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The epigenetic factor BORIS/CTCFL regulates the *NOTCH3* gene expression in cancer cells

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ABSTRACT

Aberrant upregulation of *NOTCH3* gene plays a critical role in cancer pathogenesis. However, the underlying mechanisms are still unknown. We tested here the hypothesis that aberrant epigenetic modifications in the *NOTCH3* promoter region might account for its upregulation in cancer cells. We compared DNA and histone methylation *status* of *NOTCH3* promoter region in human normal blood cells and T cell acute lymphoblastic leukemia (T-ALL) cell lines, differentially expressing *NOTCH3*. We found that histone methylation, rather than DNA hypomethylation, contributes towards establishing an active chromatin *status* of *NOTCH3* promoter in *NOTCH3* overexpressing cancer cells. We discovered that the chromatin regulator protein BORIS/CTCFL plays an important role in regulating *NOTCH3* gene expression. We observed that BORIS is present in T-ALL cell lines as well as in cell lines derived from several solid tumors overexpressing *NOTCH3*. Moreover, BORIS targets *NOTCH3* promoter in cancer cells and it is able to induce and to maintain a permissive/active chromatin conformation. Importantly, the association between *NOTCH3* overexpression and BORIS presence was confirmed in primary T-ALL samples from patients at the onset of the disease. Overall, our results provide novel insights into the determinants of *NOTCH3* overexpression in cancer cells, by revealing a key role for BORIS as the main mediator of transcriptional deregulation of *NOTCH3*.

1. Introduction

The human neurogenic locus notch homolog protein 3 (*NOTCH3*) gene encodes the third discovered mammalian member of an evolutionary conserved family of transmembrane protein receptors (NOTCH 1–4). Notch receptors are strictly required for normal embryonic development and for homeostasis of a variety of adult tissues by controlling the balance between proliferation versus differentiation of stem and/or progenitor cells [1,2].

A deregulated Notch pathway has been found to be involved in the development of a wide variety of human tumors with either an oncogenic or a tumor-suppressive role depending on the cancer type, tissue context and the member of the Notch receptor family activated [3,4].

An oncogenic role of NOTCH3 pathway was identified in T cell acute lymphoblastic leukemia (T-ALL), since constitutive expression of the activated intracellular domain of NOTCH3 (NOTCH3-IC) has been shown to induce T-cell neoplasias in transgenic mice [5] and the overexpression of NOTCH3 in the vast majority of T-ALL validates this finding in humans [6].

Aberrant NOTCH3 signaling has been also observed in several solid tumors, where NOTCH3 activation favors cell proliferation, resistance to apoptotic stimuli, metastatic capability and maintenance of stem cell-like features [7].

Moreover, a correlation between *NOTCH3* expression and poor prognostic outcome has been described in primary tumors thus confirming NOTCH3 involvement in cancer pathogenesis [8–10].

Molecular details of the genetic mechanisms (i.e. gene amplification, chromosome translocation) causing NOTCH3 receptor activation as well as the consequent activation of signaling are emerging in the context of specific tumors [11–15], while the causes of NOTCH3 gene overexpression in cancer cells remain poorly understood.

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Epigenetic processes often contribute to the deregulated expression of genes critical to the development and progression of cancer. The fact that the transcription start site (TSS) of *NOTCH3* localizes within a CpG island prompted us to investigate a possible correlation between *NOTCH3* gene DNA methylation *status* and gene expression extending the analysis to histone methylation.

We report here that the cancer testis antigen (CTA) BORIS/CTCFL (Brother Of Regulator of Imprinted Sites)/CTCF-like protein), paralog of the widely expressed chromatin insulator CTCF (CCCTC-binding factor) [16,17], is involved in the epigenetic deregulation leading to *NOTCH3* upregulation in cancer cells. In particular, BORIS presence mirrors *NOTCH3* overexpression specifically in T-ALL established cell lines as well as in primary T-ALL human samples from patients at the onset of the disease. Furthermore, BORIS binding to *NOTCH3* promoter is associated with histone modifications typical of permissive/active chromatin conformation. Overall, our data highlight a key role played by BORIS in the abnormal upregulation of *NOTCH3* in cancer cells.

2. Results

2.1. NOTCH3 gene expression and promoter epigenetic profile in normal blood cells and in T-ALL cell lines

Whether or not DNA methylation of the *NOTCH3* promoter restricts *NOTCH3* expression was investigated.

NOTCH3 mRNA level analysis was performed by real-time RT-PCR in normal blood cells [peripheral blood mononuclear cells (PBMCs), CD4+ and CD8+ T-lymphocytes] and in T-ALL cell lines differentially expressing *NOTCH3* (MOLT3 and SKW3). Fig. 1A shows that the *NOTCH3* mRNA level varied significantly with over 500-fold difference between the lowest and highest expressing cell types (MOLT3 vs. CD8+ T-lymphocytes). High *NOTCH3* mRNA levels were found in MOLT3 cells, while barely detectable *NOTCH3* expression characterized SKW3 cells. Notably, low levels of *NOTCH3* transcript were detected in all normal blood cells.

The dependence of *NOTCH3* expression on DNA methylation of its promoter, colocalizing with a CpG island (Fig. 1B), was investigated by bisulfite sequencing in CD4+ T- lymphocytes and in MOLT3 and SKW3 T-ALL cells. Fig. 1C shows that *NOTCH3* promoter was hypermethylated only in SKW3 cell line characterized by barely detectable levels of mRNA expression, while it was unmethylated in *NOTCH3* expressing MOLT3 cell line. Intriguingly, the association between promoter methylation and gene expression cannot be extended to normal blood cells, where *NOTCH3* was not expressed in spite of the absence of promoter methylation.

The data above revealed that the silencing of *NOTCH3* in normal blood cells is independent of DNA methylation, thus suggesting the involvement of other mechanism(s). In order to address this issue, our attention was focused on epigenetic changes involving histone methylation.

In fact, it is well known that histone H3 dimethylation and trimethylation at residue K4 (H3K4me2 and H3K4me3) mark transcriptional competent chromatin with H3K4me3 particularly enriched in actively transcribed promoters, while histone H3 trimethylation at residue K9 (H3K9me3) and trimethylation at residue K27 (H3K27me3) mark constitutive and facultative heterochromatin, respectively.

By chromatin immunoprecipitation (ChIP) analysis, these modifications were used to investigate the chromatin conformation *status* of *NOTCH3* promoter in cells differentially expressing *NOTCH3* (Fig. 1 D).

The presence of a high amount of H3K4me2 and H3K4me3 versus H3K9me3 and H3K27me3 indicates an active chromatin *status* in MOLT3 cells, while high H3K9me3 versus H3K4me2 and H3K4me3 indicates a non-permissive chromatin *status* in SWK3 cells. These results match with data of RT-PCR and cytosine methylation levels, showing that *NOTCH3* is expressed in MOLT3 and silenced in SKW3 cells by constitutive heterochromatization.

As with respect to PBMCs, the *NOTCH3* silencing could be explained by both the low H3K4me3 and high H3K27me3 amounts. However, this condition does not depend on the heterochromatization as indicated by the absence of H3K9me3, whose presence instead characterizes SKW3 cells, which do not express *NOTCH3*.

2.2. CTCF and BORIS differentially occupy NOTCH3 promoter in normal blood cells and T-ALL cell lines

To search for potential *trans*-acting protein factors responsible for epigenetic regulation of *NOTCH3* gene expression, the Genomatix MatInspector software was used [18,19]. The inspection of about 2000 bp of the *NOTCH3* 5-prime sequence (transcript ID ENST00000263388) revealed the presence of eight regions located in the proximity of the TSS (from −391 to +259) which match with the CTCF binding sequence (data not shown). Three of them exhibited a high degree of similarity to CTCF binding sites with a quality rating above the algorithm defined cut off value of 0.8 (Fig. 2A, sites 1, 2 and 3). Furthermore, same result was obtained by comparing the same region with previously published 20-bp binding motifs for CTCF [20] (Fig. 2B). This was also suggested by ChIP-seq data from the ENCODE consortium accessed through the UCSC genome browser (http://genome.ucsc.edu) [21–23] (Supplementary Fig. S1).

CTCF DNA-target sites could also be bound by CTCFL/BORIS, a CTCF-paralog protein that may have epigenetic CTCF-antagonistic regulative functions on CTCF binding regions of target genes [17,24–26].

To address whether CTCF and BORIS are both involved in the regulation of *NOTCH3* expression, transcript levels of *CTCF* and *BORIS* were analyzed by RT-PCR in normal blood cells and T-ALL cell lines, to compare their expression with that of *NOTCH3* (Fig. 1A).

To detect all the 23 known mRNA variants of *BORIS* [27], its expression was analyzed by quantitative RT-PCR with two sets of primers encompassing 1–2 and 3–4 exon boundaries, respectively.

CTCF transcript levels were relatively similar in both normal blood cells and T-ALL cell lines, irrespectively of *NOTCH3* expression (Fig. 3A). In contrast *BORIS* expression appeared to be restricted to T-ALL cell lines. However, the presence of *BORIS* transcript did not correlate with the expression of *NOTCH3*. Indeed, it appears to be expressed by both SKW3 cells, which do not express *NOTCH3*, and MOLT3 cells (Fig. 3B and C), which express *NOTCH3* and show permissive, unmethylated chromatin *status* of *NOTCH3* promoter (Fig. 1C and D).

To correlate the observed epigenetic chromatin *status* of *NOTCH3* promoter with the presence of CTCF and/or BORIS, chromatin immunoprecipitation (ChIP) experiments were carried out on MOLT3 and SKW3 T-ALL cells, in comparison with PBMCs.

As shown in Fig. 3D, the ChIP analysis revealed that CTCF binds to the proximal *NOTCH3* promoter fragment containing putative CTCF-binding sites 1 and 2 in PBMCs and MOLT3 cells, while no CTCF binding was detected in SKW3 cells. Concerning BORIS, the protein was associated with the same region bound by CTCF in MOLT3 cells, which overexpress *NOTCH3*. Conversely, BORIS binding was undetectable in both PBMCs and SKW3, which do not express *NOTCH3*. This finally would suggest that the binding of BORIS to the *NOTCH3* promoter directly correlates with the gene expression.

2.3. NOTCH3 expression correlates with the methylation status of its promoter and with BORIS expression in normal and cancer cell lines

In order to extend the study of the possible direct correlation between *NOTCH3* expression with the methylation *status* of its promoter, *NOTCH3* mRNA level analysis was firstly performed by real-time RT-PCR in normal blood cells (PBMCs, CD4+ and CD8+ T-lymphocytes), in immortalized normal human fibroblasts (BJ) and in cell lines derived from several cancers, including T-ALL (MOLT3, SKW3 and Jurkat), breast cancer (MCF7, T47D, MDA-MB-231, MDA-MB-453), melanoma (M14, MDA-MB-435), and colon carcinoma (HCT116, HCT15, HT29). Fig. 4A

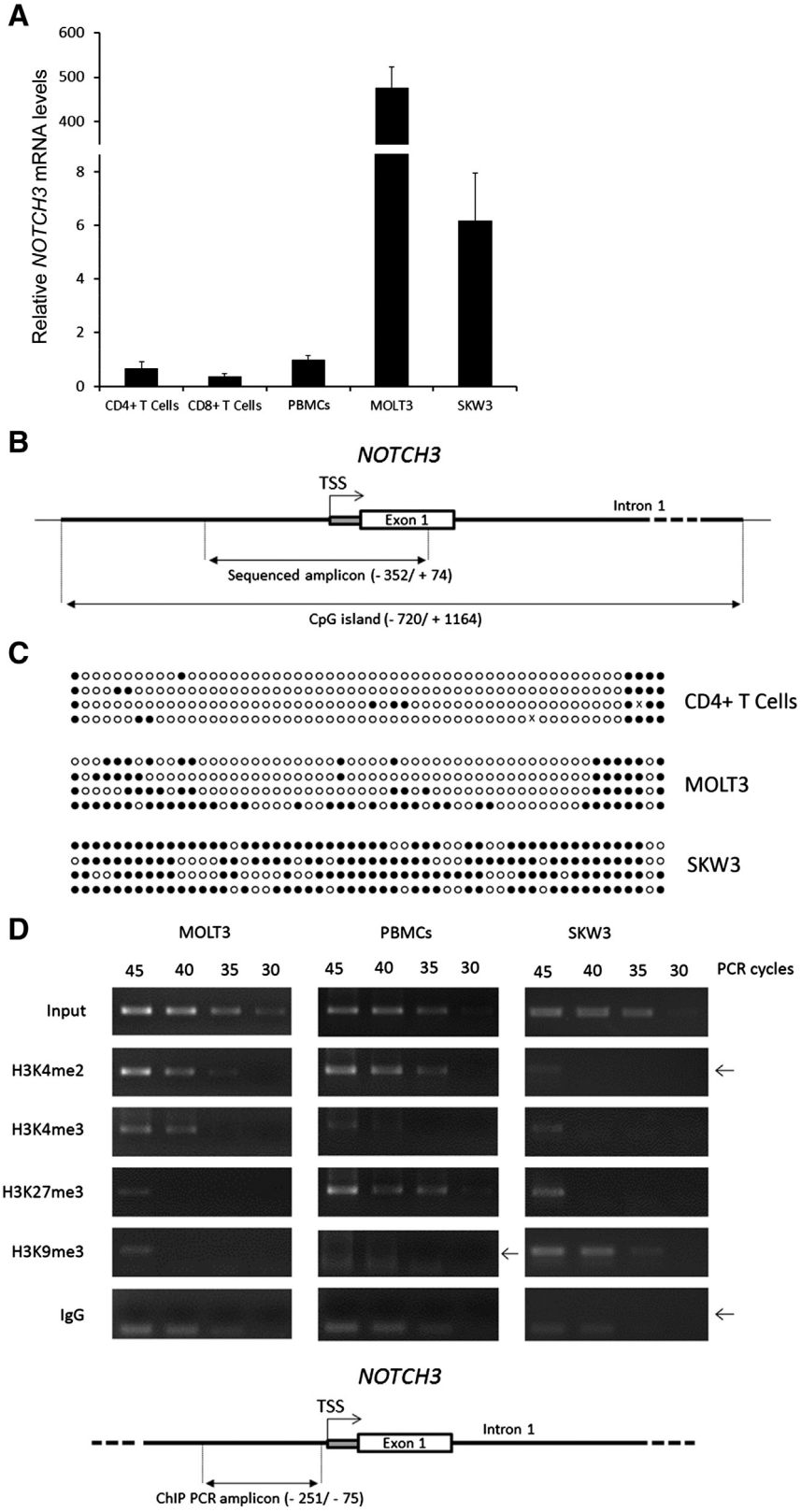

Fig. 1. Differential expression and promoter epigenetic *status* of *NOTCH3* in normal blood cells and T-ALL cell lines. (A) Expression of *NOTCH3* transcript as detected by quantitative RT-PCR in normal blood cells (CD4+, CD8+ T-lymphocytes and PBMCs,) and in T-ALL cell lines (MOLT3 and SKW3). *GUSB* transcript level was used as endogenous control. Relative levels were calculated taking the transcriptional levels of *NOTCH3* in PBMCs as 1. Results are means ± s.e.m. calculated from three experiments. (B) Schematic map of the *NOTCH3* 5′-region showing the position of the PCR product analyzed in the assay. TSS indicates the transcriptional start site. Gray box: untranslated 5′-region; open box: coding exon; black horizontal line: CpG island. Numbers in brackets indicate the nucleotide positions relative to the ATG initiator codon. (C) DNA methylation profiling of *NOTCH3* promoter as detected by bisulfite sequencing in CD4+ normal blood T-cells and in the leukemic cell lines MOLT3 and SKW3. Each circle represents a CpG dinucleotide. Open circle, unmethylated CpG; black circle, methylated CpG. X indicates cytosine positions where the sequence was indefinable. (D) ChIP analysis of *NOTCH3* promoter region for histone H3 modifications in PBMCs and in the MOLT3 and SKW3 T-ALL cell lines. Immunoprecipitation was performed by using specific antibodies to dimethyl and trimethyl lysine 4 (H3K4me2 and H3K4me3) and to trimethyl lysine 27 and 9 (H3K27me3 and H3K9me3). Normal IgG were used as negative control (IgG). Arrow indicates the expected position of the band. Diagram represents the *NOTCH3* 5′-region with the schematic illustration of the location of the PCR product analyzed in the assay.

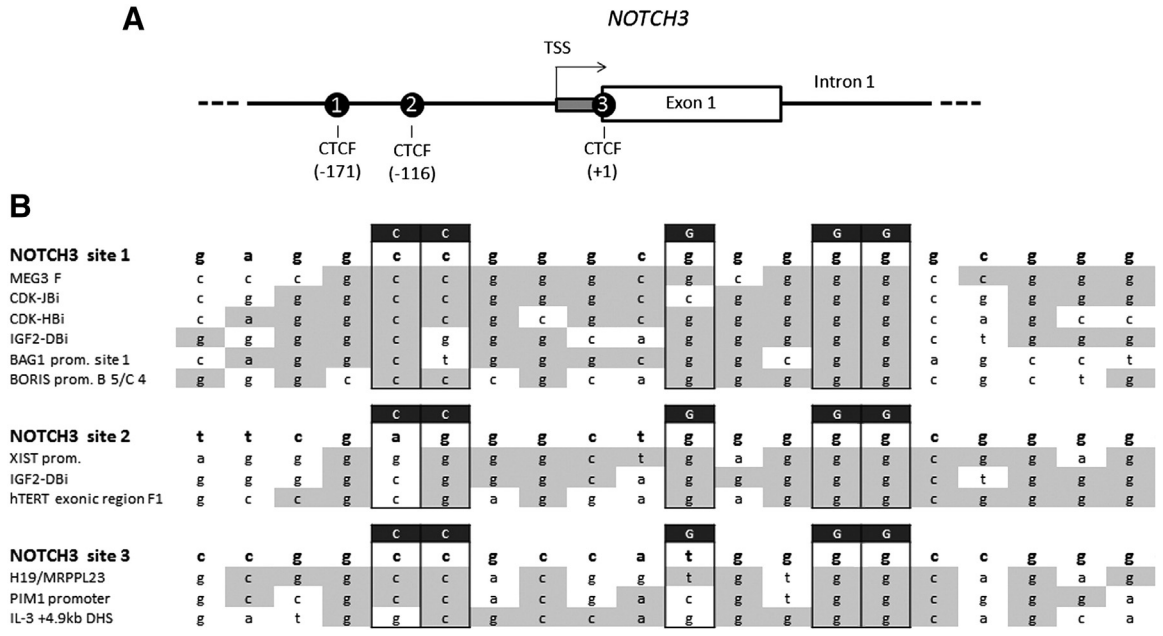

Fig. 2. In silico prediction of CTCF-binding motifs in the *NOTCH3* promoter region. (A) Schematic representation of the *NOTCH3* 5′-region with the three sites showing high degree of similarity to CTCF binding sites as predicted by the Genomatix MatInspector software. TSS indicates the transcriptional start site. Gray box: untranslated 5′-region; open box: coding exon. Numbers in brackets indicate the nucleotide positions relative to the ATG initiator codon. (B) Sequence alignment of the putative CTCF sites in the *NOTCH3* promoter with the 20-bp core motif of known human CTCF DNA binding sites. Highlighted bold text: *NOTCH3* sequence. Matching nucleotides between *NOTCH3* and CTCF target genes are highlighted in light gray. Open box indicates nucleotide positions that have been found highly conserved between functional CTCF binding sites genome-wide [20].

shows differential expression of *NOTCH3* mRNA levels in different types of tumors, as well as in immortalized and normal human fibroblasts (BJ) and in embryonic kidney HEK293T cells. Normal blood cells are included as *NOTCH3* non-expressing normal cells.

Then the DNA methylation *status* of *NOTCH3* promoter in different cells was investigated by bisulfite PCR followed by restriction analysis (COBRA).

After bisulfite modification and PCR amplification of the *NOTCH3* promoter region, the PCR product was digested with *Bst*UI restriction enzyme specific to CGCG containing sequences (Fig. 4B). Therefore, following bisulfite reaction, cleavage occurs selectively when target sequences are methylated. Fig.4C shows that *NOTCH3* promoter was hypermethylated only in cancer cell lines characterized by its low mRNA expression (SKW3, HT29, HELA, M14 and MDA-MB-231), while it was unmethylated in all *NOTCH3*-expressing T-ALL and cancer cell lines and in embryonic HEK293T cells. Notably, normal blood cells and fibroblast cell lines do not express *NOTCH3* in spite of the absence of promoter methylation.

A parallel analysis was performed on the same cell lines in order to assess the presence of *BORIS* and *CTCF* transcripts. The results have shown that while *CTCF* is expressed at similar levels in all samples (Supplementary Fig. S2), *BORIS* expression is restricted to cancer cell lines (Fig. 4D and E), irrespective of *NOTCH3* expression.

The results at mRNA levels were confirmed at the protein level by Western blots (Supplementary Fig. S3).

2.4. BORIS positively regulates NOTCH3 transcription and activity

Together, the results reported above indicate that, while *BORIS* expression alone is not sufficient, the combination of unmethylated *status* of *NOTCH3* promoter and *BORIS* expression represents the necessary condition for *NOTCH3* expression in cancer cells and in embryonic HEK293T cells.

Luciferase reporter assay was used to investigate whether and where *NOTCH3* promoter activity directly responds to BORIS. Plasmids containing the *NOTCH3* full promoter or its proximal/distal regions (Fig. 5A) were transfected into both BORIS-negative BJ and BORIS-positive HEK293T cells. According to BORIS presence, the *NOTCH3* promoter activity was significantly higher in HEK293T cells. To note, this was ascribable to the proximal promoter which contains putative CTCF binding sites (Fig. 5B). The activatory role played by BORIS on *NOTCH3* promoter was further confirmed by siRNA-mediated BORIS silencing in HEK293T cells (Fig. 5C). Taken together, these results indicate that BORIS, by targeting the proximal region of its promoter, is a positive regulator of *NOTCH3*.

To address in-depth the molecular mechanism by which BORIS is involved in *NOTCH3* upregulation, experiments of *BORIS* silencing/overexpression were performed in the BORIS-positive cell line HEK293T showing unmethylated *NOTCH3* promoter and *NOTCH3* expression (Fig. 4 and Fig. S3).

In agreement with data obtained in luciferase reporter assays (Fig. 5C) the expression of the endogenous *NOTCH3* depended on the BORIS level. In fact, endogenous NOTCH3 was downregulated or upregulated at both mRNA and protein levels following *BORIS* silencing or overexpression, respectively (Fig. 6A and B).

ChIP analysis experiments allowed us to further study what occurs on *NOTCH3* promoter following the modification of the BORIS level.

As shown in Fig. 6C, the silencing of BORIS reduced the amount of protein bound on *NOTCH3* promoter, while upregulated BORIS protein levels increased BORIS binding. Notably, these binding modifications correlate with changes in histone methylation patterns: enhanced BORIS occupancy was associated with an increased amount of H3K4me3, while BORIS depletion changed chromatin *status* of *NOTCH3* promoter towards non-permissive chromatin configuration as indicated by the decrease of the H3K4me3 and the introduction of H3K27me3.

Overall, these results show a direct relationship between BORIS binding to *NOTCH3* promoter, histone methylation *status* and the expression of *NOTCH3*. Furthermore, they indicate that the binding of BORIS to *NOTCH3* promoter was sufficient to maintain switched on its transcription.

Consistent with these findings, exogenous BORIS enforced expression was sufficient to allow *NOTCH3* expression in BORIS-negative BJ normal fibroblasts (Fig. 7A and B). Notably, *NOTCH3* upregulation associated with BORIS recruitment to *NOTCH3* promoter and with changes of promoter chromatin *status* towards permissive chromatin configuration as

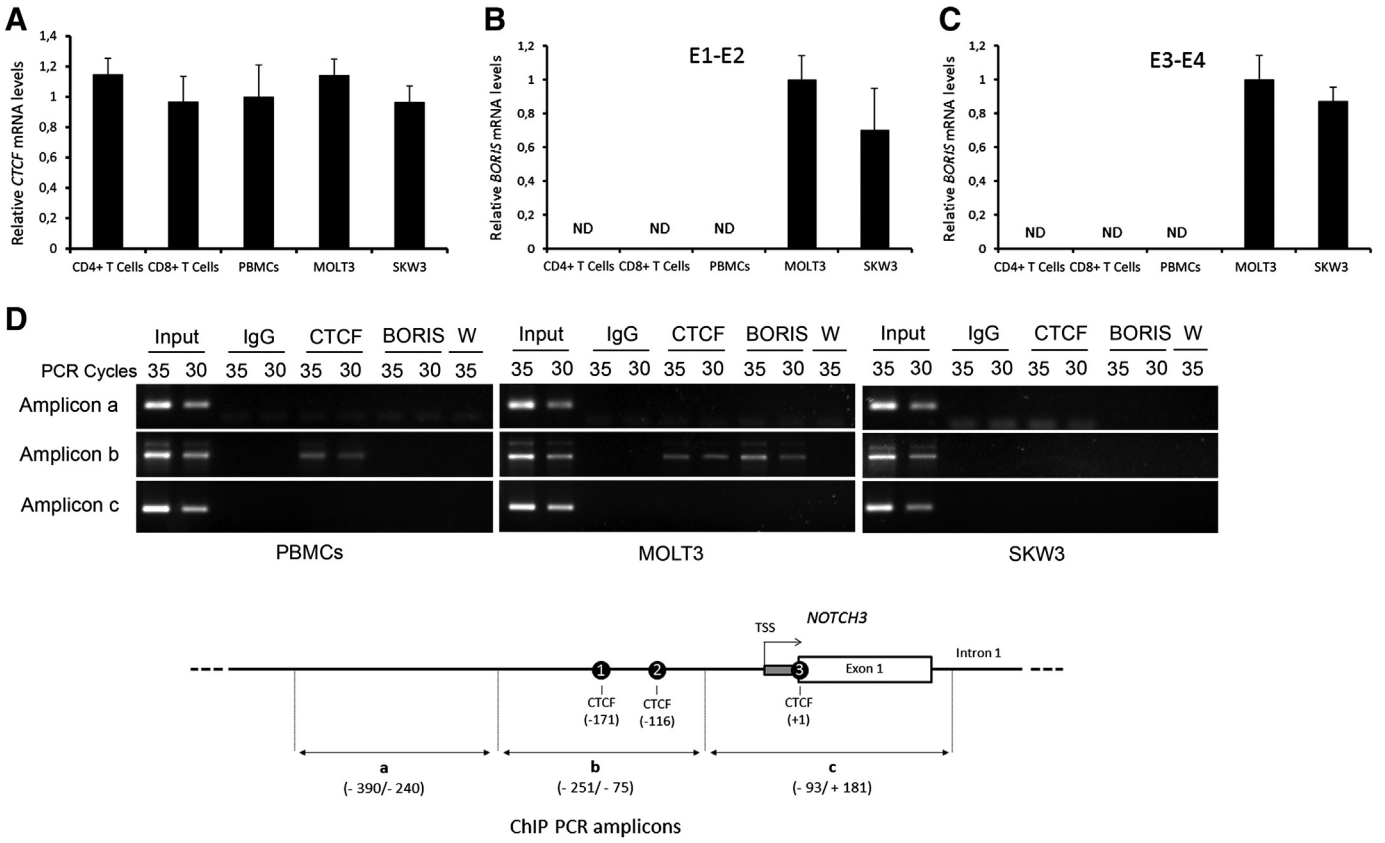

Fig. 3. CTCF and BORIS differentially occupy *NOTCH3* promoter in normal blood cells and T-ALL cell lines. (A) Expression of *CTCF* transcript as detected by quantitative RT-PCR in normal blood cells (CD4+, CD8+ T-lymphocytes and PBMCs) and in T-ALL cell lines (MOLT3 and SKW3). *GUSB* transcript level was used as endogenous control. Relative levels were calculated taking the transcriptional levels of *CTCF* in PBMCs as 1. (B and C) Expression of *BORIS* transcripts as detected by quantitative RT-PCR in normal blood cells (CD4+, CD8+ T-lymphocytes and PBMCs,) and in T-ALL cell lines (MOLT3 and SKW3). Two sets of primers encompassing 1–2 (E1–E2) and 3–4 (E3–E4) exons were used to detect all known isoforms of *BORIS* transcript. *GUSB* transcript served as loading control of total RNA. Relative levels were calculated taking the transcriptional levels of *BORIS* in MOLT3 as 1. Results shown in A, B and C are means ± s.e.m. calculated from three experiments. ND, not detectable. (D) In vivo binding of CTCF and BORIS on the 5′-region of *NOTCH3* as detected by ChIP assay in PBMCs and in the leukemic cell lines MOLT3 and SKW3. Normal IgG were used as negative ChIP control (IgG). W indicated the no-template PCR control. Diagram represents the *NOTCH3* 5′-region with the location of the putative CTCF binding sites and the PCR products analyzed in the assay. Region b corresponds to the PCR amplicon indicated in Fig. 1D. TSS indicates the transcriptional start site. Gray box: untranslated 5′-region; open box: coding exon. Numbers in brackets indicate the nucleotide positions relative to the ATG initiator codon.

indicated by the increase in H3K4me3 and the depletion in H3K27me3 histone modifications (Fig. 7C).

To determine the functional relevance of the BORIS-dependent *NOTCH3* upregulation we next investigated whether this event could initiate the NOTCH3 signaling in BJ cells. To this end, BORIS overexpression was combined with the treatment with a specific NOTCH3-neutralizing antibody able to inhibit the receptor activity. To assess the NOTCH3 signaling activity, the levels of NOTCH3-IC and of its target gene *HES1* [1,2] were monitored. We observed that upon BORIS overexpression, the NOTCH3-IC accumulation associated with a significant upregulation of *HES1* mRNA. The latter effect was clearly dependent on NOTCH3 activity as indicated by the fact that it was almost completely abrogated by the addition of the NOTCH3 neutralizing antibody (Fig. 7E), which is also able to reduce the levels of NOTCH3-IC (Fig. 7D). These results indicate that the NOTCH3 protein induced by BORIS is functional.

2.5. Co-expression of BORIS and NOTCH3 in T-ALL

Considering the association between *BORIS* expression and *NOTCH3* upregulation observed in T-ALL cell lines, whether or not this observation could be extended to human primary T-ALL samples was verified.

The mRNA levels of *NOTCH3* and *BORIS* were examined in eight cases of T-ALL and compared with normal peripheral blood T lymphocytes and with MOLT3 cells.

COBRA assay was carried out in parallel in the same samples, to verify the methylation *status* of NOTCH3 promoter. As shown in Fig. 8A and B, sustained levels of *NOTCH3* mRNA expression were associated with the unmethylated *status* of promoter in all analyzed T-ALL samples. Notably, the samples also showed the presence of the *BORIS* transcript (Fig. 8C and D), further supporting the hypothesis that aberrant expression of *BORIS* combined with the unmethylated *status* of *NOTCH3* promoter is the event responsible for *NOTCH3* expression in cancer.

3. Discussion

NOTCH3 upregulation is involved in the pathogenesis of and characterizes T-ALL [6], although the causes of such an upregulation are poorly known. Thus, the molecular mechanisms underlying *NOTCH3* transcriptional deregulation in T-ALL cells have been here investigated focusing on epigenetic events and transcriptional factors involved.

Our data firstly show that *NOTCH3* gene expression does not directly correlate with the methylation of its promoter. In fact, while promoter methylated *status* correlates with *NOTCH3* expression in T-ALL cell lines, being unmethylated in NOTCH3-expressing MOLT3 cells and hypermethylated in NOTCH3-negative SKW3 cells, its unmethylated *status* was not found to be enough to allow *NOTCH3* transcription in normal blood cells. This finding indicates that the absence of cytosine methylation is not the key factor involved in transcriptional upregulation of *NOTCH3* in T-ALL cell lines and human primary samples, thus suggesting

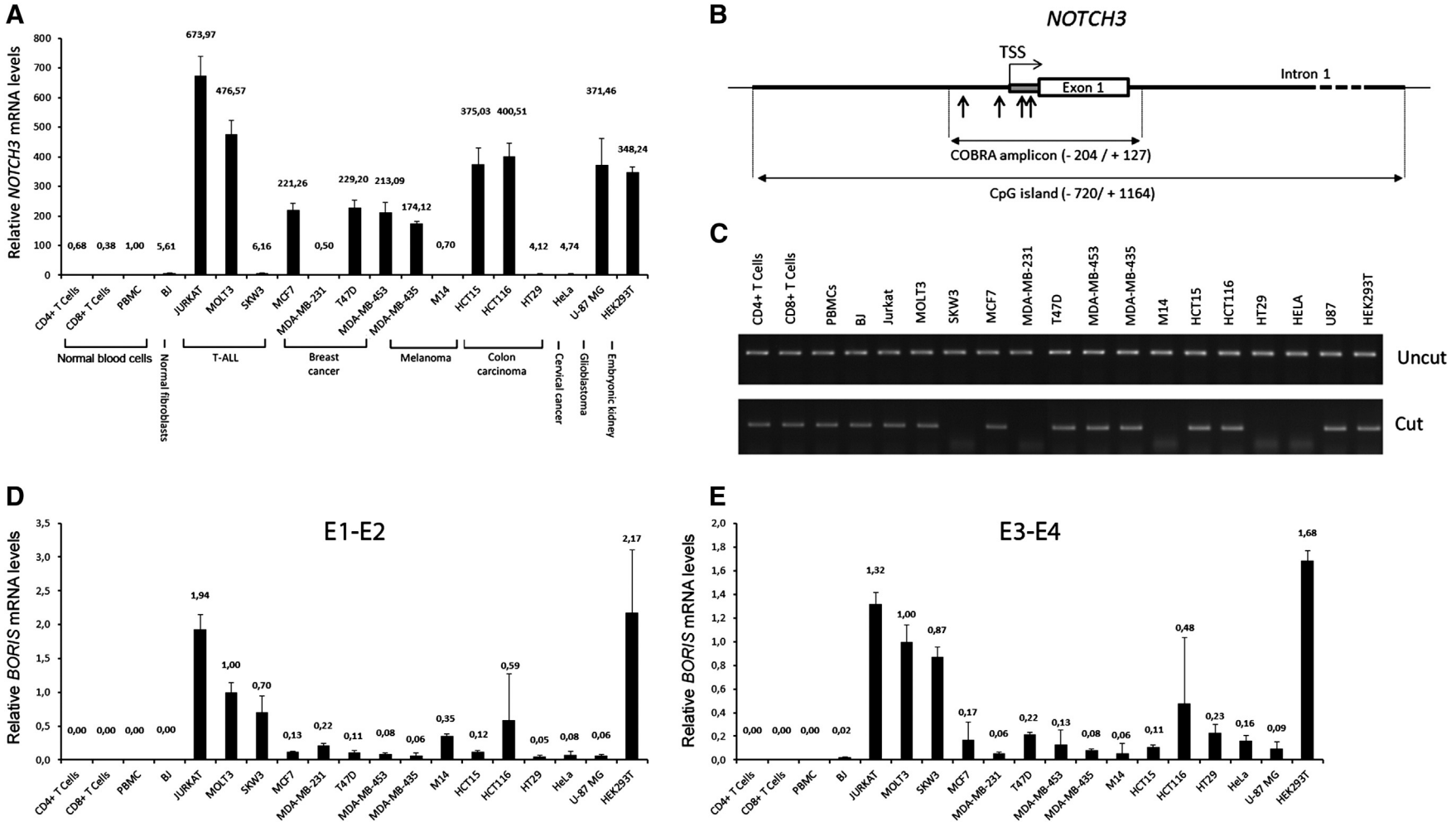

Fig. 4. Analysis of *NOTCH3* expression and promoter methylation *status* and of *BORIS* expression in normal and cancer cell lines. (A) Expression of *NOTCH3* transcript as detected by quantitative RT-PCR. Relative levels were calculated taking the transcriptional levels of *NOTCH3* in PBMCs as 1. *GUSB* transcript level was used as endogenous control. Histogram columns are labeled by the mean expression values calculated from three experiments. Error bars indicate ± s.e.m. (B) Schematic map of the *NOTCH3* 5′-region showing the position of the PCR product analyzed in the COBRA assay. Arrows indicate the approximate position of *Bst*UI sites. TSS indicates the transcriptional start site. Gray box: untranslated 5′-region; open box: coding exon. Numbers in brackets indicate the nucleotide positions relative to the ATG initiator codon. (C) *NOTCH3* promoter DNA methylation profiling as detected by COBRA assay. A 332-bp PCR fragment is cleaved by *Bst*UI when methylation is present in bisulfite-modified DNA. UNCUT and CUT indicate samples treated or not with the restriction enzyme. (D and E) Expression of *BORIS* transcript as detected by quantitative RT-PCR. Two sets of primers encompassing 1–2 (E1–E2) and 3–4 (E3–E4) exons were used to detect all known isoforms of *BORIS* transcript. Detection of *GUSB* transcript served as loading control of total RNA. Relative levels were calculated taking the transcriptional levels of *BORIS* in MOLT3 as 1. Histogram columns are labeled by the mean expression values calculated from three experiments. Error bars indicate ± s.e.m.

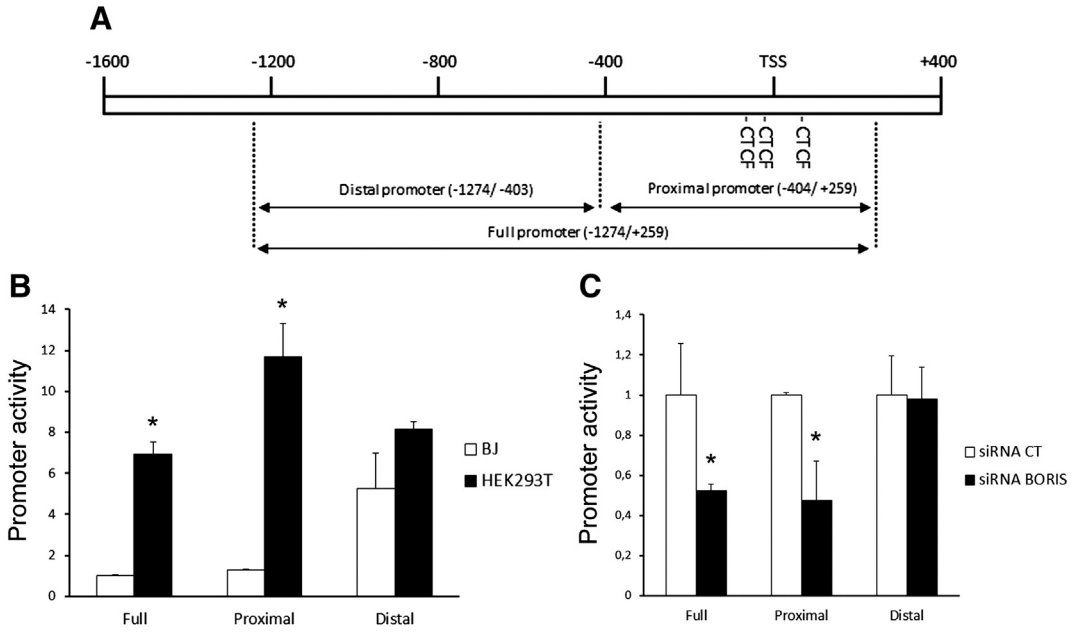

Fig. 5. BORIS stimulates the activity of proximal *NOTCH3* promoter. (A) Schematic map of the *NOTCH3* 5′-region showing the regions analyzed in the luciferase reporter assays. Numbers in brackets indicate the nucleotide position relative to the ATG initiator codon. (B) Luciferase reporter activity in BORIS-negative BJ and BORIS-positive HEK293T cells. Cells were transfected with luciferase reporter plasmids containing the fragments of *NOTCH3* 5′-region indicated in A. Cells were also transfected with CMV-β-gal to control transfection efficiency. Results are presented as ratio of luciferase/β-galactosidase relative units and represent the relative fold change compared with the activity of the full promoter construct measured in the BJ sample. (C) Luciferase reporter activity after modulation of *BORIS* expression levels by siRNA silencing in HEK293T cells. Transfection of non-targeting siRNAs (siRNA CT) was performed as control. Cells were also transfected with CMV-β-gal to control transfection efficiency. Results are presented as ratio of luciferase/β-galactosidase relative units and represent the relative fold change compared with controls. Results shown in B and C are means ± s.e.m. calculated from three experiments. *$P < 0.05$ compared with BJ (B) or controls (C) (Student's t test).

that mechanisms other than DNA methylation are involved in the up-regulation of NOTCH3 in T-ALL.

Additional details were provided by the analysis of histone H3 methylation, which is known to acts in a combinatorial fashion to specify distinct transcriptional chromatin conformation [28]. The DNA promoter hypermethylation of NOTCH3-negative cancer cells (SKW3) associates with the predominant presence of H3K9me3, which generally marks silenced heterochromatin. Conversely, unmethylated promoter in NOTCH3-positive T-ALL cells (MOLT3) and in normal blood cells shows divergent histone H3 methylation profiles. In NOTCH3-positive leukemia cells the higher presence of H3K4me3 versus H3K27me3 clearly indicate transcriptional permissive *status* of the promoter, while the higher level of H3K27me3 versus H3K4me3 in normal blood cells explains the silencing of *NOTCH3* despite the unmethylated *status* of promoter. Thus, different from DNA methylation, H3 histone methylation seems to discriminate between normal and leukemia cells.

The involvement of epigenetic factors in driving chromatin conformation changes in the *NOTCH3 locus* was then investigated. Attention was focused on CTCF [29–32] and BORIS/CTCFL [16,17] paralog proteins, as binding sites for CTCF were identified on *NOTCH3* promoter. BORIS is very similar to CTCF in the 11-zinc-finger DNA-binding domain and, sharing part of DNA target sequences with this protein, could compete with it. Conversely, CTCF and BORIS diverge in the N-and C-terminal ends, allowing interaction with distinct partners [17,24–26].

BORIS is a cancer testis antigen (CTA) as it is normally expressed in testis but aberrantly expressed in several primary tumors and established cancer cell lines [16,17,27,33–35]. Importantly, BORIS itself has a pivotal role in the expression of other CTAs (e.g. MAGE-A1) [36–39] and of some proto-oncogenes (e.g. hTERT) [40,41], generally repressed by CTCF.

Analysis of *CTCF* and *BORIS* expression shows that, while *CTCF* is expressed in both normal blood cells and T-ALL cell lines, *BORIS* is specifically expressed in T-ALL and solid tumor-derived cell lines.

Moreover, ChIP assay, used to assess the binding of CTCF and BORIS proteins to the 5′ region of the *NOTCH3* gene, demonstrated that the occupancy of *NOTCH3* promoter by CTCF and/or BORIS correlates with *NOTCH3* expression, promoter epigenetic *status* and *BORIS* expression.

In BORIS-negative normal blood cells, the presence of repressive H3 modifications coincides with CTCF binding at proximal *NOTCH3* promoter. Conversely, in MOLT3 cells the binding of both CTCF and BORIS correlates with an active chromatin *status* and upregulated *NOTCH3* expression. To note, in SKW3 cells, characterized by *NOTCH3* silencing, neither CTCF nor BORIS are bound on the hypermethylated/heterochromatic promoter. These results show that *NOTCH3* expression varies according to the binding of BORIS rather than CTCF, and that this binding depends, in turn, on promoter chromatin accessibility. This suggests that both the unmethylated *status* of *NOTCH3* promoter and *BORIS* expression and binding to the promoter are required for *NOTCH3* upregulation in T-ALL and cancer cell lines. Consistent with this hypothesis, we found that *NOTCH3* upregulation occurs in BORIS-positive cell lines derived from different cancers, showing unmethylated *NOTCH3* promoter.

To obtain information about the direct role played by BORIS in *NOTCH3* expression the highly transfectable NOTCH3- and BORIS-expressing HEK293T cell line was used. Promoter activity assays in the condition of silencing BORIS indicate that it is directly involved in the transcriptional upregulation of *NOTCH3*. Moreover, a direct analysis of what occurred on endogenous *NOTCH3 locus* following modulation of the BORIS level allowed us to establish that BORIS is directly responsible for changes in the chromatin structure necessary for transcriptional activation of *NOTCH3*. In fact, the presence of BORIS on *NOTCH3* promoter associates with an increased H3K4me3/H3K27me3 ratio.

Confirmatory finding of a role for BORIS in *NOTCH3* expression was obtained in the BORIS- and NOTCH3-negative BJ normal fibroblast where exogenous BORIS enforced expression is sufficient to induce *NOTCH3* upregulation.

Our results are in agreement with literature data showing that BORIS positively regulates gene expression by inducing an open chromatin conformation [41–44]. BORIS induced alterations in the *NOTCH3* promoter appear highly similar to those common features typical of permissive/active chromatin conformation, which have recently been reported for *Gal3st1* [43], in the physiological context of spermatogenesis, and for the activation of cancer testis antigen *TSP50* [39]. To note, genome-wide analyses revealed that BORIS co-localizes with H3K4me3 and active PolII

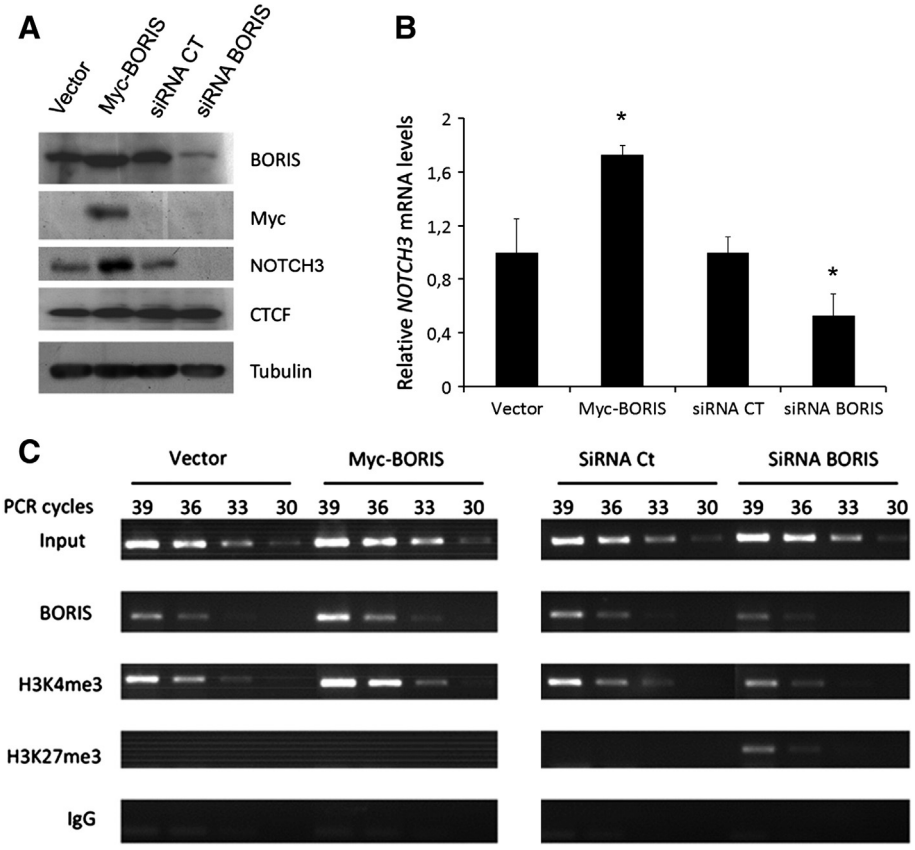

Fig. 6. BORIS is a positive epigenetic regulator of *NOTCH3* expression. Transient transfection of either siRNA (siRNA BORIS) or expression vectors for BORIS (Myc-BORIS) was performed in HEK293T cells. Transfection of non-targeting siRNAs (siRNA CT) and empty overexpression vector (Vector) was performed as control. (A) Western blot analysis of cell lysates with anti-BORIS, anti-CTCF and anti-NOTCH3 antibodies were performed to monitor endogenous protein levels. Anti-Myc antibodies were used to detect exogenous BORIS protein. Anti α-tubulin antibodies served as control for loading differences. (B) Expression of *NOTCH3* transcript as detected by quantitative RT-PCR in HEK293T cells after transfection of either siRNA or expression vectors for BORIS. Relative levels were calculated taking the transcriptional levels of *NOTCH3* in control transfection as 1. *GUSB* transcript level was used as endogenous control. Results are means ± s.e.m. calculated from three experiments. (C) ChIP analysis of *NOTCH3* promoter region for Histone H3 modifications in HEK293T cells after transfection of either siRNA or expression vectors for BORIS. Immunoprecipitation was performed by using specific antibodies to trimethyl lysine 4 (H3K4me3) and to trimethyl lysine 27 (H3K27me3). Normal IgG were used as negative control (IgG). The DNA region analyzed corresponds to the PCR amplicon indicated in Fig. 1D. *$P < 0.05$ compared with controls (Student's t test).

on the TSS of active promoters [26]. This is in agreement with data showing that SET1A H3K4 methyltransferase is a partner of BORIS [45].

Considering that abnormal *BORIS* expression has been correlated with the upregulation of proto-oncogenes in different primary tumors [35], our next aim was to gain evidence that, in T-ALL primary samples, where enhanced *NOTCH3* gene expression was found [6], BORIS was co-expressed. We found that *BORIS* expression correlated with the upregulation of *NOTCH3* in all inspected T-ALL patient samples.

4. Conclusions

Taken together our data suggest that BORIS aberrant expression in T-ALL triggers *NOTCH3* transcriptional activation most likely through an epigenetic mechanism. By binding unmethylated CTCF binding sites present on *NOTCH3* promoter, BORIS favors an active chromatin conformation by increasing the H3K4me3/H3K27me3 ratio (Fig. 9). Anyway, even though our data suggest that in cancer cell lines the *NOTCH3* promoter-region is co-bound simultaneously by both CTCF and BORIS, other alternative patterns of DNA-occupancy could not be ruled out. In fact, this region contains at least two putative target sites for the 11-zinc-finger DNA-binding domains to be independently bound either by CTCF or by BORIS.

In conclusion, this paper not only provides insights into molecular mechanisms underlying *NOTCH3* upregulation in T-ALL, but also suggests a novel molecular model for the action of BORIS that could be crucial in explaining the activation of CTAs and other proto-oncogenes in T-ALL [46].

Furthermore, this study supports the opportunity to exploit BORIS targeting for anti-cancer therapy [47–50].

5. Materials and methods

5.1. Cell culture, isolation of peripheral blood cells and patient samples

Experiments were carried out in human normal foreskin fibroblasts expressing hTERT (BJ); T-cell acute lymphoblastic leukemia cell lines MOLT3, SKW3 and Jurkat; breast cancer cell lines MCF7, T47D, MDA-MB-231 and MDA-MB-453; melanoma cell lines M14 and MDA-MB-435; colorectal cancer cell line HCT116, HCT15 and HT29; cervical cancer cell line HeLa; glioblastoma cell line U87; embryonic kidney cell line HEK293T. Cell lines were maintained as subconfluent culture in high-glucose DMEM (adherent cell culture) or RPMI (suspension cell culture) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin and 50 mg/ml streptomycin. All culture solutions were from VWR International PBI (Milan, Italy).

Peripheral blood mononuclear cells (PBMCs) were obtained from blood of normal donors by density gradient centrifugation using 'Lymphoprep' solution (Axis-Shield, Dundee, UK).

For lymphocytes preparation cells were obtained as previously described [51]. Briefly, the mature T cells, including CD8 single-positive and CD4 single-positive, were positively selected by magnetic cell separation by using the MACS (magnetic cell sorter) system (Milteny Biotec, Auburn, CA) in accordance with the manufacturer's instructions. The purity of the isolated fractions was greater than 90%, as determined by

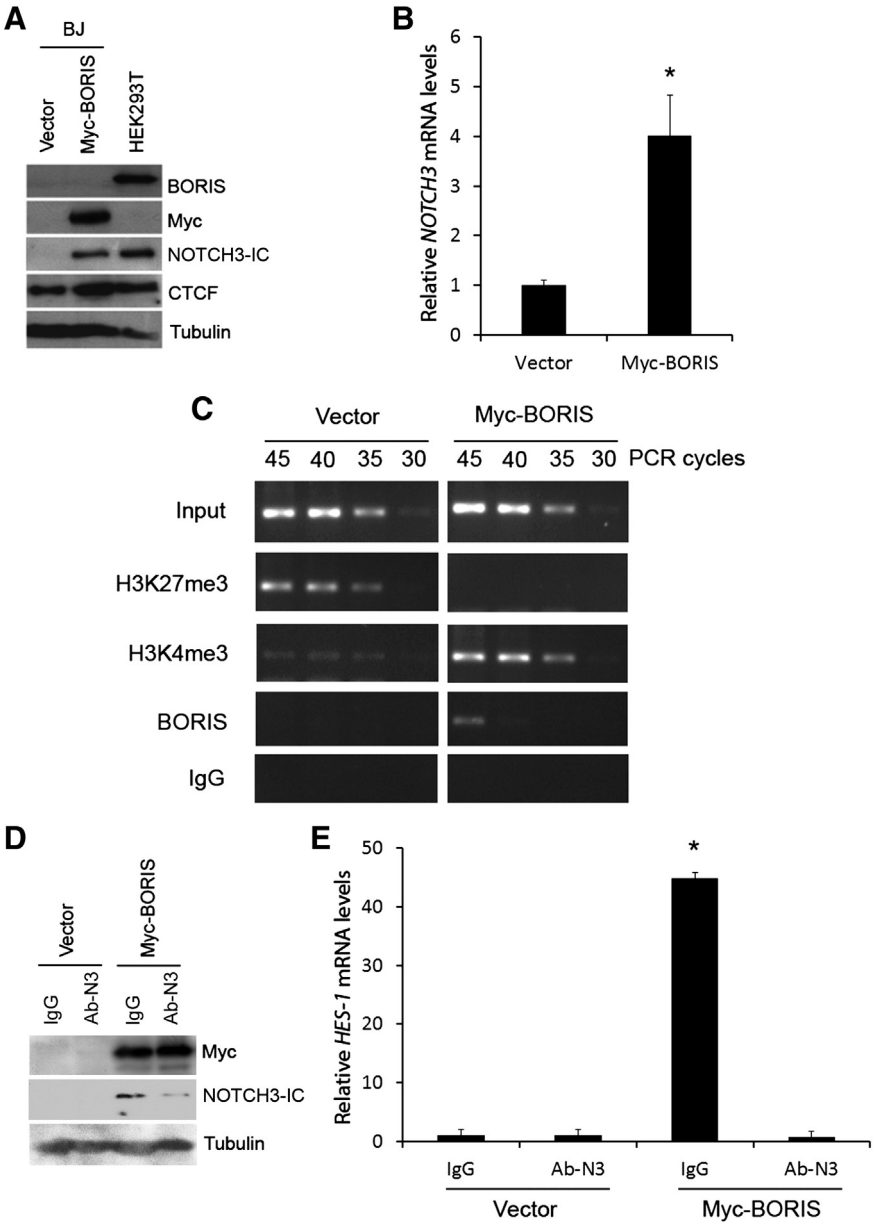

Fig. 7. BORIS enforced expression is sufficient to induce *NOTCH3* upregulation in BORIS-negative BJ normal fibroblasts. (A) Western blot analysis of cell lysates after transfection of *BORIS* expression vector (Myc-BORIS) and empty overexpression vector (Vector) as control. HEK293T samples served as positive control for BORIS and NOTCH3. Analysis was performed with anti-BORIS, anti-NOTCH3 and anti-CTCF antibodies. Anti-Myc antibodies were used to detect exogenous BORIS protein. Anti α-tubulin antibodies served as control for loading differences. (B) Expression of *NOTCH3* transcript as detected by quantitative RT-PCR after transfection of *BORIS* expression vector. Relative levels were calculated taking the transcriptional levels of *NOTCH3* in control transfection as 1. *GUSB* transcript level was used as endogenous control. Results are means ± s.e.m. calculated from three experiments. (C) ChIP analysis of *NOTCH3* promoter region for Histone H3 modifications after transfection of expression vectors for BORIS. Immunoprecipitation was performed by using specific antibodies to trimethyl lysine 4 (H3K4me3) and to trimethyl lysine 27 (H3K27me3). Normal IgG were used as negative control (IgG). The DNA region analyzed corresponds to the PCR amplicon indicated in Fig. 1D. (D) Western blot analysis of cell lysates after transfection of *BORIS* expression vector and treatment with an anti-NOTCH3 IgG, known to neutralize receptor function (Ab-N3), or with the normal control IgG (IgG). Analysis was performed with anti-Myc antibodies to detect exogenous BORIS protein and with anti-NOTCH3 antibodies. Anti α-tubulin antibodies served as control for loading differences. (E) Expression of *HES-1* transcript as detected by quantitative RT-PCR after transfection of *BORIS* expression vector and treatment with the anti-NOTCH3 neutralizing IgG. Relative levels were calculated taking the transcriptional levels of *HES-1* in control transfections as 1. *GUSB* transcript level was used as endogenous control. Results are means ± s.e.m. calculated from three experiments. *$P < 0.05$ compared with controls (Student's t test).

flow cytometry analysis (Becton Dickinson, Franklin Lakes, NJ). Anti-CD4-FITC, anti-CD8-PE, anti-CD122-FITC, and anti-CD45R/B220-PE Abs were purchased from Pharmingen (San Diego, CA).

Eight primary T-ALL samples were obtained at the onset of disease. All samples contained at least 70% leukemic cells.

5.2. Extraction of nucleic acids

Genomic DNA was prepared by the DNeasy tissue kit (Qiagen, Germantown, MD). Total RNA was purified by RNeasy mini kit (Qiagen, Germantown, MD). Concentration, purity and integrity of preparations were evaluated by spectrophotometry, followed by agarose gel–ethidium bromide electrophoresis.

5.3. Reverse transcription-PCR (RT-PCR)

Total RNA (1 μg) was subjected to reverse transcription using the SuperScript VILO cDNA synthesis kit (Life Technologies, Invitrogen, Carlsbad, CA).

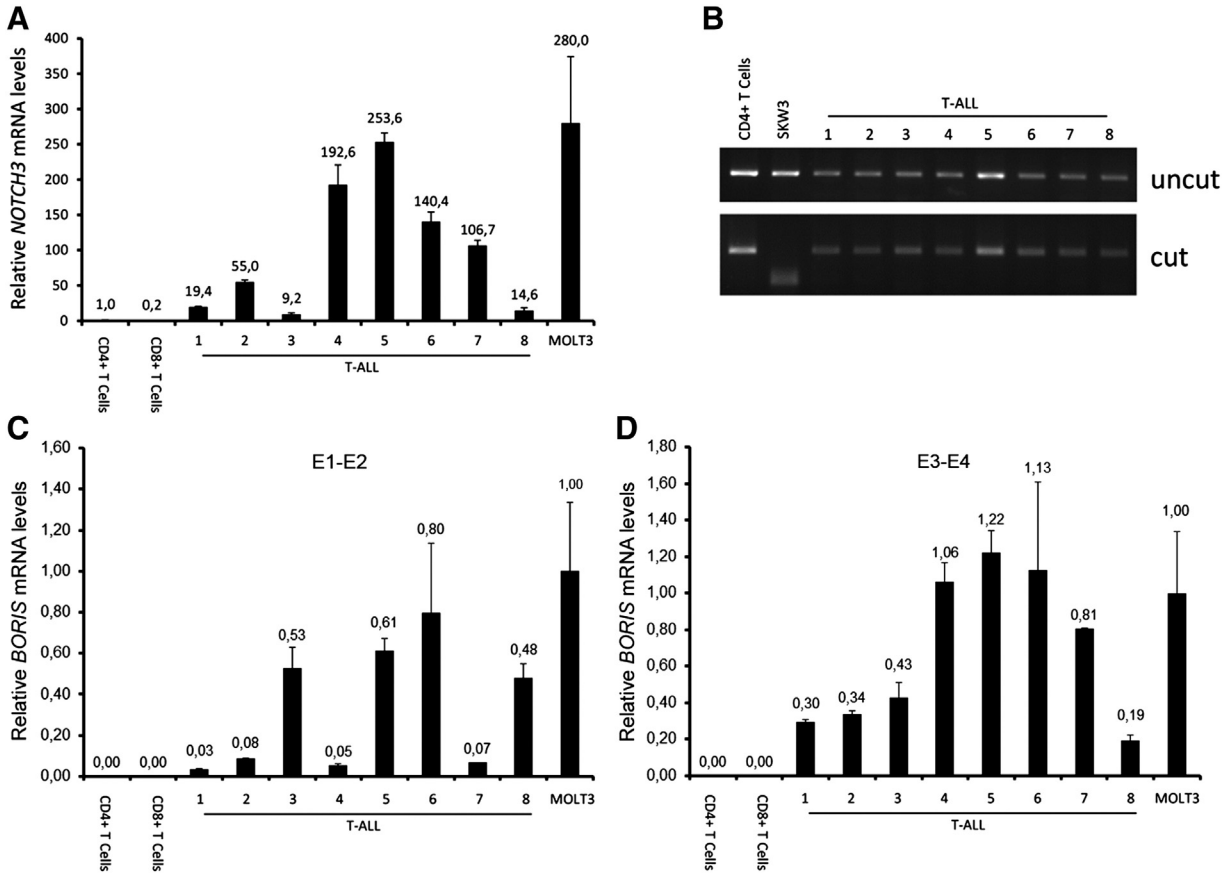

Fig. 8. Co-expression of *BORIS* and *NOTCH3* in T-ALL primary samples. (A) Expression of *NOTCH3* transcript as detected by quantitative RT-PCR in normal blood CD4 + and CD8 + T-cells, in eight cases of T-ALL, and in the leukemic cell lines MOLT3. Relative levels were calculated taking the transcriptional level of *NOTCH3* in CD4 + T-cells as 1. *GUSB* transcript level was used as endogenous control. Histogram columns are labeled by the mean expression values calculated from three experiments. Error bars indicate ± s.e.m. (B) *NOTCH3* promoter DNA methylation profiling as detected by COBRA assay. CD4 + T-cells and SKW3 served as positive and negative controls, respectively. The DNA region analyzed is indicated in Fig. 4B. (C and D) Expression of *BORIS* transcript as detected by quantitative RT-PCR in normal blood CD4 + and CD8 + T-cells, in eight cases of T-ALL, and in the leukemic cell lines MOLT3. Two sets of primers encompassing 1–2 (E1–E2) and 3–4 (E3–E4) exons were used to detect all known isoforms of *BORIS* transcript. Detection of *GUSB* transcript served as endogenous control. Relative levels were calculated taking the transcriptional levels of *BORIS* in MOLT3 as 1. Histogram columns are labeled by the mean expression values calculated from three experiments. Error bars indicate ± s.e.m.

Expression of mRNAs for *NOTCH3*, *CTCF* and *BORIS* was measured by real time PCR using Taq-Man gene expression assays (Life Technologies, Applied Biosystems, Carlsbad, CA) following the manufacturer's protocol for the comparative C_T method. The amplification reaction was performed using the TaqMan Universal PCR master Mix (Life Technologies, Invitrogen, Carlsbad, CA). All samples were run in duplicate in 96-well plates on the iCycler IQ detection system (Bio-Rad, Hercules, CA). Normalization was carried out using beta-glucuronidase (*GUSB*) as internal control gene. TaqMan gene expression assay IDs for each set of primers and probe were: Hs01128541_m1 (*NOTCH3*); Hs00902008_m1 (*CTCF*); Hs00966555_g1 (*BORIS* E1–E2); Hs00966548_g1 (*BORIS* E3–E4), Hs00172878_m1(*HES1*) and Hs99999908_m1 (*GUSB*).

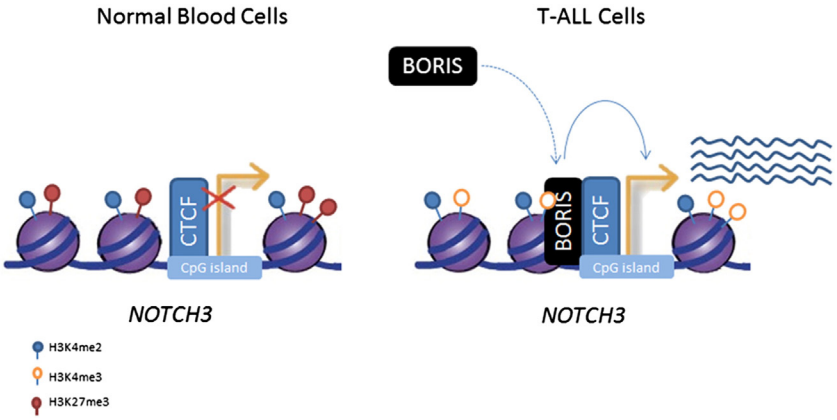

Fig. 9. Aberrant expression of BORIS in T-ALL cells favors *NOTCH3* upregulation. Figure shows a possible molecular mechanism underlying the association between *BORIS* expression and *NOTCH3* upregulation in T-ALL cells. The aberrant expression of BORIS in T-ALL cells and its recruitment on unmethylated CTCF binding sites is permissive for the transcription of *NOTCH3*. In fact, BORIS is required to maintain a local transcriptionally active chromatin configuration at the *NOTCH3* promoter.

5.4. Genomic bisulfite sequencing and combined bisulfite restriction analysis (COBRA)

Bisulfite treatment of genomic DNA (1 μg) was performed by using the EZ DNA methylation kit (Zymo Research, Irvine, CA).

For sequencing analyses the *NOTCH3* promoter region was obtained by PCR. The amplification reactions were carried out by using the AccuPrime Taq DNA polymerase system (Life Technologies, Invitrogen, Carlsbad, CA) with 10 pmol specific primers and 100 ng of bisulfite-treated DNA in a final reaction mix of 25 μl. The following cycling conditions were adopted. The initial denaturation at 94 °C for 2 min; 40 cycles of: 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s. Sequencing analysis was performed as described previously [52].

For COBRA analyses the *NOTCH3* promoter region was obtained by nested PCR.

The amplification reactions were carried out by using the AccuPrime Taq DNA polymerase system (Life Technologies, Invitrogen, Carlsbad, CA) with 10 pmol specific primers and 100 ng of bisulfite-treated DNA (first amplification) or 2 μl of the first amplification in a final reaction mix of 25 μl.

The following cycling conditions were adopted. The initial denaturation at 94 °C for 2 min; 45 cycles of: 94 °C for 30 s, 50 °C (first amplification) or 52 °C (second amplification) for 30 s, and 72 °C for 40 s. Enzyme digestion was performed with *Bst*UI restriction endonuclease (New England Biolabs, Ipswich, MA) according to manufacturer's protocol on 5 μl of the second amplification product in a final reaction mix of 20 μl. Agarose gel electrophoresis (2%) followed by ethidium bromide staining was performed to visualize digestion products (20 μl). Primers used for the amplifications are listed in the Supplementary Table.

5.5. Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously [52,53]. The cross-linking procedure was adapted for cells growing in suspension as follows. Starting sample consisted of 1×10^7 cells from subconfluent cultures. After centrifugation cells were resuspended in complete culture medium added of formaldehyde (Sigma-Aldrich, St. Louis, MO) to a final concentration of 1% and incubated 15 min at room temperature with gentle shaking. To stop cross-linking reaction, cells were recovered by centrifugation, resuspended in 0.125 M glycine (Sigma-Aldrich, St. Louis, MO) in PBS and incubated for 5 min at room temperature with gentle shaking.

The antibodies used for immunoprecipitations were: rabbit polyclonal anti-trimethyl-Histone H3 (Lys9) (Millipore, Billerica, MA); rabbit polyclonal anti-trimethyl-Histone H3 (Lys27) (Millipore, Billerica, MA); mouse monoclonal anti-trimethyl-Histone H3 (Lys4) (Millipore, Billerica, MA); rabbit polyclonal anti-dimethyl-Histone H3 (Lys4) (Millipore, Billerica, MA); rabbit polyclonal anti-CTCF (Millipore, Billerica,MA); goat polyclonal anti-BORIS (C-15) (Santa Cruz Biotechnology, Santa Cruz, CA) and normal rabbit/mouse IgGs (Santa Cruz Biotechnology, Santa Cruz, CA).

End-point PCR reactions were carried out by using the AccuPrime GC-Rich DNA polymerase system (Life Technologies, Invitrogen, Carlsbad, CA), with 10 pmol specific primers and 5 μl of immunoprecipitated DNA in a final reaction mix of 25 μl. The following cycling conditions were adopted. The initial denaturation at 94 °C for 2 min; 30–45 cycles of: 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Agarose gel electrophoresis (1.8%) followed by ethidium bromide staining was performed to visualize PCR products (8 μl). Primers used for the amplifications are listed in the Supplementary table.

5.6. Plasmid construction and transfection of cells

Human *NOTCH3* promoter-luciferase reporter plasmids were generated using the following primers: forward, 5-ATCATGCCACTGCACTTC AG-3, and reverse, 5-GTTCTTGCACTCCCCCTCTG-3; a 1533 bp fragment was amplified from human genomic DNA; the amplified insert was cloned into TA-Cloning cloning vector (Life Technologies, Invitrogen, Carlsbad, CA) and a KpnI and XhoI fragment containing the promoter was isolated and subcloned into polylinker of the plasmid PGL3 Basic vector (Promega, Madison, WI). This construct was denoted as full length *NOTCH3*-promoter. Deletion fragments of the *NOTCH3* promoter were generated using HindIII restriction site present within the *NOTCH3*-promoter which resulted in a distal fragment (−1274 to −403 bp) and a proximal fragment (−404 to +259 bp) containing CTCF sites.

Boris cDNA, containing the complete coding sequence (accession no. DQ153171), was isolated by PCR amplification using the template cDNA prepared from mouse adult testis. The following oligonucleotides were used: 5′-CACGGAATTCAATGGCAGCCACTGAGATCTCTGTCCTT-3′sense, 5′-CCTGCTCGAGTCACTTATCCATCGTGTTGAGGAGCAT-3′ antisense. The PCR amplified fragment was cloned in the myc-tag expression vector pCS2-MT that inserts 6 myc tags in-frame at the carboxyl terminus of the protein and sequenced by Eurofins MWG Operon service.

For the luciferase assays cells were transfected with Lipofectamine 2000 (Life Technologies, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were seeded in 12-well culture plates at a concentration of 2×10^5 cells/well and incubated for 24 h prior to cotransfections involving luciferase reporter constructs (30 ng/well), anti-BORIS (siGENOME SMARTpool, Thermo Fisher Scientific, 30 pmol/well) or control siRNAs (siGENOME Non-Targeting siRNA Pool, Thermo Fisher Scientific, 30 pmol/well). As internal control, plasmid pCMV-beta-gal (0.5 ng/well) (Promega, Madison, WI), containing the beta-galactosidase reporter gene, was co-transfected. Luciferase and beta-galactosidase activities were quantitated 24 h later by the luciferase or the β-galactosidase enzyme assay system (Promega, Madison, WI), respectively.

For the ChIP assays 7×10^5 cells were seeded in 100×15 mm culture dishes and incubated for 24 h prior to transfection. Transfection of siRNAs targeting BORIS and of control siRNAs (600 pmol/dish) was performed with Lipofectamine 2000 reagent using the manufacturer's protocol. Transfection of myc-Boris construct or empty myc-vector (8 μg/dish) was performed with Lipofectamine Plus reagent (Life Technologies, Invitrogen, Carlsbad, CA) using the manufacturer's protocol. After 48 h the cells were processed for ChIP, RT-PCR and for Western blot analyses.

In the NOTCH3 activity downregulation assays, cells were seeded at 1.3×10^5 cells per well in 24-well plates 24 h prior to transfections involving the myc-Boris construct or empty myc-vector (0.2 μg/well). The transfection by Lipofectamine Plus reagent followed the manufacturers' protocol. At the conclusion of the transfection procedure, cells were shifted to growth medium containing 10 μg/ml of either a sheep polyclonal anti-NOTCH3 IgG, known to neutralize receptor function (R&D Systems, Minneapolis, MN), or an equivalent concentration of the normal IgG sheep control (R&D Systems, Minneapolis, MN) and cultured for 48 h. Samples were than processed for Western blot and RT-PCR analyses.

5.7. Western blot

Western blot assays were performed as described elsewhere [54]. The antibodies used were the following: mouse monoclonal anti-Myc (9E10 clone, hybridoma-conditioned medium), mouse monoclonal anti-BORIS (Millipore, Billerica, MA), rabbit polyclonal anti-CTCF (Millipore, Billerica, MA); rabbit polyclonal anti-NOTCH3 (Cell Signaling Technology, Danvers, MA) and mouse monoclonal anti-Tubulin-α (Sigma-Aldrich, St. Louis, MO).

5.8. Statistical analysis

Statistical tests and the number of replicates performed are reported in figure legends. Data were considered to be statistically significant if $^*P < 0.05$.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagrm.2014.06.017.

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