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CBX7 is a tumor suppressor in mice and humans

Floriana Forzati,1 Antonella Federico,1,2 Pierlorenzo Pallante,1,2 Adele Abbate,1 Francesco Esposito,1 Umberto Malapelle,2,3 Romina Sepe,1 Giuseppe Palma,1,4 Giancarlo Troncone,2,3 Marzia Scarfo,5 Claudio Arra,4 Monica Fedele,1 and Alfredo Fusco1,2

1Istituto di Endocrinologia ed Oncologia Sperimentale del CNR, Dipartimento di Biologia e Patologia Cellulare e Molecolare, Facoltà di Medicina e Chirurgia, Università di Napoli “Federico II,” Naples, Italy. 2CEINGE-Centro Biotecnologie Avanzate, Naples, Italy. 3Dipartimento di Anatomia Patologica e Citopatologia, Facoltà di Medicina e Chirurgia, Università di Napoli “Federico II,” Naples, Italy. 4Istituto Nazionale dei Tumori, Fondazione Pascale, Naples, Italy. 5IRGS-Biogem, Ariano Irpino, Italy.

The CBX7 gene encodes a polycomb group protein that is known to be downregulated in many types of human cancers, although the role of this protein in carcinogenesis remains unclear. To shed light on this issue, we generated mice null for Cbx7. Mouse embryonic fibroblasts derived from these mice had a higher growth rate and reduced susceptibility to senescence compared with their WT counterparts. This was associated with upregulated expression of multiple cell cycle components, including cyclin E, which is known to play a key role in lung carcinogenesis in humans. Adult Cbx7-KO mice developed liver and lung adenomas and carcinomas. In vivo and in vitro experiments, we demonstrated that CBX7 bound to the CCNE1 promoter in a complex that included HDAC2 and negatively regulated CCNE1 expression. Finally, we found that the lack of CBX7 protein expression in human lung carcinomas correlated with CCNE1 overexpression. These data suggest that CBX7 is a tumor suppressor and that its loss plays a key role in the pathogenesis of cancer.

Introduction
CBX7 is a chromobox family protein and a member of the polycomb repressive complex 1 (PRC1) that, together with PRC2, maintains developmental regulatory genes in a silenced state (1–3). Mouse Cbx7 associates with facultative heterochromatin and with the inactive X chromosome, thereby implicating the CBX7 protein in the developmental regulatory genes in a silenced state (1–3). Mouse Cbx7 associates with facultative heterochromatin and with the inactive X chromosome, thereby implicating the CBX7 protein in the development of mouse embryos (4). It is noteworthy that, in seeming contrast with our previous data, CBX7 has been described as an oncogene (13). Indeed, it cooperates with c-Myc to produce highly aggressive B cell lymphomas and can initiate T cell lymphomagenesis (3). Moreover, CBX7 extends the lifespan of a wide range of normal human cells and immortalizes mouse fibroblasts by downregulating expression of the Ink4a (also known as Arf) locus (13, 14), and its ablation, by shRNA treatment, inhibits growth of normal cells through induction of the Ink4a locus (14). Therefore, in an attempt to better understand the role of CBX7 in the regulation of cell growth and its potential involvement in carcinogenesis, as well as to define its physiologic function in vivo, we examined the consequences of disrupting the Cbx7 gene in mice.

Here, we report that both heterozygous and homozygous Cbx7-KO mice (Cbx7+/− and Cbx7−/−, respectively) showed increased susceptibility to developing benign and malignant liver and lung neoplasias. Consistently, mouse embryonic fibroblasts (MEFs) derived from the Cbx7-KO mice had a higher growth rate and delayed senescence compared with their WT counterparts. The ability of CBX7 to counteract activation of CCNE1 expression by the HMG proteins probably accounts for some phenotypic features of the Cbx7-KO mice. We also found an inverse correlation between CBX7 and cyclin E expression in human lung carcinomas, which indicates that the loss of CBX7 gene expression plays a relevant role in lung carcinogenesis.

Results
Generation of Cbx7-KO mice. We used gene targeting techniques in ES cells to generate a null mutation at the murine Cbx7 genomic locus. The targeting vector was designed to replace exons 5 and 6 of the mouse Cbx7 gene with a neomycin cassette (Figure 1A). Exons 5 and 6 code for the C-terminal region of the protein, including the Pc-box domain required for its functions. Progeny of chimeric animals were identified by Southern blot analysis of EcoRI-digested tail DNA (Figure 1B), and matings were established to produce mice heterozygous or homozygous for

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to the potential transcript of exons 1–4 of the Cbx7 gene that are not disrupted by the target-<ref>Figure 1</ref>ing construct. Moreover, Western blot performed using antibodies able to recognize the 5′ region of the CBX7 protein did not show the expression product of the potential truncated transcript (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI58620DS1). It is likely that this short messenger is degraded and does not code for any protein.

Heterozygote matings yielded Cbx7+/+, Cbx7+/−, and Cbx7−/− offspring at roughly the expected Mendelian ratio, indicative of no marked embryonic lethality. Interestingly, Cbx7−/− mice showed a significant increase in naso-anal body length: 70% of females and 46% of males showed average increases of 6.5% (P = 0.041) and 16% (P = 0.044), respectively, compared with their WT counterparts (Figure 1, D and E).

Cbx7−/− MEFs grow faster and senesce later than do their Cbx7+/+ counterparts. To investigate the role of CBX7 in cellular proliferation, we analyzed the growth rate and cell cycle distribution of MEFs from Cbx7+/+, Cbx7+/−, and Cbx7−/− embryos at 12.5 dpc. As shown in Figure 2A, growth rate was significantly higher in Cbx7−/− MEFs than in Cbx7+/+ controls, and intermediate in Cbx7+/− MEFs. To assess whether the higher growth rate of Cbx7−/− MEFs was caused by deranged progression through the phases of the cell cycle, we examined asynchronously growing MEFs by flow cytometry. The number of Cbx7+/− MEFs was lower in G1 and higher in S phase of the cell cycle compared with Cbx7+/+ MEFs (Figure 2B). Consistently, the Cbx7+/− MEFs transiently transfected with a Myc-Hys–tagged Cbx7 expression vector showed a decreased number of cells in the S phase and an increased cell population in the G1 phase of the cell cycle compared with backbone vector–transfected Cbx7+/− MEFs (Figure 2C).
We next examined the susceptibility to senescence of the MEFs at different culture passages by measuring senescence-associated β-gal (SA-β-gal) activity. Starting from culture passage 7, SA-β-gal activity was present in WT MEFs, as evidenced by the presence of green cells (Figure 2G). Conversely, SA−β-gal activity was absent from most of the Cbx7-KO counterparts (Figure 2, H and I). These findings suggest a role of CBX7 in the induction of cellular senescence. Senescent MEFs express elevated levels of p21 and p16 consequent to replication and culture stress (15). These 2 genes may cooperate to inhibit Rb phosphorylation and maintain growth arrest in an irreversible state. Therefore, we evaluated mRNA and protein levels of p16, p53, and p21 in WT and Cbx7-KO MEFs by quantitative RT-PCR (qRT-PCR) and Western blot analysis. The mRNA and protein expression levels of p16 (starting from passage 4) and of p53 and p21 (at passage 7) were significantly lower in Cbx7-KO than in WT MEFs (Figure 2K). Consistently, the levels of S15-phosphorylated p53 paralleled those of p21, and both cyclin A and cyclin E were upregulated in Cbx7−/− versus Cbx7+/+ MEFs (Figure 2K). Moreover, Cbx7−/− MEFs in which Cbx7 expression had been restored — but not those transfected with empty vector — showed Cdkn2a and tp53 transcript levels comparable to those observed in WT MEFs, and significantly increased Cdkn1a levels compared with WT (Figure 2J).

Interestingly, MEFs isolated from 2 transgenic mouse lines overexpressing Cbx7 under the transcriptional control of a cytomegalovirus promoter (Supplemental Figure 2A) showed a behavior opposite to that of Cbx7-KO MEFs: a lower proliferation rate compared with WT (Figure 2K).
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(Supplemental Figure 2B) that was Cbx7 dose dependent, with a reduced cell population in the S phase of the cell cycle (data not shown). Consistently, decreased Ccn1, Ccn2, and Ccnb1 expression was observed in MEFs of both transgenic mouse lines compared with the WT MEFs (Supplemental Figure 2A).

CBX7 binds and negatively regulates the CCNE1 promoter. In an attempt to understand the mechanism by which CBX7 negatively regulates the G1/S transition, we focused on the CCNE1 gene for various reasons: (a) it is crucial for the G1/S transition during the mammalian cell cycle (16); (b) its promoter contains an E-box that
CCNE1

Together, these results support the hypothesis that CBX7 negatively regulates CCNE1 expression and consequently modulate cell proliferation, thereby accounting for the increased cell growth rate of Cbx7-KO fibroblasts. First, we measured Ccn1 expression in MEFs and tissues (kidney and lung) of Cbx7+/−, Cbx7−/−, and Cbx7−/+ mice by qRT-PCR. As shown in Figure 3A, Ccn1 expression levels were higher in Cbx7−/− MEFs and tissues than in the WT controls. Western blot analysis yielded the same results (Figure 2K and data not shown). Interestingly, restoration of Cbx7 expression resulted in decreased Ccn1 expression levels in Cbx7−/− MEFs, comparable to those observed in WT MEFs (Figure 3A). Moreover, Ccn1 expression was reduced in MEFs derived from mice overexpressing Cbx7 in a manner dependent on Cbx7 level (Supplemental Figure 2A). Together, these results support the hypothesis that Cbx7 negatively regulates CCNE1.

We next evaluated binding of the CBX7 protein to the CCNE1 promoter in vitro by EMSA with an oligonucleotide spanning −51 to −12 bp upstream of the transcriptional starting site (TSS) of the human CCNE1 promoter region, including an E2F-responsive element and an HMGA-responsive AT-rich stretch. Increasing amounts (5 and 20 ng) of the recombinant glutathione-S-transferase–CBX7 (GST-CBX7) protein bound to the 32P-end-labeled double-strand oligonucleotide in a dose-dependent manner (Figure 3B, lanes 2 and 3). Binding specificity was demonstrated by competition experiments that showed loss of binding with the addition of a 400-fold molar excess of the specific unlabeled oligonucleotide (Figure 3B, lane 4). No binding was obtained when an oligonucleotide corresponding to a region of the GAPDH promoter was used as a negative control (Figure 3B, lanes 11 and 12).

To identify the region of CBX7 required for CCNE1 promoter binding, we performed the same experiment with 2 recombinant mutated forms of the CBX7 protein: one containing only the chromodomain (GST-CBX7-CHROMO) and another lacking the chromodomain (GST-CBX7-NO CHROMO). Only GST-CBX7-CHROMO specifically bound to the CCNE1 promoter (Figure 3B, lanes 5–10), which indicates that the CBX7 protein is able to bind the CCNE1 promoter in vitro through its chromodomain.

We next evaluated whether the CBX7 protein could bind to a human CCNE1 promoter region, including the E-box, by performing ChIP assays. HEK 293 cells were transiently transfected with HA-tagged CBX7 expression vector or empty vector. Cells were then crosslinked, and DNA-chromatin complexes were subjected to IP with anti-HA or IgG antibodies. The IP chromatin was subsequently analyzed by 32P-PCR, using primers spanning the region of the human CCNE1 promoter (−850 to −700 bp upstream of the TSS) covering an E-box region. As shown in Figure 3C, HA-tagged CBX7 showed co-IP with the CCNE1 promoter in cells transfected with CBX7. Moreover, CBX7 also bound to CDH1 and CDKN2A, which have previously been identified as promoters (12, 20). Conversely, no amplification was observed with anti-IgG precipitates and when primers for the GAPDH promoter were used, indicative of the specificity of CBX7 binding to the CCNE1 promoter.

The same results were obtained when we performed ChIP assays on Cbx7+/− and Cbx7−/− MEFs (Figure 3D) and lung tissues (Supplemental Figure 3), which also indicated that the endogenous CBX7 protein was able to bind the CCNE1 promoter. Furthermore, the binding of Hdac2 to the Ccn1 promoter was reduced in Cbx7−/− compared with Cbx7+/− cells (Figure 3E), which indicates that the presence of Cbx7 favors Hdac2 binding to the Ccn1 promoter. Finally, to assess the functional consequences of CBX7 binding to the CCNE1 promoter, we demonstrated that CBX7 dose-dependently reduced the transcriptional activity of the CCNE1 promoter (Figure 3F).

HMGA1 displaces the HDAC2/CBX7 complex from the CCNE1 promoter. We previously demonstrated that the expression of HMGA proteins increases E2F activity by displacing HDACs from E2F-responsive promoters, including CCNE1 (21). Therefore, we hypothesized that CBX7 influences CCNE1 expression by interacting with HMGA1 and then antagonizing its activ-
To test this hypothesis, we transiently transfected HEK 293 cells with CBX7 and HMGA1b expression constructs, subjected protein extracts to IP with anti-CBX7 or anti-HMGA1 antibodies, and immunoblotted them with the reciprocal antibodies. As shown in Figure 4A, coexpression of CBX7 and HMGA1b resulted in reciprocal co-IP of the 2 proteins. Co-IP was performed in the presence of ethidium bromide to exclude that the co-IP of CBX7 and HMGA1b is dependent on contaminating DNA. The same result was obtained when co-IP was performed with the endogenous proteins extracted from WT mouse embryos (Figure 4B).
Conversely, no interaction was detected when nonspecific IgGs were used (Figure 4, A and B). To verify this interaction, we carried out a pulldown assay using a GST-CBX7 recombinant protein; as shown in Figure 4C, GST-CBX7, but not GST, was able to pull down HMGA1b. Then, because of the ability of HMGA1b to interact with CBX7 (Figure 4) and of CBX7 to interact with HDAC2 (12), we investigated whether the physical interactions between CBX7 and either HDAC2 or HMGA1b take place on the CCNE1 promoter. We performed ChIP and re-ChIP analysis on HEK 293 cells transiently transfected with HA-tagged CBX7, HMGA1b, or both expression vectors. Re-ChIP experiments were performed on exogenously expressed proteins because of the difficulty in obtaining good results when endogenous proteins are not highly expressed. The cells were crosslinked and subjected to IP with anti-HA antibodies recognizing the exogenous CBX7 protein. The CBX7 complexes were subjected to re-IP with anti-HMGA1, anti-HA, and anti-HDAC2 antibodies and then analyzed by qPCR for CCNE1 promoter amplification using the same primers as above. The results demonstrated that CBX7, HMGA1b, and HDAC2 occupied the same region on the CCNE1 promoter (Figure 5A, top). Moreover, HMGA1b expression led to a reduction of the CBX7/HDAC2 complex levels present at this promoter. The reciprocal experiment, using anti-HDAC2 antibodies for the first ChIP, yielded comparable results (data not shown), whereas the ChIP for HMGA1 showed that CBX7 expression reduced the amount of HMGA1 protein bound to the CCNE1 promoter, suggestive of mutual competition between CBX7 and HMGA1 for the binding to this promoter. Interestingly, the amount of HDAC2 present in the CCNE1 promoter–bound HMGA1b complex increased from 17% to 40% in the presence of CBX7 (Figure 5B). Taken together, these results indicate that CBX7 binds the CCNE1 promoter and participates in the DNA-bound multimeric complex containing HDAC2 and HMGA1. These data also suggest that CBX7 recruits HDAC2 on the CCNE1 promoter, whereas HMGA1 displaces the CBX7/HDAC2 complex from this promoter.

HMGA1 competes with CBX7 for binding and regulation of the CCNE1 promoter. To investigate possible functional interactions between HMGA1b and CBX7 on the CCNE1 promoter, we first analyzed the in vitro binding of both proteins to this promoter by EMSA, using the GST-CBX7 protein with increasing amounts of a histidine-tagged HMGA1b (HIS-HMGA1b) recombinant protein. Both proteins specifically bound to the oligonucleotide used as probe (Figure 5C, lanes 1–5). Importantly, the GST-CBX7/DNA complex was dose-dependently inhibited by HIS-HMGA1b (Figure 5C, lanes 6 and 7), which confirmed the competition of these proteins for binding to the CCNE1 promoter. Consistently, analysis of CCNE1 promoter activity in the presence of CBX7 and increasing amounts of HMGA1b demonstrated that HMGA1b counteracted the negative activity of CBX7 on the CCNE1 promoter (Figure 5D), thereby causing a switch from repression to activation.
CBX7 is drastically downregulated in human lung carcinomas. Because cyclin E overexpression plays a critical role in lung carcinogenesis (19, 27), we focused our attention on human lung tumors. We first evaluated CBX7 protein expression in a panel of human lung adenocarcinomas by immunohistochemistry. As shown in Table 2, CBX7 was not expressed in any of the lung carcinomas analyzed, whereas it was expressed in normal lung tissue. Moreover, analysis of CBX7- and CCNE1-specific mRNA expression by qRT-PCR revealed an inverse correlation between CBX7 and CCNE1 expression in lung carcinomas (Supplemental Figure 5).

Loss of heterozygosity (LOH) analysis at the CBX7 locus revealed LOH in 50% of the informative carcinomas (Table 2). Interestingly, when the morphologically normal lung area adjacent to the tumor was analyzed for CBX7 expression, no staining occurred in 4 of 8 cases analyzed (Figure 7). In other cases, staining was weaker compared with the normal lung (data not shown). Moreover, the 4 samples negative for CBX7 expression showed LOH4 when we analyzed the DNA extracted from the same microdissected areas (Table 2), which suggests that the reduced CBX7 expression might be involved in the transition of lung cells to a completely transformed malignant stage.

Since, as shown above, CBX7 negatively regulates CCNE1 promoter activity, we also evaluated cyclin E expression in the same lung neoplastic samples. In line with previous data (19, 27), cyclin E was overexpressed in all cases (Table 2 and Figure 7). Interestingly, cyclin E was overexpressed in the areas adjacent to the lung carcinoma only when CBX7 expression was absent and LOH was detected at the CBX7 locus (Table 2 and Figure 7).

To verify that CBX7 is a negative regulator of CCNE1 expression in lung cells, we restored CBX7 expression in 2 lung carcinoma cell lines (A549 and H1299) that did not show any expression of CBX7 (Supplemental Figure 6A). Restoration of CBX7 expression resulted in a significant decrease in CCNE1 expression, verified by qRT-PCR (Supplemental Figure 6B). Moreover, to further confirm that CBX7 downregulation could be a causative event in lung cell proliferation, we performed a colony-forming assay on the same lung carcinoma cell lines. As shown in Supplemental Figure 6C, the cells transfected with CBX7 expression vector gave rise to a lower number of colonies compared with cells transfected with empty vector.

Discussion

Polycomb group proteins function as multiprotein complexes and are part of a gene regulatory mechanism that determines cell fate during normal and pathogenic development. They form transcriptional repressor modules that can be functionally divided into at
physically associate with DNA methyltransferases (DNMTs) (32, 33) suggests a mechanism whereby PcG proteins directly contribute to the altered DNA methylation profiles that are observed in tumor cell lines (10, 11). The fact that EZH2 and CBX7 can physically associate with DNA methyltransferases (DNMTs) (32, 33) suggests a mechanism whereby PcG proteins directly contribute to the altered DNA methylation profiles that are observed in multiple cancer types. Indeed, PcG target genes are as much as 12 times more likely to be aberrantly silenced by DNA methylation in cancer than are non-PcG target genes (34–36). However, the role of CBX7 in the regulation of cell growth and tumorigenesis is still controversial, since there is also evidence that its overexpression leads to cellular immortalization in vitro and tumor development in vivo, thereby indicating that CBX7 is an oncogene (3, 14). To address this issue, we generated and characterized Cbx7–/– mice.

The data reported here propose CBX7 as a tumor suppressor gene. Consistent with our earlier finding that restoration of CBX7 expression in thyroid carcinoma cells blocks the cells in G1 phase (6), the proliferation rate of Cbx7–/– MEFs was higher than that of WT MEFs, and Cbx7+/– mice developed neoplasia. However, in contrast to a report that CBX7 extends cellular lifespan (14), Cbx7–/– MEFs went to senescence later than did WT MEFs. This suggests that the cellular context plays a critical role in the effect of the CBX7 protein on cell growth. In the case of Cbx7–/– MEFs, the context may be considered more physiological than experiments in which CBX7 was ectopically overexpressed in HprECs and WI38 cells (14). It is possible that CBX7 overexpression deranges the composition of the PRC complexes, thereby causing unbalanced cell DNA methylation. Moreover, the same authors report reduced growth of both MEFs and human lung fibroblasts (WI38) when CBX7 expression is silenced (14). The different experimental approach, ours in vivo and the previous one in vitro, may account for these contradictory results. Consistent with this hypothesis, it has been previously shown that in vivo data show significant discrepancies compared with in vitro data and transfection approaches when p53 pathway has been studied (37). Perhaps it is difficult to faithfully reproduce the relative ratios of CBX7 and its regulators and/or partners using transfection protocols. We also cannot exclude the hypothesis that the shRNA constructs used in the previous studies may have interfered with the expression of other genes, maybe of the Pc family. The behavior of MEFs derived from Cbx7–/– mice and Cbx7–/– mice described herein is supported by the drastic downregulation of CBX7 expression previously reported by several groups in malignant neoplasias — including thyroid (6), pancreatic (8), colon (11), lung (present study), gastric (10), bladder (7), and breast (9) carcinomas — and by a reduced growth rate achieved by restoration of CBX7 expression in carcinoma cells of different origin (6, 9, 11, 38).

The inhibition of Ccne1 expression by Cbx7 seems to have a critical role in the phenotype of the Cbx7–/– mice. Indeed, Ccne1 was expressed at higher levels in Cbx7–/– mice than in the WT controls, and Cbx7 binds to the CCNE1 promoter in a region that includes the E-box, thereby repressing its activity. The HMGAlb protein was a competitor of this action. The antagonistic effect of CBX7 versus HMGAlb protein on the CCNE1 promoter, and perhaps other CBX7 target genes, probably accounts for most of the phenotypic features of the Cbx7–/– mice. Interestingly, HMGAl and CBX7 act in an opposite manner in human malignancies: HMGAl overexpression and CBX7 downregulation correlate with a poor prognosis (6, 8, 11, 39, 40).

We also report that benign liver and lung adenomas were moderately frequent in Cbx7–/– mice, whereas liver and lung carcinomas appeared in Cbx7–/– mice, which suggests that the grade of malig-

Figure 7
Immunohistochemical analysis of CBX7 and cyclin E expression in human normal and neoplastic lung tissues. (A and B) Normal lung had intense immunoreactivity for CBX7 (A, arrows), whereas it was negative for cyclin E expression (B). Arrowhead in B denotes positive histiocytes, a positive internal control. (C and D) Lung carcinoma was negative for CBX7 staining (C), but strongly positive for cyclin E expression (D). Higher-magnification views of A–D are provided in Supplemental Figure 7. (E and F) In a morphologically normal lung carcinoma–adjacent tissue, the tumoral part (top left) and the adjacent tissue (bottom right) did not express CBX7 (E), whereas both expressed high levels of cyclin E (F). Original magnification: ×63 (A–D); ×10 (E and F).
nancy depends on the number of Cbx7 functional alleles. To further probe the susceptibility of Cbx7-KO mice to neoplasias and evaluate the role of Cbx7 expression in cancer progression, we plan to cross these animals with transgenic mice carrying activated oncogenes and/or induce experimental neoplasias in Cbx7-KO mice.

Interestingly, all the human lung adenocarcinomas we analyzed showed the absence of CBX7 expression, and LOH at the CBX7 locus was frequent in these neoplasias. Interestingly, LOH at the CBX7 locus also occurred in the morphologically normal tissue adjacent to lung tumor, in which CBX7 expression was significantly lower than in the normal lung tissues and cyclin E was overexpressed. These results suggest that the reduced CBX7 expression might facilitate the complete malignant lung cell transformation. Cyclin E overexpression in lung cells is probably the mechanism whereby loss of CBX7 expression contributes to lung carcinogenesis. Indeed, aberrant cyclin E expression is frequent in pulmonary dysplasia and lung cancer (19), and transgenic mice overexpressing cyclin E develop dysplasia and multiple pulmonary adenocarcinomas (41). However, in order to unequivocally demonstrate that cyclin E overexpression accounts for the phenotype of the Cbx7-KO mice, further experiments are required, specifically the generation of mice carrying the disruption of both Cbx7 and CcnE1 genes; the lack of development of lung carcinomas in mice with both alleles being deleted would validate our hypothesis.

In conclusion, our analysis of the phenotype of Cbx7-KO mice supports the concept that CBX7 is a tumor suppressor gene, in seeming contrast with other published reports that CBX7 behaves as an oncogene. It is conceivable that CBX7 can exert both oncogenic and antioncogenic functions, depending on the nature of other cellular events and on the presence of interacting proteins. Moreover, beyond defining a tumor suppressor role of the CBX7 gene, the data reported here indicate a critical role of the loss of CBX7 expression in lung carcinogenesis as a likely precursor to CCNE1 overexpression.

**Methods**

**Generation and genotyping of mutant mice.** For generation of KO mice, the Cbx7 gene targeting vector was designed to delete the fifth and sixth codon of the mouse Cbx7 gene. To clone the mouse Cbx7 genomic locus, a λXII phage library of a 129SvJ mouse strain (Stratagene) was screened. The targeting vector was constructed by subcloning, in the Bluescript plasmid (Stratagene), the 5′-flanking region (BamHI-DruI 2.6 kb fragment), the neo cassette for positive selection, the 3′-flanking region (ClaI-BamHI 2.3 kb fragment) and a TK gene cassette for negative selection. The targeting vector was linearized with NheI before electroporation into ES cells. 2 G418/ ganciclovir-resistant ES clones were injected into C57BL/6J blastocysts, and grafted into C57BL/6J Blastocyst. Each reaction was performed in duplicate. We used the ZΔCT method to calculate the relative expression levels (43). Details of primer sequences are provided in Supplemental Methods.

**Growth and cell cycle analysis of MEFs.** Primary MEFs were obtained from 12.5-day-old embryos. The MEFs were mined and used to establish single cell suspensions. They were grown in DMEM (Gibco; Invitrogen) containing 10% fetal bovine serum (HyClone), 1% glutamine (Gibco; Invitrogen), 1% penicillin/streptomycin, and 1% gentamycin (Gibco; Invitrogen). The cells (4 × 10^5 cells/dish) were plated in a series of 6-cm culture dishes and counted daily with a hemocytometer for 13 consecutive days to extrapolate growth curves. Cell cycle analysis was performed as previously described (6) using a FACS Calibur cytofluorimeter (BD). For cell cycle analysis, MEFs in logarithmic growth were trypsinized, fixed in 70% ethanol, and stored at 4°C for a few days. Cells were then washed with PBS without Ca^{2+} and Mg^{2+}, stained with 50 μg/ml propidium iodide containing RNaseA (20 μg/ml), and analyzed by FACS. Cell debris and fixation artifacts were gated out, and G1, S, and G2/M populations were quantified using CellQuest software (BD). A similar number of events was analyzed in each experiment.

**SA-β-gal assay.** 4 × 10^4 MEFs, plated 24 hours before the assay, were washed twice with PBS and immersed in fixation buffer (2% [w/v] formaldehyde, 0.2% [w/v] glutaraldehyde in PBS) for 7 minutes. After 3 additional PBS washes, the cells were allowed to stain overnight in staining solution (40 mM citric acid/sodium phosphate, pH 6.0; 150 mM NaCl; 2.0 mM MgCl2; 1 mg/ml X-gal) at 37°C without CO2 to avoid changes in pH. The next day, the staining solution was replaced with PBS, and the stained and unstained cells were counted by light microscopy (at least 24 fields).

**Plasmids, transfections, and luciferase activity assays.** Expression vectors encoding for CBX7 (12), HMGAI (44), or the same amount of the empty vectors were transfected into HEK 293 cells growing in DMEM containing 10% PBS (Gibco; Invitrogen), 1% glutamine (Gibco; Invitrogen) and 1% penicillin/streptomycin (Gibco; Invitrogen), by calcium phosphate precipitation (45). Reporter construct (0.2 μg) was pCycE-luc (donated by K. Helin, BRIC BioTech Research and Innovation Centre Copenhagen Biocenter, Copenhagen, Denmark). CMV-β-gal expression vector was used to normalize transfection efficiency according to galactosidase activity. Cells were harvested 36 hours after transfection, and lysates were analyzed for luciferase activities using the Dual Light Kit (Tropix) and a luminometer (Lumat LB9507; Berthold). All assays were performed in duplicate in 3 independent experiments.

Cbx7-restored cell lines were obtained by reintroducing a Myc-Hys-tagged murine Cbx7 in the Cbx7−/− MEFs using Neon Electroporation System (Invitrogen) according to the manufacturer’s instructions.
EMSA. Protein/DNA binding was determined by EMSA as previously described (46). 5–20 ng of WT and mutant CBX7 recombinant protein (12) was incubated with radiolabeled oligonucleotide (specific activity, 8,000–20,000 cpm/nmol). The double-strand oligonucleotide used encompassed a region spanning base 51 to 12 of the human CCNE1 promoter (5′-CCGTTTCCGGCCGGAGGGTTTTAAGTTCCCGCCTCTGAG-3′). A radiolabeled oligonucleotide spanning –23 to +4 of the human GAPDH gene was used as a negative control.

ChIP and re-ChIP assays. Chromatin samples, derived from cells and tissue, were processed for ChIP and re-ChIP experiments as reported elsewhere (12). Samples were subjected to IP with the following specific antibodies: anti-HA (sc-805; Santa Cruz), anti-V5 against the C-terminus of the human CBX7 protein (Neosystem), anti-GST (sc-126; Santa Cruz), anti–phospho-p53 (9284; Cell Signaling), anti–cyclin E (sc-481; Santa Cruz), anti-p53 (ab-54210; Abcam), anti–cyclin E (sc-481; Santa Cruz), anti-p53 (sc-397; Santa Cruz), anti-p19 anti-p16Ink4a and anti–p21 (sc-805; Santa Cruz), anti-p19 (Santa Cruz), against the beads carrying either GST or GST-HMGA1b for 2 hours in NETN buffer as nonspecific controls, and input DNA values were used to normalize the signals.

Total cell extracts from HEK 293 cells transfected or not with CBX7-V5 expression vectors were pre-incubated with the peptide against which the antibodies were raised (6) at 4°C. The protein complexes were washed 4 times in the same lysis buffer, and for each experiment, qPCR was performed in triplicate. Primer pairs specific for the Cbx7 locus and CBX7 ligase genes were used to analyze input DNA. Primer sequences are provided in Supplemental Methods.

Protein extraction, Western blotting, and co-IP. Protein extraction, Western blotting, and co-IP procedures were carried out as reported elsewhere (46). The antibodies used were as follows: anti-tubulin (sc-7649; Santa Cruz), anti-p21 (sc-379; Santa Cruz), anti-p19 anti-p16INK4a (ab-54210; Abcam), anti–cyclin E (sc-481; Santa Cruz), anti-p53 (sc-126; Santa Cruz), anti–phospho-p53 (9284; Cell Signaling), anti-cyclin A (sc-751; Santa Cruz), anti-CBX7 (a primary antibody raised against the C-terminus of the human CBX7 protein; Neosystem), anti-CBX7 (sc-70232; Santa Cruz), anti-HA (sc-805; Santa Cruz), anti-p53 (Sigma-Aldrich), and anti-HMGA1 (44).

GST pulldown assay. Bacterial expressed GST and GST-HMGA1b proteins (45) were bound to glutathione agarose and used for binding assays with total extracts from HEK 293 cells transfected or not with CBX7-V5 expression plasmid. Briefly, proteins in the extracts were allowed to associate with the beads carrying either GST or GST-HMGA1b for 2 hours in NETN buffer (12) at 4°C. The protein complexes were washed 4 times in the same buffer, disassociated by boiling in loading buffer, and electrophoresed on a 12% polyacrylamide-SDS gel. The proteins were transferred to nitrocellulose, and CBX7-V5 was visualized as described above.

Histological and immunohistochemical procedures. Immunohistochemical analysis of CBX7 and cyclin E of paraffin-embedded tissues was performed, as previously described (6), using polyclonal antibodies raised against the C-terminal region of human CBX7 protein and commercial antibodies versus cyclin E (Santa Cruz), respectively. The specificity of the antibodies used was ascertained as previously described (6); no staining was observed when normal thyroid gland and lung samples were stained with antibodies pre-incubated with the peptide against which the antibodies were raised (6) or without the primary antibodies (data not shown).

LOH analysis. LOH analysis at the Cbx7 locus on chromosome 22q13.1 was performed as previously described (6).

 Colony-forming assay. Lung carcinoma cells (A549 and H1299), growing in RPMI containing 10% FCS (Gibco; Invitrogen), 1% glutamine (Gibco; Invitrogen), and 1% penicillin/streptomycin (Gibco; Invitrogen), were plated at a density of 50,000 cells/cm² dishes, transfected with 5 μg CBX7-HA expression vector or with the backbone vector, and supplemented with geneticin (G418) 24 hours later. 2 weeks after the onset of drug selection, cells were fixed and stained with crystal violet (0.1% crystal violet in 20% methanol).

Statistics. We used 2-tailed Student’s t test for intergroup comparisons. A P value less than 0.05 was considered statistically significant.

Study approval. All animal procedures were reviewed and approved by the Institutional Ethical Committee at Centro Servizi Veterinari of the University of Naples “Federico II.” Paraffin-embedded human lung cancer samples, with paired normal adjacent alveoli, were obtained from the Department of Science Biomorofologie e Funzionali at the University of Naples “Federico II” under the guidelines of the IEOs review board. Informed consent was obtained from all patients for the scientific use of biological material.

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Address correspondence to: Alfredo Fusco, Istituto di Endocrinologia ed Oncologia Sperimentale del CNR, Via Pansini 5, 80131 Naples, Italy. Phone: 39.081.7463602; Fax: 39.081.2296674; E-mail: afusco@napoli.com or alfusco@unina.it.