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Thyroid transcription factor-1-regulated *microRNA-532-5p* targets *KRAS* and *MKL2* oncogenes and induces apoptosis in lung adenocarcinoma cells

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Key words

Characteristics and pathology of human cancer, microRNA/non-coding RNA respiratory organ, oncogenes and tumor-suppressor genes

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***Thyroid transcription factor-1 (TTF-1)*, also known as *NKX2-1*, plays a role as a lineage-survival oncogene in lung adenocarcinoma that possesses double-edged sword characteristics. Although evidence from previous studies has steadily accumulated regarding the roles of TTF-1 in transcriptional regulation of protein-coding genes, little is known about its regulatory relationship with microRNAs. Here, we utilized an integrative approach designed to extract maximal information from expression profiles of both patient tumors *in vivo* and TTF-1-inducible cell lines *in vitro*, which identified *microRNA (miR)-532-5p* as a novel transcriptional target of TTF-1. We found that *miR-532-5p* is directly regulated by TTF-1 through its binding to a genomic region located 8 kb upstream of *miR-532-5p*, which appears to impose transcriptional regulation independent of that of *CLCN5*, a protein-coding gene harboring *miR-532-5p* in its intron 3. Furthermore, our results identified *KRAS* and *MKL2* as novel direct targets of *miR-532-5p*. Introduction of *miR-532-5p* mimics markedly induced apoptosis in *KRAS*-mutant as well as *KRAS* wild-type lung adenocarcinoma cell lines. Interestingly, *miR-532-5p* showed effects on MEK-ERK pathway signaling, specifically in cell lines sensitive to siKRAS treatment, whereas those *miR-532-5p*-mediated effects were clearly rendered as phenocopies by repressing expression or inhibiting the function of *MKL2* regardless of *KRAS* mutation status. In summary, our findings show that *miR-532-5p* is a novel transcriptional target of TTF-1 that plays a tumor suppressive role by targeting *KRAS* and *MKL2* in lung adenocarcinoma.**

Lung cancer is the leading cause of cancer mortality, with more than 1.5 million deaths worldwide each year. Lung adenocarcinoma is the most prevalent subtype, accounting for approximately 50% of reported cases.[1] *KRAS* and *EGFR* are the most frequently and mutually exclusively mutated oncogenes in lung adenocarcinoma. *KRAS* mutations, thought to be an early event in molecular carcinogenesis, elicit persistent activation of downstream signaling pathways such as the RAF-MEK-ERK cascade, conferring increased proliferative capacity.[2] Although development of specific tyrosine kinase inhibitors has changed treatment strategies for patients with *EGFR* mutations,[3] *KRAS* has long been considered to be an "undruggable target", thus treatment of *KRAS*-mutant lung adenocarcinomas is a foremost clinical challenge.[4,5]

Thyroid transcription factor 1 (TTF-1), also known as NKX2-1, is indispensable for peripheral lung development and physiology, and it is used as a lineage marker for both normal and cancerous cells of the terminal respiratory unit of the lung.[6] In previous studies, we and others have found that *TTF-1* shows frequent gene amplification and overexpression, and also plays a crucial role as a lineage-survival oncogene in lung adenocarcinoma.[7–10] Furthermore, we have reported that ROR1 is a direct transcriptional target of TTF-1 that sustains a favorable balance between pro-survival and pro-apoptotic signaling in lung adenocarcinoma cells.[11,12] Interestingly, subsequent studies also revealed that TTF-1 possesses not only oncogenic, but also tumor suppressive functions, thus showing double-edged sword characteristics in cancer cells.[13]

MicroRNAs (miRNAs) are small RNA molecules of ~22 nt in length that repress gene expression by binding to a 3′-UTR of the target mRNA.[14] Following our discoveries of frequent occurrence of *let-7* downregulation and *miR-17-92* overexpression in lung cancer,[15,16] evidence for the involvement of various miRNAs in lung cancer pathogenesis has been rapidly accumulating.[17] However, little is known regarding TTF-1-mediated regulation of miRNAs, as previous studies of TTF-1 were nearly exclusively focused on transcriptional regulation of protein-coding genes.

In this study, we attempted to identify TTF-1-regulated miRNAs in lung adenocarcinoma specimens. To this end, we used

an integrative approach designed to extract information from expression profiles of lung adenocarcinoma patients *in vivo* as well as of TTF-1-inducible cell lines *in vitro* in a combinatorial fashion. We report here identification of *miR-532-5p* as a novel transcriptional target of TTF-1, and also show that *miR-532-5p* directly represses *KRAS* and *MKL2* and is capable of inducing apoptosis in lung adenocarcinoma cells.

Materials and Methods

Cell lines. The NCI-H23, NCI-H441, NCI-H1299, and NCI-H2009 lung adenocarcinoma cell lines were purchased from ATCC (Manassas, VA, USA), whereas PC-9 was obtained from RIKEN Cell Bank (Tsukuba, Japan). ACC-LC-319 and ACC-LC-94 lung adenocarcinoma cell lines were established by our group. An immortalized lung epithelial cell line, BEAS-2B, was a generous gift from Curtis C. Harris (National Cancer Institute, Bethesda, MD, USA). The conditions used to culture these cell lines have been previously reported.[18] Verification of all cell lines was carried out by short tandem repeat profiling at the Japanese Collection of Research Bioresources, National Institute of Biomedical Innovation of Japan (Osaka, Japan) in February 2015. All cell lines were confirmed to be absent of mycoplasma contamination (MycoAlert; Lonza, Tokyo, Japan).

DNA constructs. Full-length *TTF-1* cDNA was PCR-amplified from a pCMV-TTF-1 vector and inserted into a pTRE3G vector (Clontech, Shiga, Japan). A homeodomain deletion mutant of TTF-1 was generated from pCMV-TTF-1 using a KOD Plus Mutagenesis kit (Toyobo, Osaka, Japan). To generate a luciferase reporter construct, a 4549-bp fragment of the *KRAS* 3′-UTR and a 3501-bp fragment of the *MKL2* 3′-UTR were amplified from human genomic DNA (Promega, Tokyo, Japan) and cloned into a modified pGL3 vector (Promega). A KOD Plus Mutagenesis kit was then used to mutate four nucleotides in the *miR-532-5p* binding sites. For luciferase promoter assays, a 3158-bp fragment of the *CLCN5* promoter as well as a 2605-bp fragment of the *MIR532* promoter were amplified from human genomic DNA, and inserted into a pGL4.10 vector (Promega). Potential TTF-1 binding sites were then deleted using a KOD Plus Mutagenesis kit. The sequences of all primers used are listed in Table S1.

Small interfering RNA and miRNA mimics and inhibitors. The miRNA mimics, including pre-miR-532-5p (PM11553) and pre-miR-NC#2 (AM17111), were purchased from Ambion/Invitrogen (Grand Island, NY, USA). Cells were transfected with 5 nM miRNA mimics using RNAiMAX (Invitrogen). The siRNAs against *TTF-1*, *KRAS*, *MKL2*, and *SRF*, as well as a negative control siRNA, SIC002, were purchased from Sigma (St. Louis, MO, USA). For transfection experiments, 5 nM (*KRAS*, *MKL2*, and *SRF*) or 20 nM (*TTF-1*) was used. CCG-100602 and CCG-203971 were purchased from Cayman Chemical (Ann Arbor, MI, USA).

Generation of doxycycline-inducible TTF-1 cell lines. Doxycycline-inducible TTF-1 BEAS-2B, NCI-H23, NCI-H1299, and PC-9 cell lines were established by use of a Tet-On 3G Expression System (Clontech), essentially according to the manufacturer's instructions. In the resultant TTF-1-inducible cell lines, TTF-1 expression was induced in each experiment with 1 μg/mL DOX for various time periods.

Microarray analysis and TaqMan-based quantitative RT-PCR. Microarray analysis of 75 lung adenocarcinoma tumor tissues was carried out using a SurePrint G3 Human Gene Expression Microarray Kit (version 2) and GeneSpring (version 12.6), both from Agilent (Santa Clara, CA, USA), as previously described.[19] All microarray data obtained in this study are available at the Gene Expression Omnibus under the accession number GSE83839. Analysis of individual mRNA expression was undertaken using a High Capacity cDNA Reverse Transcription Kit, Power SYBR Green PCR Master Mix, and a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) with use of the gene-specific primers listed in Table S1. Expression of each gene was normalized to *18S* and *GAPDH* expression levels, and calculated using the comparative Ct method.

Global miRNA expression profiling analysis was carried out using a TaqMan MicroRNA Reverse Transcription kit, TaqMan Low Density Array Human MicroRNA Panels (A, version 2.0; B, version 3.0), and a Prism 7900HT Sequence Detection System (Thermo Fisher Scientific), as previously described.[18] All TaqMan Low Density Array analysis data obtained in this study are available at the Gene Expression Omnibus under the accession number GSE83838. The expression of individual miRNAs was determined by quantitative (q)RT–PCR analysis using TaqMan MicroRNA Assay and TaqMan MicroRNA RT kits, along with a 7500 Fast Sequence Detection System (Thermo Fisher Scientific). The non-coding RNA *RNU44* was used as an internal control for normalization.

Definition of *TTF-1* module and selection of candidate miRNAs. We defined the *TTF-1* module as a surrogate of the transcriptional activity of TTF-1 based on two-color microarray data, essentially as previously described.[18] In brief, we selected genes that were persistently up- or downregulated within 24 h after TTF-1 induction in at least two of the four TTF-1-inducible cell lines, BEAS-2B, NCI-H23, NCI-H1299, and PC-9. Consequently, 81 genes were selected as those consisting of the *TTF-1* module. Using our mRNA expression profile dataset comprised of 75 human lung adenocarcinoma tissues, we defined and calculated $act_{TTF\text{-}1}$ as described below, which was considered to reflect the *TTF-1* module activity in each tumor:

$$act_{TTF-1} = \frac{1}{n}\sum_{i=1}^{n} s_i z_i, \text{ where } s_i = \begin{cases} 1 \text{ if } i \in U \\ -1 \text{ if } i \in D \end{cases}$$

where n is the number of *TTF-1* module genes, z_i is the z-score of the $\log_2$ normalized signal ratio in each sample for gene i, s_i is the sign function of i, and U and D sets of up- and downregulated genes, respectively. We then searched for candidate miRNAs that showed a correlation with the *TTF-1* module activity using both miRNA (GSE51853, Arima *et al.*)[20] and mRNA (GSE83836, this study) expression profile datasets.

Gene ontology enrichment analysis. Gene Ontology (GO) enrichment analysis was performed using the tool available at the Gene Ontology Consortium website (http://geneontology.org/, GO database released 2015-08-06).

Western blot analysis. Western blot analysis was carried out using standard procedures with Immobilon-P filters (Millipore Japan, Tokyo, Japan) and an Enhanced Chemiluminescence system (GE Healthcare, Chicago, IL, USA). The primary antibodies used were anti-TTF-1 (8G7G3/1; Thermo Fisher Scientific), anti-KRAS (N234, Santa Cruz Biotechnology, Dallas, TX, USA), anti-MKL2) (14613), anti-ERK1/2 (9102), anti-pERK1/2 (T202/Y204) (4377), anti-MEK (9122), anti-pMEK (S217/S221) (9121) (all Cell Signaling Technology, Danvers, MA, USA), and anti-α-tubulin (T9026, Sigma).

Dual luciferase reporter assay. Dual luciferase reporter assays were undertaken to analyze *CLCN5* and *MIR532* promoter

activities using the respective reporter constructs of pGL4 with and without deletion of potential TTF-1 binding sites and a pRLTK vector, as well as a Dual Luciferase Reporter Assay System (Promega). pGL3 luciferase reporter constructs carrying the 3′-UTR of either *KRAS* or *MKL2* with wild-type or mutated sequences of the respective *miR-532-5p* target sites were utilized along with a pRLTK vector to analyze the effects of the *miR-532-5p* mimic or negative control (each 5 nM), as previously described.[18] Firefly luciferase activity was normalized to Renilla luciferase activity for each transfected well. Each assay was performed in triplicate.

Chromatin immunoprecipitation–qRT-PCR assay. NCI-H441 (2×10^7) cells were crosslinked with a 1% formaldehyde solution for 7 min at room temperature and subsequently quenched with 2.5 M glycine (Sigma) for 5 min. Cells were harvested in ice-cold PBS containing a protease inhibitor cocktail (Roche, New York, NY, USA) and processed using an M220 Ultrasonicator (Covaris, Woburn, MA, USA) for 10 min at 6°C with a 5% duty factor to obtain chromatin fragment lengths of 200–500 bp, as judged by the Bioanalyzer DNA High-Sensitivity kit (Agilent). A portion (0.1%) of the lysate was saved as an input sample, while the remainder was incubated with Dynabeads protein G (Thermo Fisher Scientific) coated with either an anti-TTF-1 antibody (H-190; Santa Cruz Biotechnology) or rabbit IgG control antibody (2729; Cell Signaling Technology) overnight at 4°C on a rotating platform. After repeated washes using a magnetic rack (Thermo Fisher Scientific), TTF-1-bound genomic DNA was recovered from Dynabeads and reverse-crosslinked, then digested with RNase A (Invitrogen) and proteinase K (Thermo Fisher Scientific) for 2 h each at 37 and 55°C, respectively. Following purification with a MinElute Reaction Cleanup Kit (Qiagen, Valencia, CA, USA), qRT-PCR analysis was undertaken using 100 ng DNA from each of the input and ChIP experiments with primers listed in Table S1. For quantification of DNA recovery, Ct values of the ChIP samples were normalized to that of the input and presented as enrichment over background. At least three independent experiments were carried out for the *CLCN5* and *MIR532* promoters, as well as for two unrelated genomic regions, *TAOK3* and *CDH10*, which served as negative controls.

Flow cytometry analysis. To determine apoptotic cell death, cells were seeded into 6-well plates and transfected with 5 nM *miR-532-5p* mimics or siRNAs against *KRAS*, *MKL2*, or *SRF*. Cells were harvested after incubation for 96 h and 1×10^4 cells were subjected to flow cytometry analysis using a FACSCalibur Cell Analyzer (BD Biosciences, San Jose, CA, USA). All assays were carried out in triplicate.

Colorimetric and colony formation assays *in vitro* and tumor growth assays *in vivo*. Cells were seeded into 12-well plates and analyzed using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) at 96 h after transfection with 5 nM *miR-532-5p* mimics, or siRNA against *KRAS*. For colony formation assays, transfected cells were seeded at a density of 1×10^3 cells per 10-cm dish and cultured for 10 (ACC-LC-94, ACC-LC-319, NCI-H1299) or 14 (NCI-H23) days. Colonies were then stained with 0.05% Crystal Violet (Sigma) and counted using a cell counting pen (AS One, Osaka, Japan). All of the *in vitro* assays were carried out in triplicate. ACC-LC-319, NCI-H1299, and ACC-LC-94 cells were transfected with either *miR-532-5p* or negative control, then 1×10^6 of each cell type in 200 μL RPMI (Sigma) with 25% Matrigel (BD Biosciences) was injected s.c. into the flanks of BALB/cSlc-nu/nu mice. Ten days after injection, the mice were killed, and tumors were harvested and weighed ($n = 10$ for ACC-LC-319; $n = 5$ for ACC-LC-94 and NCI-H1299).

Results

Identification of TTF-1-regulated miRNAs. Our search for TTF-1-regulated miRNAs was carried out according to the protocol depicted in Figure 1(a). In order to identify miRNAs commonly affected by TTF-1 in multiple cell lines, we generated four TTF-1-inducible cell lines, which included three lung

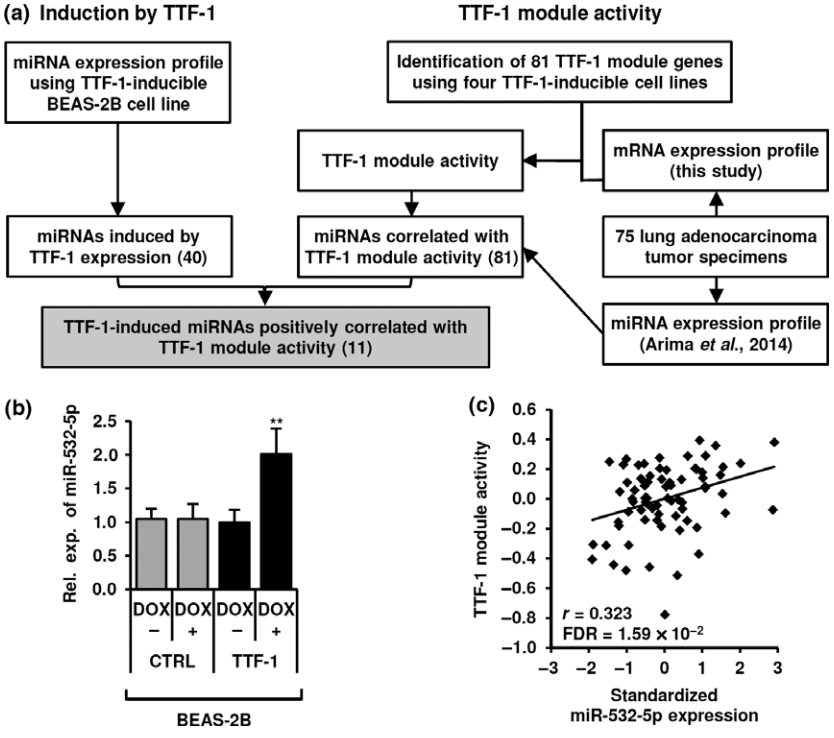

Fig. 1. Identification of thyroid transcription factor-1 (TTF-1)-regulated microRNAs (miRNAs). (a) Schematic diagram of strategy used to identify miRNAs regulated by TTF-1 *in vitro* and correlated with TTF-1 activity *in vivo*. Briefly, 81 genes commonly affected by TTF-1 in four TTF-1-inducible cell lines *in vitro* were selected to define the *TTF-1* module reflecting TTF-1 activity. We identified 81 miRNAs that showed a significant correlation with *TTF-1* module activity with the use of both miRNA and mRNA expression profile datasets of 75 lung adenocarcinoma tissues *in vivo*. Finally, 11 of the 81 miRNAs were found to be present in a panel of 40 miRNAs with 2-fold upregulation in TTF-1, but not in control (CTRL) cell line (<1.5-fold) after doxycycline (DOX) treatment *in vitro*, which we selected for further analyses. (b) Induction of *miR-532-5p* in response to DOX treatment for 72 h in TTF-1-inducible BEAS-2B cells. Data are shown as mean ± SD of three independent experiments. $^{**}P < 0.01$, two-tailed Student's *t*-test. Rel. exp., relative expression. (c) Correlation between *TTF-1* module activity and *miR-532-5p* expression in 75 lung adenocarcinoma specimens.

Table 1. MicroRNAs (miR) showing significant positive correlation with *thyroid transcription factor-1 (TTF-1)* module activity *in vivo* and more than 2-fold induction by TTF-1 *in vitro*

Candidate miRNAs	Correlation with TTF-1 module activity in tumor tissues *in vivo*		Fold induction in TTF-1-inducible BEAS-2B cells *in vitro*
	r†	FDR	
hsa-miR-532-5p	0.323	1.59×10^{-2}	3.95
hsa-miR-331	0.352	8.00×10^{-3}	3.81
hsa-miR-30b	0.527	2.69×10^{-5}	2.92
hsa-miR-30d	0.574	2.18×10^{-6}	2.88
hsa-miR-195	0.582	2.14×10^{-6}	2.76
hsa-miR-30c	0.470	2.23×10^{-4}	2.66
hsa-miR-26a	0.573	2.18×10^{-6}	2.62
hsa-miR-30a-5p	0.498	6.88×10^{-5}	2.50
hsa-let-7d	0.285	3.75×10^{-2}	2.18
hsa-miR-328	0.486	1.25×10^{-4}	2.05
hsa-miR-27b	0.379	3.98×10^{-3}	2.01

†Correlation coefficient. FDR, false discovery rate.

adenocarcinoma cell lines, NCI-H23, NCI-H1299, and PC-9, as well as the normal lung epithelial cell line BEAS-2B. TTF-1 was markedly induced in response to DOX treatment in all cell lines (Fig. S1a). Next, we analyzed changes in the miRNA expression profiles of TTF-1-inducible BEAS-2B cells in response to DOX treatment using a previously reported TaqMan array dataset,[18] which resulted in identification of 40 different miRNAs that showed greater than two-fold upregulation by TTF-1. We also attempted to identify miRNAs regulated by TTF-1 *in vivo*. As TTF-1-mediated regulation varies based on the presence or absence of cellular cofactors, and because TTF-1 expression itself may not accurately reflect TTF-1 transcriptional activity, we incorporated the *TTF-1* module for examination of a panel of 75 surgically resected lung adenocarcinoma tissues and undertook global gene expression analysis of TTF-1-inducible BEAS-2B, NCI-H23, NCI-H1299, and PC-9 cells at 24 h after induction of TTF-1 by DOX. Using those findings, we subsequently selected a panel of 51 upregulated and 30 downregulated mRNAs that were identified in at least two of the four TTF-1-inducible cell lines. Those results accordingly defined the *TTF-1* module as a set of 81 genes reflecting TTF-1 activity (Fig. S1b, Table S2). Subsequently, we determined *TTF-1* module activity in each of the 75 lung adenocarcinoma tissues and searched for miRNAs showing correlation with *TTF-1* module activity by use of our microarray datasets of both mRNA and miRNA expression in the same panel of 75 lung adenocarcinoma tissues. As a result, a set of 81 miRNAs was identified as significantly associated with *TTF-1* module activity (false discovery rate <0.05). Finally, we combined the information derived from analysis of the association between miRNA expression and experimentally defined *TTF-1* module activity in patient tumor tissues *in vivo* with that from global miRNA expression profiling in TTF-1-induced BEAS-2B cells *in vitro*. Eventually, 11 miRNAs were experimentally validated and selected as TTF-1-inducible miRNAs, and were shown to be correlated with TTF-1 activity in the present patients (Table 1). Of note, the panel of 11 miRNAs contained members of the *miR-30* family, *miR-195* and *let-7d*, which are known to function as tumor suppressor miRNAs. We selected *miR-532-5p* for further analysis, because of a lack of information in regard to its role in lung cancer development as well as a high degree of TTF-1-mediated induction shown in a confirmatory experiment (Fig. 1b) and significant correlation with *TTF-1* module activity (Fig. 1c).

***MicroRNA-532-5p* is a transcriptional target of TTF-1.** We then examined how TTF-1 regulates transcription of *miR-532-5p*, which was found to reside within intron 3 of the *CLCN5* gene (Fig. 2a). ENCODE H3K4me3 and H3K27Ac ChIP-seq data of A549 cells strongly suggested the presence of two potential genomic regions harboring active promoters, which coincided with the putative promoter of the host gene, that is, *CLCN5*, and a genomic region 8 kb upstream of *miR-532-5p*. Doxycycline treatment of TTF-1-inducible NCI-H1299 and PC-9 cells showed significant upregulation of both *miR-532-5p* and *CLCN5* mRNA (Fig. S2a). Conversely, knockdown of *TTF-1* resulted in markedly reduced *miR-532-5p* expression in both NCI-H441 and NCI-H2009 cells, whereas *CLCN5* expression was not affected in NCI-H441, and was rather unexpectedly increased in NCI-H2009 cells, showing cellular context-dependent responses (Fig. 2b). Then ChIP–qRT-PCR analysis was undertaken using NCI-H2009 cells with an anti-TTF-1 antibody and primers amplifying regions within the putative *CLCN5* and *MIR532* promoters, which contain potential TTF-1 binding sites. Those results clearly indicated binding of TTF-1 to both promoters (Fig. 2c), while similar results were obtained in NCI-H441 cells (Fig. S2b). Furthermore, a dual luciferase assay using reporter constructs of the *MIR532* promoter region revealed that deletion of the TTF-1 binding site significantly impaired promoter activity in NCI-H2009 (Fig. 2d), as well as in NCI-H441 cells (Fig. S2c). Following *TTF-1* knockdown in NCI-H2009 cells, activity of the *MIR532* promoter was also reduced (Fig. S2d). In contrast, luciferase reporter activity was significantly increased by deletion of the TTF-1 binding site within the *CLCN5* promoter, a finding consistent with increased *CLCN5* mRNA in NCI-H2009 cells knocked down for *TTF-1*. These findings indicated the existence of distinctive regulation characteristics between the *MIR532* and *CLCN5* promoters.

To further investigate this issue, PC-9 cells were co-transfected with dual luciferase reporter constructs containing the *CLCN5* or *MIR532* promoter with the TTF-1 binding site intact or deleted, along with a TTF-1 expression vector or that carrying deletion of its DNA-binding homeodomain. *MIR532* promoter activity was markedly activated in response to TTF-1 introduction, whereas it was completely abolished by deletion of the TTF-1 homeodomain (Fig. 2e). *MIR532* promoter activity was also significantly but not completely impaired by deletion of the TTF-1 binding site, suggesting possible existence of a cryptic TTF-1 binding site. Interestingly, the *CLCN5* reporter construct did not show any increase in luciferase activity in TTF-1-transfected PC-9 cells, suggesting that TTF-1 either induces or represses transcription of *CLCN5*, potentially dependent on the presence or absence of cofactor(s). Taken together, our data clearly show that TTF-1-mediated *miR-532-5p* induction is independent of regulation of *CLCN5*.

***KRAS* is a direct target of *miR-532-5p*.** We then sought to identify the target(s) of *miR-532-5p* with potential functional involvement in lung cancer development. Microarray analysis of *miR-532-5p*-introduced NCI-H23 and NCI-H1299 cells was carried out, and the resultant datasets were used to identify a set of possible direct target genes of *miR-532-5p* with the aid of TargetScan Human Release 6.2; http://www.targetscan.org/vert_61/ in a combinatorial fashion, which consequently identified 40 genes as strong candidates (Table S3). This set of 40 genes was then subjected to GO enrichment analysis, which

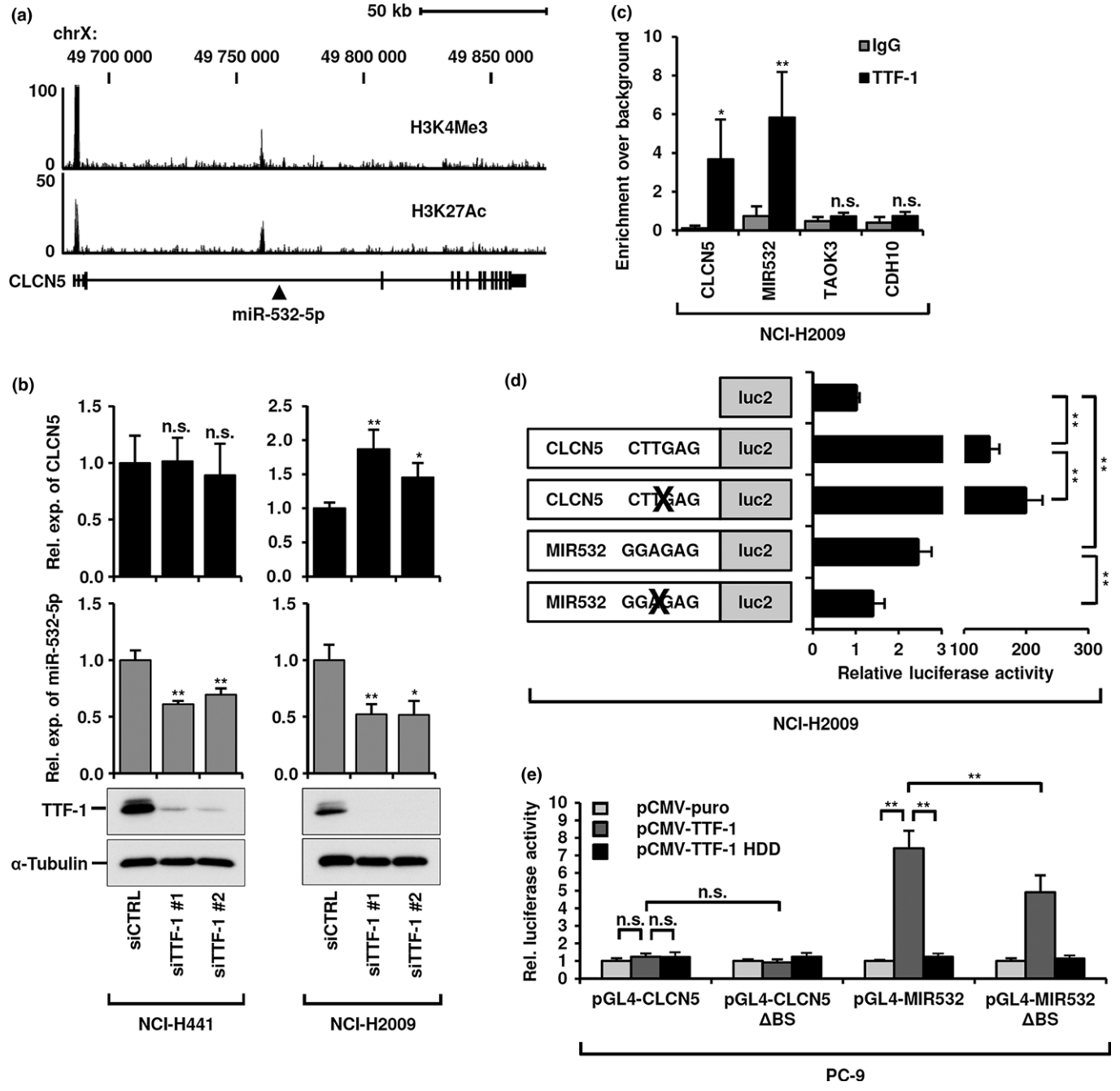

Fig. 2. Transcriptional activation of *microRNA (miR)-532-5p* by thyroid transcription factor-1 (TTF-1), independent of its host gene *CLCN5*. (a) Schematic representation of ENCODE ChIP-seq data of A549 cells. H3K4me3 and H3K27ac data suggested the existence of two distinct promoter regions at the 5′-UTR of the *CLCN5* and *MIR532* genes. (b) Quantitative RT-PCR analysis of *CLCN5* mRNA and *miR532-5p* expression, as well as Western blot analysis of TTF-1 in NCI-H441 and NCI-H2009 cells knocked down for *TTF-1*. (c) ChIP–quantitative RT-PCR analysis of TTF-1 binding to *CLCN5* and *MIR532* promoters in NCI-H2009 cells. Two unrelated genomic regions, *TAOK3* and *CDH10*, served as negative controls. (d) Dual luciferase assay of *CLCN5* and *MIR532* promoters carrying wild-type or TTF-1 binding site-deleted sequences in NCI-H2009 cells. (e) Dual luciferase assay of intact or TTF-1 binding site-deleted (ΔBS) *CLCN5* and *MIR532* promoters in PC-9 cells introduced with either wild-type (TTF-1) or homeodomain deletion mutant (TTF-1 HDD) TTF-1 expression constructs. All data shown represent mean ± SD of at least three independent experiments. **P* < 0.05; ** *P* < 0.01, two-tailed Student's *t*-test. n.s., not significant; Rel. exp., relative expression.

showed significant enrichment of genes with such biological processes as MAPK cascade, cellular response to growth factor stimulus, and the epidermal growth factor receptor signaling pathway (Table 2). We further selected genes commonly shared among biological processes enriched with the potential targets. Seven genes were found to be included in at least five of the seven biological processes (Table S4), with *KRAS* ranked on top based on context+ score; thus, it attracted much of our subsequent attention.

Quantitative RT-PCR and Western blot analyses showed a significant reduction of *KRAS* in *miR-532-5p*-introduced NCI-H23 and NCI-H1299 cells (Fig. 3a). Similar results were also obtained in two additional lung adenocarcinoma cell lines, ACC-LC-94 and ACC-LC-319 (Fig. S3a). Next, to examine

Table 2. Enriched processes among candidate *microRNA-532-5p* target genes

GO biological processes (GO database released 2015-08-06)	Number of genes	*P*-value
MAPK cascade	9	4.4×10^{-4}
Fc receptor signaling pathway	8	3.1×10^{-3}
Positive regulation of macromolecule biosynthetic process	14	9.0×10^{-3}
Cellular response to growth factor stimulus	10	1.0×10^{-2}
Epidermal growth factor receptor signaling pathway	7	1.8×10^{-2}
Positive regulation of cellular biosynthetic process	14	2.0×10^{-2}
Regulation of cellular macromolecule biosynthetic process	21	2.5×10^{-2}

GO, gene ontology.

whether *KRAS* is directly repressed by *miR-532-5p*, NCI-H23 cells were transfected with *miR-532-5p* as well as dual luciferase reporter constructs carrying the *KRAS* 3′-UTR with and without mutations at a potential *miR-532-5p* binding site. Repression of luciferase reporter activity by *miR-532-5p* was strongly impaired by the presence of mutations corresponding to the seed sequence of *miR-532-5p*, indicating that *KRAS* is a direct target of *miR-532-5p* (Fig. 3b).

We further examined whether *miR-532-5p* introduction has an effect on lung adenocarcinoma proliferation, similar to *KRAS* knockdown, using three lung adenocarcinoma cell lines with mutant *KRAS* (NCI-H23, ACC-LC-94, and ACC-LC-319), as well as NCI-H1299 with wild-type *KRAS* (Fig. 3c). In all of the lung adenocarcinoma cell lines examined, significantly reduced cell proliferation was noted when *miR-532-5p* was introduced. Similarly, *KRAS* knockdown significantly inhibited cell proliferation of NCI-H23 and ACC-LC-94 cells. In contrast, NCI-H1299 with wild-type *KRAS* showed resistance to siKRAS treatment, whereas ACC-LC-319 with mutant *KRAS* was mostly unaffected. These results suggest that *miR-532-5p* plays a suppressive role in lung adenocarcinoma, in part through direct repression of *KRAS*, and that *miR-532-5p* may also target another gene that plays a crucial role in lung adenocarcinoma development.

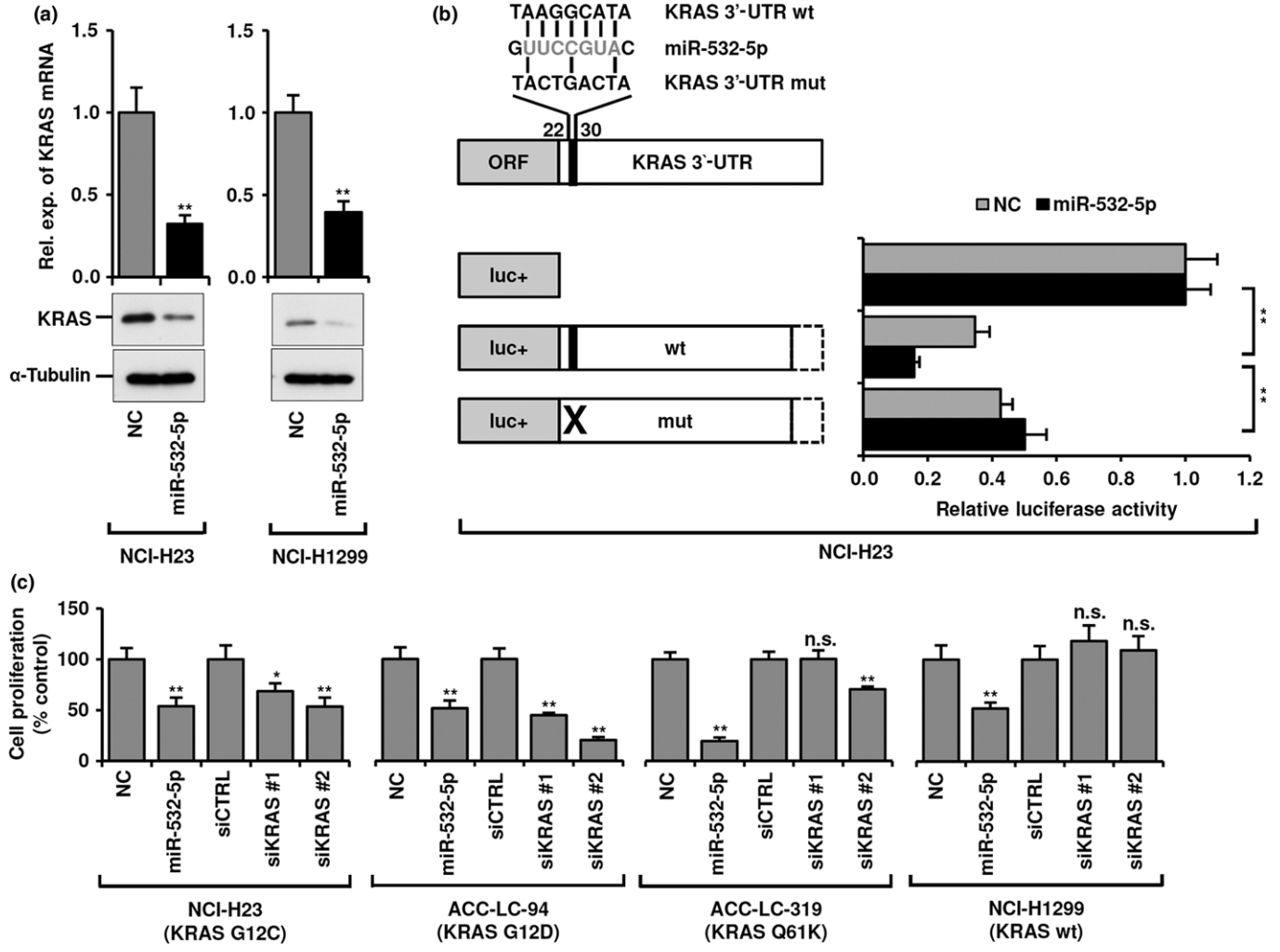

Fig. 3. Identification of *KRAS* as a target of *microRNA (miR)-532-5p* in lung adenocarcinoma cells. (a) Quantitative RT-PCR and Western blot analyses of *KRAS* in *miR-532-5p*-introduced NCI-H23 and NCI-H1299 cells. Rel. exp., relative expression. (b) Dual luciferase assay using reporter constructs carrying wild-type (wt) or mutant (mut) sequences of the *KRAS* 3′-UTR in NCI-H23 cells transiently transfected with *miR-532-5p* mimics or negative control (NC). (c) Colorimetric assay of four lung adenocarcinoma cell lines with and without *KRAS* mutations. Cells were analyzed at 96 h after transfection with either *miR-532-5p* mimics or two independent siRNAs against *KRAS*. All data shown represent mean ± SD of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$, two-tailed Student's *t*-test.

***MicroRNA-532-5p* targets *MKL2*, induces apoptosis, and inhibits lung adenocarcinoma proliferation.** Accordingly, we excluded genes constituting the same biological processes as *KRAS* from the list of 40 potential targets and searched for additional relevant targets of *miR-532-5p*. As a result, *MKL2*, a transcriptional co-activator of SRF and SMAD3,[21,22] was shown to be an intriguing candidate accounting for significant growth inhibition of siKRAS-resistant ACC-LC-319 and NCI-H1299 cells. Expression of *MKL2* was repressed at both the mRNA and protein levels following *miR-532-5p* introduction in NCI-H23 and NCI-H1299 cells (Fig. 4a), as well as in ACC-LC-94 and ACC-LC-319 cells (Fig. S4a). Also, a dual luciferase assay was carried out using reporter constructs containing the *MKL2* 3′-UTR with and without mutations at potential *miR-532-5p* binding sites. Mutations corresponding to the seed sequence of *miR-532-5p* at one of the two potential binding sites significantly increased luciferase reporter activity in NCI-H23 cells treated with *miR-532-5p* (Fig. 4b), whereas abolition of both binding sites markedly restored that activity to nearly the baseline level.

Next, we examined the effects of *miR-532-5p*, siKRAS, and siMKL2 treatment on colony formation and found significantly reduced numbers of colonies following *miR-532-5p* and siMKL2 treatment in all four cell lines (Fig. 4c). In contrast, siKRAS specifically inhibited colony formation in NCI-H23 and ACC-LC-94 cells, with a clear dependence on mutant *KRAS*-mediated signaling, whereas that was not seen in ACC-LC-319 and NCI-H1299 cells. Consistent findings were obtained in flow cytometry measurements of sub-G_1 cell populations, an indicator of apoptosis induction (Fig. 4d). These results point to the notion that TTF-1-induced *miR-532-5p* plays a role as a negative regulator of at least two biologically pertinent and distinct target genes.

We next investigated whether there were distinct responses in terms of MAPK signaling when lung adenocarcinoma cell lines were treated with either *miR-532-5p*, siKRAS, or siMKL2, with special attention given to their dependence on mutant *KRAS*-mediated signaling (Fig. 5a). NCI-H23 and ACC-LC-94 cells with apparent *KRAS* dependency showed clear inhibition of MEK and ERK phosphorylation in response to treatment with *miR-532-5p* and siKRAS, but not with siMKL2. In contrast, such inhibitory effects toward MEK and ERK activation were not observed in NCI-H1299 and ACC-LC-319 cells treated with *miR-532-5p*, siKRAS, or siMKL2. These results suggested that the repressive effect of *miR-532-5p* on lung adenocarcinoma survival is mediated not only by inhibition of the MAPK pathway through targeting of *KRAS*, but also by inhibition of another vital unidentified molecular pathway, most likely the MKL2-SRF pathway by targeting *MKL2*.

In order to investigate our speculation, we treated NCI-H23 and NCI-H1299 cells with CCG-100602[23] and CCG-203971,[24] which inhibit MKL-SRF-mediated gene transcription. Colorimetric assays revealed marked inhibition of cell proliferation in response to MKL-SRF pathway inhibition in both cell lines (Fig. 5b). In addition, we observed significant induction of apoptosis by knockdown of *SRF* in both NCI-H23 and NCI-H1299 cells (Fig. 5c), suggesting that *miR-532-5p*-induced *MKL2* repression elicits apoptosis, at least in part, due to consequential impairment of the transcriptional activating function of the MKL2–SRF complex.

Finally, we investigated the effects of *miR-532-5p* introduction in a xenograft model using ACC-LC-319, NCI-H1299, and ACC-LC-94 cells. Decreased expressions of *KRAS* and *MKL2* were observed in *miR-532-5p*-introduced ACC-LC-94 xenografts at 5 days after injection (Fig. S5), whereas the tumor weights in animals with *miR-532-5p*-introduced cells at 10 days after injection were significantly reduced as compared to those introduced with negative control in all three cell lines (Fig. 5d). Taken together, the present findings clearly indicate that *miR-532-5p* is a novel transcriptional target of TTF-1 and that *miR-532-5p* plays a role in tumor suppression, at least in part, by targeting *KRAS* and *MKL2* in lung adenocarcinomas (Fig. 5e).

Discussion

In the present study, we applied an integrative approach that combined expression profiles of patient tumor tissues *in vivo* and experimental data of cell lines *in vitro*, and our results identified *miR-532-5p* as a functionally relevant transcriptional target of TTF-1. Thyroid transcription factor-1 regulates *miR-532-5p* by binding to its own promoter rather than that of *CLCN5* harboring *miR-532-5p* in its intron 3. Along this line, it is important to note that, although *miR-532-5p* forms an miRNA cluster together with *miR-188*, *miR-500a*, *miR-500b*, *miR-362*, *miR-501*, and *miR-660*, other miRNAs were filtered out during our search because of their low expression and/or lack of correlation with *TTF-1* in patients.

TTF-1 was initially identified as a lineage-survival oncogene in lung adenocarcinoma reports;[7–10] subsequent studies, including our own, showed that it possesses double-edged sword characteristics,[13] including functions deleterious toward cancer.[25–27] The present findings clearly indicate that *miR-532-5p*, a novel target of *TTF-1*, possesses apoptosis-inducing capability, thus transcriptional activation of *miR-532-5p* is considered to reflect a deleterious function of TTF-1. In addition, multiple additional miRNAs found to be positively associated with TTF-1 activity are also known to have a tumor-suppressive function in lung cancer, including *miR-195*,[28] *let-7d*,[15] and members of the *miR-30* family,[20] providing further supporting evidence of the double-edged sword characteristics of this enigmatic oncogene. A few reports have been published regarding the reduced expression and tumor-suppressive functions of *miR-532-5p* in other types of human cancers, including renal cell,[29,30] hepatocellular,[31] and ovarian carcinomas.[32] *MicroRNA-532-5p* has also been reported to promote tumor proliferation and progression in melanomas[33] and gastric cancer cells,[34] suggesting its context-dependent roles in various cancer types. It is important to note that a sizable fraction of *TTF-1*-positive lung adenocarcinomas have low levels of *miR-532-5p* expression, despite the general and significant association of *miR-532-5p* with *TTF-1*. In our experiments, we did not find any evidence showing DNA hypermethylation of the *MIR532* promoter (data not shown), suggesting involvement of other underlying mechanism(s), such as altered histone modifications and/or lack of co-activators of TTF-1 in that cellular context.

The present results clearly revealed that *KRAS* and *MKL2* are direct targets of *miR-532-5p*. *KRAS* is a well-established oncogene that is frequently mutated and activated in lung adenocarcinomas. A functional link between *Ttf-1* and *Kras* mutations was previously reported in a study that used genetically engineered mice, in which *Ttf-1* was shown to inhibit mutant-*Kras*-driven tumorigenesis by repressing a latent gastric differentiation program in lung adenocarcinomas.[35] In the present study, we uncovered another link between *TTF-1* and *KRAS*, as TTF-1-regulated *miR-532-5p* was shown to possess a capability to directly repress *KRAS* by binding to its 3′-UTR. Along this line, *miR-532-5p* significantly decreased activation of the

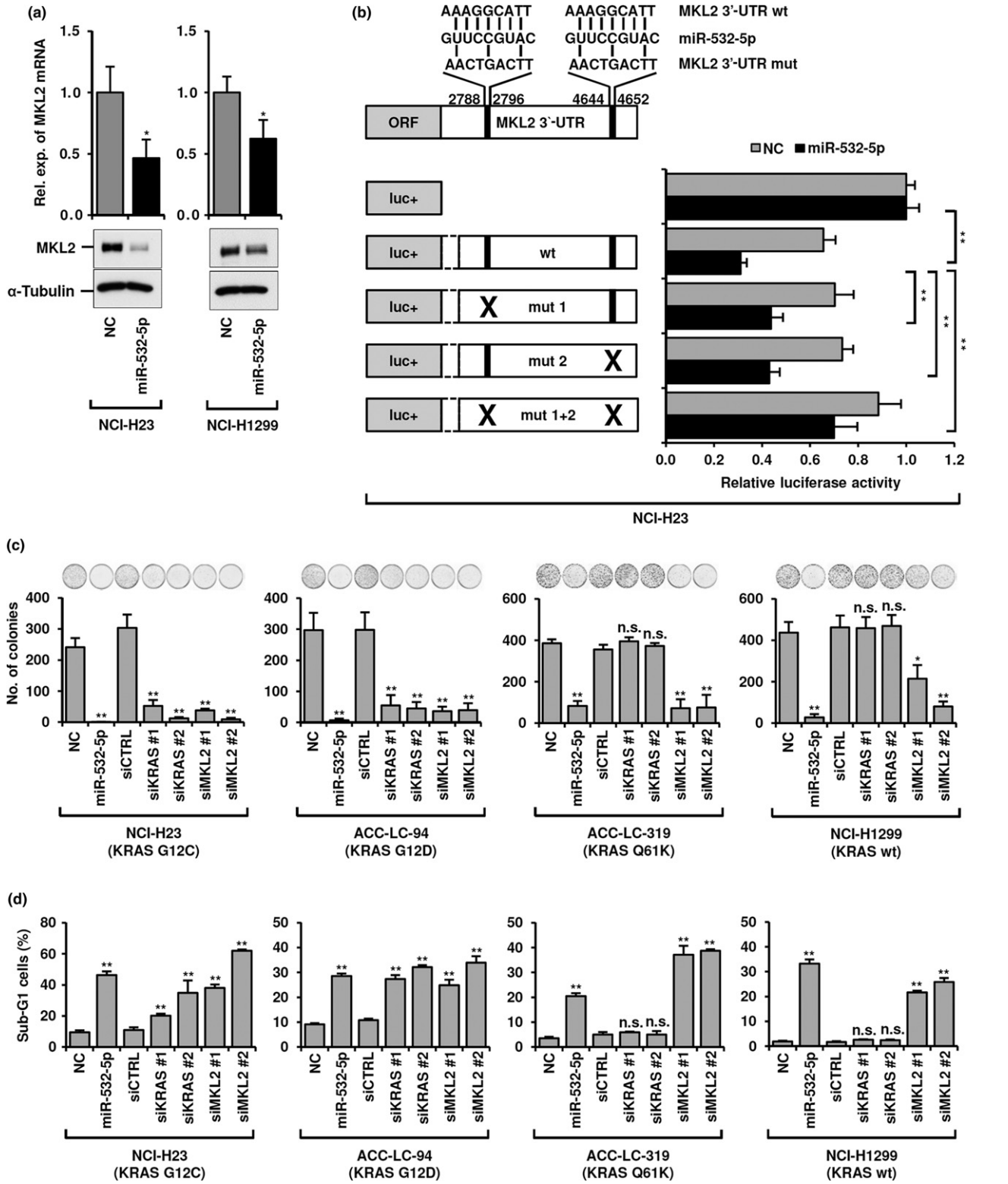

Fig. 4. *MKL2* as a *microRNA (miR)-532-5p* target and biological effects of *miR-532-5p* introduction compared with *KRAS* or *MKL2* knockdown in lung adenocarcinoma cells. (a) Quantitative RT-PCR and Western blot analyses of *MKL2* in *miR-532-5p*-introduced NCI-H23 and NCI-H1299 cells. Rel. exp., relative expression. (b) Dual luciferase assay using reporter constructs carrying wild-type (wt) or mutant (mut 1, mut 2, mut 1 + 2) sequences of the *MKL2* 3′-UTR in NCI-H23 cells transiently transfected with *miR-532-5p* mimics or negative control (NC). (c) Colony formation assay for the same four lung adenocarcinoma cell lines with and without *KRAS* mutations. Treatment with *miR-532-5p* and siMKL2 markedly inhibited colony formation in all four cell lines, whereas siKRAS treatment significantly reduced the number of colonies only in NCI-H23 and ACC-LC-94 cells. (d) Flow cytometry analysis was used to determine sub-G_1 populations in the same panel of four lung adenocarcinoma cell lines. Sub-G_1 populations were markedly increased in all four cell lines when treated with either *miR-532-5p* or siMKL2, whereas siKRAS treatment significantly induced apoptosis only in NCI-H23 and ACC-LC-94 cells. All experiments were carried out in triplicate and data shown represent mean ± SD. $*P < 0.05$; $**P < 0.01$, two-tailed Student's *t*-test. n.s., not significant.

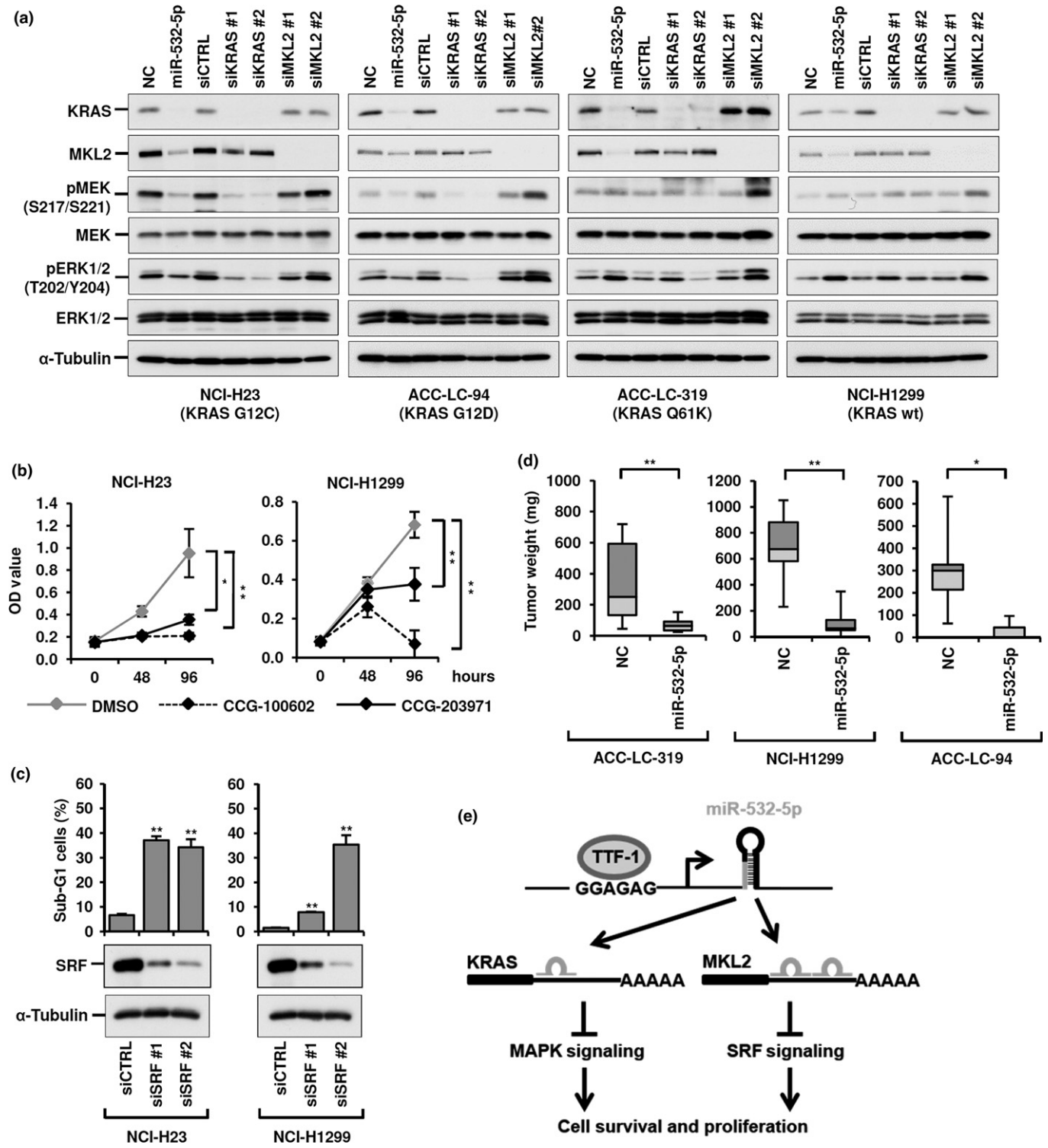

Fig. 5. Cancer cell growth decreased by *microRNA (miR)-532-5p* itself and *miR-532-5p*-affected downstream pathways. (a) Western blot analysis of MEK-ERK signaling in four lung adenocarcinoma cell lines with and without *KRAS* mutations. Note that MEK-ERK signaling was inhibited by both *miR-532-5p* and *KRAS* knockdown in NCI-H23 and ACC-LC-94, but not ACC-LC-319 or NCI-H1299 cells. (b) Colorimetric assay of effects of treatment with CCG-100602 and CCG-203971, MKL-SRF pathway inhibitors, in NCI-H23 and NCI-H1299 cells. (c) Flow cytometry and Western blot analyses of NCI-H23 and NCI-H1299 cells treated with siRNA against *SRF*, for which MKL2 plays a role as a co-activator. (d) Decreased tumor growth of *miR-532-5p*-introduced lung adenocarcinoma cell lines *in vivo*. (e) Schematic diagram summarizing the present findings: thyroid transcription factor-1 (TTF-1) induces *miR-532-5p*, which in turn targets *KRAS* and *MKL2*, thus inhibiting two crucial downstream pathways, leading to induction of apoptosis in lung adenocarcinoma cells. $^{*}P < 0.05$; $^{**}P < 0.01$, two-tailed Student's *t*-test. NC, negative control.

MEK–ERK axis in lung adenocarcinoma cells dependent on mutant *KRAS*-mediated signaling. Interestingly, though Rice *et al.* previously reported that TTF-1 transcriptionally activates *SREBF2*, a host gene of *miR-33a*,[36] *miR-33a* was excluded in the process of our search for TTF-1-regulated miRNAs because of its low level of expression.

Together, MKL2 and MKL1 form a family of co-activators of transcription factors that includes SRF[21] and SMAD3,[22] that regulates various cellular processes including cell migration, cell growth, and cytoskeleton organization.[37] To the best of our knowledge, this is the first study to identify an miRNA targeting *MKL2* in cancer. While introduction of *miR-532-5p* elicited apoptosis in lung adenocarcinoma cells regardless of *KRAS* mutation status, treatment with siRNA against *MKL2* clearly rendered a phenocopy of this effect, suggesting crucial involvement of continued MKL2 expression in this devastating cancer. Both siKRAS-sensitive NCI-H23 and -insensitive NCI-H1299 cells showed marked sensitivity to two small molecules, CCG-100602 and CCG-203971, inhibitors of MKL/SRF-dependent transcriptional activation. Consistently, *SRF* knockdown also rendered a phenocopy of the effects of *miR-532-5p* and siMKL2 treatment, indicating a vital role of MKL/SRF-mediated signaling in lung adenocarcinoma development. Muehlich and colleagues recently reported similar results of nuclear localization of MKL2, reflecting its activated state, and induction of senescence by knocking down *MKL1/2* in hepatocellular carcinoma cells.[38] A study is needed to address whether *miR-532-5p* or another small molecule can attain sufficient inhibition of MKL-SRF function without serious side-effects in lung adenocarcinomas, as well as other types of human cancer.

In conclusion, *miR-532-5p* was revealed as a novel target of TTF-1 in the present study using an integrative approach that combined both *in vitro* and *in vivo* findings. It is conceivable that *miR-532-5p* imposes intricate regulation of TTF-1 functions together with other identified miRNAs. We also showed that *miR-532-5p* is capable of inducing apoptosis in lung adenocarcinoma and targets two key oncogenes, *KRAS* and *MKL2*, suggesting that novel therapeutic strategies using *miR-532-5p* may ultimately prove effective for treating patients with this hard-to-cure cancer. Finally, we note that, although *Ttf-1*-knockout mice are born dead with severely impaired lung morphogenesis,[39] *miR-532-5p* is upregulated in their lungs from embryonic days 16–18,[40] which also warrants future investigation of its potential role in lung development.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

CLCN5	chloride voltage-gated channel 5
DOX	doxycycline
GO	gene ontology
miR	microRNA
MKL2	myocardin-like 2
qRT-PCR	quantitative RT-PCR
SRF	serum response factor
TTF-1	thyroid transcription factor-1

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Defining *thyroid transcription factor-1 (TTF-1)* module reflecting TTF-1 activity and correlation between *microRNA (miR)-532-5p* and TTF-1 expression *in vivo*.

Fig. S2. *Thyroid transcription factor-1 (TTF-1)* binds to *MIR532* promoter and transcriptionally regulates *microRNA (miR)-532-5p*.

Fig. S3. *MicroRNA (miR)-532-5p*-mediated reduction of both mRNA and protein of *KRAS* in additional lung adenocarcinoma cell lines.

Fig. S4. *MicroRNA (miR)-532-5p*-mediated reduction of both mRNA and protein of *MKL2* in additional lung adenocarcinoma cell lines.

Fig. S5. Reduction of both *KRAS* and *MKL2* mRNA in *microRNA (miR)-532-5p*-introduced ACC-LC-94 xenografts.

Table S1. Sequences of oligonucleotides used in this study.

Table S2. Genes consisting of the *thyroid transcription factor-1 (TTF-1)* module.

Table S3. List of candidates for *microRNA-532-5p* target genes.

Table S4. Top-ranked candidates for *microRNA-532-5p* target genes.