Flow cytometric analysis. Cells transfected with siRNA oligonucleotides against *MMS22L* or control siRNAs were plated at densities of $5x10^5$ per 60-mm dish. Cells were collected in PBS, and fixed in 70% cold ethanol for 30 min. After treatment with 100 μg/ml RNase (Sigma-Aldrich), the cells were stained with 50 μg/ml propidium iodide (Sigma-Aldrich) in PBS. Flow cytometric analysis was done on a Cell Lab Quanta SC (Beckman Coulter) and analyzed by CXP Analysis software (Beckman Coulter). The cells selected from at least 10,000 ungated cells were analyzed for DNA content.

Results

Expression of MMS22L in lung and esophageal cancers. We previously performed genome-wide expression profile analysis of 120 lung cancer cases using microarray consisting of 27,648 cDNAs or ESTs (5-10). Among the genes upregulated in lung and esophageal cancers, we identified *MMS22L* transcript to be frequently overexpressed in lung and esophageal cancers, and confirmed by semiquantitative RT-PCR experiments its elevated expression in all of eleven clinical lung cancers and in four clinical esophageal cancers, although its expression was not detectable in adjacent normal lung and esophagus tissues (Fig. 1a). We further confirmed by western blot analysis high levels of endogenous MMS22L protein in 11 of 12 lung cancer cell lines and in all of 9 esophageal cancer cell lines using anti-MMS22L antibody (Fig. 1b). Northern blot analysis of 16 normal tissues confirmed that *MMS22L* was hardly detectable in normal tissues except the testis (Fig. 1c).

Growth effect of MMS22L. To investigate the relevance of MMS22L to the growth and/or survival of cancer cells, we knocked down the expression of endogenous *MMS22L* in two lung cancer cell lines, LC319 and A549, by means of the RNAi technique using siRNA oligonucreotide for *MMS22L*. Semiquantitative RT-PCR experiments detected significant reduction of MMS22L expression in the cells transfected with siRNAs against *MMS22L* (si-#1 and si-#2), but not in those with control siRNAs (si-LUC and si-CNT) (Fig. 2a). Colony formation and MTT assays clearly demonstrated that the viability of lung cancer cells transfected with two effective siRNAs for *MMS22L* (si-#1 and si-#2) were reduced in correlation with the reduction of MMS22L expression level, implying essential role of MMS22L in the growth of cancer cells (Fig. 2b and c). Since our original gene expression profile database also revealed its high level of expression in clinical cervical cancers, we also knocked down the expression of *MMS22L* by siRNAs in a cervical cancer cell line, HeLa, and observed the growth suppressive effect by siRNAs for *MMS22L*.

To further examine the effect of MMS22L overexpression on the growth of mammalian cells, we transiently transfected plasmid designed to express Flag-tagged MMS22L (pCAGGSn-3xFlag-MMS22L) or mock plasmid into COS-7 or HEK293 cells that expressed endogenous MMS22L at very low level. The significant growth promoting effect was observed in the cells transfected with the MMS22L expressing vector compared to those transfected with the mock vector (Fig. 2d).

NFKBIL2 controls the nuclear localization and stability of MMS22L protein. To investigate the biological function of MMS22L protein, we screened MMS22L-interacting proteins in lung cancer cells using mass spectrometric analysis and identified the interaction between MMS22L and NFKBIL2 [nuclear factor of kappa (NFKB) light polypeptide gene enhancer in B-cells inhibitor-like 2]. Previous reports independently suggested the roles of MMS22L-NFKBIL2 interaction in genomic stability and DNA replication in immortalized cell lines (46-49), however, no study has indicated critical roles of activation of MMS22L and NFKBIL2 in clinical cancers and investigated their functional importance in carcinogenesis. Western blot analysis using cell lines derived from lung cancers and antibodies to MMS22L and NFKBIL2 revealed the co-expression of these two proteins (data not shown), suggesting some functional roles of their interaction in human carcinogenesis. Therefore, we next performed immunofluorescence analysis to determine the subcellular localization of endogenous MMS22L and NFKBIL2 in various cancer cell lines including A549, LC319 and HeLa cells, and found that endogenous MMS22L and NFKBIL2 proteins were mainly co-localized in the nucleus (representative data of HeLa cells was shown in Fig. 3a). To examine the importance of MMS22L-NFKBIL2 interaction in cellar localization of these proteins, we transiently co-expressed exogenous MMS22L and NFKBIL2 proteins using mammalian COS-7 or NIH3T3 cells that expressed these two proteins at very low levels. We found that exogenous MMS22L was mainly located in the cytoplasm and weakly in the nucleus of the cells in which exogenous NFKBIL2 protein was not introduced. However, the nuclear staining of MMS22L was significantly enhanced when both exogenous MMS22L and NFKBIL2 proteins were introduced in the cells (Fig. 3b). On the other hand, exogenous NFKBIL2 was mainly present in the nucleus of cells regardless to the presence or absence of exogenous MMS22L. In addition, we performed western blot analysis using fractionated cytoplasmic and nuclear lysates from COS-7 cells that were introduced exogenous MMS22L and NFKBIL2 proteins. When we transfected both MMS22L-Flag and NFKBIL2-HA expressing vectors, the amounts of nuclear MMS22L was significantly increased, compared with the cells transfected with MMS22L alone (Fig. 3c). Furthermore, we found that knockdown of endogenous MMS22L with siRNA for *MMS22L* (si-*MMS22L*) reduced NFKBIL2 protein level in lung cancer LC319 cells and that reduction of NFKBIL2 with si-NFKBIL2 reduced MMS22L levels and significantly suppressed cancer cell growth (Fig. 3d; data not shown). These data suggest that the expression of NFKBIL2 is likely to promote nuclear localization and stability of MMS22L protein, and a complex including these two proteins could coordinately play pivotal roles in cell growth and/or survival.

C-terminal portion of NFKIL2 protein is crucial for binding to MMS22L protein. To examine whether the MMS22L-NFKBIL2 protein complex may play important roles in carcinogenesis, we subsequently constructed various plasmids expressing partial MMS22L proteins with Flag tag or partial NFKBIL2 proteins with HA tag, and transfected them into COS-7 cells (data not shown). Immunoprecipitation and western blotting assays using antibodies to Flag- or HA-tags revealed that an N-terminal portion of MMS22L protein (M1; codon 1-414) could bind to a C-terminal region of NFKBIL2 (N3; codon

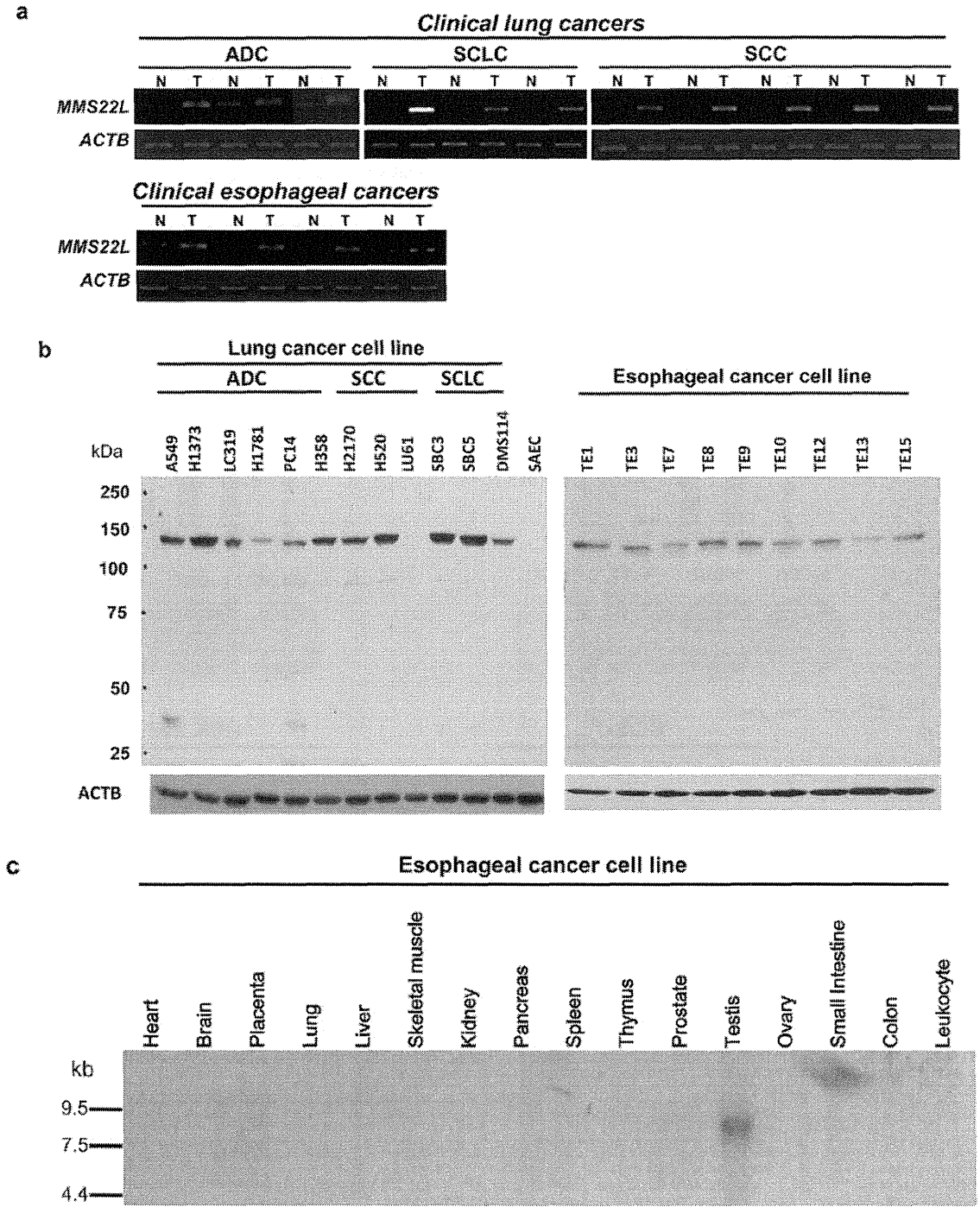

Figure 1. Expression of MMS22L in lung and esophageal cancers and normal tissues. (a) Expression of *MMS22L* gene in (T) lung and esophageal cancer tissues and (N) adjacent normal lung and esophagus tissues, detected by semiquantitative RT-PCR. (b) Expression of MMS22L protein in lung and esophageal cancer cell lines, detected by western blot analysis. (c) Northern blot analysis of the *MMS22L* transcript in 16 normal human tissues.

823-1244) (Fig. 4a). Because immunocytochemical analysis revealed that nuclear localization of MMS22L protein appeared to require the presence of NFKIL2 protein in the nucleus (Fig. 3b), we subsequently investigated which part of NFKBIL2 protein is essential for subcellular localization of MMS22L protein in cultured cells. Plasmids expressing partial proteins of NFKBIL2 were co-transfected with full-length MMS22L expression vector into COS-7 cells. Interestingly, N-terminal (N1; codon 1-450) and central part (N2; codon 403-836) of NFKBIL2 proteins could be localized in the nucleus, while aggregated MMS22L protein was mainly located in the cytoplasm of the same cells (Fig. 4b). It is concordant with the data that these two partial proteins (N1 and N2) could not bind to MMS22L protein as indicated by immunoprecipitation analyses. In contrast, MMS22L protein and C-terminal part of NFKBIL2 protein (N3; codon 823-1244) that could bind to

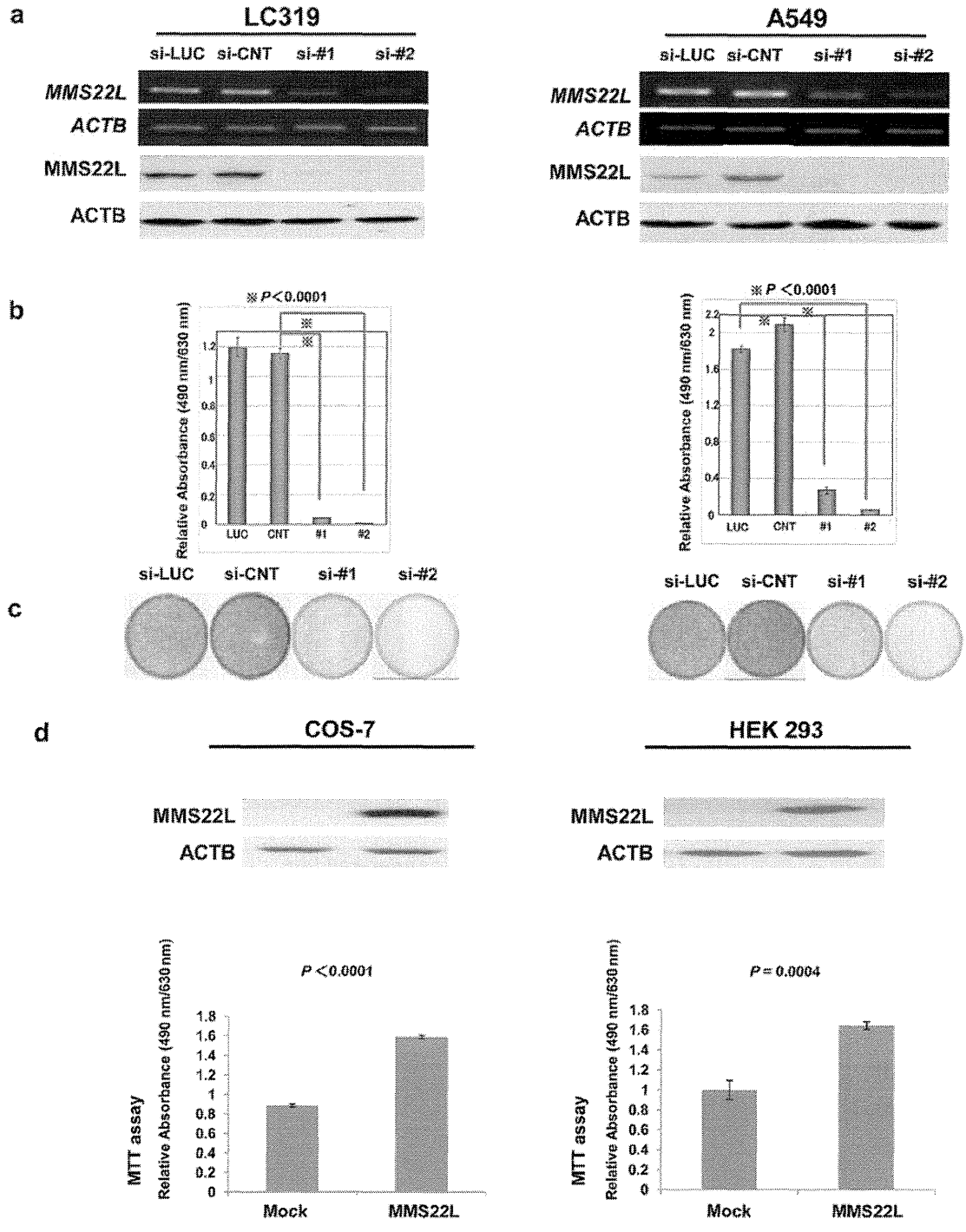

Figure 2. Growth effect of MMS22L. (a) Knockdown of MMS22L expression in lung cancer cell lines, LC319 and A549 by specific siRNA oligonucleotides for *MMS22L* (si-#1 and si-#2) or control siRNAs (si-LUC and si-CNT), confirmed by semiquantitative RT-PCR and western blot analyses. (b) Viability of A549 and LC319 cells evaluated by MTT assay in response to the siRNAs. All assays were performed in triplicate wells at three independent times. (c) Colony formation assays using LC319 and A549 cells transfected with the siRNAs. (d) Enhanced growth promoting activity of COS-7 and HEK293 cells transfected with mock or MMS22L expressing vectors as quantified by MTT assay at 7 days after transfection.

MMS22L protein were mainly localized in the cytoplasm of the cells (Fig. 4b). The data indicate that N-terminal (N1; codon 1-450) and central (N2; codon 403-836) parts of NFKBIL2 are more important for nuclear localization of NFKBIL2, while its C-terminal part (N3; codon 823-1244) is essential for binding to MMS22L.

Dominant negative growth suppressive effect of partial NFKBIL2 protein including MMS22L-binding site. According to the data above, we hypothesized that if nuclear localization of MMS22L protein is important for cancer cells growth, reduction of MMS22L protein in the nucleus by inhibiting the interaction between MMS22L and NFKBIL2 could suppress the cancer cell

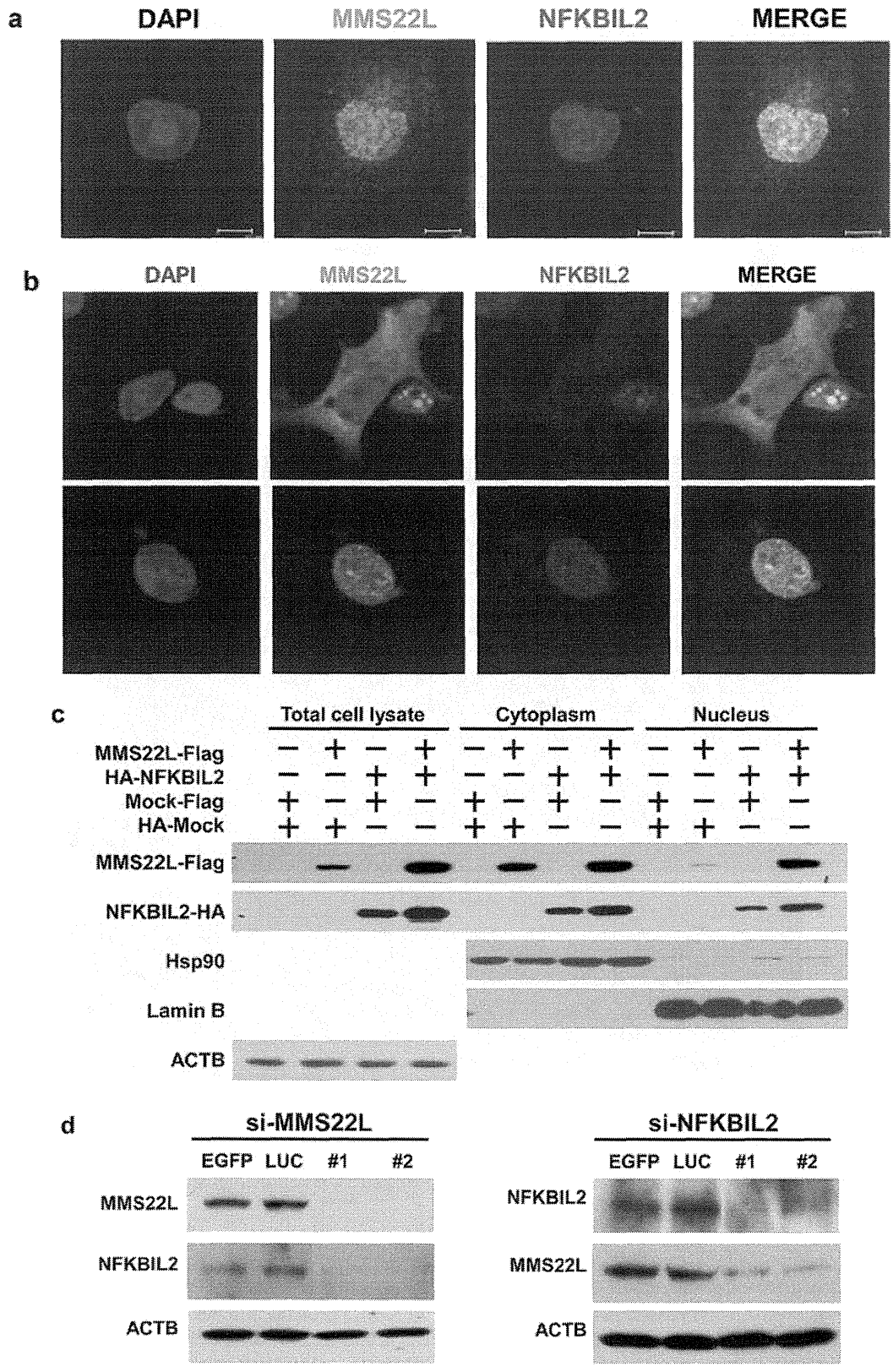

Figure 3. Nuclear localization and stability of MMS22L and its interacting protein of NFKBIL2 in cultured cells. (a) Subcellular localization of endogenous MMS22L and NFKBIL2 proteins in HeLa cells. The cells were stained with a rabbit polyclonal anti-MMS22L antibody (green); mouse polyclonal anti-NFKBIL2 (red) and with DAPI (blue). (b) Subcellular localization of MMS22L in the presence or absence of NFKBIL2 protein. COS-7 cells were co-transfected with Flag-tagged MMS22L- and HA-tagged NFKBIL2-expression vectors. MMS22L protein was stained with anti-Flag M2 antibody (green); NFKBIL2 with anti-HA (red) and nucleus with DAPI (blue). (c) Western blot analysis using cytoplasmic and nuclear fraction of COS-7 cells which were co-transfected with MMS22L- and NFKBIL2- expression vectors. MMS22L and NFKBIL2 proteins were detected using anti-Fag-M2 antibody and/or anti-HA (3F10) antibody. (d) Knockdown of MMS22L or NFKBIL2 protein expression with si-*MMS22L* or si-*NFKBIL2* oligonucleotides. The expression of endogenous MMS22L and NFKBIL2 proteins were detected by western blot analysis using anti-MMS22L antibody and anti-NFKBIL2 antibody.

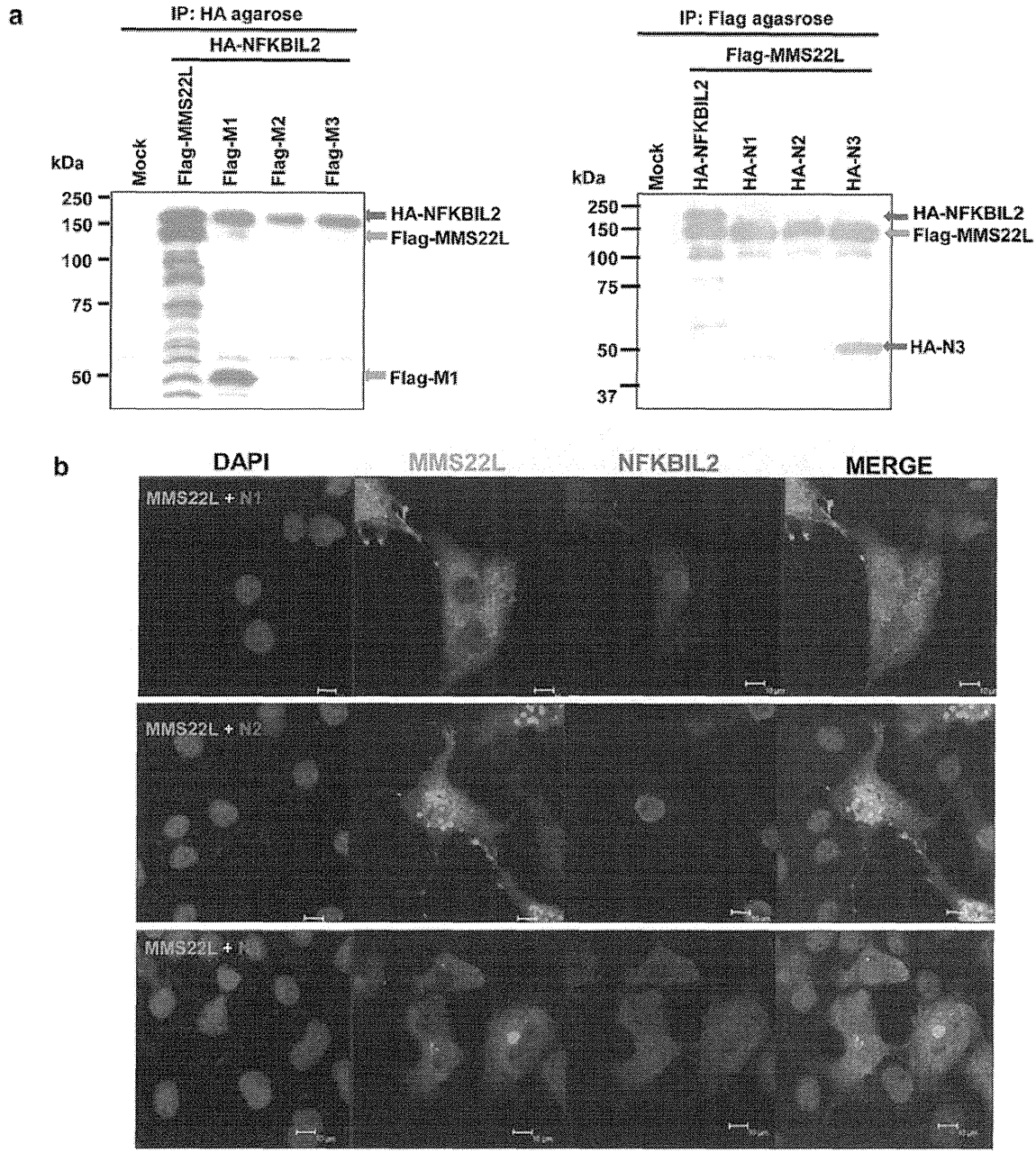

Figure 4. C-terminal portion of NFKIL2 protein is crucial for binding to MMS22L protein. (a) Binding between MMS22L and NFKBIL2 proteins detected using Flag- or HA-agarose and COS-7 cells co-transfected with full-length/partial MMS22L-Flag and full-length/partial NFKBIL2-HA proteins. (b) Subcellular localization of full-length MMS22L and three partial NFKBIL2 proteins. COS-7 cells were co-transfected with full-length MMS22L and partial NFKBIL2-expression vectors (N1-N3). MMS22L protein was stained with anti-Flag M2 antibody (green); NFKBIL2 with anti-HA antibody (red) and nucleus with DAPI (blue).

growth. To examine whether exogenous expression of partial N3 protein can inhibit the MMS22L-NFKBIL2 interaction and cell growth, we co-transfected full-length MMS22L and either of full-/partial-length NFKBIL2 expressing vectors (N1, N2 or N3) into HEK293 cells, and found that the amount of exogenous full-length NFKBIL2 protein that binds to exogenous MMS22L was significantly decreased after introduction of the partial N3 protein, as demonstrated by immunoprecipitation assays, while it was not changed in the cells transfected with N1 or N2 vectors (Fig. 5a). To investigate the functional significance of the interaction between MMS22L and NFKBIL2 for growth of cancer cells, we transfected either of vectors expressing partial NFKBIL2 proteins or mock vectors into two cancer cell lines, HeLa and LC319, which highly expressed both endogenous MMS22L and NFKBIL2 proteins and lung fibroblast CCDlu-19 cells in which MMS22L expression was hardly detectable. Expectedly, exogenous expression of the C-terminal portion of NFKIL2 protein (N3) reduced the levels of MMS22L protein in the nucleus and inhibited the growth of HeLa and LC319 cells as measured by MTT assay, while it did not affect the growth of MMS22L-negative CCDLu-19 cells (Figs. 5b-d). Our findings imply that inhibition of the interaction between the MMS22L

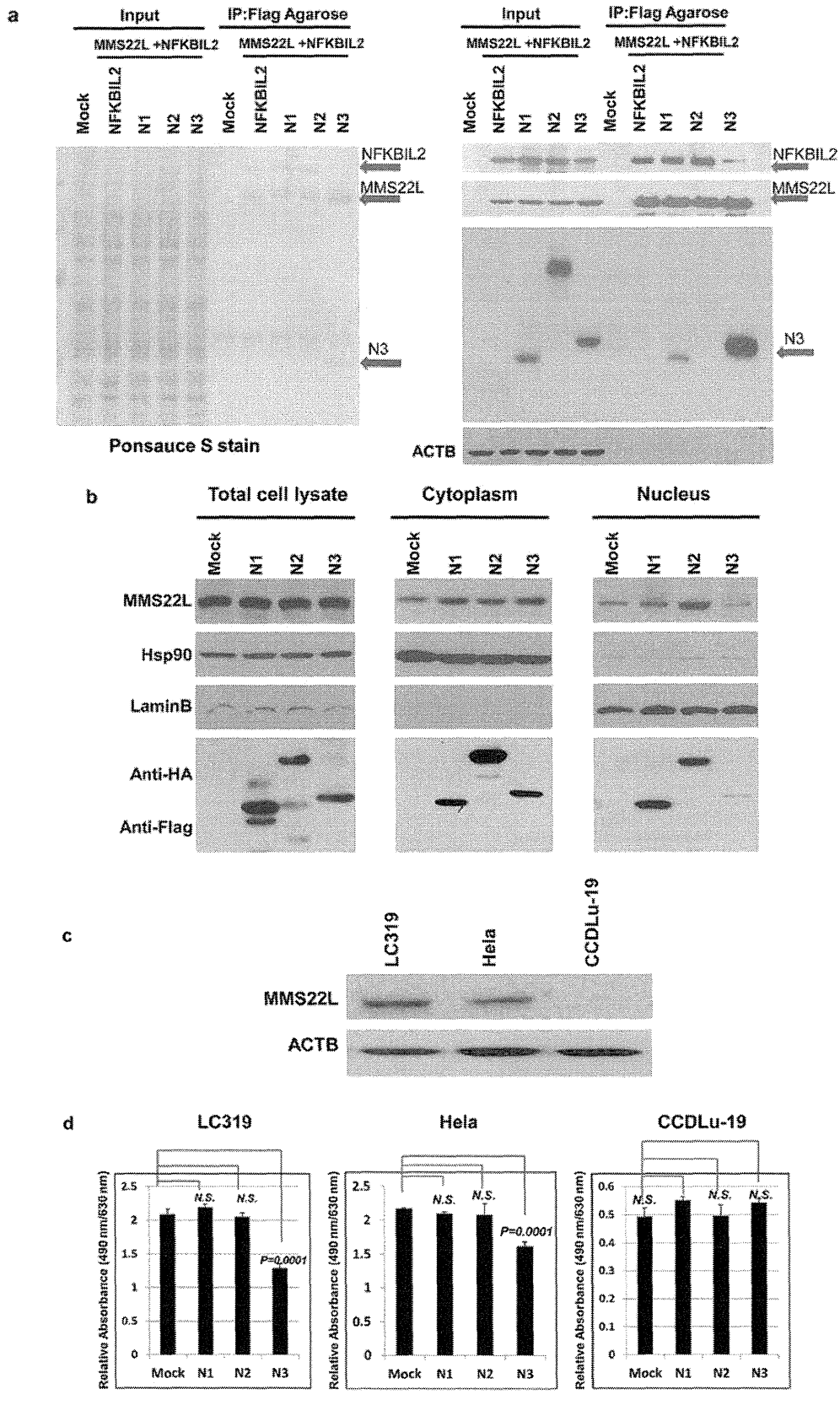

Figure 5. Dominant negative growth suppressive effect of partial NFKBIL2 protein on cancer cells. (a) HEK293 cells which were co-transfected with MMS22L- and full-length/three partial NFKBIL2-expression vectors (N1, N2, and N3). Immunoprecipitation assays were performed using Flag-M2 agarose. (b) Western blot analysis using cytoplasmic and nuclear fractionation of the lysates from HeLa cells which were transfected with partial NFKBIL2-expressing vectors. Endogenous MMS22L was detected using anti-MMS22L antibody. Partial NFKBIL2 proteins were detected with anti-rat HA (3F10) antibody. (c) The expression of MMS22L protein in HeLa, LC319 and CCDlu-19 cell lines. (d) MTT assay using MMS22L-positive HeLa and LC319 cells, and MMS22L-negative CCDlu-19 cells, which were transfected with mock plasmids or either of three partial NFKBIL2-expression vectors (N1, N2 and N3), as quantified by MTT assay at 7 days after transfection.

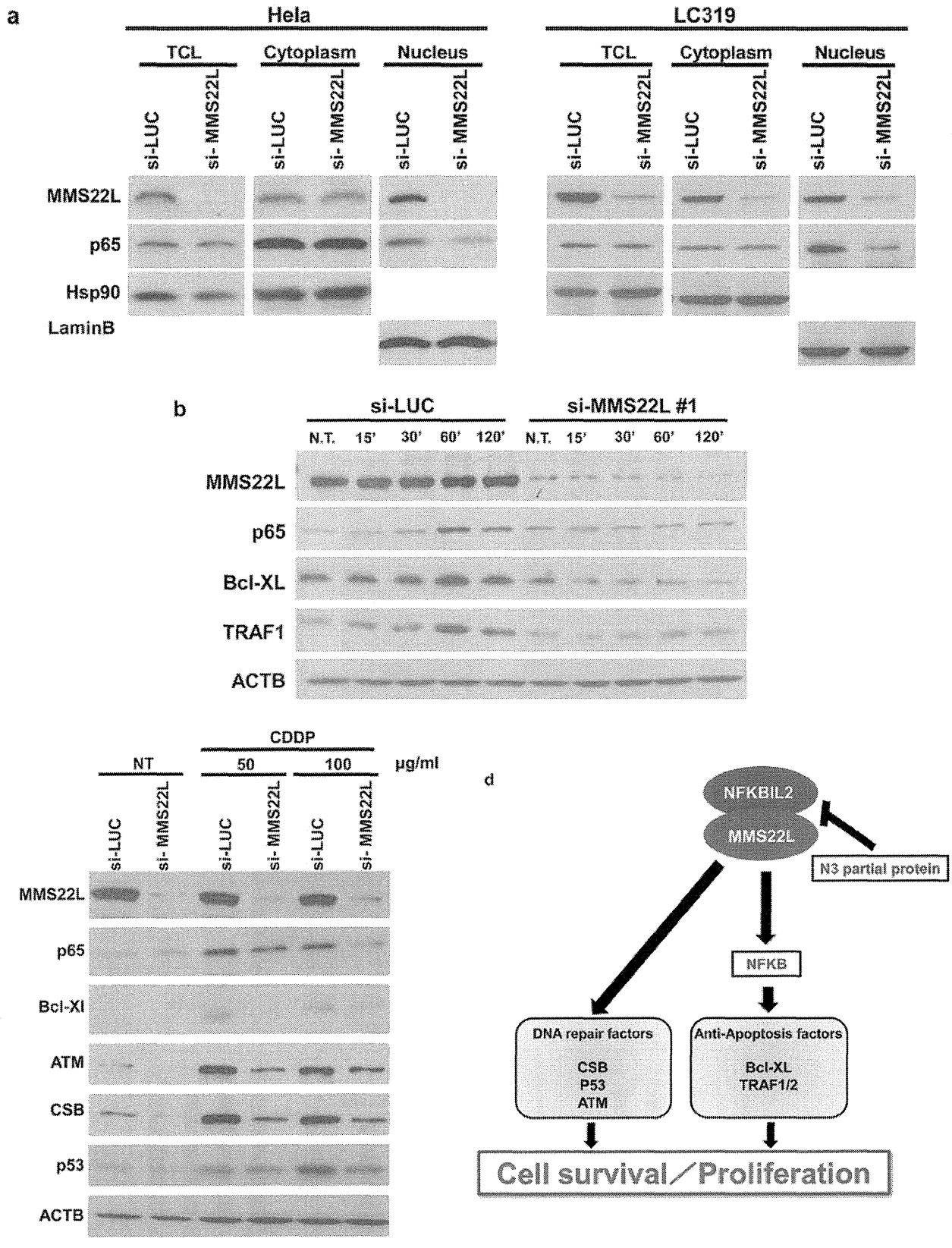

Figure 6. Involvement of MMS22L as an upstream molecule of NFKB pathway. (a) Western blot analysis using antibodies to endogenous MMS22L and RelA/p65, and HeLa and LC319 cells transfected with siRNA oligonucleotides for *MMS22L* (si-*MMS22L*) or control siRNA (si-LUC). These cell lines were stimulated with 50 ng/ml TNF-α for 15 min. The nuclear and cytoplasmic fraction was isolated using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents kit (Thermo). (b) Western blot analysis using antibodies to endogenous MMS22L, RelA/p65, Bcl-XL and TRAF1 and HeLa cells transfected with si-*MMS22L* or si-LUC. These cell lines were treated with 50 ng/ml TNF-α for 15, 30, 60 or 120 min. N.T. indicates no treatment with TNF-α. (c) Western blot analysis using antibodies to endogenous MMS22L, RelA/p65, Bcl-XL, ATM, CSB, p53 and HeLa cells which were transfected with si-*MMS22L* or si-LUC oligonucleotides, and were subsequently treated with cisplatin (CDDP; 50 or 100 μg/ml). (d) Schematic summary of MMS22L pathway.

and NFKBIL2 protein can suppress the nuclear localization of MMS22L protein, and resulted in the reduction of cancer growth, and that inhibition of the interaction in cancer cells by small molecules might be a potential therapeutic strategy for new cancer treatment.

MMS22L protein acts as an upstream molecule of NFKB pathway. Since NFKIL2 protein was indicated to be involved in the NFKB pathway that plays an essential role in the promotion of cell proliferation and anti-apoptosis (42-45), we examined the expression of NFKB p65/RelA protein in HeLa cells in which both exogenous MMS22L and NFKIL2 were introduced, and found that the level of endogenous p65/RelA protein was elevated compared with those of cells introduced NFKIL2 alone (data not shown). The result suggests that the expression of MMS22L-NFKBIL2 complex may positively regulate the NFKB pathway. Subsequently, we attempted to examine the effect of endogenous MMS22L expression on the NFKB pathway molecules using cytoplasmic and nuclear fraction of HeLa and LC319 cells that were treated with TNF-α. We first confirmed that the level of RelA/p65 was increased in the nucleus of the cells by TNF-α stimulation (data not shown), but that of endogenous RelA/p65 protein was decreased in these cells transfected with siRNA for *MMS22L* (si-*MMS22L*) after TNF-α treatment, compared to cells with control siRNA (si-LUC) (Fig. 6a). We then examined the relationship between MMS22L protein and downstream molecules of RelA/p65 such as Bcl-XL and TRAF1/2 that were the anti-apoptosis factors. When we treated the si-LUC-transfected HeLa cells with TNF-α, Bcl-XL and TRAF1 were increased in accordance with the elevation of RelA/p65 (Fig. 6b). However, the elevation of p65, Bcl-XL and TRAF1 were not detected in the TNF-α-stimulated cells transfected with si-*MMS22L*. The expression level of MMS22L protein showed good correlation with those of p65, Bcl-XL and TRAF1 proteins in lung cancer cell lines (data not shown).

To further examine the effect of MMS22L expression on apoptosis pathway in cancer cells, we cultured cancer cells that were transfected with si-*MMS22L* under DNA damage condition using DNA-damaging agents (cisplatin/CDDP or 5-fluorouracil/5-FU). After knockdown of *MMS22L* expression with si-*MMS22L* in HeLa cells, we treated the cells with CDDP (50 μg/ml) or 5-FU (50 μg/ml) for 48 h and harvested the cells for flow cytometric analysis. The sub G1 population of the cells which were transfected with si-*MMS22L* was significantly increased compared with those with control siRNA (si-LUC) under DNA damage condition (data not shown). When we exposed the cells that were transfected with si-*MMS22L* or si-LUC with 20 J of ultraviolet for 48 h, the similar results were observed (data not shown). Western blot analysis using the HeLa cells which were transfected with si-*MMS22L* or si-LUC, and subsequently treated with CDDP as mentioned above revealed that induction of DNA repair molecules such as ATM, CSB and p53 as well as RelA/p65 and its downstream anti-apoptosis factor Bcl-XL were significantly suppressed in the cells transfected with si-*MMS22L* compared with those transfected with si-LUC (Fig. 6c). The data suggest that MMS22L can function as an upstream molecule of these anti-apoptosis factors and also affect the induction of some DNA repair pathway molecules (Fig. 6d).

Discussion

Despite the recent development of surgical techniques combined with various treatment modalities such as radiotherapy and chemotherapy, clinical outcome of lung and esophageal cancer patients still remains poor. Therefore, development of new types of anticancer drugs is eagerly awaited. To identify novel target molecules for drug development, we combined genome-wide expression profile analysis of genes that were overexpressed in lung and esophageal cancer cells with high-throughput screening of loss-of-function effects by means of the RNAi technique and tumor tissue microarray analysis (5-41). Through this systematic approach we found *MMS22L* to be upregulated frequently in clinical lung and esophageal cancer samples, and showed that this gene product plays an indispensable role in the growth and/or survival of cancer cells.

We demonstrated that MMS22L is a putative oncogene and that its nuclear localization and stabilization was enhanced by binding to NFKBIL2. In addition, we revealed that introduction of the C-terminal portion of NFKBIL2 protein into cancer cells could dominant-negatively inhibit the nuclear localization of MMS22L possibly by blocking the MMS22L-NFKBIL2 interaction, and resulted in the suppression of cancer cell growth/survival. Furthermore, transfection of siRNAs against MMS22L or NFKBIL2 into cancer cells suppressed their expression and the cell growth. Therefore, inhibition of the MMS22L-NFKBIL2 interaction or suppressing MMS22L protein function can be an effective approach for development of novel cancer therapy.

To date, NFKB transcription factors are known to be the key regulators of immune, inflammatory and acute phase responses, and to be involved in the control of cell proliferation and apoptosis (42-45). Activation of NFKB activity and consequent induction of its downstream genes lead to the oncogenesis in mammalian cells. MMS22L protein appeared to act as an upstream molecule of RelA/p65 and be indispensable for induction of anti-apoptosis factors, Bcl-XL or TRAF1. Further studies on the regulation and function of MMS22L protein will contribute to the understanding of molecular mechanism of carcinogenesis through the activation of MMS22L and NFKB pathway.

In cancer chemotherapy, many kinds of DNA damaging agents are being used. The most common approach for targeting the cell cycle is to exploit the effect of DNA-damaging chemotherapeutic agents like 5-FU or CDDP, whose effects are mediated through diverse intracellular targets inducing apoptosis in various cancer cells (50). However, the toxicity of DNA-damaging drugs can be diminished by the activities of several DNA repair pathways as well as anti-apoptotic factors. Therefore, inhibitors of specific DNA repair and/or anti-apoptotic pathways might be promising therapeutic strategy for novel cancer treatments which can improve the efficacy of DNA damage-based cancer therapy (50). Our data suggested the involvement of MMS22L in cellular response to DNA damaging agents. In fact, knockdown of MMS22L expression also enhanced the apoptosis of cancer cells that were exposed to DNA-damaging agents including 5-FU and CDDP probably due to inhibition of induction of DNA repair molecules such as ATM, CSB and p53 as well as RelA/p65 and its downstream anti-apoptosis factor Bcl-XL. The combined data of our experiments suggest that MMS22L might function as an upstream molecule of these anti-apoptosis factors and DNA-repair molecules and that targeting MMS22L could

have a significant advantage in avoiding the resistance of cancer cells to anticancer treatments, although the detailed function of MMS22L in drug response of the cells and in carcinogenesis remains to be elucidated.

In summary, our data indicate that MMS22L is involved in NFKB pathway in cancer cells through its interaction with NFKBIL2 and that it might be a promising candidate target for developing highly specific anticancer drugs with minimal risk of adverse effects.

Acknowledgements

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Cancer Research

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Molecular and Cellular Pathobiology

Cancer Research

Critical Function for Nuclear Envelope Protein TMEM209 in Human Pulmonary Carcinogenesis

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Abstract

Therapeutic targets for more effective and less toxic treatments of lung cancer remain important. Here we report the identification of the integral nuclear envelope protein TMEM209 as a critical driver of human lung cancer growth and survival. TMEM209 expression was normally limited to testis, but we found that it was widely expressed in lung cancer, in which it localized to the nuclear envelope, Golgi apparatus, and the cytoplasm of lung cancer cells. Ectopic overexpression of TMEM209 promoted cell growth, whereas TMEM209 attenuation was sufficient to block growth. Mass spectrometric analysis identified the nucleoporin protein NUP205 as a TMEM209-interacting protein, stabilizing NUP205 and increasing the level of c-Myc in the nucleus. Taken together, our findings indicate that TMEM209 overexpression and TMEM209–NUP205 interaction are critical drivers of lung cancer proliferation, suggesting a promising new target for lung cancer therapy. *Cancer Res; 72(16); 4110–8. ©2012 AACR.*

Introduction

Lung cancer is one of the leading causes of death in the worldwide (1). Many genetic alterations associated with development and progression of lung cancers have been reported and contributed to the better understanding of the molecular mechanisms of pulmonary carcinogenesis (2). However, despite some advances in the early detection and recent improvements in its treatment, the prognosis of the lung cancer patients is not much improved. Over the last few decades, several newly developed cytotoxic agents such as paclitaxel, docetaxel, gemcitabine, and vinorelbine have begun to offer multiple choices for treatment of patients with advanced lung cancer, but each of those regimens confers only a modest survival benefit compared with cisplatin-based therapies (3–5). In addition to these cytotoxic drugs, several molecular targeted agents, such as monoclonal antibodies against VEGF (i.e., bevacizumab/anti-VEGF) or epidermal growth factor receptor (EGFR: i.e., cetuximab/anti-EGFR) as well as inhibitors for EGFR tyrosine kinase (i.e., gefitinib and erlotinib) and anaplastic lymphoma kinase (i.e., crizotinib) were developed and are applied in clinical practice (6, 7). However, each of the new regimens can provide survival benefits to a small subset of the patients and cause serious adverse effects, such as interstitial pneumonia and acute lung injury (8). Hence, the development of molecular targeted agents providing better clinical benefits without such adverse reactions is eagerly required.

To isolate potential molecular targets for diagnosis, treatment, and/or prevention of lung cancer, we had carried out genome-wide expression profile analysis of tumor tissues from 120 lung cancer cases by means of a cDNA microarray consisting of 27,648 genes or expressed sequence tags (EST; refs. 9–14). Among genes that were commonly transactivated in clinical lung tumor cells, we here focus on TMEM209 (Transmembrane protein 209) as a potential therapeutic target for lung cancer. TMEM209 was initially identified as an integral protein of nuclear envelope in mouse liver by high-throughput shotgun proteomics using multidimensional protein identification technology (15). However, the pathophysiologic roles or biologic functions of TMEM209 in human cancer have not been reported. Here we report that TMEM209 is indispensable for cancer cell growth and/or survival and regulate nuclear levels of c-Myc protein through its interaction with nuclear pore complex (NPC) component NUP205. Targeting TMEM209 and/or TMEM209–NUP205 interaction could be a promising therapeutic strategy for lung cancer therapy.

Materials and Methods

Lung cancer cell lines and tissue samples

The human lung cancer cell lines used in this study were as follows: lung adenocarcinoma cell lines A549, LC319, PC14, NCI-H1373, and NCI-H1781; lung squamous cell carcinoma cell lines SKMES-1, LU61, NCI-H520, NCI-H1703, and NCI-H2170; small cell lung carcinoma cell lines DMS114, DMS273, SBC-3, and SBC-5, and a large cell carcinoma cell line LX1 (Supplementary Table S1). All cells were grown in

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monolayers in appropriate medium supplemented with 10% fetal calf serum and were maintained at 37°C in atmospheres of humidified air with 5% CO_2. Human small airway epithelial cells were grown in optimized medium purchased from Cambrex BioScience, Inc. Primary lung cancer tissue samples had been obtained with informed consent as described previously (10, 14). This study and the use of all clinical materials were approved by individual institutional ethical committees.

Semiquantitative reverse transcription PCR

Total RNA was extracted from cultured cells using the TRIzol reagent (Life Technologies, Inc.) according to the manufacturer's protocol. Extracted RNAs were treated with DNase I (Nippon Gene) and reversely transcribed using oligo (dT) primer and SuperScript II. Semiquantitative reverse transcription PCR (RT-PCR) experiments were carried out with the following synthesized specific primers for *TMEM209*, *NUP205*, *CDC25A*, *CDK1*, or β-actin (*ACTB*) as follows: *TMEM209*, 5′-GCAGACTCACTAAAGTATCCCCA-3′ and 5′-CTCCATGGTGCTTTTAATGAAG-3′; *NUP205*, 5′-GAAACTTCTGGACATTGAAGGA-3′ and 5′-TGAGGATGGAACTAGGGGAAG-3′; *CDC25A*, 5′-TGAGGTGTAGGTGGGTTTTT-3′ and 5′-GCCATCCCACCTTTCTCTTT-3′; *CDK1*, 5′-ACCACTTTTCCATGGGGAT-3′ and 5′-TGGATGATTCAGTGCCATTT-3′; *ACTB*, 5′-GAGGTGATAGCATTGCTTTCG-3′, and 5′-CAAGTCAGTGTACAGGTAAGC-3′. PCR reactions were optimized for the number of cycles to ensure product intensity within the logarithmic phase of amplification.

Northern blot analysis

Human multiple tissue blots (BD Biosciences, Clontech) were hybridized with ^{32}P-labeled PCR products of *TMEM209* and *NUP205*. The cDNA probes of *TMEM209* and *NUP205* were prepared by RT-PCR using following primers: *TMEM209*, 5′-AACACTTAGATTAAATTTAG-3′ and 5′-GCTCCTTTCCTTTGGACATC-3′; *NUP205*, 5′-GCGCCCAGAAACGGACCCGC-3′ and 5′-ACTGTTTCTGAAAGGCTAGG-3′. Prehybridization, hybridization, and washing were done according to the supplier's recommendations. The blots were autoradiographed at −80°C for 14 days with intensifying BAS screens (Bio-Rad).

Western blotting

Whole cells were lysed with NP-40 buffer [150 mmol/L NaCl, 0.5% NP-40, 50 mmol/L Tris-HCl (pH 8.0)] containing Protease Inhibitor Cocktail Set III and Phosphatase Inhibitor Cocktail Set II (Calbiochem). Protein fractionation was carried out with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo). Protein samples were separated by SDS-polyacrylamide gels and electroblotted onto Hybond-ECL nitrocellulose membranes (GE Healthcare Bio-Sciences). Blots were incubated with either of antibodies to TMEM209 (catalog no. HPA031678; ATLAS Antibodies), NUP205 (catalog no. HPA024574; ATLAS Antibodies), c-Myc (catalog no. sc-40; Santa Cruz), Flag (catalog no. F3165; Sigma), or ACTB (catalog no. A5316; Sigma). Antigen–antibody complexes were detected using secondary antibodies conjugated to horseradish peroxidase (GE Healthcare Bio-Sciences). Protein bands were visualized by enhanced chemiluminescence Western blotting detection reagents (GE Healthcare Bio-Sciences).

Immunofluorescence analysis

Cells were plated onto glass coverslips (Becton Dickinson Labware), fixed with 4% paraformaldehyde, and permeablilized with 0.1% Triton X-100 in PBS for 5 minutes at room temperature. Nonspecific binding was blocked by 5% Skim milk for 30 minutes at room temperature. Cells were then incubated for 60 minutes at room temperature with primary antibodies for anti-TMEM209 antibody (catalog no. HPA031678; ATLAS Antibodies), anti-c-Myc antibody (catalog no. sc-40; Santa Cruz) or anti-Golgi 58K Protein/Formiminotransferase Cyclodeaminase antibody (catalog no. G2404; Sigma) diluted in PBS containing 1% bovine serum albumin. After being washed with PBS, the cells were stained by Alexa Fluor 488–conjugated or Alexa Fluor 594–conjugated secondary antibody (Molecular Probes) for 60 minutes at room temperature. After another wash with PBS, each specimen was mounted with Vectashield (Vector Laboratories, Inc.,) containing 4′,6′-diamidine-2′-phenylindolendihydrochrolide (DAPI) and visualized with Spectral Confocal Scanning Systems (TSC SP2 AOBS; Leica Microsystems).

RNA interference assay

To evaluate the biologic functions of TMEM209 and NUP205 in lung cancer cells, we used short interfering RNA (siRNA) duplexes against the target genes (Sigma). The target sequences of the synthetic oligonucleotides for RNA interference were as follows: control-1: [*EGFP*, enhanced GFP (eGFP) gene, a mutant of *Aequorea gictoria* GFP], 5′-GAAGCAGCACGACUUCUUC-3′; control-2 (*LUC*, luciferase gene from *Photinus pyralis*), 5′-CGUACGCGGAAUACUUCGA-3′; si-TMEM209-#1, 5′-CUACGAACUUUGGAUACUU-3′; si-TMEM209-#2, 5′-GUGUGAAUAUAUUGUGGAU-3′ si-NUP205, and 5′-CUCUCUACCUGUUGGGCUU-3′. Lung cancer cells, LC319, SBC-3, and SBC-5, were plated onto 10-cm dishes and transfected at subconfluent condition with either of the siRNA oligonucleotides (50 μmol/L) using 30 μL of Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions. After 7 days of incubation, these cells were stained by Giemsa solution to assess colony formation, and cell numbers were measured by MTT assay; briefly, cell-counting kit-8 solution (Dojindo) was added to each dish at a concentration of 1/10 volume, and the plates were incubated at 37°C for additional 30 minutes. Absorbance was then measured at 490 nm, and at 630 nm as a reference, with a Microplate Reader 550 (Bio-Rad).

Flow cytometry

Cells were collected in PBS and fixed in 70% cold ethanol for 30 minutes. After treatment with 100 μg/mL of RNase (Sigma), the cells were stained with 50 μg/mL propidium iodide (Sigma) in PBS. Flow cytometry was analyzed by using FACScan (Beckman Coulter). The cells selected from at least 20,000 ungated cells were analyzed for DNA content.

Cell growth assay

We cloned the entire coding sequence of *TMEM209* into the appropriate site of COOH-terminal Flag-tagged pCAGGS

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plasmid vector. COS-7 and SBC-3 cells transfected either with plasmid expressing Flag-tagged TMEM209 or mock plasmid were grown for 7 days in Dulbecco's Modified Eagle's Medium (COS-7) or RPMI (SBC-3) containing 10% fetal calf serum in the presence of appropriate concentrations of geneticin (G418). Cell viability was evaluated by MTT assay.

Coimmunoprecipitation and matrix-assisted laser desorption/ionizing–time of flight mass spectrometry mapping of TMEM209-associated proteins

Cell extracts from a lung cancer SBC-5 cell, which was transfected with TMEM209 expression vector or mock vector, were precleared by incubation at 4°C for 1 hour with 80 μL of protein G–agarose beads in a final volume of 200 μL of immunoprecipitation buffer (0.5% NP-40, 50 mmol/L Tris-HCl, 150 mmol/L NaCl) in the presence of Protease Inhibitor Cocktail Set III (Calbiochem). After centrifugation at 1,000 rpm for 5 minutes at 4°C, the supernatants were incubated at 4°C with anti-Flag M2 agarose (catalog no. A2220; Sigma) for 1 hour. The beads were then collected by centrifugation at 5,000 rpm for 1 minute and washed 6 times with 1 mL of immunoprecipitation buffer. The washed beads were resuspended in 30 μL of Laemmli sample buffer and boiled for 5 minutes, and the proteins were separated using 5% to 20% gradient SDS-PAGE gel (Bio-Rad). After electrophoresis, the gel was stained with SilverQuest (Invitrogen). Protein bands specifically found in extracts from the cells that were transfected with TMEM209 vector were excised and served for matrix-assisted laser desorption/ionization–time of flight mass spectrometry analysis (AXIMA-CFR plus; Shimadzu Biotech).

Immunoprecipitation assay

Cell extracts from lung cancer SBC-5 cells were precleared by incubation at 4°C for 1 hour with 80 μL of protein G–agarose beads in a final volume of 200 μL of immunoprecipitation buffer (0.5% NP40, 50 mmol/L Tris-HCl, 150 mmol/L NaCl) in the presence of Protease Inhibitor Cocktail Set III (Calbiochem). After centrifugation at 1,000 rpm for 5 minutes at 4°C, the supernatants were incubated at 4°C with normal rabbit IgG (catalog no. sc-2027; Santa Cruz) or anti-TMEM209 antibody (catalog no. 06-1020; Millipore) for overnight. The beads were then collected by centrifugation at 5,000 rpm for 1 minute and washed 6 times with 1 mL of immunoprecipitation buffer. The washed beads were resuspended in 30 μL of Laemmli sample buffer and boiled for 5 minutes. Then the proteins were separated using SDS-PAGE gel. The following procedure was carried out as described above.

Protein synthesis and proteasome inhibitors

Protein synthesis inhibitor, cycloheximide (Calbiochem) was dissolved in ethanol and added in culture medium at 100 μg/mL. Proteasome inhibitor, MG132 (Synonym: Z-Leu-Leu-Leu-al; Sigma) was dissolved in dimethyl sulfoxide and added in culture medium at 20 μmol/L.

Quantitative real-time PCR

Quantitative real-time PCR was conducted with the SYBR Green I Master Kit on a LightCycler 480 (Roche) according to the manufacturer's recommendations. Each experiment was done in triplicate. *GAPDH* was used for normalization of expression levels. cDNAs as templates were synthesized as described above. For quantitative RT-PCR reactions, specific primers for all human *TMEM209*, *CDC25A*, *CDK1*, and *GAPDH* were designed as follows: *TMEM209*, 5′-TCGCCCGTCAGTGGTTAT-3′ and 5′-CCAACAGTGGTAGGGTACGG-3′; *CDC25A*, 5′-ATCTCTTCACACAGAGGCAGAA-3′ and 5′-CCCTGGTTCACTGCTATCTCTT-3′; *CDK1*, 5′-TGGATCTGAAGAAATACTTGGATTCTA-3′ and 5′-CAATCCCCTGTAGGATTTGG-3′; *GAPDH*, 5′-GCAAATTCCATGGCACCGTC-3′ and 5′-TCGCCCCACTTGATTTTGG-3′.

Result

TMEM209 expression in lung cancers and normal tissues

To identify novel target molecules for the development of therapeutic agents and/or diagnostic biomarkers of lung cancer, we had previously carried out gene expression profile analysis of 120 lung carcinomas using cDNA microarray containing 27,648 genes or expressed sequence tags (9–14). We identified *TMEM209* that showed 3-fold or higher level of expression in the majority of 120 lung cancer samples examined and confirmed its transactivation by semiquantitative RT-PCR experiments in 10 of 15 additional lung cancer tissues and in 12 of 15 lung cancer cell lines (Figs. 1A and 1B). We also confirmed, by Western blotting analysis, high levels of TMEM209 (63 kDa) expression in lung cancer cell lines using anti-TMEM209 antibody (Fig. 1C). To examine the subcellular localization of endogenous TMEM209 in cancer cells, we carried out immunocytochemical analysis of lung cancer SBC-5 cells that overexpressed the endogenous TMEM209 protein using anti-TMEM209 antibody. TMEM209 was detected on the nuclear envelope and the Golgi apparatus and weakly in cytoplasm (Fig. 1D). Northern blot analysis with a *TMEM209* cDNA as a probe identified a 3.5-kb transcript specifically in the testis among 16 normal human tissues examined (Fig. 1E).

Inhibition of growth of lung cancer cells by siRNA against TMEM209

To assess whether TMEM209 is essential for growth or survival of lung cancer cells, we transfected synthetic oligonucleotide siRNAs against TMEM209 into lung adenocarcinoma LC319 and small cell lung cancer SBC-5 cells in which TMEM209 was highly expressed. The protein levels of TMEM209 in the cells transfected with si-TMEM209-#1 or -#2 were significantly decreased in comparison with cells transfected with either of control siRNAs (Figs. 2A and B). We also observed significant decrease in the number of colonies and the number of viable cells measured by MTT assay (Figs. 2C–F). On the other hand, we examined the effects of these siRNAs on the lung cancer SBC-3 cells in which endogenous TMEM209 was hardly detectable. MTT assay revealed that the viability of cells treated with TMEM209 siRNAs (#1, 2) was equivalent to that treated with either of control siRNAs (si-EGFP or LUC), indicating that suppression of cancer cell growth by treatment of TMEM209-specific siRNAs was not likely to be off-target effects (Fig. 2G). To further assess the

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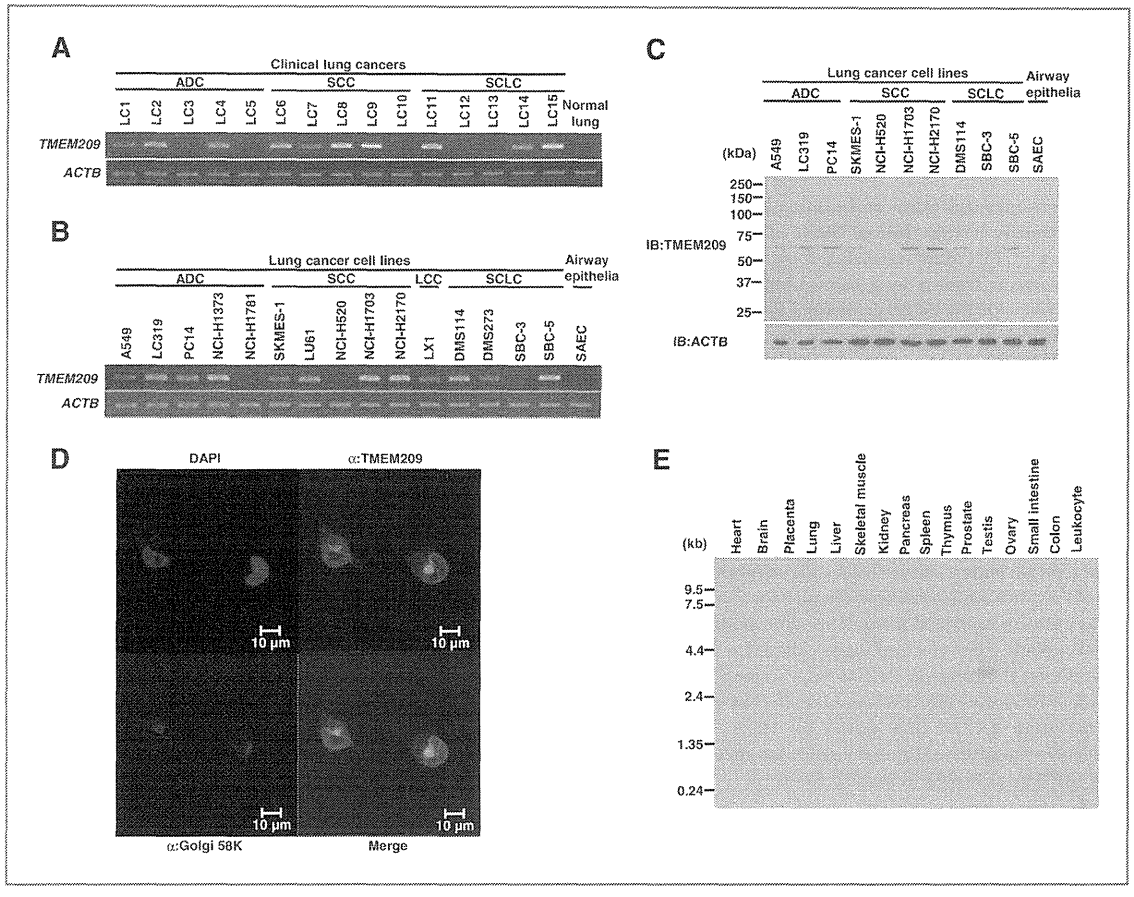

Figure 1. TMEM209 expression in lung cancers and normal tissues. A, expression of *TMEM209* in clinical samples of non-small cell lung carcinoma and small cell lung carcinoma (SCLC) and normal lung tissues analyzed by semiquantitative RT-PCR. Appropriate dilutions of each single-stranded cDNA generated from mRNAs of lung cancer samples were prepared, using β-actin (*ACTB*) expression as a quantitative control. B, expression of *TMEM209* in lung cancer cell lines examined by semiquantitative RT-PCR. C, expression of TMEM209 protein in lung cancer cell lines examined by Western blot analysis. D, subcellular localization of endogenous TMEM209 protein in lung cancer SBC-5 cells. E, expression of *TMEM209* in normal human tissues detected by Northern blot analysis. ADC, adenocarcinoma; SCC, small cell carcinoma; LCC, large cell carcinoma; SAEC, small airway epithelial cells; IB, immunoblotting.

knockdown effect of *TMEM209*, we carried out flow cytometric analysis and found the G_1 arrest of SBC-5 cells transfected with siRNA against *TMEM209* (Fig. 2H).

Growth-promoting effect of TMEM209

To further clarify a potential role of TMEM209 in carcinogenesis, we constructed plasmid vector (pCAGGS vector) expressing TMEM209 with a Flag tag at a C-terminal (TMEM209-Flag). We then transfected TMEM209-Flag vector or mock plasmid into COS-7 and SBC-3 cells, in which endogenous TMEM209 was expressed at a very low level and carried out cell growth assay. We detected the growth-promoting effect of cells overexpressing TMEM209, compared with those transfected with mock vector (Figs. 2I–L).

Interaction of TMEM209 with NUP205

To elucidate the function of TMEM209, we screened a protein(s) that interacts with TMEM209 in cancer cells. Lysates of lung cancer SBC-5 cells, which were transfected with Flag-tagged TMEM209 expression vector or mock vector, were extracted and immunoprecipitated with anti-Flag M2 agarose. The protein complex was stained with SilverQuest on the SDS-PAGE gel (Supplementary Fig. S1A). A 205-kDa band, which was observed in cell lysates transfected with TMEM209 vector, but not in those with mock vector, was successfully characterized by peptide sequencing to be a human NUP205 (Nucleoporin 205 kDa). We subsequently confirmed the interaction between endogenous TMEM209 and endogenous NUP205 in SBC-5 cells by immunoprecipitation with anti-TMEM209 antibody and subsequent immunoblotting with anti-NUP205 antibody (Fig. 3A). NUP205 is a part of subcomplex of the NPC embedded in nuclear pore and is considered as a scaffold nucleoporin that is important for the overall integrity of the NPC. In eukaryotic cells, the spatial segregation of replication and transcription in the nucleus and translation in the cytoplasm requires transport of thousands of macromolecules,

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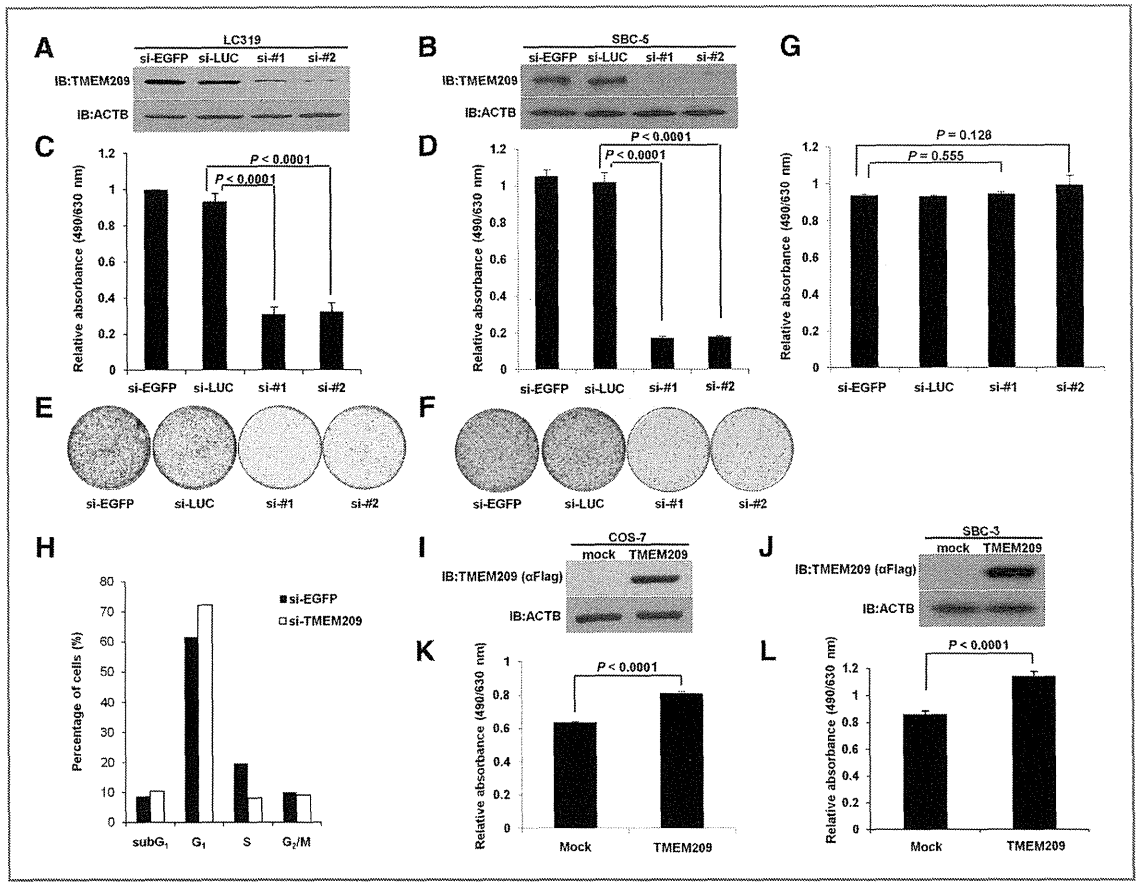

Figure 2. Growth effect of TMEM209 expression. A and B, expression of TMEM209 by the treatment with si-TMEM209 (si-#1 or si-#2) or control siRNAs (si-EGFP or si-LUC) in LC319 cells and SBC-5 cells analyzed by Western blot analysis. C and D, viability of LC319 cells and SBC-5 cells evaluated by MTT assay by the treatment with si-TMEM209 (si-#1 or si-#2), si-EGFP, or si-LUC. All assays were done in triplicate and in triplicate wells. E and F, colony formation assays of LC319 cells and SBC-5 cells transfected with si-TMEM209 (si-#1 or si-#2) or control siRNAs. G, viability of SBC-3 cells evaluated by MTT assay after treatment with si-TMEM209 (si-#1 or si-#2), si-EGFP, or si-LUC. H, cell-cycle population change in SBC-5 cells after treatment of siRNA against TMEM209. I and J, transient expression of TMEM209 in COS-7 and SBC-3 cells detected by Western blot analysis. K and L, assays showing the growth-promoting effect of transient introduction of TMEM209 in COS-7 and SBC-3 cells. Assays were done in triplicate and in triplicate wells. IB, immunoblotting.

including DNAs, RNAs, and proteins between these 2 compartments. NPCs are the gateways that facilitate this transport across the nuclear envelope in co-operation with soluble transport receptors and play a crucial and essential role in cellular event (16). We found *NUP205* expression in lung cancers, but not in normal tissues such as lung, liver, kidney, heart, and brain (Fig. 3B). Northern blot analysis with *NUP205* as a probe identified a 6.3-kb transcript in testis among 16 tissues examined, indicating that both TMEM209 and NUP205 are likely to be cancer testis antigens (Fig. 3C). To assess the functional relationship between TMEM209 and NUP205, we examined the NUP205 protein level after inhibition of TMEM209 expression by siRNA treatment in SBC-5 cells. We transfected siRNA oligonucleotides against TMEM209 (si-TMEM209) or control siRNAs (si-EGFP) into SBC-5 cells and at 24 hours after treatment with siRNAs, incubated SBC-5 cells in growth medium supplemented with protein synthesis inhibitor cycloheximide and monitored endogenous NUP205 protein levels in cells transfected with si-TMEM209 or si-EGFP. We observed the knockdown of *TMEM209* transcription in SBC-5 cells transfected with si-TMEM209, although no effect on *NUP205* transcription was observed. However, NUP205 protein levels were significantly reduced in a time-dependent manner, suggesting that the NUP205 protein was likely to be stabilized by its interaction with TMEM209 (Figs. 3D and E).

Nuclear c-Myc levels may be regulated by TMEM209–NUP205 complex

Previous reports for large-scale mapping of human protein–protein interactions by mass spectrometry suggested NUP205 to interact with c-Myc, an oncogenic transcription factor (17, 18). Therefore, we investigated the interaction between NUP205 and c-Myc in lung cancer cells using lysates of SBC-5 cells, which were transfected with Flag-tagged c-Myc

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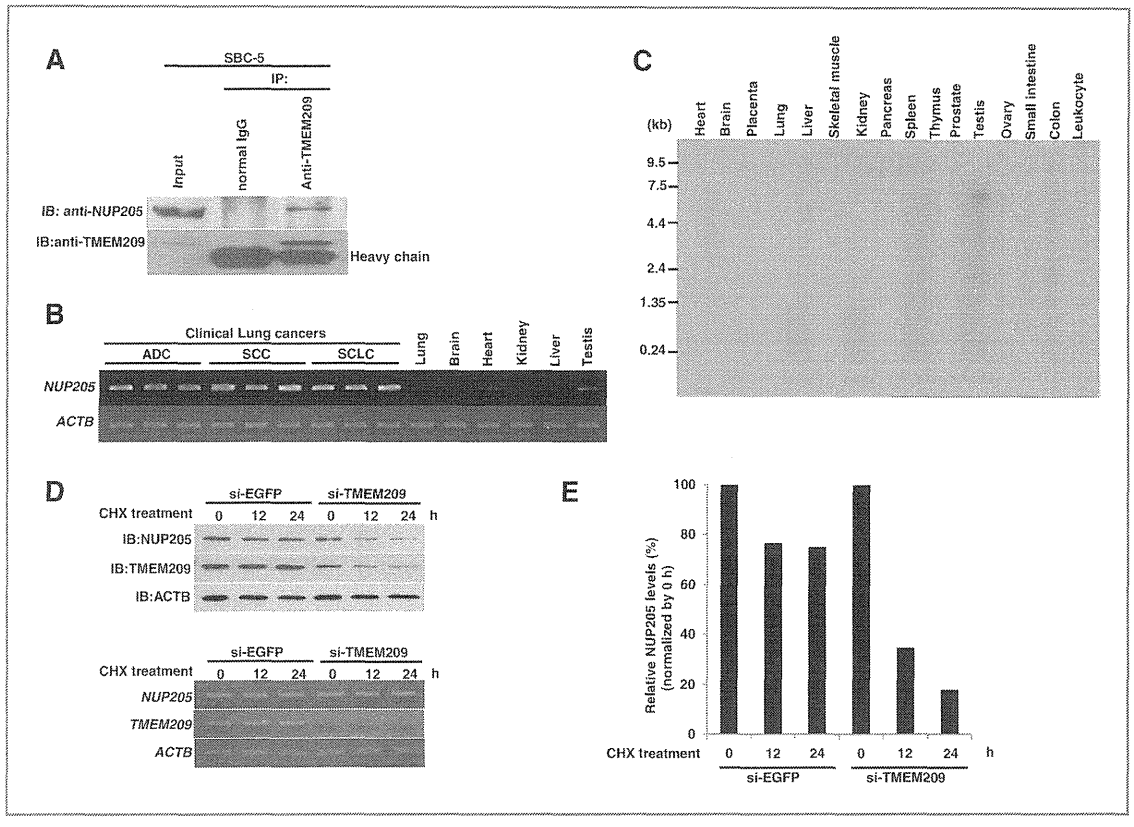

Figure 3. Interaction of TMEM209 with NUP205. A, interaction of endogenous TMEM209 with NUP205. Lysates of SBC-5 cells were immunoprecipitated with TMEM209 antibody. Precipitated proteins were separated by SDS-PAGE and Western blot analysis was carried out with NUP205 antibody. B, expression of *NUP205* in clinical samples of non-small cell lung carcinoma and small cell lung carcinoma (SCLC) and in normal tissues, analyzed by semiquantitative RT-PCR. C, expression of *NUP205* in normal human tissues detected by Northern blot analysis. D, the levels of TMEM209 and NUP205 proteins as well as *TMEM209* and *NUP205* mRNAs detected by Western blot analysis and semiquantitative RT-PCR analysis in SBC-5 cells that had been transfected with si-TMEM209 after the treatment with cycloheximide (CHX). E, relative NUP205 protein levels at each time point (0, 12, and 24 hours) quantified by imageJ software and normalized by its levels before the treatment with protein synthesis inhibitor cycloheximide (0 hour). IB, immunoblotting; IP, immunoprecipitation; ADC, adenocarcinoma; SCC, small cell carcinoma.

expression vector or mock vector. Immunoprecipitation of the cell lysates with anti-Flag M2 agarose and subsequent immunoblotting with anti-NUP205 antibody confirmed their interaction (Supplementary Fig. S1B). Because NUP family members are known to regulate nucleocytoplasmic transport of macromolecules, we assessed the effect of TMEM209–NUP205 complex on c-Myc protein localization by fractionating cell lysates to cytoplasm and nucleus. Suppression of TMEM209 or NUP205 expression by siRNAs against TMEM209 or NUP205 seemed to reduce the levels of nuclear c-Myc protein (Supplementary Fig. S2A and B). Considering that c-Myc protein stability is strictly regulated by ubiquitin–proteasome system (18), we then treated SBC-5 cells, which had been transfected with siRNAs against TMEM209 or NUP205, with proteasome inhibitor MG132. The amount of the whole c-Myc protein was not changed, whereas the level of cytoplasmic c-Myc protein was elevated and that of nuclear c-Myc was reduced in the cells treated with si-TMEM209 or si-NUP205 (Fig. 4A). These data suggested that TMEM209 and NUP205 are involved in regulation of the nuclear transport of c-Myc. Furthermore, we examined the effects of other nuclear proteins, STAT3 and p65, which were reported to translocate from the cytoplasm to the nucleus in human cancer cells. The total amounts of STAT3 and p65 proteins as well as those in the nucleus were reduced in cells transfected with si-TMEM209 or si-NUP205 (Supplementary Fig. S3). The data implied that TMEM209–NUP205 complex is associated with the nuclear import of not only c-Myc but also some nuclear proteins. To further examine whether c-Myc transcription activity could be inhibited after the knockdown of *TMEM209*, we measured the expression levels of representative c-Myc target genes, *CDC25A* and *CDK1*, which are highly expressed in lung cancers and are reported to be involved in carcinogenesis (Supplementary Fig. S4). Suppression of *TMEM209* by siRNAs reduced the expression levels of *CDC25A* and *CDK1* in LC319 and SBC-5 cells, as detected by quantitative real-time PCR (Fig. 4C). These data indicated that overexpression of TMEM209–NUP205 complex proteins may prompt the nuclear transport of c-Myc and result in

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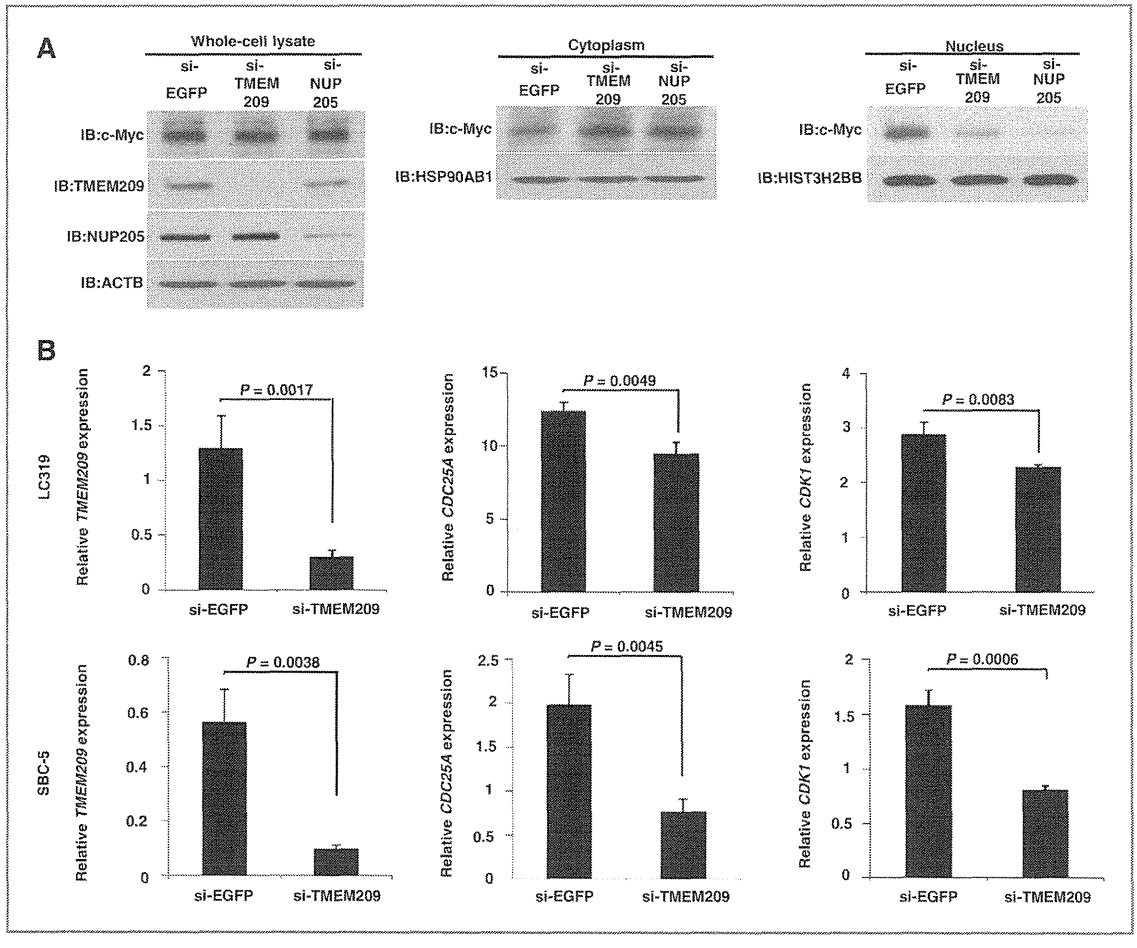

Figure 4. Nuclear levels of c-Myc may be regulated by TMEM209–NUP205 complex. A, attenuation of nuclear levels of c-Myc protein in SBC-5 cells transfected with si-TMEM209 or si-NUP205. B, the downregulation of c-Myc target genes, *CDC25A* and *CDK1*, in LC319 and SBC-5 cells after the knockdown of TMEM209 expression with siRNAs. IB, immunoblotting.

overexpression of oncogenic c-Myc target genes, such as *CDC25A* and *CDK1*.

Discussion

Recent advances in the study of the biologic mechanisms underlying cancer development have caused the paradigm shift in designing and developing a new type of therapeutic drug, termed "molecular targeted drug," that selectively interferes with molecules or pathways involved in tumor growth and/or progression. Inactivation of growth factors and their receptors in tumor cells as well as the inhibition of oncogenic pathways or specific functions in cancer cells constitutes the main rationale of novel cancer treatments (19). Molecular targeted cancer therapies are expected to treat cancer cells more selectively than normal cells, thus to be less harmful to normal cells, to reduce side effects, and to improve quality of life of cancer patients. Intensive studies to screen molecular targets for development of novel drugs identified a number of possible candidates that can be applicable for novel lung cancer therapies. However, suppression of some of such molecules also caused serious adverse reactions *in vivo* because of expression of molecules in certain types of normal tissue and/or off-target effects of compounds on nontarget molecules. Hence, the specificity of molecules in cancer cells as well as the selectivity of compounds to a certain target should be critical to develop drugs with high efficacy and minimum toxicity.

To screen more appropriate molecular targets for the drug development, we had analyzed the whole-genome expression profiles of 120 clinical lung cancer samples using cDNA microarray data containing 27,648 genes or ESTs (10–14) and investigated loss-of-function phenotypes by RNA interference systems (20–46). On the basis of this approach, we found TMEM209 to be overexpressed in the majority of clinical lung cancer cases as well as lung cancer cell lines, although its expression was hardly detectable in normal tissues, except the testis. Furthermore, we showed that the knockdown of

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TMEM209 expression resulted in inhibition of cancer cell growth, whereas transient expression of TMEM209 resulted in the significant promotion of cell growth. The data suggested that TMEM209 plays indispensable roles in the growth of lung cancer cells, indicating that TMEM209 could serve as a target for the development of anticancer agents for lung cancer, although further analysis of TMEM209 including mutational screening and/or epigenetic alteration of this gene should be required to fully address the significant role of this gene in pulmonary carcinogenesis.

TMEM209 is a 63-kDa transmembrane protein that contains a NPC component domain in its N terminus. Some proteins containing this domain are known to be components of the NPC. One member of this family is nucleoporin POM34 (budding yeast) that is thought to have a role in anchoring peripheral NUP family proteins into the pore and mediating pore formation (47). Our study also showed that TMEM209 interacted with NUP205, a component of NPC. NUP205 was identified as a component of NPC and to interact with NUP93 and NUP53 that are involved in the integrity of the NPC in *Xenopus* (48). To date, there is no report describing the involvement of TMEM209–NUP205 complex in human carcinogenesis. We also found overexpression of NUP205 in lung cancer cells and similarly to TMEM209, its expression was scarcely detectable in normal tissues except testis, suggesting that the TMEM209–NUP205 complex could be expressed specifically in lung cancer cells and testis. We also showed that TMEM209 regulated the NUP205 protein stability by its interaction. It was reported that NUP93 and NUP53 could interact with and stabilize NUP205 in Hela cells (49). Further analysis is necessary to verify the detailed relationship between the TMEM209–NUP205 complex and other NUP proteins, but it is likely that TMEM209 protein is indispensable for the function of NPC in cancer cells.

Our data also indicated that TMEM209–NUP205 complex could play important roles in nuclear levels of c-Myc and then influence to the c-Myc transcriptional activity. In *Drosophila*, Nup93 is able to preferentially interact with the phosphorylated and activated form of MAD (Human SMAD1 homolog) and could be directly involved in the nuclear import of MAD (50). One can speculate that in the process of shuttling molecules from cytoplasm to nucleus, the nuclear import of some oncogenic factors, including c-Myc protein, may be supported by the TMEM209–NUP205 complex. In fact, the amounts of STAT3 and p65 (a subunit of NF-κB) proteins were significantly reduced by the loss of TMEM209, indicating that the TMEM209–NUP205 complex is likely to be involved in nuclear transport of various nuclear proteins in addition to c-Myc.

In summary, human TMEM209 has an essential role in the growth of lung cancer cells through its interaction with NUP205 and regulation of the nuclear transport of c-Myc. Our data indicate that TMEM209 may be a good molecular target for the development of novel treatment for lung cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T. Fujitomo, Y. Daigo, Y. Nakamura
Development of methodology: T. Fujitomo, Y. Daigo, K. Ueda
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Fujitomo, Y. Daigo, K. Ueda
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Fujitomo, Y. Daigo, K. Ueda, Y. Nakamura
Writing, review, and/or revision of the manuscript: T. Fujitomo, Y. Daigo, Y. Nakamura
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Daigo
Study supervision: Y. Daigo, K. Matsuda, Y. Nakamura

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