

Dynamic interactions of high Cdt1 and geminin levels regulate S phase in early *Xenopus* embryos

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SUMMARY

Cdt1 plays a key role in licensing DNA for replication. In the somatic cells of metazoans, both Cdt1 and its natural inhibitor geminin show reciprocal fluctuations in their protein levels owing to cell cycle-dependent proteolysis. Here, we show that the protein levels of Cdt1 and geminin are persistently high during the rapid cell cycles of the early *Xenopus* embryo. Immunoprecipitation of Cdt1 and geminin complexes, together with their cell cycle spatiotemporal dynamics, strongly supports the hypothesis that Cdt1 licensing activity is regulated by periodic interaction with geminin rather than its proteolysis. Overexpression of ectopic geminin slows down, but neither arrests early embryonic cell cycles nor affects endogenous geminin levels; apparent embryonic lethality is observed around 3–4 hours after mid-blastula transition. However, functional knockdown of geminin by Δ Cdt1_193–447, which lacks licensing activity and degradation sequences, causes cell cycle arrest and DNA damage in affected cells. This contributes to subsequent developmental defects in treated embryos. Our results clearly show that rapidly proliferating early *Xenopus* embryonic cells are able to regulate replication licensing in the persistent presence of high levels of licensing proteins by relying on changing interactions between Cdt1 and geminin during the cell cycle, but not their degradation.

KEY WORDS: Cdt1:Geminin, DNA replication, *Xenopus* embryos

INTRODUCTION

To ensure that the genome is accurately duplicated during S phase and that chromosomes are correctly separated, DNA replication is under the strict control of many cell cycle pathways and checkpoints (Blow and Dutta, 2005; DePamphilis, 2005; Machida and Dutta, 2005; Sasaki and Gilbert, 2007). Initiation of replication is a crucial step in the control of DNA synthesis in eukaryotes and begins with sequential assembly of pre-replicative complex (pre-RC) proteins onto replication origins in a highly organised manner (Bell, 2002; Bell and Dutta, 2001; Blow and Dutta, 2005; Tsakraklides and Bell, 2010). First, the origin recognition complex (ORC1–6) binds to chromatin followed by recruitment of Cdc6 and Cdt1, and, finally, the clamping of the MCM2–7 around the DNA (Evrin et al., 2009; Gillespie et al., 2001; Remus et al., 2009). This process licenses the origin for future replication.

To prevent re-replication, licensing activity has to be restricted to a short time at the end of mitosis/G1 and inhibited once S phase has begun (Blow and Dutta, 2005; Dimitrova et al., 2002). In metazoans, regulation of Cdt1 activity is the major pathway that prevents re-replication and re-licensing (Blow and Dutta, 2005; DePamphilis, 2005; Lee et al., 2010; Machida and Dutta, 2005; Maiorano et al., 2000; Nishitani et al., 2000; Sasaki and Gilbert, 2007; Tada et al., 2001; Whittaker et al., 2000). In higher eukaryotes, inactivation of Cdt1 relies on two mechanisms: binding to its natural inhibitor geminin and/or its ubiquitylation and subsequent degradation (Arias and Walter, 2005; Arias and Walter, 2006; Hu and Xiong, 2006; Li and Blow, 2005; Liu et al., 2004; McGarry and Kirshner, 1998; Nishitani et al., 2006; Nishitani et al.,

2001; Senga et al., 2006; Sugimoto et al., 2004; Tada et al., 2001). In mammalian somatic cells, geminin accumulates during S and G2 phase to be degraded by the anaphase-promoting complex (APC/C) at the metaphase-anaphase transition, allowing a new round of licensing to occur (McGarry and Kirshner, 1998). However, in *Xenopus* cell-free extracts, only a proportion of the geminin is degraded and a significant amount escapes proteolysis and is imported into the nuclei upon nuclear assembly to be reactivated as a Cdt1 inhibitor during late interphase (Hodgson et al., 2002; Li and Blow, 2004; Maiorano et al., 2004). In *Xenopus* egg extracts, Cdt1 is subject to at least two types of cell cycle-dependent regulation: it is degraded on replicating chromatin during S phase in a process dependent on PCNA, Ddb1 and Cdt2 (Arias and Walter, 2005; Arias and Walter, 2006; Jin et al., 2006); and it is also degraded by the APC/C on exit from mitosis (Li and Blow, 2005).

The balance of Cdt1:geminin levels has been shown to be crucial for regulation of proper replication in somatic cells and *Xenopus* cell-free extracts. Stabilisation of Cdt1 protein levels or removal of geminin independently lead to only slight levels of re-replication; however, the abrogation of both these control systems leads to massive re-replication (Li and Blow, 2005). Geminin is recruited to chromatin at the onset of S-phase, prior to degradation of chromatin-bound Cdt1 (Gillespie et al., 2001; Oehlmann et al., 2004). Furthermore, it has been demonstrated that the stoichiometry of the Cdt1:geminin complex can regulate its activity and acts as a molecular switch between licensing and inhibition (Lutzmann et al., 2006). This may be mediated by the ability to form a fully active 2:4 Cdt1:geminin heterohexamer that is unable to engage MCMs, thus preventing new pre-RCs formation (De Marco et al., 2009).

Previous studies using *Xenopus* egg extracts show significant decline in Cdt1 levels with or without sperm DNA added and partial decline in the level of geminin upon release from metaphase (Li and Blow, 2004; Li and Blow, 2005). However, whether the embryonic system relies on such mechanisms during rapid cell cycle oscillations remains to be established. Synchronous early embryonic cleavages

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in *Xenopus* occur from the moment of fertilisation through the next 12 divisions followed by the onset of zygotic transcription at mid-blastula transition (MBT). Early embryonic cells are transcriptionally quiescent and rely on maternally derived proteins and mRNAs (Newport and Kirshner, 1982). Their cell cycles are very rapid, oscillating between DNA replication and mitosis with no apparent gap phases. Early embryos lack robust checkpoints related to incomplete DNA replication, damage or chromosome segregation (Bulavin et al., 2003; Clute and Masui, 1997). We have very limited knowledge of how licensing and therefore Cdt1 activity is regulated during such short cleavages.

A previous study in *Drosophila* has indicated the presence of high levels of dm geminin in the syncytial blastoderm (Quinn et al., 2001). Here, we address spatiotemporal dynamics of both Cdt1 and geminin in early *Xenopus* embryos, their high protein levels throughout entire pre-MBT stages and changes in their mutual interactions from the moment of fertilisation. We show that disrupting the amount of endogenous Cdt1 by neutralisation of geminin results in activation of p53 and RAD51, indicating that chromosomal breaks occur in affected cells. Cell cycle arrest after functional knock down of geminin in a number of cells before MBT inevitably affects embryonic differentiation. Our findings significantly complement results from pre-MBT stages of *Drosophila* and for the first time show in vivo that cell cycle interactions of high levels of Cdt1 and geminin are crucial for proper cleavages to occur in rapidly proliferating early embryonic cells.

MATERIALS AND METHODS

Antibodies

Primary antibodies raised in rabbit against Orc1, Orc2, Cdt1, geminin and Cdc6 were previously used (Tada et al., 2001). Anti-Mcm2 (BM28) and anti-PCNA were from BD Biosciences; anti γ -tubulin, anti RFP, anti p53 (phospho S15) and RAD51 (phosphoT309) were from AbCam; anti β -actin and anti α -tubulin, rabbit IgG and secondary antibodies (peroxidase conjugate) were from Sigma. Cyclin B1 antibody was a kind gift from J. Gannon (Cambridge, UK).

Embryo preparation

Highly synchronised embryos of *Xenopus laevis* (Linnaeus) were obtained by in vitro fertilisation (Lavery and Hoppler, 2008). Fertilisation rate was >95%. To remove the vitelline layer, embryos were placed in 2% cysteine in 0.1×MMR [10× stock: 20 mM CaCl₂, 50 mM HEPES, 20 mM KCl, 10 mM MgCl₂, 1 M NaCl (pH 7.8)] and washed with 1×MMR and 0.1×MMR. Further development continued in fresh 0.1×MMR.

Immunofluorescence

Embryos (stage 7) were fixed in MEMFA salts [10× stock: 1 M MOPS, 20 mM EGTA, 10 mM MgSO₄ (pH 7.4)] and 37% formaldehyde followed by two washes in methanol (Khokha et al., 2002). Preparation of embryos for immunofluorescence was performed as previously described (Lavery and Hoppler, 2008). Whole-mount embryos or cryosections were used. For the latter, embryos were embedded in OCT medium (RALamb) and frozen in liquid N₂. Sections (30 μ m) were cut using a cryotome E.

Alexa Fluor 488 goat anti-mouse and/or Alexa Fluor 555 Goat anti-rabbit (Invitrogen) were used as a secondary antibody.

Macroimaging

Live embryonic images were taken using a Motic stereomicroscope (Fig. 5C,D) or Leica Fluorescence Macroimaging System M205FA with digital high sensitivity DFC310FX cooled camera and LasA program (Fig. 4; Fig. 5E,F).

Confocal/2P imaging

Embryonic sections, whole embryos and *Xenopus* extracts were imaged with a Zeiss LSM_510 confocal/two-photon or Leica SP2 confocal microscope using a Plan Apochromat 63×/1.4 n.a. oil immersion objective.

FITC was excited using the 488 nm and TRITC using the 543 nm line of an Argon-Krypton laser, with emitted light bandpass filtered between 500–550 nm or 565–615 nm, respectively. For DAPI, the Zeiss LSM_510 two-photon mode was used with excitation (at 765 nm) provided by a tuneable Titanium:Sapphire Mai Tai laser (Spectraphysics). For Hoechst staining, a Leica diode laser with 405 nm excitation was used.

All images were processed using Zeiss AxioVision or ImageJ software. Statistical analysis was performed using Student's *t*-test. Each error bar represents mean±s.e.m. from indicated number of independent experiments.

Chromatin isolation

Chromatin from embryos entering stage 7 was isolated as described previously (Menut et al., 1999). Embryos (1 ml) were collected every 5 minutes. Chromatin recovery was determined by staining 2 μ l of sample with DAPI followed by imaging with confocal/2P microscopy. Additional samples were taken at 4.30 hours AF and used for DNase (Roche) treatment as follows: 3 U/ μ l of DNase was added to the sample before the final spin at 17,000 *g*. A pellet left after the final spin was used for the western blot.

Recombinant proteins and mRNAs

Recombinant Δ Cdt1_{193–447} was made as previously described (Ferenbach et al., 2005) with the difference that start and stop codons were added (primers as follows: 5' GGATTCATGTCTTCAGAGGG-CAAAGTCACC, 3' GAATTCCTAAATCAAGCCTCTAGCTTTGG). The construct was cloned between *Bam*HI and *Eco*RI into the pET32a vector. Recombinant 6×His protein was expressed in Tuner *E. coli*. The soluble protein was purified using Novagen His bind purification kit followed by mass spectrometry identification at Pinnacle services (Newcastle University, UK). For microinjection, Δ Cdt1_{193–447} was resuspended in PBS. To produce Δ Cdt1_{193–447} mRNA, the same primers were used; however, it was cloned as a *Bgl*II-*Not*I fragment into the pRN3 vector (Lemaire et al., 1995). GFP-Cdt1 was a kind gift from L. McSloy (Newcastle, UK).

Recombinant geminin^{DEL} was a kind gift from A. Li (Dundee, UK). Wide-type geminin was inserted between *Nco*I and *Bam*HI of the pET32a vector and site-directed mutagenesis was used to put cDNA in frame, which destroyed the *Nco*I restriction site.

RFP (red fluorescent protein) was linked to the C terminus of Geminin by *Bam*HI. Recombinant proteins were expressed and purified as for Δ Cdt1_{193–447}.

For mRNA, Geminin-RFP was inserted between *Bgl*II and *Not*I in the pRN3 vector. mRNA was made by in vitro transcription using high yield capped mRNA mMessage mMachine kit for T3 promoter (Ambion).

RFP-PCNA recombinant protein was purified as previously described (Kisielewska et al., 2009). RFP-PCNA mRNA was prepared as previously described with the exception that the RFP-PCNA construct was used instead of GFP-PCNA in the pRN3 vector (Kisielewska et al., 2005; Kisielewska et al., 2009).

Protein activity

To verify activity of recombinant Δ Cdt1_{193–447}, the licensing reaction was performed in *Xenopus* cell-free extracts. Unactivated egg extract (ULSS) was prepared as described previously (Chong et al., 1997). The licensing reaction was performed as previously described (Ferenbach et al., 2005). Different concentrations of Δ Cdt1_{193–447} with or without 1.8 μ g/ml Geminin^{DEL} were added to the samples. The samples were incubated at 23°C. The reaction was stopped by 160 μ l Stop C [20 mM Tris-Cl (pH 7.5); 5 mM EDTA, 0.5% SDS, 0.2 mg/ml Proteinase K] at 90 minutes.

Geminin, geminin-RFP and GFP-Cdt1 recombinant protein activity was measured as above using 1–2, 3–6 and 8 μ g/ml concentrations, respectively.

DNA titration

Xenopus extract was supplemented with different concentrations of sperm DNA (20–0.15 ng/ μ l). An additional sample of 20 ng DNA/ μ l was supplemented with 100 nM of p27 (a kind gift from Gaganmeet Singh Chadha, Dundee, UK). The extract was blotted for Cdt1 and geminin after 90 minutes. In parallel, samples containing 20 or 0.3 ng DNA/ μ l were

supplemented with 6 µg/ml of recombinant GFP-Cdt1 and fixed in 67% glycerol, 10% 10×MMR, 10% formaldehyde and 0.001% Hoechst at different time points between 30 and 120 minutes.

Immunoprecipitation

Anti-geminin, -Cdt1 and -Mock antibodies (rabbit IgG, Sigma) were coupled to protein A sepharose (Amersham) beads as described previously (Harlow and Lane, 1988). Sixty embryos were homogenised in the presence of leupeptin and aprotinin (1 mg/ml), PMSF (1 mM), 100 µM NaF and 100 µM Na₂VO₄ followed by a spin at 17,000 *g* at 4°C. Embryonic extracts from different time AF and metaphase egg extract were then incubated with 20 µl of protein A Sepharose beads overnight at 4°C in the presence of immunoprecipitation buffer [20 mM Tris (pH 8); 50 mM NaCl; 5 mM EGTA (pH 8); 1% TritonX100; 0.5% sodium deoxycholate; 100 µM NaF; 100 µM of Na₂VO₄; 1 mg/ml leupeptin and aprotinin; 1 mM PMSF]. After incubation, the beads were washed with immunoprecipitation buffer with and without 0.5 M NaCl followed by a final wash with PBS (PBS and 1 mM PMSF). After a final spin, supernatant was removed and 15 µl of 5× Laemmli Sample Buffer was added followed by 10 minutes boiling. Proteins were separated using 4–12% SDS-PAGE and immunoblotted against Cdt1 and geminin. ImageJ gel analysis was used to quantify western blots.

RESULTS

Cdt1:geminin levels and interactions

We used antibodies against *Xenopus* Cdt1 and geminin that have previously been characterised in *Xenopus* egg extracts (Tada et al., 2001) to examine the behaviour of these proteins in developing

Xenopus embryos. The Cdt1 and geminin antibodies clearly recognised their cognate proteins in both egg and embryonic extracts (supplementary material Fig. S1A). With the Cdt1 antibody, unspecific lower molecular weight bands (asterisks) were also detected. These bands were not detected on chromatin and did not co-immunoprecipitate with geminin antibody (Fig. 1C and Fig. 2B). Geminin antibody also recognised an unspecific higher molecular weight band that does not appear on chromatin or immunoprecipitation blots (Fig. 1C, Fig. 2). To address whether or not Cdt1 and geminin levels change within a single cell cycle, immunodetection of these proteins was performed using synchronised embryos from stages 1 and 7. Cyclin B1 was used to determine the cell cycle stage of embryonic cells. In *X. laevis*, the first cell cycle after fertilisation (AF) lasts about 80–90 minutes followed by 11 subsequent cycles of 30 minutes that oscillate between S and M phase until MBT (Ferrell, 1999). Cyclin B1 was clearly detected, with the exception of 20–30 minutes AF, when licensing starts in both male and female pronuclei (Ferrell, 1999; Hartley et al., 1996; Leibovici et al., 1992) (Fig. 1A). Consistent with previous reports, loss of cyclin B1 at first mitosis occurred at 70 minutes AF followed by the first division (Fig. 1A). Cyclin B1 also oscillates in stage 7 with a clear indication of mitosis at 4 and 4.4 hours, consistent with 30-minute cell cycles at this stage (Fig. 1B).

Although Cdt1, geminin and Cdc6 levels were very low or undetectable in stage VI oocytes at prophase of meiosis I, their levels increased in unfertilised eggs that are arrested in metaphase

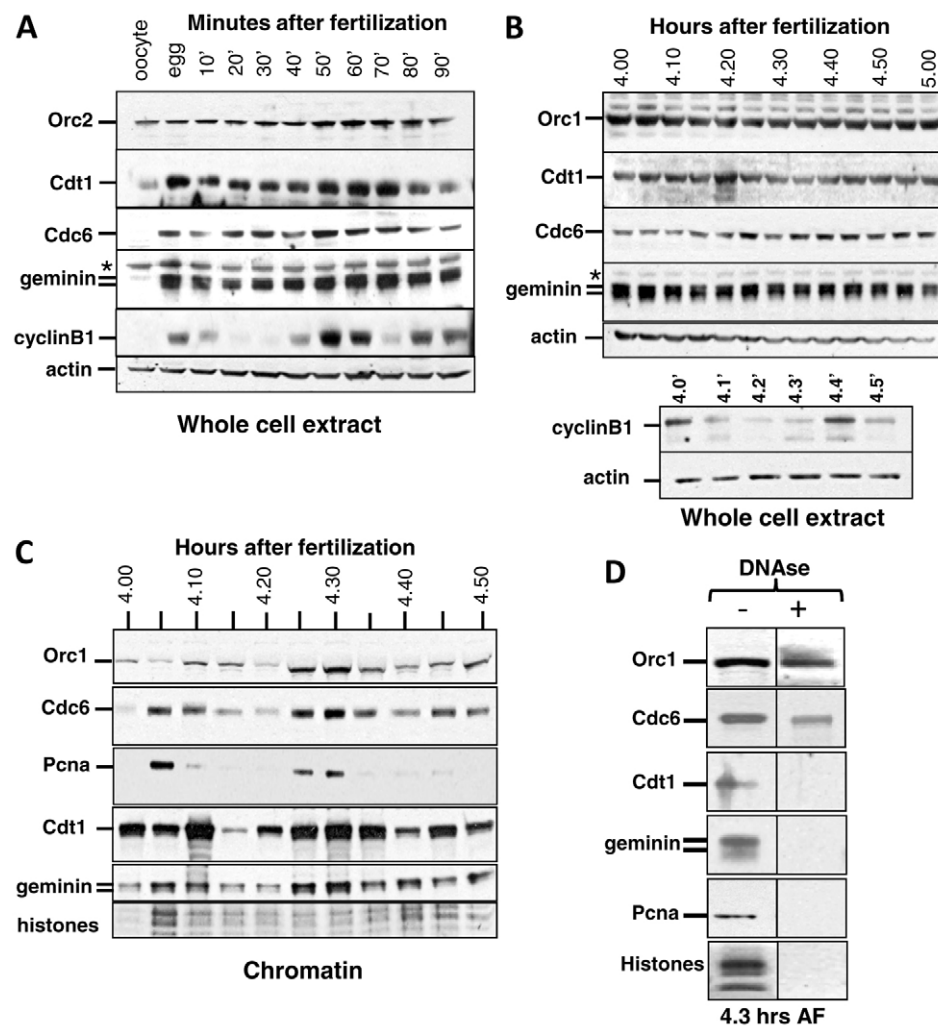


Fig. 1. Cdt1 and geminin cellular levels. (A,B) Synchronised embryos from stage 1 (A) and stage 7 (B) were used. Extract from one oocyte, egg or embryo was loaded per time point onto a gel and immunoblotted for Orc1 or Orc2, Cdt1, Cdc6 and geminin. The experiment was repeated with different batches of embryos derived from different frogs with similar results (*n*=5 for A and B separately). β -Actin is a loading control; asterisks indicate unspecific binding. (C) Isolated chromatin from synchronised embryos stage 7 was immunoblotted for Orc1, Cdc6, PCNA, Cdt1 and geminin. One full cell cycle can be detected between 4.15 and 4.40 hours. Accumulation of Cdc6 and PCNA indicates that S phase occurs between 4.25 and 4.30 hours. (D) DNase-treated and control chromatin samples from 4.30 hours AF.

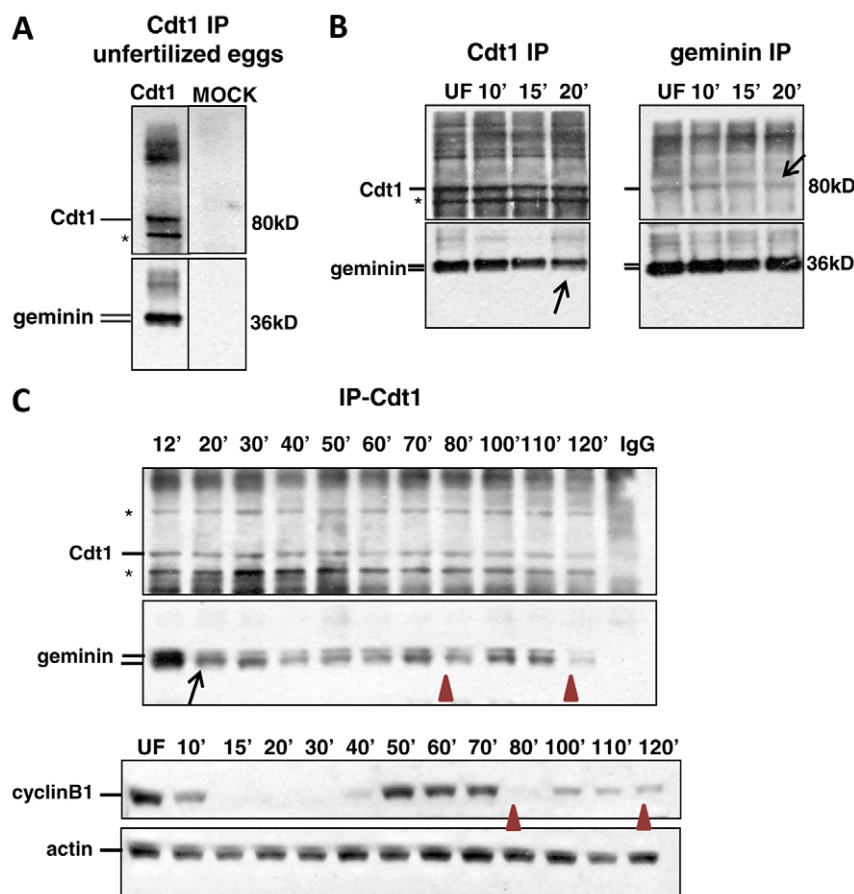


Fig. 2. Cdt1:geminin interaction during early cell cycles. (A) Cdt1:geminin complex in unfertilised eggs compared with IgG mock antibody. (B) Cdt1 and geminin immunoprecipitation showing mutual interactions during the first 20 minutes. The complex is still visible within the first 10 minutes followed by weaker interaction during the licensing at 20 minutes marked by arrows. (C) Cdt1 immunoprecipitation showing 120 minutes after fertilisation covering the first and second cell cycle, as indicated by cyclin B1 in the same cell cycle (lower panel). Black arrow indicates first decline in geminin binding. Red arrowheads indicate cytokinesis at 80 and 120 minutes. Asterisks indicate unspecific binding.

of meiosis II (Fig. 1A). Consistent with both geminin and Cdt1 being partially degraded as a consequence of APC/C activity (Hodgson et al., 2002; Li and Blow, 2005; McGarry and Kirshner, 1998) their levels decreased slightly within the first 20 minutes but returned to pre-fertilisation levels soon after (Fig. 1A). These fluctuations, however, were smaller than those typically observed in the *Xenopus* cell-free system (Li and Blow, 2005). In stage 7 embryos, Cdt1 and geminin levels remained high without the considerable changes that would indicate significant protein degradation and re-synthesis (Fig. 1B). To confirm that high Cdt1 and geminin protein levels do not depend on rapid protein turnover, we used cycloheximide to inhibit translation in embryos 4 hours AF (supplementary material Fig. S1B). Cycloheximide caused only a slight decrease in Cdt1 or geminin levels, in comparison with a total degradation of cyclin B1, suggesting that turnover rates of Cdt1 and geminin are normally low.

We then examined whether levels of licensing proteins were also high on chromatin. Embryonic development starts when one sperm fertilises an egg, which means that there is not enough DNA to be reliably isolated during the first few cleavages. Instead, chromatin was isolated from synchronised embryos in stage 7 (Fig. 1C). After DNase treatment of the chromatin, the quantity of Orc1, Cdc6, Cdt1, geminin and PCNA recovered by centrifugation was significantly reduced, consistent with the majority of the protein isolated by this technique being associated with chromosomal DNA (Fig. 1D). The periodic association of PCNA with chromatin shows the timing of S phase. Orc1, Cdc6, Cdt1 and geminin also showed a periodic association with chromatin, with minima at 4.15 and 4.40 hours, consistent with

the ~30-minute cell cycle (Fig. 1C). After the minima, Cdt1 appeared on chromatin prior to geminin, consistent with the licensing occurring during anaphase at 4.20 and 4.45 hours. The behaviour of Cdc6 and geminin on chromatin is consistent with their behaviour in *Xenopus* egg extracts. Cdc6 is recruited to ORC on DNA after origins initiate in S phase (Oehlmann et al., 2004). In egg extracts, geminin is imported into nuclei, which leads to reactivation of its ability to bind and inhibit Cdt1 (Hodgson et al., 2002; Yoshida et al., 2005). Cdt1 present on the chromatin during S phase can then recruit active geminin to chromatin (Gillespie et al., 2001; Oehlmann et al., 2004). Interestingly, there is no apparent decline in Cdt1 or geminin during the period when Cdc6 reaches its maximum (with clear detection of PCNA), suggesting that chromatin-bound Cdt1 levels are not significantly reduced during S phase as they are in *Xenopus* egg extracts (Arias and Walter, 2005). One possible explanation for the discrepancy between the in vitro and in vivo results is that at 4-5 hours, an embryo will contain the equivalent of 64-256 diploid nuclei, whereas in vitro reactions are typically performed at nuclear concentrations of >3000 nuclei/ μ l, close to the MBT or post-MBT concentrations of DNA (~4000 diploid nuclei per 0.5 μ l cytoplasm). As the major Cdt1 degradation pathway is DNA dependent (Arias and Walter, 2005; Arias and Walter, 2006; Jin et al., 2006), the lower DNA concentrations in pre-MBT embryos may lead to only partial Cdt1 degradation during S phase. We confirmed this possibility by titrating the amount of sperm in the *Xenopus* extract (supplementary material Fig. S2). Consistent with published results (Arias and Walter, 2005), Cdt1 was totally degraded when 10-20 ng/ μ l of DNA was

added; however, Cdt1 protein level was largely unaffected at lower DNA concentrations (0.15–1.2 ng/ μ l), representing conditions similar to pre-MBT DNA content.

As the licensing activity of Cdt1 is expected to oscillate during the cell cycle, we investigated whether this could be mediated in a changing affinity for geminin (Fig. 2). Using immunoprecipitation and co-immunoprecipitation, we investigated complex formation between Cdt1 and geminin during the first 120 minutes, which covers the first, long, and the second, short, cell cycle. Maximal co-precipitation of Cdt1 and geminin was seen in unfertilised eggs and during the first minutes AF, consistent with data from egg extracts showing that they form a tight complex at this stage (Li and Blow, 2004) (Fig. 2A,C). Co-precipitation of geminin by Cdt1 and of Cdt1 by geminin declined by >50% over 20 minutes (Fig. 2B,C, black arrows) as quantified by ImageJ gel analysis of band density. The parallel decline of cyclin B1 (Fig. 2C; lower panel) shows that over this period mitotic exit occurred, which is the stage of origin licensing. The lack of degradation of a significant pool of geminin suggests that early embryonic cells employ additional mechanisms for regulation of Cdt1 activity. These additional mechanisms could include already described post-translational inactivation of geminin (Hodgson et al., 2002; Li and Blow, 2004) and the localised formation of different Cdt1:geminin complexes with different

degrees of Cdt1 inhibition (De Marco et al., 2009; Lutzmann et al., 2006). DNA replication ends by 50 minutes, as indicated by increasing levels of cyclinB1. Mitosis lasts until 80 minutes when the first cleavage occurs (red arrow). At this time, geminin interaction with Cdt1 declines again, clearly indicating mitotic exit and time for DNA licensing. Interestingly, the strong Cdt1:geminin complex that is detected within the first 15 minutes AF (Fig. 2B and 12 minutes in 2C) appears weaker in the following cell cycles, which may suggest that a proportion of geminin is either inactive or in complexes with other inhibitory proteins such as Idas (Pefani et al., 2011). To gain further insight into this regulation, we examined the cell cycle-related cellular distribution of Cdt1 and geminin using immunofluorescence (Fig. 3). Both proteins were well detected in the nucleus during S-phase (Fig. 3A,B). Control experiments with non-immune IgG showed that these immunofluorescence signals were dependent on the Cdt1 and geminin-specific antibodies (Fig. 3C). To address the behaviour of Cdt1 in more detail, we used *Xenopus* extract supplemented with GFP-Cdt1 (Fig. 3D). Recombinant GFP-Cdt1 was functional as it was able to rescue DNA synthesis in extract supplemented with geminin^{DEL} (supplementary material Fig. S3A). In the presence of 20 ng DNA/ μ l (approximating MBT levels of DNA), GFP-Cdt1 was detected on chromatin only at the start of S-phase and then disappeared (Fig. 3D, 40'), consistent

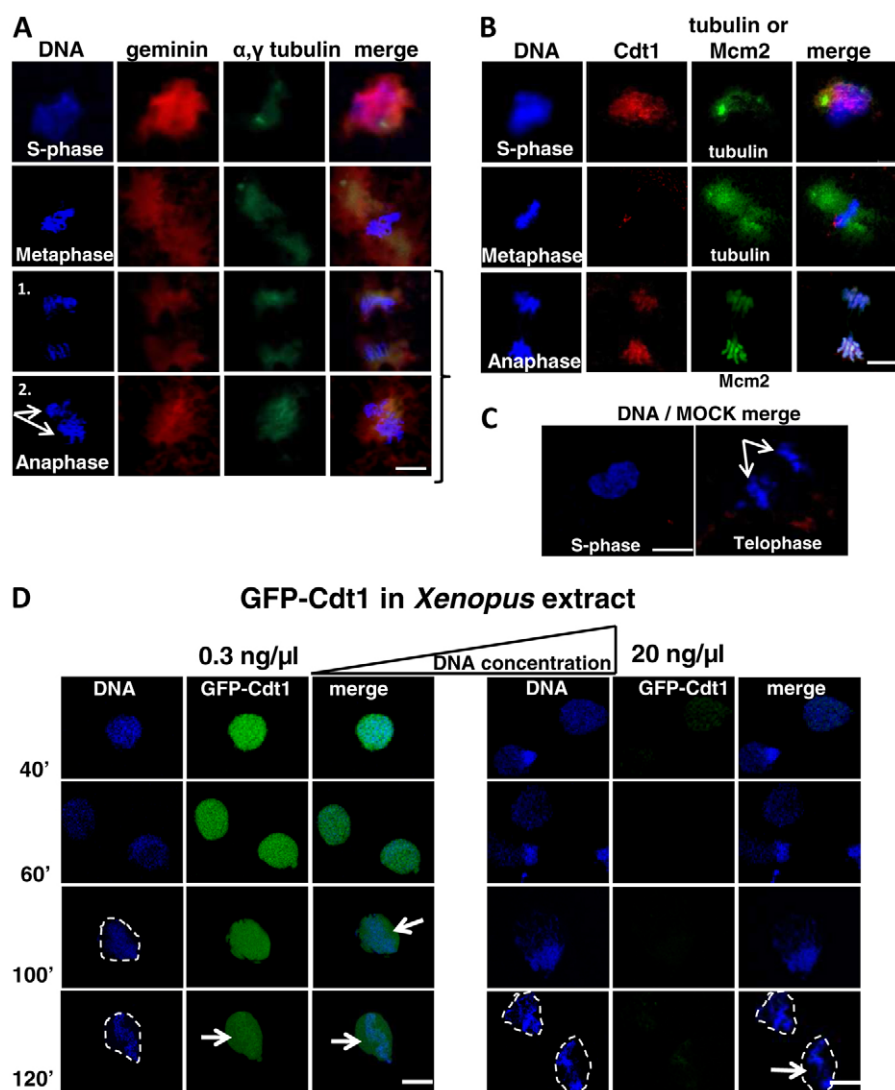


Fig. 3. Cellular distribution of Cdt1 and geminin in early embryos.

(A,B) Immunofluorescence of geminin (A) and Cdt1 (B) using embryonic slices from stage 7. (1,2) Anaphase from two different angles. Nuclear fluorescence of geminin and Cdt1 is detected during S phase. (B) Cdt1, together with Mcm2, bind strongly to chromosomes at the end of anaphase. Scale bars: 20 μ m. (C) Control for polyclonal anti-rabbit antibody with rabbit IgG. Merge between DAPI (blue) and IgG (red) is shown. Scale bar: 20 μ m. (D) GFP-Cdt1 distribution in *Xenopus* egg extract depends on sperm DNA concentration. GFP-Cdt1 (6 μ g/ml) was added to the extract supplemented either with 20 or 0.3 ng DNA/ μ l. The extract was fixed at indicated time points. White outline indicates nuclear border, arrows show GFP-Cdt1 in nucleoplasm. Scale bars: 20 μ m.

with extensive DNA-dependent Cdt1 degradation. At 0.3 ng DNA/ μ l (approximating pre-MBT DNA concentrations at 4–5 hours AF), GFP-Cdt1 fluorescence intensity was stable for 120 minutes in the extract. By 120 minutes, the extract is in G2 (mitotic entry having been inhibited by addition of cycloheximide to prevent translation of cyclin B) and the chromatin undergoes an initial 'prometaphase' condensation. At this time, much of the GFP-Cdt1 is present in the nucleoplasm not associated with chromatin (Fig. 3D, 100–120', arrows). In metaphase, geminin is detected around the mitotic spindle; however, Cdt1 is not detected probably owing to being largely dispersed into the cytoplasm of the large embryonic cell. During anaphase, when the licensing system is reactivated, Cdt1, together with Mcm2 became closely associated with the chromosomes (Fig. 3B, anaphase). Although geminin was also detected on chromosomes at this stage, its localisation to chromosomes was relatively diffuse, with significant colocalisation to the mitotic spindle (Fig. 3A, 1,2). Taken together, the cellular distribution of these proteins thus supports the idea that recruitment of Cdt1 to chromatin occurs during anaphase as licensing is activated, and that at this stage geminin is present but is not tightly associated with Cdt1.

Geminin overexpression does not stop early development

Our results suggest that geminin turnover is low and that only a small percentage of the total cellular pool of geminin is degraded to allow licensing; this level is then quickly restored. Because geminin is a Cdt1 and licensing inhibitor (Tada et al., 2001; Wohlschlegel et al., 2000), we determined whether overexpression of wild-type geminin would affect early cell cycle and embryonic progression. mRNA encoding geminin-RFP (12 ng) was injected into stage 1 embryos, into a single cell of two-cell (stage 2) embryos and into one cell of four-cell (stage 3) embryos (Fig. 4). Recombinant geminin-RFP is able to decrease the amount of DNA replicated in *Xenopus* egg extracts (supplementary material Fig. S3A,B), which suggests that the RFP tag does not interfere with this function. Injection of geminin-RFP mRNA slowed down cell cycles by about 30–60 minutes, depending on the batch of embryos (Fig. 4A). Expression of geminin-RFP protein was detected ~60 minutes after injection, as confirmed both by immunodetection and confocal/two-photon imaging (Fig. 4B–D). Endogenous geminin levels were largely unchanged in embryos expressing geminin-RFP (Fig. 4B,C). Despite the excess geminin, embryos injected at stage

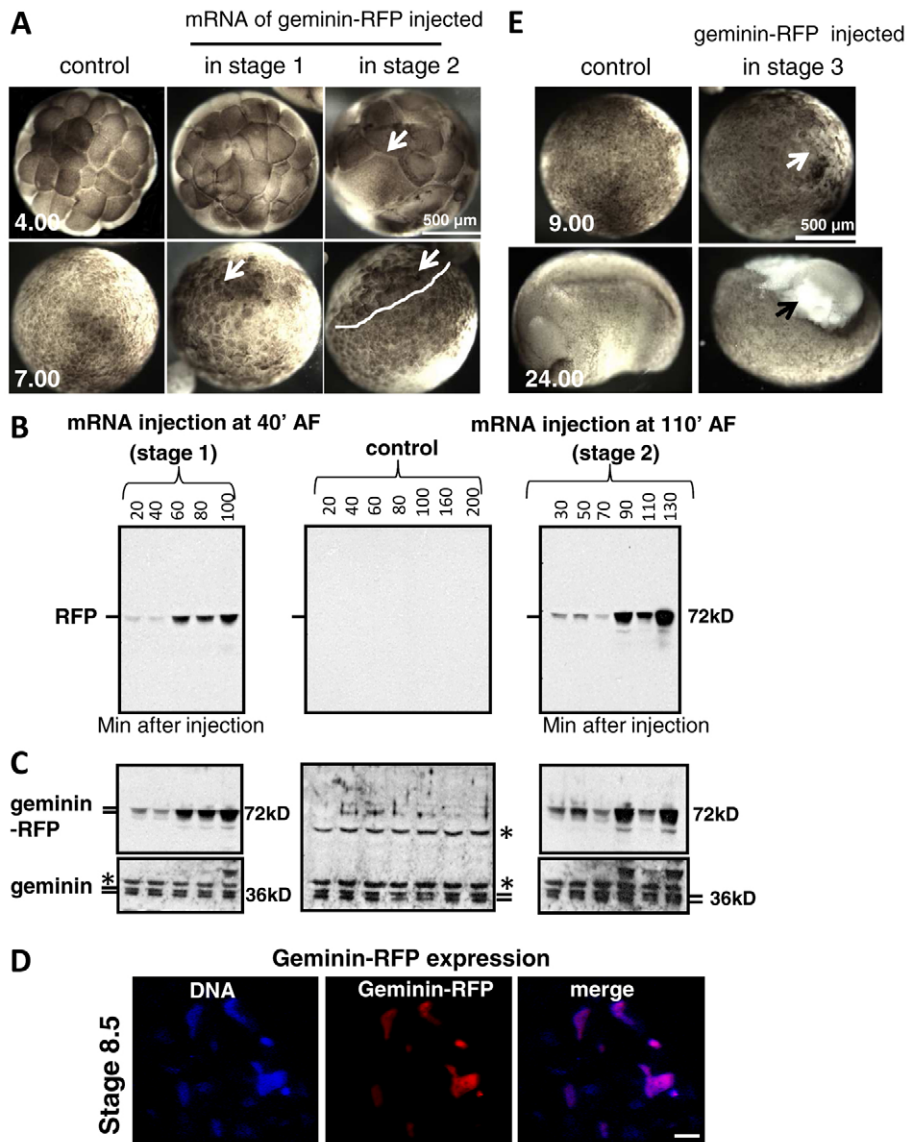


Fig. 4. Overexpression of geminin slows down but does not prevent cell cycle progression before MBT. (A) Geminin-RFP mRNA was injected into stage 1 and stage 2 embryos. Control embryos were injected with $\times 0.5$ PBS. Embryonic development was followed by live macroimaging. (B,C) Immunodetection of RFP and geminin in extracts from embryos at different times after injection. RFP was detected at ~72 kDa, suggesting that expression of mRNA occurred. Geminin antibody recognised both geminin-RFP at 72 kDa and endogenous protein at 36 kDa. (D) Injected embryos were fixed at 6 hours AF and geminin-RFP expression was detected by confocal/two-photon single cell imaging. Scale bar: 20 μ m. (E) Geminin-RFP was injected into one cell of the four-cell embryo and post-MBT embryonic progression was monitored. Arrows show an injected region that does not develop further after 24 hours.

1 and 2 progressed through the first four cleavages relatively unaffected. Fifty-nine percent of embryos injected at stage 1 ($n=37$) progressed to MBT and 45% of them reached 9 hours of development. Eighty-four percent ($n=22$) of embryos injected at stage 2 progressed to 9 hours of development. However, neither of these embryos survived beyond stage 10. When geminin-RFP mRNA was expressed in one cell of a four-cell embryo ($n=15$), 20% of embryos continued development for 24 hours but with gross abnormalities of the body corresponding to the injected region (Fig. 4E).

Cells produced from injected embryos were fixed at 6 hours AF (stage 8.5) and imaged by confocal/two-photon microscopy. We confirmed that expressed geminin-RFP was localised in the nuclei during S phase (Fig. 4D). Each cell contained a normal nucleus. This contrasts with the study reported previously where injection of a nondegradable form of geminin not recognised by the APC/C (geminin^{DEL}) resulted in inhibition of DNA replication, but did not arrest the embryonic cleavages, resulting in dividing embryos with no DNA (McGarry and Kirshner, 1998). This is consistent with previous work in egg extracts showing that although proteolysis of geminin is not required for its proper cell cycle regulation, ubiquitylation by the APC/C is required to block its inhibition of Cdt1 during anaphase (Li and Blow, 2004).

Functional neutralisation of geminin results in cell cycle arrest, chromosomal breaks and checkpoint activation in pre-MBT embryos

Earlier studies, using antisense morpholino (MO) showed that geminin mRNA can be depleted a few hours after MO injection; this does not stop pre-MBT embryonic progression, but arrests the embryo at MBT (McGarry, 2002). It has also been suggested that neither geminin nor Cdt1 play important roles in early cleavages that occur before zygotic transcription (Kerns et al., 2007; McGarry, 2002). Here, we have shown that geminin turnover is low in the early embryo and that mRNA ablation does not significantly reduce protein levels, which suggests that regulation of geminin activity may play an essential role in early cell cycles in vivo. To address this issue, we functionally depleted geminin using a truncated version of Cdt1, Δ Cdt1₁₉₃₋₄₄₇, that does not have licensing activity and has no degradation boxes, but retains the ability to bind endogenous geminin and neutralise its ability to inhibit Cdt1 (Ferenbach et al., 2005) (Fig. 5A). First, we showed that a soluble version of Δ Cdt1₁₉₃₋₄₄₇ actively prevented inhibition of licensing by geminin^{DEL} in *Xenopus* cell-free extracts (Fig. 5B). We then showed that injection of 6 ng/ μ l of Δ Cdt1₁₉₃₋₄₄₇ into stage 1 or one of a 2-cell embryo at stage 2 (where a second half of an embryo serves as a natural control) arrested

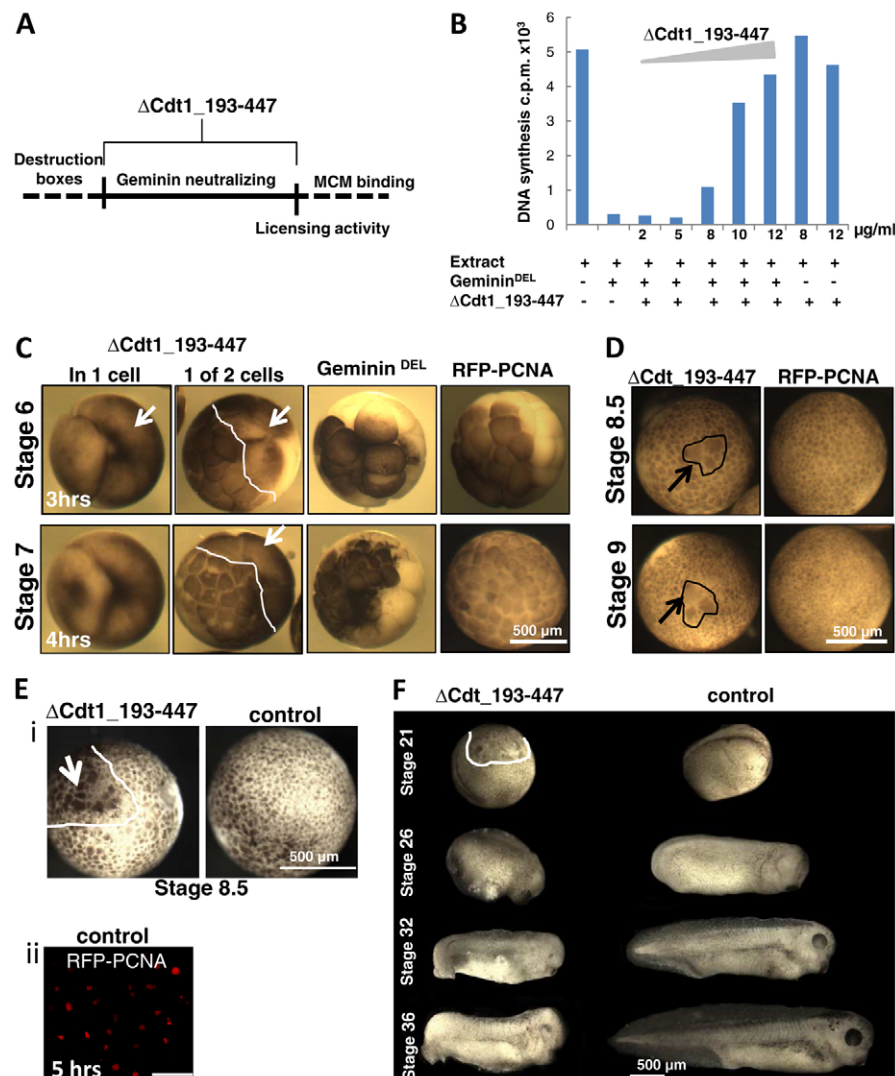


Fig. 5. Neutralisation of Geminin exposes the role of Cdt1 in early cleavages.

(A) Schematic diagram of Δ Cdt1₁₉₃₋₄₄₇ protein with activity to neutralise geminin. (B) Recombinant Δ Cdt1₁₉₃₋₄₄₇ activity was measured in *Xenopus* egg extract. Geminin^{DEL} (1.8 μ g/ml) blocked DNA replication. Different concentrations of Δ Cdt1₁₉₃₋₄₄₇ (2–12 μ g/ml) were used to restore DNA synthesis. (C) Recombinant Δ Cdt1₁₉₃₋₄₄₇ was injected into stage 1 and 2 embryos. Arrows show injected cells at stage 6 and 7. For a positive control, embryos were injected either with geminin^{DEL} (1.8 ng/ μ l) or RFP-PCNA (1.6 pM) recombinant protein into stage 1. Scale bar: 500 μ m. (D) Δ Cdt1₁₉₃₋₄₄₇ injected into animal pole triggers their arrest during the following cell cycle. Arrows indicate three affected cells on the surface of an embryo. (E) Δ Cdt1₁₉₃₋₄₄₇ mRNA (14 ng) was injected into animal pole at 4.3 hours AF and embryos were imaged at 5.3 hours AF. Arrow shows a significant region of arrested cells. Scale bar: 500 μ m. The control embryos were injected with ~15 ng of RFP-PCNA mRNA (i) and mRNA expression confirmed by confocal microscopy at 5 hours (ii). (F) Neutralisation of geminin in a number of cells before MBT results in deformation of post-MBT embryos. Scale bar: 500 μ m.

embryonic cell cycle progression (Fig. 5C). Geminin^{DEL} was used as a positive control, which as previously reported (McGarry and Kirshner, 1998) led to developmental and cell cycle arrest around the time of the MBT. By contrast, control embryos injected with RFP-PCNA protein developed unaffected until later stages (Kisielewska et al., 2005). The same amount of Δ Cdt1₁₉₃₋₄₄₇ protein injected into the cleft (beginning of blastocoele) underneath the animal pole at 4 hours resulted in a number of cells arrested during the following cell cycle (Fig. 5D). However, injection of Δ Cdt1₁₉₃₋₄₄₇ mRNA resulted in a greater number and smaller size of arrested cells after 1 hour, compared with embryos injected at 4 hours (Fig. 5E, part i, compare with 5D). As a control for mRNA expression in this experiment we used RFP-PCNA (Fig. 5E, part bii). We then followed development of embryos for the next 50 hours. All of the animal pole injected embryos (with either protein or mRNA) showed a severe reproducible phenotype with lack of development of the tail region and additional deformations, including smaller size, compared with the control at the same developmental stage, defects in eye development or abnormally twisted body (Fig. 5F). Geminin neutralisation before MBT therefore is detrimental for embryonic development independent of developmental stage.

We then established the cellular consequences of deregulating Cdt1. Nuclear DNA content was measured to determine whether cells treated with Δ Cdt1₁₉₃₋₄₄₇ had undergone re-replication. Fig.

6A shows that there was a small, but significant increase in DAPI fluorescence intensity between control pre-MBT S-phase nuclei and nuclei arrested when geminin was functionally knocked down. Although this is consistent with re-replication having occurred, it could also be a consequence of increasing the proportion of cells in late S phase. PCNA is a DNA polymerase accessory factor and can be used as a marker of ongoing DNA replication and/or DNA repair. Pre-MBT nuclei (Fig. 6Ba) show a relatively uniform pattern of PCNA on chromatin without distinguishable replication foci as previously observed in early embryos (Kisielewska et al., 2005), but post-MBT (Fig. 6Bb) nuclei show a more somatic pattern with PCNA clearly visible at replication factories (Cardoso, 1999; Leonhardt et al., 2000). In contrast to either pre- or post-MBT nuclei, nuclei from embryos treated with Δ Cdt1₁₉₃₋₄₄₇ showed visible dots of DNA throughout the entire nucleus but with uniform PCNA labelling characteristic of pre-MBT nuclei, Fig. 6Bc. In early pre-MBT embryos, all cells are synchronise; therefore, cell cycle pattern for one cell will reflect the cell cycle stage of an embryo. Control, cycling cells show clearly defined cell cycle phases expected at this time of early development [Fig. 6C (control), Fig. 6D (oscillating cells)]. At S phase, MCMs and PCNA are associated with chromatin. At prophase MCM are already dispersed from chromatin with some PCNA still being in the nucleus. Licensing of DNA for replication is completed by the end of anaphase with MCMs tightly bound to chromosomes, similar to Fig. 3B. PCNA is

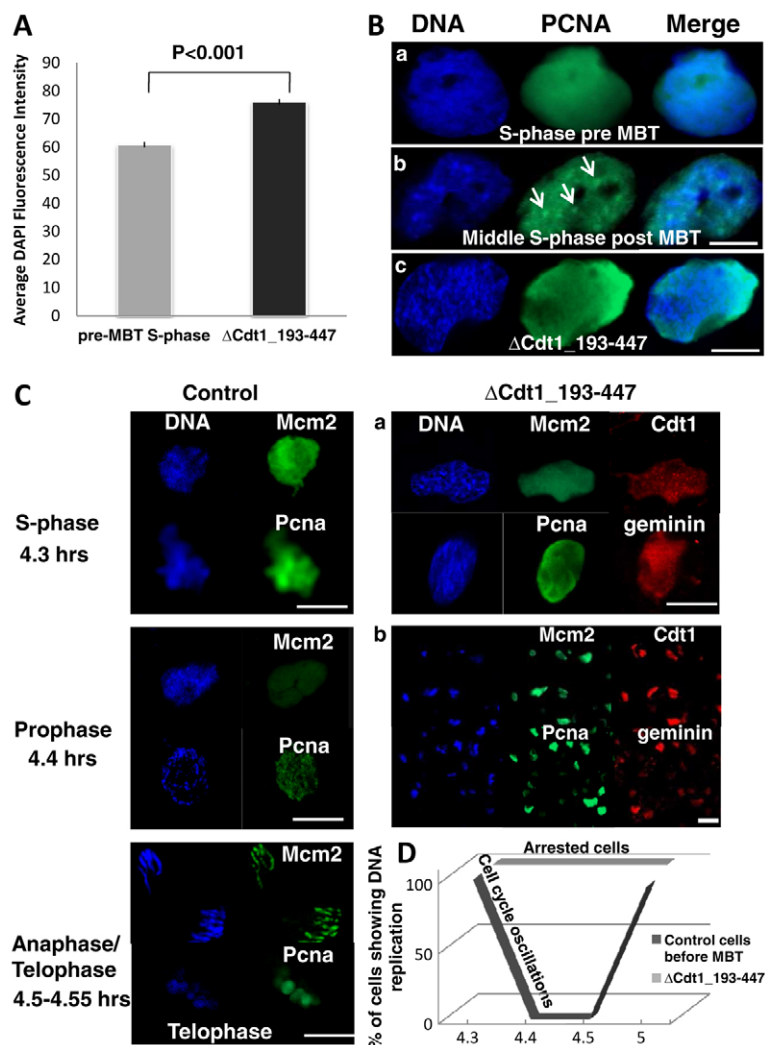


Fig. 6. Disruption of the Cdt1 protein level triggers cell cycle arrest. (A) Increase in average DAPI fluorescence intensity between arrested and pre-MBT cells. A total of 12 nuclei from three independent experiments were used. Data are mean \pm s.e.m. (B,C) Arrested cells after Δ Cdt1₁₉₃₋₄₄₇ injection at 4 hours AF were subjected to whole-mount immunofluorescence. (Bc) Nuclei of arrested cells contain particulate DNA distribution, unlike the uniform decondensed chromatin during typical S phase of early embryos at the same time (Ba), or organised replication factories (arrows) in post-MBT middle S-phase nuclei (Bb). Scale bars: 10 μ m. (C) Pre-MBT control cells oscillate between S phase and mitosis. Cell treated with Δ Cdt1₁₉₃₋₄₄₇ (a and b) are arrested in replication-like stage with Cdt1, geminin, Mcm2 and PCNA on chromatin. Scale bars: 20 μ m. (D) Graphical comparison of control cycling cells (4.3 and 5 hours, S phase; 4.4-4.5, mitosis) and geminin-depleted cells (arrested cells).

loaded on chromatin during telophase, at the time when DNA replication starts in early embryonic divisions. In contrast to control cells, those cells in which geminin was neutralised did not progress through the cell cycle into mitosis [Fig. 6Ca,Cb, Δ Cdt1_193-447; Fig. 6D (arrested cells)]. Their cell cycle was arrested with clear detection of Cdt1 and geminin within the nucleus. Moreover the presence of Mcm2 and PCNA within the nucleus suggests that this arrest occurred during DNA replication. However, the uniform pattern of PCNA might not represent chromatin-bound PCNA, which is released from the nucleus upon nuclear envelope breakdown. We therefore examined whether such nuclear phenotype could be associated with DNA damage. A maternal damage response exists in pre-MBT embryos and can be triggered by chromosomal breaks (Conn et al., 2004). Work in *Xenopus* egg extracts has shown that re-replication caused by Cdt1 de-regulation can give rise to the appearance of double-stranded DNA fragments and activation of cell cycle checkpoint kinases (Davidson et al., 2006; Li and Blow, 2005). Whole-mount immunofluorescence was performed with p53 and RAD51 antibodies (Fig. 7). Consistent with a previous report, we were able to detect basal p53 fluorescence in early nuclei of unaffected cells (Tchang and Mechali, 1999). However, arrested cells showed significantly higher levels of overlap between DNA and p53 (Fig. 7A,B). Basal levels of RAD51 were detectable in proliferating cells although, unlike p53, RAD51 was detected around mitotic chromosomes (Fig. 7D). As with p53, Δ Cdt1_193-447-treated cells showed significantly higher expression of RAD51 than controls (Fig. 7C,D). Taken together, the results shown in Figs 6 and 7 strongly suggest that neutralisation of geminin results in deregulation of Cdt1, which leads to cell cycle arrest and consequent activation of DNA damage responses.

DISCUSSION

Cdt1:geminin periodic interaction

Our work demonstrates that normal DNA replication and cell cycle progression can occur in the early embryo with only moderate changes in the abundance of either Cdt1 or geminin. Consistent with results obtained using metaphase egg extracts (Li and Blow, 2005), we show that unfertilised eggs contain consistently high protein levels of both Cdt1 and geminin that can physically interact with one another. The only noticeable decline in geminin and Cdt1 protein levels occurs within 15-20 minutes and is restored by 30 minutes AF. This may be associated with the completion of meiosis and APC/C activity before the onset of S phase of the first mitotic cycle. At this time, however, there is a major decrease in the physical interaction between these proteins, which corresponds to the ability of Cdt1 to promote origin licensing. Although they never reform such a strong a complex as occurs in unfertilised and early fertilised eggs, changes in their interaction are cell cycle dependent. The visible decrease in the amount of interacting proteins could be related to geminin involvement in other functions when embryo development starts. We have shown that whereas geminin colocalises with Cdt1 during S-phase, at the beginning of mitosis there is a dramatic change in their relative distributions. Such cellular distribution of geminin and Cdt1 in early *Xenopus* embryos is distinct from what has been reported in somatic cells (McGarry and Kirshner, 1998) and stage 11 *Drosophila* embryos (Quinn et al., 2001). In anaphase, which is the only time in the early embryo when origin licensing occurs, Cdt1, but not geminin, becomes highly enriched on chromosomes (Fig. 8A). We predict that the small amount of geminin detected on anaphase chromosomes may participate in a slow build up of the Cdt1:geminin complex and full inhibition of licensing when DNA replication begins (Fig. 8B,C).

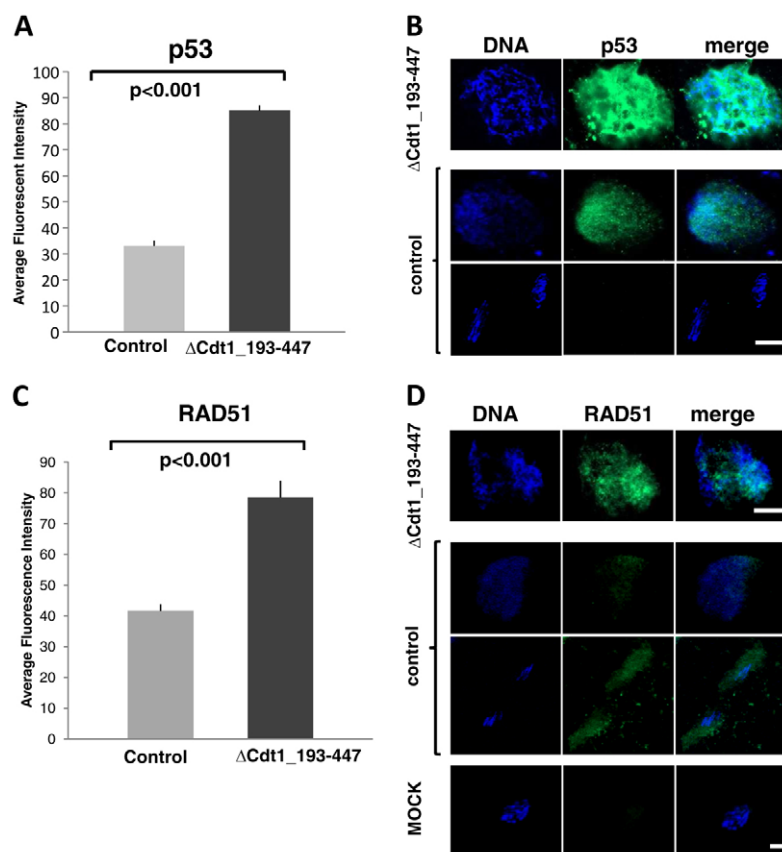


Fig. 7. Functional downregulation of geminin results in significant increase in p53 and RAD51 level on chromatin. Embryos treated with Δ Cdt1_193-447 were fixed and subjected to immunofluorescence with anti-p53 and anti-RAD51. Confocal/two-photon microscopy was performed with the same detection settings for both control and arrested cells. **(A,C)** Accumulation of p53 and RAD51 on chromatin compared with control by average fluorescent intensity or confocal/two-photon single cell imaging. Graphs represent mean \pm s.e.m. of two separate experiments with 6-8 embryos for each protein used for quantification, *** $P < 0.001$. **(B,D)** Confocal/two-photon images comparing the amount of p53 **(B)** and RAD51 **(D)** between control cycling cells and cells arrested using Δ Cdt1_193-447. Scale bars: 20 μ m.

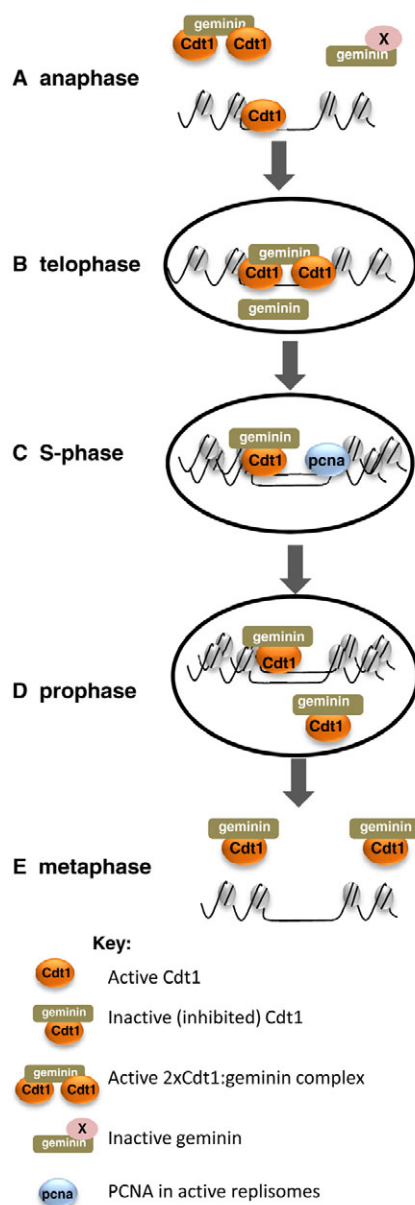


Fig. 8. Model of Cdt1:geminin interaction in early *Xenopus* embryos. (A) Licensing of DNA for replication occurs in anaphase. (B) In telophase, karyomeres appear surrounded by nuclear envelope that allows geminin nuclear import and reactivation. (C) In early S phase, both Cdt1 and geminin are associated with chromatin and at this time geminin inhibits Cdt1 to prevent re-licensing of replicated DNA. (D) During prophase, Cdt1 relocalises away from chromatin, possibly owing to a post-translational modification. (E) During metaphase, Cdt1 is displaced from chromatin and is fully inhibited by geminin.

During prophase, re-localisation of Cdt1 away from DNA occurs (Fig. 8D). Once cells enter into metaphase, Cdt1 is fully inhibited by geminin (Fig. 8E). Although the nature of the modification that inactivates geminin in early embryos has yet to be determined, it is clear that differences in the stoichiometry of geminin and Cdt1 can lead to the formation of different Cdt1:geminin complexes with different degrees of Cdt1 inhibition (De Marco et al., 2009; Lutzmann et al., 2006; Ode et al., 2011).

Proper balance between Cdt1:geminin is required for rapid embryonic cleavages

Over-expression of constitutively active geminin^{DEL} has been shown to block DNA replication in *Xenopus* (McGarry and Kirshner, 1998) and in a range of human tissue culture cells (Shreeram et al., 2002). However, it has also been reported that upregulation of wild-type geminin in some highly proliferating cells did not stop their proliferation, presumably owing to APC/C-mediated degradation or inactivation of the added geminin (Wohlschlegel et al., 2002). However, in *Drosophila* syncytial blastoderm embryos, overexpression of ectopic geminin led to inhibition of DNA replication and cellular death (Quinn et al., 2001). Another study showed that geminin-related inhibition of S phase in *Drosophila* pre-MBT early cell cycles led to an induction of an extra synchronous division and temporarily deferred MBT events (McClelland et al., 2009). In our experiments, doubling the amount of geminin by overexpression slowed down, but did not stop, early pre-MBT cell cycles. This may be mediated by the delay in ELYS-dependent nuclear pore assembly that occurs when origin licensing is inhibited (Gillespie et al., 2007; Khudoli et al., 2008). High expression of geminin-RFP also did not cause immediate cellular death even after MBT. We suggest that during rapid divisions, endogenous geminin is more stable due to translational modification or formation of complexes with other proteins, but the capacity to undergo these modifications or form these complexes is limited. Thus, the additional exogenous geminin is not susceptible to the usual modification and/or complex formation, and is more vulnerable to degradation. Consistent with this idea, cell-free extracts exiting from mitosis have limited capacity to downregulate recombinant geminin in a non-proteolytic fashion (Li and Blow, 2004).

It has been shown in a range of different cell types that loss of geminin leads to re-licensing and re-replication and subsequent checkpoint activation (Davidson et al., 2006; Li and Blow, 2005; Maiorano et al., 2005; Mihaylov et al., 2002; Yoshida et al., 2005). Previous work to investigate this in *Xenopus* early embryos used antisense morpholinos to deplete geminin mRNA (McGarry, 2002) and a non-geminin binding (NGB) Cdt1 mutant (Kerns et al., 2007). The constructs used at picogram concentration by Kerns and colleagues failed to arrest early cell cycles and induced limited post-MBT arrest (56% for NGB-Cdt1 and 3% for Cdt1). In addition, the NGB-Cdt1 mutant was apparently only part functional, as it did not restore full replication in egg extracts. However, based on these results it was concluded that *Xenopus* pre-MBT development is geminin:Cdt1 insensitive (Kerns et al., 2007; McGarry, 2002). By contrast, in our study recombinant Cdt1 (not shown) or Δ Cdt1₁₉₃₋₄₄₇ protein injected into *Xenopus* embryos caused almost immediate cellular arrest. In addition, we needed to use nanogram mRNA concentration for changes to be observed pre-MBT. We suggest that the lack of early arrest in previous studies was due to a low level of exogenous mRNA expression and slower build up of deregulated Cdt1, which showed an effect only 6 hours AF. The high levels of Cdt1 and geminin in early embryos and their low rate of proteolysis need to be counteracted by equally high levels of exogenous protein or mRNA. Furthermore, the use of MO is not a good way of reducing geminin or Cdt1 protein levels at this stage. By contrast, our approach using Δ Cdt1₁₉₃₋₄₄₇ relied on neutralising endogenous geminin in vivo and allowed its role in early cell cycle progression to be exposed. The cellular phenotype of this neutralisation was visible in the next cell cycle and included a defect in cell division, an increase in the DNA content per cell and a significant elevation of p53 and RAD51, indicators of a cellular

response to DNA damage in affected cells. This is the first direct evidence for the importance of both Cdt1 and geminin for regulation of DNA licensing and cell cycle progression in rapid embryonic divisions. This is consistent with previous reports showing strong activation of DNA damage responses and cell cycle checkpoints in response to re-replication (Li and Blow, 2005; Melixetian et al., 2004; Vaziri et al., 2003). We have shown that neutralising geminin during the pre-MBT cell cycles also has general and specific developmental effects. This may be due directly to DNA damage and cell cycle arrest, although it is also possible that it is mediated by disrupting the interaction of geminin with developmental regulators, as has been described in other systems (Del Bene et al., 2004; Kim et al., 2006; Luo and Kessel, 2004; Papanayotou et al., 2008; Pitulescu et al., 2005). The latter would be consistent with a role for geminin as a regulatory protein in directing cell proliferation and differentiation during embryonic patterning through the complexes with transcriptional regulators (Papanayotou et al., 2008) and chromatin remodelling proteins (Seo et al., 2005; Seo and Kroll, 2006). Our study suggests that a pool of licensing proteins may be involved in interactions with other proteins. Although the detailed interactions between geminin and developmental genes and their role in embryonic development remain to be established, these pathways are likely to provide a vital link between genome duplication and development of an organism.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.068676/-DC1>

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