学　位　論　文

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（p53 は little elongation complex の形成を阻害することによって *snRNA* 遺伝子の転写を抑制する）

2016 年 6 月

北　海　道　大　学

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A) LIST OF PUBLICATIONS AND PRESENTATIONS

Publications

(1) Takahashi H, Takigawa I, Watanabe M, Delnur Anwar, Shibata M, Tomomori-Sato C, Sato S, Ranjan A, Seidel CW, Tsukiyama T, Mizushima W, Hayashi M, Ohkawa Y, Conaway JW, Conaway RC, Hatakeyama S: MED26 regulates the transcription of *snRNA* genes through the recruitment of little elongation complex. Nat Commun, 5:5941 doi: 10.1038/ncomms6941, Jan 9, 2015

(2) Anwar D, Takahashi H, Watanabe M, Suzuki M, Fukuda S, Hatakeyama S: p53 represses the transcription of *snRNA* genes by preventing the formation of little elongation complex. BBA- Gene Regulatory Mechanisms. (under review)

Posters and Oral Presentations

(International conference)

(1) Takahashi H, Takigawa I, Anwar D, Shibata M, Tomomori-Sato C, Sato S, Ranjan A, Seidel CW, Tsukiyama T, Watanabe M, Hayashi M, Ohkawa Y, Conaway JW, Conaway RC, Hatakeyama S: Human Mediator subunit MED26 regulates the transcription of small nuclear RNA genes through the recruitment of little elongation complex, CSHL Meeting on Mechanisms of Eukaryotic Transcription, Cold Spring Harbor Laboratory, NY, USA, August 27-31, 2013 (Poster)

(National conference)

(1) Takahashi H, Takigawa I, Anwar D, Shibata M, Tomomori-Sato C, Sato S, Ranjan A, Seidel CW, Tsukiyama T, Watanabe M, Hayashi M, Ohkawa Y, Conaway JW, Conaway RC, Hatakeyama S: Human Mediator subunit MED26 regulates the transcription of *small*

nuclear RNA genes through the recruitment of Little elongation complex, The 37th Annual Meeting of the Molecular Biology Society of Japan, Kobe, Japan, 2013 (Poster)

(2) Anwar D, Takahashi H, Watanabe M, Tsukiyama T, Hatakeyama S, Fukuda S: Functional analysis of transcription elongation regulatory factor MED26, The 4th Hokkaido University Otolaryngology Open Seminar, Sapporo, Japan, August 28, 2014 (Presentation)

(3) Takahashi H, Takigawa I, Watanabe M, Anwar D, Shibata M, Tomomori-Sato C, Sato S, Ranjan A, Seidel CW, Tsukiyama T, Hayashi M, Ohkawa Y, Conaway JW, Conaway RC, Hatakeyama S.: Human Mediator subunit MED26 regulates the transcription of *small nuclear RNA* genes through the recruitment of Little elongation complex, The 87th Annual Meeting of the Japanese Biochemical Society, Kyoto, Japan, October 15, 2014 (Presentation)

B) INTRODUCTION

1 Gene expression

Genetic information is transmitted to the next generation through DNA replication. Gene expression is the process by which genetic information in DNA is utilized to synthesize functional gene products, which are proteins and non-protein-coding genes including transfer RNA (tRNA), ribosonal RNA (rRNA) and small nuclear RNA (snRNA) genes. The process of gene expression is almost similar in all known organisms including eukaryotes, prokaryotes and viruses. The gene expression process is modulated by several steps including transcription, translation and post-translational modification of proteins. Transcription is a copy process from DNA as a template to messenger RNA (mRNA), which is used for translation to proteins as a template.

2 Transcription and RNA polymerase

Eukaryotic cells have mainly four classes of RNA: rRNA, mRNA and tRNA, which are involved in protein synthesis (translation), and snRNA, which is involved in mRNA splicing. In humans, the synthesis of RNA from DNA is catalyzed by DNA-dependent RNA polymerases, RNA polymerases I, II and III. RNA polymerase I is responsible for transcription of *rRNA* genes. RNA polymerase II (Pol II) transcribes almost all protein-coding genes and some non-coding RNAs (e.g., snRNAs, snoRNAs and long non-coding RNAs). RNA polymerase III transcribes 5S *rRNA*, *transfer RNA* (*tRNA*) genes, and some small non-coding RNAs.

The process of RNA synthesis involves three steps: initiation, elongation and termination. Most mammalian genes contain a TATA box that is usually localized 25-30 bp upstream of the transcription start site. A TATA-binding protein (TBP) binds to a TATA box and then interacts with other TBP-associated factors (TAFs) at the promoter. The complex including

TBP and TAFs is called TFIID. Binding of TFIID to the TATA box is the first step in the formation of the transcription complex on the TATA box. The protein-DNA binding at the TATA box involving Pol II and other complexes of basal transcription factors ensures the fidelity of initiation. Once the promoter has been recognized, Pol II begins to synthesize an RNA transcript from the DNA template. The RNA chain elongation process continues until a terminal signal appears. Although research has mainly focused on mechanisms that control the pre-initiation and initiation stages of transcription[1-3], recent accumulating evidence indicates that transcription of many genes is also regulated during the transcription elongation step [4,5].

3 ELL and EAFs are key regulators of transcription elongation by Pol II

Eleven-nineteen lysine-rich leukemia (ELL), which is encoded by the *ELL* gene, is a translocation partner of mixed-lineage leukemia. ELL and its binding partners, ELL-associated factors (EAFs), are key regulators of transcription elongation by Pol II [6,7]. Super elongation complex (SEC) and little elongation complex (LEC) are two distinct ELL/EAF-containing complexes. SEC contains ELL/EAF, positive transcription elongation factor (P-TEFb) and mixed-lineage leukemia-fusion partner proteins[8-10]. SEC regulates transcription elongation of a variety of protein-coding genes, such as *c-Myc*, *Hsp90*, *Hox* and HIV provirus [11]. LEC contains ELL/EAF, NMDA receptor regulated 2 (NARG2/ICE2) and ICE1. Those components were also identified as components of *Drosophila* LEC, called ICE1 and ICE2. ICE2 was shown to interact with the C-terminus of ELL [12]. Recently, it was reported that LEC specifically regulates the transcription of Pol II-transcribed *snRNA* genes [13,14].

4 Pol II-transcribed *snRNA* genes and protein-coding genes are structurally different

There are structural differences in the transcription elongation process between Pol II-transcribed *snRNA* genes and Pol II-transcribed protein-coding genes[14]. Pol II-transcribed *snRNA* genes contain a 3' box instead of a polyadenylation signal, which is required for 3' end formation[15-18]. Transcription of Pol II-transcribed *snRNA* genes requires an integrator complex that specifically binds to the Ser7-phosphorylated form of the Pol II C-terminal domain (CTD) and proceeds to the 3' end formation [17,19]. Inhibitors of P-TEFb reduce the 3'-box-dependent 3' end processing but do not affect transcription elongation of the *snRNA* genes [20]. On the basis of these findings, we hypothesized a different regulation of transcription elongation between Pol II-dependent *snRNA* genes and protein- coding genes.

5 p53 functions as a tumor suppressor through multiple cellular processes

An anti-oncogene (tumor suppressor gene) functions as a protector in cells to inhibit cancer formation. Mutation of these genes may cause a loss or reduction of this function, leading to carcinogenesis. p53 protein was first identified in 1979 as an intracellular protein overexpressed in cancer cells [21]. p53 is an important tumor suppressor that is encoded by the *Tp53* gene in the human genome, and it functions as a tumor suppressor through multiple cellular processes [22-26]. p53 binds to DNA as a transcriptional regulator and controls gene expression to prevent mutations of the genome[27]. p53 is activated in response to alteration of normal cell homeostasis including DNA damage, heat shock, hypoxia, pH change, virus infection and nutrient starvation [28,29]. In response to DNA damage, p53 is stabilized and acts as a transcription factor that positively or negatively regulates several hundred genes[30]. p53 as a tetramer binds directly and specifically to p53-responsive elements on DNA to regulate gene expression[31]. p53 maintains genetic stability via different processes including cell-cycle arrest, DNA repair and programmed cell death.

In the absence of stress signals, p53 protein is expressed at low levels via a fine-tuning between transcription and degradation. This balance is so important, because high expression

of p53 induces apoptosis or cell cycle arrest, whereas low expression of p53 causes loss of control of carcinogenesis. Therefore, p53 protein level is tightly regulated in response to various cellular stresses at the transcriptional and translational levels and by various posttranslational modifications, such as phosphorylation, acetylation, ubiquitination, neddylation, sumoylation and methylation[32].

6 p53 plays a role in human head-neck cancer

Cancer is now one of the most common diseases in the world nowadays. Head and neck squamous-cell carcinoma (HNSCC) is the 6th most common cancer, and it accounts for about 5 percent of all malignancies in the world[33]. Although the occurrence of cancer is a multifactorially complex process, it is generally believed that tumor suppressor gene inactivation and overexpression of anti-apoptotic genes are important causes of cancer[34]. p53 mutations are commonly found also in HNSCC, whereas the presence of wild-type p53 has been shown to exert anti-cancer effects[35-37].

In the pathogenesis of thyroid follicular neoplasm, p53 has significant differences between benign tumors (adenomatous nodules and follicular adenomas) and malignant tumors (follicular carcinomas)[38]. It has been reported that matrix metalloproteinases along with SCEL, CRNN, KRT4, SPINK5, and TGM3 have significantly altered expression in HNSCC. MicroRNAs including hsa-miR-139, hsa-miR-203 and the hsa-miR-424/503 cluster have aberrant expression in HNSCC[39]. miRNA-34 is significantly upregulated in thyroid tumors[40], and p53 regulates the expression of miR-34 family members[41]. A functional single nucleotide polymorphism (SNP) on codon 72 (Arg72Pro) in the exon of the *Tp53* gene leads to a methionine proline conversion[42]. Several studies in different populations have shown an association between *Tp53* Arg72Pro polymorphism and risk of papillary thyroid carcinoma (PTC)[43-46]. In laryngeal cancer, overexpression of survivin likely upregulates the level of p53 expression [47].

7 p53 binds to ELL and inhibits the transactivation activity of ELL

Elongation factor *ELL* was originally identified as one of the genes that undergoes translocation with the *trithorax-like MLL* gene ofteen observed in acute myeloid leukemia. It has been reported that ELL functions as a Pol II-mediated elongation factor that increases the rate of transcription by Pol II by suppressing transient pausing [7]. It has also been shown that MLL-ELL is a more efficient inhibitor of p53 than is wild-type ELL. ELL extreme C terminus [ELL(eCT)] has much greater transforming activity through strong inhibition of p53[48]. Recently, it has been reported that p53 interacts with ELL via the C-terminal tail of p53 and the transcription elongation activation domain of ELL. ELL negatively regulates p53 in transcription. Conversely, p53 expression decreases the transcription elongation activity of ELL[49]. These findings suggest that p53 regulates transcriptional activity by RNA polymerase II through controlling the activity of ELL.

8 Non-coding RNAs and snRNAs

Less than 2% of the human genome is translated to protein, wheres more than 40% of the genome is thought to be transcribed to RNA. The untranslated RNA includes a remarkable number of functional non-coding RNAs (ncRNAs). The ncRNAs include microRNAs, small inference RNAs (siRNAs), repeat-associated RNAs and germline-specific RNAs. ncRNAs have been shown to be trans-acting regulators for various cellular functions[50]. The known activities of ncRNAs include endonucleolytic RNA cleavage and ligation, site-specific RNA modification, DNA methylation, DNA (telomere) synthesis and modulation of protein function. These activities are important at many levels for gene expression and also for genome stability. snRNA, also commonly referred to as U-RNA, is a class of small RNA molecules that are found within the splicing speckles and Cajal bodies in the nucleus of eukaryotic cells. The length of an average snRNA is approximately 150 nucleotides. Almost

snRNAs are highly abundant non-polyadenylated non-coding transcripts that function in the nucleoplasm[51].

9 p53 and snRNA

Target genes of DNA damage caused by exposure to ultraviolet irradiation include both proto-oncogenes and tumor suppressor genes. Mutations in the *Tp53* gene are thought to be inportant in the development of cancers. Although p53 directly regulates the transcription of protein-coding genes, recent studies have shown novel roles of p53 in regulation of the transcription of non-coding RNA genes. It has been reported that p53 represses the transcription of *snRNA* genes[52]. p53 regulates the expression of many lincRNAs in response to DNA damage in primary human fibroblasts and in primary mouse embryonic fibroblasts[53]. In addition, accumulating evidence indicates that snRNA plays a role in the carcinogenesis in lung cancer, germ cell tumors and several types of leukemia[54-56] and in the progression of human diseases including Alzheimer's disease [57-59].

10 MED26 in gene transcription

Mediator was first identified in yeast as an evolutionarily conserved coregulatory complex [60,61]. In metazoa, Mediator is composed of approximately 30 distinct subunits [62]. Mediator is a transcriptional coregulatory complex that is required for regulation between gene-specific transcription activators and the basal initiation machinery[63]. Recently, it has been reported that Mediator is involved in the activation of transcription of a number of Pol II-dependent genes at multiple steps including pre-initiation, promoter clearance, transcription elongation, transcription termination and mRNA splicing steps[64-68]. Mediator exists in multiple, functionally distinct forms that share a common core of subunits and can be distinguished by the presence or absence of a kinase module composed of cyclin C and isoforms of Med12,

Med13 and CDK8. The kinase module has been implicated in both transcriptional repression and activation[69].

In metazoa, a subset of Mediator contains an additional subunit, MED26. MED26-containing Mediator copurifies with only a small amount of the kinase module but with near-stoichiometric Pol II[62,69-71]. MED26-containing Mediator appears to play a key role in transcriptional activation[72-74]; MED26 functions in part by recruiting ELL/EAF- and P-TEFb-containing complexes including the SEC to a subset of human genes. Human Mediator complex recruits ELL/EAF- and P-TEFb-containing complexes to promoters via a direct interaction with the N-terminal domain (NTD) of MED26. The MED26 NTD is the most highly conserved region of MED26 and is similar to the NTDs of the elongation factors TFIIS and elongin A[75,76]. The MED26 NTD also binds to TFIID, and TFIID and elongation complexes interact with MED26 through its overlapping binding sites. The MED26 NTD may also function as a molecular switch that contributes to the transition of Pol II into productive elongation[64].

11 Purpose of this study

It is known that mutations of the tumor suppressor p53 are related to most human cancers including head and neck cancer (HNC). p53 has been well established as one of the targeted molecules in biomarkers and drug discoveries for cancer diagnosis and therapy, respectively. Several technologies have been developed to treat cancer, but there is still insufficient evidence and understanding of the importance of transcription in human head and neck cancers[77].

Regulation of Pol II transcriptional elongation is an important step in gene transcription regulation. Human Mediator subunit MED26 copurified with two ELL/EAF-containing complexes, SEC and LEC[64]. MED26-NTD contributes to recruitment of SEC to a subset of human protein-coding genes including c-Myc and HSP70 through direct interaction of

MED26-NTD with EAF[64]. However, the role of MED26 in recruitment of ELL/EAF-containing complexes has not been elucidated.

In a previous study, we obtained evidence that the human Mediator subunit MED26 plays a role in the recruitment of LEC to a subset of Pol II-transcribed *snRNA* genes through direct interaction of EAF and MED26-NTD. Depletion of MED26 in cells decreases the occupancy of LEC at a subset of *snRNA* genes and results in reduction in expression of the genes. Therefore, in this study, we examined the MED26-NTD-binding region in EAF1.

Since it has been reported that p53 inhibits the transcription elongation activity of ELL and that ELL negatively regulates p53 in transcription conversely, in this study we tried to clarify the molecular mechanism by which p53 functions as an oncosupressor through inhibiting the recruitment of LEC by inhibiting the interaction between EAF and ELL.

Through biochemical analysis and a ChIP assay, we showed that p53 inhibits the interaction between the EAF/ELL component and ICE1 in LEC *in vitro*. Induction of p53 triggered by UV irradiation decreased the occupancy of ICE1 at Pol II-dependent *snRNA* genes. Consistent with the results, knockdown of p53 increased both the expression of *snRNA* genes and the occupancy of Pol II and components of LEC at *snRNA* genes. Our results indicate that p53 interferes with the interaction between ELL/EAF and ICE1 and represses transcription of *snRNA* genes by Pol II.

C) LIST OF ABBREVIATIONS

APS	Ammonium peroxodisulfate
BPB	Bromophenol blue
CBB	Coomassie brilliant blue
CIAP	Calf intestine alkaline phosphatase
CS	Calf serum
CTD	C terminal domain
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EAF	ELL-associated factor
ECL	Enhanced chemiluminescence
ELL	Eleven lysine-rich leukemia
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HBS	HEPES buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IL	Interleukin
IPTG	Isopropyl β-1-thiogalactopyranoside
LEC	Little elongation complex
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLB	Passive lysis buffer
PMSF	Phenylmethylsulfonyl fluoride
Pol II	RNA polymerase II
PVDF	Polyvinylidene difluoride
RNAi	RNA interference

SEC	Super elongation complex
siRNA	Small interfering RNA
snRNA	Small nuclear RNA
TB	Transformation buffer
TBS	Tris Buffered Saline
TBST	TBS containing 0.5% Tween 20
TEMED	N,N,N',N'-tetramethylethylenediamine.
TNFα	Tumor necrosis factor α
UV	Ultraviolet
WCL	Whole cell lysate
X-gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside

Amino Acids

A	Ala	L-alanine	M	Met	L-methionine
C	Cys	L-cysteine	N	Asp	L-asparagine
D	Asp	L-aspartic acid	P	Pro	L-proline
E	Glu	L-glutamic acid	Q	Gln	L-glutamine
F	Phe	L-phenylalanine	R	Arg	L-arginine
G	Gly	glycine	S	Ser	L-serine
H	His	L-histidine	T	Thr	L-threonine
I	Ile	L-isoleucine	V	Val	L-valine
K	Lys	L-lysine	W	Trp	L-tryptophan
L	Leu	L-leucine	Y	Tyr	L-tyrosine

Nucleotides

A	adenine
C	cytosine
G	guanine
T	thymine
U	uracil

D) MATERIALS AND METHODS

1 Materials

The source of and methods for preparing the experimental materials used in this study are as follows.

1.1 Cells cultured in experiments

Sf9 cells (Invitrogen)
HCT116 cells (ATCC)
NIH3T3 cells (ATCC)
NIH3T3 SrcY527F cells (established in our laboratory)
HEK293T cells (ATCC)
HeLa cells (ATCC)
HeLa S3 cells (Invitrogen)
Flp-In 293 cells (Invitrogen)
MEF cells (MMRRC)
Codon plus Escherichia coli (Stratagene)

1.2 Equipment

PCR Machine: MJ Mini (Bio-Rad Laboratories) and StepOne^{TM} Real-Time PCR System (Applied Biosystems)
DNA sequencer: ABI PRISM 310 Genetic Analyzer (Applied Biosystems)
Electrophoresis tank:
Slab electrophoresis apparatus (Nippon Eido)
Mupid-2plus (Advance)
Transblot cell (Bio-Rad Laboratories)
Power supply: BP-T5 (Bio craft)
UV transilluminator (Agarose gel imaging apparatus):
AS-100BT (Bio cratt)
Ultrasonifer: Sonifer250 (Branson)
Slide glass: MICRO SLIDE GLASS (MATSUNAMI)
Cover glass: MICRO COVER GLASS (MATSUNAMI)
Filter paper: 3MM (Whatman)
Incubator: TVN680DA incubator (Advantec)
CO2 incubator:
Forma series II water jacketed CO_2 incubator (Thermo electron)
Heat block: Thermo Alumi bath ALB-221 (IWAKI)
Absorption spectrophotometer: Smart Spec 3000 (Bio-Rad)
Centrifugal machine:
Centrifuge 5415R (Eppendorf)

MX-300 (TOMY)
Avanti J-E centrifuge (Beckman Coulter)
Luminometer: Glomax20/20 luminometer (Promega)
Microscope: BX-51 (OLYMPUS)
CCD camera: DP71 (OLYMPUS)
pH meter: Seven Easy (Mettler Toledo)
Autoclave: KS-323 (TOMY)
Dry oven: SS-K-300 (ISUZU)
Sterilization filter: MILLEXHV (Millipore)

1.3 Kits

DNA sequence kit: BigDye Terminator Kit (Applied Biosystems)
DNA ligation kit: TaKaRa DNA ligation kit (Takara Bio)
RNA extraction kit: ISOGEN (NIPPON GENE)
cDNA synthesis kit: ReverTra plus (TOYOBO)
Plasmid extract kit:
JETSTAR2.0 (GENOMED)
Axyprep plasmid mini prep kit (Axygen)
Power SYBR Green PCR Master Mix (Applied Biosystems)
Taqman Real-Time PCR master mix (Applied Biosystems)
BacPAK system (Clontech Laboratories,
Bac-to-Bac Baculovirus Expression System (Invitrogen)
Lenti-X concentrator (Clontech)
Lenti-X p24 Rapid Titer Kit (Clontech)

1.4 Enzymes

DNA synthesis enzyme:
KOD plus (TOYOBO)
KOD FX (TOYOBO)
RNaseA (Novagen)
Trypsin (Sigma)

Restriction enzymes (BamHI, BglII, ECoRI, EcoRV, HindIII, KpnI, NotI, PstI, SacI,

SacII, SalI, SmaI, SpeI, XbaI, XhoI): all from Takara

1.5 Nucleic acids

pcDNA3 (Invitrogen)
pcDNA3-myc: pcDNA3 (Invitrogen) tag with Myc.
pCR3 (Invitrogen)
pCRFLAG: pCR3 (Invitrogen) tag with 2×FLAG.
p3×FLAG (Invitrogen)
pCGN-HA and pCGN-HA-Ub: made in our laboratory.

pFastBacHT (Invitrogen)
pFastBacHT-FLAG: pFastBacHT (Invitrogen) tag with FLAG.
pGL4.74-hRLtk (Promega)
NIH 3T3 cells Matchmaker cDNA library (Clontech)
Human Prostate Matchmaker cDNA Library (Clontech)
dNTP mixture (dATP, dCTP, dGTP, dTTP) (Takara Bio)

1.6 Membranes (filter)

Polyvinylidene difluoride (PVDF) membrane: Immobilon-P Transfer membrane (MILLIPORE) and MILLEX-HV (0.45 μm filter) (MILLIPORE)

1.7 Culture media

E.coli culture medium
Bacto tryptone (Sigma)
Bacto agar (Sigma)
Cell culture media
Dulbecco's modified Eagle's medium (DMEM) (Sigma)
Dulbecco's modified Eagle's medium /F-12 (Gibco)
Dulbecco's modified Eagle's medium, no glucose (Gibco)
Opti-MEM (Gibco)
Horse serum (Gibco)
Fetal bovine serum (FBS) (Gibco)
Dialyzed fetal bovine serum (Gibco)
Sf-900 II (Invitrogen)
McCoy’s 5a Medium (Gibco)
L-Glutamine (Sigma)
Penicillin/Streptomycin (Sigma)
Sodium pyruvate (Sigma)
Non-essential amino acid solution (Sigma)

1.8 Beads

Anti-FLAG agarose (Sigma)
Monoclonal anti-HA agarose (Sigma)
SureBeads Protein G Magnetic beads (Bio-Rad Laboratories)
Glutathione Sepharose Beads 4B (GE Healthcare)
rProtein A Sepharose Fast flow (GE Healthcare)
Protein G Sepharose 4 Fast flow (GE Healthcare)
Probond resin (Invitrogen)
Probond metal affinity beads (Invitrogen)

1.9 Antibiotics

Ampicillin (Sigma)
Kanamycin (Sigma)
Puromycin (Sigma)
Tetracyclin (Sigma)

1.10 Protein decomposing enzyme inhibitors

Aprotinin (Sigma)
Leupeptin (Sigma)
Phenylmethylsulfonyl fluoride (PMSF) (Sigma)

1.11 Antibodies

Monoclonal mouse anti-p53 (DO-1) (Santa Cruz Biotechnology)
Mouse monoclonal ANTI-FLAG antibody (M2) (Sigma-Aldrich)
Mouse monoclonal ANTI-HA antibody (HA11.11/16B12) (Covance Research Products)
Mouse monoclonal ANTI-Myc antibody (9E10) (Covance Research Products)
Mouse monoclonal ANTI-ELL antibody (Bethyl Laboratories)
Mouse monoclonal ANTI-EAF1 antibody (gift from University of Chicago)
Rabbit monoclonal ANTI-His antibody (H-33) (Santa Cruz Biotechnology)
GAPDH mouse monoclonal antibody (6C5) (Ambion)

1.12 Transfection

Gene transfer reagent: FuGENE® HD (Roche)
Gene transfer reagent: RNAiMAX (Invitrogen)

1.13 SDS-PAGE, Western blotting-related agents

Acrylamide (Sigma)
N,N'-Methylenebisacrylamide (Sigma)
Protein marker for SDS-PAGE: Prestained XL-Ladder Broad range (APRO science)
ECL Western Blotting Detection Reagents (GE Healthcare)
X ray film: CL-XPosure Film (Thermo Scientifc)
CBB R-250 (Nacalai tesque)

1.14 Others

Ethanol (Sigma)
Isopropyl alcohol (Sigma)
Phenol (Sigma)
8-Quinolinol (Sigma)
Chloroform (Sigma)
3-methyl-1-butanol (Sigma)
1 kb plus DNA ladder marker (Invitrogen)

Agarose (Sigma)
Low-melting agarose (Sigma)
Trizma base (Sigma)
PIPES (DOJINDO)
HEPES (Sigma)
HCl (Sigma)
EDTA (Sigma)
NaCI (Nacalai tesque)
KCl (Sigma)
$CaCl_2$ (Wako)
$MnCl_2$ (Sigma)
Na_2HPO_4 (Sigma)
KH_2PO_4 (Wako)
D-glucose (Sigma)
Dextrose (KANTO Chemical)
Sodium hydroxide (Sigma)
SDS (Sigma)
Triton X-100 (Sigma)
Igepal CA-630 (NP-40) (Sigma)
Tween 20 (Sigma)
Sodium deoxycholate (KANTO Chemical)
NaF (KANTO Chemical)
Na_3VO_4 (Sigma)
Sodium pyrophosphate (Nacalai tesque)
APS (Sigma)
2-mercaptoethanol (Sigma)
Glycerol (Sigma)
BPB (Sigma)
Acetic acid (Sigma)
Potassium acetate (Sigma)
Glycine (Sigma)
Imidazole (Sigma)
Dimethyl sulfoxide (DMSO) (Sigma)
Formamide (Sigma)
Isogen (NIPPON GENE)
Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma)
5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (Sigma)

1.15 Reagent preparation

MilliQ water was used as ultrapure water. Reagents were used for autoclaving or sterile filtration after being dissolved in the predetermined concentrations for guaranteed reagents,

biochemical experiments, molecular biology experiments and cell laboratory reagents. Hereinafter, % represents the volume/volume percent concentration when prepared by dissolving a liquid in a liquid and represents the weight/volume percent concentration when prepared by dissolving a solid in a liquid.

Nucleic acid reagents

0.5-2 M Tris/HCl buffer (pH 6.8-8.8)

Tris solution was modulated to an appropriate pH with hydrochloric acid (6.8-8.8) with the final Tris concentration being 0.5-2 M.

0.5 M EDTA (pH 8.0)

EDTA solution is adjusted to pH 8.0 with sodium hydroxide solution; final EDTA concentration was prepared to 0.5 M.

TE buffer (pH 8.0)

10 mM	Tris/HCl (pH 8.0)
1 mM	EDTA (pH 8.0)

Neutral phenol solution

8-Quinolinol (0.1%) was added to phenol which was melted at 65°C. An equivalent amount of 0.1 M Tris/HCl (pH 7.6) was added, and the solution was thoroughly stirred and the aqueous layer was discarded. The aqueous layer was repeatedly discarded until the solution reached neutral pH. Finally, an aqueous layer of a few cm was left, and stored in a light-shielding bottle at 4°C.

Chloroform/3-methyl-1- butanol solution

Chloroform and isoamyl alcohol were mixed at a ratio of 49:1, and the solution was stored in a light-shielding bottle at 4°C.

5 M NaCl solution

NaCl (292.5 g) was dissolved in pure water to a total volume of 1 L.

50×TAE solution

2 M Tris

2 M acetic acid

0.05 M EDTA

The solution was used at a dilution of 1×concentration.

Ethidium bromide-containing agarose gel

After preparing 0.7-2% agarose gel in 1×TAE solution, about 200 ml of 20 mg/ml ethidium bromide solution was added to prepare 1 μl gel.

Agarose electrophoresis dye: 10×loading buffer

Restriction enzyme buffer

10×buffer

Alkaline miniprep method I solution (Solution I)

50 mM	glucose
25 mM	Tris/HCl (pH 8.0)
10 mM	EDTA (pH 8.0)

Alkaline miniprep method II solution (Solution II)

0.2 M	sodium hydroxide
1%	SDS

Alkaline miniprep method III solution (Solution III)

3 M	potassium acetate
2 M	acetic acid

Culture medium for E. coli

LB plate medium

1%	Bacto tryptone
0.5%	yeast extract
170 mM	NaCl
1.5%	Bacto Agar
100 μg/ml	ampicillin
25 μg/ml	kanamycin

The medium was cooled to about 50°C after sterilization and an antibiotic (ampicillin or kanamycin) was added if desired.

LB plate medium (E. coli: DH10Bac)

1%	bacto tryptone
0.5%	yeast extract
170 mM	NaCl
1.5%	bacto agar
7 μg/ml	gentamycin

50 μg/ml	kanamycin
10 μg/ml	tetracycline
40 μg/ml	IPTG
300 μg/ml	X-gal

2×YT medium

1.6%	Bacto tryptone
1%	yeast extract
86 mM	NaCl
100 μg/ml	ampicillin
25 μg/ml	kanamycin

Antibiotics (ampicillin, kanamycin) were added if desired.

SOB medium

2%	Bacto tryptone
0.5%	yeast extract
10 mM	NaCl
2.5 mM	KCl
10 mM	MgSO
10 mM	$MgCl_2$

The medium was sterilized by autoclaving after dissolving Bacto tryptone, yeast extract, NaCl and KCl in pure water and then prepared by adding filtration-sterilized $MgSO_4$ and $MgCl_2$ mixture solution.

IPTG solution

The medium was used by dissolving IPTG in pure water to a concentration of 1 M.

Transformation buffer (TB)

10 mM	PIPES
250 mM	KCl
15 mM	$CaCl_2$
55 mM	$MnCl_2$

His6-binding buffer

50 mM	HEPES pH 7.9
150 mM	NaCl
10%	glycerol

Solnication buffer for XL-10

300 mM	NaCl
50 mM	Tris/HCl (pH 7.9)
10%	glycerol
1 mM	PMSF

Washing buffer for Probond resin

40 mM	imidazole
0.1%	Triton-X100
1 mM	PMSF

in His6-binding buffer

Washing buffer for Glutathione Sepharose Beads 4B

0.1%	Triton-X100
1 mM	PMSF

in PBS

Elution buffer for Probond resin

100 mM	NaCl
50 mM	HEPES
300 mM	imidazole
0.05%	Triton-X100
10%	glycerol

Elution buffer for Glutathione Sepharose Beads 4B

100 mM	NaCl
50 mM	Tris/HCl (pH 7.9)
0.05%	Triton-X100

20 mM glutathione

For cell culture

DMEM

Serum (FBS, CS) was inactivated at 56°C for 30 min; FBS was prepared to 10%.

Sf-900II

Sf900II medium was prepared for insect Sf9 cells with 5% FBS.

Protein electrophoresis, Western blot reagents

SDS-PAGE separation gel buffer

1.5 M	Tris/HCl (pH 8.8)
0.4%	SDS

SDS-PAGE concentration gel buffer

0.5 M	Tris/HCl (pH 6.8)
0.4%	SDS

10×SDS-PAGE electrophoresis running buffer

250 mM	Tris
1.92 M	Glycine
1%	SDS

1×SDS-PAGE electrophoresis buffer

The buffer was used by diluting 10×SDS-PAGE running buffer in 1×.

20% APS solution

After dissolving 2 g of APS in 10 ml pure water, the solution was stored at -20°C.

Coomassie brilliant blue (CBB) stain solution

50%	methanol
10%	acetic acid
0.1%	CBB R-250

The solution was stirred for 30 min to completely dissolve CBB R-250.

CBB bleaching solution

5%	methanol
7.5%	acetic acid

300 mM NaCl Cell lysis buffer

300 mM	NaCl
50 mM	Tris/HCl (pH 7.6)
0.5%	TritonX-100
1 mM	PMSF
10 μg/ml	aprotinin
10 μg/ml	leupeptin
400 μM	Na_3VO_4
400 μM	EDTA

10 mM	NaF
10 mM	sodium pyrophosphate

150 mM NaCl Cell lysis buffer

150 mM	NaCl
50 mM	Tris/HCl (pH 7.6)
0.5%	Triton-X 100
1 mM	PMSF
10 μg/ml	aprotinin
10 μg/ml	leupeptin
400 μM	Na_3VO_4
400 μM	EDTA
10 mM	NaF
10 mM	sodium pyrophosphate

Washing buffer for immunoprecipitation

150 mM	NaCl
50 mM	Tris/HCl (pH 7.6)
0.1%	Triton-X100
1 mM	PMSF
10 μg/ml	aprotinin
10 μg/ml	leupeptin
400 μM	Na_3VO_4
400 μM	EDTA
10 mM	NaF
10 mM	sodium pyrophosphate

Sf9 cell lysis buffer

300 mM	NaCl
50 mM	Tris/HCl (pH 7.6)
0.5%	Triton-X100
1 mM	PMSF
10 μg/ml	aprotinin
10 μg/ml	leupeptin
400 μM	Na_3VQ_4
400 μM	EDTA
10 mM	NaF
10 mM	sodium pyrophosphate

1×SDS sample buffer

50 mM	Tris/HCl (pH 6.8)
2%	SDS
6%	2-mercaptoethanol
10%	glycerol
0.1%	BPB

3×SDS sample buffer

150 mM	Tris/HCl (pH 6.8)
6%	SDS
18%	2-mercaptoethanol
30%	glycerol
0.1%	BPB

10×Western blot transfer buffer

0.25 M	Tris
1.92 M	Glycine

1×Western blot transfer buffer

Methanol was added to a final concentration of 10%, and 10×transcript buffer was diluted with pure water into 1×.

20×TBS

0.4 M	Tris/HCl (pH 7.6)
3 M	NaCl

1×TBST

The 20×TBS was diluted 20-fold with pure water and Tween-20 was added to be 0.05%.

10×PBS

1.37 M	NaCl
81 mM	Na_2HPO_4
27 mM	KCl
15 mM	KH_2PO_4

1×PBS

10×PBS diluted ten times with pure water was used.

Trypsin/EDTA solution

0.05% Trypsin
0.5 mM EDTA 2Na
The solution was dissolved in 1×PBS and sterile-filtered after preparation of 1 L.

2×HEPES buffered saline (HBS)
280 mM NaCl
1.5 mM Na_2HPO_4
10 mM KCl
12 mM Dextrose
50 mM HEPES
The solution was dissolved in pure water. pH was adjusted to 7.10 with 1 M NaOH, and then the solution was filtered after preparation of 500 ml.

2. Methods

Gene recombination experiments were performed in accordance with the "Hokkaido University gene recombination experiments safety management regulations"

2.1 DNA agarose gel electrophoresis

Agarose in 1×TAE solution was prepared to be 0.7-2% with ethidium bromide. After electrophoresis in 1×TAE solution, the gel was photographed using a UV transilluminator.

2.2 Ethanol precipitation and isopropanol precipitation

Five M NaCl and 2.5 volumes of ethanol were added to a 1/20 amount of a solution containing a nucleic acid, and the solution was stirred. Centrifugationwas performed at 16,200×g for 5 min at 4°C, and then ethanol was precipitated. In isopropanol precipitation, a 0.7-fold amount of isopropanol was added instead of ethanol and was precipitated in the same manner. The precipitate was dried after washing with 70% ethanol and was dissolved in pure water or TE buffer.

2.3 Phenol extraction and chloroform extraction

After adding an equal volume of neutral phenol, and the aqueous layer was recovered following centrifugation at 16,200×g for 5 minutes at room temperature. An equal volume of chloroform/isoamyl alcohol solution was added to the recovered liquid. After stirring, the aqueous layer was recovered following centrifugation at 16,200×g for 5 min at room temperature.

2.4 Recovery of DNA fragments from low-melting agarose gel

A UV transilluminator was used after electrophoresis of the samples containing the target DNA and DNA ladder markers in low melting agarose gel, and then the target fragment was cut out with a cutter knife. Low-melting agarose gel containing the fragment that had been cut out was dissolved at 100°C for 1 minute, and then phenol extraction and chloroform extraction were performed. The aqueous layer was collected and subjected to ethanol precipitation or isopropanol precipitation, dissolved in pure water or TE buffer.

2.5 *E. coli* transformation

Preparation of competent cells

E.coli was seeded in an LB plate and cultured for 14 hours in a 37°C incubator. One ml, 2 ml or 5 ml, 100 ml of the cultured bacterial solution were added to three 100 ml SOB media and cultured overnight at 18°C. The A_{600} value was measured, and culture media with A_{600} values of about 0.5 (or culture media in which cultivation was continued until A_{600} values reached that level) were selected. Then the media were cooled on ice, and cells were harvested after centrifugation at 2,500×g for 10 min at 4°C. The cells were then suspended in 40 ml of ice-cooled TB. After centrifugation at 2,500×g for 10 min at 4°C, the cells were gently suspended in 10 ml of ice-cooled TB and mixed by adding 750 μl DMSO, followed by incubation on ice for 10 min and then freezing immediately in liquid nitrogen.

Transformation

Frozen competent cells were thawed on ice and then 100 μl of competent cells was mixed with DNA samples or plasmid DNAs after the ligation reaction and incubated on ice for 30 min. Then the cells were incubated at 42°C for 45 seconds and again allowed to stand on ice for 2 min and finally incubated at 37°C for 30 min after adding 4 volumes of 2×YT medium. The cells were then seeded on LB plates containing appropriate antibiotics and cultured overnight at 37°C.

2.6 Preparation of plasmid DNA

Preparation of a small amount of plasmid by the alkaline SDS method

After culture of plasmid-containing *E. coli* in 2 ml of 2×YT medium containing an appropriate antibiotic at 37°C for 8 hours at 200 rpm, cells were harvested by centrifugation at 16,200×g for 1 min at 4°C. The cells were suspended in 200 μl of Solution I and mixed inversely by adding 200 μl of Solution II and then dissolved at room temperature for 5 min. After being inversely mixed with 200 μl of Solution III, the mixture was centrifuged at 16,200×g for 5 min. The supernatant was recovered and precipitated with isopropanol. The precipitate was dried after washing with 70% ethanol and dissolved in pure water or TE buffer.

Mini preparation of plasmid DNA for sequencing reaction

An Axyprep plasmid mini prep kit (Axygen) was used for plasmid extraction in accordance with the attached protocol.

Plasmid DNA preparation for cell transfection

JETSTAR2.0 (GENOMED) was used for plasmid extraction in accordance with the attached protocol.

2.7 Determination of the nucleotide sequence

The nucleotide sequence was determined using an ABI PRISM 310 Gentic Analyzer (Applied Biosystems).

Four hundred ng of DNA was mixed with 4 μl premix solution of the BigDye Terminator Kit, 2μl 5× sequence buffer and 1.6 pmol primer, and PCR reaction splutions were adjusted to a total amount of 20 μl with pure water. PCR was performed as follows: 96°C, 10 seconds, 50°C, 5 seconds, 60°C, 4 minutes, ×25 cycles. After PCR, the reaction solution was precipitated with ethanol, and the precipitate was dried after washing with 70% ethanol, dissolved in 25 μl formamide (Sigma), denatured for 2 min at 95°C, and quenched on ice, and sequenced by an ABI PRISM 310 Gentic Analyzer.

2.8 Primers used for qPCR analysis

Human U1 snRNA
5’-GGGAGATACCATGATCACGAAGGT-3’ (forward)
5’-ATGCAGTCGAGTTTCCCACA-3’ (reverse)

Human U2 snRNA
5’-GTTTAATATCTGATACGTCCTCTATCC-3’ (forward)
5’-TCGATGCGTGGAGTGGAC-3’ (reverse)

Human U4-1 snRNA
5’-TAGGCCCTAAACTCACCTTTGCGA-3’ (forward)
5’-AGCAATAATCGCGCCTCGGATAGA-3’ (reverse)

Human U4-2 snRNA
5’-ATGAGGTTTATCCGAGGCGCGATT-3’ (forward)
5’-CGACTATATTTCAAGTCGTCATGGCGGG-3’ (reverse)

Human U5A snRNA
5’-ACTCTGGTTTCTCTTCAGATCGCA-3’ (forward)
5’-CTTGCCAAAGCAAGGCCTCAAA-3’ (reverse)

Human U5B snRNA
5’-ACTCTGGTTTCTCTTCAGATCGT-3’ (forward)
5’-CTTGTCGGAACAAGGCCTCAAA-3’ (reverse)

Human U6 snRNA

5’-GCTCGCTTCGGCAGCACATATACTAA-3’ (forward)
5’-ACGAATTTGCGTGTCATCCTTGCG-3’ (reverse)

Human U11 snRNA
5’-TTCTGTCGTGAGTGGCACACGTA-3’ (forward)
5’-AACGATCACCAGCTGCCCAAATAC-3’ (reverse)

Human GAPDH
5’-TCGACAGTCAGCCGCATCTTCTTT-3’ (forward)
5’-GCCCAATACGACCAAATCCGTTGA-3’ (reverse)

2.9 PCR

PCR for cDNA cloning

The compositions used in PCR reactions are as follows.

PCR reaction solution composition using KOD plus (total volume: 50 μl)

Template DNA	100 ng
Primer	0.3 μM
10×PCR solution	5 μl
dNTPs	0.2 mM
$MgSO_4$	1 mM
KOD plus DNA polymerase	1 U
Sterile water	

PCR reaction solution composition using KOD FX (total volume: 50 μl)

Template DNA	100 ng
Primer	0.3 μM
2×PCR solution	25 μl
dNTPs	0.4 mM
$MgSO_4$	1 mM
KOD FX DNA polymerase	1 U
Sterile water	

Reaction conditions:

2 cycles:	98°C/10 sec→72°C/30 sec→68°C/50 sec
2 cycles:	98°C/10 sec→68°C/30 sec→68°C/50 sec
2 cycles:	98°C/10 sec→64°C/30 sec→68°C/50 sec
2 cycles:	98°C/10 sec→60°C/30 sec→68°C/50 sec
25 cycles:	98°C/10 sec→55°C/30 sec→68°C/50 sec

Real-time PCR

The amount of RNA was analyzed with Power SYBR Green PCR Master Mix (Applied Biosystems) using StepOneTM Real-Time PCR System. Data were analyzed by the included software. The PCR reaction solution was prepared as described in another section.

PCR reaction solution composition: total volume: 10 μl

Template cDNA	1 μl
Primer	0.2 μl
Power SYBR green master mix	5 μl
Sterile water	

PCR reaction conditions

40 cycles: 95°C/15 sec→60°C/1 min

2.10 Subcloning

Calf intestine alkaline phosphatase (CIAP) treatment

The vector DNA was completely decomposed by each restriction enzyme, followed by dephosphorylation of the 5' end due to the CIAP.

Preparation of insert DNA

The fragment of interest was subject to appropriate restriction enzyme treatment and phosphorylation treatment and then separated and harvested in a low melting agarose gel.

Ligation reaction

Vector DNA and insert DNA that were prepared as described above were mixed at a molar ratio of 1:5 with an equal volume of 2 × ligation solution I (TaKaRa DNA ligation kit) and incubated for 30 min at 16°C.

2.11 Polyacrylamide electrophoresis (SDS-PAGE)

To prepare a separation gel solution having the composition shown in the table below, the mixture was immediately poured into a glass plate and overlaid with isopropanol. After acrylamide had solidified, isopropanol was washed with water, and a concentrated gel solution was layered on top of the separating gel to solidify the gel. Electrophoresis was

carried out using an SDS-PAGE running buffer at a constant voltage of 100 V for about 160 min.

Separating gel solution (%)	16	14	12	10	8
30% acrylamide solution (ml)	4.00	3.50	3.00	2.50	2.00
DDW (ml)	1.55	2.05	2.55	3.05	3.55
1.5 M Tris pH 8.8 (ml)	1.88	1.88	1.88	1.88	1.88
10% SDS (μl)	75	75	75	75	75
20% APS (μl)	28.1	28.1	28.1	28.1	28.1
TEMED (μl)	7.5	7.5	7.5	7.5	7.5

Concentrated gel solution

30% acrylamide solution	0.40 ml
DDW	2.01 ml
0.5 M Tris pH 6.8	0.63 ml
10% SDS	25 μl
20% APS	12.5 μl
TEMED	2.5 μl

2.12 Western blot analysis

After SDS-PAGE, the Western blot apparatus was stacked in the order of sponge → filter paper → PVDF membrane → gel → filter paper → sponge. Electrical transfer was performed for 120 min at 100 V. The PVDF membrane after the transfer was immersed in blocking solution and it was then shaken for 30 min at room temperature. After blocking, it was washed 5 times with TBST and immersed in TBST with the first antibody overnight at 4°C. The membrane was washed 5 times for 5 min each time with TBST and then immersed in TBST with the secondary antibody for 60 min at room temperature. After washing five times for 5 min each time in TBST, the membrane was subjected to chemiluminescence reactions by ECL Western Blotting Detection Reagents.

2.13 Cell culture

HCT116 cells (ATCC CCL-247) and their derivatives were cultured under an atmosphere of 5% CO_2 at 37°C in McCoy's 5a medium (GIBCO, Grand Island, NY) supplemented with

10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA), 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Grand Island, NY). HEK293T cells and HeLa cells were cultured under an atmosphere of 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Sf-9 cells were cultured with shaking at 28°C in Sf900II medium supplemented with 10% (v/v) fetal bovine serum. Cell passaging was carried out as follows. The medium was removed and the cells were washed with PBS. PBS was removed, and an approximately one-tenth amount of trypsin/EDTA solution was added, and the cells were incubated for 5 min in 37°C. After confirmation by microscopic observation that the cells were detached from the plate, the trypsin/EDTA solution was neutralized by adding FCS-containing medium. Then the washed cells were cultured with a fresh medium.

2.14 Gene transfer into cells using lipofection

Roche's FuGENE®HD reagent was used for transfection. Briefly, cells were seeded for gene transfer on 10-cm dishes or 24-well plates and cultured overnight. Plasmid DNA (10 μg or 0.5 μg) was mixed with DMEM (500 μl or 25 μl) and FuGENE®HD (15 μl or 0.75 μl) and then incubated for 15 min. After incubation, the DNA mixture was added to the cell culture dishes. After culture for 6 hours, the medium was changed.

2.15 Preparation of cell lysates

Cells were collected with trypsin/EDTA solution or a cell scraper and centrifuged at 1,500×g for 5 min at 4°C. Then the cells were suspended in 1 ml of PBS and centrifuged at 4°C for 5 min, and the supernatant was removed. The cells was dissolved in 150 or 300 mM NaCl lysis buffer.

2.16 Immunoprecipitation

For immunoprecipitation, 1 μg of antibody was added and mixed by inversion for 1 hour at 4°C. Twenty μl of 50 (v/v) % protein G sepharose 4 fast flow was added to the solution, and the solution was further mixed by inversion for 2 hours at 4°C. After washing 5 times with a lysis buffer, 30 μl 1×SDS sample buffer was added, and the solution was heated at 100°C for 5 min and stored at -20°C until use. When anti-FLAG antibody or anti-Myc antibody was used for the immunoprecipitation, an elution buffer with 1×FLAG peptide or 1×Myc peptide (250 μg/ml) was used for elution.

2.17 RNA isolation

Cells (5×10^6) were lysed with 1 ml of Isogen and incubated for 2 min. Then 0.2 ml of chloroform was added to the lysate, and the lysate was centrifuged at 12,000×g for 15 min at 4°C. The aqueous layer was then transferred to a new tube. Then 0.5 ml of isopropanol was added and the mixture was incubated at room temperature for 5 min and centrifuged at 12,000×g for 10 min at 4°C, and the supernatant was discarded. The pellet was washed with 70% ethanol and dissolved in RNase-free sterile water (to a concentration of 1 mg/ml).

2.18 Reverse transcription

mRNA was used as a template for reverse transcription to synthesize cDNA using a ReverTra plus kit (TOYOBO). After heat-denaturation at 65°C for 5 min and rapid cooling on ice, the reaction solution was prepared as follows.

10 μl	RNase-free sterile water
1 μl	random primer (25 pmol/μl)
1 μl	total RNA (1 mg/ml)
4 μl	5×buffer
2 μl	10 mM dNTPs
1 μl	RNase inhibitor
1 μl	ReverTra Ace

Reaction: 30°C/10 min →42°C/60 min →85°C/5 min →4°C

2.19 Knockdown experiments

We transfected siRNA by using RNAiMAX reagent. Non-targeting siRNA and siRNA of interest were prepared to a final concentration of 20 nM in the culture medium.

2.20 Production of cell lines with stable expression using a retroviral expression system

PlatE cells were used as packaging cells for a retroviral expression system. PlatE cells (10-cm dish) were transfected with a retroviral vector by the lipofection method. Forty-eight hours after transfection, the culture supernatant was collected. The cells to be infected were cultured with the supernatant containing the retrovirus (with 8 μg/ml polybrene). After 48-hour incubation, the medium with the appropriate antibiotic was replaced and then stable cells were established.

2.21 Lentiviral expression system

A lentivirus vector was transfected into HEK293T cells by the lipofection method. The medium was changed after 24 hours and the culture was continued for 48 hours. The cell supernatant containing the lentivirus was collected and concentrated in a Lenti-X concentrator. After measuring virus titers by using the Lenti-X p24 Rapid Titer Kit, the virus solution was stored at -80°C.

2.22 Baculoviral expression system

The Bac-to-Bac Baculovirus Expression System (Invitrogen) was used.

(1) *E. coli* DH10Bac was transformed with the plasmid of interested. Blue/white colonies were selected to isolate recombinant bacmid DNA.

(2) The bacmid DNA was transfected to 1×10^6 Sf9 cells in a 6-well plate with FuGENE®HD, After three days, the culture supernatant was added to 1×10^6 Sf9 cells that were newly seeded in T-25 flasks. Then amplification of the virus was carried out while scaling as follows every

three days. Sf9 cells from 10-cm dishes seeded at the concentration of 2×10^6 cells were used for Western blotting to confirm the protein expression.

Culture scale for virus amplification:

1×10^6 cells/6-well plate→1×10^6 cells/T-25 flasks→2×10^6 cells/10-cm dish

→2×10^7 cells/15-cm dish→2×10^7 cells/15-cm dish (10 dishes)

(3) The collected Sf9 cells were centrifuged at 5,000×g for 20 min at 4°C. The precipitate was dissolved in 10 ml of 300 mM NaCl lysis buffer and disrupted with an ultrasonic disrupter and then centrifuged at 40,000×g for 30 min at 4°C using an ultracentrifuge, and the supernatant was recovered. Two ml 50% (v/v) Probond resin was added and mixed by inversion for 2 hours and then washed three times with 10 ml His6-binding buffer and three times with 10 ml of 50 mM imidazole-containing His6-binding buffer. Then it was eluted three times with 1 ml of 350 mM imidazole-containing His6-binding buffer. The eluates were subjected to SDS-PAGE and stained with CBB to determine the concentration of isolated proteins.

2.23 Preparation and purification of recombinant proteins using a bacterial expression system

N-terminally His6- and FLAG-tagged MED26-NTD (residues 1–113) and MED26-NTD R61A, K62A were expressed in BL21 (DE3) CodonPlus *Escherichia coli* and then purified by using ProBond metal affinity beads. Briefly, the colonies were transferred to 500 ml of a liquid medium and cultured with gentle shaking at 37°C until OD_{600} reached 0.4-0.6. When OD_{600} reached the target value, IPTG was added to a final concentration of 1 mM, and the cells were cultured overnight at 16°C. *E. coli* cells were harvested by centrifugation, washed once with PBS, and then dissolved by using *E. coli* lysis buffer. After crushing *E. coli* using a pressure crusher, the lysate was ultracentrifuged at 40,000×g for 1 hour at 4°C, and the supernatant was collected. Six-hundred μl of 50% (v/v) ProBond metal affinity beads was

added to the collected supernatant and incubated at 4°C for 4 h. The beads were washed 4 times with a binding buffer for ProBond metal affinity beads, and the recombinant protein was eluted from ProBond metal affinity beads. Eluates were subjected to SDS-PAGE and stained with CBB to confirm the concentration of purified proteins.

2.24 Immunoprecipitation and affinity purification

Protein complexes were purified from nuclear extracts or S100 fractions of cell lines stably expressing FLAG-tagged proteins using anti-FLAG M2 agarose. The cells were resuspended in Buffer A with 0.42 M NaCl and incubated on ice for 30 min. All extracts were adjusted to 0.3 M NaCl and ultracentrifuged at 100,000×g for 30 min at 4°C before incubation with anti-Flag (M2) agarose overnight. The resins were then washed four times with 0.3 M NaCl in Buffer A and once with an elution base buffer. The resins were eluted by incubation with 0.2 mg/ml of FLAG peptide in an elution buffer for 30 min at 4°C or room temperature. Two mM DTT were added to the eluates, and the eluates were frozen in liquid nitrogen.

Nuclear extracts and S100 fractions were prepared according to the method of Dignam[78] from parental HeLa cells or HEK293 FRT cells stably expressing FLAG-tagged proteins. Each of the nuclear extracts or S100 fractions was incubated with 100 μl anti-FLAG-agarose beads for 2 hours at 4°C. The beads were washed five times with a 100-fold excess of a buffer containing 50 mM HEPES-NaOH (pH 7.9), 0.3 M NaCl, 0.1% Triton X-100 and 10% (v/v) glycerol and then eluted with 100 μl of a buffer containing 0.1 M NaCl, 50 mM HEPES-NaOH (pH 7.9), 0.05% Triton X-100, 10% (v/v) glycerol and 0.25 mg/ml FLAG peptide.

2.25 Gene expression analysis

Total RNA was isolated using Isogen II (Nippon Gene, Tokyo, Japan). For RT-qPCR, total mRNA was reverse-transcribed using the iScript Select cDNA Synthesis Kit (Biorad,

Hercules, CA). The threshold cycle (Ct) values were determined by real-time PCR reactions using an Applied Biosystems StepOne Realtime PCR System and Power SYBR Green PCR Master Mix (Life Technologies) and normalized by subtracting the Ct value of the GAPDH gene from the Ct value of the respective gene ($\Delta Ct = Ct^{gene} - Ct^{GAPDH}$). The relative mRNA levels were then calculated using $2^{-\Delta Ct}$.

2.26 ChIP assays

Cells were cultured in a 10-cm dish and crosslinked with 2 mM disuccinimidyl glutarate (DSG) crosslinker in PBS for 30 min and then 1% formaldehyde in PBS for 10 min at room temperature. Then the cells were sonicated with a Bioruptor 15 times for 30 seconds each time. After sonication, micrococcal nuclease was added to digest sonicated DNA. SureBeads Protein G Magnetic beads (Bio-Rad Laboratories) and/or specific antibodies were added to the digested lysates and incubated for 2 hours at 4°C. Specific antibodies against ICE1 (Bethyl), ELL (Bethyl) and Pol II (Santa Cruz Biotechnology) were used in the assays. The beads were washed twice with CHIP buffer containing 200 mM KCl, 2 mM $CaCl_2$ and 50 mM Tris-HCl pH 8.0, twice with 500 mM KCl CHIP wash buffer and once with TE buffer. Bound complexes were eluted from the beads with 100 mM $NaHCO_3$ and 1% SDS by incubating at 50°C for 30 minutes. Crosslinking was reversed by overnight incubation at 65°C. Immunoprecipitated DNA and input DNA were treated with RNase A and proteinase K by incubation at 45°C. DNA was purified using the QIAquick PCR purification kit (28106, Qiagen, Valencia, CA) or MinElute PCR purification kit (28006, Qiagen). Immunoprecipitates and input were analyzed by quantitative PCR. The ChIP signal was normalized to the input. Three biological replicates were performed for each experiment.

E) RESULTS

1 Binding region of EAF1 for MED26-NTD

It was reported that EAF1 directly binds to MED26-NTD. We performed a binding assay to determine the region of human EAF1 responsible for interaction with MED26-NTD. We examined whether a series of HA-EAF1 C-terminal deletion mutants bind to FLAG-MED26-NTD. The full length (FL) of EAF1 bound to MED26-NTD, but the C-terminal deletion mutant N244 did not, indicating that C-terminal amino acid residues from 245 to 268 in human EAF1 are required for interaction with MED26-NTD (Figs. 1, 2). Recombinant FLAG-tagged MED26-NTD (wild-type (WT) and R61A, K62A mutant) proteins were immobilized on anti-FLAG M2 agarose and were then incubated with the lysate from Sf9 cells expressing deletion mutants of HA-tagged EAF1. After washing, bound proteins were eluted and analyzed by Western blotting. FLAG-tagged MED26-NTD (R61A, K62A) has a larger molecular weight than that of FLAG-tagged-MED26-NTD (WT) because the linker region between the N-terminal hexa-histidine-tag and FLAG-tag in MED26-NTD (R61A, K62A) is longer than that of MED26-NTD (WT).

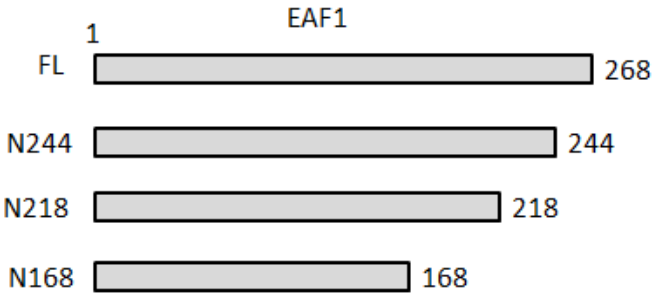

Figure 1. Schematic representation of deletion mutants of human EAF1**.**

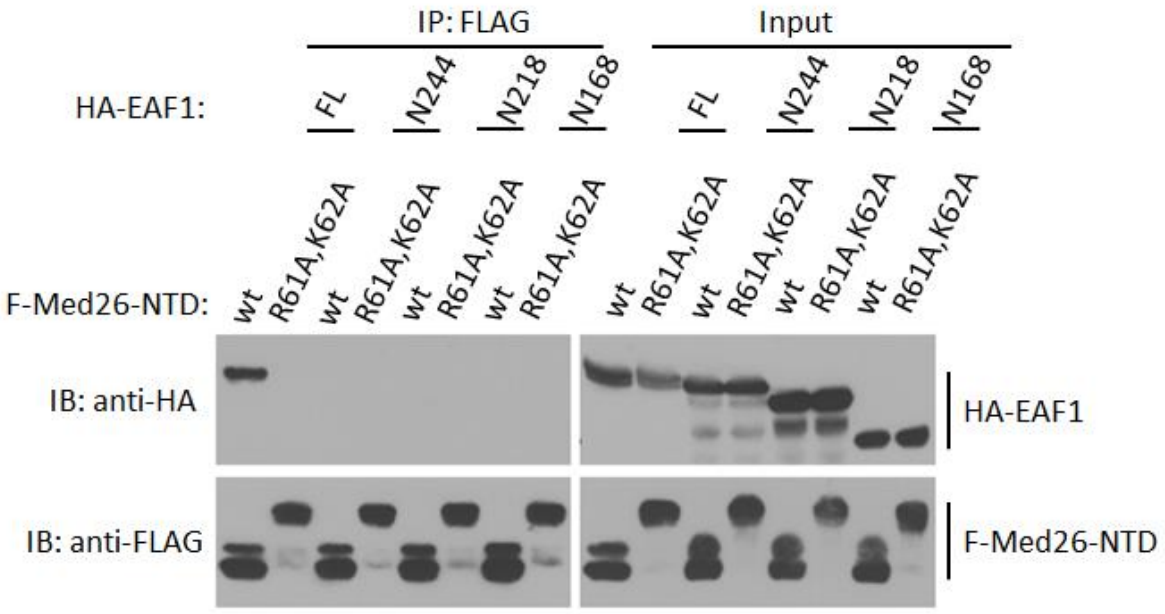

Figure 2. Binding ability of deletion mutants of EAF1 with MED26-NTD (WT or R61A/K62A mutant).

2 Depletion of EAF1 decreases the occupancy of ICE1 at Pol II-dependent *snRNA* genes

Previously, we showed that EAF family proteins function as adaptors to connect between MED26 and LEC. ICE1 functions as a core subunit of LEC and interacts with both ICE2 and ELL/EAF. The C-terminal region of ICE1 (1191-2266) is required for the interaction with LEC [64,79,80]. EAF1 increases ELL activity in activation of Pol II elongation through binding to the N-terminal region of ELL[6]. At first, we examined whether EAF1 is required for the occupancy of ICE1 at Pol II-dependent *snRNA* genes. To address this question, we performed chromatin-immunoprecipitation (ChIP) analysis using HCT116 cells that had been transfected with control small interfering RNA (siRNA) or EAF1 siRNA. The occupancy of ICE1 was decreased in *U4C* and *U5B snRNA* genes under the condition of EAF1 knockdown but not in the U5B upstream region and *U6 snRNA* gene, which is transcribed by Pol III (Fig. 3). These findings suggest that EAF1 functions as an adaptor molecule connecting between LEC and MED26 at a subset of Pol II-dependent *snRNA* genes (Fig. 4).

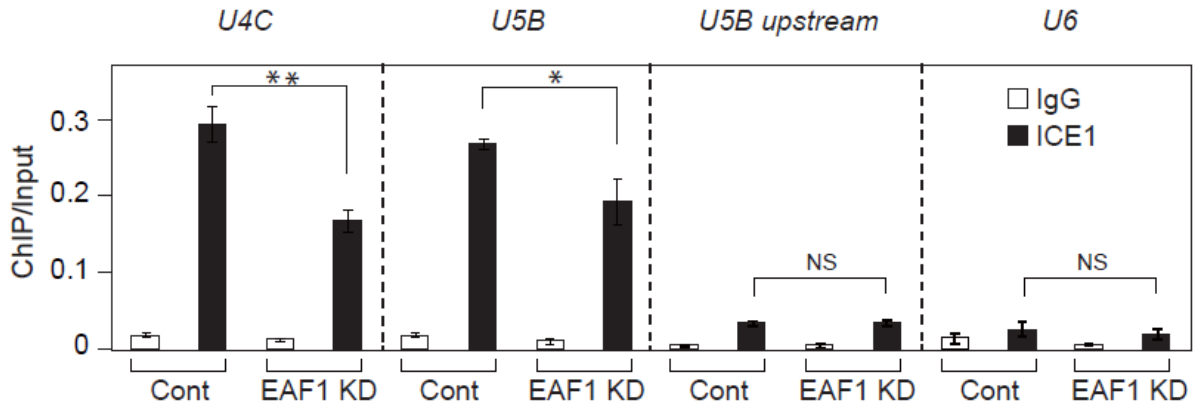

Figure 3. EAF1 is required for the occupancy of ICE1 at *snRNA* genes. Occupancy of ICE1 at regions of *U4C*, *U5B*, upstream of *U5B*, and *U6 snRNA* genes by EAF1 knockdown (KD). Standard deviations of each result were calculated from three independent experiments. *P* values were determined by Student's *t* test (*, $P < 0.05$). Error bars show s.d. NS, not specific.

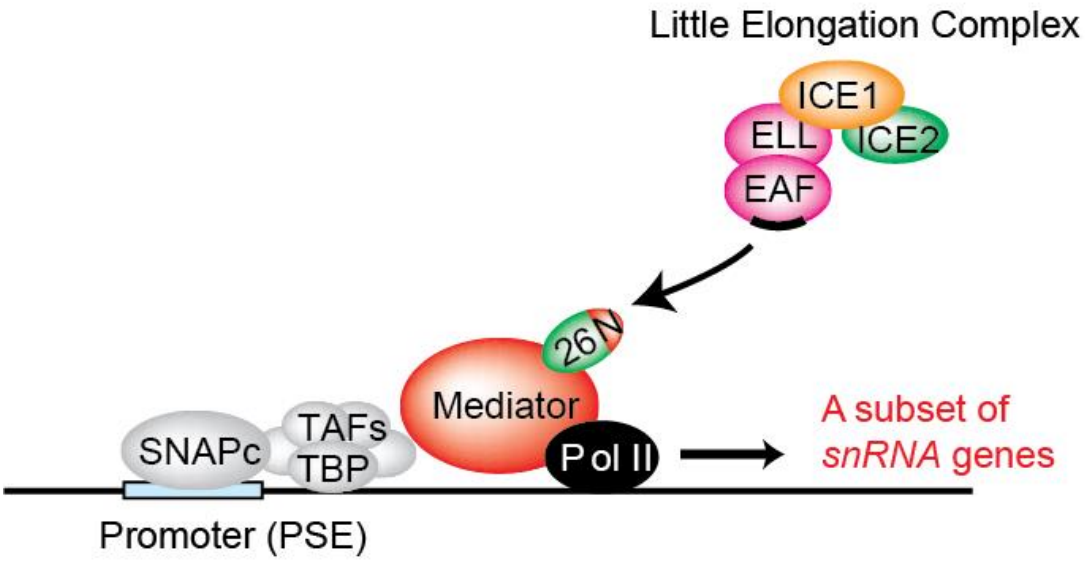

Figure 4. A putative model of the recruitment of LEC to *snRNA* genes via the MED26-containing Mediator complex.

3 p53 interferes with the interaction between EAF/ELL and ICE1

It has been reported that p53 interacts with ELL and inhibits its activity[49]. We previously showed that MED26 recruits LEC to *snRNA* genes through interaction with EAF1[79]. The N-terminal domain of MED26 (1-113) directly binds to the C-terminal region (245-268) of human EAF1 [64,79]. Previous biochemical analysis indicated that ELL/EAF binds to ICE1 in LEC through direct interaction between ELL and ICE1[79], raising the possibility that p53 interacts with ELL and interferes with the interaction between ELL and ICE1[49]. To address this possibility, we performed an *in vivo* binding assay. We expressed HA-ICE1, FLAG-ELL, Myc-EAF and His_6-p53 in the indicated combinations through a baculovirus expressing system. HA-ICE1 co-purified with FLAG-ELL or FLAG-ELL/Myc-EAF in the absence of His_6-p53, but HA-ICE1 did not copurify with FLAG-ELL or FLAG-ELL/Myc-EAF in the presence of His_6-p53. These findings suggest that p53 interacts with ELL and interferes with the binding between ELL and ICE1 (Fig. 5).

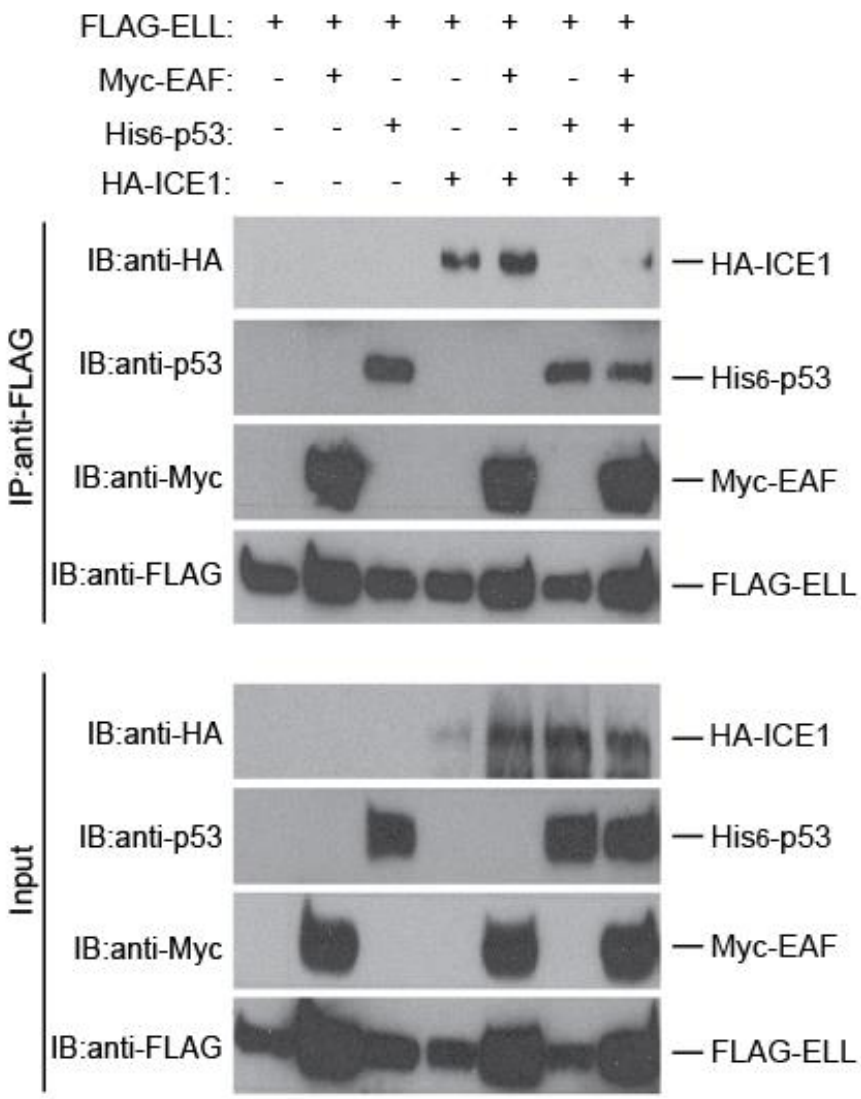

Figure 5. **p53 interferes with the interaction between ELL/EAF and ICE1.** Anti-FLAG immunoprecipitates using lysates from Sf9 cells expressing the indicated combinations of Myc-tagged EAF, FLAG-tagged ELL, HA-tagged ICE1 and His_6-tagged p53 were analyzed by immunoblotting. IP, immunoprecipitation.

4 p53 interacts with EAF1 and facilitates the interaction between ELL and EAF

p53 interacts with ELL and inhibits the activity of ELL[49], and our data showed that p53 represses the interaction of ICE1 with ELL. However, there has been no report about interactions between p53 and ELL-associated factor (EAF). We examined whether EAF interacts with p53 and whether p53 inhibits the interaction between ELL and EAF. We performed an *in vivo* binding assay. We expressed FLAG-ELL, HA-EAF and His_6-p53 in the indicated combinations through a baculovirus expressing system. We showed that p53 copurified with EAF and that ELL co-purified with EAF even in the presence of p53 (Fig. 6). These findings suggest that p53 interacts with EAF1 and does not interfere with the interaction between ELL and EAF.

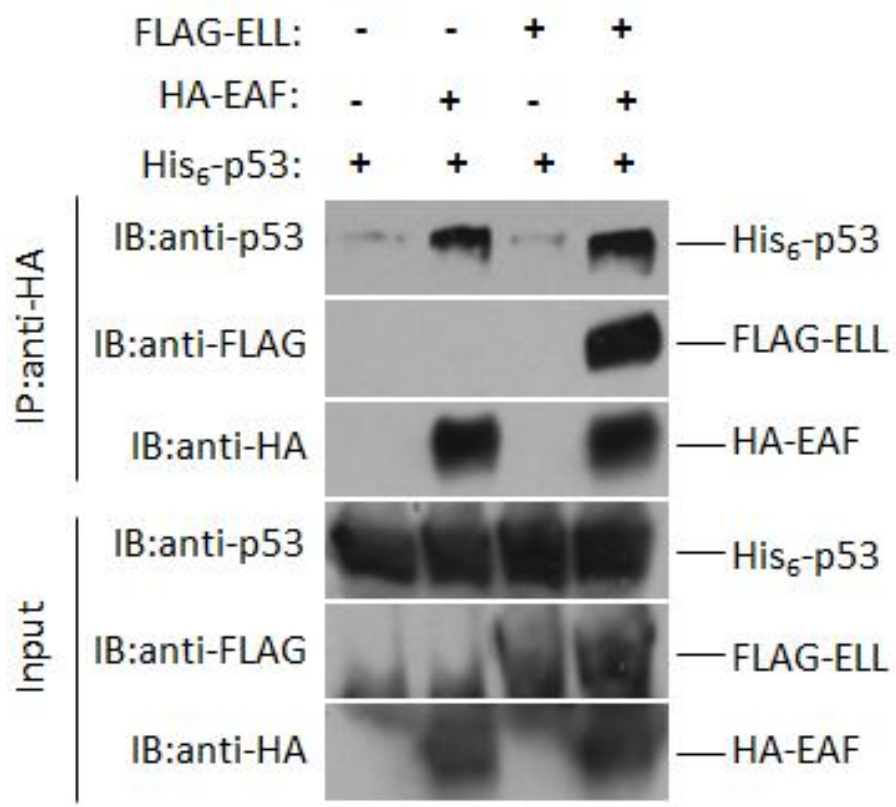

Figure 6. p53 interacts with EAF. Anti-HA immunoprecipitates lysates from Sf9 cells expressing the indicated combinations of HA-tagged EAF, FLAG-tagged ELL and His_6-tagged p53 were analyzed by immunoblotting. IP, immunoprecipitation.

5 Induction of p53 expression by UV irradiation decreases the occupancy of ICE1 at Pol II-dependent *snRNA* genes

It has been reported that several cellular stresses including DNA damage induce p53 expression[81-85]. Mutations or loss of the tumor suppressor gene *p53* are thought to play a critical role in the development of precancerous lesions. It has been reported that ICE1 functions as a scaffold protein for the localization of LEC at *snRNA* genes[86]. Since we found that p53 inhibits the interaction of ELL with ICE1, we hypothesized that induction of p53 expression by UV irradiation affects the occupancy of ICE1 at Pol II-dependent snRNAs that are regulated by LEC. We performed ChIP analysis under the condition of UV irradiation using HCT116 cells, which are known to express wild-type p53 (Fig. 7B) [87]. ChIP analysis showed that UV treatment decreased the occupancy of ICE1 at *snRNA* genes (Fig. 7A). These findings suggest that p53 activated by UV irradiation inhibits the recruitment of LEC by interfering with the binding between ELL and ICE1.

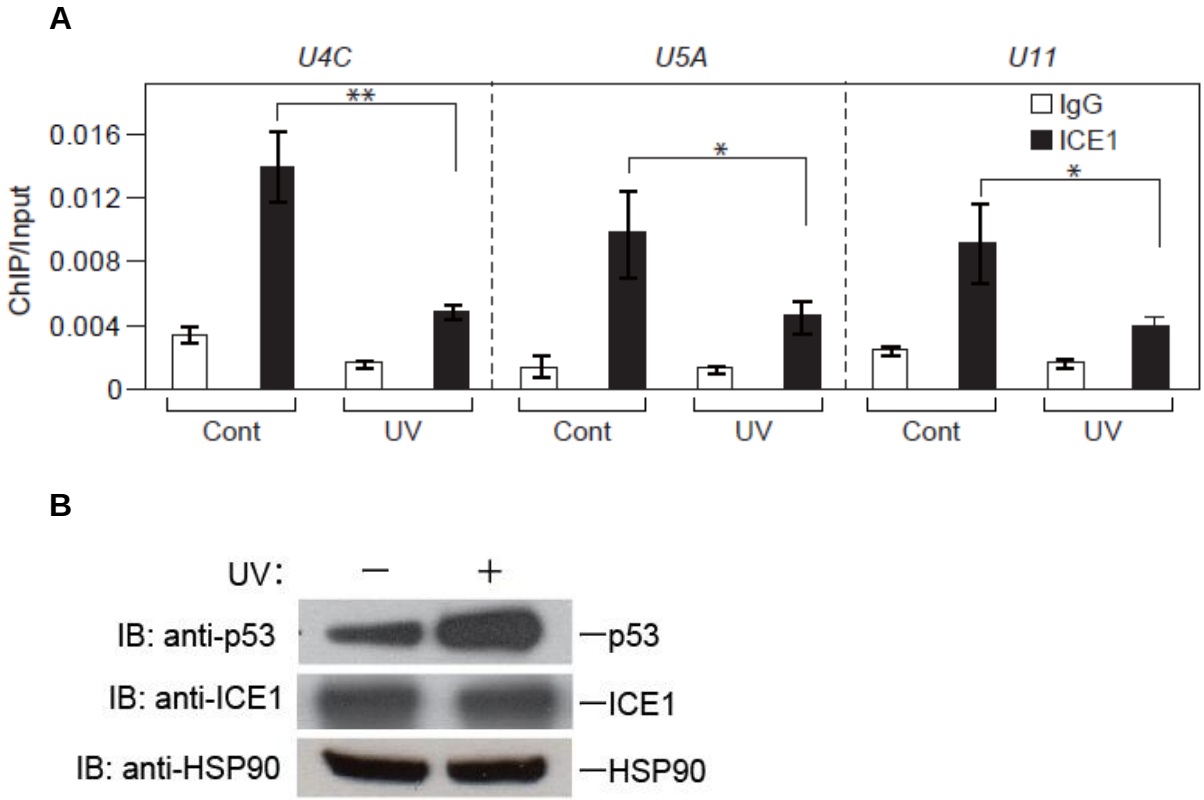

Figure 7. UV irradiation decreases the occupancy of ICE1 at Pol II-dependent *snRNA* genes. (A) ChIP assays were performed using cells treated with or without UV irradiation. The occupancy of ICE1 at Pol II-dependent *snRNA* genes including *U4C*, *U5A* and *U11 snRNA* genes was quantified after UV irradiation. (B) Western blot analysis of p53 induction and ICE1 expression after UV irradiation using HCT116 cells. Standard deviations of each result were calculated from three independent experiments. *P* values were determined by Student's *t* test (* $P<0.1$, ** $P<0.05$). Error bars show s.d.

6 Knockdown of p53 increases the occupancy of Pol II, ICE1 and ELL at *snRNA* genes.

We next tested whether p53 knockdown affected the occupancy of Pol II and LEC at *snRNA* genes. Although the expression levels of ICE1 and Hsp90 was not affected by UV irradiation and/or p53 knockdown, the expression levels of p53 is increased by UV irradiation and decreased by p53 knockdown (Fig. 8A). Consistent with our idea that p53 inhibits the recruitment of LEC at *snRNA* genes by interfering with the interaction of ELL with ICE1, p53 knockdown increased the amount of Pol II at *snRNA* genes including *U4C*, *U5B*, *U11* and *U12* (Fig. 8B). p53 knockdown also increased the occupancy of ICE1 and ELL at *snRNA* genes (Fig. 8C), however the occupancy of p53 are decreased significantly at snRNA genes including *U4C*, *U5A*, *U5B* and *U11* genes in p53 -knockdown cells (Fig. 9 and10).

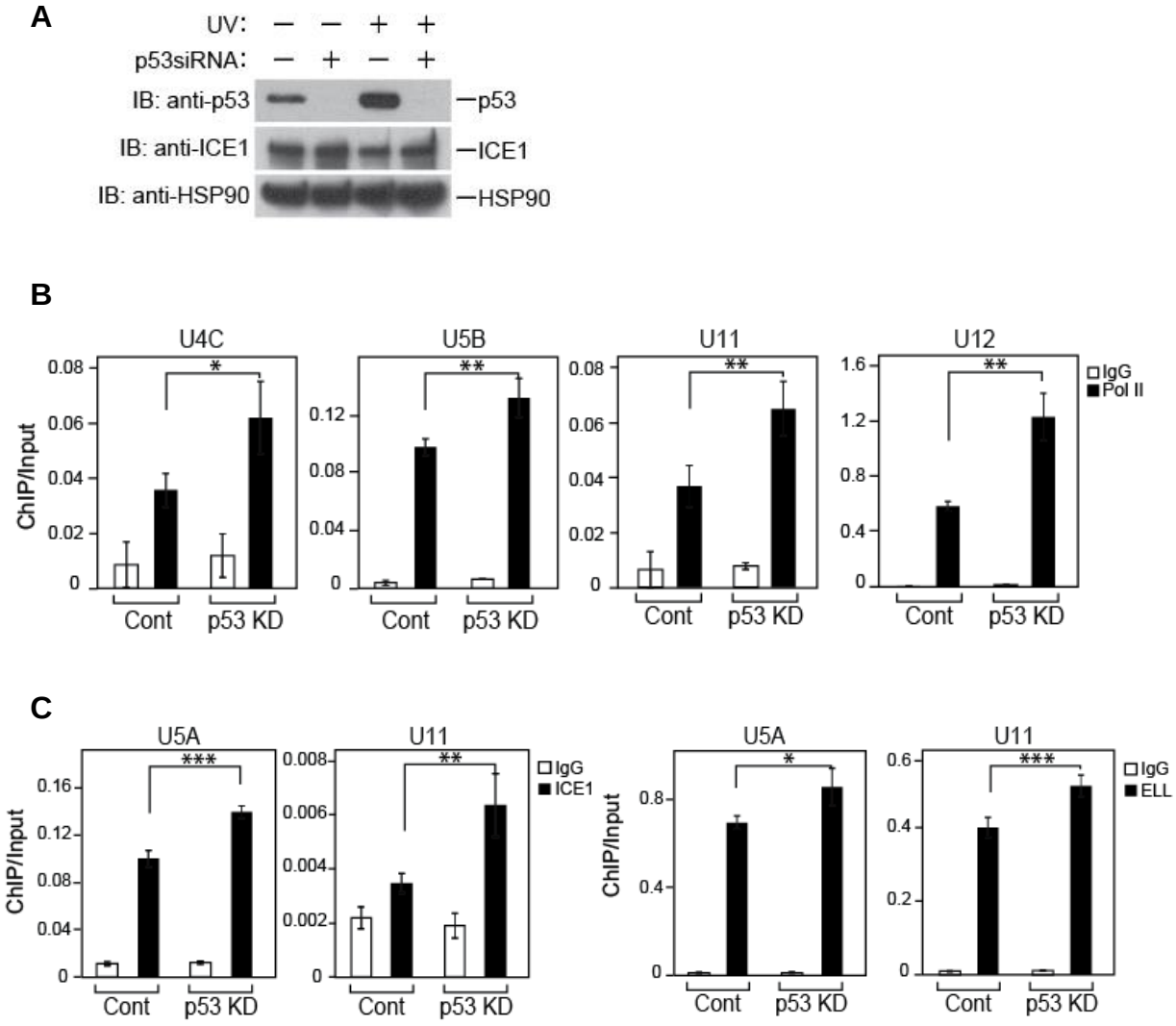

Figure 8. Knockdown of p53 increases the amounts of Pol II, ELL and ICE1 at a subset of *snRNA* genes. (A) Western blot analysis of p53 and ICE1 after p53-knockdown cells with or without UV irradiation. (B) The occupancy of Pol II at *snRNA* genes including *U4, U5B, U11* and *U12* genes was analyzed using p53-knockdown (KD) cells. (C) The occupancy of ICE1 and ELL at *snRNA* genes including *U5A* and *U11* genes was analyzed using p53-knockdown (KD) cells. Standard deviations of each result were calculated from three independent experiments. *P* values were determined by Student's *t* test (* $P<0.1$, ** $P<0.05$, *** $P<0.01$). Error bars show s.d.

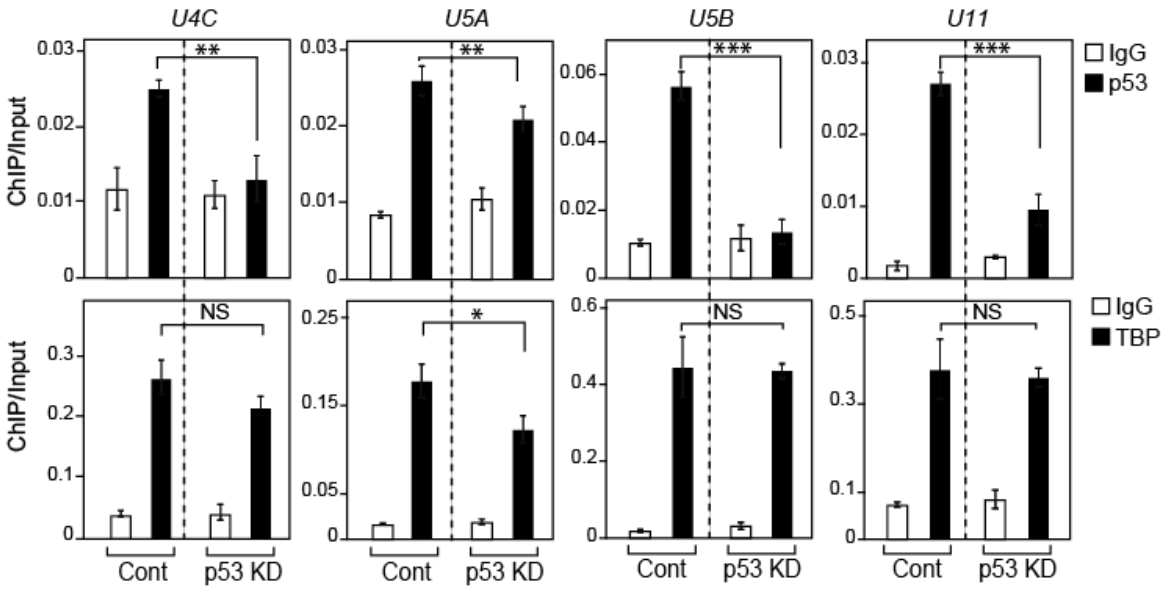

Figure 9. Knockdown of p53 decreases the occupancy of p53 at Pol II-dependent *snRNA* genes. The occupancy of p53 and TBP at the genes including *U4C*, *U5A*, *U5B* and *U11 snRNA* genes were analyzed using control and p53-knockdown (KD) cells. Standard deviations of each result were calculated from three independent experiments. *P* values were determined by Student's *t* test (*** $P<0.01$, ** $P<0.05$). Error bars show s.d. NS, not specific.

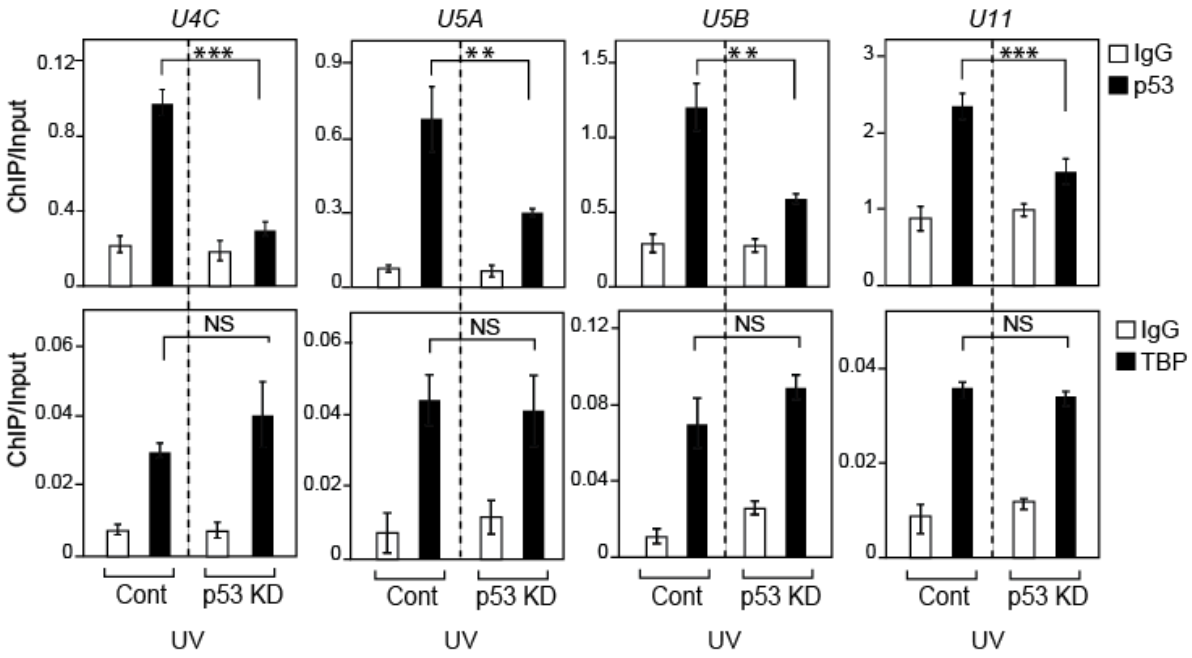

Figure 10. Knockdown of p53 decreases the occupancy of p53 after UV irradiation. After UV irradiation, the occupancy of p53 and TBP at *snRNA* genes including *U4C*, *U5A*, *U5B* and *U11 snRNA* genes were analyzed using control and p53-knockdown (KD) cells. Standard deviations of each result were calculated from three independent experiments. *P* values were determined by Student's *t* test (*** $P<0.01$, ** $P<0.05$). Error bars show s.d. NS, not specific.

It has been reported that exposure to UV irradiation stimulates the association of p53 with *snRNA* genes and that p53 represses transcription of human *snRNA* genes by Pol II[88]. However, the mechanism by which p53 inhibits the transcription of human *snRNA* genes by Pol II has not been elucidated. To clarify the function of p53 in snRNA transcription, we knocked down p53 in HCT116 cells and treated the cells with UV irradiation. ChIP analysis was then performed to examine the occupancy of Pol II at *snRNA* genes. The expression of *snRNA* genes including *U4C*, *U5B* and *U11* were increased by knockdown of p53 under the condition of exposure to UV irradiation (Fig. 11A). Consistent with these results, knockdown of p53 increased the occupancy of ICE1 and Pol II at *U5B snRNA* gene under the condition of exposure to UV irradiation (Fig, 11B). Taken together, these findings suggest that p53 repressed the expression of Pol II-dependent *snRNA* genes through inhibiting the formation of LEC at *snRNA* genes (Fig. 12).

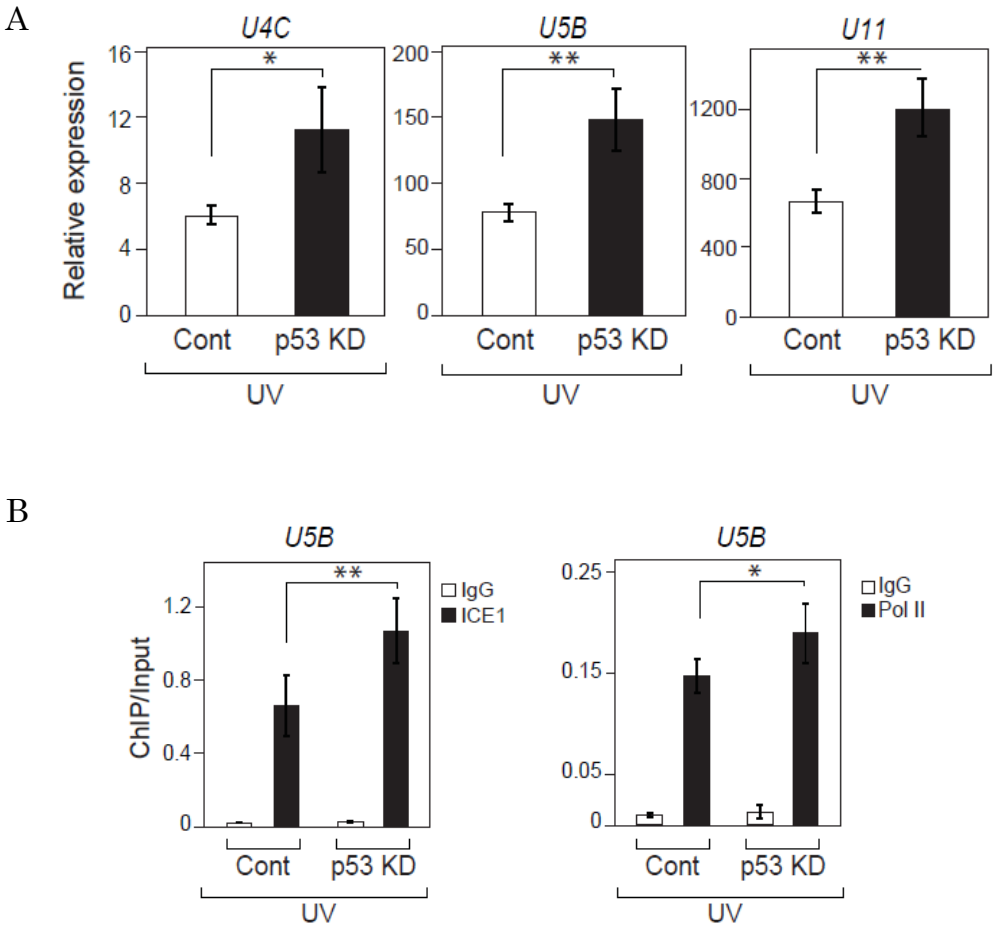

Figure 11. Knockdown of p53 increases in the expression of *snRNA* genes and the occupancy of ICE1 and Pol II after UV irradiation. (A) After UV irradiation, the expression of *snRNA* genes in p53-knockdown cells was analyzed by qPCR. (B) After UV irradiation, the occupancy of ICE1 and Pol II at the Pol II-dependent *U5B snRNA* gene was analyzed using p53-knockdown (KD) cells. Standard deviations of each result were calculated from three independent experiments. *P* values were determined by Student's *t* test (* $P<0.1$, ** $P<0.05$). Error bars show s.d.

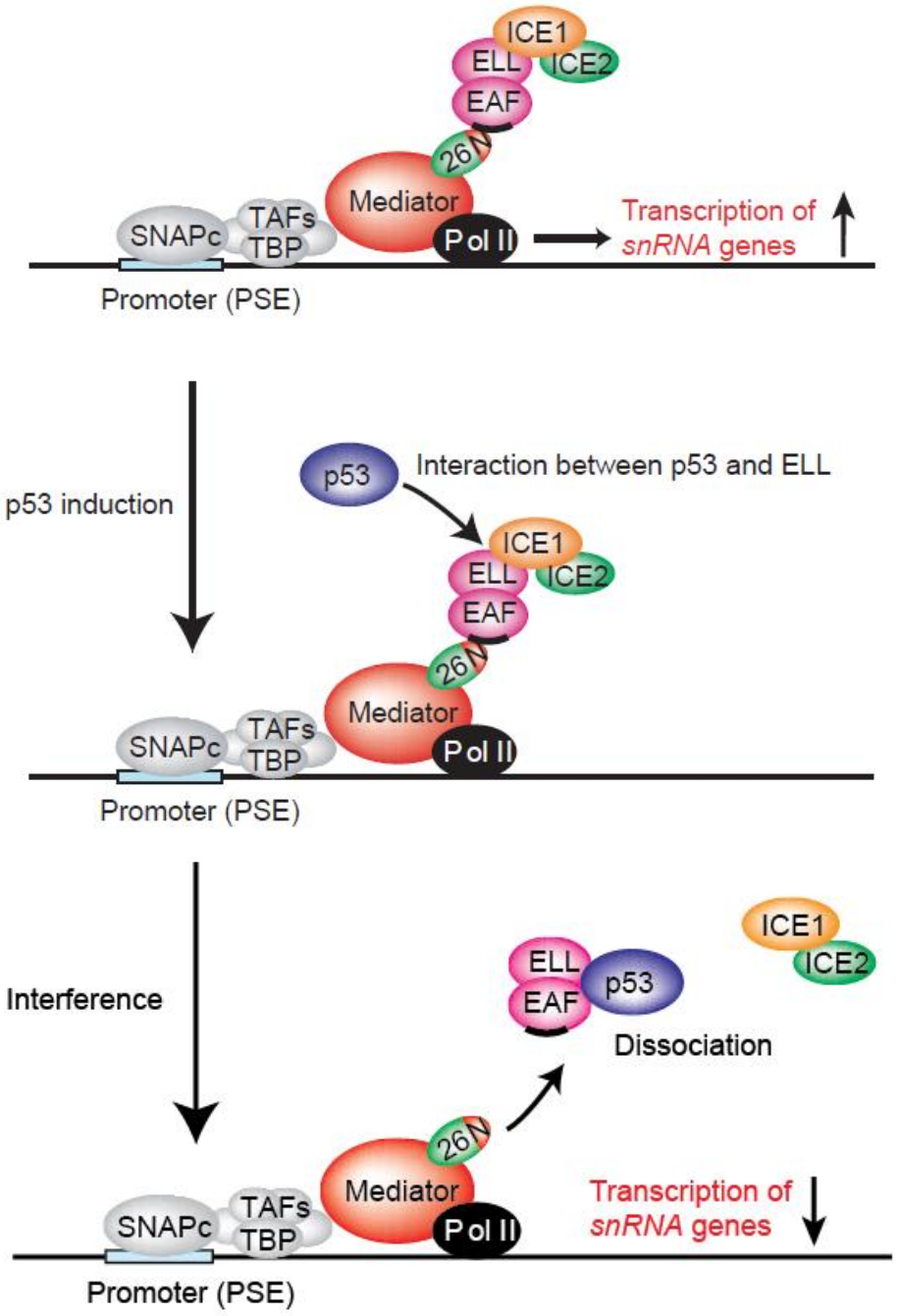

Figure 12. Model for inhibition of the transcription of a subset of Pol II-dependent *snRNA* genes by p53. p53 represses the occupancy of ICE1 at *snRNA* genes, resulting in downregulation of Pol II-dependent snRNA genes through inhibiting the formation of LEC at *snRNA* genes.

F) Discussion

In our previous work, we showed that EAF1 directly binds to the N-terminal domain of human Mediator subunit MED26 [79]. In this thesis, we showed the binding region of EAF1 to the N-terminal domain of MED26, which is the C-terminal amino-acid residues from 245 to 268 in human EAF1. We also showed that EAF family proteins function as adaptor molecules to connect between MED26 and LEC. ICE1 functions as a core subunit of LEC and interacts with both ICE2 and ELL/EAF. The C-terminal region of ICE1 (1191-2266) is required for the formation of LEC [64,79]. We showed that depletion of EAF1 decreases the occupancy of ICE1 at a subset of *snRNA* genes. Therefore, we propose a model in which EAF1 functions as an adaptor molecule connecting between LEC and MED26 at a subset of Pol II-dependent *snRNA* genes (Fig. 4).

It has been reported that p53 inhibits the activity of ELL and that p53 interacts with ELL/EAF and ICE1 of LEC. We confirmed that p53 interacts with ELL and showed that p53 interferes with the binding of ELL to ICE1.

We showed that p53 inhibits the interaction between ELL and ICE1 in LEC and that UV treatment decreases the occupancy of ICE1 at *snRNA* genes. Furthermore, we showed that knockdown of p53 increases the amount of Pol II at *snRNA* genes, including *U4*, *U5B*, *U11* and *U12*, and the occupancy of ELL and ICE1 at *snRNA* genes. To further address the function of p53 in snRNA expression, we performed knockdown of p53 in HCT116 cells treated with UV irradiation. Under the condition of UV irradiation, the expression of *snRNA* genes including *U4C, U5B* and *U11* was increased by knockdown of p53. Consistent with these results, knockdown of p53 increased the occupancy of ICE1 and Pol II at *U5B snRNA* gene under the condition of UV irradiation. Taken together, these findings suggest that p53 represses the expression of Pol II-dependent *snRNA* genes through inhibiting the formation of LEC at *snRNA* genes.

G) FINAL CONCLUSION

In this study, we obtained evidence that EAF1 is required for the recruitment of ICE1 to a subset of RNA Pol II-dependent *snRNA* genes and that EAF1 increased the occupancy of ICE1 at *snRNA* genes. We provided a model in which EAF1 functions as an adaptor molecule connecting between LEC and MED26 at a subset of Pol II-dependent *snRNA* genes (Fig. 6). We also showed that p53 interacts with ELL and interferes with the interaction between ELL and ICE1, suggesting that p53 represses the formation of LEC at *snRNA* genes. Induction of p53 expression triggered by UV irradiation decreased the occupancy of ICE1 at Pol II-dependent *snRNA* genes. p53 also repressed the occupancy of not only Pol II but also ICE1 and ELL at *snRNA* genes, resulting in down-regulation of Pol II-dependent *snRNA* genes. Taken together, the results suggest that p53 decreases a subset of Pol II-dependent *snRNA* genes by interaction with ELL.

It has been reported that p53 interacts with a key transcription elongation factor, p-TEFb[89]. p-TEFb is a component of SEC. SEC is required for proper induction of the *HSP70* gene upon stress and is involved in HIV proviral transcription. SEC also plays a role in the misactivation of *HOX* genes in leukemia and coordinates the proper induction of *Hox* genes during early developmental stages [8-10,90,91]. If p53 interacts with ELL in SEC, p53 may inhibit the formation of SEC as p53 affects LEC activity. Therefore, p53 may play a role in regulating the expression of *HSP70*, *HIV* and *HOX* genes by inhibiting the recruitment of SEC. Importantly, it has been reported that SEC is required for *MYC* expression in several leukemia cell lines, suggesting that SEC is a potential therapeutic target for the treatment of leukemia and for perhaps other cancers associated with overexpression of the *MYC* oncogene[11]. Although it has been reported that p53 represses the expression of c-Myc[92], the mechanism by which p53 regulates the expression of c-Myc has been poorly understood. Based on our results, it is possible that p53 plays a crucial role in inhibition of c-Myc expression by inhibiting the interaction between ELL and SEC.

Human Mediator subunit MED26 recruits SEC while interacting with Pol II to facilitate transformation of Pol II into the elongation stage of transcription from pausing[64]. MED26-NTD contributes to the recruitment of SEC to Pol II-transcribed protein-coding genes and the recruitment of LEC to *snRNA* genes through direct interaction with EAF[64]. snRNAs are involved in carcinogenesis of several types of leukemia, lung cancer and germ cell tumors [54-56]. It has been reported that p53 also interacts with the key regulator CDK9, which forms a complex with cyclin T1 as a positive transcription elongation complex, p-TEFb[89]. p-TEFb is composed of SEC with other components including AF4, AFF4, AF9, ENL and EAF/ELL. Taken together, the results indicate that p53 may function as an oncosupressor through inhibiting the recruitment of SEC with MED26.

In addition, we showed that C-terminal amino-acid residues from 245 to 268 in human EAF1 are required for interaction with MED26-NTD. MED26-NTD contributes to the recruitment of SEC and LEC to Pol II-transcribed protein-coding genes through direct interaction with EAF. Recently, it has been reported that ICE1 depletion decreases the occupancy of other components of LEC at *snRNA* genes, suggesting that ICE1 functions as a scaffold protein for LEC and contributes to the recruitment of LEC to the promoter[13]. The minor difference in U1 and U6 promoters is derived from a conformational difference of snRNA-activation protein complex (SNAPc); Pol II and Pol III are selected for U1 and U6 promoters by the differences, respectively. These findings suggest that a combination of MED26, ICE1, SNAPc and other factors contributes to the LEC recruitment to *snRNA* genes.

Taken together, the results indicate that p53 regulates transcription of Pol II-dependent *snRNA* genes through inhibiting the function of LEC. The *p53* gene has been shown to be mutated or lost in more than 50% of cancers. Therefore, the results of our study suggest that p53 regulates the transcription of *snRNA* genes through inhibiting transcription elongation factors such as ELL/EAF and/or LEC, and the results may contribute to an understanding of diseases associated with dysregulation of *snRNA* gene transcription.

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I) REFERENCES

1 Conaway, R. C. & Conaway, J. W. The Mediator complex and transcription elongation. *Biochim. Biophys. Acta* **1829**, 69-75 (2013).

2 Hochheimer, A. & Tjian, R. Diversified transcription initiation complexes expand promoter selectivity and tissue-specific gene expression. *Genes Dev.* **17**, 1309-1320 (2003).

3 Malik, S. & Roeder, R. G. Dynamic regulation of pol II transcription by the mammalian Mediator complex. *Trends Biochem. Sci.* **30**, 256-263 (2005).

4 Saunders, A., Core, L. J. & Lis, J. T. Breaking barriers to transcription elongation. *Nat. Rev. Mol. Cell Biol.* **7**, 557-567 (2006).

5 Kwak, H. & Lis, J. T. Control of transcriptional elongation. *Annu. Rev. Genet.* **47**, 483-508 (2013).

6 Kong, S. E. *et al.* ELL-associated factors 1 and 2 are positive regulators of RNA polymerase II elongation factor ELL. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 10094-10098 (2005).

7 Shilatifard, A. *et al.* An RNA Polymerase II Elongation Factor Encoded by the Human ELL Gene. *Science* **271**, 1873-1876 (1996).

8 Lin, C. *et al.* AFF4, a component of the ELL/P-TEFb elongation complex and a shared subunit of MLL chimeras, can link transcription elongation to leukemia. *Mol. Cell* **37**, 429-437 (2010).

9 Sobhian, B. *et al.* HIV-1 Tat assembles a multifunctional transcription elongation complex and stably associates with the 7SK snRNP. *Mol. Cell* **38**, 439-451 (2010).

10 He, N. *et al.* HIV-1 Tat and host AFF4 recruit two transcription elongation factors into a bifunctional complex for coordinated activation of HIV-1 transcription. *Mol. Cell* **38**, 428-438 (2010).

11 Luo, Z. *et al.* The Super Elongation Complex Family of RNA Polymerase II Elongation Factors: Gene Target Specificity and Transcriptional Output. *Mol. Cell. Biol.* **32**, 2608-2617 (2012).

12 Smith, Edwin R. *et al.* The Little Elongation Complex Regulates Small Nuclear RNA Transcription. *Mol. Cell* **44**, 954-965 (2011).

13 Hu, D. *et al.* The Little Elongation Complex Functions at Initiation and Elongation Phases of snRNA Gene Transcription. *Mol. Cell* **51**, 493-505 (2013).

14 Jawdekar, G. W. & Henry, R. W. Transcriptional regulation of human small nuclear RNA genes. *Biochim. Biophys. Acta* **1779**, 295-305 (2008).

15 Egloff, S. & Murphy, S. Role of the C-terminal domain of RNA polymerase II in expression of small nuclear RNA genes. *Biochem. Soc. Trans.* **36**, 537-539 (2008).

16 Egloff, S. & Murphy, S. Cracking the RNA polymerase II CTD code. *Trends Genet.* **24**, 280-288 (2008).

17 Egloff, S. *et al.* Serine-7 of the RNA polymerase II CTD is specifically required for snRNA gene expression. *Science* **318**, 1777-1779 (2007).

18 Egloff, S., Van Herreweghe, E. & Kiss, T. Regulation of polymerase II transcription by 7SK snRNA: two distinct RNA elements direct P-TEFb and HEXIM1 binding. *Mol. Cell. Biol.* **26**, 630-642 (2006).

19 Baillat, D. *et al.* Integrator, a multiprotein mediator of small nuclear RNA processing, associates with the C-terminal repeat of RNA polymerase II. *Cell* **123**, 265-276 (2005).

20 Medlin, J. *et al.* P-TEFb is not an essential elongation factor for the intronless human U2 snRNA and histone H2b genes. *EMBO J.* **24**, 4154-4165 (2005).

21 Linzer, D. I. & Levine, A. J. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* **17**, 43-52 (1979).

22 Matlashewski, G. *et al.* Isolation and characterization of a human p53 cDNA clone: expression of the human p53 gene. *EMBO J.* **3**, 3257-3262 (1984).

23 Isobe, M. *et al.* Localization of gene for human p53 tumour antigen to band 17p13. *Nature* **320**, 84-85 (1986).

24 Kern, S. E. *et al.* Identification of p53 as a sequence-specific DNA-binding protein. *Science* **252**, 1708-1711 (1991).

25 McBride, O. W., Merry, D. & Givol, D. The gene for human p53 cellular tumor antigen is located on chromosome 17 short arm (17p13). *Proc. Natl. Acad. Sci. U. S. A.* **83**, 130-134 (1986).

26 Bourdon, J. C. *et al.* p53 isoforms can regulate p53 transcriptional activity. *Genes Dev.* **19**, 2122-2137 (2005).

27 Lane, D. & Levine, A. p53 Research: the past thirty years and the next thirty years. *Cold Spring Harb. Perspect. Biol.* **2**, a000893 (2010).

28 Oren, M. Decision making by p53: life, death and cancer. *Cell Death Differ.* **10**, 431-442 (2003).

29 Su, F., Pascal, L. E., Xiao, W. & Wang, Z. Tumor suppressor U19/EAF2 regulates thrombospondin-1 expression via p53. *Oncogene* **29**, 421-431 (2010).

30 Chikayama, S. *et al.* Nuclear accumulation of p53 protein and apoptosis induced by various anticancer agents, u.v.-irradiation and heat shock in primary normal human skin fibroblasts. *Int. J. Oncol.* **16**, 1117-1124 (2000).

31 Surget, S., Khoury, M. P. & Bourdon, J. C. Uncovering the role of p53 splice variants in human malignancy: a clinical perspective. *Onco Targets Ther* **7**, 57-68 (2013).

32 Meek, D. W. & Anderson, C. W. Posttranslational modification of p53: cooperative integrators of function. *Cold Spring Harb. Perspect. Biol.* **1**, a000950 (2009).

33 Ferlay, J. *et al.* Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer* **127**, 2893-2917 (2010).

34 Mirisola, V. *et al.* A prognostic multigene classifier for squamous cell carcinomas of the larynx. *Cancer Lett.* **307**, 37-46 (2011).

35 Tergaonkar, V. *et al.* p53 stabilization is decreased upon NFκB activation: A role for NFκB in acquisition of resistance to chemotherapy. *Cancer Cell* **1**, 493-503 (2002).

36 Ikeda, A. *et al.* p300/CBP-Dependent and -Independent Transcriptional Interference between NF-κB RelA and p53. *Biochem. Biophys. Res. Commun.* **272**, 375-379 (2000).

37 Webster, G. A. & Perkins, N. D. Transcriptional Cross Talk between NF-κB and p53. *Mol. Cell. Biol.* **19**, 3485-3495 (1999).

38 Temiz, P. *et al.* Determination-of apoptosis and cell cycle modulators (p16, p21, p27, p53, BCL-2, Bax, BCL-xL, and cyclin D1) in thyroid follicular carcinoma, follicular adenoma, and adenomatous nodules via a tissue microarray method. *Turk J Med Sci* **45**, 865-871 (2015).

39 Nair, J. *et al.* Gene and miRNA expression changes in squamous cell carcinoma of larynx and hypopharynx. *Genes & Cancer* **6**, 328-340 (2015).

40 Li, X., Abdel-Mageed, A. B., Mondal, D. & Kandil, E. MicroRNA expression profiles in differentiated thyroid cancer, a review. *Int. J. Clin. Exp. Med.* **6**, 74-80 (2013).

41 Chen, P. *et al.* Pri-Mir-34b/C and Tp-53 Polymorphisms are Associated With The Susceptibility of Papillary Thyroid Carcinoma: A Case–Control Study. *Medicine (Baltimore)* **94**, e1536 (2015).

42 Storey, A. *et al.* Role of a p53 polymorphism in the development of human papillomavirus-associated cancer. *Nature* **393**, 229-234 (1998).

43 Boltze, C. *et al.* Homozygous proline at codon 72 of p53 as a potential risk factor favoring the development of undifferentiated thyroid carcinoma. *Int. J. Oncol.* **21**, 1151-1154 (2002).

44 Granja, F. *et al.* Proline homozygosity in codon 72 of p53 is a factor of susceptibility for thyroid cancer. *Cancer Lett.* **210**, 151-157 (2004).

45 Rogounovitch, T. I. *et al.* TP53 codon 72 polymorphism in radiation-associated human papillary thyroid cancer. *Oncol. Rep.* **15**, 949-956 (2006).

46 Wu, B., Guo, D. & Guo, Y. Association between p53 Arg72Pro polymorphism and thyroid cancer risk: a meta-analysis. *Tumour Biol.* **35**, 561-565 (2014).

47 Pei, S.-G. *et al.* Correlation of survivin, p53 and Ki-67 in laryngeal cancer Hep-2 cell proliferation and invasion. *Asian Pacific Journal of Tropical Medicine* **8**, 636-642 (2015).

48 Wiederschain, D. *et al.* Molecular basis of p53 functional inactivation by the leukemic protein MLL-ELL. *Mol. Cell. Biol.* **23**, 4230-4246 (2003).

49 Shinobu, N. *et al.* Physical Interaction and Functional Antagonism between the RNA Polymerase II Elongation Factor ELL and p53. *J. Biol. Chem.* **274**, 17003-17010 (1999).

50 Matera, A. G., Terns, R. M. & Terns, M. P. Non-coding RNAs: lessons from the small nuclear and small nucleolar RNAs. *Nat. Rev. Mol. Cell Biol.* **8**, 209-220 (2007).

51 Goodrich, J. A. & Kugel, J. F. Non-coding-RNA regulators of RNA polymerase II transcription. *Nat. Rev. Mol. Cell Biol.* **7**, 612-616 (2006).

52 Gridasova, A. A. & Henry, R. W. The p53 Tumor Suppressor Protein Represses Human snRNA Gene Transcription by RNA Polymerases II and III Independently of Sequence-Specific DNA Binding. *Mol. Cell. Biol.* **25**, 3247-3260 (2005).

53 Younger, S. T. *et al.* Integrative genomic analysis reveals widespread enhancer regulation by p53 in response to DNA damage. *Nucleic Acids Res* **43**, 4447-4462 (2015).

54 Köhler, J. *et al.* Circulating U2 small nuclear RNA fragments as a diagnostic and prognostic biomarker in lung cancer patients. *J. Cancer Res. Clin. Oncol.*, 1-11 (2015).

55 Holt, J. E., Stanger, S. J., Nixon, B. & McLaughlin, E. A. in *Non-coding RNA and the Reproductive System* (eds Dagmar Wilhelm & Pascal Bernard) 95-120 (Springer Netherlands, 2016).

56 Padgett, R. A. New connections between splicing and human disease. *Trends Genet.* **28**, 147-154 (2012).

57 Jia, Y., Mu, John C. & Ackerman, Susan L. Mutation of a U2 snRNA Gene Causes Global Disruption of Alternative Splicing and Neurodegeneration. *Cell* **148**, 296-308 (2012).

58 Hales, C. M. *et al.* Aggregates of Small Nuclear Ribonucleic Acids (snRNAs) in Alzheimer's Disease. *Brain Pathol.* **24**, 344-351 (2014).

59 Bai, B. *et al.* U1 small nuclear ribonucleoprotein complex and RNA splicing alterations in Alzheimer's disease. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 16562 - 16567 (2013).

60 Kim, Y. J. *et al.* A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. *Cell* **77**, 599-608 (1994).

61 Koleske, A. J. & Young, R. A. An RNA polymerase II holoenzyme responsive to activators. *Nature* **368**, 466-469 (1994).

62 Sato, S. *et al.* A set of consensus mammalian mediator subunits identified by multidimensional protein identification technology. *Mol. Cell* **14**, 685-691 (2004).

63 Malik, S. & Roeder, R. G. The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. *Nat. Rev. Genet.* **11**, 761-772 (2010).

64 Takahashi, H. *et al.* Human mediator subunit MED26 functions as a docking site for transcription elongation factors. *Cell* **146**, 92-104 (2011).

65 Huang, Y. *et al.* Mediator complex regulates alternative mRNA processing via the MED23 subunit. *Mol. Cell* **45**, 459-469 (2012).

66 Galbraith, M. D. *et al.* HIF1A employs CDK8-mediator to stimulate RNAPII elongation in response to hypoxia. *Cell* **153**, 1327-1339 (2013).

67 Donner, A. J., Ebmeier, C. C., Taatjes, D. J. & Espinosa, J. M. CDK8 is a positive regulator of transcriptional elongation within the serum response network. *Nat. Struct. Mol. Biol.* **17**, 194-201 (2010).

68 Whyte, W. A. *et al.* Master transcription factors and mediator establish super-enhancers at key cell identity genes. *Cell* **153**, 307-319 (2013).

69 Taatjes, D. J. The human Mediator complex: a versatile, genome-wide regulator of transcription. *Trends Biochem. Sci.* **35**, 315-322 (2010).

70 Mittler, G., Kremmer, E., Timmers, H. T. & Meisterernst, M. Novel critical role of a human Mediator complex for basal RNA polymerase II transcription. *EMBO Rep.* **2**, 808-813 (2001).

71 Taatjes, D. J. *et al.* Structure, function, and activator-induced conformations of the CRSP coactivator. *Science* **295**, 1058-1062 (2002).

72 Naar, A. M. *et al.* Human CRSP interacts with RNA polymerase II CTD and adopts a specific CTD-bound conformation. *Genes Dev.* **16**, 1339-1344 (2002).

73 Mo, X., Kowenz-Leutz, E., Xu, H. & Leutz, A. Ras induces mediator complex exchange on C/EBP beta. *Mol. Cell* **13**, 241-250 (2004).

74 Ryu, S., Zhou, S., Ladurner, A. G. & Tjian, R. The transcriptional cofactor complex CRSP is required for activity of the enhancer-binding protein Sp1. *Nature* **397**, 446-450 (1999).

75 Diebold, M. L. *et al.* The structure of an Iws1/Spt6 complex reveals an interaction domain conserved in TFIIS, Elongin A and Med26. *EMBO J.* **29**, 3979-3991 (2010).

76 Booth, V., Koth, C. M., Edwards, A. M. & Arrowsmith, C. H. Structure of a conserved domain common to the transcription factors TFIIS, elongin A, and CRSP70. *J. Biol. Chem.* **275**, 31266-31268 (2000).

77 Masuda, M. *et al.* Chemoprevention of Head and Neck Cancer by Green Tea Extract: EGCG-The Role of EGFR Signaling and "Lipid Raft". *J Oncol* **540148**, 2 (2011).

78 Dignam, J. D., Martin, P. L., Shastry, B. S. & Roeder, R. G. Eukaryotic gene transcription with purified components. *Methods Enzymol.* **101**, 582-598 (1983).

79 Takahashi, H. *et al.* MED26 regulates the transcription of snRNA genes through the recruitment of little elongation complex. *Nat. Commun.* **6**, (2015).

80 *U.S. House of Representatives* (1999).

81 Agarwal, M. L. *et al.* The p53 Network. *J. Biol. Chem.* **273**, 1-4 (1998).

82 Cox, L. S. & Lane, D. P. Tumour suppressors, kinases and clamps: how p53 regulates the cell cycle in response to DNA damage. *Bioessays* **17**, 501-508 (1995).

83 Harris, C. C. p53 tumor suppressor gene: from the basic research laboratory to the clinic--an abridged historical perspective. *Carcinogenesis* **17**, 1187-1198 (1996).

84 Ko, L. J. & Prives, C. p53: puzzle and paradigm. *Genes Dev.* **10**, 1054-1072 (1996).

85 Levine, A. J. p53, the cellular gatekeeper for growth and division. *Cell* **88**, 323-331 (1997).

86 Hu, D. *et al.* The little elongation complex functions at initiation and elongation phases of snRNA gene transcription. *Mol. Cell* **51**, 493-505 (2013).

87 Kaeser, M. D., Pebernard, S. & Iggo, R. D. Regulation of p53 Stability and Function in HCT116 Colon Cancer Cells. *J. Biol. Chem.* **279**, 7598-7605 (2004).

88 Gridasova, A. A. & Henry, R. W. The p53 tumor suppressor protein represses human snRNA gene transcription by RNA polymerases II and III independently of sequence-specific DNA binding. *Mol. Cell. Biol.* **25**, 3247-3260 (2005).

89 Claudio, P. P. *et al.* Cdk9 phosphorylates p53 on serine 392 independently of CKII. *J. Cell. Physiol.* **208**, 602-612 (2006).

90 Lin, C. *et al.* Dynamic transcriptional events in embryonic stem cells mediated by the super elongation complex (SEC). *Genes Dev.* **25**, 1486-1498 (2011).

91 Yokoyama, A. *et al.* A higher-order complex containing AF4 and ENL family proteins with P-TEFb facilitates oncogenic and physiologic MLL-dependent transcription. *Cancer Cell* **17**, 198-212 (2010).

92 Sachdeva, M. *et al.* p53 represses c-Myc through induction of the tumor suppressor miR-145. *Proceedings of the National Academy of Sciences* **106**, 3207-3212 (2009).