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Original Article

Cyclin A2 regulates symmetrical mitotic spindle formation and centrosome amplification in human colon cancer cells

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Abstract: Colon cancer is one of the most fatal cancers in the United States, and is characterized by the presence of chromosomal instability (CIN), causes of which are largely unclear. Emerging evidence indicates that abnormal spindle geometry and supernumerary centrosomes lead to CIN in cells. However, if and how spindle geometry defects and centrosomes amplification occur in colon cancer remains unknown. Here we show that decrease in the cell cycle regulatory protein, cyclin A2, induces spindle geometry defects in colon cancer cells. In mechanistic studies, we found that cyclin A2 is located at the centrosomes, and its depletion reduces phosphorylation of EG5, which is important for centrosome localization and movement of duplicated centrosomes to opposite poles. We also found that cyclin A2 silencing leads to centrosome amplification in the cells. Collectively, these findings demonstrate previously unrecognized role for cyclin A2 in preventing centrosomal defects in colon cancer cells and provide insights into mechanisms that may potentially cause CIN in these tumors.

Keywords: Colon cancer, cyclin A2, spindle, centrosomes

Introduction

Colon cancer is the second most lethal cancer in United States, with an estimated 50, 260 deaths in 2017 [1, 2]. Genomic instability is one of the hallmarks of cancers, and indeed, several cytogenetic studies in colon tumors have shown high degree of genomic instability and aneuploidy [3, 4]. Chromosomal instability (CIN) is the most prominent genomic instability in colon cancer, observed in about 80-85% of cases [4, 5].

CIN is defined as high degree of loss or gain, either whole or parts, of chromosomes [6]. CIN arises primarily from chromosome segregation defects in the form of chromatin bridges or lagging chromosomes [7]. Chromatin bridges occur due to stalled replication forks, DNA double-strand breaks, reduced separase activity, and/or reduced centromeric Topo 2α activity; while, lagging chromosomes occur due to merotelic attachments that arise mainly from errors in microtubule attachments, microtubule dynamics, amplification of centrosomes and the geometry of spindles [7].

Recently, it has been shown that cyclin A2 deficiency induces DNA double-strand breaks and abnormal spindle geometry that leads to chromatin bridges and lagging chromosomes, respectively, in mouse embryonic fibroblast (MEF) cells [8]. Notably, downregulation of cyclin A2 has been reported in aggressive metastatic colon tumors, compared to non-metastatic primary tumors [9]. Although chromosome instability, aneuploidy and centrosome amplification are also rarely present in non-metastatic primary tumors, the loss of cyclin A2 abundance [9] and the fact that colon tumors exhibit centrosomal defects [10], led us to investigate if cyclin A2 levels affect CIN-inducing spindle geometry defects in colon cancer cells.

Cyclin A2 is a key component of the core cell cycle machinery [11]. Cyclin A2, in complex with CDK2, regulates G1/S transition, DNA replication and repair [11]. In complex with CDK1, cyclin A2 regulates G2/M transition and kinetochore microtubule stability [11, 12]. Notably, cyclin A2 also has CDK-independent functions [8, 9]. In addition, there is evidence that cyclin A2 is redundant in some cell types because of

the compensatory roles of other cyclins that are also present in the cell cycle phases where cyclin A2 is present [13]. Accordingly, specific functions of cyclin A2 that cannot be compensated by other cyclins are largely unclear.

The present study was aimed to determine if cyclin A2 plays an essential role in preventing cellular events that may cause CIN in colon cancer cells. To this end, we depleted cyclin A2 in human colon cancer cell lines HCT-116 and DLD-1 to examine if the loss of cyclin A2 perturbs spindle geometry and centrosome duplication in the cells.

Materials and methods

Antibodies and siRNAs

Following antibodies were used in this study: mouse anti-cyclin A2 (1:5,000, E23.1, Abcam); mouse anti-actin (1:5,000, A5441/clone AC-15, Sigma); rabbit anti γ-tubulin T5192, Sigma); mouse anti-α-tubulin (1:1000, T9026/clone DM1A, Sigma); rabbit anti-Eg5 (1:100, TA30-1478, ORIGENE); rabbit anti-pEg5^{T927} (1:1000, 620502, BioLegend); horseradish peroxidase-conjugated anti-mouse/anti-rabbit secondary antibodies (Cell Signaling Technology). Alexa Fluor® 488-/Alexa Fluor® 594-conjugated anti-mouse or anti-rabbit secondary antibodies were used for immunofluorescence experiments. siRNAs designed to target human cyclin A2 was purchased from Dharmacon. An siRNA targeting luciferase that has no specificity to any human genes was used as the negative transfection control throughout the experiments.

Cell lines, cell culture, and transfections

Colon cancer cell lines HCT116 and DLD-1 were obtained from American Type Culture Collection (ATCC, Rockville, MD). HCT116 cells were cultured in McCoy's 5A medium (Sigma) with 10% (v/v) fetal bovine serum (Sigma) and 100 U/mL penicillin-streptomycin (ThermoFisher). DLD-1 cells were cultured in RPMI-1640 media with 10% (v/v) fetal bovine serum (Sigma) and 100 U/mL penicillin-streptomycin (ThermoFisher). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were transfected twice with siRNAs at every 24 hr and after 24 hr of second transfection, the cells were incubated for another 48 hr before being processed for respective experiments.

Cell cycle analysis

Cells were harvested and fixed in 95% ethanol for 5 min on ice. Cells were washed twice in ice-cold PBS, treated with RNase and stained with propidium iodide (100 μg/ml in 1% sodium citrate). After 15 min incubation in the dark, cell cycle profiles were analyzed by flow cytometry.

Quantitative real-time PCR

Total RNA was extracted from the cells using miRNeasy Mini Kit (Qiagen). cDNA synthesis was carried out with SuperScript III reverse transcription (Invitrogen). The quantitative real-time PCR performed using iTaq Universal SYBR Green Supermix (Bio-Rad). The qRT-PCR primers used were as follows: Cyclin A2: Forward: 5'-TTATTGCTGGAGCTGCCTTT-3', Reverse: 5'-CTCTGGTGGGTTGAGGAGAG-3'; GAPDH: Forward: GAGTCAACGGATTTGGTCGT, Reverse: TTGATTTTGGAGGGATCTCG. The mRNA expression levels were quantified by measuring the threshold cycle (Ct).

Immunoblotting

Cells were lysed and Western blotting was performed as described before [14, 15].

Immunofluorescence

For the co-immunostaining of cyclin A2 and γ-tubulin, cells were fixed in 3% paraformaldehyde (in PBS) at room temperature (RT) for 15 min, followed by permeabilization in 0.2% Triton X-100 (in PBS) for 10 min and blocking in BSA (3% in PBS) at RT for 30 min. For γ-tubulin and α-tubulin co-staining, fixing was done in 1% paraformaldehyde at RT for 5 min and then in ice-cold methanol at RT for 10 min. For spindle geometry analysis, ZEN software (Zeiss) was used to measure the angle between spindle and metaphase plate, after maximum intensity projection. Cells with an angle less than 85° or greater than 95°, between spindle pole axis and the metaphase plate, were considered asymmetrical.

Results

Cyclin A2 siRNA transfected cells have low levels of cyclin A2 mRNA and protein

To determine the specific cellular roles of cyclin A2 in colon cancer cells, cyclin A2 was depleted in human HCT116 and DLD-1 colon cancer cell

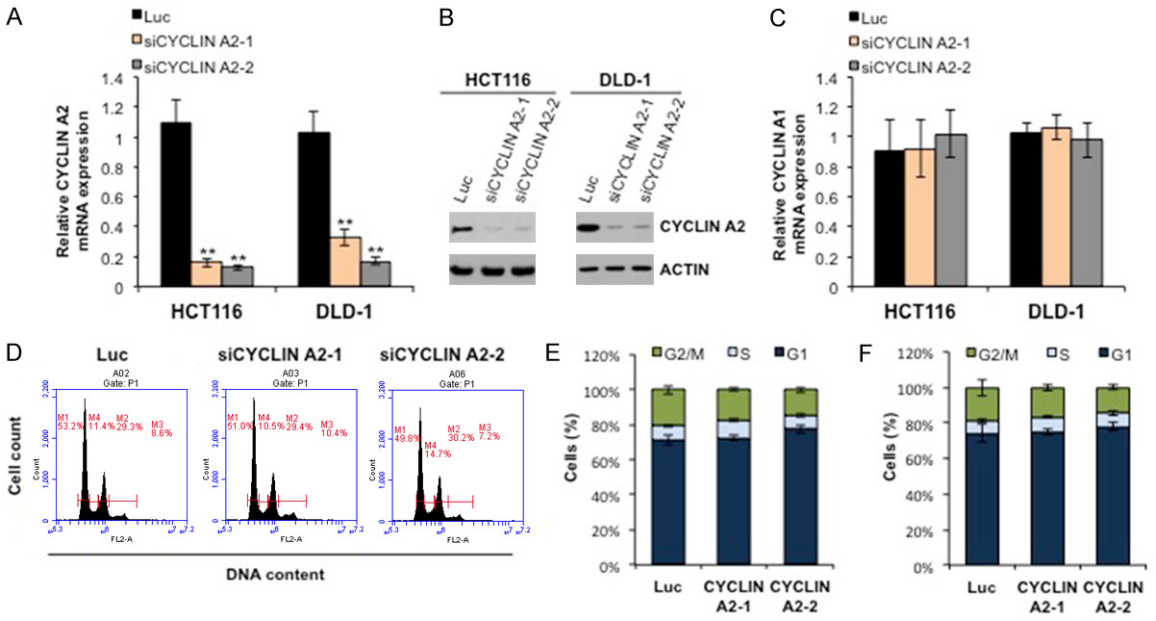

Figure 1. Cyclin A2 siRNAs reduces cyclin A2 mRNA and protein levels. The HCT116 and DLD-1 cells were transfected with control luciferase (Luc) siRNA or two independent siRNAs targeting cyclin A2 (siCYCLIN A2-1 and siCYCLINA2-2) and the cyclin A2 mRNA (A) and protein (B) abundance was determined by quantitative real-time PCR (qRT-PCR) and Western blotting, respectively. (C) mRNA expression of cyclin A1, another A-type cyclin, in the control and cyclin A2 siRNA-transfected cells was examined by qRT-PCR to confirm the specificity of the cyclin A2 siRNAs. (D) The cell cycle profile of Luc- and cyclin A2 siRNA-transfected HCT116 and DLD-1 cells determined by propidium idodide staining of the cells followed by flow cytometry. (E and F) Quantification of (D), showing distribution of cells in G1, S, and G2 phases of the cell cycle in luciferase (Luc) siRNA or cyclin A2 siRNA-transfected HCT116 (E) and DLD-1 (F) cells.

lines, using siRNAs that specifically target cyclin A2. Cyclin A2 mRNA and protein expression was markedly reduced in the cyclin A2 siRNA-transfected cells, compared to the control, non-silencing siRNA (luciferase siRNA)-transfected cells (**Figure 1A**, S1). However, the expression of cyclin A1, an alternative A-type cyclin, was not affected by the transfection of cyclin A2 siRNAs, demonstrating the specificity of the siRNAs in targeting cyclin A2 in the cells (**Figure 1A**). Cyclin A2 silencing affected G1/S and G2/M transitions in some cell types [13, 16], but, depletion of cyclin A2 in both HCT116 and DLD-1 cells did not affect their cell cycle profile (**Figure 1C-E**).

Cyclin A2 depletion induces abnormal spindle geometry

Cyclin A2 insufficiency disrupts mitotic spindle geometry in primary MEFs [8]. It is, however, unknown if this specific role of cyclin A2 is preserved in cancer cells. Towards this aim, we depleted cyclin A2 in HCT116 and DLD-1 cells and examined the geometry of the spindle poles by immunostaining with γ-tubulin, followed by visualization with confocal microscopy. We found that the geometry of the spindle pole is symmetrical in almost all control (Luc) siRNA transfected cells, but it was markedly perturbed in cyclin A2 siRNA transfected cells (**Figure 2A-C**). Only about 5% of control (Luc-transfected) HCT116 or DLD-1 metaphase cells had abnormal spindle geometry, but over 30% of cyclin A2 silenced metaphase cells had this defect (**Figure 2A-C**).

Cyclin A2 is localized at centrosomes and its depletion decreases EG5 phosphorylation

Cyclin A2 is also located at the centrosomes and regulates phosphorylation of EG5 to promote centrosome movement and symmetrical bipolar spindle formation in MEF cells [8]. To test if this is the mechanism behind cyclin A2 regulation of spindle geometry in colon cancer cells, we first tested if cyclin A2 is located at the centrosomes of HCT116 and DLD-1 cells and then examined if loss of cyclin A2 affects EG5 phosphorylation at threonine 927. Co-immunostaining of cyclin A2 and γ-tubulin revealed that cyclin A2 is present at the centrosomes and

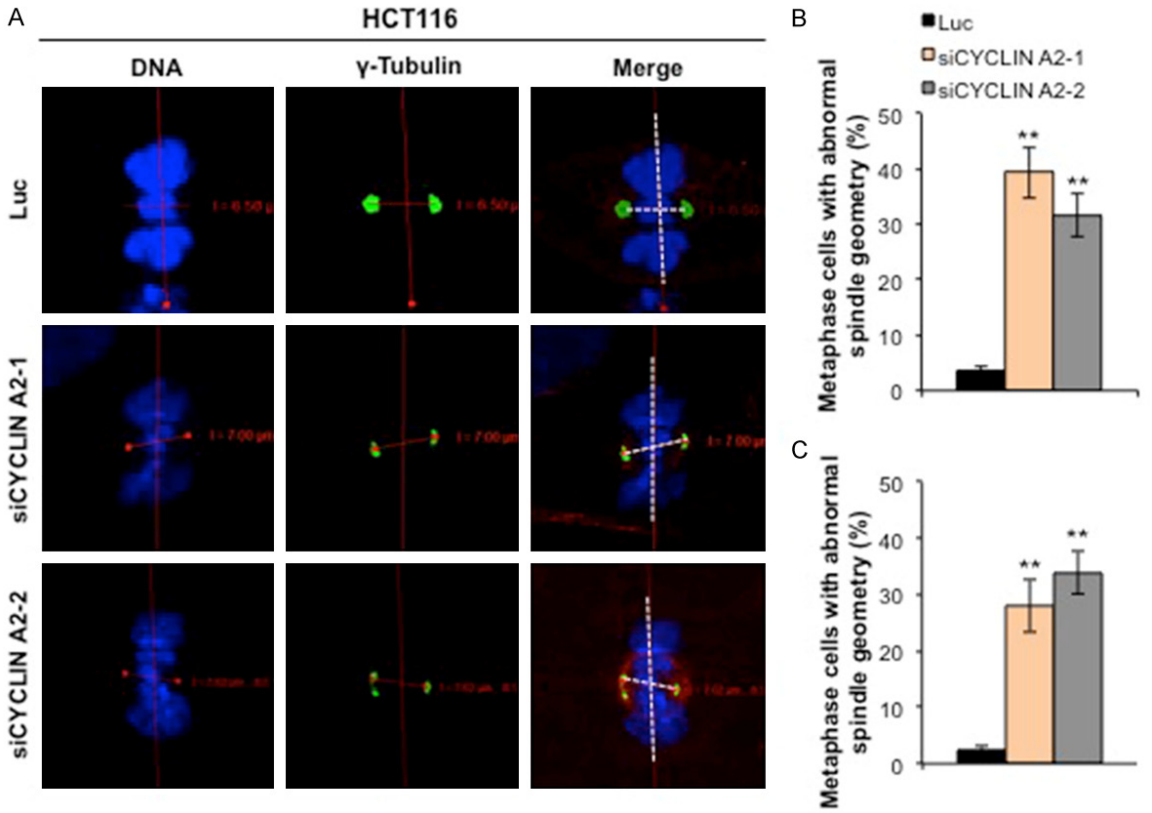

Figure 2. Cyclin A2 depletion induces spindle geometry defects. (A) Abnormal spindle geometry in cyclin A2 depleted HCT116 cells. After 48 hr siRNA transfection, HCT116 and DLD-1 cells were immunostained for γ-tubulin, and spindle geometry defects was examined by confocal microscopy. Cells with an angle less than 85° or greater than 95°, between spindle pole axis and the metaphase plate, were considered asymmetrical. Images shown are representative of at least 30 cells/each siRNA from 3 independent experiments (B) Quantification of the spindle geometry defects in HCT116 cells, (C) Quantification of the spindle geometry defects in DLD-1 cells. Mean ± SEM., n = 3 independent experiments; **P < 0.001, Unpaired *t* test.

cyclin A2 down-regulation, through siRNA transfection, reduces centrosome levels of cyclin A2 (**Figure 3A-C**). Consistent with the previous study in MEF cells[8], loss of cyclin A2 caused reduction in $EG5^{T927}$ phosphorylation without affecting total EG5 abundance (Figure S2), suggesting that reduced $EG5^{T927}$ phosphorylation may contribute to the spindle geometry defect in cyclin A2 depleted cells.

Cyclin A2 insufficiency induces centrosome amplification

Centrosome amplification is a major cause of CIN in colon cancer cells [5, 10]. While analyzing the spindle geometry defects in cyclin A2-depleted cells, we serendipitously discovered that cyclin A2 deficiency also causes centrosome over-duplication in HCT116 and DLD-1 cells. About 15-20% of cyclin A2-deficient HCT-116 and DLD-1 metaphase cells had multipolar spindles and more than two centrosomes; while only ~2% of the control siRNA transfected cells had these defects (**Figure 4A-E**), clearly indicating that cyclin A2 regulates centrosome duplication in colon cancer cells.

Discussion

Formation of bipolar spindles, a metaphase state characterized by attachment of sister kinetochores microtubules from opposite spindle poles, is essential for the accurate segregation of daughter chromosomes between the two daughter cells [17]. To ensure formation of the bi-oriented spindles, cells have several surveillance mechanisms in place, such as, spindle assembly checkpoint and kinetochore-microtubule attachment error correction machinery [18, 19]. Emerging evidence indicates that proper regulation of centrosome dynamics, which includes centrosome disjunction and poleward movement of duplicated centrosomes, is also crucial for the formation of bipo-

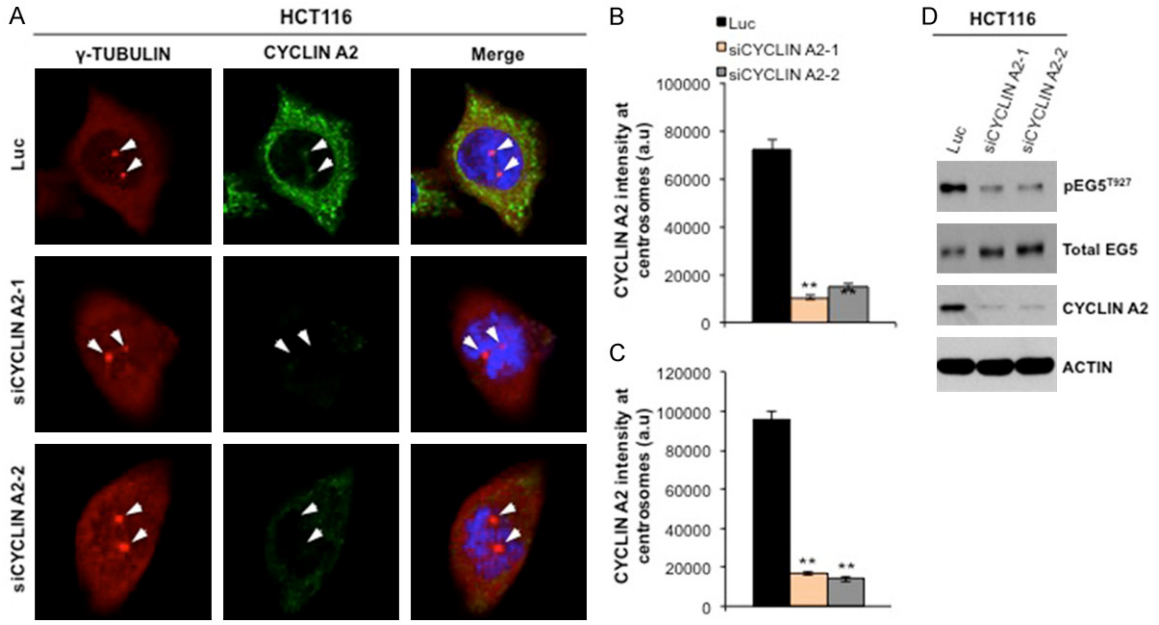

Figure 3. Cyclin A2 is located at the centrosomes and its depletion perturbs EG5^{T927} phosphorylation. (A) Representative images of HCT116 cells stained with cyclin A2 and γ-tubulin (a centrosome marker) after 48 hr control of cyclin A2 siRNA transfection. Note the centrosome localization of cyclin A2 in control (Luc) siRNA-transfected cells (white arrows). Cyclin A2 siRNA transfection reduced the cyclin A2 localization at the centrosomes, consistent with the reduction in the total cyclin A2 protein abundance by the cyclin A2 siRNAs. (B) Quantification of the cyclin A2 levels at the centrosomes of HCT116 cells, (C) Quantification of the cyclin A2 levels at the centrosomes of DLD-1 cells. Mean ± SEM., n = 3 independent experiments; **P < 0.001, Unpaired *t* test. (D) HCT116 cells were transfected with control (Luc) siRNA or cyclin A2 siRNA, and 48 hr posttransfection, the abundance of the indicated proteins were determined by Western blotting.

lar spindles [8, 20]. However, the factors that regulate centrosome dynamics remain largely unclear. A recent study in MEF cells from cyclin A2-deficient mice has shown that loss of cyclin A2 reduces poleward movement of duplicated centrosomes and causes asymmetrical spindle geometry, leading to lagging chromosomes and CIN [8]. Notably, the cyclin A2-deficient mice were highly prone to tumorigenesis [8].

Here we examined the role of cyclin A2 in symmetrical spindle geometry formation in colon cancer cells, because 1) no studies have addressed if cyclin A2 regulation of spindle geometry is preserved in transformed cells, 2) cyclin A2 is downregulated in high-grade metastatic colon tumors [9], and 3) colon cancers are characterized by CIN [4]. We found that, similar to non-transformed (or primary) cyclin A2-deficient MEF cells, cyclin A2 insufficiency induces spindle geometry defects in HCT116 and DLD-1 colon cancer cells (**Figure 2**). Mechanistically, cyclin A2 is located at the centrosomes (**Figure 3A-C**), and cyclin A2 silencing reduced phosphorylation of EG5 protein (Figure S2).

EG5 is critically involved in the movement of duplicated centrosomes to two opposite poles [21]. EG5 is a plus-end directed microtubule motor protein which, through accumulation at centrosomes and astral microtubules, creates an outward 'pushing' force by sliding antiparallel microtubules between centrosome pairs in opposite directions [22, 23]. Phosphorylation of EG5 is important for its recruitment to centrosomes, and cyclin A2-Cdk1 phosphorylates EG5 at T927 [8, 23]. Our finding that cyclin A2 depletion causes abnormal spindle geometry by decreasing EG5^{T927} phosphorylation (Figure S2) is consistent with the previous observation for cyclin A2 loss in MEFs [8]. Thus, with extending the role for cyclin A2 in bi-oriented spindle pole formation to colon cancer cells, our present study suggests that cyclin A2 downregulation may have potential implications of CIN in colon cancer cells.

Additionally, we also show that cyclin A2 depletion leads to multipolar spindles and supernumerary centrosomes in the HCT116 and DLD-1 colon cancer cells (**Figure 4**). This is an impor-

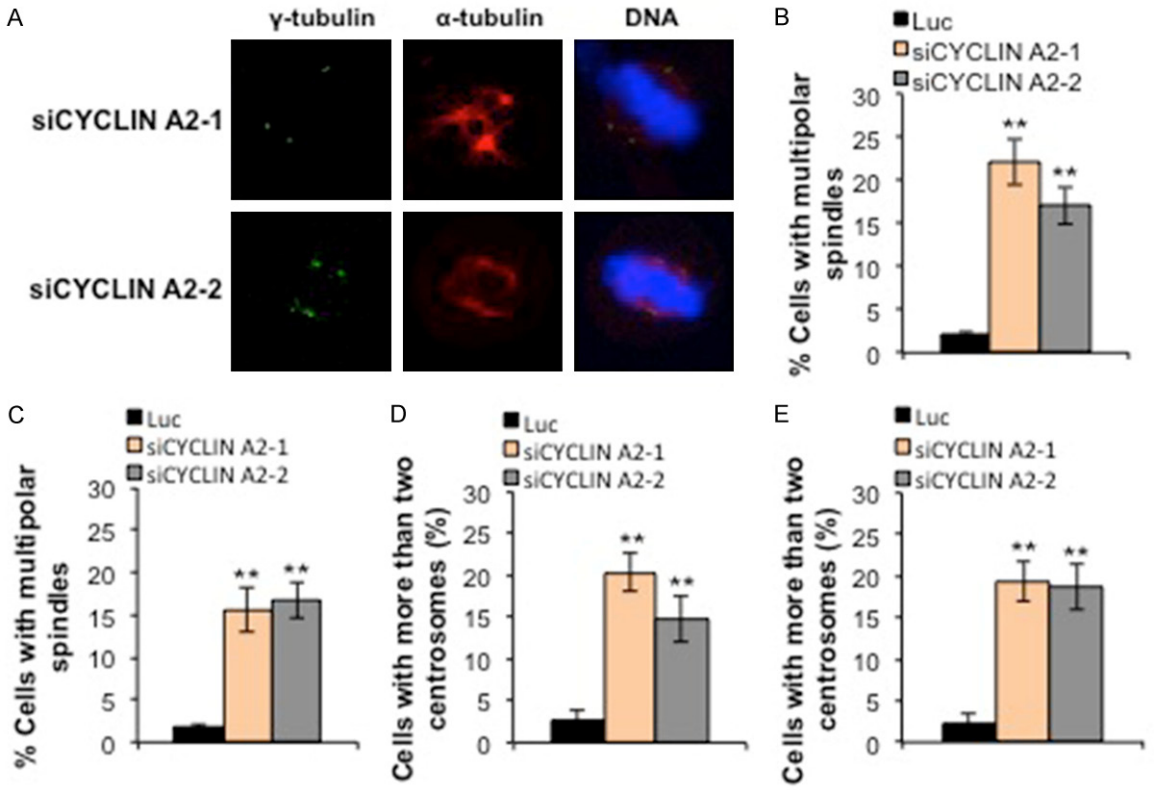

Figure 4. Cyclin A2 depletion causes centrosome amplification. A. Representative images of HCT116 cells stained with γ-tubulin and α-tubulin, after 48 hr control or cyclin A2 siRNA transfection. γ-tubulin marks the centrosomes and α-tubulin stain the spindles. B. Quantification of HCT116 cells with multipolar spindles. C. Quantification of DLD-1 cells with multipolar spindles. D. Quantification of HCT116 cells with more than two centrosomes. E. Quantification of DLD-1 cells with more than two centrosomes. Mean ± SEM, n = 3 independent experiments; **P < 0.001, Unpaired *t* test.

tant observation as centrosome amplification is a potent driver of CIN and it occurs frequently in colon cancers [10, 24, 25]. It should be noted, however, that the centrosome amplification is independent of the spindle geometry defects because, the analysis of spindle geometry defects (**Figure 2**) did not include any cells that have supernumerary centrosomes.

Centrosome over-duplication can potentially occur through multiple mechanisms, including, cytokinesis failure, deregulation of centrosome duplication, mitotic slippage, amplification of centrioles, and *de novo* centriole assembly [26]. Aberrations in proteins, including tumor suppressors and oncogenes, are associated with centrosomal anomalies [27]. A common cause for centrosome duplication is deregulation of centrosome duplication cycle. Polo-like kinase 4 is the master regulator of centrosome duplication [28, 29]. Increased Polo-like kinase 4 (Plk4) activity is associated with extra centrioles, and, conversely, its reduced activity results in decreased number of centrioles in cells [28, 29]. In addition to Plk4, activities of Plk1 and Aurora A also regulate centrosome duplication [30, 31]. Moreover, overexpression of pericentriolar material (PCM) components, such as pericentrin, causes centrosome amplification [32]. Given that cyclin A-CDK2 has been shown to affect Plk1 and Aurora A activities [33, 34], it might be possible that loss of cyclin A2 alters some of these pathways to elevate centrosome duplication. However, further studies are warranted to determine how cyclin A2 may be mechanistically involved in the regulation of centrosome duplication in colon cancer cells.

Collectively, our study demonstrates that loss of cyclin A2 causes high degree of spindle geometry defects and supernumerary centrosomes in colon cancer cells. These results may provide important insights into mechanisms that cause CIN in colon cancers.

Acknowledgements

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Disclosure of conflict of interest

None.

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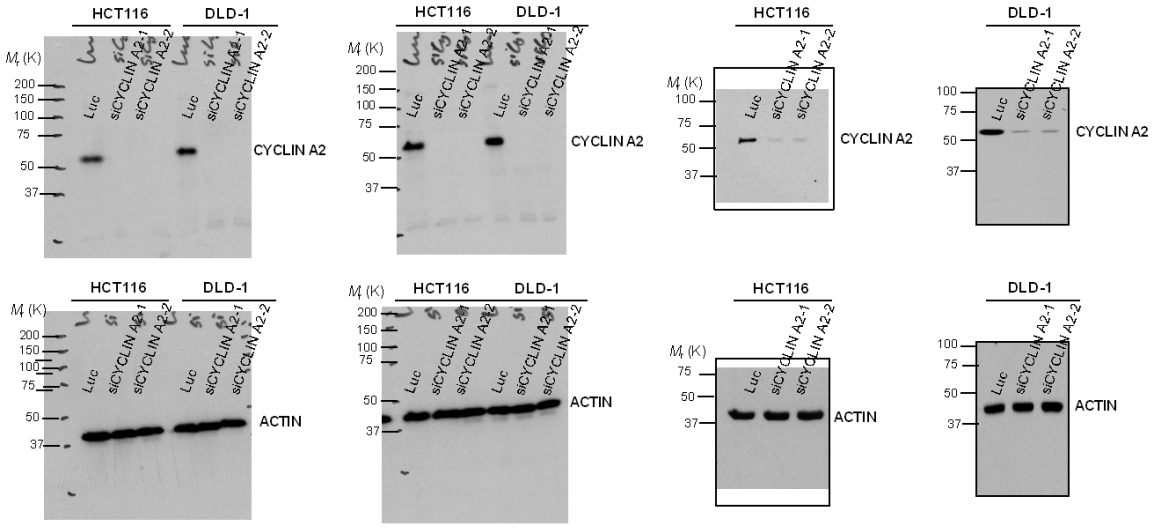

Figure S1. Uncropped western blot images.

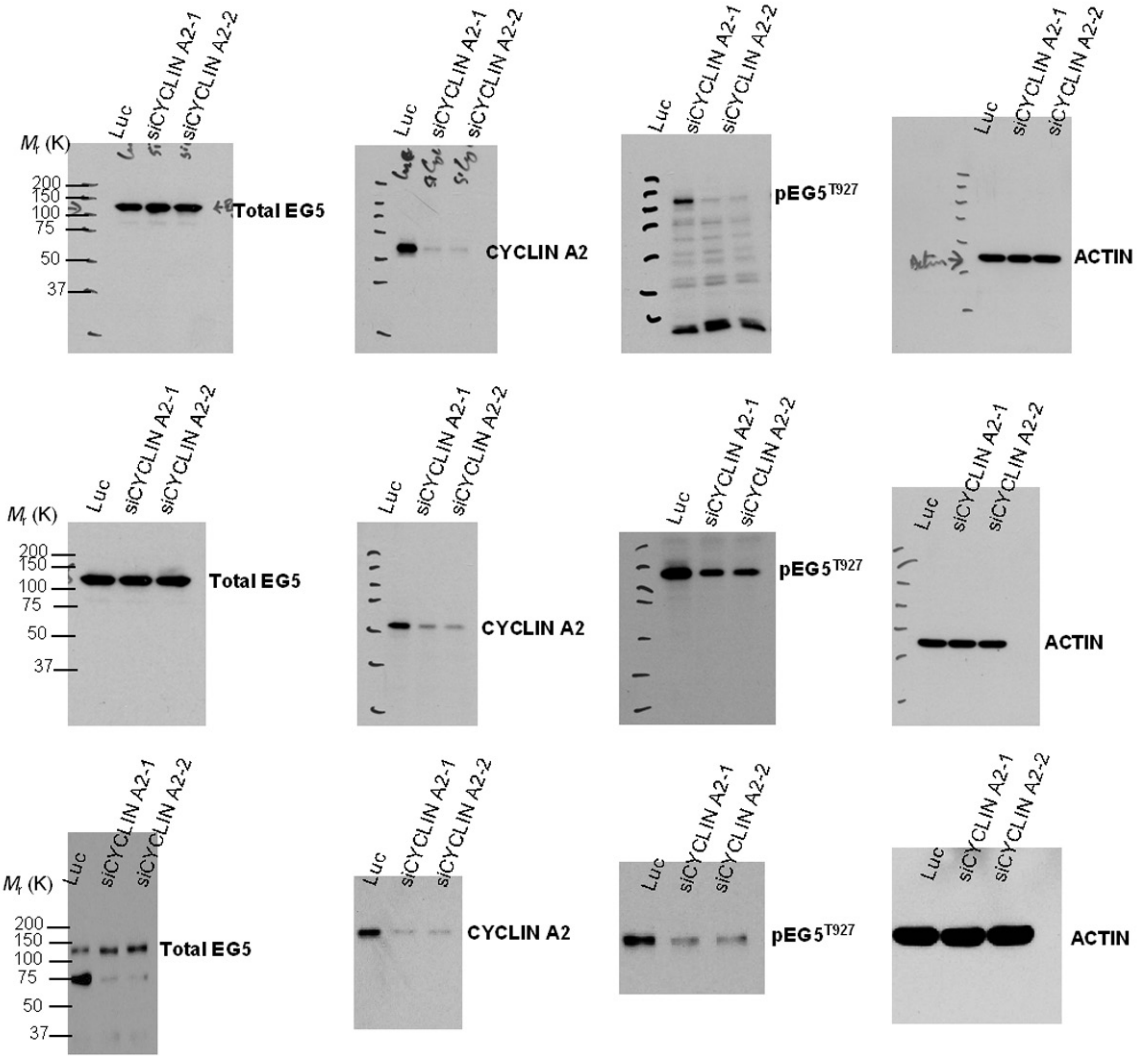

Figure S2. Uncropped western blot images.