Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin

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Eukaryotic replication origins are ‘licensed’ for replication early in the cell cycle by loading Mcm(2–7) proteins. As chromatin replicates, Mcm(2–7) are removed, thus preventing the origin from firing again. Here we report the purification of the RLF-B component of the licensing system and show that it corresponds to Cdt1. RLF-B/Cdt1 was inhibited by geminin, a protein that is degraded during late mitosis. Immunodepletion of geminin from metaphase extracts allowed them to assemble licensed replication origins. Inhibition of CDKs in metaphase stimulated origin assembly only after the depletion of geminin. These experiments suggest that geminin-mediated inhibition of RLF-B/Cdt1 is essential for repressing origin assembly late in the cell cycle of higher eukaryotes.

To ensure that chromosomal DNA is precisely duplicated and that no sections of DNA are over-replicated, eukaryotic replication origins must fire no more than once in a single cell cycle. Early in the cell cycle, origins are ‘licensed’ for replication by loading a hetero-hexameric complex of Mcm(2–7) proteins (also termed RLF-M). Mcm(2–7) are probably responsible for the larger ‘pre-replicative complex’ (pre-RC) on replication origins in the yeast Saccharomyces cerevisiae during late mitosis and G1 (ref. 10). The Mcm(2–7) proteins are essential for DNA replication, possibly providing the helicase activity required for the initiation and elongation of replication forks. As DNA replicates, Mcm(2–7) are removed from it, thus ensuring that the re-firing of replicated origins will not occur. To prevent the re-replication of DNA it is therefore necessary to prevent relicensing of DNA once S phase has begun.

A stepwise assembly reaction leads to the assembly of licensed origins, involving first the assembly of the origin recognition complex (ORC), then Cdc6 and Cdt1, and finally Mcm(2–7). Biochemical fractionation of Xenopus egg extracts has shown that at least five activities are required to assemble licensed origins on Xenopus sperm nuclei. The chromatin remodelling protein nucleoplasmin first decondenses sperm chromatin to permit the binding of ORC, and then Cdc6 (ref. 17). Once this has occurred, another currently unidentified activity termed RLF-B promotes the loading of Mcm(2–7) onto chromatin. RLF-B, which has not previously been purified, is known to be distinct from nucleoplasmin, ORC, Cdc6 and Mcm(2–7), but its relationship to Cdt1 is unknown.

The mechanisms that prevent the relicensing of replicated DNA in metazoans are currently poorly known, although in yeast, cyclin-dependent kinases (CDKs) active during S, G2 and M phases of the cell cycle are ultimately responsible. The ability of CDKs to block replication seems to be mediated by a number of redundant mechanisms, including the exclusion of Mcm(2–7) from the nucleus and the degradation of Cdc6 (refs 34,35). In mammalian cells, Cdc6 is exported from the nucleus as a consequence of CDK activation in late G1 (refs 36–38). However, in the Xenopus cell-free system, inhibition of CDK activity in G2 does not cause relicensing. Instead, regulation of RLF-B seems to have a critical role in preventing the relicensing of replicated DNA. RLF-B activity is low in metaphase, is rapidly activated on exit into anaphase, and then declines during interphase. These changes in RLF-B activity are not dependent on CDK activity.

Here we report the purification of RLF-B and show that it corresponds to Xenopus Cdt1 (ref. 20). RLF-B/Cdt1 was inhibited by geminin, a replication inhibitor found in higher eukaryotes that is degraded during late mitosis. We show that the immunodepletion of geminin from metaphase extracts rendered them competent to assemble licensed replication origins. In contrast, inhibition of CDK activity in metaphase extracts stimulated origin assembly only after geminin depletion. These experiments suggest that geminin-mediated inhibition of RLF-B/Cdt1 is essential for repressing origin assembly late in the cell cycle of higher eukaryotes.

Results

Purification of RLF-B and identification as Cdt1. Geminin was originally identified in a screen for Xenopus proteins specifically degraded at the metaphase–anaphase transition. The addition of recombinant geminin to Xenopus extracts blocked the loading of Mcm(2–7) onto chromatin. We first wished to determine whether the inhibition of DNA replication by geminin was due specifically to inhibition of origin licensing. Metaphase Xenopus egg extracts treated with the protein kinase inhibitor 6-dimethylaminopurine (6-DMAP) are specifically defective in origin licensing. A brief incubation in untreated interphase egg extract or in fractions derived from it, chromatin becomes licensed for replication and gains the ability to replicate in 6-DMAP-treated extract. Figure 1a shows that when geminin was added to the interphase extract before licensing had occurred, it abolished subsequent replication in 6-DMAP-treated extract ('after'), whereas the addition of geminin after licensing had occurred caused virtually no inhibition of replication (Fig. 1a, 'before'). This suggests that geminin inhibits DNA replication by specifically blocking replication licensing.

We next wished to determine which activity required for origin licensing is inhibited by geminin. We have been undertaking a systematic fractionation of Xenopus egg extract to identify all the proteins required for the assembly of licensed replication origins on Xenopus sperm nuclei. Figure 1b shows a diagram of the current fractionation scheme. To determine which of the licensing activities interact with geminin, we coupled recombinant geminin...
to agarose beads and incubated them with interphase Xenopus extract. Figures 1c, d show that this geminin bead-treated extract was unable to license origins on sperm nuclei and could be rescued only by fractions containing RLF-B, and not by other fractions containing nucleoplasmin, ORC, Mcm(2–7) or Cdc6. This suggests that geminin specifically binds RLF-B.

We next investigated whether we could use recombinant geminin to affinity-purify RLF-B from the ‘BPAS’ fraction (Fig. 1b), which contains RLF-B enriched ~220-fold by three purification steps18,23,24; BPAS also contains ORC, Cdc6 and Cdt1 (Fig. 2a, inset). After application to a geminin column, BPAS lost all RLF-B activity but retained ORC, Cdc6 and ~50% of the Cdt1 (Fig. 2a, inset). Neither RLF-B nor the bound Cdt1 were eluted from the geminin beads by 1 M KCl, but both were efficiently eluted in buffer containing 4 M urea. This eluate contained RLF-B activity enriched >20,000-fold. Further fractionation by gel filtration in 4 M urea gave a peak of RLF-B activity at an apparent relative molecular mass of ~350 kDa (Mr ~350K) (Fig. 2b). SDS–PAGE and silver staining showed a single major band of Mr ~75K (the size of Xenopus Cdt1) that was co-eluted with RLF-B (Fig. 2c, arrowhead). Because of the low concentration of protein present, contaminating keratin bands were also evident (arrows). The Mr ~75K protein was recognized by anti-Cdt1 antibody (Fig. 2c, bottom panel), suggesting that RLF-B is identical to Cdt1.

To confirm this identity, we showed that recombinant Cdt1 (ref. 20) could substitute for purified RLF-B in replication licensing (Fig. 3a). As we have shown previously for RLF-B4,23, licensing induced by recombinant Cdt1 also required the Mcm(2–7) proteins. Conversely, purified RLF-B was capable of fully restoring licensing activity to extracts immunodepleted of Cdt1 (Figure 3b). Extract that had been affinity-depleted of RLF-B by geminin beads was also fully rescued by purified RLF-B (Figure 3b). Similarly, depletion of Cdt1 from RLF-B fractions abolished RLF-B activity (data not shown). Taken together, these results prove conclusively that RLF-B activity is provided by the Cdt1 protein.

To show that geminin and RLF-B/Cdt1 specifically antagonize one another, we inhibited replication by adding recombinant geminin to interphase Xenopus extract41. This inhibition was completely overcome by the addition of recombinant Cdt1 (Fig. 3c) or by the addition of purified RLF-B (Fig. 3d). These results suggest that RLF-B/Cdt1 is the only essential replication activity inhibited by geminin. Geminin is a physiological inhibitor of RLF-B/Cdt1. The above results show that recombinant geminin can specifically inhibit RLF-B/Cdt1 but do not indicate whether geminin normally performs
licensing was assayed by the addition of 6-DMAP-treated extract containing Mcm(2–7) activity (RLF-M) and 10 nM recombinant Cdt1. After 20 min, the extent of DMAP chromatin was incubated in interphase extract, a crude fraction containing Cdt1 antibody or with recombinant geminin beads, in the presence or absence of purified RLF-B. After 20 min, the extent of licensing was assayed by addition of 6-DMAP-treated extract containing [α-32P]dATP and incubation for a further 90 min. b, Unlicensed '6-DMAP chromatin' was incubated in interphase extract depleted with non-immune antibody, with anti-Cdt1 antibody or with recombinant geminin beads, in the presence or absence of purified RLF-B. After 20 min, the extent of licensing was assayed by addition of 6-DMAP-treated extract containing [α-32P]dATP and incubation for a further 90 min. c, d, Interphase extract containing [α-32P]dATP and sperm nuclei was supplemented with 35 nM recombinant geminin, 20 nM recombinant Cdt1 or 0.25 volumes purified RLF-B. After 90 min, total DNA synthesis was measured.

Figure 3. Recombinant Cdt1 can provide RLF-B activity. a, Unlicensed '6-DMAP chromatin' was incubated in interphase extract, a crude fraction containing Mcm(2–7) activity (RLF-M) and 10 nM recombinant Cdt1. After 20 min, the extent of licensing was assayed by the addition of 6-DMAP-treated extract containing [α-32P]dATP and incubation for a further 90 min. b, Unlicensed '6-DMAP chromatin' was incubated in interphase extract depleted with non-immune antibody, with anti-Cdt1 antibody or with recombinant geminin beads, in the presence or absence of purified RLF-B. After 20 min, the extent of licensing was assayed by addition of 6-DMAP-treated extract containing [α-32P]dATP and incubation for a further 90 min. c, d, Interphase extract containing [α-32P]dATP and sperm nuclei was supplemented with 35 nM recombinant geminin, 20 nM recombinant Cdt1 or 0.25 volumes purified RLF-B. After 90 min, total DNA synthesis was measured.

Figure 4. RLF-B/Cdt1 can associate with geminin in interphase extracts. a, Interphase extract was supplemented with 35 nM recombinant geminin and then immunodepleted with either non-immune (1) or anti-geminin (2) antibodies. Inset: samples were blotted for either Cdt1 or geminin. Unlicensed '6-DMAP chromatin' was incubated in these depleted extracts or interphase extract or the BPAS fraction, with or without a crude fraction containing Mcm(2–7) activity (RLF-M). After 20 min the extent of licensing was assayed by the addition of 6-DMAP-treated extract containing [α-32P]dATP and incubation for a further 90 min. b, BPAS fraction containing partly purified RLF-B/Cdt1 was fractionated on phenyl-Sepharose. Fractions were assayed for RLF-B (open circles) and for the ability to rescue licensing in Cdt1-depleted extracts (filled triangles), or were immunoblotted for Cdt1 and geminin.

this function in vivo. We next set out to determine whether endogenous geminin is a physiological inhibitor of RLF-B/Cdt1. First we investigated whether geminin could associate with endogenous RLF-B/Cdt1 in whole Xenopus egg extract. Figure 4a shows that when recombinant geminin was added to interphase extract and was then depleted again, almost all (~95%) of the endogenous RLF-B/Cdt1 was removed from the extract. This suggests that endogenous RLF-B/Cdt1 is capable of forming a complex with recombinant geminin in whole extract. The residual RLF-B activity remaining in the extract after the addition and removal of geminin was probably sufficient to account for the DNA replication reported by McGarry and Kirschner after this treatment. We next investigated whether endogenous geminin present during interphase of the cell cycle was associated with endogenous RLF-B/Cdt1. We have previously described the behaviour of RLF-B activity on phenyl-Sepharose chromatography. Figure 4b shows that whereas RLF-B activity was eluted in a single peak, Cdt1 was eluted in two overlapping peaks. One peak corresponded to active RLF-B; the other peak contained endogenous geminin. This co-purification of Cdt1 and geminin probably represents a physical interaction because immunodepletion of geminin from the inactive fractions depleted Cdt1 simultaneously (data not shown). Interestingly, only fractions with RLF-B activity could rescue licensing in Cdt1-depleted extracts (Fig. 4b, triangles). These results suggest that geminin is an important physiological inhibitor of RLF-B/Cdt1. We propose that Xenopus egg extracts contain two forms of Cdt1: an inactive form associated with geminin, and a form not associated with geminin that has RLF-B activity. The inactive geminin-associated form probably accounts for the Cdt1 that did not bind recombinant geminin beads (Fig. 1a).

Support for this idea comes from analysing proteins assembled onto chromatin under different conditions (Fig. 5). In interphase extracts that support origin licensing, X Orc1, Xcdc6, X Cdt1 and XMcm7 are found associated with sperm chromatin. In contrast, metaphase extracts support the loading of much lower levels of these proteins. When metaphase extracts are treated with 3 mM 6-DMAP, they enter an interphase-like state in which origin licensing
is inhibited\textsuperscript{4,23}, and so XMcm7 loading onto chromatin remains low. However, this ‘6-DMAP chromatin’ contained high levels of XORC, XCdc6, XCdt1 and geminin (Fig. 5). The Cdt1 loaded onto ‘6-DMAP chromatin’ is probably in the inactive geminin-bound form, because this chromatin requires incubation with both RLF-B and Mmc(2–7) for licensing to occur\textsuperscript{4,23}. The addition of recombinant geminin to interphase extract also blocked the assembly of XMcm7 onto chromatin (Fig. 5) but had a different effect on the other origin proteins. Although levels of XCdc6 on the chromatin were increased, there was virtually no stimulation of XCdt1 loading and only a modest stimulation of geminin loading. Addition of geminin or 6-DMAP to extracts at levels insufficient to block licensing (0.5 mM 6-DMAP or 7 nM geminin) produced chromatin similar to that assembled in untreated extract (Fig. 5). We therefore conclude that although 6-DMAP and geminin can both block licensing in the Xenopus system, they do not do so in exactly the same way.

Geminin is the major inhibitor of origin assembly in metaphase extracts. Metaphase Xenopus extracts contain an inhibitor of licensing that is lost on exit from mitosis\textsuperscript{10,15}. This inhibitor does not seem to target Mmc(2–7), because Mmc(2–7) activity can be detected in metaphase extracts\textsuperscript{40}. Figure 6a shows that when unlicensed ‘6-DMAP chromatin’ (which contained ORC, Cdc6, Cdt1 and geminin) was incubated in metaphase extract, no licensing occurred. However, the addition of purified RLF-B/Cdt1 to the metaphase extract promoted licensing. This suggests that metaphase extract cannot license DNA because it contains an inhibitor of RLF-B/Cdt1. Because geminin is degraded on exit from metaphase just as RLF-B becomes active\textsuperscript{40,41}, and as we have shown above that geminin inhibits RLF-B/Cdt1, geminin is a good candidate for this inhibitor. We therefore immunodepleted metaphase extracts of geminin and tested its ability to inhibit licensing. Figure 6b shows that unlike control-depleted metaphase extract, high concentrations of geminin-depleted extract did not inhibit licensing, but instead stimulated it. Although there is an unavoidable ~2-fold dilution of extracts as a consequence of the immunodepletion protocol, geminin-depleted and control-depleted extracts had similar CDK activities as judged by H1 kinase levels (Fig. 6c), and geminin-depleted extract still condensed sperm DNA into mitotic chromatids (Fig. 6d). This suggests that the depleted extracts were still arrested in metaphase and that the loss of the licensing inhibitor after the depletion of geminin was not due simply to a loss of CDK activity. As well as having lost its licensing inhibitor, geminin-depleted metaphase extract was competent to license ‘6-DMAP chromatin’ by itself (Fig. 6e). This suggests that metaphase extracts contain both RLF-B/Cdt1 and Mmc(2–7) activities, with RLF-B/Cdt1 normally being restrained by geminin.

For chromatin to become licensed by the combined activity of RLF-B/Cdt1 and Mmc(2–7), it must already contain bound ORC and Cdc6 (refs 16–19). Figure 7a shows that the depletion of geminin from metaphase extract allowed it to license naive sperm nuclei containing none of these proteins. Geminin is therefore essential for blocking the assembly of licensed origins in metaphase. However, serial dilution revealed that the licensing activity of geminin-depleted metaphase extracts was less than that of untreated control extract (open circles) were then assayed for their ability to inhibit licensing of ‘6-DMAP chromatin’ by an equal volume of interphase extract. Inset, extract depleted with control antibodies (lane 1) or anti-geminin antibodies (lane 2) immunoblotted for geminin. c, Histone H1 kinase activity of different extracts: lane 1, interphase extract; lane 2, metaphase extract; lane 3, geminin-depleted metaphase extract; lane 4, control-depleted metaphase extract. d, Morphology of sperm nuclei incubated in metaphase extract (i, ii), geminin-depleted metaphase extract (iii, iv) or interphase extract (v, vi), stained with Hoechst 33258 and viewed under ultraviolet fluorescence (i, iii, v) or phase-contrast optics (ii, iv, vi). Scale bar, 10 μm. e, The indicated dilution of depleted metaphase extract (open triangles, control-depleted; filled triangles, geminin-depleted), or untreated metaphase (open circles) or interphase (filled circles) extract was incubated for 20 min with ‘6-DMAP chromatin’, after which licensing was assessed.

Figure 5. Proteins associated with chromatin in different extracts. Sperm nuclei were incubated for 20 min in metaphase extract, in interphase extract, in metaphase extract supplemented with 0.5 or 3 mM 6-DMAP and 0.3 mM CaCl\textsubscript{2} (metaphase+6-DMAP) or in interphase extract supplemented with 7 or 35 nM geminin. Chromatin was isolated and immunoblotted for Orc1, Cdc6, Cdt1, Mmc7 and geminin.

Figure 6. Characterization of the licensing inhibitor in metaphase extracts. a, Metaphase-arrested extract was mixed with either geminin bead-puriﬁed RLF-B or interphase extract. The mixture was incubated for 20 min with ‘6-DMAP chromatin’, after which licensing was assessed. b, Metaphase extract was immunodepleted with anti-geminin antibodies (filled triangles) or non-immune antibodies (open triangles). Serial dilutions in LFB2/50 buffer of depleted extract or untreated metaphase extract (open circles) were then assayed for their ability to inhibit licensing of ‘6-DMAP chromatin’ by an equal volume of interphase extract. Inset, extract depleted with control antibodies (lane 1) or anti-geminin antibodies (lane 2) immunoblotted for geminin. c, Histone H1 kinase activity of different extracts: lane 1, interphase extract; lane 2, metaphase extract; lane 3, geminin-depleted metaphase extract; lane 4, control-depleted metaphase extract. d, Morphology of sperm nuclei incubated in metaphase extract (i, ii), geminin-depleted metaphase extract (iii, iv) or interphase extract (v, vi), stained with Hoechst 33258 and viewed under ultraviolet fluorescence (i, iii, v) or phase-contrast optics (ii, iv, vi). Scale bar, 10 μm. e, The indicated dilution of depleted metaphase extract (open triangles, control-depleted; filled triangles, geminin-depleted), or untreated metaphase (open circles) or interphase (filled circles) extract was incubated for 20 min with ‘6-DMAP chromatin’, after which licensing was assessed.
Discussion

In this paper we have shown that the previously unidentified RLF-B activity is provided by Cdt1 protein. We have shown that RLF-B/Cdt1 is specifically inhibited by geminin and that the two proteins can interact tightly with one another. We also show that, in *Xenopus* egg extracts, geminin is the major inhibitor of origin assembly in metaphase. When depleted of geminin, metaphase extracts become competent to assemble functional licensed replication origins. These results are in contrast with experiments in yeast showing that the inhibition of cyclin-dependent kinases late in the cell cycle is necessary and sufficient to permit origin reassembly.

We have purified the RLF-B component of the replication licensing system by exploiting its high affinity for recombinant geminin protein. Purified RLF-B consisted of a single major polypeptide of Mₐ ~75K that was recognized by antibodies against the *Xenopus* Cdt1 protein. Recombinant Cdt1 could provide RLF-B activity, whereas depletion of Cdt1 from extracts also removed RLF-B activity. Recent work has shown that, like RLF-B/Cdt1, Cdt1 is required for loading Mcm(2–7) proteins onto DNA²⁰,²¹. We have also demonstrated here that purified RLF-B can restore licensing activity to Cdt1-depleted extracts. Taken together, these results provide strong evidence for the identity of RLF-B and Cdt1.

We also provide evidence that RLF-B/Cdt1 is the major target of replication inhibition by geminin. The addition of recombinant geminin to *Xenopus* egg extracts blocks the loading of Mcm(2–7) onto chromatin and functional origin licensing. The inhibition of replication caused by recombinant geminin can be overcome by the addition of recombinant Cdt1 or purified RLF-B, whereas endogenous RLF-B/Cdt1 binds specifically to recombinant geminin. Further, ~50% Cdt1 in interphase *Xenopus* egg extracts is in an inactive form that seems to be in a complex with endogenous geminin. These results suggest that geminin is a highly specific inhibitor of RLF-B/Cdt1.

The simplest interpretation for the inhibition of RLF-B/Cdt1 by geminin is that the two proteins interact via their coiled-coil domains, leaving Cdt1 unable to provide RLF-B activity. This is consistent with the strong interaction seen between purified RLF-B/Cdt1 and recombinant geminin, which required 4 M urea to release purified RLF-B/Cdt1. A similar sort of interaction between recombinant geminin and endogenous RLF-B/Cdt1 probably occurs in vivo because the addition of recombinant geminin to interphase extract can be used to remove most endogenous RLF-B/Cdt1 (Figs 3b, 4a). However, residual RLF-B activity remained in these extracts after the removal of geminin, suggesting that there is something in whole extract that is capable of reducing the affinity
of these proteins for one another. In metaphase extracts the affinity of endogenous geminin and endogenous RLF-B/Cdt1 seems to be even weaker, because immunodepletion of geminin from metaphase extract left a significant proportion of RLF-B/Cdt1 behind. We are currently investigating the possible existence of factors that can modulate the interaction between RLF-B/Cdt1 and geminin. However, in the Xenopus cell-free system, unlike other components of the replication licensing system, RLF-B is absent during metaphase but is abruptly activated on exit into anaphase; it then declines again during interphase. The absence of RLF-B activity in metaphase is due to the presence of an RLF-B inhibitor, and the activation of RLF-B on progression into anaphase corresponds to the time when geminin undergoes cell-cycle-specific degradation. We show here that this inhibition is dependent on the presence of geminin and that, when geminin was immunodepleted from metaphase extracts, the extract became competent to assemble licensed replication origins on sperm chromatin. A similar effect was achieved by the addition of RLF-B/Cdt1 to metaphase extract. This suggests that the main reason for the inhibition of origin assembly in these metaphase extracts is the geminin-mediated repression of RLF-B/Cdt1 (Fig. 8a). This conclusion seems to contrast with results obtained in yeast. When CDK activity is abolished in G2 of the yeast cell cycle, chromosomal DNA is re-replicated, presumably as a consequence of the rebinding of Mcm(2–7) to origins. Further, the formation of pre-RCs (licensed origins) in yeast can only occur in G1, when CDK activity is low. Importantly, the ability to activate pre-RCs reassembly after CDK inactivation does not require the activity of the anaphase-promoting complex (APC/C) that is required for the cell-cycle-dependent degradation of proteins during anaphase. This suggests that, in yeast, relicensing that occurs as a consequence of CDK inactivation does not depend on the degradation of (currently unidentified) geminin homologues.

Previous work in the Xenopus system has also shown that the inhibition of CDK activity alone is not sufficient to induce relicensing of replicated DNA. When extracts arrested in G2 phase were treated with the CDK inhibitor p21Cip1/Waf1, relicensing of replicated DNA was not observed. When CDK inhibitors (6-DMAF, staurosporine or olomoucine) are added to metaphase extracts, they spontaneously exit into an interphase-like state while maintaining their metaphase-specific RLF-B inhibition. These observations are consistent with the conclusion of the present study, which shows that only when geminin is removed from metaphase extracts do the extracts lose their RLF-B/Cdt1 inhibitory activity and become competent to assemble licensed replication origins. Addition of 6-DMAF to metaphase extracts is known to block the degradation of cyclins that normally occurs on exit from metaphase. The lack of RLF-B activity in 6-DMAF-treated extracts is therefore likely to be due, at least in part, to a failure to degrade geminin, although other effects might also operate.

Although geminin seems to be the major activity responsible for blocking origin assembly in metaphase Xenopus extracts, CDKs clearly have a role. The inhibition of CDK activity in geminin-depleted metaphase extracts strongly stimulated origin assembly. Further, addition of large quantities of recombinant CDKs to Xenopus extracts inhibited origin assembly. Nevertheless, on exit from metaphase, both geminin and the mitotic cyclins are degraded, thus removing both inhibitory components of the origin assembly pathway (Fig. 8b).

The experiments presented here have addressed the role of geminin during meiosis II (unfertilized Xenopus eggs). However, a similar inhibition of RLF-B activity is seen in interphase of the embryonic (mitotic) cell cycle that is dependent on new protein synthesis but not on CDK activity. Further, a human geminin homologue in HeLa cells accumulates during interphase and disappears abruptly on exit from metaphase. We therefore propose that the interaction between geminin and RLF-B/Cdt1 has a conserved role in the mitotic cell cycles of higher eukaryotes.

Methods
Preparation of egg extracts
Metaphase-arrested and interphase Xenopus egg extracts were prepared as described. 6-DMAF-treat ed extracts were prepared by supplementing mitotic extracts with 10 μg/ml cycloheximide, 25 mM phosphocreatine, 15 μg/ml creatine phosphokinase, 3 mM MgATP and 20 μM A23187, before being spin-crushed in buffer lacking EGTA. Recovered cytoplasm was diluted 1:5 with LFB1 (40 mM Hepes–KOH (pH 8.0), 20 mM K2HPO4/KH2PO4 pH 8.0, 2 mM MgCl2, 1 mM EGTA, 2 mM DTT, 10% (v/v) sucrose, and 1 μg/ml each of leupeptin, pepstatin and aprotinin) supplemented with 30 mM KCl (i.e., LFB1/30) and reconflated at 20,000 r.p.m. for 30 min in an SW41 swinging-bucket rotor (Beckman). The clarified supernatant (LFE) was frozen and stored at –80°C.

Immunodepletion of the extracts was performed as described. In brief, Protein A-Sepharose beads were incubated with 2 volumes of serum for 1 h, and the beads were externally washed. Metaphase-arrested or interphase Xenopus egg extract (100 μl) was incubated with 36–50 μl Protein A–Sepharose beads for 45 min at 4°C. After removal of the beads by centrifugation through a 1.5 μm nylon filter, the process was repeated. This procedure led to a twofold dilution of the extract, and was therefore designated ‘×2.5’ in titration experiments. Geminin-bead-depleted extract was prepared in a similar way, except that 100 μl extract was incubated twice with 25 μl of geminin-coupled beads (see below) for 15 min.

Chromatin templates
Demembranated Xenopus sperm nuclei were prepared as described and frozen in aliquots in liquid nitrogen. Unlicensed 6-DMAF-treated chromatin was prepared by incubating sperm nuclei for 15 min at 23°C in 6-DMAF-treated extracts at 30,000 nuclei ml–1. The extract was then diluted tenfold in nuclear isolation buffer (20 mM Hepes-KOH (pH 7.6), 5 mM MgCl2, 1 mM dithiothreitol, 0.5 mM spermidine HCl, 0.15 mM spermine 4HCl, 1 μg/ml each of leupeptin, pepstatin and aprotinin) supplemented with 0.01% Triton X-100, and then underlayered with the same buffer containing 15% (v/v) sucrose. The chromatin was pelleted at 6000g in a swinging-bucket rotor at 4°C for 5 min. The diluted extract was then removed and the chromatin pellet was resuspended in NIB and frozen in aliquots in liquid nitrogen. Isolation of chromatin for immunoblotting was performed in a similar way, except that the Triton X-100 concentration was 1%.

Purification of RLF-B
All chromatographic procedures were performed at 4°C. After lysis, LFE was supplemented with 4.25% (v/v) PEG6000 (BDH), incubated on ice for 30 min, and centrifuged at 12,000g for 10 min in a fixed-angle rotor. The pellet was resuspended in LFB1 containing 150 mM KCl, and absorbed batch-wise for 15 min at 4°C onto freshly prepared, pre-cycled phosphocellulose (Whatman) equilibrated in LFB1 containing 150 mM KCl. After the phosphocellulose had been packed into a column, activity was eluted stepwise in LFB1 containing 500 mM KCl. The eluate was then supplemented with saturated ammonium sulphate in 50 mM Tris–HCl (pH 8) to a final concentration of 40% and incubated on ice for 40 min. Precipitated material was pelleted by centrifugation (12,000g for 10 min in a fixed-angle rotor) and was resuspended in LFB1 at 1/5 the volume of undiluted extract.

Recombinant protein and antibodies
Recombinant Xenopus geminin-DEL was produced from an expression plasmid kindly provided by T. McGarry, and was purified on Ni-agarose by standard techniques. Recombinant protein was used to generate rabbit antisera. Recombinant Xenopus Cdt1 (XcCdt1) was produced as described previously. Anti-XcCdt1 antibodies were as described previously.

Licensing assay
To assay licensing, 2 μl fractions (typically containing 1 μl RLF-B and 1 μl RLF-M, or 2 μl whole egg extract) were incubated with 0.3 μl of either RLF-M/Cdt1 or 0.3 μl demembranated Xenopus sperm nuclei at 80 ng/ml DNA and incubated for 20 min at 23°C. 6-
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