Supplementary Materials and Methods

siRNA sequences used in this study

The sequences of Stealth Select RNAi for *ALK* and *FLOT-1* were as follows: *ALK* sense no.1 (ALK): 5′ -AAUACUGACAGCCACAGGCAAUGUC-3′ ; *ALK* antisense no.1 (ALK): 5′ -GACAUUGCCUGUGGCUGUCAGUAUU-3′ ; *ALK* sense no.2 (ALK′): 5′ -UUAGGUGGGACAGUACAGCUUCCCU-3′ ; *ALK* antisense no.2 (ALK′): 5′ -AGGGAAGCUGUACUGUCCCACCUAA-3′ ; *FLOT-1* sense no.1 (ALK): 5′ -GGGCAUCAGUGUGGUUAGCUACACU-3′ ; *FLOT-1* antisense no.1 (ALK): 5′ -AGUGUAGCUAACCACACUGAUGCCC-3′ ; *FLOT-1* sense no.2 (ALK′): 5′ -CGGGAAGCUAAAGCCAAGCAGGAAA-3′ ; *FLOT-1* antisense no.2 (ALK′): 5′ -UUUCCUGCUUGGCUUUAGCUUCCCG-3′ . Stealth RNAi^{TM} siRNA Negative Control GC Dulpex #2 (Invitrogen) was used as control.

Immunoblotting

Cells were washed twice with ice-cold PBS and lysed in a lysis buffer. Protein concentration was determined using the BCA protein assay reagents (Thermo Scientific). The samples were resolved by SDS-PAGE, transferred to PVDF membranes (Millipore), and blocked with Blocking One (Nakarai Tesque). The membranes were incubated with primary antibodies for 2 hours at room temperature (RT) followed by incubation with HRP-conjugated secondary antibodies (GE healthcare) for 30 minutes at RT. Then, the immunoreactive bands were visualized using ECL Plus Western Blotting Substrate (GE Helathcare) on an X-ray film.

Immunoprecipitation

For immunoprecipitation, total cell lysates (0.5–2 mg) were incubated with the indicated antibodies (1–2 μg) for 2 hours at 4 °C with continuous rotation. Subsequently, Protein G Sepharose 4 Fast Flow (GE Healthcare; 40 μL, 50 % slurry) was added to each sample and incubated with continuous rotation at 4 °C for 30 minutes. Samples were washed four times using a lysis buffer and then the sample buffer was added (2 % SDS, 0.1 M Tris-HCl, pH 6.5, 10 % glycerol, 0.01 % bromophenol blue, 0.1 M dithiothreitol). Obtained samples were analyzed by immunoblotting.

Establishment of shRNA stable clones

Clones of TNB-1 cells stably expressing control or *FLOT1* shRNA were generated using lentiviral particles system (Santa Cruz Biotechnology; sc-108080 and sc-35391-V, respectively) which consists of a pool of lentiviral particles containing 3 sets of target specific shRNA. TNB-1 cells cultures in 12-well plate were treated by 20 µl of lentiviral solution and selected by 4 µg/ml puromycin for 30 days. Resultant stable clones were picked-up, and knockdown of FLOT1 was verified by immunoblotting. Two independent clones per gene were used (control; Con1 and Con2, *FLOT1*; FL1 and FL2).

Immunofluorescence

Cells grown on coverslips were fixed with ice-cold methanol for 5 minutes followed by permeabilization with 0.3 % Triton-X100 in PBS for 5 minutes at RT. After treatment with blocking solution (5 % skim milk in PBS) for 1 hour at RT, the cells were incubated with the appropriate primary antibody (diluted in blocking solution, 1:100) at 4 °C overnight. Then, the cells were washed three times using PBS for 10 minutes and incubated with fluorescence-conjugated secondary antibodies (Invitrogen) for 60 minutes at RT. After washing three times with PBS for 10 minutes at RT, the cells were incubated with DAPI (1 µg/mL, Invitrogen) at RT for 10 minutes. The coverslips were mounted using Slow Fade Gold antifade agent (Invitrogen). Samples were observed using an Olympus IX81-ZDC-DSU microscope equipped with a cooled CCD camera (ORCA-ER, Hamamatsu), and the imaging system was driven by MetaMorph software (Universal Imaging).

Purification of ALK-binding tyrosine-phosphorylated proteins

TNB-1 cells stably expressing ALK constructs (250 mg protein/sample) were collected and lysed in a lysis buffer (50 mM Hepes-NaOH (pH 7.0), 150 mM NaCl, 10 % glycerol, 1 % Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM Na_3VO_4, 100 mM NaF, and a protease inhibitor cocktail). The ALK proteins were affinity purified using an immunoaffinity (ALK-antibody sepharose) column conjugated with CNBr sepharose (GE healthcare) for 6 hours at 4 °C. After washing four times with lysis buffer, antibody-bound proteins were eluted using an 8 M urea buffer (8 M urea, 1 % Triton X-100, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4). After

denaturation by dialysis against a solution of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride, the samples were affinity purified with a phosphotyrosine 4G10-antibody conjugated with CNBr sepharose for 5 hours at 4 °C. After washing with lysis buffer four times, the antibody-bound proteins were eluted using 0.1 M phenylphosphate buffer (0.1 M phenylphosphate, 0.1 % heptylglucoside, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl). The purified proteins were concentrated and separated by SDS-PAGE. After visualization of the proteins by silver stain, the bands including the 47-kDa proteins were analyzed by mass spectrometry.

Supplementary Figure Legends

Supplementary Figure S1. Schematic of two-step immunoaffinity purification of tyrosine-phosphorylated proteins associated with ALK

The cell lysates from TNB-1 cells expressing ALK constructs were subjected to sequential immunoaffinity purification, first with anti-Flag antibody column and then with phosphotyrosine antibody 4G10 column. The purified proteins were separated by SDS-PAGE and analyzed by mass spectrometry. Flotillin-1 (FLOT1) was identified as a protein in the 47-kDa band.

Supplementary Figure S2. Expression of FLOT1 and ALK in selected human neuroblastoma samples

Protein samples obtained from clinical neuroblastoma specimens classified by Bredeur's clinical prognosis classification (good, 5 cases; intermediate, 5 cases; poor, 5 cases) were analyzed by western blot using the indicated antibodies.

Supplementary Figure S3. ALK regulates tyrosine-phosphorylation of FLOT1

NB-39-nu cells were treated with control (con), *ALK* (ALK1 or ALK2), or *FLOT1* (FL1) siRNA for 16 hours and then treated with DMSO or ALK inhibitor NVP-TAE-684 (TAE; 20 nM) for 2 hours. The cell lysates were immunoprecipitated with control IgG or anti-phosphotyrosine 4G10 antibody and analyzed by immunoblotting.

Supplementary Figure S4. FLOT1, but not clathrin and caveolin, regulates ALK endocytosis upstream of lysosomal degradation.

(A) NB-39-nu cells were treated with control siRNA (con) or *FLOT1* siRNA (FL1) for 72 hours and additionally cultured in the presence of lysosomal inhibitor

concanamycin (Cc, 10 μM) for 8 hours. The plasma membrane-localized proteins were purified and analyzed by immunoblotting using the ALK antibody or N-cadherin antibody. Total cell lysates were also analyzed by immunoblotting using the indicated antibodies.

(B) NB-39-nu cells were pulse-chased with ALK-antibody for 15 minutes to monitor the early phase of endocytosis. The cells were stained with DAPI (blue) and antibodies against the major endosomal markers (green), including FLOT1, clathrin heavy chain (HC), and caveolin-1. The internalized-ALK antibodies were also stained (red). Arrowheads indicate cells in which internalized ALK co-localizes with FLOT1 at the submembrane regions.

(C) The percentage of cells that showed colocalization of internalized ALK and each endosomal marker was calculated as described in the Material and Methods**.**

Supplementary Figure S5. FLOT1 downregulates malignant phenotypes of neuroblastoma cells

(A) NB-39-nu cells were transfected with control (con) or *FLOT1* siRNA (FL1 or FL2) for 48 hours. The cells were further treated with CDDP (300 μM) in combination with DMSO or ALK inhibitor NVP-TAE-684 (TAE; 20 nM) for 48 hours. The extent of cell death was determined as described in the Materials and Methods.

(B) NB-39-nu cells transfected with indicated siRNAs were treated with DMSO or ALK inhibitor NVP-TAE-684 (TAE; 20 μM) for 2 hours. Cell migration was assayed as described in the Materials and Methods under continuous treatment with DMSO or TAE.

(C) NB-39-nu cells transfected with the empty vector or HA-tagged FLOT1 were cultured with DMSO or the caspase inhibitor z-VAD-FMK (100 μM; Z-VAD) for 72 hours. The extent of cell death was determined.

(D) NB-39-nu cells transfected with the empty vector or HA-tagged FLOT1 for 24 hours were subjected to ALK-internalization assays (chase time; 60 minutes). The avidin-bounded (internalized) proteins and total cell lysates were analyzed by immunoblotting using the indicated antibodies.

(E) The anchorage-independent cell growth of TNB-Con1 cells and TNB-FL1 cells was analyzed under continuous treatment with the vehicle (DMSO) or ALK inhibitor NVP-TAE-684 (TAE; 20 nM).

Supplementary figure S6. FLOT1 regulates ALK expression and activation as well as oncogenicity by regulating ALK-endocytosis in Nagai neuroblastoma cells harboring amplified wild-type ALK

(A) Nagai cells were treated with control (con) or *FLOT1* (FL1 or FL2) siRNA for 72 hours. The treated cells were subjected to ALK-internalization assay (chase time; 30 minutes). The internalized proteins and total cell lysates were analyzed by immunoblotting.

(B) Nagai cells transfected with indicated siRNAs were treated with DMSO or ALK inhibitor NVP-TAE-684 (TAE; 20 μM) for 2 hours. Cell migration was assayed under continuous treatment with DMSO or TAE.

Supplementary Figure S7. FLOT1 regulates migration and resistance to CDDP-induced cell death of neuroblastoma cells expressing ALK mutants

(A) The stable TNB-1 transfectants of the ALK mutants were transfected with control siRNA (con) or *FLOT1* (FL1 or FL2) siRNA for 24 hours. The cells were further treated with CDDP (300 μM) in combination with DMSO or TAE (20 μM) for 48 hours. The extent of cell death was then determined.

(B) The stable TNB-1 transfectants were transfected with indicated siRNAs for 48 hours and subjected to migration assay in the presence or absence of DMSO or TAE.

Supplementary Figure S8. The summary

Schematic overview of results. Cell surface expression of wild-type ALK, and probably some ALK mutants that have high affinity to FLOT1, is downregulated by FLOT-mediated endocytosis and subsequent degradation at lysosomes. In neuroblastoma cells with lower expression of FLOT1 or harboring mutant ALKs, such as F1174L and K1062M that have lower affinity to FLOT1, internalization and degradation of ALK were impaired. This may lead to constitutive activation of ALK at the plasma membrane and of downstream signaling pathways, thereby promoting ALK-dependent malignant phenotypes of neuroblastoma.