

# Calyculin A induces prematurely condensed chromosomes without histone H1 phosphorylation in mammalian G1-phase cells

James R. Paulson, Erica R. Vander Mause

Department of Chemistry, University of Wisconsin-Oshkosh, Oshkosh, USA  
Email: [paulson@uwosh.edu](mailto:paulson@uwosh.edu)

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## ABSTRACT

**It is shown here that one can induce prematurely condensed chromosomes (PCCs) in G1-phase human (HeLa) and mouse (FT210) cells by treating them with the protein phosphatase inhibitor calyculin A. However, histone H1 is not phosphorylated in these G1-PCCs. It has previously been proposed that histone H1 phosphorylation is responsible for mitotic chromosome condensation, but our results suggest that this is not the case. They indicate instead that phosphorylation of histone H1 is not required for chromosome condensation. It is known that the Cdk1 protein kinase, which triggers mitosis and also phosphorylates histone H1, cannot be activated in G1-phase because mitotic cyclins are not present. Since calyculin A induces PCCs in G1-phase in the absence of active Cdk1, our results suggest that inactivation of protein phosphatases may be just as important for the onset of chromosome condensation and other mitotic events as the activation of protein kinases.**

**Keywords:** Mitosis; Chromosome Condensation; Histones; Protein Phosphatases

## 1. INTRODUCTION

It is well known that mitosis is triggered by activation of the protein kinase Cdk1/Cyclin B, also known as M-phase Promoting Factor or MPF [1]. Many proteins are phosphorylated at mitosis either by Cdk1 itself or by secondary protein kinases, and this presumably changes the functions of those proteins so as to produce the characteristic events of mitosis.

In some cases, the roles played by particular mitotic phosphoproteins are known. For example, nuclear envelope breakdown at mitosis involves the phosphorylation of

nuclear lamins, leading to depolymerization of lamin filaments and consequent disassembly of the nuclear lamina and nuclear envelope [2-4]. As another example, cessation of transcription at mitosis is due at least in part to phosphorylation of certain transcription factors by Cdk1 [5]. For some mitotic phosphoproteins, however, the function of their phosphorylation is not known. This is the case for the histones.

Histones are the major proteins associated with DNA in eukaryotic chromosomes, packaging the DNA into nucleosomes [6,7] and 30 nm chromatin fibers [8-10]. Histones H1 and H3 are extensively phosphorylated at mitosis. In fact, histone H1 was the first mitotic phosphoprotein to be identified [11]. Histone H1 is phosphorylated at several sites during mitosis, primarily but not exclusively by Cdk1 [12,13], whereas histone H3 is phosphorylated at Ser10 and Ser28, mainly by Aurora B kinase [14].

It was proposed more than 30 years ago that histone H1 phosphorylation is involved in chromosome condensation [15-17] and numerous subsequent studies have also pointed out this possibility (reviewed in [12]). However, these proposals have generally been based on the temporal correlation between H1 phosphorylation and chromosome condensation, without clear evidence for a mechanism. It must therefore be said that at this point the function of histone H1 phosphorylation at mitosis remains unknown.

Prematurely condensed chromosomes (PCCs) are the result of triggering interphase cells to enter a mitosis-like state. This phenomenon was first studied by Johnson and Rao [18] who showed that nuclear envelope breakdown and chromosome condensation could be induced in a G1-, S- or G2-phase cell by fusing it with a metaphase-arrested cell. The premature mitosis-like state presumably occurs because the interphase nucleus is exposed to ac-

tive Cdk1/cyclin B provided by the mitotic cell.

In the course of our studies on protein dephosphorylation during exit from mitosis [19], we observed that cell-permeable protein phosphatase inhibitors such as calyculin A and cantharidin are able to induce prematurely condensed chromosomes (PCCs) in interphase mammalian cells. The ability of calyculin A to induce PCCs has also been observed by others [20,21] and it has been put to use in the study of chromosome dynamics, breakage and repair, and for cytogenetic analysis (e.g., [22-24]).

In the work reported here, we have used PCCs to explore the possible involvement of histone H1 phosphorylation in chromosome condensation. We show that calyculin A induces chromosome condensation in G1-phase HeLa and FT210 cells, but that histone H1 is not phosphorylated in these condensed chromosomes. This result suggests that H1 phosphorylation is not required for chromosome condensation.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and Media

Media and components were obtained from Invitrogen and Atlanta Biologicals. All other reagents were obtained from Sigma-Aldrich unless otherwise noted. Calyculin A (LC Laboratories, Woburn, MA) was prepared as a 50  $\mu$ M stock solution in dimethylsulfoxide (DMSO) and stored at  $-20^{\circ}\text{C}$ . Nocodazole was prepared as a 5 mg/mL solution in DMSO and ZM447439 (Santa Cruz Biotechnology) was prepared as a 10 mM solution in DMSO. Both were also stored at  $-20^{\circ}\text{C}$ . Thymidine was dissolved to 100 mM in 0.9% NaCl, filter sterilized and stored at  $2^{\circ}\text{C}$ .

### 2.2. Cell Culture and Metaphase Arrest

Mouse FT210 cells [25] were grown in suspension at  $32^{\circ}\text{C}$  in RPMI-1640 medium and arrested in metaphase with nocodazole as previously described [26]. HeLa S3 cells were grown in suspension at  $37^{\circ}\text{C}$  in RPMI-1640 medium supplemented with penicillin/streptomycin and 10% fetal bovine serum and diluted daily to  $2.0 - 2.5 \times 10^5/\text{mL}$  [27]. For metaphase arrest, HeLa cells were first synchronized in S-phase by treatment with 2.5 mM thymidine for 20 - 24 hrs [28], then released from the thymidine block and arrested with nocodazole as previously described [29]. Mitotic indices for both FT210 and HeLa cells were determined by the method used previously [26] and were typically 80% - 85% for FT210 and 80% - 95% for HeLa S3. The same method was used to determine the percentage of cells exhibiting PCCs. For each determination, at least 200 cells were counted.

### 2.3. Histone Extraction and Polyacrylamide Gel Electrophoresis

Metaphase chromosomes and/or interphase nuclei were

prepared using the procedure for isolating metaphase chromosome clusters [19,30]. Lysis solutions contained either 2 mM p-chloromercuriphenyl sulfonate (PCMPS) or 5 mM Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), or DTNB) to block histone dephosphorylation [31]. Histones were extracted from pelleted chromosomes or nuclei with 0.2 M  $\text{H}_2\text{SO}_4$ , precipitated with ethanol, washed with acetone, dried and redissolved in 3 mM HCl [31]. For analysis of histone H1 phosphorylation by acid-urea gel electrophoresis, aliquots of acid-extracted histones were dried using a CentriVap (Labconco) and redissolved in 4  $\mu$ L of sample buffer [32]. Gels 16 cm long containing 15% acrylamide, 0.1% N,N'-methylene-bis-acrylamide, 2.5 M urea and 5.4% acetic acid were overlain with a polyethylene glycol solution during pre-electrophoresis overnight to prevent distortion of the wells [33]. After samples were loaded, electrophoresis was run at 300 V for 5 - 6 hrs until the blue component of the methyl green marker reached the bottom of the gel. 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.

### 2.4. 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  $37^{\circ}\text{C}$  they were fixed by addition of 200  $\mu$ L of fresh fixative (3 volumes methanol: 1 volume acetic acid). Fixed cells were then pelleted and gently resuspended in fresh fixative three times. Droplets of the final fixed cell suspension were then dropped from a height of 20 cm onto cold, wet microscope slides to spread the chromosomes and the slides were air dried [34]. Chromosomes were stained using a solution of 40  $\mu\text{g}/\text{mL}$  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. Calyculin A Induces PCCs without Histone H1 Phosphorylation in G1-Phase Mouse FT210 Cells

For a first test of the ability of calyculin A to induce prematurely condensed chromosomes (PCCs) in G1-phase, we used mouse FT210 cells. It is known that Cdk1 is temperature-sensitive in these cells [35] and we have previously shown that metaphase-arrested FT210 cells can be induced to exit mitosis and return to interphase by treating them at their non-permissive temperature to inactivate Cdk1 [26]. After this treatment, the cells appear to be biochemically normal in the sense that they can complete another cell cycle, traversing G1-, S- and

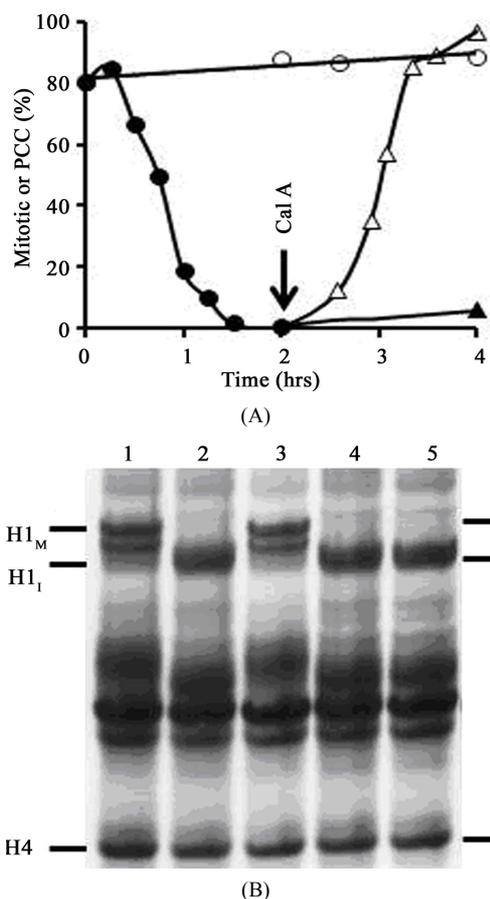
G2-phases and eventually reaching the next mitosis. However, when arrested at the next metaphase they are found to have twice the normal number of chromosomes because the heat treatment caused them to leave mitosis without undergoing cytokinesis [26].

To obtain early G1-phase cells, therefore, FT210 cultures were first arrested in metaphase and then treated with heat to induce exit from mitosis. A typical experiment is shown in **Figure 1**. FT210 cells growing at their permissive temperature (32°C) were blocked in metaphase (mitotic index, 81%) with 0.25 µg/mL nocodazole. An aliquot of the culture was then shifted to 41°C and the mitotic index was followed as a function of time. As shown in **Figure 1(A)**, the mitotic index dropped significantly during the 1.5 hr heat treatment, from 81% to less than 2%, as cells reassembled nuclear envelopes and decondensed their chromosomes. In an aliquot of the culture that was not heat-treated, the mitotic index remained above 80%. Cell viability remained high (above 95%) in all samples throughout the 4 hrs of the experiment.

After 1.5 hr, the portion of the culture that had been treated at 41°C was shifted back to 32°C and incubation was continued. At T = 2 hr, half of these cells were treated with 100 nM calyculin A while the other half received no further treatment. In the untreated aliquot, the mitotic index remained low (less than 5%) throughout the rest of the incubation, whereas in the aliquot treated with calyculin A, condensed chromosomes were seen in virtually all cells within 2 hr (**Figure 1(A)**). In short, calyculin A induced G1-phase PCCs.

Eventually more than 97% of the cells displayed condensed chromosomes in the calyculin A-treated culture aliquot (**Figure 1(A)**). However, histone H1 was not phosphorylated, as shown by the acid-urea polyacrylamide gel in **Figure 1(B)**. Acid-urea gels, unlike the more familiar SDS polyacrylamide gels, separate basic proteins largely on the basis of charge. Histones are highly positively charged and migrate toward the cathode, but at the pH of the gel each phosphate group contributes one negative charge, reducing the overall positive charge on the protein and decreasing its electrophoretic mobility. Since mitotic histone H1 is phosphorylated at several sites, it migrates significantly more slowly than unphosphorylated (interphase) H1 in acid-urea gels [31,36].

The gel in **Figure 1(B)** shows that H1 extracted from metaphase-arrested cells at the start of the experiment (lane 1) runs with the lower mobility characteristic of hyperphosphorylated (mitotic) histone H1 (H1<sub>M</sub>). H1 extracted at the end of the experiment (T = 4 hr) from cells that never received the heat treatment is also hyperphosphorylated (lane 3). On the other hand, cells that were treated for 1.5 hrs at 41°C and exited mitosis (**Figure 1(A)**) contain histone H1 that runs with the mobility



**Figure 1.** Induction of PCC without histone H1 phosphorylation in early G1-phase mouse FT210 cells. (A) FT210 cells, which have temperature-sensitive Cdk1, were arrested in metaphase at 32°C (mitotic index, 81%). Aliquots were then either shifted to 41°C (●-●) to induce exit from mitosis [26] or continued at 32°C (○-○). At T = 1.5 hr after the start of the heat treatment, the cells at 41°C were shifted back to 32°C and at T = 2 hr (“Cal A”) aliquots were either treated with 100 nM calyculin A (▲-▲) or not (▲-▲). The percentage of mitotic or PCC cells is shown as a function of time. (B) Samples were taken at various times and histones analyzed on an acid-urea gel. Lane 1, metaphase-arrested cells taken at T = 0 hr. Lane 2, cells treated at 41°C for 1.5 hr, sample taken at T = 2 hr. Note that histone H1 has become dephosphorylated. Lane 3, control cells left at 32°C, sample taken at T = 2 hr. Lane 4, cells treated at 41°C but not treated with calyculin A, sample taken at T = 4 hr. Lane 5, cells treated at 41°C, then treated with 100 nM calyculin A for 2 hr, sample taken at T = 4 hr. The positions of mitotic (phosphorylated) histone H1 (H1<sub>M</sub>), interphase histone H1 (H1<sub>I</sub>) and histone H4 are indicated.

characteristic of interphase H1 (H1<sub>I</sub>), both at the 2 hr (lane 2) and 4 hr (lane 4) time points.

Most importantly, cells that were induced to leave mi-

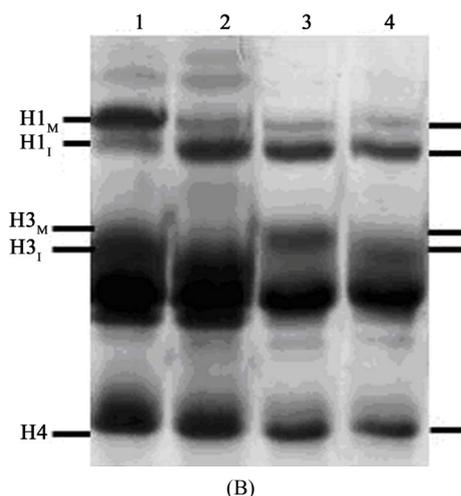
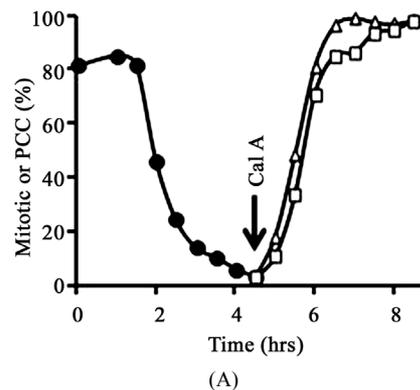
tosis and afterwards treated with 100 nM calyculin A also contain histone H1 that runs in the interphase (unphosphorylated) position (**Figure 1(B)**, lane 5), even though nearly all the cells in this sample contain PCCs. We conclude that calyculin A can induce prematurely condensed chromosomes in G1-phase FT210 cells, but that histone H1 is not phosphorylated in these condensed chromosomes.

### 3.2. PCCs Induced by Calyculin A in G1-Phase HeLa Cells Also Lack Phosphorylated Histone H1

An experiment similar to the one described above was carried out with HeLa cells, but in this case G1-phase cells were obtained by first arresting the cells in metaphase with nocodazole and then releasing them from the nocodazole block. For this experiment we used the minimum concentration of nocodazole sufficient for a good metaphase arrest (60 ng/mL) and exposed the cells to nocodazole only until a point 16 hrs after thymidine had been removed. This procedure resulted in a lower mitotic index (80% - 85%) than that attained with a longer block, but reversal of the block and exit from mitosis were more reliably achieved. To reverse the nocodazole block, metaphase-arrested cells were pelleted in the centrifuge, washed twice by resuspending in isotonic saline and pelleting again, and finally resuspended in fresh medium.

After removal of nocodazole, the metaphase-arrested cells completed mitosis and underwent cytokinesis gradually and asynchronously over the course of about 4 hours. At T = 0, the mitotic index was 81% (**Figure 2(A)**), most of the cells displayed condensed mitotic chromosomes (**Figure 3(A)**) and histone H1 was mainly in its mitotic, hyperphosphorylated form (H1<sub>M</sub>) (**Figure 2(B)**, lane 1). However, by T = 4.5 hr the mitotic index had fallen to about 5% (**Figure 2(A)**), nearly all cells contained interphase nuclei (**Figure 3(B)**) and histone H1 was mainly in its dephosphorylated, interphase form (H1<sub>I</sub>) (**Figure 2(B)**, lane 2). Note that in **Figure 2**, lane 2, a minor band remains at the H1<sub>M</sub> position. This is because at T = 4.5 hr about 5% of the cells remained in mitosis. With a mitotic index of 5%, about 10% of the histone H1 should be H1<sub>M</sub> because mitotic cells have twice the amount of DNA and histones as G1 cells.

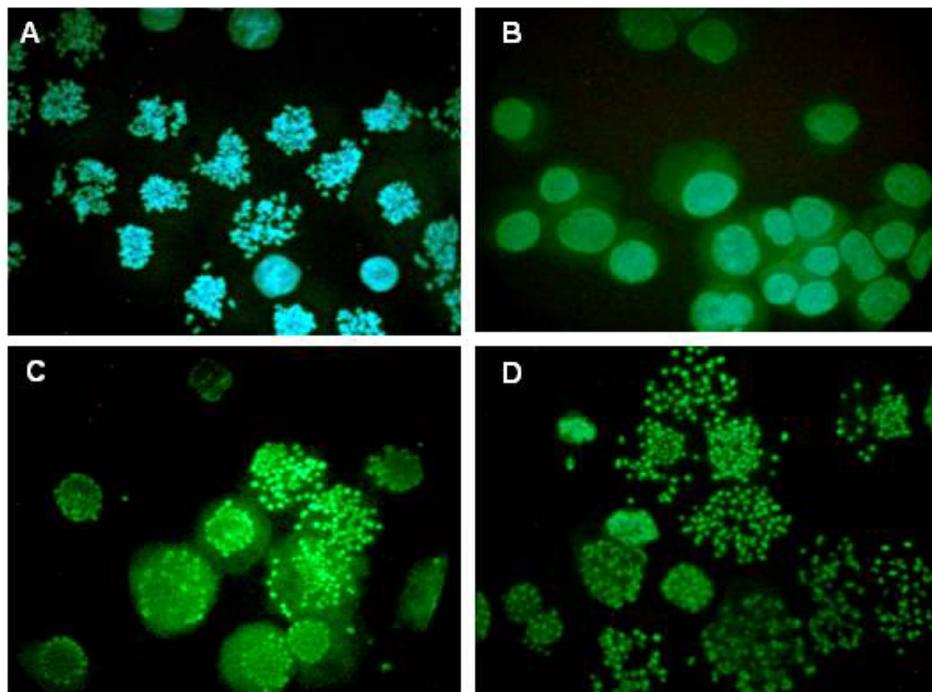
At T = 4.5 hr, a portion of the now mainly G1-phase culture was treated with 100 nM calyculin A. After exposure to calyculin A, the percentage of cells with condensed chromosomes increased to more than 95% by T = 8.5 hr (**Figure 2(A)**; **Figure 3(C)**). However, histone H1 remained primarily in its dephosphorylated, interphase form, H1<sub>I</sub> (**Figure 2(B)**, lane 3), with no noticeable change from **Figure 2(B)**, lane 2. This result shows that G1-phase PCCs which lack histone H1 phosphorylation can



**Figure 2.** Induction of PCC without histone H1 phosphorylation in G1-phase HeLa S3 cells. (A) At T = 0, cells blocked with 60 ng/mL nocodazole (mitotic index, 81%) were released from metaphase arrest by pelleting, washing with saline, and then continuing incubation in fresh medium (●-●). After 4.5 hr ("Cal A"), when the mitotic index had dropped to 5%, one aliquot of the culture was treated with 100 nM calyculin A (Δ-Δ) while another aliquot was treated with both 100 nM calyculin A and 10 μM ZM447439 (□-□). The percentage of mitotic or PCC cells is shown as a function of time. (B) Histones were extracted from the various samples and separated on an acid-urea gel. Lane 1, metaphase-arrested cells at T = 0. Lane 2, a sample taken at 4.5 hr after removal of nocodazole. Lane 3, cells released from nocodazole (as in lane 2) and then treated with 100 nM calyculin A for 4 hr. Lane 4, cells released from nocodazole and then treated with both 100 nM calyculin A and 10 μM ZM447439 for 4 hr. The positions of mitotic (phosphorylated) histone H1 and H3 (H1<sub>M</sub> and H3<sub>M</sub>), interphase histone H1 and H3 (H1<sub>I</sub> and H3<sub>I</sub>), and histone H4 are indicated.

be induced in HeLa cells as well as in FT210 cells.

Another portion of the G1-phase culture was treated at T = 4.5 hr with 100 nM calyculin A and also with 10 μM ZM447439. In this case, too, PCCs were induced in more



**Figure 3.** Microscopy of G1-phase PCC induced in HeLa cells by calyculin A. Cells from the experiment shown in **Figure 2** were spread on slides, stained with Hoechst 33342 and viewed and photographed by epifluorescence. (A) The initial metaphase-arrested culture (mitotic index, 81%) at  $T = 0$ . (B) At 4.5 hr after removal of nocodazole. (C) After release from nocodazole for 4.5 hr and treatment with 100 nM calyculin A for 4 hr. (D) After release from nocodazole and treatment with both 100 nM calyculin A and 10  $\mu$ M ZM447439 for 4 hr.

than 95% of cells (**Figure 2(A)**; **Figure 3(D)**) but histone H1 was not phosphorylated (**Figure 2(B)**, lane 4). One difference seen here, however, is that histone H3 appears to run with higher mobility than in **Figure 2(B)**, lane 3. This suggests that although H3 may be phosphorylated in calyculin A-induced G1-PCCs (**Figure 2(B)**, lane 3), it is not phosphorylated when the PCCs are induced in the presence of ZM447439 (**Figure 2(B)**, lane 4). This is in fact expected since ZM447439 inhibits Aurora B [37], the protein kinase that phosphorylates histone H3 at Ser10 in mitosis [14]. Further analysis is needed to verify that H3 is not phosphorylated in this situation, but if this can be confirmed it would lend weight to the notion that neither H1 nor H3 phosphorylation is required for chromosome condensation.

#### 4. DISCUSSION

It has previously been shown that calyculin A, a cell-permeable inhibitor of Protein Phosphatases 1 and 2A [38], can induce prematurely condensed chromosomes (PCCs) in interphase mammalian cells [20]. The work we have presented here confirms this and shows further that treatment of HeLa or mouse FT210 cells with calyculin A during G1 phase leads to PCCs that lack phosphorylated histone H1.

These results clearly indicate that histone H1 phosphorylation is not required for formation of G1-PCCs. Since premature chromosome condensation is, as far as we know, very similar or identical to mitotic chromosome condensation, our results suggest that H1 phosphorylation may not be essential in mitosis. The same may also be true of histone H3, since calyculin A can induce G1-PCCs in HeLa cells even in the presence of ZM447439 (**Figure 2**; **Figure 3(D)**), an inhibitor of Aurora B, the protein kinase that phosphorylates histone H3 at serine 10 [14,37]. However, further investigation is necessary regarding the phosphorylation of H3 or the lack thereof in G1-PCCs induced in the presence of ZM447439.

Numerous studies have proposed that histone H1 phosphorylation is involved in mitotic chromosome condensation (e.g., [15-17,39]) and our results may seem to be at odds with them. However, these proposals were based only on the correlation between the two events. As noted by Banerjee and Chakravarti in 2011 [40], it was still unclear how H1 phosphorylation could contribute to chromosome condensation. The situation is similar in the case of histone H3, where temporal correlation between H3 phosphorylation and mitotic chromosome condensation has been demonstrated [41,42]. It has also been shown in tetrahymena micronuclei that mutation of the

serine 10 phosphorylation sites in H3 to alanine disrupts chromosome condensation [43]. Nevertheless, the role of H3 phosphorylation at mitosis is not clear [44].

Our results do not necessarily contradict those of earlier studies. Although they suggest that H1 phosphorylation is not essential for chromosome condensation, we cannot rule out that it plays some role. It may be, for example, that there are multiple, redundant mechanisms of chromosome condensation and that histone H1 phosphorylation, when it is present, does make a contribution, even though it is dispensable. Perhaps H1 phosphorylation contributes to changes in chromosomes that initiate or facilitate condensation, for example chromatin remodeling [45]. If those changes persist into early G1, then H1 phosphorylation may not be required for G1-PCC. Alternatively, H1 phosphorylation could serve as a "label" [46] for the recruitment of other factors, and perhaps those factors are still present in the chromosomes in early G1. Finally, we cannot rule out the possibility that there may be significant differences between condensed mitotic chromosomes and G1-PCCs that are not evident at the level of resolution provided by the light microscope.

An intriguing aspect of our results is that PCCs and a premature mitosis-like state can be induced in G1-phase in the absence of Cdk1, the protein kinase associated with initiation of mitosis. It should not be possible to activate Cdk1 in G1-phase because the mitotic cyclins necessary for its activation should have been degraded during exit from the previous mitosis and will not be synthesized again until the next S-phase. Since Cdk1/cyclin B phosphorylates histone H1 [12], the absence of phosphorylated H1 in the calyculin A-induced G1-PCCs confirms that active Cdk1/cyclin B is not present. This suggests that many of the effects of Cdk1/cyclin B at the onset of mitosis may be mediated through the inactivation of protein phosphatases and that protein phosphatase inhibitors such as calyculin A can mimic those effects. This in turn suggests that the inactivation of protein phosphatases may be as important a part of the initiation of mitosis as the activation of protein kinases.

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