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Non-proteolytic inactivation of geminin requires CDK-dependent ubiquitination

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Abstract

In late mitosis and G1, Mcm2-7 are assembled onto replication origins to “license” them for initiation. At other times licensing is inhibited by cyclin dependent kinases (CDKs) and geminin, thus ensuring that origins fire only once per cell cycle. We show here that, paradoxically, CDKs are also required to inactivate geminin and activate the licensing system. On exit from metaphase in *Xenopus* egg extracts, CDK-dependent activation of the Anaphase Promoting Complex (APC/C) results in the polyubiquitination of geminin. This ubiquitination triggers geminin inactivation without requiring ubiquitin-dependent proteolysis, and is essential for replication origins to become licensed.

Keywords

DNA replication; replication licensing; *Xenopus*; geminin; ubiquitination

Introduction

For most eukaryotic cells, chromosomal DNA must be replicated once and only once in each cell cycle. This is ensured by separating DNA replication into two distinct phases. In the first phase, which normally occurs in late mitosis and early G1, replication origins are “licensed” for a single initiation event by loading heterohexamers of the Mcm2-7 proteins, thus forming the pre-replicative complex (pre-RC) (reviewed in 1,2,3). The second phase occurs during S phase, when replication forks are initiated at licensed origins. As each origin initiates Mcm2-7 are displaced from it. So long as the ability to license origins ceases before DNA replication commences, then no replication origin will initiate replication more than once.

The central role of Mcm2-7 regulation in preventing re-replication of DNA was first demonstrated in *Xenopus* egg extracts⁴⁻⁶. Chong et al⁴ and Kubota et al⁵ both exploited the observation that the replication licensing system, as originally defined⁷, could be specifically inhibited by treating metaphase extracts with certain protein kinase inhibitors such as staurosporine and 6-dimethylaminopurine (6-DMAP). Both these treatments inhibit licensing by ultimately preventing the loading of Mcm2-7 onto chromatin. However, 6-DMAP-treated extracts can efficiently replicate chromatin that has already been licensed. The ability to assay licensing activity using a 6-DMAP-based assay has permitted a systematic chromatographic fractionation of the *Xenopus* licensing system and the complete reconstitution of the licensing of *Xenopus* sperm chromatin with purified proteins⁸.

Despite the effectiveness of this assay, it has remained unclear how 6-DMAP and staurosporine selectively inhibit the licensing system. Inhibition only occurs if 6-DMAP or staurosporine are added to extracts arrested in metaphase; no inhibition of licensing occurs when they are added to extracts already in interphase^{9,10}. Further, 6-DMAP and staurosporine only inhibit licensing at concentrations that are sufficient to cause the extracts to spontaneously exit from metaphase arrest and enter interphase⁹⁻¹¹. They are therefore effective at concentrations that inhibit the metaphase-inducing activity of Cdk1-cyclin B, originally defined in *Xenopus* as MPF (Maturation Promoting Factor)^{12,13}. Olomoucine, an inhibitor that is more specific for CDKs, is able to inhibit licensing in the same way as 6-DMAP and staurosporine¹⁴. These results raise the possibility that in *Xenopus*, the activation of the licensing system that normally occurs on exit from metaphase depends on CDK function.

This suggestion appears at odds with much data showing that CDKs inhibit the licensing system and normally act to prevent inappropriate origin licensing late in the cell cycle¹⁻³. In yeasts, inhibition of CDKs in G2 phase is sufficient to permit re-licensing and hence re-replication of DNA¹⁵⁻¹⁸. CDKs block licensing in a number of different ways that appears to vary from organism to organism¹⁹⁻²¹. In metazoans, inappropriate licensing of replication origins late in the cell cycle is also inhibited by a small protein called geminin. In *Xenopus* eggs, geminin provides the major activity preventing DNA re-replication^{22,23}. Geminin specifically binds and inhibits the Cdt1/RLF-B component of the licensing system^{22,24-27}. However, no geminin homologue has so far been identified in yeasts.

In this paper, we demonstrate a new link between these two licensing inhibitors in *Xenopus* egg extracts. We show that CDKs play an essential role in activating the licensing system on exit from metaphase, being required for the non-proteolytic inactivation of geminin mediated by its ubiquitination.

Results

Xenopus eggs are naturally arrested at meiotic metaphase II; on fertilization a transient increase in cytosolic calcium induces them to complete mitosis and enter interphase. Extracts prepared from *Xenopus* eggs can likewise be released into interphase by addition of calcium. As these extracts exit metaphase, there is a rapid activation of the replication licensing system (Fig 1A, left). If metaphase-arrested egg extracts are treated with CDK inhibitors such as 6-DMAP, staurosporine or olomoucine, MPF is inhibited and the extracts enter an interphase-like state where DNA is assembled into functional nuclei^{9,10,14}. Even if stimulated with calcium, these extracts are unable to support replication licensing, though they can efficiently replicate DNA templates that have already been licensed (Fig 1A, right). To provide further evidence that the failure of licensing in such extracts is due to CDK inhibition we repeated these experiments with roscovitine, an adenine derivative with a high specificity for CDKs²⁸. We first determined the ability of roscovitine to inhibit MPF by titrating roscovitine into metaphase-arrested extracts and monitoring whether they could assemble DNA into interphase nuclei in the absence of calcium stimulation. Figure 1B shows that 50 - 200 μ M roscovitine inhibited MPF and caused spontaneous mitotic exit, similar to the concentration of roscovitine required to block entry into mitosis in cycling extracts²⁸. This corresponded to a decline in the total histone H1 kinase activity present in the extracts (Figure 1C). Metaphase extracts treated with 200 μ M roscovitine prior to calcium addition were severely compromised in their ability to load Mcm7 onto chromatin (Fig 1D). As has been previously shown with 6-DMAP and staurosporine^{9,10}, inhibition of Mcm7 loading was only seen when roscovitine was added to metaphase extract, and did not occur when roscovitine was added to extract after release into interphase (Fig 1D). The close correlation between the ability of roscovitine to inhibit MPF and replication licensing

(which is also seen with 6-DMAP, staurosporine and olomoucine^{9,10,14}) provides strong evidence that CDK activity is required for the activation of the licensing system that normally occurs on exit from metaphase.

Metaphase extracts treated with 6-DMAP (“6-DMAP-treated extracts”) not only lack licensing activity but also contain an inhibitor of the Cdt1/RLF-B component of the licensing system¹¹. It has recently been shown that the major inhibitor of licensing in *Xenopus* egg extracts is geminin, a specific inhibitor of Cdt1/RLF-B^{22-24,27}. We therefore asked whether geminin is responsible for the licensing defect seen in metaphase extract treated with CDK inhibitors. Gel filtration of metaphase extracts showed two peaks of geminin (Fig 2A), a high molecular weight peak (fractions 3-5) consisting of complexes between geminin and Cdt1, and a low molecular weight peak (fractions 7-10) consisting of free geminin²³. On exit from metaphase, a proportion of the geminin was degraded (Fig 2B), but the remaining geminin no longer formed a complex with Cdt1 (Fig 2A, “+Ca”) ²³. In order to determine whether the lack of complex formation was due to a change in Cdt1 or a change in geminin, we supplemented extract with excess recombinant geminin (geminin^{DEL})²⁴ after release into interphase. The recombinant geminin complexed with all of the Cdt1 in the extract (Fig 2A). Previous experiments have shown that in early interphase, endogenous geminin is incapable of interacting with recombinant Cdt1²³. Together these results strongly suggest that in early interphase, endogenous Cdt1 is fully competent to bind to geminin and that the lack of complex formation is due to a defect in geminin’s Cdt1-binding ability.

We next examined the effect of CDK inhibitors on complex formation. When metaphase extracts were treated with 6-DMAP prior to calcium addition so as to block activation of the licensing system, geminin levels remained high (Fig 2B) and a significant proportion remained bound to Cdt1 (Fig 2A, “+6-DMAP+Ca”). A similar effect was seen when metaphase extracts were treated with roscovitine prior to calcium addition (Figs 2A and B, “+rosco.+Ca”). Immunodepletion of the high molecular weight fractions 3-5 confirmed that the geminin and Cdt1 were complexed together, since all the Cdt1 was co-depleted with anti-geminin antibodies (Fig 2C, “gem-”) and all the geminin was co-depleted with anti-Cdt1 antibodies (Fig 2C, “Cdt1-”). To confirm an essential role for CDKs in inactivating geminin, we immunodepleted metaphase extracts of Cdk1 before addition of calcium. Although some Cdt1 and geminin were co-depleted with the anti-Cdk1 antibodies (data not shown), the remaining Cdt1 was fully complexed to geminin (Fig 2B, “Cdk1- + Ca”). These Cdk1-depleted extracts were devoid of licensing activity, though licensing activity could be restored by addition of Cdt1 (data not shown). This result is consistent with previous observations showing that immunodepletion of Cdk1 (Cdc2) from metaphase-arrested *Xenopus* extracts blocks subsequent DNA replication²⁹.

To address whether the presence of active geminin explains the ability of 6-DMAP-treated extracts to inhibit licensing, we performed a functional assay for licensing inhibitors. As expected from previous results^{11,23}, 6-DMAP-treated extract inhibited the licensing activity of normal interphase extract (Fig 3A, ‘6-DMAP extract’). However, immunodepletion of geminin from the 6-DMAP-treated extract almost completely abolished its inhibitory activity, whilst control depletion with non-immune antibodies had no effect. This shows that geminin (or a geminin-associated protein) is the major licensing inhibitor in 6-DMAP-treated extract. Next, we supplemented 6-DMAP-treated extracts with recombinant Cdt1 to titrate out the inhibitory activity of geminin. Fig 3B shows that this completely restored the ability of 6-DMAP-treated extracts to support DNA replication. These results suggest that the only replication defect in 6-DMAP-treated extracts is the presence of active geminin.

Analogous replication assays could not be performed with roscovitine-treated extracts because roscovitine, as well as inhibiting replication licensing, also inhibits CDK activity required for the initiation of DNA replication (ref 28 and data not shown). Instead, we investigated whether the addition of recombinant Cdt1 to licensing-defective extracts would restore their ability to load Mcm2-7 onto chromatin. Metaphase extracts were treated with 6-DMAP or roscovitine before addition of calcium, and as expected, these extracts did not load Mcm7 onto chromatin (Fig 3C). However, subsequent addition of recombinant Cdt1 to the extract induced efficient Mcm7 loading. Taken together, these results suggest that on exit from metaphase, CDKs have an essential function in inactivating geminin and activating the licensing system. This contrasts with other well-documented roles of CDKs in inhibiting the licensing system at other cell cycle stages¹⁻³.

One known function of CDKs on exit from metaphase is the activation of the Anaphase Promoting Complex (APC/C)^{30,31}, and it has been shown that addition of 6-DMAP to metaphase extracts prevents the APC/C-mediated proteolysis of cyclin B^{11,32}. We therefore investigated whether inactivation of geminin is dependent on APC/C function. To do this we used a peptide representing the consensus APC/C-recognition sequence (D-box peptide) that is a competitive inhibitor of the APC/C³³. Addition of the D-box peptide to metaphase extracts before addition of calcium blocked the degradation of cyclin B and geminin, and blocked the ability of the extracts to load Mcm7 onto chromatin (Fig 4A). Gel filtration of D-box peptide-treated extract showed Cdt1 and geminin still complexed together (Fig 4C). Thus the D-box peptide prevents the activation of the licensing system and the inactivation of geminin that normally occur on exit from mitosis. Similar results were obtained by supplementing extracts with a mutant form of ubiquitin (ubiquitin K48R) that prevents formation of polyubiquitin chains (Fig 4B, C). These results suggest that the ubiquitin ligase activity of the APC/C (which in turn is dependent on CDK activity) is essential for inactivating geminin on metaphase exit. The effect of ubiquitin K48R suggests that polyubiquitination via lysine 48 is likely to be required for geminin inactivation.

Proteins known to be ubiquitinated by the APC/C, such as cyclin B and securin, are subsequently degraded by the 26S proteasome. We therefore used MG-132, a potent inhibitor of the 26S proteasome, to investigate whether proteasome function is required for the inactivation of geminin. Although proteasome mediated degradation of cyclin B and geminin was inhibited by MG-132 (Fig 5A) it had no effect on the activation of the licensing system (Fig 5B) or on the inactivation of geminin (Fig 5E). Similar results were obtained with epoxomicin, another highly-specific proteasome inhibitor (Fig 5D, E). Remarkably, metaphase extracts treated with MG-132 were still able to support efficient DNA replication (Fig 5C); under these conditions Cdk1-cyclin B activity is inhibited by ubiquitination in the absence of proteolysis (ref ³⁴ and data not shown; see also 35). Taken together, the results presented here suggest that whilst activity of the APC/C is essential for geminin inactivation, proteasome-mediated degradation is not.

Geminin has previously been reported to be ubiquitinated by the APC/C on exit from metaphase²⁴. Since we have shown that APC/C activity is essential for geminin inactivation, we investigated whether geminin itself is an essential APC/C substrate in this process. We prepared two forms of ³⁵S-labelled recombinant geminin: the first wild-type and the second with a deletion of 9 amino acids in the APC/C recognition motif (geminin^{DEL}) so that it is no longer a substrate for the APC/C²⁴. In order to prevent degradation of the wild-type recombinant protein, proteolysis was inhibited with MG-132. Fig 6A shows that on incubation in *Xenopus* extract exiting from mitosis, about half of the recombinant wild-type geminin was inactivated so that it did not associate with Cdt1. In contrast, the geminin^{DEL} mutant was completely refractory to inactivation. This suggests that APC/C-mediated ubiquitination of geminin is required for it to be inactivated.

No significant proportion of the inactive geminin seen in interphase extracts is itself ubiquitinated, as ubiquitination would cause an increase in molecular weight of >8 kDa that is clearly not observed (Figs 2, 4 and 5 and data not shown). Since geminin forms octomeric or decameric complexes (ref²³ and S. Shreeram, data not shown) we entertained the possibility that inactive complexes of geminin contain a mixture of both ubiquitinated and non-ubiquitinated forms. However, examination by SDS PAGE of gel filtration fractions containing ³⁵S-labelled wild-type geminin in the inactive complex revealed no significant quantity of high molecular weight ubiquitinated geminin (Fig 6A, right panel).

Xenopus eggs contain high levels of isopeptidases that remove ubiquitin from proteins³⁶, so an alternative explanation for these results is that geminin is only transiently ubiquitinated on exit from metaphase, but de-ubiquitination leaves geminin locked into an inactive conformation. To test this idea, metaphase extracts supplemented with GST-ubiquitin, recombinant geminin and MG-132 were released into interphase by calcium addition and incubated for various times. GST-tagged proteins were then collected on glutathione beads, separated by SDS PAGE and immunoblotted with anti-geminin antibodies. 5-10 minutes after calcium release, a ladder of high molecular weight geminin was detected (Fig 6B, left panel). The lowest band in this ladder was ~70 kDa, the expected size of geminin conjugated to GST-ubiquitin. By 30 min, this ladder had almost completely disappeared, suggesting that the geminin had been de-ubiquitinated (proteolysis having been blocked by MG-132). A similar ladder, though considerably fainter, was seen without addition of recombinant geminin, which suggests that the same reaction is taking place on endogenous geminin (Fig 6B, right panel). The intensity of the ladder was increased by addition of ubiquitin aldehyde, an inhibitor of deubiquitinating enzymes. Taken together, these results suggest that geminin is transiently ubiquitinated on exit from metaphase, but the ubiquitin is subsequently removed leaving the geminin in an inactive form.

Discussion

We have detailed here an unexpected role for CDKs in activating the licensing system in *Xenopus* extracts. As these extracts exit from metaphase, the licensing system is abruptly activated, and this requires geminin to no longer complex with Cdt1. We show that CDKs are required for this inactivation of geminin by a pathway that depends on the APC/C-mediated ubiquitination of geminin, but does not depend on proteasome-mediated proteolysis. Therefore inhibition of CDK activity in metaphase extracts prevents the activation of the licensing system that normally occurs on exit from metaphase. When the MPF activity of metaphase extracts is inhibited by rather non-specific CDK inhibitors such as 6-DMAP, activation of the licensing system is completely inhibited but the treated extracts can still efficiently replicate DNA templates that have previously been licensed⁹. The ability of 6-DMAP to inhibit MPF without inhibiting CDK activity required for the initiation of S phase (SPF activity³⁷) may reflect the ability of 6-DMAP to also inhibit other kinases such as MAP kinases¹⁴ that play a role in maintaining metaphase arrest³⁸. These results therefore explain how treatment of metaphase extracts with CDK inhibitors can provide a functional assay for licensing system components^{4,5,8-10}. Reconstitution experiments based on this assay have shown that *Xenopus* sperm nuclei must be incubated with a combination of nucleoplasmin, ORC, Cdc6, Cdt1 and Mcm2-7 before the chromatin becomes competent to replicate when transferred to 6-DMAP-treated extract^{8,39}.

The results presented here provide strong support for the model outlined in Fig 7. In metaphase-arrested *Xenopus* egg extracts, geminin binds and inhibits Cdt1, whilst active CDKs reduce the affinity of ORC and Cdc6 for chromatin. After calcium addition, the APC/C is activated and this activation requires ongoing CDK activity³⁰⁻³². The active APC/C then polyubiquitinates geminin²⁴. Some of the ubiquitinated geminin is degraded, but this is

not essential for activation of the licensing system. In order for the licensing system to be activated, the remaining geminin must be inactivated. This occurs as a consequence of APC/C-mediated ubiquitination. The polyubiquitination of geminin is only transient, but after deubiquitination geminin remains in an inactive state. One possible way this could occur might be by a second covalent modification that is dependent on prior geminin ubiquitination. 2-dimensional gels indeed show many different geminin isoforms, though none seem to correlate precisely with activity (data not shown). Thus geminin joins an expanding collection of proteins whose activity is known to be regulated by ubiquitination independently of proteolysis^{40,41}.

The inhibitory role of CDKs on replication licensing and DNA re-replication has been extensively documented¹⁻³. In contrast, we show here that in *Xenopus* egg extracts CDKs are required to inactivate geminin on exit from metaphase, thus playing an essential positive role in replication licensing. There are, however, precedents for CDKs playing a positive role in this process. During *Drosophila* endocycles, Mcm2-7 are re-loaded onto DNA only as cyclin E levels rise at the start of each endocycle⁴². In cell free extracts prepared from human cells, the loading of Mcm2 onto DNA is stimulated by cyclin E/Cdk2⁴³. It is possible that these observations also reflect CDK-dependent ubiquitination and inactivation of geminin, since the function of other SCF-type ubiquitin ligases also depends on CDK activity. The ubiquitination-dependent inactivation of geminin may therefore be a widespread means of regulating the licensing system.

Materials and Methods

Preparation and use of egg extracts

Metaphase-arrested and interphase low speed supernatant *Xenopus* egg extracts were prepared as described⁴⁴. All extracts were supplemented with 250 μ g/ml cycloheximide, 25 mM phosphocreatine, 10 μ g/ml creatine phosphokinase before use, and incubations were performed at 23°C. *Xenopus* sperm nuclei were demembrated with lyssolecithin as described⁴⁴ and frozen in aliquots in liquid nitrogen. For DNA synthesis experiments, sperm nuclei were incubated at a final concentration of 3 ng DNA/ μ l extract (~1,000 nuclei/ μ l), and extracts (typically 10 μ l) were supplemented with 50 μ Ci/ml [α -³²P]dATP. DNA synthesis was assessed after 90 min incubation by TCA precipitation as described⁴⁴. The histone H1 kinase assay was performed essentially as described⁴⁵. Gel filtration of *Xenopus* extracts was performed as described²³.

Immunodepletion of Cdk1 from extract was performed essentially as described⁴⁴. Affinity-purified anti-Cdk1 antibody was coupled to protein A agarose beads at 15mg antibody/ml beads and washed in EDB-S buffer (50 mM Hepes, 50 mM KCl, 2 mM DTT, 0.4 mM MgCl₂, 0.4 mM EGTA, 10% sucrose, 10 μ g/ml each of leupeptin, pepstatin and aprotinin, pH 7.6). Metaphase-arrested extract was mixed with 0.2 volumes of antibody beads for 1 hr at 4°C, after which the beads were removed. The process was repeated two further times. Immunoblotting of the depleted extract showed no detectable Cdk1 or cyclin B. The depleted extract spontaneously assembled sperm DNA into interphase nuclei, consistent with the removal of MPF activity.

Recombinant protein and antibodies

Recombinant 6xHis-tagged *Xenopus* Cdt125 was produced as described⁸. ³⁵S-labelled geminin was produced from pET28(a) plasmid DNA containing wild-type geminin or geminin^{DEL} 24 using the TnT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. Antibodies against *Xenopus* geminin, Cdt1 and Mcm7 were as previously described^{22,46}. Antibody against *Xenopus* cyclin B2 was a kind gift of Tim

Hunt. The D-box peptide (RRTALGDVTNKVSE) as described³³ was a kind gift of Jim Hutchins and Paul Clarke. Antibody against Cdk1 was raised against a C-terminal peptide (CFDDLKSSLPANQIRN) and was a kind gift of Johannes Walter and Tanya Prokhorova. Immunoblots were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford IL) and pre-flashed film.

Chromatin isolation

For immunoblotting experiments, extracts were treated in various ways, then 20 μ l aliquots were supplemented with demembranated sperm nuclei at a final concentration of 10 ng DNA/ μ l extract (~3,000 nuclei/ μ l). After 20 min incubation, each reaction was diluted in 500 μ l NIBA (50 mM KCl, 50 mM Hepes KOH pH 7.6, 5 mM MgCl₂, 2 mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 2.5 mM Mg-ATP, 1 μ g/ml each of leupeptin, pepstatin and aprotinin) supplemented with 0.1% Triton X-100 and underlayered with 100 μ l of the same buffer containing 15% sucrose. The chromatin was pelleted at 6,000g in a swinging bucket rotor at 4°C followed by SDS PAGE and immunoblotting.

Licensing inhibition assay

Licensing inhibition assays were performed essentially as described²³. Briefly, 1.5 μ l fractions of interest were incubated with 0.5 μ l interphase extract and 0.3 μ l of sperm nuclei (80 ng DNA/ μ l) for 30 min. The degree of licensing was then assessed by addition of 6 μ l interphase extract containing 1.5 ng/ μ l geminin^{DEL} and [α -³²P]dATP and incubation for a further 90 min; total DNA synthesis was measured by TCA precipitation as described above.

Ubiquitination assay

20 μ l metaphase arrested extract was supplemented with 800 μ M MG-132 and 25 μ g/ml GST-ubiquitin, 50 μ g/ml ubiquitin-aldehyde, or 5 μ g/ml recombinant wild-type geminin as appropriate. Extracts were released into interphase with 0.3 mM CaCl₂ and incubated at 23°C. Reactions were stopped by dilution in 30 μ l of ice cold Bead Binding Buffer (50 mM K phosphate, pH8, 0.05 % Tween 20) supplemented with 150 mM NaCl, 10 mM N-ethyl maleimide and 10 μ l glutathione beads. After 30 min incubation on rotating wheel at 4° C the beads were washed three times in 200 μ l Bead Binding Buffer containing 500 mM NaCl and once in 200 μ l Bead Binding Buffer without NaCl. Beads were then resuspended in SDS gel loading buffer followed by SDS PAGE and immunoblotting for geminin.

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Figure 1.

Roscovitine inhibits licensing at concentrations where MPF is inhibited.

A, Schematic picture showing events occurring after calcium release of metaphase-arrested *Xenopus* egg extracts. In the left hand panel, calcium addition causes inactivation of mitotic CDK activity, and activates the licensing system. When S phase CDKs are activated the licensed DNA can replicate. In the right hand panel, 6-DMAP is added to the metaphase extract before calcium. The licensing system fails to become active, so no DNA replication takes place. **B**, Metaphase extracts were supplemented with *Xenopus* sperm and the indicated concentrations of roscovitine. The percentage of sperm heads assembled into interphase nuclei possessing an intact nuclear envelope was measured. **C**, Metaphase extracts were incubated for 15 min plus and minus calcium and roscovitine as indicated, before being used for a histone H1 kinase assay. Roscovitine concentrations were: 10, 20, 50, 100, 200, 500 and 1000 μM. **D**, Metaphase extracts were supplemented with 3 mM 6-DMAP or various concentrations of roscovitine prior to addition of 0.3 mM calcium ("metaphase + 6-DMAP + Ca" or "metaphase + roscov. + Ca"). Alternatively, 6-DMAP or roscovitine were added after calcium addition ("metaphase + Ca + 6-DMAP" or "metaphase + Ca + roscov."). *Xenopus* sperm were then added to the extract, and after incubation for 20 min the chromatin was isolated and immunoblotted for Mcm7.

Figure 2.

6-DMAP and roscovitine prevent release of geminin from Cdt1.

Metaphase extracts were supplemented with or without 3 mM 6-DMAP or 500 μ M roscovitine or were immunodepleted of Cdk1, and were then supplemented with 0.3 mM CaCl_2 and incubated for 15 min. Alternatively, metaphase extract was supplemented with 0.3 mM calcium, followed by addition of recombinant geminin^{DEL} and incubation for 15 min. Metaphase extract without addition is also shown. **A**, Extracts were separated by gel filtration and fractions immunoblotted for Cdt1 and geminin. The migration of molecular weight markers (in kDa) is indicated above. 'r-geminin' indicates the migration of geminin^{DEL}. **B**, Immunoblot of total Cdt1 and geminin in: 1, metaphase extract alone; 2, metaphase extract + calcium; 3, metaphase extract + 6-DMAP + calcium; 4, metaphase extract + roscovitine + calcium. Endogenous *Xenopus* geminin migrates as a doublet, presumably reflecting the existence of two closely related geminin genes (geminin-H and geminin-L24). **C**, Gel filtration fractions 3-5 from extract supplemented with 500 μ M roscovitine prior to CaCl_2 addition were pooled together. They were then immunodepleted with antibodies against geminin ("gem.-"), Cdt1 or non-immune antibodies ("NI-"). Immunodepleted extract was then separated by SDS PAGE and immunoblotted for geminin and Cdt1. The starting pool without any immunodepletion is also shown ("pool").

Figure 3.

The only inhibitory activity in treated extracts is geminin.

A, Metaphase extract was supplemented with 3 mM 6-DMAP and then 0.3 mM calcium ("6-DMAP extract"). 6-DMAP extracts were immunodepleted with antibodies to geminin, or with non-immune antibodies. The resultant extracts were assayed for their ability to inhibit the licensing of interphase *Xenopus* extracts. Licensing is expressed as the % of that seen with no inhibitory extract. Dashed lines, background licensing of sperm nuclei incubated in buffer alone. **B**, Metaphase extract was supplemented with or without 3 mM 6-DMAP and then 0.3 mM calcium. Various concentrations of recombinant Cdt1 (1.8, 5.4 and 16 nM) were then added, and the extracts were assayed for their ability to replicate sperm nuclei. **C**, Metaphase extract was supplemented plus or minus 3 mM 6-DMAP or 500 μM roscovitine or 300 nM recombinant Cdt1, prior to addition of 0.3 mM calcium. Untreated metaphase extract is also shown. Sperm nuclei were then added to the extract, and after 20 min incubation chromatin was isolated and immunoblotted for Mcm7.

Figure 4.

Inhibition of the APC/C prevents activation of the licensing system.

Metaphase extracts were supplemented with D-box peptide (2 mM; A, C) or ubiquitin K48R (1.2 mM; B, C) then plus or minus 0.3 mM calcium as indicated. **A**, After incubation for 20 min, extracts were immunoblotted for cyclin B and geminin (bottom panels). Alternatively, sperm nuclei were incubated in the extracts for 20 min, chromatin was then isolated and immunoblotted for Mcm7 (top panel). **B**, After incubation for 20 min, extracts were immunoblotted for geminin. **C**, After incubation for 20 min extracts were gel filtered and immunoblotted for Cdt1 and geminin.

Figure 5.

Proteasome-mediated proteolysis is not required for activation of licensing.

A, Metaphase extracts were supplemented with various concentrations of MG-132 (0, 0.51, 0.256, 1, 6, 32, 160, 800 μ M) and then plus or minus 0.3 mM calcium. After incubation for 20 min, extracts were immunoblotted for cyclin B and geminin. **B**, Metaphase extracts were supplemented plus or minus 800 μ M MG-132 or 0.3 mM calcium. Sperm nuclei were then added, and after incubation for 20 min, chromatin was isolated and immunoblotted for Mcm7. **C**, Metaphase extracts were supplemented with or without 800 μ M MG-132 or 0.3 mM calcium. Extracts were then assayed for their ability to replicate sperm nuclei. **D**, Metaphase extracts were supplemented with epoxymycin (500 μ M) and then plus or minus 0.3 mM calcium. After incubation for 20 min, extracts were immunoblotted for cyclin B and geminin. **E**, Metaphase extracts were supplemented with MG-132 (800 μ M) or epoxymycin (500 μ M) and then 0.3 mM calcium, after which they were gel filtered and immunoblotted for Cdt1 and geminin.

Figure 6.

Geminin is only transiently ubiquitinated on exit from metaphase.

A, Recombinant ^{35}S -labelled wild-type geminin or geminin^{DEL} was incubated for 15 min in metaphase extract supplemented with 800 μM MG-132 before being released into interphase with 0.3 mM calcium; after a further 15 min incubation, extracts were gel filtered and fractions examined by SDS PAGE and fluorography (bottom panels). The entire lane containing fraction 8 of inactive ^{35}S -labelled wild-type geminin is also shown, with the migration of molecular weight markers (kDa) to the right. **B**, Metaphase extract was supplemented with 800 μM MG-132 and then the indicated combinations of recombinant wild-type geminin, GST-ubiquitin and ubiquitin-aldehyde. At the indicated time, GST-conjugated proteins were isolated on glutathione beads, run on SDS PAGE and immunoblotted with anti-geminin antibodies. Molecular weight markers (kDa) are shown on the right.

Figure 7.

Positive and negative effects of CDKs on the licensing system.

In metaphase (top part of figure), CDKs inhibit the licensing system by reducing the affinity of ORC and Cdc6 for chromatin, whilst geminin inhibits Cdt1. On exit from metaphase (lower part of figure) CDKs activate the licensing system via APC/C-dependent ubiquitination of geminin. See text for further details.