

Figure 2. Involvement of LRRC42 in growth and/or survival of lung cancer cells. (A and B) Expression levels of *LRRC42* in response to treatment with si-*LRRC42* (si-#1 or si-#2) or control siRNAs (EGFP or LUC) in LC319 and SBC-3 cells, analyzed by quantitative real-time PCR. The columns and bars represent the mean and SE, respectively. (C and D) Viability of LC319 and SBC-3 cells evaluated by MTT assay in response to treatment with si-*LRRC42* (si-#1 or si-#2), si-EGFP, or si-LUC. All assays were performed in triplicate using triplicate wells. The columns and bars represent the mean and SE, respectively. (E and F) Colony formation assays of LC319 and SBC-3 cells transfected with specific siRNAs or control siRNAs. (G) Induction of LRRC42 expression in T-REx™ 293 cells with Dox (Doxycycline) by western blot analysis. (H) Assays demonstrating the growth promoting effect of induced *LRRC42* expression in T-REx 293 cells. Assays were performed in triplicate using triplicate wells. The columns and bars represent the mean and SE, respectively.

Tris-HCl, 150 mmol/l NaCl) in the presence of phosphatase and proteinase inhibitors. After centrifugation at 1,000 rpm for 5 min at 4°C, the supernatants were incubated at 4°C with anti-Flag M2 agarose for 3 h. The beads were then collected by centrifugation at 1,000 rpm for 1 min and washed six times with 1 ml of each immunoprecipitation buffer. The washed beads were resuspended in 30 μ l of Laemmli sample buffer and boiled for 5 min, and the proteins were separated using 5% to 20% SDS PAGE gels (Bio-Rad Laboratories, Marnes-la-Coquette, France). After electrophoresis, the gels were stained with SilverQuest (Invitrogen). Protein bands specifically found in extracts which was transfected with LRRC42 vector were excised and served for matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) analysis (AXIMA-CFR, Shimadzu-Biotech, Kyoto, Japan).

Flow cytometry. After transfection of each siRNAs, cells were treated with Aphidicolin (Sigma) at 1 μ g/ml for 24 h. Then, the

cells were washed with PBS four times and growth medium was added into the dish. The cells were collected in PBS every 3 h and fixed in 70% cold ethanol for 30 min. After treatment with 100 μ g/ml of RNase (Sigma), the cells were stained with 50 μ g/ml propidium iodide (Sigma) in PBS. Flow cytometry was analyzed by using FACScan (Beckman Coulter, Brea, CA, USA). The cells selected from at least 20,000 ungated cells were analyzed for DNA content.

Results

***LRRC42* expression in lung cancers and normal tissues.** To identify novel target molecules for the development of therapeutic agents and/or diagnostic biomarkers for lung cancer, we previously performed gene expression profile analysis of 120 lung carcinoma samples using cDNA microarray containing 27,648 genes or expressed sequence tags (6-10), and identified that LRRC42 was significantly transactivated (more than 3 times higher than in their corresponding normal tissues) in

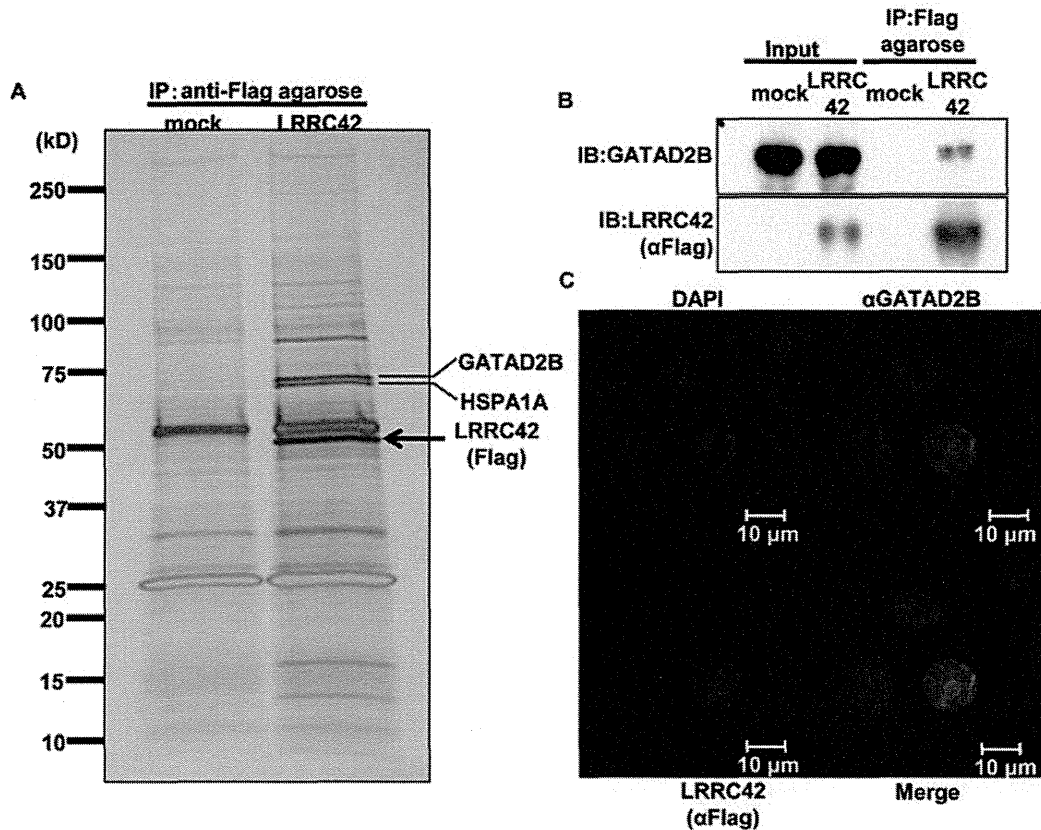


Figure 3. Interaction of LRRC42 with GATAD2B. (A) Silver staining of SDS-PAGE gels that contained immunoprecipitated lysates of lung cancer SBC-3 cells, which were transfected with Flag-tagged LRRC42 expression vector or mock vector, using anti-Flag M2 agarose. (B) Interaction between exogenous LRRC42 and endogenous GATAD2B in SBC-3 cells transfected with LRRC42 expression vector by immunoprecipitation and western blot analysis. (C) Colocalization of LRRC42 and GATAD2B in the nucleus of SBC-3 cells transfected with LRRC42 expression vector, detected by immunocytochemical staining.

>50% of 120 lung cancer samples examined. We subsequently confirmed its transactivation by quantitative real-time PCR experiments in lung cancer tissues as well as lung cancer cell lines (Fig. 1A and B). Northern blot analysis with the *LRRC42*-specific probe identified a 1.7-kb transcript only in testis among 16 normal human tissues examined (Fig. 1C). To determine the subcellular localization of LRRC42 protein, we constructed a plasmid expressing LRRC42 (carboxyl-terminal Flag-tagged pCAGGS plasmid vector), transfected it into COS-7 cells and detected exogenous LRRC42 protein in the nucleus of the cells using an anti-flag antibody (Fig. 1D).

Effect of LRRC42 on cell growth. To assess whether LRRC42 is essential for growth or survival of lung cancer cells, we transfected synthetic oligonucleotide siRNAs against LRRC42 into lung adenocarcinoma LC319 and small cell lung cancer SBC-3 cells in which *LRRC42* was highly expressed. The mRNA levels of *LRRC42* in the cells transfected with si-LRRC42-#1 or -#2 were significantly decreased in comparison with those transfected with either of the control siRNAs (Fig. 2A and B). MTT and colony formation assays revealed a significant reduction of cell viability as well as the number of colonies in si-LRRC42-transfected cells (Fig. 2C-F).

To further clarify a potential role of LRRC42 in carcinogenesis, we established HEK293 cells using the Flp-In T-Rex expression system, where LRRC42 expression was under the control of the tetracycline-regulated cytomegalovirus/tetO₂ hybrid promoter. MTT assay demonstrated that the growth

of the cells treated with doxycycline was enhanced compared with that without doxycycline, indicating the growth promoting activity of LRRC42 protein (Fig. 2G and H).

Interaction and colocalization of LRRC42 with GATAD2B.

To elucidate the molecular mechanism of LRRC42 in lung carcinogenesis, we screened a protein(s) that could interact with LRRC42. Lysates of SBC-3 cells which was transfected with LRRC42 expression vector (carboxyl-terminal Flag-tagged pCAGGS plasmid vector) or mock vector were extracted, and immunoprecipitated with anti-Flag M2 agarose. The protein complex was separated by SDS-PAGE and visualized by silver staining (Fig. 3A). A 65-kDa band, which was detectable in lysates of cells transfected with LRRC42 vector, but not in those with mock vector, was extracted. The peptide sequence analysis determined by mass spectrometry indicated the protein to be GATAD2B (GATA zinc finger domain containing 2B) that is known to be a component of the MeCP1 complex that represses transcription through preferential binding, remodeling and deacetylation of methylated nucleosomes (15). We subsequently confirmed interaction between exogenous LRRC42 and endogenous GATAD2B in SBC-3 cells using anti-GATAD2B antibody by co-immunoprecipitation experiment (Fig. 3B). We also conducted immunofluorescence analysis and found colocalization of exogenous LRRC42 with endogenous GATAD2B in the nucleus of SBC-3 cells which were transfected with the LRRC42 expression vector (Fig. 3C).

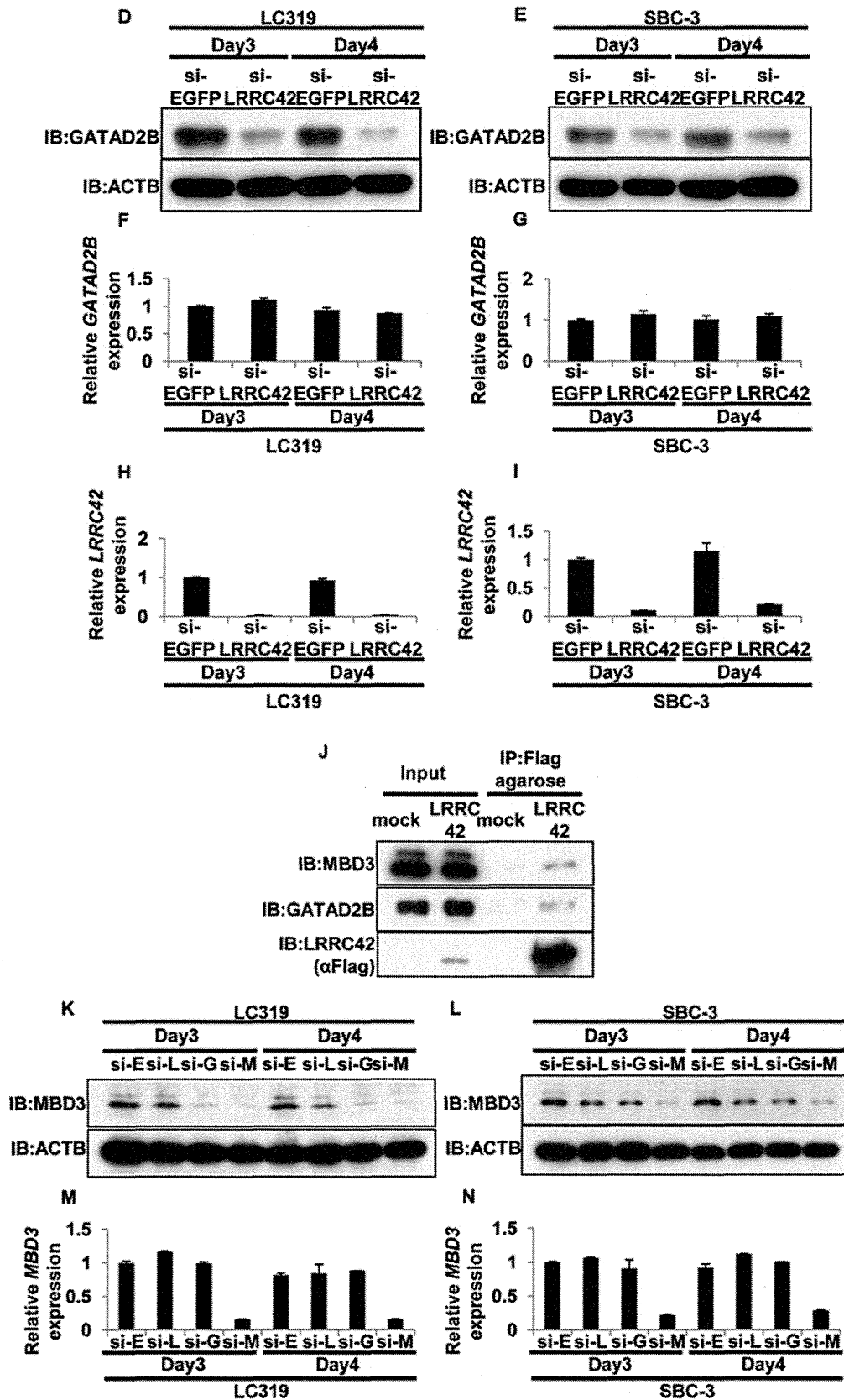


Figure 3 Continued. Interaction of LRRC42 with GATAD2B. (D and E) The level of GATAD2B proteins detected by western blot analysis in LC319 and SBC-3 cells transfected with si-EGFP or si-LRRC42. The columns and bars represent the mean and SE, respectively. (F and G) The level of GATAD2B mRNAs detected by quantitative real-time PCR analysis in LC319 and SBC-3 cells transfected with si-EGFP or si-LRRC42. The columns and bars represent the mean and SE, respectively. (H and I) The level of LRRC42 mRNAs detected by quantitative real-time PCR analysis in LC319 and SBC-3 cells transfected with si-EGFP or si-LRRC42. The columns and bars represent the mean and SE, respectively. (J) Interaction between exogenous LRRC42 and endogenous GATAD2B or MBD3 in SBC-3 cells transfected with LRRC42 expression vector by immunoprecipitation and western blot analysis. (K and L) The level of MBD3 proteins detected by western blot analysis in LC319 and SBC-3 cells transfected with si-EGFP, si-LRRC42, GATAD2B or MBD3. (M and N) The level of MBD3 mRNAs detected by quantitative real-time PCR analysis in LC319 and SBC-3 cells transfected with si-EGFP, si-LRRC42, si-GATAD2B or si-MBD3. The columns and bars represent the mean and SE, respectively.

To further investigate the biological significance of the interaction between LRRC42 and GATAD2B in cancer cells, we examined the protein level of GATAD2B after suppressing LRRC42 expression in LC319 and SBC-3 cells. Treatment of siRNA oligonucleotides against LRRC42 (si-LRRC42) effectively knocked down the expression of endogenous LRRC42, compared to the control siRNA (si-EGFP). Interestingly, the protein level of GATAD2B was also significantly decreased in cells transfected with si-LRRC42, while the transcript level of GATAD2B was unchanged (Fig. 3D-I). A previous study indicated GATAD2B as a key component of the MeCP1 complex that interacted with MBD3 (15). Furthermore, GATAD2B was shown to possess an ability to target MBD3 protein to specific nuclear loci (15,46). MBD3 was indicated to be recruited to a promoter region of the $p21^{Waf1/Cip1}$ tumor suppressor gene and silence its expression (47). Hence, we have hypothesized that LRRC42 might have a very significant effect on the function of the MeCP1 complex through the interaction with GATAD2B as well as MBD3 (Fig. 3J). We found that knockdown of LRRC42 or GATAD2B reduced the amount of MBD3 protein while no change was observed in mRNA level of MBD3 (Fig. 3K-N).

LRRC42-GATAD2B-MBD3 axis regulates $p21^{Waf1/Cip1}$ expression. We then examined the downstream target of the LRRC42-GATAD2B-MBD3 complex in lung cancer cells. As described above, MBD3 might be recruited at the $p21^{Waf1/Cip1}$ promoter and silence its expression (47). Therefore, we firstly assessed the knockdown effect of either of LRRC42, GATAD2B or MBD3 on $p21^{Waf1/Cip1}$ expression by quantitative real-time PCR and western blot analysis. Suppression of either of LRRC42, GATAD2B or MBD3 by siRNA appeared to increase $p21^{Waf1/Cip1}$ at transcriptional and protein levels in LC319 (p53 null) and SBC-3 (p53 wild-type) cells (Fig. 4A-D). However, this effect on $p21^{Waf1/Cip1}$ was not clear in other lung cancer cell lines examined (NCI-H1781, NCI-H358, NCI-H1299 and DMS273; data not shown).

Since $p21^{Waf1/Cip1}$ expression was known to cause the G_0/G_1 arrest, we performed FACS (Fluorescence Activated Cell Sorter) analysis to evaluate the knockdown effect on the cell cycle in these cell lines. After synchronization of the cancer cells at G_1 phase by aphidicolin, we removed aphidicolin from the cell culture medium and monitored the cell cycle progression process (Fig. 4E). The cells transfected with si-EGFP progressed rapidly into the S and G_2/M phases. However, the cells treated with siRNA against LRRC42, GATAD2B or MBD3 revealed significant delay in entering into S phase although the delay from the G_1 to S transition in the cells treated with si-MBD3 was less significant to those treated with siLRRC42 or si-GATAD2B. These data implied that the LRRC42-GATAD2B interaction may regulate MBD3 protein as well as the MeCP1 complex, and enhance the growth of cancer cells.

Discussion

Recent advances in understanding the molecular mechanisms underlying cancer development/progression have driven the design of new therapeutic approaches, termed 'molecular targeted therapies', that selectively interfere with molecules or pathways involved in tumor growth and progression.

Inactivation of growth factors and/or their receptors on tumor cells as well as the inhibition of oncogenic tyrosine kinase pathways that play crucial roles in cancer cells constitute the main rationale of new cancer treatments and also lead to the way for the personalized treatment for individual patients. Small-molecule inhibitors and monoclonal antibodies are at present major components of these targeted approaches for various types of human cancer (16). Molecular targeted cancer therapies hold the promise of being very selective to cancer cells, but not affecting normal cells. Hence, they are expected to be less harmful to normal cells, reduce severe side effects, and improve the quality of life of cancer patients. Toward identification of molecular targets for drug development, we had performed whole-genome expression profiles of 120 clinical lung cancer samples using cDNA microarray data and subsequent loss-of-function phenotype analyses by means of RNA interference systems (17-38). On the basis of this approach, we found LRRC42 to be highly over-expressed in the majority of clinical lung cancer cases as well as in most of the lung cancer cell lines examined, while its expression was absent in normal tissues except testis.

Furthermore, we demonstrated that knockdown of LRRC42 expression suppressed the growth of lung cancer cells. In addition, induction of LRRC42 expression by a T-REx system resulted in enhancement of cell growth, suggesting that LRRC42 is likely to be an important growth promoting factor in lung cancer cells and could serve as a valuable target for the development of an anticancer agent for lung cancer.

LRR family members were reported to participate in many biologically important processes such as hormone-receptor interactions, enzyme inhibition, cell adhesion and cellular trafficking. A number of studies clarified the involvement of LRR proteins in early mammalian development (39), development of neuron (40), cell polarization (41), regulation of gene expression (42) and apoptosis (43). It was also shown that LRR domains may be critical for the cell morphology as well as the cytoskeleton dynamics (44,45). In these processes, the LRR motifs are probably essential in mediating protein-protein interactions. However, there has been no report describing the involvement of LRRC42 in human carcinogenesis.

Our data indicated that LRRC42 was able to interact with GATAD2B. GATAD2B is an important component of the MeCP1 complex that represses transcription of genes through preferential binding to, remodeling, and deacetylating methylated nucleosomes. It has the ability to translocate MBD3 protein to specific nuclear foci (15,46). We demonstrated that LRRC42 could activate this transcription-repressive complex through interacting with and stabilizing GATAD2B and MBD3 proteins. We also revealed that the LRRC42-GATAD2B-MBD3 interaction is likely to play a significant role in transcriptional regulation of the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ which is a well-known tumor suppressor and an inhibitor of cell cycle progression from G_1 to S phase. $p21^{Waf1/Cip1}$ negatively regulates DNA replication through the interaction with PCNA (Proliferative cell nuclear antigen), and also binds to the CDK (Cyclin-dependent kinase) complex and inhibits the G_1 -S transition (48).

Several transcriptional regulators, activators or repressors of $p21^{Waf1/Cip1}$ have been reported. p53 is an activator of $p21^{Waf1/Cip1}$ and the activation of the p53- $p21^{Waf1/Cip1}$ pathway is

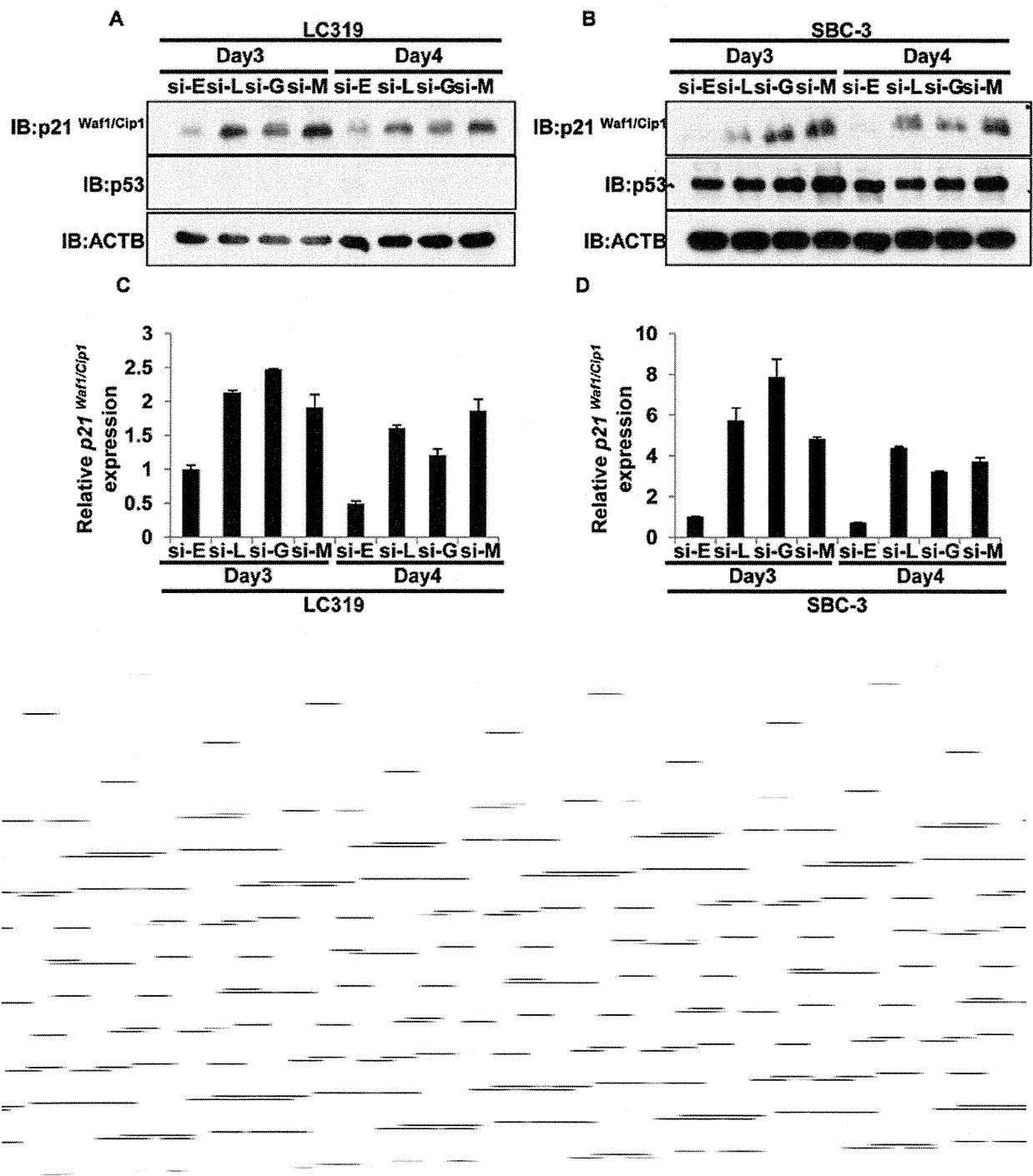


Figure 4. Induction of p21^{Waf1/Cip1} and the effect on cell cycle progression by knockdown of LRRC42, GATAD2B or MBD3. (A and B) Expression levels of p21^{Waf1/Cip1} protein after suppression of LRRC42, GATAD2B or MBD3 by siRNA in LC319 and SBC-3 cells. (C and D) Expression levels of p21^{Waf1/Cip1} mRNA after suppression of LRRC42, GATAD2B or MBD3 by siRNA in LC319 and SBC-3 cells. The columns and bars represent the mean and SE, respectively. (E) Histogram of the cell cycle phases after treatment with si-EGFP, si-LRRC42, si-GATAD2B or MBD3 in SBC-3 cells.

critically important when cells need to arrest the cell cycle and repair the DNA damage. Myc, one of p21^{Waf1/Cip1} repressors, was reported to induce the expression of AP4 (Transcription Factor AP-4) that has the ability to repress p21^{Waf1/Cip1} expression. Myc may also repress p21^{Waf1/Cip1} expression through

the interaction with MIZ1 (ZBTB17) (49). MBD3 is known to be one of the repressors which directly regulate p21^{Waf1/Cip1} expression and play important roles in oncogenic transformation and proliferation (50). FACS analysis clearly demonstrated that suppression of *MBD3* caused the delay of G₁-S transition

1 although its effect was not as significant as the knockdown of
2 LRRC42 or GATAD2B.

3 In conclusion, human LRRC42 is essential for growth and
4 survival of lung cancer cells. Our data imply that targeting
5 LRRC42 and/or the LRRC42-GATAD2B interaction may be
6 a good approach for development of new treatment of lung
7 cancer with specific activity and minimum toxicity.

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