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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 immunoafffinity 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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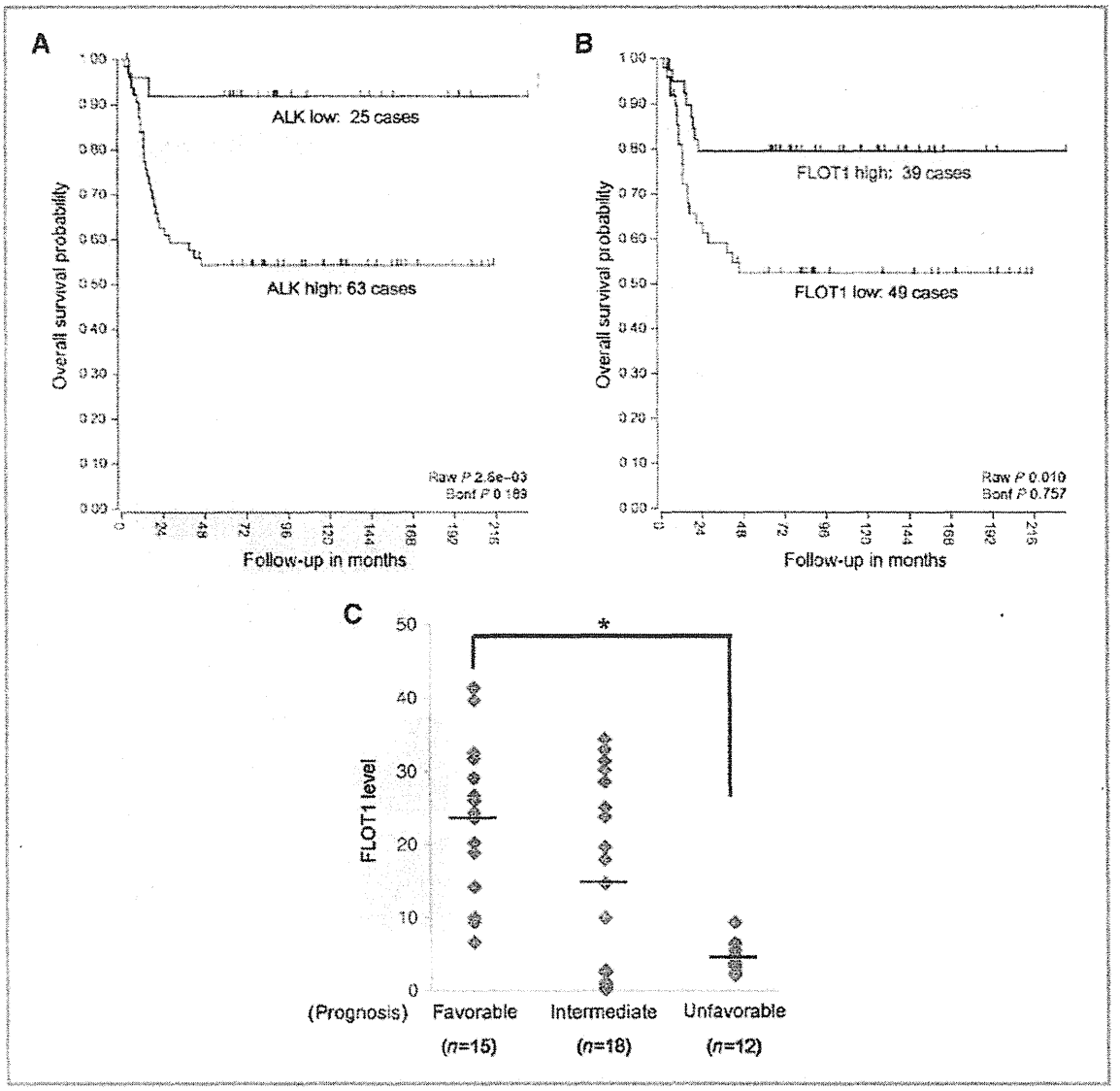

Figure 1. Clinical impact of FLOT1 expression in neuroblastoma cases. A and B, Kaplan–Meier analysis of overall survival in patients with neuroblastoma with the classifications based on *ALK* (A) and *FLOT1* (B) mRNA expression. The data were obtained from the R2 microarray public database (http://r2.amc.nl). 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 × 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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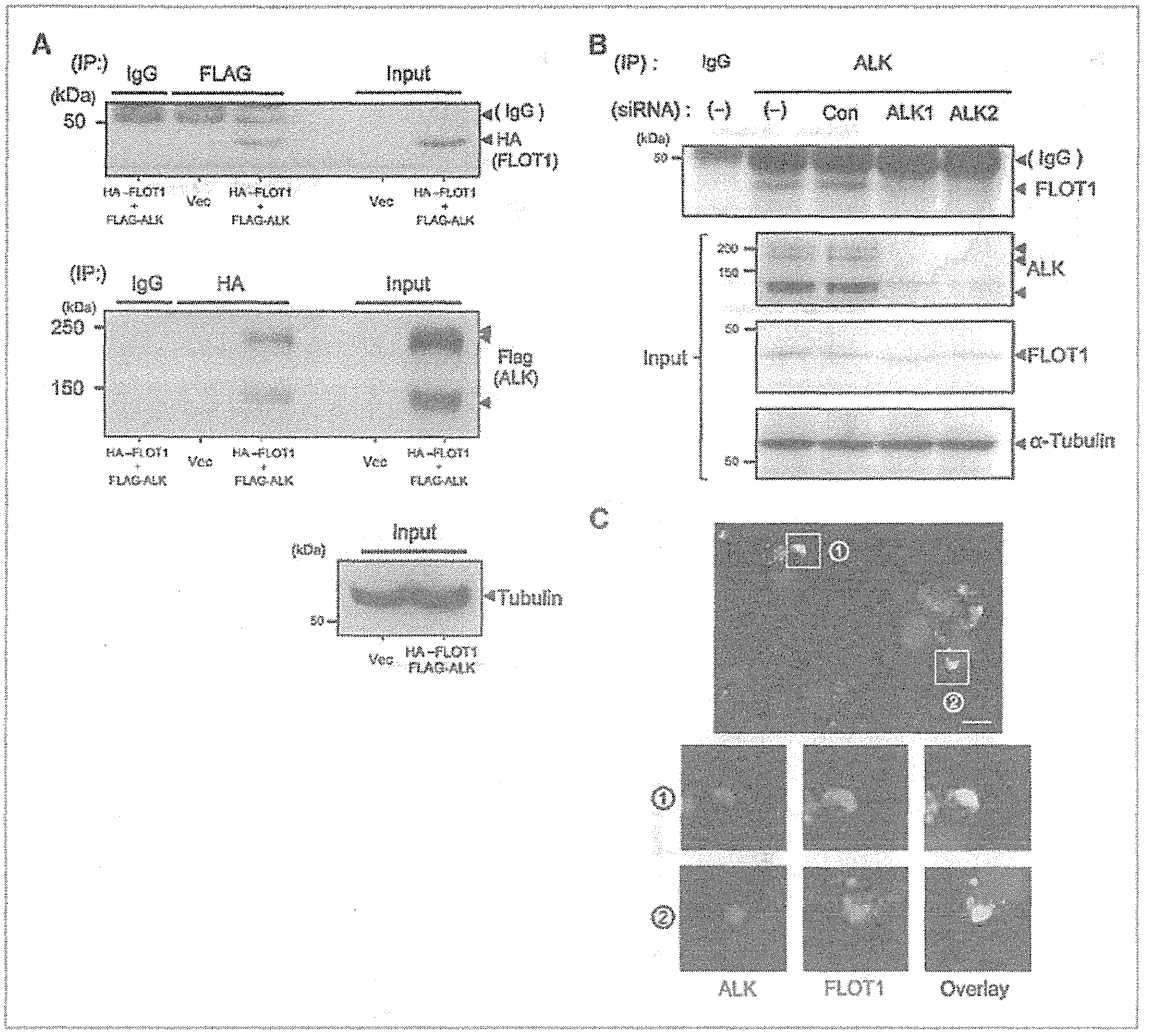

Figure 2. FLOT1 interacts with ALK in neuroblastoma cells. A, Cos-7 cells were transiently transfected with an empty vector (vector), flag-tagged WT *ALK*, or HA-tagged WT *FLOT1* for 12 hours. The cell lysates were immunoprecipitated with control IgG, anti-FLAG antibody (ALK), or anti-HA antibody (FLOT1) 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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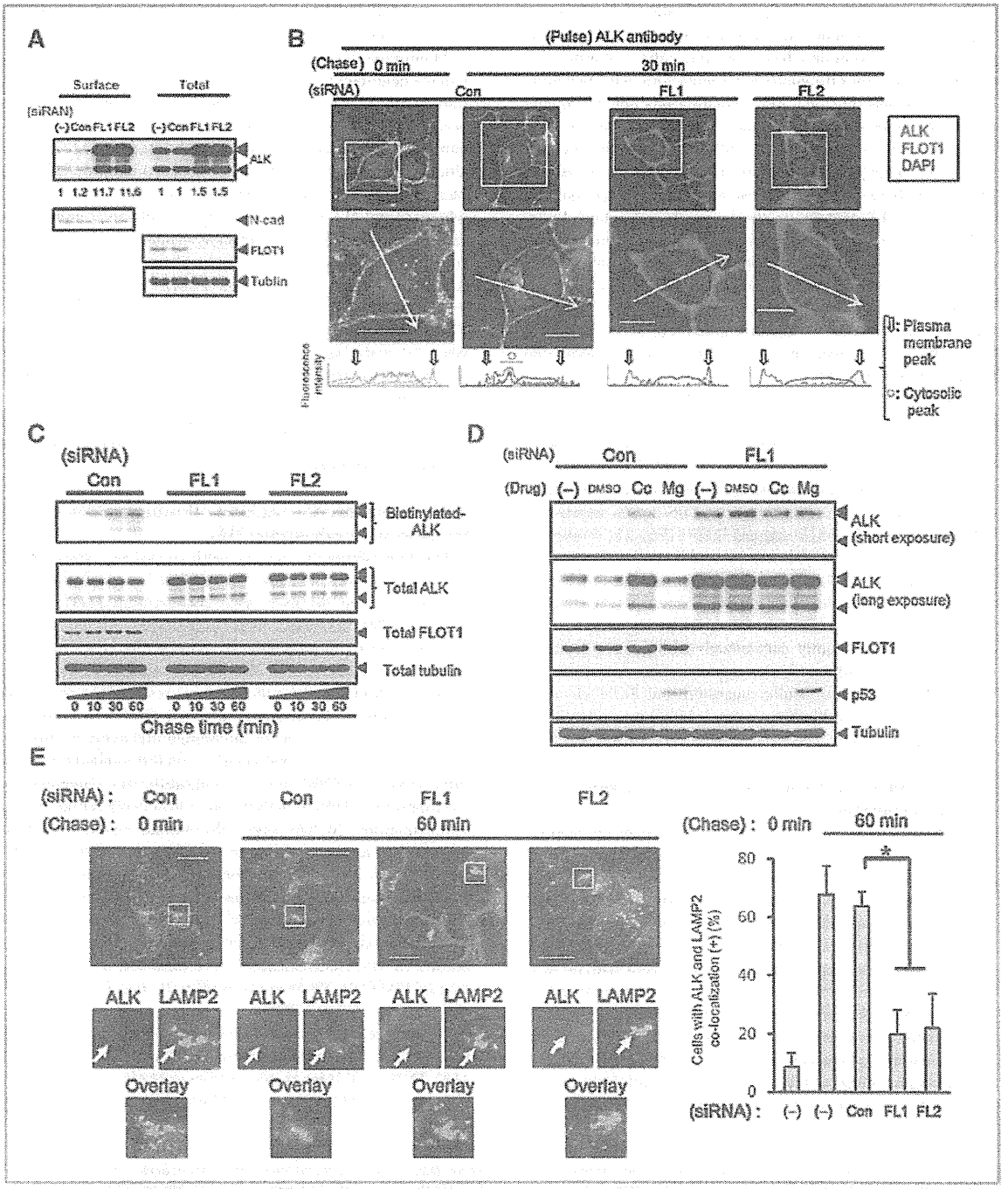

Figure 3. FLOT1 regulates endocytosis and cell surface expression of ALK. A, NB-39-nu cells were treated with control siRNA (con) or *FLOT1* siRNA (FL1 and FL2) for 72 hours. The plasma membrane–localized proteins were purified as described in Materials and Methods and analyzed by immunoblotting using 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

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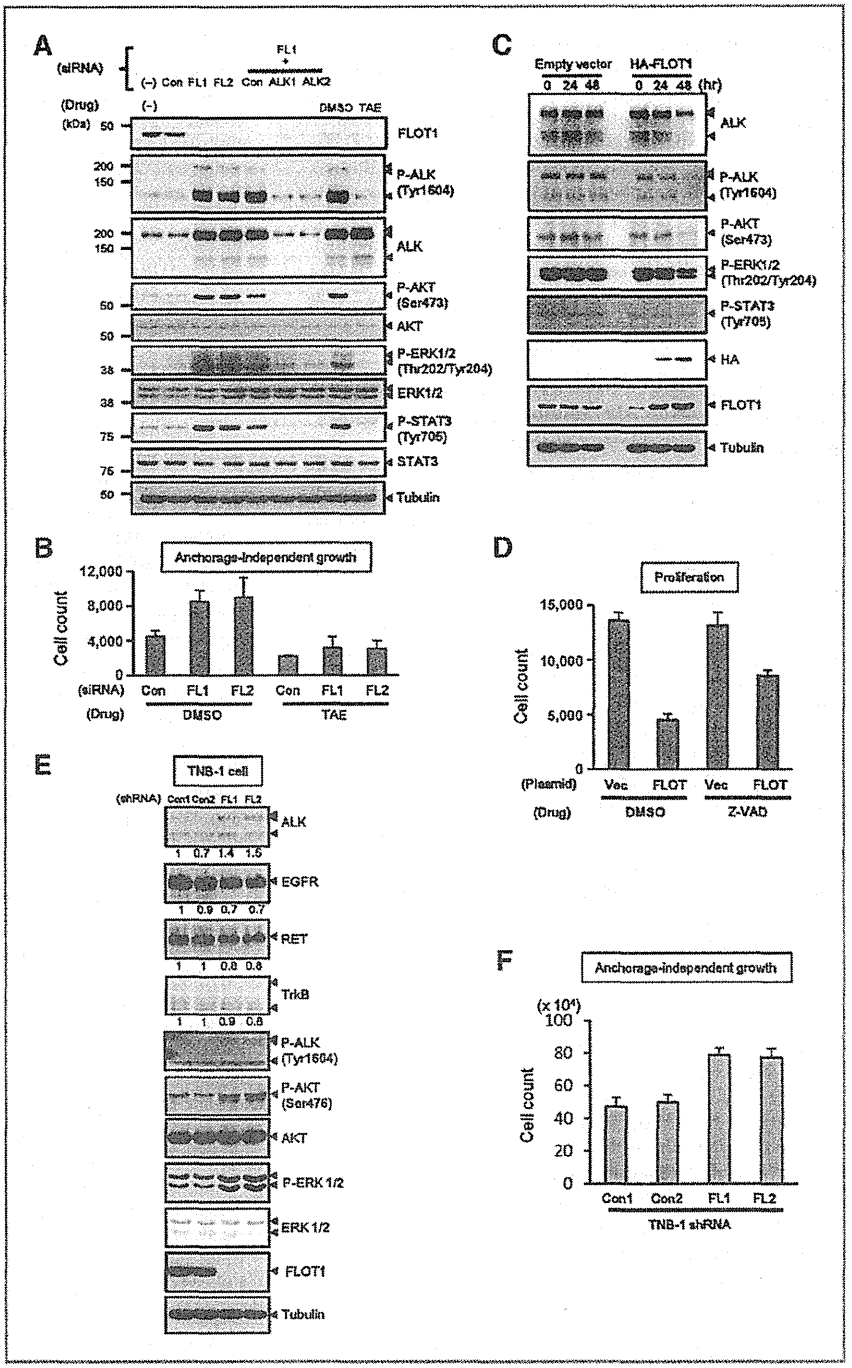

Figure 4. FLOT1 regulates ALK signaling and oncogenicity of neuroblastoma cells. A, NB-39-nu cells were treated with control (Con) or *FLOT1* siRNA (FL1 or FL2) alone or in combination with *ALK* siRNA (ALK1 or ALK2) for 72 hours. *FLOT1* siRNA-treated cells were further cultured in the presence of DMSO 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 down-regulation 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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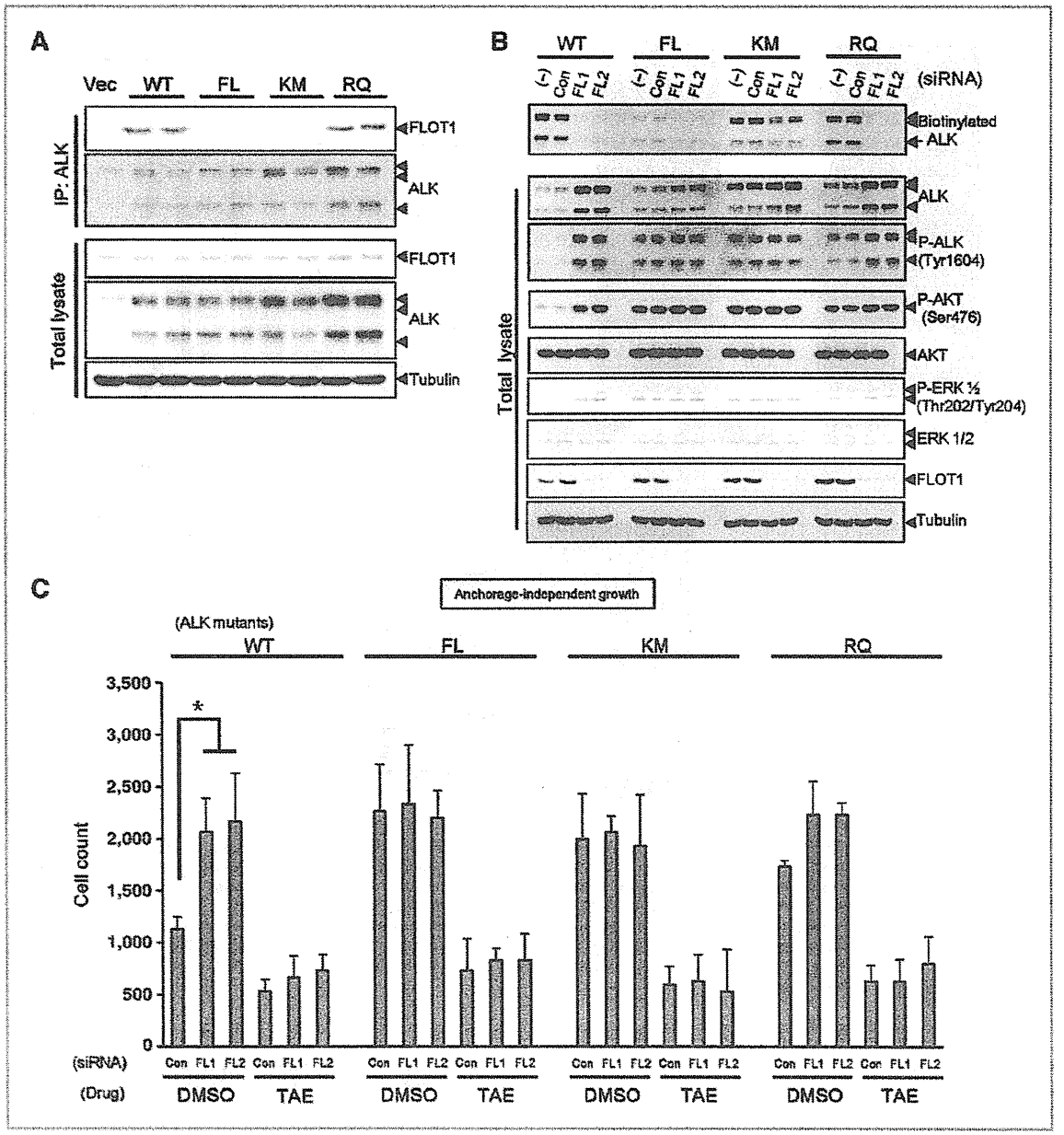

Figure 5. Activating mutations of ALK confer resistance to downregulation by FLOT1. A, cell lysates from each pair of different stable TNB-1 transfectants of empty vector and ALK mutants (WT; FL, F1174L; KM, K1062M; RQ, R1275Q) were immunoprecipitated using the anti-ALK antibody. The immunocomplexes 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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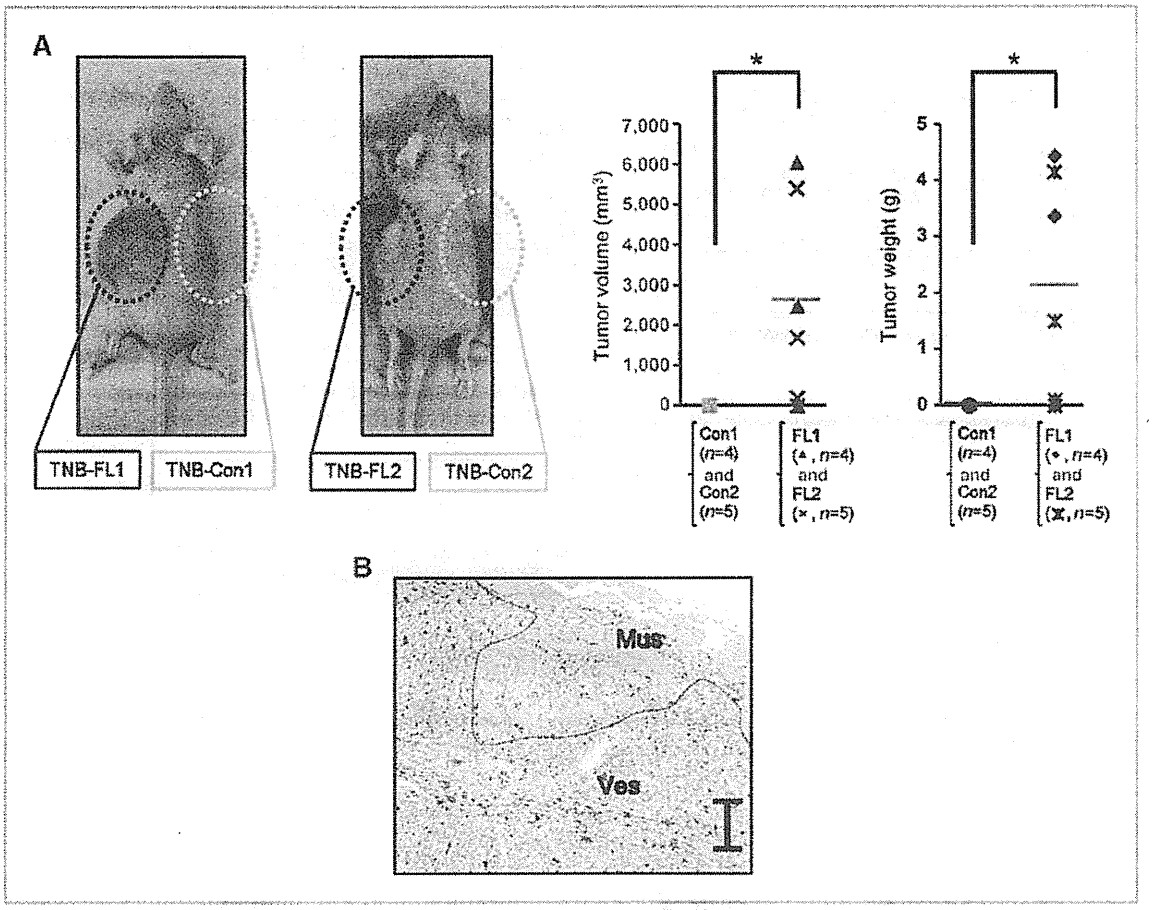

Figure 6. FLOT1 negatively regulates tumorigenicity of neuroblastoma cells. A, left, macroscopic images of the mouse xenograft experiment. The TNB-1 cells stably expressing control (TNB-Con1 and -Con2, yellow circle) or FLOT1 shRNA (TNB-FL1 and -FL2, red circle) were subcutaneously inoculated into both 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 RJ275Q 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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■ 特集　小児固形腫瘍の分子生物学（その4）：次世代シーケンサーの時代

神経芽腫の次世代シーケンサーによる解析

滝田順子*

はじめに

神経芽腫は，胎生期の神経堤（neural crest）由来の悪性腫瘍であり，乳幼児期に好発する。小児腫瘍のなかでは，白血病，脳腫瘍についで頻度が高い[1]。発症年齢が1歳未満の症例は予後良好であるのに対し，1歳以上の年長症例はほとんどが進行例であり，現在の集学的治療を行ってもきわめて予後不良である[1]。また救命できた例では，強力な化学療法や放射線療法による重篤な晩期障害が深刻な問題となっている[1]。したがって，神経芽腫の治療成績向上と長期生存患者のQOLの向上のためには，分子病態に立脚した合理的かつより副作用の少ない新規治療法の開発が必要である。

従来より，予後不良群の神経芽腫の30～50%に*MYCN*の増幅がみられることが知られていたが[1]，*MYCN*は転写因子であることから，それを標的とした治療法の開発は困難をきわめていた。一方，筆者らは，SNPアレイを用いた網羅的ゲノム解析により，*ALK*の異常な酵素活性の上昇が病因の一つであることをつきとめた[2]。海外ではすでに，神経芽腫を含む難治性小児固形腫瘍に対する*ALK*阻害薬の第I/II相試験が終了したが[3]，*ALK*変異を有する神経芽腫は全体の約10%であることから，*ALK*阻害薬につぐ新たな治療標的の探索が急務と考えられる。

近年，がんの基礎研究の分野では，次世代シーケンサー（next generation sequencing：NGS）の登場により，さまざまながん腫の分子病態が急速な勢いで解明されつつある[4]。神経芽腫においても，NGSによる解析が進められ，いくつかの新知見が得られている。本稿では，NGSを用いた神経芽腫におけるゲノム解析につき概説する。

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I．神経芽腫における全ゲノム解析

オランダのグループは，すべての病期を含む神経芽腫87例の患者検体を用いて，全ゲノム解析を行った[5]。すべての腫瘍は，免疫組織染色の結果から80%以上の腫瘍含有量が確認されたものとした。ペアエンドシーケンスアッセイにより，1検体につき全ゲノム領域の96.6%をカバーする領域の情報が得られた。その結果，1検体当たり平均3,347,592個のsingle nucleotide variations（SNVs）を検出した。このうち1,014個の候補somatic変異につき検証を行ったところ，1検体当たり平均12個のアミノ酸置換を伴うsomatic変異が確認された。このなかでもっとも頻度が高い変異は*ALK*変異であり，約6%の検体に見いだされた。*ALK*変異を上回る頻度の高いsomatic変異は検出されなかった。

また，構造異常に関する解析から，進行神経芽腫の18%で，chromothripsisとして知られる染色体の局所的な粉砕を同定した（図1 A）。chromothripsisを伴う腫瘍は予後不良群に多くみられた（図1 B）。chromothripsisによる構造変化は，ニューロンの成長円錐の安定化に関与する遺伝子群*ODZ3*，*PTPRD*および*CSMD1*の領域に頻発しており，それにより遺伝子の機能障害が予想された。それに加えて，*ATRX*，*TIAM1*およびRac/Rho経路の一連の調節因子にも変異がみられたことから，神経芽腫の発症には神経突起生成

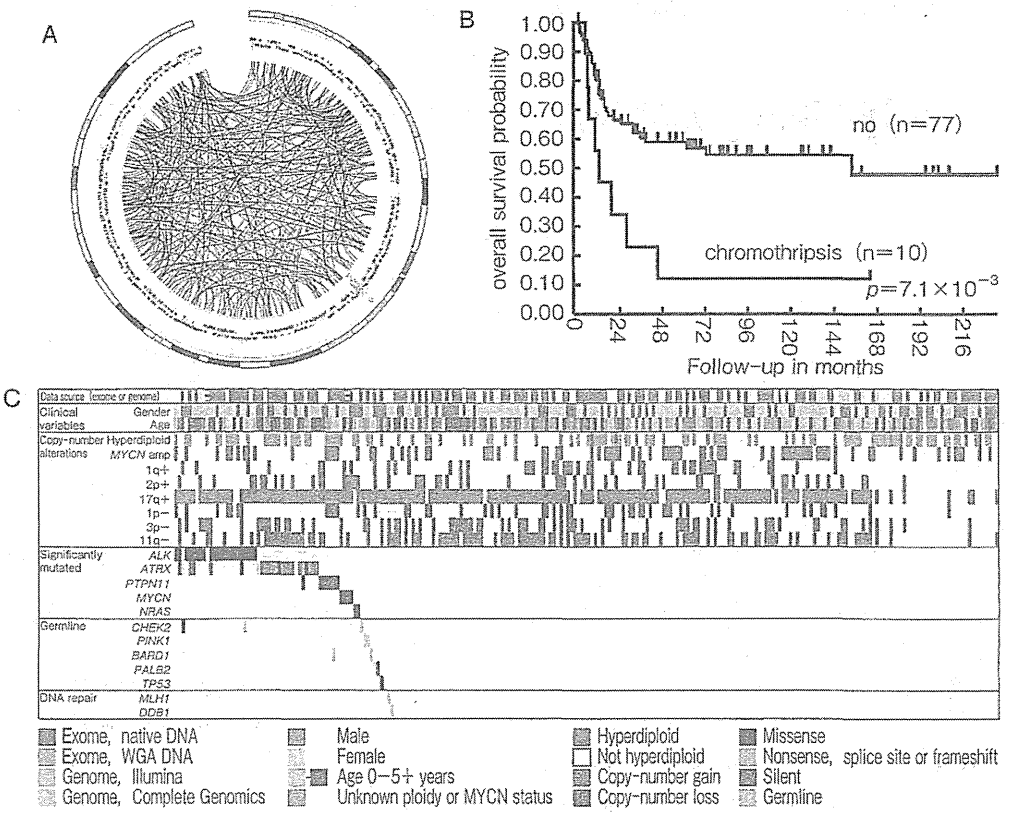

図 1 神経芽腫でみられた chromothripsis と genetic landscape

A. circos plot で示された染色体 5 における chromothripsis。全ゲノム解析により，進行神経芽腫の 1 例で，染色体 5 内に複雑な構造異常が検出された。

B. chromothripsis のある，なしによる生存曲線。chromothripsis を伴う例は，そうでない例に比べて有意に予後不良であった。

C. 神経芽腫 240 例における genetic landscape。全体の約 2/3 の例は，遺伝子変異陰性の例であった。

(Molenaar ら[5]，2012；Pugh ら[10]，2013 より引用改変)

の異常が関与することが示唆された。以上の結果より，chromothripsis と神経突起生成遺伝子の変化という 2 つの新規の分子異常が神経芽腫の病態に関与していることが明らかとなった。

II. 年長児における *ATRX* の重複変異

米国の St. Junde 小児病院とワシントン大学のグループは，Pediatric Cancer Genome Project の一環として，乳児から思春期発症の神経芽腫 40 例の臨床検体を用いて，全 exome 解析を行った[6]。その結果，思春期神経芽腫 5 例全例（100%）において，*ATRX* の変異を検出した。*ATRX* 変異は，1 歳以上の神経芽腫 5/29 例（17%）にも検出されたが，乳児神経芽腫の 6 例には見いだされなかった。さらに 64 例のコホートで検証を行った結果，全体として思春期神経芽腫の約 44%，1 歳以上例の約 17% に *ATRX* 変異が認められたが，乳児例では全く変異は検出されなかった。

ATRX は，SNF2 ファミリーに属する ATP 依存的クロマチンリモデリング蛋白をコードする遺伝子であり，染色体 Xq13.3 に存在する[7]。この遺伝子の生殖細胞系列変異は，重篤な精神遅滞とサラセミアの合併を特徴とする X 連鎖性 α サラセミア・精神遅滞症候群の原因と考えられている[8]。また *ATRX* は，テロメア伸長機序にも関与しており，その機能喪失により，がん細胞ではテロメ

アの短縮が起こらないことが知られている[9]。したがって，*ATRX* 変異によるクロマチンリモデリングの障害やテロメア長の保持が，神経芽腫の発症に関与している可能性が示された。

Ⅲ．進行神経芽腫における genetic landscape

フィラデルフィア小児病院の Pugh ら[10]は，高リスク神経芽腫の somatic 変異の全体像を明らかにするために，進行神経芽腫 240 例につき，全 exome，transcriptome および全ゲノム解析を行った。その結果，中央値 1 Mb 当たり 0.60 個と低頻度の exon 内変異（1 Mb 当たり 0.48 個の非サイレント変異）を検出した。また，神経芽腫においては高頻度な変異を有する遺伝子がほとんどないことを見いだした。somatic 変異の頻度が有意に高い遺伝子には，*ALK*（9.2%），*PTPN11*（2.9%），*ATRX*（2.5%，さらに 7.1%に局所的な欠失あり），*MYCN*（1.7%），*NRAS*（0.83%）が含まれていた。本症の病因となる可能性がある生殖細胞系列変異は，*ALK*，*CHEK2*，*PINK1*，*BARD1* に有意に多く存在した。以上より，神経芽腫において高頻度な somatic 変異が相対的に少ないことが明らかとなり，発がん性ドライバー遺伝子の高頻度な後天的変異をターゲットとした治療戦略は，神経芽腫には必ずしもそぐわないことが示唆された（図 1 C）。

Ⅳ．神経芽腫における *ARID1A* と *ARID1B* 変異

テキサス大学の Sausen ら[11]は，神経芽腫の遺伝的基盤を解析するために NGS を用いて，6 症例について全ゲノム解析，16 症例には全 exome 解析，32 症例についてはゲノムワイドな再編成の検出，さらに 40 症例について特定のゲノム座位の標的解析を行った。1 検体に見いだされた coding region の somatic 変異は，平均して 19 個（3〜70 個）であった。神経芽腫に関する新規遺伝子異常としては，クロマチンリモデリング関連の *ARID1A* と *ARID1B* の染色体欠損や変異が 8/71 例（11%）に検出された。興味深いことに，両者の異常は予後不良と有意に相関していた。

さらに，NGS による特定のゲノム座位の標的解析を用いて，血清中に浮遊する腫瘍由来の DNA 断片に含まれる *MYCN* や *ALK* などに生じている腫瘍特異的ゲノム再編成を検出することに成功した。これらの結果は，神経芽腫の発症にクロマチンリモデリング制御の異常が重要な役割を果たしている可能性を示したものであり，また，NGS の活用が原因検索のみならず，微小残存腫瘍の検出による治療の効果判定にも有用であることを明らかにしたものである。

Ⅴ．神経芽腫におけるエピジェネティック関連遺伝子のターゲットキャプチャー

前述の NGS を用いた神経芽腫における大規模ゲノムシーケンスの結果から，神経芽腫において 10%を超える高頻度の変異は認められずに，本腫瘍の大半は特有の遺伝子変異をもたない腫瘍であることが明らかとなった。この理由の一つとして，神経芽腫の heterogeneity により，アレル頻度が低い変異が，検出されていない可能性も考えられる。そこで筆者らは，特定の遺伝子に焦点を絞って，NGS を用いた超深々度シークエンスを行い，マイナーアレル変異の検出を試みた。

一方，ポリコーム（PcG）蛋白群複合体，トリソラックス群複合体は，Hox 群遺伝子，細胞増殖機構，造血および神経幹細胞の自己再生機能のエピジェネティック制御に関与している[12]。神経芽腫の発症にこれらのエピジェネティック関連遺伝子が関与しているか否かを検討するために，PcG 関連遺伝子群計 80 個につき進行神経芽腫 24 例を用いてターゲットキャプチャーを行った（図 2 A）。検出された変異の検証には，正常組織が得られた 50 例の正常 DNA とさらに神経芽腫 96 検体を用いた。その結果，再発腫瘍 2 例において，トリソラックス群複合体の一つである *ASH1L* の変異を検出した。神経芽腫 96 検体を用いた変異の検証では，新鮮腫瘍の 7.5%，細胞株の 10%にこの遺伝子の変異を検出した（図 2 B）。さらに新鮮腫瘍を用いた *ASH1L* の発現定量解析では，stage 1 群と比べて，stage 4 群の腫瘍において有意な発現低下が検出された（$p=0.005$）。以上の結果から，*ASH1L* は神経芽腫の発症・進展に関与する標的分子の一つである可能性が示唆された。

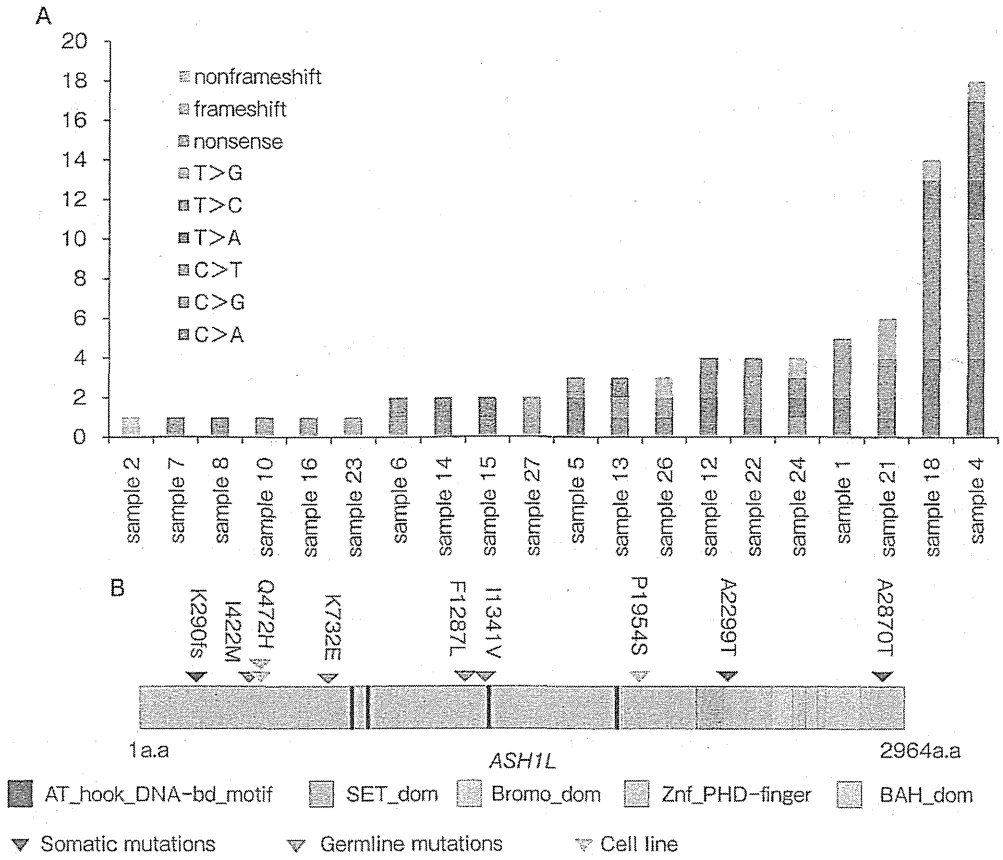

図 2 神経芽腫におけるエピジェネティック関連遺伝子のターゲットキャプチャー

A. discovery cohort 24 例で検出された変異。変異総数は 78 個であり，1 検体当たり平均 3.9 個の変異が検出された。

B. 神経芽腫 136 検体（細胞株 40 株）で検出された *ASH1L* の変異部位。検出された変異は遺伝子の全長にわたっており，変異による機能喪失が推定された。

おわりに

NGS による近年の新たなゲノム研究の展開をふまえて，神経芽腫における最新の知見を概説した。これらの研究により，神経芽腫は，多くの成人がんとは異なり，ドライバー遺伝子変異の蓄積により生じる疾患ではないことが確認された。現状では，神経芽腫における分子病態に立脚した新規治療法の開発というゴールには，まだまだ先は遠い。今後，さらに NGS を用いた統合的ゲノム解析を推進し，本症の分子病態の解明に新たな展開がみられることを期待したい。

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