Molecules and Cells

The Tumor Suppressor, p53, Negatively Regulates Non-Canonical NF-κB Signaling through miRNA-Induced Silencing of NF-κB–Inducing Kinase

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NF-κB signaling through both canonical and non-canonical pathways plays a central role in immune responses and inflammation. NF-κB–inducing kinase (NIK) stabilization is a key step in activation of the non-canonical pathway and its dysregulation implicated in various hematologic malignancies. The tumor suppressor, p53, is an established cellular gatekeeper of proliferation. Abnormalities of the *TP53* gene have been detected in more than half of all human cancers. While the non-canonical NF-κB and p53 pathways have been explored for several decades, no studies to date have documented potential cross-talk between these two cancer-related mechanisms. Here, we demonstrate that p53 negatively regulates NIK in an miRNA-dependent manner. Overexpression of p53 decreased the levels of NIK, leading to inhibition of the non-canonical NF-κB pathway. Conversely, its knockdown led to increased levels of NIK, IKKα phosphorylation, and p100 processing. Additionally, miR-34b induced by nutlin-3 directly targeted the coding sequences (CDS) of NIK. Treatment with anti-miR-34b-5p augmented NIK levels and subsequent non-canonical NF-κB signaling. Our collective findings support a novel cross-talk mechanism between non-canonical NF-κB and p53.

Keywords: cancer, microRNA, NF-κB, NF-κB-inducing kinase, p53, tumor suppressor gene

INTRODUCTION

The transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), regulates genes involved in immune responses, inflammation, cell differentiation, proliferation and survival in response to cytokines and other stimuli (Vallabhapurapu and Karin, 2009; Zhang et al., 2017). NF-κB signaling is classified into canonical and non-canonical pathways. The canonical pathway is triggered by signals from a variety of immune receptors, such as tumor necrosis factor-alpha (TNF-α) receptor (Sun, 2017). Association of these receptors with their specific ligands activates TGF-β-activated kinase 1 (TAK1) and stimulates activity of the IKK complex

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composed of IKKα, IKKβ, and IKKγ. The active IKK complex promotes phosphorylation and proteasomal degradation of IκBα, resulting in nuclear translocation of p65 (RelA)/p50 (NF-κB1) or c-Rel/p50. On the other hand, NF-κB–inducing kinase (NIK) has been highlighted as a key player in the non-canonical NF-κB pathway. In genetic studies on *MAP3K14* (encoding NIK), NIK-defective mice showed impaired antibody production and proliferation of B cells, supporting a critical role in regulation of the immune response (Yamada et al., 2000). Stabilization of NIK is a central regulatory step in the initiation of non-canonical NF-κB signaling (Sun, 2017). Under physiological conditions, *de novo* synthesized NIK is bound to TNFR-associated factor 3 (TRAF3) and recruited to the cellular inhibitor of apoptosis (cIAP)-TRAF2-TRAF3 E3 ubiquitin ligase complex. cIAPs catalyze ubiquitination and subsequent degradation of NIK. Several ligands, such as CD40 ligand (CD40L), B-cell activating factor and TNF superfamily member 14 (LIGHT/TNFSF14), promote recruitment of cIAP-TRAF2-TRAF3 to its receptor. Degradation of TRAF3 by cIAPs facilitates its dissociation from NIK, resulting in stabilization and activation of NIK. Accumulating NIK phosphorylates and activates IKKα, inducing processing of p100 associated with RelB. RelB/p52 (NF-κB2) complexes subsequently localize to the nucleus and activate target genes. Genetic loss of *TRAF3* or *BIRC2/3* encoding cIAP1/2 as negative regulators of NIK results in stabilization of NIK and constitutive non-canonical NF-κB processing in multiple myeloma, B cell lymphoma or T cell lymphoma (Keats et al., 2007; Rahal et al., 2014). Therefore, targeting of oncogenic NIK may be a particularly effective strategy in treatment of these cancer types.

Tumor protein p53 (TP53) is a transcription factor characterized as a tumor suppressor. Stress conditions, such as DNA damage and exposure to ultraviolet light, stimulate production of p53 protein. Increased expression of p53 leads to inhibition of the cell cycle and blood vessel formation or apoptosis (Kastenhuber and Lowe, 2017; Vogelstein et al., 2000). Abnormalities of the *TP53* gene have been detected in more than half of all human cancers. The p53 polypeptide contains several functional domains to achieve DNA binding and transactivation, including two transactivation domains (TAD1 and TAD2), proline-rich domain (Pro), DNA-binding domain (DBD), oligomerization domain (OD) and C-terminal regulatory domain (CTD) (Sullivan et al., 2018). Recent studies further suggest that TP53 regulates not only numerous protein-coding genes but also microRNAs (miRNAs) both transcriptionally and post-transcriptionally (Hermeking, 2012).

miRNAs are ~22 nucleotide-long RNA molecules that direct post-transcriptional repression of mRNA targets via base-pairing to 3' untranslated regions (3' UTR) (Bartel, 2018). Several studies indicate that miRNAs bind coding sequences (CDS) of their target transcripts (Chi et al., 2009; Forman et al., 2008; Tay et al., 2008). miRNAs are transcribed by RNA polymerase II as part of a several hundred nucleotide-long RNA chain, known as primary miRNA (pri-miRNA). Mature miRNAs are generated from sequential two-step cleavage of pri-miRNAs and precursor miRNAs (pre-miRNAs) by *DROSHA* and *DICER*, respectively. One strand of the miRNA duplex (mature miRNA) is loaded onto the Argonaute protein (AGO) to form the RNA-induced silencing complex (RISC), which mediates the degradation or inhibition of translation of respective mRNA targets (Bartel, 2018; Ha and Kim, 2014).

Tumor suppressor genes negatively regulate expression of specific proto-oncogenes (Benitez et al., 2017; Kim et al., 2011) and inhibit the tumor-promoting activities of NF-κB subunits (Mayo et al., 2002; Rocha et al., 2003). p53 inhibits the canonical NF-κB pathway by competing out the transcriptional coactivator, p300/CBP, from p65 (RelA) (Ravi et al., 1998). In addition, *TP53* deficiency promotes O-GlcNAcylation of IKKβ, resulting in enhancement of canonical NF-κB signaling (Kawauchi et al., 2009). On the other hand, IKKα-mediated CBP phosphorylation switches the protein binding preference of CBP from p53 to canonical NF-κB (Huang et al., 2007). However, crosstalk between p53 and non-canonical NF-κB pathways remains to be established. In this study, we have identified a novel role of the tumor suppressor p53 as a regulator of the non-canonical NF-κB pathway. Our results additionally suggest that miR-34b upregulated by nutlin-3 targets the CDS of NIK, thereby inhibiting the non-canonical NF-κB signaling pathway. These collective findings reveal a novel link between p53 and non-canonical NF-κB signaling in cancer cells.

MATERIALS AND METHODS

Plasmids and reagents

pCMV-MYC-NIK-CDS and plasmid containing the NF-κB luciferase reporter were obtained from Dr. Choi (Choi et al., 2014). CDS of NIK was amplified via polymerase chain reaction (PCR) from pCMV-MYC-NIK-CDS, followed by cloning into pCMV-pGL3-luciferase plasmid (#17186; Addgene, USA) digested with *NotI*. Phusion High-Fidelity DNA polymerase (M0530; NEB, USA) was used for amplification of NIK-CDS. Mutations of p53-mTAD (L22Q, W23S), p53-mNLS (K305A, R306A, K319A, K320A, K321A) or p53-R175H and NIK-T559A were generated via site-directed mutagenesis using pcDNA3.1-FLAG-TP53 and pCMV-MYC-NIK-CDS, respectively. MG-132 (C2211), chloroquine (C6628), bafilomycin A1 (B1793), 3-methyladenine (M9281), and E-64 (trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane) (3132) were obtained from Sigma-Aldrich (USA). Bortezomib (Velcade) (S1013) and recombinant human TNFSF14/LIGHT (664-LI) were obtained from Selleck Chemicals (USA) and R&D Systems (USA), respectively. Primers used for PCR are summarized in Supplementary Table S1.

Cell culture and DNA transfection

293T, HeLa or U-2 OS and NCI-H1299 or Z-138 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Welgene, Korea) and RPMI 1640 (Welgene), respectively. Cell lines were cultured in media supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, USA), 1× antibiotic-antimycotic (Gibco) in a humidity-controlled environment (37°C, 5% CO_2). Transient transfection of cells with plasmids was performed using Lipofectamine 2000 (Thermo Fisher Scientific, USA) or X-tremeGENE HP (Roche, USA) reagent according to the manufacturer's instructions.

RNA interference

HeLa or H1299 cells were electroporated with siRNAs (GenePharma, China) of tumor suppressor genes or other genes (Bioneer, Korea) using the Neon transfection system (Thermo Fisher Scientific) according to the manufacturer's protocol. For knockdown of tumor suppressor genes, we used a mixture of two different siRNAs targeting a single gene. The target sequence of the validated si-TP53 (Bioneer) and pLKO.1-shTP53-puro #1 (SHCLND-NM_000546; Sigma-Aldrich) for stable knockdown was the same. pLKO.1-shTP53-puro was transfected into 293T cells together with psPAX2 and pMD2G. After three days, viral particles were harvested from the culture medium via filtration. Lentiviruses were used to infect HeLa or Z138 cells in the presence of 8 μg/ml polybrene, and stable cell lines obtained via antibiotic selection with 2 μg/ml and 0.2 μg/ml puromycin (A11138; Gibco), respectively. To supplement endogenous miRNA activity, AccuTarget miRNA mimic or miRNA mimic negative control #1, chemically synthesized double-stranded RNA oligonucleotides purchased from Bioneer were cotransfected with plasmids into 293T cells using Lipofectamine 2000. For functional inhibition of endogenous miRNA, AccuTarget miRNA inhibitor, a single-stranded synthetic inhibitor targeting human miRNAs, or miRNA inhibitor negative control #1 (Bioneer) was cotransfected with plasmids into H1299 cells using Lipofectamine 2000. Cells were lysed 48 h after transfection.

Immunoblot analysis

Cell lysates were prepared with RIPA lysis buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, cOmplete EDTA-free protease inhibitor cocktail (5056489001; Roche), and a PhosSTOP phosphatase inhibitor (4906837001; Roche). Protein samples were loaded on to 8-10% SDS gels for electrophoresis followed by transfer onto nitrocellulose membranes. The primary antibodies for immunoblot analyses included those specific for NIK (#4994), Argonaute2 (#2897), IKKα (#2682), IKKβ (#2684), p-IKKα/β Ser176/180 (#2697), NF-κB2 p100/p52 (#3017) (all from Cell Signaling Technology, USA), p53 (sc-126), LAMP2 (sc-18822), β-actin (sc-1616, sc-47778), and GAPDH (sc-47724) (all from Santa Cruz Biotechnology, USA), p62 (Sequestosome-1) (MABC32), FLAG M2 (F1804), and tubulin (T5168) (all from Sigma-Aldrich), and LC3 (PM036; MBL International, USA). Primary and secondary antibody dilutions were prepared according to the manufacturer's instructions.

Luciferase assay

H1299 cells were transfected with NF-κB luciferase reporter and pRL-TK at a 3:1 (*Firefly*:Renilla) ratio. Other cell lines were transfected with the indicated luciferase reporter plasmids and pRL-CMV (5:1 or 10:1). After 24 or 48 h, cells were harvested, lysed, and assayed with the Dual-Luciferase Reporter Assay System (E1910; Promega, USA) according to the manufacturer's instructions.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted using TRI Reagent solution (AM9738; Invitrogen, USA) or the RNeasy Plus Mini Kit (74136; Qiagen, Germany) in keeping with the manufacturer's protocol. For RNA extraction using TRI Reagent, contaminating genomic DNA was removed using the TURBO DNA-free Kit (AM1907; Invitrogen). cDNA was synthesized via reverse transcription using 1 to 2 μg total RNA and the RevertAid H Minus First Strand cDNA Synthesis Kit (K1632; Thermo Fisher Scientific). For miRNA detection, small RNA was isolated using the mirVana miRNA Isolation Kit (AM1561; Invitrogen) according to the manufacturer's protocol. cDNA was synthesized via polyadenylation and reverse transcription using the Mir-X miRNA First-Strand Synthesis Kit (638313; Takara Bio, Japan). qPCR was performed using a real-time PCR Kit with EvaGreen (SRH71-M40h; SolGent, Korea) and gene-specific primers. The primers for qPCR are summarized in Supplementary Table S1. Quantification of mRNA and miRNA levels was performed by calculating the Cq value normalized to β-actin and U6 expression, respectively.

Small RNA sequencing

RNA was isolated for miRNA expression profiling with the aid of the mirVana miRNA isolation kit. RNA extracts from duplicate samples were sent to Macrogen (Korea) for small RNA sequencing. After performing quality control, sequencing libraries were generated using the Illumina TruSeq Small RNA Library Prep kit (Illumina, USA). Next, the libraries were size-selected for sequencing of 18 to 30 nucleotide RNA fragments. Sequencing was performed on an Illumina HiSeq-2000 platform. Differentially expressed miRNAs between groups treated for 6 h with DMSO and nutlin-3a were identified based on ≥ 1.5 fold change and P values < 0.05.

Statistical analysis

Results are representative of at least two or three independent repeat experiments. Data are presented as mean ± SEM and P values determined using Student's t-test on Microsoft Excel 2016 (Microsoft, USA). P values < 0.05 were considered statistically significant.

RESULTS

Identification of p53 as a negative regulator of NIK

To identify novel regulators of NIK, we performed knockdown screening using tumor suppressor-specific siRNAs. In total, 15 tumor suppressor genes were selected based on a previous report (Walker et al., 2012). HeLa cells were electroporated with control scrambled or tumor suppressor-specific siRNAs, followed by transfection of NIK-encoding plasmid, and cell lysates examined for NIK expression. Under unstimulated conditions, endogenous NIK was hardly detected owing to continuous degradation by the cIAP-TRAF2/3 complex. Among the 15 tumor suppressor genes, knockdown of *CDH1* and *TP53* led to a significant increase in NIK protein levels (> 2-fold), compared with scrambled control (P < 0.05) (Fig. 1A, Supplementary Figs. S1A-S1D). As expected, knockdown of *BIRC3* induced a > 2-fold increase in NIK. Knockdown of *TP53* using validated si-*TP53* oligonucleotides additionally augmented NIK protein levels in HeLa and U2OS cells (Figs. 1B and 1C). Z138 cells, mantle cell lymphoma, harbor the

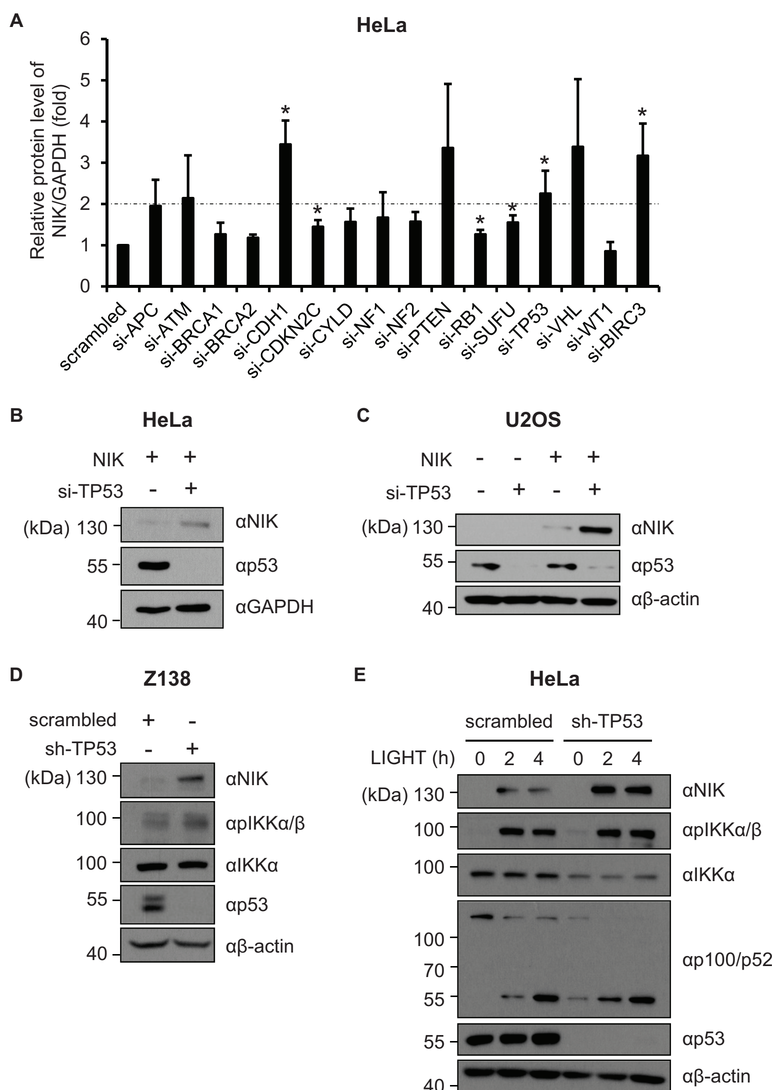

Fig. 1. Identification of p53 as a negative regulator of NIK protein and the non-canonical NF-κB pathway via RNAi screening. (A) Quantification of NIK protein abundance upon tumor suppressor gene knockdown. HeLa cells were electroporated individually with control scrambled siRNA, 50 nM siRNAs targeting 15 tumor suppressor genes or 50 nM siRNAs targeting BIRC3 for 24 h, followed by transfection with a NIK expression plasmid for 24 h. The relative protein levels of gene-specific knockdown cells were normalized to those of control scrambled siRNA-treated cells. Tumor suppressor genes; APC, adenomatous polyposis coli; ATM, ataxia telangiectasia mutated; BRCA1, breast cancer 1; BRCA2, breast cancer 2; CDH1, E-cadherin; CDKN2C, cyclin-dependent kinase inhibitor 2C; CYLD, cylindromatosis; NF1, neurofibromin 1; NF2, neurofibromin 2; PTEN, phosphatase and tensin homolog; RB1, retinoblastoma 1; SUFU, suppressor of fused homolog; TP53, tumor protein p53; VHL, von Hippel-Lindau tumor suppressor; WT1, Wilms tumor 1. Data represent mean ± SEM. $*P \leq 0.05$ with Student's *t*-test. (B) HeLa cells were electroporated with control scrambled siRNA or 50 nM si-TP53 for 24 h, followed by transfection with NIK expression plasmid for 24 h. Lysates of HeLa cells depleted of p53 were analyzed via immunoblotting with antibodies against NIK, p53 or GAPDH as loading control. (C) U2OS cells were electroporated with control scrambled siRNA or 50 nM si-TP53 for 24 h, followed by transfection with control vector or NIK expression plasmid for 48 h. U2OS cell lysates depleted of p53 were analyzed via immunoblotting with antibodies against NIK, p53 or β-actin as a loading control. (D) Z138 cells stably expressing control scrambled shRNA or shRNA specific for p53 were lysed and subjected to immunoblot analysis. (E) HeLa cells stably expressing control scrambled shRNA or shRNA specific for p53 were either left untreated or treated with LIGHT (100 ng/ml) for the indicated time-periods.

non-sense mutation in TRAF2 and endogenous NIK is stabilized and can be detected in Z138 cells (Rahal et al., 2014). *TP53* depletion using specific shRNA #1 enhanced endogenous NIK levels and phosphorylation of IKKα in Z138 cells (Fig. 1D, Supplementary Fig. S1E). Furthermore, upon LIGHT treatment, NIK stabilization, IKKα phosphorylation and p100

processing were further increased in *TP53*-depleted HeLa cells, supporting the idea that p53 regulates the NIK level (Fig. 1E). It was of note that knockdown of *TP53* increased p100 processing even in the absence of LIGHT, although endogenous NIK was not detected in the immunoblotting (Fig. 1E).

Overexpression of p53 suppresses NIK protein levels

We then examined whether overexpression of p53 decreases NIK level. Overexpression of p53 led to a dose-dependent decrease in NIK protein (Fig. 2A). To further clarify the effects of p53 on endogenous protein levels, Z138 cells were treated with the non-genotoxic MDM2 antagonist, nutlin-3a, as a stabilizer of p53. Nutlin-3a treatment led to rapid accumulation of p53 and a concomitant decrease in the levels of endogenous NIK, but not IKKα nor IKKβ, which are key players in NF-κB signaling (Fig. 2B, Supplementary Fig. S2A). IKKα, a downstream kinase of NIK, is required for the non-canonical NF-κB pathway while IKKβ is critical for the canonical NF-κB pathway (Zhang et al., 2017). Notably, IKKα phosphorylation and p100 processing, downstream events of NIK stabilization, were slightly decreased upon prolonged nutlin-3a treatment (Fig. 2B).

Next, we investigated whether overexpression of p53 affects the transcriptional activity of NF-κB with the aid of NF-κB reporter assays. Expression of NIK stimulated NF-κB transcriptional activity about 20-fold and expression of wild-type p53 (p53-WT) inhibited NIK-induced NF-κB luciferase activity (Fig. 2C). Interestingly, the p53-transactivation domain (TAD) mutant (p53-L22Q/W23S; p53-mTAD) could not inhibit NIK induced NF-κB activity (Fig. 2C). To further examine whether the transcriptional activity of p53 is required for NIK stabilization, plasmids expressing the p53-TAD mutant (p53-L22Q/W23S; p53-mTAD) and p53-nuclear localization signal (NLS) mutant (p53-KRKKK/AAAAA; p53-mNLS) were generated (Supplementary Fig. S2B) (Lin et al., 1994; O'Keefe et al., 2003). p53-mTAD was found to be severely compromised in terms of transactivation and p53-mNLS led to accumulation of p53 in the cytoplasm, resulting in defective transactivation (Supplementary Fig. S2C). In contrast to p53-WT, overexpression of p53-mTAD or p53-mNLS had no effect on the NIK level (Fig. 2D). Moreover, overexpression of p53 C-terminal deletion mutant (p53-ΔC-term) without the oligomerization domain essential for transcriptional activation did not influence the NIK level (Supplementary Fig. S2D), indicating that p53 transcriptional activity is required for downregulation of

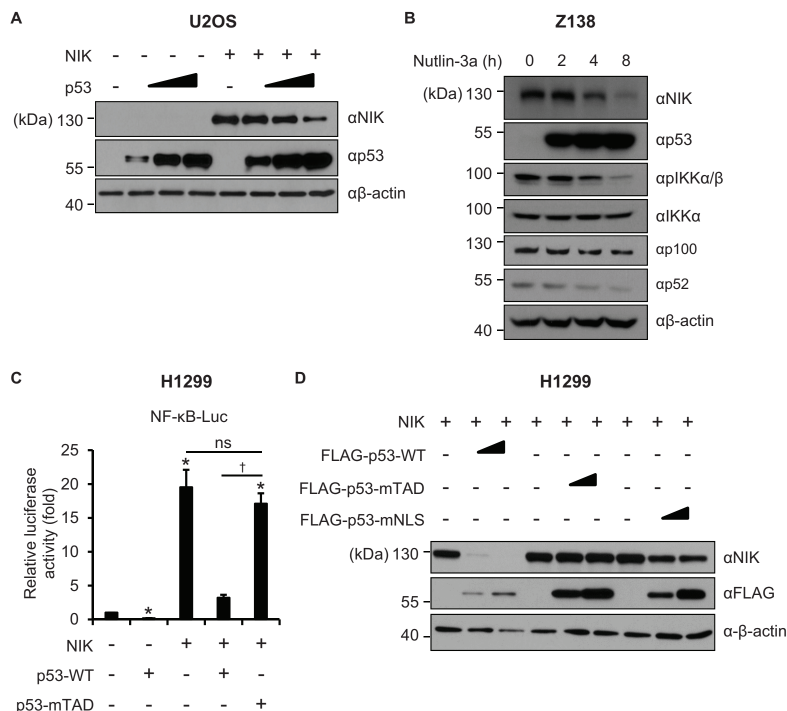

Fig. 2. p53 suppresses protein levels of NIK and non-canonical NF-κB activity. (A) U2OS cells were transiently transfected with control vector, NIK expression plasmid or various amounts of p53 expression plasmid for 24 h. (B) Z138 cells treated with 10 μM nutlin-3a for indicated time-periods were lysed and subjected to immunoblot analysis. (C) H1299 cells were cotransfected with control vector, a NF-κB-luciferase reporter gene plasmid and expression plasmids for NIK, p53-WT or p53-mTAD as indicated. At 24 h after transfection, H1299 cells were lysed and monitored via the dual-luciferase assay. *Firefly* luciferase activities were normalized to that of Renilla luciferase. Values are presented as an average of duplicates for a representative experiment. Data represent mean ± SEM. **P* < 0.05 versus the corresponding control; †*P* < 0.05 for the indicated comparisons; ns, not significant with Student's *t*-test. (D) H1299 cells were transfected with control vector and expression plasmids for NIK, FLAG-p53-WT, FLAG-p53-mTAD or FLAG-p53-mNLS. Various amounts of the p53 constructs were transfected into H1299 cells. After 48 h, H1299 cell lysates were analyzed via immunoblotting with antibodies against FLAG for detection of p53-WT or p53 mutants.

NIK.

p53 suppresses expression of NIK through the miRNA pathway but not UPS or lysosomal degradation

As NIK levels are known to be primarily controlled by the ubiquitin-proteasome system (UPS) in physiological conditions, we initially examined the involvement of UPS in NIK downregulation by p53. However, treatment with bortezomib (BZM), a proteasome inhibitor, did not lead to recovery of NIK (Fig. 3A). We further determined whether p53 suppresses NIK through the lysosomal degradation pathway, the major system other than UPS responsible for protein

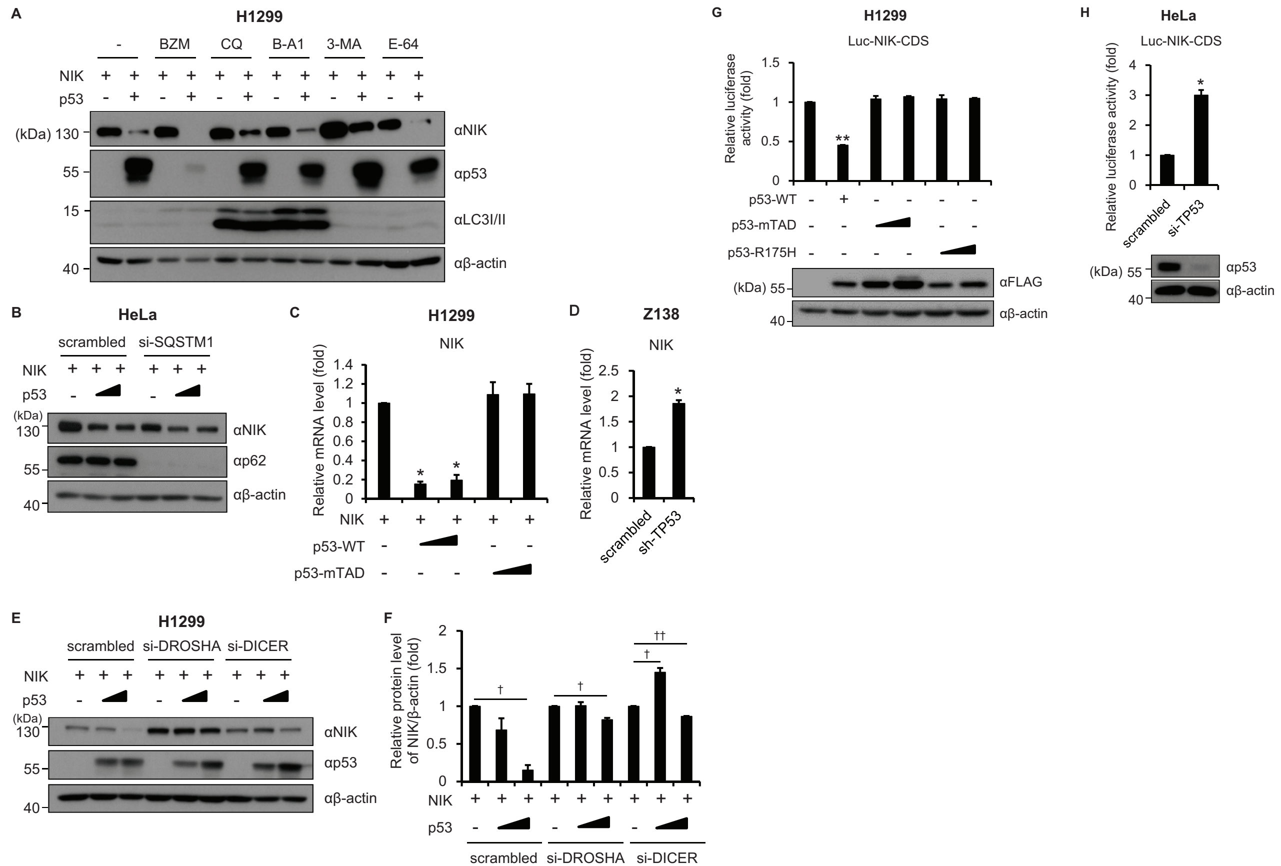

Fig. 3. p53 suppresses the expression of NIK through miRNA-mediated gene silencing, but not UPS or lysosomal degradation. (A) H1299 cells were transfected with control vector and expression plasmids for NIK or p53. After 6 h, cells were incubated in the absence or presence of 2 μM bortezomib (BZM), 100 μM chloroquine (CQ), 0.1 μM bafilomycin A1 (B-A1), 5 mM 3-methyladenine (3-MA) or 10 μM E-64. After 12 h, H1299 cells were lysed and subjected to immunoblot analysis. (B) HeLa cells were electroporated with control scrambled siRNA or 50 nM si-SQSTM1 for 24 h, followed by transfection with control vector or expression plasmids for NIK or various amounts of p53 for 24 h. (C) H1299 cells were transfected with control vector and expression plasmids for NIK or various amounts of p53 for 48 h. Total RNA isolated from H1299 cells was subjected to RT-qPCR analysis for NIK mRNA. Levels of NIK mRNA were normalized to β-actin in the same sample. (D) Total RNA was isolated from Z138 cells stably expressing control scrambled shRNA or shRNA specific for p53 and subjected to RT-qPCR analysis of mRNA for NIK. (E) H1299 cells were electroporated with control scrambled siRNA or siRNAs targeting DROSHA or DICER for 36 h, followed by transfection with control vector or expression plasmids for NIK or various amounts of p53 for 24 h. (F) Densitometric analysis of the immunoblot shown in Figure 3E. Intensity of NIK bands normalized to that of β-actin. Values are presented as an average of two independent experiments. (G) Upper panels: Full-length CDS of NIK was attached to the 3' end of *Firefly* luciferase of pCMV-pGL3-Luciferase plasmid (Luc-NIK-CDS) and cotransfected into H1299 cells with control vector and expression plasmids for FLAG-p53-WT, FLAG-p53-mTAD or FLAG-p53-R175H. After 48 h transfection, H1299 cells were lysed, followed by the dual luciferase assay. Activities of the Luc-NIK-CDS reporter were normalized to that of cotransfected CMV-Renilla construct. Lower panels: H1299 cell lysates used for luciferase assays were analyzed via immunoblotting with antibodies against FLAG for detection of p53-WT or p53 mutants. (H) Upper panels: HeLa cells were electroporated with control scrambled siRNA or 50 nM si-TP53 for 24 h, followed by transfection with the Luc-NIK-CDS reporter construct, and subjected to the dual luciferase assay. Activity of the Luc-NIK-CDS reporter was normalized to that of the cotransfected CMV-Renilla construct after 24 h transfection. Lower panels: HeLa cells were analyzed via immunoblotting with antibodies against p53. Quantitative data in Figures 3C, 3D, 3F, 3G, and 3H represent mean ± SEM. $*P < 0.05$; $**P < 0.005$ versus the corresponding control; $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.005$ for the indicated comparisons with Student's *t*-test.

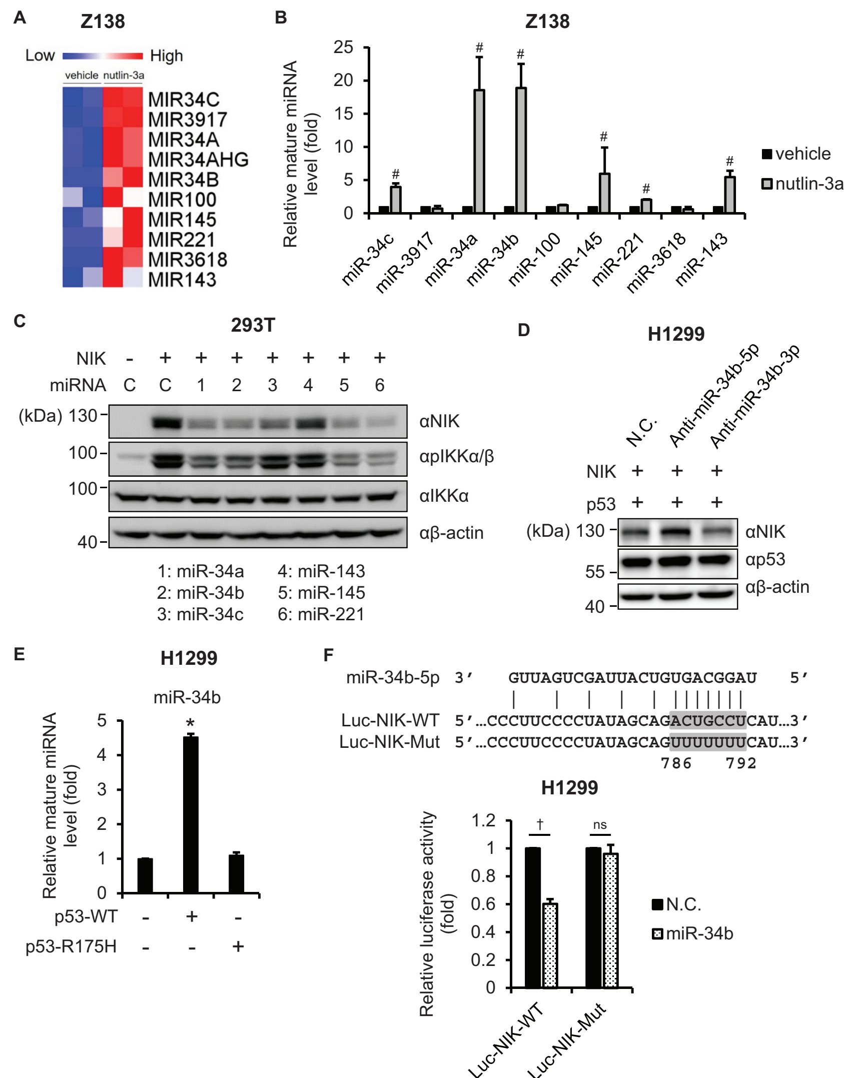

Fig. 4. p53-induced miR-34b negatively regulates the non-canonical NF-κB pathway by targeting the CDS of NIK. (A) Heat map for upregulated miRNAs in Z138 treated with 10 μM nutlin-3a for 6 h identified via small RNA sequencing. Total RNA isolated from Z138 cells using the mirVana miRNA isolation kit was used for preparation of small RNA libraries, followed by small RNA sequencing. Genes showing fold change ≥ 1.5 and $P < 0.05$ were selected for depiction in a heat map, scaled from low (blue) to high (red). (B) Z138 cells were treated with DMSO (vehicle) or 10 μM nutlin-3a. After 6 h, small RNA isolated from Z138 cells using the mirVana miRNA isolation kit was subjected to RT-qPCR analysis of mature miRNA. Levels of mature miRNA were normalized to those of U6 RNA in the same sample. Data are presented as mean ± SEM. #, genes with fold change ≥ 2 were selected. (C) 293T cells were cotransfected with control vector or expression plasmid for NIK and miRNA mimic negative control (C) or 100 nM miRNA mimics. After 48 h, cell lysates were analyzed via immunoblotting. (D) H1299 cells were cotransfected with expression plasmids for NIK or p53 and miRNA inhibitor negative control (N.C.), 100 nM anti-miR-34b-5p or 100 nM anti-miR-34b-3p. After 48 h, cell lysates were analyzed via immunoblotting. (E) H1299 cells were transfected with control vector, expression plasmids for p53-WT or p53-R175H, followed by miRNA isolation and RT-qPCR analysis. Levels of miR-34b-5p were normalized to U6 RNA in the same sample. (F) Upper panels; Location of the seed binding site of miR-34b-5p was predicted at 786-792 nt of CDS of NIK. The 123 bp partial NIK-CDS containing this putative binding site was inserted into 3' end of *Firefly* luciferase of pCMV-pGL3-Luciferase plasmid (Luc-NIK-WT). Mutations were generated at the seed binding site as indicated (Luc-NIK-Mut). Lower panels; H1299 cells were cotransfected with the Luc-NIK-WT or Luc-NIK-Mut reporter construct and miRNA mimic negative control (N.C.) or 100 nM miR-34b mimic and subjected to the dual luciferase assay. Activities of Luc-NIK-WT or Luc-NIK-Mut reporter were normalized to those of cotransfected CMV-Renilla construct after 48 h transfection. (G) HeLa cells were transfected with miRNA inhibitor negative control (N.C.) or 100 nM anti-miR-34b-5p. After 24 h transfection, HeLa cells were either left untreated or treated with 100 ng/ml LIGHT for the indicated time-periods. (H) Image depicting the involvement of miR-34b-induced silencing of NIK in crosstalk between the non-canonical NF-κB and p53 pathways. Quantitative data in Figures 4E and 4F represent mean ± SEM. $*P < 0.05$ versus the corresponding control; $^{\dagger}P < 0.05$ for the indicated comparisons; ns, not significant via Student's *t*-test.

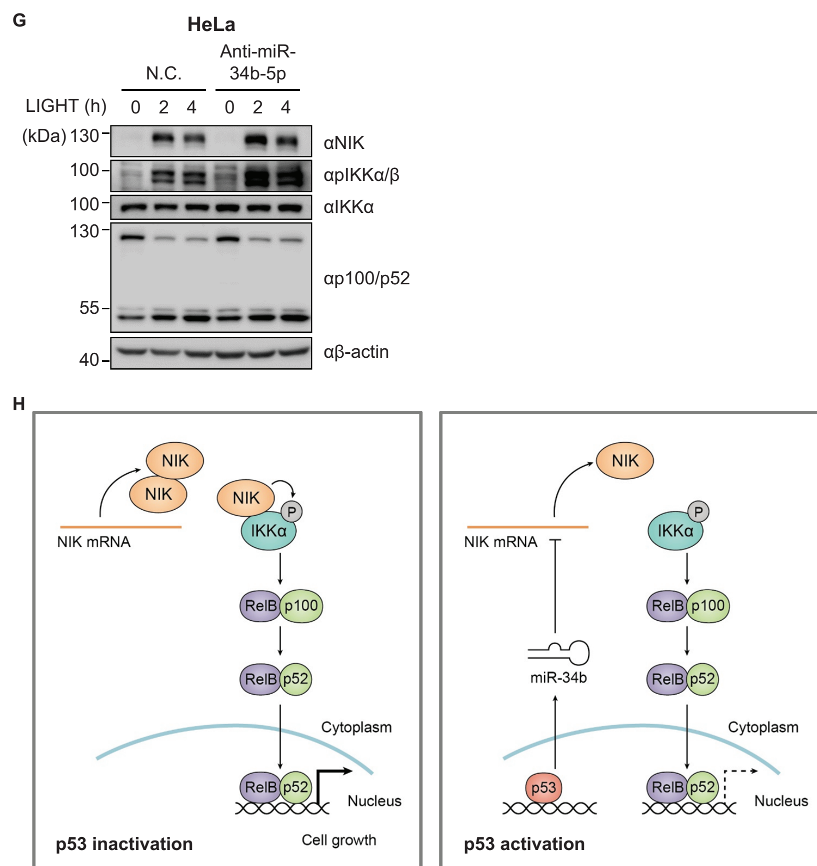

Fig. 4. Continued.

degradation. Treatment with autophagy inhibitors, such as the lysosomotropic agent chloroquine (CQ), bafilomycin A1 (B-A1), 3-methyladenine (3-MA), and the cysteine protease inhibitor, E-64, did not affect the p53-mediated decrease in NIK (Fig. 3A), which was additionally confirmed in HeLa and Z138 cells (Supplementary Figs. S3A-S3C). Furthermore, siRNA-mediated knockdown of p62/SQSTM1 did not induce recovery of NIK, confirming that the regulatory function of p53 on NIK is independent of autophagy (Fig. 3B). The data collectively indicate that the mechanism of p53-mediated decrease of NIK is not associated with UPS or autophagy, the major systems controlling NIK levels.

Next, we ascertained whether p53 regulates NIK mRNA. Unexpectedly, upon overexpression of p53-WT, but not p53-mTAD, NIK mRNA levels were decreased in H1299 cells (Fig. 3C). Conversely, knockdown of *TP53* induced upregulation of NIK mRNA (about two-fold) in Z138 (Fig. 3D, Supplementary Fig. S3D), clearly suggesting that p53 regulates NIK mRNA. This finding highlights the possibility that p53 regulates NIK mRNA through the miRNA pathway, since direct repression of transcription of exogenously expressed NIK by p53 is not feasible and p53 is reported to transcriptionally upregulate miRNAs and promote processing of specific pri-miRNAs to pre-miRNAs to increase mature miRNA levels (Hermeking, 2012). To examine this possibility, the NIK level was examined following knockdown of *DROSHA* and *DICER,* two key nucleases in miRNA processing machinery. Knockdown of either gene led to recovery of the p53-induced decrease in NIK (Figs. 3E and 3F, Supplementary Fig. S4A). In keeping with this finding, knockdown of AGO1/2, essential miRNA binding proteins in miRNA-mediated silencing, suppressed the p53-mediated decrease in NIK (Supplementary Figs. S4B and S4C). miRNAs are known to mainly bind 3' UTRs of mRNA and thereby inhibit translation or induce mRNA decay. However, several miRNAs have recently been reported to interact directly with the CDS of specific target mRNAs in addition to 3' UTR, inducing miRNA-mediated silencing (Tay et al., 2008). To establish whether p53-regulated miRNAs directly target the CDS of NIK, we generated *Firefly* luciferase reporter plasmid (Luc-NIK-CDS) with CDS of NIK attached to the 3' end of the *Firefly* luciferase gene. Expression of *Firefly* luciferase is driven by the CMV promoter and supposedly suppressed by cellular miRNAs (Jin et al., 2013). Overexpression of p53-WT but not p53-mTAD and p53-R175H mutants suppressed the relative luciferase activities of Luc-NIK-CDS (Fig. 3G). We employed p53-R175H as the control, since this is the most common p53 mutant allele that not only lacks DNA binding activity but also interferes with functional assembly between the DROSHA complex and p68, leading to attenuation of pri-miRNA processing (Suzuki et al., 2009). Consistent with data obtained with the p53-mTAD mutant, overexpression of the p53-R175H mutant did not suppress NIK levels

(Supplementary Fig. S4D). Furthermore, knockdown of p53 increased the relative luciferase activities of Luc-NIK-CDS in HeLa cells (Fig. 3H). The collective results demonstrate that p53-regulated miRNAs suppress NIK expression by targeting the protein-coding region of the transcript.

p53-induced miR-34b targets NIK leading to suppression of non-canonical NF-κB signaling

To identify the specific miRNAs involved in NIK silencing, high-throughput small RNA sequencing analysis was performed. Specifically, small RNAs isolated from Z138 cells treated with DMSO or nutlin-3a for 6 h were analyzed via small RNA sequencing. Based on criteria of $P < 0.05$ and ≥ 1.5-fold change, 10 upregulated miRNAs and 441 downregulated miRNAs were identified (Fig. 4A, Supplementary Table S2). In view of previous results, we assumed that miRNAs upregulated by nutlin-3a could target NIK mRNA. Accordingly, the above upregulated miRNAs were validated via RT-qPCR, which revealed > 2-fold increase in six of the miRNAs (miR-34c, miR-34a, miR-34b, miR-145, miR-221, and miR-143) by nutlin-3a (Fig. 4B). Next, we investigated whether these miRNAs suppress NIK expression and IKKα phosphorylation. Notably, transfection of four miRNA mimics (miR-34a, miR-34b, miR-145, and miR-221) led to reduced levels of NIK as well as IKKα phosphorylation (Fig. 4C). Specific inhibitors for these miRNAs were examined for suppression of NIK levels. Interestingly, the inhibitor of miR-34b-5p blocked the p53-mediated decrease of NIK in H1299 cells to a significant extent (Fig. 4D, Supplementary Fig. S5). Furthermore, overexpression of p53-WT, but not the p53 mutant R175H promoted miR-34b-5p levels in H1299 cells (Fig. 4E).

To establish whether miR-34b-5p directly targets CDS of NIK, we focused on identifying the putative binding site of miR-34b-5p in the CDS region. The seed sequences of miR-34b-5p (AGGCAGU) corresponded to “ACUGCCU” (+786~+792) in NIK-CDS. Accordingly, we generated *Firefly* luciferase reporter plasmids in which 123 bp partial NIK-CDS containing either “ACTGCCT” (Luc-NIK-WT) or “TTTTTTT” (Luc-NIK-Mut) is attached to the 3’ end of *Firefly* luciferase gene. Transfection of the miR-34b mimic decreased the relative luciferase activities of Luc-NIK-WT, but not Luc-NIK-Mut (Fig. 4F). Furthermore, treatment with an anti-miR-34b-5p inhibitor augmented LIGHT-induced NIK stabilization and subsequent non-canonical NF-κB signaling in HeLa cells (Fig. 4G). Based on the collective results, we suggest that p53-induced miR-34b-5p is a key factor in silencing of NIK and downregulation of non-canonical NF-κB signaling.

DISCUSSION

Constitutive activation of non-canonical NF-κB promotes cell proliferation in multiple cancer types (Balaji et al., 2018; Yamaguchi et al., 2009). Genetic alterations of TRAF2, TRAF3, BIRC2 and BIRC3, genomic gain of MAP3K14 and epigenetic alterations of MAP3K14 are involved in enhanced NIK expression in cancer cells (Keats et al., 2007; Otto et al., 2012; Rahal et al., 2014; Yamamoto et al., 2010). In the current study, we identified the tumor suppressor p53 as a negative regulator of non-canonical NF-κB signaling and showed that p53-induced miR-34b directly targets the CDS of NIK, thereby silencing NIK expression (Fig. 4H).

Previous reports have revealed that two miRNAs (miR-31 and miR-520e) target the 3’ UTR of NIK to suppress cell growth in adult T cell leukemia and hepatocellular carcinoma, respectively (Yamagishi et al., 2012; Zhang et al., 2012). However, data from the current study indicate that p53-induced miR-34b targets the CDS of NIK and suppresses non-canonical NF-κB signaling. The miR-34 family functions as tumor suppressors via induction of apoptosis, cell cycle arrest or senescence (Hermeking, 2010; Slabakova et al., 2017). Three miR-34 family members, miR-34a, miR-34b and miR-34c, which are direct targets of p53, share the same seed sequences and a common set of target genes but display different sequences, leading to differences in target affinity (Hermeking, 2010; Kim et al., 2019). miR-34b is reported to directly target two oncogenes, c-MYC and cyclic AMP-responsive element binding protein (CREB), and its downregulation promotes tumorigenesis in *MYC* translocation-negative Burkitt lymphoma and acute myeloid leukemia, respectively (Leucci et al., 2008; Pigazzi et al., 2009). Regarding miRNA-based therapeutic interventions, considerable efforts have been made to identify tumor suppressor miRNA signatures associated with diagnosis and therapy (Van Roosbroeck and Calin, 2017) and MRX34, a liposomal miR-34a mimic, has been developed for miRNA replacement therapy of cancer (Beg et al., 2017).

Tumor suppressor RNAi screening led to the identification of E-cadherin (CDH1) as a negative regulator of NIK abundance (Fig. 1A). E-cadherin, a calcium-dependent cell adhesion protein, regulates the mobility and proliferation of epithelial cells. Loss of E-cadherin promotes metastasis through induction of epithelial-to-mesenchymal transition (EMT) (Onder et al., 2008). Non-canonical NF-κB signaling is reported to promote the function of tumor-initiating cells by stimulating EMT in addition to the strong contribution of NIK to the stem cell-like phenotype in breast cancer (Kendellen et al., 2014; Vazquez-Santillan et al., 2016). It would be interesting to determine whether E-cadherin actually affects the abundance of NIK and non-canonical NF-κB signaling to promote metastasis.

As our findings were obtained under situations whereby exogenous NIK was expressed or endogenous NIK accumulated via LIGHT treatment, it is plausible that the catalytic activity of NIK is required for p53-mediated regulation of NIK abundance. To address this issue, an NIK catalytic mutant in which threonine 559 was substituted with alanine (NIK-T559A) was generated (Lin et al., 1998). In view of the observed decrease in NIK-T559A under conditions of p53 overexpression, we propose that p53-mediated NIK downregulation is independent of catalytic activity (Supplementary Figs. S2E and S2F).

Another lysosomal protein degradation, chaperone-mediated autophagy (CMA), involves selective degradation of specific cytosolic proteins distinct from macro-autophagy (Dice, 2007). To determine whether CMA is involved in the p53-induced NIK decrease, NIK levels were examined following depletion of *LAMP-2A*, a critical component of CMA. The results suggest that CMA is not involved in p53-induced NIK

reduction (Supplementary Fig. S3E). Moreover, p53 induces several genes that function in regulation of the endosomal sorting complexes required for transport (ESCRT) machinery in the endosomal compartment and enhances internalization of receptors for targeting for degradation (Yu et al., 2009). Based on this finding, we hypothesized that the endocytic pathway may be involved in p53-induced decrease of NIK. However, knockdown of the ESCRT proteins charged multivesicular body protein 4c (*CHMP4C*) or ubiquitin associated protein 1 (*UBAP1*) had no effect on p53-mediated NIK regulation (Supplementary Figs. S3F and S3G).

To our knowledge, this study is the first to provide evidence that oncogenic NIK is a target of tumor suppressive miR-34b. The observed crosstalk between non-canonical NF-κB and p53 signaling through NIK-miR-34b interactions expands our understanding of the mechanisms underlying cancer cell growth and tumorigenesis. The finding that miR-34b inhibits non-canonical NF-κB signaling in cancer cells supports its potential application in cancer therapy.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

Disclosure

The authors have no potential conflicts of interest to disclose.

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