Targeting the Chromosomal Passenger Complex Subunit INCENP Induces Polyploidy, Apoptosis, and Senescence in Neuroblastoma

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Abstract

Chromosomal passenger complex (CPC) has been demonstrated to be a potential target of cancer therapy by inhibiting Aurora B or survivin in different types of cancer including neuroblastoma. However, chemical inhibition of either Aurora B or survivin does not target CPC specifically due to off-target effects or CPC-independent activities of these two components. In a previous chromatin-focused siRNA screen, we found that neuroblastoma cells were particularly vulnerable to loss of INCENP, a gene encoding a key scaffolding component of the CPC. In this study, INCENP was highly expressed by neuroblastoma cells, and its expression decreased following retinoic acid–induced neuroblastoma differentiation. Elevated levels of INCENP were significantly associated with poor prognosis in primary tumors of neuroblastoma patients with high-risk disease. Genetic silencing of INCENP reduced the growth of both MYCN–wild-type and MYCN-amplified neuroblastoma cell lines in vitro and decreased the growth of neuroblastoma xenografts in vivo, with significant increases in murine survival. Mechanistically, INCENP depletion suppressed neuroblastoma cell growth by inducing polyploidization, apoptosis, and senescence. In most neuroblastoma cell lines tested in vitro, apoptosis was the primary cell fate after INCENP silencing due to induction of DNA damage response and activation of the p53–p21 axis. These results confirm that CPC is a therapeutic target in neuroblastoma, and targeting INCENP is a novel way to disrupt the activity of CPC and inhibit tumor progression in neuroblastoma.

Significance: Dysregulation of INCENP contributes to neuroblastoma tumorigenesis and targeting INCENP presents a novel strategy to disrupt the activity of chromosomal passenger complex and inhibit neuroblastoma progression.

Introduction

Neuroblastoma is the most common extracranial solid tumor in early childhood. It accounts for more than 7% of pediatric malignancies in patients under the age of 15 yet causes 15% of all pediatric cancer deaths (1, 2). Neuroblastoma is a neural crest–derived malignancy and is presumed to arise from a failure of sympathoadrenal progenitor differentiation during the normal development of sympathetic nervous system (3). During the last few decades, there has been significant progress in delineating the genetic underpinnings involved in neuroblastoma tumorigenesis, leading to better risk stratification. This and the addition of immunotherapy have contributed to the increased survival rate of neuroblastoma patients (4–6). However, overall survival of high-risk neuroblastoma patients is still <50% despite intensive multimodal treatment (2, 6). Therefore, a better understanding of the functional consequences of the genetic alterations in high-risk neuroblastoma patients and a function-based target selection strategy should lead to novel therapeutics.

In a chromatin-focused siRNA screen, we identified 53 targets whose loss of function led to decreased neuroblastoma cell growth and/or increased neuroblastoma differentiation. INCENP was identified as one of those candidate genes whose silencing significantly inhibits neuroblastoma cell proliferation (7, 8). INCENP encodes the inner centromere protein (INCENP), which is the structural and regulatory component of the chromosomal passenger complex (CPC) comprised of INCENP, survivin, Borealin, and the Aurora B kinase (9). CPC is responsible for proper chromosomal alignment, segregation, and cytokinesis during the mitosis (9). In the CPC, INCENP plays two critical roles: firstly, it functions as a scaffold protein coordinating assembly of this complex by interacting with all the other three components; and secondarily, the interaction between INCENP and Aurora B is necessary for activation of the Aurora B kinase, the catalytic subunit of this complex (9). Thus, disruption of INCENP expression leads to dissociation of the whole complex and limits Aurora B kinase activity (10, 11). Targeting the CPC has been led by strategies aimed at targeting survivin or Aurora B kinase in neuroblastoma because inhibition of either of them significantly blocks neuroblastoma tumor cell growth in vitro and xenograft growth in vivo (12–15). Recent genome-wide meta-analyses
(GWAS) studies have identified SNPs in INCENP that are linked to increased susceptibility and risk in breast, ovarian, and prostate cancer (16, 17). In addition, INCENP has been found to be overexpressed in high-grade non–Hodgkin B-cell lymphomas and non–small-cell lung cancer, and proposed to be a biomarker for poor prognosis in these types of cancer (18, 19). However, the function and therapeutic potential of targeting INCENP in neuroblastoma remains unclear.

In this study, we investigated how genetic targeting of INCENP affected neuroblastoma cell growth. We find that decreased INCENP levels are associated with increases in polypliodization, apoptosis, and senescence in vitro and decreased neuroblastoma tumor growth in vivo.

Materials and Methods

Cell culture

All the human neuroblastoma cell lines used in this study were obtained from the cell line bank of the Pediatric Oncology Branch of the NCI and have been genetically verified by short tandem repeat analysis and were routinely tested to be free of mycoplasma. Neuroblastoma cells were routinely cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (termed complete RPMI-1640). Stable shRNA neuroblastoma cell lines were maintained in complete RPMI-1640 containing 0.5 or 1 µg/mL puromycin. Tet21N cells were maintained in complete RPMI-1640 supplemented with 1 µg/mL Tetracycline (Tet). HeLa, MDA-MB-231, and A549 cells were cultured in DMEM with 10% FBS, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. For retinoic acid (RA) treatment experiments, neuroblastoma cells were treated with 5 µM for 14 days. Neuroblastoma cell lines were treated with or without 1 µg/mL doxycycline. For the rescue experiment, we used a stable BE2C cell line with doxycycline-inducible expression of siRNA-resistant form of INCENP (still encoded the same protein). To perform the rescue experiment, the expression of siRNA-resistant form of INCENP was induced immediately by adding 0.5 µg/mL doxycycline after shRNAs were transfected into this cell line.

Cell growth and clonogenicity assays

To assess the effect of INCENP knockdown on neuroblastoma cell growth, neuroblastoma cells transfected with siCTRL or siINCENP were plated in 24-well plates, and the growth kinetics were monitored in IncuCyte ZOOM (Essen Bioscience) using the integrated confluence algorithm as a surrogate for cell number. MTS assay (Promega) was performed according to the manufacturer’s instructions in 96-well plate at day 3 after transfection. To investigate the effects of INCENP knockdown on clonogenic ability of neuroblastoma cells, we performed both colony formation assays (adherent colony formation) and soft-agar assay (anchor-age-independent colony formation) using inducible shRNA neuroblastoma cell lines. For colony formation assays, 10^4 neuroblastoma cells were plated into each well of 6-well plates and cultured in complete RPMI-1640 medium containing puromycin (0.5 µg/mL for SY5Y and NGP or 1 µg/mL for BE2C). For the rescue experiment, we used a stable BE2C cell line with doxycycline-inducible expression of siRNA-resistant form of INCENP (still encoded the same protein). To perform the rescue experiment, the expression of siRNA-resistant form of INCENP was induced immediately by adding 0.5 µg/mL doxycycline after shRNAs were transfected into this cell line.

Immunofluorescent cytochemical staining

Subconfluent cells were seeded on 8-well chamber slide after nucleofection with siRNAs. The cells were fixed in 4% paraformaldehyde/PBS for 10 minutes at 48 hours after transfection. Immunostaining was performed as previously described (7). Antibodies used for immunostaining are listed as follow:
expression levels were analyzed by the 2 \( \Delta \Delta CT \) method. Transcriptional changes in BE2C cells following 24 hours of transfection with INCENP siRNAs were analyzed by RNA-seq (HiSeq4000, Illumina). Total proteins were extracted from cultured cell lines or tumor tissues in RIPA buffer (50 mmol/L Tris pH 8, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, 1 mmol/L EDTA, and a mix of protease/phosphatase inhibitors). Western blot was performed as previously described (7).

**RNA and protein analyses**

Total RNA was isolated with the RNeasy Plus Mini Kit (Qiagen Inc.). The reverse transcription reactions were conducted with the High-Capacity RNA-to-cDNA Kit (Applied Biosystems). Housekeeping gene GAPDH was used to normalize the gene expression. Primers used in this study were as follows: INCENP-Forward 5′-AGGCTCTGAAATGTTGAGGTGC-3′ and INCENP-reverse 5′-GTGTCCTGTTGGCAATCTCCGT-3′; GAPDH-forward 5′-AGGAAGGCTCTGGCATTGG-3′. GAPDH-reverse 5′-AGGGGCCATCCTACAGGTCATC-3′. Quantitative real-time PCR was performed on CFX384 Touch Real-Time PCR Detection System (Biorad) using SYBR Green SuperMix as described previously (7). The gene expression levels were analyzed by the 2 \( \Delta \Delta CT \) method. Transcriptional changes in BE2C cells following 24 hours of transfection with INCENP siRNAs were analyzed by RNA-seq (HiSeq4000, Illumina). Total proteins were extracted from cultured cell lines or tumor tissues in RIPA buffer (50 mmol/L Tris pH 8, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, 1 mmol/L EDTA, and a mix of protease/phosphatase inhibitors). Western blot was performed as previously described (7).

**Caspase-Glo 3/7 assay**

Neuroblastoma cells nucleofected with siRNAs were plated in triplicate in 96-well plates. At day 3 after transfection, caspase-3/7 activity was detected using Caspase-Glo 3/7 Assay Systems (Promega) according to the manufacturer's instruction.

**Xenograft assay**

All animal studies were performed at NCI and approved by the Animal Care and Use Committee of the NCI (PB-023). SY5Y shCTRL, SY5Y shINCENP, BE2C shCTRL, and BE2C shINCENP cells were reseeded in Hank's Balanced Salt Solution and Matrigel (1:1, Trevigen), respectively. Cell suspension (100 μL) containing 2 × 10^6 cells was injected s.c. into the left flank of 5- to 6-week-old female athymic nude mice (Frederick Animal Facility, NCI) using a 28-gauge needle (Becton Dickinson). When tumors reached approximately 100 to 200 mm^3, mice were given chow containing doxycycline (Bioserv) or regular chow. The dimensions, length (L) and width (W), of the tumors were measured thrice weekly using a digital caliper, and the tumor volume (mm^3) was calculated as \((L \times W^2)/4\). Mice were euthanized once the tumor diameter reached 2 cm.

**Statistical analysis**

Statistical analyses were performed by Student t test using GraphPad Prism 7.0 software. Kaplan–Meier survival analysis was used to estimate murine survival in the animal studies. To evaluate the differences between the survival curves of control group and experiment group, we used a log-rank test method (Mantel–Cox). All \( P \) values of less than 0.05 are considered to be statistically significant, and ns stands for not statistically significant.

**Results**

**INCENP is overexpressed in neuroblastoma cells, and high INCENP level correlates with poor outcome in high-risk neuroblastoma patients**

From our previous siRNA screen (7), we found silencing of INCENP using either a pool of siRNAs or any of four individual siRNAs targeting INCENP significantly decreased the cell number in SY5Y cells. To understand the nature of this dependency, we first evaluated the expression level of INCENP in 14 commonly used neuroblastoma cell lines and one nontransformed cell line (ARPE-19). Western blot showed that INCENP protein was highly expressed in both MYCN–wild-type (MYCN-WT) and MYCN-amplified (MYCN-ampl) neuroblastoma cell lines (Fig. 1A). This pattern of INCENP expression is similar to levels of the other components of the CPC-Aurora B, survivin, and Borealin, which are also expressed in neuroblastoma cells (Supplementary Fig. S1A). In addition, we detected the expression pattern of all the components of CPC during RA-induced differentiation of neuroblastoma cells and found that RA treatment decreased MYC level in SY5Y cells (Fig. 1B, left) and MYCN level in both KCNR and BE2C cells as early as day 2 (Fig. 1B, right). Meanwhile, decreased INCENP was observed in all selected neuroblastoma cell lines (SY5Y, KCNR, and BE2C) upon RA treatment (Fig. 1B). Notably, different from further decrease of MYC or MYCN level in SY5Y and NGP cells, MYCN was decreased at day 2 but sustained at a stable level in BE2C cells despite of continuous RA treatment (Fig. 1B). The expression of other CPC components (Aurora B, survivin, and Borealin) decreased in RA-treated SY5Y and KCNR cells, but their levels did not show obvious change in RA-treated BE2C cells (Supplementary Fig. S1B). Next, we evaluated publicly available microarray or RNA-seq data of primary neuroblastoma tumor tissues in R2 database (r2.amc.nl); ref. 20. We chose and analyzed two different datasets—TARGET with 249 patient samples (21) and Kocak with 649 patient samples (22)—and found that there was higher expression of INCENP in stage 4 tumors compared with stage 1 tumors (Fig. 1C, left and Supplementary Fig. S1C, left). In addition, we found significantly higher INCENP mRNA levels in MYCN-ampl tumors compared with MYCN-WT tumors (Fig. 1C, right and Supplementary Fig. S1C, right). Consistent with a prevailing view that MYCN is an amplifier of transcriptionally active genes (23, 24), MYCN chromatin immunoprecipitation sequencing (ChiP-seq) data (24) show that MYCN binds to the promoter regions of all the CPC component genes including INCENP in BE2C cells, indicating an involvement of MYCN in
the transcriptional regulation of INCENP and other CPC components (Supplementary Fig. S1D). Consistently, in Tet21N neuroblastoma cell line with tetracycline (Tet)-regulated expression of MYCN (Tet-off), Tet removal induced the expression of MYCN and upregulated expression of CPC components (Supplementary Fig. S1E, left). In contrast, knockdown of MYCN led to a downregulation of the expression of CPC components in BE2C cells (Supplementary Fig. S1E, right). These results indicate that in neuroblastoma cells MYCN directly regulates the expression of CPC components, and sustained CPC levels in RA-treated BE2C cells may be due to residual MYCN expression. A Kaplan–Meier analysis of all neuroblastoma stages showed high INCENP expression was associated with worse prognosis, whereas low level of INCENP was associated with a better outcome (Fig. 1D and E; Supplementary Fig. S1F and S1G). Moreover, in high-risk neuroblastoma patients, including stage 4 patients >18 months and patients whose tumors harbor MYCN amplification, high INCENP expression was associated with poor prognosis, which was statistically significant in both TARGET dataset and Kocak dataset (Fig. 1F and G; Supplementary Fig. S1H and S1I). Taken together, our results show INCENP is highly expressed in neuroblastoma cells and patients whose tumors have elevated levels of INCENP have worse prognoses.

INCENP depletion inhibits neuroblastoma cell growth and tumorigenicity in vitro

To evaluate the biological effects of loss of INCENP in neuroblastoma cells, we utilized short interfering RNAs (siRNA) to transiently knock down the expression of endogenous INCENP in 3 genetically distinct neuroblastoma cell lines: BE2C...
(MYCN-amp and p53-mutant), NGP (MYCN-amp and p53-WT), and SY5Y (MYCN-WT and p53-WT). These neuroblastoma cell lines were transfected with control siRNA (siCTRL) or INCENP siRNAs (siINCENP-#2 and siINCENP-#4) targeting two different regions of INCENP mRNA and cultured for 3 to 5 days. Western blot showed that INCENP protein levels were significantly downregulated in either of INCENP siRNA–transfected neuroblastoma cells compared with control siRNA–transfected neuroblastoma cells (Fig. 2A). Phenotypically, we observed INCENP silencing resulted in significant cell growth inhibition as both cell confluence and the number of attached live cells in the siINCENP-transfected neuroblastoma cells significantly decreased (Fig. 2B–D). A rescue experiment was performed to exclude the possibility that these siRNA-induced phenotypes were due to off-target effects. Briefly, we first generated a stable BE2C cell line with doxycycline-inducible expression of siINCENP-#2–resistant form of INCENP that still encoded the same protein (Supplementary Fig. S2A). We found that overexpression of siRNA-resistant form of INCENP could rescue all of the siINCENP-mediated phenotypes including cell growth inhibition (Supplementary Fig. S2B and S2C). This finding indicates that functional activities due to INCENP depletion could be rescued by INCENP overexpression. In addition, the inhibitory effects of INCENP silencing in neuroblastoma cells have also been verified.

Figure 2. 
INCENP depletion inhibits neuroblastoma cell growth in vitro. 
A, Western blot analysis of INCENP knockdown efficiency in BE2C, NGP, and SYSY cells transfected with two different siRNAs targeting INCENP (#2, siINCENP-#2; #4, siINCENP-#4) and control siRNA (C, siCTRL) for 72 hours. B, The bright-field pictures of BE2C, NGP, and SYSY cells at 72 hours (BE2C and NGP) or 96 hours (SYSY) after siRNA transfection. Scale bars, 100 μm. C, The confluence analysis of siCTRL, siINCENP-#2, and #4-transfected BE2C, NGP, and SYSY cells in the IncuCyte ZOOM. The values of cell confluence at 96 hours were used for statistical analysis by standard two-tailed Student t test (p1, siCTRL vs. siINCENP-#2; p2, siCTRL vs. siINCENP-#4). Bars, average of three replicates ± SD. D, Live-cell number counting of siCTRL, siINCENP-#2, and #4-transfected BE2C, NGP, and SYSY cells at 72 hours (BE2C and NGP) or 96 hours (SYSY) after transfection. Data are represented as mean ± SD of triplicates.
in the KCNR and Kelly neuroblastoma cell lines using pool siRNAs (Supplementary Fig. S2D and S2E). Consistent with above results, gene dependency analysis in multiple neuroblastoma cell lines based on CRISPR-Cas9 screen data from Project Achilles (http://www.broadinstitute.org/achilles, Broad Institute) further demonstrates that neuroblastoma cells are dependent on INCENP as well as other CPC components for their growth (Supplementary Fig. S2F).

To investigate whether INCENP depletion could affect the clonogenicity and tumorigenicity of neuroblastoma cells, we utilized doxycycline-inducible lentiviral shRNA knockdown system to generate stable neuroblastoma cell lines expressing doxycycline-regulated shRNAs targeting INCENP (hereinafter refer to as shINCENP). We found that doxycycline-mediated INCENP silencing caused a significant decrease in INCENP levels (Fig. 3A), and this was accompanied by a significant inhibition of neuroblastoma cell growth (Fig. 3A). In clonogenic assays, we found that INCENP knockdown significantly decreased colony numbers in all three neuroblastoma cell lines tested (Fig. 3B). In soft-agar colony formation assays, both the size and the number of the colonies generated from INCENP-depleted cells were reduced compared with the colonies from control-treated cells (Fig. 3C). However, control shRNA (hereinafter refer to as shCTRL) had no effects on cell growth and the anchorage-dependent (clonogenic assay) or -independent colony formation (soft-agar assay) ability of neuroblastoma cells (Supplementary Fig. S3A–S3C). These results show that INCENP is important for neuroblastoma cell clonogenicity and tumorigenicity in vitro.

**INCENP depletion inhibits neuroblastoma cell growth and tumorigenesis in vivo**

To investigate the effects of INCENP depletion in vivo, we implanted doxycycline-inducible shCTRL or shINCENP BE2C cells (BE2C-doxycycline-shCTRL or BE2C-doxycycline-shINCENP) and SY5Y cells (SY5Y-doxycycline-shCTRL or SY5Y-doxycycline-shINCENP) into nude mice subcutaneously. When tumor volume reached 100 to 200 mm³, mice were stratified from control-treated cells (Fig. 3C). However, control shRNA (hereinafter refer to as shCTRL) had no effects on cell growth and the anchorage-dependent (clonogenic assay) or -independent colony formation (soft-agar assay) ability of neuroblastoma cells (Supplementary Fig. S3A–S3C). These results show that INCENP is important for neuroblastoma cell clonogenicity and tumorigenicity in vitro.

**INCENP depletion in neuroblastoma cells leads to multinucleation and polyploidy**

Next we investigated the mechanisms of how INCENP depletion affected neuroblastoma growth and tumorigenesis. As a critical scaffold protein and regulatory component of the CPC, INCENP silencing would disrupt the normal function of CPC in mitosis and cytokinesis by causing complex dissociation and mislocalization and inactivation of Aurora B kinase, which could lead to multinucleation and generation of polyploid cells (9–11). As a result, we hypothesized that INCENP knockdown–induced polyploidy contributes to cell growth inhibition in neuroblastoma cells. To examine this, we cotained siRNA-transfected neuroblastoma cells with the nuclear dye DAPI and cytoskeleton marker α-tubulin. After transfection with siINCENP, we observed an increase in multinucleated and polyploid cells compared with siCTRL-treated BE2C cells (Fig. 5A) and NGP cells (Fig. 5B). Consistently, hematoxylin and eosin staining of tumor xenografts showed cells with irregular nuclear shape from doxycycline-chow–fed mice bearing BE2C shINCENP xenografts compared with controls (Fig. 5C). Cell-cycle analysis of NGP cells transfected with siINCENP-#2 or -#4 showed significant increases in the percentage of cells at the G2-M phase and increases in the population of cells with DNA content ≥ 4N (Fig. 5D). In INCENP-silenced SY5Y cells, we also observed cells with polyploidy or abnormal nuclei (Supplementary Fig. S5A–S5C). As expected, multinucleated cells have also been found in HeLa and two nontumorigenic cell lines-293T and ARPE19 cells after transfection of siRNAs targeting INCENP (Supplementary Fig. SSD). Taken together, these data support our hypothesis that INCENP depletion increases the percentage of G2-M population and induces the generation of multinucleated and polyploid neuroblastoma cells, which contributes to decreased cell growth.

**INCENP silencing induces DNA damage and apoptosis in neuroblastoma cells**

To further identify mechanisms involved in the growth inhibition after INCENP silencing, we performed RNA-seq analysis after INCENP knockdown for 24 hours. Gene ontology analysis of differentially expressed genes showed enrichment of a series of genes associated with cell growth and differentiation in INCENP-silenced BE2C cells (Table S1; Supplementary Fig. S6A and S6B). Among the differentially expressed genes, GADD45B was upregulated significantly upon INCENP knockdown. Previous studies show that the upregulation of GADD45B frequently occurs in response to different cell stressors and implicates DNA damage as a prelude to an apoptotic cellular response (25–27). We found that INCENP knockdown increased the expression of DNA damage markers (pH2A.X and pCHK2) in all three neuroblastoma cell lines (Fig. 6A and Supplementary Fig. S6C) with some 30% to 50% of the cells exhibiting activation of DNA damage signaling as evidenced by pH2A.X immunostaining (Fig. 6B). Next, we determined the apoptosis level in these control and INCENP-silenced cells and found there was a significant increase in caspase-3/7 activity when INCENP expression was silenced (Fig. 6C and Supplementary Fig. S6D). Western blot analysis further confirmed that INCENP depletion was accompanied by increases in p53-p21 signaling in p53-WT neuroblastoma cell lines (NGP, SY5Y) and upregulation of apoptosis markers including cleaved caspase-3 and cleaved PARP-1 in all three neuroblastoma cell lines (Fig. 6D). Notably, p53 and p21 were significantly increased in INCENP-depleted NGP cells and SY5Y cells, both of which had wild-type p53. In p53-mutant BE2C cells, p53 levels did not change, but the levels of p21 slightly increased upon INCENP knockdown (Fig. 6D left and Supplementary Fig. S6E). To verify whether p21 upregulation is p53 dependent in BE2C cells, we performed INCENP and p53 double knockdown experiment. We found that knockdown of p53 did not block p21 upregulation in INCENP-silenced BE2C.
Figure 3.
INCENP depletion suppresses the colony formation ability and tumorigenesis of neuroblastoma cells in vitro. A, Top, the confluence analysis of BE2C-, NGP-, and SY5Y-inducible shINCENP cells treated with doxycycline (dox) or vehicle in the IncuCyte ZOOM. Bottom, the representative pictures for each cell type under different treatment are shown. Western blot analysis of INCENP knockdown level after 72-hour doxycycline treatment is also shown. The values of cell confluence at 120 hours was used for statistical analysis by standard two-tailed Student t test. Bars, average of three replicates ± SD. Scale bars, 100 μm. B, Clonogenicity of BE2C-, NGP-, and SY5Y-inducible shINCENP cells was determined by colony formation assays. The relative number of colonies (top) and the representative pictures (bottom) for each cell type after crystal violet staining are shown. Bars show the mean ± SD of triplicates. C, The tumorigenicity of BE2C-, NGP-, and SY5Y-inducible shINCENP cells was determined by soft-agar assays in vitro. The relative colony numbers (top) and the representative pictures (bottom) of colonies grown in soft agar for each cell type upon different treatment are shown. Data are represented as mean ± SD of three replicates.
cells but resulted in a slight increase of the protein levels of p21, which is consistent with mutant p53 playing a dominant-negative role in BE2C cells (Supplementary Fig. S6F). This result suggests that induction of p21 in INCENP-depleted BE2C cells is p53 independent. Thus, our results indicate that loss of INCENP activates p21 in both p53-WT neuroblastoma cells and p53-mutant cells through different mechanisms. Moreover, treatment of INCENP-silenced BE2C cells with the pan-caspase inhibitor Z-VAD-fmk (Z-VAD) partially rescued the BE2C cell numbers, and this finding further demonstrates that knockdown INCENP induces cell apoptosis (Supplementary Fig. S6G, left). Western blot results also showed Z-VAD treatment inhibited the upregulation of pH2A.X and cleaved caspase-3 upon INCENP silencing (Supplementary Fig. S6G, right). Interestingly, different from our observation in neuroblastoma cells, we found that INCENP knockdown could only induce polyploidy, but no obvious apoptosis was detected in 293T, ARPE19, and even HeLa cells, whereas massive apoptosis has been observed in siINCENP-silenced neuroblastoma cells (Supplementary Fig. S6H). This observation indicates that neuroblastoma cells are more sensitive to INCENP knockdown. Collectively, these data show that INCENP silencing increases the DNA damage signaling and induces apoptosis in neuroblastoma cells.
INCENP depletion induces a cellular senescence phenotype in neuroblastoma cells

Because several studies have reported that inhibition of Aurora kinase activity using specific chemical inhibitors or genetic silencing induced significant cellular senescence in different types of cancer cell lines (28–32), we tested whether INCENP knockdown would also induce cellular senescence in neuroblastoma cell lines. Western blot results showed dynamic regulation of two markers of cell-cycle exit 5 days after INCENP silencing with increased levels of p21, whereas levels of

Figure 5. Genetic inhibition of INCENP in neuroblastoma cells leads to multinucleation and polyploidy. A, The results of immunostaining (DAPI, cyan; α-tubulin, magenta) showing the irregular nuclear shape of INCENP-depleted BE2C cells at 48 hours after transfection. White arrows, multinucleated cells with enlarged and abnormal nuclei. Scale bars, 20 μm. B, The results of immunostaining (DAPI, cyan; α-tubulin, magenta) showing the irregular nuclear shape of INCENP-depleted NGP cells at 48 hours after transfection. White arrows, multinucleated cells with enlarged and abnormal nuclei. Scale bars, 20 μm. C, Hematoxylin and eosin (H&E) staining results of BE2C-shINCENP xenografts without (top) or with (bottom) doxycycline (dox) treatment. Scale bars, 50 μm. D, Cell-cycle analysis of NGP cells transfected with siCTRL, siINCENP-#2, and siINCENP-#4 for 48 hours. Data represent mean ± SD of three replicates.
phosphorylated forms of RB (pRB) decreased (Fig. 7A). Treatment of BE2C cells with Barasertib, a specific Aurora B kinase inhibitor, for 7 days significantly increased senescence-associated β-galactosidase (SA-β-gal) activity, a marker of senescent cells (Supplementary Fig. S7A). Similarly, INCENP knockdown in BE2C, NGP, and SY5Y cells also induced a 30% to 50% increase in the fraction of cells with SA-β-gal activity (Fig. 7B–D, Supplementary Fig. S7B and S7C).

Discussion

In this study, we find that high expression of INCENP mRNA levels is associated with poor prognosis of neuroblastoma patients. Our genetic inhibition studies show that INCENP is indispensable for the growth of both MYCN-WT and MYCN-amp neuroblastoma cell lines in vitro and in vivo. The functional response of neuroblastoma cells to INCENP silencing is heterogeneous with the evidence of induction of
Figure 7.
INCENP depletion induces cellular senescence phenotype in neuroblastoma cells. A, Western blot analysis of senescence markers' downregulation of pRB and upregulation of p21 in BE2C, NGP, and SY5Y cells transfected with two different siRNAs targeting INCENP (#2, siINCENP-#2; #4, siINCENP-#4) and control siRNA (C, siCTRL) for 5 days. B–D, β-Gal staining of senescent cells in siCTRL, siINCENP-#2, and #4-transfected BE2C (B), NGP (C), and SY5Y (D) at day 7 after transfection to determine the cellular senescence in each cell population. Bright-field pictures (left) and quantification data (right) are shown. Bars, mean ± SD of triplicates. Scale bars, 50 μm.
multinucleated cells, increased apoptosis, and cellular senescence, which all contribute to decreased cell numbers. Our findings suggest that strategies aimed at decreasing INCENP levels selectively target the CPC and inhibit neuroblastoma tumor growth.

High INCENP expression has been reported in some cancer types. In high-grade non-Hodgkin B-cell lymphomas (18), there is significantly higher nuclear IHC signal of INCENP compared with low-grade ones. High expression of three components of CPC (Aurora B, INCENP, and survivin) except Borealin is associated with poor survival rate in patients with NSCLC (19). Recent evidence shows both Aurora B and survivin are highly expressed in neuroblastoma cells, and high levels of either Aurora B or survivin are associated with poor prognosis (12–15). We find that INCENP is highly expressed and required for cell growth in both MYCN-AMP and MYCN-WT neuroblastoma cell lines, although MYCN-amplified neuroblastoma patient tumors have higher INCENP mRNA levels (Fig. 1C, right; and Supplementary Fig. S1C, right). High INCENP level is associated with poor prognosis even in high-risk neuroblastoma patients (Fig. 1F and G, Supplementary Fig. S1H and S1I). The results of MYCN knockdown and overexpression experiments as well as analysis of publically available ChIP-seq data support a model in which MYCN directly activates INCENP expression and thus contribute to its elevated levels in neuroblastoma cells expressing high levels of MYCN. However, the mechanisms leading to dysregulation of INCENP in the high-risk non–MYCN-amplified tumors have not been delineated. It is known that some MYCN-WT neuroblastoma tumors have an elevated MYC level or MYCN/MYC transcripts signature (33, 34), which might contribute to elevated INCENP level in these non–MYCN–amplified tumors. Another intriguing possibility is that INCENP is located at the chromosomal location at 11q12.3 adjacent to the commonly deleted region on chromosome 11q, which is a characteristic of the high-risk MYCN subgroup and may affect INCENP regulation (35, 36). GWAS identified several SNPs in INCENP, which contributed to the susceptibility of breast, ovarian, and prostate cancer (16, 17), but GWAS analyses in neuroblastoma have not revealed any susceptibility loci in INCENP’s chromosomal region (37, 38).

Similar to the functions of the other two components of CPC-Aurora B and survivin in neuroblastoma, we find INCENP is also indispensable for neuroblastoma growth in vitro and in vivo. Previously, Aurora B and survivin have been shown to be necessary for neuroblastoma growth (12–15). Survivin was firstly described as a member of the inhibitor of apoptosis protein (IAP) family (39), and the subsequent finding that survivin was expressed in a cell-cycle–dependent manner led to its involvement in the CPC (40–42). A combined siRNA and drug screen identified Aurora Kinase B as a potent and selective target in neuroblastoma cells (15). Targeting either survivin or Aurora B with small-molecule inhibitors or siRNAs inhibits neuroblastoma cell growth by induction of multinucleation and apoptosis (12–15). In the present study, we observed similar phenotypes in INCENP-depleted neuroblastoma cells. Consistent with the important function of INCENP and CPC, DAPI staining and cell-cycle analysis showed that INCENP knockdown induced multinucleation and generated more cells with DNA content ≥ 4N. In addition, in INCENP-silenced neuroblastoma cells, there was massive apoptosis marked by activation of p53–p21 pathway (only in p53-WT neuroblastoma cells), upregulation of caspase-3/7 activity, and increases in cleaved caspase-3 and cleaved PARP-1 (Fig. 6C and D and Supplementary Fig. S6D) and increases in the sub-G1 fraction of neuroblastoma cells in the cell-cycle analysis (Fig. 5D and Supplementary Fig. S5C). In contrast, there was no obvious cell death in INCENP-silenced nontumorigenic 293T and ARPE-19 cells or tumorigenic HeLa cells (Supplementary Fig. S6H). These findings suggest that neuroblastoma cells are more sensitive to INCENP silencing than nontumorigenic cells and some other types of cancer cells. One possible explanation why neuroblastoma cells respond so dramatically to INCENP silencing is that neuroblastoma cells have a dysregulated postmitotic checkpoint due to disruption of the p53–p21 axis (43, 44) or loss of pRb or amplification/overexpression of MYC family genes (44–46). The significant increase in DNA damage signaling in INCENP knockdown cells supports a model in which polyplody neuroblastoma cells arising after INCENP depletion are able to re-enter the next cell cycle and undergo S-phase and subsequent mitosis, and these events eventually lead to endoreduplication, increased genomic instability, and apoptosis (47).

In this study, we report that both Aurora B inhibition and INCENP knockdown significantly increase the number of senescent cells in neuroblastoma, as judged by cell morphology, SA-β-gal expression, induction of p53–p21cip1 signaling pathway, hypophosphorylated RB, and reduction of cell proliferation. Aurora B kinase inhibition has been associated with an increase in senescent cells in several different types of cancer cells and chemotherapeutic cells (28–32), which is variably reported to be dependent on p53 (29, 31). Here, we find INCENP knockdown induces senescence in both p53–WT NGP and p53-mutant BE2C cells, and this indicates that senescence induced by INCENP depletion in neuroblastoma is not p53 dependent.

CPC has been proved as a very potential therapeutic target for cancer by inhibiting either Aurora B or survivin. However, targeting these two components could not always specifically inhibit CPC function for following reasons. Firstly, to date, many Aurora kinase inhibitors have been developed to interfere with the activation of these kinases by binding ATP-binding pocket competitively, including Baraerinib (AZD1152; ref. 48) used in this study. As many kinases share similar ATP-binding pocket, these chemical inhibitors have nonspecific activities against many other kinases (49). Furthermore, it is relatively straightforward to generate mutant cells resistant to these Aurora inhibitors after long-term treatment, and the utility of these inhibitors in the cancer therapy may therefore be limited (49, 50). Secondly, survivin also plays important roles in regulating apoptosis independent on CPC (39). Therefore, inhibition of survivin could suppress tumor cell growth through both CPC-dependent and CPC-independent mechanism. Targeting INCENP or the interaction between INCENP and Aurora B kinase may provide a novel and alternative strategy to inhibit the CPC function and block neuroblastoma cell growth (51, 52). However, alternative strategies to limit the CPC function by targeting INCENP need to be further tested. The identification of peptides specifically interacting with INCENP may enable a PROTAC strategy (53–55) to be developed that selectively degrades INCENP and destabilizes the CPC complex. In addition, an antisense oligo-mediated gene silencing approach has been shown to effectively target nuclear protein-STAT3 in lymphomas and lung cancer (56) as well as neuroblastoma (57), and such an approach could also...
provide another feasible strategy to selectively target CPC by inhibiting INCENP.

In conclusion, our study demonstrated the importance of INCENP for neuroblastoma cell growth and the association of high INCENP expression with poor prognosis for neuroblastoma patients. We further showed INCENP silencing inhibited neuroblastoma tumor growth in neuroblastoma xenograft model. Finally, we demonstrated INCENP silencing inhibited neuroblastoma growth by induction of polypliodization, DNA damage response, massive cell apoptosis, and cellular senescence.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: M. Sun, V. Veschi, Z. Liu, C.J. Thiele
Development of methodology: V. Veschi, C.J. Thiele
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Sun, S. Bagchi, M. Xu, A. Mendoza, C.J. Thiele
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Sun, S. Bagchi, M. Xu, C.J. Thiele

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Acknowledgments
We would like to thank Dr. Karen M. Wolcott and Norris Lam for their technical assistance with cell-cycle analysis. We are grateful to Dr. Alexander E. Kelly for his helpful suggestions about this work. We thank all members of the Cell and Molecular Biology Section (CMBS), NCI, for helpful discussions and comments during the course of all the experiments and preparation of this article. This research was supported by the Center for Cancer Research, NCI, NIH Intramural Research program.

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Received February 28, 2019; revised July 3, 2019; accepted August 7, 2019; published first August 15, 2019.
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Targeting the Chromosomal Passenger Complex Subunit INCENP Induces Polyploidization, Apoptosis, and Senescence in Neuroblastoma

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doi:10.1158/0008-5472.CAN-19-0695

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