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# Epigenetic alteration of the NF-κB-inducing kinase (*NIK*) gene is involved in enhanced NIK expression in basal-like breast cancer

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**Basal-like breast cancers are triple-negative (estrogen receptor negative, progesterone receptor negative, erythroblastic leukemia viral oncogene homolog 2 (ERBB2) negative) tumors with an aggressive clinical behavior that lacks effective molecular targets for therapy. We reported previously that the basal-like subtype cell lines display high constitutive nuclear factor (NF)-κB activation, whose inhibition in the basal-like subtypes suppressed their proliferation. Moreover, NF-κB-inducing kinase (NIK) is involved in the constitutive NF-κB activation. Here, we report that enhanced NIK expression, which is exclusively observed in the basal-like subtype rather than the luminal-like subtype or non-tumorigenic mammary epithelial cells, is caused by epigenetic alteration of the *NIK* gene. The stability of *NIK* mRNA and transcriptional activity driven by the *NIK* promoter are similar in the basal-like and luminal-like subtypes. However, histone H3 acetylation levels were up-regulated in the basal-like subtype. Furthermore, treatment of the luminal-like subtype with a histone deacetylase inhibitor, valproic acid, significantly increased NIK expression. Although DNA methylation of the *NIK* locus was not detected, NIK expression also increased when the luminal-like subtype was treated with 5-azacytidine, which inhibits histone H3-Lys-9 dimethylation in addition to DNA methylation. Taken together, these results suggest that the closed chromatin structure mediated by histone H3 methylation and deacetylation suppresses NIK expression in the luminal-like subtype, whereas disruption of these suppression mechanisms leads to enhanced NIK expression and the constitutive NF-κB activation in the basal-like subtype. Thus, NIK and genes induced by the NIK-mediated constitutive NF-κB activation could be therapeutic targets of basal-like breast cancer. (*Cancer Sci* 2010; 101: 2391–2397)**

Breast cancers are categorized according to their profiles of gene expression as follows: luminal-like, ERBB2 overexpressing, basal-like, and normal breast like subtypes.[1] The luminal-like subtype is characterized as an estrogen receptor-positive ($ER^+$) and a progesterone receptor-positive ($PR^+$) phenotype. The basal-like subtype, which represents the $ER^-$, $PR^-$, $ERBB2^-$ phenotypes, shows a higher malignancy than other subtypes and exhibits a poor prognosis against various methods of therapy.[2,3] Therefore, it is necessary to find molecular signatures and signaling pathways that contribute to the malignancy of basal-like breast cancer.

The NF-κB family of transcriptional factors plays a critical role in inflammation, immunoregulation and cell differentiation.[4,5] This family consists of five members, including p50, p52, RelA, RelB and c-Rel, which form homomeric or heteromeric dimers to activate transcription of the target genes. NF-κB is made transcriptionally inactive by being sequestered in the cytoplasm when it forms complexes with the IκB family, including IκBα, IκBβ, IκBε and the p105 and p100 precursors of p50 and p52, respectively. Nuclear translocation of NF-κB can be driven by two distinct signaling pathways. In the canonical pathway, a large number of stimuli including various cytokines and bacterial and viral products induces IκB kinase (IKK) β-catalyzed phosphorylation and proteasomal degradation of IκBα, followed by nuclear translocation of mainly p50-RelA heterodimers. The non-canonical pathway is activated by receptors that are crucial in the formation of lymphoid organs and lymphocyte development such as the lymphotoxin (LT) β receptor, the receptor activator of NF-κB (RANK), and CD40. This pathway induces the IKKα-catalyzed phosphorylation of the C-terminal half of p100 that sequesters RelB in the cytoplasm, which leads to polyubiquitination-dependent processing of p100 to p52 and the nuclear translocation of the p52-RelB heterodimers.

Accumulating evidence indicates that aberrant NF-κB activation leads to tumorigenesis and cancer malignancy through the expression of genes involved in survival, metastasis and angiogenesis.[5,6] We have previously reported that the basal-like subtype, but not the luminal-like subtype, undergoes constitutive and strong activation of NF-κB, whose activation is transient in normal cells upon various physiological stimuli. Furthermore, reduction of the NF-κB activity of the basal-like subtype resulted in growth inhibition.[7] These results indicate that constitutive activation of NF-κB is involved in the malignancy of basal-like breast cancers and could be an effective therapeutic target.

Recently, several groups reported that NF-κB-inducing kinase (NIK), an activator of IKKα in the non-canonical pathway, is constitutively activated in a number of neoplasias. Multiple myeloma cells have several mutations and deletions in the *TRAF2*, *TRAF3* and *cIAP* genes, whose products are normally involved in the degradation of NIK, resulting in the accumulation and activation of NIK proteins and thereby leading to constitutive activation of the non-canonical NF-κB pathway.[8] Aside from the post-translational regulation of NIK, *NIK* mRNA is significantly increased to constitutively activate the non-canonical pathway in adult T-cell leukemia and Hodgkin Reed–Sternberg cells.[9] We have also reported that the basal-like subtypes preferentially show high *NIK* mRNA expression, which is involved in the constitutive NF-κB activation. This result strongly suggests that inhibition of NIK expression is an effective therapeutic strategy to down-regulate the constitutive NF-κB activation in basal-like breast cancers. Therefore, the molecular mechanisms of regulation of the *NIK* mRNA expression in breast cancer need to be elucidated.

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## Materials and Methods

**Cell culture, chemicals and adenovirus infection.** The culture condition of all breast cancer cell lines has been described previously.[7,10] MG-132 (Calbiochem, San Diego, CA, USA), valproic acid (VPA) (Wako, Osaka, Japan) and 5-azacytidine (Sigma, St Louis, MO, USA) were purchased. Methods for adenovirus infection have been described previously.[7]

**RT-PCR.** RNA isolation and RT-PCR were performed as described previously.[11] The primer sets used were as follows: 5′-TTG GTT GGG GAG ATC GGC GCT TG and 5′-GGG GCT GAA CTC TTG GCT ATT CTC for *NIK*; 5′-GGT CAG AAG GAT TCC TAT GT and 5′-GGT GTT GAA GGT CTC AAA CA for *β-actin*; 5′-ATT GAG CGG AAG ATT CAA CT and 5′-TGT GGA TTT CTT GTC ATA GA for *RelB*.

**Western blotting.** Western blotting analysis was performed as described previously.[11] The antibodies used were anti-NIK (Cell Signaling Technologies, Beverly, MA, USA), anti-p52 (Millipore, Bedford, MA, USA) and anti-α-tubulin (Calbiochem).

**Reporter construct and luciferase assay.** The *NIK* promoter-driven luciferase reporter construct was generated by the insertion of several *NIK* promoter regions, which was determined based on the transcriptional start site of the *NIK (MAP3K14)* gene (NM_003954.2), into the pGL4.10 vector (Promega, Madison, WI, USA). Various *NIK* promoter regions were amplified from the human sperm genome DNA pool by PCR with appropriate primers. Luciferase assays were performed as described previously.[11]

**Bisulfite sequencing analysis.** Bisulfite sequencing analysis was performed as described previously.[12] The mixture of salmon sperm DNA (5 μg) and untreated or *in vitro* methylated *NIK* promoter (−8017/+76) in pGL4.10 (40 pg) using SssI methylase and S-adenosylmethionine (New England BioLabs, Beverly, MA, USA) was used as a negative or positive control, respectively. The *NIK* promoter region (−684/−313, −338/−79, −97/+451 and +432/783) of bisulfite modified MCF-7 cells genomic DNA (5 μg) and control samples were amplified using AccuPrime Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) with appropriate primers and subcloned in pT7blue vector (Novagen, Madison, WI, USA). Three independent clones of each region were sequenced.

**Chromatin immunoprecipitation (ChIP) assay.** A ChIP assay was performed as described previously.[13] The antibody against acetylated histone-H3 was purchased from Millipore. The immunoprecipitation efficiency of the *NIK* promoter region (−263 to −152) was analyzed by real-time PCR using a FastStart Universal SYBR Green Master (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. The primer set used was 5′-ACA TCG TCC GGA AAT AGT GC and 5′-CCT ACG CCA ACC AAT GAG AC.

**Statistics.** Statistical significance was evaluated using a two-tailed Student *t* test. *P*-values <0.05 were considered significant.

## Results

**NIK is highly expressed in basal-like subtype breast cancer cell lines.** We have previously analyzed *NIK* mRNA expression in

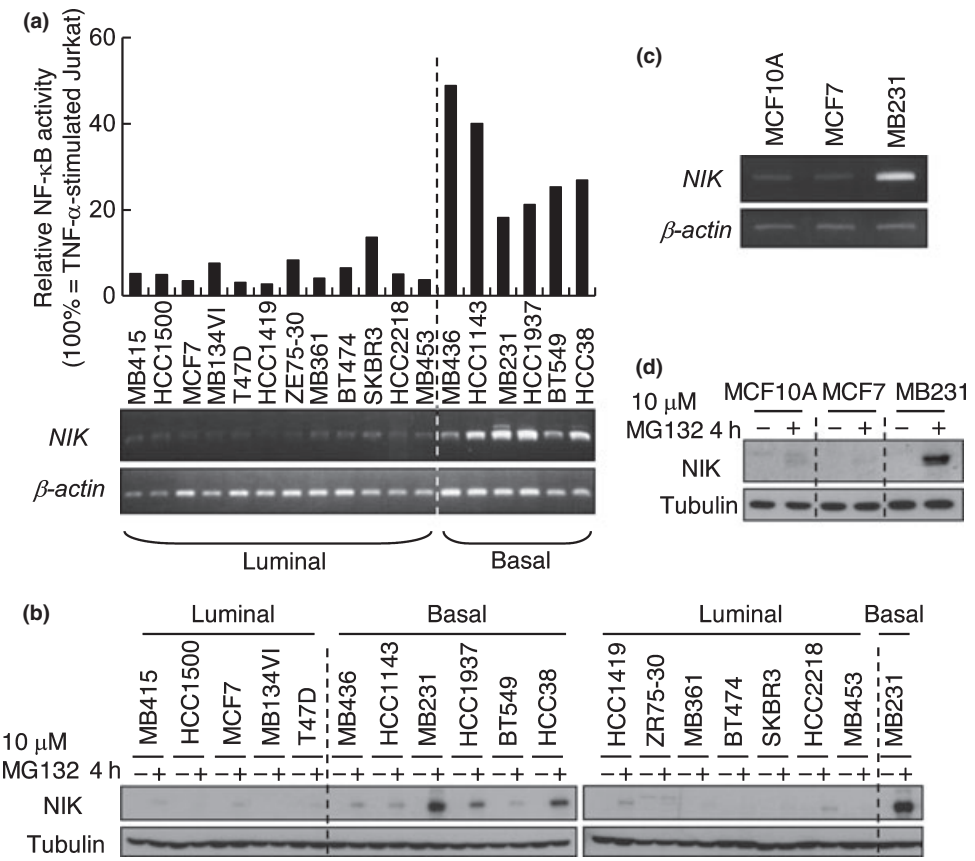


**Fig. 1.** Enhanced NF-κB-inducing kinase (NIK) expression is a hallmark of the basal-like subtypes. (a) Basal NF-κB activation and *NIK* mRNA expression in various breast cancer cell lines. The NF-κB activity was measured by electrophoretic mobility shift assays, as previously described.[7] *NIK* mRNA expression was measured by RT-PCR. (b) Expression levels of NIK protein in various breast cancer cell lines were measured by western blotting in the presence or absence of the proteasomal inhibitor MG-132. (c,d) NIK expression was measured by RT-PCR (c) and western blotting in the presence or absence of the proteasomal inhibitor MG-132 (d).


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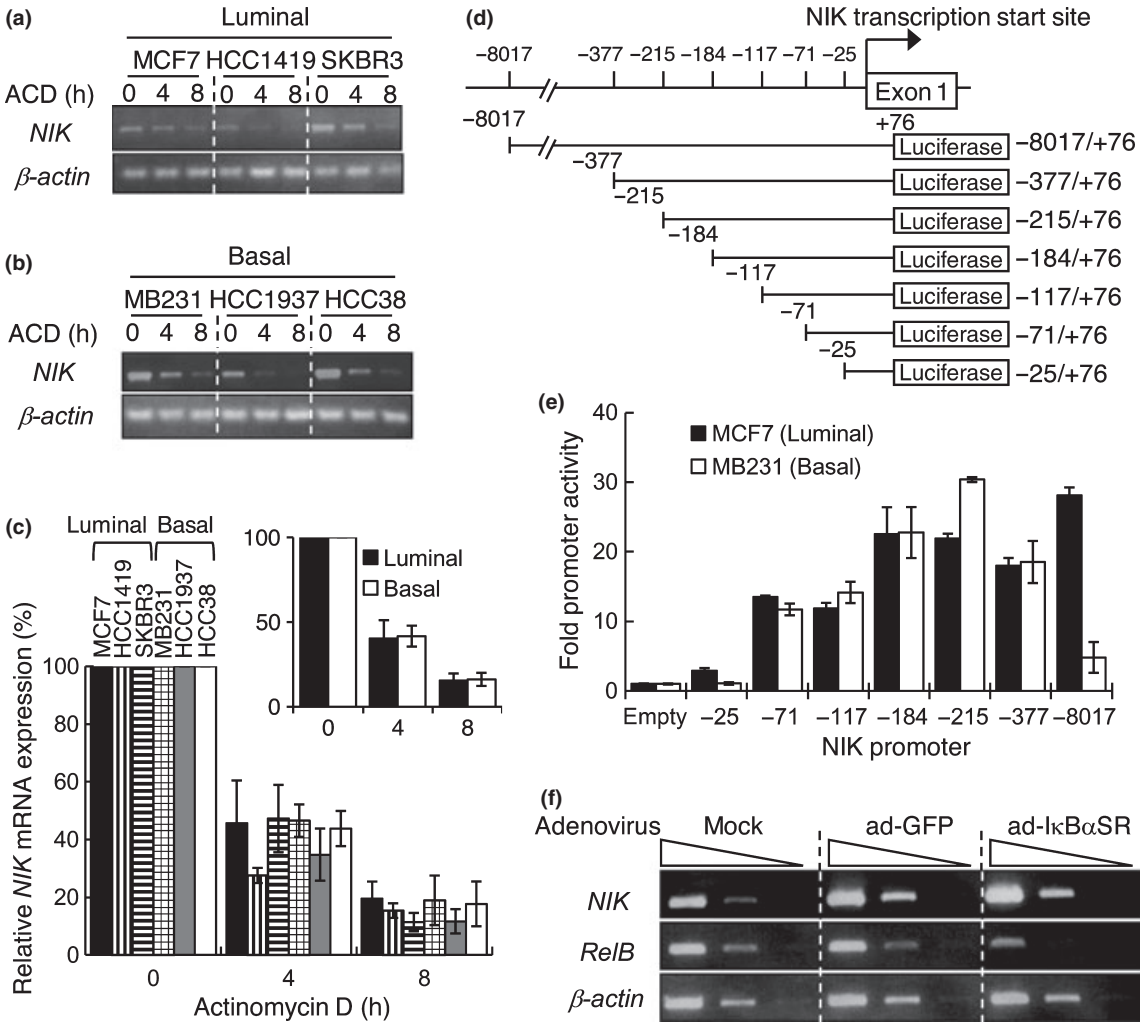


**Fig. 2.** Similar NF-κB-inducing kinase (*NIK*) mRNA stability and transcriptional activity driven by the 5′ flanking region of the *NIK* gene in the luminal-like and the basal-like subtypes. (a,b) The *NIK* mRNA expression was measured by RT-PCR in the luminal-like (a) and the basal-like (b) subtypes in the presence of actinomycin D (ACD, 5 μg/mL). (c) Quantification of band intensity in (a) and (b) using an image analyzer. Expression levels of *NIK* mRNA are shown as the percentages relative to that of each cell line without actinomycin D treatment (0 h). Data represent the mean ± SD of three independent experiments. The insert represents *NIK* mRNA stability in each subtype. (d) Schematic representation of the progressive 5′ deletion mutants of the *NIK* promoter luciferase reporter constructs. (e) Transcriptional activity of various mutant *NIK* promoters was measured by luciferase assay in MCF7 (luminal) and MB231 (basal) cells. (f) Reduced activation of NF-κB did not affect *NIK* mRNA expression. MB436 (basal) cells were either mock-infected or infected with adenovirus expressing GFP or IκBαSR. Total RNA was extracted 24 h after infection and a semiquantitative RT-PCR (fivefold serial dilutions) was performed.

eight basal-like and two luminal-like subtypes of breast cancer cell lines and proposed that enhanced expression of *NIK* mRNA is observed more frequently in the basal-like subtypes.[7] Because the number of luminal-like subtypes analyzed in the previous paper was not sufficient to conclude that enhanced NIK expression is characteristic of the basal-like subtypes, we analyzed 12 luminal-like subtypes together with six basal-like subtypes. The basal-like subtypes showed higher *NIK* mRNA expression than all of the luminal-like subtypes tested (Fig. 1a, lower). Moreover, the expression levels of the *NIK* mRNA are closely correlated with the extent of the constitutive NF-κB activation reported in our previous data[7] (Fig. 1a). We then analyzed NIK protein levels in the presence of the proteasome inhibitor MG-132, because NIK protein is normally degraded so that it is kept at low levels by the TRAF2/TRAF3/cIAP complex in a proteasome-dependent manner.[14] The NIK protein is easily detected in the basal-like subtypes by the MG-132 treatment, whereas the NIK protein was scarcely detected in the luminal-like subtypes even in the presence of MG-132 (Fig. 1b). To further examine the correlation between NIK expression and the malignancy of the breast cells, we then compared NIK expression in a non-tumorigenic mammary epithelial cell line, MCF10A, with that of two breast cancer cell lines. Neither the *NIK* mRNA nor the NIK protein was detected in MCF10A, a non-tumorigenic mammary epithelial cell line, and MCF7 (luminal) cells, whereas NIK expression was dramatically enhanced at both mRNA and protein levels in MB231 (basal) cells (Fig. 1c,d). These results strongly suggest that the enhanced expression of NIK is a hallmark of basal-like subtype cell lines and is involved in the constitutive activation of NF-κB and in the aggressiveness of basal-like breast cancers.

**Luminal-like and basal-like subtype cell lines exhibit similar *NIK* mRNA stability and transcriptional activity driven by the 5′ flanking region of the *NIK* gene.** To elucidate the molecular mechanisms of enhanced *NIK* mRNA expression in the basal-like subtype, we first compared the stability of *NIK* mRNA

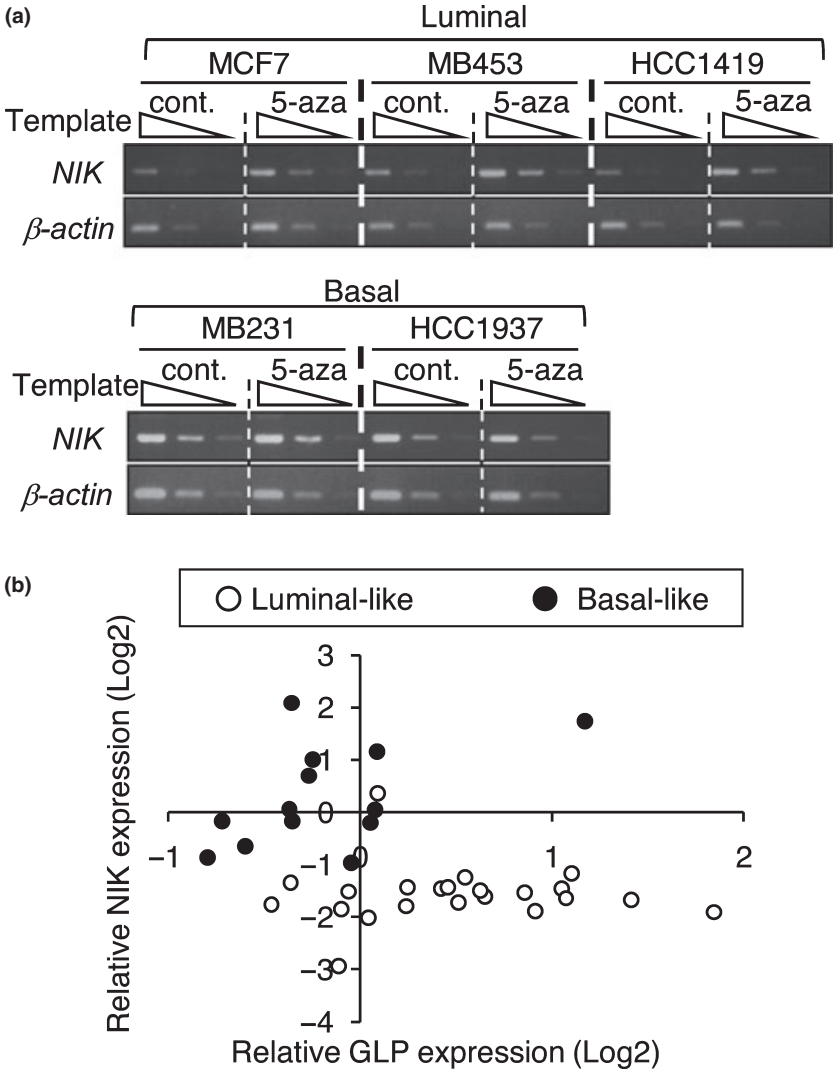


**Fig. 3.** Treatment with 5-azacytidine induced NF-κB-inducing kinase (NIK) expression in a CpG methylation-independent manner in the luminal-like subtypes. (a) Effect of 5-azacytidine on *NIK* mRNA expression in breast cancer cell lines. Cells were treated with or without 5-azacytidine (20 μM) for 24 h. Total RNA was extracted and a semiquantitative RT-PCR (fivefold serial dilutions) was performed. (b) Expression profiles of *NIK* and *GLP* in 35 breast cancer cell lines. Open or filled circles represent luminal-like or basal-like cell lines, respectively. Expression levels were estimated based on previous microarray data.[17]

between the luminal-like and the basal-like subtypes using actinomycin D to block new mRNA synthesis (Fig. 2a–c). The half-lives of *NIK* mRNA were approximately 3–4 h for both subtypes (Fig. 2c), indicating that mRNA stability is not significantly different between the luminal-like and basal-like subtypes. Additionally, we used published CGH-array data[15] to investigate *NIK* gene amplification. There are two probes (58.5 kbp upstream and 341.2 kbp downstream of the *NIK* gene) whose copy number may indicate that of the *NIK* gene. Intensities of these two probes indicate the normal copy number in several basal-like cell lines such as MB436, HCC1143, MB231, HCC1937, BT549 and HCC38, which are used in this study. Thus, these data suggest that the *NIK* gene is not amplified in the basal-like subtypes.

We then compared the transcriptional activity driven by the 5′ flanking region of the *NIK* gene. Progressive 5′ deletion mutations of the 8.1 kbp promoter were used to generate various reporter constructs (Fig. 2d). The two distinct regions, located between −71 and −25 and between −117 and −184, positively regulate the *NIK* promoter. However, transcriptional activities driven by the mutant promoters other than the −8017/+76 luc were similar between both subtypes (Fig. 2e). The −8017/+76 luc was activated more in the luminal-like than the basal-like subtype (Fig. 2e). However, we could not find any basal-like subtype-specific enhancer elements, which account for the enhanced NIK expression in the basal-like subtype, within the 8.1 kbp *NIK* promoter. Additionally, we examined the effect of NF-κB activity on *NIK* mRNA expression in the basal-like subtype because the activity of NF-κB is constitutively high in the basal-like subtype but not in the luminal-like subtype. While the expression of RelB, a known NF-κB target gene, was significantly reduced by the non-degradable IκBα super repressor (IκBαSR)-mediated inhibition of NF-κB in the MB436 (basal) cells,[7] NIK expression was hardly affected (Fig. 2f). Thus, the constitutive NF-κB activation is not a cause of enhanced NIK expression in the basal-like subtypes. Taken together, these data strongly suggest that transcriptional activity driven by the *NIK* promoter in the basal-like subtypes is similar to that in the luminal-like subtypes.

**5-azacytidine and VPA induce NIK expression in the luminal-like subtype breast cancer cell lines.** We did not detect any significant differences in the stability of *NIK* mRNA or in the transcriptional activity of the *NIK* promoter between the basal-like and the luminal-like subtypes, and thus speculated that *NIK* mRNA expression might be regulated by epigenetic alterations. To address this question, we first investigated CpG methylation on the *NIK* promoter. *NIK* mRNA expression was increased approximately fivefold by treatment with 5-azacytidine, a DNA methyltransferase inhibitor, in the luminal-like but not in the basal-like subtypes (Fig. 3a). However, bisulfite sequence analysis on the 1.4 kbp (−684/+783) fragment, which covers approximately 70% of the CpG island surrounding the *NIK* promoter, in


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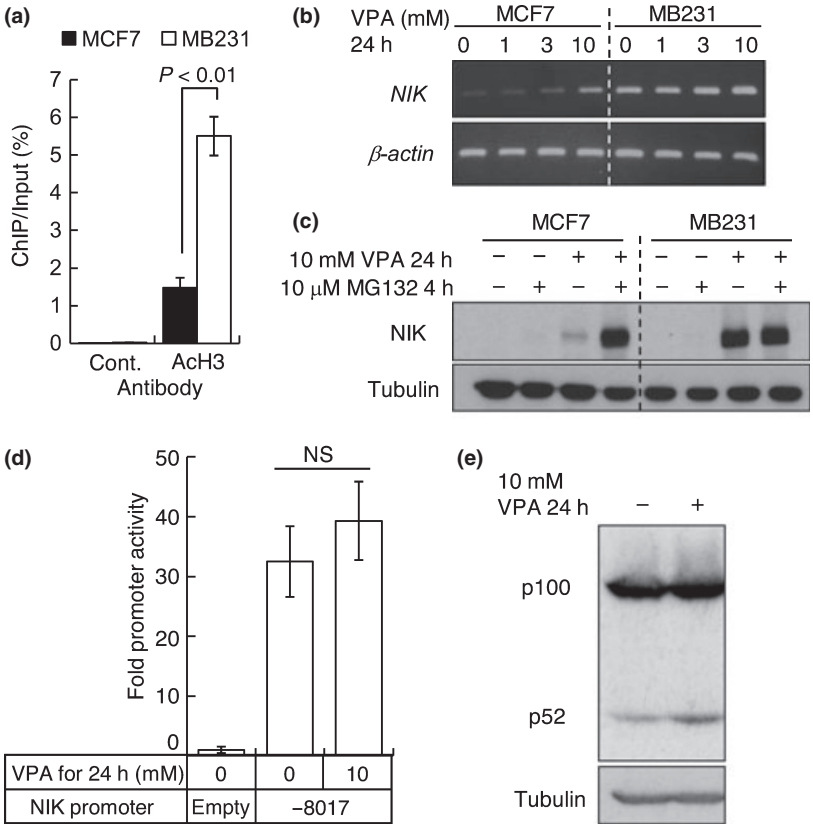


**Fig. 4.** Histone H3 acetylation in the NF-κB-inducing kinase (*NIK*) promoter is involved in enhanced NIK expression in the basal-like subtype. (a) The histone H3 acetylation profile in the *NIK* promoter region was analyzed by a chromatin immunoprecipitation (ChIP) assay in the MCF7 and MB231 cells. (b) The effect of valproic acid (VPA) on *NIK* mRNA expression was examined by RT-PCR in MCF7 and MB231 cells. (c) The effect of VPA on NIK protein levels was examined by western blotting in the MCF7 and MB231 cells. (d) Effect of VPA on *NIK* promoter activity. One day after transfection of empty or *NIK* promoter reporter plasmid into MCF7 cells, the cells were treated with VPA for 24 h. (e) The effect of VPA on p100 processing to p52 was analyzed by western blotting in the MCF7 cells. NS, not significant.

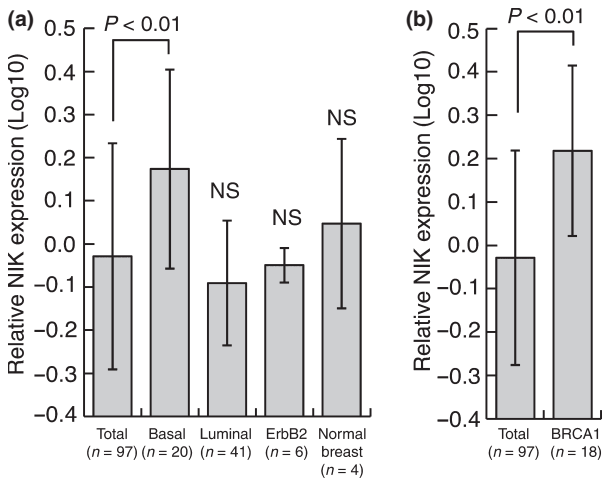


**Fig. 5.** Enhanced NF-κB-inducing kinase (NIK) expression in the basal-like subtype of primary breast cancer specimens. (a) Comparison of *NIK* mRNA levels among various subtypes of primary breast cancer specimens. Based on the gene expression profiles of breast cancers published by van't Veer *et al.*[19] Sørlie *et al.*[1] classified 97 samples into 20 basal-like, 41 luminal-like, six ERRB2-overexpressing, and four normal breast-like subtypes. *P*-values were determined for the difference between each group and unclassified data. Basal-like, 0.00130; luminal-like, 0.173; ErbB2, 0.839; and normal breast-like, 0.543. (b) Comparison of *NIK* mRNA levels between sporadic and *BRCA1*-mutated tumor samples. Expression levels of *NIK* mRNA were estimated based on the dataset of van't Veer *et al.*

MCF7 (luminal) cells revealed no CpG methylation (Fig. S1). It has recently been reported that *RUNX3*, a tumor suppressor gene, is suppressed by histone H3-Lys-9 dimethylation catalyzed by the G9a/G9a like protein 1 (GLP) complex without involving CpG methylation.[16] Furthermore, treatment of cells with a DNA methyltransferase inhibitor, 5-aza-2-deoxycytidine, reduced the level of histone H3-Lys-9 dimethylation and abolished this hypoxic RUNX3 silencing independently of cytosine methylation. Interestingly, our microarray data[17] indicate that expression of *GLP*, which is necessary for G9a/GLP histone methyltransferase activity,[18] was significantly reduced in the basal-like subtype when compared with the luminal-like subtype (Fig. 3b). These results strongly suggest that NIK expression is suppressed by histone H3-Lys-9 dimethylation in the luminal-like subtypes.

On the other hand, a ChIP assay revealed that histone acetylation in the 5′-regulatory region of the *NIK* promoter was significantly higher in MB231 (basal) cells than in MCF7 (luminal) cells (Fig. 4a). Moreover, treatment with the histone deacetylase (HDAC) inhibitor VPA significantly induced *NIK* mRNA expression in MCF7 (luminal) cells, but not in MB231 (basal) cells (Fig. 4b). Co-treatment with MG132 and VPA caused significant accumulation of the NIK protein in MCF7 (luminal) cells to a level similar to that in MG132-treated MB231 (basal) cells (Fig. 4c). Moreover, the VPA treatment scarcely affected activity of the *NIK* promoter in the transiently transfected reporter plasmid in MCF7 (luminal) cells (Fig. 4d). Processing of p100 to p52 was enhanced by the VPA treatment in MCF7 (luminal) cells (Fig. 4e), indicating that accumulated NIK protein by the VPA treatment activates the non-canonical NF-κB pathway. These data suggest that NIK expression is suppressed by HDAC-mediated histone deacetylation of the *NIK* promoter region in MCF7 (luminal) cells.

**NIK expression is increased in sporadic basal-like subtype and BRCA-1-mutated primary breast cancers.** To investigate NIK expression levels among the subtypes of primary breast cancer, we used a published dataset of human breast cancer specimens. Sørlie *et al.*[1] classified 97 breast cancer specimens by hierarchical clustering based on gene expression profiles that had been

published previously.[19] These specimens were classified into 20 basal-like, 41 luminal-like, six ERBB2 over-expressing and four normal breast-like subtypes. Compared with total specimens, only the basal-like subtypes showed significantly elevated NIK expression (Fig. 5a). We then compared NIK expression in the total specimens with that in the specimens carrying *BRCA1* germline mutations. Tumors with *BRCA1* mutations showed a higher expression of *NIK* mRNA (Fig. 5b), which is consistent with the report that tumors with *BRCA1* mutations are classified as basal-like subtypes based on their gene expression profiles.[20]

## Discussion

It is becoming increasingly apparent that gene expression is governed by epigenetic changes even without genetic abnormalities. Various aberrant epigenetic alterations result in gene expression patterns that are critical for cancer initiation and development.[21] Whether the chromatin structure takes an ''open'' or ''closed'' form is determined by histone acetyltransferases (HAT), HDAC and histone methyltransferases (HMT). Histone acetyltransferase-mediated acetylation of lysine residues in histones such as Lys-9 and Lys-14 in histone-H3 results in an open chromatin structure.[22] Histone deacetylase-mediated deacetylation leads to closed chromatin structure and transcriptional repression.[23] Histone methyltransferases transfer methyl groups to the lysine residues in histones such as Lys-4 and Lys-9 in histone-H3. In particular, Lys-9 methylation controlled by the G9a/GLP complex is implicated in gene repression.[24] Moreover, CpG dinucleotides in genome DNA can be methylated by DNA methyltransferases after the formation of the closed chromatin.[25]

In this paper, we demonstrate that NIK expression in the luminal-like subtype is significantly increased by treatment with VPA, a class I HDAC inhibitor (Fig. 4b,c), and that histone-H3 acetylation in the *NIK* promoter region is significantly higher in the basal-like rather than the luminal-like subtype (Fig. 4a). These data strongly suggest that NIK expression is constitutively suppressed by class I HDAC activity in the luminal-like subtype. We also demonstrate that treatment of 5-azacytidine, an inhibitor of CpG methylation and the G9a/GLP-catalyzed histone H3-Lys-9 methylation, increases *NIK* mRNA expression in the luminal-like subtypes (Fig. 3a), while no CpG methylation was observed in the *NIK* promoter region (Fig. S1). Moreover, expression of GLP was significantly reduced in the basal-like subtypes (Fig. 3b). Taken together, our results strongly suggest that NIK expression is suppressed by histone H3 deacetylation and histone H3-Lys-9 dimethylation in the luminal-like subtypes. NIK expression appears to be induced due to chromatin opening mediated by demethylation and acetylation of histone H3 in the basal-like subtype.

We analyzed NIK expression in primary breast cancer specimens in addition to the breast cancer cell lines. A significant augmentation of NIK expression was observed in the basal-like subtype specimens and tumors carrying *BRCA1* mutations, which frequently display a basal-like phenotype.[20] There are several potential applications of this finding in the therapeutic strategy of human breast cancer. One strategy is to suppress NIK expression in basal-like breast cancers. We have previously reported that siRNA-mediated silencing of NIK resulted in reduced NF-κB activation, which is important for cell-cycle promotion in basal-like subtypes.[7] Therefore, proliferation of sporadic and *BRCA1* mutation-induced basal-like breast cancers is expected to be inhibited by NIK suppression. The other strategy is to treat the basal-like subtypes with cIAP antagonists. cIAP is necessary for tumor necrosis factor-α–induced NF-κB activation in the canonical pathway, whereas cIAP induces degradation of NIK in the non-canonical pathway. cIAP antagonists induce degradation of the cIAP protein, which results in the accumulation of NIK proteins and non-canonical NF-κB activation, leading to the induction of TNF-α. Secreted TNF-α in turn induces apoptosis since NF-κB-mediated anti-apoptotic signaling in the canonical pathway is blocked by cIAP antagonists.[26,27] Our data showed that NIK is significantly accumulated by treatment with proteasome inhibitor MG132 only in the basal-like subtypes (Fig. 1d). Therefore, it is possible that cIAP antagonists induce sufficient accumulation of the NIK protein to activate the non-canonical pathway in the basal-like subtypes, which results in the abundant secretion of TNF-α to induce their apoptosis.

Finally, our results strongly suggest that expression of the *NIK* gene is regulated by its epigenetic alterations mediated by acetylation and methylation of histone H3. Further studies on the regulation of NIK expression in breast cancer cells are required to develop therapeutic strategies against basal-like breast cancers.

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## Disclosure Statement

The authors have no conflict of interest.

## References

1 Sorlie T, Tibshirani R, Parker J *et al.* Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci USA* 2003; **100**: 8418–23.
2 Rakha E, Reis-Filho J, Ellis I. Basal-like breast cancer: a critical review. *J Clin Oncol* 2008; **26**: 2568–81.
3 Vargo-Gogola T, Rosen J. Modelling breast cancer: one size does not fit all. *Nat Rev Cancer* 2007; **7**: 659–72.
4 Schmitz M, Mattioli I, Buss H, Kracht M. NF-kappaB: a multifaceted transcription factor regulated at several levels. *Chembiochem* 2004; **5**: 1348–58.
5 Inoue J, Gohda J, Akiyama T, Semba K. NF-kappaB activation in development and progression of cancer. *Cancer Sci* 2007; **98**: 268–74.
6 Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature* 2006; **441**: 431–6.
7 Yamaguchi N, Ito T, Azuma S *et al.* Constitutive activation of nuclear factor-kappaB is preferentially involved in the proliferation of basal-like subtype breast cancer cell lines. *Cancer Sci* 2009; **100**: 1668–74.
8 Keats J, Fonseca R, Chesi M *et al.* Promiscuous mutations activate the noncanonical NF-kappaB pathway in multiple myeloma. *Cancer Cell* 2007; **12**: 131–44.
9 Saitoh Y, Yamamoto N, Dewan MZ *et al.* Overexpressed NF-kappa B-inducing kinase contributes to the tumorigenesis of adult T-cell leukemia and Hodgkin Reed-Sternberg cells. *Blood* 2008; **111**: 5118–29.
10 Soule H, Maloney T, Wolman S *et al.* Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res* 1990; **50**: 6075–86.


doi: 10.1111/j.1349-7006.2010.01685.x

11 Yamaguchi N, Oyama T, Ito E *et al.* NOTCH3 signaling pathway plays crucial roles in the proliferation of ErbB2-negative human breast cancer cells. *Cancer Res* 2008; **68**: 1881–8.
12 Frommer M, McDonald L, Millar D *et al.* A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci USA* 1992; **89**: 1827–31.
13 Shang Y, Hu X, DiRenzo J, Lazar M, Brown M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell* 2000; **103**: 843–52.
14 Zarnegar B, Wang Y, Mahoney D *et al.* Noncanonical NF-kappaB activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and TRAF3 and the kinase NIK. *Nat Immunol* 2008; **9**: 1371–8.
15 Neve R, Chin K, Fridlyand J *et al.* A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* 2006; **10**: 515–27.
16 Lee S, Kim J, Kim W, Lee Y. Hypoxic silencing of tumor suppressor RUNX3 by histone modification in gastric cancer cells. *Oncogene* 2009; **28**: 184–94.
17 Ito E, Honma R, Yanagisawa Y *et al.* Novel clusters of highly expressed genes accompany genomic amplification in breast cancers. *FEBS Lett* 2007; **581**: 3909–14.
18 Tachibana M, Ueda J, Fukuda M *et al.* Histone methyltransferases G9a and GLP form heteromeric complexes and are both crucial for methylation of euchromatin at H3-K9. *Genes Dev* 2005; **19**: 815–26.
19 van 't Veer L, Dai H, van de Vijver M *et al.* Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002; **415**: 530–6.
20 Foulkes W, Stefansson I, Chappuis P *et al.* Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. *J Natl Cancer Inst* 2003; **95**: 1482–5.
21 Lund A, van Lohuizen M. Epigenetics and cancer. *Genes Dev* 2004; **18**: 2315–35.
22 Roth S, Denu J, Allis C. Histone acetyltransferases. *Annu Rev Biochem* 2001; **70**: 81–120.
23 Thiagalingam S, Cheng K, Lee H, Mineva N, Thiagalingam A, Ponte J. Histone deacetylases: unique players in shaping the epigenetic histone code. *Ann N Y Acad Sci* 2003; **983**: 84–100.
24 Dambacher S, Hahn M, Schotta G. Epigenetic regulation of development by histone lysine methylation. *Heredity* 2010; **105**: 24–37.
25 Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev* 2002; **16**: 6–21.
26 Varfolomeev E, Blankenship J, Wayson S *et al.* IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. *Cell* 2007; **131**: 669–81.
27 Vince J, Wong W, Khan N *et al.* IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. *Cell* 2007; **131**: 682–93.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** CpG methylation status in the NF-κB-inducing kinase (*NIK*) promoter region of the MCF7 cells. Bisulfite sequencing analysis was performed, as described in the Materials and Methods. Open or filled circles represent unmethylated or methylated CpG dinucleotides, respectively. The −684/−313 fragment amplified from untreated or *in vitro* methylated *NIK* promoter (−8017/+76) was used as a negative or positive control, respectively.

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