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Research Article

CCDC134, a novel secretory protein, inhibits activation of ERK and JNK, but not p38 MAPK

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Abstract. In this study, we report a novel gene, *CCDC134* (coiled-coil domain containing 134), that encodes a secretory protein that can inhibit the MAPK pathway as a novel human MAPK-regulating protein. The CCDC134 mRNA contains 1280 nucleotides, encoding a protein of 229 amino acids. CCDC134 is a classical secretory protein. Expression profile analysis by Northern blot, RT-PCR, immunohistochemistry and Western blot reveals that *CCDC134* is widely expressed in normal adult tissues, tumor tissues and cell lines. Functional investigation reveals that overexpression of *CCDC134* and its purified protein significantly inhibit transcriptional activity of Elk1 and phosphorylation of Erk and JNK/SAPK but not p38 MAPK. Conversely, specific siRNA against CCDC134 activates Elk1 transcriptional activity and promotes Erk and JNK/SAPK phosphorylation. These results clearly indicate that *CCDC134* is a novel member of the secretory family and down-regulates the Raf-1/MEK/ERK and JNK/SAPK pathways.

Keywords. *CCDC134*, secretory protein, inhibit, Elk1, MAPK pathway.

Introduction

The completion of the human genome project has resulted in the identification of thousands of novel genes that have been deposited in the human Refseq and EST databases in GenBank, most of them with unknown or poorly understood functions. To identify novel functional genes involved in cell proliferation, cell apoptosis, or cell survival, we have established several high-throughput functional gene screening systems based on overexpression in human cell lines, including the cell-based assays using reporter genes [1] and cell microarray system [2], etc.

To detect functional genes involved in the Elk1 signal pathway, several mammalian cell lines were cotransfected with expression plasmids containing the full-length open reading frame (ORF) of a novel gene in combination with an Elk1 trans-reporting system. In the high-throughput assays, we found a novel human coiled-coil domain containing 134 gene termed *CCDC134*, which significantly inhibited Elk1 transcriptional activity. The coiled-coil domain is a ubiquitous protein motif that is often involved in oligomerization. It has been found in many types of proteins, including transcription factors, intermediate filaments and certain tRNA synthetases [3].

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Within the Elk1 signaling pathway, the evolutionarily conserved mitogen-activated protein kinase (MAPK) family consists of serine/threonine-specific protein kinases, involved in signal transduction pathways between the membrane and the nucleus. MAPKs transduce diverse extracellular stimuli (mitogenic growth factors, environmental stresses and proapoptotic agents) to the nucleus *via* kinase cascades to regulate proliferation, DNA synthesis arrest, differentiation, and apoptosis [4–8]. MAPKs become activated through phosphorylation of specific threonines and tyrosines by an upstream dual specificity kinase. Regulation of the signaling pathway occurs *via* the sequential phosphorylation and activation of each member of the kinase cascade [9]: an MAP4K (such as TAB1) phosphorylates and activates a MAP3K (such as Raf or TAK), which activates an MAP2K (such as MEK), which activates MAPK (such as Erk1/2, JNK, or p38). Elk-1 (also known as p62 ternary complex factor, TCF), is an Ets-domain transcription factor activated by MAPK that binds to the serum response element (SRE) to induce immediate early gene transcription in response to extracellular stimuli such as serum and growth factors.

In this paper, we describe CCDC134, a novel secretory protein, and investigate its functional role in the Elk1 signaling pathway. Based on the gene sequence, human *CCDC134* encodes a 229-amino acid protein with a putative signal peptide at its N terminus and one coiled-coil domain. Functional studies indicated that overexpression of *CCDC134* and its purified protein significantly inhibited transcriptional activity of Elk1 and phosphorylation of Erk and JNK/SAPK but not p38 MAPK. Conversely, specific siRNA against *CCDC134* activated Elk1 transcriptional activity and promoted Erk and JNK/SAPK phosphorylation, suggesting a potential inhibiting role of CCDC134 in MAPK-mediated Elk1 transcription. To our knowledge, no functional study has been performed on this hypothetical gene. Data are presented suggesting that CCDC134 might be a novel human MAPK regulating protein.

Materials and methods

Reagents. Restricted endonucleases *Eco*RI, *Hin*dIII and *Bam*HI were purchased from TaKaRa (Japan). Phorbolmyrlstate acetate (PMA), PD98059 (MEK1/2 inhibitor) and SP600125 (JNK/SAPK inhibitor), wortmannin (PI3 kinase inhibitor), Brefeldin A (BFA), monoclonal antibodies against c-myc and β-actin were purchased from Sigma-Aldrich (USA). Cell lysis buffer (10×), polyclonal antibodies against phosphorylated Elk1 at Ser383, MEK1/2 at Ser217/221, Erk1/2 at Thr202/Tyr204, JNK/SAPK at Thr183/Tyr185 and p38 at Thr180/Tyr182, Erk1/2, JNK/SAPK and p38 were purchased from Cell Signaling Technology (USA). IRDye™ 800-conjugated secondary antibodies against mouse and rabbit IgG, IRDye™ 800-conjugated antibody against GFP were purchased from LI-COR Bioscience (USA). Endoplasmic reticulum (ER)-specific fluorescent dye ER Tracker was purchased from Molecular Probes (USA). Protease inhibitor cocktail tablets were obtained from Roche Applied Science (Switzerland); and Ionomycin was from Santa Cruz Biotechnology (USA).

Cell lines and transfections. HEK293T (human embryonic kidney cell line, a kind gift from T. Matsuda, Japan) and HeLa (human cervical carcinoma cell line) cells were maintained in Dulbecco's modified Eagle medium (Life Technologies, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and L-glutamine (2 mM) at 37 °C in a humidified 5% CO_2. DNA transfection was performed using VigoFect (Vigorous, USA), a non-liposomal cationic formula, according to the instructions provided by the manufacturer or by electroporation at a single pulse of 120 V, 20 ms, with 10 μg plasmid/10^6 cells in 2-mm gap cuvettes using an ECM 830 Square Wave Electroporation System (BTX, USA).

cDNA cloning and vector construction. The full-length coding region of CCDC134 was amplified from a human lung cDNA library (Clontech, USA) by PCR using the specific primers. The N-terminal truncated mutation, CCDC134-ΔSP, was amplified by the primers with enzyme cut sites. The purified PCR product was ligated into the pGEM-T Easy vector (Promega, USA). The insert was released by *Eco*RI and *Bam*HI and subcloned into pcDNA.3.1/myc-his(-)B (Invitrogen, USA) to construct plasmids of pcDB-CCDC134 and pcDB-CCDC134-ΔSP. To construct pEGFP-CCDC134 and pEGFP-CCDC134-ΔSP plasmids, the corresponding primers were used for fusion with C-terminal GFP tag in the pEGFP-N1 (Clontech, USA) vector with *Hin*dIII and *Bam*HI. All clones were confirmed by sequencing using an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, USA). All the primers used for these PCRs were listed in Table 1. After confirmation by sequencing, all mammalian expression plasmids were extracted and purified for transfection using EndoFree Plasmid Maxi Kit (Qiagen, USA).

Northern blot and RT-PCR assay. A 758-bp CCDC134 PCR product amplified by the specific primers was purified and labeled with fluorescein

Table 1. Sequences of primers used for PCR experiments.

Number	Sets of primers: forward//reverse
CCDC134	5′-CTGCTCGCACAGGACTCGG-3′//5′-GGGCTATAACTCAGACTGGGATCTG-3′
pEGFP-CCDC134	5′-AAGCTTATGGACCTTCTTCAATTC-3′//5′-GGATCCAGTAACTCAGACTGGGATCTG-3′
pEGFP-CCDC134-ΔSP	5′-AAGCTTATGTCCCTGGACCCAAGC-3′//5′-GGATCCAGTAACTCAGACTGGGATCTG-3′
pcDB-CCDC134	5′-GAATTCATGGACCTTCTTCAATTC-3′//5′-GGATCCAGTAACTCAGACTGGGATCTG-3′
pcDB-CCDC134-ΔSP	5′-GAATTCATGTCCCTGGACCCAAGC-3′//5′-AGTAACTCAGACTGGGATCTGGGATCC-3′

using a Gene Images Random Prime Labeling Kit (Amersham Biosciences, Sweden) according to the manufacturer's instructions. Total RNA of human adult tissues for Northern blot was provided from Chinese National Human Genome Center, Beijing, China (CHGB). Samples (20 μg of each tissue specimen) were separated by electrophoresis and transferred onto a nylon membrane (Amersham Biosciences), which was subsequently hybridized with the probe at 65 °C overnight. After washing with saline sodium citrate buffer, the membrane was incubated with anti-fluorescein-AP conjugate and images were developed using a Gene Images CDP-Star Detection Module (Amersham Biosciences). Two panels of human multiple normal tissue cDNA (human MTC TM panel I, catalog No.636742; human MTC TM panel II, cat. no. 636743; Clontech, USA) and human multiple cancer tissue cDNA (Shanghai Genomics, China) containing mixed cDNA libraries from different individuals were used. Total RNA of CCDC134 was extracted from cell lines using TRIzol reagent (Invitrogen, USA). RT-PCR was performed with ThermoScript RT-PCR System (Invitrogen, USA) using the specific primers for CCDC134 and GAPDH.

Protein extraction and Western blot analysis. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in cell lysis buffer (10 mM HEPES pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 0.5 % NP-40, with freshly added proteinase inhibitor cocktail) for 30 min at 4 °C. Cell lysate was clarified by centrifugation at 4 °C at 16 000 *g* for 15 min. Protein concentration was determined using the BCA protein assay reagent (Pierce, USA). Equal amounts of protein were separated by 12.5 % SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Pharmacia, UK). Membranes were blocked in Tris-buffered saline containing 0.1 % Tween-20 (TBS-T) and 5 % nonfat milk or 5 % BSA for 2 h and incubated overnight at 4 °C with the appropriate primary antibody. After washing in TBS-T buffer, membranes were incubated for 1 h in the dark with the appropriate IRDye™ 800-conjugated secondary antibodies. Signals were detected on an Odyssey Infrared Imager (LI-COR Bioscience, USA).

Polyclonal anti-CCDC134 antibody preparation. Antibodies against CCDC134 were generated by immunization of rabbits (our lab) with two KLH-coupled CCDC134 peptides (peptide 1: CTGTLRTSLDPSLEIYK; peptide 2: CRKEIRKGPRISRSQSE), which were synthesized by solid phase synthesis and purified by HPLC to 90 % purity (Chinese Peptide, China). Rabbit polyclonal antibody was purified using CNBR Sepharose 4B coupled with specific CCDC134 peptides. The antibodies were validated by ELISA, Immunohistochemistry, Immunofluorescence and Western blot analysis.

Tissue specimens and Immunohistochemistry. Tissue chips were purchased from Beijing Friendship Hospital. The tissue chips were deparaffinized and rehydrated. Antigen retrieval was performed in 0.01 M citrate buffer (pH 6.0) twice in a microwave oven for 5 min each. The sections were incubated with 3 % H_2O_2 at room temperature for 10 min, rinsed twice and blocked in PBS plus 10 % normal goat serum for 10 min. The slides were then incubated with a rabbit anti-CCDC134 polyclonal antibody (1:50 in PBS) at 37 °C for 1 h. After thorough washing, Dakocytomation Envision^{+TM} System HRP (DakoCytomation, USA) was applied for 30 min. After rinsing in PBS, all sections were visualized with 0.05 % 3,3′-diaminobenzidine (DAB). The sections were then counterstained with hematoxylin. Sections with anti-CCDC134 antibody pre-incubated with the specific CCDC134 peptides at room temperature for 2 h were also used as preabsorption control.

Expression of mammalian recombinant protein and N-terminal sequence analyzing. pcDB-CCDC134 plasmid (20 μg) containing a His^6-tag at the C terminus was transiently transfected into HeLa cells (3×10^6) by electroporation as previously described. The transient transfected cells were cultured in the media without serum (Gibco, USA) for 3 days, and then harvested for the next step of purification. The transient transfected cell supernatant was purified with a Ni-Sepharose™ 6 Fast Flow column (GE

Healthcare, USA) according to manufacturer's instruction. The purified CCDC134 protein was dialyzed in PBS at 4 °C and analyzed by capillary electrophoresis prior to bioassay. Peak fractions of >85 % pure CCDC134 protein were quantified by SDS-PAGE and Coomassie blue staining with BSA as a standard. The purified protein was measured by N-terminal sequence analysis using Tandem-MS technology (Shanghai GeneCore BioTechnologies Co., Ltd, China). Fluorescein isothiocyanate (FITC) labeling of purified CCDC134 protein was prepared as described previously [10].

Fluorescence microscopy. Transiently transfected HeLa cells expressing pEGFP-CCDC134 or pEGFP-CCDC134-ΔSP were cultured on the coverslips, stained with 1 μM ER Tracker for 30 min at 37 °C, and imaged using a TCS-SP laser-scanning confocal microscope with a 40× or 63× oil immersion lens (Leica Microsystems, Germany).

For different fluorescent protein treatments, HeLa cells were grown in specialized glass-bottom microwell dishes (MatTek Corp, USA) to about 50 % confluence, and then fresh, 10 % fetal bovine serum medium containing different fluorescent molecules was added. Final concentrations of fluorescent molecules were 1 μM purified CCDC134-FITC protein; 1 μM recombinant PDCD5-FITC (a kind gift from Wang Y, China) was used as positive control. After 5 h of treatment with these proteins at 37 °C or 4 °C separately, cells were rinsed twice with PBS buffer and fixed with 2 % paraformaldehyde in PBS for 15 min at room temperature. Cells were rinsed three times, all fluid was removed, and samples were mounted with 90 % glycerin (Sigma) in PBS. Finally, cells were imaged using a TCS-SP laser-scanning confocal microscope.

BFA and wortmannin block assay. HeLa cells were transfected with pcDB-CCDC134 plasmid and incubated in 37 °C, 5 % CO_2 incubator. After 24 h, 10 μg/ml Brefeldin-A, 1 μM wortmannin or DMSO/ethanol used as negative control, was added to cell culture supernatant, and the cells were incubated at 37 °C for 24 h. Finally, HeLa cell lysate and cell supernatant were harvested for Western blot analysis.

Neuraminidase and endoglycosidase-H digestion. Purified CCDC134 protein was treated with neuraminidase and endoglycosidase-H (Endo-H; Roche Molecular Biochemicals, Germany) in 0.5 % SDS, 0.5 M DTT, with freshly added proteinase inhibitor cocktail for 4 h at 37 °C and analyzed by Western blot.

Transient expression dual-luciferase reporter assay. Elk1 luciferase activity was measured using Elk-1 trans-reporting system. Approximately 1.0×10^4 HEK293T cells/well were seeded into a 96-well culture plate. After 24 h, the cells in each well were cotransfected with 50 ng pcDB-CCDC134 plasmid, 45 ng pFR-Luc plasmid containing a synthetic promoter of the yeast Gal4-binding sites that control expression of the firefly luciferase gene, 5 ng pFA-Elk1 fusion transactivator plasmid expressing a fusion protein consisting of the activation domain of Elk1 fused with the yeast Gal4 DNA-binding domain, which is driven by a cytomegalovirus promoter (Stratagene, USA) and 4 ng pRL-TK plasmids as the internal control containing Renilla luciferase gene (Promega, USA). The positive and negative controls were performed with GFP-MKP (a kind gift from Anton M. Bennett, USA) and pcDB plasmids, respectively. Each transfection experiment was performed in triplicate wells. At 36 h after transfection, the cells were stimulated with PMA (100 ng/ml) and ionomycin (1 μM) for 4 h, and then lysed in standard lysis buffer. Using a GENios Pro reader (Tecan, Switzerland) the cell lysate was assayed for both firefly and renilla luciferase activities with the dual-luciferase reporter (DLR) assay system (Promega, USA) according to the manufacturer's instructions.

To detect Elk1 luciferase activity of purified CCDC134 protein, only 45 ng pFR-Luc plasmid, 5 ng pFA-Elk1 plasmid and 4 ng pRL-TK plasmids were cotransfected into cells in each well. After 12 h, the purified CCDC134 protein was added into the supernatant of cells, and then the protocol described above was followed.

siRNAs synthesis and electroporation transfection. Specific siRNA against CCDC134 with targeting sequence 5′-CTTCCAGAACCCATTTAAA-3′ were designed, chemically synthesized and PAGE-purified free of RNase contamination according to manufacturer's instructions (Genechem Corporation, China). The non-silencing siRNA was confirmed to have no matches with the complete human genome by a BLAST search in NCBI (www.ncbi.nlm.nih.gov). All siRNAs were dissolved to a concentration of 20 mM with buffer containing 20 mM KCl, 6 mM HEPES pH 7.5 and 0.2 mM $MgCl_2$. Cell density was adjusted to 5×10^5/350 μl and an indicated amount of siRNA alone or combined with plasmid were electroporated into cells.

Phosphorylation of MAPKs and Immunoassay. Following incubation (24 h, 37 °C), transfected cells were serum deprived for 20 h, then stimulated with PMA (100 ng/ml) and ionomycin (1 μM) for the detection of

phospho-Erk1/2, with UV (80 mJ) treatment for phospho-JNK/SAPK and phospho-p38 detection for the indicated times. Treated cells were washed with pre-chilled PBS and incubated in cell lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2 EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4,1 μg/ml leupeptin, 1% Triton X-100, with freshly added proteinase inhibitor cocktail) for 30 min at 4 °C. Cell lysate was analyzed by Western blot.

To detect affection of purified CCDC134 protein, the cells were plated. After 12 h, purified CCDC134 protein was added into cell culture supernatant, and then the immunoassay protocol described above was followed.

Results

Cloning and bioinformatics analysis of human CCDC134. The full-length human CCDC134 cDNA clone (GenBank accession no. NM_024821; FLJ22349) was directly isolated from a human lung cDNA library. It is 1280 bp long with an in-frame stop codon upstream of the putative ATG start codon and a 3′-poly (A) tail. The ORF encodes 229 amino acids with a predicted molecular mass of 26.4 kDa and a p*I* of 9.4. The full-length cDNA and predicted amino acid sequences of CCDC134 are shown in Fig. 1A. Human *CCDC134* is located on chromosome 22q13, and encompasses 7 exons and 6 introns (Fig. 1B). *CCDC134* is conserved in human, macacamulatta, rat, mouse and canis familiaris, etc. (Fig. 1C), but shares no obvious homology to any known genes or proteins. SignalP analysis (http://www.cbs.dtu.dk/services/SignalP) suggests that there is a putative signal peptide near the N terminus of the protein.

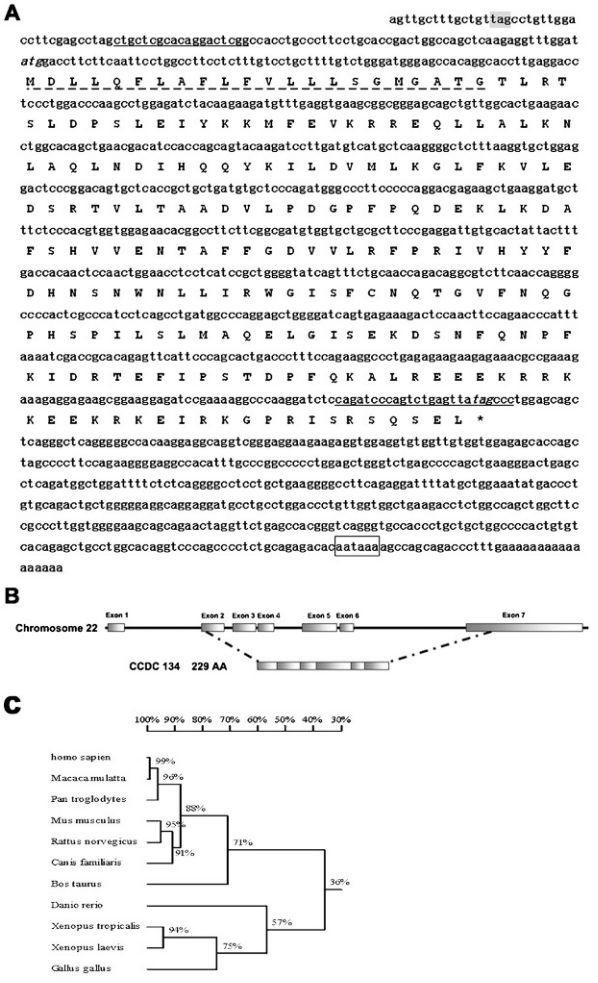

Figure 1. Identification and sequence analysis of CCDC134. (*A*) cDNA and predicted amino acid sequences of human CCDC134. Primers used to amplify the ORF are underlined. The start and stop codons are italicized. An in-frame stop codon in front of the start ATG is shaded. Bold letters in a frame at the 3′ end indicate the poly(A) signal sequence. The putative signal peptide (amino acid residues 1–22) is indicated with a broken line. (*B*) The sketch map of the *CCDC134* gene and cDNA structure. The boxes show the exons with their relative size and the positions in the *CCDC134* gene. (*C*) Phylogenetic analysis of *CCDC134*.

Expression profiles of CDDC134. The presence of CCDC134 on the mRNA level was confirmed by Northern blot and RT-PCR in various human tissues and human cell lines, respectively. As shown in Figure 2A, Northern blot analysis revealed that a ~1.3-kb transcript specific for CCDC134, which was consistent with the bioinformatics analysis (1280 bp), was expressed in lung and placenta, especially at a higher level in the testis of human adult normal tissues. Additionally, RT-PCR analysis revealed CCDC134 transcript was expressed in some normal adult tissues, including spleen, placenta, ovary, leukocyte and lung, etc. (Fig. 2B), a variety of tumor tissues examined (Fig. 2C) and cell lines (Fig. 2D).

In addition, Western blot analysis identified expression of CCDC134 protein in various human cell lines, a band of ~26 kDa could be detected, which was consistent with the calculated molecular weight of CCDC134 protein. The result also revealed the existence of bands of ~34 and ~36 kDa in several cell lines. These results suggest that CCDC134 was synthesized as a polypeptide with an apparent molecular mass of ~26 kDa, and then modified, possibly by some glycosylation modification, finally existing as a ~34-kDa form, which was the protein without signal peptide and an ~36-kDa form, which was the protein with signal peptide (Fig. 2E). Moreover, immunohistochemical staining using rabbit anti-CCDC134 specific antibody confirmed that CCDC134 protein was also expressed in different adult normal tissues. As shown in Figure 2F, in some normal gland epithelial

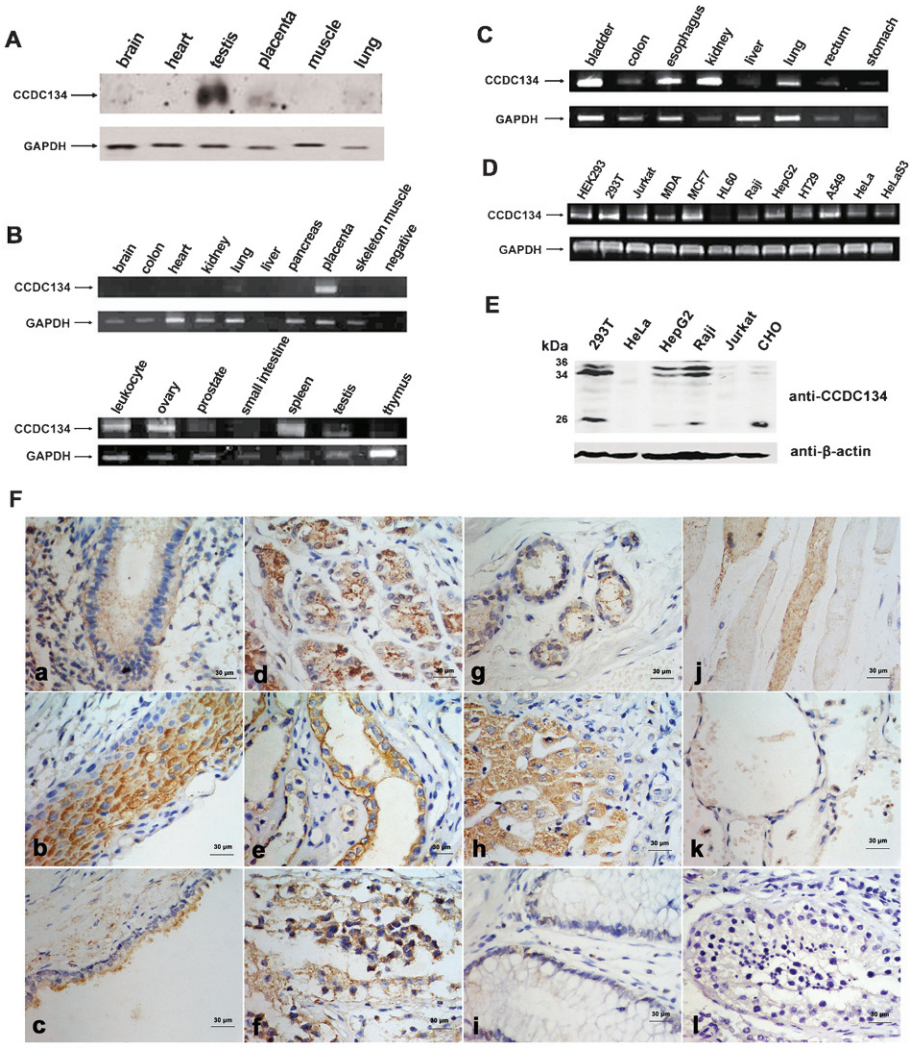

Figure 2. Expression profiles of human CCDC134. (*A*) Northern blot analysis of CCDC134 expression in adult human normal tissues. The membrane was hybridized with fluorescein-labeled CCDC134. GAPDH probe was used as an internal control. CCDC134 mRNA expression was also analyzed by RT-PCR in human normal tissues (*B*), human tumor tissues (*C*) and human cell lines (*D*). GAPDH was used as an internal control. (*E*) CCDC134 endogenous protein expression was also indicated by Western blot using rabbit polyclonal anti-CCDC134 antibody in various human cell lines. β-actin expression was detected as an internal control. (*F*) Immunohistochemical staining with a rabbit polyclonal anti-CCDC134 antibody. Expression of CCDC134 were analyzed in normal human cervical gland (a), cervical squamous epithelial cell (b), endometrium (c), stomach (d), kidney distal convoluted tubule (e), testis spermatogenic cells (f), mammary gland (g), liver (h), colon (i), striated muscle (j) and lung (k). No immunoreactivity was seen in the preabsorption control for CCDC134 (l). Peroxidase activity was visualized with diaminobenzidine as the substrate, and the sections were counterstained with hematoxylin. Scale bars = 30 μm.

cells, including cervical glands, endometrial glands, stomach and mammary glands, CCDC134 was located at the apical surface of epithelial cell by apocrine form; and in cervical squamous epithelial cell, kidney distal convoluted tubule, testis, liver and striated muscle, CCDC134 staining was mostly observed at the cytoplasm. However, no positive staining was observed in small intestine and lung tissues. In addition, no immunoreactivity was seen in the preabsorption control for CCDC134. These results supported CCDC134 could be widely expressed and secreted in numerous normal adult tissues.

CCDC134 is a classical secretory protein. To confirm that CCDC134 is a secretory protein, we constructed different truncated CCDC134, including pcDB-CCDC134-ΔSP and pEGFP-CCDC134-ΔSP (Fig. 3A), and performed Western blot analysis of cell lysate and culture supernatant of HeLa cells transiently transfected with these mammalian expression constructs expressing a c-myc-tagged or GFP-tagged human CCDC134 protein. Using mouse monoclonal anti-human c-myc and rabbit polyclonal anti-human GFP antibody, we detected specific protein bands in cell lysate transfected with pcDB-CCDC134, pEGFP-CCDC134, pcDB-CCDC134-ΔSP and pEGFP-CCDC134-ΔSP, but not in cell lysate transfected with pcDB. We also detected specific protein bands in cell culture supernatant transfected with pcDB-CCDC134 and pEGFP-CCDC134; but not in cell culture supernatant transfected with pcDB-CCDC134-ΔSP, pEGFP-CCDC134-ΔSP and pcDB (Fig. 3B). These results suggest CCDC134 could be modified and secreted into cell culture supernatant. In addition, to examine the subcellular location of pEGFP-CCDC134 and pEGFP-CCDC134-ΔSP, HeLa cells were transiently transfected with these expression vectors. After 36 h, pEGFP-CCDC134 exhibited a diffuse cytoplasmic distribution. Confocal microscopic analysis revealed that pEGFP-CCDC134

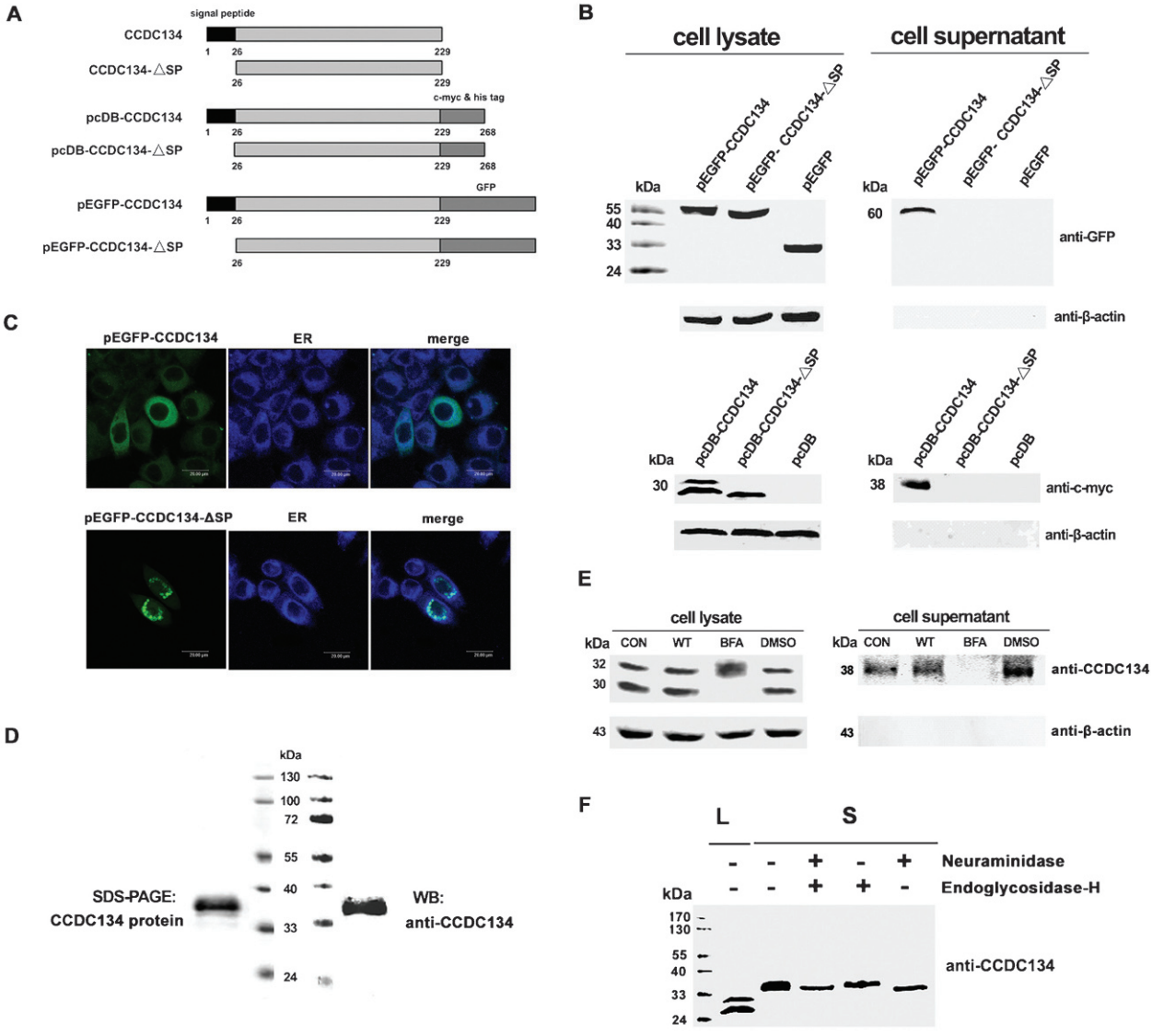

Figure 3. CCDC134 is identified to be a classical secretory protein. (*A*) Schematic representation of human CCDC134 and constructs used in this study. (*B*) Detection of overexpressed CCDC134 secreted into culture supernatant by Western blot. Cell lysate and culture supernatant from HeLa cells transfected with pEGFP-CCDC134, pEGFP-CCDC134-ΔSP and pEGEP were detected using anti-GFP antibody (upper) and anti-c-myc monoclonal antibody (lower). (*C*) Subcellular localization of pEGFP-CCDC134 and pEGFP-CCDC134-ΔSP. Two-color confocal microscopy analysis of pEGFP-CCDC134, pEGFP-CCDC134-ΔSP fusion protein (green) and ER-specific fluorescent dye ER Tracker (blue) was performed after 36h transfection. (*D*) SDS-PAGE and Western blot analysis of purified CCDC134 protein with a Ni-Sepharose^{TM} 6 Fast Flow column. (*E*) Western blot analysis of BFA and wortmannin block assay. CON indicates the cells without treatment, and BFA blocks CCDC134 secretion, whereas wortmannin and DMSO, as a negative control, have no effect. (*F*) The effect on CCDC134 detection of neuraminidase and endoglycosidase-H digestion. Purified CCDC134 protein (S) and transfected cell lysates with pcDB-CCDC134 plasmid (L) were treated as indicated and subjected to Western blot analysis with rabbit polyclonal anti-CCDC134 antibody.

co-localized with ER-specific fluorescent dye ER Tracker. No co-localization with the lysosome-specific fluorescent dye Lyso Tracker, the mitochondria-specific fluorescent dye Mito Tracker or the Golgi-specific fluorescent dye Golgi Tracker was observed (data not shown). However, pEGFP-CCDC134-ΔSP exhibited a punctate cytoplasmic distribution and concentrated in the perinuclear region compared with pEGFP-CCDC134. No co-localization with ER-specific fluorescent dye ER Tracker was observed (Fig. 3C).

Next, the pcDB-CCDC134 expression protein in HeLa cells was purified using a Ni-Sepharose^{TM} 6 Fast Flow column. The purified protein was validated by SDS-PAGE and Western blot analysis (Fig. 3D), and then was detected by N-terminal sequence analysis. The result showed that purified protein had an molecular mass of 38 kDa, a p*I* of 9.4, and the

analysis of N-terminal 5 amino acids showed TLRTS, which was at position 22 and 23 of CCDC134, suggesting that cleavage site of CCDC134 protein was located at position 22 and 23 of CCDC134 N terminus. These results all validated that CCDC134 was a secretory protein.

Additionally, the secretion form of CCDC134 was also investigated to determine whether CCDC134 was secreted by classical secretion pathway. As shown in Figure 3E, BFA treatment, which can block classical secretion pathway, markedly blocked the CCDC134 secretion in the transfected cells with pCDB-CCDC134, whereas wortmannin, an inhibitor of phosphoinositol-3-kinase, which inhibits nonclassical secretion pathway including exosome secretion, showed no effect on CCDC134 secretion. These results indicate CCDC134 is a classical secretory protein, and its secretion into the cell culture supernatant can be blocked by BFA.

These findings suggested that CCDC134 exhibited two CCDC134 species of 30 and 32 kDa in transfected cell lysate and a single 38-kDa form in transfected cell culture supernatant (Fig. 3F). Neuraminidase treatment slightly reduced the apparent molecular mass of purified CCDC134 protein from 38 to 35 kDa, and additional treatment with *N*-glycosidase caused a similar effect with reduction of the apparent molecular mass to around 35 kDa, whereas *N*-glycosidase treatment alone did not have any effect. These results indicate that purified CCDC134 protein has *O*-linked sugars that are highly modified by sialic acids, and CCDC134 protein has other modification.

CCDC134 inhibits Elk1 transcriptional activity. According to the result of high-throughput functional screening systems for Elk1, *CCDC134* overexpression inhibited transcriptional activity of Elk1 in HEK293T cells. To further identify whether CCDC134 could prevent MAPK-mediated activation of Elk1 in various cell lines, we overexpressed *CCDC134* in HEK293T and HeLa cells separately, and measured Elk1 transcription activity in response to PMA and ionomycin stimulation. GFP-MKP, which was reported to inhibit Elk1 activation, was used as a positive control [11]. PMA and ionomycin activated Elk1 by approximately fivefold in HeLa and HEK293T cells, while CCDC134 inhibited Elk1 activation significantly in cells stimulated with PMA and ionomycin (Fig. 4A).

To further detect the effect of purified exogenous CCDC134 protein on Elk1 activity, HEK293T and HeLa were treated with purified CCDC134 fusion protein. MEK1-specific inhibitor, PD98059, which inhibits PMA and ionomycin-induced Elk1 activation, was used as positive control. The result showed that CCDC134 protein inhibited Elk1 activity at appropriate concentrations, i.e., 100 or 200 ng/ml, whereas at high or low concentrations, i.e., 40 or 800 ng/ml, purified CCDC134 protein had no significant effect for Elk1 activity (Fig. 4B). To gain conclusive evidence that CCDC134 was a secretory protein, we therefore sought to identify the mechanism through which CCDC134 mediated its effects and acted extracellularly. Using purified CCDC134-FITC protein, we performed CCDC134 protein binding assays *in vitro* in HeLa cells. Recombinant PDCD5-FITC protein, which has been reported to have a remarkable role in intercellular transport, was used as control [12]. As depicted in Figure 4C, cell endocytosis was blocked at 4 °C, and both CCDC134-FITC and PDCD5-FITC located on cell membrane; at 37 °C, while CCDC134-FITC was still localized on cell membrane, PDCD5-FITC was localized to discrete compartments in the cytoplasm, suggesting that CCDC134 mediated its effects through binding unidentified protein on cell membrane.

Next, to further determine the role of CCDC134 in Elk1 activity, siRNA was designed to silence the expression of *CCDC134* in HEK293T cells. Non-silencing siRNA or siRNA against *CCDC134* (si-CCDC134) was transfected into HEK293T cells alone or combined with the pEGFP-CCDC134 vector. At 40 h and 66 h after transfection, CCDC134 mRNA and protein levels were significantly decreased in cells transfected with si-CCDC134, as assessed by RT-PCR (Fig. 5A), Western blot (Fig. 5B) and fluorescence microscopy (data not shown). Furthermore, we evaluated whether si-CCDC134 treatment could activate Elk1 activity. As illustrated in Figure 5C, si-CCDC134 activated Elk1 transcriptional activity compared with non-silencing siRNA. These data suggest that CCDC134 might play a key role in the regulation of transcription factor Elk1.

CCDC134 inhibits Erk1/2 and SAPK/JNK but not p38 MAPK signaling pathway. To further define the mechanism by which CCDC134 inhibited PMA and ionomycin-induced Elk1 activation, we assessed the phosphorylation status of Elk-1 Ser383. Because the mammalian MAPK family that affected Elk1 included three main subgroups, Erk, JNK/SAPK and p38 MAPK [13], we detected the protein levels of Elk1, MEK and Erk1/2 as well as their phosphorylation levels. As shown in Figure 6A, *CCDC134*-overexpressing cells had a lower phosphorylation level of Elk1 compared with cells transfected with pCDB. Similar differences in the phosphorylation of MEK and Erk1/2 were found. Furthermore, JNK1/2 and p38 MAPK phosphorylation were also detected. In addition to Erk1/2, CCDC134 also inhibited phosphoryla-

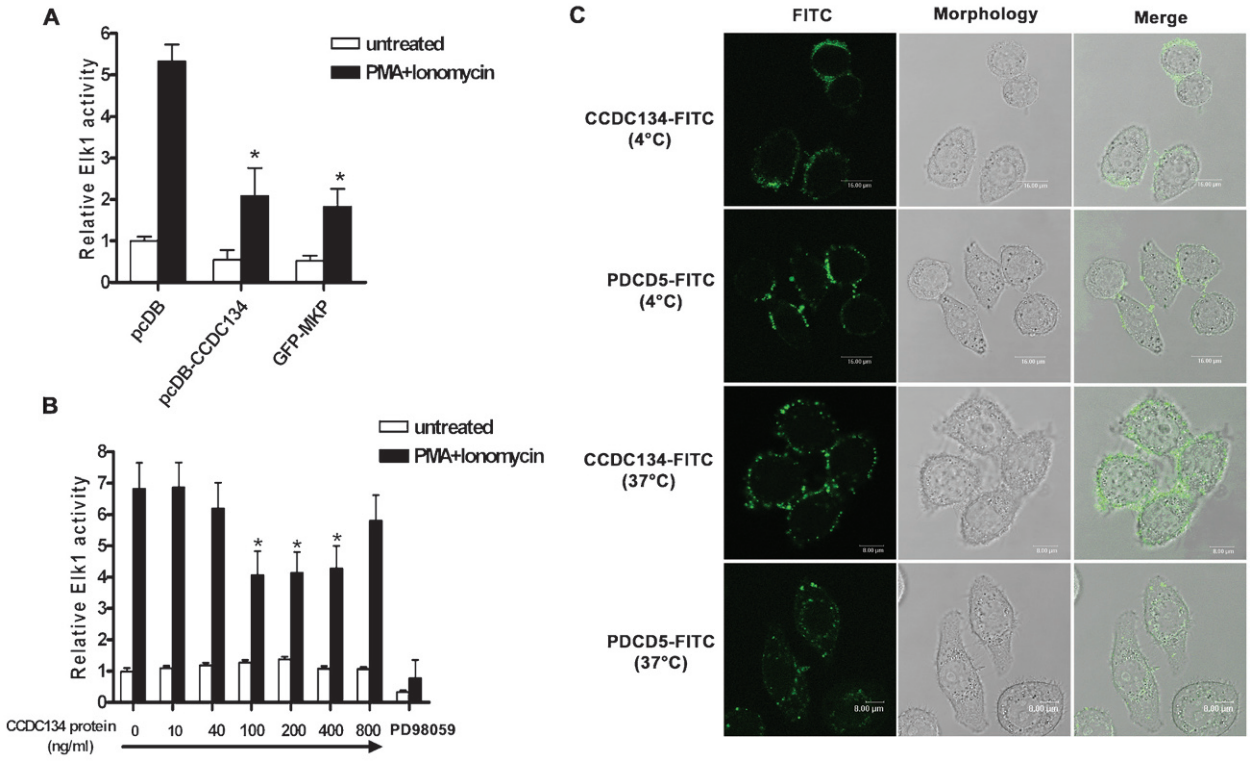

Figure 4. HEK293T cells were transiently cotransfected with the pFR-luc reporter plasmid, pFA-Elk1 fusion activator plasmid and pRL-TK plasmid. Relative luciferase activity was normalized by co-transfection with pRL-TK plasmid (internal control). (*A*) Relative Elk1 activity of CCDC134 overexpression. GFP-MKP was used as positive control. (*B*) Relative Elk1 activity of purified CCDC134 protein at different concentrations; PD98059 as a positive control. Transfected HEK293T cells were either left untreated or were stimulated with PMA and ionomycin for 4 h. Data shown are relative luciferase activities normalized based on Renilla luciferase levels and are means of three repeats in a single transfection experiment. The error bars indicate standard deviations for three assays. Each transfection experiment was performed at least three times. (*C*) Binding assays done with CCDC134-FITC in HeLa cells. After the cells were incubated for 5 h with purified CCDC134-FITC protein and PDCD5-FITC protein as control, the location of CCDC134-FITC and PDCD5-FITC was detected using a TCS-SP laser-scanning confocal microscope.

tion of JNK/SAPK, although it had no effect on phosphorylation of p38 MAPK. Conversely, si-CCDC134-transfected cells had a higher level of phosphorylation of Erk1/2 and JNK/SAPK compared with cells transfected with non-silencing siRNA, while for phosphorylation of p38, si-CCDC134 had no effect (Fig. 6B).

To test the effect of purified exogenous CCDC134 protein on phosphorylation of Erk1/2, HEK293T and HeLa cells were treated with CCDC134 protein. MEK1-specific inhibitor, PD98059, was used as positive control. CCDC134 protein inhibited phosphorylation of Erk1/2 at appropriate concentrations, 100 or 200 ng/ml, whereas CCDC134 protein had no obvious inhibition effect at a higher or lower concentration, 40 or 400 ng/ml (Fig. 6C). We used 100 ng/ml CCDC134 protein to monitor the phosphorylation of various MAPKs in HEK293T and HeLa cells. Phosphorylation of Erk1/2 was decreased at 30 min after treatment with CCDC134 protein, and this protein also induced inhibition of phospho-JNK/SAPK (Fig. 6D).

Discussion

In the present study, we identified *CCDC134* as a potentially novel gene based on a search of EST sequences in GenBank. Through a series of experiments we identified *CCDC134* as being a functional gene, and it is widely expressed in various adult cancer tissues, normal tissues and cell lines. Bioinformatics analysis indicated the presence of a potential signal peptide at the N terminus of CCDC134, suggesting that it might be a secretory protein. Using an enzyme-linked immunosorbent assay, CCDC134 was detected in cell culture supernatant when overexpressed in HEK293T and HeLa cells. To confirm that the CCDC134 is a secretory protein, we purified and sequenced the CCDC134 protein from cell culture supernatant, and showed that CCDC134 lacked the 22-amino acid of N-terminal region, consistent with the bioinformatics analysis. Further expression studies indicated that truncated CCDC134 (lacking 26 amino acids at the N terminus) could not be secreted into cell culture supernatant; and subcellular location of

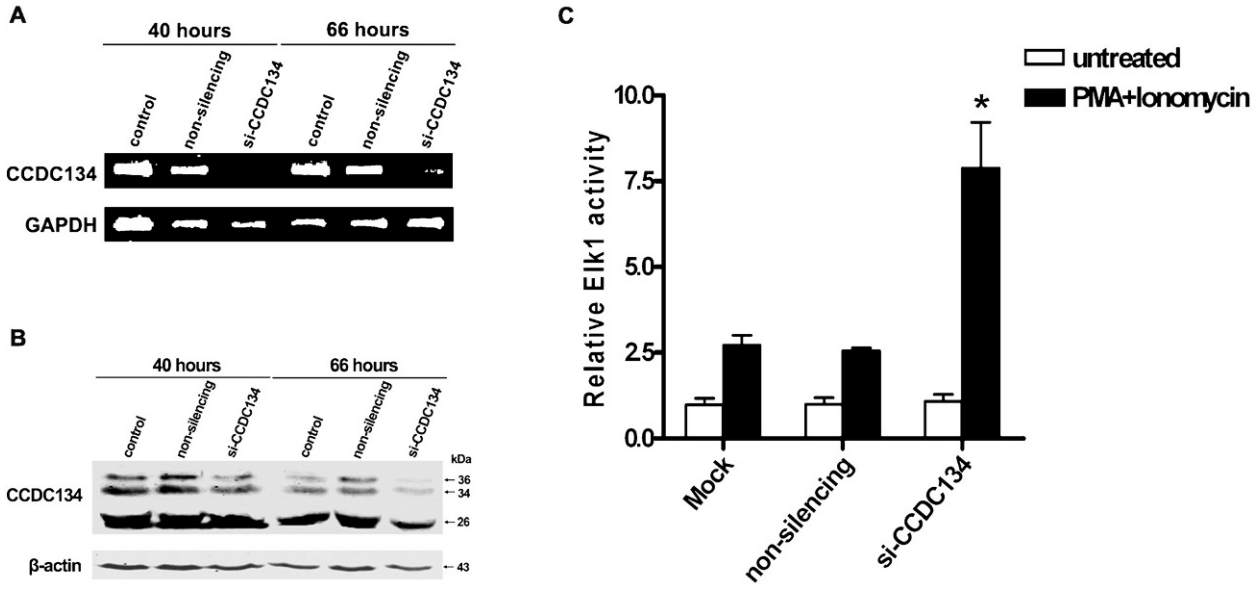

Figure 5. Silencing of CCDC134 activated Elk1 transcriptional activity. (*A*) RT-PCR results for CCDC134 mRNA expression. Si-CCDC134 shows a strong inhibitory effect for CCDC134 mRNA expression at 66 h after transfection with CCDC134 siRNA compared with non-silencing siRNA and wild type (control) in HEK293T cells. (*B*) The protein expression level of CCDC134 detected by Western blot. Si-CCDC134 inhibits CCDC134 expression at protein level compared with non-silencing siRNA and wild type (control) in HEK293T cells. (*C*) Activation effect of si-CCDC134 on Elk1 activity. Transfected HEK293T cells were either left untreated or were stimulated with PMA and ionomycin for 4 h. Data shown are relative luciferase activities normalized based on Renilla luciferase levels and are means of three repeats in a single transfection experiment. The error bars indicate standard deviations for three assays. Each transfection experiment was performed at least three times.

pEGFP-CCDC134-ΔSP exhibited a punctate cytoplasmic distribution and concentrated in the perinuclear region compared with pEGFP-CCDC134, confirming the presence of a typical signal peptide at the N terminus. Further evidence that CCDC134 is a secretory protein in its natural state was shown by immunohistochemistry analysis, which revealed that CCDC134 was located at the apical surface of epithelial cells by the apocrine form. CCDC134 was also detected in culture supernatant of phytohemagglutinin-activated human peripheral blood mononuclear cells (data not shown).

We also looked for evidence that CCDC134 traversed the classical secretory pathway through the Golgi apparatus *en route* to its final extracellular destinations. Many secretory proteins are thought to rely upon transmembrane cargo receptors for efficient ER-to-Golgi transport [14]. Western blot analysis of CCDC134 revealed a single high molecular mass band in cell culture supernatant, which was different from the molecular mass of CCDC134 found in cell lysate, suggesting that the soluble form of CCDC134 had undergone post-translational modification to result in the higher molecular mass form. Proteins can undergo various post-translational modifications that affect their final molecular mass, *e.g.*, glycosylation and proteolytic cleavage. Based on bioinformatics analysis, CCDC134 was predicted to have an *O*-glycosylation site at position 192, and two C-mannosylation sites at positions 136 and 142, so these modifications can be made as the protein passes through the ER and the Golgi apparatus [15]. Neuraminidase treatment slightly reduced the apparent molecular mass of purified CCDC134 protein from 38 to 35 kDa, although treatment of *N*-glycosidase had no effect. These studies suggested that CCDC134 might be synthesized as a polypeptide of 26 kDa, which is modified by the addition of *O*-linked sugars and secreted into the extracellular space as a 38-kDa form.

We also studied the effect of CCDC134 on the transcriptional activity of Elk1. First, we demonstrated that overexpression of CCDC134 inhibited Elk1 activity in HEK293T and HeLa cells. Additionally, overexpression of *CCDC134* and purified CCDC134 protein significantly inhibited phosphorylation of Erk1/2 and JNK/SAPK but not p38 MAPK. The modifying effect of CCDC134 was further confirmed using siRNA-mediated knockdown of *CCDC134*, which activated Elk1 through up-regulating phosphorylation of Raf-1/MEK/ERK and JNK/SAPK in HEK293T and HeLa cells. There are two possible ways by which CCDC134 could inhibit the MAPK pathway. First, as a typical secretory protein, CCDC134 might inhibit MAPK phosphorylation *via* an unidentified receptor on the plasma membrane. In this study, we found that cell culture supernatant

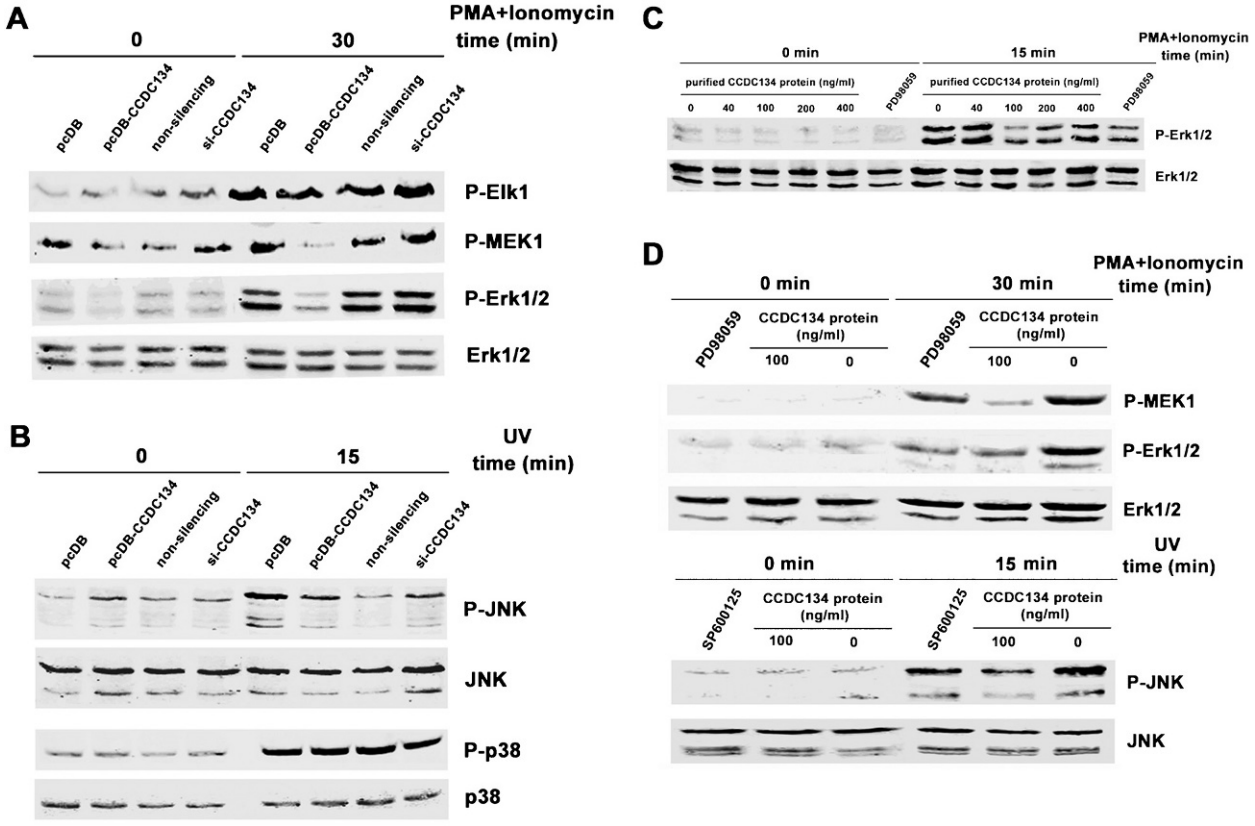

Figure 6. CCDC134 inhibits MAPK phosphorylation. HEK293T cells were transfected with pcDB, pcDB-CCDC134 plasmid, and nonsilencing and CCDC134 siRNA, respectively. (*A*) CCDC134 inhibits the Erk1/2 pathway. Cells were stimulated with PMA and ionomycin for 30 min, or were left unstimulated as indicated. (*B*) The effect of CCDC134 on phosphorylation of JNK and p38 MAPK. Cells were stimulated with UV for 15 min, or were left unstimulated as indicated. (*C*) HEK293T cells were preincubated with CCDC134 protein at different concentration. Cells were stimulated with PMA and ionomycin for 30 min and phospho-Erk1/2 was detected. (*D*) HEK293T cells were preincubated with 100 ng/ml CCDC134 protein. PD98059 was used as positive control for Erk1/2 pathway. SP600125 was used as positive control for JNK pathway. The presence of phosphorylated proteins was monitored by Western blot using phospho-specific antibodies as indicated. Loading of equal amounts of proteins was determined by restaining of the blots with antibodies directed against Erk1/2, JNK or p38.

containing CCDC134 had significant inhibition activity on Elk1 transcription and Erk phosphorylation by trans-well assay. Furthermore, we detected an unidentified protein (possibly a specific receptor of CCDC134), which bound CCDC134-FITC protein. There are many known growth factor receptors on the plasma membrane that, when activated, initiate biochemical cascades or signal transduction pathways that ultimately lead to changes in gene expression and induction of mitogenic response [16]. As an example, TGF-β down-regulates ERK2 (and JNK) to mediate negative-growth control of human breast cancer cells *via* its receptor on the plasma membrane [17, 18]. Second, intracellular CCDC134 also has the ability to inhibit Erk and JNK. This phenomenon has been observed in this study by showing that overexpression of CCDC134 inhibited Elk1 activity and the phosphorylation of Erk1/2 and JNK/SAPK. However, the mechanism of CCDC134-mediated MAPK pathway requires further study.

Because the majority of ERK stimuli lead to cell proliferation or differentiation, we predicted that CCDC134 might play a role in these processes. However, in our unpublished experiments neither CCDC134 overexpression nor si-CCDC134 had a significant effect on cell growth or differentiation in HEK293T and HeLa cells, the effect of CCDC134 at the cellular level is not yet clear.

In conclusion, the *CCDC134* gene was detected through high-throughput functional screening systems, and was identified as a secretory protein that played an important role in transcription regulation and MAPK signal transduction through the Raf-1/MEK/Erk and JNK/SAPK, but not p38 MAPK pathways. These results make a contribution to a better understanding of the complex mechanism of CCDC134.

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