1 **Aberrant accumulation of NIK promotes tumorigenicity by** 2 **dysregulating post-translational modifications in breast cancer**

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28

29 **Keywords**

30 NIK, Non-canonical NF- κ B, Post-translational regulation, in vivo selection, Orthotopic
31 xenograft, Breast cancer

32

33 **Abstract**

34 Post-translational modifications and mRNA translation are frequently altered in human cancers.
35 However, investigations to understand their roles in the cancer progression mechanism remain
36 insufficient. In this research, we explored protein levels altered by translational or
37 post-translational regulation by analyzing transcriptome and western blotting data of the highly
38 malignant breast cancer cell lines. From these analyses, NIK was found to be upregulated at the
39 protein level to predominantly activate the non-canonical NF- κ B pathway in a breast cancer cell
40 line. Furthermore, the increase in NIK protein production was attributed to the dysregulation of

41 ubiquitin-proteasome system caused by a decrease in the translation of cIAP1. NIK upregulation
42 contributed to tumorigenicity by regulating the expression of inflammatory response-related
43 genes. Collectively, our study suggests that NIK is post-translationally modified and has the
44 potential to be a therapeutic target and diagnostic marker for breast cancer.
45

46 **Introduction**

47 Breast cancer is the most common cancer among women worldwide ¹. Although breast
48 cancer mortality has decreased for three decades since the 1990s, the decreasing trend in
49 mortality has slowed in recent years ^{1,2}. Breast cancer has been traditionally categorized into
50 four molecular subtypes (luminal A, luminal B, human epidermal growth factor receptor type 2
51 (HER2) -enriched, and triple-negative) based on the expression of pathological marker proteins
52 such as HER2, estrogen receptor (ER), and progesterone receptor (PR). In particular,
53 triple-negative breast cancer (TNBC) constitutes approximately 10%~20% of breast cancers and
54 is characterized by defects in HER2, ER, and PR expression ^{3,4}. TNBC presents a relatively poor
55 prognosis, with metastases occurring more frequently than in other subtypes despite the limited
56 molecular therapeutic targets ^{3,5}. Therefore, it is necessary to explore innovative therapeutic
57 targets by further elucidating the malignant mechanism of TNBC.

58 In normal cells, signal transductions are tightly regulated through translational and
59 post-translational modification mechanisms, while , in cancer cells, abnormalities in
60 translational and post-translational modifications contribute to their malignancy ^{6,7}. During the
61 translation process, oncogenic signaling activation, such as through the mTOR and RAS-MAPK
62 pathways, induces an enhancement in eIF4F complex expression and phosphorylation, thereby
63 promoting global translation and contributing to cancer malignancy ⁶. On the other hand,
64 unusual tRNA modification and RNA conformations also contribute to cancer progression by
65 promoting the translation of specific genes ⁸⁻¹⁰. Post-translational modifications involve a large
66 variety of mechanisms. For example, ubiquitination, one of the major post-translational
67 modifications, has a large number of regulatory functions, including proteostasis, signaling
68 complex assembly, chromatin remodeling, and protein secretion ⁷. In particular, protein

69 degradation by ubiquitination is essential for the regulation of signaling pathways, such as
 70 MAPK, NF- κ B and PI3K-AKT-mTOR, which play important roles in cell growth and survival ⁷.
 71 Disruption of ubiquitin-modifying machinery can lead to the malignant transformation of cancer
 72 and a variety of other diseases ^{7,11}. To elucidate mechanisms of cancer malignancy,
 73 transcriptome analysis is frequently used, and the development of analysis at single cell level
 74 has made it possible to conduct more detailed analysis ¹². However, it is also necessary to
 75 conduct analyses integrating transcriptome analysis, translational and post-translational
 76 modification evaluations at the protein level.

77 *In vivo* investigations with cancer cell line-derived models have contributed to the
 78 understanding of cancer biology and cancer hallmarks ¹³. Animal models enable to analyze the
 79 functions of certain genes involved in cancer malignancy and the evaluation of the antitumor
 80 properties of preclinical candidate drugs. Among these models, xenograft models have been
 81 frequently utilized to assess tumorigenicity at particular organs and metastases to distant organs.
 82 Xenograft models are classified into two categories: orthotopic and ectopic. In particular, an
 83 orthotopic xenograft (OX) model, which mimics early cancer progression, is appropriate to
 84 comprehensively understand tumorigenesis and metastatic mechanisms. Recently we established
 85 a cell line, LM05, with high tumorigenic and lung-metastatic properties from a TNBC cell line
 86 MDA-MB-231 using an OX model ¹⁴. Interestingly, this cell line showed an different expression
 87 profile from that of another lung-metastatic cell line, LM1-2-1, established by tail vein injection
 88 (TVI) that corresponds to an ectopic xenograft model ^{14,15}. However, the molecular mechanisms
 89 of high tumorigenic and lung-metastatic properties of LM05 cells have not yet been studied in
 90 detail.

91 In this research, we comparatively analyzed western blot and microarray data of LM05
92 cells to discover their specific activating signals in comparison with parental MDA-MB-231
93 cells and LM1-2-1 cells. As a result, we identified that the non-canonical NF- κ B pathway was
94 constitutively activated in LM05 cells via NIK upregulation at the protein level. The aberrant
95 accumulation of NIK due to dysregulation of post-translational modifications promoted
96 tumorigenicity via a cancer-inducing inflammatory response.

97

98

99 **Materials and Methods**

100 *Cell culture*

101 MDA-MB-231-*mSlc7a1-luc2* (parental cells), LM05 and LM1-2-1 cells were established
102 as previously described ¹⁴. These cell lines were cultured in RPMI-1640 (Fujifilm Wako Pure
103 Chemical Corporation, Osaka, Japan) supplemented with 10% heat-inactivated FBS (Nichirei
104 Biosciences Inc., Tokyo, Japan), 100 U/mL penicillin (Meiji Seika Pharma Co., Ltd., Tokyo,
105 Japan) and 100 μ g/mL streptomycin (Meiji-Seika Pharma) at 37°C in 5% CO₂. Plat-E packaging
106 cells were kindly provided from T. Kitamura (Institute of Medical Science, University of Tokyo),
107 and TIG-3 cells were kindly provided from B. Shiotani. These cells were cultured in DMEM
108 (Fujifilm Wako Pure Chemical Corporation) supplemented with 10% heat-inactivated FBS, 100
109 U/mL penicillin, and 100 μ g/mL streptomycin.

110

111 *Western blotting*

Western blotting was performed as previously described¹⁶. Cells were lysed in 1× SDS sample buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 0.1% BPB, 10% glycerol) and then boiled at 95°C for 5min. To examine NIK protein level, cells were treated with 10 μM MG132 (Peptide Institute, Inc., Osaka, Japan) for 4 hours. To assess cIAP1 protein stability, cells were treated with 10 μg/mL cycloheximide (CHX) (Fujifilm Wako Pure Chemical Corporation). In addition, hypotonic buffer (10 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, protease inhibitor cocktail, and 0.15 mM DTT) was used to fractionate nuclear lysates and cytoplasmic lysates. Then, 3× SDS sample buffer was added to each lysate. The lysates were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore, Darmstadt, Germany). Finally, the target proteins were detected using Immobilon™ Western (Millipore). Information regarding the primary antibodies and secondary antibodies is described in Supplementary Table S1.

Immunoprecipitation

Breast cancer cells were lysed in TNE buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 2 mM EDTA, 25 mM NaF, 17.5 mM β-glycerophosphate, 1 mM Na₃VO₄, cOmplete (Roche, Basel, Switzerland)). The protein concentrations of the cell lysates were measured by the BCA method (Thermo Fisher Scientific, Waltham, MA, USA). Then, each protein lysate was added to a NIK antibody (#4994, Cell Signaling Technology, Danvers, MA, USA) or a rabbit IgG antibody (12-370, Millipore) and mixed with rotation overnight. After mixing, 20 μL of Protein A Sepharose 4 FF (GE Healthcare Japan, Tokyo, Japan) was added and mixed with rotation at 4°C for 3 hours. Subsequently, the supernatant was removed after centrifugation, and the Protein A Sepharose was washed five times with TNE Buffer. Finally, 1× SDS sample buffer

135 was added after removing the supernatant followed by heating at 95°C for 5 minutes. This
136 extract solution was used as the immunoprecipitation sample.

137

138 *Animal experiments*

139 Animal experiments were performed in compliance with the guidelines of the Institute for
140 Laboratory Animal Research, National Cancer Center Research Institute (experimental number:
141 T18-009) and the Animal Committee of Waseda University (accession numbers: WD19-058,
142 2019-A068, WD20-005, 2020-A067, WD21-082, 2021-A074). Female
143 NOD.CB-17-*Prkdc*-scid/J mice (NOD-SCID, 5–6 weeks old, Charles River Laboratories Japan,
144 Inc., Kanagawa, Japan) were used for the orthotopic xenograft and tail vein injection models.
145 The methods for establishing the xenograft metastasis model and tail vein injection model and
146 performance of bioluminescence imaging were previously described¹⁴. For orthotopic
147 xenografts, a total of 1.0×10^6 cancer cells in 10 μ l of D-PBS(-) (Fujifilm Wako Pure Chemical
148 Corporation) were implanted into the fourth fat pad of each mouse using a 28-gauge syringe.
149 When the primary tumor volumes reached 300 mm³, they were resected under anesthesia with
150 2.5% isoflurane (Fujifilm Wako Pure Chemical Corporation). After the primary tumors were
151 resected, mice were periodically monitored for metastasis formation using an *in vivo* imaging
152 system (IVIS) -Lumina XRMS (Perkin-Elmer, Waltham, MA, USA) for 2 weeks. For the tail
153 vein injection model, a total of 5.0×10^5 cancer cells in 100 μ l of D-PBS(-) (Wako Pure
154 Chemical Industries) were injected into the tail vein of each mouse using a 27-gauge needle. To
155 monitor these xenograft mice, 200 μ L of D-luciferin (15 mg/mL) (Gold Biotechnology, Inc., St.
156 Louis, MO, USA) was injected intraperitoneally into each mouse, and bioluminescence imaging
157 was performed with the IVIS every week. In addition, the bioluminescence of the lung

158 metastatic tissue was measured ex vivo using the IVIS after intraperitoneal administration of
159 200 μ L of D-luciferin to assess the metastatic potential of the cancer cells.

160

161 *Histochemical analyses*

162 The dissected primary tumors and lung metastasis tissues from the orthotopic mouse model
163 were fixed with 4% paraformaldehyde–PBS and embedded in paraffin. The paraffin-embedded
164 human breast cancer cells with a breast tissue microarray (BC081116d, US Biomax, MD, USA)
165 were baked for 2 hours at 60°C before proceeding with the following steps. The paraffin
166 sections were deparaffinized and rehydrated in xylene, a graded ethanol series that decreased
167 stepwise from 100% to 50%, and distilled water.

168 For immunohistochemistry (IHC) analysis, antigen retrieval was performed in 10 mM
169 citrate buffer (pH 6.0) at 120°C or 95°C for 20 minutes. After cooling to room temperature, the
170 endogenous peroxidase activity was blocked with 3% H₂O₂ in 10% methanol for 30 min. Then,
171 these sections were incubated in 2.5% normal horse serum (Vector Laboratories, CA, USA) for
172 1hour at room temperature. The specimens were incubated with primary antibodies against
173 α -SMA (A2547, Sigma-Aldrich Co., MO, USA), Ki-67 (ab16667, Abcam, Cambridge, UK),
174 CD206 (AF2535, R&D Systems, Inc., MN, USA), CAM5.2 (#349205, BD Bioscience, CA,
175 USA), and NIK (HPA027269, Sigma-Aldrich Co.) at 4°C overnight. Then, the sections were
176 incubated with the secondary antibody solutions for 1hour at room temperature. The sections
177 were stained with an ImmPACT® DAB EqV substrate kit (Vector Laboratories) followed by
178 staining with hematoxylin, dehydration and mounting. Images were acquired with a BZ-X700
179 microscope (Keyence Corporation, Osaka, Japan) and analyzed using the image analysis

180 application for BZ-X700 (Keyence Corporation) and ImageJ software (National Institutes of
181 Health).

182 For hematoxylin and eosin (HE) staining, deparaffinized and rehydrated sections were
183 stained in Mayer's hematoxylin solution (Muto Pure Chemicals Co., Tokyo, Japan) for 10min.
184 Then, the sections were soaked in 0.1% saturated lithium carbonate at 37°C for 5min. After
185 washing in distilled H₂O, the sections were stained with eosin solution (Muto Pure Chemicals
186 Co.) for 10min. Then, the sections were immediately washed with 100% ethanol, 90% ethanol
187 and xylene. Finally, the sections were mounted, and images were acquired with a BZ-X700
188 microscope.

189 For terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining,
190 deparaffinized and rehydrated sections were stained with a TUNEL Assay Kit (ab206386,
191 Abcam) according to the manufacturer's protocols. After staining with hematoxylin,
192 dehydration and mounting, images were acquired with a BZ-X700 microscope (Keyence
193 Corporation).

194

195 *RNA-seq analysis*

196 Total RNA was extracted with QIAzol (Qiagen, CA, USA) according to the manufacturer's
197 protocol. After purification of the total RNA, the quantity and quality of the RNA were
198 evaluated with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific). cDNA
199 libraries for RNA sequencing were established from total RNA using NEBNext Poly(A) mRNA
200 Magnetic Isolation Module (New England Biolabs, MA, USA) to select poly-A mRNA
201 followed by strand-specific library preparation using MGIEasy RNA Directional Library Prep

202 Set V2.0 (MGITech Co. Shenzhen, China). Paired-end sequencing with a lead length of 150
203 bases was performed on a DNBSEQ-G400 (MGI tech) platform following the manufacturer's
204 instructions.

205 The raw sequence data (fastq files) were trimmed to remove adapter sequences by
206 Trim-Galore (v0.6.4), and then, the trimmed read data were mapped on GRCh38/hg38
207 (GENCODE) using HISAT2 (v2.2.0). After the SAM files were converted Bam files using
208 SAMtool (v1.10), the gene expression FPKM was quantified by StringTie (v2.1.2). Then, read
209 count matrices were generated for each gene by prepDE.py. After removing low-expression
210 genes with less than 10 counts per million (CPM), differentially expressed genes were identified
211 using edgeR in R. Statistical cutoffs based on a p-value <0.05 and a log fold change (Log2(FC))
212 ± 1 were used to filter DEGs between LM05-shGFP cells and LM05-shNIK cells. The z scores
213 of these DEGs were calculated by gene filtering, and hierarchical clustering heatmaps were
214 created using pheatmap in R (v3.6.2). For pathway analysis, the expression data were analyzed
215 with gene set enrichment analysis (GSEA; <https://www.gsea-msigdb.org/gsea/index.jsp>) and
216 ingenuity pathway analysis
217 (IPA; [https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis](https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-ipa/)
218 [-and-visualization/qiagen-ipa/](https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-ipa/)).

219

220 *Statistical analysis*

221 All statistical analyses were performed with GraphPad Prism 7 (GraphPad Software, San
222 Diego, CA, USA). Statistical results are presented as the mean $\pm$ SEM. Welch's t test and
223 one-way or two-way ANOVA were employed for comparisons between two or three groups of
224 data.

225

226 *Data availability*

227 RNA-seq data were submitted to the NCBI GEO database under accession number
228 GSE182261. The microarray expression data of the breast cancer cells that can generate lung
229 metastases were obtained from our previous research ¹⁴. These expression data and other
230 supporting data of this research are available from the corresponding authors upon reasonable
231 request.

232

233 Other experimental methods are included in Supplementary Methods.

234

235

236 **Results**

237 *The non-canonical NF- κ B pathway is constitutively activated in LM05 cells*

238 We previously established two lung metastatic breast cancer cell lines (LM05 and
239 LM1-2-1) ¹⁴. LM05 cells were established from MDA-MB-231 cells (parental cells) by two
240 cycles of the generation of OX and subsequent extraction from the lung metastatic tissue (Fig.
241 1A). On the other hand, LM1-2-1 cells were established from parental cells by two cycles of
242 TVI and subsequent extraction from the lung metastatic tissue (Fig. 1A). LM05 cells have a
243 high potential for tumorigenicity in the fat pads compared with the parental cells and LM1-2-1
244 cells (Fig. 1B) ¹⁴. In previous studies, transcriptome analysis of these highly malignant cell lines
245 was performed as a conventional research strategy ¹⁷⁻¹⁹. In this research, considering the
246 importance of translational and post-translational modifications in cancer malignancy, we

investigated proteins that showed changes in amount and/or modification after the translation process by comparison between western blotting and the microarray expression data to discover the factors that enhanced tumorigenicity. We employed LM1-2-1 cells as the comparison subject to search for specific factors in LM05 cells. The analysis results showed that the amounts of mRNA and protein related with EGFR-MAPK, AKT-mTOR and JAK-STAT pathways were not changed (Fig. 1C, D), although the protein level of NF- κ B2 (p52) increased (Fig. 1D). The activation of NF- κ B signaling results in nuclear translocation of the NF- κ B family of proteins: p105/p50 (NF- κ B1), p100/p52 (NF- κ B2), RelA (p65), and RelB^{20,21}. Therefore, we investigated the nuclear translocation of the NF- κ B family proteins by western blotting to evaluate the activity of NF- κ B signaling in LM05 cells. Signaling analysis revealed that NF- κ B2 (p52) and RelB increased in the nuclear fraction of LM05 cells compared with the parental cells and LM1-2-1 cells (Fig. 1E). On the other hand, p50 and RelA in the nuclear fraction were unchanged in LM05 cells (Fig. 1E). Since p52 and RelB are classified as part of the non-canonical NF- κ B pathway, we examined the amounts of NF-kappa- β -inducing kinase (NIK), a key regulator of the non-canonical NF- κ B pathway. NIK is rapidly degraded by ubiquitin-proteasome system after ubiquitinated with TRAF2-TRAF3-cIAP1/2 complex^{22,23}. Therefore, we investigated the amounts of NIK protein in the presence of a proteasome inhibitor, MG132. As a result, NIK was found to be increased at the protein level even though NIK mRNA expression was not significantly changed in LM05 cells (Fig. 1F). These results indicated that the non-canonical NF- κ B pathway was constitutively activated in LM05 cells via NIK upregulation at the protein level.

268

269 *NIK upregulation was induced by suppression of nascent cIAP1 protein production*

270 NIK is constantly degraded in the normal state by the TRAF2-TRAF3-cIAP1/2 complex in
 271 the ubiquitin-proteasome system^{22,23}. We therefore examined the ubiquitination of NIK by
 272 immunoprecipitation with a NIK antibody. Ubiquitination of NIK was found to be decreased in
 273 LM05 cells compared with parental cells (Fig. 2A). Next, we examined the amounts of cIAP1,
 274 which acts as an E3 ligase to ubiquitinate NIK^{22,23}. cIAP1 was found to be decreased at the
 275 protein level even though cIAP1 mRNA expression was unchanged compared to the parental
 276 and LM05 cells (Fig. 2B). Notably, NIK upregulation in LM05 cells was suppressed by cIAP1
 277 overexpression (Supplementary Fig. S1A). From these results, the mechanism of NIK
 278 upregulation in LM05 cells was dependent on cIAP1 reduction. Next, we evaluated the protein
 279 stability of cIAP1 by cycloheximide chase analysis. These results demonstrated that the cIAP1
 280 reduction at the protein level was not ascribed to protein degradation in LM05 cells (Fig. 2C). In
 281 previous studies, it was established that nascent cIAP1 production was occasionally regulated
 282 by translational mechanisms^{24–26}. Hence, we evaluated the production of the nascent cIAP1
 283 protein pulse-labeled with L-homopropargylglycine (HPG) as a methionine analog conjugated
 284 to a biotin tag using a click reaction. The results showed that HPG-labeled cIAP1 protein
 285 production decreased in LM05 cells compared with parental cells (Fig. 2D). All of these results
 286 indicated that the downregulation of cIAP1 translation caused NIK stabilization, which
 287 contributed to the constitutive activation of the non-canonical NF-κB pathway.

288

289 *NIK knockdown reduced colony formation activity in LM05 cells*

290 To determine the contribution of NIK to cancer malignancy, we characterized the
 291 phenotype of LM05 cells after NIK knockdown *in vitro* (Fig. 3A). NIK knockdown cells
 292 depressed the non-canonical NF-κB pathway by suppressing the nuclear localization of p52 and

293 RelB (Fig. 3B). We next evaluated cell proliferation and anchorage-independent growth with a
 294 soft agar assay. NIK expression did not affect cell growth in planar culture (Fig. 3C), although
 295 the colony formation activity was decreased by NIK knockdown (Fig. 3D). We therefore
 296 assessed the contribution of NIK knockdown to cancer stemness, since NIK is known to be a
 297 supportive factor of cancer stemness from the results of previous studies^{27,28}. Notably, NIK
 298 knockdown did not bring about changes in the stemness population rate (Fig. 3E) or sphere
 299 formation in the mammosphere culture assay (Fig. 3F). From these results, NIK upregulation in
 300 LM05 cells contributed to colony formation but not cancer stemness.

301

302 *Upregulation of NIK contributed to tumorigenicity in LM05 cells*

303 To investigate whether NIK upregulation contributes to the inherent high tumorigenicity
 304 and potential of LM05 cells to metastasize to the lungs, NIK knockdown cells were
 305 orthotopically injected into the mammary fat pads of NOD-SCID mice. NIK knockdown in
 306 LM05 cells significantly decreased the primary tumor weights and volumes (Fig. 4A and B). In
 307 addition, the reduction in tumorigenicity caused by NIK knockdown was partially rescued by
 308 recovering the ectopic expression of NIK (Supplementary Fig. S2A, B and C). In addition, we
 309 examined the effects of NIK knockdown on metastatic potential by performing a metastasis
 310 assay in an orthotopic xenograft model. To remove the effects from tumorigenicity reduction
 311 created by NIK knockdown, we uniformly resected the primary tumors after they reached a size
 312 of 300 mm³. Under these conditions, NIK knockdown did not affect the potential of LM05 cells
 313 to form lung metastases (Fig. 4C). Furthermore, IHC analysis of lung metastases using an
 314 anti-CAM5.2 antibody, a human-specific cytokeratin, indicated that there were no differences in
 315 the metastatic node areas between the control and NIK knockdown groups (Fig. 4D). These

316 results indicated that NIK upregulation facilitated the inherent tumorigenicity but not lung
317 metastatic potential of LM05 cells.

318 Next, we performed immunohistochemical staining of the primary tumor to investigate the
319 role of NIK in the primary tumor. The number of TUNEL-positive cells (apoptosis marker)
320 increased in the primary tumor after NIK knockdown (Fig. 4E). In addition, the α -SMA-positive
321 region (a cancer-associated fibroblast (CAF) marker) decreased in the primary tumors after NIK
322 knockdown (Fig. 4E). Therefore, we utilized a co-culture system in a Boyden chamber to
323 investigate the effects of NIK on the human fibroblast cell line TIG-3. From the experimental
324 results, it was found that NIK knockdown suppressed the attraction of TIG-3 cells to the cancer
325 cells in the co-culture system (Fig. 4F). On the other hand, α -SMA protein in TIG-3 cells did not
326 change when co-cultured with control and NIK knockdown cells (Fig. 4G), indicating that NIK
327 is involved in attraction of fibroblasts but not induce their activation to CAFs. These results
328 suggest that the suppression of tumorigenesis caused by NIK knockdown is due to increased
329 apoptosis of the tumor cells and decreased induction of CAFs.

330

331 *NIK knockdown suppressed cancer-inducing inflammatory signaling*

332 To determine the NIK-related pathway and genes that contribute to the phenotypes altered
333 by NIK knockdown (Fig. 4A, B and E), we performed transcriptome analysis by RNA-seq. We
334 extracted the differentially expressed genes (DEGs) between LM05-shGFP cells and
335 LM05-shNIK cells using the following criteria: FDR <0.05 (Benjamini-Hochberg method) and
336 log fold change ($\text{Log}_2(\text{FC})$) ± 1 (Fig. 5A). To determine the NIK-related pathways, the common
337 DEGs in the NIK knockdown cells were analyzed by gene set enrichment analysis (GSEA) and
338 ingenuity pathway analysis (IPA). GSEA using the MSigDB Hallmark gene set collection

revealed that the genes with decreased expression in the NIK knockdown cells were significantly enriched for pathways related to inflammation, interferon response, and TNF α signaling via NF- κ B (Fig. 5B, Supplementary Table S2). Similarly, pathways related to the interferon response and TNF secretion were suppressed in NIK knockdown cells using Gene Ontology gene sets (Supplementary Table S3). Furthermore, upstream regulator analysis by IPA contained components of NF- κ B signaling as an upstream regulator of the downregulated pathway (Supplementary Fig. S3A). These transcriptome analyses showed that the inflammatory-related genes regulated by the NF- κ B pathway were suppressed in LM05 NIK knockdown cells. Indeed, NIK knockdown decreased the expression of *IL6* and *CXCL1*, which are important for the migration of fibroblasts (Fig. 5C)^{29,30}. Furthermore, *BIRC3* expression, which contributes to apoptosis resistance, was markedly reduced by NIK knockdown (Fig. 5C)^{31,32}. Based on these results, the reduction in *BIRC3* expression and fibroblast-attracted cytokines and chemokines caused by NIK knockdown inherently result in decreased tumorigenicity.

353

354 *NIK protein production increased in malignant breast cancer tissue*

We then investigated the relationship between NIK production and breast cancer malignancy. We characterized NIK production by immunohistochemistry in a tissue microarray from breast cancer patients (Fig 6A). IHC analysis showed that NIK production was significantly increased in stage I-III patients compared with normal breast tissues (Supplementary Table S4, Fig. 6B). In addition, NIK protein production was not correlated with the expression scores of breast cancer marker genes such as ER, PR, and HER2 (Supplementary Fig. S4A, B and C). From

361 these results, NIK has the potential to be used as a diagnostic marker across other breast cancer
362 subtypes.

363

364

365 **Discussion**

366 Constitutive activation of the non-canonical NF- κ B pathway has been reported in breast
367 cancer cell lines and multiple myeloma cell lines^{33–35}. In breast cancer, basal-like subtype cell
368 lines have shown increased NIK mRNA expression by epigenetic dysregulation of NIK gene,
369 which induces constitutive activation of the non-canonical pathway^{35,36}. On the other hand,
370 certain multiple myeloma cell lines have genetic defects in TRAF2/3 and cIAP1/2^{33,34}, which
371 are repressors of the non-canonical pathway. However, constitutive activation of the
372 non-canonical pathway due to reduced translation of cIAP1, as revealed in this study, is a novel
373 activation mechanism of the non-canonical pathway in breast cancer cell lines (Fig. 6C). In
374 previously, the activation of the non-canonical NF- κ B pathway by NIK upregulation contributes
375 to the enhancement in cell proliferation and maintenance of cancer stemness properties via the
376 NOTCH pathway, especially in basal-like breast cancer cell lines^{27,35}. Although claudin-low cell
377 lines exhibit high levels of NF- κ B activation with increased NIK mRNA expression^{35,36}, the
378 function of NIK in tumorigenesis and metastasis has not been investigated. Our results
379 suggested that NIK upregulation contributed to tumorigenicity by attracting CAFs and
380 inhibiting apoptosis through regulation of the expression of genes related to inflammatory
381 responses in LM05 cells, which is classified as the claudin-low subtype (Fig. 6C). Therefore,
382 there are novel function and aberrant accumulation mechanism of NIK in breast cancer
383 malignancy that differs from the conventional researches.

384 From our results, a reason for the reduction of tumorigenicity by NIK knockdown is the
 385 expansion of the apoptotic area in the primary tumor. In addition, our results suggest that the
 386 decrease in *BIRC3* expression, which has antiapoptotic properties, and CAFs may contribute to
 387 the increase in the apoptotic area. CAFs have been reported to contribute to the enhancement of
 388 tumor malignancy by promoting the antiapoptotic potential and chemoresistance of cancer cells
 389 ^{37,38}. It is possible that a reduction in the number of CAFs in tumors by NIK knockdown
 390 contributes to the decline in the antiapoptotic potency of cancer cells. CXCL12/SDF-1 secreted
 391 by CAFs contributes to tumor growth and anti-apoptosis in cancer cells via CXCR4 ^{39,40}. Indeed,
 392 the expression of *CXCR4* was reduced in NIK knockdown cells; thus, there was a deficiency in
 393 the positive feedback through this paracrine mechanism. Therefore, NIK plays an important role
 394 in the interaction with stromal cells in the cancer microenvironment.

395 NIK is crucial for the maintenance of various tissue functions, including the immune
 396 system, bone formation, the kidneys, the liver, glucose homeostasis and hematopoiesis ⁴¹.
 397 Therefore, abnormal NIK activation has been implicated in a variety of autoimmune diseases,
 398 such as systemic lupus erythematosus, acute kidney injury and cancer ⁴¹. Recently, exploratory
 399 studies of NIK inhibitors have expanded ⁴¹. NIK inhibitors have been developed by modifying
 400 inhibitors of similar kinase family members and conventional NIK inhibitors based on
 401 structure-activity relationships and docking simulations using NIK crystallographic data ^{42,43}.
 402 These studies have identified that NIK inhibitors have therapeutic effects in certain mouse
 403 models of inflammatory hepatic diseases and systemic lupus erythematosus ^{44,45}. In addition,
 404 mangiferin, which is a natural compound with NIK inhibitory activity, has been reported to
 405 inhibit tumorigenesis and metastatic potential in melanoma cell lines ⁴⁶. However, the efficacy
 406 of these NIK inhibitors against other types of cancer is unclear; thus, verification of the activity
 407 of these NIK inhibitors is needed to examine this possibility. In contrast, inactivating NIK

408 mutations have been shown to cause immunodeficiency, such as decreased numbers of mature B
409 cells and T cells their functional impairment⁴⁷. NIK plays an important role in antitumor
410 immunity by regulating metabolism in cytotoxic CD8⁺ T cells⁴⁸. Therefore, it is necessary to
411 develop NIK inhibitors through further comprehension of the reciprocal NIK functions to avoid
412 interfering with the homeostatic role that NIK plays in the body. Our experimental results
413 indicated that NIK knockdown critically affected tumorigenicity rather than the potential to
414 form lung metastases in LM05 cells. From this result, we hypothesized that NIK may play an
415 important role in tumorigenesis mechanisms in the mammary gland. To address this hypothesis,
416 we evaluated the proliferative potential of parental and LM05 cells in lung tissue using tail vein
417 injection. The results showed that there was no significant difference in the proliferative
418 potential of the lung tissues between the parental and LM05 cells (Supplementary Fig. S5A). In
419 addition, LM05 cells were repeatedly xenografted into the mammary gland and grown at this
420 site. Thus, the enhanced tumorigenicity of LM05 is probably mammary gland-specific. Indeed,
421 TWEAK and RANKL, ligands of the non-canonical NF- κ B pathway, are expressed and have
422 important functions in mammary gland development and tumorigenesis^{49–52}. In short, LM05
423 cells may be more susceptible to the activation of the non-canonical pathway by these ligands in
424 breast tissue because of the accumulation of NIK proteins. Meanwhile, we were not able to
425 identify the genes that contribute to the enhanced potential of LM05 cells to metastasize to the
426 lungs. However, we identified a group of genes that were upregulated in LM05 cells compared
427 to the parental strain but not downregulated after NIK knockdown, including known lung
428 metastasis regulatory genes such as *IL13RA2*, *TNSI*, and *EMPI*^{18,53,54} (Supplementary Fig.
429 S6A). Further studies of those genes may reveal the NIK-independent metastasis enhancement
430 mechanism in LM05 cells.

431 In conclusion, our results demonstrated a novel role and mechanism of NIK after its
432 upregulation during breast cancer progression. In addition, the amount of NIK protein was
433 increased in tumor tissues compared to normal tissues; thus, NIK has the potential to be a
434 diagnostic marker of breast cancer. Elucidating the functions and regulatory mechanisms of NIK
435 can lead to a deeper understanding of NIK as a potential biomarker or therapeutic target in
436 breast cancer.

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449

450 **Authors' contributions**

451 J.N. and K.S. conceived and designed the study. Y.H. and J.N. performed the analyses and the
452 experiments. M.Y., M.M., S.W., S.H., J.I. and Y.Y. interpreted the data. Y.H. wrote the original
453 paper. J.N., Y.Y. and K.S. revised the manuscripts. All authors reviewed and edited the
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455

456 **Conflicts of interest**

457 The authors declare that they have no competing interests.

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- 586

587 **Figure legends**

588 **Figure 1. The non-canonical NF- κ B pathway was constitutively activated in LM05 cells via**
 589 **upregulation of NIK at the protein level.**

590 **A.** Schematic representation of the *in vivo* selection process using an orthotopic xenograft (OX)
 591 and tail vein injection (TVI). Luciferase-expressing MDA-MB-231 (parental cells) cells were
 592 transplanted into NOD-SCID mice by OX or TVI. Subsequently, lung metastatic cells were
 593 collected and established from lung tissue with metastases. These cells were reinjected into
 594 NOD-SCID mice using the same xenograft model to concentrate these cells with higher lung
 595 metastatic activity. **B.** Representative *in vivo* bioluminescent images (upper) and tumor growth
 596 curves (lower, n=4 per group, two-way ANOVA followed by Tukey's multiple comparison test)
 597 of NOD-SCID mice orthotopically injected with parental or LM05 cells at 0 week and 5 weeks.
 598 **C.** Integrative signaling analysis of LM05 cells analyzed by microarray expression data. The
 599 microarray expression data results are shown as a heatmap of the log₂-fold change in each cell
 600 compared with the parental cells. **D.** Integrative signaling analysis of LM05 cells analyzed by
 601 western blotting. The western blotting results had four evaluation criteria (yellow: upregulated,
 602 green: downregulated, and gray: unchanged protein production levels compared with parental
 603 cells, diagonal line: undetected protein). **E.** Western blotting analysis of NF- κ B1 (p50), NF- κ B2
 604 (p100/52), RelA and RelB in whole cells and the nuclear and cytoplasmic extracts of the
 605 parental, LM05, and LM1-2-1 cells. **F.** Western blotting (upper) and qRT-PCR (lower, n=3,
 606 Welch's t test) results of NIK protein and mRNA in the parental, LM05, and LM1-2-1 cells. For
 607 western blotting, all cell lines were either untreated or treated with MG132 (10 μ M for 4 hr). All
 608 data are representative of three independent experiments and are shown as the mean $\pm$ SEM. NS,
 609 not significant. * P<0.05.

610

611 **Figure 2. Downregulation of cIAP1 translation caused NIK stabilization, which**
 612 **contributed to constitutive activation of the non-classical NF- κ B pathway.**

613 **A.** Western blotting analysis of ubiquitinated NIK in HA-tagged ubiquitin (HA-UB)-expressing
 614 parental or LM05 cells after treatment with 10 μ M MG132 for 4 hr. The protein lysates of
 615 HA-UB expressed in parental or LM05 cells were immunoprecipitated with an anti-NIK
 616 antibody and then immunoblotted with an anti-HA antibody. **B.** Western blotting (upper) and
 617 qRT-PCR (lower, n=3, Welch's t test) analysis of cIAP1 expression in parental and LM05 cells.
 618 **C.** Assessment of cIAP1 protein stability in parental and LM05 cells treated with 10 μ g/mL
 619 cycloheximide (CHX). Quantification of cIAP1 protein level normalized by α -Tubulin protein
 620 level in each time point (n=3, two-way ANOVA followed by Bonferroni's multiple comparisons
 621 test). **D.** Assessment of nascent cIAP1 protein levels in parental and LM05 cells using a click
 622 reaction. Parental and LM05 cells were treated with 50 μ M L-homopropargyl glycine (HPG) for
 623 24 hr. The nascent proteins labeled with HPG were conjugated to biotin using a click reaction.
 624 The biotinylated proteins were purified with streptavidin beads and subjected to western blotting.
 625 All data are representative of three independent experiments and are shown as the mean $\pm$ SEM.
 626 NS, not significant.

627

628 **Figure 3. NIK upregulation in LM05 cells contributed to colony formation activity but not**
 629 **cancer stemness.**

630 **A.** Validation of NIK knockdown efficiency via Western blot (upper) and qRT-PCR (lower, n=3,
 631 one-way ANOVA followed by Tukey's multiple comparison test) analyses in LM05-shGFP,

shNIK no.1 and shNIK no.2 cells. For western blotting, all cell lines were either untreated or treated with MG132 (10 μ M for 4 hr). **B.** Western blotting analysis of NF- κ B1 (p50), NF- κ B2 (p100/52), RelA and RelB in the nuclear extracts of LM05-shGFP, shNIK no.1 and shNIK no.2 cells. **C.** Cell growth curves of LM05-shGFP, shNIK no.1 and shNIK no.2 cells from planar culture (n=3, one-way ANOVA followed by Tukey's multiple comparison test). **D.** Representative images (upper) and quantification data (lower) (n=5, one-way ANOVA followed by Tukey's multiple comparison test) of the soft agar colony formation assay in LM05-shGFP, shNIK no.1 and shNIK no.2 cells. The scale bar is 5mm. **E.** Representative images (upper) and quantification data (lower) (n=3, one-way ANOVA followed by Tukey's multiple comparison test) from the flow cytometry analysis of the cancer stem cell populations in LM05-shGFP, shNIK no.1 and shNIK no.2 cells. **F.** Representative images (upper) and quantification data (lower) (n=3, one-way ANOVA followed by Tukey's multiple comparison test) of the mammosphere culture assay in LM05-shGFP, shNIK no.1 and shNIK no.2 cells. All data are representative of three independent experiments and are shown as the mean $\pm$ SEM. NS, not significant. * P<0.05.

647

Figure 4. NIK upregulation facilitated inherent tumorigenicity but not the lung metastatic potential of LM05 cells.

A. Representative images of primary tumors (upper) and quantification data of the primary tumor weights (lower) (n=4, one-way ANOVA followed by Tukey's multiple comparison test) in LM05-shGFP, shNIK no.1 and shNIK no.2 cells. The scale bar is 1cm. **B.** Representative *in vivo* bioluminescent images of LM05-shGFP and shNIK no.2 cells (upper). Tumor growth curves (lower) (n=6 per group, two-way ANOVA followed by Tukey's multiple comparison test) of

655 NOD-SCID mice orthotopically injected with LM05-shGFP, shNIK no.1 and shNIK no.2 cells.

656 **C.** Representative *in vivo* bioluminescent images (upper) and quantification data of the lung

657 metastasis tissue (lower) (one-way ANOVA followed by Tukey's multiple comparison test)

658 derived from NOD-SCID mice orthotopically injected with LM05-shGFP (n=5), shNIK no.1

659 (n=4) and shNIK no.2 cells (n=5). **D.** Representative HE and IHC staining images (upper) and

660 quantification data (lower) (one-way ANOVA followed by Tukey's multiple comparison test) of

661 NIK protein production in lung metastasis tissue derived from NOD-SCID mice orthotopically

662 injected with LM05-shGFP, shNIK no.1 and shNIK no.2 cells. The average CAM5.2-positive

663 percentages were calculated from 5 fields of view from n=4 individual lung metastasis slides.

664 The scale bar is 500 μ m. **E.** Representative HE and IHC staining images (upper) and

665 quantification data (lower) (one-way ANOVA followed by Tukey's multiple comparison test)

666 from the TUNEL assay and α -SMA protein production in primary tumor tissues derived from

667 NOD-SCID mice orthotopically injected with LM05-shGFP, shNIK no.1 and shNIK no.2 cells.

668 The average number of TUNEL- and α -SMA-positive cells were calculated from 5 fields of

669 view from n=4 individual primary tumor slides. The scale bar is 500 μ m. **F.** Representative

670 images (upper) and quantification data (lower) (n=5, one-way ANOVA followed by Tukey's

671 multiple comparison test) of the Boyden chamber assay with TIG-3 cells that were co-cultured

672 with LM05-shGFP, shNIK no.1, and shNIK no.2 cells. The scale bar is 500 μ m. **G.** Western

673 blotting (upper) and immunofluorescence staining (lower) of α -SMA expression in TIG-3 cells

674 that were co-cultured with LM05-shGFP, shNIK no.1 and shNIK no.2 cells or noncells (NC).

675 The scale bar is 50 μ m. All data are shown as the mean $\pm$ SEM. NS, not significant. * P<0.05.

676

677 **Figure 5. NIK regulated cancer-inducing inflammatory signaling in LM05 cells.**

678 **A.** Heatmap and Venn diagram of the DEGs from the LM05-shGFP, LM05-shNIK no.1 and
 679 LM05-shNIK no.2 RNA-seq data, each performed in duplicate. Hierarchical analysis of the
 680 heatmap was performed by the complete linkage method. Venn diagram of the signature genes
 681 in LM05-shNIK cells. **B.** GSEA enrichment plot of the hallmark gene sets for the differentially
 682 expressed genes in LM05-shNIK cells compared with LM05-shGFP cells. **C.** Validation of NIK
 683 signature gene expression related to tumor inflammation using qRT-PCR (lower) (n=3, one-way
 684 ANOVA followed by Tukey's multiple comparison test) in LM05-shGFP, shNIK no.1 and
 685 shNIK no.2 cells. All data are shown as the mean±SEM. NS, not significant. * P<0.05.

686

687 **Figure 6. NIK protein production increased in malignant breast cancer tissue.**

688 **A.** Representative IHC staining images of NIK protein production in normal breast tissue and
 689 breast tumors. The scale bar is 500 µm. **B.** Quantification data of the NIK IHC staining images
 690 in normal breast tissue and breast tumors ((n=10 normal, n=6 stage I, n=72 stage II, and n=22
 691 stage III); one-way ANOVA followed by Tukey's multiple comparison test). **C.** The graphical
 692 summary indicates that the abnormal accumulation of NIK due to reduced translation of cIAP1
 693 enhances tumorigenesis by promoting an inflammatory cancer microenvironment. All data are
 694 shown as the mean±SEM. NS, not significant. * P<0.05.

695

696

697 **Supplementary Figure 1. cIAP1 ectopic expression reduced NIK protein production in**
 698 **LM05 cells.**

699 Western blotting analysis of NIK protein production in parental, LM05-Venus and
700 LM05-TAP-cIAP1 (murine) cells. For western blotting, all cell lines were either untreated or
701 treated with MG132 (10 μ M for 4 hr). All data are representative of three independent
702 experiments.

703

704 **Supplementary Figure 2. NIK ectopic expression partially rescued the reduction in**
705 **tumorigenicity induced by NIK knockdown.**

706 **A.** Validation of the NIK rescue efficiency via Western blot analysis in LM05-shGFP, shNIK
707 no.2 and NIK rescue cells. For western blotting, all cell lines were either untreated or treated
708 with MG132 (10 μ M for 4 hr). **B.** Western blotting analysis of NF- κ B1 (p50), NF- κ B2
709 (p100/52), RelA and RelB in the nuclear extracts of LM05-shGFP, shNIK no.2 and NIK rescue
710 cells. **C.** Tumor growth curves (two-way ANOVA followed by Tukey's multiple comparison
711 test) of NOD-SCID mice orthotopically injected with LM05-shGFP (n=6), shNIK no.2 (n=6)
712 and NIK (n=4) rescue cells. All data are representative of three independent experiments and are
713 shown as the mean $\pm$ SEM. NS, not significant. * P<0.05.

714

715 **Supplementary Figure 3. The upstream regulators of the downregulated common DEGs in**
716 **NIK knockdown cells contained some genes associated with NF- κ B signaling.**

717 **A.** Ingenuity pathway analysis suggested statistically significant upstream regulators of the
718 downregulated common DEGs in NIK knockdown cells (selected activation z-score < -2). The
719 bar color indicates the molecule type of the upstream regulator (orange: cytokine, blue:
720 transcription regulator, yellow: kinase, green: receptor). The dotted line shows a p-value = 0.05.

721

722 **Supplementary Figure 4. NIK expression was not correlated with PR, ER, or HER2 scores.**

723 **A.** Quantification data of the NIK IHC staining images of normal breast tissue and breast tumors
 724 for each PR score (n=47 - (negative), n=26 +, n=12 ++, and n=24 +++; one-way ANOVA
 725 followed by Tukey's multiple comparison test). **B.** Quantification data of the NIK IHC staining
 726 images of normal breast tissue and breast tumors for each ER score (n=35 - (negative), n=17 +,
 727 n=26 ++, and n=41 +++; one-way ANOVA followed by Tukey's multiple comparison test). **C.**
 728 Quantification data of the NIK IHC staining images of normal breast tissue and breast tumor for
 729 each HER2 score (n=72 - (negative), n=3 +, n=3 ++, and n=31 +++; one-way ANOVA followed
 730 by Tukey's multiple comparison test).

731

732 **Supplementary Figure 5. The TVI model showed that the lung metastatic potential of**
 733 **LM05 cells was not enhanced compared with parental cells.**

734 **A.** Representative *in vivo* bioluminescent images (left) and quantification data of lung
 735 metastases (right) (one-way ANOVA followed by Tukey's multiple comparison test) derived
 736 from NOD-SCID mouse tail vein injection with parental, LM05, or LM1-2-1 cells (n=5).

737

738 **Supplementary Figure 6. Some known lung metastasis-promoting genes are highly**
 739 **expressed in the LM05 cell line independent of NIK knockdown.**

740 **A.** Venn diagram of the upregulated genes in LM05 cells based on previous microarray
 741 expression data and the downregulated genes found in the NIK KD cell line based on the

742 RNA-seq data from this study. Expression of the known metastasis-promoting genes *IL13RA2*,

743 *TNSI*, and *EMPI* is independent of NIK knockdown.

744

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Fig.S1

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Fig.S2

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Fig.S3

Fig.S4

Fig.S5

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Fig.S6