The hierarchical structure of the perichromosomal layer comprises Ki67, ribosomal RNAs, and nucleolar proteins

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Abstract

The perichromosomal layer (PCL) is a structure that surrounds mitotic chromosomes, found in both animal and plant cells. It comprises various proteins and RNAs, mainly derived from the nucleolus. Several functions for the PCL have been suggested; however, the mechanism of PCL organization during mitosis remains unclear. The localization of several nucleolar proteins to the PCL is reportedly dependent on pre-ribosomal RNAs and the marker of proliferation, Ki67, which is a major PCL-localized protein. Here we demonstrate that, although the removal of pre-ribosomal RNAs from the PCL causes PCL delocalization of several nucleolar proteins, it does not affect the localization of Ki67. Conversely, Ki67 depletion results in the dissociation of both pre-ribosomal RNAs and nucleolar proteins from the PCL, which indicates that Ki67 is required for the PCL accumulation of pre-ribosomal RNAs, to which several nucleolar proteins are associated. Given these findings, we propose a model for PCL organization that comprises three essential layers: the scaffolding protein Ki67, pre-ribosomal RNAs for linkage, and outer nucleolar proteins.

Keywords: perichromosomal layer; nucleolus; rRNA; Ki67

Abbreviations: PCL, perichromosomal layer; rRNA, ribosomal RNA; pre-rRNA, precursor ribosomal RNA; NCL, nucleolin; Act D, actinomycin D; siRNA, small interfering RNA; Ki67, marker of proliferation Ki-67; TIF-IA, RRN3 homolog, polymerase I transcription factor; PFA, paraformaldehyde; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindole; EU, 5-ethynyl uridine; NPM, nucleophosmin; α-ama, α-amanitin; siTIF-IA, siRNA for TIF-IA

1. Introduction

During the cell cycle, chromatin undergoes dynamic structural changes to ensure the faithful transmission of genomic material from mother to daughter cells. During mitosis, entangled interphase chromatin fibers are resolved into rod-shaped structures known as mitotic chromosomes, which are comprised of four structural/functional domains: centromeres, telomeres, arm chromatin, and the periphery [1]. A recent study revealed that the periphery constitutes more than 33% of the protein mass of mitotic chromosomes [2], but, unlike for the other three domains, little is known regarding its composition and function. The less well-characterized peripheral structure, called the perichromosomal layer (PCL), persists from prophase to telophase in various animal and plant cells [3]. Although the exact role of the PCL is yet to be elucidated, several functions have been proposed: it may act as a barrier between mitotic chromosomes and the cytoplasm, provide a binding site for multiple proteins, or help ensure the equal distribution of processing components to daughter cells [4].

The PCL consists of various proteins and RNAs, most of which are derived from the nucleolus [5]. At the onset of mitosis, the nucleolus is disassembled by CDK1

activation, which suppresses ribosomal RNA (rRNA) transcription [6-8]. Subsequently, several nucleolar proteins and RNAs associate with the chromosome surface to establish the PCL. A recent report demonstrated that newly synthesized precursor rRNAs (pre-rRNAs) associate with the PCL and act as binding sites for several pre-rRNA processing proteins. Pre-rRNA processing proteins, such as fibrillarin, nucleolin (NCL), and NOP58, are delocalized from the chromosome fraction after treatment with actinomycin D (Act D), an inhibitor of rRNA transcription [9]. In addition, a recent small interfering RNA (siRNA) study revealed that the marker of proliferation, Ki67, a protein that localizes to the nucleolus during interphase and binds to the chromosome surface during mitosis [10], is also required for the association of several nucleolar proteins with the PCL; siRNA depletion of Ki67 causes dissociation of the nucleolar proteins from the PCL [11,12]. In fact, the thickness of the Ki67-depleted metaphase chromosomes is approximately half that of the normal metaphase chromosomes [2].

Thus, the PCL localization of several nucleolar proteins is dependent on Ki67 and/or pre-rRNAs; however, it is unclear how pre-rRNAs and Ki67 affect the association of nucleolar proteins with the PCL. In this study, we investigated the roles of Ki67 and

RNAs, particularly pre-rRNAs, in the regulation of the association of nucleolar proteins with the PCL; we observed that the removal of pre-rRNAs from the PCL led to the dissociation of several nucleolar proteins, without affecting Ki67 localization. This suggests that pre-rRNAs constitute binding sites for nucleolar proteins on the PCL. In addition, the depletion of Ki67 caused delocalization of both nucleolar proteins and pre-rRNAs from the PCL. Taken together, these findings suggest a model for the PCL structure, in which Ki67 functions as a binding scaffold for pre-rRNAs, which subsequently act as an intermediate glue to anchor other nucleolar proteins to the PCL.

2. Materials and methods

2.1. Cell culture

HeLa cells were grown at 37°C in Dulbecco's modified eagle medium with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin.

2.2. Antibodies

The following antibodies were used in the study: rabbit polyclonal antibodies to

Ki67 (Santa Cruz Biotechnology); goat polyclonal antibodies to RRN3 homolog, polymerase I transcription factor (TIF-IA; Santa Cruz Biotechnology); and mouse monoclonal antibodies to nucleophosmin (NPM; Thermo Fisher Scientific), NCL (MBL), and UBF (Santa Cruz Biotechnology) were used. The rabbit polyclonal anti-MYBBP1A was prepared as previously described [13].

2.3. siRNA transfection

Cells were transfected with siRNAs using Lipofectamine® RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's protocol. The siRNA constructs were purchased from Thermo Fisher Scientific. The sequences of the siRNA duplexes were as follows: TIF-IA, 5′-CGACACCGUGGUUUCUCAUGCCAAU-3′ and Ki67, 5′-GCAUUUAAGCAACCUGCAA-3′. Stealth RNAi™ siRNA Luciferase Reporter Control (Thermo Fisher Scientific) was used as the negative control.

2.4. Immunofluorescence

Cells were grown on a poly-L-lysine-coated coverslip. For immunostaining, the

cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4) for 15 min and permeabilized with 0.1% Triton X-100 in PBS for 3 min. For immunostaining using anti-NPM, cells were incubated for 2 min in 0.1% Triton X-100 in PBS before fixation.

Fixed cells were blocked with 3% bovine serum albumin (BSA)/PBS for 1 h at room temperature. Subsequently, the cells were incubated with the indicated primary antibodies diluted in 3% BSA/PBS for 1 h in a humidity box, stained with Alexa Fluor®-488 or 594 conjugated secondary antibodies for 1 h, counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min, and mounted with VECTASHIELD (Vector Laboratories). Images were captured using a Biorevo (Keyence) or Axio Observer.Z1 (Carl Zeiss) microscope, and processed using ImageJ software (National Institutes of Health).

Quantification of signal intensities of proteins in the PCL was performed using ImageJ software using images obtained at identical illumination settings. The average pixel intensity of NPM, MYBBPIA, or Ki67 within chromosomal region was calculated, and the average pixel intensity within cytosol (outside region of DAPI in

cells) was used as background. After background subtraction, the relative ratio of signal intensity for each protein was normalized to the control cells. We repeated three independent experiments.

2.5. Nascent RNA staining

Cells were treated with 1 mM 5-ethynyl uridine (EU) and cultured for 4 h. EU-treated cells were pre-fixed with 4% PFA/PBS for 5 s, permeabilized with warmed PBS containing 0.1% Triton X-100 for 5 min, then fixed with 4% PFA/PBS for 15 min. EU incorporation was detected using the Click-iT™ Cell Reaction Buffer Kit (Thermo Fisher Scientific), and Alexa Fluor® 488 dye was introduced following the manufacturer's protocol.

2.5. Chromosome preparation and RNase A treatment

Mitotic chromosomes for RNase A treatment were prepared as described previously [9]. Mitotic HeLa cells were collected by 75 nM nocodazole treatment for 12–16 h, followed by mitotic shake-off. Mitotic cells were suspended in 0.5× buffer A (15 mM

Tris-HCl [pH 7.4], 0.2 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 80 mM KCl, protease inhibitor cocktail [Nacalai Tesque], and 1 mM DTT) and incubated at room temperature for 10 min. Swollen cells were centrifuged and resuspended in chilled buffer A containing 0.1% NP40 and disrupted by passage through a 23-gauge needle. The suspension was centrifuged on a 0.25 M sucrose cushion in buffer A containing 0.1% NP40 at 250 *g* for 5 min to discard the contaminating cells and nuclei. The supernatant was centrifuged again on a 0.5 M sucrose cushion in buffer A containing 0.1% NP40 at 2000 *g* for 20 min to obtain the pure chromosomal fraction. The chromosomal fraction was treated with buffer A containing 100 μg/ml of BSA or RNase A for 10 min at room temperature and washed once with buffer A.

We fractionated the HeLa cells treated with RNA polymerase inhibitors or siRNAs. Mitotic HeLa cells were collected by nocodazole block and mitotic shake-off. Mitotic cells were lysed in fractionation buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% [v/v] glycerol, 0.25% Triton X-100, 1 mM DTT, phosphatase inhibitor cocktail [Nacalai Tesque], and protease inhibitor cocktail) and incubated on ice for 10 min. Chromosome-enriched fractions were obtained as pellets

after centrifugation at 1,500 *g* for 5 min at 4ºC, and washed three times with the same buffer.

3. Results

3.1. Pre-rRNAs are required for the PCL localization of several nucleolar proteins, but not Ki67

To determine if RNAs were required for the PCL localization of nucleolar proteins, we treated mitotic HeLa cells with PBS containing 1 mg/ml BSA (control) or RNase A before fixation, followed by immunofluorescence staining. The nucleolar proteins NPM and MYBBP1A, which were localized to the PCL in control cells, were diffused throughout the cytoplasm following RNase A treatment (Fig. 1A). This suggests that RNAs are essential for the PCL localization of these proteins. In contrast, Ki67 remained in the PCL following RNase A treatment (Fig. 1B), which indicates that RNAs are dispensable for the PCL localization of Ki67. We quantified signal intensities of the above mentioned proteins in the PCL (Fig. 1C). Signal intensities of NPM and MYBBPIA in the PCL were decreased about 80% but that of Ki67 was hardly affected

following RNase A treatment (Fig. 1C). To further analyze the effect of RNase A treatment on the chromosome association of these proteins, we isolated mitotic chromosomes, and then we treated them with BSA (control) or RNase A (Fig. 1D). Immunoblotting showed that RNase A treatment reduced the amount of NCL, NPM, and MYBBP1A in the chromosomal fraction, whereas it scarcely affected Ki67 localization (Fig. 1D). This was consistent with the data from the immunofluorescence staining.

Previous reports have revealed that pre-rRNAs localize to the PCL during mitosis and are required for the PCL localization of several nucleolar proteins [4,9]. We treated HeLa cells with specific inhibitors for RNA polymerases and tested the PCL localization of nucleolar proteins. Simultaneously, we visualized intracellular RNAs by culturing the cells in medium containing EU, a compound that is incorporated into newly synthesized RNAs. In control cells, we observed intense EU signals around mitotic chromosomes, which indicated the accumulation of nascent RNAs to the PCL during mitosis (Fig. 2A). Treatment with a low dose of Act D, which specifically inhibits RNA polymerase I, efficiently diminished the EU signals from the PCL. On the

other hand, the RNA polymerase II inhibitor, α-amanitin (α-ama), did not affect the EU signals (Fig. 2A). The PCL localization of Ki67 was not affected (Fig. 2A), even when the EU signals disappeared from the PCL after Act D treatment. In contrast, the PCL localization of MYBBP1A and NPM was compromised by Act D treatment, but not by α-ama treatment (Fig. 2B and C). Quantification of the signal intensity in the PCL supported above results: The signal intensity of Ki67 in the PCL was little changed by treatment with either Act D or α-ama, whereas those of NPM and MYBBBP1A were reduced by Act D treatment, but not α-ama treatment (Fig. 2D). We further examined the effects of Act D and α-ama on the chromosomal localization of nucleolar proteins by chromosome fractionation. Consistent with the immunofluorescence staining results, the amount of Ki67 in the chromosomal fraction was scarcely changed by treatment with either Act D or α-ama, whereas the amounts of NCL, NPM, and MYBBBP1A were reduced by Act D treatment, but not α-ama treatment (Fig. 2E).

To more specifically inhibit rRNA transcription, we treated HeLa cells with an siRNA for TIF-IA (siTIF-IA), a basal initiation factor for RNA polymerase I [14]. We found, by immunofluorescence staining, that signals for NPM and MYBBP1A, but not

for Ki67, were abrogated in the PCLs prepared from HeLa cells treated with siTIF-IA (Fig. 2F and G).

Taken together, these findings indicate that PCL-localized pre-rRNAs are required for the localization of several nucleolar proteins to the PCL, but are not essential for Ki67 localization.

3.2. Ki67 is indispensable for the PCL localization of pre-rRNAs and nucleolar proteins

Immunofluorescence staining analysis indicated that siRNA-mediated Ki67 depletion prevented the localization of NPM and MYBBP1A to the PCL (Fig. 3A–C), which is consistent with previous reports that Ki67 depletion abrogates the localization of nucleolar proteins, such as NCL, NIFK, PES1, and cPERPs, to the PCL [2,11,12]. Next, we performed cell fractionations to examine the levels of nucleolar proteins on the mitotic chromosomes after the depletion of Ki67 or TIF-IA. We found, by immunoblotting, that the depletion of Ki67 or TIF-IA reduced the amounts of NCL, NPM, and MYBBP1A in the chromosomal fraction (Fig. 3D), which supports the data

from the immunofluorescence staining.

However, our experimental results suggested that Ki67 is unlikely to function as a direct binding site for nucleolar proteins in the PCL because both RNase A and Act D treatments blocked the PCL localization of NPM and MYBBP1A, but not Ki67 (Figs. 1 and 2A–E). In addition, the depletion of TIF-IA, as well as Ki67 depletion, reduced the levels of nucleolar proteins in the mitotic chromosomes (Figs. 2F, G and 3A–D). Therefore, we speculate that Ki67 depletion may affect rRNA localization, which in turn influences the PCL localization of these proteins.

To test this hypothesis, we performed EU labeling to visualize nascent RNAs in the context of Ki67 depletion. During interphase, nascent RNAs in the nucleolus largely co-localized with Ki67, the depletion of which did not appear to affect EU signals in the nucleolus (Fig. 3E), which indicates that Ki67 is not essential for rRNA transcription and localization during interphase. In contrast, in most mitotic cells, Ki67 depletion caused the translocation of EU signals from the PCL and caused aggregation in nearly 80% of cells (Fig. 3F). Thus, Ki67 is required for the PCL localization of pre-rRNAs.

4. Discussion

In this study, we examined the role of Ki67 and pre-rRNAs in the formation of the PCL. Our study provides a model for the PCL: it comprises the scaffolding protein Ki67, intermediate pre-rRNAs, and other nucleolar proteins (Fig. 4). This model is supported by our findings and previous reports. First, the PCL localization of several nucleolar proteins was compromised when total RNAs were removed by RNase A treatment or when rRNA transcription was inhibited by Act D treatment or the depletion of TIF-IA, without affecting Ki67 localization. Thus, it is likely that pre-rRNAs, but not Ki67, function as direct binding sites for nucleolar proteins in the PCL. Second, Ki67 depletion caused the dissociation of pre-rRNAs from the PCL in most mitotic cells, and led to aggregation in nearly 80% of mitotic cells. This localization of pre-rRNAs in the Ki67-depleted cells is reminiscent of reports in which Ki67 depletion induced cytoplasmic aggregation of nucleolar proteins such as NCL and NIFK [11]. Third, given that Ki67 localizes to the PCL prior to other nucleolar proteins at prophase [4], it may be required for the PCL localization of rRNAs and nucleolar proteins. Taken together, these data suggest that the PCL comprises a scaffolding protein Ki67, pre-rRNAs that

act as an intermediate glue, and other nucleolar proteins that are anchored to pre-rRNAs. However, we cannot exclude the possibility that several proteins directly bind to Ki67 in the PCL because Ki67 reportedly interacts with proteins like Hklp2, NIFK, and protein phosphatase 1γ [11,15,16].

Ki67 is a huge protein comprising approximately 3,000 amino acids; it contains several conserved structural domains. At the C-terminus, the LR domain, which is comprised of leucine and arginine pairs with irregular spacing, is required for binding to the mitotic chromosome surface [10]. However, no RNA binding motif has been reported in Ki67. Thus, the mechanism by which Ki67 interacts with pre-rRNAs and controls their localization during mitosis is a subject for future investigation. A recent study revealed that Ki67 functions as a biological surfactant to disperse mitotic chromosomes [17]. However, the physiological relevance of other PCL-associated nucleolar proteins in mitosis remains unknown. It is possible that they coordinately function with Ki67 as surfactants required for the individualization of mitotic chromosomes. Alternatively, each PCL-associated nucleolar protein may have a specific mitotic function. In the future, analyzing the roles of PCL proteins may provide new

insights into the structure and dynamics of mitotic chromosomes.

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6. References

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Figure Legends

Figure 1. RNase A treatment causes delocalization of several nucleolar proteins without affecting Ki67 localization.

(A) Dissociation of nucleolar proteins from the PCL by RNase A treatment. HeLa cells were treated with PBS containing 1 mg/ml BSA (control) or RNase A (RNase A) for 2 min before fixation. Cells were stained with antibodies to NPM (green) and MYBBP1A (red), and DNA was counterstained with DAPI (blue). Scale bar, 5 μm.

(B) Stable PCL localization of Ki67 after RNase A treatment. HeLa cells were treated as described in Fig. 1A, and cells were stained with antibodies for NPM (green) and Ki67 (red). DNA was counterstained with DAPI (blue). Scale bar, 5 μm.

(C) Relative signal intensity of NPM, MYBBP1A, and Ki67 in the PCL following RNase A treatment (RNase A), normalized to the untreated cells (Cont.). Values are shown as mean ± s.d. Three independent experiments.

(D) Immunoblot analysis of the chromosomal fraction. Mitotic chromosomes prepared

from HeLa cells were incubated with buffer containing 100 μg/ml BSA (control) or RNase A (RNase A) for 10 min, and the treated chromosomal fractions were examined by immunoblotting using the indicated antibodies.

Figure 2. Inhibition of RNA polymerase I delocalizes several PCL proteins without affecting Ki67 localization.

(A) Stable PCL localization of Ki67 after Act D treatment. Cells were treated with 1 mM EU along with 2 μg/mL α-ama or 5 nM Act D. After 4 h, cells were treated with Click-iT™ reagents (EU; green) to visualize nascent RNAs and stained with an antibody to Ki67 (red). DNA was counterstained with DAPI (blue). Scale bar, 5 μm.

(B and C) Dissociation of nucleolar proteins from the PCL by Act D treatment. HeLa cells were treated with α-ama or Act D for 4 h and stained with antibodies to MYBBP1A (B; red), anti-UBF (B; green), and NPM (C; green), and DNA was counterstained with DAPI (blue). UBF, which directly binds to the rDNA promoter, is a negative control, as its localization is not affected by the inhibitors. Scale bar, 5 μm.

(D) Relative signal intensity of NPM, MYBBP1A, and Ki67 in the PCL following α-

ama or Act D treatment, normalized to the untreated cells (Cont.). Values are shown as mean ± s.d. Three independent experiments.

(E) Reduction of nucleolar proteins in the mitotic chromosome fraction by Act D treatment. HeLa cells were treated with 2 μg/ml α-ama or 5 nM Act D for 12 h; mitosis was synchronized by the addition of 75 nM nocodazole for another 12 h. Whole cell extracts (Whole) or chromosome fractions (Chr.) were examined by immunoblotting using the indicated antibodies. HSP90 is a negative control that is not present in the chromosomal fraction. Histone H3 is a positive control for chromosomal proteins.

(F) Dissociation of nucleolar proteins from the PCL upon treatment with siTIF-IA. HeLa cells were treated with siRNA for luciferase (siCont.) or siTIF-IA for 48 h and stained with anti-NPM, anti-MYBBP1A, or anti-Ki67 antibodies. Scale bar, 5 μm.

(G) Relative signal intensity of NPM, MYBBP1A, and Ki67 in the PCL following the treatment with siTIF-IA, normalized to siCont. Values are shown as mean ± s.d. Three independent experiments.

Figure 3. Ki67 depletion causes delocalization of pre-rRNAs and nucleolar proteins

from the PCL.

(A–C) Dissociation of nucleolar proteins from the PCL by Ki67 depletion. HeLa cells were transfected with siRNA for Ki67 and cultured for 48 h. The cells were then fixed and stained with antibodies to NPM (A; green), Ki67 (A; red), MYBBP1A (B; red), and UBF (B; green). DNA was counterstained with DAPI (blue). UBF, which directly binds to rDNA, is a negative control, as its localization is not changed by Ki67 depletion. Scale bar, 5 μm. (C) Relative signal intensity of NPM and MYBBP1A in the PCL following siKi67 treatment, normalized to siCont. Values are shown as mean ± s.d. Three independent experiments.

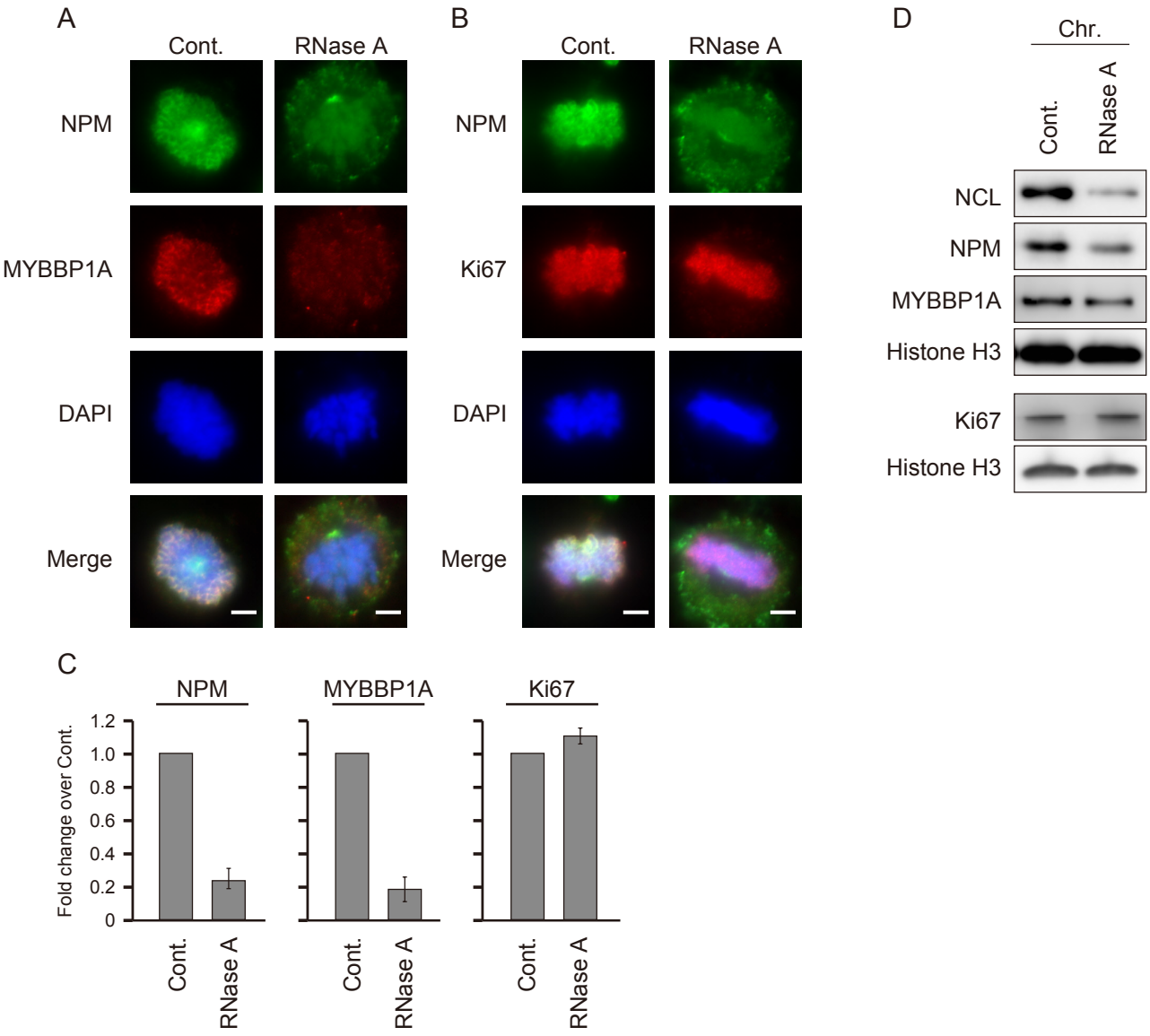
(D) Reduction of nucleolar proteins in the mitotic chromosome fraction after depletion of Ki67 or TIF-IA. HeLa cells were treated with siRNA for Ki67 or TIF-IA for 48 h, and subsequently synchronized in mitosis with 75 nM nocodazole treatment for 12 h. Whole cell extracts (Whole) or chromosomes fractions (Chr.) were examined by immunoblotting using the indicated antibodies.

(E, F) Delocalization of pre-rRNAs from the PCL by Ki67 depletion. HeLa cells were transfected with Ki67 siRNA, cultured for 48 h, then further cultured in 1 mM EU for 4

h. Cells were treated with Click-iT™ reagents (EU; green), to visualize nascent RNAs, as well as antibodies to Ki67 (red). (E) Interphase cell and (F) mitotic cell images. Mitotic cells were divided into three classes based on nascent RNA localization (E; right graph): nascent RNAs present in the PCL, those forming aggregates (Aggregate), or those absent from the PCL (Absence). More than 100 cells were counted from three independent experiments. Scale bar, 5 μm.

Figure 4. Model for the PCL structure

The PCL comprises three layers: the inner Ki67 scaffold layer, the intermediate pre-rRNA linkage layer, and the outer layer of various nucleolar proteins.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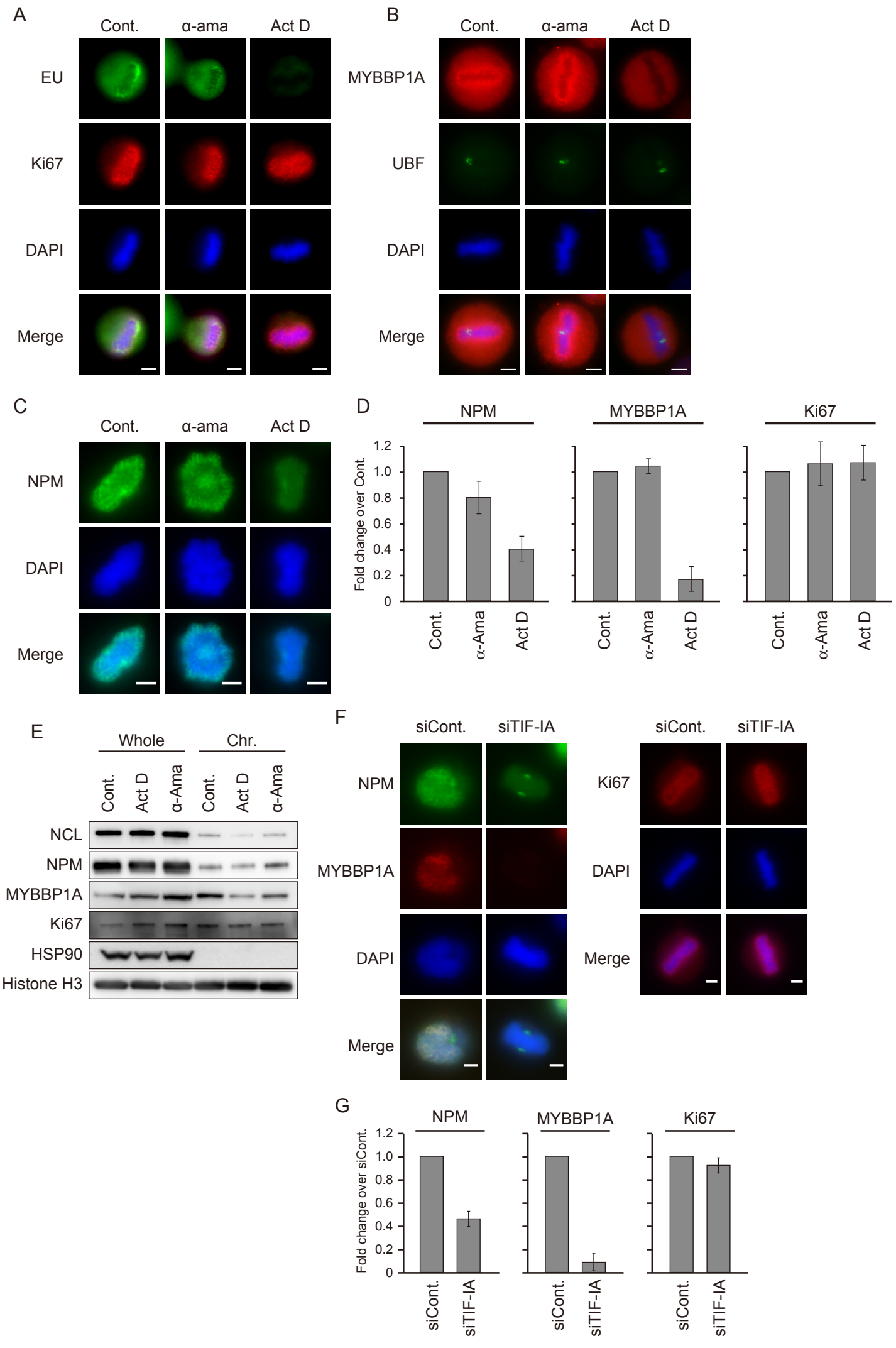
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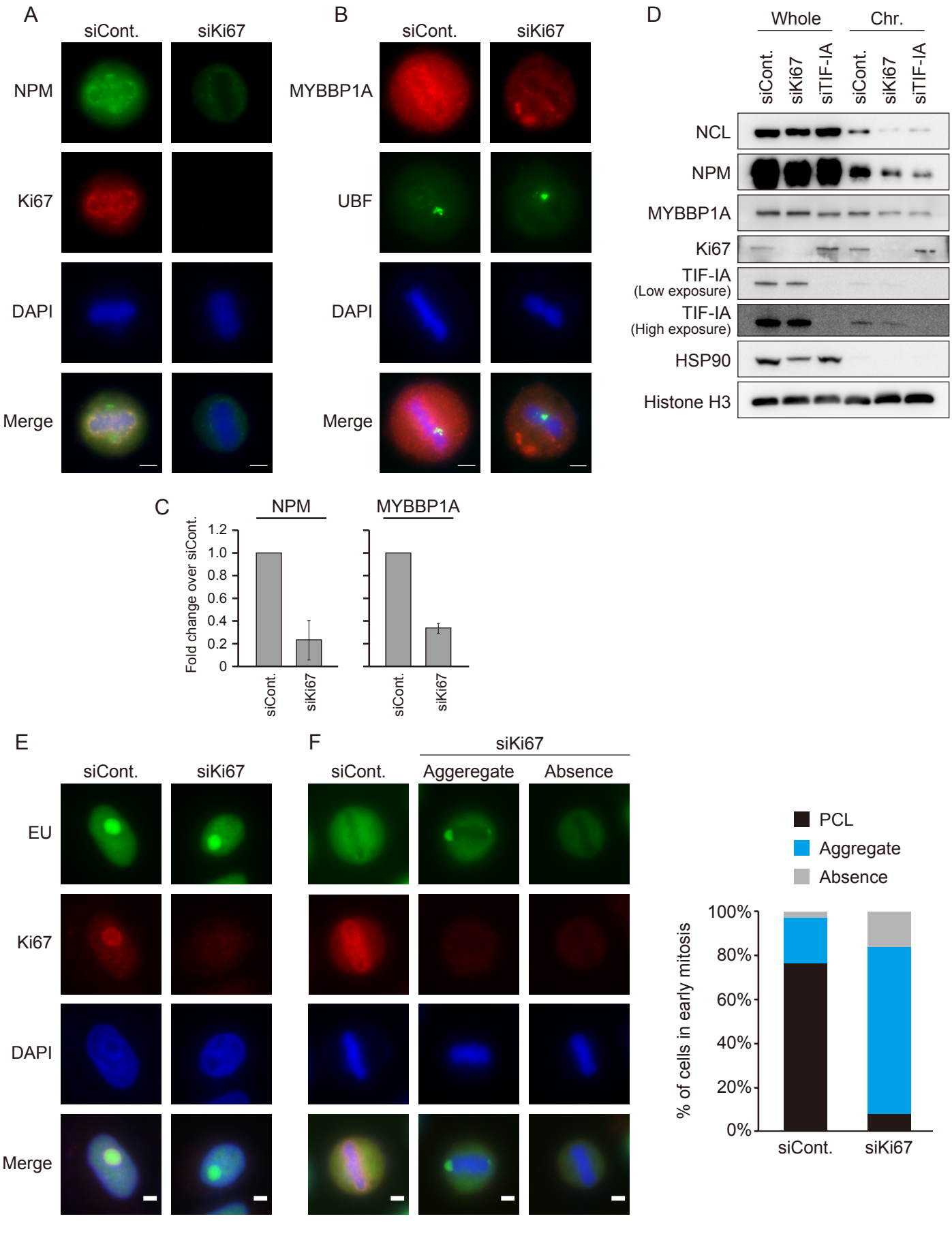
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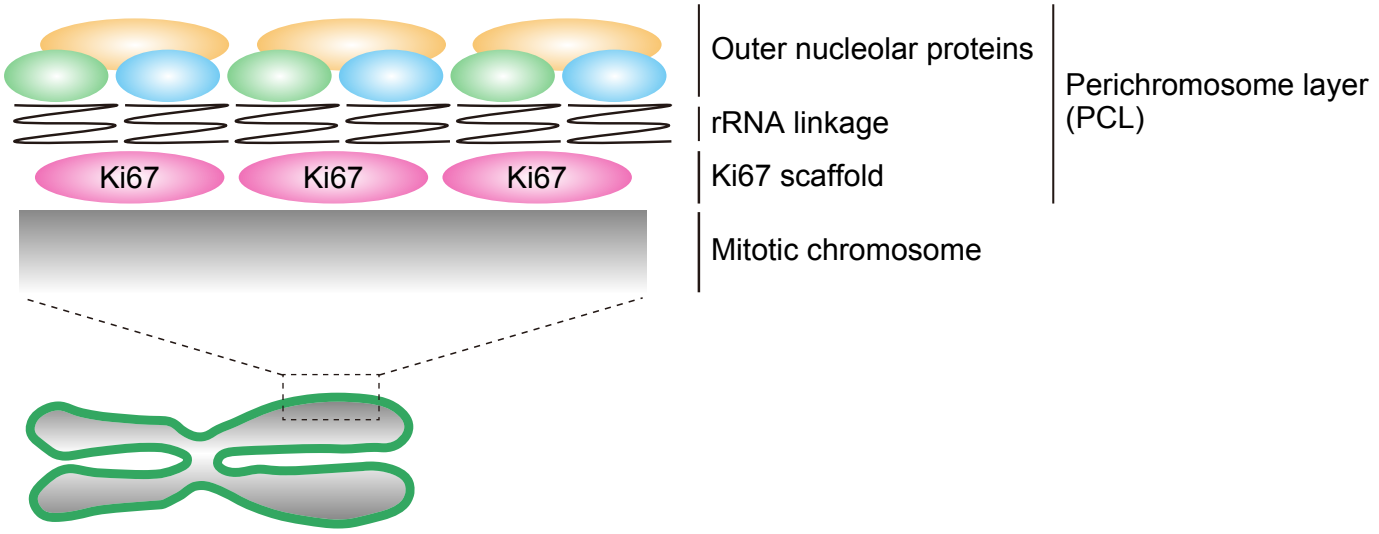
(High exposure)
HSP90
Histone H3
C
NPM
MYBBP1A
Fold change over siCont.
1.2
1.0
0.8
0.6
0.4
0.2
0
siCont.
siKi67
siCont.
siKi67
E
siCont.
siKi67
EU
Ki67
DAPI
Merge
F
siKi67
siCont.
Aggeregate
Absence
PCL
Aggregate
Absence
% of cells in early mitosis
100%
80%
60%
40%
20%
0%
siCont.
siKi67


Ki67
Ki67
Ki67
Outer nucleolar proteins
rRNA linkage
Ki67 scaffold
Mitotic chromosome
Perichromosome layer (PCL)