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The Role of Histone Phosphorylation in Chromosome Condensation

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Abstract

A cell spends most of its life in interphase, during which time chromatin (DNA combined with histone proteins) is loosely packed in the cell nucleus and the DNA is duplicated. During mitosis, the process of division, the chromatin condenses into discrete chromosomes. This allows one copy of each chromosome to be distributed to each new daughter cell. Mitosis is triggered by the activation of the enzyme Cdk1, a protein kinase that phosphorylates histone H1 (and other proteins) and activates Aurora B, which phosphorylates histone H3. Prematurely condensed chromosomes (PCCs) can be induced at any stage of the cell cycle by introducing calyculin A (Cal A), a protein phosphatase inhibitor that prevents phosphates from being removed from histones. This project explored the role of histone phosphorylation in chromosome condensation by treating HeLa cells at various stages of the cell cycle with Cal A to induce PCCs. Some cells were also treated with ZM447439 (ZM), an inhibitor of Aurora B. Formation of PCCs was checked using fluorescence microscopy. Phosphorylation was checked by gel electrophoresis. PCCs in G1-phase lack H1 phosphorylation and can form even when ZM is present to block H3 phosphorylation. This result indicates that histone phosphorylation is not essential for chromosome condensation.

Keywords: chromosomes, histone, phosphorylation

1. Introduction

A cell spends most of its life in interphase, during which time chromatin (DNA combined with histone proteins) is loosely packed in the nucleus and DNA is duplicated. Interphase begins with G1-phase, which is followed by S-phase and then G2-phase. At the end of G2-phase, cell division (mitosis) commences. The cell undergoes many changes in mitosis including nuclear envelope breakdown and chromosome condensation. Chromosomes condense into compact, discrete "X" like formations, which allow for the organized distribution of identical genetic materials to the two new daughter cells. Mitosis is triggered by the activation of the enzyme Cdk1/Cyclin B[1], a protein kinase that phosphorylates histone H1 (and other proteins) and activates Aurora B, which phosphorylates histone H3[2]. Cdk1/Cyclin B is inactivated upon entry into G1 phase by destruction of its Cyclin B subunit. Production of Cyclin B begins again in S-phase and continues through G2-phase

Histones are the major chromosomal proteins. They make up about half the chromosomal mass and are responsible for packaging DNA and neutralizing its negative charge. Histones H1 and H3 are heavily phosphorylated by Cdk1 and Aurora B, respectively, during mitosis[2,3.] The function of histone phosphorylation is not known, but it has been suggested that it plays a role in chromosome condensation[4,5,6].

Loosely packed interphase chromatin can be induced to form prematurely condensed chromosomes (PCCs). This was first done by fusing interphase cells with mitotic cells[7], which exposes interphase cells to active Cdk1/Cyclin B and triggers chromosome condensation and nuclear envelope breakdown. PCCs can also be induced by treating cells with calyculin A (Cal A)[8], an inhibitor of Protein Phosphatases 1 and 2A[9]. Protein phosphatases catalyze the

removal of phosphate groups from proteins by hydrolysis; i.e., they do the opposite of protein kinases like Cdk1 which use ATP to add phosphate groups to proteins.

In the work reported here we found, surprisingly, that PCCs form after treatment with Cal A, even in situations where it should not be possible for Cdk1/Cyclin B to be activated. Lack of active Cdk1/Cyclin B in these situations was confirmed by protein gels showing that H1 remained unphosphorylated. First, calyculin A was used to induce PCCs during early G1-phase, and in this stage of the cell cycle Cdk1 cannot be activated because Cyclin B is not available. Second, calyculin A was used to induce PCCs in G2-phase in the presence of Roscovitine, an inhibitor of Cdk1. PCCs were also induced in the presence of ZM447439 (ZM), an inhibitor of Aurora B, the protein kinase which phosphorylates histone H3. These results suggest that histone phosphorylation is not essential for chromosome condensation.

2. Methods

2.1. Cell Culture And Synchronization

HeLa S3 cells were grown in suspension at 37°C in RPMI-1640 medium supplemented with penicillin/streptomycin and 10% fetal bovine serum and diluted daily to 2.0-2.5 × 10^5 cells/mL[10]. For synchronization in G2-phase, cells were first blocked in S-phase by treatment with 2.5 mM thymidine (TdR) for 20-24 hrs[11]. They were then released from TdR by pelleting, resuspending in isotonic saline, pelleting again and resuspending in fresh medium, and incubated further for 6 hrs. For arrest in mitosis, nocodazole was added 4 hrs after TdR removal. For the experiment shown in Fig. 1, 0.25 µg/mL nocodazole was used and metaphase-arrested cells were obtained at 18 hrs after the release from TdR. For the experiment in Fig. 2, cells were treated with 60 ng/mL nocodazole and used at 16 hrs after TdR release.

For determination of mitotic index, 200 µL of cells were mixed with an equal volume of water containing 20 µg/mL Hoechst 33342, allowed to swell for 5 min, fixed with 40 µL of methanol-acetic acid (3:1), and viewed by epifluorescence in a Nikon Labophot microscope. The same method was used to determine the percentage of cells exhibiting PCCs. For each determination, at least 200 cells were counted.

2.2. Histone Extraction And Polyacrylamide Gel Electrophoresis

Metaphase chromosomes and/or interphase nuclei were prepared using the procedure for isolating metaphase chromosome clusters[12]. Lysis solutions contained 5 mM DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) to block histone dephosphorylation[13]. Histones were extracted with 0.2 M H_2SO_4 and analyzed by acid-urea gel electrophoresis[14]. All gels were stained with 0.1% Coomassie Brilliant Blue R250 (BioRad) in 50% methanol, 10% acetic acid and destained in 5% methanol, 10% acetic acid.

2.3. Light Microscopy

For microphotography, cells in a 5 mL culture aliquot were pelleted and resuspended in 2 mL 75 mM KCl. After incubation for 15 min at 37ºC they were fixed by addition of 200 µL methanol-acetic acid (3:1) and slides were prepared as previously described[15]. Chromosomes were stained using a solution of 40 µg/mL Hoechst 33342 in water, and viewed and photographed by epifluorescence in a Nikon Labophot microscope equipped with a Nikon Coolpix 990 camera.

3. Results

3.1 Induction of PCCs in G1 Cells Obtained by Treatment of M-arrested Cells with Roscovitine

Our first experiment (Fig. 1) was designed to test whether Cal A can induce PCCs in G1-phase cells and if so, is histone H1 phosphorylated in the resulting condensed chromosomes? G1-phase cells were obtained by arresting in mitosis with nocodazole (see Methods) and then treating the cells with 200 µM Roscovitine, an inhibitor of Cdk1

kinase. It has been shown that inhibition or inactivation of Cdk1 in mitosis triggers exit from mitosis and passage of the cells into G1-phase[16].

At 18 hours after release from thymidine (defined here as T=0), the nocodazole-treated culture was about 90% mitotic (Fig. 1a, T=0). As a control, one aliquot of cells was incubated without further treatment throughout the 4.5 hr experiment, and these stayed above 90% mitotic (Fig. 1a. Sample C). The remaining cells were treated with Roscovitine for 1 hr, inducing exit from mitosis, chromosome decondensation and nuclear envelope reassembly. At T=1 the mitotic index was less than 1% (Fig. 1a, Sample B). Note that in the absence of a mitotic spindle the cells exit mitosis without undergoing cytokinesis.

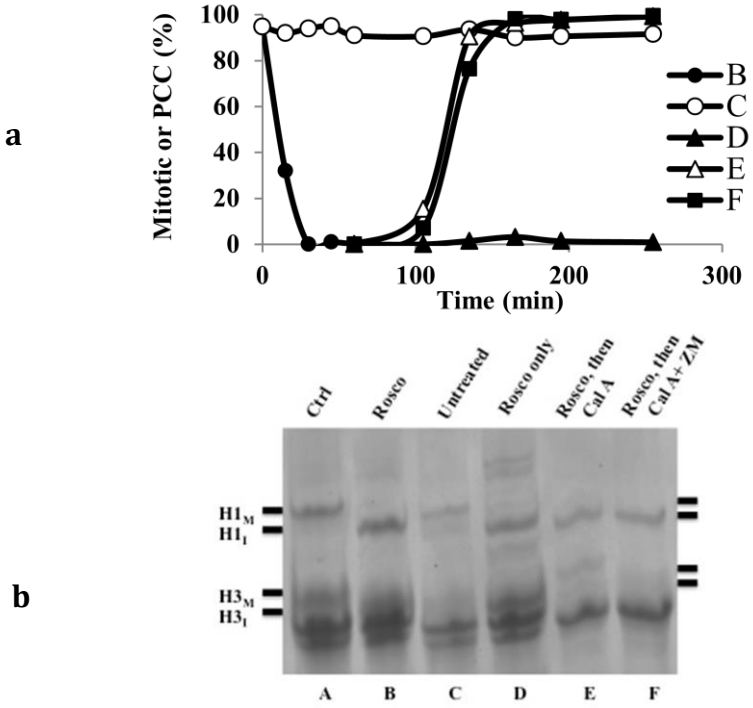


Fig. 1. Induction of PCCs in G1-phase cells obtained by treatment of metaphase-arrested cells with Roscovitine. (a) Time course showing the percentage of cells containing condensed chromosomes as a function of time. (b) Histones from the various samples analyzed on an acid-urea gel. The positions of mitotic and interphase histones H1 and H3 are indicated by $H1_M$, $H1_I$, $H3_M$ and $H3_I$, respectively. Sample A was from the initial metaphase-arrested culture at T=0 which was divided into Samples B and C. Sample B was treated with Roscovitine (Rosco) for 1 hr. Sample C was untreated for 4 hr. At T=1hr., Sample B was washed out of Rosco and divided into Samples D, E and F. For an 3 additional hours, Sample D was left untreated, Sample E was treated with calyculin A, and Sample F was treated with both calyculin A and ZM.

The cells that had been induced to leave mitosis and enter G1-phase were washed with isotonic saline to remove residual Roscovitine and then divided into three aliquots which were incubated for an additional 3 hrs. The first aliquot received no further treatment, and these cells remained in interphase with nuclei that appeared normal (Fig. 1a, Sample D). The second aliquot received 100nM calyculin A. At T=4, after 3 hrs with calyculin A, 99.2% of these cells exhibited condensed chromosomes (Fig. 1a, Sample E). The third aliquot was treated with 100nM calyculin A and 10µM ZM. Nuclear envelope breakdown occurred in this sample and the percentage of cells with condensed chromosomes lagged only slightly lagged behind the sample that had been treated with calyculin A only (Fig. 1a, Sample F).

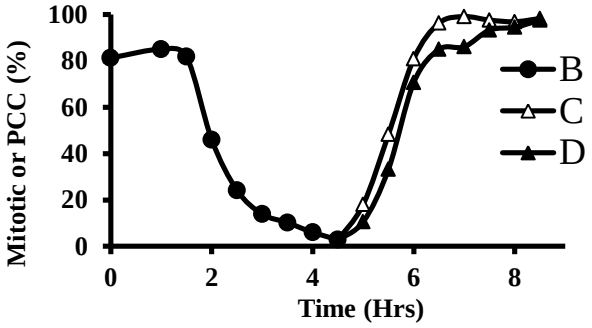
Examination of histone phosphorylation using acid-urea polyacrylamide gel electrophoresis (Fig. 1b), shows that in the initial metaphase-arrested culture at T=0 (Fig. 1b, lane A), histone H1 is mainly in its mitotic, highly phosphorylated form, $H1_M$. The same is true for the culture aliquot that was never treated with Roscovitine, from which histones were extracted at the end of the experiment (Fig. 1b, lane C). But after treatment with Roscovitine for 1 hr, histone H1 runs in the position characteristic of interphase $H1_I$, indicating that it has been dephosphorylated (Fig. 1b, lane B). This is expected, since histone H1 is normally dephosphorylated during exit from mitosis.

The samples treated with calyculin A might be expected to contain phosphorylated histone H1 because they have condensed chromosomes. However, they do not. In both of the calyculin A-treated samples (Fig. 1b lanes E and F), histone H1 runs in the $H1_I$ position, indicating that it is not phosphorylated. This observation is consistent with the absence of Cyclin B during G1-phase and shows that phosphorylation of H1 is not required for PCCs. Comparison of lanes E and F in Fig. 1b suggests further that histone H3 phosphorylation may not be necessary for PCCs. H3 appears to run slightly lower in the PCCs induced in the presence of ZM (Fig. 1b, lane F).

3.2 Induction of PCC in G1 Cells Obtained by Release From Nocodazole

One could argue that the cells obtained by treatment of metaphase-arrested HeLa cells with Roscovitine are not in a normal G1-state. We therefore repeated the experiment described above using G1-phase cells obtained in another way. In this case, nocodazole-blocked cells were pelleted and washed with isotonic saline to remove residual nocodazole and incubated in fresh medium (see Methods). After several hours, they completed mitosis, though rather asynchronously. We found that the best reversal of the nocodazole block was achieved when the concentration of nocodazole and the time of exposure to it were minimal. Cells were treated with 60ng/mL nocodazole, which was found to be the minimum concentration of nocodazole sufficient to obtain good metaphase arrest. They were removed from nocodazole after 12 hrs exposure (i.e., 16 hrs after removal from thymidine).

a



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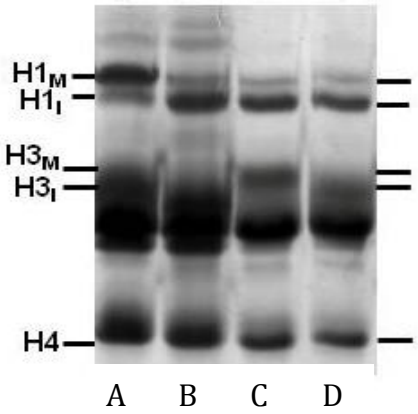

Figure 2. Induction of PCCs in G1 cells obtained by release from nocodazole. (a) The percentage of cells with condensed chromosomes as a function of time. (b) Histones from various samples on an acid urea gel. The positions of interphase and mitotic histones H1 and H3 are marked $H1_I$, $H1_M$, $H3_I$ and $H3_M$ respectively. Sample A was taken at T=0 (nocodazole arrested). Sample B shows cells incubated for 4.5 hours after being removed from nocodazole. At T=4.5 hr., Sample B was divided into Samples C and D. Sample C was treated with calyculin A and Sample D was treated with both calyculin A and ZM for an additional 4 hrs.

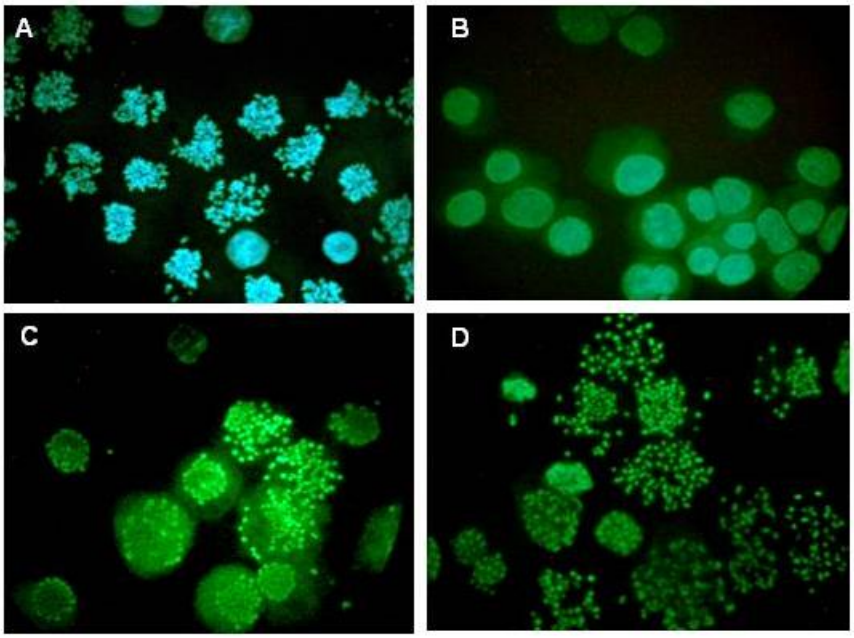

Figure 3. Micrographs of samples from induction of PCCs in G1 cells obtained by release from nocodazole (see Fig. 2). Sample A (T=0) cells are mostly mitotic. Sample B was taken at T=4 hours after removal from nocodazole and most cells progressed into early G1-phase. The G1-phase cells were treated for 4 hr with Cal A (Sample C) or Cal A and ZM (Sample D). Samples C and D both displayed condensed chromosomes.

After being released from the Nocodazole block, the cells were incubated for 4.5 hrs. During this period, they finished mitosis and entered into G1-phase. The culture was 80% mitotic at T=0, before removal of nocodazole (Figs. 2a and 3a), but by T=4.5 hrs the mitotic index had dropped to about 5% (Figs. 2a and 3b). At T=4.5 hrs, the cells were divided into two groups. Group C was treated with 100nM Cal A only, while Group D was treated with 100nM Cal A and 10μM ZM. These cells were allowed to incubate for an additional 4 hrs after treatment. Both groups C and D progressed to having over 95% of the cells with condensed chromosomes (Figs. 2a, 3c and 3d).

Examination of the histones from this experiment on acid-urea gels showed, as expected, that the metaphase-arrested cells at T=0 contained mainly mitotic, phosphorylated histone $H1_M$ (Fig. 2b, lane A). However, at T=4.5 hrs after removal from nocodazole, histone H1 was mostly in the higher mobility interphase form, $H1_I$ (Fig. 2b, lane B). There is some residual histone $H1_M$ in lane B because about 5% of the cells in Sample B were still in metaphase arrest (Fig. 2a). With a mitotic index of 5%, about 10% of the histone H1 would be expected to be in the form of $H1_M$ because mitotic cells have twice the amount of DNA and histones as G1 cells.

Cells treated with calyculin A (Samples C and D) had a high percentage of condensed chromosomes (Fig. 2a). However, in both samples histone H1 ran in the interphase position ($H1_I$) on the acid urea gel (Fig. 2b, lanes C and D). As with Fig. 1b lanes E and F, this result is in line with what is known about cyclin B production not beginning until early S-phase. Thus, Cdk1 kinase cannot be activated and cannot phosphorylate histone H1.

On the other hand, during treatment of the G1-cells with calyculin A, histone H3 returned to the mitotic position (Fig. 2b, lane C). This suggests that there was nothing in place to deter Aurora B, the protein kinase that phosphorylates histone H3 at mitosis. However, histone H3 from cells treated with both calyculin A and ZM moved farther down the gel (Fig. 2b, lane D) than in the sample with calyculin A alone (Fig. 2b, lane C). This is consistent with ZM blocking Aurora B, which phosphorylates histone H3. Although, the change in mobility on the acid urea gel is suggestive. Further confirmation is needed to determine if histone H3 phosphorylation was indeed successfully blocked by ZM in these calyculin A-induced PCCs.

3.3 Cal A-induced PCC in G2-phase Cells also Treated with Roscovitine and ZM

After treatment with thymidine for 22 hrs, cells synchronized in G2-phase were obtained via a timed release from 2.5μM thymidine. After being washed with isotonic solution and resuspended in fresh medium, the cells were allowed to incubate for 6 hrs. At that time, which is called T=0, Sample A was taken and the cells were found to be 3% mitotic (Fig. 4a). Analysis by acid-urea gel electrophoresis showed that histones H1 and H3 were not phosphorylated (Fig. 4b, lane A). The remaining cells were divided into six aliquots (B-G), treated in various ways and then incubated further for 3 hours at 37°C. At the end of this time, a portion of each sample was used to determine the percentage of cells with condensed chromosomes (mitotic or G2-PCCs) (Fig. 4a). The remainder of each was used for histone extraction and analysis on acid-urea gels (Fig. 4b).

Sample B was treated with 100nM calyculin A. After 3 hrs of treatment, 99.1% of cells had condensed chromosomes (Fig. 4a) and histones H1 and H3 were in their mitotic forms (Fig. 4b, lane B). This is consistent with what is known about Ckd1/Cyclin B being present and ready to be activated in G2-phase. Sample C was treated similarly with 100nM calyculin A, but it was also treated with 10μM ZM. After 3 hrs, 96.7% of cells had condensed chromosomes and histone H1 was phosphorylated (Fig. 4b, lane C). Histone H3 appears to have advanced farther down the gel than in Sample B suggesting that it is phosphorylated after Cal A treatment (Sample B) but is in its dephosphorylated interphase form after treatment with both Cal A and ZM. This would be consistent with ZM blocking Aurora B, but more evidence is needed for confirmation.

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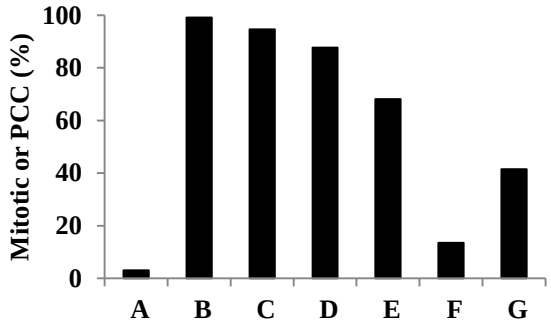

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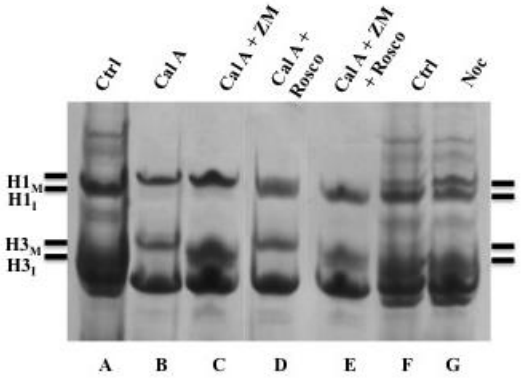

Figure 4. Cal A-induced PCC in G2-phase Cells Also Treated with Roscovitine and ZM. (a) Percentage of cells with condensed chromosomes. (b) Histones from various samples on an acid urea gel. The positions of interphase and mitotic histones H1 and H3 are marked $H1_I$, $H1_M$, $H3_I$ and $H3_M$ respectively. Sample A was taken after G2 synchronization at T=0. All other samples were treated for 3 hours after G2 synchronization. Sample B was treated with calyculin A. Sample C was treated with calyculin A and ZM. Sample D was treated with calyculin A and Roscovitine. Sample E was treated with calyculin A, ZM and Roscovitine. Sample F was untreated and sample G was treated with nocodazole.

Sample D was treated with 100nM calyculin A and 100µM Roscovitine, an inhibitor of Cdk1. In this case, 87.7% percent of cells had condensed chromosomes after treatment (Fig. 4a) and histone H3 was phosphorylated, but histone H1 was not (Fig. 4b, lane D).

Sample E was treated with 100nM calyculin A, 100µM Roscovitine and 10µM ZM. After 3 hrs, 68.1% of the cells had condensed chromosomes after 3 hrs of treatment (Fig. 4a) and histone H1 was unphosphorylated. It also appears that a majority of histone H3 is unphosphorylated, but there are clear bands at both the phosphorylated and unphosphorylated positions (Fig. 4b, lane E).

Sample F received no treatment after the G2 synchronization. These cells were 13.5% mitotic after 3 hrs of incubation (Fig. 4a) and contained mainly unphosphorylated histones (Fig. 4b, lane F). Sample G was treated only with Nocodazole. These cells were 41.5% mitotic after three hrs (Fig. 4a) and contained a mixture of phosphorylated and unphosphorylated histone H1 (Fig. 4b, lane G). Samples F and G were controls which show that the high percentage of cells with condensed chromosomes in the calyculin A-treated samples cannot simply be attributed to cells passing through G2-phase and entering mitosis.

4. Conclusions

These experiments show that calyculin A can induce PCCs without histone H1 phosphorylation and suggest that it can also induce PCCs without histone H3 phosphorylation if the Aurora B inhibitor ZM is included. In the experiments shown in Figs. 1 and 2, Cal A induced PCCs in G1-phase cells. Presumably histone H1 was not phosphorylated in this situation because its phosphorylation depends on Cdk1/Cyclin B which is not active and cannot be activated in G1-phase because Cyclin B has been degraded during exit from mitosis and entry into G1.

In the experiment shown in Fig. 3, PCCs lacking H1 phosphorylation were induced with Cal A in G2-phase by simultaneously treating the cells with Roscovitine, an inhibitor of Cdk1. In all three experiments, PCCs also formed in the presence of ZM, an inhibitor of Aurora B kinase, which phosphorylates histone H3 in mitotic chromosomes. It is likely that ZM has blocked H3 phosphorylation in these PCCs, although this remains to be confirmed by "western" immunoblotting and other methods.

Our results suggest that histone phosphorylation is not required for chromosome condensation. They also suggest that induction of mitosis and chromosome condensation may depend as much on protein phosphatase inactivation as on protein kinase activation.

5. Acknowledgements

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