Molecular Cancer Research

Centriole Overduplication is the Predominant Mechanism Leading to Centrosome Amplification in Melanoma

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Abstract

Centrosome amplification (CA) is common in cancer and can arise by centriole overduplication or by cell doubling events, including the failure of cell division and cell–cell fusion. To assess the relative contributions of these two mechanisms, the number of centrosomes with mature/mother centrioles was examined by immunofluorescence in a tissue microarray of human melanomas and benign nevi ($n = 79$ and 17, respectively). The centrosomal protein 170 (CEP170) was used to identify centrosomes with mature centrioles; this is expected to be present in most centrosomes with cell doubling, but on fewer centrosomes with overduplication. Using this method, it was determined that the majority of CA in melanoma can be attributed to centriole overduplication rather than cell doubling events. As Polo-like kinase 4 (PLK4) is the master regulator of centriole duplication, the hypothesis that PLK4 overexpression contributes to centriole overduplication was evaluated. PLK4 is significantly overexpressed in melanoma compared with benign nevi and in a panel of human melanoma cell lines (A375, Hs294T, G361, WM35, WM115, 451Lu, and SK-MEL-28) compared with normal human melanocytes. Interestingly, although PLK4 expression did not correlate with CA in most cases, treatment of melanoma cells with a selective small-molecule PLK4 inhibitor (centrinone B) significantly decreased cell proliferation. The antiproliferative effects of centrinone B were also accompanied by induction of apoptosis.

Implications: This study demonstrates that centriole overduplication is the predominant mechanism leading to centrosome amplification in melanoma and that PLK4 should be further evaluated as a potential therapeutic target for melanoma treatment. *Mol Cancer Res; 16(3); 517–27. ©2018 AACR.*

Introduction

Centrosomes, the major microtubule-organizing centers of the cell, are composed of two orthogonal centrioles embedded in a protein-rich pericentriolar material. During interphase, centrosomes organize cytoplasmic microtubules and anchor cilia. In mitosis, centrosomes organize the mitotic spindle. Structural and functional defects of centrosomes are associated with cancer (1, 2). Among these, the most common is centrosome amplification (CA), the numerical increase in centrosome number, which has been reported in nearly all human cancers, both solid and hematologic (1, 3–8), and in some contexts is sufficient for tumorigenesis (9). CA has a number of unwanted consequences. First, cells with supernumerary centrosomes generate genetic diversity through asymmetric cell divisions on abnormal spindles with chromosome missegregation (3, 6, 10). Consistent with this, CA correlates with aneuploidy and chromosomal instability in cancer (4, 11). In addition, CA enhances invasiveness, attenuates cilia signaling, increases activity of Rac and Rho GTPases, induces dedifferentiation, and increases microtubule-directed polarization (4, 12, 13).

Polo-like kinase 4 (PLK4) is the master regulator of centriole duplication (14–16). When overexpressed, PLK4 can induce CA through the generation of multiple procentrioles adjoining each parental centriole (17, 18) and can enhance cancer cell migration via actin reorganization (19). PLK4 inhibition impairs centriole duplication and enhances genomic instability of cancer cells, leading to cell death (20). On the basis of recent research, PLK4 is emerging as a potential target for cancer treatment. PLK4 is overexpressed in colorectal cancer tissue compared with the adjacent normal intestinal mucosa (21). In addition, PLK4 is overexpressed in breast cancer, which correlates with worse outcomes (22), and predicts resistance to taxane-based chemotherapy (23). However, the role of PLK4 in melanoma and its association with CA are not known.

Melanoma is one of the most aggressive human cancers with approximately 87,110 new melanoma cases and 9,730 melanoma-related deaths predicted in the United States in

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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doi: 10.1158/1541-7786.MCR-17-0197

2017 (24). The role of CA in melanoma and its underlying causes have not been well studied. The activating mutation *B-RAF*^{V600E} can induce CA by abrogating a negative feedback regulation loop that disrupts centrosome duplication (25, 26); however, CA does not correlate with *B-RAF* mutations in melanoma cell lines (27), suggesting that other mechanisms are responsible. The two possible mechanisms leading to CA are cell doubling events (cytokinesis failure or cell–cell fusion) and centriole overduplication (12). The relative contribution of these mechanisms is yet to be determined.

In this study, we evaluated the prevalence and mechanism of CA in melanoma, and the possible role of PLK4 overexpression in CA and as a therapeutic target.

Materials and Methods

Tissue microarray and IHC analysis

A melanoma tissue microarray (TMA) was purchased from US Biomax (ME1004c, 100 cases/cores), containing 62 cases of malignant melanoma, 20 metastatic melanoma, and 18 nevus tissue. IHC was carried out as described previously (4, 28). Briefly, the slide was heated at 60°C for 30 minutes to melt the paraffin, deparaffinized with xylenes (10 minutes × 3), and rehydrated with serially diluted ethanol washes (100%, 95%, 80%, 50%, 2 minutes each) followed by water. Antigen retrieval was performed in a pressure cooker at 121°C with citrate buffer (pH 6.0) for 5 minutes. Blocking was done for 1 hour in 10% FBS in PBS. Tissues were probed with anti-PLK4 (Abcam, ab137398, 1:200), anti-pericentrin (Abcam, ab4448, 1:200), anti-CEP170 (Life Technologies, 72-413-1, 1:100), anti–γ-tubulin (Abcam, ab27074), and anti-tyrosinase (Thermo Fisher Scientific, MS-800, 1:200) antibodies diluted in 1% FBS and 0.1% Triton X-100 in PBS overnight in a humidified chamber at 4°C. The slide was then incubated with Alexa Fluor–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) for 1 hour at room temperature. Slides were washed 3 times after primary and secondary antibody incubations with PBS + 0.1% Triton X-100. Slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted with ProLong Gold antifade reagent (Life Technologies). Scoring of centrosome phenotypes was performed using a Nikon Eclipse Ti inverted microscope, 100× objective, and CoolSNAP HQ2 charge-coupled device camera (Photometrics). The observer was blinded to clinical data and analyzed centrosomes in an average of 29.7 cells per case from at least 3 different regions of the tumor core. We examined the number of pericentrin foci as well as foci that overlapped with CEP170 in cells expressing tyrosinase, a melanocyte marker. A focus was defined as a region of signal intensity that exceeded a set threshold. Tyrosinase staining also allowed for delineation of individual cells. Tissue quality was poor for 3 melanomas and 1 benign sample, so these were excluded from analysis.

To quantify PLK4 expression, the TMA was imaged using a Vectra automated quantitative pathology imaging system (PerkinElmer Life Sciences) with a 40× objective. Tissue images were segmented and scored using inForm (version 1.4.0). We quantified total PLK4 expression and PLK4 expression colocalizing with pericentrin in tyrosinase-positive cells.

Cell culture

The human melanoma cell lines A375, Hs294T, G361, WM35, WM115, 451Lu, and SK-MEL-28 and adult human epidermal melanocytes (HEMa) cells were obtained from ATCC. The HEMa cells were cultured in Dermal Cell Basal Medium supplemented with Melanocyte Growth Kit (ATCC). The melanoma cell lines were maintained in specified media supplemented with 10% FBS (Sigma-Aldrich) in a humidified chamber with 5% CO_2 at 37°C. G361 was maintained in McCoy's 5a medium, A375 and Hs294T in DMEM and 451Lu, WM35, WM115, and SK-MEL-28 in Minimum Essential Medium, procured from Corning Cellgro. Melanoma cells were authenticated by STR analysis using the Promega PowerPlex 16 HS System Kit (DC2101) at the University of Wisconsin Translational Research Initiatives in Pathology laboratory (TRIP Lab). The cells were routinely tested for mycoplasma using the Mycoplasma Detection Kit (Lonza) according to the vendor's protocol. The effects of PLK4 inhibitor centrinone B (Tocris Biosciences) were analyzed at multiple concentrations for 48 hours.

To make PLK4 resistant to centrinone B, we used Phusion site-directed mutagenesis of pcDNA3XFLAG-PLK4 vector to introduce the G95L mutation (29). PLK4^{G95L} was transfected into melanoma cell lines using Lipofectamine 2000 (Thermo Fisher Scientific). Cells were plated in 6-well plates and transfected at 60% confluency. DNA lipid complex was prepared using 4 μg plasmid in 250 μL serum-free medium and 10 μL Lipofectamine 2000 in 250 μL serum-free medium. Each was mixed separately for 5 minutes, then combined together and incubated for 20 minutes at room temperature. During incubation, medium was removed from wells, rinsed once with PBS, and replaced with 2 mL of serum-free medium and 500 μL of DNA–Lipofectamine 2000 complex to appropriate wells. Cells were incubated for 24 hours, and transfection media were removed and replaced with regular media. Selection of stable clones was done using 2 μg/mL G418.

Immunoblotting

Control and treated cell pellets were lysed in 1× RIPA buffer (EMD Millipore Corp.) containing 10 μL/mL protease inhibitor cocktail (Thermo Fisher Scientific) and 1 mmol/L PMSF (Amresco, LLC) and protein was isolated. For Western blot analysis, 40 μg protein was electrophoresed on SDS-PAGE, transferred to a nitrocellulose membrane, blocked with 5% nonfat dry milk, incubated with primary antibodies PLK4 (Abcam ab56752), PARP (Cell Signaling Technology 9542), and β-actin (Cell Signaling Technology 4970S), followed by incubation with secondary HRP-conjugated antibody and chemiluminescent detection using Kodak Image Station 4000 MM (Carestream Health).

qRT-PCR

RNA was extracted from the cells using RNeasy Mini Kit (Qiagen), and cDNA was transcribed with M-MLV reverse transcriptase (Promega). qRT-PCR was performed using StepOnePlus PCR system (Life Technologies) and SYBR Premix Ex Taq II (TaKaRa). PLK4 primer pair was procured from Sigma-Aldrich and GAPDH primer pair was selected from the PrimerBank database (30). Relative quantification was analyzed using GAPDH as endogenous control and ΔΔCT algorithm to assess PLK4 mRNA level.

Flow cytometry

Detection of apoptotic cells in centrinone B–treated samples was carried out using Vybrant Apoptosis Assay Kit (Molecular Probes). Briefly, A375 and Hs294T cells (10^5) were plated in 6-

well plates and treated the next day with centrinone B (50 and 100 nmol/L) for 48 hours. Cells were trypsinized and resuspended in Annexin V–binding buffer, followed by incubation with 5 μL Annexin V-FITC for 15 minutes at 4°C in the dark. Cells were further stained with 5 μL of propidium iodide (PI; 50 μg/mL) for another 5 minutes at 4°C in the dark. Apoptotic cells were evaluated immediately on a flow cytometer (BD Biosciences) and analyzed with FlowJo software.

Cell proliferation and viability assays

The effect of centrinone B on melanoma cell line and normal melanocyte viability was determined using the CytoTox-Glo assay (Promega). Briefly, cells were counted and plated in a 96-well plate and next day, treated with centrinone B for 48 hours, followed by incubation for 15 minutes with AAF-Glo substrate (alanyl-alanylphenylalanyl-aminoluciferin), which determines a distinct intracellular protease activity related with cytotoxicity (dead cell protease) via a luminescent signal. Cell viability was determined by subtracting the luminescent signals of dead cells (due to centrinone B) from total dead cells (after addition of digitonin to lyse remaining viable cells). Data are represented as relative light units for viable cells.

To assess whether CA sensitizes to centrinone B, we utilized RPE-1 and MCF10A (two immortalized, nontransformed human epithelial cell lines) with doxycycline-inducible PLK4. These cell lines were a kind gift from Dr. David Pellman, Harvard University, Boston, MA. Cells were plated in 96-well plates (1,000 cells in 100 μL media/well) and allowed to attach overnight; then, doxycycline was added the next morning to a concentration of 2 μg/mL. Centrinone B was added the following day. After 4 days of incubation with centrinone B, CCK-8 vital stain (Biotool) was added to each well, incubated for 1 hour at 37°C, and absorbance was analyzed with a spectrophotometer. From each experimental absorbance value, we subtracted the absorbance value of wells with media plus CCK-8 reagent (indicating background absorbance without cells). To further assess proliferation, we utilized crystal violet staining in 24-well plates. For crystal violet staining, cells were plated at a density of 5,000/well. Doxycycline was added the next day, and centrinone B was added the following day. Cells were incubated for 4 days after addition of centrinone B and then stained with crystal violet.

Immunofluorescence and microscopy

Immunofluorescence and imaging were carried out as described previously (31, 32). Cells were seeded on glass coverslips in 24-well plates and fixed with 100% ice-cold methanol for 15 minutes. Fixed cells were then blocked for 30 minutes in 3% BSA and 0.1% Triton X-100 in PBS (PBSTx + BSA). Primary antibodies were incubated in PBSTx + BSA for 1 hour at room temperature and washed three times in PBSTx, followed by secondary antibody incubation in PBSTx + BSA for 30 minutes at room temperature and two washes with PBSTx. Primary antibodies used were: pericentrin (Abcam, ab4448), centrin (Millipore, 04-1624), γ-tubulin (Abcam, ab27074), and CEP170 (Life Technologies, 72-413-1). Alexa Fluor–conjugated secondary antibodies were used at 1:350 (Invitrogen). Cells were counterstained with DAPI and mounted on glass slides with Prolong Diamond antifade medium (Invitrogen). Images were acquired on a Nikon Eclipse Ti inverted microscope using a 100× objective and CoolSNAP HQ2 charge-coupled device camera (Photometrics). Optical sections were taken at 0.2-μm intervals and deconvolved using Nikon Elements. Images were processed and analyzed using Nikon Elements software. The observer was blinded to information about each sample in the TMA.

Statistical analysis

ANOVA and *t* tests were used to compare means. The correlation of centrosomes with PLK4 expression was assessed with Pearson correlation. Dunnett multiple comparison and Fisher least significant difference tests were used to compare the control with all the treatment groups for experiments involving centrinone B treatment. Two-sided, unpaired statistical tests were used to calculate statistical differences. $P < 0.05$ was considered statistically significant for all statistical tests. Indications are made in the figures and legends for statistical significance.

Results

CA is prevalent in melanoma

To determine the frequency of CA in melanoma, we analyzed centrosomes by immunofluorescence staining of a melanoma TMA for pericentrin (Fig. 1A). We analyzed CA in 79 melanomas and 17 benign nevi (Fig. 1B). The mean centrosome number was 2.0 (median 1.9) in melanoma compared with 1.1 (median 1.2) in benign tissue (Fig. 1C). We also analyzed CA by calculating the percentage of tumor cells within each sample that had greater than 2 centrosomes (Fig. 1D), as proliferating cells in late G_2 can have duplicated and separated centrosomes without CA. The rate of CA ranged from 0% to 83.3% with a mean of 33.5% (median 31.4%) for melanomas compared with benign samples that had a range of 0% to 20.0% with mean 6.5 (median 3.3). Of 79, 35 melanoma samples had an average of greater than 2 centrosomes per cell compared with 0/17 benign samples. There was no significant difference based on stage ($P = 0.40$) or comparing primary with metastatic samples ($P = 0.33$; Fig. 1E). We conclude that CA is common in malignant melanoma but does not appear to have a strong correlation with disease progression.

CA arises predominantly from centriole overduplication

Next, we sought to determine the contributions of the two principal mechanisms of CA: centriole overduplication versus cell doubling events (e.g., cytokinesis failure or cell–cell fusion). CEP170 marks mother centrioles and is recruited to centrosomes in late G_2 (33). If centriole overduplication is the predominant mechanism leading to CA, we expect only one centrosome to costain with CEP170; conversely, if cell doubling events were predominant, then we expect all centrosomes would have mature centrioles, as identified by CEP170 (Fig. 2A). Staining for CEP170 can distinguish between these two mechanisms of CA (Fig. 2B; ref. 33). Before employing this strategy, we confirmed this by assessing CEP170 in cells that failed cytokinesis compared with cells that overexpressed PLK4 to cause centriole overduplication (Supplementary Fig. S1). In the TMA, we assessed the percent of centrosomes in each melanoma cell (determined by tyrosinase expression, see Supplementary Fig. S2) that costained for CEP170 (Fig. 2C). The mean percentage of centrosomes with CEP170 was 18.0% for melanoma compared with 56.8% for benign samples. This demonstrates that the majority of centrosome-amplified melanomas arise from overduplication rather than cell doubling events. There was no significant difference based on stage or comparing primary with metastatic samples (Fig. 2D).

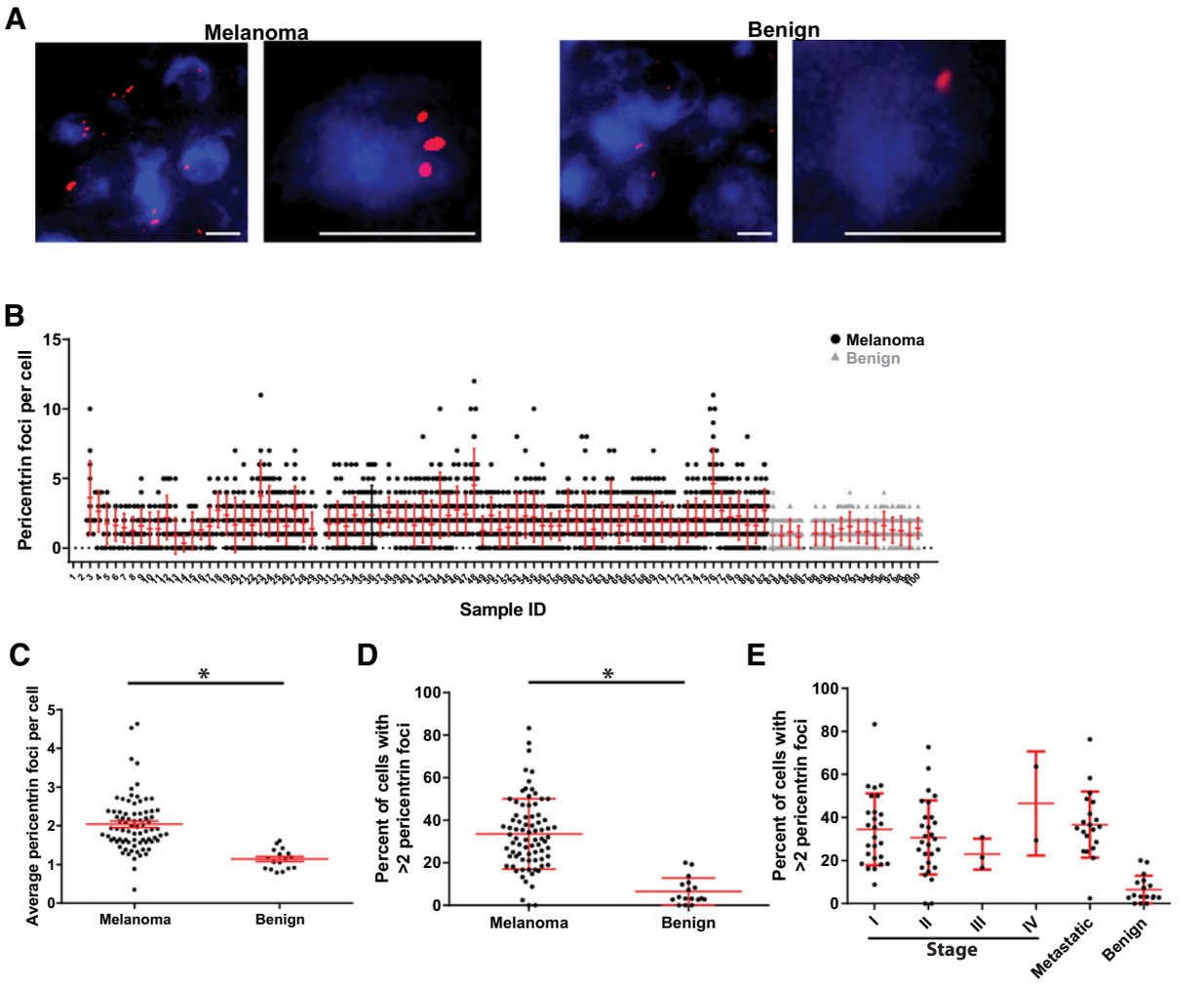

Figure 1.

CA is prevalent in melanoma. **A,** Micrographs demonstrating CA in melanoma but not in benign melanocytes from a melanoma TMA (US Biomax TMA# ME1004c). Blue, DNA/DAPI; red, pericentrin. Scale bar, 5 μm. **B,** Dot plot demonstrating the number of pericentrin foci observed in each individual cell in every sample in the TMA. Melanoma samples are demonstrated with black circles, and benign samples are demonstrated with gray triangles. Each dot represents one cell. **C,** The data from **B** are combined to demonstrate aggregate differences in CA between melanoma and benign samples. **D,** Dot plot quantifying the percent of cells displaying CA, defined as having more than two pericentrin foci, in melanoma samples ($n = 79$) versus benign samples ($n = 17$). **E,** Dot plot demonstrating differences in CA based on primary melanoma tumors of stage I–IV ($n = 59$) versus metastatic tissue ($n = 20$) versus benign tissue ($n = 17$). Bars, means ± SD. Statistical significance is indicated as *, $P < 0.05$. *t* tests were used in **C** and **D**, and an ANOVA was used in **E**.

PLK4 overexpression is associated with CA in clinical melanoma cases

Given that centriole overduplication is the dominant cause of CA, we next investigated underlying mechanisms. *B-RAF*^{V600E} mutations do not adequately explain all cases of CA in melanoma (27). Given that PLK4 is the master regulator of centriole duplication, we hypothesized that its overexpression could be responsible for overduplication. To test this, we quantified PLK4 expression in the same melanoma TMA and costained with two centrosome markers, pericentrin and γ-tubulin (Fig. 3A). We empirically tested 4 different antibodies before choosing the best (Supplementary Fig. S3). To validate the PLK4 antibody used in this study, we first stained untreated and PLK4-overexpressing cell lines by immunofluorescence and also embedded formalin-fixed cell lines in paraffin for fluorescence IHC; indeed, this antibody labels centrosomes (Supplementary Fig. S4). Furthermore, we utilized RPE PLK4 floxed conditional knockout cell lines (unpublished results). These cells were treated with Cre recombinase to cause deletion of exons 3 and 4 of PLK4; then, the efficiency of PLK4 knockout was assessed by qRT-PCR, followed by immunofluorescent staining with the PLK4 antibody. We find that knockout of PLK4 was accomplished and resulted in reduced PLK4 staining by this antibody (Supplementary Fig. S4), and therefore proceeded to stain the melanoma TMA.

In the TMA, melanoma samples had significantly greater PLK4 expression at the centrosomes compared with benign nevi (Fig. 3B). However, PLK4 expression did not correlate well with CA (Fig. 3C and D). Because PLK4 overexpression is known to drive CA via centriole overduplication, we correlated PLK4 expression with our CEP170 mature centriole analysis. Interestingly, many of

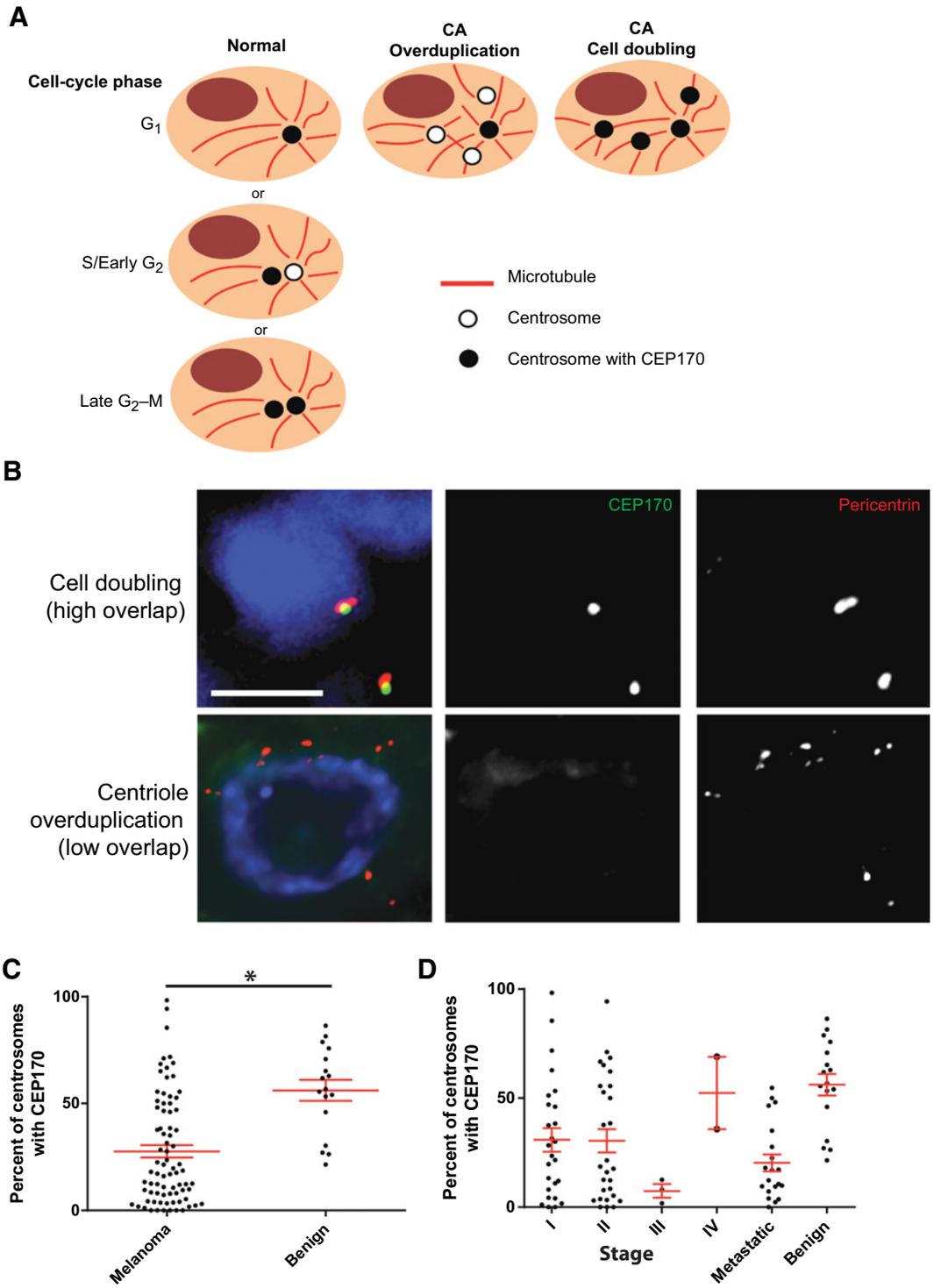

Figure 2.

CA arises predominantly from centriole overduplication. **A,** Normally, cells have one centrosome during G_1 with one mother centriole expressing CEP170, two centrosomes after duplication in early S-phase, and CEP170 is recruited to one centriole in each centrosome late in G_2. If centriole overduplication was predominant, then one would expect many centrosomes with only one staining for CEP170. Conversely, if doubling events predominated, then one would expect many centrosomes that all stain for CEP170. **B,** Micrographs demonstrating centrosomes lacking CEP170 (low overlap, more consistent with centriole overduplication hypothesis) versus centrosomes expressing CEP170 (high overlap, more consistent with cell doubling hypothesis). Blue, DNA/DAPI; green, CEP170; red, pericentrin. Scale bar, 5 μm. **C,** Dot plot quantifying the percent of centrosomes staining for CEP170 in melanoma samples ($n = 79$) versus benign samples ($n = 17$). **D,** Dot plot demonstrating differences in the percent of centrosomes staining for CEP170 based on primary melanoma tumors of stage I–IV ($n = 59$) versus metastatic tissue ($n = 20$) versus benign tissue ($n = 17$). Bars, means ± SD. Statistical significance is indicated as *, $P < 0.05$. A *t* test was used in **C**.

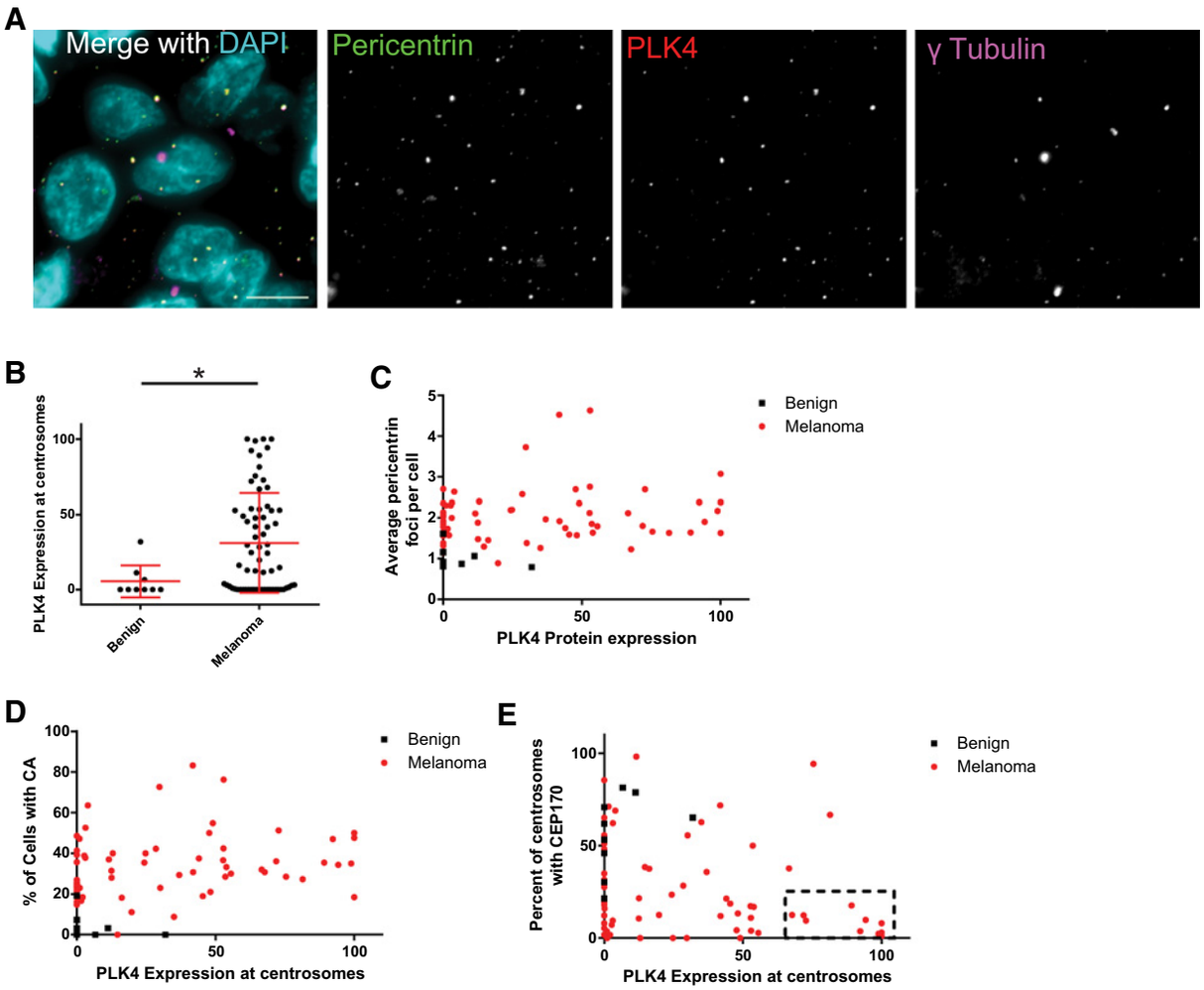

Figure 3.

PLK4 is required for centriole overduplication, but its overexpression does not drive most instances of CA in human melanoma. **A,** Representative micrograph of a melanoma immunostained using PLK4, pericentrin, and γ-tubulin antibodies. Scale bar, 10 μm. **B,** Quantification of PLK4 expression that overlapped with pericentrin. Bars represent means ± SD with statistical significance *, $P < 0.05$. **C,** Correlation of PLK4 expression with centrosomes (average pericentrin foci per cell in each sample). **D,** Correlation of PLK4 expression with CA, defined as the percent of cells with more than two centrosomes (Pearson R for melanomas = 0.26, $P = 0.04$). **E,** Correlation of PLK4 expression with the average percent of centrosomes expressing CEP170 in each sample (Pearson R for melanomas = −0.22, $P = 0.07$). The dotted rectangle indicates cases where we anticipated that PLK4 overexpression caused CA by centriole overduplication.

the melanomas with high PLK4 expression demonstrated a low percent of centrosomes with CEP170, suggesting that PLK4 overexpression may be responsible for centriole overduplication–induced CA in these 15% (10/66 cases) of melanomas (Fig. 3E, outlined by dotted rectangle).

We then assessed the correlation between PLK4 expression and survival in the TCGA melanoma dataset. Interestingly, PLK4 expression varied among tumors by nearly 7 log expressions (Supplementary Fig. S5A). Although there was a trend toward worse survival with higher PLK4 expression, the differences were not significant (Supplementary Fig. S5B and S5C). We conclude that PLK4 expression is the best candidate as a principal driver of centriole overduplication in melanoma but is not itself prognostic.

There are two scores to determine CA in clinical samples that have previously been reported: the centrosome index (CI; refs. 34, 35) and the centrosome amplification 20 (CA20; ref. 36). Both of these scores are calculated from mRNA expression values of certain genes required for centriole duplication. We assessed whether or not these scores correlated with PLK4 expression and whether they were prognostic in the melanoma TCGA cohort; cBioPortal was used to query the data (37). Both scores trended toward correlating with worse overall survival and disease-free survival in this cohort. Overall survival was significantly worse in patients with both high CI and CA20 (Supplementary Fig. S5D–S5I). CA20 and CI significantly correlated with each other, although with limited strength ($r = 0.23$, $P < 0.001$, Supplementary Fig. S5J). CA20 also correlated strongly with PLK4, but CI did not (Supplementary Fig. S5K and S5L). Next, we assessed the p53 status of tumors overexpressing PLK4, as loss of p53 has been shown to permit the growth of cells with CA, which would otherwise arrest (38). Of the 20 melanomas with increased

PLK4 expression, 4 had mutant or deleted p53 (Supplementary Fig. S5M). Taken together, these melanoma TCGA data suggest that PLK4 overexpression may explain some cases of CA in melanoma.

Inhibition of PLK4 exerts antiproliferative effects and depletes centrioles in human melanoma cells

To evaluate the potential of PLK4 as a drug target in melanoma, we first examined its expression profile in a series of human melanoma cell lines (A375, Hs294T, G361, WM35, WM115, 451Lu, and SK-MEL-28) and compared with normal HEMa. All of these cell lines have intact p53 activity except for SK-MEL-28. Compared with HEMa, melanoma cell lines showed a significantly higher level of PLK4 protein and mRNA (Fig. 4A and B). Next, melanoma cells were treated with a small-molecule inhibitor of PLK4, centrinone B (29), and stained for centrin and pericentrin (Fig. 4C and D). We then assessed centriole numbers in these cell lines and correlated with PLK4 expression by immunofluorescent staining of centrioles (Fig. 4E–G). Although the data suggest a positive trend between centriole number and PLK4 expression, the correlation is not statistically significant. To further assess the link between PLK4 expression and CA, melanoma cell lines were treated with centrinone B, resulting in a marked decrease in cell viability in melanoma cells except p53-mutant SK-MEL-28 and 451Lu (Fig. 4H). Interestingly, normal human melanocytes (HEMa) were much less sensitive to centrinone B (Fig. 4H). Our data demonstrate that treatment of human melanoma cell lines with centrinone B reduces cell proliferation.

To ensure that the observed effects of centrinone B on these melanoma cell lines are dependent on centrinone B targeting PLK4, we overexpressed a centrinone B–resistant allele of PLK4 (G95L mutation; ref. 29) in these cell lines (Supplementary Fig. S6). We find that overexpression of $PLK4^{G95L}$ makes the cell lines more resistant to centrinone B, suggesting that the observed effects of centrinone B on the melanoma cell lines are dependent on inhibiting PLK4.

We hypothesized that CA is a biomarker for sensitivity to PLK4 inhibition. To test this hypothesis, we correlated centriole numbers with sensitivity to centrinone B in the aforementioned melanoma cell lines. We find no significant correlation between CA and centrinone B sensitivity. Furthermore, we utilized inducible PLK4 overexpression in RPE-1 and MCF10A immortalized, nontransformed human cell lines to model CA and treated these cells with a range of concentrations of centrinone B. By both crystal violet staining and assessment of cell viability, we find no difference in centrinone B sensitivity in cells with CA compared with controls (Supplementary Fig. S7).

PLK4 inhibition induces apoptosis in human melanoma cells

Reduced cell viability in response to centrinone B could be attributable to slowed proliferation, apoptosis, or to other mechanisms of cell death. To assess apoptosis, centrinone B–treated cells were stained with Annexin V and PI and examined by flow cytometry. The number of Annexin V and PI-stained cells exhibited a significant concentration-dependent increase in A375 and Hs294T cells, indicating an increase in apoptosis following PLK4 inhibition; however, this increase in apoptosis was not observed in normal melanocytes (Fig. 5A and B). Also, PARP cleavage was assessed in centrinone B–treated cells, as this is a hallmark of cellular apoptosis. Cleaved PARP bands are seen in centrinone B–treated A375 and Hs294T cells, but these cleaved PARP bands are not as pronounced in HEMa (Fig. 5C). We conclude that PLK4 inhibition by centrinone B reduces cell viability and induces apoptosis in human melanoma cell lines.

Discussion

A century ago, Theodor Boveri proposed that increased centrosome numbers can cause cancer (39). This is supported by evidence demonstrating that CA is found in precursor lesions and could be an early or even initiating event in carcinogenesis (11, 40, 41). Furthermore, CA is elevated in higher tumor stages and grades, is a prognostic biomarker (4), and may predict paclitaxel resistance (42). Therefore, it is important to understand the underlying mechanisms leading to CA.

Our findings address several important questions regarding CA in human melanoma. Our analysis of a melanoma TMA revealed that essentially all melanomas exhibit some degree of CA, but there is great heterogeneity in the percentage of cells with CA in each tumor. Herein, we have proposed that most of CA is due to centriole overduplication, whereas a minority of CA is due to doubling events. There are some limitations to this analysis using a mother centriole marker. First, if cells with centriole overduplication progressed through late G_2 when CEP170 is recruited to the nascent mature centriole (33), we would underestimate the amount of CA due to centriole overduplication and overestimate the amount of CA due to doubling events. However, we actually observed a decrease in the percentage of centrosomes containing CEP170 in melanomas compared with benign controls, suggesting that this has not had a substantial impact on our analysis. Another limitation is the difficulty in assessing centrioles in formalin-fixed paraffin-embedded tissue. There are instances where the pericentrin staining may underestimate the number of centrioles present in a sample; therefore, there may be instances where we have underestimated the degree of CA. In addition, a cell's centrosomes may not be present in the analyzed tissue section, and this sectioning artifact can also contribute to an underestimation of CA. There are also instances where we may have overestimated CA, as not every pericentrin focus may represent a real centrosome with centrioles.

We hypothesized that overexpression of PLK4, the master regulator of centriole duplication, was responsible for most of these overduplication events. Our results suggest that PLK4 is required for centriole overduplication in melanoma, as inhibition of PLK4 with centrinone B reduces CA in melanoma cell lines; however, overexpression of PLK4 does not appear to be the driver of most cases of centriole overduplication in melanoma, and there may be other mechanisms at play. One previously reported mechanism is $B\text{-}RAF^{V600E}$ mutation (25, 26), but the relative contribution of this mechanism to CA in melanoma is unclear (27). Further study of these other drivers of centriole overduplication is warranted. *In vitro* experimental evidence has suggested that overexpression of PLK4 (13), SAS6 (43), STIL (44), and pericentrin (45), to name a few, can result in centriole overduplication. There is a dearth of reports of the main centrosome components being genetically altered in cancer and leading to CA. We analyzed melanoma TCGA data and also found no obvious causative mutations, copy number variations, or expression changes in the 366 proteins known to localize to the centrosome (data not shown).

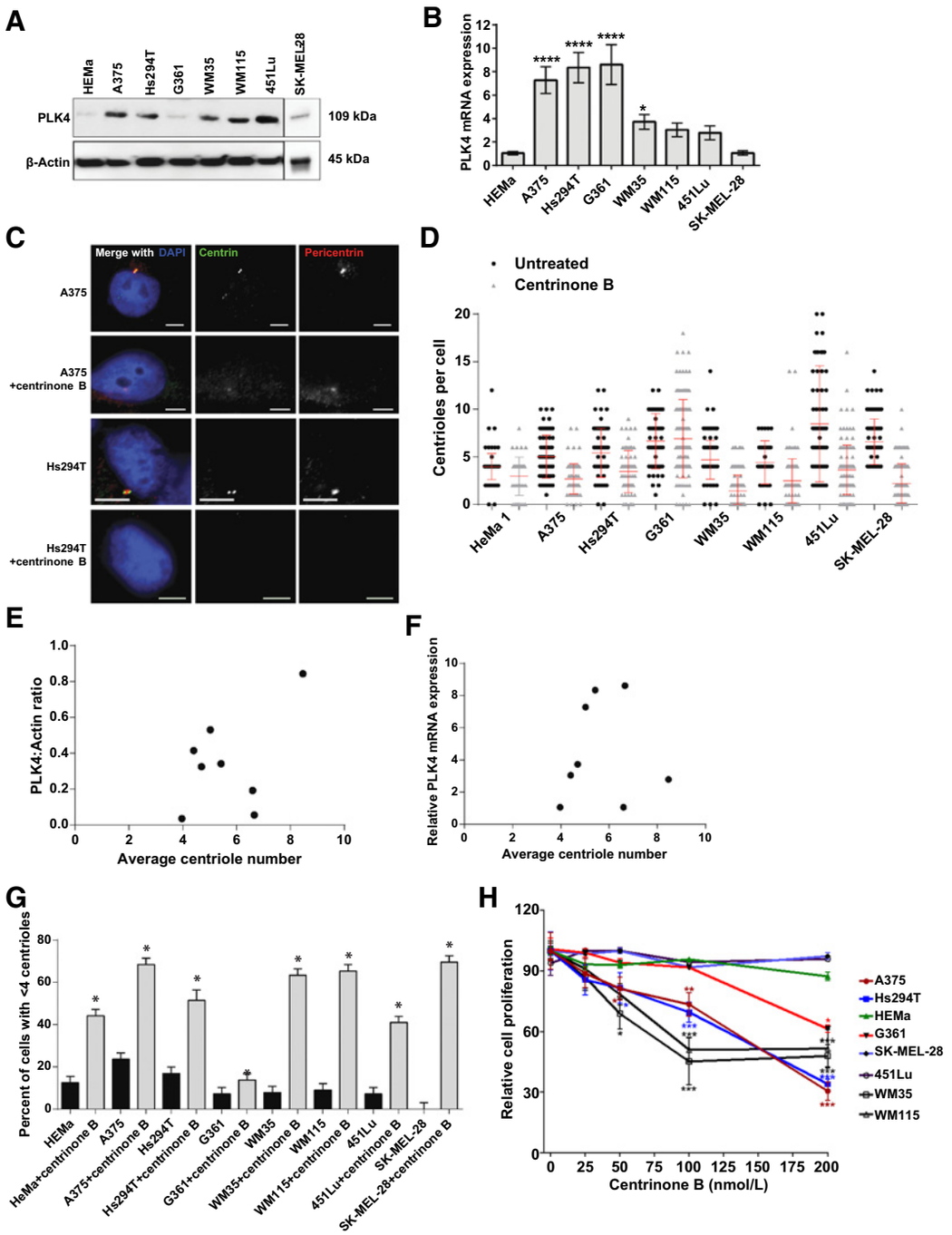

Figure 4.

PLK4 is overexpressed in human melanoma cell lines, and its inhibition with small-molecule inhibitor centrinone B significantly reduces cell viability in human melanoma cells. **A** and **B,** Western blot analysis (**A**) and qRT-PCR analysis (**B**) for PLK4 expression in normal HEMa and seven human melanoma cell lines (A375, Hs294T, G361, WM35, WM115, 451Lu, and SK-MEL-28). **C,** Centrioles were assessed before and after centrinone B treatment by immunofluorescence. Representative images of A375 and Hs294T melanoma cells with or without centrinone B treatment (100 nmol/L for 48 hours). Centrin labels individual centrioles, whereas pericentrin labels the entire centrosome or PCM. Scale, 5 μm. **D,** Quantification of centrioles in each cell line before and after treatment with centrinone B. **E,** Correlation of centrioles with PLK4 protein expression in the melanoma cell lines. PLK4 expression was normalized to actin. Pearson $R = 0.47$, $P = 0.24$. **F,** Correlation of centrioles with PLK4 mRNA expression in the melanoma cell lines. Pearson $R = 0.05$, $P = 0.90$. **G,** Quantification of the percent of cells with <4 centrioles before and after treatment with centrinone B. **H,** The antiproliferative potential of the PLK4 inhibitor centrinone B (treated for 48 hours) was assessed using CytoTox-Glo assay in melanoma cell lines compared to normal human melanocytes. Proliferation values were normalized to untreated cells for each cell line. All the data are representative of at least three independent experiments. Quantitative results are presented as means ± SEM. Statistical significance are indicated as *, $P < 0.05$; **, $P < 0.1$; ***, $P < 0.001$; ****, $P < 0.0001$.

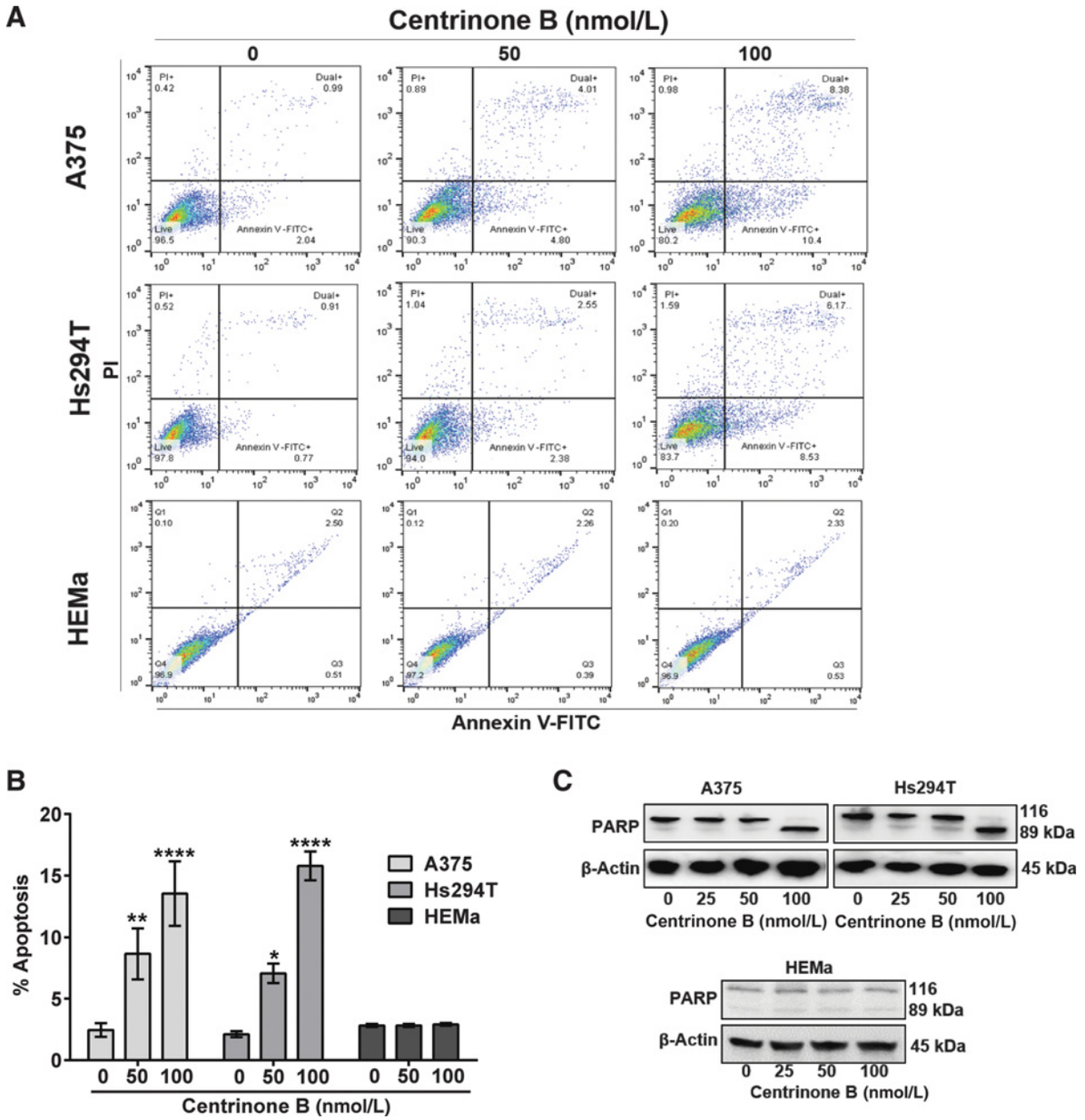

Figure 5.

PLK4 inhibition with centrinone B causes apoptosis in human melanoma cells. **A,** Melanoma cells were grown to 70% confluency and then treated for 48 hours with 50 and 100 nmol/L of centrinone B. The number of apoptotic cells was assessed by flow cytometric analysis of Annexin V/propidium iodide (PI) staining. Representative two-dimensional dot plots of Annexin V-FITC and PI fluorescence are shown here. **B,** Total Annexin V–positive and Annexin V–positive plus PI-positive (Q2 + Q3 quadrants/apoptotic) cells are plotted. Data represent means ± SEM of three replicates. Statistical significance is indicated as *, $P < 0.05$; **, $P < 0.1$; ***, $P < 0.001$; ****, $P < 0.0001$. **C,** Western blot analysis of PARP cleavage in 25, 50, and 100 nmol/L centrinone B–treated A375, Hs294T, and HEMa cells. β-Actin was used as a loading control.

Our results show that PLK4 is significantly overexpressed in clinical human melanoma tissues compared with nevi tissues, and in human melanoma cell lines compared with normal primary melanocytes. Previous reports have similarly found increased PLK4 expression in medulloblastoma, breast, colorectal, prostate, and ovarian cancers (21–23, 46–48). In some melanoma cell lines, the PLK4 mRNA and protein levels did not correlate well. This may suggest some additional posttranscriptional and/or posttranslational regulation of PLK4 that has heretofore been undetermined for PLK4, such as the presence of upstream open reading frames (49, 50), and represents a potentially interesting area of future research. Furthermore, we find that PLK4 inhibition reduces cellular growth and viability and increases apoptosis in human melanoma, suggesting a potent pro-proliferative function of PLK4. These effects appear to be on-target effects of centrinone B on PLK4, as exogenous expression of the $PLK4^{G95L}$ centrinone

B–resistant mutant partially reversed the centrinone B–mediated inhibition of cell proliferation. One caveat to this experiment is that overexpression of PLK4 should cause CA, which may actually reduce cell proliferation and cause cell-cycle arrest; given our results, our cell lines expressing PLK4^{G95L} continued to proliferate, so it is likely that PLK4 was not overexpressed to such a level to induce CA.

CA from PLK4 overexpression causes invasive acini formation and greater cell invasiveness in *in vitro* cell-based assays (13, 19), so we hypothesized that metastatic melanoma samples would have higher CA compared with primary melanoma samples. However, there was not a clear difference in CA between primary and metastatic samples in the TMA we analyzed. This study was not adequately powered to reject the hypothesis that CA is more common in metastatic disease, and further investigation comparing CA in primary versus metastatic patient samples is warranted.

PLK4 has grown in interest as a therapeutic target to treat cancer (29, 46). One PLK4 inhibitor, CFI-400945 (46), has completed phase I clinical trial for advanced cancer (51). However, it is important to note that this compound has also demonstrated activity against the Aurora kinases, so it is difficult to determine whether the potential clinical efficacy of this drug is due to inhibition of PLK4, the Aurora kinases, or both. Given the data presented herein, we suggest that CFI-400945 or other PLK4 inhibitors be studied in a cohort expansion of melanoma patients. A remaining question to be answered is what biomarker predicts sensitivity to PLK4 inhibitors. It appears that CA does not independently predict sensitivity to PLK4 inhibition; however, our data suggest that PLK4 may be a potential biomarker, and further study will be required to answer this question. In conclusion, our data suggest that CA is prevalent in human melanoma, CA predominantly arises by overduplication of centrioles, and PLK4 is a potential biomarker and drug target in melanoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

Research reported in this publication was supported by the NIH grants R01AR059130, R01CA176748, F30CA203271, T32GM008692, UL1TR000427, and TL1TR000429 and the Department of Veterans Affairs (VA Merit Review Award number 1I01BX00100). Also, we acknowledge the core facilities supported by the Skin Diseases Research Center (SDRC) Core Grant P30AR066524 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS). The authors thank the University of Wisconsin Carbone Cancer Center's Experimental Pathology Laboratory, supported by University of Wisconsin Carbone Cancer Center Cancer Center Support Grant P30 CA014520. Finally, the authors thank Karla Esbona and Sally Drew for help with imaging and analysis.

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Received April 12, 2017; revised October 4, 2017; accepted November 29, 2017; published OnlineFirst January 12, 2018.

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