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Molecular Behavior of CENP-A and PCNA throughout the Cell Cycle in Living Human HT-1080 Cells

Chika Nakagawa[1] Kaori Senda-Murata[2], Ai Kawakita-Yamaguchi[2], Takashi Fukada[1], and Kenji Sugimoto[1,2]*

[1]*Laboratory of Applied Molecular Biology, Division of Bioscience and Informatics, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka, 599-8531 Japan*
[2]*Live Cell Imaging Institute, Osaka Prefecture University, Osaka, 599-8531 Japan*

Abstract

Centromere protein A (CENP-A) is a centromere-specific histone H3 variant present in prekinetochores in nuclei and kinetochores of mitotic chromosomes. The proliferating cell nuclear antigen (PCNA) forms replication foci in S phase but diffuses in nuclei except for nucleoli in other phases. Here to elucidate the molecular behavior of CENP-A and PCNA throughout the cell cycle, we performed a prolong time-lapse imaging of HT-1080 cell line stably expressing mKO-CENP-A and EGFP-PCNA with a highly sensitive EM-CCD camera. We followed the mKO-CENP-A dots in interphase nuclei from G1 through to G2 phase, monitoring the cell cycle progression by the appearance of replication foci of EGFP-PCNA in S phase.

Keywords: Centromere protein A (CENP-A); Proliferating cell nuclear antigen (PCNA); Replication focus; Cell cycle; Live-cell imaging

Introduction

Centromere is the chromosomal region to which spindle microtubules attach in mitosis. Centromere protein A (CENP-A), a centromere-specific histone H3 variant [1], was originally identified by anti-centromere autoantibodies [2]. Human CENP-A is assembled into prekinetochores in G1 phase, and is stably maintained through multiple cell divisions [3]. We previously reported the relative position of three centromere auto antigens, CENP-A, CENP-B, and CENP-C, in human MDA435 cells in interphase nuclei and metaphase chromosomes [4]. We then characterized the molecular behavior of CENP-A and heterochromatin protein 1α (HP1α) during mitosis in living MDA435 cells [5]. Recently, Lidsky et al. reported the dynamics of Cid/CENP-A, CENP-C, and Cal 1 in *Drosophila* S2R+ cells, and presented the live-cell images stably expressing the Cid-EGFP and mCherry-PCNA through the cell cycle [6]. However, the localization of mammalian CENP-A throughout the cell cycle in living cells is unclear.

Proliferating cell nuclear antigen (PCNA) is one of the components of the replication machinery that is conserved from yeast to mammals. The localization of PCNA changes in S phase according to the progression of DNA replication [7]. Thus, PCNA has been used as an S phase marker in living cells [8, 9]. Chagin et al. also reported the progression of DNA replication in living HeLa cells stably expressing GFP-PCNA [10]. Here to elucidate the molecular behavior of CENP-A and PCNA throughout the cell cycle, we performed time-lapse imaging of human HT-1080 cells with a modal number of 46 chromosomes [11], stably expressing mKO-CENP-A and EGFP-PCNA.

Materials and Methods

Construction of pmKO-CENP-A, pEGFP-PCNA and pmPlum-histone H3

Plasmid pmKO-CENP-A was described previously [12]. Plasmid pEGFP-PCNA was constructed by inserting the amplified 0.7 kb fragment of PCNA cDNA from pCMV6-XL5-PCNA (OriGene, Rockville, MD, USA) into the XhoI-BamHI site of pEGFP-C1. Plasmid pmPlum-C1 was constructed by inserting the amplified DNA fragment encoding mPlum of pBAD-mPlum (provided by Professor Roger Y. Tsien, UCSD) into the Nhe I-Bgl II site of pEGFP-C1 and then pmPlum-histone H3 was constructed by inserting the 0.4kb fragment encoding histone H3 of pECFP-histone H3 [13] into the BamHI-EcoRI site of pmPlum-C1.

Construction of a stable cell line and live-cell imaging

The human fibrosarcoma HT-1080 cell line expressing mKO-CENP-A, pEGFP-PCNA and mPlum-histone H3 was obtained and cultured in DMEM essentially as described previously [13]. Cells were inoculated into a 35 mm glass bottom dish (Iwaki, Funabashi, Chiba, Japan) and grown in an INUG2-ZILCS stage top incubator (Tokai Hit, Fujinomiya, Shizuoka, Japan).

An Eclipse Ti-S fluorescent microscope (Nikon, Tokyo, Japan) was equipped with PlanApo VC object lens (60x, Nikon), a ProScan III H117N1 XY-axis stage controller (Prior, Cambridge, UK), and a MAC5000 controller with excitation and emission filter wheels and a Z-axis motor (Ludl Electronic Products, Hawthorne, NY, USA). A 100W halogen lamp was used as a light source to obtain the light of an appropriate wavelength for mKO-CENP-A and EGFP-PCNA at 8 and 9 μW, respectively [14]. We used the excitation and emission filters for mKO-CENP-A (543/3 and 572/28 nm, respectively) and used for EGFP-PCNA (480/17 and 520/35 nm, respectively) with a dichroic mirror (Di01-R488/543/594, Semrock, Rochester, NY, USA). Time-lapsed images (15 optical sections with 1 μm distance) at eight points were captured by an ImagEM C9100-13 CCD camera (Hamamatsu Photonics, Hamamatsu, Shizuoka, Japan) exposing for 800 millisecond for mKO-CENP-A and 100 milliseconds for EGFP-PCNA at 3 min intervals, operating a Volocity software (ver. 5.3.3, Improvision, Coventry, UK) [15]. The Z-axis image stacks were converted to maximum-intensity projection. Images were processed with a LuminaVision software for Mac OSX (Mitani Corporation, Fukui, Japan).

Results

Time-lapse analysis of mKO-CENP-A and EGFP-PCNA localization throughout the cell cycle

PCNA has been shown to localize in the nucleus except for the

***Corresponding author:** Kenji Sugimoto, Live Cell Imaging Institute, Osaka Prefecture University, Osaka, 599-8531 Japan, E-mail: sugimoto@bioinfo.osakafu-u.ac.jp

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nucleoli (nucleolar exclusion) in early S phase [7,16]. We performed long-term multipoint time-lapse imaging of the HT-1080 cell line stably expressing mKO-CENP-A and EGFP-PCNA with an extremely sensitive EM-CCD camera [15]. As shown in Figure 1, PCNA was distributed in the cytoplasm in M phase, but the fluorescence of PCNA was weaker in the chromosomal region where CENP-A was localized. After the cytokinesis, PCNA was gradually concentrated into the nucleus (60-120 min). We could recognize the "nucleolar exclusion"

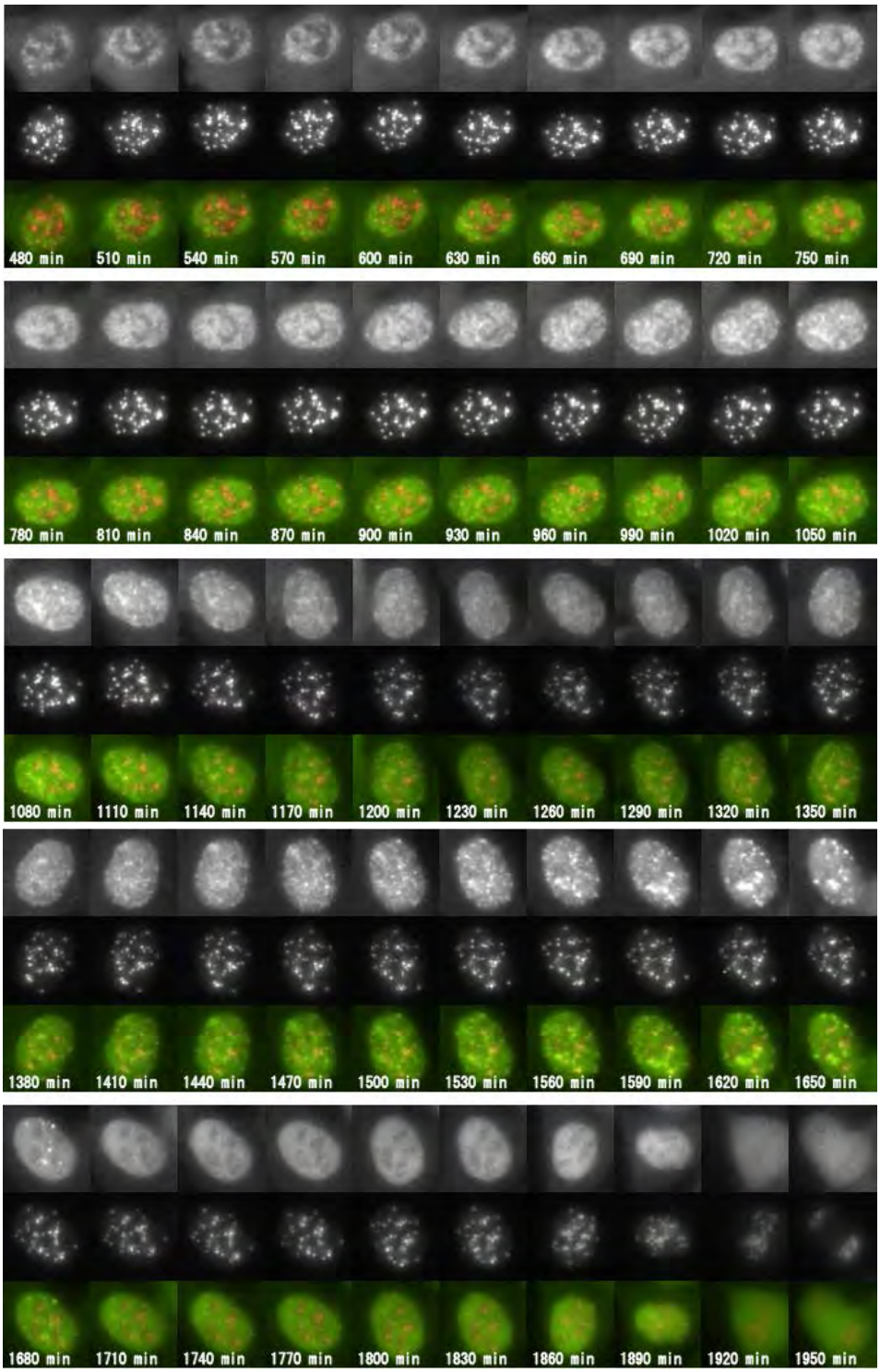

Figure 1: Molecular behavior of mKO-CENP-A and EGFP-PCNA of HT-1080 cells throughout the cell cycle.

Each image of the HT-1080 cell stably expressing EGFP-PCNA (upper) and mKO-CENP-A (middle) and was captured at 3 min intervals but only the representative images were shown here. The merged images of PCNA (green) and CENP-A (orange) are also shown (lower). The elapsed time before or after G1 phase onset (0 min) is indicated at the bottom.

pattern in the subsequent cell images of G1 phase (90-450 min) as well as early S phase (453-990 min).

The S phase onset was identified at 453 min when replication foci of PCNA appeared. Many foci of PCNA were distributed in the nucleus except for the nucleoli (480-720 min), so called "granular pattern" in early S phase [7]. The dark, weak fluorescent areas of PCNA in the nucleus gradually decreased in size, and could hardly be recognized after 1080 min (mid S phase). Large aggregates appeared in the nucleus at 1560-1620 min (late S phase). We could recognize the end of S phase when PCNA foci suddenly disappeared in the nucleoplasm (1710 min). The "nucleolar exclusion" pattern was observed again in G2 phase and the relative positions of these areas were maintained until the nuclear envelope breakdown (1890 min). PCNA was distributed again into the cytoplasm in M phase (1920-1950 min).

In contrast, CENP-A dots were separated to the opposite poles in anaphase (-3 min). After the cytokinesis, CENP-A dots, condensed in the daughter cells, gradually dispersed in the nucleus as the daughter nuclei grew in size (6-30 min, early G1 phase). When we carefully compared the distribution patterns of CENP-A dots in the subsequent images, we noticed that relative positions of CENP-A dots were maintained.

As shown in Figure 2, we focused on seven groups of CENP-A dot(s), enclosed by colored circles, indicated by the letters A-G at 1080 min. Four groups, including A (green), B (magenda), C (green) and D (blue), are located in one half side of the nucleus, while three groups including E (green), F (red) and G (yellow) are located in the other half side of the nucleus. We followed the positions of colored circles in the subsequent cell images in G1 phase (60-450 min), S phase (480-1680 min) and G2 phase

(1710-1860 min). Their relative positions in the nucleus were well maintained until 1860 min, just before chromosome condensation (1890 min). These results indicated that the relative positions of CENP-A dots changed little in interphase nuclei.

Co-localization of mKO-CENP-A and EGFP-PCNA in late S phase

Figure 3 shows the localization of mKO-CENP-A and EGFP-PCNA in late S phase with 12 min intervals (1542-1698 min). As shown within the rectangle, CENP-A dot 1 was localized in the nuclear periphery. Three small replication foci of PCNA, found in the boundary of this CENP-A dot in the rectangle, were changing their shape. When carefully compared with CENP-A dot 1, a replication focus of PCNA appeared near the CENP-A dot at 1614 min. It enlarged, changing its shape from 1626 to 1650 min, and became defined at 1650 min. The focus decreased in size at 1674 min and disappeared at 1686 min. The co-localization of PCNA with CENP-A dot 1 continued for 60 min (1614-1674 min). During this period, another replication focus, without a corresponding CENP-A dot, appeared in the upper nuclear periphery (indicated by alphabet a) at 1590 min, and increased in size, changing its shape between 1614-1650 min. The replication focus decreased in size and disappeared at 1698 min. It stayed for about 1 h.

Comparison of the localization pattern of mKO-CENP-A dots and EGFP-PCNA between daughter cells

Figure 4 shows the localization patterns of CENP-A dots in two daughter cells originating from the same parental cell. The nucleolar exclusion pattern was observed until the nuclear envelope breakdown (-30 min). Then PCNA was distributed in the cytoplasm in M phase. After the cytokinesis, PCNA was gradually concentrated into the nucleus and the nucleolar exclusion pattern appeared in daughter nuclei after 120 min. Replication foci appeared at 435 min in upper daughter cell and at 420 min in the lower daughter cell. Interestingly, the distribution pattern of the presumptive nucleoli was apparently

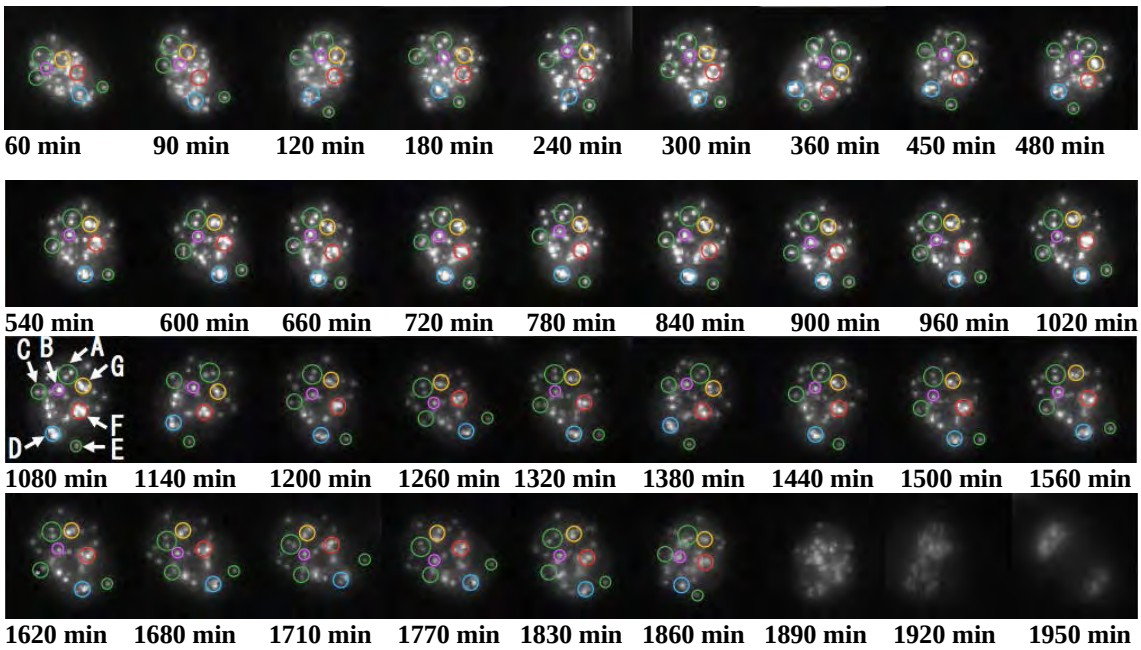

Figure 2: Localization pattern of CENP-A dots was maintained from early G1 phase through to late G2 phase.

Fluorescent images of mKO-CENP-A from late G1 phase through to late G2 phase. Some CENP-A dot(s) were grouped into seven: four marked with yellow, red, orange, and cyan circles located inside the nucleus, while three with green circles are in the nuclear periphery. Each group of CENP-A dot was chased from early G1 phase (60 min) through to late G2 phase (1860 min).

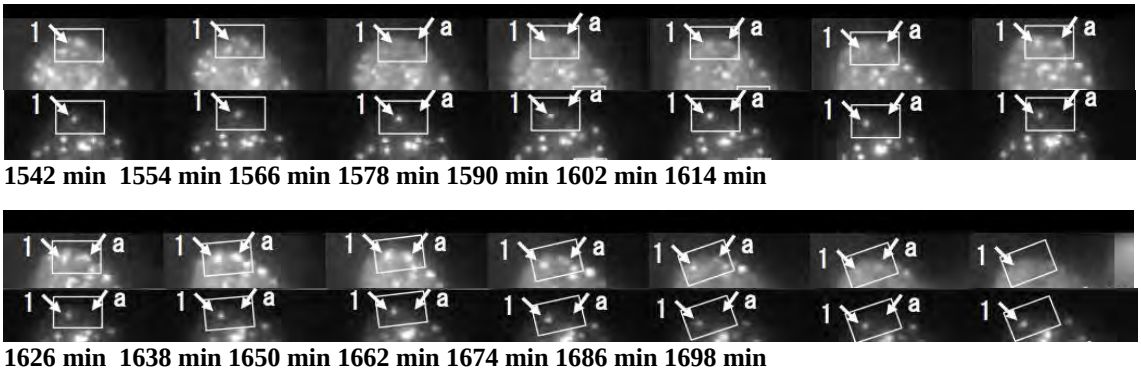

Figure 3: Co-localization of CENP-A dot with replication focus of PCNA in late S phase. Each image of EGFP-PCNA (upper) and mKO-CENP-A (lower) was presented at 12 min intervals. Note that PCNA was apparently co-localized with CENP-A dot 1 from 1614 min to 1650 min.

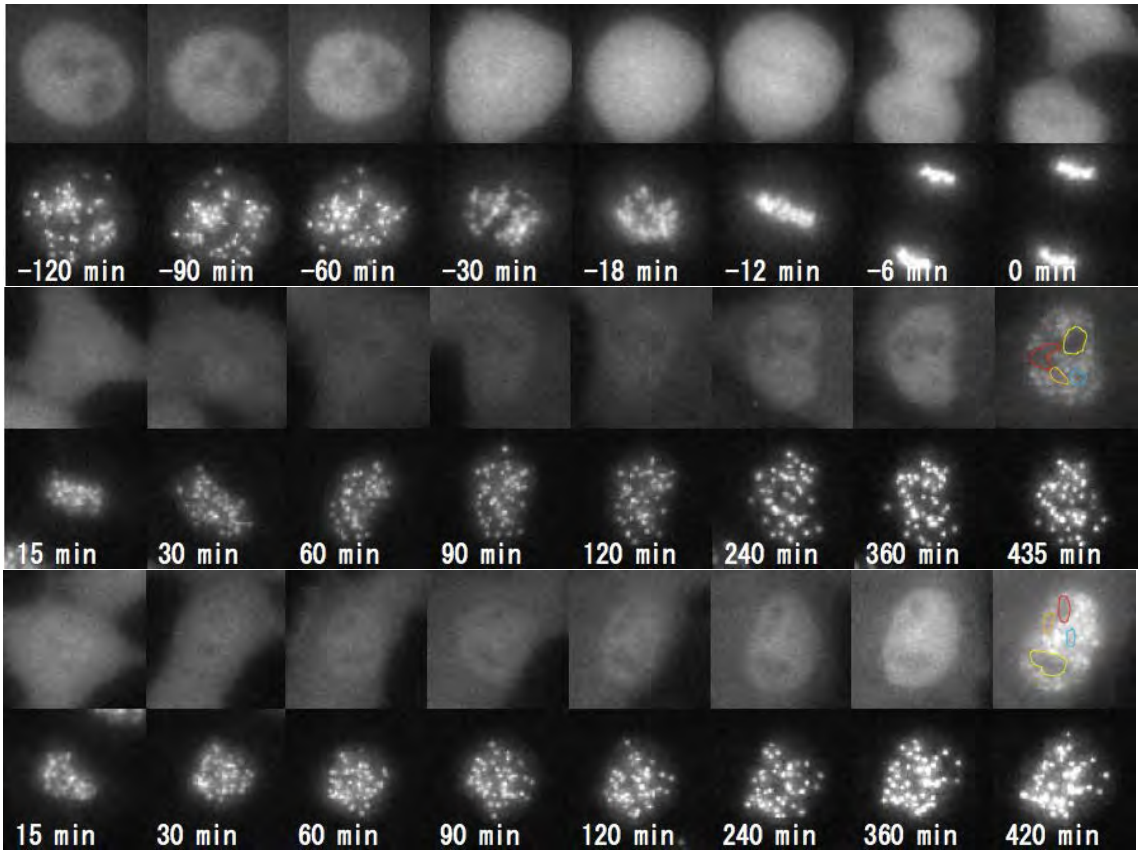

Figure 4: Localization patterns of CENP-A and PCNA are different between two daughter cells.

Each image of EGFP-PCNA (upper) and mKO-CENP-A (lower) of another fluorescent HT-1080 cell was captured from late G2 phase through to the entry into S phase in the second cell cycle. The elapsed time before and after G1 phase onset (0 min) is indicated at the bottom. Note that the localization patterns of CENP-A dots and the nucleolar exclusion pattern of PCNA were different between two daughter cells as well as among the parental and two daughter cells.

different between the two daughter cells. In addition, the localization patterns of CENP-A dots in the daughter cells was different form each other.

Discussion

In this study, we characterized the molecular behavior of human CENP-A and PCNA throughout the cell cycle. To express mKO-CENP-A and mCherry-PCNA, we used the HT-1080 fibrosarcoma cell line with a few chromosomal changes from the normal diploid karyotype [11]. We found that some CENP-A dots were co-localized with PCNA foci in late S phase (Figure 3), probably reflecting the replication process of CENP-A-associated DNA in the living state. CENP-A dots were often found in the region showing the nucleolar exclusion pattern of PCNA in G1, early S and G2 phases [16-18]. Hemmerich et al. [8] also showed that CENP-C dots were also localized in the nucleoli from early S phase throughout late S phase (Figure 4) [8].

The localization pattern of CENP-A dots was different between the

daughter cells (Figure 4). After the cell division, however, the relative positions of CENP-A dots seemed to be maintained or to be fixed in each daughter nucleus in the subsequent S and G2 phases. Although HT-1080 cells showed a modal number of 46 chromosomes with the distribution between 44 and 48 [11], the karyotype of this fluorescent cell line was not so stable (data no shown). It is likely that the chromosome miss-segregation changed the localization pattern of CENP-A dots in the daughter nuclei. If a certain chromosome delays in the chromosome segregation process for example, the relative position of other CENP-A dots would be affected by the lagging chromosome in the daughter nuclei. Alternatively, it might be affected by the asymmetry of the mitotic spindle or spindle poles, somehow disturbed in the parental cells. Anyway, it would be interesting to know if the relative position of CENP-A dots as well as the nucleolar exclusion pattern of PCNA are maintained in the cell cycles in non-transformed cell lines or primary cells with the normal karyotype.

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