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Research Report

# The oncoprotein LMO3 interacts with calcium- and integrin-binding protein CIB

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ABSTRACT

The protein LMO3 belongs to the LIM only (LMO) group of transcriptional regulators, which act as molecular adaptors for protein–protein interactions. However, little is known about its interactive proteins and functions. Evaluating LMO3 in a yeast two-hybrid screen, we identified the calcium- and integrin-binding protein CIB as an LMO3-binding protein, which binds via the second LIM domain (LIM2) of LMO3. Cotransfection of LMO3 and CIB resulted in a shift in LMO3 protein from the nucleus to the cytoplasm. In functional assays, LMO3 induced C8 astrocyte proliferation was suppressed by the overexpression of CIB. This study demonstrates one function for LMO3 in C8 cells and suggests that one role of the CIB/LMO3 complex is to inhibit cell proliferation.



## 1. Introduction

The nuclear LIM-domain-only proteins (LMOs), which are composed of only two tandem LIM domains, are a subfamily of LIM-containing proteins. The LIM domain is characterized by a double zinc finger structure. Unlike many other zinc finger structures, the primary function of the LIM domain is to mediate the interaction of DNA with other proteins but not to bind to DNA (Retaux and Bachy, 2002). LMOs, which consist of four transcription regulatory factors LMO1–4, have roles in determining cell fate, controlling cell growth, and differentiation. There is also increasing evidence that LMOs are involved in transcriptional regulation by forming complexes with other protein factors and then altering the transcription of target genes.

LMO1 and LMO2 were originally identified by their translocation in acute T-cell leukemia, and their overexpression in transgenic mice leads to T-cell tumors. It has been established that LMO2 interacts directly with a number of proteins and has a critical function in early hematopoiesis and angiogenesis by participating in a number of multiprotein complexes (Yamada et al., 2002, 1998). Like LMO2, LMO4 is upregulated in breast cancer, and its overexpression in mice leads to hyperplasia and tumor formation (Visvader et al., 2001). LMO4 also forms a complex with the corepressor CtIP and the breast tumor suppressor BRCA1 in breast epithelial cells, and represses BRCA1-mediated transcriptional activation (Sum et al., 2002). LMO3 is the least understood member of the LMO family. It was first cloned on the basis of its sequence homology and was found to be highly expressed in the brain and some

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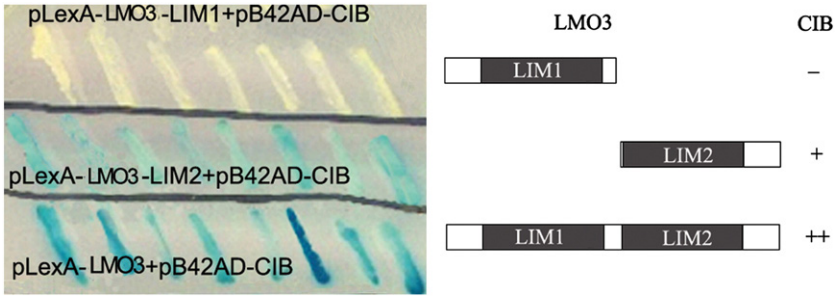


**Fig. 1 – Specific interaction between LMO3 and CIB. The full-length CIB cDNA fused in frame with the transcription activator B42 cDNA in the pB42AD vector was transformed into the EGY48 yeast strain and mating assays were performed, with YM4271 containing one of the following baits fused to the pLexA DNA-binding domain: LMO3 (full-length LMO3), LIM1 (the first LIM domain of LMO3), or LIM2 (the second LIM domain of LMO3). Transformants were tested for specific interaction on galactose X-gal plates. The blue colour indicates positive protein–protein interactions.**

vestibular ganglion cells (Boehm et al., 1991; Foroni et al., 1992; Hinks et al., 1997). Then the expression of LMO3 was found to be upregulated in astrocytes treated with dopamine (Shi et al., 2001). A recent study has shown that LMO3 is expressed at higher levels in unfavorable subsets of neuroblastoma and is a neuronal-specific oncogene in neuroblastoma cells (Aoyama et al., 2005). Despite the identification of a variety of binding proteins for LMOs, including LMO1, LMO2, and LMO4, no protein that binds LMO3 has been identified with yeast two-hybrid analysis. To investigate the function of LMO3, a yeast two-hybrid study was performed to identify interacting protein partners that may suggest the biological role of this protein. The calcium- and integrin-binding protein (CIB) was identified and the interacting domains were delineated.

## 2. Results

### 2.1. *Library screening identifies CIB as an LMO3-binding protein*

The *LIM* domain of LIM proteins is believed to function as a specific protein-binding interface. To identify proteins that interact with LMO3, a yeast two-hybrid assay was performed using the second LIM domain of LMO3 as bait. CIB was identified as a putative protein-binding candidate. To quantify the binding specificity of CIB to LMO3, we tested the interaction in a yeast mating assay. As shown in Fig. 1, the second LIM domain of LMO3, but not the first LIM domain (amino acids 1–73), associated with CIB in yeast. The full-length LMO3 coding region was also subcloned into the pLexA vector to produce the construct pLexA-LMO3. When this construct was tested in the yeast two-hybrid assay, full-length LMO3 was also shown to bind to CIB.

### 2.2. *Coimmunoprecipitation experiments confirm that LMO3 interacts with CIB in vivo*

A coimmunoprecipitation assay was used to investigate whether CIB and LMO3 interact *in vivo*. Plasmids encoding myc-CIB and His-LMO3 were cotransfected into C8 cells, and cell lysates were prepared after 48 h incubation. The lysates were subjected to immunoprecipitation using monoclonal antibody specific for a Myc or His epitope, and His-LMO3 and myc-CIB were detected in the immunocomplexes by western blot analysis with anti-His and anti-myc antibody, respectively. Western blot analysis showed that myc-CIB was present in the immunocomplex precipitated with anti-His antibody, and that His-LMO3 was present in the anti-Myc-precipitated immunocomplex (Fig. 2). These results suggest an association between the two proteins in C8 cells.

### 2.3. *Subcellular colocalization of LMO3 and CIB*

The physiological relevance of LMO3 and CIB was also assessed by colocalization assays in C8 cells and NIH3T3 cells. Immunofluorescence revealed that exogenously expressed His-LMO3 localized primarily in the nucleus of C8 cells (Fig. 3A), whereas intense cytoplasmic staining with some nuclear staining was observed in NIH3T3 cells (data not shown). Unlike LMO3, myc-CIB showed predominant cytoplasmic staining, with less staining in the nuclei of the C8 cell lines (Fig. 3B). After cotransfection of LMO3- and CIB-expressing constructs, LMO3 was restricted to the cytoplasm and CIB also localized to the cytoplasm (Fig. 3C). The

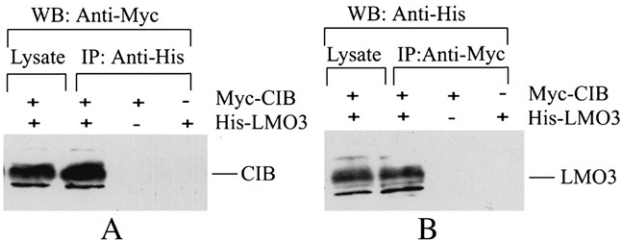


**Fig. 2 – Coimmunoprecipitation experiment confirmed that LMO3 and CIB interact *in vivo*. C8 cells were transfected with pCMV-myc-CIB and/or pcDNA4/HisA-LMO3. The expression of Myc-CIB and His-LMO3 in the cotransfected cells was analyzed by western blotting (A, left lane, and B, left lane). (A) Cell lysates were immunoprecipitated with anti-His antibody and immunoblotted with anti-Myc antibody. (B) Cell lysates were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-His antibody.**

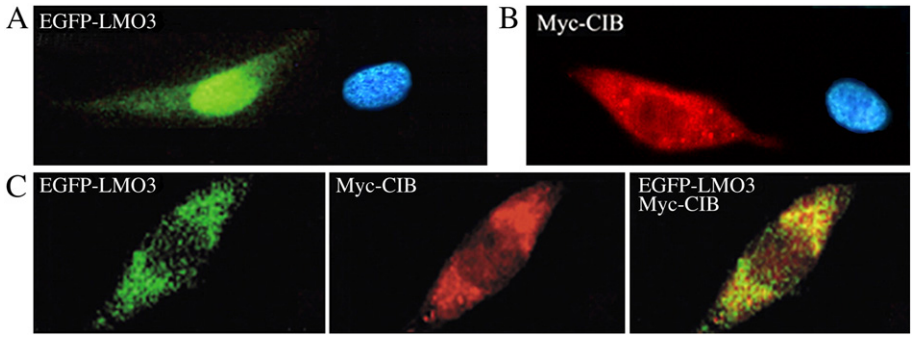


**Fig. 3 – Location of immunofluorescent LMO3 and CIB in transfected C8 cell. Optical sections of singly and doubly transfected C8 cell were analyzed with confocal microscopy or fluorescence microscopy for the expression of enhanced green fluorescent protein (EGFP)-LMO3 (green) or myc-tagged CIB detected with TRITC-coupled secondary antibody (red). (A) Singly transfected C8 cell expressing the EGFP-LMO3 fusion protein (left panel). Nucleus was labeled by hochest33258 (right panel). (B) Singly transfected C8 cell expressing the myc-tagged CIB fusion protein (left panel). Nucleus was labeled by hochest33258 (right panel). (C) Doubly transfected C8 cell expressing the EGFP-LMO3 fusion protein and the myc-tagged CIB fusion protein. The localization of the EGFP-LMO3 fusion protein is shown (left panel). The localization of the myc-tagged CIB fusion protein is shown (middle panel). Superimposition of the images was shown in the right panel.**

superimposition of the LMO3 and CIB immunostaining patterns indicated that their cytoplasmic distributions were largely identical (Fig. 3C). The shift in the localization of LMO3 when coexpressed with CIB also indicated that LMO3 and CIB interact *in vivo*.

### 2.4. *LMO3-induced cell proliferation is attenuated by CIB*

A recent study has shown that the overexpression of CIB and its interacting protein PS2 promotes cell death (Stabler et al., 1999). Because CIB is a binding partner of LMO3, its effect on cell proliferation was studied. Stably transfected sublines C8-LMO3 and C8-HisA were established, and MTT assays were performed. The overexpression of LMO3 in the C8 subline was confirmed by RT-PCR and western blotting (Fig. 4A, top panel). In the MTT assay, C8-LMO3 cells were shown to have a higher proliferation rate than C8-HisA cells (2nd–4th day: $p<0.05$; 5th day: $p\leq0.05$) (Fig. 4A, bottom panel), suggesting that the overexpression of LMO3 promoted cell growth. The effect of LMO3 on proliferation may be explained by its role in cell-cycle progression. In a flow-cytometric cell-cycle analysis, the overexpression of LMO3 increased the population of cells in S phase from 26.19% to 32.1% and reduced the population of cells in G0/G1 phase from 65.74.6% to 60.47% in C8-LMO3 cells compared with C8-HisA control cells (Fig. 4B). LMO3 overexpression increased the population of U251 cells in S phase from 16.19% to 25.77% and reduced the population of U251 cells in G0/G1 phase from 74.63% to 62.49% (Fig. 4B).To further investigate the effect of CIB on LMO3-induced cell proliferation, the stably transfected sublines C8-LMO3 and C8-HisA were transfected transiently with the pCMV-myc-CIB or pCMV-myc vector and subjected to the MTT assay. The expression of CIB in the sublines was examined by western blotting (Fig. 4C, left panel). The C8-HisA cells transfected with CIB had a lower proliferation rate than that of the C8-HisA cells transfected with empty vector ($p<0.05$). Although C8-LMO3 cells transfected with empty vector still displayed accelerated cell growth, the proliferation rate of the C8-LMO3 cells transfected with CIB was significantly reduced (2nd day: $p<0.05$; 3rd–5th day: $p<0.01$) (Fig. 4C, right panel). These results indicate that CIB overexpression attenuates LMO3-induced proliferation.

## 3. Discussion

Increasing evidence suggests that the LIM domain acts as a docking site for the assembly of multiprotein complexes, including transcription factors and other modeling protein (Retaux and Bachy, 2002). In this work, we provide evidence that LMO3 interacts with the EF-hand-containing protein calcium- and integrin-binding protein CIB (also known as CIB1, calmyrin, and KIP1). First, LMO3 interacted with CIB in a yeast two-hybrid assay. Second, the two proteins were coimmunoprecipitated. Third, the two full-length proteins colocalized when coexpressed *in vivo*. Fourth, LMO3-induced cell proliferation was suppressed by CIB. The interaction between CIB and LMO3 is noteworthy because, to the best of our knowledge, CIB is the first protein demonstrated to interact with LMO3 by yeast two-hybrid screening.

The LMO proteins are composed of two LIM domains, which provide surfaces for protein interactions. The binding affinities of the two LIM domains of the LMOs for different target proteins are distinct. A recent study showed that both LIM domains of LMO4 can interact with its binding partner, the glycoprotein 130 subunit (Novotny-Diermayr et al., 2005). Furthermore, the interaction of LMO2 with GATA appears to require both LIM domains, but especially the LIM2 domain (Terano et al., 2005). However, other studies have found that LMO4 mediated its interaction with cofactor CtBP-interacting protein and 5S-labeled metastasis tumor antigen 1 (MTA1) via its first LIM domain but not its second LIM domain (Singh et al., 2005; Sum et al., 2002). A similar result was reported for LMO2 and LMO4 when interacting with Ldb1 (Ryan et al., 2006). Our data show that CIB interacts directly with LMO3 and that the second LIM domain is sufficient to bind CIB. It is notable

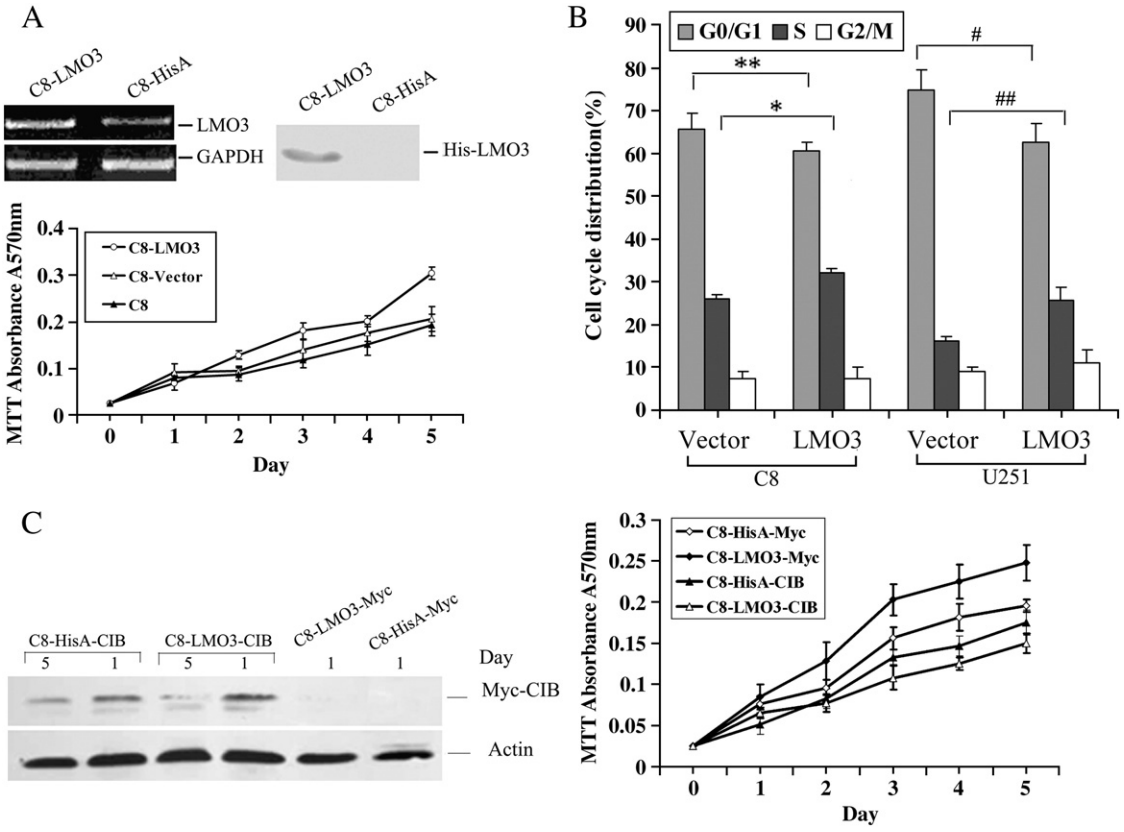


**Fig. 4 – Cell proliferation was analyzed with MTT and flow-cytometric assays. (A) LMO3 promoted C8 cell proliferation, as shown in the MTT assay. The overexpression of LMO3 in the stably transfected subline C8-LMO3 and C8-HisA was analyzed by RT-PCR (top panel). The MTT assay was performed as six replicates, and the symbol error bars indicate the standard deviations. (B) Comparison of the cell-cycle distribution of C8 cells and U251 cells transfected with pcDNA4/HisA empty vector or pcDNA4/HisA-LMO3 by flow-cytometric cell-cycle analysis. The assay was performed in triplicate, and the symbol error bars indicate the standard deviations. The statistical significance of the increase in the S phase population and the reduction in the $G_0/G_1$ phase population in the LMO3-transfected cells compared with those of the vector-transfected cells was confirmed with a paired Student's *t* test. ($^{*}p<0.01$ versus vector, $^{**}p<0.05$ versus vector, $^{\#}p<0.01$ versus vector, $^{\#\#}p<0.05$ versus vector) (C) LMO3-induced cell proliferation was suppressed by CIB. The stably transfected sublines C8-LMO3 and C8-HisA were transiently transfected with pCMV-myc-CIB or the empty pCMV-myc vector. The expression of CIB was analyzed on the days indicated by western blotting using anti-Myc antibody, with actin as the control (left penal). At 24 h posttransfection, the cells were seeded to perform the MTT assay. The MTT assay was performed as six replicates and the symbol error bars indicate the standard deviations.**

that we did not identify Ldb1 protein in our two-hybrid screen. It does not seem to be consistent with the fact that Ldb1 binds LMO1, LMO2, and LMO4. However, instead of the whole LMO3 protein, only the second LIM domain (LIM2) was used as the bait to screen for LMO3-binding proteins. Consequently, only the C-terminal-LIM-binding proteins were isolated in our study and not the N-terminal partners.

Although LMO proteins are thought to function predominantly in the nucleus, mediating protein interactions with various transcription factors, they do not contain a nuclear localization sequence. Therefore, the subcellular localization of LMOs is different. Previous work has shown that LMO2 and LMO4 localize in both the nucleus and cytoplasm in normal tissues (Arber and Caroni, 1996; Neale et al., 1995b). However, under conditions of forced LMO2 expression in T-cells, LMO2 was detected predominantly in the nucleus (Neale et al., 1995a). The research of Aoyama et al. has shown that LMO3 physically interacts with HEN2, a neuronal basic helix-loop-helix protein, and that the two proteins colocalize in the nuclei of COS7 cells in which endogenous LMO3 and HEN2 are coexpressed. Their results suggest that LMO3 markedly promotes the growth of SH-SY5Y neuroblastoma cells, probably by interacting with HEN2 (Aoyama et al., 2005). Our study shows that overexpressed LMO3 primarily localized in the nuclei of C8 cells. Therefore, it seems that HEN2 and LMO3 are assembled into functional protein complexes to promote C8 cell proliferation. It is interesting that the overexpression of LMO3 in NIH3T3 cells results in a cytoplasmic localization pattern. The discrepancy in the localization of LMO3 in C8 and NIH3T3 cells may be attributed to the nature of the different cell lines. Variations in the nuclear and cytoplasmic localization of LMO2 are also dependent on cell type (Neale et al., 1995a). Future work on the subcellular localization of LMO3 should clarify this.

CIB itself was cloned in a yeast two-hybrid study using the integrin α-IIb subunit as bait (Naik et al., 1997). Subsequently, CIB has been shown to interact with a number of other proteins (Kauselmann et al., 1999; Lee et al., 2004; Tahara et al., 2005). Both transfected and endogenous CIB protein have been shown to accumulate in the nucleus and the cytoplasm (Lee et al., 2004; Stabler et al., 1999; Tahara et al., 2005). In fact, CIB may play several roles, including in hemostasis, apoptosis, and the response to DNA damage in diverse or intersecting pathways, by binding to different proteins and shuttling between the nucleus and the cytoplasm (Yamniuk et al., 2007). The overexpression of CIB and its interacting protein PS2 in HeLa cells, which colocalized in the cytoplasm, promoted cell apoptosis *in vitro* (Stabler et al., 1999). With the exogenously expression of CIB and its binding protein 6–16 in TMK-1 and MKN-28 cells, both proteins colocalized in the cytoplasm, and also induced apoptosis (Tahara et al., 2005). Conversely, when CIB interacted with the catalytic subunit of hTERT, they colocalized in the cell nucleus, positively regulating telomerase activity and telomere length (Lee et al., 2004). In this study, CIB suppressed LMO3-induced proliferation and the transfection of the CIB cDNA into C8 and SHG44 cells also induced apoptosis (L. Hui, unpublished observations). Furthermore, the coexpression of LMO3 and CIB in C8 cells did not cause LMO3 to remain the nucleus, where LMO3 might have interacted with HEN2 and/or other factors to accelerate cell growth, but resulted in the accumulation of both proteins in the cytoplasm. It is possible that the overexpression of CIB suppresses cell growth by interfering with the formation of the physiological nuclear complex HEN2/LMO3 and causing LMO3 to leave the complex and enter the cytoplasm, where LMO3 and CIB act synergistically to alter the transcription of target genes by forming a new complex with other factors. Overall, the interaction between LMO3 and CIB is interesting because LMO3 may function as a cell proliferation protein, whereas CIB is a cell death protein. Further study of LMO3 and its relationship with CIB will increase our understanding of proliferation and apoptosis in both normal and pathological conditions, including tumors.

## 4. Experimental procedures

### 4.1. *Yeast two-hybrid library screening*

The yeast two-hybrid assay was performed using the Matchmaker pLexA Two-Hybrid System (Clontech). The sequence encoding the second LIM domain (residues 73–145) of mouse LMO3 was amplified by PCR and cloned into the EcoRI and BamHI restriction sites of the pLexA vector to act as the bait in the yeast two-hybrid screen. The nucleotide sequence of the DNA insert was confirmed by sequence analysis to verify that it did not contain mutations. This bait construct and the LacZ reporter plasmid p8op-lacZ were cotransformed into yeast strain EGY48, which was then transformed with a mouse fetal brain cDNA library in pB42AD. The transformants were selected on plates without histidine, leucine, or uracil. Putative positive colonies were restreaked onto fresh master plates for β-galactosidase assays, according to standard protocols. The pB42AD library plasmid DNA from candidate clones was recovered and the cDNA inserts were analyzed by nucleotide sequencing to ensure that the inserts were in frame with the B42 activation domain. The positive clones were analyzed and verified by yeast mating, with standard protocols.

### 4.2. *Delineation of interacting regions by yeast mating*

The full-length cDNA of LMO3 (encoding 145 amino acids) and the cDNA encoding the first LIM domain, LIM1 (1–73 amino acids), were subcloned into the EcoRI–BamHI sites of the pLexA vector to generate pLexA-LMO3 and pLexA-LIM1, respectively. All constructs were sequenced before testing with yeast mating and the interactions were scored as ++ (moderately positive), + (slightly positive), or – (negative), where ++ denoted growth on leu-trp-his-ura plates for four days.

### 4.3. *Plasmid construction and transfection*

Eukaryotic expression vectors pEGFP-LMO3, pcDNA4/HisA-LMO3, and pCMV-myc-CIB were constructed using molecular biological techniques. All constructs were sequenced before use. Then C8-D1A (C8) astrocytes or NIH3T3 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. To construct stably transfected sublines, C8 cells transfected with pcDNA4/HisA-LMO3 (C8-LMO3) or pcDNA4/HisA (C8-HisA) were cultured in medium containing 0.4 mg/mL Zeocin (Invitrogen) until clone formation. Well-separated clones were picked three weeks later and screened for the expression of LMO3 by western blotting using mouse monoclonal anti-His antibody (Santa Cruz Biotechnology). The stably transfected sublines were incubated in medium containing 0.2 mg/mL Zeocin.

### 4.4. *Immunoprecipitation*

C8 cells were transfected singly with pcDNA4/HisA-LMO3 or pCMV-myc-CIB or cotransfected with pcDNA4/HisA-LMO3 and pCMV-myc-CIB. Whole-cell extracts were prepared 48 h after transfection. Proteins were immunoprecipitated with either anti-His or anti-Myc antibody (9E10; Santa Cruz Biotechnology) and protein A/G-agarose (Santa Cruz Biotechnology), and separated by SDS-PAGE. After the proteins had been transferred to PVDF membranes (Amersham Biosciences, Inc.), the filters were blocked and incubated with anti-Myc or anti-His antibody. The filters were then incubated with horseradish-peroxidase-coupled secondary antibodies and developed with enhanced chemiluminescence (ECL; Amersham Biosciences, Inc.).

### 4.5. *Subcellular localization and immunofluorescence colocalization assay*

C8 or NIH3T3 cells grown on coverslips were transfected with pEGFP-LMO3 or pCMV-myc-CIB. At 48 h posttransfection, the cells were fixed and stained with mouse anti-Myc antibody and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse secondary antibody (1:50; Sigma). After a PBS wash, cells were counterstained in hochest33258 (Beyotime) for 10 min. Cell immunofluorescence was analyzed with a TCS-NT laser scanning confocal microscope or a fluorescence microscope.

### 4.6. *MTT cell proliferation assay*

For the 3-(4,5-dimethylthiazol-2-yl-)2, 5-diphenyltetrazolium bromide (MTT) assay, cells were seeded at a density of $1\times10^4$ cells per well in a 96-well plate in 100 μL of medium and were cultured for five days. MTT (10 μL, 10 mg/mL; Roche) was added to each well and the cells were incubated at 37 °C for 4 h. After the medium had been removed, the purple precipitate in each well was dissolved in 200 μL of DMSO (Sigma) and the optical density at 570 nm was measured. The assays were performed as six replicates.

### 4.7. *Flow-cytometric cell-cycle analysis*

The stably transfected C8-LMO3 and C8-HisA sublines were cultured, expanded with trypsin-EDTA treatment, and then subcultured at a split ratio of 1:5. The cells were washed with phosphate-buffered saline (PBS), trypsinized, and fixed with 70% ethanol. The cells were later stained for 20 min at 37 °C with a 50 μg/mL propidium iodide (Sigma) solution in PBS containing 0.1 mg/mL DNase-free RNase A (Sigma). The cells were analyzed on a FACSCalibur flow cytometer (Becton and Dickenson). The assay was performed in triplicate.

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