Trim32 Facilitates Degradation of MYCN on Spindle Poles and Induces Asymmetric Cell Division in Human Neuroblastoma Cells

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Abstract

Asymmetric cell division (ACD) is a physiologic process during development and tissue homeostasis. ACD produces two unequal daughter cells: one has stem/progenitor cell activity and the other has potential for differentiation. Recent studies showed that misregulation of the balance between self-renewal and differentiation by ACD may lead to tumorigenesis in Drosophila neuroblasts. However, it is still largely unknown whether human cancer stem-like cells exhibit ACD or not. Here, using human neuroblastoma cells as an ACD model, we found that MYCN accumulates at spindle poles by GSK-3β phosphorylation during mitosis. In parallel, the ACD-related ubiquitin ligase Trim32 was recruited to spindle poles by CDK1/cyclin B–mediated phosphorylation. Trim32 interacted with MYCN at spindle poles during mitosis, facilitating proteasomal degradation of MYCN at spindle poles and inducing ACD. Trim32 also suppressed sphere formation of neuroblastoma-initiating cells, suggesting that the mechanisms of ACD produce differentiated neuroblastoma cells that will eventually die. Thus, Trim32 is a positive regulator of ACD that acts against MYCN and should be considered as a tumor-suppressor candidate. Our findings offer novel insights into the mechanisms of ACD and clarify its contributions to human tumorigenesis. Cancer Res; 74(19); 1–11. ©2014 AACR.

Introduction

Asymmetric cell division (ACD) is a physiologic process that occurs during development and tissue homeostasis in a large variety of organisms. ACD produces two unequal daughter cells; one has multipotent stem and/or progenitor cell activity and the other has potential for differentiation. Recent ACD studies using model organism systems revealed that the balance between self-renewal and differentiation by ACD is robustly controlled and that misregulation of this balance may lead to tumorigenesis in Drosophila neuroblasts (1–3).

Neuroblastoma is one of the major childhood tumors (4–6) and is derived from normal neural crest cells, which serve as multipotent stem cells that differentiate into mature tissues, including peripheral neurons (7). Of the many genetic and biochemical features of neuroblastoma, MYCN oncogene amplification has been shown to correlate with an aggressive phenotype and a poor outcome (4–6). Recent studies have shown that MYCN shows not only oncogenic activity but also plays a central role in self-renewal growth of normal neural stem and precursor cells (8–12). Although the precise role of MYCN in control of the balance between cell self-renewal and differentiation is still unknown, it is now suspected that neuroblastoma has a cancer stem cell–like property due to aberrant MYCN expression in multipotent neural crest cells (7).

Human neuroblastoma cell lines have a unique property in that, although they show unlimited cell proliferation, they are easily induced to become mature neuronal cells by drugs such as retinoic acids (7). Thus, we considered that a human neuroblastoma cell line is the most suitable system for understanding the mechanism of ACD in human cells because it has both self-renewal and differentiation abilities. In fact, we previously reported that NuMA, one of the conserved ACD-related polarity cues, distributed to one side of the cell cortex during cell division, was detected in many MYCN-nonamplified human neuroblastoma cell lines, and that MYCN powerfully induced self-renewal division against ACD in MYCN-amplified neuroblastoma cells (13). In this study, we attempted to identify the cellular components that abolish the self-renewal proliferation function by MYCN.

Materials and Methods

Cell lines and transfections

All cell lines except TGW were obtained from the American Type Culture Collection (ATCC). TGW was obtained from Japan Health Science Research Resources Bank, Osaka, Japan. All cell lines have been validated by short tandem repeat analysis. These cell lines were maintained in complete medium [Dulbecco's Modified Eagle Medium, supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and
streptomycin (100 µg/mL) in an atmosphere containing 5% CO₂ at 37°C. For transient knockdown in cells, short interfering RNAs targeting the GSK-3β (sc-35527), Cyclin B (sc-29284), and Trim32 (sc-61714) cDNA sequences and control siRNA (sc-37007) were obtained from Santa Cruz Biotechnology. The pCMV, pCMV-Myc, pCMV-Trim32, pCMV-Flag, and pCMV-Flag-Trim32 vectors were obtained from Origene Technologies. These siRNAs and the plasmid DNAs were transfected using Oligofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection at 37°C for 6 hours, cells were supplied with complete medium for 24 hours and then analyzed. For constructing MYCN-T58A and Flag-Trim32/3A plasmids, the QuikChange Site-Directed Mutagenesis Kit (#200519-5; Agilent Technologies) and QuikChange Lightning Multi-Site-Directed Mutagenesis Kit (#210515-5, Agilent Technologies), respectively, were used. MG-132 (a proteasome inhibitor, #474790, final 1 µmol/L for 5 hours), BIO (GSK-3 inhibitor-IX, #361556, final 1 µmol/L for 3 hours), and RO-3306 (a Cdk1/cyclin B inhibitor, #217699, final 5 µmol/L for 3 hours) were obtained from Calbiochem Inc.

Sphere-forming assay
Stable transfectants were selected with G418 (800 µg/mL) for 10 days. After G418 selection, 1 × 10⁴ transfectants were plated onto Ultra-low cluster 6-well dishes (Corning), and cultured in SFM [DMEM-F12, 1:1 (Wako)], 50 µg/mL penicillin/streptomycin, 2% B27 supplement (Invitrogen), 1% N-2 supplement (Wako), 25 ng/mL epidermal growth factor (Wako), and 25 ng/mL fibroblast growth factor basic (Wako). Half of the medium was replaced with fresh culture medium every 7 days. Spheres were counted and measured under a microscope with an eyepiece micrometer.

Indirect immunofluorescence
Cells grown on coverslips were briefly washed in PBS three times, and then fixed with 100% methanol at 20°C. The cells were treated with 1% NP-40 in PBS solution for 10 minutes, and then incubated with blocking solution [15% bovine serum albumin (BSA) in PBS] for 1 hour at 37°C. The cells were then probed with primary antibodies for 1 hour, and antibody–antigen complexes were detected with either Alexa Fluor594– or Alexa Fluor488–conjugated donkey secondary antibody (Molecular Probes, Invitrogen) by incubation for 1 hour at room temperature. The samples were washed three times with PBS after each incubation and then counterstained with 4’,6’-diamidino-2-phenylindole (DAPI). Immunostained cells were examined under a fluorescence microscope (Nikon Eclipse E400) using a 100× objective lens. The fluorescence images were captured with a CCD camera (Leica DFC350FX) and processed with Adobe Photoshop (Adobe Systems). The primary antibodies used were as follows: anti-NuMA antibody (NR500-174; Novus Biologicals), anti-MYCN antibody (sc-53993; Santa Cruz Biotechnology), anti-MYCN antibody (#94058; Cell Signaling Technology), anti-Trim32 antibody (H00022954-M09; Abnova), anti-Fbxw7 antibody (ab71961; Abcam), anti-Huwe1 antibody (ab70161; Abcam), anti-pericentrin antibody (NB100-68277; Novus Biologicals), anti-centrin 2 antibody (sc-2793R; Santa Cruz Biotechnology), anti-phospho-T58 Myc antibody (ab28842; Abcam), anti-phospho-S62 Myc antibody (ab51156; Abcam), anti-c-Myc antibody (9E10, MAI-980; Thermo Scientific), and anti-Flag antibody (#2368S; Cell Signaling Technology).

Immunoblot and immunoprecipitation analyses
Cells were lysed in SDS/Nonidet P-40 lysis buffer [1% SDS, 1% Nonidet P-40, 50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 2 µg/mL leupeptin, 2 µg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 5 mmol/L NaF, and 100 µmol/L Na₃VO₄]. The lysates were boiled for 5 minutes and then cleared by centrifugation at 15,000 rpm and 4°C. Protein concentration of the supernatant was determined using a BCA Protein Assay Reagent (Pierce). The lysates were further boiled for 5 minutes in sample buffer. Samples were then resolved by SDS-PAGE and transferred onto Immobilon-P (Millipore Corp.) sheets. The blots were first incubated in blocking buffer [5% (v/v) nonfat dry milk in Tris-buffered saline (TBS) plus 0.05% Tween 20] for 1 hour. The blots were then incubated with a primary antibody for 16 hours at 4°C, followed by incubation with a horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature. The antibody–antigen complex was visualized by ECL-plus chemiluminescence (Amersham Pharmacia Biotech). For immunoprecipitations, cells were lysed in 0.5% Nonidet P-40 lysis buffer. One milligram of lysates was pre-cleared by incubation with 20 µL of protein G- or A-conjugated agarose for 1 hour at 4°C and incubated on a platform shaker for 3 hours with the primary antibody (4 µg) at 4°C. Protein G- or A-conjugated agarose (40 µL of protein) was then added to the lysate, and the mixture was further incubated on a platform shaker for 1 hour at 4°C, spun down, and washed three times in wash buffer [0.1% Nonidet P-40; 50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 2 µg/mL leupeptin, 2 µg/mL aprotinin, 1 mmol/L PMSF, 5 mmol/L NaF, and 100 µmol/L Na₃VO₄]. After these washes, proteins bound to the beads were eluted with sample buffer by boiling at 95°C for 5 minutes, separated by SDS-PAGE, and analyzed by immunoblotting. The primary antibodies used were anti-GSK-3β antibody (sc-7291; Santa Cruz Biotechnology), anti-cyclin B antibody (sc-245; Santa Cruz Biotechnology), anti-β-actin antibody (#612656; BD Biosciences), anti-Trim32 antibody (H00022954-M09; Abnova), anti-Trim32 antibody (sc-53993; Santa Cruz Biotechnology), anti-Trim32 antibody (sc-9011; Santa Cruz Biotechnology), anti-MYCN antibody (sc-53993; Santa Cruz Biotechnology), anti-MYCN antibody (sc-791; Santa Cruz Biotechnology), anti-GST antibody (PM013 and MBL), and anti-γ-tubulin monoclonal antibody (clone GTU-88; Sigma).

Measurement of proteasome activity and in vitro ubiquitinylation assay
For the measurement of proteasome activity in individual cell lines, the Proteasome Activity Assay Kit (ab107921; Abcam) was used according to the manufacturer's instructions. For ubiquitinylation assay, the lysate from TGW cells was immunoprecipitated by anti-Trim32 antibody, and then an auto-ubiquitinylation kit (#BML-UW0970; Enzo Life Sciences) was used according to the manufacturer's instructions.
Figure 1. MYCN is localized at spindle poles during mitosis via the GSK-3β phosphorylation signaling pathway. A and B, representative images of accumulation of MYCN protein at spindle poles during mitosis in TGW and SK-N-DZ cells upon exposure to 10 μmol/L MG132 for 5 hours. DMSO (0.1%)-treated cells were used as a control. Spindle pole marker NuMA, green; MYCN, red; DAPI (DNA), blue. Arrows, spindle poles. C and D, percentage of cells with MYCN accumulation at spindle poles in TGW or SK-N-DZ cells. Error bars, SEM from three experiments; \( P = 0.0002 \) for TGW and \( P = 0.0004 \) for SK-N-DZ. Y-axis shows percentage of cells with MYCN-accumulated spindle poles. E, representative images of phospho-T58-MYCN but not phospho-S62-MYCN accumulation at spindle poles during mitosis in TGW cells. (Continued on the following page.)
**Statistical analysis**

Comparisons of the frequencies of polarity between different groups were carried out using the unpaired Student t test, owing to the binary nature of the datasets (asymmetric vs. symmetric). Statistical analysis was performed using a statistical software package (StatView, JMP). Probability values less than 0.05 were considered significant.

**Results**

**MYCN is accumulated at spindle poles during mitosis through GSK-3β phosphorylation signaling**

Because ACD is a highlight event during mitosis, we performed immunostaining experiments using MYCN-amplified human neuroblastoma cell lines to address where MYCN localized during mitosis. MYCN was present all over the cells and did not localize to any particular organelle (Fig. 1A and B). However, when we treated the cells with a proteasome inhibitor, MG-132, for 5 hours, surprisingly, we found that a large amount of MYCN protein accumulated at spindle poles in TGW and SK-N-DZ cells (Fig. 1A and B). In fact, a significant number of mitotic cells showed MYCN accumulation at spindle poles (Fig. 1C and D). We also attempted the same experiment using other MYCN-amplified human neuroblastoma cell lines such as SK-N-NE and CHP-212 (Supplementary Fig. S1A). In these cell lines, MYCN physiologically accumulated at spindle poles during mitosis in a proteasome inhibitor–independent manner. From these results, we speculated that spindle poles are a site of physiologic degradation for MYCN, and detection of MYCN localization at spindle poles depends on the level of endogenous proteasome activity in individual cell lines. Therefore, we measured proteasome activity in these cell lines (Supplementary Fig. S1B). As expected, in the cells with high proteasome activity (TGW and SK-N-DZ), proteasome inhibitor treatment was necessary for the detection of MYCN localization at spindle poles. On the other hand, in the cells with low proteasome activity (SK-N-NE and CHP-212), accumulation of MYCN at spindle poles was physiologically detected without proteasome inhibitor treatment. Thus, spindle poles are an important site for the degradation of MYCN. What about the case for c-MYC protein? We examined whether c-MYC also accumulated at spindle poles during mitosis by using a c-MYC–amplified cell line (SK-BR-3) and c-MYC–overexpressed cell lines (HeLa and U251-MG; Supplementary Fig. S2). As a result, c-MYC did not accumulate at spindle poles even when treated with a proteasome inhibitor. Thus, the degradation kinetics of c-MYC differs from that of MYCN, as reported previously (14), and the degradation of MYCN is a unique property during mitosis.

Next, we examined the phosphorylation status of MYCN accumulated at spindle poles. Immunostaining experiments showed that the majority of accumulated MYCN at spindle poles was in the threonine (T) 58-phosphorylated form and the proportion of the Ser62-phosphorylated form was very low (Fig. 1E–G, and Supplementary Fig. S3). Because GSK-3β is known to phosphorylate MYCN at T58 (6, 15), we treated the cells with MG-132 plus GSK-3 kinase inhibitor, BIO. Immunostaining experiments revealed that the proportion of accumulated MYCN significantly decreased (Fig. 1H and I and Supplementary Fig. S4). Therefore, we speculated that GSK-3β phosphorylation signaling might recruit MYCN to spindle poles. To address this point precisely, we silenced GSK-3β expression with siRNA, treated with MG-132 for 3 hours, and then performed an immunostaining experiment (Fig. 1J and K). As expected, the proportion of MYCN accumulation at spindle poles was reduced despite high MYCN protein levels (Fig. 1J–L). These findings provide the evidence that GSK-3β phosphorylation signaling recruits MYCN to spindle poles in human neuroblastoma cells.

**Trim32 is recruited to spindle poles during mitosis through Cdk1/cyclin B phosphorylation signaling**

Because it is now known that proteasome is involved in MYCN/c-MYC degradation, we searched for MYC-related ubiquitin ligases such as Trim32 (16, 17), Fbxw7 (18, 19), and Huwe1 (20) that facilitate the degradation of MYCN in proteasome, by localizing at spindle poles during mitosis. We found that Trim32 localized at spindle poles during mitosis, but not in interphase (Fig. 2A). On the other hand, other MYC-related ubiquitin ligases such as Fbxw7 and Huwe1 did not localize at spindle poles during mitosis (Supplementary Fig. S5A and S5B). Thus, Trim32 is a plausible ubiquitin ligase for MYCN degradation. As mentioned above, Trim32 does not localize at centrosomes in interphase, but localizes at spindle poles during mitosis. In addition, it is reported that Trim32 is phosphorylated at its serine residues (S328, S335, and S339) by Cdk1/cyclin B (21, 22). Therefore, we speculated that phosphorylation of Cdk1/cyclin B is important for Trim32 to localize at spindle poles. To test this hypothesis, we treated the cells with a low dose (5 μmol/L) of a Cdk1/cyclin B inhibitor, RO-3306 (Supplementary Fig. S6), and then performed immunostaining. Interestingly, RO-3306 treatment abolished the spindle pole localization of Trim32 (Fig. 2B and C and Supplementary Fig. S7). Therefore, we next silenced cyclin B expression with siRNA (Fig. 2D). Knockdown of cyclin B caused displacement of Trim32 from spindle poles (Fig. 2E and F). In addition, we constructed a phosphorylation-deficient mutant

(Continued) MYCN, red; phospho-T58-MYCN or phospho-S62-MYCN, green; DAPI (DNA), blue. Arrows, spindle poles. F and G, percentage of cells with phosphorylated or unphosphorylated MYCN at spindle poles in TGW and SK-N-DZ cells. Error bars, SEM from three experiments. H and I, representative images of MYCN accumulation at spindle poles during mitosis in TGW and SK-N-DZ cells upon exposure to 10 μmol/L MG132 plus 0.1% DMSO or 1 μmol/L BIO for 3 hours. NuMA, green; MYCN, red; DAPI (DNA), blue. Arrows, spindle poles. J, immunoblot of GSK-3β and MYCN expression in SK-N-DZ cells transfected with control siRNA or GSK3β siRNA. Immunoblot of β-actin served as a loading control. K, representative images of MYCN accumulation at spindle poles during mitosis in SK-N-DZ cells transfected with control siRNA or GSK3β siRNA. After transfection of siRNAs, the cells were treated with 10 μmol/L MG132 for 3 hours. NuMA, green; MYCN, red; DAPI (DNA), blue. Arrows, spindle poles. L, percentage of cells with MYCN accumulation at spindle poles in SK-N-DZ cells transfected with control siRNA or GSK3β siRNA. Error bars, SEM from three experiments. Localization statuses of MYCN on spindle poles were categorized into three types (intense, weak, or no localization). Scale bars, 10 μm.
Figure 2. Trim32 is recruited to spindle poles during mitosis through Cdk1/cyclin B phosphorylation signaling. A, representative images of localization of Trim32 to spindle poles during mitosis but not in interphase in SH-SY5Y, TGW, and SK-N-DZ cells. Centrosome marker pericentrin, green; Trim32, red; DAPI (DNA), blue. Arrows, spindle poles. B and C, representative images of Trim32 localization to spindle poles during mitosis in TGW and SK-N-DZ cells upon exposure to 5 μmol/L RO-3306 for 3 hours. DMSO (0.1%)-treated cells were used as a control. Centrosome (centriole) marker centrin-2, is green; Trim32, red; DAPI (DNA), blue. Arrows, spindle poles. D, immunoblot of cyclin B expression in SK-N-DZ cells transfected with control siRNA or Cyclin B siRNA. Immunoblot of β-actin served as a loading control. E, representative images of Trim32 localization to spindle poles during mitosis in SK-N-DZ cells transfected with control siRNA or Cyclin B siRNA. Centrin-2, green; Trim32, red; DAPI (DNA), blue. Arrows, spindle poles. F, percentage of cells with Trim32 localization to spindle poles in SK-N-DZ cells transfected with control siRNA or Cyclin B siRNA. Error bars, SEM from three experiments. Localization statuses of Trim32 at spindle poles were categorized into three types (intense, weak, or no localization). G, representative images of Flag-Trim32 or Flag-Trim32/3A localization to spindle poles during mitosis in SK-N-DZ cells transfected with Flag-Trim32 or Flag-Trim32/3A expression vector. Pericentrin, green; Flag, red; DAPI (DNA), blue. Arrows, spindle poles. Flag-Trim32 localizes to spindle poles, but Flag-Trim32/3A does not. Scale bars, 10 μm.
of Trim32 [Trim32-S328A, S335A, and S339A (Trim32/3A)], and then transfected this mutant plasmid into the cells. As expected, the phosphorylation-deficient mutant of Trim32 did not localize at spindle poles during mitosis, whereas the wild-type Trim32 did. Thus, the Cdk1/cyclin B phosphorylation signal recruits Trim32 to spindle poles (Fig. 2G).

**MYCN is a physiologic substrate of Trim32 ubiquitin ligase**

We next examined whether Trim32 interacts with MYCN. Immunostaining experiments showed that Trim32 colocalized with MYCN at spindle poles under proteasome inhibitor treatment (Supplementary Fig. S8A and S8B). Therefore, the cell lysate from MYCN-amplified cells was immunoprecipitated using an anti-MYCN antibody, and then the immunoprecipitates were blotted using an anti-Trim32 antibody. As a result, a Trim32 band was detected (Fig. 3A). Alternatively, the lysate was immunoprecipitated using an anti-Trim32 antibody, and then the immunoprecipitates were blotted using an anti-MYCN antibody. Interestingly, an MYCN band was detected only in the proteasome inhibitor (MG-132) – treated sample, but not in the untreated one (Fig. 3A). This result strongly suggests that Trim32 interacts with MYCN in vivo, and then rapidly ubiquitinylates MYCN protein, followed by degradation.
Trim32 Targets MYCN for Inducing Asymmetric Cell Division

via the proteasome system. We also addressed whether Trim32 interacts with MYCN in a T58 phosphorylation–dependent manner. We transfected MYCN (wild-type) or MYCN-T58 phosphorylation-deficient mutant (MYCN-T58A) into MYCN-nonamplified cells (SH-SY5Y), and then performed an immunoprecipitation experiment. As a result, Trim32 interacted with MYCN in a phosphorylation-independent manner (Supplementary Fig. S9), suggesting that phosphorylation is dispensable for MYCN to bind Trim32, and that spindle poles are important sites for the interaction between Trim32 and MYCN because both proteins localize to spindle poles during mitosis. Moreover, we attempted an in vitro ubiquitinylation assay to examine whether Trim32 ubiquitinylates MYCN. Trim32 was immunoprecipitated by an anti-Trim32 antibody, and then E1, E2 ubiquitin ligase complex, ATP, ubiquitin, and GST or GST-MYC were added in the Trim32 immunoprecipitates. The result clearly showed that GST-MYC was polyubiquitinylated by Trim32 in vitro (Fig. 3B). In addition, we addressed the ubiquitinylation activity of Trim32 during mitosis. As a result, the ubiquitin ligase of Trim32 was shown to be active during both interphase and mitosis (Supplementary Fig. S10).

Next, when we silenced Trim32 expression with siRNA, as expected, the immunoblotting experiment showed that the MYCN protein level increased (Fig. 3C). In addition, importantly, in the Trim32-knockdown cells, an immunostaining experiment revealed that MYCN protein significantly accumulated at spindle poles without proteasome inhibitor treatment (Fig. 3D and E). These results strongly suggest that MYCN is a physiologic substrate of Trim32 in human neuroblastoma cells.

We recently reported that, while MYCN-nonamplified neuroblastoma cells showed NuMA cortex-based asymmetric cell division (NuMA-ACD) at a high frequency (~30%), MYCN-amplified cells showed NuMA-ACD at a low frequency (3% to 10%; ref. 13). Among MYCN-amplified cells, TGW cells showed NuMA-ACD at approximately 10% (13). Therefore, we examined the proportion of NuMA-ACD with or without Trim32 knockdown. The results showed that the proportion of NuMA-ACD was significantly reduced in Trim32-knockdown TGW cells (Fig. 4A and B), suggesting that Trim32 might be an inducer of ACD against MYCN.

We subsequently transfected the Trim32 or Trim32/3A expression vector into the MYCN-amplified cells (SK-N-DZ) to determine whether Trim32 and its mutant have ubiquitinylation function. The results showed that Trim32/3A as well as Trim32 had ubiquitinylation activity because MYCN protein level decreased in both Flag-Trim32- and Flag-Trim32/3A-transfected cells (Fig. 5A). However, importantly, the proportion of Flag-Trim32/3A cells with aggresome significantly increased compared with that of Flag-Trim32 cells (Fig. 5B). It is known that aggresomes represent a misconnection between ubiquitin ligase and other components of the proteasome complex (23–27). These results suggest that, although the
Trim32/3A mutant still has ubiquitinylation activity for MYCN, it does not have reliable signaling interaction for the proteasome system.

Trim32 suppresses sphere-forming ability in human neuroblastoma cells

We also transfected the Trim32 or Trim32/3A expression vector into MYCN-amplified cells (SK-N-DZ) and performed the sphere-forming assay to determine whether Trim32 affects the self-renewal growth of neuroblastoma cells. After selection of transfectants, each transfectant was plated onto ultra-low attachment dishes and, after 7 days, the large spheres of ≥100 μm in diameter were counted (Fig. 6A). Interestingly, SK-N-DZ transfected with a control vector showed significantly higher numbers of large spheres than the Flag-Trim32–transfected cells (Fig. 6A and B). This strongly suggests that Trim32 has the ability to block self-renewal division (symmetric cell division) of cancer-initiating/stem cells. In addition, the Flag-Trim32/3A–transfected cells somewhat recovered the ability of sphere formation compared with the Flag-Trim32–transfected cells (Fig. 6A and B). These findings suggest that spindle pole localization of Trim32 may be important for blocking self-renewal growth in neuroblastoma cells.

Trim32 induces ACD in human neuroblastoma cells

We finally transfected the Trim32 or Trim32/3A expression vector into MYCN-amplified cells (SK-N-DZ; Fig. 7A), and examined the status of ACD by immunostaining (Fig. 7B). As reported previously (13), SK-N-DZ rarely showed NuMA-ACD (~3%). However, in Trim32-transfected cells, MYCN protein level decreased and complete ACD (opposite distributions of NuMA and Trim32) was detected (complete...
ACD; Fig. 7B and C). We additionally found ACD, in which Trim32 was oppositely distributed from MYCN at the end of cell division (Supplementary Fig. S11). We believe that this ACD status occurs in a neuroblastoma-specific manner. In Trim32-transfected cells, it was also detected that both NuMA and Trim32 localized to the same side of the daughter cell during anaphase (Fig. 7B). We termed this aberrant ACD “sympatric ACD.” Although it is unknown why sympatric ACD was detected, correct distribution of other ACD-related components might be necessary for establishing complete ACD. In Trim32/3A-transfected cells, NuMA-ACD was detected but Trim32 distribution was not asymmetric (Fig. 7B and C). We also performed the transfection of the Trim32/3A vector with MYCN shRNA into SK-N-DZ cells (Supplementary Fig. S12A). Interestingly, in Trim32/3A-transfected and MYCN-knockdown cells, the percentage of NuMA-ACD cells significantly increased (Supplementary Fig. S12B). This result indicates that MYCN interferes with NuMA-ACD. Together, these results suggest that spindle pole localization of Trim32 may be important for both degradation of MYCN and reliable induction of complete ACD (Fig. 7D).

Discussion

In the present study, we found that Trim32 may be an inducer of ACD in our human neuroblastoma cell system. Recent studies showed that human neuroblastoma cells contain tumor-initiating cells whose phenotype resembles cancer stem cells, including features such as self-renewal, induction of multilineage cell differentiation, and high drug efflux capacity (7, 24). ACD is another important characteristic of cancer stem cells, and may cause tumor cell heterogeneity. Because Trim32 degrades MYCN, produces differentiated neuroblastoma cells that will eventually die, and suppresses sphere formation, we consider it to be a tumor suppressor in neuroblastoma. In fact, a public microarray database (R2) for human neuroblastoma using 88 clinical samples showed that patients with high Trim32 expression in neuroblastoma tumors had better relapse-free survival than those with low expression ($P = 0.05$; Supplementary Fig. S13). Therapies enhancing Trim32 activity may, thus, lead to a cure of refractory neuroblastoma with MYCN amplification.

In this study, although the forced expression of Trim32 targeted MYCN for degradation in MYCN-amplified
neuroblastoma cells, we found that Trim32 also localized at spindle poles even in MYCN-nonamplified neuroblastoma cells (Fig. 2A, SH-SY5Y cells). This result suggests that Trim32 has not only ubiquitinylation activity for MYCN but may also have other functions. For example, Trim32 is known to function in the translation of mRNAs and to activate microRNAs, such as let-7 (17). Thus, activation of microRNAs and translation of cell fate–related mRNAs by Trim32 might also be necessary for establishing complete ACD in neuroblastoma cells.

Spindle poles are organelles that ensure reliable segregation of chromosomes during mitosis. Our results show that the spindle pole localization of Trim32 itself may also be important for the suppression of self-renewal growth and the establishment of asymmetric cell polarity during mitosis. It is also known that spindle orientation and asymmetric segregation of cell-fate determinants are important for the establishment of ACD (3). Our results suggest that the spindle pole–associated ubiquitin–proteasome system is also indispensable for ACD. In fact, it is
known that proteasome localizes to centrosomes/spindle poles and functions for cell homeostasis in many mammalian cells (25, 26, 28). In addition, some proteasome components are known to segregate asymmetrically during mitosis in T lymphocytes (29) and human pancreatic cancer cells (30). Thus, we believe that the ubiquitin–proteasome system may largely contribute to the reliable establishment of ACD.

In summary, we showed here that human neuroblastoma cell lines are a very suitable model system for analyzing the mechanism of ACD in human cells. Our study may also provide new therapeutic clues for targeting cancer stem cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

Authors' Contributions

Conception and design: H. Izumi, Y. Kaneko

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Izumi

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Cancer Res  Published OnlineFirst August 6, 2014.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-14-0169

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