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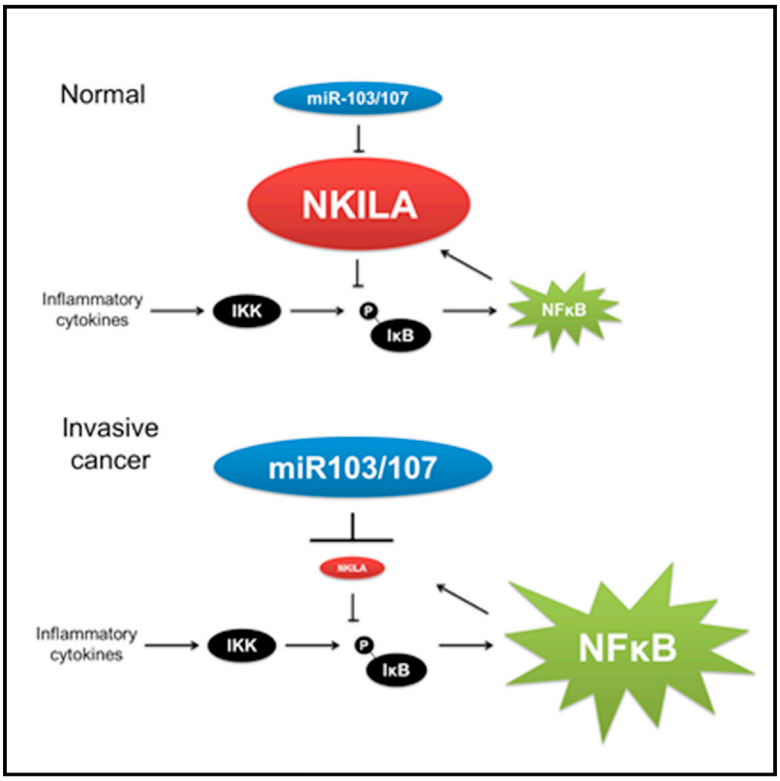
A Cytoplasmic NF-κB Interacting Long Noncoding RNA Blocks IκB Phosphorylation and Suppresses Breast Cancer Metastasis

Graphical Abstract



Highlights

- NF-κB interacting long noncoding RNA (NKILA) directly blocks IκB phosphorylation
- NKILA interacts with NF-κB/IκB to form a stable ternary complex
- NKILA is a negative feedback regulator of NF-κB in both resting and activated cells
- Decreased NKILA in invasive breast cancer is associated with poor patient outcome

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In Brief

Liu et al. identify an NF-KappaB Interacting LncRNA (NKILA) that binds to NF-κB/IκB complex and represses NF-κB signaling and cancer-associated inflammation. Low NKILA expression is associated with breast cancer metastasis and poor patient prognosis.

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A Cytoplasmic NF-κB Interacting Long Noncoding RNA Blocks IκB Phosphorylation and Suppresses Breast Cancer Metastasis

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SUMMARY

NF-κB is a critical link between inflammation and cancer, but whether long non-coding RNAs (lncRNAs) regulate its activation remains unknown. Here, we identify an NF-KappaB Interacting LncRNA (NKILA), which is upregulated by NF-κB, binds to NF-κB/IκB, and directly masks phosphorylation motifs of IκB, thereby inhibiting IKK-induced IκB phosphorylation and NF-κB activation. Unlike DNA that is dissociated from NF-κB by IκB, NKILA interacts with NF-κB/IκB to form a stable complex. Importantly, NKILA is essential to prevent over-activation of NF-κB pathway in inflammation-stimulated breast epithelial cells. Furthermore, low NKILA expression is associated with breast cancer metastasis and poor patient prognosis. Therefore, lncRNAs can directly interact with functional domains of signaling proteins, serving as a class of NF-κB modulators to suppress cancer metastasis.

INTRODUCTION

Nuclear factor-κB (NF-κB) is a family of transcription factors that play critical roles in inflammation, immunity, cell proliferation, differentiation, and survival (Ruland, 2011). Aberrant NF-κB activation, a consequence of underlying inflammation in tumor microenvironment that can promote cancer invasion and metastasis, has been observed in many tumors, including breast cancer (Chaturvedi et al., 2011; Fornier et al., 2007). Hence, NF-κB is considered as a critical link between inflammation and cancer. A number of negative regulators, including regulatory proteins and miRNAs, inhibit overactivation of NF-κB signaling and function as tumor suppressors (Chaturvedi et al., 2011; Ruland, 2011). Among them, IκB (inhibitor of NF-κB) that binds to and sequesters NF-κB in the cytoplasm is considered as a major brake on NF-κB signaling. IκB kinases (IKK), which can be activated by various stimuli, can phosphorylate IκB, leading to its degradation and subsequent NF-κB activation. However, previous studies have shown that elevated IKK activity induced by inflammatory stimuli persists much longer than NF-κB activation (Ojha et al., 2008) and basal IKK activity is also sufficient to phosphorylate NF-κB-bound IκB (O'Dea et al., 2008), suggesting that some unknown mechanisms may protect NF-κB-bound IκB from being phosphorylated by the persistently elevated or basal IKK activity.

Long non-coding RNAs (lncRNAs) are a large class of non-protein-coding transcripts that are greater than 200 bases in length and are involved in numerous physiological and pathological processes (Wang and Chang, 2011). Presently, only a small

Significance

Emerging evidences indicate that long non-coding RNAs (lncRNAs) regulate various hallmarks of cancer. However, whether lncRNAs play a role in cancer-associated inflammation is not known yet. Here we report that NKILA is an essential lncRNA that regulates NF-κB signaling and represses cancer-associated inflammation. NKILA binds to NF-κB/IκB complex and inhibits NF-κB signaling by masking the phosphorylation sites of IκB and stabilizing the complex. Nevertheless, NKILA expression is significantly decreased in many breast cancers, which is associated with cancer metastasis and poor patient prognosis. Our study has discovered a class of lncRNAs that directly regulate signaling pathways.

number of lncRNAs have been characterized functionally, and most of them are shown to control gene expression by regulating various aspects of gene expression (Ankö and Neugebauer, 2010; Carrieri et al., 2012; Gong and Maquat, 2011; Hung et al., 2011; Lee, 2012; Wang et al., 2011; Yoon et al., 2012). Many lncRNAs are shown to regulate important cancer hallmarks, including proliferation (Yang et al., 2013b), apoptosis (Huarte et al., 2010), metastasis (Gupta et al., 2010; Yang et al., 2013a; Yuan et al., 2014), metabolism (Yang et al., 2014), senescence (Yap et al., 2010), and drug-resistance (Fan et al., 2014). Nevertheless, whether lncRNA plays a role in cancer-related inflammation is not clear yet.

Although the precise mechanisms by which lncRNAs function remain poorly understood, a widely proposed model is that lncRNAs may play their role via discrete modules that decoy, guide, or scaffold other regulatory proteins (Guttman and Rinn, 2012; Wang and Chang, 2011). The "guiding" lncRNAs, like KCNQ1OT1 (Pandey et al., 2008) and lincRNA-p21 (Huarte et al., 2010), are associated with transcriptional co-regulators or chromatin regulatory protein complexes and recruit them to specific genomic DNA regions to regulate transcription. Additionally, "decoying" lncRNAs, such as GAS5 (Kino et al., 2010), Lethe (Rapicavoli et al., 2013), and PANDA (Hung et al., 2011), mimic and compete with their consensus DNA binding motifs for binding nuclear receptors or transcription factors in the nuclei, or act as decoy microRNA binding sites like ceRNAs to control the functions of regulatory miRNAs (Salmena et al., 2011). Furthermore, lncRNAs can serve as molecular scaffolds, bringing specific regulatory proteins into proximity with each other to function as a unique complex, such as HOTAIR (Tsai et al., 2010), XIST (Jeon and Lee, 2011), and NRON (Sharma et al., 2011). Collectively, these studies indicate that the function of most lncRNAs depends on their ability to interact with proteins, implying that lncRNAs may also directly interact with functional domains of signaling proteins and thus regulate signal transduction. Indeed, it has recently been reported that a lncRNA regulates STAT6 signal transduction by interacting with STAT6 in the cytoplasm of dendritic cells and modulating its phosphorylation (Wang et al., 2014). However, it remains unclear whether lncRNAs can regulate NF-κB activation by interacting with and post-translationally modifying NF-κB signaling proteins. Because it is widely accepted in the field of NF-κB that IκB can rapidly dissociate NF-κB from its cognate DNA and a stable NF-κB:IκBα:DNA complex is unlikely to form (Zabel and Baeuerle, 1990), whether lncRNA can interact with NF-κB in the presence of IκB needs mechanistic investigation. In this study, we aimed to investigate the contributions of lncRNAs in NF-κB signaling pathway of breast cancer cells and their roles in breast cancer metastasis.

RESULTS

NKILA Is an lncRNA Upregulated by Inflammatory Cytokines via NF-κB Signaling

Aberrant NF-κB activation underlies the development of many cancers (Chaturvedi et al., 2011). Inflammatory cytokines abundant in breast cancer, including tumor necrosis factor (TNF)-α and IL-1β, have been shown to activate NF-κB signaling in tumor cells (Chaturvedi et al., 2011; Goldberg and Schwertfeger, 2010). To investigate the role of lncRNA in this process, we used microarray to establish lncRNA expression profiles in MDA-MB-231 breast cancer cells treated by these cytokines. A classical NF-κB activator, lipopolysaccharide (LPS), was also used. We found that 23 lncRNAs were upregulated and 28 lncRNAs downregulated by all the above stimuli by more than 10-fold (Table S1). Among them, NKILA, a lncRNA encoded by a gene at chromosome 20q13, was consistently found upregulated by more than 12-fold. Northern blotting and quantitative RT-PCR (qRT-PCR) confirmed the significantly increased NKILA expression in both MDA-MB-231 and MCF7 breast cancer lines (Figure 1A and Figure S1A). We also estimated the number of NKILA RNA molecules per MCF7 cell by Northern blotting (Figure S1B) and absolute quantitative PCR (Figure S1C and Table S2). The expression level of NKILA in MCF7 cells was similar to Gas5 in HeLa cells (Kino et al., 2010). The result also showed TNF-α treatment upregulated NKILA copy number by 5.8-fold (Figures S1B and S1C and Table S2). Using 5′- and 3′- rapid amplification of cDNA ends, NKILA was found to be a 2,570 nucleotide (nt) intronless transcript that is identical to AK056098 in GeneBank or uc002xyu in the UCSC database. Apart from a few short potential open reading frames (ORF) that have limited chance (<1%) of encoding short ORF encoded polypeptides (Slavoff et al., 2013), NKILA does not have a typical protein-coding ORF that is longer than 300 nt. Confocal microscopy for fluorescent in situ hybridization (FISH) showed that NKILA located primarily in the cytoplasm (Figure 1B), which was confirmed by nuclear/cytoplasm fractionation (data not shown), suggesting that NKILA may exert its biological function in the cytoplasm.

We then investigated whether NF-κB signaling is responsible for the induced NKILA transcription. Sc-3060 and JSH-23, two inhibitors for NF-κB nuclear translocation, abrogated the stimuli-induced NKILA upregulation in MDA-MB-231 cells (Figure 1C). Further, a constitutively active IKKβ (CA-IKK2) (Huber et al., 2004) in MDA-MB-231 cells induced NF-κB activation and upregulated NKILA expression, which could be completely inhibited by Sc-3060 or JSH-23 (Figure 1D). Using a series of pGL3 reporter plasmids containing deleted (Figure S1D) or mutated (Figure 1E) 5′-flanking region of NKILA, we identified a κB binding site located at −104 to −95 bp upstream of NKILA TSS (Figure S1D). This was further confirmed by chromatin immunoprecipitation (ChIP) analysis with anti-p65 antibody (Figure 1F) and electrophoretic mobility shift assay (EMSA) using a biotin-labeled DNA probe containing the κB site of NKILA promoter (Figure S1E).

NKILA Is a Negative Regulator of NF-κB Signaling

Because elevated NF-κB activity is associated with enhanced invasiveness of tumor cells (Huber et al., 2004), we examined the correlation between NKILA expression and basal NF-κB activities in nine breast cancer cell lines with different metastatic potentials. Surprisingly, NKILA expression was much higher in the five low-metastatic breast cancer lines, including MCF7, ZR-75-1, T47D, MDA-MB-453, and BT-474 with low NF-κB activities, than in three high-metastatic lines, including BT-549, MDA-MB-436, and MDA-MB-231 with high NF-κB activities, and SK-BR-3 line with moderate NF-κB activity (Figure 2A and Figure S2A). These data indicate a negative correlation between NKILA expression and NF-κB activity. Exogenous NKILA

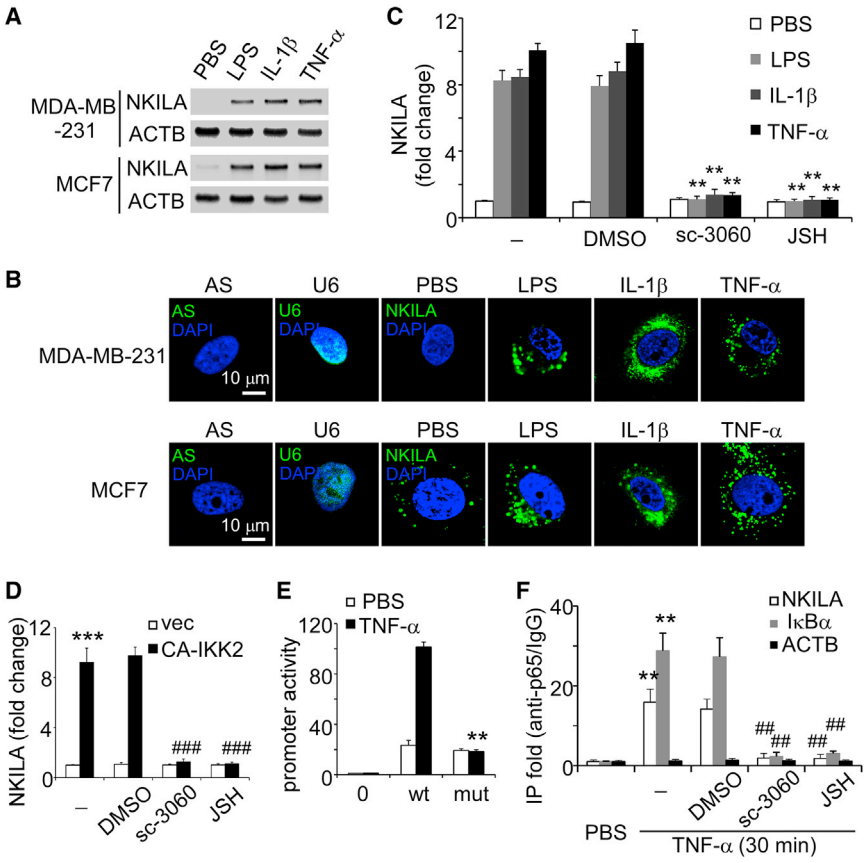

Figure 1. Inflammatory Cytokines Upregulate NKILA via NF-κB Signaling

(A) Expression of NKILA in MDA-MB-231 and MCF7 cells treated with inflammatory mediators for 24 hr, assayed by Northern blotting.

(B) Confocal FISH images showing cytoplasmic localization of NKILA in MDA-MB-231 and MCF7 cells. AS, probe for antisense NKILA; U6, probe for U6 snRNA.

(C) Expression of NKILA assayed by qRT-PCR in MDA-MB-231 cells induced by inflammatory stimuli (mean ± SD, n = 3, **p < 0.01, versus DMSO), with or without NF-κB inhibition by sc-3060 or JSH-23 (JSH).

(D) Expression of NKILA assayed by qRT-PCR in MDA-MB-231 cells with or without NF-κB inhibition by sc-3060 or JSH-23 (JSH) (mean ± SD, n = 3, ***p < 0.001, versus vec, ###p < 0.001, versus DMSO).

(E) Luciferase reporter assays for HEK293 cells transfected with reporter plasmids containing NKILA promoter and treated with PBS or cytokines for 24 hr (mean ± SD, n = 8, **p < 0.01, versus wt; wt: wild-type −2,000∼+1 construct, mut: −2,000∼+1 construct with point mutation in κB binding site).

(F) Localization of p65 to NKILA promoter, IκBα promoter (positive control), or ACTB promoter (negative control) in MDA-MB-231 cells treated with TNF-α for 30 min with or without NF-κB inhibition, analyzed by ChIP (mean ± SD, n = 3, **p < 0.01 versus PBS, ##p < 0.01 versus DMSO). Treatment for 30 min was chosen due to optimal p65 nuclear translocation.

See also Figure S1 and Table S1 and Table S2.

(Figure S1F) in MDA-MB-231 cells reduced the basal activity by 60%, while silencing NKILA (Figure S1G) in MCF7 cells increased NF-κB activity by 3- to 4-fold (Figure 2B and Figure S2B). Furthermore, NKILA inhibited TNF-α-induced p65 nuclear translocation in MDA-MB-231 cells, while silencing NKILA significantly prolonged p65 translocation to 24 hr (Figure 2C). TNF-α-enhanced NF-κB transcriptional activity in MDA-MB-231 cells was suppressed by 80% in cells with exogenous NKILA, but was further increased by 3-fold upon NKILA silencing (Figure 2D). Additionally, NKILA inhibited TNF-α-induced expression of several NF-κB target genes (Figure S2C). Collectively, these data suggest that NKILA acts as a negative regulator to suppress both the basal and cytokine-stimulated NF-κB activities in breast cancer cells.

To understand what leads to low NKILA expression and high NF-κB activity in metastatic breast cancer cells, we monitored the kinetics of NKILA expression after TNF-α treatment. NKILA expression in MDA-MB-231 cells peaked at 12 hr after TNF-α treatment, but dropped rapidly to baseline at 48 hr. In contrast, NKILA expression in MCF7 cells plateaued at 12–24 hr after TNF-α treatment and remained high for up to 72 hr (Figure 2E). The half-life of NKILA is much shorter in MDA-MB-231 (∼3.8 hr) than in MCF7 cells (∼8.8 hr) upon transcriptional inhibition by α-amanitin (Figure 2F), indicating that NKILA is decaying more rapidly in the former. It is known that miRNAs may degrade mRNAs with poly A tails via a miRNA-directed deadenylation pathway (Huntzinger and Izaurralde, 2011) and NKILA is such an adenylated RNA. Therefore, we explored whether NKILA is degraded by specific miRNAs in high-metastatic breast cancer cells. By screening a comprehensive library of 434 miRNAs predicted to target NKILA by RegRNA (Huang et al., 2006) (Table S3), we identified seven miRNAs that markedly inhibited NKILA expression in MCF7 cells with a *Z* score <−3 (Figure S2D). Among them, miR-103 and miR-107 displayed an opposite expression pattern versus NKILA, as their levels were much higher in high-metastatic than low-metastatic breast cancer cells (Figure S2E), which is consistent with a previous report (Martello et al., 2010). More importantly, antisense oligonucleotides for miR-103 or miR-107 increased NKILA expression in MDA-MB-231 cells by 3-fold, while mimics of miR-103 or miR-107 reduced NKILA expression in MCF7 cells by 80% (Figure 2G), which could be restored by silencing key mediators for miRNA-mediated RNA degradation, including Argonaute protein EIF2C2 and GW182 protein TRNC6A (Huntzinger and Izaurralde, 2011) (Figure S2F). Indeed, a miR-103/107 target site was found at 1,203–1,234 nt of NKILA (Figure S2G). Furthermore, luciferase reporter assay using pMIR-REPORT constructs showed that co-transfection of miR-103 or miR-107 significantly reduced luciferase activity of the reporter gene cloned with wild-type but not mutant miR-103/107 target sequence at its 3′-UTR (Figure S2H). Therefore, overexpression of miR-103 and miR-107 in high-metastatic breast cancer cells is responsible for rapid degradation of NKILA, which offsets the enhanced transcription of NKILA caused by high NF-κB activity.

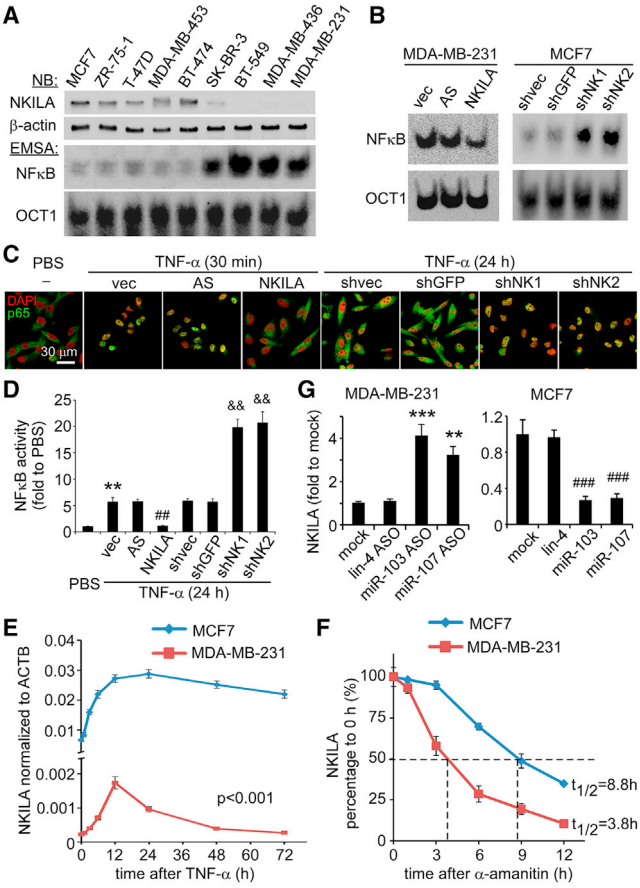

Figure 2. NKILA Is a Negative Regulator of NF-κB Signaling

(A) NKILA expressions and basal NF-κB activities in various breast cancer cell lines, shown by Northern blotting (NB) and EMSA.

(B) NF-κB activities of MDA-MB-231 cells stably expressing exogenous NKILA or MCF7 cells expressing NKILA shRNAs, examined by EMSA. (vec, pcDNA 3.1 vector; AS, antisense NKILA; shvec, pMKO.1-puro shRNA vector; shGFP, GFP-shRNA; shNK1, 2, NKILA-shRNA1, 2).

(C) p65 nuclear translocation in MDA-MB-231 expressing NKILA or NKILA shRNA treated with TNF-α, assayed by immunofluorescent confocal microscopy.

(D) NF-κB activity of TNF-α-treated MDA-MB-231 cells, examined by luciferase reporter assay (mean ± SD, n = 8, ***p < 0.001 versus PBS, ##p < 0.01 versus vec, &&p < 0.01 versus shvec).

(E and F) NKILA expression kinetics in MCF7 and MDA-MB-231 cells following TNF-α stimulation without (E) or with subsequent α-amanitin treatment (F).

(G) NKILA expression in MDA-MB-231 cells transfected with miR-103/107 antisense oligonucleotides (ASO) or in MCF7 cells transfected with miR-103/107 mimics, examined by qRT-PCR (mean ± SD, n = 3, **p < 0.01, ***p < 0.001, ###p < 0.001 versus mock).

See also Figure S2 and Table S3.

NKILA Inhibits IκB Phosphorylation by Interacting with the NF-κB:IκB Complex

To study the mechanisms by which NKILA suppresses NF-κB activation, we examined the effect of NKILA on the phosphorylation of IκBα and IκB kinase (IKK). IκBα phosphorylation was inhibited in MDA-MB-231 cells by ectopic NKILA expression, but was enhanced in MCF7 cells by silencing NKILA. However, basal IKK phosphorylation was not influenced by altering NKILA in both cell lines (Figure 3A). Furthermore, enforcing or silencing

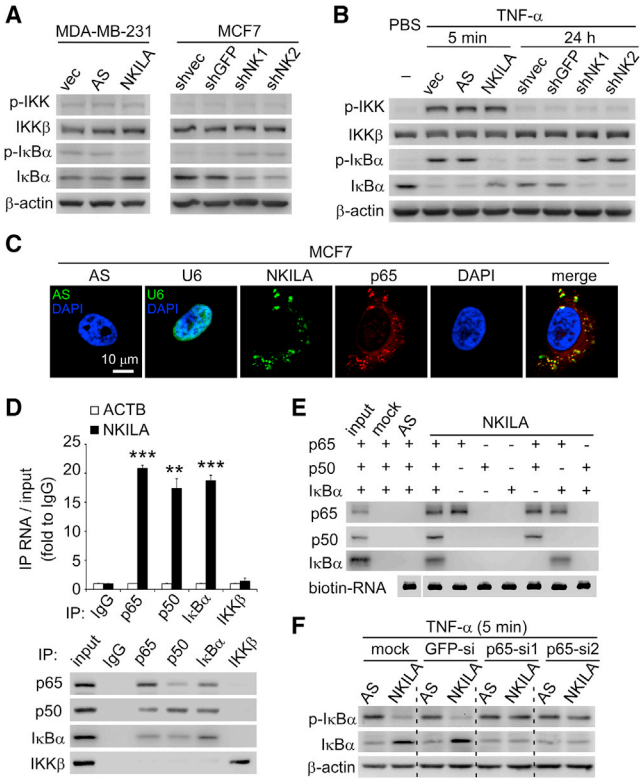

Figure 3. NKILA Inhibits IκB Phosphorylation by Interacting with the NF-κB:IκB Complex

(A) Western blotting showing total and phosphorylated IKK and IκBα in MDA-MB-231 or MCF7.

(B) Western blotting showing total and phosphorylated proteins of IKK and IκBα in TNF-α-treated MDA-MB-231 cells.

(C) Confocal FISH images showing colocalization of NKILA and p65 in MCF7.

(D) Binding of NKILA to p65/p50/IκBα complex in MCF7 cells, shown by RNA immunoprecipitation and qRT-PCR (mean ± SD, n = 3, **p < 0.01, ***p < 0.001 versus IgG).

(E) In vitro interaction between NKILA and p65, p50, or IκBα, shown by RNA pulldown.

(F) Western blot analysis for phosphorylated and total IκBα in NKILA-expressing MDA-MB-231 cells treated with TNF-α and transfected with siRNAs targeting p65 (GFP-si, GFP siRNA; p65-si1, 2, p65 siRNA1, 2).

See also Figure S3.

NKILA expression in MDA-MB-231 cells reduced or increased TNF-α-induced IκBα phosphorylation respectively, but did not influence IKK phosphorylation (Figure 3B), suggesting that NKILA mainly affects IκBα phosphorylation but not IKK activation. Inhibiting IKK kinase activity by Bay-117082 or Wedelolactone abolished the effects of NKILA-shRNA on IκBα phosphorylation and NF-κB activation (Figures S3A and S3B), indicating that IKK is upstream of NKILA in the pathway. Furthermore, silencing NKILA had no effect on NF-κB activation in cells with trans-dominant IκBα (TD-IκBα) (Huber et al., 2004) or with IκBα silenced (Figure S3C). These results suggest that NKILA suppresses NF-κB activation by inhibiting IKK-induced IκBα phosphorylation without influencing IKK activity.

The cytoplasmic localization of NKILA implies that the lncRNA may function by interacting with cytoplasmic NF-κB:IκBcomplex. Indeed, confocal microscopy for NKILA FISH and p65

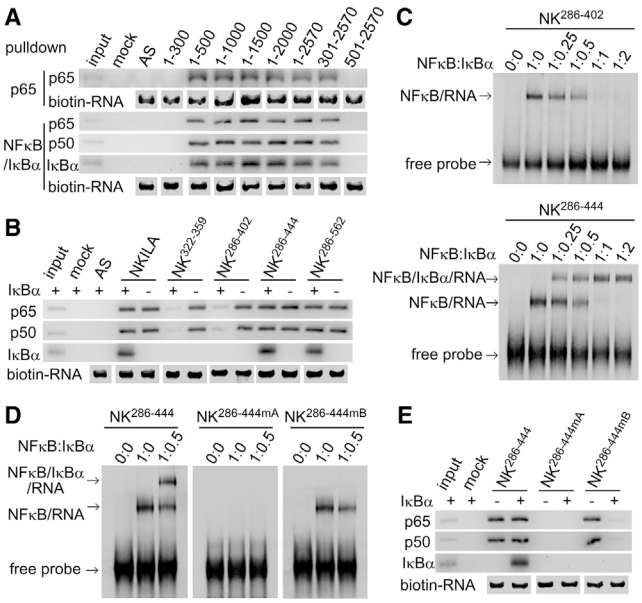

Figure 4. NKILA Stably Associates with the NF-κB:IκB Complex via Two Motifs

(A) In vitro interaction of sequentially deleted NKILA mutants with p65 alone or p65/p50/IκBα (NF-κB/IκBα) complex, assayed by RNA pulldown.

(B) Interaction of NKILA truncation mutants with p65/p50 alone or in the presence of IκBα, assayed by RNA pulldown (NK, full-length NKILA; $NK^{322–359}$, NKILA truncation mutant containing nt 322–359; $NK^{286–402}$, nt286–402; $NK^{286–444}$, nt286–444; $NK^{286–562}$, nt286–562).

(C) RNA EMSA determining interaction of $NK^{286–402}$ (left) or $NK^{286–444}$ (right) with NF-κB (p65/p50 heterodimer) and IκBα. (NF-κB:IκBα indicates the molar ratio between p65/p50 heterodimer and IκBα.)

(D) RNA pulldown showing effects of mutations of hairpin A ($NK^{286–444mA}$) or hairpin B ($NK^{286–444mB}$) in $NK^{286–444}$ on its binding capacity to p65/p50 alone or in presence of IκBα. (NF-κB:IκBα indicates molar ratio.)

(E) RNA pulldown showing effects of mutations of hairpin A ($NK^{286–444mA}$) or hairpin B ($NK^{286–444mB}$) in $NK^{286–444}$ on its binding capacity to p65/p50 alone or in presence of IκBα.

See also Figure S4 and Table S4.

immunostaining showed ~80% co-localization of NKILA and p65 in the cytoplasm of MCF7 cells (Figure 3C and Figure S3D). Immunoprecipitation (IP) of p65, p50, or IκBα, but not IKKβ, specifically retrieved NKILA (Figure 3D). Silencing p65 eliminated NKILA retrieval by p50 or IκBα, but silencing p50 or IκBα did not reduce NKILA enrichment in p65 immunoprecipitates (Figure S3E), suggesting that NKILA has strong affinity to p65, but not to p50 or IκBα. This was confirmed by retrieval of p65, p50, and IκBα with biotinylated NKILA from MCF7 only in the presence of p65 (Figure S3F). In vitro RNA/protein interaction assay also showed that NKILA binds to p65, but not p50 or IκBα, and NKILA can only retrieve p50 or IκBα from complexes containing p65 (Figure 3E). Furthermore, silencing p65 abolished the inhibitory effect of NKILA on TNF-α-induced IκBα phosphorylation (Figure 3F). These results suggest that NKILA binds p65 to associate with NF-κB:IκBα complex, which is essential for its function.

NKILA Binds to p65 at Two Different Sites to Stably Associate with the NF-κB:IκB Complex

Nevertheless, it has been shown that IκBα can rapidly dissociate the complex of NF-κB transcription factor from its cognate DNA (Zabel and Baeuerle, 1990). Thus, it is generally assumed that an NF-κB:IκBα:DNA complex is unlikely to be stable, although such a transient NF-κB:IκBα:DNA complex was demonstrated recently (Sue et al., 2011). Therefore, we investigated how NKILA binds to p65 in the presence of IκBα to form a stable NF-κB:IκBα:NKILA complex. A series of NKILA deletion mutants was used to determine the nucleotides in NKILA that binds to p65 in the presence or absence of IκBα. NKILA mutants retaining nt 300–500 bound to p65 alone or p65:p50:IκBα complex as efficiently as full-length NKILA, whereas other mutants completely lost their binding capacity (Figure 4A), suggesting that nt 300–500 of NKILA is responsible for the association with p65:p50:IκBα complex via p65. Two independent sets of software, Mfold (Zuker, 2003) and RNAfold (Hofacker, 2003), predicted two hairpin structures within nt 300–500, named hairpin A (nt 322–359) and hairpin B (nt 395–418), respectively (Figure S4A). Hairpin A, analogous to NF-κB binding DNA motif (NBM), is formed by two NF-κB binding consensus sequences (nt 322–332 and nt 350–359 of NKILA) that fold back to complement with each other (Table S4). Both hairpin structures were confirmed by RNA structure probing (Figure S4B).

EMSA using a 3′-biotin-labeled RNA probe containing hairpin A, (nt 322–359, $NK^{322–359}$) revealed an RNA:p65 complex that was super-shifted by anti-p65 antibody and competed by a non-labeled consensus κB-binding DNA probe (Figure S4C), indicating that hairpin A mimics NBM and binds to NF-κB. Additionally, the hairpin probe also formed complex with c-rel, but not with p50 (Figure S4C). Therefore, hairpin A at nucleotides 322 to 359 of NKILA mimics κB binding motif and binds to NF-κB. To determine if hairpin A binds to NF-κB in vivo, we generated a hairpin A ($NK^{322–359}$) tagged with a fragmented GFP mRNA (162–278 nt of NC_011521.1) and transfected it into MDA-MB-231 cells. RNA-IP using anti-p65 antibody showed that NF-κB p65 binds to the tagged hairpin A, but not the tagged hairpin A mutated at nt 331 and its complementary nt 351 (Table S4 and Figure S4D).

To further evaluate how hairpins A and B contribute to p65 binding in the presence of IκBα, we used another series of deletion mutants predicted to preserve secondary structure of hairpins. Mutants retaining only hairpin A, nt 322–359 ($NK^{322–359}$) and nt 286–402 ($NK^{286–402}$), bound to the p65:p50 heterodimer, as well as the p65 homodimer in the absence of IκBα, but not in the presence of IκBα. Nevertheless, mutants retaining both hairpins, nt 286–444 ($NK^{286–444}$) and nt 286–562 ($NK^{286–562}$), bound to both the p65:p50 and the p65:p50:IκBα complexes as efficiently as full-length NKILA (Figure 4B). This was confirmed by EMSAs using $NK^{286–402}$ harboring hairpin A or $NK^{286–444}$ harboring both hairpins (Figure 4C). Consistent with previous findings of NF-κB-binding DNA motif (Zabel and Baeuerle, 1990), IκBα can dissociate the RNA:NF-κB complex with hairpin A alone. Nevertheless, RNA:NF-κB complex with both hairpin A and B was not dissociated by IκBα, but instead formed a complex with IκBα, suggesting that hairpin B of NKILA is critical in forming a stable RNA:NF-κB:IκBα complex.

Next, we compared the affinity of $NK^{286–444}$ containing both hairpins A and B with a consensus κB-binding DNA fragment to bind p65 in EMSA competition assays. Addition of a RNA probe containing $NK^{286–444}$ at an RNA:DNA ratio of 1:1 efficiently decreased the DNA:NF-κB complex, while a similar level of competition could only be achieved by DNA probe at 5:1 ratio

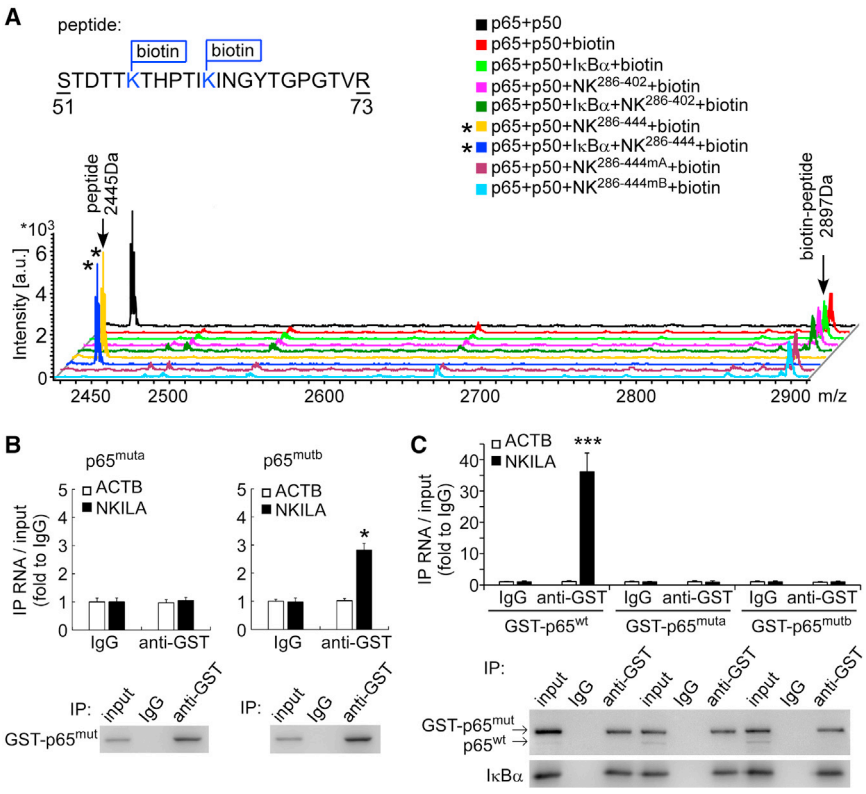

Figure 5. NKILA Binds to p65 at Two Different Sites

(A) The effects of wild-type or mutant NK$^{286–444}$ on the accessibility of amino acids in NF-κB p65, determined by biotin-labeling assay (mass spectra of NF-κB p65 with indicated treatments; numbers indicate positions of amino acids; *biotin labeling inhibited by RNA).

(B and C) MCF-7 cells were co-transfected with siRNA targeting 3′-UTR of endogenous p65 and pcDNA3.1 vectors expressing wild-type or p65 mutants disrupting its interaction with hairpin A (muta: R33A, R35A, Y36A, and E39A) or hairpin B (mutb: K56A and K62A). (B) RNA-IP of GST-tagged p65 (mean ± SD, n = 3, *p < 0.05 versus IgG). (C) Sequential RNA-IP of IκBα and then GST-tagged p65 (mean ± SD, n = 3, ***p < 0.001 versus IgG).

See also Figure S5 and Table S5.

(Figure S4E). Furthermore, it took 5× the amount of DNA probe to compete NF-κB:NK$^{286–444}$ complex to the level competed by 1× the non-labeled NK$^{286–444}$ probe (Figure S4F). Therefore, the affinity of NK$^{286–444}$ containing both hairpins A and B to bind p65 is much stronger than consensus κB-binding DNA.

RNA footprinting assay identified two different sites in NK$^{286–444}$ that belong to hairpins A and B, respectively, and corresponding bands disappeared when binding to p65 or p65 and IκBα (Figure S4G). Furthermore, mutation of hairpin A at nt 331 and its complementary nt 351 (NK$^{286–444mA}$, NK$^{322–359mA}$, and NK^{mA}, Table S4) abrogated the binding of RNA to NF-κB in the presence or absence of IκBα without disrupting the hairpin structure (Figures 4D and 4E and Figures S4C and S4H). However, probes with hairpin B mutated by deleting nt 403–409 (NK$^{286–444mB}$ and NK^{mB}, Table S4) bind NF-κB alone but failed to do so in the presence of IκBα (Figures 4D and 4E and Figure S4H). These data suggested that hairpin A is essential for NKILA to bind p65 and hairpin B that also binds p65 prevents the disruption of p65:NKILA complex by IκBα, so that a stable NKILA:NF-κB:IκBα complex could be formed.

By analyzing differential accessibility of lysine residues in p65 to biotin with or without RNA engagement (Kvaratskhelia and Le Grice, 2008), we identified the amino acids in p65 that physically interact with RNA. The 2445 Da peptide (S51 to R73, Table S5A) was biotinylated with the peak shifting to 2,897 Da (Table S5B), which was substantially reduced by NK$^{286–444}$ that contains both hairpin A and B, but not by NK$^{286–402}$ that contains only hairpin A (Figure 5A), suggesting an interaction between hairpin B and the peptide that is nine amino acids downstream of the consensus DNA-binding sites (Chen et al., 1998; Ghosh et al., 2012). Moreover, mutation of hairpin B (NK$^{286–444mB}$) abrogated the RNA/peptide interaction, whereas mutation of hairpin A (NK$^{286–444mA}$) disrupted the essential RNA/p65 binding and thus also abrogated the RNA/peptide interaction (Figure 5A). Similar findings were obtained when full-length NKILA with mutation of hairpin A (NK^{mA}) or B (NK^{mB}) was used (Figure S5A). On the other hand, point mutations at the hairpin A (R33A, R35A, Y36A, and E39A) or the hairpin B-binding site (K56A and K62A) of p65 disrupted its interaction with NKILA in vivo, which was independent and dependent of IκBα binding, respectively (Figures 5B and 5C).In contrast, although hairpin A also binds to c-rel (Figure S4C), RNA footprinting assay demonstrated that c-rel did not interact with the hairpin B of NK$^{286–444}$ (Figure S5B). This is probably due to different amino acids in the c-rel site corresponding to the hairpin B-binding site in p65 (STDTTKTHPTIKINGYTGPGTVR for p65 and STDNNRTYPSI QIMNYYGKGKVR for c-rel). More importantly, in vitro RNA/protein interaction assay showed that NKILA can only retrieve c-rel in the experiments without IκBα, whereas the presence of IκBα abrogated the interaction between NKILA and c-rel (Figure S5C), which is consistent with previous reports that IκBα can dissociate DNA:NF-κB interaction (Zabel and Baeuerle, 1990). Collectively, these data suggest that hairpin B binding to S51-R73 of p65 is necessary for NKILA to stably associate with NF-κB:IκB complex.

NKILA Directly Inhibits IκB Phosphorylation by Masking the IKK Phosphorylating Sites

To explore how p65-bound NKILA inhibits IκB phosphorylation, we used an in vitro kinase assay, in which purified active IKKβ was added to p65-prebound IκBα. The addition of NKILA into p65-prebound IκBα prior to active IKKβ prevented in vitro IκBα phosphorylation, but adding NKILA after IKKβ did not inhibit IκBα phosphorylation (Figure 6A and Figure S6A), suggesting that NKILA inhibits IKKβ-induced IκBα phosphorylation, but does not dephosphorylate IκBα. NKILA mutated at hairpin A

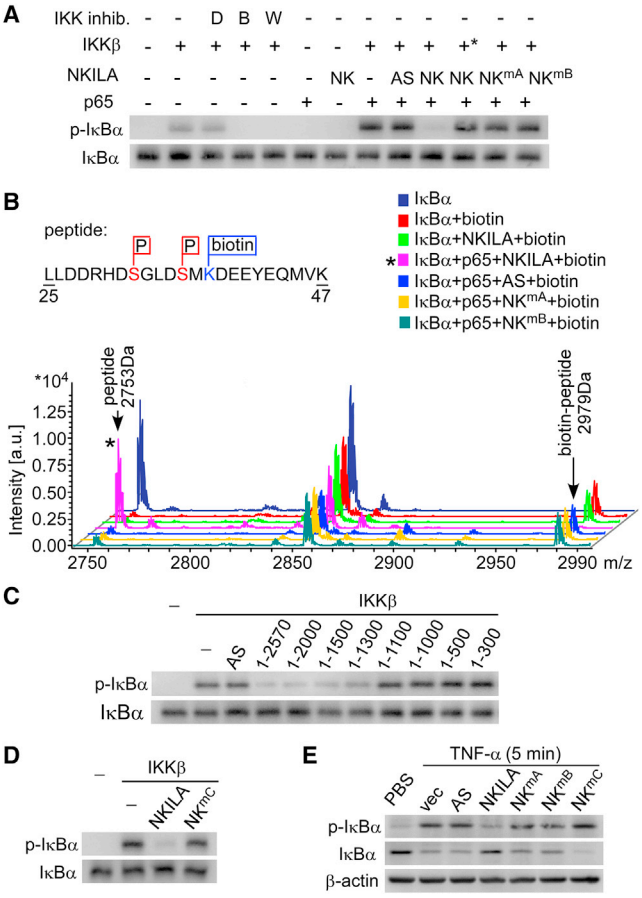

Figure 6. NKILA Directly Inhibits IκB Phosphorylation by Masking the IKK Phosphorylating Sites

(A) In vitro phosphorylation assay showing the effects of NKILA on IKK-mediated IκBα phosphorylation. inhib, inhibitor; D, DMSO; B, Bay-117082; W, Wedelolactone; +*, IKKβ was added to reaction mixture 20 min prior to NKILA; NK^{mA}, mutation of hairpin A in NKILA; NK^{mB}, mutation of hairpin B in NKILA.

(B) The effects of NKILA on the accessibility of amino acids in IκBα to IKK, determined by biotin-labeling assay followed by mass spectrometry (mass spectra of IκBα with indicated treatment were shown. Numbers indicate the position of amino acids. *Assay in which biotin labeling was inhibited by respective RNA.

(C and D) Inhibition of in vitro IκBα phosphorylation by NKILA and its sequentially deleted mutants (C), or NK^{mC} mutant (D). NK^{mC}, NKILA mutant deleting the hairpin C.

(E) The ability of NKILA mutants to inhibit TNF-α-induced IκBα phosphorylation in MDA-MB-231 cells.

See also Figure S6 and Table S6 and Table S7.

(NK^{mA}) or B (NK^{mB}) that fails to interact with p65:IκBα complex did not inhibit the IKKβ-induced IκBα phosphorylation (Figure 6A and Figure S6A), indicating that binding with the p65:IκBα complex is prerequisite for NKILA to inhibit the IKKβ-induced IκBα phosphorylation.

Mass spectrum analysis for trypsin-digested peptides from IκBα revealed that a peptide peak from free IκBα (L25 to K47, Figure 6B and Table S6A) that harbors both of the IKKβ-mediated phosphorylation sites (S32 and S36) was biotinylated and shifted (Table S6B). Importantly, the biotinylation of this peak was substantially reduced in the presence of p65-bound NKILA (Figure 6B), suggesting a close contact between NKILA and phosphorylation sites of IκBα that leads to reduced IKKβ accessibility to its target sites on IκBα. Thus, NKILA inhibits IKKβ-induced IκBα phosphorylation by physically masking the phosphorylation sites of IκBα and blocking its accessibility to IKK.

Further, NKILA deletion to nt 1,300 from its 3′-end preserved its ability to inhibit IκBα phosphorylation, but deletion to nt 1,100 abrogated such effect (Figure 6C), suggesting that nt 1,100–1,300 are critical to its suppressive function on IκBα phosphorylation. Moreover, Mfold and RNAfold (Figure S6B) predicted a triple-loop hairpin structure at nt 1,121–1,216 of NKILA (hairpin C), as further verified by RNA structure probing (Figure S6C). To confirm if hairpin C directly interact with IκBα, NKILA was incubated with p65 and p50 in the presence or absence of IκBα and the RNA-protein complex was subjected to RNase T1 digestion and PCR amplification. A qRT-PCR based RNA protection assay demonstrated that the region from nt 1,135 to 1,195, which resides within hairpin C (nt 1,121–1,216), was enriched when IκBα was incubated with the complex, indicating a direct interaction between hairpin C and IκBα (Figure S6D and Table S7). Although mutation to disrupt hairpin C in NKILA (NK^{mC}, Table S4) did not reduce its binding to p65 (Figure S6E), NK^{mC} failed to mask the phosphorylation motif on IκBα (Figure S6F) and thus could not inhibit the IKKβ-induced IκBα phosphorylation in vitro (Figure 6D). These observations indicate that nt 1,121–1,216 is necessary for NKILA to physically interact and mask the phosphorylation motif of IκBα.

NKILA with mutation at each of the hairpins (NK^{mA}, NK^{mB}, NK^{mC}) all failed to inhibit IκBα phosphorylation and NF-κB activation in MDA-MB-231 cells (Figure 6E and Figure S6G), indicating that all three hairpins are essential for NKILA function. On the other hand, replacement of endogenous wild-type p65 or IκBα with their mutants, by RNAi-targeting the 3′UTR of endogenous gene and ectopic expression of p65 or IκBα mutants that failed to interact with NKILA, abrogated the effect of NKILA to inhibit NF-κB activity in MCF7 cells (Figures S6H and S6I). Taken together, hairpins A, B, and C of NKILA play different roles to bind p65, assist the formation of the NF-κB:IκBα:NKILA complex and mask phosphorylation sites of IκBα, respectively, which coordinately inhibit IκBα phosphorylation.

NKILA Inhibits NF-κB-Mediated Breast Cancer Metastasis

Aberrant NF-κB signaling can promote tumorigenesis by inhibiting apoptosis (Baldwin, 2012) and increasing invasion (Huber et al., 2004). Consistent with its anti-NF-κB role, NKILA increased apoptosis and reduced invasion in MDA-MB-231 cells (Figures 7A and 7B). Conversely, silencing NKILA in MCF7 cells reduced apoptosis and enhanced invasiveness (Figures 7A and 7B), suggesting that NKILA antagonizes the anti-apoptotic and pro-invasive effects of NF-κB. However, in the presence of sc-3060 or JSH-23 that abrogates NF-κB nuclear translocation, NKILA could not further increase apoptosis or reduce invasion in MDA-MB-231 cells (Figures 7A and 7B), suggesting that NKILA exerts its effect via inhibiting NF-κB. Further, breast cancer cell invasion was not enhanced by silencing NKILA when an IKK-resistant TD-IκBα was expressed (Figure S7A), and was not inhibited by NKILA when IκBα was silenced (Figure S7B), indicating that the effects of NKILA are dependent on IκBα.

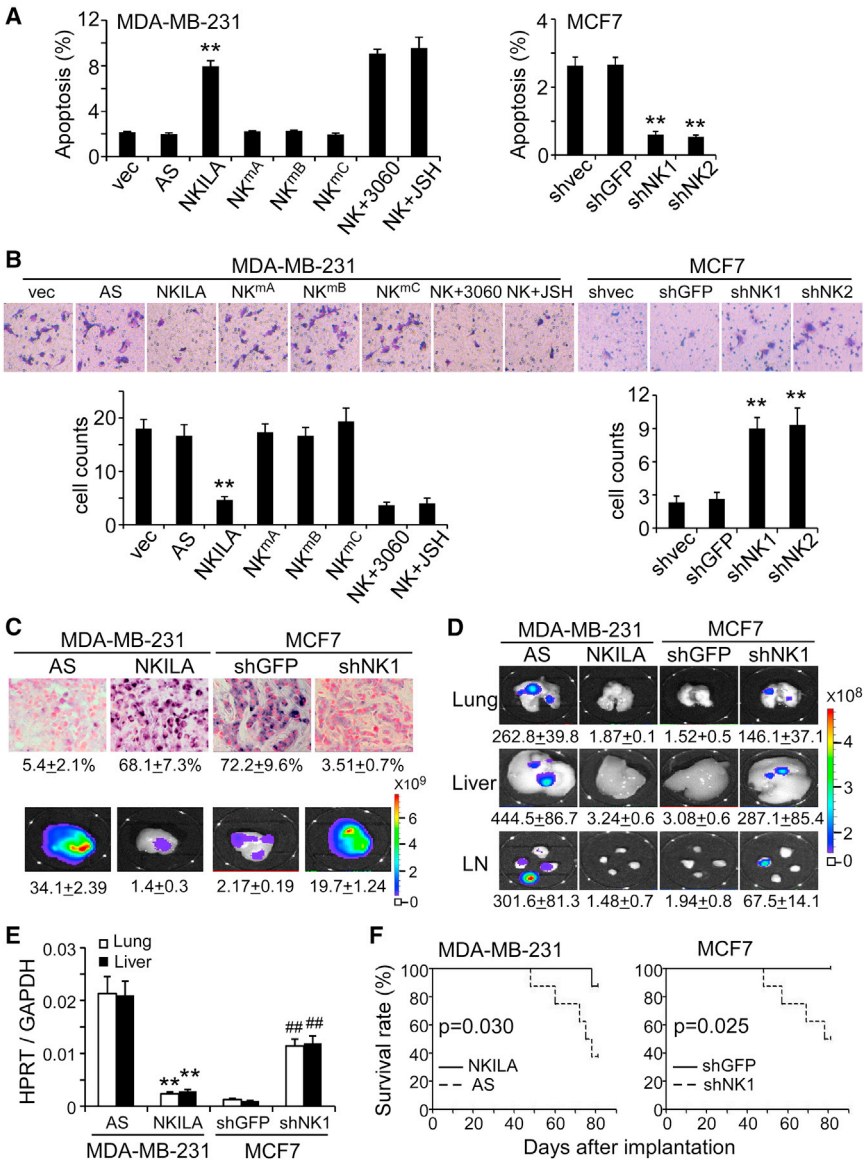

Figure 7. NKILA Inhibits NF-κB-Mediated Breast Cancer Metastasis

(A and B) Apoptosis (A) and invasion (B) of MDA-MB-231 or MCF7 cells stably expressing antisense, wild-type or mutant NKILA, or expressing shRNA targeting NKILA, assayed by Annexin-V/PI staining (48 hr after seeding) and Boyden Chamber assay (8 hr for MDA-MB-231 and 16 hr for MCF7 after seeding, respectively). 3060, 10 μM sc-3060; JSH, 5 μM JSH-23; mean ± SD; n = 3; **p < 0.01 versus vec; #p < 0.05 versus shvec; ###p < 0.001 versus shvec.

(C) In situ hybridization (ISH) staining for NKILA (upper; the percentage of positive cells is indicated under the picture, mean ± SD, n = 3) and luminal imaging for NF-κB activities (lower; the photon intensities are indicated under the picture [$\times 10^8$, mean ± SD, n = 3]) in xenografts of MDA-MB-231 stably expressing NKILA or MCF7 stably expressing NKILA-shRNA bearing a κB-dependent luciferase reporter construct showing NF-κB activity.

(D) Luminal imaging for lung, liver, and lymph node (LN) metastasis of MDA-MB-231 or MCF7 xenografts stably expressing NKILA or NKILA-shRNA. The metastasis was observed when primary tumors grew to similar size (1.5 cm in diameter when sacrificed to examine metastasis). The photon intensities in the organ are indicated ($\times 10^6$, mean ± SD, n = 3).

(E) qRT-PCR for hHPRT mRNA normalized to GADPH mRNA in the lungs and livers of mice orthotopically implanted with MDA-MB-231 or MCF7 cells (mean ± SD, n = 8, ***p < 0.001 versus vec, ###p < 0.001 versus shGFP).

(F) Kaplan-Meier survival curve of mice orthotopically implanted with MDA-MB-231 or MCF7 cells (n = 8 per group).

See also Figure S7.

In xenografts of breast cancer cell lines, NKILA expression in low-metastatic MCF7 xenografts was much higher than that in high-metastatic MBA-MD-231 xenografts, which was negatively correlated with their NF-κB activities (Figure 7C). Ectopic NKILA expression in MBA-MD-231 xenografts reduced NF-κB activities (Figure 7C), inhibited cancer metastasis to the lungs, liver, and lymph nodes (Figures 7D and 7E and Figure S7C), and prolonged mice survival (Figure 7F). Conversely, silencing NKILA in MCF7 xenografts increased their NF-κB activities (Figure 7C) and significantly promoted cancer metastasis (Figures 7D and 7E and Figure S7C) and reduced mice survival (Figure 7F). Taken together, these results indicate that NKILA inhibits breast cancer metastasis by suppressing NF-κB activation.

NKILA Reduction Predicts Poor Clinical Outcome in Patients with Breast Cancer

To further evaluate the clinical significance of NKILA in breast cancer progression, we used in situ hybridization to examine NKILA expression in 124 cases of benign breast tissues, 81 ductal carcinomas in situ (DCIS) and 942 invasive ductal carcinomas (IDC) of the breast. Abundant NKILA expression was observed in the epithelia of normal breast tissues and DCIS that are localized within the basement membrane (Figure 8A and Figure S8A). In comparison, NKILA expression was obviously lower in the invasive carcinomas without distal or regional lymph node metastasis, and further reduced in those with metastasis (Figure 8A and Figure S8A). In contrast, the pattern of miR-103 and miR-107 expression was opposite to that of NKILA in normal and malignant breast tissues (Figure 8A) and a negative correlation was observed between NKILA and the miRNA expression in the invasive breast cancers ($p < 0.001$, Figure S8B). This was further quantified by qRT-PCR showing that NKILA in normal breast tissues was comparable to DCIS ($p > 0.05$), but was approximately 6.6-fold higher than the IDCs without metastasis ($p < 0.001$) and approximately 19.7-fold higher than those with metastasis ($p < 0.001$, Figure 8B). Likewise, qRT-PCR also revealed that NKILA was negatively correlated with miR-103 ($p < 0.001$) and miR-107 ($p < 0.001$, Figure S8C). These data suggest that reduced NKILA expression

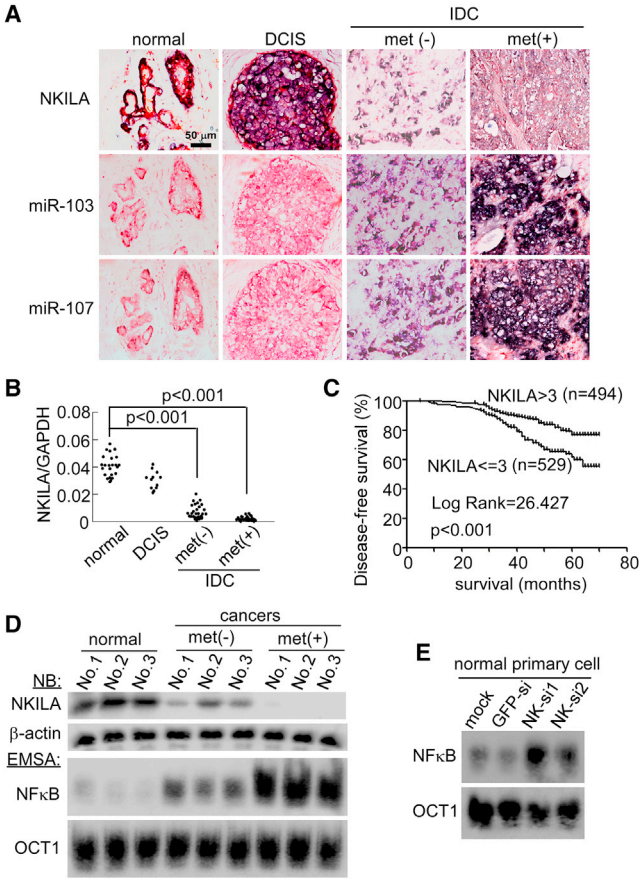

Figure 8. Low NKILA Expression Predicts Poor Clinical Outcome in Patients with Breast Cancer

(A) ISH for NKILA, miR-103, and miR-107 in paraffin-embedded tissue of normal breast, ductal carcinoma in situ (DCIS), and invasive ductal carcinoma (IDC) with and without metastasis.

(B) The expression of NKILA, normalized to GAPDH, in fresh-frozen samples, determined by qRT-PCR.

(C) Kaplan-Meier survival curve of patients with breast cancer with low (staining index, [SI] $\leq$ 3, n = 529) and high NKILA (SI > 3, n = 494; $p < 0.001$) with a median follow-up period of 40 months.

(D) NKILA expressions (NB, Northern blotting) and basal NF-κB activity (EMSA) in primary normal breast epithelial cells and primary breast cancer cells with or without metastasis (met(−)/met(+); nos. 1, 2, 3 represent different individuals).

(E) NF-κB of normal primary breast cells transfected with indicated siRNAs, examined by EMSA (GFP-si, GFPsiRNA; NK-si1, 2, NKILA siRNA1, 2).

See also Figure S8 and Table S8.

is associated with clinical invasion and metastasis of breast cancer.

We next correlated NKILA expression with the clinicopathological status of patients with breast cancers (Table S8A). In addition to the association with lymph node ($p < 0.001$) and distal metastasis ($p < 0.001$), reduced NKILA in breast cancer cells was also correlated with advanced disease staging ($p < 0.001$), higher histopathological grading ($p < 0.001$), and larger tumor ($p < 0.001$). However, there was no observed difference in NKILA expression regarding ER status, as well as Ki67 and PCNA expression ($p > 0.05$). More importantly, NKILA reduction was associated with poor patient survival ($p < 0.001$, Figure 8C), and could serve as an independent prognostic factor for breast cancers in a multivariate Cox regression analysis ($p = 0.002$, Table S8B).

To confirm the correlation of NKILA expression and NF-κB activities identified in breast cancer cell lines, we isolated primary breast epithelial cells from three cases of normal breast tissues, three cases of invasive breast cancers without metastasis and three cases with metastasis. Northern blot analysis demonstrated that NKILA expression was abundant in normal breast epithelial cells, but was obviously lower in invasive cancer cells from breast tumors without metastasis and further reduced in those with metastasis (Figure 8D). In agreement with findings of the cell lines, basal NF-κB activities in these cells, determined by EMSA, were negatively correlated with NKILA expression, as lowest NF-κB activities were observed in normal breast epithelial cells and highest in breast cancer cells from patients with metastasis (Figure 8D). More importantly, silencing NKILA in normal breast epithelial cells dramatically elevated NF-κB activities (Figure 8E), suggesting that NKILA is essential to prevent aberrant NF-κB activation even under normal physiological conditions. In addition, NKILA was upregulated by the inflammatory cytokine TNF-α, not only in breast cancer cells with or without metastasis, but also in normal breast epithelial cells determined by qRT-PCR (Figure S8D) and FISH assay (Figure S8E), suggesting that the negative feedback loop of NF-κB mediated by NKILA generally exists in normal and malignant breast epithelia. Finally, we examined the expression of NKILA in normal and cancer tissues of prostate, colon, lung, and liver in addition to breast. The results showed that NKILA was abundantly expressed in normal prostate, colon, and lung tissue, but its expression was much lower in normal liver tissue. Similar to the breast, NKILA expression was reduced in cancer versus normal tissues across tissue types (Figure S8F).

DISCUSSION

NF-κB is constitutively active in many tumors and considered to be a key factor in cancer development (DiDonato et al., 2012; Fornier et al., 2007). A number of negative regulators for NF-κB pathway, for example deubiquitinase (DUB) A20 (Cake et al., 2003) and CYLD (Brummelkamp et al., 2003), ubiquitin ligase SOCS-1 (Ruland, 2011), and a group of miRNAs (Boldin and Baltimore, 2012), etc, have been shown to be tumor suppressors. Many of these regulators are transcribed by NF-κB, but participate in the negative feedback loops to prevent sustained or excessive activation of NF-κB pathway (Ruland, 2011). We found that NKILA, a NF-κB-induced lncRNA, is also involved in the negative feedback loop of NF-κB regulation, and serves as a tumor suppressor by inhibiting breast cancer progression and metastasis. Different from most previously identified NF-κB suppressors that either inhibit signaling upstream of IKK complex or affect the amount of NF-κB or IκB (Ruland, 2011; Tse et al., 2007), NKILA operates at the level of IκB by directly inhibiting IKK-induced IκB phosphorylation without influencing IKK activity, which represents a different layer of negative regulation on NF-κB pathway.

Only a fraction of malignancies with aberrant NF-κB activities harbors gene mutations that affect components of NF-κB pathway and activate NF-κB. Constitutive NF-κB activation in

the malignant cells of many solid tumors was thought to be mainly driven by inflammatory cytokines in the tumor microenvironment (DiDonato et al., 2012; Fornier et al., 2007). Herein, our data indicate that NKILA is capable of restraining NF-κB activity in both normal and malignant breast epithelial cells, while its downregulation leads to aberrant NF-κB activation even without inflammatory stimulation, suggesting that NKILA is an essential "gatekeeper" of NF-κB signaling in resting cells. In normal breast epithelia or non-invasive breast cancers, NKILA is abundantly expressed and silencing its expression results in appreciable phosphorylation and degradation of IκB, probably mediated by the basal activity of IKK. Indeed, previous studies demonstrated that basal IKK activity was critical and sufficient to degrade NF-κB-bound IκB and may lead to NF-κB activation when synthesis of IκB molecules was suppressed (Ghosh and Dasgupta, 1996; O'Dea et al., 2008). Therefore, our findings suggest that stable NF-κB-bound IκB that maintains normal NF-κB activity in resting cells depends not only on IκB replenishment (Ghosh and Dasgupta, 1996) but also on NKILA-mediated protection of NF-κB-bound IκB from being phosphorylated by basal active IKK (Figure S8G). However, in invasive cancer cells, NKILA is significantly reduced by miR-103/107-mediated degradation, which results in abnormal NF-κB activation and thus contributes to cancer metastasis and poor patient outcome (Figure S8H). Moreover, because NF-κB signaling plays a pivotal role in many physiological and pathological processes, the importance of NKILA-mediated regulation on NF-κB activation is not limited to cancer progression and metastasis, but it may also underlie the process of embryo and tissue development, immune and stress responses, inflammation and other diseases related to NF-κB activation (Ghosh and Dasgupta, 1996).

Our findings reveal that a stable NKILA:NF-κB:IκBα complex in the cytoplasm is prerequisite for NKILA to prevent IKK-induced IκB phosphorylation. However, it has been well established that stable DNA:NF-κB:IκBα complexes are unlikely to form because IκBα actively destabilizes and disrupts the complex of NF-κB and its cognate DNA. In agreement, our results also show that the 322–359 fragment of NKILA that contains only hairpin A, a mimic of the κB DNA motif, is rapidly dissociated from p65 by IκBα. However, with the aid of a neighboring hairpin B that binds to the amino acid residues of p65 in the vicinity of its consensus DNA binding site, hairpin A is no longer dissociated and a stable RNA:NF-κB:IκBα complex is formed. The crystal structure of the NF-κB:IκBα complex has documented that the presence of IκBα leads to en bloc movement and conformational change of the p65 subunit amino-terminal domain mainly due to electrostatic interaction. This switches the p65 subunit from a DNA-bound "open" to a "closed" conformation, and thus induces allosteric inhibition of NF-κB DNA binding. Nevertheless, a recent study detects a transient complex of NF-κB and IκBα with DNA upon stepwise addition of a κB binding DNA fragment to the NF-κB:IκBα complex, suggesting that the DNA binding site of p65 is not totally blocked by IκBα (Sue et al., 2011). Here, our present findings indicate that additional binding of NKILA hairpin B to the amino acids vicinal to the consensus DNA binding motif of p65 not only enhances the affinity of NKILA to p65, but also abolishes IκBα-mediated inhibition of NKILA/NF-κB interaction and plays an essential role in forming a stable NKILA:NF-κB:IκBα complex. In fact, the amino acids that bind NKILA hairpin B locate at loop L1 of the p65 N-terminal domain, while two affinity- and specificity-determining DNA-binding residues of p65, Arg-33 and Arg-35, are from this same loop. Therefore, this additional binding may help to stabilize the interaction between NKILA hairpin A and p65 amino-terminal domain against the electrostatic attraction by IκBα. On the other hand, although c-rel has been shown to bind a large variety of target DNA sequences, it bears variation in the amino acids at the hairpin B binding site and therefore cannot bind hairpin B. Consequently, c-rel alone is able to bind NKILA, but cannot form a stable complex with NKILA in the presence of IκBα. Therefore, our findings may provide an exceptional example for nucleic acids to bind the NF-κB:IκB complex and suggest that the flexible and versatile secondary structure of lncRNA may extend their capability to interact with protein complexes.

In addition to the two adjacent hairpins A and B that act in concert for the formation of a stable NKILA:NF-κB:IκBα complex, NKILA activity also depends on a discrete triple-loop hairpin C that physically interacts with IκB and masks its phosphorylation sites (Figure S8I). Although serving as molecular scaffolds that interact with multiple regulatory proteins is one of the major functions for many lncRNAs (Guttman and Rinn, 2012), NKILA differs from these scaffold lncRNAs by directly regulating signal transduction without mobilizing other regulatory proteins. A scaffold lncRNA assembles other regulatory proteins to form a unique functional complex and the protein components could not form a complex without the lncRNA. In addition, the scaffold lncRNA itself does not carry out any regulatory effect, but relies on the recruited proteins to function. For example, it was shown that NRON was reported as a molecular scaffold that is necessary for the assembly of NFAT and several NFAT kinases, including CK1, GSK3β, and DYRK2, which are responsible for NFAT phosphorylation and its sequestration in the cytosol (Sharma et al., 2011). NRON depends on their interacting proteins to carry out the function. In contrast, formation of the NF-κB:IκB complex does not depend on NKILA, suggesting the lncRNA does not act as a scaffold to assemble NF-κB and IκB. Furthermore, p65-bound NKILA inhibits IKK-induced IκB phosphorylation by directly masking the phosphorylation sites of IκB from IKK, revealing a underappreciated function of lncRNAs that regulate signal transduction by directly acting on active domains of signaling proteins without mobilizing other regulatory molecules. Align with our findings, another cytoplasmic lncRNA, lnc-DC, directly blocks STAT3 phosphorylation by SHP1 and inhibits STAT3 activation (Wang et al., 2014). Therefore, our data and others may indicate the existence of a class of lncRNAs that regulate signal transduction at post-translational level, and our present study has further illustrated the in-depth mechanisms how these lncRNAs interact with the signaling proteins.

Finally, our findings have therapeutic implications. It has long been acknowledged that RNA is a malleable evolutionary substrate compared with protein, and mutation or deletion of the regions outside its functional domains may not interfere with its core functionality (Wutz et al., 2002). Therefore, synthetically engineered ncRNAs containing the functional domains that act on the active regions of signaling proteins can be tested for their therapeutic effects on diseases with aberrant activation of major signaling pathways, which may thus offer a category of RNA therapy.

EXPERIMENTAL PROCEDURES

RNAI Immunoprecipitation, RNA Pulldown, and RNA EMSA

RNA immunoprecipitation was performed using Magna RIP RNA-Binding Protein Immunoprecipitation Kit (17-700, Millipore) according to manufacturer's instructions. RNA for in vitro experiments was transcribed using T7 High Yield RNA Synthesis Kit (E2040S, NEB) according to manufacturer's instructions. The 3′-end biotin-labeled RNA probes used in RNA pulldown and RNA EMSA were generated using RNA 3′ End Biotinylation Kit (20160, Pierce) according to manufacturer's instructions. RNA pulldown was performed as described (Tsai et al., 2010). Five picomoles of 3′-biotinylated RNA was used in each pulldown assay. RNA EMSA was performed using LightShift Chemiluminescent RNA EMSA Kit (20158, Pierce) according to manufacturer's instructions. To be specific, RNA samples used in RNA EMSA were re-natured in 1× RNA EMSA buffer by heating to 95°C for 3 min, placed on ice for 5 min, and placed at room temperature for 20 min. Detection of biotinylated RNA in blots were performed using Chemiluminescent Nucleic Acid Detection Module Kit (89880, Pierce).

In Vitro Phosphorylation Assay

Briefly, GST-labeled recombinant human IKKβ was purified in SF9 insect cells. Recombinant human IκBα (ab113133) was purchased from Abcam. In vitro phosphorylation was performed in 1× kinase buffer (25 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.1 mM Na_3VO_4, 5 mM β-glycerophosphate, and 2 mM DTT). Phosphorylation of IκBα was detected by immunoblotting or ELISA using PathScan Phospho-IκBα (Ser32) Sandwich ELISA Kit (#7355, Cell Signaling Technology).

Structural Analysis of Protein-RNA Interactions with Biotin-Labeling Assay and Mass Spectrometry

Biotin-labeling assay was performed as described elsewhere (Kvaratskhelia and Le Grice, 2008). Briefly, RNA and the indicated proteins were added into RIP buffer and incubated for 30 min, followed by adding of 400 μM NHS-biotin (20217, Pierce) with 20 min incubation. Proteins were then separated by SDS-PAGE and in-gel proteolysis was performed using trypsin (89871, Pierce). Mass-spectrum analysis was then performed on MALDI-TOF (Bruker Daltonics) as described elsewhere (Kvaratskhelia and Le Grice, 2008).

Tumor Xenografts

Six-week-old female athymic nude mice were purchased from Vital River Laboratories, housed under standard conditions at the animal care facility at Center of Experimental Animal of Sun Yat-sen University. The procedures were approved by SunYat-sen University Animal Care and Use Committee.

Patients and Tumor Specimens

Paraffin-embedded samples of normal breast tissue and carcinomas were obtained from 1,147 patients (normal 124, cancer 1,023) in the breast tumor center, Sun Yat-sen Memorial Hospital, Sun Yat-sen University from February 2006 to October 2012. Fresh tumor tissues were obtained from 84 breast cancer cases (DCIS 12, IDC 72) before any treatment during the surgery. Fresh normal breast tissue was from samples from breast reduction surgery (eight cases) or quadrantectomy (16 cases). All samples were collected with signed informed consent according to the internal review and ethics boards of Sun Yat-sen Memorial Hospital.

ACCESSION NUMBERS

The Gene Expression Omnibus database accession number for the array data reported in this paper is GSE57539.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, eight figures, and eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.ccell.2015.02.004.

AUTHOR CONTRIBUTIONS

E.S., B.L., and Q.L. conceived the project. B.L. and L.S. conducted experiments on functions and mechanisms of NKILA and analyzed the data. C.G. conducted experiments on clinical samples and analyzed the data. Y.Y. and H.Y. conducted animal experiments and analyzed the data. X.L. constructed most of plasmids and purified the proteins. L.L. conducted FISH assay. F.S. collected and prepared samples for clinical study. The manuscript was written by E.S., Q.L., B.L., D.L., M.Z., and L.S. All authors discussed the results and commented on the manuscript.

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