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CTCF and BORIS Regulate *Rb2/p130* Gene Transcription: A Novel Mechanism and a New Paradigm for Understanding the Biology of Lung Cancer

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Abstract

Although innumerable investigations regarding the biology of lung cancer have been carried out, many aspects thereof remain to be addressed, including the role played by the retinoblastoma-related protein Rb2/p130 during the evolution of this disease. Here we report novel findings on the mechanisms that control *Rb2/p130* gene expression in lung fibroblasts and characterize the effects of Rb2/p130 deregulation on the proliferative features of lung cancer cells. We revealed for the first time that in lung fibroblasts the expression of *Rb2/p130* gene is directly controlled by the chromatin insulator CCCTC-binding factor, CTCF, which by binding to the *Rb2/p130* gene promoter induces, and/or maintains, a specific local chromatin organization that in turn governs the transcriptional activity of *Rb2/p130* gene. However, in lung cancer cells the activity of CTCF in controlling *Rb2/p130* gene expression is impaired by BORIS, a CTCF-paralogue, which by binding to the *Rb2/p130* gene could trigger changes in the chromatin asset established by CTCF, thereby affecting CTCF regulatory activity on *Rb2/p130* transcription. These studies not only provide essential basic insights into the molecular mechanisms that control *Rb2/p130* gene expression in lung cancer, but also offer a potential paradigm for the actions of other activators and/or corepressors, such as CTCF and BORIS, that could be crucial in explaining how alterations in the mechanism regulating *Rb2/p130* gene expression may accelerate the progression of lung tumors, or favor the onset of recurrence after cancer treatment. *Mol Cancer Res; 9(2); 225–33. ©2011 AACR.*

Introduction

Lung cancer is one of the most frequent human cancers in both males and females, and only 13% to 15% of lung cancer patients survive more than 5 years (1). This inadequate prognosis is due to lack of effective screening modalities for lung cancer, which results in patients presenting with advanced stage disease that is not responsive to current therapies. Lung cancers are divided into small cell (SCLC) and nonsmall cell (NSCLC) lung cancer depending on their pathological and histological features and, from the standpoint of genetic alterations SCLC and NSCLC are two markedly different entities. Approximately 80% of lung cancer cases are NSCLC (which includes squamous cell carcinoma, adenocarcinoma and large cell carcinoma), and the remaining fraction is SCLC. SCLC is the most aggressive type of lung cancer and is defined not only by its characteristic neuroendocrine differentiation but also by a specific set of genetic aberrations, including the loss of the tumor suppressor gene *p53* (>75%) and *Rb1* (>90%), and the amplification of any member of the *Myc* family of oncogenes (2). Oncogenic mutations of Ras protein are exclusively found in NSCLC tumors (3). Of interest is the specificity observed in the mutation patterns affecting the p16INK4a/CyCD1/RB1 pathway in NSCL and SCLC. Alteration of this pathway is found in virtually all tumor types however, despite the mutual exclusiveness of the mutations in these genes, alterations in *p16INK4a* (30% to 70%), *CycD1* or *CDK4* are most commonly seen in NSCLC, whereas *Rb1* gene inactivation is a typical feature of SCLC (4). This information suggests that distinct molecular pathways might be constitutively active in NSCLC and SCLC cells contributing to their phenotypic differences and cellular responses to proliferation, differentiation, and survival signals.

Importantly, the diversity of these responses may be due at least in part to a distinct activity of Rb2/p130- and p107-retinoblastoma related proteins. Rb1, Rb2/p130, and p107 share extensive overlapping functions in cells in culture, but they also have distinct functions *in vivo* (5, 6). Importantly, it has been indicated that Rb family proteins have cell type-specific functions in the lung epithelium important for development and tumor suppression. Although Rb1 plays a critical unique role in regulating epithelial proliferation and survival, Rb2/p130 and p107 have distinct roles in Rb1-deficient cells: Rb2/p130 promotes apoptosis, whereas p107 induces cell cycle arrest (7). Furthermore, it has been reported that Rb2/p130- and p107-deficient cells are insensitive to p16-induced growth arrest, supporting a role for these Rb1-related proteins in protecting against cancer (8).

Although innumerable investigations have been carried out, many issues regarding the biology of lung cancer remain to be addressed, including the role played by Rb2/p130 and p107 during the evolution of this disease. The contribution of Rb2/p130 to lung tumorigenesis has been indicated by us and other investigators however, it remains controversial and under debate. Supporting a Rb2/p130 tumor suppressor activity there are studies showing mutations of this gene in SCLC cell lines and primary lung tumors, as well as studies demonstrating that the ectopic overexpression of Rb2/p130 in lung cancer cell lines suppresses cell growth, and studies indicating an independent role of Rb2/p130 expression in the development and/or progression of human lung cancer (9, 10, 11). On the other hand, challenging a role for Rb2/p130 in lung tumorigenesis, there are other studies reporting a functional Rb2/p130 expression in both lung tumor cell lines and primary tumors, and studies reporting reduced expression of Rb2/p130 in SCLC cell lines but absence of *Rb2/p130* gene mutation (12, 13).

Clearly, there are still insufficient and conflicting data to understand the role played by *Rb2/p130* in lung cancer and link the altered *Rb2/p130* expression observed in NSCLC and SCLC cells with the genetic mutation of this gene.

Importantly, our studies on *RB2/p130* in lung cancer have revealed that this gene is not a "classic tumor suppressor" but its activity, as a strong or weak oncosuppressor, strongly depends on the cellular context, the tumor microenviroment, and other genetic and epigenetic alterations, such as *Rb1* and *p107* loss of function, *Ras* activation and altered function of *Rb2/p130* itself. For instance, Rb2/p130 activity could rely on specific alterations in the p16INK4a/CyCD1/RB1 or p14^{ARF}/mdm2/p53 pathway in nonsmall and small cell lung cancer. An intricate feedback loop between Rb2/p130 activity and antiproliferative and proliferative signaling (e.g., MAPK estrogen and TGF-b signaling) in turn could trigger tumor suppression or tumor progression, raising the important question of what other cellular function(s) of Rb2/p130 are at play in lung cancer.

The chromatin insulator CCCTC-binding factor (CTCF) is a ubiquitously expressed nuclear protein binding approximately 20,000 sites on the human genome through eleven DNA-binding zing finger domains (14). CTCF possesses many DNA-regulation functions, including acting as a chromatin insulator, enhancer blocker, transcription activator, and nuclear organizer (15, 16, 17). Recently, a CTCF-binding sites database (CTCFBSDB) has been created to characterize experimentally identified and computationally predicted CTCF-binding sites (18). Interestingly, it has been demonstrated that BORIS, a CTCF-paralogue normally present only in male germ cells, is overexpressed in several lung cancer cell lines, and it seems to deregulate CTCF-target genes (19). In the present study, we report a novel finding on *Rb2/p130* gene regulation in lung cancer. We have disclosed the mechanisms and "players" controlling *Rb2/p130* gene expression in normal lung cells, characterizing the alterations impairing *Rb2/p130* expression in NSCL and SCLC cells. We found that CTCF directly regulates *Rb2/p130* gene expression in MRC-5 lung fibroblasts, and that in both NSCLC and SCLC cell lines, which exhibit increased and reduced levels of Rb2/p130, respectively, the function of CTCF in regulating *Rb2/p130* gene expression is impaired. Importantly, our findings support the hypothesis that the activity of CTCF in regulating Rb2/p130 could be disturbed by binding of BORIS to Rb2/p130 in NSCLC and SCLC. In fact, the analysis of *Rb2/p130* gene occupancy, using specific primers spanning a region of the *Rb2/p130* gene containing two putative CTCF/BORIS binding sites, revealed that while this region is bound only by CTCF in MRC-5 fibroblast cells, in NSCLC cell lines the same region is bound by both CTCF and BORIS, and in SCLC cells only by BORIS. Furthermore, we observed that both CTCF and BORIS proteins are overexpressed in SCLC and NSCLC cells with respect to the nonimmortalized lung fibroblast cells MRC5, which express CTCF only. We also found overexpression of both CTCF and BORIS in the immortalized lung fibroblast cells NL-20.

Taken together, our results show that CTCF directly controls *Rb2/p130* gene expression in lung fibroblasts. We believe that the binding of CTCF to *Rb2/p130* induces, and/or maintains, a specific local chromatin organization, which in turn may govern the transcriptional activity of this gene. However, in lung cancer cells the activity of CTCF in controlling *Rb2/p130* gene expression appears to be impaired by BORIS binding to the *Rb2/p130* gene, which in turn could trigger changes in the chromatin asset established by CTCF, then affecting the regulation of *Rb2/p130* transcription.

Materials and Methods

Cell culture

The following cell lines were used: A549 and H358 (NSCLC), H82 and H69 (SCLC), and MRC-5 (normal lung fibroblasts). The cell lines were purchased from American Type Culture Collection (ATCC) and cultured according to the manufacturer's protocols.

Western blot analysis

Cells were trypsinized, washed in PBS and resuspended in lysis buffer (50 mM Tris-Cl pH7.4, 5 mM EDTA, 250 mM

NaCl, 50 mM NaF, 0.1% Triton X-100, 0.1 mM Na_3VO_4) with fresh EDTA-free complete protease inhibitor cocktail (ROCHE). After incubation in ice, lysates were centrifuged (13,000 × g, 15 minutes) and supernatants were collected.

Protein concentrations were determined using Bio-Rad Protein Assay (Bio-Rad). Total lysates were resolved by SDS-polyacrylamide electrophoresis and then transferred to nitrocellulose membranes. The membranes were probed for Rb2/p130 (sc-317, Santa Cruz), CTCF (sc-28198, Santa Cruz), or BORIS (sc-51038, Santa Cruz). The expression of HSP70 and Actin protein was assessed to normalize protein loading. Antibody binding was detected by incubation with horseradish peroxidase-coniugated secondary antibodies followed by chemiluminescence (ECL Plus, Amersham Biosciences), and visualized by exposing the membrane to X-ray films.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (XChIP) assays were performed using the ChIP-IT Express Enzymatic kit (Active Motif). For each immunoprecipitation, 10 μg of sheared chromatin were incubated overnight with 2 μg of polyclonal antibody specific for CTCF (Millipore, DAM1421463), BORIS (Rockland, 600–401-907) or normal rabbit IgG (Santa Cruz, sc-2027). PCR were performed on 1/10 of immunoprecipitated DNA using specific primers for *Rb2/p130* core promoter region (Fig. 1A and B). As positive control for CTCF and CTCFL/BORIS DNA-protein binding, PCR were performed amplifing the *H19/DMR* locus (20). Input represents the 0.25% of total pre-immunoprecipitated chromatin. The results were confirmed by three independent experiments.

Transfection and reporter gene assay

Rb2/p130 Plasmids *r1*, *r12*, *r123* and *r23* were generated by PCR-amplification of different regions of *RBL2* promoter, by digestion with BglII and HindIII and by cloning into pGL3 *basic* (Promega) vector. The following primers have been used: *agatctgtttaaaatgcggggaaggt* and *gcccaagcttc-cattcaaacggcgaagc* (r1), *agatctgtttaaaatgcggggaaggt* and *cggta-agcttactggtcacctcccgacg* (r12), *agatctgtttaaaatgcggggaaggt* and *aagcttgctggatctgaggggtg* (r123), *ggcagatctgcttcgccgtttgaatgg* and *aagcttgctggatctgaggggtg* (r23). Plasmids r123Δ1 and r123Δ2 were generated by site-directed mutagenesis with the QuickChange II site-directed Mutagenesis Kit (Stratagene), following manufacturer's instructions. r123 plasmid was used as template and primers used were *gcggggaaggtgcttt-tatttgaaactaggggagc* and *gctcccctagtttcaaataaaagcaccttccccgc* (r123Δ1), *ggaggaggaggacgacaagacgccgcgccgcctgc* and *gcaggc-ggcgcggcgtcttgtcgtcctcctcctcc* (r123Δ2). Plasmid CMV-CTCF, containing the complete cDNA of CTCF, was generated by digestion of pOTB7/CTCF with EcoRI and XhoI and ligation with pCI-neo (Promega) digested with EcoRI and SalI. Plasmid CMV-BORIS, containing the complete cDNA of CTCFL/BORIS, was generated by digestion of pCR4-TOPO/BORIS with EcoRI and ligation with pCI-neo (Promega) digested with EcoRI. All plasmid constructs were checked by DNA sequencing. The expression of proteins following transient transfection was confirmed by Western blot analysis. Dual luciferase reporter assay
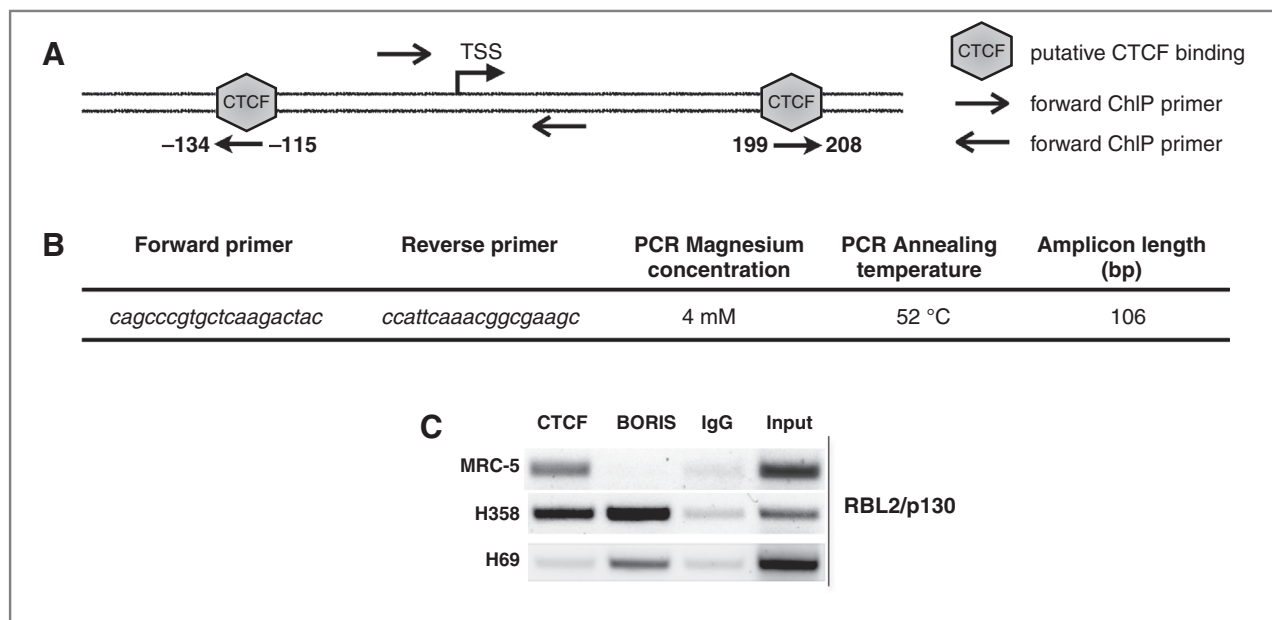

Figure 1. A, schematic representation of *Rb2/p130* gene region containing CTCF putative binding sites. B, nucleotide sequence of the primers spanning the *Rb2/p130* region investigated. C, *in vivo* binding (XChIP) of CTCF and Boris to *Rb2/p130* (*RBL2/P130*) promoter in MRC-5 lung fibroblasts, and H358 (NCSCL) and H69 (SCLC)lung cancer cell lines. The XChIPs were performed using the ChIP-IT Express Enzymatic kit (Active Motif). For each immunoprecipitation, 10 μg of sheared chromatin was incubated overnight with 2 μg of polyclonal antibody specific for CTCF (Millipore, DAM1421463), BORIS (Rockland, 600–401-907) or normal rabbit IgG (Santa Cruz, sc-2027). PCR was then performed on 1/10 of immunoprecipitated DNA using specific primers for Rb2/p130 core promoter region (B). As positive control for CTCF and BORIS DNA–protein binding, PCR was performed amplifing the *H19/DMR* locus (Nguyen, 2008; ref. 21). Input represents the 0.25% of total preimmunoprecipitated chromatin. The results were confirmed by three independent experiments.

(Promega) was used to measure the firefly luciferase and renilla luciferase activity within the transfected cells. pRL-TK renilla luciferase vector was used in each transfection as internal control of transfection efficiency in a ratio 1:20 respect to firefly luciferase vector. Each experiments was conducted as suggested by the manufacturer. Luciferase assays were performed with Sirius Luminometer (Berthold detection systems). Relative light units (RLU) of firefly luciferase activity was normalized against that of the renilla luciferase, and this ration was set to 1 for the control (basal activity of each promoter). Results represent the average of at least three separate biological experiments ± standard error.

PCR nucleosome mapping

Nuclei were isolated from MRC-5, H69 and H358 cells using a modified protocol of ChIP-IT Express Enzymatic kit (Active Motif). Briefly, cells were grown to 70%–80% confluence and fixed 5′-10′ with formaldehyde 1%. The fixation reaction was stopped by adding glycine 0.125 M in PBS to each of the plates for 5′. Cellular pellets were resuspended in 1 mL of lysis buffer supplemented with PMSF and PIC (Active motif) and incubated for 30′ at 4°C in constant agitation. The lysate was centrifuged at $2,400 \times g$ for 10′ at 4°C and the pellet obtained was resuspended again in 1 mL of lysis buffer. The lysate was transfered in an ice-cold homogenizer and 10 strokes of tight pestle were applied. The lysate was centrifuged again at $2,400 \times g$ for 10′ at 4°C to obtain pelleted nuclei. Nuclei were resuspended and incubated for 20′ in ice in 96 μL of Microccal Nuclease (MNase) buffer (Tris-Cl pH 7.5 10.4 mM, NaCl 10.4 mM, $MgCl_2$ 3.01 mM, $CaCl_2$ 1.04 mM, NP-40 4.02%) supplemented with PMSF and PIC. After an initial incubation for 5′ at 37°C, 4 μL of MNase sigma (2 U, 0.5 U/μL) were added and nuclei were incubated for 10′ at 37°C. MNase reaction was stopped incubating each sample in ice after adding EDTA 10 mM. Protocol was continued following kit instructions for the elution of sheared chromatin. The mononucleosomal DNA fraction was gel extracted using the Gel band extraction kit (QIAGEN), and 10 ng was used as a template in each PCR. Undigested genomic DNA was used as the positive control. No template was added to the PCR negative control. Primer pairs were designed to amplify areas of 80 to 120 bp on the Rb2/p130 promoter. Each PCR product overlaps the neighboring amplified regions by approximately 20 to 40 bp.

Results

Rb2/p130, CTCF, and BORIS expression in NSCLC and SCLC cells

To study the protein expression pattern of Rb2/p130, CTCF and BORIS in lung cancer we performed Western blotting analyses in cycling NSCLC (A549 and H358) and SCLC (H82 and H69) cell lines and in MRC-5 lung fibroblasts. Interestingly, we observed low levels of Rb2/p130 in SCLC cells, whereas in NSCLC Rb2/p130 was
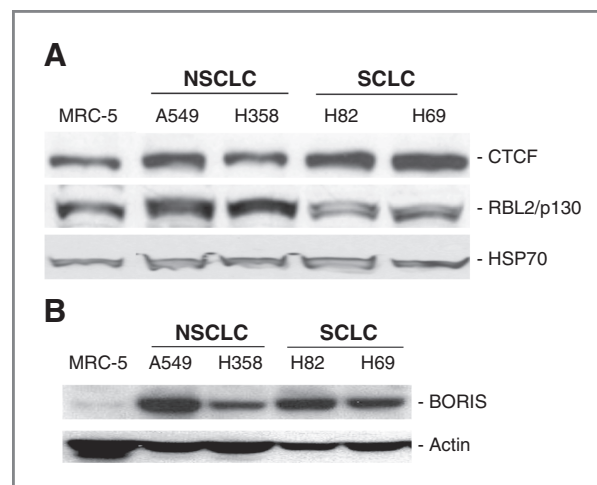
Figure 2. Protein expression level of (A) CTCF, and Rb2/p130 (RBL2/p130), and (B) BORIS, in nonimmortalized lung fibroblast (MRC-5), in A549 and H358 (NSCLC), and in H82 and H69 (SCLC) cells. The relative protein levels were detected by Western blotting using whole lysate. The expression of HSP70 and Actin protein was assessed to normalize protein loading.

significantly overexpressed with respect to the nonimmortalized lung fibroblast cells MRC-5 (Fig. 2A). Overexpression of CTCF and BORIS proteins was observed in both SCLC and NSCLC cells with respect to the nonimmortalized lung fibroblast cells MRC-5, which expressed CTCF only (Fig. 2A and B). The expression of HSP70 and β-Actin protein was assessed to normalize protein loading.

Ectopic overexpression of BORIS and its effect on CTCF and Rb2/p130 protein expression in MRC-5 lung fibroblasts

Plasmid CMV-BORIS, containing the complete cDNA of BORIS, was generated by digestion of pCR4-TOPO/BORIS with EcoRI and ligation with pCI-neo (Promega) digested with EcoRI. Ectopic overexpression of BORIS in MRC-5, which physiologically expresses CTCF only, induced a significant decrease in Rb2/p130 protein level whereas CTCF expression was only slightly altered (Fig. 3).

X-ChIP revealed that CTCF binds to *Rb2/p130* in lung fibroblasts, the ability of CTCF to bind *Rb2/p130* is lost and acquired by BORIS in SCLC cells, and both BORIS and CTCF bind to *Rb2/p130* in NSCLC cells

We performed crosslinking chromatin immunoprecipitation assays (XChIP) to investigate *Rb2/p130* promoter occupancy by CTCF and BORIS in MRC-5 lung fibroblasts, H358 and A549 (NSCLC), and in H82 and H69 (SCLC) cells. Our analysis was focused on the intergenic region of *Rb2/p130* between the nucleotides −155 and +159 containing two putative CTCF/BORIS binding sites (ref. 21; Fig. 1A and B). XChIP assays were performed using the ChIP-IT Express Enzymatic kit (Active Motif) as
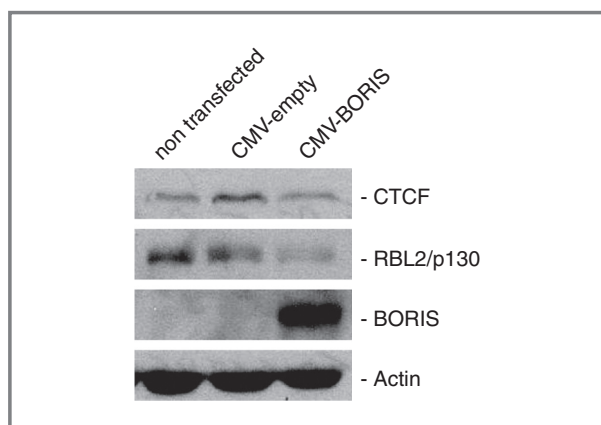
Figure 3. Ectopic overexpression of Boris and its effect on CTCF and Rb2/p130 protein expression in MRC-5 lung fibroblasts. MRC-5 lung fibroblasts were transfected with CMV-BORIS plasmid. After 72 hours from transfection the level of CTCF and RB2/p130 proteins were detected by Western analysis. The expression of Actin protein was assessed to normalize protein loading.

described in the Materials and Methods section. For each immunoprecipitation, 10 μg of sheared chromatin was incubated overnight with 2 μg of polyclonal antibody specific for CTCF, BORIS (Rockland, 600–401-907) or normal rabbit IgG. PCRs were performed on 1/10 of immunoprecipitated DNA using specific primers for Rb2/p130 core promoter region (Fig. 1B). As a positive control for CTCF and CTCFL/BORIS DNA-protein binding, PCR was performed by amplyfying the H19/DMR locus (20). Input represented the 0.25% of total preimmunoprecipitated chromatin. The results were confirmed by three independent experiments.

Importantly, the results from these experiments revealed a different pattern of binding of CTCF and BORIS to Rb2/p130 in MRC-5 fibroblasts, NSCLC and SCLC. As shown in Figure 3C, we found that Rb2/p130 is bound (a) exclusively by CTCF in MRC-5 fibroblast cells, (b) by both CTCF and BORIS in H358 and A549 NSCLC cell lines, and (c) exclusively by BORIS in H82 and H69 SCLC cell lines (Fig. 1C). Our idea is that the differential binding of CTCF and BORIS to Rb2/p130 may affect the transcription of this gene.

Interestingly, silencing of CTCF by siRNA did not affect BORIS expression whereas it induced a significant decrease in both Rb2/p130 transcript and protein level, indicating a key role for CTCF in controlling Rb2/p130 expression (data not shown). Physiologically, the expression of CTCF and BORIS could be controlled by independent mechanisms. During the neoplastic process, events altering the mechanisms controlling CTCF and BORIS expression may induce a "forced aberrant increase" in both CTCF and BORIS protein levels triggering changes in the binding affinity of these proteins to Rb2/p130 gene that in turn may differentially affect the expression of Rb2/p130 gene in nonsmall and small lung cancer cells.

CTCF controls Rb2/p130 gene expression in MRC-5 lung fibroblasts, and its regulatory function is impaired in lung cancer cells

We evaluated the effect of ectopic expression of CTCF and BORIS on Rb2/p130 gene expression in MRC-5 lung fibroblasts, and in H358 (NSCLC) and H69 (SCLC) lung cancer cell lines. We traced changes in the Rb2/p130 promoter activity by luciferase assay. Several Rb2/p130 plasmid constructs were generated and cotransfected with CTCF or BORIS. As shown in Figure 4A, r123 construct was designed to contain the Rb2/p130 gene region from −155 to +259, and contains two putative CTCF binding sites (−134 to −115; +198 to +217). r1 and r12 constructs were designed to contain only one CTCF binding site (−134 to −115) and the Rb2/p130 gene region from −155 to +57 and +142, respectively. r123Δ1 construct contained the Rb2/p130 gene region from −155 to +259, but the CTCF binding site located between −125 to −116 was mutated. r123Δ4 construct contained Rb2/p130 gene region from −155 to +259, but the CTCF binding site located between +198 to +217 was mutated. r23 construct contained the Rb2/p130 gene region from +49 to + 259, but contained only the CTCF binding site located between +198 to 217. As shown in Figure 1B, the cotransfection of CTCF and r123 construct increased Rb2/p130 promoter activity about 3.4-fold 48 hours after transfection and only in MRC-5 fibroblasts cells, indicating that the regulatory function of CTCF on Rb2/p130 expression must be impaired in both H358 and H69 cells. On the other hand, cotransfection of BORIS and r123 construct induced an increase in Rb2/p130 promoter activity 1.9-fold 48 hours after transfection, and only in MRC-5.

Moreover, cotransfection of BORIS or CTCF and r123Δ4 constructs induced a slight increase in Rb2/p130 promoter activity (1.2–1.7-fold), while cotransfection of BORIS or CTCF and r123Δ1 induced a slight decrease (0.5–0.8-fold) in all cell lines investigated. Interestingly, deletion of the region between -155 to +40 (construct r23) had a significant effect on Rb2/p130 expression inducing a tremendous increase in Rb2/p130 promoter activity in response to both CTCF and BORIS transfection, and in all the cell lines investigated (Fig. 4A and B), suggesting that the Rb2/p130 region between −155 to +40 contains important elements for the regulatory activity of CTCF and BORIS.

Importantly, the increase in CTCF and BORIS protein levels after ectopic expression of CTCF and Boris was detected in all cell lines investigated, even when no change in Rb2/p130 promoter activity was observed, confirming the reliability of the obtained evidence.

Determination of nucleosome positioning on Rb2/p130 promoter and 5′-coding regions

To disclose the mechanisms responsible for the aberrant Rb2/p130 expression in NSCLC and SCLC we performed both genetic and epigenetic studies. Mutational analysis did not show Rb2/p130 gene mutation in almost all the NSCLC and SCLC cell lines we investigated, supporting
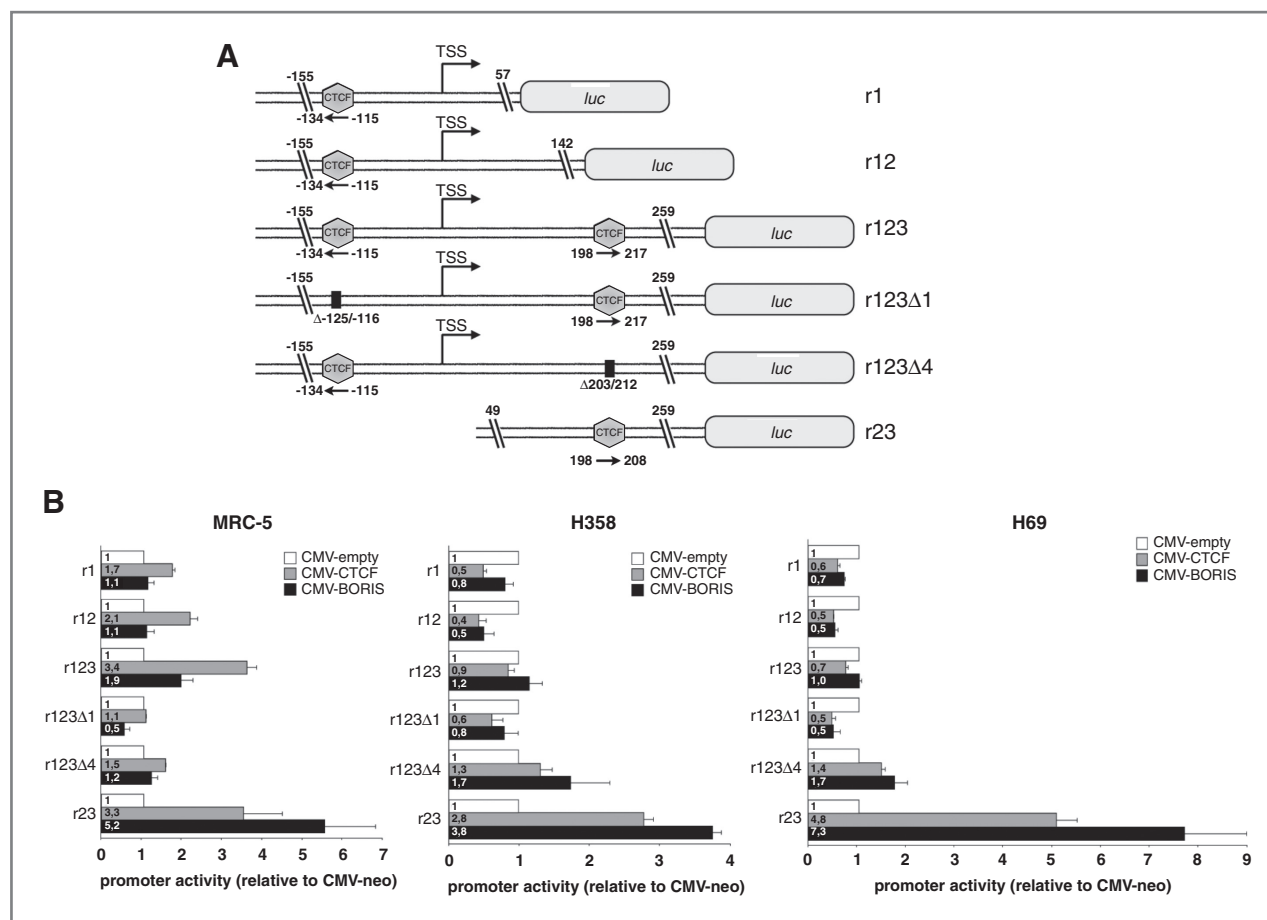
Figure 4. Effect of CTCT and BORIS on *Rb2/p130* promoter activity. A, Rb2/p130 Plasmids r1, r12, r123, and r23 were generated by PCR-amplification of different regions of *Rb2/p130* promoter, by digestion with BglII and HindIII and by cloning into pGL3 *basic* (Promega) vector. The following primers have been used: *agatctgtttaaaatgcggggaaggt* and *gcccaagcttccattcaaacggcgaagc* (r1), *agatctgtttaaaaatgcggggaaggt* and *cggtaagcttactggtcacctcccgacg* (r12), *agatctgtttaaaatgcggggaaggt* and *aagcttgctggatctgaggggtg* (r123), *ggcagatctgcttcgccgtttgaatgg*, and *aagcttgctggatctgaggggtg* (r23). Plasmids r123Δ1 and r123Δ2 were generated by site-directed mutagenesis with the QuickChange II site-directed Mutagenesis Kit (Stratagene), following manufacturer's instructions. r123 plasmid was used as template and primers used were *gcggggaaggtgctttatttgaaactaggggagc* and *gctcccctagtttcaaataaaagcaccttccccgc* (r123Δ1), *ggaggaggaggacgacaagacgccgcgccgcctgc* and *gcaggcggcgcggcgtcttgtcgtcctcctcctcc* (r123Δ2). B, plasmid CMV-CTCF, containing the complete cDNA of CTCF, was generated by digestion of pOTB7/CTCF with EcoRI and XhoI and ligation with pCI-neo (Promega) digested with EcoRI and SalI. Plasmid CMV-BORIS, containing the complete cDNA of CTCFL/BORIS, was generated by digestion of pCR4-TOPO/BORIS with EcoRI and ligation with pCI-neo (Promega) digested with EcoRI. All plasmid constructs were checked by DNA sequencing. The expression of proteins following transient transfection was confirmed by Western blot analysis. Dual luciferase reporter assay (Promega) was used to measure the firefly luciferase and renilla luciferase activity within the transfected cells. pRL-TK renilla luciferase vector was used in each transfection as internal control of transfection efficiency in a ratio 1:20 respect to firefly luciferase vector. Each experiments was conducted as suggested by the manufacturer. Luciferase assays were performed with Sirius Luminometer (Berthold detection systems). Relative light units (RLU) of firefly luciferase acitiviy was normalized against that of the renilla luciferase, and this ration was set to 1 for the control (basal activity of each promoter). Results represent the average of at least three separate biological experiments ± standard error.

the idea that *Rb2/p130* mutation is a rare event in lung cancer and importantly, other mechanisms, such as epigenetic mechanisms mediated by CTCF and BORIS, must contribute to impairing its expression. It is our idea that *Rb2/p130* expression is physiologically controlled by CTCF, which by binding to the *Rb2/p130* gene induces and/or maintains a specific local chromatin organization that governs *Rb2/p130* transcriptional activity. However, in lung cancer cells the control of CTCF on *Rb2/p130* expression might be impaired by BORIS binding to the *Rb2/p130* gene, which in turn could trigger changes in the chromatin asset established by CTCF. To test our hypoth-

esis, and since nucleosome positioning is essential for understanding mechanisms of gene expression regulation, we performed nucleosome mapping on *Rb2/p130* promoter and 5′-coding regions (−600 to +700) using a PCR-based method (22), in MRC5 lung fibroblasts, and in H358 (NSCLC) and H69 (SCLC) lung cancer cells (Fig. 5).

Briefly, MRC-5, H69 and H358 nuclei were obtained using a modified protocol of ChIP-IT Express Enzymatic kit (Active Motif). Cells were grown to 70% to 80% confluence and fixed 5′ to 10′ with formaldehyde 1%. The fixation reaction was stopped by adding glycine 0.125

Figure 5. Determination of nucleosome positioning on the human *Rb2/p130* promoter and 5′-coding regions in MRC-5, H358, and H69 cell lines. (A) Illustration of the *Rb2/p130* promoter and 5′-coding regions. The amplified region of each PCR product is shown with its corresponding number. The resulting nucleosomal regions are diagrammed as solid black bars. (B) Results of PCR analyses with the number corresponding to the amplified PCR product listed in Table 1. Samples were from formaldehyde cross-linked chromatin that either was not digested with micrococcal nuclease (undigested chromatin, 500 ng) or was subjected to micrococcal nuclease digestion to produce mononucleome-size chromatin (digested chromatin, 500 ng). Nuclease-free water without template was used as negative control.
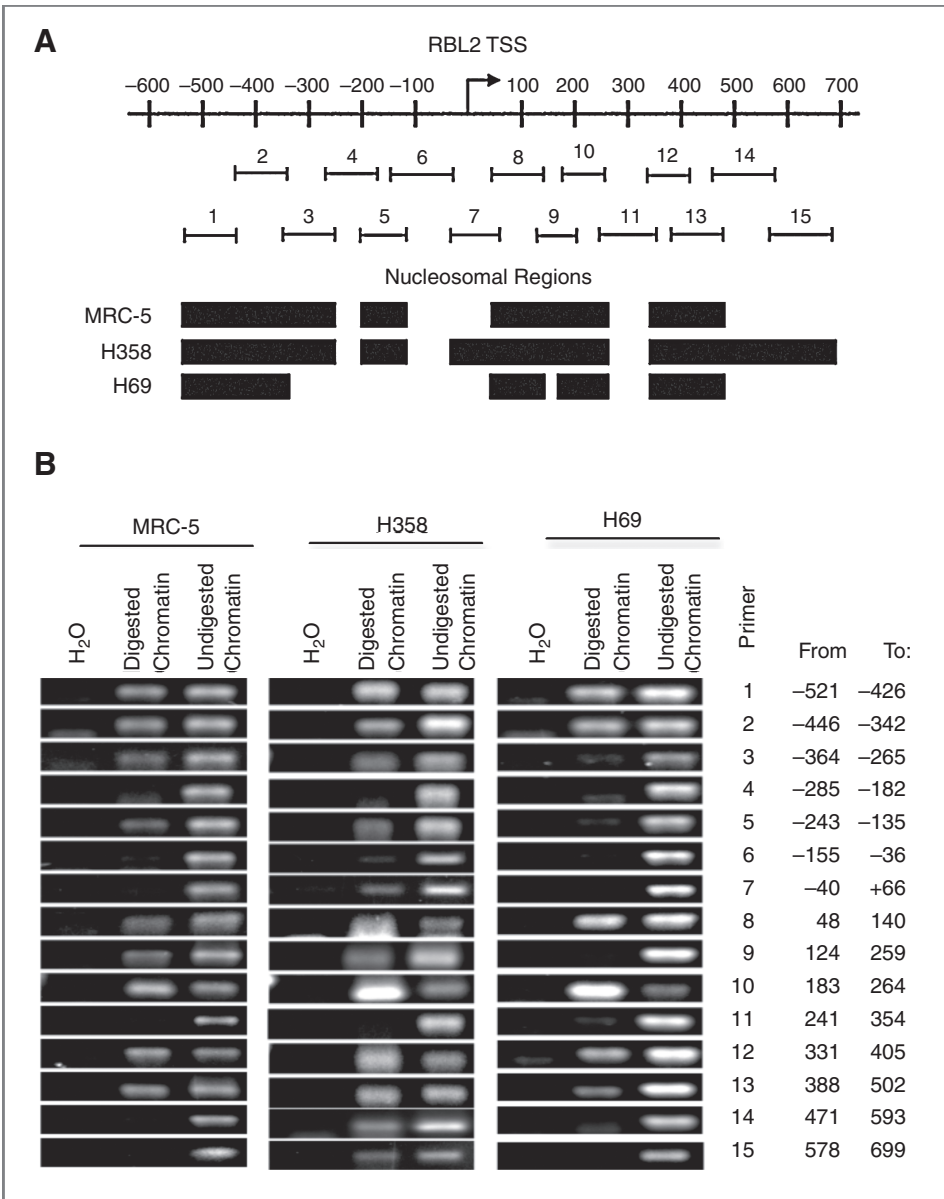
M in PBS to each of the plates for 5′. Cellular pellets were resuspended in 1 mL of lysis buffer supplemented with PMSF and PIC (Active motif) and incubated for 30′ at 4°C in constant agitation. The lysate was centrifuged at 2,400 × *g* for 10′ at 4°C and the pellet obtained was resuspended again in 1 mL of lysis buffer. The lysate was transferred into an ice-cold homogenizer and 10 strokes of tight pestle were applied. The lysate was centrifuged again at 2,400 × *g* for 10′ at 4°C to obtain pelleted nuclei. Nuclei were resuspended and incubated for 20′ in ice in 96 μL Microccocal Nuclease (MNase) buffer (Tris-Cl pH 7.5 10.4 mM, NaCl 10.4 mM, MgCl$_2$ 3.01 mM, CaCl$_2$ 1.04 mM, NP-40 4.02%) supplemented with PMSF and PIC. After an initial incubation for 5′ at 37°C, 4 μL of MNase (2 U, 0.5 U/μL) were added and nuclei were

incubated for 10′ at 37°C. MNase reaction was stopped incubating each sample in ice after adding EDTA 10 mM. Protocol was continued following kit instructions for the elution of sheared chromatin. The mononucleosomal DNA fraction was gel extracted using the Gel band extraction kit (QIAGEN), and 10 ng was used as a template in each PCR. Undigested genomic DNA was used as the positive control. No template was added to the PCR negative control. In order to deal with the different amplification efficiencies of the primer sets, each was extensively and independently characterized to determine appropriate cycle numbers. Primer pairs were designed to amplify areas of 80 to 120 bp on the *Rb2/p130* promoter. Each PCR product overlapped the neighboring amplified regions by approximately 20–40 bp. The PCR products were numbered, and the regions

Table 1. Amplified PCR product

Region amplified	Forward primer	Reverse primer	PCR Magnesium concentration	PCR Annealing temperature	Amplicon length (bp)
1	ggaaaggactgttacaatctttg	tgcccttgttcattcctggg	1.8 mM	52°C	95
2	cccaggaatgaacaagggca	gcctttgcaaaaatcctaacagt	1.8 mM	54°C	105
3	actgttaggattttgcaaaggc	ttcctcatcgcctggatatc	3 mM	56°C	99
4	gatatccaggcgatgaggaa	caacagggctgtactgacga	3 mM	56°C	104
5	ggatacgaaaccttcacacgtc	accttcccgcattttaaac	3 mM	52°C	108
6	gtttaaaatgcggggaaggt	ggdgctgggaaacgtagt	1.8 mM	56°C	120
7	cagcccgtgctcaagactac	ccattcaaacggcgaagc	3 mM	52°C	106
8	gcttcgccgtttgaatgg	actggtcacctcccgacg	3 mM	55°C	93
9	cgtcgggaggtgaccagt	tgctggatctgaggggtg	1.8 mM	55°C	136
10	atgaggaggaggaggacgac	aaccgctgctggattctgagg	1.8 mM	56°C	81
11	cacccctcagatccagca	agcgtgtagctttcgctcat	3 mM	55°C	115
12	cagcatgagcgaaagctacac	ggttcacgccaactaggc	3 mM	52°C	75
13	gcctagttggcgtgaacc	ggaatagcgcagaggaagtc	1.8 mM	52°C	114
14	gagaggggtggggacttcct	gtgctactttagggcaagacg	3 mM	57°C	123
15	tgccctaaagtagcacagcaa	ttgacaacgacactagttcattc	3 mM	55°C	121

amplified were as follows: 1, −521 to −426; 2, −446 to −342; 3, −364 to −265; 4, −285 to −182; 5, −243 to −135; 6, −155 to −36; 7, −40 to +66; 8, +48 to +140; 9, +124 to +259; 10, +183 to +264; 11, +241 to +354; 12, +331 to +405; 13, +388 to +502; 14, +471 to +593; 15, +578 to +699 (Fig. 5).

We observed interesting variations in the positioning of nucleosomes on *Rb2/p130* promoter and 5′-coding regions between H69 (SCLC) and H358 (NSCLC) lung cancer cell lines, as well as between H69 and H358 cells and MRC-5 lung fibroblasts, which greatly supports our idea that the binding of CTCF and/or BORIS to the *Rb2/p130* gene leads to changes in the local chromatin organization affecting *Rb2/p130* transcriptional activity. Nucleosome-free regions were centered at about position 4, 6, 7, 11, 14 and 15 in MRC5 lung fibroblasts, at about position 4, 6 and 11 in H358 (NSCLC) cells, and at about position 3, 4, 5, 6, 7, 11, 14 and 15 in H69 (SCLC) cells (Fig. 5).

Currently, we are further investigating the phenomena observed, and also determining the relative levels of histone H3 and H4 acetylation on the single nucleosomes identified.

Discussion

Until now, the contribution of Rb2/p130-retinoblastoma related protein to lung tumorigenesis remains controversial and under debate. However, our previous studies regarding *Rb2/p130* in lung cancer have suggested that *Rb2/p130* epigenetic modifications, and/or post-translation modification, rather than *Rb2/p130* genetic alterations, might contribute to impairing Rb2/p130 activity during the neoplastic process.

Here, we report a novel finding on *Rb2/p130* gene regulation in lung cancer by disclosing the mechanisms and the "players" that directly control *Rb2/p130* gene expression in normal lung cells, and by characterizing the events responsible for the deregulated expression of *Rb2/p130* gene in NSCL and SCLC cells. Specifically, our studies indicate that the chromatin insulator CCCTC-binding factor (CTCF) binds to the *Rb2/p130* gene and controls its expression in normal lung fibroblasts. However, during the neoplastic process defects occurring in the mechanisms by which CTCF controls *Rb2/p130* gene expression seem to trigger the binding of BORIS (CTCF-paralogue) to the *Rb2/p130* gene in nonsmall (NSCLC) and small (SCLC) lung cancer cells. It is our idea that the binding of BORIS to *Rb2/p130* affects the transcription of this gene by altering the chromatin asset established by CTCF through mechanisms that involve changes in nucleosome positioning.

Importantly, the activities of CTCF and BORIS in controlling *Rb2/p130* gene expression could specifically rely on specific alterations in p16INK4a/CyCD1/RB1, p14^ARF/mdm2/p53, and PI3K/AKT signaling, as well as on the presence of alterations in the other Rb family members, in nonsmall and small cell lung cancer, which in turn may critically influence Rb2/p130 functions in suppressing epithelial cell growth.

These studies provide essential basic insights into the mechanisms that control *Rb2/p130* gene expression in lung cancer, offering a potential paradigm for the actions of other activators and/or corepressors, such as CTCF and BORIS, that could be crucial in explaining how alterations in the mechanisms regulating *Rb2/p130* gene expression may accelerate the progression of lung tumor, or favor the onset of recurrences after cancer treatment, perhaps by affecting the apoptosis and senescence signaling that are Rb2/p130-dependent.

The characterization of the mechanisms regulating *Rb2/p130* gene expression, and the identification of the

alterations that affect this regulation (as well as the biological effect) in lung cancer offer the opportunity to disclose the distinct role played by Rb2/p130 in executing its onco-suppressive functions when anti-proliferative Rb1- and p53- mediated signaling is impaired. Insights into the molecular pathways that are strictly correlated to Rb2/p130 expression and constitutively active in NSCLC and SCLC cells may provide new biomarkers for an early diagnosis of lung cancer and/or predictive factors to determine the effect on tumor treatment, as well as may lead to the development of novel therapies based upon clinical modulation of Rb2/p130, CTCF and/or BORIS expression. We are currently investigating the "molecular signatures" underlying the NSCLC and SCLC phenotype through an integrated survey of the changes in gene expression profiles, Rb2/p130-dependent and-independent, that occur in NSCLC and SCLC during the evolution of this disease, with the goal of understanding the relationship between these changes and the proliferative and metastatic potential of lung cancer cells.

Disclosures of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Correction: CTCF and BORIS Regulate *Rb2/p130* Gene Transcription: A Novel Mechanism and a New Paradigm for Understanding the Biology of Lung Cancer

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