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NCYM promotes calpain-mediated Myc-nick production in human *MYCN*-amplified neuroblastoma cells

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ABSTRACT

NCYM is a *cis*-antisense gene of *MYCN* and is amplified in human neuroblastomas. High *NCYM* expression is associated with poor prognoses, and the NCYM protein stabilizes MYCN to promote proliferation of neuroblastoma cells. However, the molecular mechanisms of NCYM in the regulation of cell survival have remained poorly characterized. Here we show that NCYM promotes cleavage of MYCN to produce the anti-apoptotic protein, Myc-nick, both *in vitro* and *in vivo*. NCYM and Myc-nick were induced at G2/M phase, and NCYM knockdown induced apoptotic cell death accompanied by Myc-nick downregulation. These results reveal a novel function of NCYM as a regulator of Myc-nick production in human neuroblastomas.

1. Introduction

Neuroblastoma is one of common solid childhood tumors that originate from sympatho-adrenal tissues [1]. *MYCN* oncogene is frequently amplified in high-risk neuroblastomas [2,3], and the MYCN oncoprotein regulates transcription of various target genes to promote the tumorigenesis [4]. Overexpression of MYCN in the sympathetic nervous system is sufficient to develop neuroblastoma in mice [5], and concomitant activation of cell survival signals or inhibition of apoptosis further induces metastatic tumors [6–8].

NCYM is a *cis*-antisense transcript of *MYCN* oncogene [9–11], which has been thought to be a long non-coding RNA [11]. Recently we reported that *NCYM* is a newly evolved coding gene which is conserved only in a taxonomically-restricted group including humans and monkeys [8]. *NCYM* is co-amplified with *MYCN* in human neuroblastomas [8,10], and high *NCYM* expression is associated with poor prognoses of the tumors [8]. NCYM stabilizes MYCN through inhibition of GSK3β, and MYCN stimulates *NCYM* transcription [8]. *MYCN* amplification in human neuroblastomas induces both *MYCN* and *NCYM*, and the positive auto-regulatory loop between MYCN and NCYM keeps their expression at high levels [8,12]. Furthermore, overexpression of MYCN and NCYM in the sympathetic nervous system of mice frequently develops metastatic tumors, inhibiting apoptotic cell death within the tumors [8]; however the molecular mechanisms by which NCYM regulates cell survival have remained elusive.

MYC family oncogenes encode transcription factors which regulate a wide variety of biological phenomena, including development and tumorigenesis [13,14]. Myc-nick has been identified as the cleaved product of MYC family members [15], MYC and MYCN, and function as anti-apoptotic proteins in transcriptional regulation-independent manners [16]. MYC and MYCN are cleaved by the Ca^{2+} dependent protease calpain to produce Myc-nick, a cytoplasmic 42 kDa protein lacking the DNA binding domains [15].

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Myc-nick inhibits apoptosis and promotes the formation of mature autophagosomes which are implicated in autophagy, leading to cancer cell survival [16]. Moreover, Myc-nick increases acetylation of α-tubulin through the recruitment of GCN5, an acetyltransferase, and promotes migration of cancer cells [16]. Because NCYM directly binds to MYCN and augments aggressiveness of neuroblastomas by inhibiting apoptosis [8], we hypothesized that NCYM may regulate Myc-nick in human neuroblastoma cells. In this study, we found that NCYM promotes calpain-mediated Myc-nick production and reduces apoptotic cell death at G2/M phase in *MYCN*-amplified neuroblastoma cells.

2. Materials and methods

2.1. In vitro cleavage of MYC or MYCN

Purified MYCN (50 ng, Abnova, Taipei, Taiwan) was incubated with 25 μM MG132, 250 ng of calpain (Abcam, San Francisco, CA, USA) and increasing amounts (0, 25, 50, 100 ng) of purified NCYM protein in buffer G [15] (30 mM Tris–HCl pH 7.6, 100 mM NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 50 μM ATP, 5% glycerol) for 1 h on ice, and the samples were analyzed by western blot. Purification of NCYM protein was done as previously described [8]. Cleavage of MYC (50 ng, Abnova) and MYCL (50 ng, Abnova) was performed as described above with 100 ng of purified NCYM protein. For the time course assays, MYC (50 ng, Abnova) or MYCN (50 ng, Abnova) was incubated with 25 μM MG132 and 250 ng of calpain (Abcam), with or without 100 ng purified NCYM protein, in buffer G for indicated times and samples were analyzed by western blot. For the inhibition experiments, 50 ng of purified MYC or MYCN (Abnova) was incubated with 25 μM MG132, with or without 2000 ng of calpain (Abcam), 100 ng of purified NCYM protein and 100 μM calpeptin (Calbiochem, San Diego, CA, USA) in buffer G for 3 h on ice and the samples were analyzed by western blot.

2.2. GST-pulldown assay

For GST-pulldown assay, 0.5 μg of calpain protein (Abcam) was incubated with 0.5 μg of GST protein or MYCN (Abnova) with or without 0.5 μg of purified NCYM protein for 2 h at 4 °C. Bound complexes were recovered on glutathione-sepharose beads coated with 1 μg/μl BSA, washed with buffer G (30 mM Tris–HCl pH 7.6, 100 mM NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 50 μM ATP, 5% glycerol and protease inhibitors), boiled in Laemmli sample buffer and analyzed by immunoblotting. PIC (Roche, Mannheim, Germany) was used for protease inhibition.

2.3. Cell culture, infection, transfection, and RNA interference

Human neuroblastoma cell line CHP134 was maintained in RPMI-1640 medium supplemented with heat-inactivated 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA). HEK293T cells were cultivated in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented with 10% heat-inactivated FBS. CHP134 has amplified *MYCN*. Lentivirus was produced by co-transfecting cDNA or shRNA expression plasmids with pCMVR and pMDG plasmids into HEK293T cells using FuGene HD transfection reagent (Roche). The NCYM shRNA expression plasmids contained pLKO.1-puro as the backbone (Sigma, St Louis, MO, USA). At 24 and 48 h after transfection, the viral supernatants were collected and mixed with CHP134 cells. Construction of the expression vector pcDNA3-FLAG-NCYM was previously described [8]. CHP134 cells were transfected with pcDNA3-FLAG-NCYM using Lipofectamine 2000 transfection reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The target sequences of the shRNAs used were as follows: NCYM sh-1 (N-cym 1 custom shRNA, Sigma) 5′-tggcaattgcttgtcattaaa-3′, NCYM sh-2 (N-cym 2 custom shRNA, Sigma) 5′-gaggttgctcctgtgtaatta-3′. The control shRNA (SHC002) was purchased from Sigma.

2.4. Immunoblotting

We resolved cell proteins by SDS-PAGE before electro-blotting onto PVDF membranes. We incubated the membranes with the following primary antibodies overnight at 4 °C: anti-MYCN (1:1000 dilution; Calbiochem and Cell Signaling, Danvers, MA, USA), anti-MYC (1:1000 dilution; Santa Cruz, CA, USA), anti-NCYM (1:1000 dilution [8]), anti-acetyl α-tubulin (1:1000 dilution; Abcam), anti-α-tubulin (1:1000 dilution; Cell Signaling). The membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG at 1:2000–1:4000 dilution or anti-mouse IgG at 1:2000 dilution; both from Cell Signaling) and the bound proteins were visualized using a chemiluminescence-based detection kit (ECL and ECL pro kit, Amersham, Piscataway, NJ, USA; ImmunoStar LD, Wako). For quantification of immunoblotting analysis, we used Molecular Imager® VersaDoc™ MP 5000 System (Bio-Rad laboratories Inc. CA, USA).

2.5. Double thymidine block and cell distribution

CHP134 cells were washed twice with 1 × PBS and RPMI-1640 containing 2 mM thymidine was added for 18 h. The medium was removed and cells were washed with 1 × PBS and maintained in RPMI-1640 for 9 h to release cells. The medium was replaced with RPMI-1640 containing 2 mM thymidine for 17 h. After the second block, thymidine was removed by washing with 1 × PBS, and cells were released by adding fresh medium. Cells were harvested at indicated times and subjected to western blot and cell cycle distribution analyses. For cell cycle distribution analysis, cells were collected at indicated times after the double thymidine block, stained with propidium iodide (PI) and examined by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA).

2.6. RNA isolation and quantitative real-time RT-PCR

Total RNA from CHP134 neuroblastoma cells was prepared using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instruction. cDNA was synthesized using SuperScript II with random primers (Invitrogen). Quantitative real-time RT-PCR (qRT-PCR) using an ABI PRISM 7500 System (Applied Biosystems, Foster City, CA, USA) was carried out using a SYBR green PCR. The primer sets used were as follows: human *NCYM*, 5′-cgccccctaggaacaagac-3′ and 5′-gcgcccctcttctttcaatt-3′. The mRNA levels of each of the genes were standardized by *β-actin*.

2.7. Terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) assays

CHP134 cells were transfected with the indicated shRNA with 50% lentiviral supernatant. Seventy-two hours after transfection, all cells were collected by centrifugation at indicated times after release of double thymidine block, attached onto the coverslips by CYTOSPIN 4 (Thermo Fisher Scientific, Wilmington, DE, USA), and fixed in 4% paraformaldehyde for 1 h. Apoptotic cells were detected by using an *in situ* cell death detection kit (Roche Applied Science GmbH, Mannheim, Germany) according to the manufacturer's protocol. The coverslips were mounted with DAPI-containing mounting medium (Vector Laboratories, Burlingame, CA, USA) and observed under a laser scanning confocal microscope (DMI 4000B; Leica, Wetzlar, Germany).

2.8. Statistical analysis

All statements are based on replicated experiments (a minimum of three times). All statistical significance was tested by Student's *t*-test.

3. Results

3.1. NCYM promotes the cleavage of MYC and MYCN in vitro

We investigated whether NCYM directly promotes the cleavage of Myc family members *in vitro*. Calpain alone cleaved MYCN, and Myc-nick levels increased with the addition of increasing amounts of NCYM (Fig. 1A). Calpain at 250 ng/μl alone could not cleave MYC, but the combination of NCYM and calpain resulted in Myc-nick production (Fig. 1B, left panel). MYCL, which lacks the cleavage site of calpain, didn't show band shifts by addition of calpain and NCYM (Fig. 1B, right panel). Myc-nick production by calpain occurred at earlier time points in the presence of NCYM (Fig. 1C, D) and the NCYM-mediated Myc-nick production was partially inhibited by the calpain inhibitor, calpeptin (Fig. 1E, F). Moreover, GST-pulldown assays revealed that GST-fused MYCN interacted with NCYM and calpain in the buffer of the *in vitro* cleavage assay (Fig. 1G, right panel). Thus, NCYM promotes calpain-mediated Myc-nick production *in vitro*, forming complex with MYCN.

3.2. NCYM positively regulates Myc-nick production

We next investigated Myc-nick production in CHP134 neuroblastoma cells using NCYM knockdown and overexpression. As described previously [15,16], the cleaved form of MYCN, a cytoplasmic 42 kDa protein, was observed in the *MYCN*-amplified neuroblastoma CHP134 cells. We also detected phosphorylated Myc-nick [16], as indicated in Fig. 2A. In *MYCN*-amplified neuroblastoma cell line CHP134, knockdown of NCYM decreased Myc-nick production (Fig. 2A, B), whereas overexpression of NCYM increased production of Myc-nick (Fig. 2C, D).

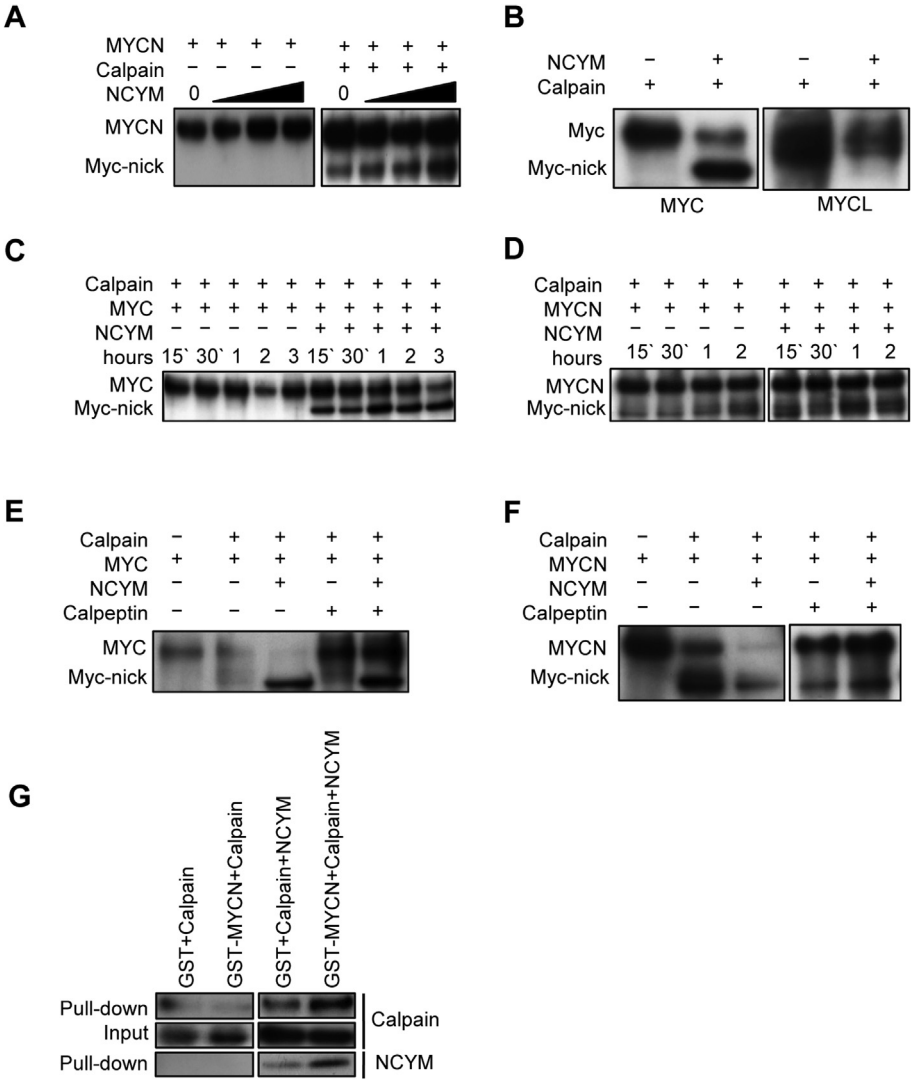

Fig. 1. NCYM promotes cleavages of MYC and MYCN to produce Myc-nick *in vitro*. (A) NCYM promoted Myc-nick production in a dose-dependent manner. Western blot. Results are representative of three independent experiments. (B) MYC, but not MYCL was cleaved in the presence of NCYM. Results are representative for three independent experiments. (C, D) Cleavage of MYC or MYCN was increased in a time-dependent manner in the presence of NCYM. Western blot. Results are representative of three independent experiments. (E, F) Calpeptin inhibits Myc-nick production induced by NCYM. Western blot. Results are representative of three independent experiments. (G) GST-pulldown assay. Purified NCYM and Calpain proteins were pulled down with GST-fused MYCN. Results are representative of three independent experiments.

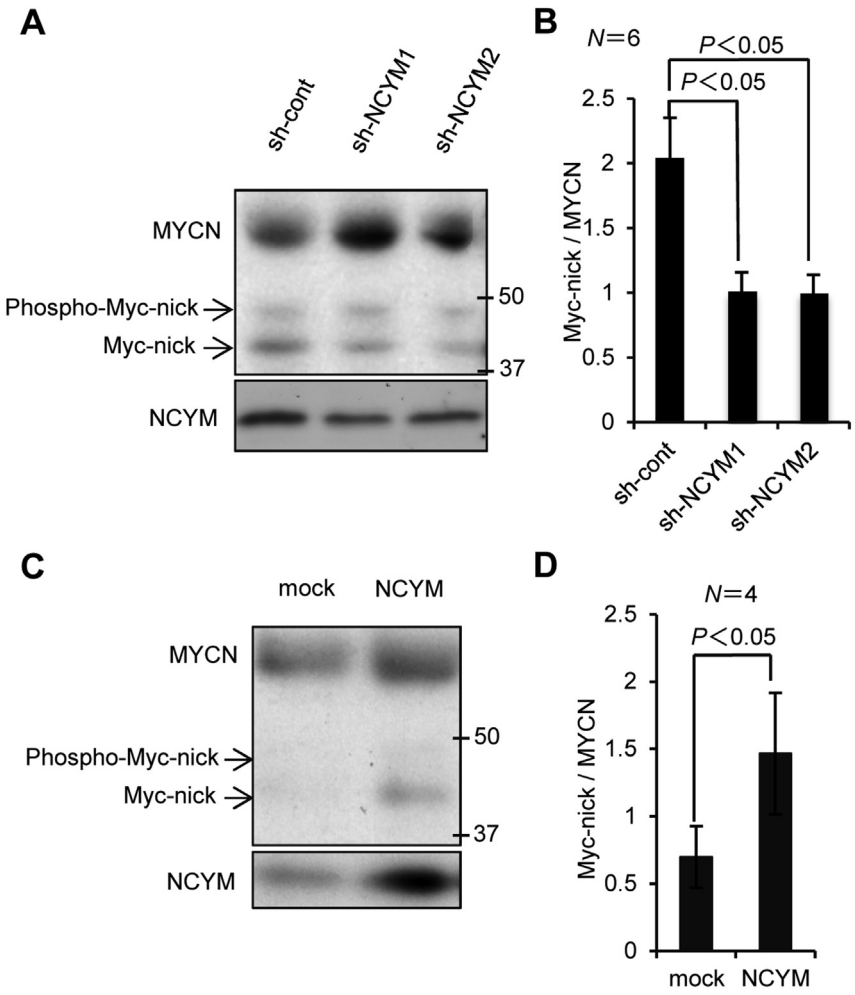

Fig. 2. NCYM increases Myc-nick production in human neuroblastoma cells. (A) Knockdown of NCYM decreased Myc-nick production. Western blot. The amounts of MYC or MYCN were adjusted to normalize Myc-nick. Results are representative of six independent experiments. (B) Quantification of western blotting analysis in NCYM knockdown cells. Myc-nick levels were normalized to MYC or MYCN. Data represent the mean ± SEM from six independent experiments. (C) Overexpression of NCYM increased Myc-nick production. The amounts of MYC or MYCN were adjusted to normalize Myc-nick. Results are representative of four independent experiments. (D) Quantification of (C). Data represent the mean ± SEM from four independent experiments.

3.3. Increased NCYM and Myc-nick in G2/M phase

To investigate NCYM and Myc-nick protein levels throughout the cell cycle, we synchronized CHP134 cells by double thymidine block. Fluorescence activated cell sorting showed that CHP134 cells were synchronized in G2/M phase at 7 h after double thymidine block treatment (Fig. 3A, bottom panel). NCYM and Myc-nick protein levels rose at G2/M phase, accompanied by increased acetylated α-tubulin, whereas α-tubulin levels remained unchanged throughout the cell cycle (Fig. 3A). No changes in *NCYM* mRNA levels were detected in CHP134 cells (Fig. 3B).

3.4. Knockdown of NCYM induces apoptosis at G2/M

We next examined whether NCYM influences the levels of Myc-nick and acetylated α-tubulin by using shRNA-mediated NCYM knockdown. At 72 h after transfection, cells were collected at 0, 4 or 7 h after the double thymidine block. Knockdown of *NCYM* decreased Myc-nick production, accompanied by decreased level of acetyl α-tubulin at 7 h after the double thymidine block (Fig. 3C). NCYM knockdown significantly increased the number of apoptotic cells relative to controls at G2/M phase (Fig. 3D, E). These data indicate that NCYM downregulation is associated with decreased levels of acetylated α-tubulin and Myc-nick at G2/M phase.

4. Discussion

Myc-nick contributes to aggressiveness of cancer cells by inhibiting apoptosis [16]; however, the upstream regulatory mechanisms of Myc-nick production have been poorly characterized. In this study, we identified NCYM as a regulator of Myc-nick in *MYCN*-amplified neuroblastoma cells. Purified NCYM and calpain directly bound to MYCN, and NCYM augmented calpain-mediated Myc-nick production. Since NCYM alone did not produce Myc-nick, the interaction between MYCN, NCYM, and calpain may facilitate efficient Myc-nick production. NCYM-mediated Myc-nick production was partially blocked by the treatment of a calpain inhibitor, calpeptin. Thus, calpain activity is one of the key determinants for NCYM-dependent Myc-nick production.

High MYCN expression in mitotic phase maintains self-renewal of *MYCN*-amplified neuroblastoma cells [17,18], and degradation of MYCN induces asymmetric cell division [17,18], producing differentiated neuroblastoma cells that will eventually die [18]. MYCN degradation requires sequential phosphorylation by cyclin B/Cdk1

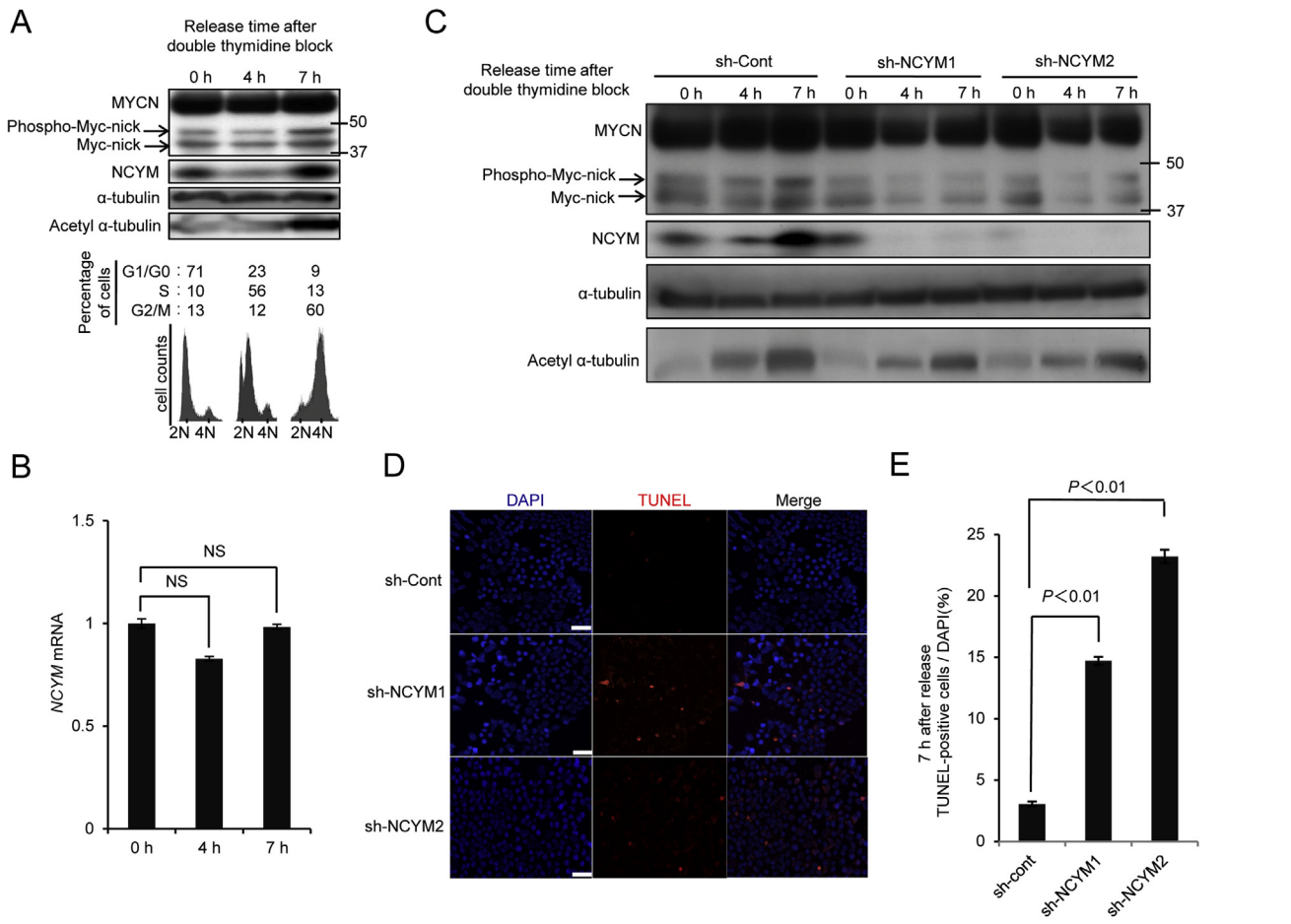

Fig. 3. NCYM knockdown decreases Myc-nick and induces apoptosis at G2/M phase. (A) The expressions of both Myc-nick and NCYM were increased and were accompanied by the induction of acetyl α-tubulin. CHP134 human neuroblastoma cells were synchronized using double thymidine block, harvested at indicated times and subjected to western blot and cell cycle distribution analyses. α-tubulin was used as a loading control. Results are representative of three independent experiments. (B) Quantitative real-time PCR (qRT-PCR) of *NCYM* mRNA in CHP134 cells after double thymidine block. Relative mRNA expression was normalized to *β-actin*. Data show mean ± SEM of triplicated wells. Statistical significance was tested by Student's *t*-test. NS: not significant. (C) Western blot analysis in shRNA-mediated *NCYM* knockdown cells at indicated times after double thymidine block. α-tubulin was used as a loading control. Results are representative of three independent experiments. (D) Representative TUNEL assay images at G2/M phase. Results are representative of three independent experiments. (E) Quantification of apoptotic cells (D) presented as percentage of TUNEL-positive cells. Data represent the mean ± SEM from three independent experiments. NS: not significant. Scale bar, 50 μm.

and GSK3β [19]. The phosphorylation by GSK3β in mitotic phase promotes accumulation of MYCN at spindle poles where MYCN is destabilized by the ubiquitin-proteasome system [18]. Aurora kinase A, which is an essential regulator of mitosis [20], interacts with both MYCN and SCF ubiquitin ligase and stabilizes MYCN in mitotic phase [21]. Aurora A inhibitors destabilize MYCN and suppress progression of *MYCN*-amplified neuroblastomas [22,23]. Therefore, targeting mitotic MYCN is a promising strategy for treatment of *MYCN*-amplified neuroblastomas. In this study, we found that NCYM increases Myc-nick at G2/M phase. Since NCYM stabilizes MYCN by inhibiting GSK3β [8], NCYM at G2/M may regulate the amount of Myc-nick by two mechanisms: increasing the substrate of calpain (full-length MYCN) and facilitating calpain-mediated cleavage by the interaction with MYCN. Previous reports have shown the significance of Myc-nick-related genes in G2/M transition [24–28]. Inhibition of calpain induces aberrant mitosis and prometaphase arrest due to activation of the spindle assembly checkpoint [24]. GCN5 promotes the G2/M transition by inducing α-tubulin acetylation [25] and the deletion of GCN5 augment cell apoptosis and G2/M retardation [26]. Furthermore, acetylated α-tubulin is involved in stabilization of α-tubulin, promoting antimitotic drug resistance [27] and aggressiveness of cancers [28]. Since Myc-nick promotes acetylation of α-tubulin via recruitment of GCN5 [15], NCYM may inhibit apoptosis through Myc-nick-mediated α-tubulin acetylation at G2/M phase. In accordance with this idea, NCYM knockdown decreased acetylated α-tubulin at G2/M phase and induced apoptotic cell death in CHP134 cells.

In conclusion, our findings suggest that NCYM promotes calpain-mediated cleavage of MYCN and subsequent Myc-nick production in neuroblastoma cells. This study provides a new insight into the function of NCYM as a regulator of Myc-nick production. Further studies are needed to examine whether NCYM-mediated Myc-nick production could regulate polar ejection forces through increased acetyl α-tubulin to override the mitotic checkpoint.

Conflict of interest

We report grants from the Ministry of Health, Labour and Welfare for the Third Term Comprehensive Control Research for Cancer, Japan (AN), grants from the Japan Society for the Promotion of Science (JSPS) (YS and AN), during the conduct of the study; grants from Takeda Science Foundation (SY and AN), grants from GlaxoSmithKline, outside the submitted work; In addition, YS and

AN have a patent neuroblastoma mouse model pending. This does not alter the authors' adherence to all policies of Biochemical and Biophysical Research Communications. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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