGGAGCC	GATGGTTTCCACTTCGCAGC
BIRC5	AGGACCACCGCATCTCTACA	TGTTCTCTATGGGGTCGTCA
POU5F1	TCGCAAGCCCTCATTTACACC	TTGGAAGCTTAGCCAGGTCC
S100A4	TCTTGTTTTGATCCTGACTGCT	ACTTGTCACCCTCTTTGCCC
cJUN	GGAGACAAGTGGCAGAGTCC	CCAAGTTCAACAACCGGTGC
FRA1	AACCTCCTCGCTTTGTGAG	GAAACAGTGGGCAGCTTTGG
FOS	CTGGCGTTGTGAAGACCATGA	TCTAGTTGGTCTGTCTCCGCT
AXIN2	ACACCGATAGAGGGGTGGAA	CCACCGACCACGTTTCTGTA
GZMB	AGCCTGCACCAAAGTCTCAA	TTTCATTACAGCGGGGGCTT
PRDM1	AGTGTTGCGGAGAGGCAAG	CCCACACGTTTTTCCAAGCAA
NKD1	TGAGGACCACTCTCCTACGG	AGGGTCCTCTACTGTCGTCC
SFRP1	CTTCTACTGGCCCGAGATGC	ATCCTCAGTGCAAACCTCGCTG
MAP3K7	TGGTGCTGAACCATTGCCA	CTGCAACCAGCAGTAAGTTTGG
WNT11	GCCCACCACATGGAATCACT	AGTTCACTTGACGAGGCCG
AXIN1	CAAGCAGAGGTATGTGCAGGA	CACAACGATGCTGTACACAG
PSEN1	AAGCGTATACCTAATCTGGGAGC	CTGCCGTTCTCTATTGTCATTCTG
Genes	DNA promoter primers (5' to 3')	
	Forward	Reverse
CCND1	ATTCTTTGGCCGTCTGTCCG	GAGACCACGAGAAGGGGTGA
BIRC5	TTTGAAAGCAGTCGAGGGGG	GTGTGCCGGGAGTTGTAGTC
POU5F1	GAGGTCAAGGCTAGTGGGTG	CGAGAAGGCGAAATCCGAAG
S100A4	CCCCCTAGCTTTTGTGTCACC	ATGGGGGACAGGGAGTAGT
cJUN	GGGAAACAACAACCACCCCT	GACTGTCTAGTGACGGTGGC
FRA1	CAGAAACGGAGGTTAGCCCA	CTGACGTAGCTGCCCATACA
FOS	AACGATCCTGCCACGGACAT	CTGAAGGGATAACGGGAACGC
AXIN2	CTGTGGGTGGAGAAATGCCC	CCCTACTCCACCCCTCTAGC
GZMB	AAAGCGTCTTCAAAATCATAGCTGA	GCTGGAATTCTACAGGGTAGGAG
PRDM1	GGGCGATCTGGAGTGTTTAGA	GAGAGCTTCCTCTCTTCGCAT
NKD1	GGGAAGACTGGGAAAAGGCA	TCCGCTCCACGTTCTCTCTA