Supplementary Information

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

Md. Kamrul Hasan, Asmaa Nafady, Atsushi Takatori, Satoshi Kishida, Miki Ohira, Yusuke Suenaga, Shamim Hossain, Jesmin Akter, Atsushi Ogura, Yohko Nakamura, Kenji Kadomatsu and Akira Nakagawara

Supplemental Materials and methods

Wound healing assays. Cells (1×10^5) were seeded in six-well plates and allowed to adhere overnight. Cells were then transfected with the expression plasmids. Forty-eight hours after transfection, confluent cell monolayers were manually scratched with a micropipette tip, and allowed to migrate for 16 h.

Supplementary Figure Legends

Supplementary Figure S1. MYC proteins enhance the expression of ALK. (A) MYC proteins enhanced the endogenous mRNA expression of *ALK*. HeLa cells were transfected with *MYCN* or *c-Myc* expression vector. Twenty-four hours after transfection the expression of *ALK*, *MYCN* or *c-Myc* was checked by RT-PCR. (B) and (C) Ectopic expression of MYCN induced endogenous protein expression of ALK. NBL-S (B) and NLF (C) cells (1×10^5) were seeded in six-well plates and allowed to adhere overnight. Cells were then transfected with empty plasmid or *MYCN* expression plasmid in a dose-dependent manner. Forty-eight hours after transfection, cells were lysed and the resulting lysates were subjected to immunoblotting to verify the expression of ALK and MYCN using anti-ALK and anti-MYCN antibodies, respectively. Actin was used as control for protein loading.

Supplementary Figure S2. MYCN and c-Myc both regulates the transcription of *ALK*. (A) U2OS cells were transfected with *ALK* (-2056 bp) luciferase reporter construct or empty plasmid. Luciferase reporter assays showed *ALK* promoter activity. (B) Overexpression of *MYCN* enhanced the basal promoter activity of *ALK*. U2OS cells were transfected with *ALK* (-2056 bp) luciferase reporter construct and co-transfected with increasing amounts of *MYCN* expression vector. Luciferase assays were then performed to measure promoter activity. (C) E-box1 and 2 are important for the transcriptional activation of *ALK* gene. Site-specific deletions were introduced into the parental core promoter (-350 bp) luciferase reporter construct at the indicated MYCN-binding sites, E1 and E2 (left panel). HeLa 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 results of luciferase activity driven by the expression of MYC proteins.

Supplementary Figure S3. Delineation of the promoter region of *ALK*. SK-N-AS (A) and HeLa (B) cells were simultaneously transfected with different deletion luciferase reporter constructs of *ALK* promoter region together with *MYCN* or *c-Myc* expression vector. The graph shows the results of luciferase activity driven by the expression of MYC proteins. Primer sets used for cloning of these *ALK* promoter constructs are as follows: forward primers; For -2056–+30 construct, Fw 5'-GCTCGCTAGCCTCGAACTGTGTGATGTGTTAG-3'; For -984–+30 construct, Fw 5'-GCTCGCTAGCCTCGAGAACCACTTGTTATAA-3'; For -350–+30 construct, Fw 5'-GCTCGCTAGCCTCGAAGTTCTCACATTTGCTCC-3'; For -113–+30 construct, Fw 5'-GCTCGCTAGCCTCGAAGTTGGCTTGTGAGC-3'; For -51–+30 construct, Fw 5'-GCTCGCTAGCCTCGATCAGCCAGCTGCAAGTGG-3' and same reverse primer was used for all constructs, Re 5'-TCTTGATATCCTCGAGTACCAGCTGCTACC-3'.

Supplementary Figure S4. ALK expression contributes activation of AKT. (A) Overexpression of both wild-type (W/T) and mutated ALK enhanced constitutive phosphorylation of ALK and AKT. SK-N-AS, SK-N-DZ, NLF and NBL-S cells (1×10^5) were seeded in six-well plates and allowed to adhere overnight. Cells were then transfected with the expression plasmid of wild-type or mutated (F1174L) ALK or empty plasmid. Forty-eight hours after transfection, cells were lysed and analyzed by immunoblotting with anti-ALK, anti-phosphorylated ALK Tyr-1604, anti-AKT, anti-phosphorylated AKT Ser-473 and anti-Actin antibody. (B) siRNA-mediated knockdown of endogenous ALK suppressed phosphorylation of AKT. NBL-S cells (1×10^5) were seeded in six-well plates and allowed to adhere overnight. Cells were then transfected with control siRNA or siRNA targeting ALK. Seventy-two hours after transfection, cells were lysed and the cell lysates were subjected to immunoblotting using anti-ALK, anti-phosphorylated ALK, anti-AKT and anti-phosphorylated AKT antibody. Actin was used as control for protein loading.

Supplementary Figure S5. ALK enhances cell migration after wounding. SH-SY5Y (A), SK-N-AS (B) and HeLa (C) cells were transfected with the expression plasmids for *ALK* or *MYCN* or empty plasmid and cultured to monolayer confluence. Forty-eight hours after transfection cells were wounded and incubated for 16 h, and then migrating cells were counted. Single plus (+) indicates 1 μg and double plus (++) indicates 2 μg expression plasmid used for transfection. All experiments were performed in triplicate.

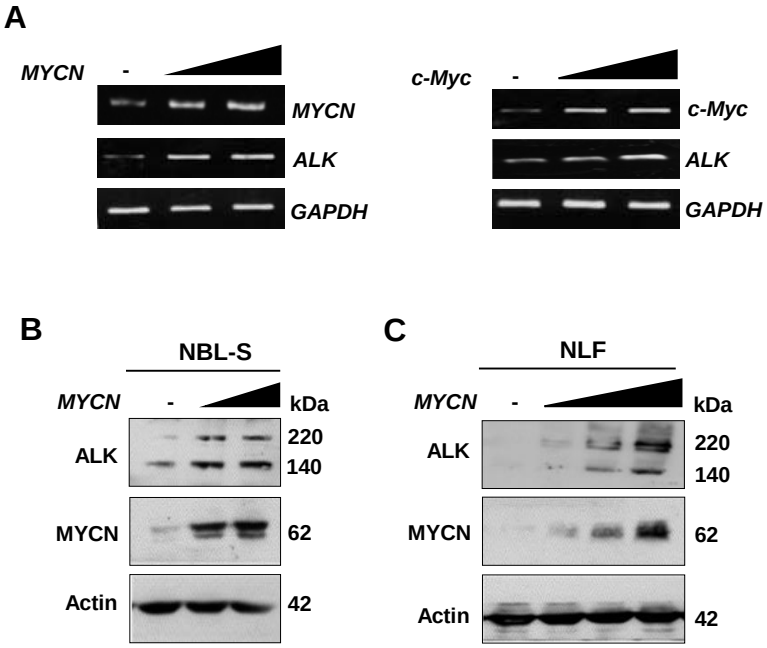
Supplementary Figure S6. ALK contributes to cell migration and invasion. (A) Overexpression of *ALK* enhanced cell migration and invasion. HeLa cells were transfected with pcDNA3-*ALK* or empty plasmid, and ALK ectopic expression (220 kDa) was determined by immunoblotting (left). Migration assays (middle) and invasion assays (right) were performed in Boyden chambers. (B) siRNA-mediated knockdown of *ALK* suppressed cell migration and invasion. SK-N-DZ cells were transfected with control siRNA or with siRNA targeting *ALK*. The knockdown of *ALK* mRNA expression in SK-N-DZ cells was confirmed by qRT-PCR (left). Migration assays (middle) and invasion assays (right) were performed as indicated above. All experiments were performed in triplicate.

Supplementary Figure S7. Endogenous expression of *ALK* in NBL and non-NBL cell lines. (A) mRNA expression level and missense mutations of *ALK* in NBL cell lines. qRT-PCR and DNA sequencing of *ALK* gene was applied to 27 indicated human neuroblastoma tumor cell lines. (B) mRNA expression level of *ALK* in non-NBL cell lines. qRT-PCR was performed on 19 indicated human non-neuroblastoma tumor cell lines.

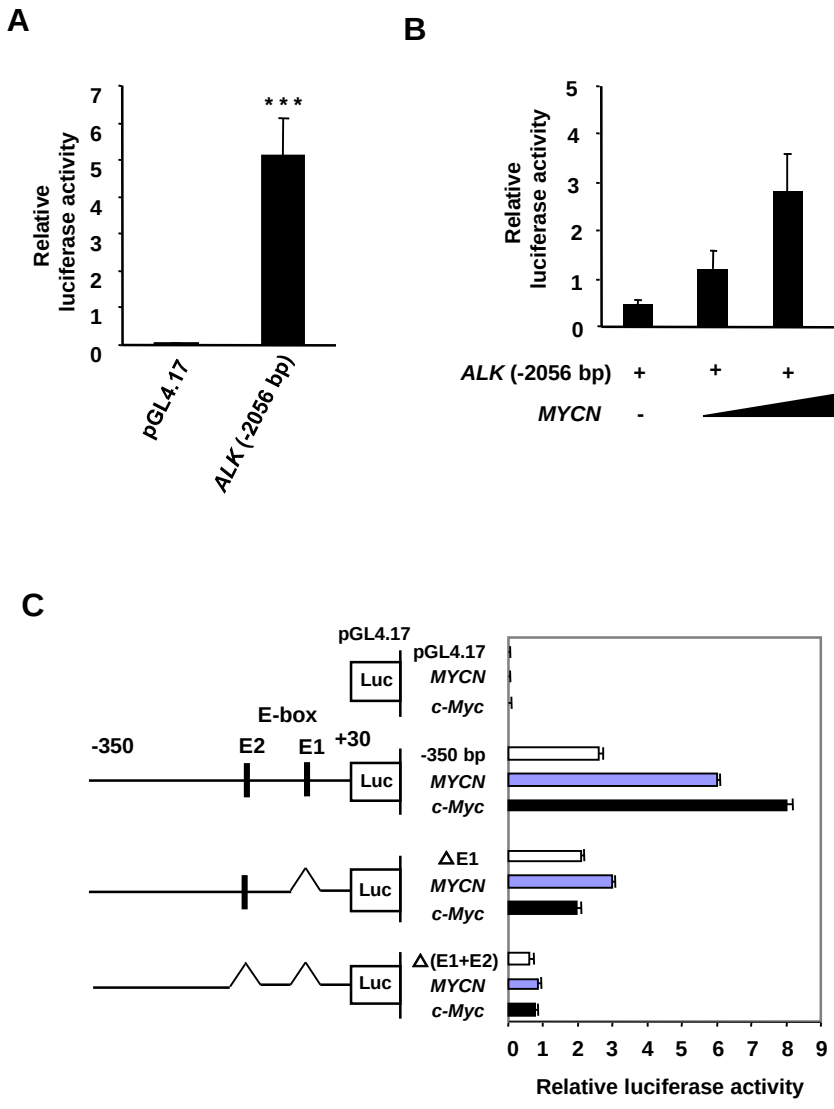
Supplementary Figure S8. Effects of crizotinib and CH5424802 on NBL cells. (a) Crizotinib (left) or CH5424802 (right) inhibited the proliferation of NBL cells. *MYCN*-non-amplified (SK-N-AS) or amplified (NLF and SK-N-DZ) NBL cells were treated with various concentrations of crizotinib or CH5424802 for 72h and cell proliferation was measured. The values are the mean ± SD of triplicate experiments.

(b) Crizotinib or CH5424802 suppressed cell migration of MYCN amplified NBL cells. SK-N-DZ cells were treated with 50 nM or 200 nM of crizotinib, CH5424802 or DMSO as control, and cell migration assay was performed. The values are the mean ± SD of triplicate experiments.

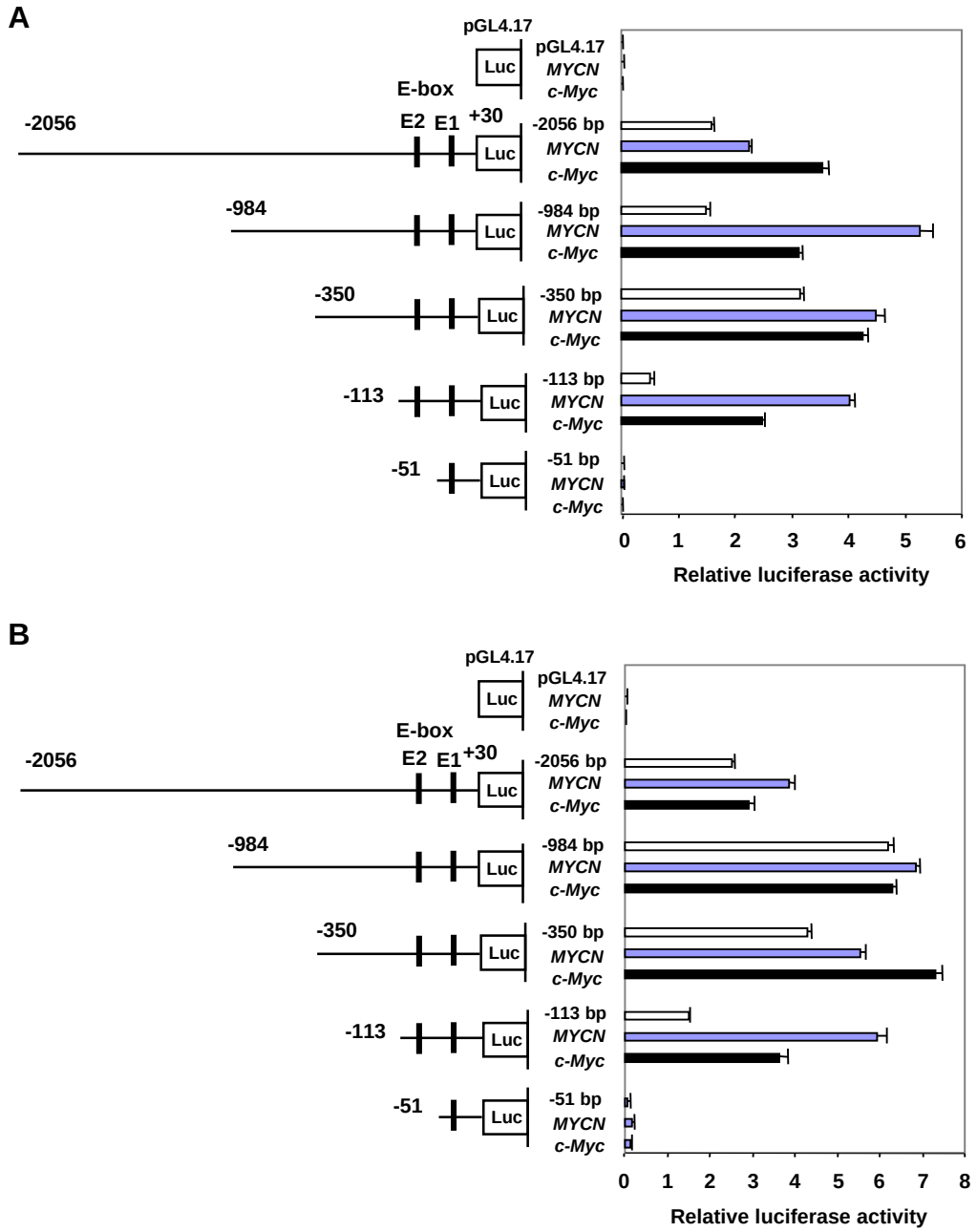
Supplementary Figure S1



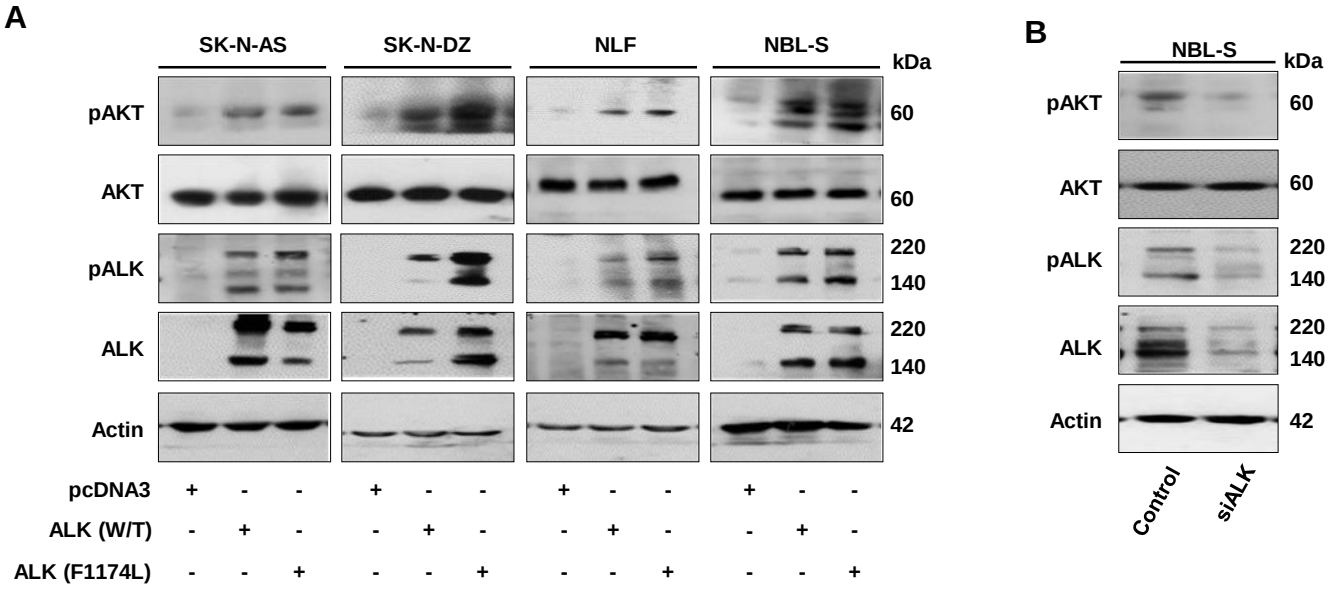
Supplementary Figure S2



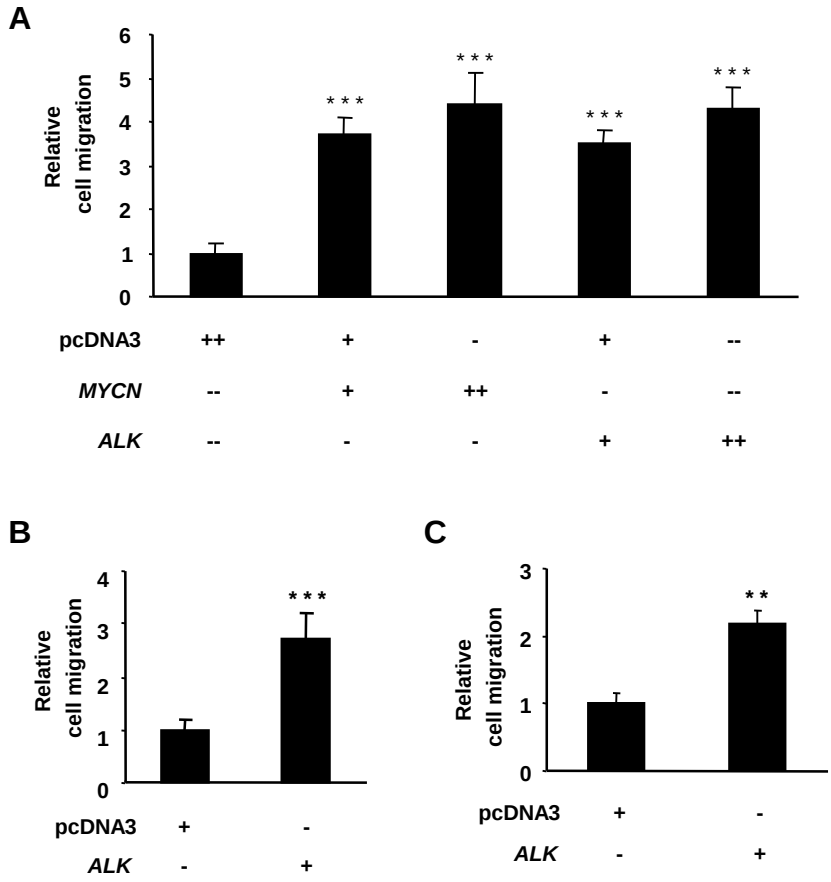
Supplementary Figure S3



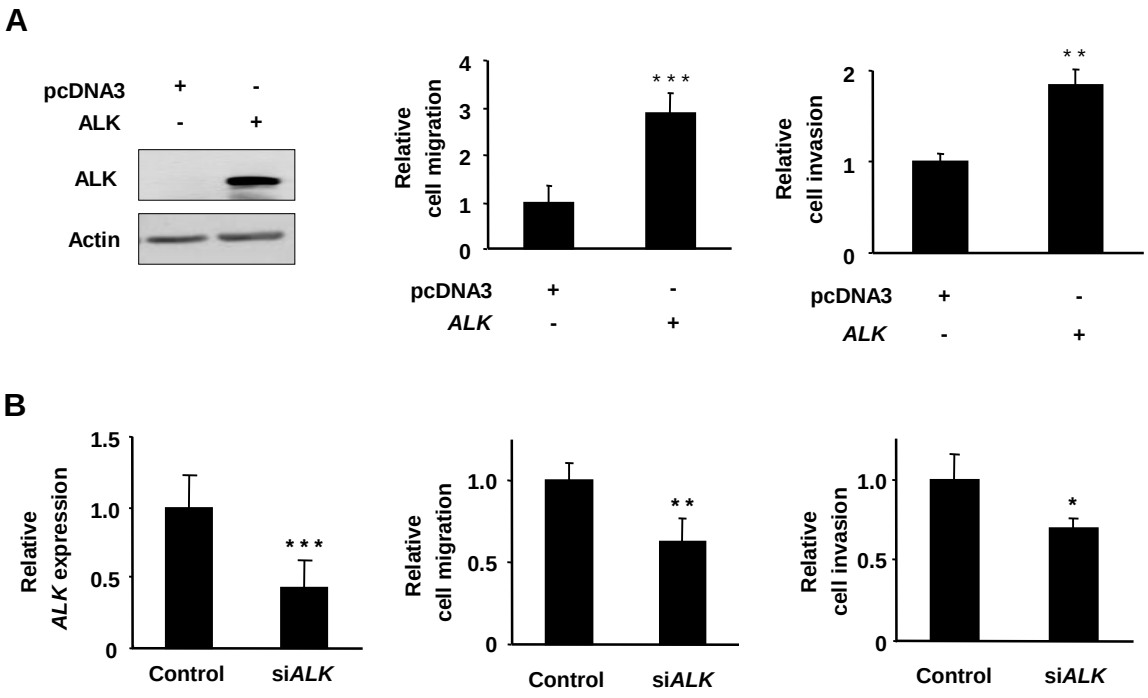
Supplementary Figure S4



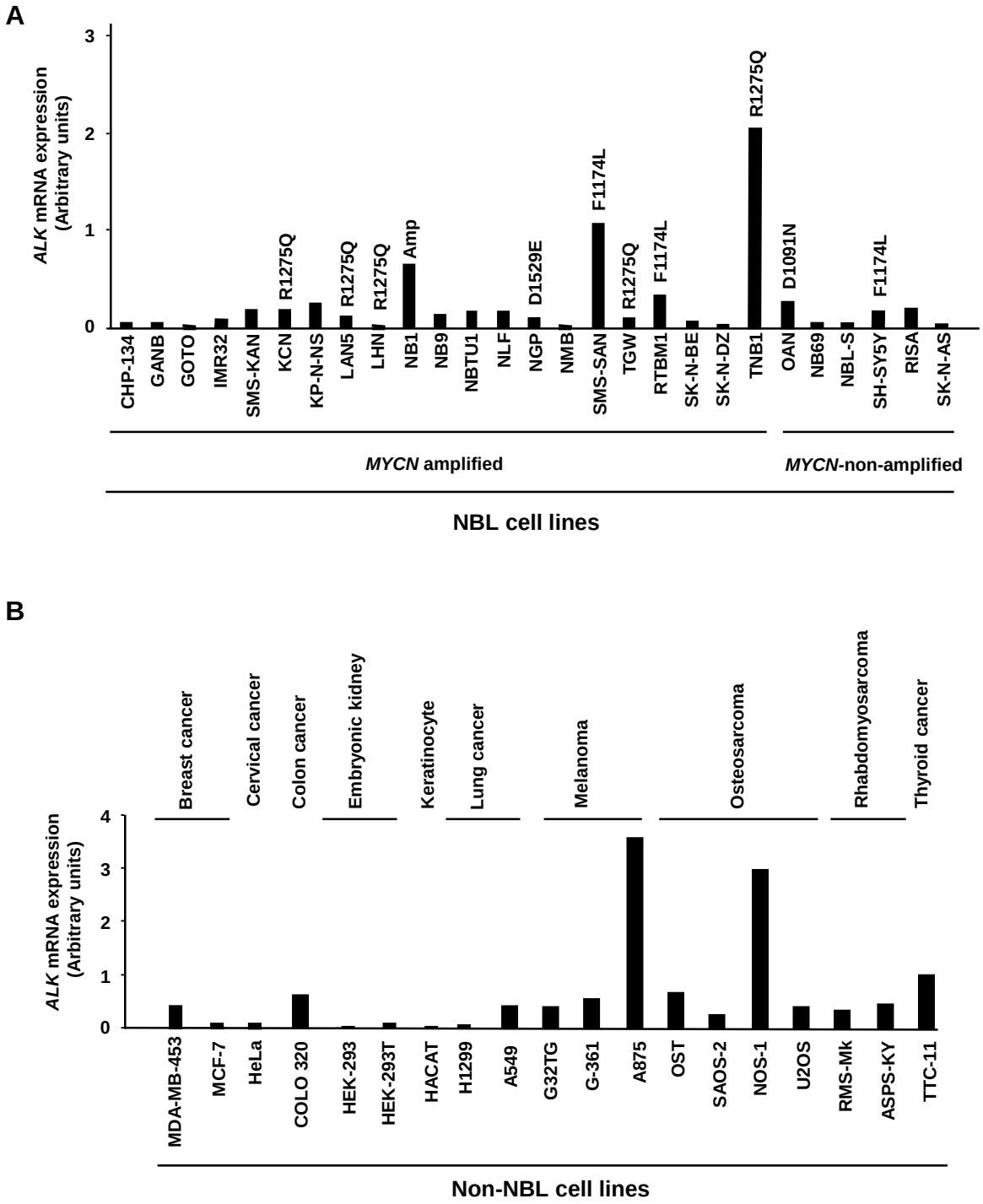
Supplementary Figure S5



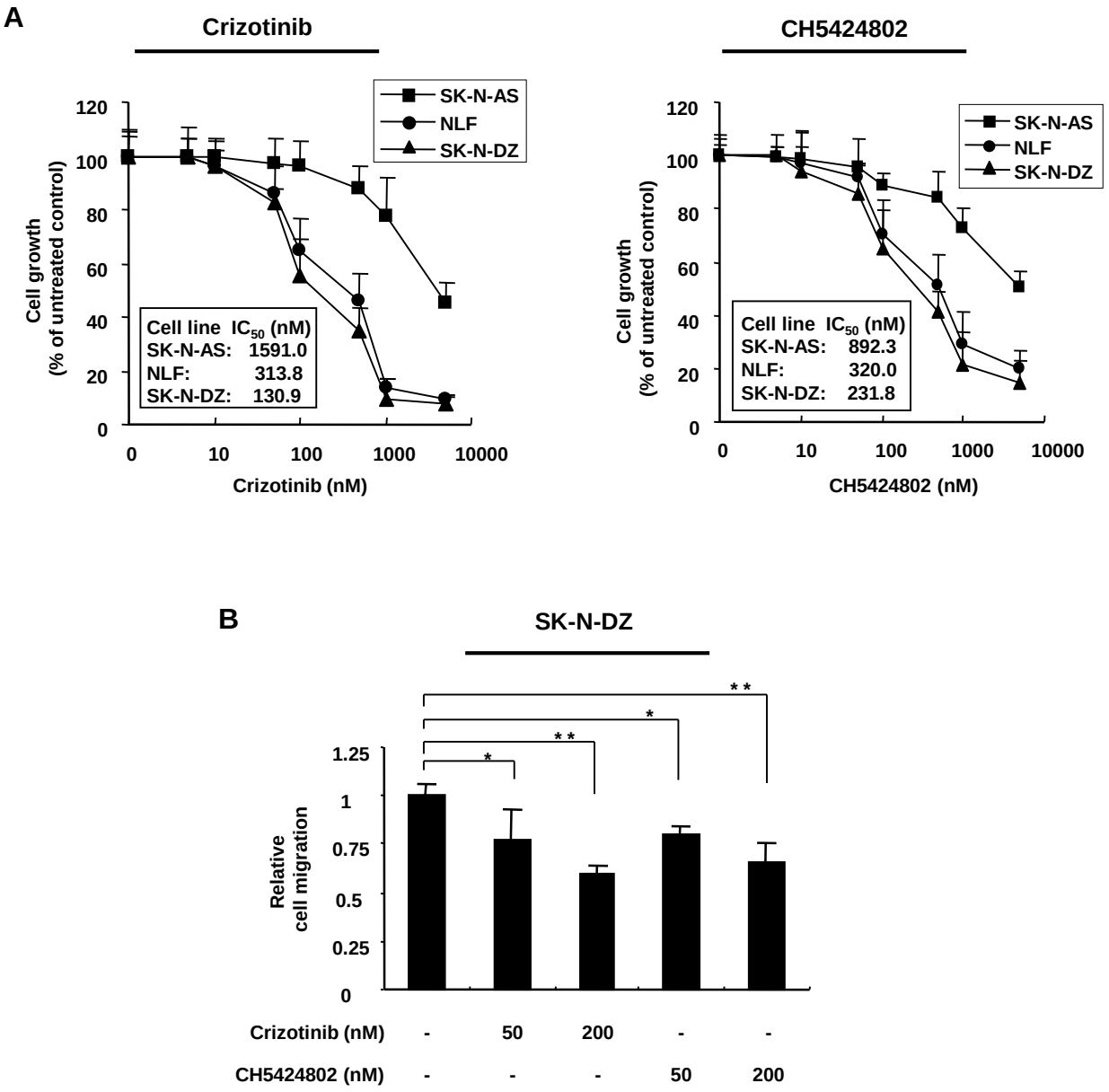
Supplementary Figure S6



Supplementary Figure S7



Supplementary Figure S8



Supplementary Figure S9. **Full-length images of the blots presented in the main figure.**

For Figure 5a