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Replication origins are already licensed in G1 arrested unfertilized sea urchin eggs

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ABSTRACT

Fertilization relieves the oocyte from a cell cycle arrest, inducing progression towards mitotic cycles. While the signalling pathways involved in oocyte to embryo transition have been widely investigated, how they specifically trigger DNA replication is still unclear. We used sea urchin eggs whose oocytes are arrested in G1 to investigate *in vivo* the molecular mechanisms regulating initiation of replication after fertilization. Unexpectedly, we found that CDC6, Cdt1 and MCM3, components of the pre-replication complexes (pre-RC) which license origins for replication, were already loaded on female chromatin before fertilization. This is the first demonstration of a cell cycle arrest in metazoan in which chromatin is already licensed for replication. In contrast pre-RC assemble on chromatin post-fertilization as in other organisms. These differences in the timing of pre-RC assembly are accompanied by differences in Cdk2 requirement for DNA replication initiation between female and male chromatin post-fertilization. Finally, we demonstrated that a concomitant inhibition of MAP kinase and ATM/ATR pathways releases the block to DNA synthesis. Our findings provide new insight into the mechanisms contributing to the release of G1 arrest and the control of S-phase entry at fertilization.

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Introduction

Oocytes and eggs have evolved natural cell cycle pauses from which they are released by an external signal, maturation-inducing hormone or fertilization. The cell cycle stage at which the egg is paused until it is fertilized varies among species; for example, first meiotic prophase in the clam *spisula*, metaphase of first meiosis in insects and tunicates, metaphase of second meiotic division (MII) in *Xenopus* and almost all vertebrates, and G1 of the first mitosis in cnidarians and in a number of echinoderms as sea urchin or certain starfishes, (Stricker, 1999). Whether unfertilized (UF) eggs are arrested in meiosis II or in G1, maintenance of the arrest requires a high level of ERK Mitogen Activated Protein Kinase (MAPK) activity (reviewed in Perry and Verlhac, 2008). The role of this MAPK activity would be to prevent eggs from entering a program of mitotic divisions. In *Xenopus*, on release from MII arrest at fertilization, the anaphase-promoting complex/cyclosome (APC/C), previously inhibited by the high MAPK activity, is activated causing cyclin B degradation, output of meiosis II and ensuing first mitotic S-phase. When fertilization occurs after completion of meiosis II in eggs of the starfish *Patiria pectinifera*, ERK inactivation has been directly correlated with DNA synthesis stimulation (Tachibana et al., 1997, 2000). In sea urchin, in which UF eggs are arrested in G1, discrepancies remain about the activity of this cascade at fertilization. While reports described a rapid ERK1 activation after fertilization (Philipova et al., 2005; Philipova and Whitaker, 1998) other authors mentioned a decrease in ERK activity (Carroll et al., 2000; Zhang et al., 2006, 2005). Moreover, whereas Carroll et al. have shown that UF *Litechinus variegatus* eggs underwent DNA replication after MEK inhibition, Zhang et al. (2006) demonstrated that inactivation of this pathway in mature *Paracentrotus lividus* arrested eggs, by altering the internal calcium level, generates M-phase entry and recurrent cyclin B/Cdk1 oscillations without evidence of full DNA synthesis.

DNA replication is a tightly controlled process. One critical step is the licensing reaction, which assembles pre-replication complexes (pre-RC) onto origins of replication. A consensus scheme has been drawn from studies of this cell cycle step in different models, mainly yeast, mammalian cells and cell-free extracts from *Xenopus* oocytes (reviewed in Bell, 2002; Cvetic and Walter, 2005; DePamphilis et al., 2006; Spradling, 1999) First, ORC (for origin recognition complex) binds to the origins, recruiting CDC6 and Cdt1. Assembly of pre-RC is then completed by loading of the hexameric MCM2-7 helicase complex. Once the MCMs are loaded, Cdks together with a second kinase Dbf4/Cdc7 (Ddk) promote pre-RC activation and initiation of DNA replication. Regulation of pre-RC component assembly on chromatin is a crucial step to limit replication to once per cell cycle, thus assembly of pre-RC is restricted to late mitosis and G1 phase of the cell cycle. In metazoan, Cdt1 binding to chromatin is a key target of this control and geminin its negative regulator, prevents illegitimate re-replication. Recruited on chromatin via Cdt1, geminin forms a

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complex that is unable to recruit MCM to origins (Tada et al., 2001; Xouri et al., 2007). Geminin inactivation and partial degradation at the metaphase–anaphase transition mark the reinitiation of the licensing period (Li and Blow, 2004; Tada et al., 2001).

When the cell cycle is arrested for a long period of time, the competence to replicate is usually lost. This is the case for vertebrate oocytes arrested at meiosis I. Upon hormonal stimulation, arrested oocytes resume meiosis, and re-establish the ability to DNA licensing through accumulation of CDC6 (Lemaitre et al., 2004), pre-RC assembly being repressed until fertilization through a sustained high level of cyclin B/Cdk1 activity. In contrast, in *S. pombe*, the synthesis of cdc18 (the *S. pombe* CDC6 ortholog) is down regulated during meiosis to prevent DNA replication from occurring in spores. When yeasts *S. cerevisiae* are arrested in G1 with the mating pheromone α factor prior to conjugation, pre-RC have assembled at chromatin origins in the absence of Cdk activities (Diffley et al., 1994).

A very limited number of higher eukaryotes present such a G1 natural arrest and until now the status of the origin of replication has not been investigated in these few animal models. The sea urchin, representative of this unusual arrest, occupies an important phylogenetical position. Echinoderms are with hemichordates and invertebrate-chordates, the organisms closest from vertebrates in the deuterostome subgroup (Delsuc et al., 2006). Therefore, this model is useful to study the mechanisms of regulation of DNA replication from an evolutionary perspective. Moreover, as natural arrests are often adaptations of cell cycle checkpoint present in dividing cells, this reinforces the interest in uncovering the underlying mechanism of this G1 pause.

Mature sea urchin eggs are thus haploid when stored in the ovarium lumen and the external fertilization directly triggers entry into mitotic cycles rather than meiosis resumption as in most vertebrate eggs. A transient calcium signal (Steinhardt et al., 1977; Whitaker, 2006) and a rise in pHi (Johnson and Epel, 1976) mark sperm entry and the short G1 phase post-fertilization (p.f.) allows time for male pronucleus formation and migration of male and female pronuclei, steps which are accompanied by deep remodelling of male chromatin (Imschenetzky et al., 2003). These events culminate in pronuclear union, fusion of decondensed chromatins and S-phase entry, yet karyogamy is not a prerequisite for DNA replication (Sluder et al., 1995). In fertilized eggs, Cdk1 activity remains low during the short G1 period, only beginning to increase at the onset of DNA replication (Geneviere-Garrigues et al., 1995; Meijer et al., 1991). Chromatin is therefore in a permissive state for licensing during this period. In contrast, a Cdk2 activity is present in UF eggs which does not cycle during cleavage cycles (Moreau et al., 1998; Sumerel et al., 2001).

We aimed to investigate how the licensing of replication origins at fertilization is regulated in sea urchin eggs. As the first DNA replication is independent of protein synthesis, we reasoned that pre-RC components would be proteins from maternal origin, present in UF eggs, and would assemble on male and female chromatin during the licensing permissive periods, where Cdk1 activity is low. Unexpectedly, we uncovered that pre-RC are already present on female chromatin in mature arrested eggs. We then explored which mechanisms could prevent the firing of origins and which signalling pathways are requested to activate DNA replication initiation.

Materials and methods

Materials

Aphidicoline, colchicine, caffeine, U0126, A23187 and roscovitine were obtained from Sigma.

Animals and handling of gametes

The sea urchins *P. lividus* were collected in the Mediterranean Sea (Banyuls-sur-mer, France) and maintained until use in running sea water. Spawning was induced by intracoelomic injection of 0.2 M acetylcholine. Eggs were collected in sea water, filtered through a 100 mesh nylon sieve and washed three times with filtered (0.22 µm) sea water (FSW). Eggs were stored at 19 °C until use while sperm was collected and kept concentrated at 4 °C. For fertilization, sperm was diluted 10^5 fold in a 5% (v/v) egg suspension in FSW under slow agitation. Only batches with at least 95% fertilized eggs were further used. S-phase entry is observed 25 min p.f. as previously reported (Geneviere-Garrigues et al., 1995). Ammonium activation was performed as described (Mazia and Ruby, 1974).

Expression of recombinant proteins

CDC6, Cdt1 and geminin mRNAs were identified in the *P. lividus* EST database (http://goblet.molgen.mpg.de/cgi-bin/webapps/paracentrotus.cgi). These ESTs were generated from clones of 4 organized libraries constructed in pSPORT within the European *P. lividus* Genome Initiative of the Network of excellence Marine Genomics. These libraries contain full-length cDNAs from several stages of development (UF eggs, and pools of cleavage and early blastula stages, mid and swimming blastula stages and gastrula-pluteus stages) independently cloned in pSPORT. The coding regions of the cDNAs of interest were retrieved from UF library and fully sequenced (GENOME express).

The nucleotide sequences encoding the C-terminal domain of Cdt1 (aa 534–691) and the full-length geminin were amplified by PCR from the corresponding constructs in pSPORT and inserted into pET21b (Novagen). The fused histidine recombinant proteins were expressed in *E. coli* (strain BL21) and purified with TALON Metal Affinity Resins according to the manufacturer's instructions (Clontech). Purified proteins were concentrated with Microcon (Millipore). To produce the GFP-tagged Cdt1 and geminin proteins, the corresponding coding sequences were first inserted into pEGFP-C1 vector (Clontech). Cdt1 and geminin tagged at their N-terminus were further excised and inserted into pCal-n (Stratagene). The recombinant GFP-tagged proteins fused with calmodulin-binding peptide (CBP) in N-ter were expressed in BL21 and purified using calmodulin affinity resin according to the manufacturer's instructions (Stratagene).

Antibody characterization

Polyclonal antibodies against the recombinant His-geminin and the His-tagged C-terminal domain of Cdt1 were raised in rabbit by standard immunization protocol (Eurogentec). The anti-geminin antibodies were affinity purified by Eurogentec using the immobilized geminin recombinant protein. The antibodies were kept in aliquots at −80 °C.

A rabbit polyclonal anti-CDC6 was raised against a pool of the two following peptides designed from N-ter and C-ter CDC6 sequences respectively: H2N-PVQ TRR GRQ STI PFQ C-COOH and H2N-CEA GLP TNT EKK GKK L-COOH (Eurogentec). The serum was purified on AminoLink Column following the manufacturer's instructions (Pierce).

The selectivity of these antibodies was checked by immunoblotting experiments using whole egg extract (Cdt1, geminin) or a purified chromatin fraction in case of low abundant protein (CDC6) (Fig. 1). The specificity was verified by comparing signals obtained from immunoblots using antibodies previously incubated or not with the corresponding recombinant protein (Cdt1 and geminin) or the synthetic peptides (CDC6). Anti-MCM3 antibody raised against the *Xenopus laevis* protein was a gift from P. Romanowski (Romanowski et al., 1996).

Immunoprecipitation, immunoblotting and pulldown assay

To prepare whole egg extract, samples of egg suspension containing 50 µl of cell volume were centrifuged (1000 × *g*, 10 s) and the pellets were homogenized by sonication in 500 µl of an ice-cold RIPA buffer (9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4 pH 7.4, 150 mM NaCl (PBS 1×) supplemented with 1% NP-40, 0.5% sodium deoxycholate, 0.1%

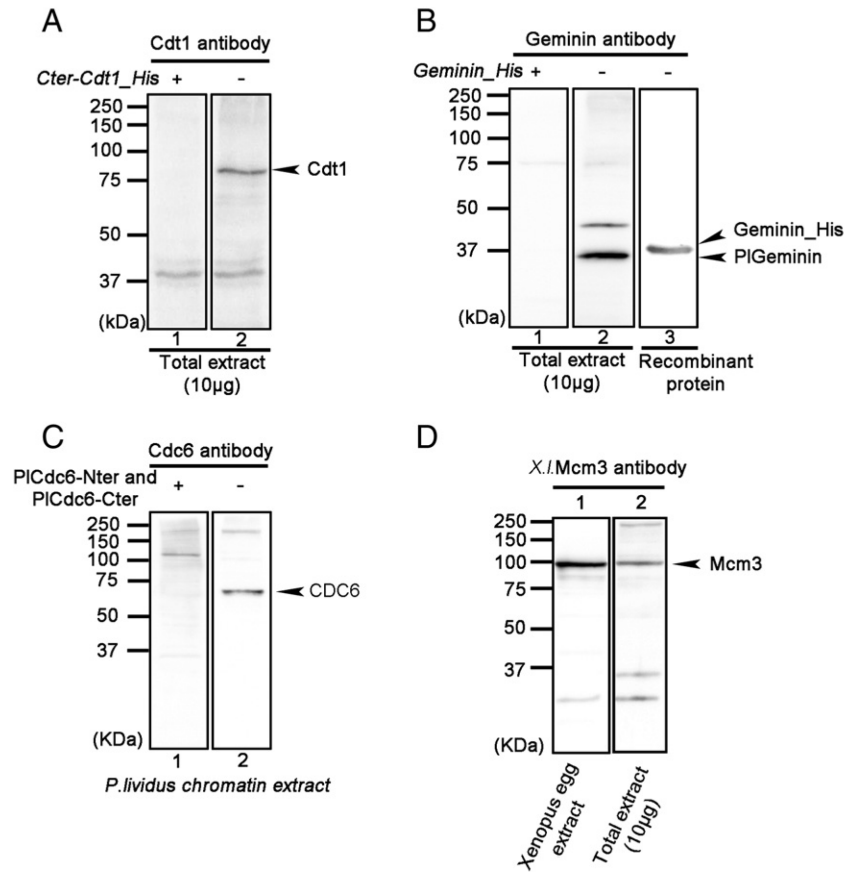


Fig. 1. Characterization of the antibodies. (A) Specificity of the Cdt1 antibody. Proteins (10 μg) from whole egg extracts were resolved by SDS-PAGE (12%) and immunoblotted using anti-Cdt1 antibody previously incubated (lane 1) or not (lane 2) with immobilized recombinant Cdt1 (1 mg). A band of 78 kDa (the predicted molecular weight of Cdt1) is specifically recognized. (B) Specificity of anti-geminin antibody. The geminin antibody recognizes two bands (lane 2) in proteins from whole egg extracts, both being displaced by previous incubation of antibody with the recombinant protein (lane 1). The lower band at 35 kDa is relevant for geminin regarding the mobility observed for the recombinant His-geminin (lane 3). The upper band is not found in chromatin-bound proteins and remains invariant along the cell cycle (data not shown) while the signal corresponding to the 35 kDa band raises (Fig. 3). We conclude that this last band corresponds to the geminin protein. (C) Specificity of CDC6 antibody. Chromatin samples were immunoblotted using anti-CDC6 antibody (lane 2) or anti-CDC6 antibody pre-incubated with 1 mg of combined peptides (lane 1). The antibody recognizes specifically a major band at 68 kDa (lane 2), the molecular weight predicted for *P. lividus* CDC6. (D) The anti-*Xenopus laevis* MCM3 antibody was previously described (Romanowski et al., 1996), it recognizes in sea urchin whole egg extract a major band (90 kDa) migrating with the same mobility than in *Xenopus* and corresponding to the molecular mass predicted for *P. lividus* MCM3.

SDS, 10 μg/ml PMSF, 20 μg/ml Aprotinin, 100 mM sodium orthovanadate). After centrifugation (10,000 $\times g$, 1 min) the supernatants were processed for immunoprecipitation, pulldown experiment, or immunoblotting.

Immunoprecipitations were performed using 5 μg of antibodies coated on protein A-agarose beads. After overnight incubation at 4 °C, the beads were centrifuged and washed three times with RIPA, once with Tris 20 mM pH 7.5 and eluted with 15 μl of Laemmli 2×.

For immunoblotting, proteins were loaded on SDS-PAGE gels and transferred to PVDF membranes (Millipore). Membranes were saturated overnight in Tris–HCl 50 mM pH 7.5, NaCl 150 mM (TBS), 0.1% Tween containing 5% milk and incubated 2 h at room temperature in the relevant antibodies (CDC6 1:500, Cdt1 1:200, geminin 1:1000, MCM3 1:1000). After washing three times 10 min in TBS–0.1% Tween, membranes were incubated with a secondary antibody (Pierce-goat anti-rabbit-1:8000) conjugated to peroxydase. The chemiluminescence signal was visualized using ECL^+ kit (GE Healtcare) and captured with a camera (Vilber Lourmat). Immunoblot for cleavage stage or sperm histones were used as loading control except for whole extracts where tubulin was employed.

For pulldown experiments, 1 μg of CBP tagged GFP-geminin was incubated with 20 μl of calmodulin affinity resin in 500 μl of 1× binding buffer according to the manufacturer's instruction (Stratagene) for 2 h at 4 °C. The beads were washed three times with 1× washing buffer and added to 500 μl of egg extract. After an overnight incubation at 4 °C, beads were washed three times with RIPA buffer and once with Tris 20 mM pH 7.5. The bound proteins were eluted with Laemmli buffer and analysed by immunoblotting.

Subcellular fractionation and chromatin isolation

Chromatin was prepared as described (Imschenetzky et al., 1990). UF eggs and embryos were collected by centrifugation (500 $\times g$, 5 min) and washed three times in 3 volumes of 1.5 M dextrose. The eggs or zygotes were then suspended in 10 volumes of a 20 mM sodium phosphate pH 6.0, 10 mM EDTA, 150 mM NaCl and 0.5 mM PMSF buffer (CLB) supplemented with 0.2% Triton X-100 and homogenized by passing several times through a 25 gauge needle. The suspension was filtered through a 40 μm-pore nylon sieve and centrifuged 10 min at 3000 $\times g$. While the supernatant was frozen in liquid nitrogen, the chromatin pellet was washed with CLB and chromatin-bound proteins were solubilized in Laemmli buffer. Chromatin-bound proteins represent 5–7% of total proteins. Salt resistance was also tested by washing chromatin with CLB containing NaCl 0.4 M or 0.8 M and the remaining bound proteins were eluted in Laemmli buffer.

To obtain chromatin from purified nuclei, the nuclear fraction was prepared from a modification of protocols previously described (Geneviere-Garrigues et al., 1995; Hinegardner, 1962). UF eggs were washed 4 times in 1.5 M dextrose and lysis was performed by adding 5 volumes of 2 mM MgCl2, 10 mM EGTA. After addition of one volume of

solution A containing 6 mM MgCl2, 10 mM EGTA, 1 mM spermidine, 0.3 mM spermine, 1 mM PMSF supplemented with 0.1% Triton X-100 and 1 M dextrose. The suspension was filtered through a 40 μm pore-size nylon filter and layered over a 0.4 M sucrose cushion in solution A and centrifuged 10 min at 1000 ×*g*. The pellet was suspended in 0.2 M sucrose in solution A and centrifuged again through a 0.4 M sucrose cushion. This last step was repeated 3 times and the chromatin was finally extracted from the isolated nuclei as described above.

Chromatin was isolated from sperm head as described (Collas and Poccia, 1998). Proteins were extracted from sperm chromatin by micrococcal nuclease digestion in the presence of 0.8 M NaCl.

Immunofluorescence and monitoring of DNA replication

Eggs were fertilized as described above while in the presence of 1 mM 3-amino-1,2,4-triazole (ATA) to avoid hardening of the fertilization membrane (Showman and Foerder, 1979). At indicated times, fertilized or UF eggs were transferred to a pronase solution (0.2 mg/ml in FSW) in order to remove the fertilization membrane. The reaction was stopped by transferring eggs to 1% bovine serum albumin in FSW and subsequently washing them once in FSW. Eggs were incubated during 2 h in 25 mM 2-(N-morpholino)-ethanesulfonic acid (MES) pH 6.8, 10 mM EGTA, 1 mM $MgCl_2$, 1% NP-40 and 25% glycerol. Treated eggs were fixed for 1 h in a methanol-glycerol solution (75:25, v/v), washed twice in TBS–0.05% Tween and transferred for 1 h to a blocking solution containing 5% goat serum in TBS–Tween. Eggs were then incubated overnight at 4 °C in the first antibody, washed twice in TBS–Tween for 1 h and finally incubated in 0.2% FITC-conjugated anti-rabbit antibody (Sigma) during 2 h in darkness. After washing three times in TBS-Tween, the eggs were mounted on pre-washed glass-plates in Moewiol and observed with a fluorescence microscope Olympus with 60× objective.

DNA replication was monitored by incorporation of BrdU. Eggs were fertilized in the presence of 1 mM ATA and 0.1 mg/ml BrdU. At indicated times fertilized membranes were removed as described above and embryos were fixed 2 h in 4 M HCl. After 30 min of post-fixation in methanol 100%, embryos were washed 15 min in TBS–Tween and incubated overnight at 4 °C in undiluted anti-BrdU antibody (GE Healthcare). Eggs were washed twice during 1 h in TBS–Tween, incubated 2 h in FITC or TRITC (when indicated)-conjugated anti-mouse antibody and then washed, mounted and observed as described above.

Microinjection procedure

Injection were conducted as already described (Zhang et al., 2006) using an Eppendorf-Femtojet microinjector.

Results

Expression of pre-RC components in SU eggs

In a previous publication (Fernandez-Guerra et al., 2006) we showed that the whole repertoire of genes regulating DNA synthesis was present in the genome of the sea urchin *Strongylocentrotus purpuratus*, the first echinoderm genome to be sequenced. Furthermore, a tilling array analysis suggested that most of these genes were expressed during early embryogenesis (Samanta et al., 2006). A blast analysis of an extensive EST database constructed from mRNA of *P. lividus* (a European sea urchin) embryos from one-cell to late gastrulae stages confirms this conclusion (data not shown).

With the aim to describe the stepwise assembly of the pre-RC proteins in fertilized eggs we isolated in *P. lividus* two components required for licensing, CDC6 and Cdt1 as well as the licensing regulator geminin. The cDNAs encoding these three proteins were retrieved from an organized cDNA library constructed from UF eggs, confirming the expression of those genes among maternal mRNAs.

Analysis of sequences demonstrated conservation of most of the protein motifs present in the vertebrate proteins (Fig. 2A). CDC6, encodes a protein of 592 amino acids (aa) which displays an AAA+ domain (ATPase associated with cellular activities), also identified as a clamp loader domain (Neuwald et al., 1999). This AAA+ domain is limited by the highly conserved walker A and B motifs (Walker et al., 1982) , required for the loading of Mcm_{2-7} onto chromatin (Cook et al., 2002). Following this segment, a winged helix domain known to be a DNA localisation factor (Gajiwala and Burley, 2000) was predicted in the C-terminal part. The AAA+ and winged helix domains are present in all CDC6 orthologs (Liu et al., 2000). Two Nuclear Localisation Signal (NLS) were also predicted in the *P. lividus* CDC6 sequence. Cdt1 encodes a protein of 691 aa. The N-terminal sequence contains a conserved motif of interaction with the proliferating cell nuclear antigen (PCNA), a PIP box (Maga and Hubscher, 2003), which was shown in *Xenopus* to be involved in PCNA-dependent Cdt1 degradation (Arias and Walter, 2006). Two coiled coils domains, required for protein–protein interaction, were predicted (aa 151–190 and 507–589). The first coiled coil domain, conserved in *Xenopus*, human, and mouse is included in a region of Cdt1 involved in geminin binding,

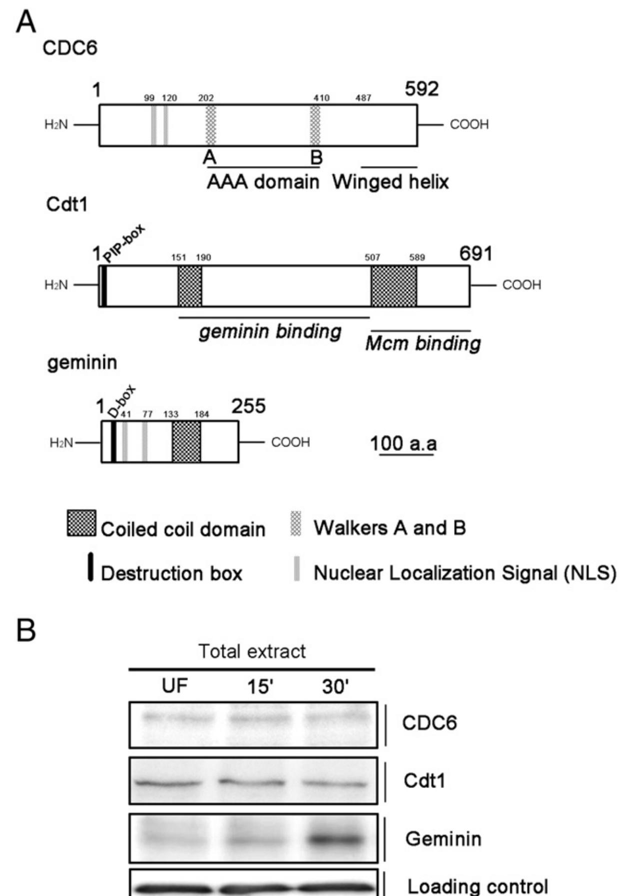


Fig. 2. Structure and expression of Cdt1, CDC6 and geminin. (A) Schematic representation of the proteins. Pfam analysis of the *P. lividus* CDC6 sequence predicted an AAA+ and a winged helix domain. The walkers A and B are represented by hatched bars. Two NLS, identified using pSORT II are indicated in grey bars. The *P. lividus* Cdt1 sequence contains domains that were predicted in other species to bind respectively geminin and MCM2-7. The PIP box is represented by a black bar. The coiled coils domains were predicted using COILS program and represented by black hatched boxes. The COILS program predicted a coiled coil domain in the geminin sequence. Two NLS have been identified with pSORT II. The D-box Finder program found one destruction box motif (black bar). The scale bar represents 100 aa. (B) CDC6, Cdt1 and geminin proteins are expressed in sea urchin UF eggs and early embryos. Whole extracts were prepared from UF eggs and embryos sampled 15 min and 30 min p.f. Protein samples (10 μg) were resolved on SDS-PAGE and immunoblotted with the indicated antibody. Tubulin content was assessed as loading control.

while the second domain is part of the minimum domain necessary for MCM binding in *Xenopus* (Ferenbach et al., 2005). *P. lividus* geminin sequence encodes a 255 amino acids protein containing a conserved coiled coil domain known to be involved in geminin dimerisation in other metazoan. A conserved D-box, a target motif of the APC (McGarry and Kirschner, 1998) and two putative NLS were also identified in the geminin N-terminus.

To monitor expression of these proteins, we raised polyclonal antibodies (see Materials and methods) and demonstrated the presence of CDC6, Cdt1 and also geminin in sea urchin UF eggs using immunoblot from whole extracts (Fig. 2B). These results confirm that pre-RC components critical for DNA licensing are proteins of maternal origin. While CDC6 and Cdt1 concentrations were constant until S-phase entry, the amount of geminin progressively increased suggesting that the geminin regulator is actively synthesized post-fertilization.

Pre-RC components are already loaded on female chromatin in unfertilized eggs

In order to survey the assembly of pre-RC onto replication origins, we first compared the association of CDC6 and Cdt1 to chromatin in UF eggs, G1 (15 min p.f.) and S-phase (30 min p.f.) embryos (Fig. 3A).

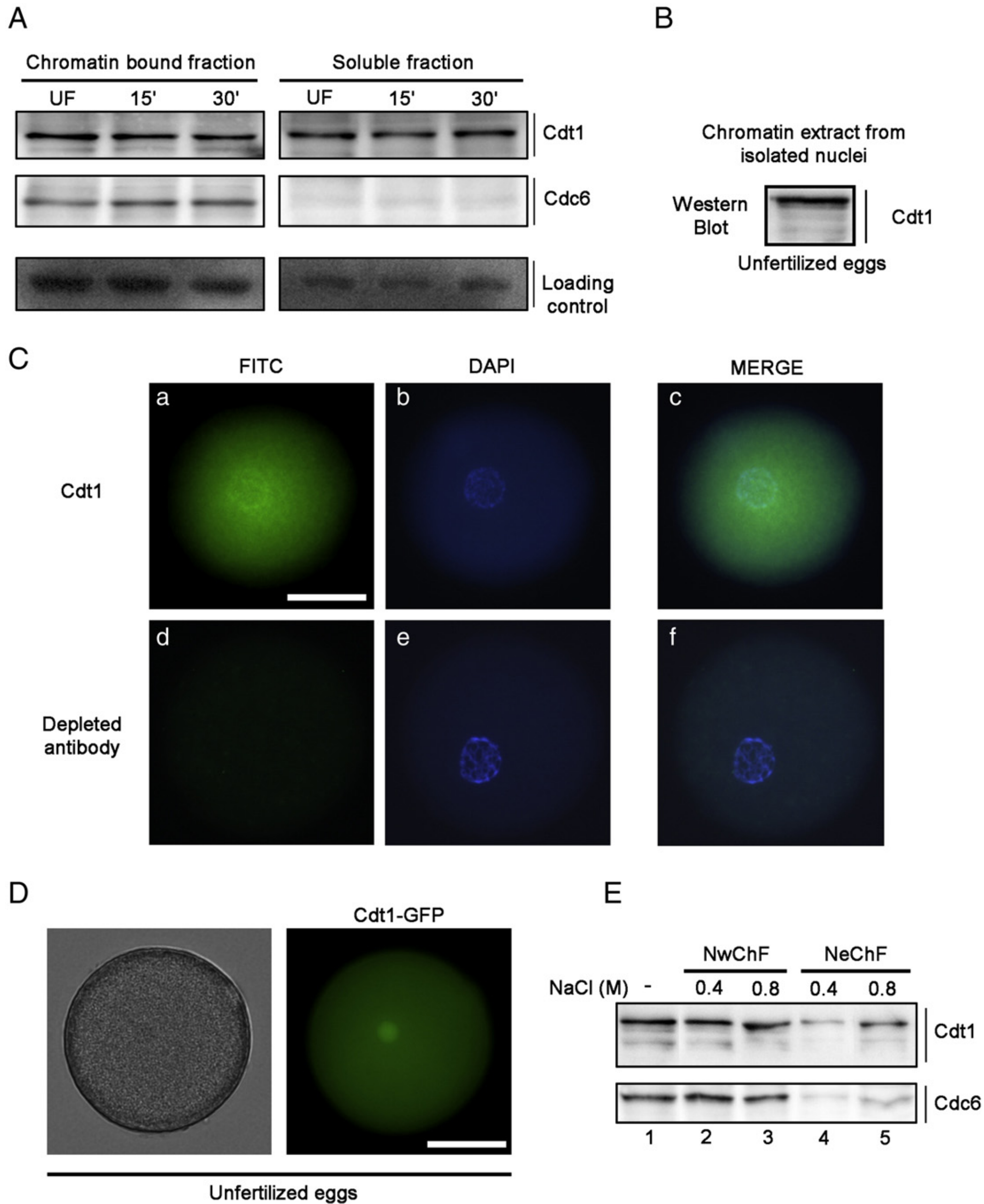


Fig. 3. Pre-RC components are tightly associated to female chromatin in unfertilized sea urchin eggs. (A) Detection of the pre-RC components on chromatin. Chromatin was isolated from UF eggs and early embryos and chromatin-bound proteins were obtained as described in Materials and methods. Chromatin-bound and soluble proteins were resolved with SDS-PAGE and immunoblotted with the indicated antibodies. Immunolabeling with antibodies to cleavage stage histones was used as loading control. (B) Nuclei were isolated from UF sea urchin eggs and chromatin prepared from these nuclei was analysed by immunoblot using anti-Cdt1 antibody. (C) Immunostaining shows the presence of Cdt1 in UF eggs. Immunostaining was performed with anti-Cdt1 antibodies previously incubated (d, f) or not (a, c) with recombinant Cdt1 C-terminal domain. (D) Accumulation of Cdt1-GFP in UF eggs pronuclei. The GFP-tagged recombinant protein was microinjected in UF eggs and immediately observed under microscope with phase contrast (left) or epifluorescence (right). Scale bar: 30 μm. (E) The tight association of pre-RC components to chromatin was tested through resistance to salt wash (see Materials and methods). (NwChF = NaCl washed chromatin fraction. NeChF = NaCl eluted chromatin fraction).

As expected, both proteins were found in the chromatin fraction prepared from embryos at the onset of S-phase. However, more surprisingly, we found that the two pre-RC components were already present in female chromatin fraction before fertilization. While CDC6 is mainly found in chromatin-bound proteins, Cdt1 is also found abundant in the soluble fraction. To exclude a putative association of Cdt1 from the soluble pool to chromatin during purification, we prepared female chromatin from previously isolated oocyte nuclei. Under these conditions Cdt1 still co-purified with chromatin (Fig. 3B). Moreover, immunofluorescence analysis of UF eggs shows that Cdt1 is abundant in the cytoplasm but localizes as well on the maternal chromatin (Fig. 3C). In addition, recombinant GFP-Cdt1 microinjected in arrested eggs rapidly accumulates in female pronuclei, spreading in that case in the entire nucleoplasm (Fig. 3D).

To confirm the tight loading of pre-RC components on female chromatin, we tested the resistance of this binding to salt wash (Fig. 3E). CDC6 and Cdt1 were retained in the chromatin pellet after a NaCl 0.8 M wash, demonstrating their strong association to chromatin in UF eggs. Accordingly, washing chromatin with another chaotropic salt, KCl 150 mM, neither removed CDC6 nor Cdt1 (data not shown).

Data obtained in *Xenopus* show that licensing of male chromatin only occurs post-fertilization (Blow and Laskey, 1986; Lohka and Masui, 1983). We thus examine the status of male chromatin before fertilization in sea urchin. Neither CDC6 nor Cdt1 could be observed by western blot analysis in sperm nuclei (Fig. 4A). In contrast, after egg fertilization, an immunofluorescence analysis showed that Cdt1 is rapidly detected in male pronuclei during their migration toward the centre of the egg (Fig. 4B and S1).

These data suggested that while fertilization triggers stepwise assembly of pre-RC on male chromatin, the licensing process of female chromatin already began before fertilization.

In metazoan, geminin through interaction with Cdt1, has been shown to inhibit the loading of MCM2–7 to chromatin thereby preventing completion of licensing, (Tada et al., 2001; Wohlschlegel et al., 2000). More recently, it was reported that geminin is able to bind to Cdt1 on chromatin (Maiorano et al., 2004; Xouri et al., 2007). To investigate whether geminin could preclude MCM2–7 loading on chromatin in UF eggs or whether pre-RC are already fully assembled, we tested by immunoblot the association of geminin and MCM3 on female chromatin. Both geminin and MCM3 were bound to chromatin in G1 arrested eggs (Figs. 5A, B). Moreover, MCM3 association to female chromatin is resistant to high salt wash (NaCl 0.8 M) while geminin is already displaced by a medium (NaCl 0.4 M) salt wash (Fig. 5C). The tight association of MCM3 to chromatin supports the view that pre-RC assembly is already completed on female chromatin in UF eggs.

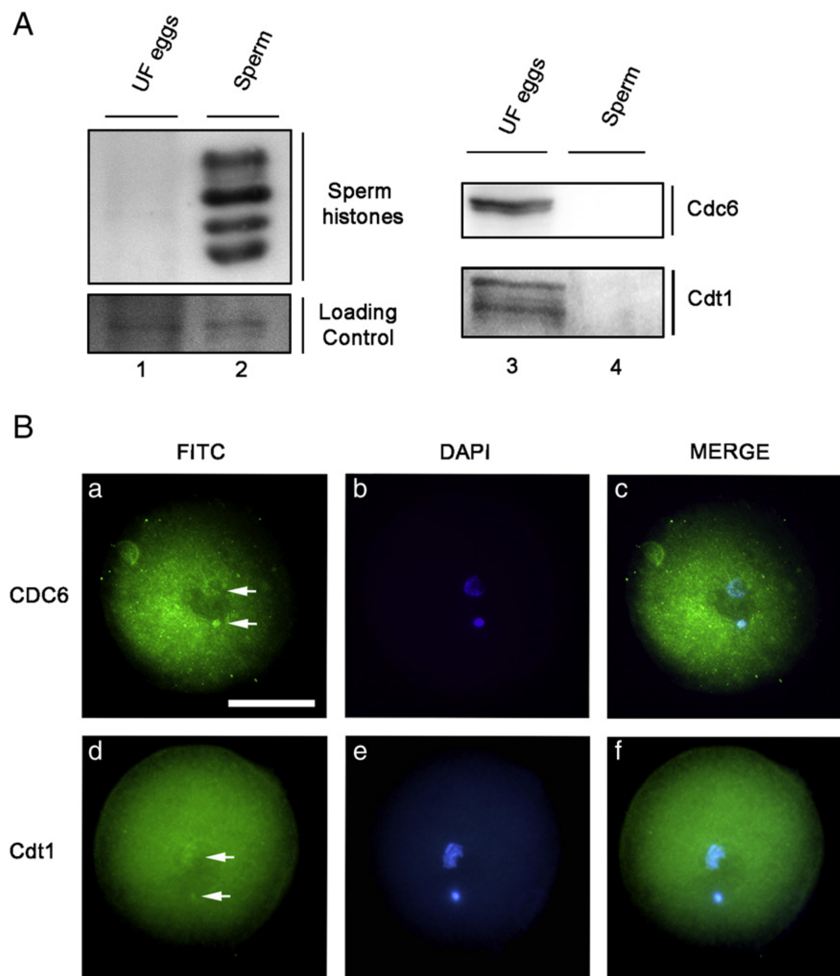


Fig. 4. The pre-RC components are assembled on male chromatin after fertilization. (A) Absence of pre-RC proteins in sperm nuclei. Sperm chromatin was isolated as described in Materials and methods and 20 µg of proteins was analysed by immunoblot (lanes 2 and 4). Proteins extracted from egg chromatin were loaded as control (lanes 1 and 3). Immunoblot using anti-spermatic histones (lane 2) was used to confirm the efficiency of protein extraction from sperm nuclei and a non specific band stained with amido black was used as loading control. (B) CDC6 and Cdt1 colocalize with male and female chromatin post-fertilization. Embryos were fixed 10 min after insemination and immunostained as described in Materials and methods with anti-CDC6 (a) or anti-Cdt1 (d) antibodies and a secondary antibody linked to FITC. The DNA was visualized with DAPI (b, e) and the merge images were displayed (c, f). Scale bar: 30 µm.

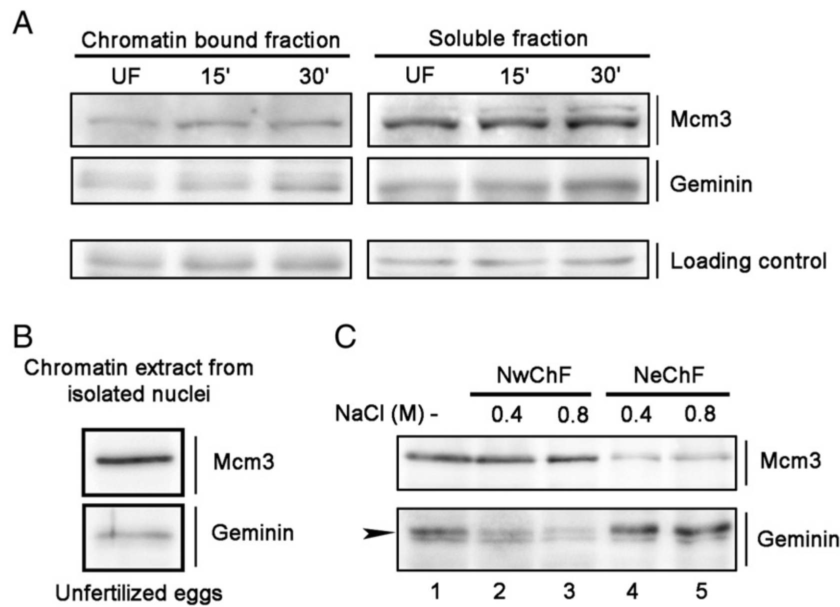


Fig. 5. MCM3 and geminin are present on unfertilized sea urchin egg chromatin. (A) Detection of MCM3 and geminin on female chromatin. The chromatin-bound and soluble proteins were prepared as described in Materials and methods and 10 µg of proteins was analysed as in Fig. 3. Immunoblots were detected using the indicated antibodies. Immunolabeling with antibodies to cleavage stage histones was used as loading control. (B) Chromatin was purified from nuclei isolated from UF sea urchin eggs and analysed by immunoblot. (C) MCM3 is more tightly associated to chromatin than geminin. The pellet of chromatin isolated from UF eggs was treated with salt buffer as described in Materials and methods and Fig. 3 and immunoblotted with anti-MCM3 or anti-geminin antibodies. 20 µg of proteins was loaded in lane 1 and the corresponding amount of washed or eluted chromatin fraction in lanes 2 to 5.

Geminin is present on chromatin in vivo during licensing

The simultaneous detection of Cdt1 and geminin on the female licensed chromatin of sea urchin eggs suggests that the loading factor responsible for binding of MCM2–7 helicase to chromatin *in vivo* is the complex Cdt1 associated to geminin, as previously indicated from *in vitro* experiments in *Xenopus* extracts (Lutzmann et al., 2006). Based on this it was proposed that Cdt1–geminin complexes only become inactivated upon origin firing by recruiting additional geminin monomers. In agreement, the geminin concentration on female chromatin in UF eggs and embryos just after fertilization is low and further recruitment on zygotic chromatin only begins at S-phase onset (Fig. 5A). We also observed by immunofluorescent experiments that geminin is translocated to the male pronucleus early after sperm penetration as already illustrated for Cdt1 (Fig. 6A). This strongly suggests that licensing of male chromatin also involves a Cdt1–geminin complex.

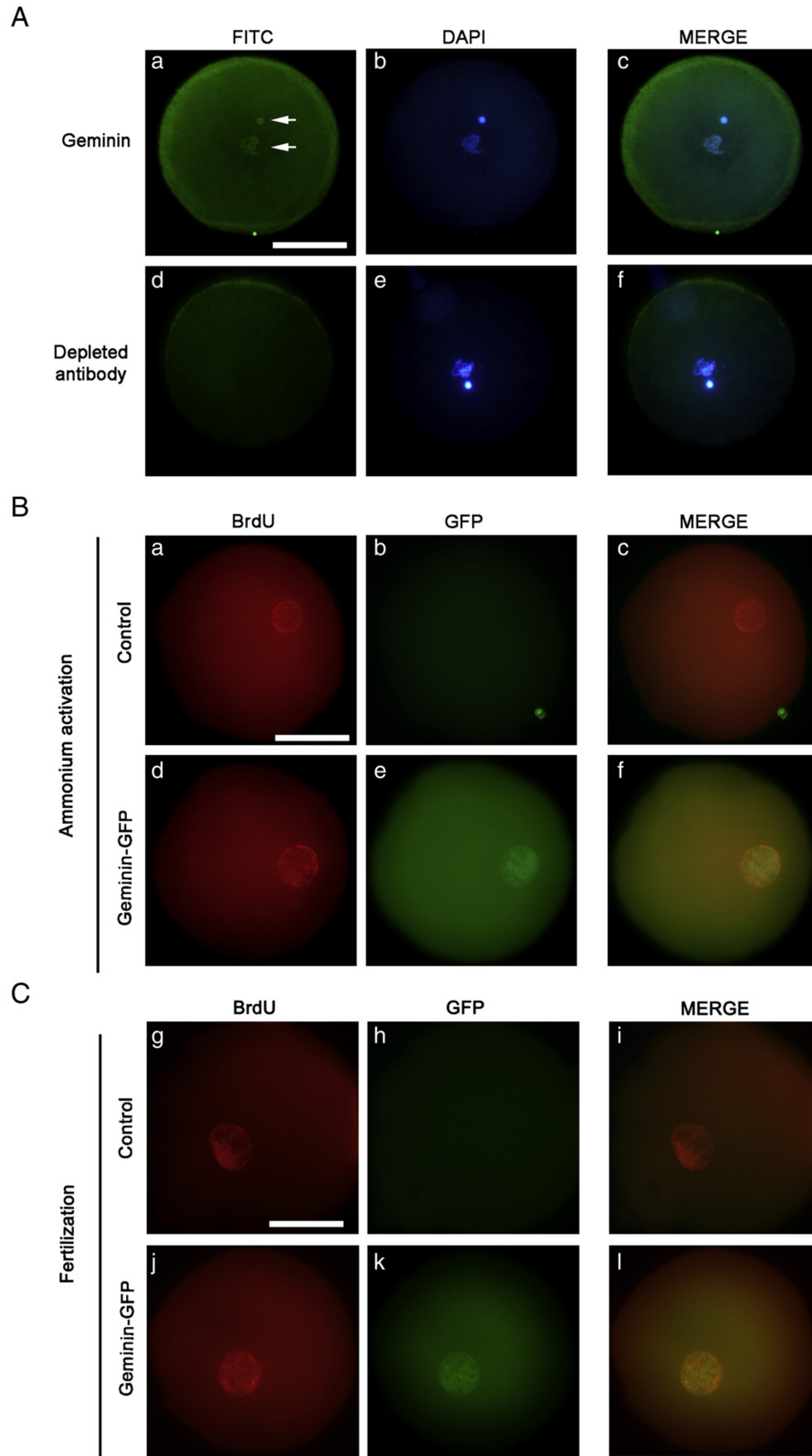
To investigate the effect of increased geminin concentration on the firing of already licensed female origin, we injected recombinant GFP-geminin in UF eggs and monitored BrdU incorporation after activating eggs by an ammonium treatment. Ammonia which induces increase in intracellular pHi has been shown to activate UF eggs and to trigger DNA replication (Dube and Epel, 1986; Epel, 1967; Mazia and Ruby, 1974). A similar incorporation of BrdU was observed in control and activated eggs (Fig. 6B), implying that the increased concentration of geminin does not impede maternal DNA replication. We verified that the GFP-geminin microinjected in the cytoplasm penetrates the nucleus after ammonia treatment (Fig. 6B-e). To further examine the effect of additional geminin during the male licensing period, we measured BrdU incorporation in fertilized eggs microinjected with the recombinant protein before fertilization. As evidenced in (Fig. 6C), DNA replication is not affected by an increased concentration of geminin. The capacity of GFP-geminin to interact with endogenous Cdt1 was confirmed by geminin pulldown and co-immunoprecipitation assay with Cdt1 antibodies (Fig. 7). Moreover, we verify that the recombinant GFP-geminin was able to inhibit DNA replication using *Xenopus* extracts (Supplementary data S2).

The above findings suggest that *in vivo* in sea urchin eggs, Cdt1–geminin complexes serve as positive molecular switch to trigger pre-RC assembly on female and male chromatin, as already proposed from data obtained *in vitro* from *Xenopus* extracts (Lutzmann et al., 2006). In addition, the inability of additional recombinant geminin brought during licensing period to impede DNA replication *in vivo* suggests that active Cdt1–geminin complexes become inactivated by binding additional geminin only upon origin firing.

A time differential Cdk activity requirement for pre-RC to pre-IC conversion in male and female chromatin

Upon entry into S-phase, DNA replication is initiated by the conversion of pre-RCs into pre-initiation complex (pre-IC) by further addition of proteins, including CDC45 and GINS, necessary for activation of replication forks and for recruitment of the DNA-polymerase (Labib and Gambus, 2007). This transformation requires the activity of two families of protein kinases, the Cdks and the Cdc7/Dbf4 kinases which cooperate to recruit Cdc45 to origins of DNA replication (Jares and Blow, 2000; Takisawa et al., 2000; Walter, 2000; Zou and Stillman, 2000). In order to investigate the timing of pre-RC to pre-IC transition on female and male chromatin of sea urchin eggs we first examined the Cdk-dependence of replication initiation. In sea urchin Cdk2 activity is already present in the G1 arrested eggs and is kept on after fertilization. In contrast, the low residual cycline B/Cdk1 kinase activity present in UF eggs still decreases immediately p.f. (Supplementary data S3 and Zhang et al., 2006). While cyclin E-Cdk2 activity is required for male pronuclear maturation after sperm penetration, we and others have shown that inhibition of Cdk activities from a few minutes p.f. to S-phase does not prevent BrdU incorporation into zygotic nucleus (Moreau et al., 1998; Schnackenberg et al., 2007). These results lead to the conclusion that replication origins can fire whereas Cdk activity has been inhibited from a few minutes p.f. onwards. Since pre-RC are already assembled on female chromatin while they are not formed on male chromatin this prompted us to re-investigate if the Cdk requirement for the firing of origin could differ between male and female chromatin. We thus

examined the effect of roscovitine, a selective inhibitor of Cdks (Meijer et al., 1997), on DNA replication in fertilized eggs in which fusion of pronuclei was prevented by addition of colchicine. It has been previously demonstrated that the absence of syngamy does not affect DNA replication (Zimmerman and Zimmerman, 1967). Roscovitine (20 μM) was added to egg culture at different times from



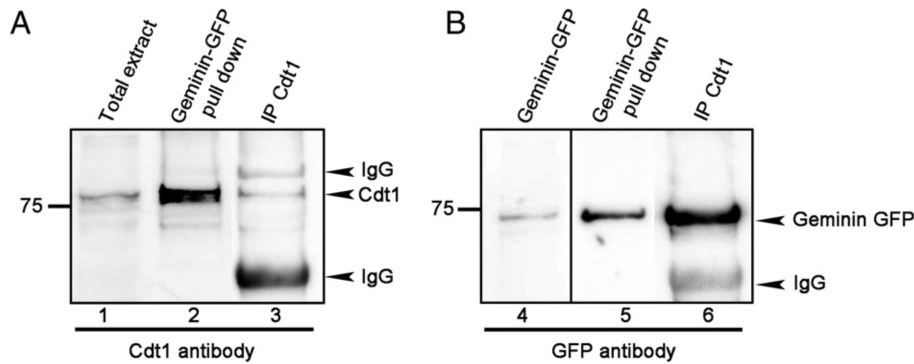


Fig. 7. Geminin-GFP is able to interact with endogenous Cdt1. GFP-geminin tagged with CBP was added to whole extracts of UF eggs. The mixture was then pulled down with calmodulin beads (lanes 2 and 5) or immunoprecipitated with the anti-Cdt1 antibody (lanes 3 and 6) as described in Materials and methods. Samples were resolved by SDS-PAGE and immunoblotted using anti-Cdt1 and anti-GFP antibodies after striping. Proteins from whole extracts (lane 1) or purified GFP-geminin (line 4) were loaded as control.

20 min before fertilization (b.f.) to 25 min p.f. and cultures were supplemented with BrdU and colchicine at fertilization. Control experiment without colchicine was run in parallel. At the concentration used, roscovitine is known to inhibit Cdk2 activity *in vitro* (Schnackenberg et al., 2007) and in the present experiment we verified it prevents M-phase entry in fertilized eggs (data not shown). This treatment of eggs with roscovitine before fertilization completely blocked paternal chromatin decondensation, either in the presence or absence of colchicine (Fig. 8B and Supplementary data S4), reinforcing the previous observation done when roscovitine alone was added to fertilized eggs 1.5 min p.f. (Schnackenberg et al., 2007). In this condition DNA replication of male chromatin is suppressed (Fig. 8B). However, we also observed a simultaneous inhibition of maternal chromatin replication. In contrast, when roscovitine was applied from 5 min p.f. onward, significant BrdU staining was observed in non-fused male and female pronuclei even if paternal chromatin is not fully decondensed. In eggs treated with colchicine alone, maturing male pronuclei had undergone a morphological transformation and are observed as a sphere by 10 min p.f. From that time, inhibition of Cdk activity ceases to decrease BrdU incorporation in paternal chromatin. In contrast, BrdU staining in female pronuclei remained affected, a level of BrdU incorporation identical to control being only recovered in samples treated with roscovitine 25 min p.f.

These results confirm that Cdk2 activity is essential for male chromatin maturation. However, as soon as male chromatin is fully decondensed, replication does not necessitate Cdk activity anymore suggesting that the Cdk-dependent phosphorylations required for activation of male DNA replication are fulfilled early p.f., during DNA decondensation and well before BrdU incorporation can be observed in chromatin. In addition, replication of female chromatin is only prevented when the Cdk activities are inhibited in UF eggs and maintained hampered after fertilization. From fertilization onward inhibition of Cdk activities does not preclude BrdU incorporation in maternal chromatin however it reduces its efficiency.

Simultaneous inhibition of MAP kinases and checkpoint kinases triggers initiation of DNA replication in unfertilized eggs

The above data prompted us to determine which mechanism inhibits the firing of the licensed origins in sea urchin UF eggs. As mentioned in introduction, cell cycle arrest after meiotic maturation is associated with a high level of MAP kinase activity which is relieved at fertilization and in oocytes of the starfish *P. pectinifera*, ERK inactivation directly induces DNA synthesis (Tachibana et al., 1997, 2000). In contrast, in the sea urchin *P. lividus*, a treatment of G1-arrested eggs with U0126, a potent inhibitor of MEK (Favata et al., 1998), while inducing different mitotic-like events (microtubule polymerization, nuclear envelope breakdown, oscillations in the phosphorylation of Cdk1 on tyrosine), did not produce full DNA replication (Zhang et al., 2006). Indeed, U0126 treatment of UF eggs only generated a slight incorporation of BrdU into DNA, not sensitive to aphidicoline. Alteration of an additional signalling pathway would thus be necessary to trigger complete S-phase from sea urchin G1 arrested eggs. Recent experiments in *Xenopus* cell-free extracts and in mammalian cells have demonstrated that the DNA damage checkpoint kinases ATM/ATR and Chk1 regulate replication origin firing in normal S-phase (Sorensen et al., 2003; Zhao et al., 2002). We thus hypothesized that such a mechanism could be involved in arresting eggs in G1 with pre-RC assembled on chromatin. To address this question, we simultaneously treated UF eggs with U0126 (1 µM) and caffeine (10 mM), an inhibitor of the ATM/ATR checkpoint kinases (Blasina et al., 1999; Hall-Jackson et al., 1999), and compared the level of BrdU incorporation to the one obtained in eggs activated with ammonium or calcium ionophore (A23187). As shown in Fig. 9(e, f), low levels of BrdU incorporation are observed in the presence of U0126 or caffeine alone. However, simultaneous treatment with both drugs (Fig. 9g) triggered a BrdU incorporation similar to the one observed in ammonium or A23187 treated eggs (Fig. 9b and c). Even though caffeine is also known to raise intracellular level of calcium in certain cells, elevation of the fertilization envelope was never observed in caffeine- or U0126 plus caffeine-treated sea urchin eggs (compare Fig. 9d and h). Moreover, Patel et al. reported that caffeine addition which rescued aphidicolin-arrested *Lytechinus pictus* sea urchin embryos did not induce intracellular calcium change (Patel et al., 1997). Finally, caffeine treatment only was unable to promote DNA replication as did calcium ionophore (Fig. 9f and c). These data suggest that replication of female DNA is repressed simultaneously by MAP kinase and checkpoint kinase pathways. On the other hand, as ammonia treatment triggers DNA synthesis in UF eggs without altering Phospho-MAPK level (Zhang et al., 2006), we can hypothesize that ammonia directly act on a downstream target in the MAPK signalling pathway to trigger DNA replication.

Fig. 6. Geminin is present on chromatin during licensing. (A) Geminin is recruited to male pronucleus soon after fertilization. Embryos were fixed 10 min p.f. and treated for immunostaining as described in Materials and methods, using anti-geminin antibodies previously incubated (d–f) or not (a–c) with immobilized His-geminin. Secondary antibodies were coupled to FITC. The DNA was visualized with DAPI (b, e). Merge pictures are also represented (c, f). Scale bar: 30 µm. (B) Increasing geminin concentration in UF sea urchin eggs didn't impede DNA replication. UF eggs were microinjected either with buffer as control (a–c, g–i) or with GFP-geminin (d–e, j–l). Eggs were either activated by ammonium treatment in FSW or fertilized, in the presence of BrdU (0.1 mg/ml) and fixed 50 min later. Replication was monitored through BrdU incorporation detected with TRITC-conjugated antibodies as described in Materials and methods (a, d, g, j), and GFP-geminin was visualized by epifluorescence (b, e, h, k). Superposition of the two signals is displayed (c, f, i, l). Scale bar: 30 µm.

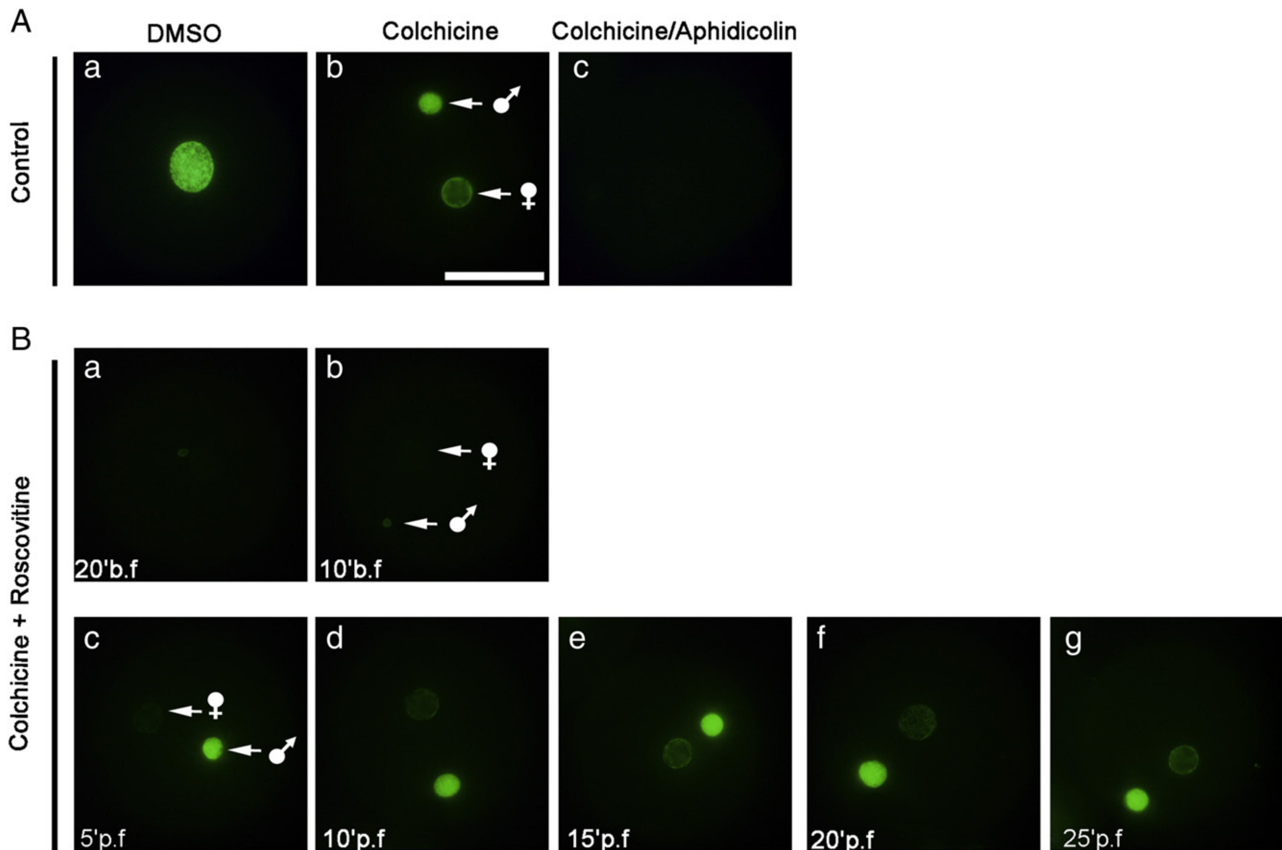


Fig. 8. Inhibition of Cdk activity at the time of fertilization alters DNA replication. DNA replication was monitored by BrdU incorporation as described in Materials and methods. (A) Colchicine did not prevent DNA replication. Eggs were fertilized in the presence of BrdU and indicated drugs (colchicine 0.5 µg/ml, aphidicolin 20 µg/ml) and were fixed 50 min p.f. It was verified that in the presence of aphidicolin BrdU is not incorporated (c). (B) A differential BrdU incorporation is observed according to the moment when roscovitine is supplied. Roscovitine (20 µM) was added to eggs cultured in the presence of BrdU and colchicine at different times from 20 min before fertilization to 25′ after fertilization as indicated.

Discussion

Our present data show that sea urchin oocytes arrested in G1 have already assembled pre-RC on replication origins. To our knowledge, this is the first demonstration of a cell cycle arrest in metazoan in which chromatin is already licensed for replication. As natural cell cycle arrests are often reminiscent of checkpoint occurring in dividing cells, this suggests that a mechanism should control the completion of pre-RC assembly in cells. Our data further show that signalling pathways involving both MAP kinases and checkpoint kinases participate in preventing activation of DNA replication in G1-arrested sea urchin eggs.

Initiation of embryonic mitotic cell cycles after fertilization is a key event for the harmonious development of an embryo and the first DNA replication a critical step for accurate duplication of parental genome. In proliferating somatic cells, DNA origins can be licensed from M-phase output to the beginning of S-phase when Cdk activities are low. When embryos are fertilized in metaphase of meiosis II, as in *Xenopus*, progression towards S-phase follows the release from meiosis arrest and licensing of origins occurred on chromatin when Cdk activities have decreased. Until now, it remained unclear how and when takes place the licensing when fertilization occurs in G1-arrested eggs as in several echinoderms and cnidarian. Experiments were sought in sea urchin to investigate if licensing of female and male chromatin occurs concomitantly post-fertilization or if female chromatin is already licensed before fertilization. We first identified mRNAs of the pre-RC components CDC6, Cdt1 and MCM3 as well as the regulator of their assembly, geminin, and confirmed that the different proteins are expressed in UF eggs and early embryos. While CDC6, Cdt1 and MCM3 associate to male chromatin post-fertilization they were found already loaded on female chromatin before fertilization. Thus, male chromatin is licensed post-fertilization as observed in *Xenopus* (Blow and Laskey, 1986; Lohka and Masui, 1983). In contrast, pre-RC assembled on female chromatin before eggs paused in G1 presumably when Cdk activities decrease at meiosis II output. This leads to an unexpected situation in metazoan in which cells are arrested with already licensed chromatin and suggests that a specific mechanism should be activated to prevent initiation of DNA replication. Our data demonstrate that this mechanism does not rely on the inhibitory function of geminin, which in contrast seems to participate in loading Cdt1 on female and male origins. Indeed, a low concentration of geminin is present with pre-RC components on chromatin of UF eggs arguing that *in vivo*, as already proposed from data obtained *in vitro* in *Xenopus* extracts, a Cdt1–geminin complex could facilitate pre-RC assembly. Moreover, we observed that an excess of geminin produced by cytoplasmic microinjection of a recombinant GFP-geminin able to interact with Cdt1, does not prevent initiation of DNA replication even if the protein is translocated to the nucleus. As the same recombinant protein inhibits sperm DNA replication in *Xenopus* extract, this suggests that *in vivo* in sea urchin, some molecular event should take place upon firing of origin to allow further association of geminin to Cdt1 to form an inactive complex inhibiting new pre-RC assembly. MCM9 was proposed in *Xenopus* to transiently protect Cdt1 from geminin additional binding (Lutzmann and Mechali, 2008). As we previously reported, MCM9 mRNAs are expressed in sea urchin embryo (Fernandez-Guerra et al., 2006), suggesting that the protein, which is not vertebrate-specific as initially believed, could play the same role.

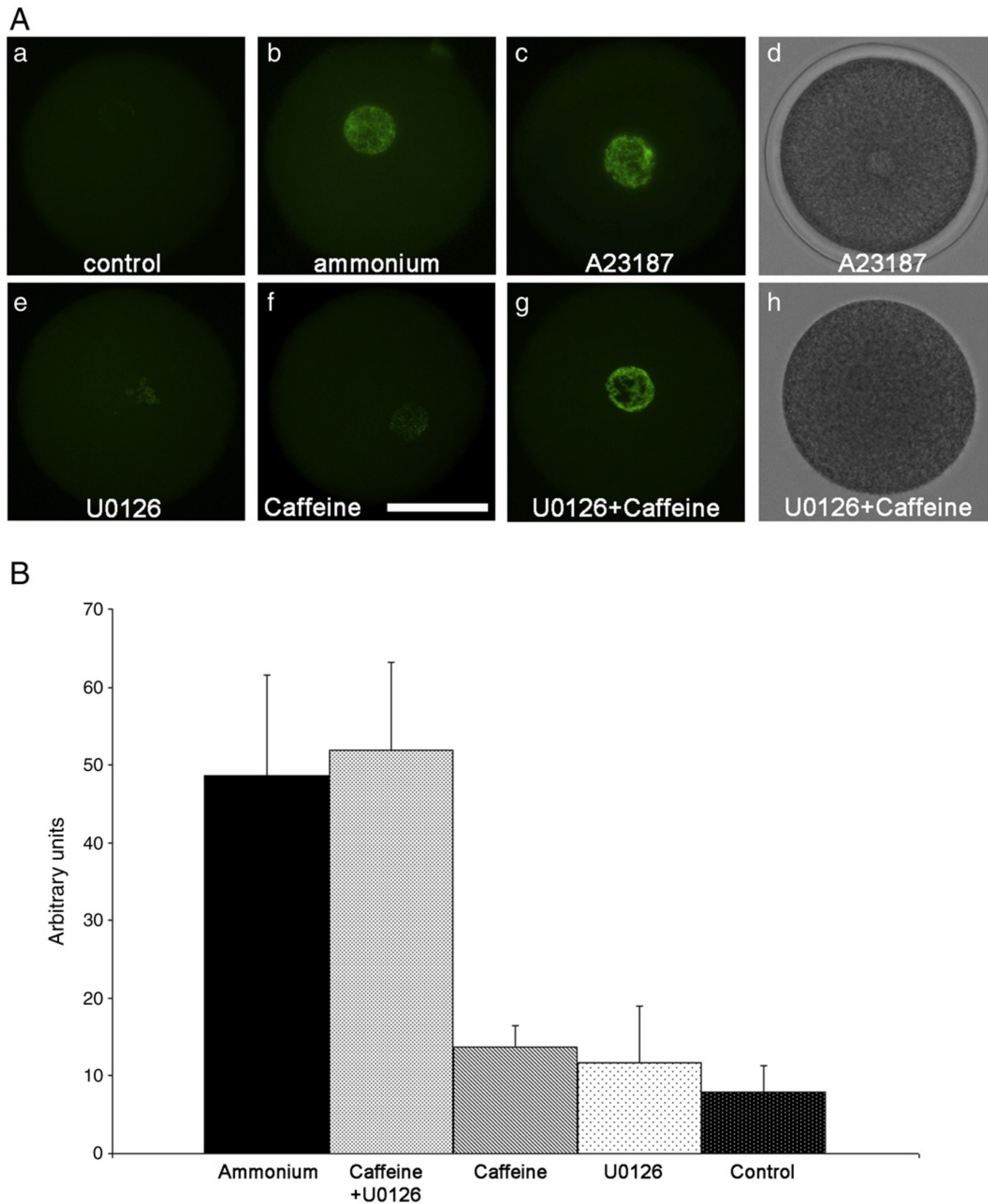


Fig. 9. Synergistic effect of caffeine and U0126 in promoting DNA synthesis in unfertilized sea urchin eggs. (A) DNA replication was monitored by BrdU incorporation as described in Materials and methods. UF eggs parthenogenetically activated by ammonium or calcium ionophore (A23187) and analysed 1 h after incubation show a major BrdU incorporation (b, c) which was not observed in untreated eggs (a). UF eggs treated with 1 µM U0126 (e) or 5 mM caffeine (f) during 1 h show weak BrdU incorporation while a simultaneous treatment (g) promotes a BrdU incorporation similar to the one observed in the presence of ammonium. Light microscopy images of eggs treated with A23187 (d) or caffeine and U0126 (h). Scale bar = 30 µm. (B) Statistical analysis of fluorescence. The amount of fluorescence in individual nuclei was quantified with Bio 1D software (Vilbert Lourmat). For each treatment condition, the mean value from 20 representative eggs coming from two independent experiments was reported.

After chromatin became competent, conversion of pre-RC to pre-ICs depends on Cdk2 and CDC7 activities which will allow recruitment of CDC45 at the G1/S transition (Lei and Tye, 2001; Nougarede et al., 2000; Walter, 2000). However, in *S. granularis* and *S. purpuratus* sea urchin embryos, we and others have shown that Cdk2 activity can be inhibited from a few minutes p.f. onwards without precluding initiation of the first DNA replication (Moreau et al., 1998; Schnackenberg et al., 2007). In this report, we further observed that addition of roscovitine to *P. lividus* eggs 5 min p.f. does not prevent DNA replication neither of female nor of male chromatin in fertilized eggs treated with colchicine to prevent syngamy. In contrast, when roscovitine is applied 20 min before fertilization DNA replication is completely blocked. Thus, as in other metazoan, a Cdk2-dependent phosphorylation is necessary for pre-RC activation. However, the Cdk2-dependent phosphorylation required for pre-IC formation is already fulfilled, at least on a set of origins, in a time window close to fertilization, well before S-phase entrance. While we were writing this manuscript, Kisielewska et al. reported in *L. variegatus* a transient Cdk2 activity peaking 4 min p.f. (Kisielewska et al., 2009). They also show that a treatment of roscovitine applied 30 min b.f. inhibits male chromatin decondensation and replication of fused zygotic chromatin. Our findings confirm and extend their results showing that the timing of Cdk2 requirement is different for male and female chromatin. Our observations could also be consistent with the potential presence of pre-IC on chromatin of UF eggs. Further experiments are thus needed to determine if the stepwise assembly of the replication complexes in sea urchin oocytes culminates with loading of the MCM2–7 helicase or if some components of the pre-IC complexes are already associated to chromatin. Even if replication can be observed

in the presence of Cdk inhibitors during S-phase, previous experiments (Moreau et al., 1998) and present data show that inhibition of Cdk activity alters S-phase progression, preferentially affecting female chromatin replication (Fig. 8). This can be explained either by an inhibition of the firing of origins that would have been progressively recruited in unperturbed embryos or by a requirement of Cdk activity at a step following DNA synthesis initiation. In most metazoan somatic cells, origin firing follows a defined spatiotemporal program (Zink, 2006), i.e. transcriptionally active domain replicating generally before transcriptionally silent domains. Unexpectedly, recent data shown that, replication timing is also deterministic in *Xenopus* egg extracts at the level of large chromosomic domain, which suggests that a replication timing program is already established before MBT in early vertebrate embryos, even if transcription does not occur (Labit et al., 2008). It is possible that a similar staggered program of replication activation exists in echinoderm, in particular in sea urchin where transcription is already active in fertilized eggs even if it does not concern genes essential during early cleavages. In that case origins could be found at different stages in G1 arrested eggs, in preparation for a sequential firing post-fertilization, what would explain the pattern of BrdU incorporation we observed after Cdk2 inhibition. On the other hand, it has been recently reported that Cdk2 is recruited to replication foci in a CDC45-dependent way to phosphorylate histone H1 and promote a DNA remodelling that facilitates fork progression (Alexandrow and Hamlin, 2005). This late Cdk2 requirement can also explain a slow down of replication in the presence of roscovitine.

The signalling pathways that control release of cell cycle arrests have been investigated mainly in *Xenopus* and mouse, two examples of eggs arrested in meiosis MII and in the starfish eggs of *P. pectinifera* paused in G1 (for reviews see Kishimoto, 2003; Perry and Verlhac, 2008). In the three models, the Mos-MAPK cascade is responsible for the cell cycle arrest and its activity is inhibited at fertilization, however, the downstream effectors of Mos vary significantly from one model to the other. While Rsk is an essential component of the MII cytostatic factor in frog and of the G1 arresting mechanism in starfish, it is not required in mouse. Whatever is the downstream substrate of MAPK, inhibition of MAPK signalling pathway in MII arrested eggs ends up in MPF inhibition through activation of the cyclin B proteolysis. In contrast, in G1-paused starfish eggs MAPK-pathway deactivation relieves two independent blocks, one sensitive to the Rsk transducer which controls S-phase entry and promote destruction of cyclin A and B, the other-one involving an independent pathway negatively regulating cyclin B synthesis and consequently activation of MPF for M-phase entry (Hara et al., 2009). In the sea urchin *P. lividus*, inhibition of the MAPK signalling pathway also triggers mitotic-like events with oscillations of phosphorylation of the Tyr15 residue of Cdk1 and the correlated H1-kinase activity, although it does not induce the completion of DNA replication (Zhang et al., 2006).

In this report we show that the simultaneous inhibition of the MAPK and ATM/ATR signalling pathways in UF eggs trigger the same extent of DNA replication than the one generated by ammonium or calcium ionophore treatment. The ATM/ATR signalling pathway plays a prominent role in mediating cellular response to DNA damage (for reviews see Bartek and Lukas, 2007; Clemenson and Marsolier-Kergoat, 2009). However, either in mammalian cells or in *Xenopus* extract it is also implicated in regulating different aspects of unperturbed S-phase in particular the timing and spacing of DNA replication origins (Marheineke and Hyrien, 2004; Maya-Mendoza et al., 2007; Petermann and Caldecott, 2006; Shechter et al., 2004; Sorensen et al., 2004). Indeed, the nuclear ATM/ATR kinases are part of a sensor mechanism that detect DNA damage or stalled replication forks. While ATM preferentially recognizes DNA double strand breaks, ATR is activated in response to formation of single-stranded DNA (ssDNA). SsDNA is recognized and become coated with the ssDNA binding protein replication protein A (RPA), which subsequently recruits and activates the ATR complex (Byun et al., 2005; MacDougall et al., 2007; Michael et al., 2000; Zou and Elledge, 2003). In sea urchin one-cell embryo, caffeine, an ATM/ATR inhibitor (Blasina et al., 1999; Hall-Jackson et al., 1999), has been shown to reverse the nuclear breakdown inhibition imposed by aphidicolin (Patel et al., 1997), suggesting that these kinases are expressed in eggs. Consistently, two homologues of ATM and ATR genes have been identified in the sea urchin genome, however, according to the results of a tilling array only ATR would be expressed in early embryos (Fernandez-Guerra et al., 2006). While the mechanism of activation of the checkpoint kinase pathway in UF eggs is currently difficult to predict, different hypotheses can be proposed. Our data show that MCM are physically associated to maternal chromatin in UF eggs. We thus cannot exclude that a helicase activity could be associated to certain origins that would have also loaded CDC45, leading to an RPA coating sufficient to trigger checkpoint activation. We should mention that two subunits of RPA (34 and 70 kDa) have been found associated to chromatin in UF eggs (data not shown). On the other hand, a direct interaction has been demonstrated between MCM7 and ATRIP, a factor essential for ATR-dependent signalling and there is evidences that MCM are direct targets of the ATM/ATR kinases in human cell lines where an excess of MCM loading could be important for checkpoint function (Cortez et al., 2004). It is thus possible that loading of MCM2–7 on sea urchin female chromatin can somehow trigger a checkpoint activation leading to cell cycle arrest.

On the other hand, ERK activity has been shown to regulate ATR function by promoting cytoplasm to nucleus ATR transport therefore facilitating activation of the S-phase DNA damage checkpoint in human cell lines (Wu et al., 2006). Part of the cytostatic effect of MAPK cascade in G1-arrested sea urchin eggs could rely on this mechanism.

In G1, S or G2/M DNA damage checkpoints ATM/ATR kinases act primarily by activating a pair of effector kinases Chk1 or Chk2 which then control the activation of the checkpoint responses. Immediate cell cycle arrest is mediated by the inactivation of CDC25 Tyr-phosphatases which in turn impede activation of Cdks. Both Chk1 and Chk2 as well as one CDC25 genes have been identified in the sea urchin genome, all being expressed in embryos (Fernandez-Guerra et al., 2006). While cyclin E has been located in the female pronuleus (Schnackenberg and Marzluff, 2002) as well as Cdk2 (personal data), this does not imply that the complex cyclin E/Cdk2 is active inside the nucleus of the arrested eggs. It is possible that the recruitment of ATR to competent chromatin by turning on Chk1/2 locally inactivates CDC25 hampering Cdk2 activity in order to prevent origin firing. Fertilization by reversing ATR stimulation of Chk1/2 could lead to Cdk2 activation in the female pronucleus allowing DNA replication to be initiated. Other effectors independent of Cdk2 activity that have been implicated in transducing the Chk1/2 signal could also be involved. Ddk activity which is required with Cdk for CDC45 loading on chromatin (Jares and Blow, 2000; Walter, 2000) has been proposed as a potential target of the checkpoint in response to replication stress (Costanzo et al., 2003). More recent data have shown that CDC7/Dbf4 would not be a target but an upstream regulator of Chk1 the activity of which would be critical for checkpoint release (Tsuji et al., 2008).

Our results demonstrate that a cell can be arrested for a long period with pre-RC already assembled on chromatin, pointing out the existence of a long-term established mechanism that prevent the firing of replication origins. New insight is also provided into the mechanisms contributing to the G1 arrest and the control of S-phase entry at fertilization. Further experiments are needed to understand the relationship between the kinases participating in the oocyte arrest and those involved in pre-RC to pre-IC transition.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.02.009.

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