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ALK is a MYCN target gene and regulates cell migration and invasion in neuroblastoma

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Human anaplastic lymphoma kinase (*ALK*) has been identified as an oncogene that is mutated or amplified in NBLs. To obtain a better understanding of the molecular events associated with *ALK* in the pathogenesis of NBL, it is necessary to clarify how *ALK* gene contributes to NBL progression. In the present study, we found that *ALK* expression was significantly high in NBL clinical samples with amplified *MYCN* ($n = 126$, $P < 0.01$) and in developing tumors of *MYCN*-transgenic mice. Indeed, promoter analysis revealed that *ALK* is a direct transcriptional target of *MYCN*. Overexpression and knockdown of *ALK* demonstrated its function in cell proliferation, migration and invasion. Moreover, treatment with an *ALK* inhibitor, TAE-684, efficiently suppressed such biological effects in *MYCN* amplified cells and tumor growth of the xenograft in mice. Our present findings explore the fundamental understanding of *ALK* in order to develop novel therapeutic tools by targeting *ALK* for aggressive NBL treatment.

Neuroblastoma (NBL) is an embryonal malignancy derived from precursor cells of the sympathetic nervous system, and accounts for 7–10% of childhood cancers and around 15% of cancer deaths in children¹. Though some subsets of NBL undergo spontaneous regression without therapy, about 60–70% of high-risk NBL patients are resistant to currently available therapies and have poor prognoses^{1–3}. The genetic feature most consistently associated with treatment failure is an amplification of the *MYCN* proto-oncogene, which is strongly correlated with advanced disease^{4–7}. Even in otherwise favorable localized disease, *MYCN* amplification indicates poor outcome, underscoring its biological importance. Indeed, upregulation of *MYCN* in NBL cells resulted in accelerated proliferation, migration and invasion^{8–11}. Consistent with these observations, transgenic mice overexpressing *MYCN* in neural crest-derived tissues displayed frequent development of NBL¹², suggesting that upregulated expression of *MYCN* is causative in the genesis and development of NBL *in vivo*. However, the role of *MYCN* expression and its molecular mechanisms to induce an aggressive phenotype are still unclear. Identification of its direct transcriptional target gene(s) may provide a novel insight into understanding the functional contribution of *MYCN* in malignant phenotypes of aggressive NBL.

The *MYC* family of proto-oncogenes belongs to the basic helix-loop-helix leucine-zipper class of transcription factors. *MYC* proteins (*MYCN* and *c-Myc*) share several regions of homology and similar cellular functions that target proliferative pathways vital for cancer progression. Members of this family function as heterodimers with *MAX*, and exert transcriptional activity by specifically binding to a consensus E-box motif (CACGTG) located within the promoter regions of a diverse set of target genes^{13–15}. Although a handful of *MYCN* target genes involved in *MYCN*-driven cell proliferation and apoptosis have been identified, the target genes responsible for *MYCN*-mediated cell migration and invasion remain elusive.

Anaplastic lymphoma kinase (*ALK*) has been identified as a gene upregulated in unfavorable NBL, suggesting a possible oncogenic role for this receptor tyrosine kinase, which was previously linked with NBL^{16,17}. Recently, *ALK* point mutations were described in 3–11% of sporadic NBL, and were found to be one of the most important



types of mutations in hereditary NBL^{18–22}. More recently, Passoni *et al.* described NBL patients with high levels of ALK expression without ALK gene mutations. They showed that regardless of mutation status, high ALK levels were strongly correlated with poor prognosis²³. This correlation between high ALK levels and unfavorable prognosis was also confirmed by some other investigators^{24–26}. Moreover, Di Paolo *et al.* demonstrated that RNA interference (RNAi)-based knockdown of ALK, regardless of its genetic status, showed reduced proliferation and increased apoptosis in NBL cells and inhibited NBL tumor growth as well as prolonged survival *in vivo*²⁷.

In the present study, we found that ALK directly mediates MYCN-induced oncogenic properties. The promoter region of ALK gene contains a non-canonical E-box located upstream of the transcription initiation site, and MYC proteins bind onto the promoter region and regulate its transcription. Wild-type ALK functions as a modulator of proliferation as well as cell migration and invasion. In addition, those biological activities and tumor growth in xenograft model derived from NBL cell lines with MYCN amplification were inhibited by targeting wild-type ALK with TAE-684, suggesting that highly expressed ALK in MYCN amplified cells could be inhibited by ALK inhibitor in the same manner as mutated or amplified ALK. These findings may be beneficial to the understanding of the molecular mechanism of wild-type ALK function, and contribute to the development of a possible therapeutic strategy for ALK-expressing NBLs.

Results

ALK mRNA expression is associated with MYCN amplification and expression in neuroblastomas. The expression of MYCN and ALK mRNA was measured by quantitative real-time PCR (qRT-PCR) for cDNA samples obtained from NBL clinical tissues. MYCN expression was significantly higher in tumors with MYCN amplification than MYCN-non-amplified tumors ($P < 0.001$; Figure 1a). In this subset of NBLs with MYCN amplification, mRNA expression of ALK was significantly higher as compared with MYCN-non-amplified tumors ($P < 0.01$; Figure 1a). High expression of ALK was also observed in NBLs at stages 3, 4 and 4S (Figure 1b), suggesting that ALK might contribute to an aggressiveness and metastasis of NBL.

Both MYCN and c-Myc regulates ALK expression. In a MYCN transgenic mice model, the expression of the human MYCN oncogene was targeted to neural crest cells with the use of a tyrosine hydroxylase promoter¹². This promoter is active in migrating cells of the neural crest early in the development of sympathetic ganglia and the adrenal medulla from which NBLs often arise²⁸. Expression of both human MYCN and endogenous *Alk* mRNA was induced in superior mesenteric ganglion (SMG) tissues of 2-week-old MYCN-hemizygous mice (Figure 1c), which continued in adrenal tumor tissues until the mice were at least 11 weeks old. Consistent with the transgenic mice data, overexpression of MYCN or *c-Myc* in NBL (wild-type ALK cell line; NBL-S and NLF, and mutated ALK cell line; SH-SY5Y) and non-NBL (U2OS and HeLa) cells induced ALK expression in dose- and time-dependent manners at the mRNA (Figures 2a–c and Supplementary Figure S1A) and protein (Supplementary Figures S1B and C) levels. Next, we performed siRNA-mediated knockdown of MYCN experiment in SK-N-AS cells as described previously²⁹, and found that MYCN knockdown decreased expression of ALK (Figure 2d). To confirm a possible relationship between MYCN and ALK, we employed MYCN-inducible neuroblastoma cells (Tet21/N) derived from a parental neuroblastoma cell line, SHEP⁸. The Tet21/N cells constitutively expressed MYCN in the absence of tetracycline (Tc), whereas the addition of Tc to the culture decreased MYCN expression levels. At the indicated time points after Tc depletion, total RNA was prepared and subjected to RT-PCR. As shown in Figure 2e, Tc deprivation led

to an induction of MYCN in association with a significant increase in the expression levels of ALK. In contrast, addition of Tc significantly reduced the expression levels of MYCN with a concomitant decrease in ALK expression levels (Figure 2e), suggesting that ALK might be a direct transcriptional target of MYCN.

ALK is a direct transcriptional target of MYCN and c-Myc. To identify a possible promoter region of ALK gene, we generated luciferase reporter constructs containing –2056 bp to +30 bp fragments of ALK gene. We performed promoter study using NBL (mutated ALK cell line; SH-SY5Y and wild-type ALK cell line; SK-N-AS) and non-NBL (U2OS and HeLa) cells, which showed correlated regulation of ALK gene with MYCN or c-MYC expression (Figures 2b–d and Supplementary Figure S1A). An increase in luciferase activity was observed in cells transfected with pGL4.17 ALK (–2056 bp) compared with empty pGL4.17-basic vector (Figure 3a and Supplementary Figure S2A). The luciferase activity with pGL4.17 ALK (–2056 bp) was enhanced by co-expression of increasing amounts of MYCN or *c-Myc* expression vector (Figure 3b and Supplementary Figure S2B).

Next, we performed ChIP assays with anti-MYCN and anti-c-Myc antibodies to determine whether they could directly bind to the ALK promoter. As shown in Figure 3c, MYCN was recruited onto the ALK promoter region. Endogenous MYCN was recruited onto the same region in TNB1 cells (MYCN amplification and high expression of ALK, Supplementary Figure S7A) but not in RISA cells (MYCN-non-amplified and low ALK expression). Moreover, in non-NBL cell lines, the recruitment of endogenous c-Myc was more obvious in A875 cells (high ALK expression, Supplementary Figure S7B) than HeLa cells (low ALK expression) (Figure 3c). Since the region to which MYC proteins were recruited contains two possible E-boxes (E-box1 and E-box2), E-box deletions were introduced in the luciferase reporter construct (–350 bp). Deletion of the E-box1 and/or E-box2 resulted in a significant reduction of ALK gene promoter activity in SK-N-AS (Figure 3d) and HeLa cells (Supplementary Figure S2C).

ALK shows oncogenic potential in NBL cells. Consistent with previous reports^{23,30}, our results also showed that ectopic expression of wild-type or mutated (F1174L) ALK induced the phosphorylation of both ALK and an ALK-associated signaling molecule, AKT (Supplementary Figure S4A). The siRNA-mediated knockdown of endogenous ALK resulted in reduced phosphorylation of AKT in NBL cells harboring a wild-type allele (Supplementary Figure S4B). Next, we investigated cell growth study using NBL cells (SK-N-DZ, SK-N-AS and NBL-S), which showed activation of the downstream signaling molecule AKT by ALK overexpression (Supplementary Figure S4A). We also performed siRNA mediated knockdown of ALK experiment in MYCN amplified NLF cells. Figure 4a and 4b shows that overexpression of ALK enhanced cell proliferation, whereas siRNA-mediated knockdown of ALK was correlated with an inhibition of proliferation of NBL cells. Colony formation assays also revealed that the number and size of ALK-expressing viable clones were higher than those of vector control cells (Figures 4c and 4d).

To examine whether ALK contributes to metastatic activity of NBL cells, we performed wound healing assays in MYCN-non-amplified NBL (mutated ALK cell line; SH-SY5Y and wild-type ALK cell line; SK-N-AS) and low ALK expressing non-NBL (HeLa) cells, and found that ALK expression enhanced cell migration (Supplementary Figure S5). Consistent with these results, Boyden chamber migration and invasion assays also showed a significant increase in the number of migrated and invaded cells arising from ALK-expressing cells compared with the vector control cells (Figure 5a and Supplementary Figure S6A). This study we performed using MYCN-non-amplified cell line SK-N-AS, as shown downstream signaling molecule AKT was activated upon ALK overexpression (Supplementary Figure S4A), and low ALK expressing non-NBL cell line HeLa.

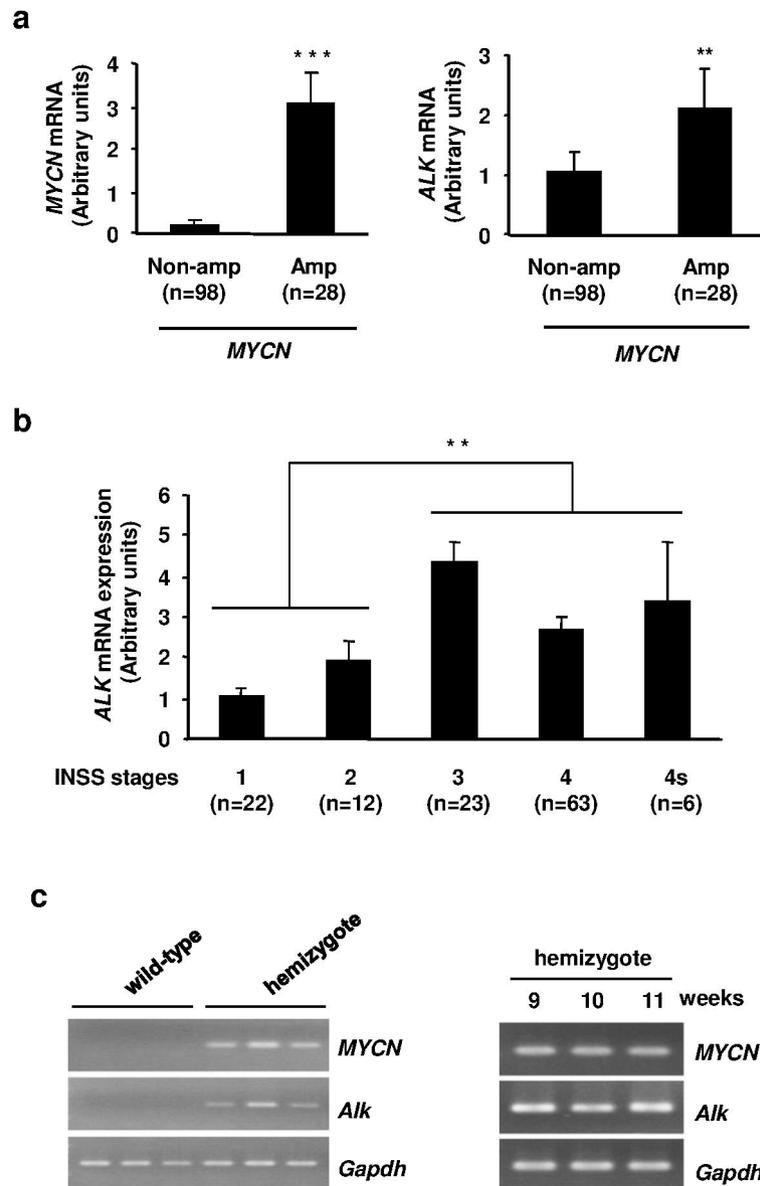


Figure 1 | Endogenous *ALK* expression is correlated with *MYCN* expression levels. (a) *ALK* expression in NBL clinical samples. The expression levels of *MYCN* (left) and *ALK* (right) mRNA in subsets of *MYCN*-non-amplified and amplified NBL clinical samples. (b) *ALK* mRNA in different stages of NBL (INSS stages). (c) High expression of *MYCN* and *Alk* mRNA in *MYCN*-transgenic mice. *MYCN* and *Alk* expression were investigated by RT-PCR in SMG tissues of 2-week-old wild-type and *MYCN*-hemizygous mice (left) and in adrenal gland tumor tissues of *MYCN*-hemizygous mice at 9, 10 and 11 weeks old (right).

Migration and invasion of NBL cells were significantly suppressed by knockdown of *ALK* expression (Figure 5b and Supplementary Figure S6B). For knockdown of *ALK* experiment, we used *MYCN*-non-amplified NBL-S cells, which have a high expression of *MYCN* protein and invasive potency as described previously¹⁰, and phosphorylation of the downstream molecule AKT was reduced with *ALK* knockdown (Supplementary Figure S4B). We also performed knockdown of *ALK* study in *MYCN* amplified cell line SK-N-DZ. Both cell lines have a wild-type *ALK* allele.

***ALK* mediates *MYCN*-induced oncogenesis.** We next tested the effect of growth inhibitory stimulus such as retinoic acid (RA) on the proliferative and colony-forming ability of cells expressing wild-type or F1174L-mutant *ALK*. RA is a well known *MYCN* suppressor, and the addition of RA to proliferating NBL cells halts their division and leads to either differentiation or apoptosis^{31–33}. We analyzed RA treatment experiment using *MYCN* amplified NBL cells (GOTO), which have a wild-type *ALK* allele and showed sensitivity to RA as

previously described³³. Figures 6a and b showed that after treatment with RA, the proliferation as well as the number and size of colony of NBL cells were significantly suppressed. As expected, *MYCN* expression was decreased after RA treatment, and *ALK* expression was also downregulated (Figure 6c). However, both wild-type and F1174L mutated *ALK*-overexpressing cells had a higher number of live clones compared with the control cells following treatment with RA, suggesting that the prior *ALK* expression partially prevented the effect of RA on cell proliferation.

We also examined the effect of *MYCN*-induced cell migration in the presence or absence of *ALK* expression. This study we performed using NBL-S cells, which showed upregulation of endogenous *ALK* with *MYCN* overexpression (Figure 2a and Supplementary Figure S1B). Consistent with previous reports^{10,11}, *MYCN* expression enhanced cell migration. As expected, the migration was suppressed by siRNA-mediated knockdown of *ALK* in NBL cells (Figure 6d), suggesting that *MYCN* function in cell migration is at least partly regulated by *ALK* expression.

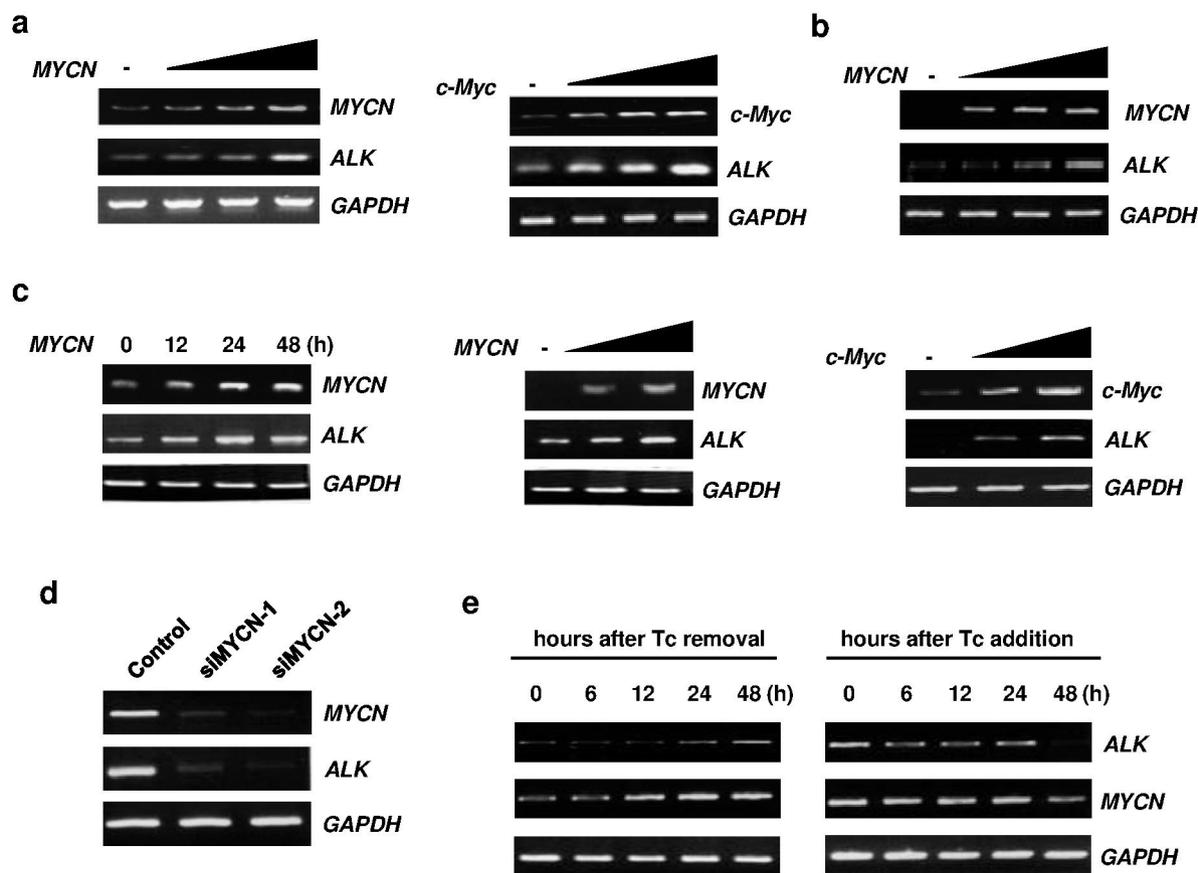


Figure 2 | MYC proteins regulate the expression of *ALK*. (a) NBL-S cells were transfected with different amounts of *MYCN* or *c-Myc* expression vector. (b) SH-SY5Y (F1174L *ALK* mutation) cells were transfected with *MYCN* expression vector as (a). (c) U2OS cells were transfected with *MYCN* or *c-Myc* expression vector, and the expression of *ALK* mRNA was examined in a time- (left) or dose- (middle and right) dependent manner. At the indicated time points (for time-dependent) or at 24 h (for dose-dependent) after transfection, the expression of *ALK*, *MYCN* or *c-MYC* was checked by RT-PCR. (d) siRNA-mediated knockdown of *MYCN* downregulated *ALK* expression. SK-N-AS cells were transfected with control siRNA or siRNA targeting *MYCN* (siMYCN-1 and -2). Seventy-two hours after transfection, total RNA was prepared and processed for RT-PCR. (e) Induction of *ALK* in *MYCN*-inducible SHEP Tet21/N cells (F1174L *ALK* mutation). RT-PCR of *ALK* and *MYCN* expression was performed after the removal (left) or addition (right) of tetracycline (Tc, 100 ng/ml) at the indicated time intervals.

ALK inhibitors suppressed NBL cell growth, migration and invasion, and inhibited tumor growth in xenograft model. Finally, we analyzed the effect of ALK inhibition on NBL cell proliferation, migration and invasion. Cell proliferation of NBL cells with *MYCN* amplification (SK-N-DZ, NLF and GOTO) were effectively inhibited by TAE-684 compared to *MYCN*-non-amplified cells (SK-N-AS and RISA) (Figure 7a). The IC₅₀ values of SK-N-DZ, NLF, GOTO, RISA and SK-N-AS cells were 75.6 nM, 95.5 nM, 132.7 nM, 349.0 nM and 894.7 nM, respectively. Consistence with these results, efficient suppression of cell migration and invasion were observed in *MYCN* amplified NBL cells (SK-N-DZ and NLF) after TAE-684 treatment, whereas no significant suppression was detected in *MYCN*-non-amplified SK-N-AS cells (Figure 7b). Similar results in cell growth and migration assays using NBL cells with *MYCN* amplification were obtained by two selective ALK inhibitors, crizotinib and CH5424802 (Supplementary Figure S8). In addition, TAE-684 treatment significantly suppressed tumor growth of xenograft generated from *MYCN* amplified NBL cells (SK-N-DZ and NLF) (Figure 7c).

Discussion

MYCN amplification occurs in approximately 25% of primary NBLs and is one of the most reliable prognostic factors identified to date^{1,5-7}. It is significantly associated with advanced disease stages, rapid tumor progression and poor prognosis. However, the molecular mechanisms

how *MYCN* induces aggressive NBL have not yet been fully elucidated. Our present findings clearly provided the evidence that *MYCN*-mediated *ALK* induction promotes cell proliferation, migration and invasion.

To our knowledge, this is the first report showing a direct role of *MYCN* in the transcriptional regulation of *ALK* in NBL. Consistent with the evidence that *ALK* expression was significantly correlated with *MYCN* amplification in primary NBLs, overexpression of *MYCN* induced promoter activity of the *ALK* gene, leading to a high level of *ALK* expression in NBL cells. The induction of *ALK* expression was also observed in non-NBL cells, suggesting that the transcription of *ALK* gene is generally regulated by *MYCN*. Moreover, in agreement with the previous results showing that *c-Myc* recognizes and binds to the E-box in the same manner as *MYCN*^{14,15,34}, *ALK* expression was also transcriptionally regulated by *c-Myc* in non-NBL cells. Taking this into account, *ALK* is a direct target gene of MYC proteins. Our data are also informative to explain why *ALK* expression is high in *MYCN* transgenic mice. High levels of *ALK* expression were observed in 2-week-old *MYCN*-hemizygous mice, indicating that a spontaneously arising NBL expresses *ALK* in the early stage of tumor development driven by *MYCN*. Recently, PHOX2b has been reported as a transcription factor targeting *ALK*³⁵. While the researchers identified transcriptional activity in the promoter construct of *ALK* gene from -672 to +384 bp, we observed comparable basal promoter activity in the -350 to +30 bp promoter region that

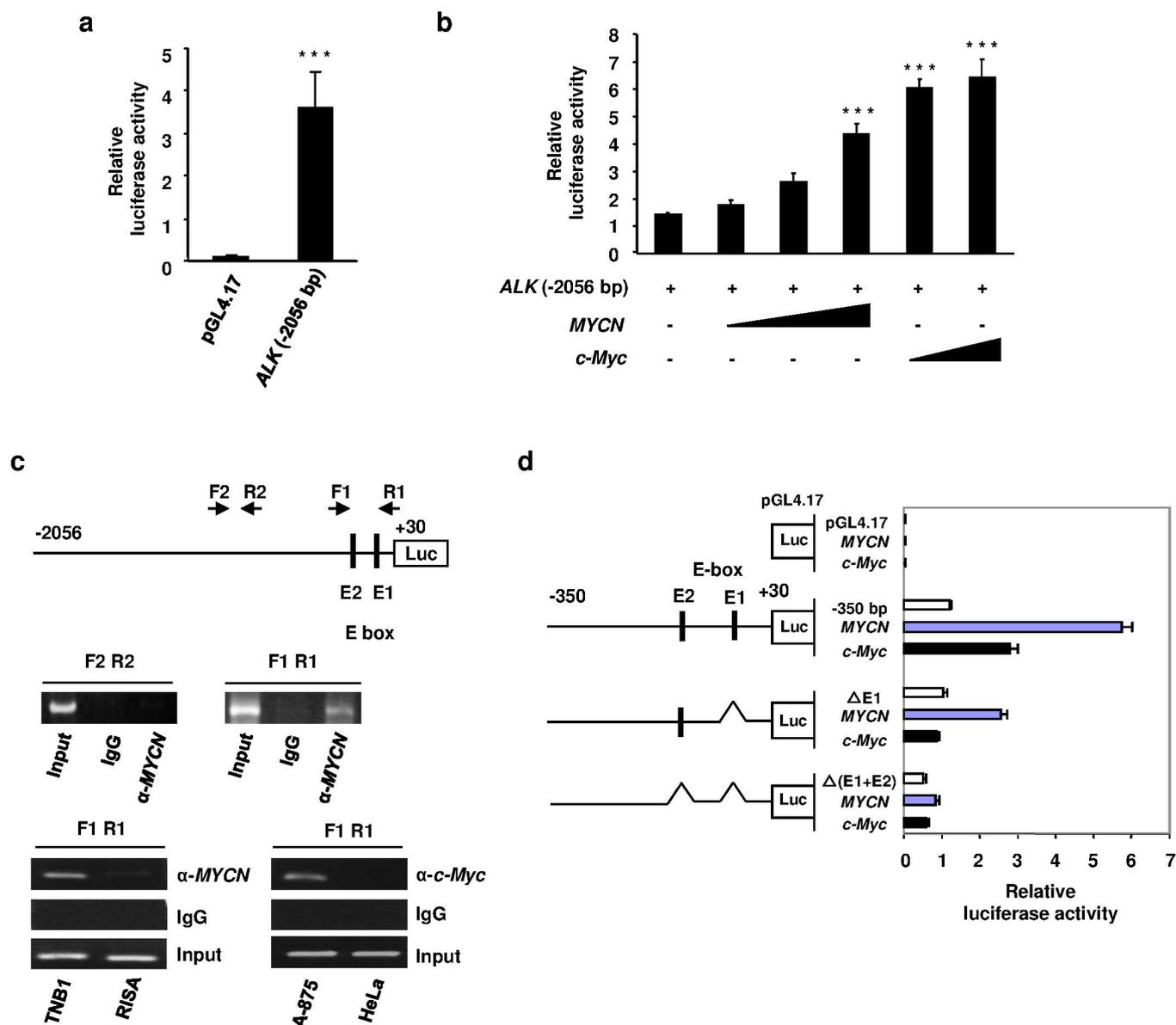


Figure 3 | Transcription of *ALK* is directly regulated by MYC proteins. (a) SH-SY5Y cells were transfected with *ALK* luciferase reporter construct (-2056 bp) or empty vector and subjected to luciferase reporter assays. (b) Overexpression of both *MYCN* and *c-Myc* enhanced the basal promoter activity of *ALK*. SH-SY5Y cells were co-transfected with *ALK* (-2056 bp) and increasing amounts of *MYCN* or *c-Myc* expression vector. Luciferase assays were then performed to measure the promoter activity. (c) Both *MYCN* and *c-Myc* were recruited onto the *ALK* promoter region. Schematic drawing of the 5'-upstream region of human *ALK* indicates the positions of putative E-boxes (E1 and E2). Primer sets (F1 R1 and F2 R2) used for ChIP assays are indicated by arrows (top). HeLa cells were transiently transfected with *MYCN* expression plasmid. Forty-eight hours after transfection, ChIP assays were performed using anti-MYC antibody (middle). To detect the recruitment of endogenous *MYCN* (bottom left) and *c-Myc* (bottom right), ChIP assays were carried out in the indicated cell lines using anti-MYC or anti-*c-Myc* antibodies. (d) E1 and E2 are important for the transcriptional activation of *ALK*. Site-specific deletions were introduced into the parental core promoter (-350 bp) of the luciferase reporter construct at the indicated E-boxes (left panel). SK-N-AS cells were simultaneously transfected with parental or deletion mutants of luciferase reporter constructs together with *MYCN* or *c-Myc* expression vector. The graph shows the relative luciferase activity driven by the expression of MYC proteins.

contains no PHOX2b binding motif. According to our results, this minimum region of the *ALK* promoter possesses MYC-binding sites and transcriptional activity of *ALK* gene.

More recently, Schonheer *et al.* showed that both wild-type and gain-of-function *ALK* mutants were able to stimulate transcription at the *MYCN* promoter through the activation of a downstream molecule, ERK, and initiate mRNA transcription of *MYCN* in both neuronal and NBL cells³⁶. Furthermore, Berry *et al.* demonstrated that the F1174L mutation of *ALK* enhanced *MYCN* protein stabilization and found that endogenous *Mycn* mRNA was upregulated in the tumors of *MYCN/ALKF1174L* transgenic mice³⁷. Taken together, these data

suggest a positive feedback loop for *MYCN* which, in turn, directly regulates *ALK* expression to potentiate the oncogenic activity of *MYCN*, leading to rapid malignant transformation.

The theory that *ALK* overexpression contributes to oncogenic activity is supported by the data obtained from patients with NBL. As described previously, high expression of *ALK* mRNA^{25,26} and/or protein^{23,24} was significantly correlated with poor outcome of NBL. Consistent with these earlier reports, our present data clearly showed that aggressive and metastatic NBLs (stages 3, 4 and 4s) exhibited a significantly higher expression level of *ALK* mRNA compared with localized and favorable NBLs (stages 1 and 2), suggesting an onco-

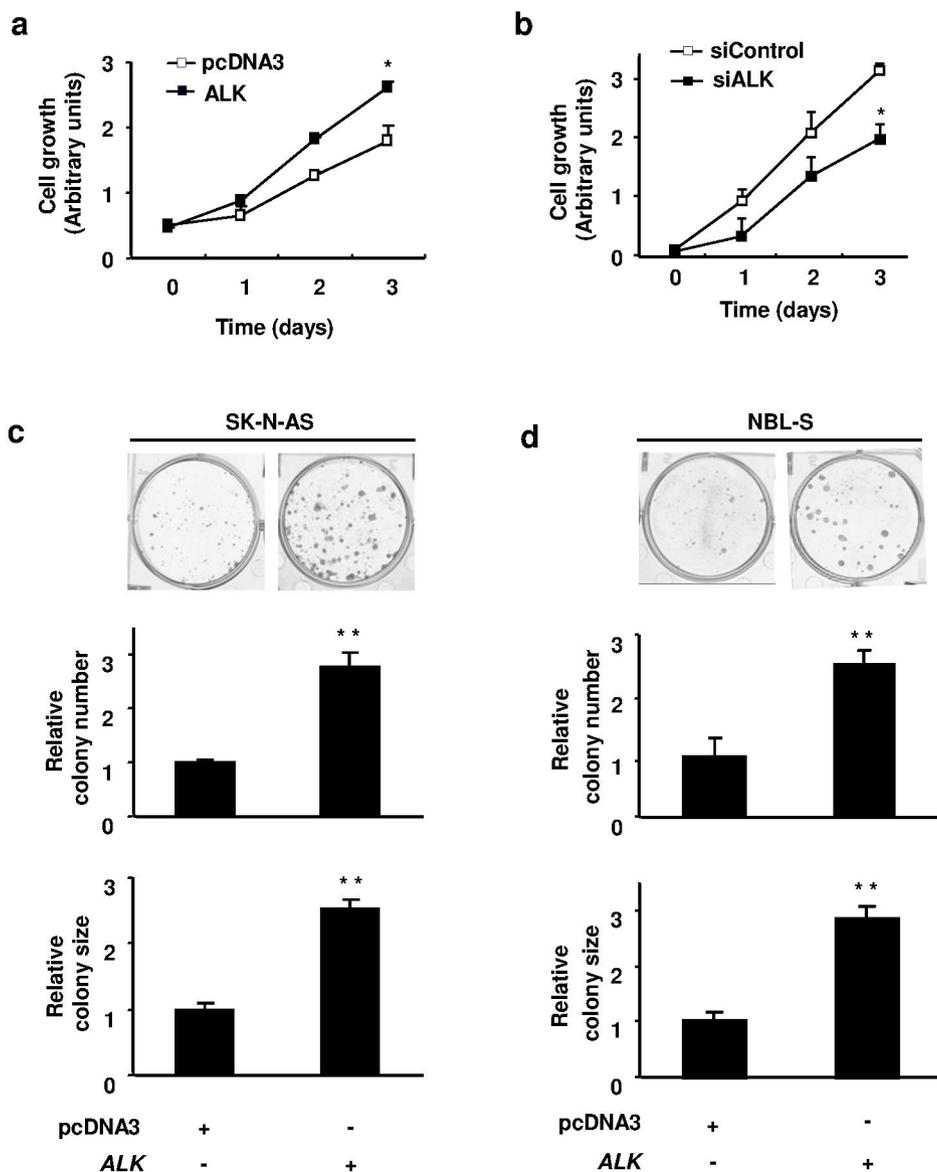


Figure 4 | ALK promotes cell proliferation. (a) SK-N-DZ cells were transfected with the expression plasmid for *ALK* or empty plasmid and subjected to WST-8 assays at the indicated times after transfection. (b) NLF cells were transfected with control siRNA or siRNA against *ALK*. Twenty-four hours after transfection, cells were seeded in 96-well cell culture plates. At the indicated time points, the numbers of viable cells were measured by WST-8 assays. (c) and (d) Colony formation assays. SK-N-AS (c) and NBL-S (d) cells were transfected with pcDNA3-*ALK* or empty plasmid. Forty-eight hours after transfection, cells were transferred to fresh medium containing G418 (400 $\mu\text{g}/\text{ml}$ for SK-N-AS and 500 $\mu\text{g}/\text{ml}$ for NBL-S cells). Images were taken after crystal violet staining. Numbers and sizes of colonies were counted. All experiments were performed in triplicate.

genic relevance of *ALK* in NBL. Previously obtained evidence has indicated an important role of *ALK* in both familial and sporadic NBL pathogenesis^{18–22}. However, the contribution of wild-type *ALK* to NBL development was not well understood. Intensive studies have been mainly performed examining a correlation between activating mutations in the tyrosine kinase (TK) domain of *ALK* and poor clinical outcome in NBLs. However, mutations in the TK domain are observed in limited cases. According to De Brouwer and colleagues, only 6.9% missense mutations and 1.7% focal amplifications in *ALK* gene were detected among 709 NBL patients²⁵. They have also revealed that there were no significant survival differences observed in tumors with or without *ALK* mutations or amplifications²⁵, suggesting that wild-type *ALK* might have an important role in NBL pathogenesis. Consistent with Passoni *et al.* and Di Paolo *et al.*, our present findings indicate that wild-type *ALK* can exert oncogenic activity in NBL cells, in addition to its mutated isoforms.

In the current study, overexpression of either wild-type or mutated *ALK* partially restored the decreased cell proliferation caused by RA treatment, implicating that RA-mediated reduction of *MYCN* expression resulted in decreased cell proliferation partially through the downregulation of *ALK* expression. The contribution of native *ALK* to migration and invasion implies a role for *ALK* in tumor progression and metastasis of NBLs. *MYCN*-induced cell migration was also inhibited by siRNA-mediated knockdown of *ALK*, suggesting that *ALK* is one of the key target genes of *MYCN* to conduct NBL cell migration. Moreover, treatment with TAE-684 effectively inhibited cell proliferation, migration and invasion of NBL cells with *MYCN* amplification compared to *MYCN*-non-amplified cells. Xenograft tumors derived from *MYCN* amplified NBL cells were significantly suppressed with TAE-684 treatment, indicating that *ALK* had a pivotal role in the development of NBL with *MYCN* amplification. Taken together, these data provide an important

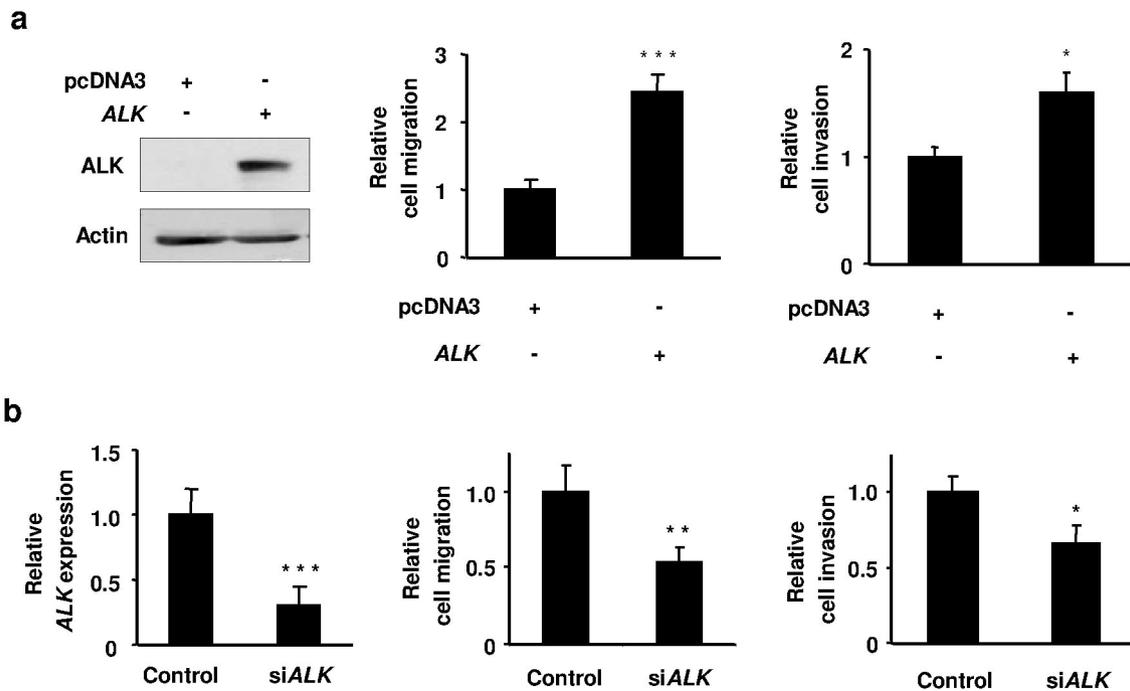


Figure 5 | ALK contributes to cell migration and invasion. (a) Overexpression of ALK enhanced NBL cell migration and invasion. SK-N-AS cells were transfected with pcDNA3-*ALK* or empty plasmid, and ALK ectopic expression (220 kDa) was determined by immunoblotting (left, full-length blots are presented in Supplementary Figure S9). Migration assays (middle) and invasion assays (right) were performed in Boyden chambers. (b) siRNA-mediated knockdown of *ALK* suppressed NBL cell migration and invasion. NBL-S cells were transfected with control siRNA or siRNA targeting *ALK*. Knockdown of *ALK* mRNA expression in NBL-S cells was confirmed by qRT-PCR (left). Migration assays (middle) and invasion assays (right) were performed as (a). All experiments were performed in triplicate.

and direct mechanism by which *MYCN* is able to sensitize cells and tumors to aggressive characteristics through the regulation of *ALK* expression.

In conclusion, this study provides several lines of evidence that *ALK* is a direct transcriptional target of *MYC* proteins and a key molecule for *MYC* proteins to exert influence towards oncogenesis. Thus, our present findings might help to explain a novel molecular mechanism for the development and progression of aggressive NBL with or without *MYCN* amplification, and suggest that a therapy targeting *ALK* should be considered in combination with more conventional agents to treat NBLs with high expression of *ALK*.

Methods

Patient population. One hundred and twenty-six patients with NBL were diagnosed clinically and histologically, using surgically removed tumor specimens according to the International Neuroblastoma Pathological Classification (INPC). According to the International NBL Staging System (INSS)³⁸, 22 patients were diagnosed as stage 1, 12 were stage 2, 23 were stage 3, 63 were stage 4, and 6 were stage 4S. *MYCN* and *ALK* amplification were determined using fluorescence *in situ* hybridization (FISH). This study was approved by the Ethics Committee of the Faculty of Biology and Medicine at the Chiba Cancer Center, and appropriate informed consent was obtained from all patients.

Transgenic mice samples collection and RT-PCR. Tyrosine hydroxylase (TH)-*MYCN* mice were maintained through hemizygous matings, as previously described³⁹. Superior mesenteric ganglion (SMG) tissues were obtained from 2-week-old wild-type or *MYCN*-hemizygous mice ($n = 3$ mice per group). Adrenal gland tumor tissues were collected from 9-, 10- or 11-week-old *MYCN*-hemizygous mice ($n = 3$ mice per group). All animals were handled in accordance with institutional guidelines for safe and ethical treatment of mice, and this study was approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine. Total RNA extraction was performed using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. cDNA was generated from total RNA using SuperScript III reverse transcriptase and random primers following the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). The resultant cDNAs were subjected to PCR-based amplification using the following primer sets and annealing temperatures (Ta): human *MYCN*, 5'-CGACCACAAGGCCCTC AGTA-3' (sense) and 5'-CAGCCTTGGTGTGGAGAG-3' (antisense), Ta, 56°C; mice *Alk*, 5'-GACAGGATGGCTCCACCACA-3' (sense) and 5'-CGGAAGCA

GAGCGCACACAA-3' (antisense), Ta, 56°C; mice *Gapdh*, 5'-GGTGGTGAAGCA GGCATCTG-3' (sense) and 5'-GGAGCCATGTAGCCATGA-3' (antisense), Ta, 57°C.

Cell culture. Human-derived NBL cell lines harboring wild-type *ALK*, including SK-N-AS, SK-N-DZ, NBL-S, NLF, RISA, NB69, SK-N-BE, NMB, NBTU1, NB9, KP-N-NS, SMS-KAN, GOTO, IMR32, GANB, CHP-134, and mutated *ALK*, including SH-SY5Y (F1174L), TNB1 (R1275Q) and SHEP Tet-21/N (F1174L), OAN (D1091N), RTBM1 (F1174L), TGW (R1275Q), SMS-SAN (F1174L), NGP (D1529E), LHN (R1275Q), LAN5 (R1275Q), KCN (R1275Q) and *ALK* amplified NB1 (Amp), were cultivated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and penicillin (100 IU/ml)/streptomycin (100 µg/ml). Non-NBL cell lines including HeLa, U2OS, A-875, TTC-11, ASPS-KY, RMS-Mk, NOS-1, SAOS-2, OST, G-361, G32TG, A549, H1299, HACAT, HEK-293T, HEK-293, COLO 320, MCF-7 and MDA-MB-453, were maintained in Dulbecco's modified Eagle's medium with the same supplements. Cells were grown at 37°C in a water-saturated atmosphere of 95% air and 5% CO₂. NBL cell lines were obtained from the CHOP cell line bank (Philadelphia, PA, USA). SHEP Tet 21/N cell line was kindly provided by Dr. M. Schwab (German Cancer Research Center, Heidelberg, Germany) and RISA cell line was established at Chiba Cancer Center Research Institute, Chiba, Japan. Non-NBL cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). For transient transfection, cells were transfected with the indicated expression of plasmids using Lipofectamine 2000 transfection reagent (Invitrogen), according to the manufacturer's recommendations.

Plasmid constructs. *ALK* promoter construct and different deletion constructs of *ALK* promoter were generated using an In-Fusion HD Cloning Kit (Clontech Laboratories, Mountain View, CA, USA) and inserted into the luciferase pGL4.17-basic plasmid (Promega, Madison, WI, USA). The protein coding region of *c-Myc* was inserted into the pcDNA3 plasmid (Invitrogen). pcDNA3-*ALK* wild-type and pcDNA3-*ALK* (F1174L *ALK* mutation) mutated expression plasmids were kindly provided by Dr. J. Takita (The University of Tokyo, Tokyo, Japan). pUHD-*MYCN* expression plasmid was kindly provided by Dr. M. Schwab (German Cancer Research Center, Heidelberg, Germany). All constructs were verified by DNA sequencing.

RNA isolation and RT-PCR. Total RNA was prepared from the indicated cell lines using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. cDNA was generated from total RNA using SuperScript II reverse transcriptase and random primers following the manufacturer's conditions (Invitrogen). The resultant cDNAs were subjected to PCR-based amplification using the following primer sets and annealing temperatures

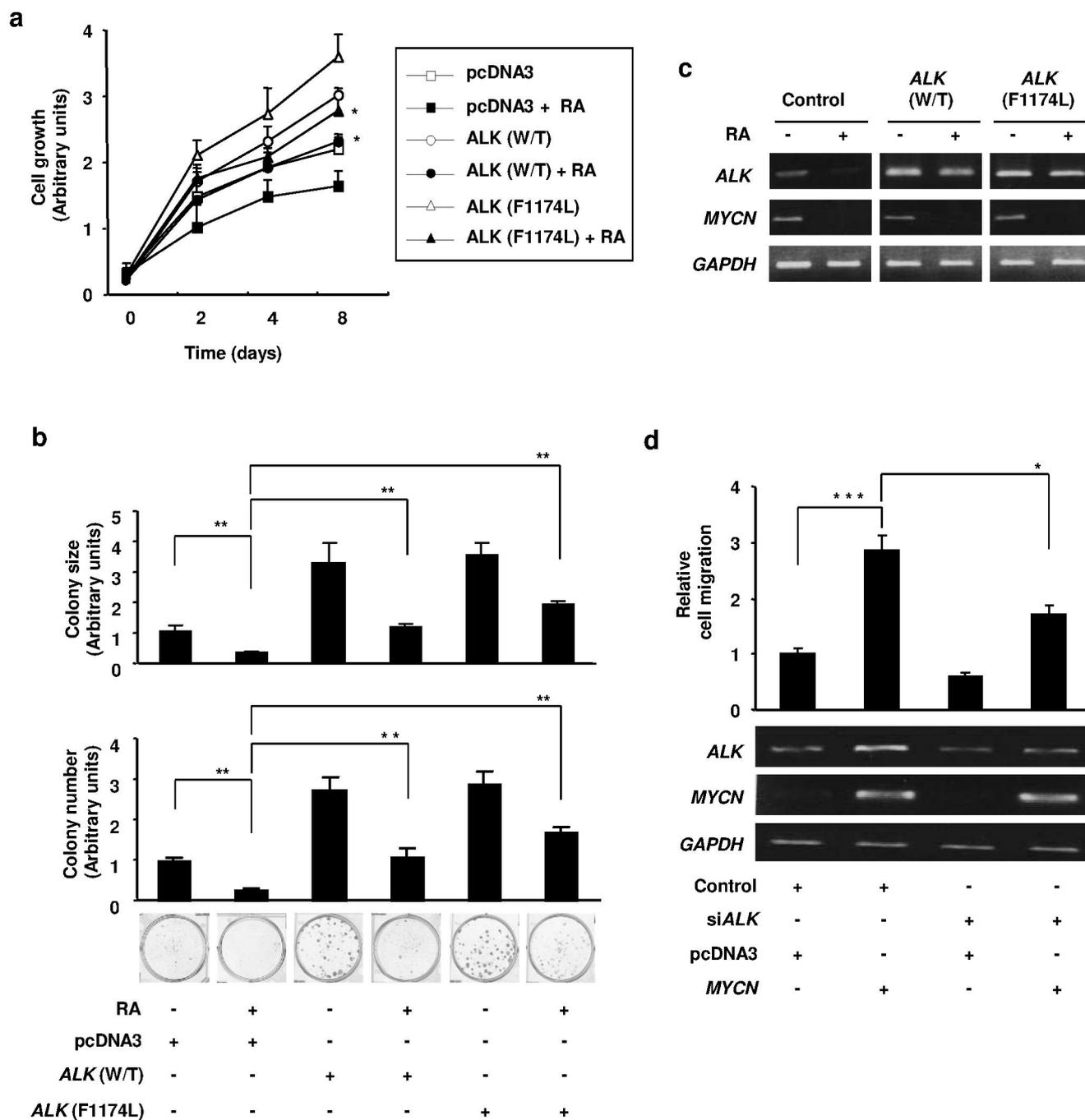


Figure 6 | MYCN induces oncogenesis through regulating ALK expression. (a) and (b) Effect of RA on the proliferative and colony forming abilities of NBL cells overexpressing *ALK*. Cell proliferation assays (a) and colony formation assays (b) to examine the proliferation of GOTO cells with wild-type or F1174L mutated *ALK* showed a significant difference in cell proliferation following treatment with RA (10 μ M). (c) GOTO cells were transfected with pcDNA3-*ALK* (wild-type or mutated) or empty plasmid. Twenty-four hours after transfection, cells were treated with or without RA. Forty-eight hours after RA treatment, the expression of *ALK* and *MYCN* mRNA was verified by RT-PCR. (d) Cell migration assay. NBL-S cells were transfected with siRNA against *ALK*, followed by the transfection of *MYCN* expression vector or empty vector 24 h after siRNA transfection. Seventy-two hours after the first transfection, cell migration was examined (upper panel) and the expression levels of *ALK* and *MYCN* were determined (lower panel) by RT-PCR. All experiments were performed in triplicate.

(Ta): human *MYCN*, 5'-CTTCGGTCCAGCTTTCTCAC-3' (sense) and 5'-GTCCGAGCGTGTTC AATTTT-3' (antisense), Ta, 58°C; human *GAPDH*, 5'-ACCTGACCTGCGCTAGAA-3' (sense) and 5'-TCCACCACCTGTTGCTGTA-3' (antisense), Ta, 58°C; human *ALK*, 5'-AGGACCCGGATGTAATCAAC-3' (sense) and 5'-CTGTGCAACTCCGAAGGAG-3' (antisense), Ta, 58°C; human *c-Myc*, 5'-CTCGACTACGACTCGGTGCA-3' (sense) and 5'-TGGTGGCGGTGTCTCCTCA-3' (antisense), Ta, 60°C. To control for the integrity and uniformity of the sample preparation, *GAPDH* mRNA was amplified. All PCR amplifications were carried out with a GeneAmp PCR 9700 (Applied Biosystems, Foster City, CA, USA), using rTaq

DNA polymerase (Takara, Shiga, Japan). PCR products were separated by 2.0% agarose gel electrophoresis and stained with ethidium bromide.

Quantitative real-time PCR (qRT-PCR). Total RNA was extracted from clinical samples and NBL as well as non-NBL cell lines using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and reverse transcription was performed with SuperScript II reverse transcriptase (Invitrogen). qRT-PCR was carried out using an ABI Prism 7700 sequence detection system (Applied Biosystems), according to the manufacturer's protocol. TaqMan probe for *ALK*

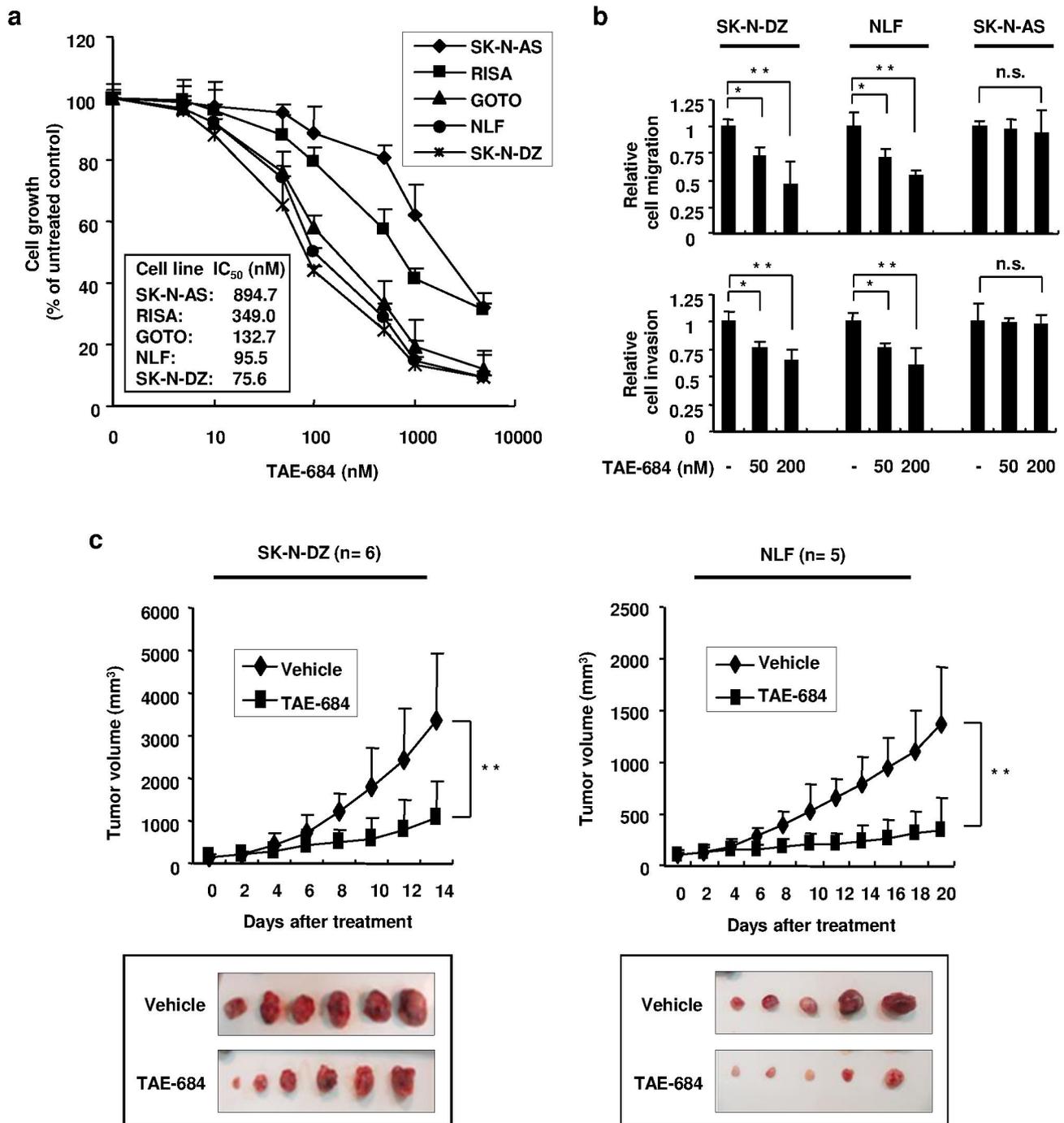


Figure 7 | Effects of ALK inhibitor TAE-684 on NBL cells and xenograft tumors. (a) TAE-684 reduced the proliferation of NBL cells. *MYCN*-non-amplified (SK-N-AS and RISA) or amplified (GOTO, NLF and SK-N-DZ) NBL cells were cultured with varying concentrations of TAE-684 for 72 h and cell proliferation was measured. The values are mean \pm SD of triplicate experiments. (b) TAE-684 suppressed cell migration and invasion of NBL cells with *MYCN* amplification. NBL cells with *MYCN* amplification (SK-N-DZ and NLF) or without amplification (SK-N-AS) were treated with 50 nM or 200 nM of TAE-684 or DMSO as control, and cell migration (upper panel) or invasion (lower panel) assays were performed. The values are mean \pm SD of triplicate experiments. (c) TAE-684 suppressed tumor growth in mice. SK-N-DZ (left panel) and NLF (right panel) cells were subcutaneously injected into mice. When palpable tumors appeared, mice were orally treated with TAE-684 or carrier solution (vehicle), and tumor sizes were measured. Tumor sizes are displayed as mean \pm SD at the indicated time interval of TAE-684 treatment (upper panel). Pictures of subcutaneous tumors for each group are shown (lower panel). $n = 6$ for each group of SK-N-DZ cells and $n = 5$ for each group of NLF cells.

(Assay ID: Hs00608292_m1) and β -actin control reagent kit were purchased from Applied Biosystems. *MYCN* mRNA expression was measured by the SYBR green real-time PCR system using the following primer set: 5'-GGACACCTGAGCG ATTCAGA-3' (sense) 5'-AGGAGGAACGCCGCTTCT-3' (antisense). The mRNA levels of each gene were standardized by β -actin.

siRNA-mediated knockdown. Cells were transiently transfected with 10 nM siRNA targeting *MYCN*, siRNA-1: 5'-GAACCCAGACCUCGAGUUUUU-3' (sense);

5'-PAAACUCGAGGUCUGGGUUCUU-3' (antisense) and siRNA-2: 5'-UCACGG AGAUGCUGCUUGAUU-3' (sense), 5'-PUCAAGCAGCAUCUCCGUGAUU-3' (antisense) or a control non-targeting siRNA (Thermo Fisher Scientific, Waltham, MA, USA) using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. Seventy-two hours after transfection, total RNA was prepared and subjected to RT-PCR. To knockdown endogenous *ALK* expression, cells were transfected with 100 nM of control siRNA or with siRNA against *ALK* target sequences: 5'-CCUGUAUACCGGAUUAUGA-3',



5'-GUUGGGGUCAUAGAUGUUU-3', 5'-UGAUUAUUUUACAUGGAAU-3', 5'-GGAGUGGCGGAAUGAUUA-3' (Thermo Fisher Scientific) according to the manufacturer's recommendations. Seventy-two hours after transfection, cell lysates were prepared and analyzed for *ALK* and *GAPDH* expression levels by qRT-PCR or the expression levels of *ALK*, *pALK*, *AKT* and *pAKT* by immunoblotting analysis.

Site-specific deletion. The luciferase reporter constructs (−350 bp)−Δ E1 and (−350 bp)−Δ E 1/2 deletion constructs were generated with QuikChange Site-Directed Mutagenesis System (Invitrogen) on the basis of the parental construct (−350 bp), according to the manufacturer's instructions. The following primer sets were used: (−350 bp)−ΔE1, 5'-GCGGGCTCAGCCAGCTGGCGGGCGCCAG-3' (sense) and 5'-CATCTGCCTGGGCGCCGCCAGCTGGCTGAG-3' (antisense); (−350 bp)−ΔE2, 5'-GCAGCAGCGCGAGTTGGAGCCCCGCC-3' (sense) and 5'-CCCGGAGGGGGCGGGCTCCAACCTCCGCG-3' (antisense). The deletions were verified by DNA sequencing.

Luciferase reporter assays. Cells (5×10^4) were seeded in 12-well cell culture plates (Becton Dickinson, NJ, USA) and allowed to adhere overnight. Cells were then co-transfected with expression plasmid, luciferase reporter construct and pRL-TK Renilla luciferase cDNA. Total amounts of plasmid DNA per transfection were kept constant with empty plasmid pcDNA3 and/or pGL4.17. Forty-eight hours after transfection, cells were lysed, and both firefly and Renilla luciferase activities were measured with the Dual-Luciferase Reporter Assay system (Promega), according to the manufacturer's instructions. The firefly luminescence signal was normalized based on the Renilla luminescence signal.

ChIP assays. Chromatin immunoprecipitation (ChIP) assays were performed using a chromatin immunoprecipitation assay kit according to the protocol provided by Millipore (Bedford, MA, USA). In brief, TNB1 (high *ALK* expression), RISA (low *ALK* expression), A-875 (high *ALK* expression) and HeLa cells (low *ALK* expression) were cross-linked with formaldehyde, and cross-linked chromatin was sonicated followed by immunoprecipitation with anti-IgG (Cell Signaling), polyclonal anti-MYC and/or monoclonal anti-c-Myc (anti-MYC-Tag (9B11)) antibody. DNA of the immunoprecipitates and control input DNA were purified and then analyzed by standard PCR using the following primer sets: F1 (−350), 5'-GCTCGCTAGCCCTC GAAGTTCTCACATTTGCTCC-3' (sense); R1 (+30), 5'-TCTTGATATCCTCG AGTACCAGCTGCTACC-3' (antisense) and F2(-1813), 5'-GGAGAGGGGTATTA TTAGAGAACG-3' (sense); R2 (−1433), 5'-GGCAAAGAATTATCTACCC-3' (antisense).

Cell proliferation assays. Cells (1×10^3) were seeded in 96-well cell culture plates (Becton Dickinson, NJ, USA) and allowed to adhere overnight. At the indicated time points, cell proliferation was measured using a cell counting kit-8 (Dojindo, Kumamoto, Japan) according to manufacturer's instructions.

Colony formation assays. Cells were seeded at a final density of 2×10^4 cells per well in 6-well cell culture plates (Becton Dickinson, NJ, USA) and allowed to attach overnight. Cells were then transfected with the empty or pcDNA3-*ALK* expression plasmid. Forty-eight hours after transfection, cells were transferred to the fresh medium containing G418. After 14 days, viable colonies were washed in PBS, fixed with 4% paraformaldehyde (PFA) and stained with crystal violet solution.

Retinoic acid treatment. To study the effect of retinoic acid (RA, Sigma, St. Louis, MO, USA) on the proliferation or colony formation of GOTO cells expressing wild-type or mutated *ALK*, transfected cells were grown in normal growth medium, and dimethylsulfoxide (DMSO) or 10 μ M RA (with 50 μ g/ml of G418 for colony formation assays) was added to the medium the following day. The culture medium was changed every 48 h to replenish the RA and G418. Cell viability and colony formation assays were performed as described above.

Cell migration and invasion assays. Cell motility was measured using 8- μ m pore size FluoroBlok Transwell chambers (BD Biosciences, San Diego, CA, USA). Cells were collected by a brief treatment with trypsin/EDTA solution, washed once with serum-containing medium, centrifuged, resuspended in medium containing 0.1% bovine serum albumin, and then placed in the inserts at a concentration of 2.5×10^4 cells in 250 μ l (migration assays) or 5×10^4 cells in 250 μ l (invasion assays). In the lower compartments of the chambers, 750 μ l of medium containing 10% FBS was added as a chemoattractant. Invasion assays were carried out by the same procedure except that the filters of the Transwell chambers were coated with 50 μ g Matrigel (BD Biosciences). After incubation at 37°C for 16 h (migration assays) or 24 h (invasion assays), cells that migrated through the pores to the lower chamber were stained with Giemsa solution or 1 μ M Calcein AM (Invitrogen). Stained cells were analyzed by either counting under the microscope or by detecting the fluorescence intensity using microplate reader (Perkin-Elmer, Waltham, MA, USA).

Immunoblot analysis. Immunoblotting was performed as previously described⁴⁰ using the following antibodies: monoclonal anti-*ALK* was from Beckman Coulter (Marseille, France), polyclonal anti-phosphorylated Tyr-1604 *ALK*, polyclonal anti-*AKT*, polyclonal anti-phosphorylated *AKT* Ser-473, polyclonal anti-MYC and monoclonal anti-MYC-Tag (9B11) were from Cell Signaling (Danvers, MA, USA), polyclonal anti-Actin was from Sigma-Aldrich (St. Louis, MO, USA). All antibodies were used at a dilution of 1:1000 in immunoblot analysis.

Drug treatment. *ALK* inhibitors, TAE-684^{41,42} and CH5424802⁴³ were purchased from Active Biochemicals (Hong Kong, China), and crizotinib^{36,37,44} was purchased from Selleck (Houston, TX, USA). For cell proliferation assay; NBL cells (3×10^3) were seeded in 96-well cell culture plates and treated with varying concentrations of TAE-684, crizotinib or CH5424802 for 72 h and cell proliferation was measured as described above. The IC₅₀ (half maximal inhibitory concentration) values of NBL cells were calculated by nonlinear regression (variable slope) using the GraphPad Prism 6 software (La Jolla, CA, USA). Cell migration and invasion assay; cell migration and invasion assays were performed as described above with or without the treatment of 50 nM or 200 nM of TAE-684, crizotinib or CH5424802.

Xenograft study; NBL cells with *MYCN* amplification (SK-N-DZ and NLF) were subcutaneously injected (5×10^6 cells/inoculate in RPMI 1640 together with an equal volume of Matrigel, Becton Dickinson) in the right flank of C.B-17-SCID female mice at 6 weeks old. After implantation, tumor sizes were measured using the following formula: [(width)² × length]/2. Treatment of TAE-684 was initiated at the time when tumor size exceeds 85 mm³. TAE-684 was resuspended in 10% N-methyl-2-pyrrolidone (Wako, Osaka, Japan)-90% PEG (polyethylene glycol, molecular weight 300) (Wako) solution. Mice were administered TAE-684 (10 mg/kg) by oral gavage once daily. All mice were housed in a pathogen-free conditions strictly following the Chiba Cancer Center Research Institute guidelines, and these studies were approved by the Institutional Animal Care and Use Committee of Chiba Cancer Center Research Institute.

Data analysis. Results were expressed as the mean ± SD. Student's *t* tests and two-independent samples *t* tests were used to compare the differences in the means of different groups. *P* values of <0.05 were considered statistically significant. *P* values of <0.05, <0.01 and <0.001 were denoted by a single asterisk, double asterisks and triple asterisks, respectively. n.s. indicates not significant at the 0.05 probability level.

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Author contributions

M.K.H. and As.N. designed, performed and analyzed cellular and animal experiments, clinical data, and wrote the manuscript. A.T. performed and analyzed cellular and animal experiments, and wrote the manuscript. S.K. and K.K. performed and analyzed animal experiments and wrote the manuscript. M.O. performed and analyzed clinical data. Y.S., S.H., J.A., A.O. and Y.N. assisted in the experiments and figures. Ak.N. designed and supervised the experiments, analyzed data, wrote and edited the manuscript.

Additional information

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