

# C/EBP $\delta$ Gene Targets in Human Keratinocytes

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## Abstract

C/EBPs are a family of B-Zip transcription factors -TFs- involved in the regulation of differentiation in several tissues. The two most studied members -C/EBP $\alpha$  and C/EBP $\beta$ - play important roles in skin homeostasis and their ablation reveals cells with stem cells signatures. Much less is known about C/EBP $\delta$  which is highly expressed in the granular layer of interfollicular epidermis and is a direct target of p63, the master regulator of multilayered epithelia. We identified C/EBP $\delta$  target genes in human primary keratinocytes by ChIP on chip and profiling of cells functionally inactivated with siRNA. Categorization suggests a role in differentiation and control of cell-cycle, particularly of G2/M genes. Among positively controlled targets are numerous genes involved in barrier function. Functional inactivation of C/EBP $\delta$  as well as overexpressions of two TF targets -MafB and SOX2- affect expression of markers of keratinocyte differentiation. We performed IHC on skin tumor tissue arrays: expression of C/EBP $\delta$  is lost in Basal Cell Carcinomas, but a majority of Squamous Cell Carcinomas showed elevated levels of the protein. Our data indicate that C/EBP $\delta$  plays a role in late stages of keratinocyte differentiation.

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## Introduction

The skin is a multilayered epithelium that enables organisms to be protected from the exterior, while keeping homeostasis of fluids. Skin differentiation is a lifelong process that leads to the expansion of cells with specific, exquisite features from a relatively few progenitors that guarantee the maintenance of the stem cells pool. Renewal of stem cells and ongoing active terminal differentiation require the progressive fine tuning of transcriptional programs, which are masterminded by Transcription Factors (TFs). Several TFs have a role in keratinocytes physiology. Specifically, genetic experiments established that p63, IRF-6, KLF4, Gata3 and C/EBPs are important [1].

C/EBPs are a family of six B-Zip TFs that activate and repress transcription of genes involved in differentiation and growth control [2]. The two most studied members of the family, C/EBP $\alpha$  and C/EBP $\beta$  are required for differentiation of adipocytes and hematopoietic cells [3–6]. Inactivation of C/EBP $\epsilon$  leads to lack of natural killer cells [7]. C/EBP $\zeta$  -CHOP, DDIT3- is involved in the response to noxious signals, such as ER-stress and DNA-damage [8]. C/EBP $\delta$  KO mice have a mammary phenotype, with an alteration in the involution of the mammary glands upon lactation [9,10]. Similarly to C/EBP $\beta$  adipocyte differentiation is impaired in cells that lack C/EBP $\delta$  when cultured *in vitro* [6].

In the skin, C/EBP $\alpha$  is expressed mostly in the upper layers [10] and mice with selective ablation of C/EBP $\alpha$  in the skin have no

apparent phenotype in normal conditions, but are subject to increased RAS-mediated tumorigenesis [11]. C/EBP $\beta$  is expressed in the cytoplasm of basal keratinocytes [10] and in the nuclei of the spinous layer [12]. Mice with selective ablation of C/EBP $\beta$  in the skin have epidermal hyperplasia with downplay of Keratin 1 and 10 expression [13]. Contrary to C/EBP $\alpha$ , C/EBP $\beta$  ablation in the skin suppresses RAS-mediated tumorigenesis [14–17]. Importantly, the combined ablation of C/EBP $\alpha$  and C/EBP $\beta$  leads to profound alteration in interfollicular epidermis due to increased proliferation of basal cells, defective differentiation and insufficient barrier function [18]. These mice show suprabasal p63 expression. Vinson's group reported on a transgenic model expressing a dominant negative C/EBP -termed A-C/EBP- in basal keratinocytes: these mice have hyperplasia of the basal epidermis and increased apoptosis of the upper layers [19]: p53 and pro-apoptotic markers are induced and C/EBP $\beta$  dramatically reduced. A-C/EBP is not C/EBP $\beta$ -specific, thus C/EBP $\delta$  might contribute to the observed phenotype. Although C/EBP $\delta$  KO mice have no apparent skin alterations [20], expression is high in human interfollicular skin, and negatively regulated by p63 [21]. Importantly, in the multi-layered epithelia of the corneal limbus, C/EBP $\delta$  is upstream of p63 and thought to be a marker of stemness [22].

Recent reports reveal alterations in the structure or expression levels of C/EBP family members in a variety of human cancers. For example, down-regulation of C/EBP $\alpha$  is seen in several

human malignancies, including skin [23], whereas C/EBP $\beta$  is overexpressed in carcinogen-induced skin tumors [24] and breast cancer [25]. Moreover, “loss of function” alterations of CEBP $\delta$  and promoter methylation of the C/EBP $\delta$  locus have been observed in primary human breast tumors [26,27]. Through ChIP on chip experiments, C/EBP $\alpha$  and C/EBP $\beta$  targets were identified in 3T3-L1 adipocytes [28], and C/EBP $\delta$  in mammary cells undergoing growth arrest [29]. To reconstruct the C/EBP $\delta$  network in keratinocytes, we identified targets, through the use of RNAi inactivation coupled to gene expression profiling, and ChIP on chip technology.

## Materials and Methods

### Cells and culture conditions

First passage human primary keratinocytes were derived from breast of healthy individuals undergoing plastic surgery and grown on a feeder-layer of lethally irradiated 3T3 cells in DMEM F12 added of Insulin (5  $\mu$ g/ml), EGF-R (10 ng/ml), hydrocortisone (0.4  $\mu$ g/ml), T3 (2 nM), Cholera toxin (0.1 nM) and transferrin (5  $\mu$ g/ml). The preparation of primary keratinocytes was performed at the “Banca della Cute”-CTO Hospital in Torino (I), and the procedure was approved by the Ethics Committee “Comitato Etico Interaziendale S. Giovanni Battista CTO-M. Adelaide”. Informed verbal consent from the patients was obtained as stated in procedure, for which approval was granted by the Ethics Committee.

### RT-PCR and transfections

For RNA profilings, 10<sup>6</sup> first passage primary keratinocytes from healthy individuals were transfected with Nucleofector (Amaxa, D) according to the Manufacturer’ conditions with the off-target control siRNA oligos (Sigma), or with an oligonucleotide targeting human C/EBP $\delta$  GACUCAGCAACGCCATuu [21]. The mRNAs were extracted 48 hours after transfections with the RNA-Easy kit (Qiagen, D). For cDNA synthesis, 2  $\mu$ g of RNA were used with M-MLV-RT kit (Invitrogen, USA). Semi-quantitative PCR analysis of the C/EBP $\delta$  mRNA were performed with specific primers (AGTTCCTGGGACATAGGAGCGCA; GTACCTTAGCTGCATCAACAGGAG). qRT-PCR analysis was used to validate the profiling arrays, with a Biorad MyIQ single colour thermal cycler and a SYBR Green PCR Master mix. Specificity of products was monitored with a heat dissociation curve, fold enrichment was calculated with the formula  $2^{-\Delta C_t}$  where the  $C_t$  represented the threshold cycles of the input, the specific antibody and the negative antibody; a further normalization with the enrichment obtained on a negative genomic region (Centromeric Satellite 11) was applied. A list of the RT-PCR primers is provided in Table S1. Transfections with 4  $\mu$ g of Sox2 and MafB expression vectors, or empty pcDNA3 control, were used; cells were split in two after 24 hours, and one of the culture was differentiated by addition of 1,4 mM CaCl<sub>2</sub>.

### ChIP on chip

ChIP analysis was carried out with the method described in Ref. 21 with an anti-C/EBP $\delta$  antibody (Active Motif #39006). The immunoprecipitated DNA was analyzed with specific primers in semi-quantitative PCR assays with the primers listed in Table S2.

The generation of amplicons from the individual ChIPs was performed following the protocol of LM-PCR described in Refs. 30, 31. Briefly, two unidirectional linkers were annealed and ligated to the chromatin IPs, previously blunted by T4 DNA polymerase. The first amplicons were generated by PCR (15 cycles). The reaction was purified using the GFX PCR purification

kit (Amersham Biosciences) according to the Manufacturer’s instructions. A fraction of these initial reactions was used to generate more amplicons for 30 additional cycles. After purification, the DNA was quantified and examined by gene specific PCR to ensure that the initial enrichment was maintained. 5  $\mu$ g of amplicons for Flag and input DNA (subjected to the same number of PCR manipulations) were labeled using the LabelIT Cy5/Cy3 Nucleic acid labeling kit (Mirus), following the manufacturer’s instructions, with a reagent to DNA ratio of 2.5 for Cy5 (IPs) and 1.5 for Cy3 (Input). The hybridization and washing conditions for these slides were described previously [30,31]. The CpG 21K slides were purchased from University Health Network, Toronto, Canada. The hybridized microarrays were scanned and analyzed using a ScanArray 4000 and QuantArray analysis software (Packard). Features of poor intensity (<500) and those which did not meet the quality control criteria (visual inspection, spot circularity, spot uniformity and background uniformity for both channels) were discarded. After the background subtraction for each spot, the data were normalized to median, that is, the ratio of the median value of all spots in the Cy5 channel (IP DNA) and the ratio of the median value of the control channel (Cy3 Input). The 21K CpG island array was described in Ref. 31. We considered positive all the clones which satisfied the described criteria: >1.5-fold enrichment in the C/EBP $\delta$  samples over the negative antibody -Flag- samples in each of the performed experiments. The sequences of the positive CpG islands clones were retrieved from both the Sanger center (<http://www.sanger.ac.uk/HGP/cgi.shtm>) and the UHN Cancer Center (<http://derlab.med.utoronto.ca/CpGISlands.htm>) and mapped on the human genome using Blat (<http://genome.ucsc.edu>). Approximately 2 kb of genomic regions 5’ and 3’ of the CpG islands clones were considered and one or more Gene ID were assigned to each clone, if they laid in these regions. GO enrichment analysis was performed at <http://bioinfo.vanderbilt.edu/webgestalt>.

### RNA profiling

Keratinocytes from three independent healthy donors were transfected to inactivate C/EBP $\delta$ . 2  $\mu$ g of each sample were pooled, for further processing. For each sample, 500 ng of total RNA were synthesized to biotinylated cRNA using the Illumina RNA Amplification Kit (Ambion, USA). Synthesis was carried out according to the Manufacturers’ instructions. From each sample, three technical replicates were produced and 750 ng cRNA were hybridized for 18 hrs to HumanHT12 v. 3.0 Expression BeadChips (Illumina Inc., USA) according to the protocol provided by the manufacturer. Hybridized chips were washed and stained with streptavidin-conjugated Cy3 (GE Healthcare, USA). BeadChips were dried and scanned with an Illumina BeadArray Reader (Illumina, USA). For data analysis, the intensity files were loaded into the Illumina BeadStudio v. 3.1.3.0 software for quality control and gene expression analysis. First, the quantile normalization algorithm was applied on the dataset to correct systematic errors. Background is subtracted. For differential expression analysis, technical replicates of each sample were grouped together and genes with a detection  $p$ -value below 0.01, corresponding to a false positive rate of 1%, were considered as detected. Differently expressed genes were selected with Diff Score cutoff set at  $\pm 30$ ; genes with a differential expression of >1.5 between siC/EBP $\delta$  and siScramble were retrieved.

### Western blot and Immunohistochemistry

Western blot analysis was performed using standard procedure with a Pierce secondary antibody and PDS detection system (Genespin, I).

For IHC, tissue samples were fixed in buffered formalin, dehydrated, embedded in paraffin wax and sectioned. After deparaffinizing and rehydrating, each tissue section was immersed in citrate buffer 0.01M pH 6 or EDTA 0.05M pH 8 or Tris-HCl 0.05M pH 9.9, boiled 3 times for 5 minutes in a pressure cooker and washed with TBS buffer. Each section was placed on the Dako cytostation automated immunostainer and incubated with the specific monoclonal antibody at room temperature for 45 minutes, washed with TBS pH 7.6 and incubated in a biotinylated goat anti-mouse anti-rabbit immunoglobulins (Dako REAL<sup>TM</sup>, Dako, Dn) at room temperature for 30 minutes. After incubation with the secondary antibody and a new wash with TBS pH 7.6, sections were incubated with streptavidin conjugated to alkaline phosphatase at room temperature for 30'. A red chromogen solution was prepared as indicated by Dako REAL<sup>TM</sup> datasheet. Each section was counterstained in Mayer's Hematoxylin solution and coverslipped. Skin cancer tissue array included 48 cases of normal, benign and cancerous tissue of the skin and subcutaneous tissues in duplicates. All the tissues were from surgical resection. They were fixed in 10% neutral buffered formalin for 24 hours and processed using identical SOPs. Sections were picked onto Superfrost Plus or Apes coated Superfrost slides. (Skin Tumor Tissue Array, BioChain, cat. Z7020093). Anti-C/EBP $\delta$  antibody (Active Motif, USA) was used in this analysis, together with the 4A4 anti-p63 as a control.

## Results

### Identification of C/EBP $\delta$ loci in human keratinocytes

C/EBP $\delta$  emerged in expression profilings of human HaCaT and primary keratinocytes in which p63 was functionally inactivated by RNAi [21,32]. To identify C/EBP $\delta$  targets *in vivo*, we performed ChIP on chip analysis with a specific antibody on chromatin derived from keratinocytes of three healthy individuals undergoing plastic surgery. The enrichment over the Flag control was first tested on three promoters previously identified as *bona fide* C/EBP $\delta$  targets [21]: Figure 1A shows that TGF $\beta$ II-R, ESR and Zeb1 promoters, unlike satellite DNA, are specifically enriched in the C/EBP $\delta$  IP with respect to control IP. We hybridized DNA immunoprecipitated with C/EBP $\delta$  and Flag control antibodies to the 21K CpG islands array, together with DNAs from Input controls. With a *ratio* of 1.5 enrichment over Inputs, 136 C/EBP $\delta$  loci were identified. The sequences of these CpG islands spots were retrieved and mapped on the human genome (Table S3). For validation of the ChIP on chip, we selected 13 loci near gene promoters and located potential C/EBP binding sites by rVista and ConSite [33,34] with JASPAR. Amplicons were designed in the area and ChIPs from independent human primary keratinocytes performed. A schematic representation of the selected promoters, with the position of the predicted C/EBP sites and the results of the ChIP experiments are shown in Fig. 2. Ten loci were enriched, one -Gata3- was modestly enriched, and LTBP and BCOR did not show credible binding, at least in the region we analyzed. It should be noted that this analysis is complex, as the bound site(s) could be within 0.5 Kb on either side of the CpG island clone identified.

Functional mining of the associated genes was performed by GO analysis and a few terms emerged with a good degree of robustness: *cell cycle*, *transcription* and *DNA-damage checkpoint* in the Biological Process category; *transcription factor* and *DNA-binding* in Molecular Function, and *nucleus* in the Cellular Component category (Fig. 1B). Comparison with ChIP on chip experiments performed in mammary cells upon overexpression of C/EBP $\delta$  [29] yielded no overlap. The difference in the arrays -12K vs 21K arrays used in our study- the relative paucity of the targets identified (100 vs 136) and the difference of the cellular context -

the MCF12A mammary cell line transfected with C/EBP $\delta$  vs normal keratinocytes- might account for this finding. Nevertheless, many GO enriched categories were similar: *cell adhesion*, *cell cycle*, *transcription*, *DNA-damage* [29].

### Identification of C/EBP $\delta$ -regulated genes in human primary keratinocytes

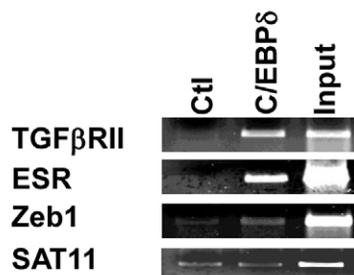
We also sought to identify genes whose expression varies inactivating C/EBP $\delta$  in human primary keratinocytes. We RNAi-inactivated C/EBP $\delta$  with one of the siRNAs previously tested [21]: the process was efficient, as checked in RT-PCR and Western blot analysis (Fig. 3A). Keratinocytes derived from three healthy individuals undergoing plastic surgery, independent from those used in ChIP on chips, were transfected with control and C/EBP $\delta$  siRNAs, followed by gene expression profiling with the Illumina platform, in triplicate. For subsequent analysis, we considered a threshold of 1.5-fold over scramble siRNA samples. Heat maps of these experiments are shown in Fig. 3B: 420 up-regulated and 163 down-regulated genes were identified (Table S4).

We validated by qRT-PCR 22 genes that decrease upon C/EBP $\delta$  removal and 9 that increase: Fig. 3C shows that all genes were regulated in the expected way. Variation were quantitatively greater in qRT-PCR compared to the array data, an indication that the threshold considered is relatively stringent and the cohort of truly regulated genes is larger. To ascertain whether the genes are direct targets of ChIPs, we performed ChIPs with chromatin from adult and neonatal primary keratinocytes on five promoters -Sox2, TEGT, MafB, TopoII $\alpha$  and Jag1- with antibodies against C/EBP $\delta$ ; p63 was used as a positive control, since these genes are also p63 targets. Fig. 3D (Left Panels) shows that most targets are positive, with the exception of Jag1 in neonatal keratinocytes. In the Right Panels qPCR analysis was performed on additional targets in adult keratinocytes and two targets -TIG1 and Hes1- were not validated in the core promoter region considered. Overall the validation data support the results of the profiling analysis.

We performed functional mining of the C/EBP $\delta$  regulated genes by GO analysis (Figure S1). Among the genes whose expression increased after C/EBP $\delta$  removal, hence repressed, the *epidermis development*, *transcription* and *cell migration* classes emerged with p values lower than E-05. Among the genes decreased by C/EBP $\delta$  removal, therefore normally activated, *cell cycle* and *mitosis* were at the top of the Biological Process list, with *lipid biosynthesis*, *development (ectoderm)*, *muscle*, *immune system* having p values lower than E-07. In the Cellular Component category, we found *chromosomal part* and *spindle*, and in the Molecular Function few terms were enriched. In conclusion, this analysis is in good accordance with the terms retrieved from the ChIP on chip experiments, since terms like *cell cycle* and *transcription* predominate. Note, however, that only one gene -MafB- was found in both lists, possibly due to the use of different genomic platforms.

### Comparison between the C/EBP $\delta$ and p63 targets in keratinocytes

We have recently shown that some of the targets of p63 are also influenced by inactivation or overexpression of C/EBP $\delta$  [21]. We were therefore interested in verifying the overlap between the p63 targets and the C/EBP $\delta$  targets identified here. For this analysis, shown in Figure 4, we considered collectively the targets identified in various p63 profiling experiments performed with siRNA [32,36-39]. The coregulated genes are grouped according to the p63 and C/EBP $\delta$  effects on them: the larger cohort is represented by genes that are repressed by p63 and activated by C/EBP $\delta$ , with only three genes behaving in the opposite way. Interestingly, several genes are either repressed or activated by both TFs. These data are in

**A****B****C/EBPδ target genes**

BIOLOGICAL PROCESS	p.value	ANALYZED GENES
regulation of cell cycle	0.009	NF2, ING4
regulation of progression through cell cycle	0.009	NF2, ING4
regulation of transcription	0.009	ING4, GATA3, HOXB4, ING4, BCOR
transcription	0.006	GATA3, HOXB4, ING4, BCOR, PTTG1
transcription\, DNA-dependent	0.003	GATA3,HOXB4, ING4, BCOR
hemopoietic or lymphoid organ development	0.001	
hemopoiesis	0.001	
sensory organ development	0.004	
protein complex assembly	0.004	
immune system development	0.002	
DNA integrity checkpoint	0.005	
DNA damage checkpoint	0.004	
DNA damage response\, signal transduction	0.009	
<b>MOLECULAR FUNCTIONS</b>		
transcription regulator activity	0.008	GATA3, HOXB3, BCOR, PTTG1
transcription factor activity	0.001	GATA3, HOXB3, HOXB4,PTTG1
binding	0.003	PGRMC1
nucleic acid binding	0.001	GATA3, HOXB4, LBR, TBRG1, PTTG1
DNA binding	0.0001	GATA3, HOXB4, LBR, TBRG1, PTTG1
sequence-specific DNA binding	0.002	GATA3, HOXB3
protein binding	0.008	CDH12,CLIC4, LBR, NF2, ING4, BCOR, PTTG1
sodium channel activity	0.009	TRPM2
<b>CELLULAR COMPONENT</b>		
nucleus	0.01	

**Figure 1. Identification of C/EBPδ targets by ChIP on chip.** **A.** Generation of the ChIP amplicons for ChIP on chip experiments. ChIP of human primary keratinocytes with C/EBPδ and control antibodies. The known targets tested are indicated. The normalization of the immunoprecipitated material was performed by evaluating a negative genomic region (Centromeric Satellite 11). **B.** Gene Ontology analysis of C/EBPδ targets genes from the ChIP on chip screening.

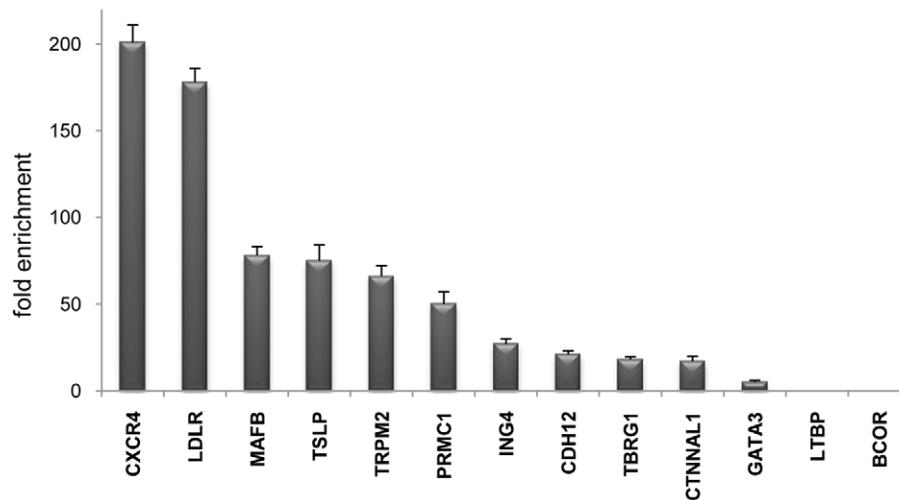
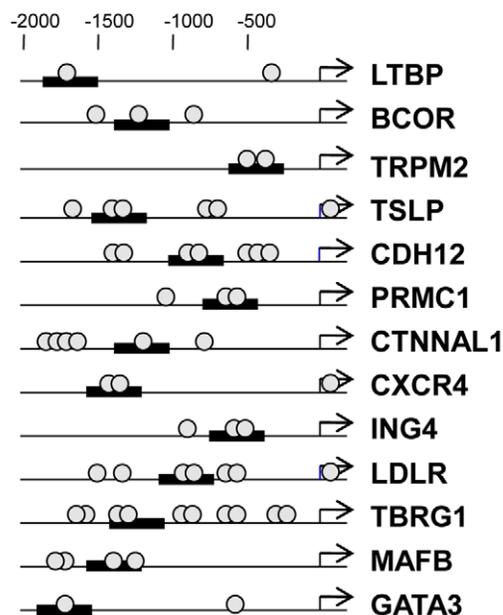
doi:10.1371/journal.pone.0013789.g001

agreement with a different role of the two TFs in keratinocyte differentiation and with the overlapping expression patterns of p63 and C/EBPδ in human interfollicular epidermis [21].

#### C/EBPδ and keratinocyte differentiation

We analyzed the specific role of C/EBPδ in keratinocyte differentiation by transfecting primary keratinocytes with C/EBPδ and scramble siRNAs and inducing differentiation *in vitro*; we

compared the mRNA levels of differentiation markers in silenced and control cells. As shown in Fig. 5, qRT-PCR analysis reveals a substantial increase of the mRNA levels of filaggrin and KRT10 upon differentiation of control cells, but not in cells inactivated for C/EBPδ. On the other hand, the levels of KRT14 modestly decrease after differentiation in control cells, but not in siRNA-treated cells, suggesting that C/EBPδ indeed affects the expression of genes that mark keratinocytes differentiation.

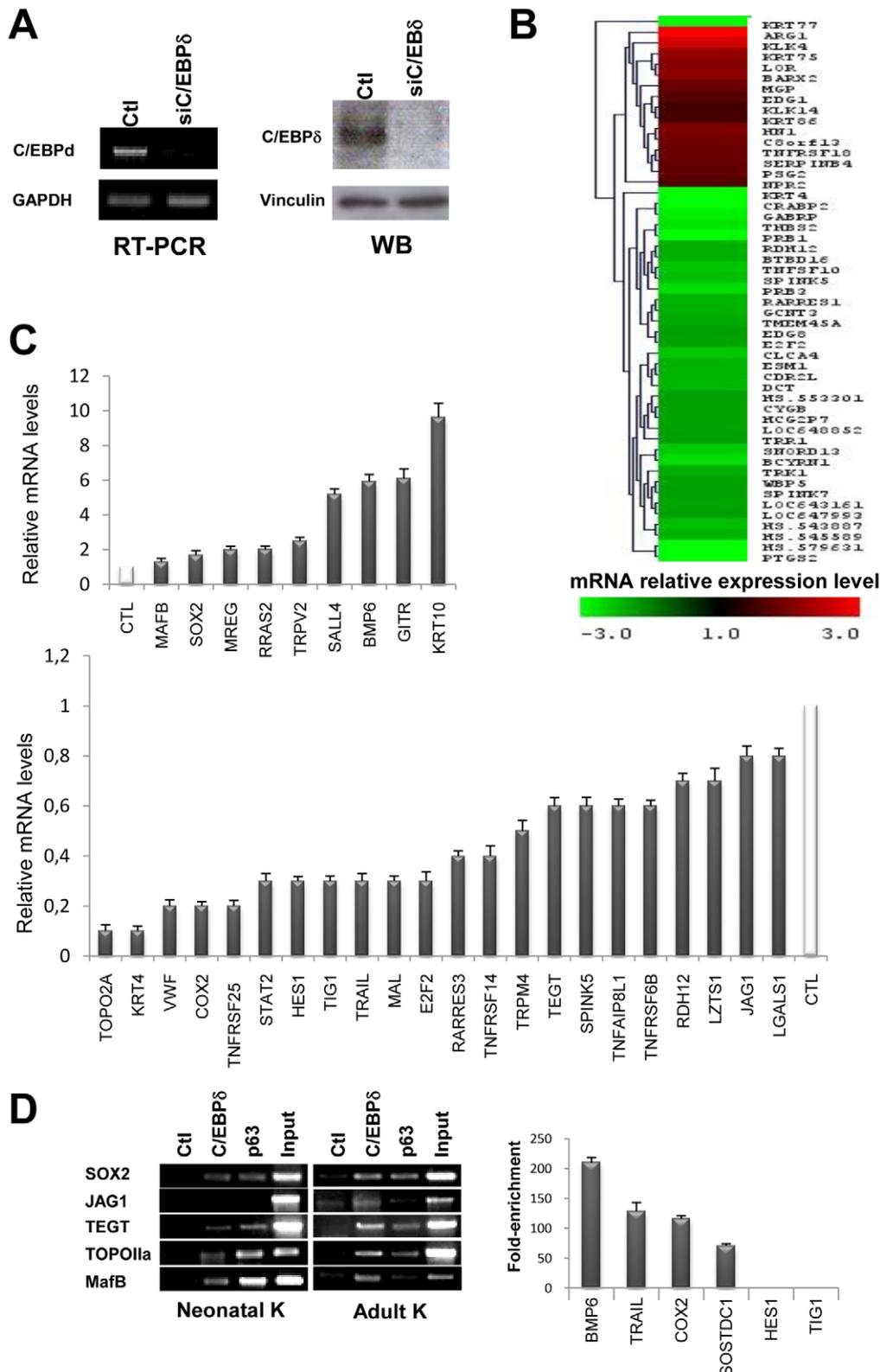
**A****B**

**Figure 2. Validation of C/EBP $\delta$  targets in primary keratinocytes.** **A.** Analysis of the *loci* targeted derived from the ChIP on chip experiments by qPCR analysis of ChIPs with anti-C/EBP $\delta$  and control antibodies from human primary keratinocytes. **B.** The position of the amplicons (black bars) and of the putative C/EBP consensus site (circle) are indicated for targets analyzed in A. doi:10.1371/journal.pone.0013789.g002

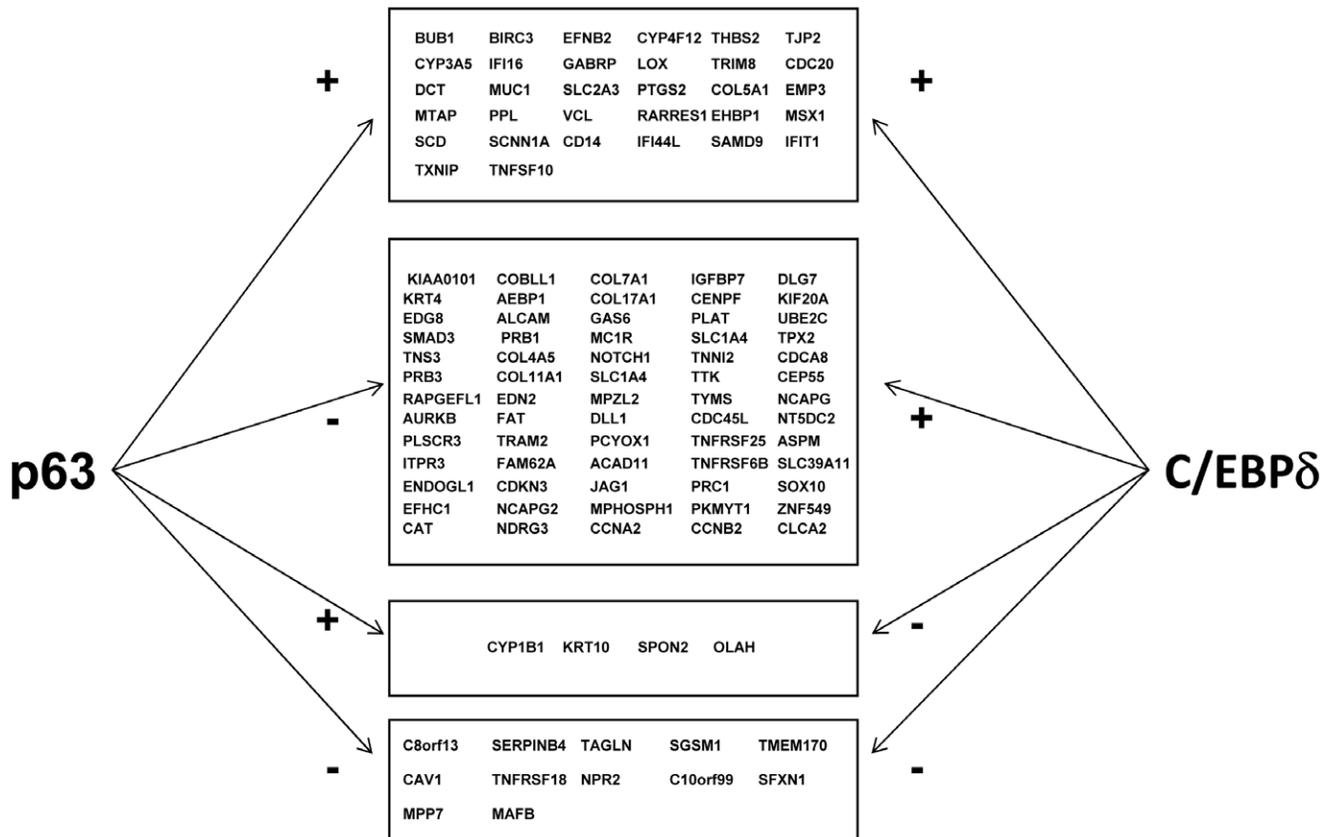
### Role of C/EBP $\delta$ targets Sox2 and MafB, in keratinocyte differentiation

We further analyzed two C/EBP $\delta$  target genes -Sox2 and MafB- to verify their function in keratinocytes differentiation. Sox2 is one of the genes that leads to reprogramming of differentiated cells to pluripotent ES-like cells [40]. MafB is a TF with a role in the development and differentiation of several tissues [41]. The former is repressed by C/EBP $\delta$ , the latter activated. We

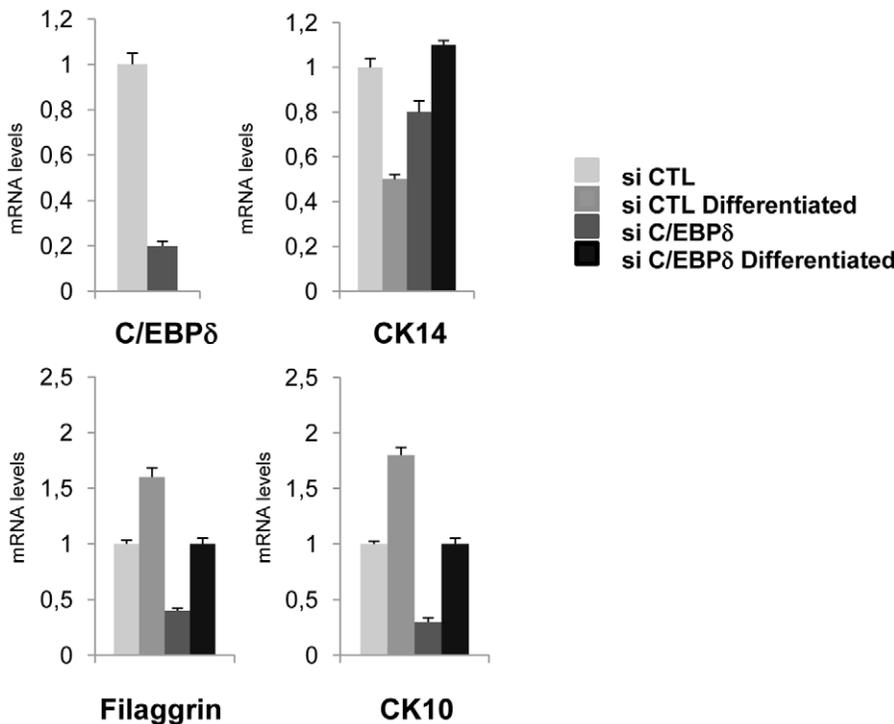
transiently transfected primary keratinocytes with Sox2 and MafB expression vectors, evaluating by qRT-PCR the levels of endogenous markers of keratinocytes differentiation (Fig. 6): Sox2 caused a decrease of filaggrin and KRT10, whereas MafB overexpression resulted in higher levels of filaggrin and KRT10. The changes in the basal layer marker KRT14 were modest. These data suggest the levels of two TFs regulated by C/EBP $\delta$  impact on the expression of markers of differentiation.



**Figure 3. Identification of C/EBPδ-regulated genes in human keratinocytes.** **A.** Left Panels, RT-PCR analysis of human primary keratinocytes after C/EBPδ RNAi inactivation at 48 hours post-transfection. cDNA normalization was performed with GAPDH. Right Panels, Western Blot analysis of the same C/EBPδ-inactivated human primary keratinocytes with the C/EBPδ antibody. Vinculin was used as a loading control. **B.** Heat map showing the mRNA expression levels of several classes of genes after C/EBPδ inactivation. **C.** qRT-PCR analysis of C/EBPδ regulated genes that emerged from the expression profiling. **D.** ChIP analysis of promoter regions of C/EBPδ-regulated genes with chromatin from human primary keratinocytes, with  $\alpha$ -C/EBPδ and control antibodies. In the Left Panel, adult and neonatal keratinocytes were analyzed in semi-quantitative PCR. In the Right Panel, the targets were validated by qPCR in adult keratinocytes. doi:10.1371/journal.pone.0013789.g003

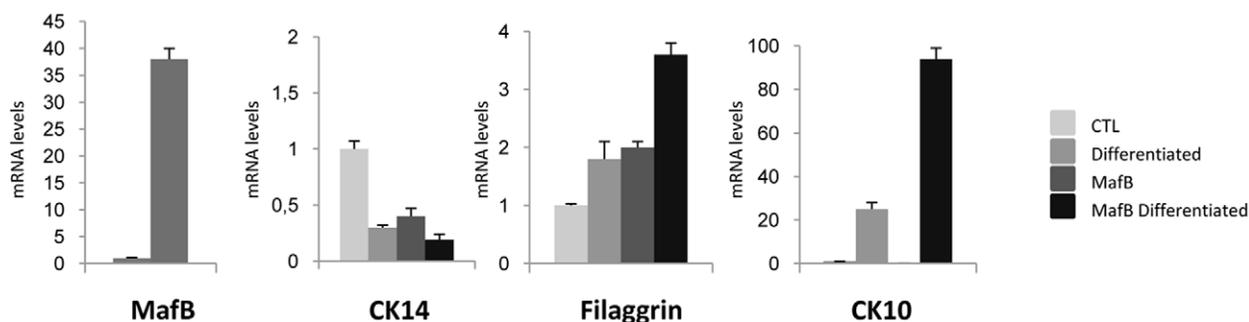


**Figure 4. C/EBP $\delta$  and p63 regulated genes.** Genes coregulated by p63 and C/EBP $\delta$ , either activated or repressed (Table S2). The data on p63 were derived from References 32, 36–39. doi:10.1371/journal.pone.0013789.g004

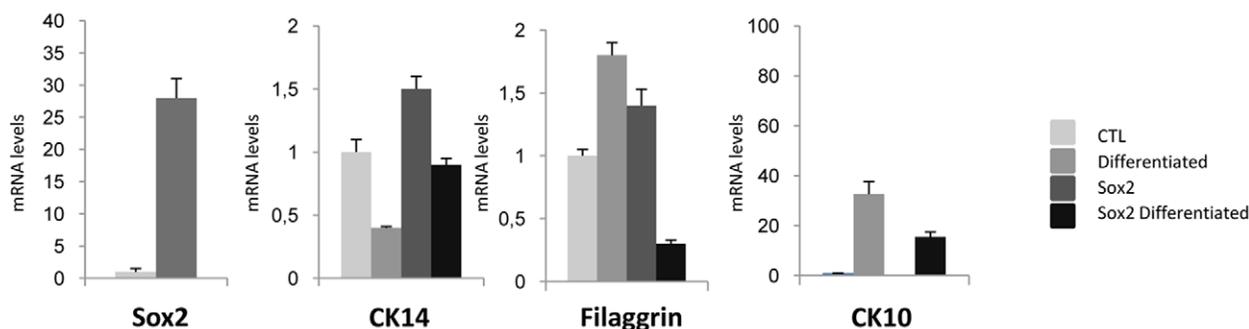


**Figure 5. C/EBP $\delta$  is important for keratinocyte differentiation.** Analysis of differentiation markers by qRT-PCR of C/EBP $\delta$ -inactivated cells induced to differentiate. Primary keratinocytes were transfected with scramble and C/EBP $\delta$  siRNAs. After 24 h, half of the cells were induced to differentiate by addition of calcium. Samples were harvested after 3 days, RNAs prepared and qPCR performed on the indicated genes. Values are normalized to GAPDH, used as an internal control. doi:10.1371/journal.pone.0013789.g005

A



B



**Figure 6. Role of C/EBP $\delta$  target genes in keratinocyte differentiation.** **A.** Analysis of differentiation markers by qRT-PCR of primary keratinocytes induced to differentiate after transient transfection of MafB expression plasmid and empty vector as control. After 24 h, half of the cells were induced to differentiate by addition of calcium, as in Fig. 5. **B.** Same as A, except that Sox2 was overexpressed. doi:10.1371/journal.pone.0013789.g006

### C/EBP $\delta$ is differentially expressed in human skin tumors

The expression of C/EBP $\alpha$  and  $\beta$  is altered in several tumors, including those of epithelial origin [23–27]. Nothing is known about the expression of C/EBP $\delta$  in various types of skin tumors. We decided to analyze its expression in a panel of different skin tumors by immunohistochemistry (IHC); the results are shown in Figure S2: 0 is no expression, and 3 is maximum positivity. Most Squamous Cell Carcinomas -SCC- show overexpression of C/EBP $\delta$  (14 out of 20); as expected, p63 levels were also generally high [42]. On the other hand, Basal Cell Carcinomas were negative for C/EBP $\delta$  expression, whereas p63 expression was very high. Figure 7 shows the IHC staining of representative cases of SCC and BCC, and comparison with normal skin. In conclusion, these data indicate that, unlike p63, C/EBP $\delta$  is differentially expressed in human BCC and SCC tumors and, like the related C/EBP $\beta$ , overexpressed in SCC.

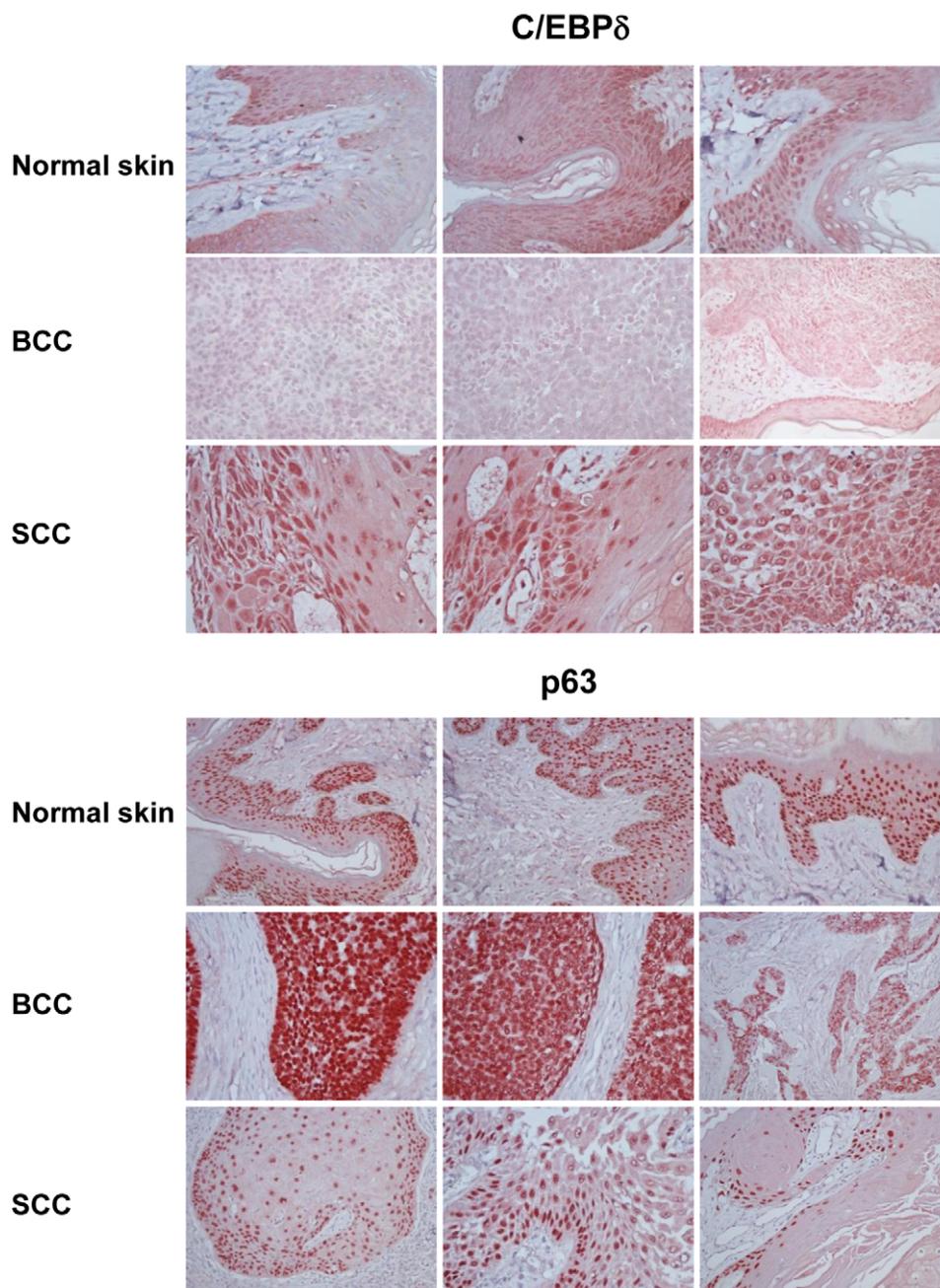
### Discussion

The modulation of gene expression in growth and differentiation is controlled by transcription factors. In tissues in which these processes are continuously active to provide the required mass of specialized cells, many TFs contribute, with distinct yet partially overlapping programs. Hierarchical models have been suggested that specific TFs account for differentiation programs in a multistep way. This matter is complicated by the fact that TFs come in

families, with members often being apparently redundant. In multilayered epithelia, it is clear that p63 plays a role that cannot be vicariate by other members of the family, p53 or p73. The recent identification of p63 targets provided links with other TFs that play an important role in skin biology, notably C/EBPs [21,35].

### C/EBP $\delta$ and keratinocyte differentiation

C/EBP $\delta$  belongs to a family of TFs important for several differentiation pathways, including adipocytes, liver and haematopoietic lineages [2]. C/EBP $\delta$  and C/EBP $\beta$  are structurally the two closest members and genetic experiments have linked them to the early phases of adipocyte differentiation, with redundant roles [6]. Neither C/EBP $\beta$  nor C/EBP $\delta$  KO mice show a skin phenotype, possibly because of redundancy. Double C/EBP $\alpha$ /C/EBP $\beta$  KO have profound skin defects, with lack of complete differentiation and increased proliferation of basal layer cells, associated to increased p63 expression [18]. Among the established targets of C/EBPs are desmocollins, which are required for skin desmosome formation. Desmocollin 3, which is expressed in basal cells, is transactivated by C/EBP $\delta$  and C/EBP $\beta$ , the suprabasal Desmocollin 1 by C/EBP $\delta$  and C/EBP $\alpha$  [43]. In humans, little variation of expression of C/EBP $\delta$  was detected in the different phases of maturation of skin annexes, the hair follicle and the sebaceous gland, unlike other members of the family [44]. However, we observed that C/EBP $\delta$  staining is not uniform in human interfollicular skin. Co-expression with the growth

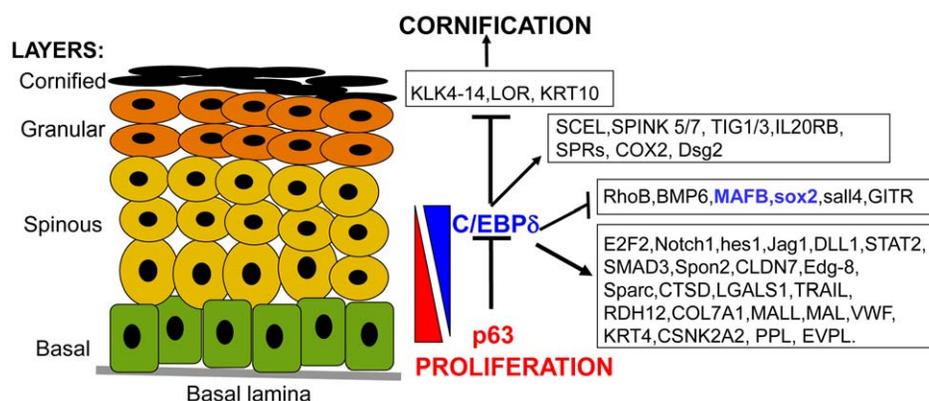


**Figure 7. Overexpression of C/EBP $\delta$  in human non-melanoma skin tumors.** Immunohistochemistry analysis of p63 and C/EBP $\delta$  in normal skin (Upper Panel), BCC (Middle Panel) and SCC (Lower Panel).  
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promoting p63 is seen only in selected basal cells, it is balanced in the spinous layer, and in the upper granular layer, C/EBP $\delta$  predominates [21]. Among the many genes targeted by C/EBP $\delta$  and p63, the vast majority is controlled in an opposite way by the two TFs, which is consistent with their overlapping expression patterns in the skin.

Many genes activated by C/EBP $\delta$  in our experiments are involved in functions of the granular layer and in the formation of the skin barrier (Fig. 8). SPRRs, RARRES3 and COX-2 are expressed in the granular layer and involved in differentiation [44–49]. KRT4 is a developmentally regulated Keratin, required for proper differentiation of internal multi-layered epithelia (50).

Spink proteins are serine protease inhibitors involved in skin barrier formation: mutations in the Spink5 –LEKTI- gene in humans cause the Netherton syndrome, an ichthyosiform dermatosis (51); Spink5 KO mice have severe alteration in the Stratum Corneum (SC) [52–54]. Spink targets, such as KLK4, a serine protease implicated in the desquamation, are repressed by C/EBP $\delta$  [55]. BMP6 is a signaling molecule that inhibits stem cell proliferation and triggers cell cycle exit from transit amplifying cells [56]. KRT10, Loricrin and Late Cornified Envelope group, LCE1F, LCE3A and LCE3C are markers of the suprabasal layers [57]. The experiments of Fig. 5 confirm that KRT10 expression is affected by C/EBP $\delta$  removal. In summary, C/EBP $\delta$  joins the list



**Figure 8. Scheme of C/EBP $\delta$  targets in human skin.**

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of other TFs involved in barrier formation, such as IRF6 [58,59], KLF4 [60], Gata3 [61,62] and HBP1 [63]. Each is likely to promote the expression of subsets of differentiation genes. Importantly, KLF4, Gata3 and HBP1 are directly controlled by p63 [62–64].

### C/EBP $\delta$ and control of proliferation

To induce terminal differentiation, proliferation must be stopped: C/EBPs are largely thought to be growth suppressors. Experiments performed in cultured cells *in vitro* as well as in mice *in vivo* have generally supported this idea. Mechanisms related to control of cell cycle progression, rather than induction of apoptosis or senescence, have been suggested. The mechanistic details are apparently multiple, and range from a direct role on CDKs, to activation of CDKI genes, to repression of growth stimulatory genes [65]. Comparatively less is known about C/EBP $\delta$ , but the available data fully support this idea: it is induced upon several environmental changes that lead to growth arrest in mammary and lung epithelial cells [66]. Overexpression leads to growth arrest in mammary and prostate epithelial cell lines [67–70]. It was therefore somewhat expected that cell cycle terms would emerge from our analysis on C/EBP $\delta$  targets. However, the strong emphasis on G2/M genes is novel and unexpected, genes involved in kinetokore function during mitosis, such as BUB1, Aurora Kinase B and Borealin-CDCA8, as part of the CPC complex, KNTC1-ROD, genes recruited by AurKB, CENPF, consensins/NCAPD2 [71–73], as well as Cyclin B2, Cyclin A, CDKN3-KAP, CDC20. These findings could explain the chromosomal instability in mammary cells upon removal of C/EBP $\delta$  [74].

The Notch, Wnt and TGF $\beta$  pathways are well represented - Notch1, Hes1, JAG1, SMAD3, BMP6, FRAT1, LTBP1, Sall4- as expected for a TF with a global outlook. Growth suppressor genes are activated by C/EBP $\delta$  in primary keratinocytes: EDG-8 [75], STAT2, a TF expressed in the granular layer of the skin [76], E2F2 and IRF1. Specifically, E2F2 functions as a tumor suppressor in the skin, since inactivation cooperates with transgenic expression of Myc to enhance tumor development. Hemizygous E2f2<sup>+/-</sup> mice showed increased tumor incidence. E2F2 inactivation apparently modifies gene expression patterns of some Myc targets [77]. IRF-1, also a target of p63, together with another member of the family, IRF-3 [35], is expressed throughout human skin [78] and it is generally considered a growth suppressor [79]. Yet, IRF1 is overexpressed in profiling experiments of Squamous Cell Carcinomas, amid other IFN-activated signature genes [80].

Among the genes repressed by C/EBP $\delta$ , three genes, apparently not expressed in the skin, stand out because of their role in stem cells: Sox2, Sall4 and BCOR. Sox2 and Sall4 are TFs that reprogram differentiated cells, including keratinocytes, to Embryonic Stem cells-like iPS, [81,82]. BCOR is a marker of mesenchymal stem cells [83]. We specifically tested Sox2 and indeed overexpression has negative consequences on the expression of markers of terminal differentiation, Filaggrin and KRT10. An opposite result was obtained with MafB. This latter TF was more efficient in repressing the levels of the basal marker KRT14 before and after differentiation. MafB is a member of the large leucine zipper AP1 family, and specifically of the MAF subfamily [41]. MafB, as well as c-Maf, are highly expressed in basal keratinocytes and in the hair follicle at the late embryo stage, dropping after birth [84]. Genetic data generated by analysis of KO mice of other MAFs -Nrf2, MafG, MafK and the negative regulator Keap1- indicate that several members play a role in multilayered epithelia, notably of the esophagus [85].

C/EBPs expression is altered in many tumors, notably of epithelial origin [23–27]. In general, lack of expression has been reported which, in the case of C/EBP $\delta$  in cervical carcinomas, is caused by epigenetic silencing due to both DNA methylation of CpG islands and PcG-mediated deposition of negative histone marks [86]. Our analysis of non-melanoma skin cancers show that BCC are negative, but the majority of SCCs have relatively high levels of C/EBP $\delta$ , especially in areas of ongoing differentiation. Indeed, oncogenes, such as FRAT1, MALT1 and the TFs HOXB4, Sall4 and PTTG1 [87–89] are among the targets. KLF4 is another example of a TF with strong growth suppressive and pro-differentiation activities, that is nevertheless associated to tumor promoting functions in specific contexts. Our findings suggest that C/EBP $\delta$  could play different roles, depending upon the tissue context. More generally, it could serve a dual role in skin homeostasis, both in very early and late stages. In the former, it could limit, through its suppressive properties, the growth potential of early progenitors. In the later stages, it may coordinate cell-cycle exit and induce differentiation markers.

### Supporting Information

**Figure S1** GO analysis of the C/EBP $\delta$  regulated genes identified by ChIP on chip.

Found at: doi:10.1371/journal.pone.0013789.s001 (2.36 MB TIF)

**Figure S2** Skin cancer tissue array results obtained by IHC.

Found at: doi:10.1371/journal.pone.0013789.s002 (2.98 MB TIF)

**Table S1** RT-PCR primers list.

Found at: doi:10.1371/journal.pone.0013789.s003 (0.06 MB DOC)

**Table S2** List of the primers used in ChIP assay.

Found at: doi:10.1371/journal.pone.0013789.s004 (0.04 MB DOC)

**Table S3** List of the C/EBP $\delta$  target genes identified by ChIP on chip analysis.

Found at: doi:10.1371/journal.pone.0013789.s005 (0.04 MB XLS)

**Table S4** List of the C/EBP $\delta$  target genes identified by expression profiling analysis.

Found at: doi:10.1371/journal.pone.0013789.s006 (0.06 MB XLS)

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Conceived and designed the experiments: SB RM. Performed the experiments: SB DF MR OMVG. Analyzed the data: SB DD MR OMVG AW EB MAV. Contributed reagents/materials/analysis tools: DF DA CC. Wrote the paper: RM. Post-doc of the U. degli Studi di Milano: SB. Student of the PhD School in Toxicology/Doctorate in Molecular Oncology and Molecular Pathology, University of Napoli (Italy): MR. Student of the PhD School in Environment, Biological, Biotechnology, Physics, Mathematics and Informatics Sciences/Doctorate in Pathology of Cell Signal Transduction: OMVG.

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