lines of TNB-1 cells, TNB-Con1 and TNB-Con2 cells, were also established using the control vector. Enhanced expression of ALK and phosphorylation of AKT and ERK1/2 was observed in TNB-FL1 and TNB-FL2 cells, whereas no significant changes in the expression of other RTKs, such as EGFR, RET, and TrkB, were observed (Fig. 4E). In addition, increased anchorage-independent growth was detected in the TNB-FL1 and TNB-FL2 cells, which was blocked by treatment with the ALK inhibitor (Fig. 4F and Supplementary Fig. S5E). These results demonstrated that FLOT1 inhibits the malignant phenotype of neuroblastoma cells through endocytosis of ALK.

Activating mutations of ALK have low binding affinities to FLOT-1 and cause ALK stabilization and malignant phenotypes in neuroblastoma cells

It is reported that some of the activating mutations of ALK such as the common F1174L mutation exhibit more malignant phenotypes and poor prognosis than others (19), while the mechanisms causing the differences are still not clear. We investigated differences in the binding affinities between mutant ALK proteins and FLOT1 by using TNB-1 neuroblastoma cells stably expressing WT, F1174L (FL; mutation near the c-helix loop), K1268M (KM; mutation in the juxtamembrane domain), and R1275Q (RQ; mutation near the ATP-binding domain) mutants of ALK (8–11). FLOT1 steadily associated with the WT and RQ mutants of ALK, but not as efficiently with the FL and KM mutants (Fig. 5A). Furthermore, knockdown of FLOT1 affected the internalization of biotinylated ALK in the WT and RQ mutants but not in the FL and KM mutants (Fig. 5B). In addition, the phosphorylation levels of ALK, AKT, and ERK1/2 were not obviously elevated by treatment with FLOT1 siRNA in the TNB-1 cells with the FL or KM mutation (Fig. 5B).

Anchorage-independent growth was significantly enhanced by FLOT1 siRNA in cells expressing WT or RQ mutant, whereas no obvious changes were observed in the cells expressing the FL and KM mutants, which originally showed enhanced anchorage-independent growth. Anchorage-independent growth of all the cells analyzed was reduced by treatment with the ALK inhibitor (Fig. 5C). Similar difference in ALK mutants were also confirmed using the TNB-1 cells expressing WT ALK, the FL mutant, and the KM mutant as for resistance to CDDP and cell migration (Supplementary Fig. S7A and S7B). These results suggested that some of the activating mutations of ALK might have enhanced stability at the cell membrane by reduced affinity to FLOT1, which leads to further enhancement of the malignancy of neuroblastoma.

FLOT1 regulates tumorigenicity of neuroblastoma cells

To investigate the role of FLOT1 in the tumorigenicity of neuroblastoma, TNB-Con1/2, and TNB-FL1/2 cells were subcutaneously injected into nude mice (Fig. 6A). Because of the low tumorigenicity of the original TNB-1 cells, tumors were not detectably formed at 6 weeks following injection of TNB-Con cells. On the other hand, tumors as large as 10 to 40 mm in diameter were clearly formed by the TNB-FL1 and TNB-FL2

cells in this period (Fig. 6A). Histologic study of these tumors revealed that the tumor cells had infiltrated into the muscle layers and formed large intratumoral vessels, which reflects the malignant phenotype of the tumors (Fig. 6B). These results indicated that FLOT1 might be a negative regulator of the malignant characteristics of neuroblastoma *in vivo*. Along with the results indicating that the low expression of FLOT1 is significantly associated with poor prognosis and unfavorable histologic grades of neuroblastoma (Fig. 1B and C), it was indicated that deregulation of FLOT1 expression is involved in the progression of neuroblastoma through enhancement of ALK signaling.
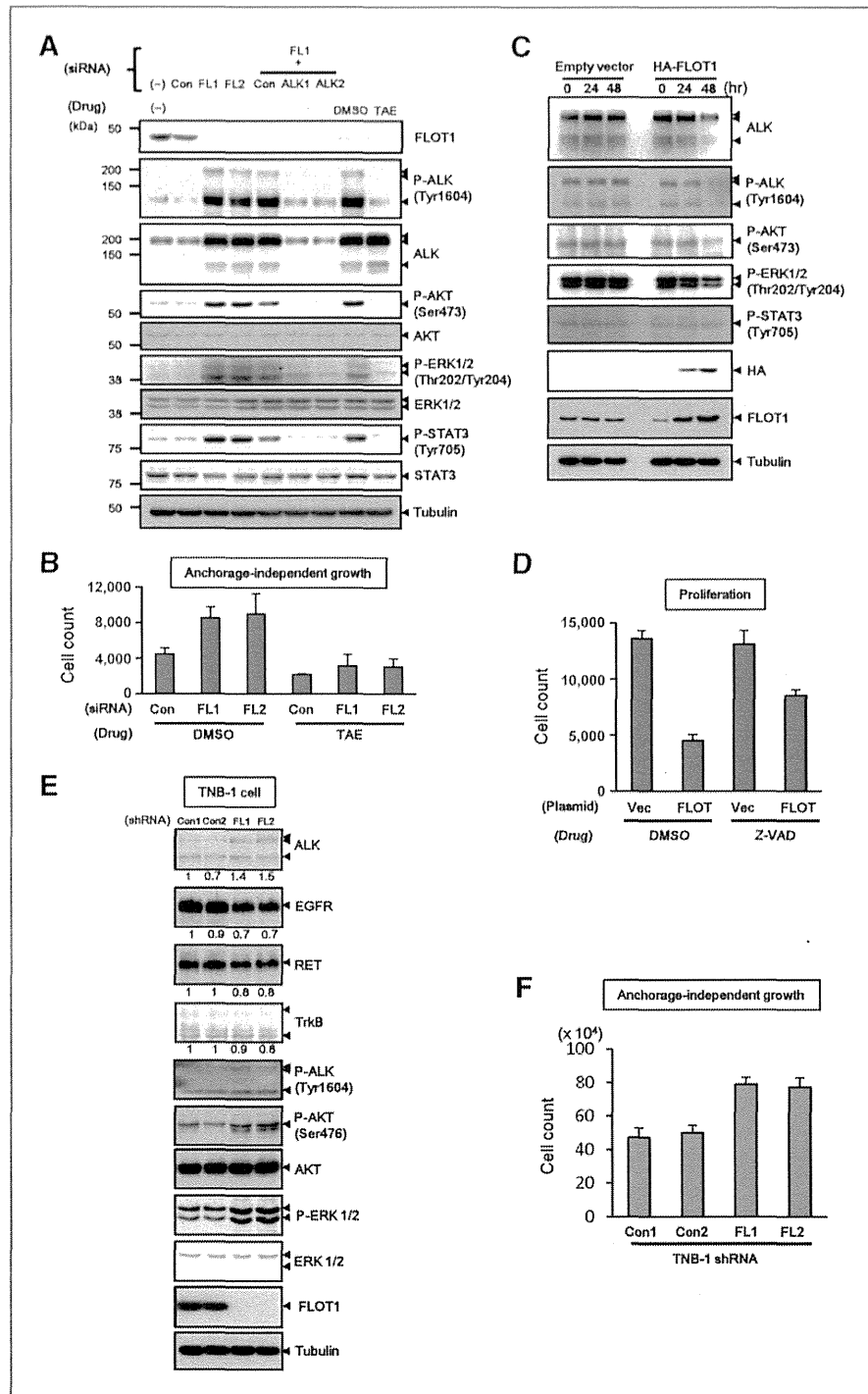
Discussion

Deregulation of the RTK ALK by amplification or activating mutation of the *ALK* gene has been reported in 10% to 15% of human neuroblastoma cases, in which the relationship between ALK signaling and oncogenesis of neuroblastoma is indicated. Although the association between ALK expression and poor prognosis of neuroblastoma is observed (Fig. 1A), it is not clear whether different modes of activation of ALK signaling are involved in the progression of the other neuroblastoma cases. In this study, we provided *in vitro* and *in vivo* evidence that activation of ALK signaling caused by impaired FLOT1-mediated endocytosis is associated with malignancy of neuroblastoma cells. This finding was supported by the observation that FLOT1 expression levels in the clinical samples is inversely correlated with prognosis of the disease in a public database (Fig. 1B) and grades of malignancy in tissue samples (Fig. 1C). Taken together, the novel tumor-suppressing role of FLOT1 in the majority of neuroblastoma that lacks genetic alterations of *ALK* was implied.

There was tendency that the low expression level of FLOT1 is associated with high expression levels of ALK in the neuroblastoma tissues used for Fig. 1C, while it was not statistically significant possibly due to limited numbers of tissues examined (data not shown). Therefore, we could not completely exclude the possibility that FLOT1 also degrades signaling molecules other than ALK, which are associated with malignancy of neuroblastoma, although it was confirmed that FLOT1 preferably regulates the ALK protein among several other RTKs expressed in neuroblastoma (Fig. 4E). The information about the involvement of FLOT1 in cancer development is still limited (29, 40–42). It was recently suggested that FLOT1 is associated with poor prognosis of breast cancer as a result of stabilization of ErbB2 (27) and also plays oncogenic roles in esophageal cancer and hepatocellular carcinoma (28, 40). It is speculated that FLOT1 might regulate the organ-dependent target proteins and functions in the development of cancers and the regulation is rather selective to ALK in neuroblastoma. Considering that activated ALK found in other types of cancers lack transmembrane domain, the accumulation of membranous ALK by deficient FLOT1 might be etiological only in neuroblastoma.

It has been reported that FLOT1 physiologically acts as an endosomal transporter of membrane proteins (23–25). Further study is required to clarify the precise mechanisms

Figure 4. FLOT1 regulates ALK signaling and oncogenicity of neuroblastoma cells. A, NB-39-nu cells were treated with control (Con) or *FLOT1* siRNA (FL1 or FL2) alone or in combination with *ALK* siRNA (ALK1 or ALK2) for 72 hours. *FLOT1* siRNA-treated cells were further cultured in the presence of DMSO or ALK inhibitor NVP-TAE-684 (TAE; 20 nmol/L) for 2 hours. The cell lysates were analyzed by immunoblot analysis using the indicated antibodies. B, NB-39-nu cells were treated with indicated siRNAs and their anchorage-independent growth under continuous treatment with DMSO or TAE was tested as described in Materials and Methods. C, NB-39-nu cells were transiently transfected with an empty vector or HA-tagged FLOT1 for the indicated times. The cell lysates were analyzed by immunoblot analysis using the indicated antibodies. D, NB-39-nu cells transfected with vector alone or HA-tagged FLOT1 were cultured with DMSO or the caspase inhibitor z-VAD-FMK (Z-VAD; 100 μmol/L) for 72 hours. Cell proliferation was measured as described in Materials and Methods. E, stable transfectants of TNB-1 cells expressing control shRNA (TNB-Con1 and TNB-Con2) or FLOT1 shRNA (TNB-FL1 and TNB-FL2) were analyzed by immunoblotting. The expression levels of RTKs were quantified and denoted as relative value (TNB-Con1 = 1) under each immunoblotting data. F, the anchorage-independent growth of TNB-1 cells expressing control or FLOT1 shRNA was tested as described in Materials and Methods.

of endocytosis of ALK, including mode of association between FLOT1 and ALK and the role of tyrosine phosphorylation of FLOT1 in this process. It has been reported that another RTK-binding protein Cbl is involved in the downregulation of RTKs through receptor ubiquitination followed

by endocytosis. Indeed, some mutations in the RTKs, Met and EGFR, decrease the affinity of RTKs with Cbl, which results in impaired endocytosis and oncogenic accumulation of RTKs in several cancers, including lung cancer and glioblastoma (43).
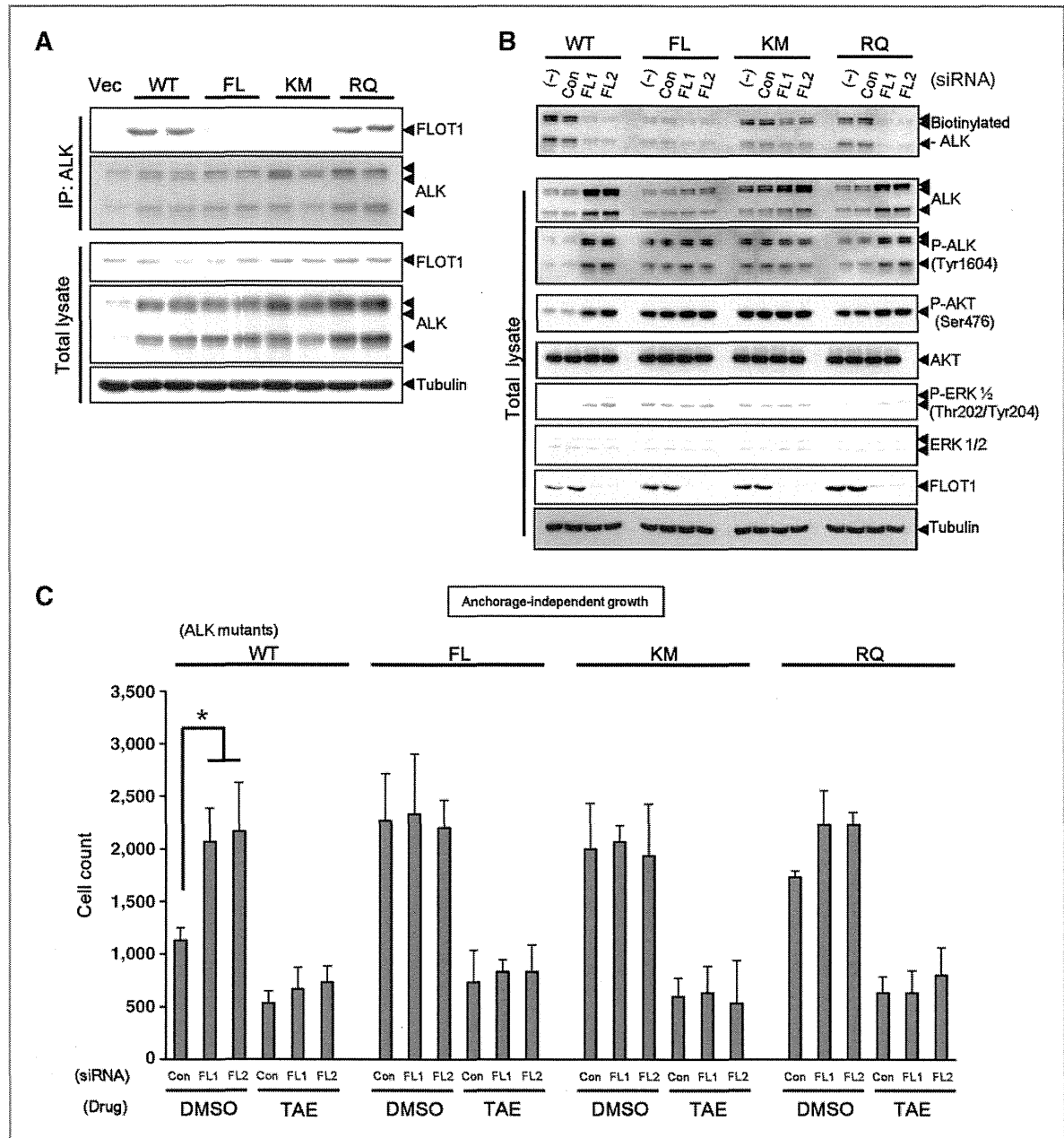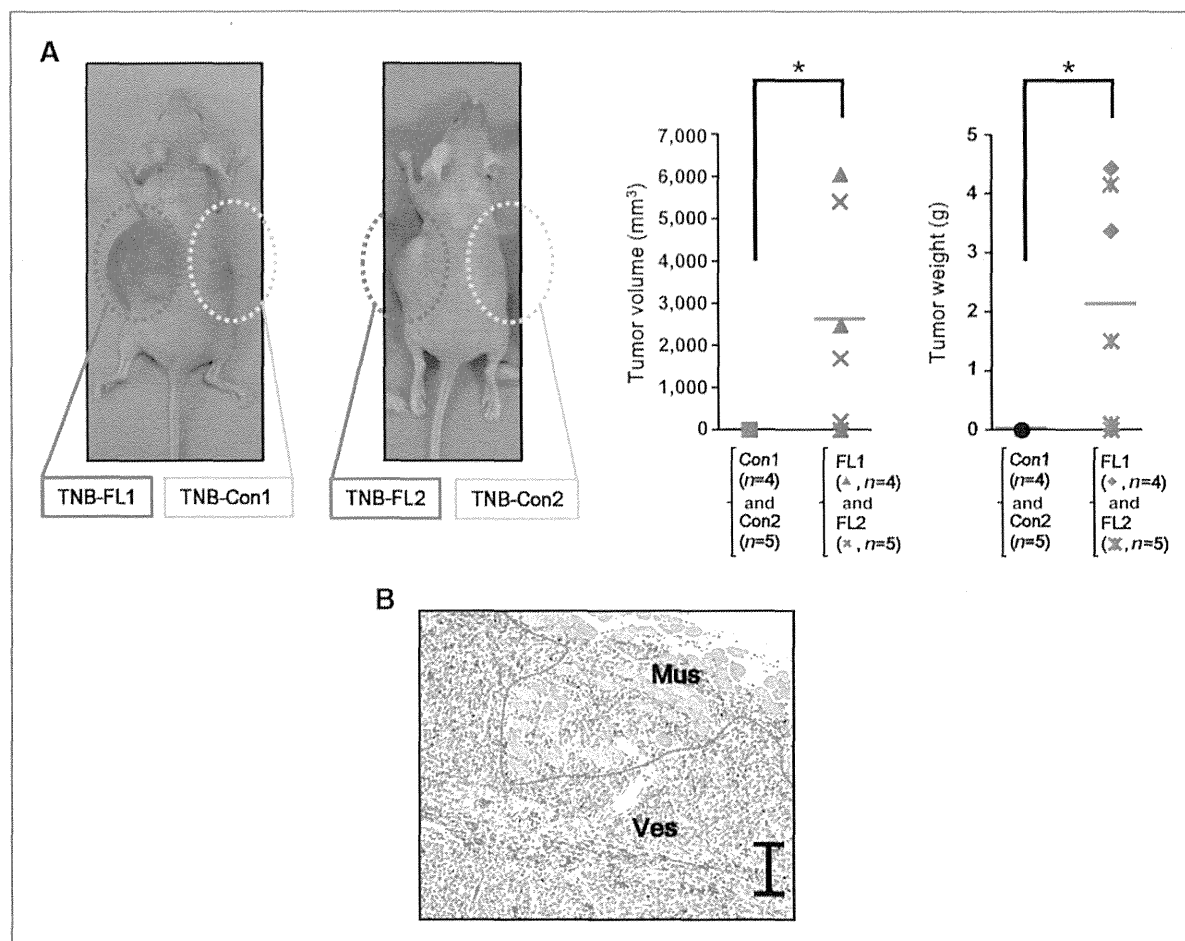
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Figure 5. Activating mutations of ALK confer resistance to downregulation by FLOT1. A, cell lysates from each pair of different stable TNB-1 transfectants of empty vector and ALK mutants (WT; FL, F1174L; KM, K1062M; RQ, R1275Q) were immunoprecipitated using the anti-ALK antibody. The immunocomplexes and total cell lysates were analyzed by immunoblotting. B, the stable TNB-1 transfectants were transfected with control siRNA (con) or FLOT1 siRNA (FL1 or FL2) for 48 hours. The cells were subjected to the ALK internalization assay for 60 minutes, and the internalized proteins and total cell lysates were analyzed by immunoblotting. C, the stable TNB-1 cells transfectants were transfected with indicated siRNAs for 48 hours and cultured in the presence of DMSO or ALK inhibitor NVP-TAE-684 (TAE; 20 nmol/L) for 2 hours. The cells were subjected to the anchorage-independent cell growth assay under continuous treatment with DMSO or TAE. *, $P < 0.01$.

It was recently reported that neuroblastoma cases harboring certain activation mutations of ALK exhibit higher refractoriness (19). For example, neuroblastoma cases harboring the F1174L mutant are also reported to have higher resistance than cases with amplified or R1275Q-mutant ALK when treated with an ALK inhibitor (18). Both the F1174L and R1275Q mutations of

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Figure 6. FLOT1 negatively regulates tumorigenicity of neuroblastoma cells. A, left, macroscopic images of the mouse xenograft experiment. The TNB-1 cells stably expressing control (TNB-Con1 and -Con2, yellow circle) or FLOT1 shRNA (TNB-FL1 and -FL2, red circle) were subcutaneously inoculated into both sides of the flank of 4-week-old nude mice as shown (see Supplementary Materials and Methods). In the first group (group 1) TNB-Con1 and -FL1 cells, and in the second group (group 2) TNB-Con2 and -FL2 cells were inoculated. The images of one of the 5 mice from each group 1 and group 2 at 6 weeks after inoculation were presented. Right, the total tumor volume and weight were measured and plotted as scatter grams. Green bars, average values. *, $P < 0.01$. B, high magnification image of the tumor tissue stained with H&E from the mouse (group 1, TNB-FL1 cells). Tumor invasion into the subcutaneous muscle layer (Mus) and the formation of intratumoral large vessels (Ves) were observed. The dotted line denotes the margin of the muscle layer. Bar, 100 μm.

ALK are frequently observed, while F1174L mutant is more aggressive and resistant to ALK inhibitors than R1275Q mutants even in a transgenic fly system (20). We demonstrated that the FL and KM mutants of ALK have less affinity to FLOT1 and therefore less affected by FLOT1-mediated endocytosis than the WT ALK (Fig. 5B). The role of oncogenic potential of ALK mutants has previously been analyzed with respect to ATP-binding potential and impaired receptor trafficking (44, 45), while our results suggest that impaired downregulation of ALK by FLOT1 might contribute to the aggressiveness of some mutations of ALK (Supplementary Fig. S8). It should be emphasized that expression levels of FLOT1 might also become one of the efficient clinical markers determining prognosis and therapeutic effectiveness of ALK inhibitors in neuroblastoma cases.

Disclosure of Potential Conflicts of Interest

Authors' Contributions

Conception and design: A. Tomiyama, T. Uekita, R. Kamata, J. Takita, R. Sakai
Development of methodology: A. Tomiyama, J. Takita
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Tomiyama, K. Sasaki, J. Takita, A. Nakagawara
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Tomiyama, T. Uekita, R. Kamata, K. Sasaki, J. Takita, R. Sakai
Writing, review, and/or revision of the manuscript: A. Tomiyama, T. Uekita, J. Takita, H. Yamaguchi, R. Sakai
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Tomiyama, T. Uekita, R. Kamata, J. Takita, M. Ohira, A. Nakagawara, C. Kitanaka, R. Sakai
Study supervision: T. Uekita, J. Takita, K. Mori, H. Yamaguchi, R. Sakai

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Molecular and Cellular Pathobiology

Cancer Research

Flotillin-1 Regulates Oncogenic Signaling in Neuroblastoma Cells by Regulating ALK Membrane Association

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Abstract

Neuroblastomas harbor mutations in the nonreceptor anaplastic lymphoma kinase (ALK) in 8% to 9% of cases where they serve as oncogenic drivers. Strategies to reduce ALK activity offer clinical interest based on initial findings with ALK kinase inhibitors. In this study, we characterized phosphotyrosine-containing proteins associated with ALK to gain mechanistic insights in this setting. Flotillin-1 (FLOT1), a plasma membrane protein involved in endocytosis, was identified as a binding partner of ALK. RNAi-mediated attenuation of FLOT1 expression in neuroblastoma cells caused ALK dissociation from endosomes along with membrane accumulation of ALK, thereby triggering activation of ALK and downstream effector signals. These features enhanced the malignant properties of neuroblastoma cells *in vitro* and *in vivo*. Conversely, oncogenic ALK mutants showed less binding affinity to FLOT1 than wild-type ALK. Clinically, lower expression levels of FLOT1 were documented in highly malignant subgroups of human neuroblastoma specimens. Taken together, our findings suggest that attenuation of FLOT1-ALK binding drives malignant phenotypes of neuroblastoma by activating ALK signaling. *Cancer Res; 74(14); 3790–801. ©2014 AACR.*

Introduction

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase (RTK) that is rather specifically expressed in the nervous system during development in mice (1). ALK was first identified in anaplastic large cell lymphoma as the fusion protein NPM-ALK caused by chromosomal translocation (2). Recently, ALK was highlighted as a therapeutic target of several cancers such as non–small cell lung cancers and colon cancers, which possess oncogenic fusion ALK proteins such as EML4-ALK (3–6). Genetic alterations of ALK have also been identified in cell lines and clinical samples of neuroblastoma, which consist of gene amplifications, activating mutations, or N-terminus truncations (7–12). Activated ALK proteins in neuroblastoma

are distinct from other tumors as for the point that they retain the transmembrane domain. The survival of neuroblastoma cells with activated ALK is dependent on the ALK protein in some cases, which highlights the so called oncogene addiction to activated ALK (13).

Neuroblastoma is one of the most refractory solid tumors in children with 5-year survival rates of less than 40% following conventional treatments (14–16). To this end, clinical trials involving patients with neuroblastoma and ALK inhibitors such as crizotinib have already begun (17). However, it was reported that neuroblastoma harboring certain types of activation mutations of ALK show greater resistance to the ALK inhibitors (18) and that there are differences in the malignancy grades among neuroblastoma cases with mutant ALK depending on the type of mutations (19, 20). Therefore, further investigation is necessary to elucidate what aspects of the mutant ALK protein determine the clinicopathological features of neuroblastoma.

As ALK is a RTK, it is essential to understand the signal transduction pathways that mediate the activation of this kinase. In addition to the common downstream mediators of RTKs, such as Akt, Erk, and STAT3, we have shown the critical role of ShcC as a binding partner of ALK in neuroblastoma (21, 22). Further identification of the tyrosine-phosphorylated binding partners of ALK and analysis of their functions in neuroblastoma will aid understanding of the unique oncogenic roles of ALK signaling.

Flotillin-1 (FLOT1) is a plasma membrane lipid raft-localizing protein that is involved in internalization of membrane-localizing proteins into the cytosol by endocytosis. In addition, FLOT1 plays a role in the regulation of actin organization and neuronal regeneration (23, 24), and phosphorylation of FLOT1

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at the tyrosine or serine is necessary during internalization (25, 26). At present, there is only limited information about the involvement of FLOT1 in the oncogenicity of solid cancers other than neuroblastoma (27–29). In this report, we identified FLOT1 during the screening of ALK-binding tyrosine-phosphorylated proteins in neuroblastoma cells by using mass spectrometry analysis. Functional analysis revealed that FLOT1 controls the malignant properties of neuroblastoma by regulating the endocytosis and degradation of membrane-localizing ALK protein. It was also suggested that alterations to the binding affinity to FLOT1 in some of the ALK mutants might contribute to the enhancement of oncogenic ALK signaling in neuroblastoma.

Materials and Methods

Antibodies and plasmids

The rabbit ALK antibody was previously described (22). The antibodies against phospho-ALK, Akt, phospho-Akt, p44/42 MAPK (ERK1/2), phospho-ERK1/2, STAT3, phospho-STAT3, and p53 were purchased from Cell Signaling Technology. Other antibodies used are: ALK (H260), clathrin HC, and LAMP2 (Santa Cruz Biotechnology); FLOT1, N-cadherin, and caveolin-1 (BD Transduction Laboratories); FLAG M2 and α-tubulin (Sigma); HA (Nakarai Tesque); and phosphotyrosine (4G10; Upstate Biotechnology).

The cDNAs of human wild-type (WT) *ALK*, the activating mutants of *ALK* (F1174L, K1062M, and R1275Q) and WT *FLOT1* were subcloned into the pcDNA3.1 vector.

Cell culture and tissue samples

NB-39-nu and Nagai human neuroblastoma cell lines were provided by Carcinogenesis Division, National Cancer Center Research Institute (Tokyo, Japan) in 2001 (30). TNB-1 human neuroblastoma cell line was obtained from Human Science Research Resource Bank in 2001 (31). Gene amplification of *MYCN* in these three lines and of *ALK* in NB-39-nu and Nagai is periodically checked to confirm the neuroblastoma origin of these cell lines, most recently in March 2014 (22). The cells were maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS, 10 U/mL penicillin, and 10 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Human neuroblastoma tissue samples were prepared as previously described (32).

Transfection and establishment of stable clones

Of note, 20 nmol/L of Stealth Select RNAi (Invitrogen) or 4 μg of plasmid was transfected by electroporation using the NEON system (Invitrogen). The siRNA sequences are described in Supplementary Materials and Methods. For establishment of stable ALK-mutant clones, TNB-1 cells were continuously treated with 400 μg/mL of G418. TNB-1 cells stably expressing control or *FLOT1* shRNA were established using lentiviral particles according to the manufacturer's instructions (Santa Cruz Biotechnology).

Purification of ALK-binding tyrosine-phosphorylated proteins

The immunoaffinity purification methods previously described (33) were modified and used for isolation of the ALK-binding tyrosine-phosphorylated proteins. The detailed protocol is described in Supplementary Materials and Methods.

Immunoblotting, immunoprecipitation, and immunofluorescence

The immunoblotting, immunoprecipitation, and immunofluorescence were done as described previously (26, 32) with modifications. The detailed protocols are described in Supplementary Materials and Methods.

Pulse-chase analysis of ALK internalization

Cells cultured on coverslips were incubated with cold complete medium for 5 minutes at 4°C and then with medium containing 4 μg/mL of anti-ALK (H260) antibody for 30 minutes at 4°C. After removing the medium, the cells were cultured in fresh medium at 37°C for the indicated time period. The cells were fixed and stained with the fluorescence-conjugated secondary antibody. For colocalization analysis, the cells were also stained for FLOT1, clathrin, or caveolin-1. The cells have cytosolic colocalization signals (diameter > 2 μm) and were counted using fluorescence images and ImageJ software. At least 200 cells per sample were counted, and the percentage of positive cells was calculated.

Biotinylation and purification of plasma membrane–localized proteins

A total of 5×10^7 cells were incubated with cold complete medium for 5 minutes at 4°C. The cell surface proteins were labeled with 200 μg sulfo-NHSS-biotin (Thermo Scientific) for 40 minutes at 4°C. After cell lysis, biotinylated proteins were immunoprecipitated using Ultralink Immobilized NeutrAvidin protein (Thermo Scientific). For internalization assay, the labeled cells were cultured in fresh complete medium at 37°C for 60 minutes. The cell surface biotin was stripped by incubation with 180 mmol/L sodium 2-mercaptoethane sulfonate (MesNa; Sigma). After quenching the MesNa by the addition of 180 mmol/L iodoacetamide (Sigma) for 10 minutes, the biotinylated proteins were immunoprecipitated.

Cell migration assay

The cells (1×10^4) were seeded onto the upper part of the Transwell inserts (BD Falcon) coated with fibronectin. The migrated cells on the lower surface of the filter were fixed and stained with Giemsa's stain solution. The number of migrated cells was counted using a BX51 microscope (Olympus).

Cell death assay

The cellular nuclei stained with 100 μmol/L Hoechst 33342 and 4.0 μg/mL propidium iodide (PI; Thermo Scientific) were independently counted using a fluorescence microscope (IX81-ZDC-DSU; Olympus). At least 500 cells per sample were examined and the percentage of PI-positive cells to total Hoechst-positive cells was calculated.

Anchorage-independent cell proliferation assay

Cells were cultured on MPC-coated plates (Thermo Scientific) at 1×10^3 cells per 6 wells for 7 days and the total numbers of cells were counted.
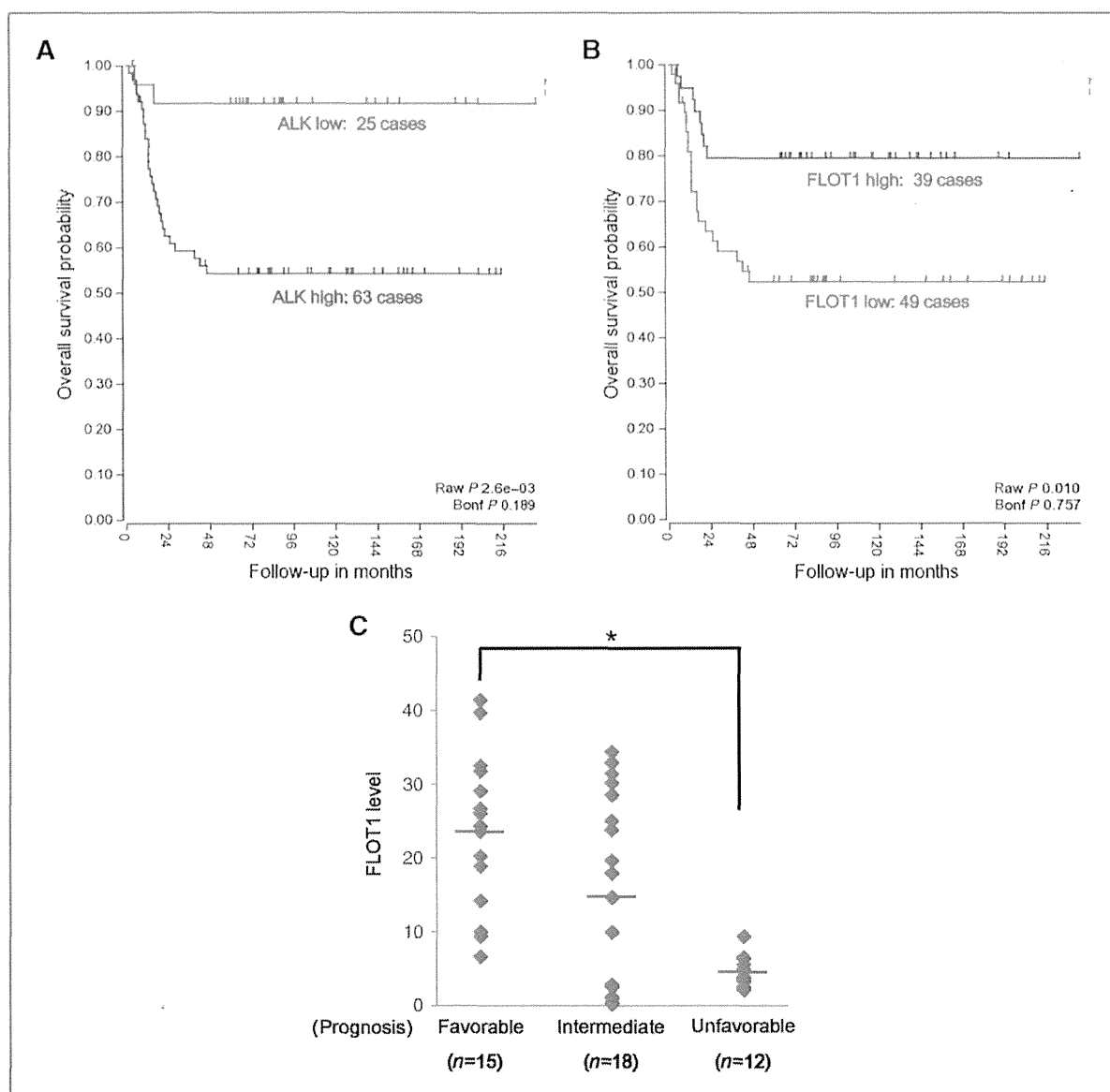
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Figure 1. Clinical impact of FLOT1 expression in neuroblastoma cases. A and B, Kaplan–Meier analysis of overall survival in patients with neuroblastoma with the classifications based on *ALK* (A) and *FLOT1* (B) mRNA expression. The data were obtained from the R2 microarray public database (http://r2.amc.nl). C, ten protein samples from clinical neuroblastoma specimens classified by Brodeur's classification (favorable, 15 cases; intermediate, 18 cases; unfavorable, 12 cases) were subjected to immunoblotting using the FLOT1 antibody and the expression levels of FLOT1 were quantified. Red bars, average values. *, $P < 0.01$.

Tumor xenograft assay

The animal experimental protocols were approved by the Committee for Ethics of Animal Experimentation, and the experiments were conducted in accordance with the guidelines for animal experiments in the National Cancer Center. TNB-1 cells (5×10^6) were subcutaneously injected into the bilateral flank of female 6-week-old BALB/c nude mice (Clea Japan). At 6 weeks after tumor inoculation, the mice were sacrificed, and the subcutaneous tumors were excised with the attached muscle layers. The tumor volume was calculated with the equation (length $\times$ width^2)/2 and the tumor weight (g) was measured. The tumor tissue was stained by hematoxylin and eosin (H&E).

Statistical analysis

The data for all the quantitative results are expressed as mean and SD from three independent experiments. Plotting of scatter graphs and testing of difference of means by

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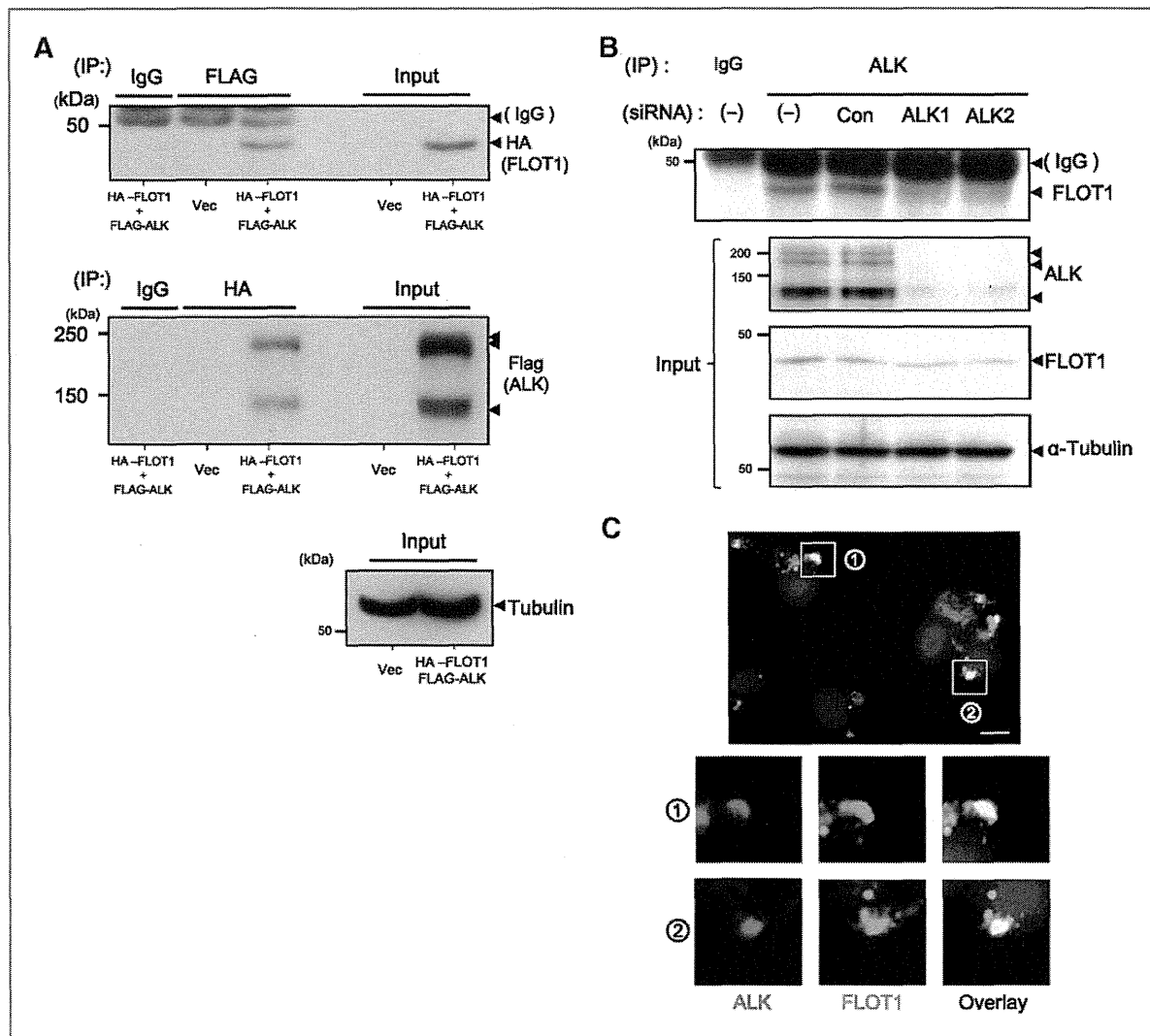
Figure 2. FLOT1 interacts with ALK in neuroblastoma cells. A, Cos-7 cells were transiently transfected with an empty vector (vector), flag-tagged WT *ALK*, or HA-tagged WT *FLOT1* for 12 hours. The cell lysates were immunoprecipitated with control IgG, anti-FLAG antibody (ALK), or anti-HA antibody (FLOT1) and the immunocomplexes and total cell lysates (input) were analyzed by immunoblotting using the indicated antibodies. B, NB-39-nu neuroblastoma cells were transiently transfected with control siRNA (Con) or one of the two siRNAs against *ALK* (ALK1 and ALK2) for 72 hours. The cell lysates were immunoprecipitated with the indicated antibodies and subjected to immunoblotting. C, NB-39-nu cells were stained with DAPI (blue) and antibodies against ALK (red) and FLOT1 (green), and were observed by confocal microscopy. The submembrane regions of the dorsal cell membrane were imaged. Bottom panels are magnified images of the boxed regions. Arrows, colocalization of ALK and FLOT1. Bar, 10 μm.

Student *t* test were achieved using Microsoft Excel 2007 software. *P* values of <0.01 were considered as statistically significant.

Results

Identification of FLOT1 as a binding partner and kinase substrate of ALK in neuroblastoma

To identify the phosphotyrosine-containing proteins associated with ALK, we performed two-step affinity purification using TNB-1 neuroblastoma cells, which stably expresses the

ALK protein tagged with FLAG at the C-terminus as described in Supplementary Fig. S1. Mass spectrometry analysis identified several reported binding partners of ALK such as ShcA, ShcC, and IRS1 (22, 34) along with numbers of novel candidates of ALK-binding phosphoproteins. Association of these novel candidates with ALK was confirmed by immunoprecipitation analysis using available antibodies and further association with prognosis of neuroblastoma was checked using public database to estimate clinical impact. In this study, we focused on FLOT1 among these ALK-binding proteins through these screening.
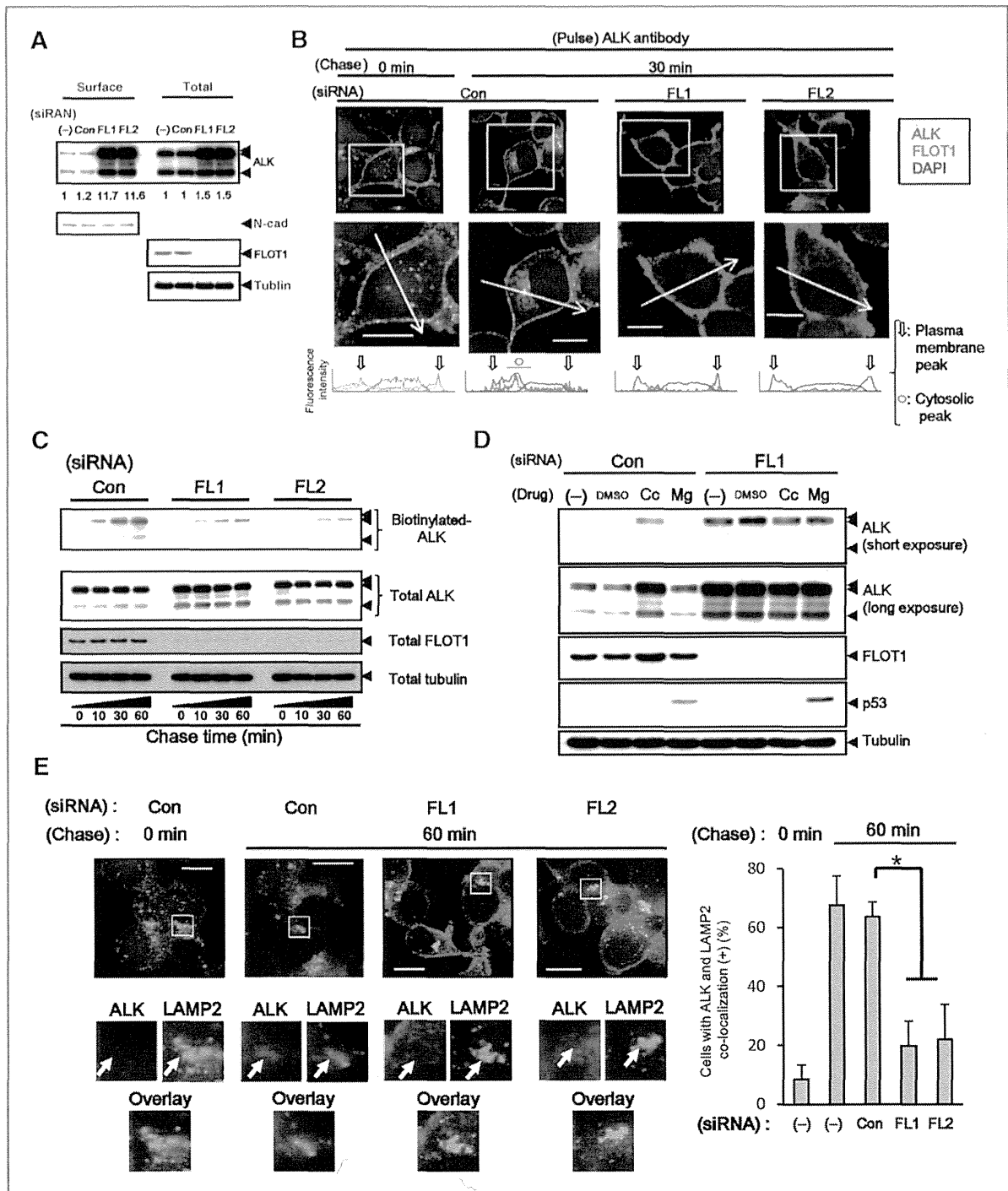
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Figure 3. FLOT1 regulates endocytosis and cell surface expression of ALK. A, NB-39-nu cells were treated with control siRNA (con) or *FLOT1* siRNA (FL1 and FL2) for 72 hours. The plasma membrane–localized proteins were purified as described in Materials and Methods and analyzed by immunoblotting using the ALK antibody or N-cadherin antibody. Total cell lysates were also analyzed by immunoblotting using the indicated antibodies. The levels of ALK in each sample were quantified and denoted as relative value [siRNA (−) = 1] under immunoblotting data. B, NB-39-nu cells treated with indicated siRNAs were subjected to pulse-chase analyses by using an ALK antibody (red). The cells were stained with DAPI (blue) and FLOT1 antibody (green) and observed by confocal microscopy. The lower images are magnified images of the boxed regions. Fluorescent intensity of each signal was quantified by line scan analysis as indicated by yellow arrows and depicted as histograms. (*Continued on the following page.*)

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The R2 database, one of the largest public databases of microarray in neuroblastoma cases (http://r2.amc.nl), indicated that high expression levels of ALK mRNA significantly correlates with poor prognosis in patients with neuroblastoma (Fig. 1A), suggesting that ALK signaling has clinical impact even in patients without genetic alteration of ALK. On the other hand, low expression of FLOT1 mRNA was positively correlated with poor prognosis of clinical neuroblastoma cases in the R2 database (Fig. 1B). We also analyzed the expression of FLOT1 and ALK proteins in specimens from 45 clinical neuroblastoma cases, which belong to three clinical malignancy grades (favorable, 15 cases; intermediate, 18 cases; unfavorable, 12 cases) as classified by Brodeur's classification (35, 36), and demonstrated that the levels of FLOT1 expression inversely correlate with clinical malignancy grade (Fig. 1C). A representative blot of five samples from each group is shown in Supplementary Fig. S2.

Because FLOT1 expression has apparent association with prognosis and clinical grades of neuroblastoma, we hypothesized that FLOT1 regulates oncogenic potentials of neuroblastoma through association of ALK. The binding of ALK to FLOT1 was confirmed by immunoprecipitation analysis using anti-FLAG or anti-HA antibodies in COS-7 cells expressing FLAG-tagged ALK and HA-tagged FLOT1 (Fig. 2A). Binding of ALK to endogenous FLOT1, as well as ALK-mediated tyrosine-phosphorylation of FLOT1, was also demonstrated in NB-39-nu neuroblastoma cells harboring amplified ALK (Fig. 2B and Supplementary Fig. S3). By immunocytostaining analysis, ALK and FLOT1 were mainly colocalized within the cytoplasm, especially at the submembrane regions of the ventral membrane (Fig. 2C). These results suggested that FLOT1 is associated with ALK as a binding partner and kinase substrate in neuroblastoma cells.

FLOT1 regulates degradation of ALK in lysosome through endocytosis

Considering that FLOT1 is reported to be involved in endocytosis of membrane proteins, we investigated the effect of FLOT1 knockdown on the amount of membrane-localizing ALK. The amount of ALK protein at the plasma membrane was markedly increased by treatment with either of two FLOT1 siRNAs, which resulted in rather moderate increases in total ALK protein levels (Fig. 3A). Pulse-chase analysis with an ALK antibody revealed marked reduction in the amount of internalized ALK in the NB-39-nu cells treated with each FLOT1 siRNA at the time point of 30 minutes (Fig. 3B). Biotinylation internalization analysis confirmed that gradual increase in the total amount of internalized ALK was significantly impaired by treatment with each FLOT1 siRNA (Fig. 3C). These results

suggested that FLOT1 regulates the amount of ALK on the cell surface through endocytosis.

Membrane proteins that are internalized by endocytosis are usually degraded by the proteasome or lysosome (37). Degradation of ALK was inhibited following treatment with the lysosomal inhibitor concanamycin, while it was not significantly affected by the proteasomal inhibitor MG132 (Fig. 3D). Under the presence of concanamycin, accumulation of ALK at plasma membrane was observed by knockdown of FLOT1, whereas total ALK protein level was less affected (Supplementary Fig. S4A). Pulse-chase analysis visualized by immunocytostaining demonstrated the colocalization of internalized ALK with the lysosomal marker LAMP2 that was disrupted by treatment with FLOT1 siRNA (Fig. 3E). Colocalization of FLOT1 with internalized ALK was also observed at the early phase of endocytosis, whereas no obvious colocalization of ALK with the other known endosomal transporters, clathrin heavy chain and caveolin-1 (38, 39), was observed (Supplementary Fig. S4B and S4C). These results indicated that FLOT1 regulates lysosomal degradation of ALK through clathrin/caveolin-independent endocytosis.

FLOT1 regulates ALK signaling through modulation of the amount of cell-surface ALK

Phosphorylation of ALK as well as known downstream mediators of ALK such as AKT, ERK1/2, and STAT3, was increased in the NB-39-nu cells treated with FLOT1 siRNA (Fig. 4A). The increased levels of phosphorylation of these molecules were all subsequently reduced by treatment with either ALK siRNA or NVP-TAE-684, an inhibitor of ALK. We further analyzed whether the expression of FLOT1 affects the oncogenic properties of activated ALK in NB-39-nu neuroblastoma (13, 22). Induction of anchorage-independent growth, resistance to the anticancer agent cis-diamminedichloroplatinum (cisplatin; CDDP), and cell migration were enhanced by the treatment of NB-39-nu cells with FLOT1 siRNA (Fig. 4B and Supplementary Fig. S5A and S5B). Similar results were also obtained using Nagai, another neuroblastoma cell line harboring amplified WT ALK (Supplementary Fig. S6A and S6B). On the other hand, reduced expression and phosphorylation of ALK, and phosphorylation of AKT, ERK1/2, and STAT3 as well as induction of cell death, decreased proliferation, and acceleration of ALK internalization were observed by overexpression of FLOT1 in NB-39-nu cells (Fig. 4C and D and Supplementary Fig. S5C and S5D).

To investigate whether FLOT1 has the same regulatory roles of ALK in neuroblastoma cells harboring single-copy ALK, TNB-1 cell lines stably expresses FLOT1 shRNA, TNB-FL1 and TNB-FL2 were established (Fig. 4E). Two control

(Continued.) Open arrows and circles indicate peaks of the fluorescence signals for the plasma membrane and the cytosol, respectively. Bar, 10 μm. C, the siRNA-treated NB-39-nu cells were subjected to ALK-internalization assays at the indicated time points as described in Materials and Methods. The avidin-bounded (internalized) proteins and total cell lysates were analyzed by immunoblotting using the indicated antibodies. D, NB-39-nu cells treated with siRNAs were cultured in the presence of DMSO, lysosomal inhibitor concanamycin (Cc, 10 μmol/L), or proteasomal inhibitor MG132 (MG, 15 μmol/L) for 8 hours and subjected to immunoblotting. p53 was analyzed as a representative protein degraded by proteasome. E, NB-39-nu cells were treated with siRNAs and pulse-chased with an ALK antibody (red). The cells were stained with DAPI (blue) and LAMP2 antibody (green). Bottom panels are magnified images of the boxed regions. Cells with ALK positive and LAMP2-positive dots were quantified as described in Materials and Methods (right bar graph). Bar, 10 μm. *, $P < 0.01$.

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lines of TNB-1 cells, TNB-Con1 and TNB-Con2 cells, were also established using the control vector. Enhanced expression of ALK and phosphorylation of AKT and ERK1/2 was observed in TNB-FL1 and TNB-FL2 cells, whereas no significant changes in the expression of other RTKs, such as EGFR, RET, and TrkB, were observed (Fig. 4E). In addition, increased anchorage-independent growth was detected in the TNB-FL1 and TNB-FL2 cells, which was blocked by treatment with the ALK inhibitor (Fig. 4F and Supplementary Fig. S5E). These results demonstrated that FLOT1 inhibits the malignant phenotype of neuroblastoma cells through endocytosis of ALK.

Activating mutations of ALK have low binding affinities to FLOT-1 and cause ALK stabilization and malignant phenotypes in neuroblastoma cells

It is reported that some of the activating mutations of ALK such as the common F1174L mutation exhibit more malignant phenotypes and poor prognosis than others (19), while the mechanisms causing the differences are still not clear. We investigated differences in the binding affinities between mutant ALK proteins and FLOT1 by using TNB-1 neuroblastoma cells stably expressing WT, F1174L (FL; mutation near the c-helix loop), K1268M (KM; mutation in the juxtamembrane domain), and R1275Q (RQ; mutation near the ATP-binding domain) mutants of ALK (8–11). FLOT1 steadily associated with the WT and RQ mutants of ALK, but not as efficiently with the FL and KM mutants (Fig. 5A). Furthermore, knockdown of FLOT1 affected the internalization of biotinylated ALK in the WT and RQ mutants but not in the FL and KM mutants (Fig. 5B). In addition, the phosphorylation levels of ALK, AKT, and ERK1/2 were not obviously elevated by treatment with FLOT1 siRNA in the TNB-1 cells with the FL or KM mutation (Fig. 5B).

Anchorage-independent growth was significantly enhanced by FLOT1 siRNA in cells expressing WT or RQ mutant, whereas no obvious changes were observed in the cells expressing the FL and KM mutants, which originally showed enhanced anchorage-independent growth. Anchorage-independent growth of all the cells analyzed was reduced by treatment with the ALK inhibitor (Fig. 5C). Similar difference in ALK mutants were also confirmed using the TNB-1 cells expressing WT ALK, the FL mutant, and the KM mutant as for resistance to CDDP and cell migration (Supplementary Fig. S7A and S7B). These results suggested that some of the activating mutations of ALK might have enhanced stability at the cell membrane by reduced affinity to FLOT1, which leads to further enhancement of the malignancy of neuroblastoma.

FLOT1 regulates tumorigenicity of neuroblastoma cells

To investigate the role of FLOT1 in the tumorigenicity of neuroblastoma, TNB-Con1/2, and TNB-FL1/2 cells were subcutaneously injected into nude mice (Fig. 6A). Because of the low tumorigenicity of the original TNB-1 cells, tumors were not detectably formed at 6 weeks following injection of TNB-Con cells. On the other hand, tumors as large as 10 to 40 mm in diameter were clearly formed by the TNB-FL1 and TNB-FL2

cells in this period (Fig. 6A). Histologic study of these tumors revealed that the tumor cells had infiltrated into the muscle layers and formed large intratumoral vessels, which reflects the malignant phenotype of the tumors (Fig. 6B). These results indicated that FLOT1 might be a negative regulator of the malignant characteristics of neuroblastoma *in vivo*. Along with the results indicating that the low expression of FLOT1 is significantly associated with poor prognosis and unfavorable histologic grades of neuroblastoma (Fig. 1B and C), it was indicated that deregulation of FLOT1 expression is involved in the progression of neuroblastoma through enhancement of ALK signaling.
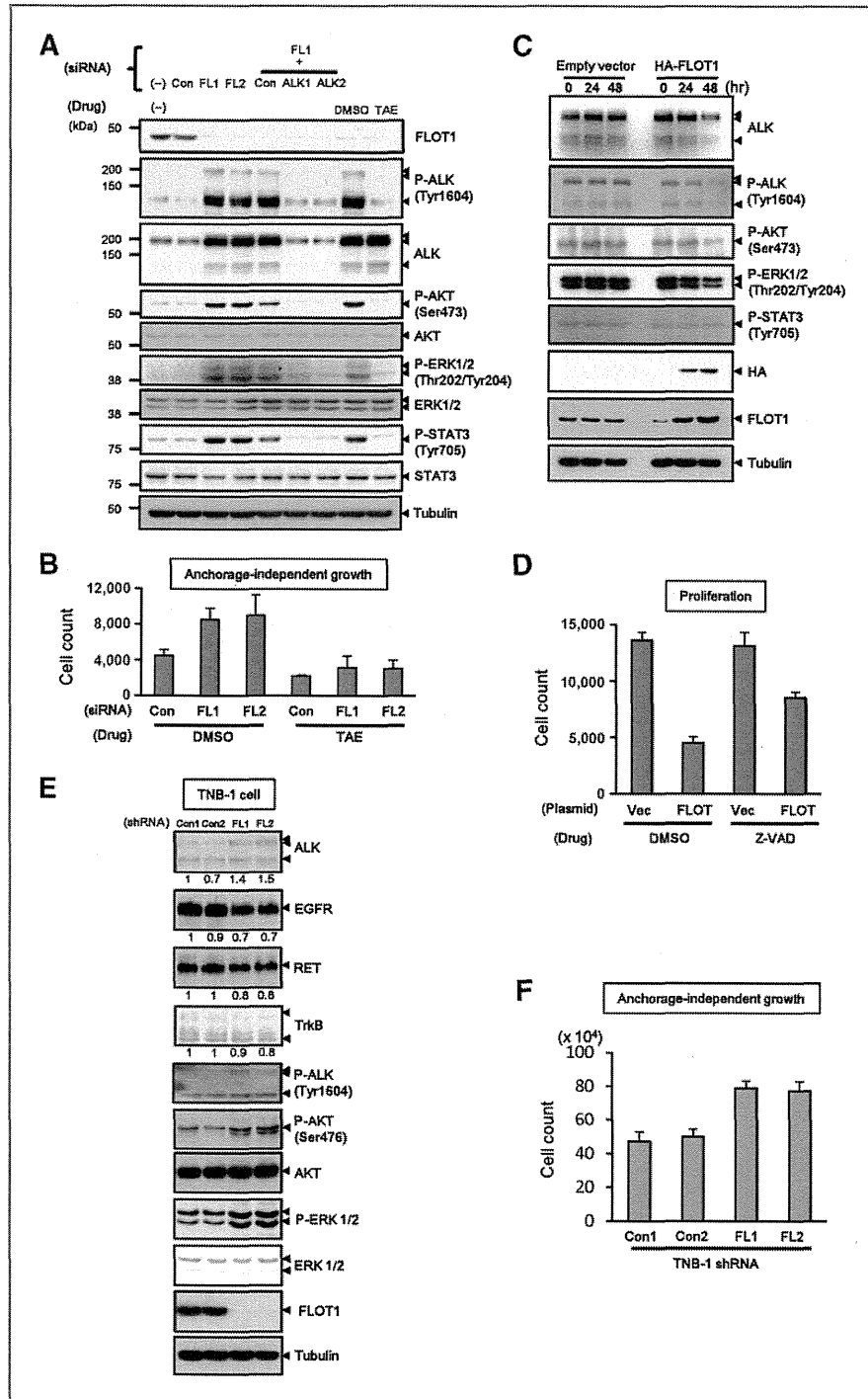
Discussion

Deregulation of the RTK ALK by amplification or activating mutation of the *ALK* gene has been reported in 10% to 15% of human neuroblastoma cases, in which the relationship between ALK signaling and oncogenesis of neuroblastoma is indicated. Although the association between ALK expression and poor prognosis of neuroblastoma is observed (Fig. 1A), it is not clear whether different modes of activation of ALK signaling are involved in the progression of the other neuroblastoma cases. In this study, we provided *in vitro* and *in vivo* evidence that activation of ALK signaling caused by impaired FLOT1-mediated endocytosis is associated with malignancy of neuroblastoma cells. This finding was supported by the observation that FLOT1 expression levels in the clinical samples is inversely correlated with prognosis of the disease in a public database (Fig. 1B) and grades of malignancy in tissue samples (Fig. 1C). Taken together, the novel tumor-suppressing role of FLOT1 in the majority of neuroblastoma that lacks genetic alterations of *ALK* was implied.

There was tendency that the low expression level of FLOT1 is associated with high expression levels of ALK in the neuroblastoma tissues used for Fig. 1C, while it was not statistically significant possibly due to limited numbers of tissues examined (data not shown). Therefore, we could not completely exclude the possibility that FLOT1 also degrades signaling molecules other than ALK, which are associated with malignancy of neuroblastoma, although it was confirmed that FLOT1 preferably regulates the ALK protein among several other RTKs expressed in neuroblastoma (Fig. 4E). The information about the involvement of FLOT1 in cancer development is still limited (29, 40–42). It was recently suggested that FLOT1 is associated with poor prognosis of breast cancer as a result of stabilization of ErbB2 (27) and also plays oncogenic roles in esophageal cancer and hepatocellular carcinoma (28, 40). It is speculated that FLOT1 might regulate the organ-dependent target proteins and functions in the development of cancers and the regulation is rather selective to ALK in neuroblastoma. Considering that activated ALK found in other types of cancers lack transmembrane domain, the accumulation of membranous ALK by deficient FLOT1 might be etiological only in neuroblastoma.

It has been reported that FLOT1 physiologically acts as an endosomal transporter of membrane proteins (23–25). Further study is required to clarify the precise mechanisms

Figure 4. FLOT1 regulates ALK signaling and oncogenicity of neuroblastoma cells. A, NB-39-nu cells were treated with control (Con) or *FLOT1* siRNA (FL1 or FL2) alone or in combination with *ALK* siRNA (ALK1 or ALK2) for 72 hours. *FLOT1* siRNA-treated cells were further cultured in the presence of DMSO or ALK inhibitor NVP-TAE-684 (TAE; 20 nmol/L) for 2 hours. The cell lysates were analyzed by immunoblot analysis using the indicated antibodies. B, NB-39-nu cells were treated with indicated siRNAs and their anchorage-independent growth under continuous treatment with DMSO or TAE was tested as described in Materials and Methods. C, NB-39-nu cells were transiently transfected with an empty vector or HA-tagged FLOT1 for the indicated times. The cell lysates were analyzed by immunoblot analysis using the indicated antibodies. D, NB-39-nu cells transfected with vector alone or HA-tagged FLOT1 were cultured with DMSO or the caspase inhibitor z-VAD-FMK (Z-VAD; 100 μmol/L) for 72 hours. Cell proliferation was measured as described in Materials and Methods. E, stable transfectants of TNB-1 cells expressing control shRNA (TNB-Con1 and TNB-Con2) or FLOT1 shRNA (TNB-FL1 and TNB-FL2) were analyzed by immunoblotting. The expression levels of RTKs were quantified and denoted as relative value (TNB-Con1 = 1) under each immunoblotting data. F, the anchorage-independent growth of TNB-1 cells expressing control or FLOT1 shRNA was tested as described in Materials and Methods.

of endocytosis of ALK, including mode of association between FLOT1 and ALK and the role of tyrosine phosphorylation of FLOT1 in this process. It has been reported that another RTK-binding protein Cbl is involved in the downregulation of RTKs through receptor ubiquitination followed by endocytosis. Indeed, some mutations in the RTKs, Met and EGFR, decrease the affinity of RTKs with Cbl, which results in impaired endocytosis and oncogenic accumulation of RTKs in several cancers, including lung cancer and glioblastoma (43).
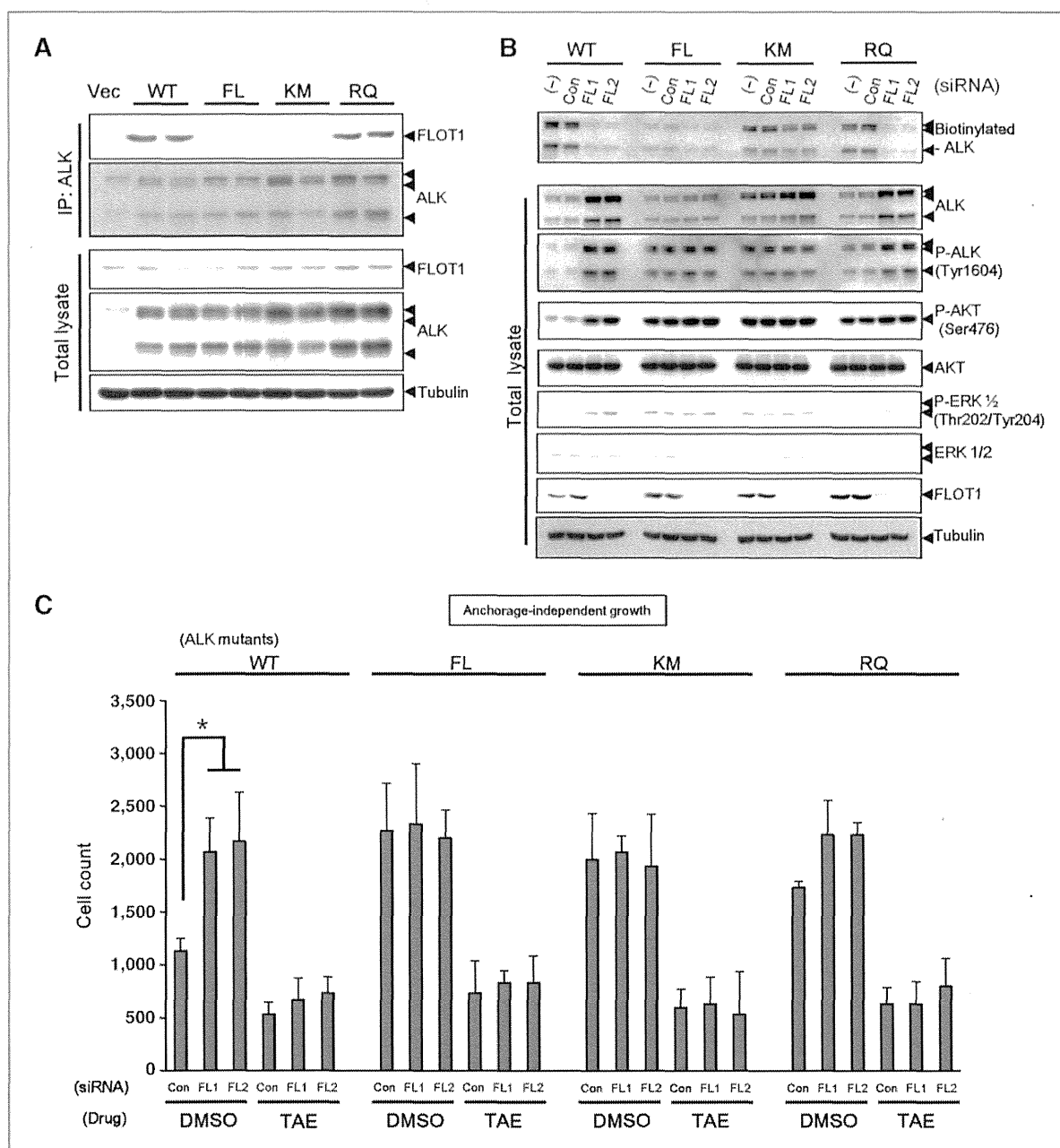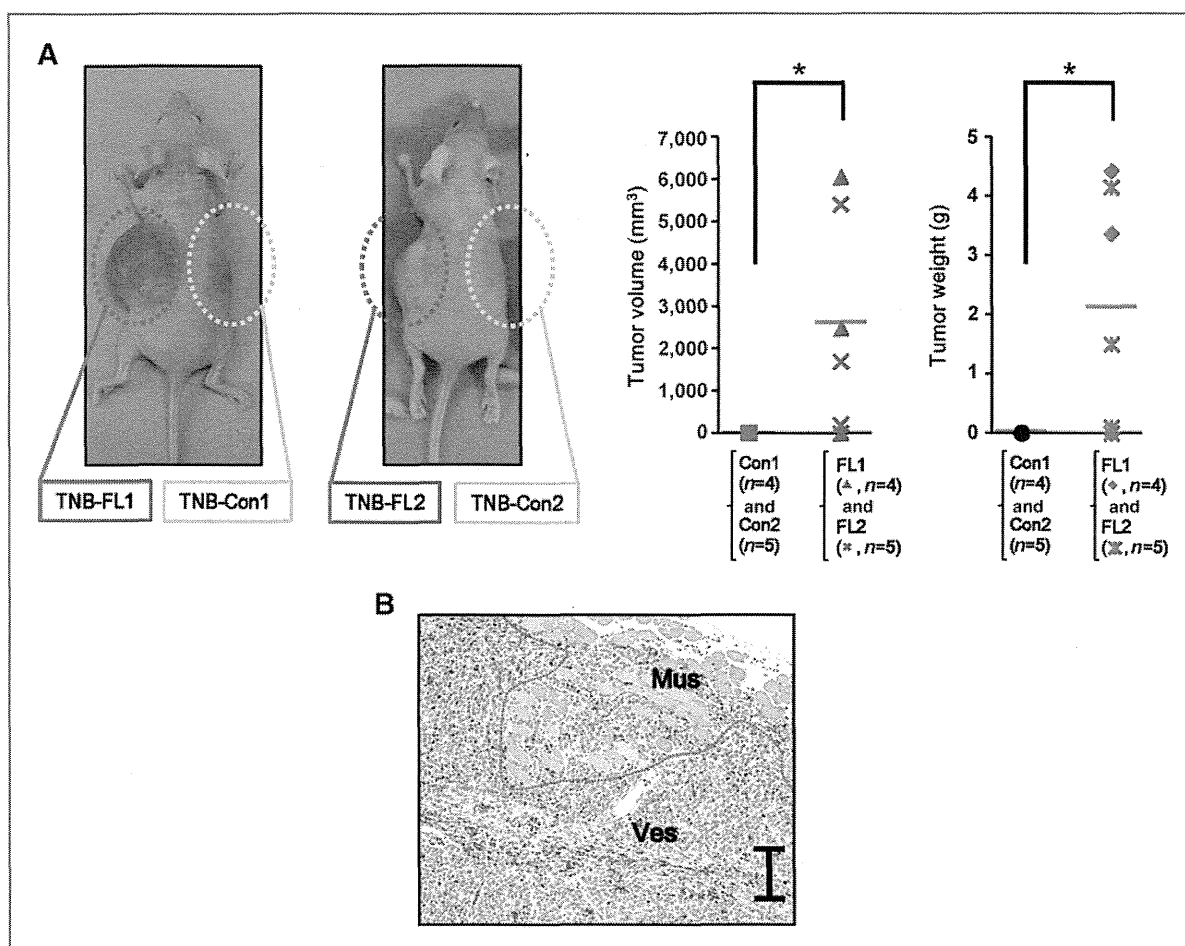
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Figure 5. Activating mutations of ALK confer resistance to downregulation by FLOT1. A, cell lysates from each pair of different stable TNB-1 transfectants of empty vector and ALK mutants (WT; FL, F1174L; KM, K1062M; RQ, R1275Q) were immunoprecipitated using the anti-ALK antibody. The immunocomplexes and total cell lysates were analyzed by immunoblotting. B, the stable TNB-1 transfectants were transfected with control siRNA (con) or FLOT1 siRNA (FL1 or FL2) for 48 hours. The cells were subjected to the ALK internalization assay for 60 minutes, and the internalized proteins and total cell lysates were analyzed by immunoblotting. C, the stable TNB-1 cells transfectants were transfected with indicated siRNAs for 48 hours and cultured in the presence of DMSO or ALK inhibitor NVP-TAE-684 (TAE; 20 nmol/L) for 2 hours. The cells were subjected to the anchorage-independent cell growth assay under continuous treatment with DMSO or TAE. *, $P < 0.01$.

It was recently reported that neuroblastoma cases harboring certain activation mutations of ALK exhibit higher refractoriness (19). For example, neuroblastoma cases harboring the F1174L mutant are also reported to have higher resistance than cases with amplified or R1275Q-mutant ALK when treated with an ALK inhibitor (18). Both the F1174L and R1275Q mutations of

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Figure 6. FLOT1 negatively regulates tumorigenicity of neuroblastoma cells. A, left, macroscopic images of the mouse xenograft experiment. The TNB-1 cells stably expressing control (TNB-Con1 and -Con2, yellow circle) or FLOT1 shRNA (TNB-FL1 and -FL2, red circle) were subcutaneously inoculated into both sides of the flank of 4-week-old nude mice as shown (see Supplementary Materials and Methods). In the first group (group 1) TNB-Con1 and -FL1 cells, and in the second group (group 2) TNB-Con2 and -FL2 cells were inoculated. The images of one of the 5 mice from each group 1 and group 2 at 6 weeks after inoculation were presented. Right, the total tumor volume and weight were measured and plotted as scatter grams. Green bars, average values. *, $P < 0.01$. B, high magnification image of the tumor tissue stained with H&E from the mouse (group 1, TNB-FL1 cells). Tumor invasion into the subcutaneous muscle layer (Mus) and the formation of intratumoral large vessels (Ves) were observed. The dotted line denotes the margin of the muscle layer. Bar, 100 μm.

ALK are frequently observed, while F1174L mutant is more aggressive and resistant to ALK inhibitors than R1275Q mutants even in a transgenic fly system (20). We demonstrated that the FL and KM mutants of ALK have less affinity to FLOT1 and therefore less affected by FLOT1-mediated endocytosis than the WT ALK (Fig. 5B). The role of oncogenic potential of ALK mutants has previously been analyzed with respect to ATP-binding potential and impaired receptor trafficking (44, 45), while our results suggest that impaired downregulation of ALK by FLOT1 might contribute to the aggressiveness of some mutations of ALK (Supplementary Fig. S8). It should be emphasized that expression levels of FLOT1 might also become one of the efficient clinical markers determining prognosis and therapeutic effectiveness of ALK inhibitors in neuroblastoma cases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

Authors' Contributions

Conception and design: A. Tomiyama, T. Uekita, R. Kamata, J. Takita, R. Sakai
Development of methodology: A. Tomiyama, J. Takita
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Tomiyama, K. Sasaki, J. Takita, A. Nakagawara
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Tomiyama, T. Uekita, R. Kamata, K. Sasaki, J. Takita, R. Sakai
Writing, review, and/or revision of the manuscript: A. Tomiyama, T. Uekita, J. Takita, H. Yamaguchi, R. Sakai
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Tomiyama, T. Uekita, R. Kamata, J. Takita, M. Ohira, A. Nakagawara, C. Kitanaka, R. Sakai
Study supervision: T. Uekita, J. Takita, K. Mori, H. Yamaguchi, R. Sakai

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