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Reconstitution of licensed replication origins on *Xenopus* sperm nuclei using purified proteins

Peter J Gillespie[1,2], Anatoliy Li[1] and J Julian Blow*[1]

Address: [1]CRC Chromosome Replication Research Group, Wellcome Trust Biocentre, University of Dundee, Dundee DD1 5EH, UK and [2]Current address: Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, New York 11724, USA

E-mail: Peter J Gillespie - peter.gillespie@cshl.org; Anatoliy Li - a.li@dundee.ac.uk; J Julian Blow* - j.j.blow@dundee.ac.uk

*Corresponding author

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Abstract

Background: In order to ensure precise chromosome duplication, eukaryotes "license" their replication origins during late mitosis and early G1 by assembling complexes of Mcm2-7 onto them. Mcm2-7 are essential for DNA replication, but are displaced from origins as they initiate, thus ensuring that no origin fires more than once in a single cell cycle.

Results: Here we show that a combination of purified nucleoplasmin, the origin recognition complex (ORC), Cdc6, RLF-B/Cdt1 and Mcm2-7 can promote functional origin licensing and the assembly of Mcm2-7 onto *Xenopus* sperm nuclei. The reconstituted reaction is inhibited by geminin, a specific RLF-B/Cdt1 inhibitor. Interestingly, the purified ORC used in the reconstitution had apparently lost the Orc6 subunit, suggesting that Orc6 is not essential for replication licensing. We use the reconstituted system to make a preliminary analysis of the different events occuring during origin assembly, and examine their nucleotide requirements. We show that the loading of *Xenopus* ORC onto chromatin is strongly stimulated by both ADP, ATP and ATP-γ-S whilst the loading of Cdc6 and Cdt1 is stimulated only by ATP or ATP-γ-S.

Conclusions: Nucleoplasmin, ORC, Cdc6, RLF-B/Cdt1 and Mcm2-7 are the only proteins required for functional licensing and the loading of Mcm2-7 onto chromatin. The requirement for nucleoplasmin probably only reflects a requirement to decondense sperm chromatin before ORC can bind to it. Use of this reconstituted system should allow a full biochemical analysis of origin licensing and Mcm2-7 loading.

Background

During S phase of the eukaryotic cell division cycle the entire genome must be faithfully duplicated. The many thousands of replication forks involved in this process must be co-ordinated to ensure that, despite the very large quantities of DNA involved, no section of DNA is left unreplicated and no section of DNA is replicated more than once. Cells achieve this by dividing the process of replication origin activation into two distinct phases [1–3]. During late mitosis and early G1, proteins are assembled onto replication origins which culminates in the origin becoming 'licensed' for a single round of DNA replication by loading complexes of Mcm2-7 proteins [4–8]. In yeast, a 'pre-replicative complex' (pre-RC) forms a footprint over replication origins during G1 [9] which may well correspond to the Mcm2-7 loading that

represents the licensed state, although it is possible that other factors in addition to those required for origin licensing are involved. During S phase, replication forks are initiated at licensed replication origins / pre-RCs following activation of the Cdc7 kinase and S phase cyclin-dependent kinases (CDKs). Mcm2-7 are displaced from the origin as initiation occurs, thereby ensuring that each origin fires no more than once in each cell cycle. Following displacement from origins, Mcm2-7 appear to move along with the replication fork [10], consistent with their providing essential helicase activity for the progression of the replication fork [11].

Three other proteins are known to be required for licensing replication origins and loading of Mcm2-7 onto them [1,2,12]: the Origin Recognition Complex (ORC), Cdc6 and RLF-B/Cdt1 [13–19]. ORC is first required on the DNA in order to recruit Cdc6 and RLF-B/Cdt1. The continued presence of ORC, Cdc6 and RLF-B/Cdt1 are not required for Mcm2-7 to remain bound to DNA once licensing has occurred [18,20–22]. By restricting the activity of ORC, Cdc6 or RLF-B/Cdt1 to a period between late mitosis and the end of G1, the relicensing of replicated origins can therefore be prevented. At least two activities present at other periods of the cell cycle, geminin (an RLF-B/Cdt1 inhibitor) and CDKs, are known to inhibit origin licensing and pre-RC assembly [1,2,12,19,23].

In order to understand how origin licensing and Mcm2-7 loading are regulated, we have been purifying from *Xenopus* egg extracts all the proteins required to support functional origin licensing [3]. We chose *Xenopus* sperm nuclei as the DNA template because it is the natural substrate for replication in *Xenopus* eggs and because it is replicated very efficiently in the cell-free system. Using this assay we have previously shown that the licensing of *Xenopus* sperm nuclei requires ORC, Cdc6, RLF-B/Cdt1 and Mcm2-7 [5,15,16,19]. We have also shown that this process requires nucleoplasmin [24], which is needed to remove the sperm basic proteins from sperm nuclei and decondense the chromatin [25,26], which in turn is required for ORC to bind to the chromatin [24].

In the present report we show that a combination of these purified proteins (nucleoplasmin, ORC, Cdc6, RLF-B/Cdt1 and Mcm2-7) can efficiently license *Xenopus* sperm nuclei and assemble Mcm2-7 onto it. We show that the reconstituted reaction is dependent on ATP and is inhibited by geminin. We use the reconstituted system to make a preliminary analysis of the different events occuring during the licensing of replication origins.

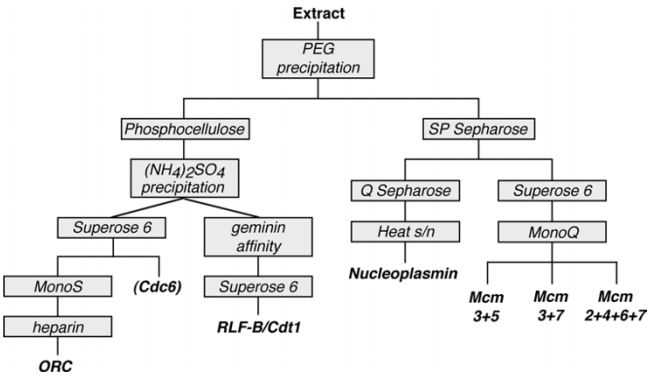

Figure 1
Fractionation scheme for proteins used in this study. Interphase Xenopus egg extract was fractionated by the methods outlined. See Materials and Methods for more details. Cdc6 was not purified to homogeneity but was replaced in the reconstituted reaction with recombinant material produced in insect cells.

Results

Characterization of protein fractions

We have been performing a systematic fractionation of *Xenopus* egg extracts to identify all the proteins required to reconstitute functionally licensed replication origins on *Xenopus* sperm nuclei [5,8,15,16,19,24,27]. The sequential separation of activities present in whole egg extract is summarised in Figure 1. Preliminary reconstitution experiments suggested that a combination of nucleoplasmin, ORC, Cdc6, RLF-B/Cdt1, and Mcm2-7 were sufficient to support efficient origin licensing of *Xenopus* sperm nuclei (PJG, data not shown). During the fractionation, no additional activities that stimulate origin licensing were observed [[5,8,15,16,19,24,27] and data not shown]. In addition to this scheme, we also prepared three of the proteins (nucleoplasmin, Cdc6 and RLF-B/Cdt1) by different means. Nucleoplasmin was purified by a different published protocol [24,28], recombinant Cdc6 was prepared from baculovirus-infected insect cells [14] and recombinant RLF-B/Cdt1 was prepared from *Escherichia coli*[18,19].

Figure 2 shows Coomassie gels of the purified proteins. Purified nucleoplasmin showed a number of different bands that correspond to different phosphorylated forms [28,29] (Fig 2a). Purified ORC yielded 5 specific bands, corresponding to XOrc1, 2, 3, 4 and 5 [15,30–32] (Fig 2b). No band corresponding to XOrc6 was observed, and to date no *Xenopus ORC6* gene has been cloned. ORC purified from HeLa cell chromatin has also been reported to lack Orc6 [33], and in *S. cerevisiae* Orc6 is not required for ORC to bind origin DNA [34]. We also observed two additional proteins that contaminate the *Xenopus* ORC preparation [15]. These proteins were

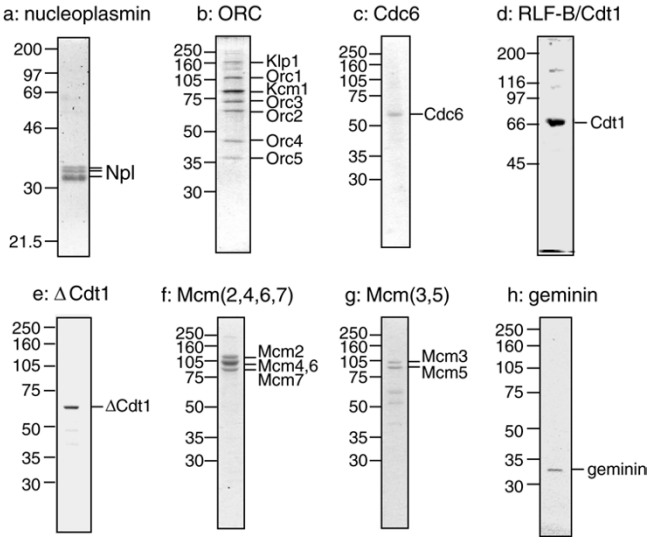

Figure 2
Purified proteins used in this study. Purified proteins were run on SDS polyacrylamide gels, and visualized with either Coomassie Blue (a-c, e-h) or silver staining (d). **a,** Nucleoplasmin purified from *Xenopus* eggs. **b,** ORC purified from *Xenopus* eggs. **c,** Recombinant *Xenopus* Cdc6 purified from baculovirus-infected insect cells. **d,** RLF-B/Cdt1 purified from *Xenopus* eggs. **e,** Recombinant *Xenopus* ΔCdt1 purified from *E. coli*.**f,** Mcm(2+4+6+7) purified from *Xenopus* eggs. **g,** Mcm(3+5) purified from *Xenopus* eggs. **h,** Recombinant *Xenopus* geminin purified from *E. coll.*

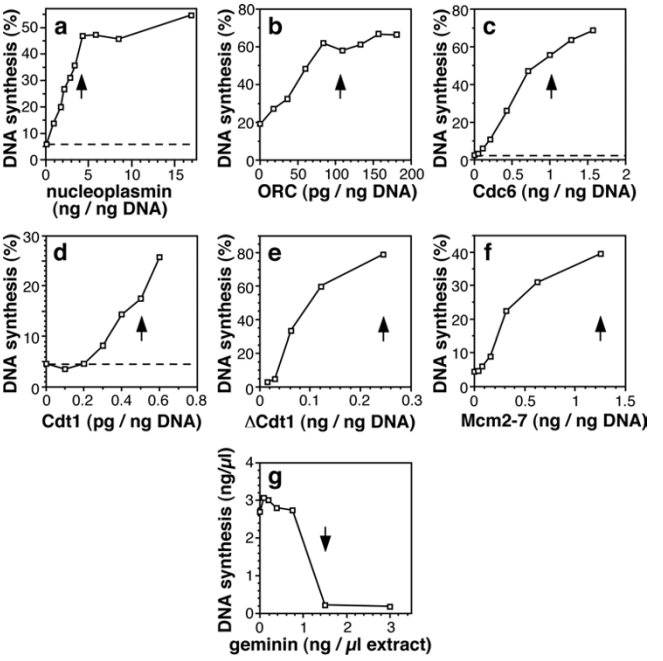

Figure 3
Activity of the purified proteins. Different quantities of purified proteins were subjected to specific individual assays, using *Xenopus* sperm nuclei as substrate. Protein quantity is given as mass of protein per ng template DNA (a-f) or mass of protein per μl extract (g). DNA synthesis is expressed as % of the DNA template replicated (a-f), or ng DNA synthesised per μl extract (g). **a,** Nucleoplasmin activity required in a crude licensing assay. **b,** ORC activity required for licensing in an ORC-depleted extract. **c,** Cdc6 activity required for licensing in an Cdc6-depleted extract. **d, e,** RLF-B activity required in a crude licensing assay. **f,** RLF-M/Mcm2-7 activity required in an Mcm2-7-depleted extract. **g,** Replication inhibition by geminin in whole egg extract containing 3 ng/μl DNA. Arrows show the protein quantity used in subsequent reconstitution experiments.

subjected to MALDI-TOF analysis and were identified as *Xenopus* Klp1 and Kcm1 [35,36]. The chromatographic behaviour of these proteins suggests that they do not form complexes with ORC [[15] and data not shown]. Further, immunodepletion of ORC from *Xenopus* egg extracts did not co-deplete XKlp1 (data not shown). We therefore conclude that Klp1 and Kcm1 are fortuitous contaminants of our ORC preparation. Recombinant *Xenopus* Cdc6 (Fig 2c) [14], purified RLF-B/Cdt1 (Fig 2d) [19] and recombinant ΔCdt1 (amino acids 141–621 of Xenopus Cdt1, Fig 2e) [18] showed a single band of the expected molecular weight. Complexes of *Xenopus* Mcm(2+4+6+7) and Mcm(3+5) were purified from *Xenopus* egg extracts as described [24,27] (Figs 2f and 2g). The Mcm(3+5) contained two contaminating bands of ~52 and ~58 kDa, which we were unable to identify by mass spectrometry. However, their chromatographic behaviour suggests that they are not components of the Mcm2-7 complex [24,27]. Recombinant geminin was prepared from *E. coli* as described (Fig 2h) [37].

Specific assays for individual proteins

In order to minimise the risk that any of these fractions were contaminated with other activities required for origin licensing, we wanted to ensure that each of them was used in the reconstitution at limiting quantities. We therefore titrated each of the purified fractions into a separate assay that was specific for each activity (Fig 3). Nucleoplasmin reached an optimum at ~4 ng / ng template DNA for both a crude licensing reaction (Fig 2a) and sperm decondensation [24]. This is approximately equal to 1 nucleoplasmin pentamer for each 64 bp DNA, close to the expected density of protamines on sperm DNA and consistent with the role of nucleoplasmin being to decondense the chromatin by removal of protamines [24].

ORC was assayed by titrating into an extract previously immunodepleted with anti-Orc1 antibodies, an assay specific for licensing activities co-depleted with ORC (Fig 3b). Purified ORC reached an optimum at ~85 pg / ng DNA, equal to one molecule of ORC per ~6 kb template

DNA. This is close to the expected density of ORC on *Xenopus* sperm chromatin in the early embryo (~1 ORC per 10–15 kb) [15] and close to the spacing of replication origins on sperm chromatin (1 origin every ~10 kb) [38]. It is notable that the purified ORC, lacking detectable Orc6, can efficiently restore activity to ORC-depleted extracts.

Recombinant Cdc6 was assayed by titrating into an extract previously immunodepleted with anti-Cdc6 antibodies, an assay specific for Cdc6 or activities co-depleted with it (Fig 3c). Recombinant Cdc6 restored licensing (50% of the template) at 0.75 ng / ng template DNA. This represents 1 molecule of Cdc6 per ~125 bp DNA, somewhat higher than is available in whole egg extract [14]. This suggests that, consistent with previous reports [14], the recombinant protein is only partially active.

When purified RLF-B/Cdt1 was titrated into a crude licensing assay, no plateau of activity was reached value, with 20–25% of template becoming licensed with ~0.6 pg / ng DNA template, equal to one molecule of RLF-B/Cdt1 per ~35 kb template DNA (Fig 3d). In a similar assay, recombinant ΔCdt1 supported licensing (~60% of the template) at ~0.12 ng / ng template DNA (Fig 2e), which represents 1 molecule ΔCdt1 per ~700 bp template DNA. Like the recombinant Cdc6, recombinant ΔCdt1 therefore appears to have lower specific activity than the protein purified from whole egg extract.

Purified Mcm2-7 was produced by mixing equal quantities of the Mcm(3+5) dimer and the Mcm(2+4+6+7) tetramer [24,27]. It was assayed by titration into an extract previously immunodepleted with anti-Mcm3 and Mcm7 antibodies, an assay specific for Mcm2-7 or activities co-depleted with them. The activity of purified Mcm2-7 started to plateau at about 1.25 ng / ng template DNA (Fig 3f). This corresponds to 1 heterohexamer per ~700 bp template DNA, close to the Mcm2-7 density found on sperm chromatin in whole egg extract [39]. The difficulty in providing enough Mcm2-7 activity appears largely to be due to having to keep the proteins at high enough concentration (~600 μg / ml in whole egg extract), whilst the specific activity of the purified proteins remains fairly high [24,39].

Recombinant geminin was assayed by its ability to inhibit licensing in whole egg extract. Since this appears to be related more to the concentration of RLF-B/Cdt1 in whole extract (~200 pg / μl) rather than the concentration of DNA (data not shown), we have expressed the titration in terms of ng geminin / μl extract. Fig. 2g shows that geminin inhibits origin licensing in whole egg extract at ~1.5 ng / μl, consistent with previous reports [19,37].

Reconstitution of origin assembly with purified proteins

We next incubated the purified proteins together with *Xenopus* sperm nuclei to see if they were sufficient to support efficient origin licensing. Since our major concern here was to reduce the risk of undetected contaminants playing an essential role in the assay, we used all the purified proteins at limiting concentrations as indicated by the arrows in Figure 3. Origin licensing was assessed in two different ways. The first was a functional assay, based on the ability of the kinase inhibitor 6-dimethylaminopurine (6-DMAP) to specifically block origin licensing activity as originally defined in *Xenopus* egg extracts [4,40,41]. Egg extracts treated with 6-DMAP prior to exit from metaphase ("6-DMAP extracts") fail to degrade geminin, a specific inhibitor of origin licensing [19,23,37,42] and so will only replicate chromatin that contains licensed replication origins. Previous work has shown that under the assay conditions used, there is close correlation between the number of licensed origins and the degree of replication [38,39]. Mcm2-7 were first shown to be involved in once-per-cell cycle replication using this type of assay [5–8]. The second assay we used was to detect the quantity of Mcm2-7 loaded onto the sperm chromatin. This binding assay is slightly less satisfactory than the functional assay for two reasons: first, up to 10–20 Mcm2-7 heterohexamers can load onto each origin, although only ~2 per origin are required to give maximal replication rates [27,39]; second, intermediates in the Mcm2-7 assembly pathway can bind to chromatin, but these intermediates appear to be non-functional and their assembly is independent of other origin proteins [27]. We have therefore concentrated most effort on the functional assay.

Figure 4 shows the results of assays showing that licensed origins can be assembled with good efficiency (corresponding to 18–29% of origins) with a combination of purified nucleoplasmin, ORC, Cdc6, RLF-B/Cdt1 and Mcm2-7. When any one of these components was omitted from the reaction, licensing dropped to background levels. Further, functional origin licensing correlated with the assembly of Mcm7 onto the sperm chromatin (Fig 4b; see also Fig 6 for Mcm3). The highest activity was obtained with a preparation of RLF-B/Cdt1 taken prior to the final gel filtration step which has very high specific activity [19] (Fig 4a). However, both the completely purified RLF-B/Cdt1 (Fig 4c) and recombinant ΔCdt1 (Fig 4d) supported efficient origin licensing. Because the efficiency of the reconstitution (Fig 4) was not greatly lower than the activity of the individual proteins put into the reaction (Fig 3), we conclude that the licensing of sperm nuclei depends only on the five pu-

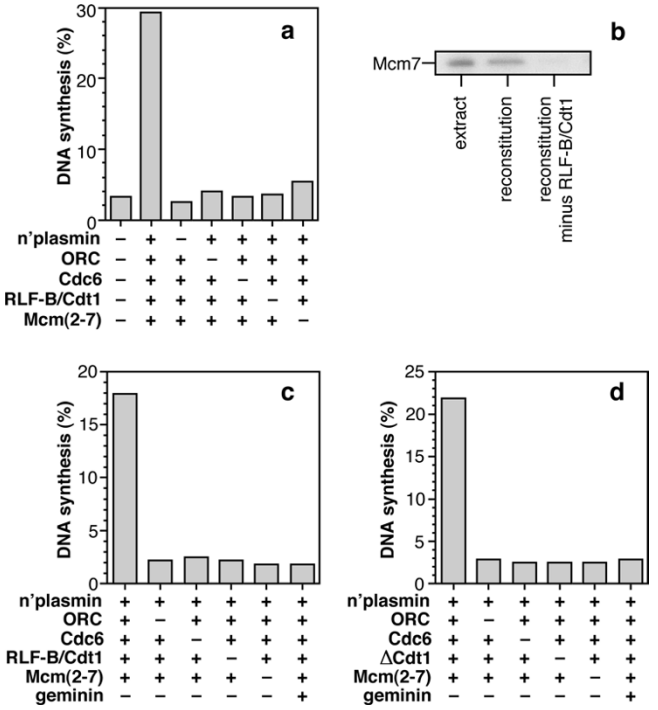

Figure 4
Reconstitution of licensing using purified proteins. **a, c, d,** Licensing activity of different combinations of purified proteins incubated in the presence of 2.5 mM ATP. DNA synthesis is expressed as % of the DNA template replicated. **b,** Western blot of Mcm7 loaded onto sperm chromatin in whole *Xenopus* extract, the full reconstituted reaction and the reconstituted reaction lacking RLF-B/Cdt1. The reconstituted reactions contained 2.5 mM ATP.

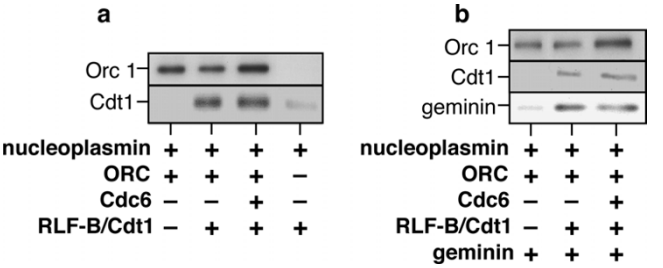

Figure 5
Requirements for Cdt1 and geminin association with chromatin. **a, b,***Xenopus* sperm nuclei were incubated with the indicated proteins in the presence of 2.5 mM ATP. Chromatin was then isolated and immunoblotted for the presence of bound Orc1, Cdt1 and geminin. The RLF-B/Cdt1 was a slightly more active fraction taken from the geminin bead eluate.

rified activities and is unlikely to depend on any other contaminating activity.

Geminin has recently been shown to form a complex with RLF-B/Cdt1 and inhibit its function [19,23]. Fig 2 shows that complete inhibition of licensing in whole extract was achieved with geminin at 1.5 ng / μl. Figures 4c and 4d show that at the same concentration recombinant geminin also inhibited licensing in the reconstituted reaction. This suggests that the inhibition of RLF-B/Cdt1 by geminin is a direct consequence of their interaction and does not require any additional factors.

Order of assembly

We next asked whether the binding of Cdt1 and geminin to chromatin was dependent on the other proteins. Although Cdt1 has been shown to bind to chromatin in the absence of Cdc6 in *Schizosaccharomyces pombe,* this has not been shown in metazoans. Figure 5a shows that whilst the binding of Cdt1 to chromatin depended on the presence of ORC, it was not affected by the presence of Cdc6. Unfortunately we could not determine whether the binding of Cdc6 to chromatin was dependent on the presence of RLF-B/Cdt1, because some recombinant Cdc6 bound to decondensed chromatin even in the absence of ORC. This is different to its behaviour in whole extract, where the binding of Cdc6 to chromatin is dependent on ORC [14,16]. Two possible explanations are that our recombinant Cdc6 preparation may contain denatured or precipitated protein (see above) or that the non-specific binding of Cdc6 to sperm chromatin is normally competed by the binding of other chromatin-associated proteins not present in our reconstitution.

We have previously shown that when recombinant geminin is added to *Xenopus* egg extract, it associates with sperm chromatin [19]. In Figure 5b we have investigated the requirement for this association using purified proteins. Geminin associated only weakly with decondensed chromatin in the presence of ORC. Recruitment of geminin to the chromatin was greatly stimulated by the presence of ORC and RLF-B/Cdt1. The presence of Cdc6 did not affect the quantity of geminin bound to the chromatin. This suggests that the recruitment of geminin to chromatin is mediated by its tight association with RLF-B/Cdt1 [19,23], which in turn is independent of Cdc6 (Fig 5a).

Nucleotide requirements

We have previously shown that the licensing of *Xenopus* sperm chromatin by crude fractions requires ATP, and this ATP requirement cannot be fulfilled by non-hydrolysable nucleotide analogues such as ATP-γ-S or AMP-PNP [5]. Figure 6a shows a similar requirement for ATP with the reconstituted system. Neither ADP nor ATP-γ-S could substitute for ATP. Figure 6b shows the proteins loaded onto chromatin in reactions containing these dif-

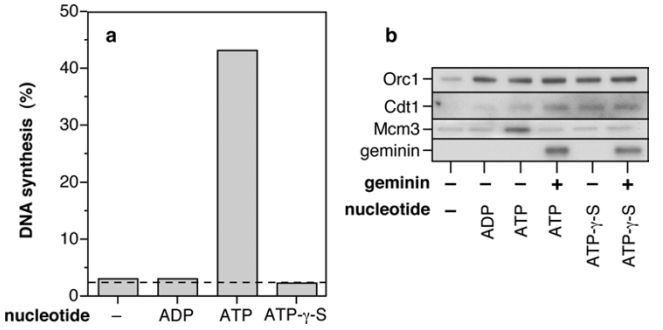

Figure 6
Nucleotide requirements of the reconstituted reaction. *Xenopus* sperm nuclei were incubated with nucleoplasmin, ORC, Cdc6, RLF-B/Cdt1, Mcm2-7 plus or minus geminin in the presence of either ADP, ATP, ATP-γ-S at 2.5 mM, or no added nucleotide. **a,** The degree of functional licensing was assessed, expressed as % of the DNA template replicated. **b,** Chromatin was isolated and immunoblotted for the presence of Orc1, Cdt1, Mcm3 and geminin. The Mcm2-7 was a slightly more active fraction taken from the gel filtration step in the purification, and the RLF-B/Cdt1 was from the geminin bead eluate.

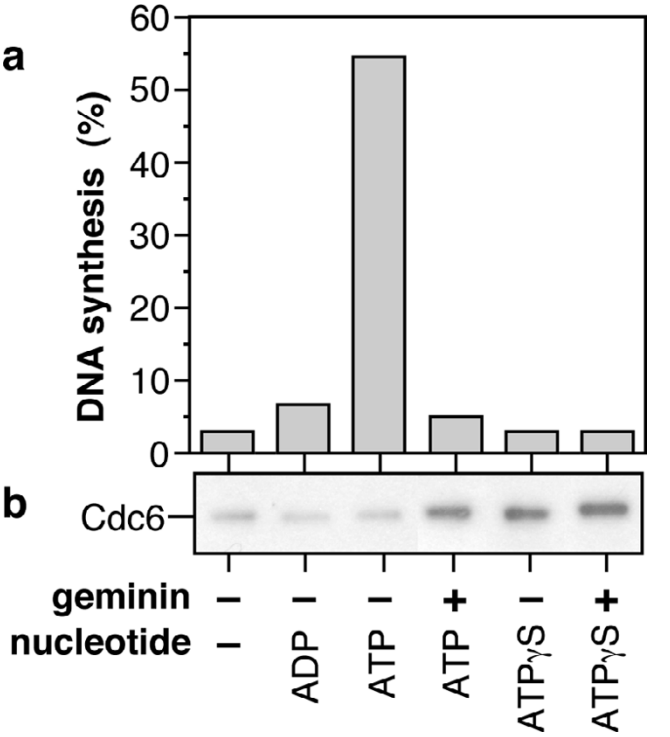

Figure 7
Nucleotide requirement for Cdc6 chromatin association in desalted extracts. Sperm nuclei were incubated in desalted whole egg extract plus or minus geminin in the presence of either ADP, ATP, ATP-γ-S at 2.5 mM, or no added nucleotide. **a,** The degree of functional licensing was assessed, expressed as % of the DNA template replicated. **b,** Chromatin was isolated and immunoblotted for the presence of Cdc6.

ferent nucleotides. ORC loading was weak in the absence of any nucleotide, but was strongly stimulated by ADP, ATP or ATP-γ-S. In *S. cerevisiae,* ORC binds specifically to origin DNA in the presence of ATP or ATP-γ-S [43,44], though data about ADP has not been published. Cdt1 binding to chromatin was low in the absence of nucleotide, and was only slightly stimulated by the presence of ADP, despite ORC binding becoming maximal. Further stimulation of Cdt1 binding was seen in the presence of ATP, as origin licensing occurred. However, the highest level of Cdt1 binding was seen in samples containing both ATP and geminin, or in samples containing ATP-γ-S, conditions when licensing did not occur (Fig 6b). This is similar to previous results suggesting that ORC and Cdc6 become destabilised at origins once licensing has occurred [22].

In order to investigate the requirement for nucleotides in the binding of Cdc6 to chromatin, we used a crude extract, from which most nucleotides and other small molecules had been removed by gel filtration. Consistent with the results obtained with the purified proteins, gel-filtered extracts only supported efficient origin licensing when supplemented with hydrolysable ATP (Fig 7a). High levels of Cdc6 binding to chromatin were seen only in samples containing both ATP and geminin, or in samples containing ATP-γ-S (Fig 7b). Under these conditions no licensing occurred (Fig 6a, 7a) and high levels of Cdt1 were bound to chromatin (Fig 6b). The lack of strong Cdc6 binding in the presence of ATP is consistent with our previous results showing that Cdc6 does not bind tightly to chromatin once licensing has occurred ("licensing-dependent origin inactivation") [22,45]. Taken together, these results suggest a model where high levels of Cdc6 and Cdt1 bind to chromatin only in the presence of nucleotide triphosphate (ATP or ATP-γ-S), but where the affinity of both proteins is reduced as a consequence of licensing.

Discussion

A significant amount of work in both *Xenopus* and yeast has detailed the sequential deposition of ORC, Cdc6, RLF-B/Cdt1 and finally Mcm2-7 onto replication origins early in the cell cycle [5–8,10,14,15,17–20,24,46–49]. However, the licensing reaction has not before been reconstituted with purified proteins, which is a pre-requisite for a proper biochemical analysis of the reaction. Here we describe the results of a systematic purification of the proteins which have allowed us to license *Xenopus* sperm nuclei with purified proteins.

Reconstitution of licensed origins with purified proteins

We have shown that licensed replication origins can be efficiently assembled on *Xenopus* sperm nuclei using a combination of nucleoplasmin, ORC, Cdc6, RLF-B/Cdt1 and Mcm2-7. The reconstitution was assessed in two separate ways: first by a functional assay where the initiation of DNA replication is strictly dependent on the presence of licensed replication origins as originally defined in *Xenopus* egg extracts [4,40,41], and secondly by assessing the loading of Mcm3 and Mcm7 onto chromatin. The requirement of all these proteins is consistent with previous work on the assembly of replication origins in a number of eukaryotic organisms. However, it is currently unclear whether this set of proteins (excluding nucleoplasmin) will also generate the pre-RC footprint over replication origins in *S. cerevisiae,* as this may well involve other proteins [13,47].

We provide three lines of evidence strongly suggesting that no other undetected activities contaminating our purified proteins play an essential role in origin licensing. First, during our extensive fractionation of *Xenopus* egg extracts [[5,8,15,16,19,24,27] and data not shown], we have not detected any unidentified fractions that significantly stimulate origin licensing. Second, the proteins we used have been purified in a number of different ways and contained only a small number of visible contaminating proteins. Third, the efficiency of the reconstituted reaction was high, resulting in ~18–28% of potential origins being functionally licensed. In order to minimise the risk of introducing contaminants into the assay, all the individual activities were used at limiting quantities, giving 20–80% licensing of template DNA. The efficiency of the reconstituted reaction was therefore not significantly lower than the activities of the individual components, strongly suggesting that no other activities are required. Although it is never possible to prove that purified proteins do not contain functionally important contaminants, we believe that these three independent lines of evidence strongly argue against a requirement for other proteins in the reconstitution.

The purified ORC we used was devoid of visible Orc6, but despite this, could efficiently support replication licensing in both crude and purified assays. This strongly suggests that Orc6 is not required for origin licensing. It is also noteworthy that no homologue of the *S. cerevisiae* Mcm10/Dna43 protein was required for the reconstitution. Mcm10/Dna43 is required for the initiation of DNA replication, in *S. cerevisiae*, and a recent report has shown that when temperature-sensitive mutants of *MCM10/DNA43* are moved to the restrictive temperature in G1, Mcm2 is released from the chromatin [50]. Since we find no requirement for Mcm10/Dna43 for Mcm2-7 loading, one possible interpretation of this result is that Mcm10/Dna43 is required for stabilisation, rather than establishment, of Mcm2-7 complexes on chromatin.

Stages of origin assembly

The DNA in *Xenopus* sperm nuclei is held in a tightly condensed state by the sperm basic proteins. Removal of these protamines from the sperm DNA, mediated by nucleoplasmin [25], is necessary for ORC to bind to the DNA [24] (Fig 8a,b). This step is almost certainly unique for sperm nuclei, as most other nuclear templates do not contain abundant protamines. We have shown that the binding of ORC to decondensed sperm chromatin required the presence of nucleotides (Fig 8c). This requirement could be fulfilled by ATP, ATP-γ-S or ADP. In *S. cerevisiae* and *Drosophila,* ORC, the specific binding of ORC to origin DNA is dependent on ATP or non-hydrolysable ATP analogues [43,44,51], though the ability of ADP to substitute for this function has not been described.

Once ORC binding has occurred, Cdc6 and RLF-B/Cdt1 can be recruited to the DNA (Fig 8d) [14,17,18,52]. The binding of Cdt1 and Cdc6 to ORC-containing DNA can occur independently of one another [[17,18] and this paper]. We show that the recruitment of *Xenopus* Cdc6 to chromatin required ATP or a non-hydrolysable ATP analogue, consistent with results obtained with *S. cerevisiae* Cdc6 [52,53]. However, it is currently unclear whether the ATP needs to bind ORC or Cdc6 to promote Cdc6 binding [47,48]. We also show for the first time that the binding of RLF-B/Cdt1 to chromatin is stimulated by ATP or ATP-γ-S. Quantitation of the purified RLF-B/Cdt1 suggested that one molecule could license ~35 kb DNA, which is expected to contain 3–4 origins. One possible explanation for the full activity of sub-stoichiometric amounts of RLF-B/Cdt1 is that it can be rapidly released from replication origins (perhaps coupled to ATP hydrolysis), so that one molecule of RLF-B/Cdt1 can act sequentially at a number of different origins.

The final step in assembling a licensed origin is the assembly of Mcm2-7 onto chromatin (Fig 8e). This step requires ATP hydrolysis [5,53] and can lead to the binding of 10–20 copies of Mcm2-7 to each origin [39,54,55]. Once Mcm2-7 loading has occurred (which depends on ORC, Cdc6 and RLF-B/Cdt1), their continued binding to DNA does not require the continued presence of ORC or Cdc6 [20–22]. Indeed, the affinity of Cdc6 [22] and RLF-B/Cdt1 (this paper) for chromatin appears to drop once licensing is complete, and maximal levels of these proteins on chromatin are only seen when origin licensing is blocked and in the presence of ATP or ATP-γ-S (Fig 8f). This process, which we have termed "licensing-dependent origin inactivation" [22] does not depend on later

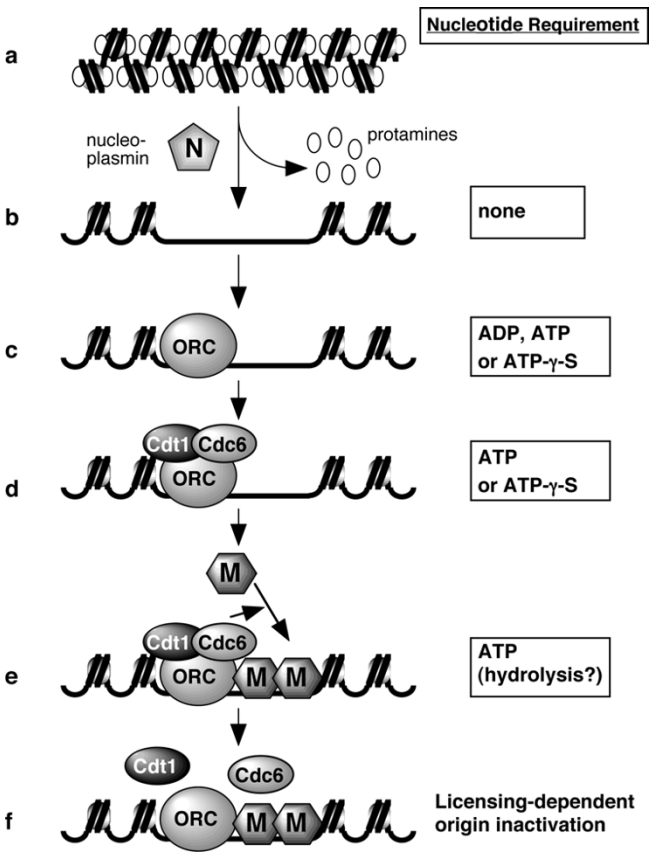

Figure 8
Stages in the assembly of licensed replication origins on sperm nuclei. A cartoon of a small segment of *Xenopus* sperm DNA is shown following exposure to egg cytoplasm. Nucleotide requirements are shown on the right. **a,** Condensed sperm chromatin, with H2A and H2B replaced by protamines. **b,** Removal of protamines by nucleoplasmin. **c,** Assembly of ORC onto origins, requiring ADP, ATP or ATP-γ-S. **d,** Assembly of Cdc6 and RLF-B/Cdt1 onto ORC-containing DNA, requiring ATP or ATP-γ-S. **e,** The loading of Mcm2-7 hetero-hexamers onto chromatin represents origin licensing. 10–20 copies of Mcm2-7 can be assembled onto each origin. This step requires hydrolysable ATP. **f,** Once licensing is complete, the affinity of Cdc6 and RLF-B/Cdt1 for origins is decreased (licensing-dependent origin inactivation).

steps in initiation such as CDK or Cdc7 activation [45], and seems to occur in the reconstituted reaction.

Regulation of Origin Licensing

Origin licensing can be inhibited in at least two ways [1,2,12]: work in yeast has shown that Cdk activity late in the cell cycle is necessary to prevent the re-licensing (and hence re-firing) of replicated origins, whereas recent work in *Xenopus* has shown that inhibition of RLF-B/Cdt1 by geminin is also a physiologically important mechanism in regulating the replication licensing system during the cell cycle [19,23,37]. We show here that geminin can inhibit origin licensing in the fully reconstituted system, suggesting that inhibition by geminin does not require the presence of any other factor. We also show that geminin is also recruited to chromatin in the reconstituted system, and that this is dependent on ORC and RLF-B/Cdt1, but not on Cdc6. The reconstituted reaction should allow us to explore in detail the way that geminin and CDKs regulate the assembly of different origin components during the cell division cycle.

Conclusions

This work shows that origin licensing and Mcm2-7 loading can be reconstituted on *Xenopus* sperm nuclei with a combination of nucleoplasmin, ORC, Cdc6, RLF-B/Cdt1 and Mcm2-7. The ORC used in the reconstituted reaction apparently lacked the Orc6 subunit, suggesting that it is not required for replication licensing. The reaction required hydrolysable ATP, though ORC loading could be promoted by ADP, and the loading of Cdc6 and RLF-B/Cdt1 could be promoted by ATP-γ-S. The reconstituted reaction could also be directly inhibited by geminin. The demonstration that the origin licensing and Mcm2-7 loading can be reconstituted with purified proteins now opens the way for a detailed biochemical analysis of this reaction.

Materials and Methods

Preparation of extracts and DNA templates

Metaphase-arrested *Xenopus* egg extracts were prepared as described [56]. They were supplemented with 250 μg/ml cycloheximide, 25 mM phosphocreatine, 15 μg/ml creatine phosphokinase, and [α-^{32}P]dATP as appropriate, and were then released into interphase with 0.3 mM $CaCl_2$. To make "6-DMAP extract" for licensing assays, extracts were supplemented with 3 mM 6-dimethylaminopurine (6-DMAP) prior to being released into interphase with $CaCl_2$[40,56]. Immunodepletion of interphase extracts with antibodies against Orc1, Cdc6, Mcm3+7 was performed as described [56].

Desalted interphase extract was prepared as follows: 2 ml pre-swollen Sephadex G-25 (Amersham Pharmacia Biotech) was packed into a column and equilibrated in LFB1 (40 mM Hepes KOH pH 8.0, 20 mM K_2HPO_4/KH_2PO_4 pH 8.0, 2 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 10% (w/v) sucrose and 1 μg/ml each of leupeptin, pepstatin and aprotinin) supplemented with 50 mM KCl (i.e. LFB1/50). The column was tested for the separation of bulk protein from [α-^{32}P]dATP. 200 μl of metaphase extract supplemented with cycloheximide was released into interphase with $CaCl_2$ for 15 min at 23°C. Extract was then applied to the Sephadex G-25 column under gravity and 80 μl drip fractions were collected. 5 fractions, from the peak of protein elution were collected,

pooled and stored under liquid nitrogen in 20 µl beads until required. The final extract recovered was diluted ~3-fold.

"Licensing factor extract", which was used as the starting point for all the purifications was prepared as described [5,56]. Briefly, eggs were activated for 5 min by the calcium ionophore A23187, before being spin-crushed in buffer lacking EGTA. Recovered cytoplasm was diluted 1:5 with LFB1/50 and re-centrifuged at 20,000 rpm for 30 min in an SW41 swinging bucket rotor (Beckman). The clarified supernatant was frozen and stored at -80°C until required.

Demembranated *Xenopus* sperm nuclei were prepared as described [56] and frozen in aliquots in liquid nitrogen. Unlicensed "6-DMAP chromatin" [56] was assembled by incubating sperm nuclei for 15 min at 23°C in 6-DMAP treated extracts at 30,000 nuclei / µl; extract was then diluted 10-fold in nuclear isolation buffer (NIB: 50 mM KCl, 50 mM Hepes KOH pH 7.6, 5 mM $MgCl_2$, 2 mM DTT, 0.5 mM spermidine 3HCl, 0.15 mM spermine 4HCl, 1 µg/ml each of leupeptin, pepstatin and aprotinin), and underlayered with 100 µl of the same buffer containing 15% sucrose. The chromatin was pelleted at 6,000 *g* 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.

Purification of proteins

Nucleoplasmin was purified from "licensing factor extract" by the protocol of Sealy et al. [28]. RLF-B/Cdt1 was purified as described by Tada et al. [19]. Because of the very low quantity of RLF-B/Cdt1 produced, protein concentration was roughly estimated using quantitative Western blotting. For some assays (as indicated) the eluate from the geminin affinity column was used instead of the final gel filtration material. Recombinant *Xenopus* ΔCdt1 was prepared using the bacterial expression construct described by Maiorano et al. [18]. Inclusion bodies prepared from *E. coli* 4 hr after induction were solubilised in 8 M urea and purified on Nickel NTA agarose (Qiagen) in the presence of 8 M urea. The eluate was dialysed sequentially against THED/400 buffer (20 mM Hepes-KOH pH 8, 20% ethylene glycol, 1 mM dithiothreitol, 0.03% Triton X-100 plus 400 mM KCl) containing urea at 8 M, 4 M, 2 M, 1 M, and finally 0 M. Recombinant *Xenopus* Cdc6 was prepared using the baculovirus expression construct described by Coleman et al. [14]. Cell lysate prepared from infected Sf21 insect cells was purified on Nickel NTA agarose as described for ΔCdt1, and finally dialysed into 150 mM NaCl, 20 mM Tris Cl pH 7.5, 1 mM DTT, 5% glycerol, 0.01% Triton X-100. *Xenopus* Mcm2-7 protein was purified as described by Gillespie and Blow [24]. MQ1 and MQ3 peaks were mixed in a ratio of 62:38. For some assays (as indicated) the fractions from the gel filtration column were used instead of the MonoQ eluate. Recombinant geminin was prepared in *E. coli* as described by McGarry and Kirschner [37].

Xenopus ORC was purified by a modification of the protocol described by Rowles et al. [15]. 160 ml of "licensing factor extract" was precipitated with 4.25% polyethylene glycol, resuspended in 50 ml LFB1 plus 150 mM KCl (LFB1/150), and mixed for 15 min at 4°C with 12.5 ml phosphocellulose (Whatman P11) equilibrated in LFB1/150. The slurry was poured into a column, washed with LFB1/150 until the UV signal dropped, and activity was eluted by step in LFB1/500. The eluate was then precipitated with 43% (w:v) ammonium sulphate, and the pellet resuspended in 910 µl LFB1/50 plus 2.5 mM Mg-ATP and 0.01% Triton X-100. 4 × 220 µl aliquots were fractionated on a 24 ml Superose 6 gel filtration column (Amersham Pharmacia Biotech) run at 0.25 ml / min in LFB1/50 plus 0.01% Triton X-100. 2 × 500 µl fractions from each run containing the UV absorbance peak at ~550 kDa were pooled. The combined gel filtration fractions were applied at 100 µl / min to a 1 ml MonoQ column (Amersham Pharmacia Biotech) equilibrated in LFB1/130, and protein eluted in a gradient to LFB1/330 over 20 ml (500 µl fraction size). Fractions containing Orc1 at ~225 mM KCl (confirmed by dot-blotting) were pooled, diluted 1:1 with LFB1 plus 0.01% Triton X-100, and loaded onto a column prepared with 800 µl heparin-agarose FF (Amersham Pharmacia Biotech) equilibrated in LFB1/100. The column was developed with a 2.4 ml gradient to LFB1/450 (flow rate 100 µl/min, fraction size 100 µl). Fractions at ~310 mM KCl containing purified ORC (as determined by SDS-PAGE and Coomassie staining) were pooled and diluted 1:3 in LFB1/10 (4.23 ng ORC / µl).

Assays for individual activities

The assays for ORC, Cdc6 and Mcm2-7 activity were performed by incubating 2.5 µl test samples (purified protein diluted in 20 mM Hepes KOH pH 8.0, 10 mM KCl, 2 mM DTT, 10% (w:v) sucrose and 1 µg/ml each of leupeptin, pepstatin and aprotinin) with 2.5 µl egg extract depleted of ORC, Cdc6 or Mcm2-7 and 0.5 µl *Xenopus* sperm nuclei (70 ng DNA / µl) for 30 min; the reaction was then added to 12.5 µl 6-DMAP-treated extract [40,56] supplemented with [α-^{32}P]dATP, and the incubation continued for a further 90 min. Total DNA synthesis was determined by TCA precipitation [56]. The assay for nucleoplasmin activity was performed exactly as described [24]. In brief, 0.3 µl *Xenopus* sperm nuclei (70 ng DNA / µl) plus 1 µl of a crude fraction containing ORC, Cdc6 and RLF-B/Cdt1 (1.5× BPAS diluted in LFB1/50 + 5 mM Mg-ATP) [16,24,56] plus 1 µl of a crude frac-

tion containing Mcm2-7 (SP Sepharose eluate) [24] and 1 μl purified nucleoplasmin were incubated for 30 min; the reaction was then added to 6 μl 6-DMAP-treated extract and processed as above. The assay for RLF-B activity was performed by incubating 3 μl test samples (purified RLF-B diluted in TAB/I 00 buffer) (TAB/100: 2.5 mM Mg-ATP, 4 mg / ml bovine serum albumin, 0.02% Triton-X 100 plus 100 mM KCl) with 2 μl crude Mcm2-7 (Q Sepharose eluate) [24] and 0.5 μl "6-DMAP chromatin" (50 ng DNA / μl) for 30 min; the reaction was then added to 6-DMAP-treated extract and processed as above.

Reconstitution

The reconstituted reaction consisted of 0.4 μl purified nucleoplasmin (340 ng / μl), 0.9 μl purified ORC (4.2 ng / μl), 0.7 μl purified Cdc6 (50 ng / μl), 2 μl Mcm2-7 (17 ng / μl), and 1 μl of either purified RLF-B/Cdt1 or ΔCdt1 (8.7 ng / μl) and 0.5 μl demembranated *Xenopus* sperm nuclei (70 ng DNA / μl). ATP, ATP-γ-S and ADP were added to a final concentration of 2.5 mM as required. Some reactions were supplemented with 0.5 μl geminin (15 ng / μl). When individual components were removed, the volume was made up with the appropriate buffer for each protein. Reactions were performed for 30 min at 23°C. For assessment of functional licensing, the reactions were added to 12.5 μl 6-DMAP-treated extract supplemented with [α-^{32}P]dATP, and the incubation continued for a further 90 min. Total DNA synthesis [dependent on the degree of licensing that had occurred, [40]] was determined by TCA precipitation [56].

For chromatin blotting experiments, the reconstitution reactions were scaled up 10× to 50 μl. After the 30 min licensing incubation, each reaction was diluted in 400 μl NIB supplemented with 0.1% Triton X-100, and underlayered with 120 μl of the same buffer containing 10% sucrose. The chromatin was pelleted at 2,500 *g* for 15 min in a swinging bucket rotor at 4°C (Megafuge; Heraeus). The diluted extract was removed and the chromatin pellet was resuspended in 6× SDS-PAGE loading buffer. The samples were run on 4-12% Bis-Tris gradient gels (Novex / Invitrogen). The portion of the gel below the 25 kDa marker was cut off and stained with Coomassie to check the level of histones H3 and H4 as a control for chromatin recovery; the remainder of the gel was analysed by immunoblotting.

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