ABSTRACT

Polyploidy, a common characteristic of cancer cell lines, puts extra pressure on the regulatory components of mitosis. Specifically, the process of chromatin condensation becomes especially critical due to the challenge of accurately aligning and segregating a multifold increase of DNA on the mitotic spindle. The regulator of chromosome condensation 1 (RCC1) is a protein bound to DNA throughout the cell cycle, which we have shown to be necessary for chromosome size scaling in C. elegans. We hypothesize that RCC1 levels increase in polyploid cells to compensate for this excess DNA content and promote adequate DNA condensation. We isolated populations of highly polyploid HeLa human cancer cells and found that RCC1 levels scale with DNA content in both highly polyploid and control HeLa cells. We believe that this scaling relationship is what affords highly polyploid cells the ability to maintain effective chromatin condensation and perhaps mitotic progression. Highly polyploid HeLa cells that have been stably transfected with a non-DNA-binding RCC1 mutant maintain their polyploidy through more rounds of division than do control polyploid populations. Analysis of metaphase chromosomes has revealed that these cells contain over-condensed chromosomes. We now believe that the loss of RCC1’s DNA-binding ability, which allows over-condensation of chromosomes, is what allows these highly polyploid cells to maintain effective rounds of mitosis, as smaller chromosomes are likely more easily segregated in highly polyploid cells.
INTRODUCTION

Cancer is a complex and constantly adapting disease that has captured the interest of researchers for decades. Its most notable feature is unchecked growth, which occurs from the ability of cancer cells to ignore checkpoints on its way through the cell cycle, allowing them to divide with little to no checkpoint regulation. This is the basis for the majority of chemotherapy drugs in use, which are most effective against cells that proliferate rapidly. However, this causes a number of side effects for many somatic tissues whose cells are also unintentionally inhibited (Niu et al., 2015).

Another defining characteristic of cancer is abnormally high amounts of DNA, which is a result of mitotic defects that lead to cells with extra chromosomes, also known as aneuploidy (Weaver et al., 2007). This phenomenon has long been known and has even been proposed as the cause of cancer (Boveri et al., 1914). However, aneuploidy is unique to cancer cells, and thus offers a novel approach to selectively target cancerous cells without producing the harmful side effects seen in contemporary chemotherapies.

Upon entry into mitosis nuclear DNA must condense into highly ordered structures called chromosomes. This process is called chromosome condensation and is a necessary checkpoint to proceed through mitosis (Koshland et al., 1996). Errors in chromosome condensation and subsequent problems with the mitotic spindle can lead to improper segregation of chromosomes and aneuploidy (Hegyi et al., 2012). Although cancer cells progress through mitosis in an anarchic way, we believe that chromosome condensation must be maintained to keep up with the rapid cellular divisions.
The regulator of chromatin condensation 1 (RCC1) was first discovered in a cell line that carried a temperature sensitive RCC1 mutant (tsBN2), which induced premature chromatin condensation at the nonpermissive temperature (Ohtsubo et al., 1987). It was later discovered that RCC1 is a nuclear-localized protein that is DNA bound (Ohtsubo et al., 1989). RCC1 also has a known function as a Ran guanine nucleotide exchange factor (Ran-GEF). RCC1 exchanges GDP for GTP on Ran creating a Ran-GTP/GDP gradient, which drives nuclear import of various cargoes (Klebe et al., 1995 & Clarke et al., 2008). During assembly of the mitotic spindle, the Ran-GTP/GDP gradient attracts growing microtubules to the metaphase plate (Carazo-Salas et al., 1999). Other than this phenotypic evidence, RCC1’s exact mechanism in regulating chromosome condensation remains a mystery. In this study, we use a non-DNA-bound RCC1 mutant (RCC1^KRR) to explore how RCC1 regulates chromosome condensation in a polyploid system.

Changes in chromosome condensation have been well characterized in multiple developmental systems. This phenomenon is known as chromosome size scaling, where rapid divisions create smaller cells, which necessitate smaller chromosomes to maintain effective divisions. This has been observed in both C. elegans (Ladouceur et al., 2015) and X. laevis (Kieserman et al., 2011). In C. elegans, RCC1 is required for this scaling relationship (Ladouceur et al., 2015). We want to explore how chromosome size is regulated in polyploid human cancer cells. We hypothesize that RCC1 levels increase in polyploid human cancer cells to compensate for excess DNA and promote adequate chromatin condensation.
METHODS

Cell Culture

The HeLa cell line and its derivatives, which expresses RCC1-GFP and triple-mutant-RCC1-GFP, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and penicillin-streptomycin (Invitrogen) at 37°C in a humidified 5% CO₂, 95% air incubator. For shRNA digestions, cells were grown to 70-80% confluency. 100 μL Opti-MEM (ThermFisher Scientific) and 6 μL Lipofectamine (Invitrogen) were incubated with 1 μg of both shRNA plasmids targeted for the 3’ UTR of RCC1 for 15 minutes. 100 μL of this was added dropwise to each plate and incubated for 24 hours at 37°C before immunostaining or metaphase chromosome spreads were performed. Stable cell lines were created by following the preceding transfection process with a plasmid containing the wildtype or mutated RCC1 with a GFP tag and a kanamycin resistance gene. Cultures were grown in g418 (Gibco) and sorted to monoclonal cell lines using the FACSAría II (BD Biosciences) with help from the UNC Flow Cytometry Core.

Flow Cytometry

HeLa cells were grown in standard culture medium described above in 10-cm cell culture dishes to 80-90% confluency. On the day of the experiment, cells are detached using Trypsin-EDTA 0.05% and spun down to a pellet. Cells were resuspended in phosphate buffered saline (PBS) containing 2% (v/v) fetal bovine serum. DNA was incubated with Hoechst 33342 (Thermo Scientific) at a working concentration of 5 μg/mL. Fluorescence-activated cell sorting was done with the help of the UNC Flow Cytometry Core on the FACSAría II (BD Biosciences). Flow cytometry analysis was
done on an LSRII (BD Biosciences). Flow Cytometry data were collected using FlowJo X.0.7 software.

**Immunostaining**

HeLa cells, plated on a EtOH-cleaned coverslip, to be stained for fixed cell imaging were fixed at 4°C with a 1:1 solution of methanol:acetone for 20 minutes. They were then permeabilized with a solution of 0.5% Triton-X in PBS for 5 minutes. The coverslips were then blocked in Abdil-milk solution (0.1% Triton-X, 0.1% NaN₃ and 2% milk in PBS) for 30 minutes. Primary antibodies used include anti-RCC1 N-19 (Santa Cruz) and anti-alpha tubulin (LSBio), both at a dilution of 1:300 in the blocking solution for 2 hours. Fluorophore-conjugated secondary antibodies were diluted at 1:300 and were allowed to react for 45 minutes. Coverslips were then incubated with 0.5 μg/mL DAPI in PBS for 15 minutes. Coverslips were then mounted onto slides with mounting media (90% glycerol and 5 mg/mL n-propyl gallate in diH₂O) and sealed using nail polish.

**Metaphase Chromosome Spreads**

Chromosome spreads were prepared according to the protocol described by Earnshaw et al. 1989. Cells plated on a coverslip were swollen in a hypotonic solution (75 mM KCl) for 25 minutes. Equal volume fixative (3:1 Methanol:Glacial Acetic Acid) was added to the hypotonic solution for 2 minutes. The liquid was aspirated and fixative was added again for 5 minutes. Fixative was aspirated and the coverslips were blown dry using a cut 1000 μL pipet tip. Chromosomes were rehydrated in PBS Azide (1 mM EGTA and 0.01% NaN₃ in PBS) for 10-15 minutes. The spreads were then incubated in 0.5 μg/mL DAPI in PBS Azide for 10 minutes. Coverslips were mounted onto slides with
mounting media (90% glycerol and 5 mg/mL n-propyl gallate in diH₂O) and sealed using nail polish.

Microscopy

Imaging was performed at room temperature on a DeltaVision microscope using Softworx software (Applied Precision) equipped with a CoolSnap HQ2 camera (Photometrics) at 1 × 1 binning and a ×60 Nikon oil-immersion objective using the 45 μm pinhole setting. All images were deconvolved using Softworx software (Applied Precision).

Data Analysis

All image analysis was done using Fiji (ImageJ) and statistics were analyzed with Microsoft Excel (Microsoft) and Prism (CenturyLink). Figures we created using Adobe Illustrator.

RESULTS

RCC1 scales with DNA content in polyploid HeLa cells

RCC1 is a Ran-GEF, and is necessary for nuclear import by loading Ran with GTP, which initiates cargo release (Edens et al., 2012). We originally hypothesized that maintaining extra DNA content would require more nuclear import, which would be dependent on increased activity or expression of RCC1. Furthermore, previous published work from our lab has demonstrated that RCC1 is necessary to maintain chromosome size scaling with cell and nuclear size in early C. elegans embryonic development (Ladouceur et al., 2015). Since RCC1 is also a DNA bound protein, we first hypothesized that RCC1 protein levels would scale with the total amount of DNA. To test this, we labeled HeLa cells with Hoechst 33342 to determine a wildtype
distribution of DNA content in a population of HeLa cells using flow cytometry (Figure 1B). We were able to use fluorescence-activated cell sorting (FACS) to gate the population as shown in Figure 1B into control and polyploid populations, which we stably maintained in culture. To determine the relationship between DNA content and RCC1 protein levels, we fixed cells from both gated populations and probed with an RCC1 antibody, co-staining with DAPI to quantify DNA content. We found that RCC1 levels scaled with the amount of DNA content in both our control and polyploid sorted populations (Figure 1C-D).

**Loss of RCC1’s DNA-binding activity maintains polyploidy levels within a population**

We wanted to determine RCC1’s role in polyploid HeLa cells and decouple the protein from its scaling relationship with DNA. We achieved this by creating a triple-point mutant RCC1 protein, whose DNA-binding residues (Makde et al., 2010) were mutated to alanines (Figure 3A, RCC1^KRR::GFP). This protein was tagged with GFP and no longer localized to the metaphase plate during mitosis (Figure 3B). This construct, along with a RCC1::GFP construct, were stably transfected into HeLa cells. These HeLa cell lines were again sorted into control and polyploid populations, while gating for GFP-positive cells only (Figure 2). Following the experimental protocol established for the first FACS experiments, these populations were grown out for two passages and analyzed using flow cytometry to determine how the distribution of DNA content had changed. Non-transfected polyploid HeLa cells almost immediately returned to the wildtype distribution of DNA content (Figure 2A). Polyploid HeLa cells that were transfected with the RCC1::GFP construct did not support a highly polyploid population,
but centered around a lower chromosome number (Figure 2B). The polyploid population that was isolated from HeLa cells transfected with RCC1KRR::GFP maintained a large number of polyploid cells compared to isolated populations of HeLa cells and HeLa cells transfected with RCC1::GFP (Figure 2C).

To explore this phenomenon on a cellular level, we performed fixed-cell staining of our isolated control and polyploid HeLa cell lines. We also used shRNA directed to the 3' UTR of RCC1 to deplete the endogenous protein and leave only our transfected constructs. Depletion of RCC1 using two different constructs directed at the 3' UTR of RCC1 led to an approximate 75% knockdown of endogenous RCC1 protein levels as shown by Western blot analysis (Figure 4C-D). Interestingly, metaphase plate lengths increase when endogenous RCC1 is depleted in all cell lines (Figure 5).

**Disruption of RCC1’s DNA binding ability decreases chromosome area**

We wanted to explore how HeLa cells containing the RCC1KRR::GFP construct were able to maintain their polyploidy. Previous work from our lab has shown that RCC1 depletion decreased chromosome size in *C. elegans* (Ladouceur et al., 2015). Therefore, to determine how chromosome size is regulated by our RCC1KRR::GFP construct, metaphase chromosome spreads were analyzed to measure how chromosome size scaling is disrupted. Chromosome area was measured consistent with the quantification method outlined in Kieserman et al., 2011. We found that chromosome area decreases in response to RCC1 depletion in HeLa cells (Figure 4). We also found that chromosome area significantly decreases in HeLa cells that carry the non-DNA-binding RCC1, even without depletion of the endogenous RCC1 (Figure 4).
DISCUSSION

We have demonstrated that RCC1 protein levels scale in response to increased DNA content, or polyploidy. However, contrary to our predictions, RCC1_{KRR}::GFP was able to afford maintenance of ploidy better than wildtype RCC1, as evidenced by the maintenance of a large population of polyploid cells in sorted RCC1_{KRR}::GFP HeLa cell lines. Our lab previously reported that upon partial depletion of RCC1 in *C. elegans*, chromosomes were significantly shorter (Ladouceur et al., 2015). We observed that in HeLa cells also, chromosome size decreased in response to both RCC1 depletion and disruption of the DNA-binding ability of RCC1. This contradicts our hypothesis predicting that the scaling relationship between RCC1 and DNA provides a mechanism to ensure effective chromatin condensation. In response to this confounding data we hypothesize that decreased chromosome size in RCC1_{KRR}::GFP cells explains their ability to maintain effective rounds of mitosis despite the abnormally large amount of DNA, as smaller chromosomes are likely more efficiently and accurately segregated in polyploid cells. This could explain how highly polyploid HeLa cells that contain the RCC1_{KRR}::GFP maintain the polyploidy through more rounds of division than do HeLa cells containing the wildtype RCC1.

An interesting observation that we made that would seem opposed to this theory is that metaphase plate lengths increase in response to RCC1 depletion. However, we believe that this is due to irregular spindle assembly. RCC1_{KRR}::GFP likely creates a mislocalized RanGTP gradient that is not located at the metaphase plate. Therefore, growing microtubules will not be accurately directed to chromosomes, creating a larger metaphase plate as observed.
However, our data still poses many questions about how exactly RCC1 is regulating chromatin condensation. The disruption of DNA binding facilitates the decrease in chromosome size, but RCC1 has a role in nuclear transport, which could also explain how it affects condensation. Potentially, RCC1 could regulate the import of other factors into the nucleus, such as epigenetic modifiers or other known components of the condensation machinery. Further study of chromosome structure using super resolution microscopy could elucidate how exactly RCC1 regulates chromosome size. Further work needs to be down to determine how our RCC1\textsuperscript{KRR}::GFP protein disrupts this regulation. Potentially, the residues that we have mutated could have a wider range of effects on RCC1’s function than just disrupting DNA binding. It is easy to imagine that non-DNA-bound RCC1 cannot properly facilitate the import of critical condensation factors, due to loss of Ran-GEF ability; however, this is currently unknown. We believe that our RCC1\textsuperscript{KRR}::GFP is a separation of function mutation, and that nuclear import is still occurring at near-wildtype efficiency, which is supported by the data presented here. Further study of our RCC1\textsuperscript{KRR} mutant will be vital to understanding RCC1’s exact role in chromatin condensation and also chromosome size scaling.

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**FIGURES**

**Figure 1.** RCC1 levels scale with DNA content in control and aneuploid HeLa cells. (A) DNA content (Hoechst Indo-1) is plotted as a function of cell size (FSC-A). (B) A histogram of DNA content (Hoechst Indo-1) in a population of HeLa cells. (C-D) Relative RCC1 and DAPI intensity are plotted. Representative fluorescent images are shown below each graph.
Figure 2. Non-DNA-bound RCC1 facilitates maintenance of polyploid cells through more rounds of division than control cells. (Top Panel) Sorted populations of control (blue box) and polyploid (red box) cells from HeLa cells (A) and HeLa cells stably transfected with RCC1::GFP (B) or RCC1\textsuperscript{KRR}::GFP (C). (Bottom Panel) Populations were maintained in culture for one week and the DNA content profiles were measured using flow cytometry.

Figure 3. RCC1\textsuperscript{KRR}::GFP (K232A-R234A-R237A) does not locate to the metaphase plate. (A) A schematic of the mutated residues of RCC1. (B) Live-cell fluorescent imaging of RCC1\textsuperscript{KRR}::GFP construct in HeLa cells.
Figure 4. Chromosome area decreases upon RCC1 depletion and in HeLa cells transfected with \textit{RCC1}\textsuperscript{KRR::GFP}. (A) Chromosome area (chromosome length x chromosome width) is plotted based on control treatment (blue dots) and RCC1-depletion (red dots). (B) A representative structural illumination microscopy (SIM) image of a metaphase chromosome spread. (C) Western blot showing the depletion of RCC1 protein levels upon treatment with two separate (or combined) shRNA constructs directed to the 3' UTR of the human RCC1 gene. Asterisks indicate treatment with MNase. (D) Graphical representation of RCC1 depletion as graphed by the ratio of RCC1 intensity to H2B (control) intensity.

Figure 5. Metaphase plate lengths increase upon depletion of endogenous RCC1 with shRNA. The length of the longest axis of the metaphase plate was measured by fitting an ellipse to a segmented image of DAPI. Each cell line was measured with (red dots) and without (blue dots) RCC1 depletion. Error bars show 95% confidence interval.
REFERENCES


cDNA of the human cell cycle gene (RCC1) involved in the regulation of onset of chromosome condensation. *Genes & Development, 1*(6), 585 - 593.
