Regulation of the DNA licensing protein Cdt1 in *Xenopus laevis* embryos

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"Nothing that has value in the world can be had without effort"

- **Thomas A. Edison**

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Abstract

During each cell cycle the DNA must be replicated accurately in order to maintain genomic integrity. To ensure faithful replication of the entire genome, DNA replication must be tightly controlled. This control is achieved through the process of DNA licensing in which pre-replicative complexes are assembled to prime the DNA for replication in the coming S-phase. To prevent re-licensing and subsequent re-replication, which would lead to genomic instability, DNA licensing must also be tightly controlled. The main mechanism of regulation of DNA licensing is through regulation of Cdt1 activity, a key component of DNA licensing. During the metazoan somatic cell cycle Cdt1 is regulated by proteolysis and inhibition by geminin. However there is evidence that the mechanisms of Cdt1 regulation during the short, rapid cleavage cell cycles of the early pre-MBT *Xenopus* embryo may differ.

The results presented here show that upon expression of a deregulated, truncated version of Cdt1 in pre-MBT *Xenopus* embryos the cell cycle arrests with damaged DNA and evidence of checkpoint activation. This demonstrates that correct Cdt1 regulation is crucial for proper DNA licensing and pre-MBT embryonic cell cycle progression. There was no evidence of ubiquitination, degradation or phosphorylation of endogenous Cdt1. This suggests that changing interactions with geminin rather than proteolysis or post-translational modification provides the main mechanism of Cdt1 regulation in pre-MBT *Xenopus* embryos.

The highly regulated N-terminal region of Cdt1 is capable of binding to DNA and the licensing component Orc1. This suggests that domains for DNA and Orc1 binding are also located at this region of the Cdt1 protein. However, a truncated Cdt1 construct lacking the N-terminal domain is still capable of licensing the DNA. Since the regulation of Cdt1 is crucial for correct DNA licensing, these interactions may therefore constitute redundant mechanisms to ensure the proper activity of Cdt1.

Overall the results presented in this thesis show that in early *Xenopus* embryos Cdt1 regulation is crucial for faithful DNA licensing and cell cycle progression. In addition the main mechanism for regulation of Cdt1 is through dynamic interactions with geminin rather than post-translational modification or degradation during the pre-MBT embryonic cell cycle.

Publications and Reviewed Abstracts

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List of Abbreviations

Abbreviation	Full Term
ANCOVA	Analysis of Covariance
APC/C	Anaphase Promoting Complex/Cyclosome
ARS	Autonomous Replication Sequence
ATP	Adenosine Triphosphate
bp	Base Pair
Cdc6	Cell Division Cycle 6
CDK	Cyclin-Dependant Kinase
Cdt1	Cdc10-dependant Transcript
Chk 1	Checkpoint Kinase 1
CMG	Cdc45-Mcm2-7-GINS
CNS	Central Nervous System
Co-IP	Co-immunoprecipitation
DMP	Dimethylpimelimidate
DNA	Deoxyribonucleic Acid
dsDNA	Double Stranded DNA
DTT	Dithiothreitol
ER	Energy Regenerator
GFP	Green Fluorescent Protein
GINS	Go, Ichi, Nii and San (5, 1, 2, and 3 in Japanese)
hCG	Human Chorionic Gonadotropin
HRP	Horseradish Peroxidase
INK	Inhibitor of CDK
IP	Immunoprecipitation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	Kilobase
MBT	Mid-blastula Transition
MCM	Mini Chromosome Maintenance Complex
MPF	Maturation-Promoting Factor
mRNA	Messenger Ribonucleic Acid
NEM	N-ethylmaleimide

ng	Nanogram
ORC	Origin Recognition Complex
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PEST	Peptide sequence rich in Proline, glutamic acid, serine and threonine
pg	Picogram
PIP	PCNA Interacting Protein
PMSG	Pregnant Mare's Serum Gonadotropin
pRB	Retinoblastoma Protein
Pre-RC	Pre-replicative Complex
RFC	Replication Factor C
RFP	Red Fluorescent Protein
RNA	Ribonucleic Acid
RNAi	RNA Interference
RPA	Replication Protein A
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
siRNA	Small Interfering RNA
ssDNA	Single Stranded DNA
TGF-β	Transforming Growth Factor-beta
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1.

Introduction

1.1 The Cell Cycle

The cell cycle is a highly coordinated and regulated process in which the genetic material is duplicated and transferred through cell division to each generation of new cells. During the cell cycle the entire genome must be faithfully replicated and identical copies of the chromosomes distributed to opposite poles of the cell prior to cytokinesis which divides the cytoplasm to form two daughter cells (Israels and Israels, 2000).

1.1.1 Cell Cycle Structure

The somatic cell cycle is composed of four distinct phases: G1, S-phase, G2 and M-phase. During S-phase the entire genome is accurately duplicated while in M-phase (mitosis) the duplicated chromosomes are separated and cell division occurs. The S- and M- phases of the cell cycle are separated by the gap phases G1 and G2. The gap phases serve multiple purposes; firstly they allow time for the cell to grow and the cytoplasm, with associated organelles and proteins, to double in mass. They also allow time for extracellular signals to be monitored to ensure the suitability of the environment for cell division. Finally, the gap phases contain checkpoints which ensure accurate completion of each cell cycle phase prior to progression and completion of the cell cycle (Alberts *et al.*, 2002b).

There is a further optional cell cycle phase, known as G0, in which the cells may enter a resting state if the conditions are deemed unfavourable for cell division. Some cell types such as neurons enter G0 permanently once terminally differentiated whereas other cells can enter and exit G0 under certain conditions (Alberts *et al.*, 2002a). Cells may enter the G0 resting state during G1 due to checkpoint activation in response to DNA damage. It has also been shown that the p53 checkpoint may be activated under other conditions such as metabolite depletion causing an arrest of the cell cycle in the G0 phase (Linke *et al.*, 1996). The resting phase allows the cell to address any issues before returning to the cell cycle as division in the presence of DNA damage may contribute to the development of cancerous cells.

At M-phase the replicated chromosomes are separated before cyokinesis in which the cell is divided into two. M-phase is further divided into prophase, metaphase, anaphase and teleophase. During prophase the chromatin is condensed into chromosomes of which there are two copies, known as the sister chromatids, which

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are joined at the centromere. In parallel during prophase the mitotic spindle begins to form in the cytoplasm of the cell. The mitotic spindle is a cytoskeleton of microtubules which act to separate the chromosomes to opposite poles of the cell. At the end of prophase (prometaphase) the nuclear envelop begins to break down. Once the nuclear envelope is degraded the chromosomes attach to the mitotic spindle and during metaphase line up at the equator of the spindle. Anaphase then follows in which the sister chromatids are separated along the spindle. During telophase the mitotic spindle elongates to leave one complete copy of the genome at each pole of the cell. Once at the poles the chromosomes are released from the mitotic spindle before reformation of the nuclear envelope. Through the process of cytokinesis the cytoplasm of the cell is then divided to produce two daughter cells each with an identical copy of the entire genome (Alberts *et al.*, 2002b). The structure and events of the somatic cell cycle are highlighted in Figure 1.1 adapted from (Alberts *et al.*, 2002b).
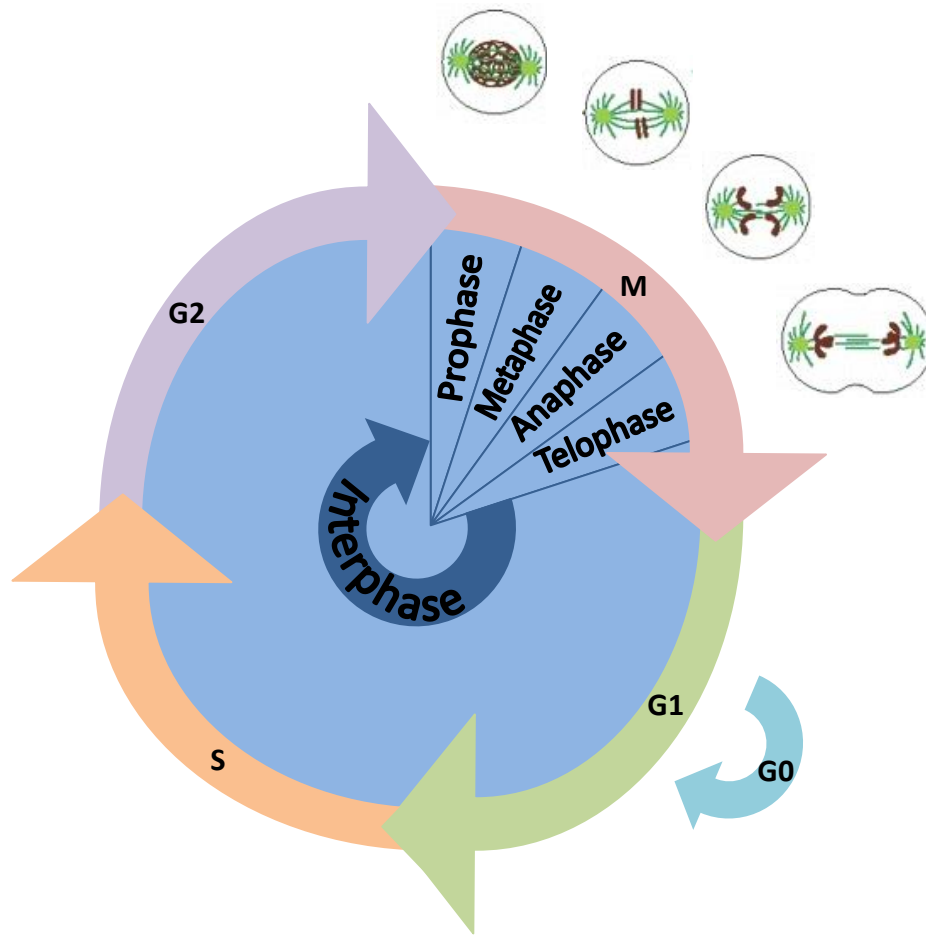
Figure 1.1. The structure and events of the somatic cell cycle. The cell cycle is comprised of the distinct phases G1, S-phase, G2 and M-phase with an optional resting state known as G0. During S-phase DNA replication occurs. During M-phase the events of prophase, metaphase, anaphase and telophase lead to separation of the replicated chromosomes. Following telophase the cytoplasm is divided through cytokinesis to produce two genetically identical daughter cells.

1.1.2 Regulation of the Cell Cycle by Cyclin-Dependant Kinases

The universal regulators of the eukaryotic cell cycle are the cyclin-dependant kinases (CDKs) which pair with specific cyclins to allow progression through the distinct cell cycle phases. Progression through each cell cycle phase is controlled by a different cyclin-CDK complex. In vertebrates the CDKs CDK4 and CDK6 coupled with the cyclins D1, D2 and D3 are responsible for the transition between G1 and S-phase. Then progression through S-phase is controlled by CDK2 coupled with the cyclins E1, E2 or A2. The transition between G2 and M-phase is regulated by CDK1 coupled with the cyclins B1 and B2 (Sanchez and Dynlacht, 2005).

The cyclin-CDK complexes function to regulate the cell cycle by phosphorylation of downstream target proteins which are required for progression through to the next cell cycle phase. The cyclin-CDK complex which has been the most extensively studied is the G1 CDK4/6-cylin D complex (Grana and Reddy, 1995). Once activated in early G1 the CDK4/6-cylin D complex goes on to phosphorylate the retinoblastoma protein (pRB) which is then further phosphorylated by the cyclin E-CDK2 complex leading to release and activation of the pRB binding protein E2F (Harbour *et al.*, 1999). E2F is a transcription factor which once activated causes transcription of specific genes whose protein products are necessary for entry into S-phase (Lundberg and Weinberg, 1998).

1.1.3 Cell Cycle Checkpoints

To ensure faithful DNA replication and cell division eukaryotic cells possess checkpoints which are activated in response to errors such as DNA damage. Activation of the checkpoints leads to cell cycle arrest to allow time for any errors to be corrected or death of the cell through apoptosis to prevent propagation of mutant progeny. The G1 checkpoint ensures cells with damaged DNA do not enter S-phase and replicate the damaged DNA as well as ensuring that the environmental conditions are adequate for cell proliferation. Meanwhile the G2 checkpoint prevents cells with damaged DNA following S-phase from entering mitosis. There is also an M-phase checkpoint to certify correct assembly of the mitotic spindle (Murray, 1994).

In G1 the p53 checkpoint is activated in response to DNA damage and mediates cell cycle arrest or apoptosis. The p53 protein activates expression of $p21^{CIP1}$, a CDK inhibitor which prevents phosphorylation of pRB through inhibition of the G1 cyclin-

CDK complexes cyclin D1/D2-CDK4 and the S-phase cyclin-CDK complex cyclin E-CDK. Through prevention of pRB phosphorylation, transcription of the genes required for S-phase entry is prevented and the cell therefore arrests in G1 (Harper *et al.*, 1993). There is also evidence that p53 may play a role in G2/M cell cycle arrest as the CDK inhibitor p21, which is expressed following p53 activation, is also an inhibitor of the G2 CDKs (Agarwal *et al.*, 1995). Another CDK inhibitor from the CIP/KIP family, p27^{KIP1}, is responsible for cell cycle arrest in G1 in response to cell to cell contact and TGF-β in growth arrested cells. An excess of p27^{KIP1} causes inhibition of the cyclin E-CDK2 complex triggering the G1 arrest (Polyak *et al.*, 1994).

There is also a further family of CDK inhibitors known as the INK4/ARF family which act to arrest cells in G1 (Canepa *et al.*, 2007). The INK4a/ARF gene generates two protein products, p16^{INK4a} and p19^{ARF}. The protein p16^{INK4a} competes with cyclin D for binding of the CDK4 and CDK6 kinases thereby inhibiting the cyclin D-CDK4/6 kinase activity. This prevents phosphorylation of pRB and thus inhibits the transition from G1 into S-phase. The p19^{ARF} protein prevents the inhibition of p53 by MDM2 and therefore allows the activity of p53 to induce G1 and G2 arrest (Chin *et al.*, 1998).

1.2   DNA Licensing and Replication

1.2.1   DNA Licensing

It is essential that DNA replication is accurate in order to maintain genomic integrity and prevent potentially cancerous alterations to the genome. Metazoans possess a large genome and so DNA replication is enlisted from multiple replication origins. Therefore this process must be co-ordinated and controlled to ensure complete and accurate replication of the entire genome only once per cell cycle. This tight control is achieved by the process of DNA licensing in which each replication origin is primed and committed to replication (Blow and Dutta, 2005).

The first step of DNA licensing is the binding of the six subunit origin recognition complex (ORC) to the origins of DNA. Once ORC is bound Cdc6 and Cdt1 are recruited to the origin. To complete licensing the Mcm2-7 complex is loaded to the origin by Cdt1. Together ORC, Cdc6, Cdt1 and Mcm2-7 form the pre-replicative complex (pre-RC) (Gillespie *et al.*, 2001). DNA licensing is believed to be a dynamic process with the

key step being the loading of Mcm2-7 to the DNA as once this step is complete ORC, Cdc6 and Cdt1 become dispensable (Tsakraklides and Bell, 2010).

In the yeast *S.cerevisiae* ORC binds to specific DNA sequences, the ARS (autonomous replication sequence) (Bell and Stillman, 1992). In *Xenopus* however, there is no such ORC binding consensus sequence and the ORC complexes are instead organised spatially approximately 5-15 kb apart. The spacing of DNA origins was dependant on ORC abundance as a reduction in ORC lead to an increase in the average distance between origins (Blow *et al.*, 2001). The human ORC protein complex also binds DNA via a mechanism that is independent of DNA sequence (Vashee *et al.*, 2003). Also in *Drosophila,* ORC binding to DNA is independent of sequence and instead binding may be dependent on DNA topology with *Drosophila* ORC showing greater affinity for negatively supercoiled DNA (Remus *et al.*, 2004). In addition ORC DNA binding is dependent on ATP binding but not its hydrolysis in *Drosophila* (Chesnokov *et al.*, 2001). Similarly in human cells, *Xenopus* egg extract and the yeast *S.cerevisiae* ORC DNA binding is also dependant on ATP binding but not hydrolysis (Klemm *et al.*, 1997; Gillespie *et al.*, 2001; Giordano-Coltart *et al.*, 2005).

It has been demonstrated that the licensing proteins ORC, Cdc6 and Cdt1 are all required to load the MCM complex to DNA and therefore complete DNA licensing (Gillespie *et al.*, 2001). However the exact mechanism and sequence of chromatin loading of the licensing proteins is unclear and may differ slightly between species. In the budding yeast *S.cerevisiae* the ORC complex protein Orc6 is required for an interaction between the ORC complex and Cdt1 which then facilitates Mcm2-7 loading and completion of DNA licensing (Chen *et al.*, 2007). In contrast it has been suggested that in *Xenopus* egg extract Orc6 is not essential for DNA licensing (Gillespie *et al.*, 2001). Instead prior loading of Cdc6 to DNA is essential in *Xenopus* egg extract to allow chromatin bound Cdt1 to be active in DNA licensing (Tsuyama *et al.*, 2005). In fission yeast however, Cdt1 recruitment to DNA appears to be independent of Cdc6 (Nishitani *et al.*, 2000). It is therefore possible that in different species the order and mechanism of DNA loading of the licensing proteins also differs.

Recently, studies have shown that there may be multiple intermediate complexes formed between the licensing proteins to allow Mcm2-7 chromatin binding during

DNA licensing. In *S.cerevisiae* chromatin bound ORC and Cdc6 form an ORC-Cdc6 complex which then recruits multiple Cdt1 proteins to the DNA. The loading of multiple Cdt1 proteins then triggers formation of a double hexamer containing two Mcm2-7 complexes which is then loaded to DNA to complete DNA licensing (Takara and Bell, 2011). It has also been shown that the ORC-Cdc6 complex is capable of recruiting Mcm2-7 which is in turn in complex with Cdt1 as a Cdt1-Mcm2-7 complex. This mechanism produces an ORC-Cdc6-Cdt1-Mcm2-7 complex as an intermediate in DNA licensing (Sun *et al.*, 2013). Following production of the intermediate it is thought that Cdt1 is released from the complex due to ATP hydrolysis of Orc1 and Cdc6 to leave an ORC-Cdc6-Mcm2-7 protein complex bound to DNA. It is believed that Cdt1 is then involved in loading a second Mcm2-7 hexamer to the DNA to form a Mcm2-7 double hexamer and therefore complete DNA licensing, however the exact mechanism of Mcm2-7 double hexamer formation remains unknown (Fernandez-Cid *et al.*, 2013).

In all eukaryotes the Mcm2-7 complex is essential for DNA licensing with the loading of the Mcm2-7 complex being the critical step which allows completion of DNA licensing. In both mammalian cells and *Xenopus* egg extract knock down of the MCM complex proteins leads to a block in DNA replication (Kimura *et al.*, 1994; Madine *et al.*, 1995). The Mcm2-7 complex was originally thought to act as a DNA helicase to unwind the DNA double helix during S-phase due to its parallel movement with the replication fork during S-phase (Aparicio *et al.*, 1997). However it has since been shown that the DNA helicase is only active when the Mcm2-7 complex binds Cdc45 and the GINS complex proteins to form a Cdc45-Mcm2-7-GINS (CMG) complex (Kang *et al.*, 2012).

In somatic cells, DNA licensing occurs at the end of M-phase and into early G1 of the cell cycle (Gillespie *et al.*, 2001). Several mechanisms contribute to the sanctioning of licensing only at this cell cycle stage; firstly the APC/C (anaphase promoting complex) is activated towards the end of M-phase leading to the destruction of M-phase regulatory proteins including CDK1, securin and geminin (Lei and Tye, 2001; Peters, 2002). Secondly there is accumulation of the crucial licensing proteins Cdc6 and Cdt1. Finally, in metazoans only, there is down regulation of the licensing inhibitor geminin, a protein which inhibits the DNA licensing activity of Cdt1 during S-phase, G2 and early M-phase (Lei and Tye, 2001).

CDKs, which have various crucial roles throughout the cell cycle, have both positive and negative roles in promoting DNA licensing at the correct cell cycle stage. It has been shown that towards the end of M-phase CDKs activate the APC/C which then inhibits geminin activity therefore encouraging DNA licensing (Li and Blow, 2004). However extensive evidence suggests that CDK activity also has an inhibitory effect on licensing with high CDK levels throughout the cell cycle except for a window of low CDK levels during late M to early G1 phase in which licensing is sanctioned (Nishitani and Lygerou, 2002).

1.2.2 DNA Replication

Once DNA licensing is complete the origin is sufficiently equipped to allow DNA replication in S-phase. The trigger for recruitment of the replication machinery and initiation of DNA replication is an increase in the levels of two kinases: CDKs and DDKs (Dbf4-dependent kinase) during S-phase (Bell and Dutta, 2002). One of the first steps in initiation of DNA replication is phosphorylation of the MCM complex by DDK (Lei *et al.*, 1997). It is thought that the action of the kinases at the beginning of S-phase causes a switch in the conformation of the Mcm2-7 helicase from an inactive to an active conformation. The GINS complex, which is named after the Japanese for 5, 1, 2 and 3 (Go-Ichi-Nii-San) and consists of the four proteins Sld5, Psf1, Psf2, and Psf3, is then necessary for DNA replication (Takayama *et al.*, 2003). The GINS complex and Cdc45, which are essential for initiation and elongation of DNA replication, then bind to Mcm2-7 to form a Cdc45-Mcm2-7-GINS (CMG) complex which activates the helicase activity of the Mcm2-7 complex. A strong association of Cdc45, part of the CMG, with the chromatin is also dependant on the activity of CDKs (Zou and Stillman, 1998). However GINS binding to chromatin is also important for DNA replication initiation with the chromatin binding of Cdc45 and GINS being mutually dependant and the Psf1 component of GINS being essential for GINS chromatin binding and activity during DNA replication (Kubota *et al.*, 2003; Kamada *et al.*, 2007).

When the Mcm2-7 complex is bound as part of the CMG complex the Mcm2-7 helicase has greater affinity for the DNA and ATP hydrolysis is increased in order to drive the Mcm2-7 helicase motor to unwind the DNA for replication (Ilves *et al.*, 2010). Although the Mcm2-7 complex binds to double stranded DNA (dsDNA) during pre-RC formation, at initiation of replication there is remodelling of the Mcm2-7 complex to allow binding

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to ssDNA during replication. The Mcm2-7 helicase complex then translocates in the 3'
to 5' direction on the leading strand of the DNA template with exclusion of the lagging
strand from the centre of the helicase (Fu *et al.*, 2011).

The Mcm10 protein, although not part of the Mcm2-7 helicase complex, is also
essential for the initiation of DNA replication. Mcm10 binds to the DNA following pre-
RC formation and is required for loading of Cdc45, a component of the CMG complex,
and RPA (Replication Protein A) binding (Wohlschlegel *et al.*, 2002). RPA is essential for
DNA replication as a single stranded DNA (ssDNA) binding protein which protects
ssDNA from degradation during replication. It is also possible that RPA has roles in
coordinating the binding of other DNA replication proteins to the ssDNA (Fanning *et
al.*, 2006).

Once the Mcm2-7 as part of the CMG has unwound the DNA at the origin, replication
of the DNA can begin. DNA polymerase α is then recruited to the unwound DNA
(Walter and Newport, 2000). DNA polymerase α forms a complex with primase which
is an RNA polymerase required to initiate *de novo* DNA synthesis. The primase
generates a small RNA primer of which the pol α component of the complex recognises
the 3'OH and extends the primer to produce an RNA-DNA primer. By this mechanism
the pol α-primase initiates DNA synthesis at both the leading strand and each Okazaki
fragment of the lagging strand (MacNeill, 2012). The RNA-DNA primers are then
extended by DNA polymerase ε in the case of the leading strand (Pursell *et al.*, 2007)
and DNA polymerase δ in the case of the lagging strand (Nick McElhinny *et al.*, 2008).

The processivity of the DNA polymerase enzymes is increased by the DNA sliding clamp
protein PCNA (proliferating cell nuclear antigen) which acts as a clamp to tether the
polymerase enzymes to the DNA (Zhang *et al.*, 1998). PCNA consists of three PCNA
molecules which are bound together to form a ring structure with a central pore which
encircles dsDNA (Krishna *et al.*, 1994). The PCNA ring is loaded onto DNA by the sliding
clamp loader Replication Factor C (RFC). The RFC protein binds PCNA to allow the
PCNA ring to open and bind DNA in a mechanism dependant on ATP binding to RFC.
The ATP is then hydrolysed and RFC is released from the PCNA-DNA complex to allow
binding of the polymerase enzyme (Sakato *et al.*, 2012).

As well as tethering the polymerase to the DNA, PCNA also acts as a platform to recruit other proteins to the sites of DNA replication. During replication of the lagging strand of the DNA, Okazaki fragments are produced due to the 5' to 3' direction of the polymerase enzymes. The Okazaki fragments must then be processed to remove the RNA-DNA primer flap and seal the nick in the DNA. To achieve this FEN-1 and DNA ligase I are recruited to DNA via PCNA. In addition PCNA recruits binding partners to the replication forks during other processes linked to replication such as DNA damage repair (Moldovan *et al.*, 2007). Figure 1.2 shows a schematic representation of DNA licensing and replication in the somatic cell cycle adapted from (Nishitani and Lygerou, 2002; Li and Blow, 2004; DePamphilis *et al.*, 2006).
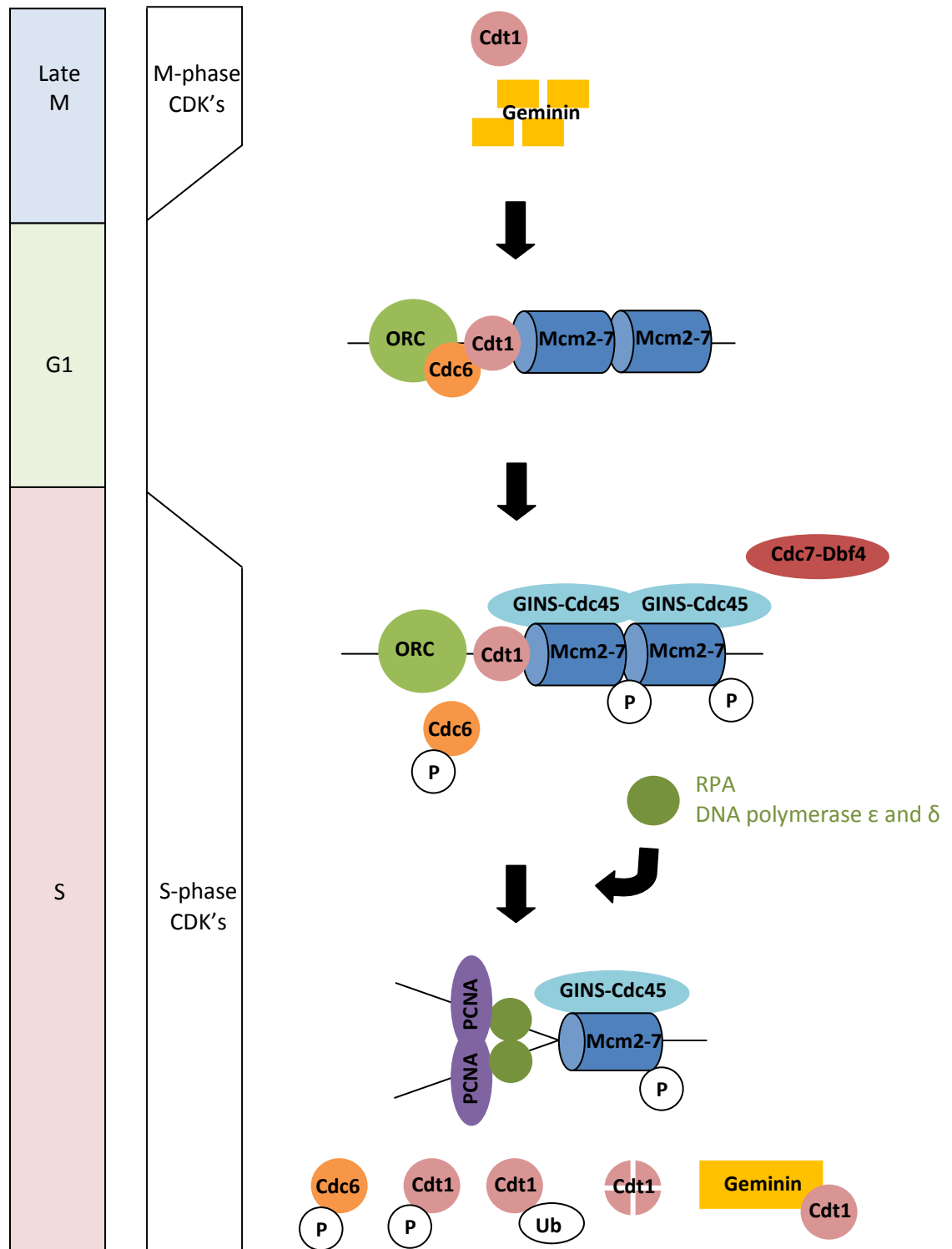
Figure 1.2. DNA licensing and replication initiation in somatic cells. A licensed DNA origin is shown in G1. In S-phase DNA replication is initiated, the MCM complex is phosphorylated by Cdc7-Dbf4 before binding GINS and Cdc45 to form an active helicase complex and RPA and the DNA polymerase enzymes are recruited to the replication fork. PCNA is recruited to tether the polymerase to the DNA and further licensing is inhibited by downregulation of licensing components. During G2 licensing inhibition is maintained and the high CDK level continues (not shown). Towards the end of M-phase the licensing block is released and geminin is degraded releasing Cdt1 from inhibition to allow DNA licensing in the forthcoming G1.

1.3 Prevention of DNA Re-replication

The licensing activity of the pre-RC is under strict control of the cell cycle. Mis-regulation of pre-RC activity leads to a second round of licensing and replication on already replicated DNA (re-licensing and re-replication). Re-replication of the DNA can lead to an imbalance between oncogenes and their suppressors and contribute to development of abnormal proliferation and cancer if the errors in the DNA are not repaired (Tachibana *et al.*, 2005a). DNA licensing must therefore be regulated so that it is only sanctioned during late M-phase to G1 and for the remainder of the cell cycle, S-phase, G2 and early M-phase, further licensing is inhibited (Blow and Dutta, 2005).

1.3.1 CDK Activity

In yeast there is extensive evidence that CDK activity is important not only for cell cycle progression but also for ensuring that re-replication of DNA does not occur. In the fission yeast *S.pombe* overexpression of the Rum1 gene, which encodes a CDK inhibitor, in G2 of the cell cycle causes extensive re-replication (Moreno and Nurse, 1994). The rum1 gene encodes a cdc2 kinase inhibitor which when overexpressed causes inhibition of the kinase activity of the $p34^{cdc2}$-$p56^{cdc13}$ complex. The resulting re-replication is due to relinquishing of the block preventing unsolicited S-phase entry (Correa-Bordes and Nurse, 1995). In agreement with this when the $p34^{cdc2}$-$p56^{cdc13}$ mitotic kinase complex is defective in G2 *S.pombe* cells, re-replication results as the cells revert back to a G1 state and undergo repeated entry into S-phase (Hayles *et al.*, 1994). It was proposed that the CDK activity at S-phase not only contributes to initiation of DNA replication but also inhibits new pre-RC formation. Indeed in the budding yeast *S.cerevisae,* when the kinase activity of the cyclin B-Cdk complex is inhibited during G2, new pre-RC complexes are formed on the already replicated DNA (Dahmann *et al.*, 1995).

Cyclin-dependant kinases have been shown to prevent re-replication of the DNA via multiple pathways in *S.cerevisae*. In this system the Clb-Cdc28 B-type CDK complex blocks re-assembly of pre-RCs during G2 and M-phase through multiple mechanisms in order to prevent re-replication (Nguyen and Li, 2001). Firstly, the Clb-Cdc28 CDK complex phosphorylates Cdc6 targeting the protein for ubiquitin-mediated proteolysis (Drury *et al.*, 2000). The Clb-Cdc28 CDK complex also promotes export of the Mcm2-7 complex form the nucleus during G2 and M-phases thereby preventing access of the

Mcm2-7 complex to the DNA (Nguyen *et al.*, 2000). In a third mechanism the Clb-Cdc28 CDK complex further prevents re-replication by hyperphosphorylation of the ORC complex which inhibits the function of ORC. It is only when all three of these mechanisms are inhibited that re-replication of the DNA is observed. At the end of M-phase the kinase activity is inactivated to allow pre-RC formation during G1 thus ensuring that the DNA is licensed and replicated only once during each cell cycle (Nguyen and Li, 2001).

It has been suggested that CDK activity may also play a role in limiting DNA replication to once per cell cycle in higher eukaryotes. This was based on evidence that in *Xenopus* egg extract a high Cdk2-cyclin E concentration is capable of inhibiting licensing by preventing the association of the Mcm2-7 complex with the DNA post-replication (Hua *et al.*, 1997). However in the same *Xenopus* cell free system treatment of G2 nuclei with p21^{Cip1}, an inhibitor of CDK activity, did not cause Mcm2-7 loading to the DNA and therefore did not induce re-licensing. This shows that CDK activity alone is not sufficient to prevent re-licensing and therefore other mechanisms are present in higher eukaryotes to limit DNA licensing and replication to once in a single cell cycle (Sun *et al.*, 2000).

1.3.2 Cdc6 Regulation

Cdc6 is essential for DNA licensing as in the absence of Cdc6 no licensing occurs (Coleman *et al.*, 1996). In the yeast *S.cerevisae*, Cdc6 is highly unstable and levels fluctuate during the cell cycle. Cdc6 is regulated by a pattern of degradation during S-phase and re-synthesis during late M-phase ready for the next cell cycle. The CDK cdc28 is responsible for Cdc6 degradation during late G1 and the degradation is mediated by the SCF complex, a multi-protein (Skp, Cullin and F-box proteins) E3 ubiquitin ligase complex (Drury *et al.*, 2000). In contrast in metazoans Cdc6 can be detected bound to chromatin during both S-phase and G2 (Mendez and Stillman, 2000). However Cdc6 is degraded in human cells during early G1 by the APC/C in association with the APC/C activator protein CDH1. Cdc6 undergoes polyubiquitination *in vivo* which is believed to mediate the degradation. Although Cdc6 is degraded in early G1, Cdc6 mRNA is present throughout the cell cycle in Hela cells and Cdc6 is resynthesised during late G1. The Cdc6 protein levels then persist through S-phase, G2

and M-phase which suggests that Cdc6 may play additional roles during the cell cycle in addition to the role in DNA licensing (Petersen *et al.*, 2000).

In contrast to yeast cells, Cdc6 levels remain persistent during S-phase, G2 and M-phase in mammalian cells. This suggests that regulation of other licensing components limits DNA licensing and replication to once per cell cycle in this system with Cdc6 required for further cell cycle events (Petersen *et al.*, 2000). One possible additional function of Cdc6 is ensuring replication of the entire genome prior to exit from G2 into M-phase. There is evidence of this function in human cells where overexpression of Cdc6 in G2 cells causes cell cycle arrest preventing entry into mitosis. The arrest was due to checkpoint activation as evidenced by Chk1 (checkpoint kinase 1) phosphorylation. It is therefore believed that human Cdc6 may have a role in regulating entry into M-phase (Clay-Farrace *et al.*, 2003).

In *S.pombe* over expression of Cdc18 (the homologue to Cdc6) caused repeated rounds of DNA replication without an intervening M-phase (re-duplication). This suggested that Cdc6 regulation is important for preventing re-duplication in the yeast *S.pombe* (Nishitani and Nurse, 1995). However this observation appears to be unique to *S.pombe*. In *C.elegans* Cdc6 undergoes CUL4 (cullin 4, a component of the E3 ubiqutin ligase complex) mediated phosphorylation followed by exportation from the nucleus during S-phase. When this export of Cdc6 is blocked the embryos of the hermaphrodite worms are less viable compared to wild type controls. However the lethality is increased when the export insensitive Cdc6 mutant is expressed alongside a non-degradable Cdt1 mutant. Re-replication was observed when both the Cdc6 mutant and the Cdt1 mutant were co-expressed. This suggests that although Cdc6 may be targeted for regulation to prevent re-replication, this is a redundant mechanism, with Cdt1 regulation being the main mechanism to prevent re-replication (Kim *et al.*, 2007). In Drosophila Cdc6 was shown to remain nuclear during S-phase and again overexpression of Cdc6 did not cause re-replication however, it did cause a slight delay in S-phase suggesting that Cdc6 may have role in S-phase coordination (Crevel *et al.*, 2005).

1.3.3   Cdt1 Regulation

Cdt1 is an important component of the DNA licensing machinery and its regulation is crucial to ensure that licensing of DNA and therefore replication occur only once in a given cell cycle. Maiorano *et al.*, have shown that addition of recombinant Cdt1 to G2 nuclei causes DNA re-licensing and subsequent re-replication. This provides evidence that during G2 mechanisms are in place to exclude Cdt1 from the DNA (Maiorano *et al.*, 2005). The addition of Cdt1 to G2 nuclei in *Xenopus* egg extract actually causes uncontrolled re-replication. This leads to the production of fragments of dsDNA due to the collision of replication forks in a head-to-tail manner. The Chk1 checkpoint is also activated as a result (Davidson *et al.*, 2006). This shows that unsolicited Cdt1 activity following DNA replication in S-phase is sufficient to cause re-licensing and highlights the importance of correct Cdt1 regulation.

Cdt1 degradation is an important mechanism for regulating Cdt1 activity in several model systems. In human cells Cdt1 undergoes proteasome mediated degradation. In this system the N-terminal region of phophorylated Cdt1 is recognised by Skp2. Skp2 is a component of the SCF ubiquitin ligase complex which polyubiquitinates multiple target proteins to target them for degradation by the proteasome. Following interaction with Skp2, Cdt1 undergoes SCF^{Skp2} mediated polyubiquitination which leads to Cdt1 degradation (Li *et al.*, 2003). In addition in human cells a further ubiquitin ligase, the APC/C also plays a role in Cdt1 degradation. The Cdt1 N-terminal region contains three destruction boxes which interact with the APC/C triggering Cdt1 degradation. Upon deletion of the three destruction boxes of Cdt1 re-replication occurs demonstrating the importance of APC/C mediated Cdt1 degradation in mammalian cells (Sugimoto *et al.*, 2008).

In *C. elegans* Cdt1 degradation also occurs, however the E3 ubiquitin ligase responsible is CUL-4/DDB-1 rather than SCF^{Skp2} as in humans. It has been shown that not only does DDB-1 interact with Cdt1 and CUL-4 directly, but also when DDB-1 is knocked down re-replication is observed. This highlights the importance of regulating Cdt1 activity by degradation for correctly controlled DNA replication in *C. elegans* (Kim and Kipreos, 2007). There is also a role for the CUL-4/DDB-1 pathway in Cdt1 degradation in humans but only after DNA damage induced by UV irradiation. After DNA damage, Cdt1 is ubiquitinated and degraded in a process dependant on PCNA as a co-factor in

order to inhibit licensing until the DNA is repaired (Senga *et al.*, 2006). Interestingly in *Xenopus* egg extract the CUL-4/DDB-1 E3 ubiquitin ligase is also responsible for Cdt1 ubiquitination and degradation in a process dependent on PCNA. In this pathway a complex of Cdt1 and CUL-4/DDB-1 loaded on chromatin is likely activated by PCNA loading in S-phase to trigger Cdt1 destruction. By this mechanism chromatin bound Cdt1 is degraded during ongoing DNA replication in S-phase in a replication dependant manner (Arias and Walter, 2005a).

Metazoan cells also possess a unique Cdt1 inhibitor protein: geminin. During the cell cycle in human cells geminin activity is required to regulate Cdt1 activity to prevent re-replication in G2 and subsequent cell cycle arrest at the G2 to M-phase transition (Klotz-Noack *et al.*, 2012). However, there is evidence that geminin has a more complex role with Cdt1 other than simply as an inhibitor. It has been reported that in human cells along with its role as a Cdt1 inhibitor, geminin also promotes licensing by protecting Cdt1 from degradation during M-phase thereby allowing Cdt1 to accumulate (Ballabeni *et al.*, 2004; Ballabeni *et al.*, 2013). In agreement with this Lutzmann *et al.,* have shown that a Cdt1-geminin complex is not only capable of preventing DNA re-replication but is also capable of licensing the DNA. This suggests that rather than simply blocking the activity of Cdt1, geminin may form a complex with Cdt1 to function as a molecular switch to shift between states of licensing -active and -inactive (Lutzmann *et al.*, 2006).

One possible mechanism which would allow the Cdt1:geminin complex to switch between licensing -active and -inactive states is a change in stoichiometry of the complex. It has been shown that Cdt1 and geminin are able to form complexes together with two different stoichiometries, a heterotrimer 1:2 Cdt1:geminin complex and a heterohexamer consisting of two heterotrimers. When Cdt1 forms a 2:4 Cdt1:geminin heterohexamer the complex is unable to license the DNA due to concealment of the MCM binding residues of Cdt1 which are crucial for DNA licensing. This suggests that the Cdt1 heterotrimer acts as the licensing active "permissive" switch while the heterohexamer acts as the licensing inactive "inhibitory" switch (De Marco *et al.*, 2009). The Cdt1:geminin molecular switch model also explains the co-localisation of Cdt1 and geminin on chromatin in human cell lines (Xouri *et al.*, 2007). A

recent study has also shown that a Cdt1-geminin complex acts as a molecular switch in sea urchin eggs to trigger licensing of the male pronucleus (Aze *et al.*, 2010).

In *Drosophila* correct geminin levels are essential for DNA replication (Quinn *et al.*, 2001). Loss of geminin in *Drosophila* cells causes re-replication which is dependent on the presence of Cdt1 suggesting it to be the result of re-licensing due to Cdt1 activity (Melixetian *et al.*, 2004). This highlights the importance of Cdt1 regulation by geminin in this system. There is also evidence that geminin loss has an effect on other events dependent on the cell cycle. In both normal and cancerous human cells geminin loss can cause overduplication of the centrosomes (Tachibana *et al.*, 2005b). In mouse cells loss of geminin causes genome duplication in the absence of mitosis (Gonzalez *et al.*, 2006a). This suggests wide ranging functions for geminin in cell cycle and cell division processes as well as its well documented role as a Cdt1 inhibitor. In addition there is evidence that geminin may play a role in cell proliferation and embryonic patterning by inhibiting both Hox gene transcription and protein function, which play a role in embryonic development (Pitulescu *et al.*, 2005).

There is variation between different species of the significance of each regulatory mechanism in preventing DNA re-licensing and therefore re-replication. When geminin is depleted from *Xenopus* egg extract there is no re-replication observed even though geminin is capable of inhibiting DNA replication (McGarry and Kirschner, 1998). In *Xenopus* egg extract both geminin and Cdt1 proteolysis must be inhibited for uncontrolled Cdt1 activity to trigger re-licensing and re-replication (Li and Blow, 2005). However in the human cancer cell lines HCT116 and H1299 when geminin is depleted re-replication does occur (Zhu *et al.*, 2004). Correct geminin function is essential in human cells to prevent re-replication. When the APC/C inhibitor Emi1 is depleted in human cell lines the APC/C is prematurely activated and goes on to inhibit geminin function. The result is re-replication (Machida and Dutta, 2007). However, in HeLa cells Cdt1 is degraded before geminin is expressed which suggests that in this case geminin inhibition acts as a redundant mechanism while degradation is the primary mechanism for Cdt1 regulation (Nishitani *et al.*, 2001).

Both degradation of Cdt1 and inhibition by geminin have been shown to be important for regulation of Cdt1 activity to prevent re-replication in *Xenopus* egg extract (Arias

and Walter, 2005b; Li and Blow, 2005). It has been shown in extract that upon entry into S-phase, the recruitment of Cdk2/cyclin E, Cdc45, RPA and DNA polymerase to the DNA triggers ubiquitin mediated degradation of Cdt1. Addition of recombinant Cdt1 to the extract following DNA replication resulted in re-replication and the extent of re-replication was increased upon depletion of geminin. This is evidence that Cdt1 degradation and inhibition by geminin act redundantly in the extract to prevent re-licensing and re-replication (Arias and Walter, 2005b). In agreement with this, Li and Blow (2005) demonstrated that in *Xenopus* egg extract both Cdt1 degradation, mediated by the APC/C, and geminin inhibition of Cdt1 were necessary to avert re-licensing and re-replication of the DNA (Li and Blow, 2005).

1.3.4 Cdt1 Structure

The Cdt1 protein has a domain structure with 3 main functional regions. It has previously been shown that the smallest section of Cdt1 capable of DNA licensing is a construct containing amino acids 243-620 (Ferenbach *et al.*, 2005) with extensive evidence showing that the N-terminal region contains sites for regulation of Cdt1 via degradation (Li *et al.*, 2003; Nishitani *et al.*, 2004; Arias and Walter, 2005b; Arias and Walter, 2005a; Senga *et al.*, 2006). The first 21 N-terminal amino acids contain a highly conserved PIP box which is essential for Cdt1 interaction with PCNA required for replication dependant Cdt1 degradation (Arias and Walter, 2005a). The N-terminal region also contains domains required for interaction with E3 ubiquitin ligase complexes. The PIP box is utilised for PCNA dependant degradation of Cdt1 mediated by the Cul4-DDB-1 E3 ubiquitin ligase (Nishitani *et al.*, 2006). A cy motif is also located within the first 100 amino acids which is essential for the cyclin/Cdk dependant phosphorylation of Cdt1. The phosphorylated Cdt1 is then recognised by the SCF^{Skp2} E3 ubiquitin ligase complex and targeted for proteolysis (Li *et al.*, 2003; Liu *et al.*, 2004; Nishitani *et al.*, 2004; Nishitani *et al.*, 2006). There are also several N-terminal destruction boxes which are recognised by the APC/C to allow ubiquitination and subsequent degradation of Cdt1 (Sugimoto *et al.*, 2008).

Although the N-terminal region of Cdt1 shows weak binding activity the main geminin binding region lies between amino acids 193-447. This region lies between two predicted coiled-coil domains of Cdt1. When the N-terminal coiled-coil domain is lost, as with the smallest Cdt1 construct capable of licensing DNA (ΔCdt1$^{243-620}$), partial

geminin resistance results (Ferenbach *et al.*, 2005). Coiled-coil domains are protein tertiary structure motifs consisting of 2-5 α-helical coils further coiled together (Mason and Arndt, 2004). The coiled-coil domains of Cdt1 are highly conserved between species and it has been suggested that the coiled-coil interacts with geminin although the coiled-coil domains and the central region of Cdt1 between these domains are both capable of interaction with geminin (Ferenbach *et al.*, 2005). In addition to the degradation and geminin binding domains, the N-terminal region also contains a nuclear localisation signal (NLS) (Nishitani *et al.*, 2004) to allow entry to the nucleus through NLS receptors on the nuclear envelope (Lange *et al.*, 2010).

The C-terminal 173 amino acids of Cdt1 bind to a complex containing the MCM complex proteins Mcm 2, 4, 6 and 7. Loading of the Mcm2-7 complex to DNA completes DNA licensing and therefore the MCM binding region is crucial for the licensing activity of Cdt1 (Ferenbach *et al.*, 2005). A schematic representation of the domain structure of Cdt1 is shown in Figure 1.3.
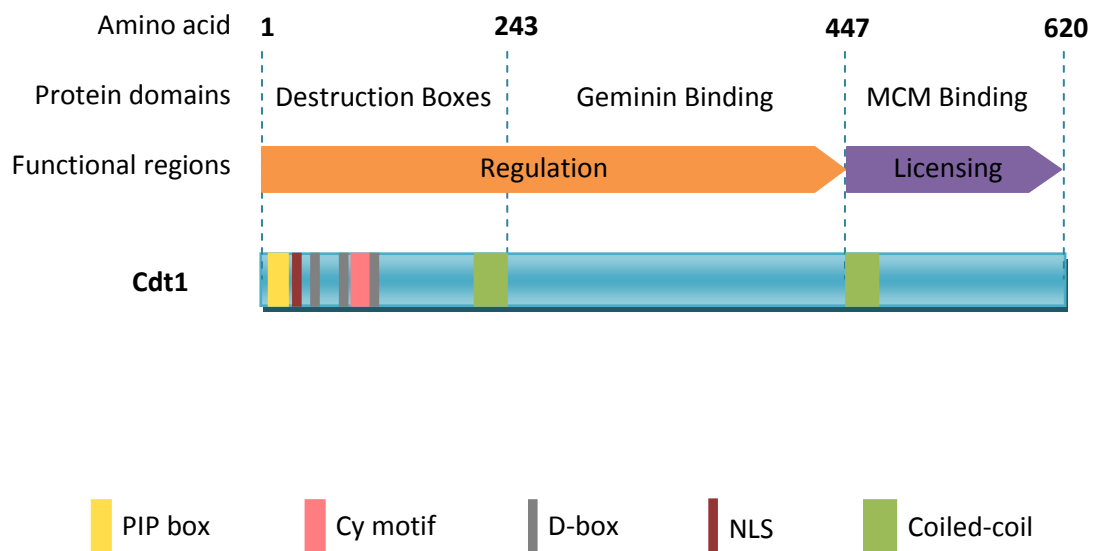
Figure 1.3. Cdt1 structure and protein domains. Cdt1 has a domain structure and can be separated into a regulatory region and a DNA licensing region. The N-terminal 1-243 amino acids of Cdt1 contain D-box sequences for ubiquitin mediated degradation, a PIP box for PCNA dependant degradation, a cy motif for cyclin/Cdk dependant Cdt1 phosphorylation as well as a nuclear localisation sequence (NLS). The central region is essential for geminin binding while the green regions represent the coiled-coil domains also thought to be important for geminin binding. The C-terminal contains the MCM binding region which is necessary for the licensing activity of Cdt1.

1.3.5 The Importance of Correct DNA Licensing and Replication

Due to the fact that the licensing proteins are intricately linked to DNA replication it is unsurprising that a connection between aberrant licensing activity and cancer development has been suggested. Inappropriate expression of the DNA licensing proteins or an insufficient number of licensed origins could lead to genomic instability which is a hallmark of cancer (Blow and Gillespie, 2008).

Both Cdc6 and Cdt1 have been implicated in cancer development. It has been suggested that the genes for both Cdc6 and Cdt1 may function as oncogenes. During cancer development an oncogene is a mutated gene encoding a protein involved in the cell cycle which leads to increased cell division (Chial, 2008). It has been shown that integration of retroviral DNA into the primitive erythroid EB-PE cell line caused activation of the Cdt1 gene and this coincided with the EB-PE cells becoming immortal with immortalisation being a key step in cancer development (Arentson *et al.*, 2002). In addition it has also been shown that Cdt1 overexpression causes development of DNA double strand breaks which activate the DNA damage checkpoint although the cells subsequently undergo senescence or apoptosis. However prolonged overexpression of Cdt1 in U2OS cells eventually led to a situation in which the cells were able to bypass the checkpoint. These cells showed significant genomic instability and also showed the potential to be potently invasive, an aggressive cancerous phenotype (Liontos *et al.*, 2007). This provides evidence that Cdt1 may function as an oncogene (Arentson *et al.*, 2002).

The licensing protein Cdc6 has also been shown to exhibit oncogenic potential. There is evidence that overexpression of Cdc6 results in transcriptional repression of the INK4/ARF gene locus and a subsequent downregulation of the tumour suppressors encoded at this region, namely p16^{INK4a}. Alongside activation of this oncogenic pathway, Cdc6 overexpression can also cooperate with the Ras oncogene to induce immortalisation and neoplastic transformation thus providing further proof of the oncogenic potential of Cdc6 (Gonzalez *et al.*, 2006b).

Although Cdt1 may contribute to cancer development it is also possible that Cdt1 may be a suitable candidate for targeted cancer therapy. A recent study has shown that the re-replication induced by inappropriate Cdt1 activity can be exploited to contribute to

the cell death of cancer cells. The NAE (NEDD8-activating enzyme) inhibitor MLN4924, which is currently undergoing phase I trials, causes Cdt1 accumulation by preventing the CRL-mediated (cullin ring ligase of an E3 ubiquitin ligase complex) mechanism of Cdt1 degradation. The elevated Cdt1 levels leads to re-replication and DNA damage which ultimately triggers apoptosis (Milhollen *et al.*, 2011). It has also been suggested that cancerous cells can be selectively killed by suppressing geminin activity. Again unsolicited Cdt1 activity caused re-replication and DNA damage which subsequently led to apoptosis. The selectivity of this method to cancer cells was suggested to be due to the presence of additional mechanisms for the prevention of re-replication in normal cells compared to cancer cells (Zhu and DePamphilis, 2009).

In addition to the association of inappropriate DNA licensing and caner, some of the DNA licensing proteins have also been linked to other diseases. Since proper DNA licensing and replication is important for correct cell cycle progression and cell division it is unsurprising that appropriate activity of licensing proteins is vital for development, a time when cell division is particularly significant. In line with this mutations of Orc1 and other licensing proteins including Orc4, Orc6, Cdc6 and Cdt1 have been associated with the developmental growth defect Meier Gorlin syndrome, a form of primordial dwarfism (Bicknell *et al.*, 2011a). It has been shown that in *Zebrafish* embryos depletion of Orc1 causes significant reductions in body size likely due to delays in S-phase entry as a result of inadequate origin licensing (Bicknell *et al.*, 2011b).

With mutations in DNA licensing proteins including Cdt1, Cdc6 and the ORC proteins linked to diseases such as cancer and developmental growth defects, a thorough understanding of regulation of DNA licensing is essential. Through understanding how the licensing proteins are regulated and how mutations lead to disease new therapies may evolve. Indeed suppression of geminin activity to selectively kill cancer cells (Zhu and DePamphilis, 2009) may provide just one future treatment of certain cancers which involve targeting of the DNA licensing system.

1.4 *Xenopus laevis Model System*

There are several advantages to using *Xenopus* embryos as a model system. The first reasons are practical considerations; the embryo is large and develops externally making it easily manipulated by microinjection or dissection and the animal cap cells

can be removed and cultured (Jones and Smith, 2008). Also, manipulation of the embryo allows the effects on not just one cell cycle but development of the entire organism to be investigated.

In addition the eggs of the *Xenopus laevis* toad can be utilised to produce *Xenopus* egg extract. The egg extract system was developed almost 30 years ago and as such has been well characterised. *Xenopus* extract supports many of the cell cycle events such as DNA licensing, replication and separation of the sister chromatids *in vitro*. These properties make the *Xenopus* egg extract system a useful model with which to study the mechanisms of cell cycle and DNA replication regulation (Gillespie *et al.*, 2012).

1.4.1 *Xenopus laevis* Embryo Development

Embryonic development begins when one sperm from the male of the species fertilises one oocyte (egg) from the female to form the zygote. The zygote is the initial cell produced when two gamete cells, the sperm and the egg, fuse. Following fertilisation cleavage cell cycles occur which increase the total number of cells without a parallel increase in size of the zygote. At this stage in development the zygote forms a blastocyst in mammals and a blastula in other vertebrates. Gastrulation then follows in which the cells of the embryo are rearranged into three layers: the endoderm, ectoderm and mesoderm. Through differentiation, the cells of each layer then give rise to different tissues and organs leading to the development of a viable organism (Gilbert, 2000a).

The eggs of the African clawed toad *Xenopus laevis* have a distinct polarity consisting of a dark pigmented animal pole and a pale vegetal pole which contains much of the yolk of the egg. The eggs are laid encased in a jelly and vitelline membrane which acts to both protect the egg and to attract and activate sperm cells to allow fertilisation (Gilbert, 2000a). The vitelline membrane is composed of a minimum of four glycoproteins which are required for the interaction between the sperm and the egg (Vo and Hedrick, 2000; Miwa *et al.*, 2010).

Xenopus laevis eggs are deposited from the female into the external environment prior to fertilisation and subsequent embryonic development. With no outside source of nutrition the reserves of the egg must be sufficient to produce an organism capable of self-feeding. The *Xenopus* eggs are therefore large, at approximately 1 mm in

diameter, to allow sufficient nutritional reserves for embryonic development. Due to the large size of the egg in comparison to the amount of DNA the first 12 cell divisions consist of rapid cleavages during which the DNA content increases exponentially to provide the necessary transcriptional output for later embryonic development (O'Farrell *et al.*, 2004).

Upon fertilisation the *Xenopus* zygote completes the meiotic cell cycle before entry into the mitotic cleavage cell cycles. The first cell division therefore takes longer than the subsequent cleavage divisions. Division from the single cell zygote to the two cell embryo therefore takes 1 hour 30 minutes following fertilisation whereas each cleavage cell cycles takes approximately 30 minutes. The cleavage cycles continue until approximately 6 hours after fertilisation at which point the mid-blastula transition (MBT) occurs and the cell cycle length is increased before gastrulation begins (O'Farrell *et al.*, 2004).

The development of the *Xenopus* embryo is classified into developmental stages (Nieuwkoop and Faber, 1967b). The blastula stage in which the cells form a layer over the blastocoelic cavity begins at stage 7 until stage 9 of development. The blastocoelic cavity, also known as the blastocoel, likely serves to provide space for the cell movements of gastrulation and to prevent the cells of the animal and vegetal poles from prematurely interacting (Gilbert, 2000b). Gastrulation begins at stage 10 during which the cells migrate and rearrange to form endoderm, ectoderm and mesoderm. The surface cells of the animal pole form the ectoderm while the cells of the vegetal pole form the endoderm and the cells of the mesoderm form from the deeper layers of cells within the embryo (Gilbert, 2000b). In later embryonic development the cells of the ectoderm give rise to the skin and nerve cells, the endoderm gives rise to the gut and organs associated with the gut and the mesoderm gives rise to blood cells and the vascular and connective tissues (Gilbert, 2000b; Fehling *et al.*, 2003).

Gastrulation is completed by stage 13.5 at which point the neurula stages begin which are characterized by formation of the neural plate. The early development of the CNS and associated nerves, ganglia and sense organs including the eyes and ears develop approximately up to stage 28. Up to stage 38 the axis of the embryo is developed along with the alimentary system of digestive organs, visceral and muscle tissue. By stage 53

the brain is well developed with much of the epidermis, connective tissue and patterns of skin pigmentation developed up to stage 60. The *Xenopus* embryo resembles a tadpole by stage 34 at approximately 48 hours following fertilisation and as such is motile by this stage of development (Nieuwkoop and Faber, 1967b). The developmental stages from stage 1 to 11 and stages 22, 34 and 40 are shown in Figure 1.4 adapted from (Nieuwkoop and Faber, 1967a) along with the approximate times post-fertilisation of each stage.

Stage 1, 1 hr   Stage 2, 1 hr 30 min   Stage 3, 2 hr   Stage 4, 2 hr 30 min

Stage 5, 2 hr 45 min   Stage 6, 3 hr 5 min   Stage 6.5, 3 hr 30 min   Stage 7, 4 hr

Stage 8, 5 hr   Stage 9, 7 hr   Stage 10, 9 hr   Stage 10.5, 11 hr

Stage 11, 12 hr   Stage 22, 24 hr   Stage 34, 48 hr

Stage 40, 72 hr
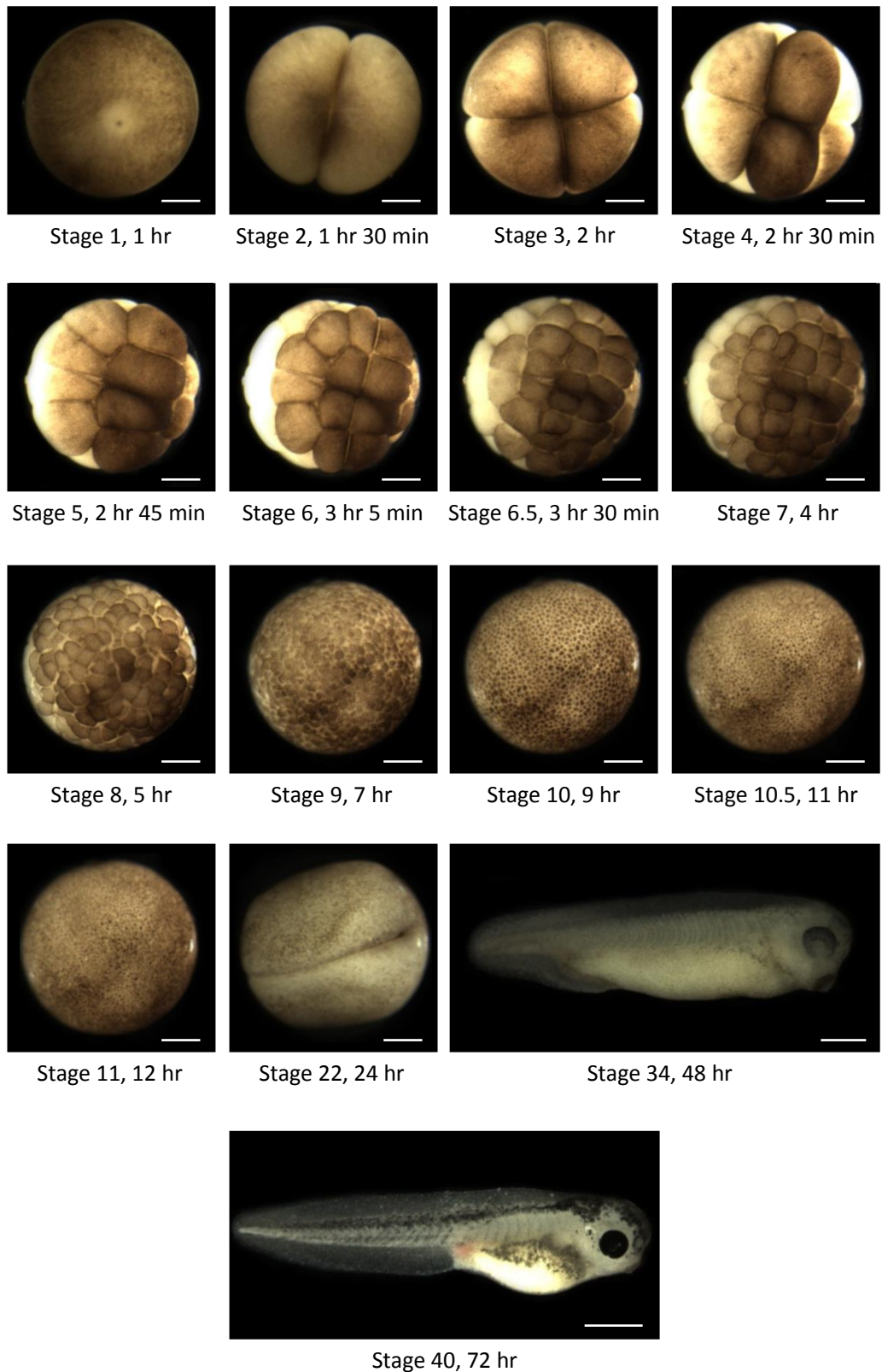
Figure 1.4. Developmental Stages of *Xenopus laevis* Embryos. *Xenopus* eggs were fertilised and the vitalline envelope removed before normal development was monitored using a Leica M205 FA macro imaging system. For each stage of development depicted, the time post-fertilisation of the embryo is indicated. The embryo development shown is at 23°C. Scale bars for stages 1 through to 22 are 250μm, scale bar for stage 34 is 900μm and scale bar for stage 40 is 1200μm.

1.4.2 The Embryonic Cell Cycle

The mature oocytes of *Xenopus* and *Zebrafish* are arrested in metaphase of meiosis II, the cell cycle which produces the haploid gamete cells. Meanwhile the oocytes of the fruit fly *Drosophila* are arrested in meiosis I and in the nematode worm *C.elegans,* completion of meiosis I and II only occurs upon fertilization (Von Stetina and Orr-Weaver, 2011). In sea urchin the oocytes complete the meiotic cell cycle prior to fertilisation and arrest in G1-G0 (Voronina *et al.*, 2003). Regardless of the stage of the cell cycle arrest, upon fertilisation a calcium wave releases the oocyte from the arrest prior to completion of meiosis and entry into the mitotic cell cycles (Whitaker, 2008).

The first mitotic cell cycles of the *Xenopus*, *Drosophila*, and *Zebrafish* embryos consist of rapid cleavage cell cycles in which the number of cells and amount of DNA increases exponentially with each division while the size of the embryo remains constant (O'Farrell *et al.*, 2004). To allow the rapid synchronous cleavages, the embryonic cell cycle consists of alternating S- and M- phases and lacks the distinct gap phases, G1 and G2, which are present in the somatic cell cycle (Graham and Morgan, 1966). Also, during the cleavage cell cycles the embryo is transcriptionally silent and instead relies on maternally derived stores of mRNA and proteins (Newport and Kirschner, 1982; Edgar and Schubiger, 1986).

The rapid cleavages of *Xenopus* and sea urchin embryos are driven by oscillations in CDK activity to drive cell cycle events including nuclear membrane breakdown, mitosis and DNA replication (Newport and Kirschner, 1984). It has been shown that MPF is a complex between B type cyclins and CDK1 (Hartley *et al.*, 1996; Doree and Hunt, 2002). During the first 16 cell divisions of the *Xenopus* embryo the levels of cyclin B1 and B2 oscillate in parallel with the activity of CDK1 to regulate embryonic cell cycle timing (Hartley *et al.*, 1996). However in *Drosophila* embryos the cyclin B and CDK1 levels remain constant during the first 7 cell cycles with the onset of fluctuations in cyclin levels and CDK1 activity between cycles 8 and 13 (Edgar *et al.*, 1993). However, although the total levels of the B type cyclins and CDK1 activity in the syncytial *Drosophila* embryo do not fluctuate, there is local proteolysis of small pools of cyclin B which is required for mitosis exit during the cell cycle (Su *et al.*, 1998).

It has been shown that during the cleavage cell cycles in *Drosophila* embryos, accumulation of cyclins is necessary for some of the events of mitosis but is not directly responsible for timing the cell cycle (McClealand *et al.*, 2009a). Instead during the cleavage cycles, S-phase acts as a mitotic timer to time the cell cycle with checkpoints which couple S-phase completion to M-phase only essential prior to the MBT and becoming dispensable during the post-MBT cell cycles (McClealand *et al.*, 2009b). In sea urchin embryos during the early cleavage cycles progression into S-phase is triggered by the activity of the cyclin E-CDK2 complex with high levels of cyclin E-CDK2 throughout the cell cycle unlike in somatic cells where the cyclin E-CDK2 levels oscillate (Sumerel *et al.*, 2001).

The cleavage cell cycles constitute the first 12 cell divisions in the *Xenopus* embryo until the onset of the mid-blastula transition (MBT) which occurs at approximately 6 hours after fertilisation (O'Farrell *et al.*, 2004). The MBT is triggered when the DNA to cytoplasm ratio reaches a critical level. Following the MBT the cell cycles of the embryo closer resemble the somatic cell cycles as they become asynchronous, gap phases are introduced, the cells acquire motility and zygotic transcription begins (Newport and Kirschner, 1982). The sea urchin embryo also undergoes cleavage cell cycles consisting only of oscillating S- and M- phases however unlike with *Xenopus* and *Drosophila* embryos, there is zygotic transcription throughout the cleavage cell cycles and an absence of a defined MBT (Yasuda and Schubiger, 1992).

During the cleavage cell cycles the entire genome is replicated during the short and rapid S-phase prior to entry into mitosis. In the cleavage cycles of *Drosophila* embryos the 180 million base pair (bp) genome is replicated in 3.4 minutes while in *Xenopus* the 1.7 billion bp genome is replicated in 15 minutes (O'Farrell *et al.*, 2004). In order to achieve complete replication of the genome in such a short period of time the cells enlist many more DNA replication origins compared to in the longer later embryonic- and somatic- cell cycles (McKnight and Miller Jr, 1977; Walter and Newport, 1997).

Due to the short rapid cleavage cycles of the early embryo and the lack of gap phases, DNA licensing occurs earlier in M-phase in time for the onset of S-phase. In *C.elegans* embryos Mcm2-7 loading and therefore DNA licensing occurs in anaphase of meiosis II and at metaphase and anaphase of the following mitotic cell cycles. Rapid turnover of

Orc1 and Cdc6 allows these proteins to license multiple origins to ensure adequate DNA licensing in the short *C.elegans* embryonic cell cycle (Sonneville *et al.*, 2012). During the *Drosophila* embryonic cell cycle, prior to introduction of gap phases, Mcm2 also binds DNA during anaphase (Su and O'Farrell, 1997). In early *Xenopus* embryos, karyomeres of nuclear membrane lamina form around the chromosomes to allow DNA licensing to occur during anaphase. PCNA is detected on such chromosomes during telophase suggesting initiation of DNA replication. This pattern of DNA licensing and replication terminates after the MBT (Lemaitre *et al.*, 1998; Kisielewska and Blow, 2012). Figure 1.5 shows a schematic representation of DNA licensing and regulation of licensing during the pre-MBT cell cycle in *Xenopus* embryos.

Uniquely to sea urchin the DNA of the unfertilized G1 arrested egg is already licensed for replication with Cdc6, Cdt1 and Mcm3 all bound to chromatin. The male chromatin is then licensed post-fertilisation. In the unfertilized egg, the repression of DNA replication from the licensed DNA origins is thought to be achieved via pathways involving the MAP and checkpoint kinases (Aze *et al.*, 2010).

Considering that the structure of the cell cycle during the embryonic cleavage cell cycles differs significantly from the somatic cell cycle, it is also possible that regulation of DNA licensing and replication may differ considerably in early embryos. Indeed there is evidence that in early embryos the predominant methods of regulation differ, likely due to the rapid nature of the cell cycles. In early *Xenopus* embryos Cdt1 degradation is minimal with the levels of both Cdt1 and geminin remaining persistently high throughout the cleavage cycles (Kisielewska and Blow, 2012). A persistently high level of geminin is also present in early *Drosophila* embryos during the syncitial divisions, regardless of the stage of the cell cycle (Quinn *et al.*, 2001). In *C.elegans* embryos, Orc1 and Cdc6 are excluded from the nucleus during S-phase, possibly providing an alternative method to prevent re-licensing when there is insufficient time for protein degradation and re-synthesis (Sonneville *et al.*, 2012).

The importance of Cdt1 regulation for correct cell cycle progression in early pre-MBT *Xenopus* embryos has previously been disputed. It has been shown that depletion of endogenous geminin from the *Xenopus* embryo using morpholinos has no effect on pre-MBT cell division with cell cycle arrest only occurring at the MBT (McGarry, 2002).

However it has since been shown that functional knockdown of the geminin protein and therefore uncontrolled Cdt1 activity does lead to cell cycle arrest in pre-MBT *Xenopus* embryos (Kisielewska and Blow, 2012). Along with evidence that siRNA and morpholinos may be inadequate to produce RNAi in *Xenopus* embryos (Lund *et al.*, 2011) this suggests that Cdt1 regulation may be crucial for proper cell division in pre-MBT embryos. The role and mechanisms of Cdt1 regulation during the pre-MBT cell cycle therefore requires clarification (highlighted in the red box of Figure 1.5).
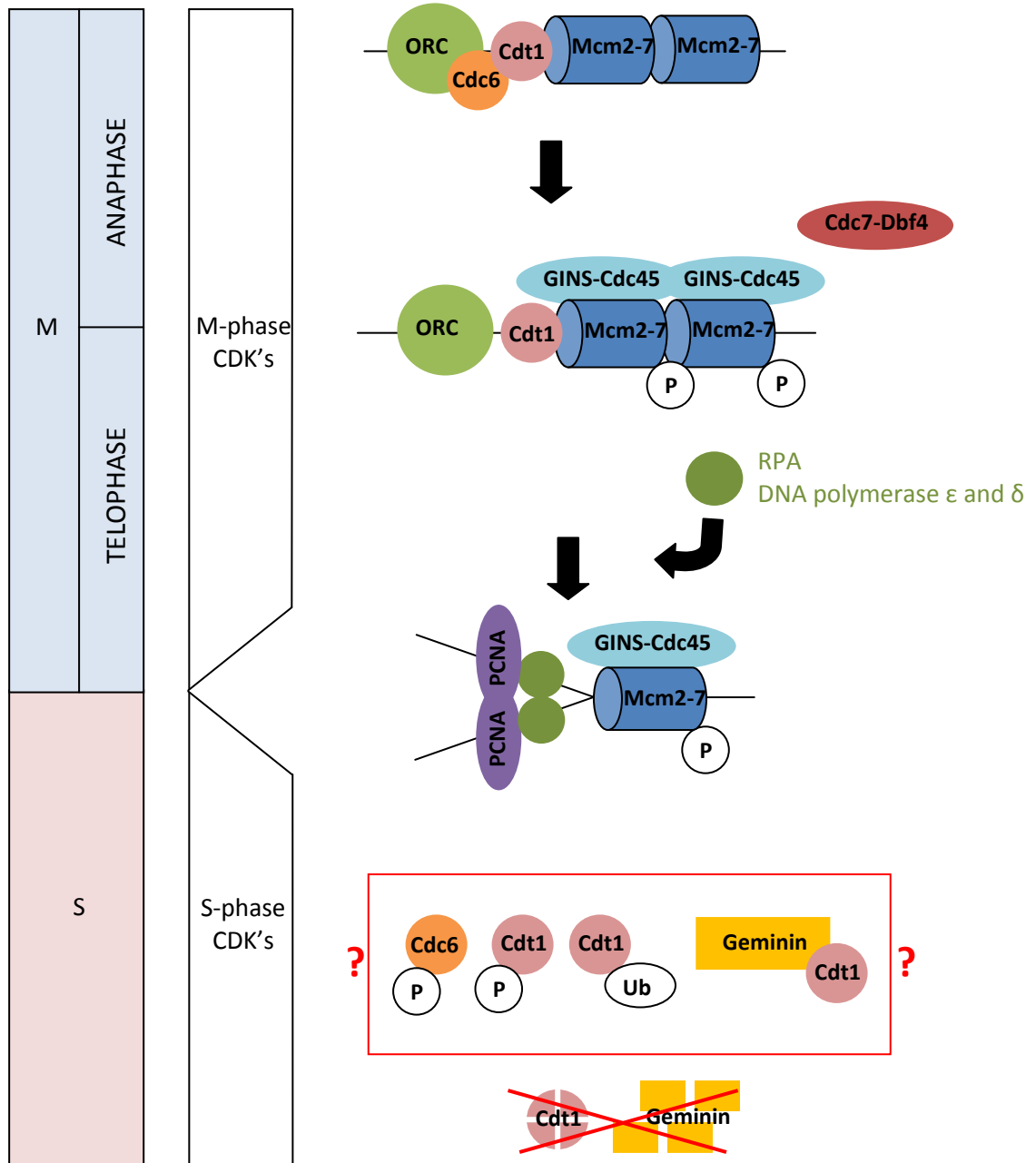
Figure 1.5. DNA licensing and replication in the pre-MBT *Xenopus* embryonic cell cycle. The pre-MBT embryonic cell cycle consists of alternating S- and M- phases and DNA licensing occurs in late M-phase, specifically anaphase. In telophase PCNA can be detected on chromatin suggesting initiation of DNA replication (Philipova *et al.*, 2005). During S-phase further licensing must be inhibited however the mechanisms behind the inhibition are currently unknown (red box). Although the exact mechanisms of regulation of the licensing proteins Cdt1 and geminin remain unclear there is evidence that they do not undergo degradation in the pre-MBT cell cycle as they do in somatic cells.

1.4.3   Additional Roles for the Pre-RC Proteins during Embryonic Development

Some of the proteins involved in DNA licensing also interact with other proteins and form important interactions during embryonic development to provide roles distinct from licensing. Geminin in particular interacts with multiple proteins during embryonic development. Geminin not only associates with Hox proteins, which are important in regulating development of embryonic axis and structures, but can also interact with the regulator elements of the DNA at the Hox gene thereby functioning to coordinate development (Luo *et al.*, 2004). Geminin has also been shown to play a role in cell commitment and ensuring the germ layers of the *Xenopus* embryo form at the correct locations (Lim *et al.*, 2011).

In *Drosophila* embryos, overexpression of geminin caused neural differentiation suggesting that geminin activity may play a role in neurogenesis during development. In addition, geminin overexpression resulted in a decrease in size of the eye discs of the larvae and adult eye (Quinn *et al.*, 2001). A role for geminin in eye development has also been shown in fish embryos (Medaka fish). During Medaka development geminin interacts with the transcription factor Six3, known to be involved in development of the eye, to regulate a balance between cell differentiation and proliferation which is essential for normal eye development (Del Bene *et al.*, 2004).

There is also evidence that Cdc6 has diverse roles during the cell cycle and embryo development in addition to the well established role in DNA licensing. In mouse oocytes Cdc6 activity is essential for spindle formation during progression through meiosis (Anger *et al.*, 2005). It has also been shown that in *Xenopus* oocytes an increase in Cdc6 activity may be sufficient to induce sperm binding and therefore aid in fertilisation (Tian *et al.*, 1997).

Geminin has been shown to be crucial in many pathways during embryonic development across different species in parallel to the roles in regulation of DNA licensing (Pitulescu *et al.*, 2005). In addition, it has also been shown that Cdc6 may have roles in oocyte maturation and fertilisation (Tian *et al.*, 1997; Anger *et al.*, 2005). Some of the licensing proteins therefore play an important role during embryonic development as well as DNA licensing and replication. Therefore a comprehensive

understanding of regulation of the licensing proteins in the unique cell cycle of the early embryo is crucial.

1.4.4 *Xenopus* Egg Extract

Xenopus egg extract is a cell free *in vitro* model prepared from the eggs of the *Xenopus laevis* toad. The egg extract is produced from the *Xenopus* eggs using centrifugation which purifies the extract to leave only the membranes, cytoplasm and cytoplasmic proteins, including each of the proteins required for DNA licensing. During the centrifugation process materials which are not essential for extract function such as the yolk platelets, lipids and mitochondria are separated from the cytoplasm and discarded (Gillespie *et al.*, 2012).

The eggs of *Xenopus laevis* are held at metaphase arrest of meiosis II until fertilisation (Kanki and Donoghue, 1991). The arrest can be released through addition of Ca^{2+} to mimic the calcium spike which occurs at fertilisation. The egg extract can therefore be maintained in the metaphase arrest by addition of EGTA which sequesters any exogenous calcium to prevent activation of the eggs (Lohka and Masui, 1984). Extract produced in the presence of EGTA is therefore known as metaphase arrested egg extract (Gillespie *et al.*, 2012).

Upon addition of Ca^{2+} and demembranated *Xenopus* sperm DNA, the extract is released from the metaphase arrest into mitotic interphase and the sperm chromatin is decondensed. In addition a nuclear structure forms within the extract and the DNA licensing and replication machinery is also activated to allow efficient semi-conservative replication of the sperm DNA. Within the extract the DNA is decondensed before being licensed and replicated to produce a complete set of paired sister chromatids. The extract can then be advanced into mitosis where the chromatids are condensed and separated along a mitotic spindle as is the case during the cell cycle *in vivo*. The events of the cell cycle in the egg extract are also regulated via the same control mechanisms of the *in vivo* cell cycle. These properties make the egg extract system an excellent model with which to study the events of DNA licensing and replication *in vitro* (Gillespie *et al.*, 2012).

1.5 Aims

Much of the current data regarding Cdt1 regulation relates to the somatic cell cycle. Even data acquired using *Xenopus* egg extract is more representative of the somatic or post-MBT cell cycle as the extract contains gap phases (Li and Blow, 2004; Maiorano *et al.*, 2005). Given the differences between the early (pre-MBT) embryonic cell cycle and the somatic cell cycle it is also likely that the regulatory mechanisms may differ. Due to the short and rapid nature of the pre-MBT cell cycle it is likely that there is insufficient time for protein degradation and re-synthesis to be ready for the forthcoming cell cycle stage and therefore alternative mechanisms are utilised for Cdt1 regulation. Indeed, it has recently been shown that in pre-MBT *Xenopus* embryos, Cdt1 and geminin levels remain persistently high throughout the cell cycle with little degradation of Cdt1 (Kisielewska and Blow, 2012).

The main aim of the thesis was therefore to elucidate the mechanisms which regulate DNA licensing to prevent re-replication of the DNA during the early cell cycle in *Xenopus* embryos. In order to achieve this goal the specific aims were:

1. To investigate the effect of mis-regulation of Cdt1 on the *Xenopus laevis* embryonic cell cycle and embryo development *in vivo.*

2. To determine the importance of correct Cdt1 regulation for proper DNA licensing and cell cycle progression in pre-MBT embryos.

3. To establish the mechanisms of Cdt1 regulation in pre-MBT *Xenopus* embryos *in vivo.*

4. To investigate whether or not the N-terminal domain of Cdt1 participates in protein-protein interactions with other members of the licensing machinery which may contribute to Cdt1 regulation

Chapter 2.

Materials and Methods

2.1 Plasmids and Cloning

2.1.1 PCR

The DNA sequence for the gene of the protein of interest, either *Xenopus* Cdt1 or RFP, was obtained from the National Centre for Biotechnology Information (NCBI). The DNA sequence was then aligned with the protein sequence and the start codon determined. Using this information appropriate PCR primers were designed in order to amplify the DNA sequence for the protein region required. The primers were also designed to include an ATG start codon in the 5' forward primer before the start of the construct sequence to allow initiation of mRNA translation as well as either one or two restriction enzyme sites at the start and end of the sequence to allow insertion of the DNA into a plasmid vector. The primer sequences are listed in Table 2.1.

For the PCR reaction DNA template (a minimum of 200 ng of circular plasmid DNA containing the DNA sequence of interest), 10 pmol start primer, 10 pmol end primer, 1 mM dNTP's (250 µM per dNTP, Promega dNTP mix), reaction buffer (1x concentrated), 2.5 mM $MgCl_2$ and either the Mol Taq polymerase, which possesses 5' to 3' exonuclease activity and produces a 3' A overhang, or Velocity polymerase, which possesses proofreading 3' to 5' exonuclease activity and produces amplicons with blunt ends, were made up to a volume of 50 µl in dH_2O and placed in a PCR thermal cycler. The reaction conditions for the PCR were as follows; 1 cycle at 95°C for 5 minutes to ensure denaturation of the double stranded DNA template, followed by 30 cycles of 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute to amplify the target sequence with one final cycle of 95°C for 5 minutes, 55°C for 30 seconds and 70°C for 20 minutes to ensure completed DNA synthesis.

Construct	Start Restriction Site	End Restriction Site	Primer Sequence (5' to 3')
ΔCdt1$^{243-620}$	Bam HI, Bgl II	Not I, Eco RI	GGATCCAGATCTATGCCAGCCTATCAACGTTTTCAT
			GAATTCGCGGCCGCCTAGAGAGACTCTTCTTCCTTTGT
ΔCdt1$^{1-243}$	Bam HI	Eco RI	GGATCCATGCCAGCCTATCAACGTTTTCAT
			GAATTCTGGGGCCTTTTCACTTTCCTG
ΔCdt1$^{1-243}$	Bgl II	Eco RI	AGATCTATGGCAGACATGTCGCAAATG
			GAATTCTGGGGCCTTTTCACTTTCCTG
ΔCdt1$^{243-570}$	Bam HI, Bgl II	Eco RI	GGATCCAGATCTATGCCAGCCTATCAACGTTTTCAT
			GAATTCTTCCATCTCACCTGGAGACAT
RFP	Eco RI	Not I	GAATTCATGGCCTCCTCCGAGGACGTC
			GCGGCCGCGGCGCCGGTGGAGTGGCGGCC

Table 2.1. PCR primer sequences for production of Cdt1 and RFP constructs. For the Cdt1 constructs listed the numbers in superscript represent the amino acids of the Cdt1 protein which are encompassed by the primers. The restriction sites to be incorporated at the start and end of the DNA region of interest are listed. All primer sequences are in the 5' to 3' configuration.

2.1.2   Agarose Gel Electrophoresis

To analyse DNA samples generated by PCR or restriction enzyme digested plasmid DNA samples, agarose gel electrophoresis was used. The DNA samples were prepared by addition of x6 DNA loading dye before running on a 1% agarose gel, covered with 1x TAE buffer (40 mM TRIS BASE, 5 mM EDTA pH 8, 0.114% acetic acid) at 130 V for 35 minutes. Ethidium bromide (0.25 µg/ml) was added to the gel to allow detection of the DNA via UV. To allow identification of the DNA a 1 Kb DNA ladder (Promega) was loaded into one lane of the gel. DNA bands observed to be at the appropriate size were then cut from the gel and purified using the Qiagen QIAquick Gel Extraction Kit according to manufacturer's instructions. Purified DNA samples were stored at -20°C to prevent degradation.

2.1.3   Plasmids and Transformation

Construct DNA produced by PCR and subsequently purified from the agarose gel was then inserted into the pGEM-T vector which contains a single T overhang at the 3' end. This allows the pGEM-T vector to accept insertion of PCR generated DNA which contains a single A overhang at the 3' end created by the polymerase enzyme. To insert the DNA into the plasmid an overnight ligation at 16°C was used which consisted of the construct DNA, 50-100 ng pGEM-T vector, 1x reaction buffer and 1-3 units T4 DNA ligase enzyme. The pGEM-T vector is compatible with the blue and white screening technique as the inserted DNA disrupts the gene for the α-peptide of β-galactosidase. When transformed with the ligation mix, white bacterial colonies indicate pGEM-T containing the construct DNA. Blue colonies do not contain the construct DNA and therefore produce functional β-galactosidase which is activated by IPTG to cleave X-gal on the plates producing a blue by-product.

The overnight ligation mixture was used to transform either Nova Blue or JM109 *E.coli* strains using heat shock. For the transformation 5 µl of the overnight ligation mix was added to 20-100 µl of bacteria, depending on the competency of the cells, and incubated on ice for 30 minutes. The bacteria were then subjected to heat shock at 42°C for 1 minute and incubation on ice for a further 2 minutes before addition of 200 µl of SOC media (Sigma). The bacterial culture was then incubated at 37°C for 45 minutes before spreading on agar plates containing 100 µg/ml ampicillin and coated with 100 µl 200 mM IPTG and 2.5 mg X-gal. The plates were incubated overnight at

37°C. White colonies were then removed from the plate and cultured small scale in 10 ml LB media containing ampicillin (100 µg/ml), overnight at 37°C, 180 rpm. The plasmid DNA was then purified from 1.5 ml of the small scale culture using the Promega Wizard Plus SV Minipreps DNA Purification System according to manufacturer's instructions.

The purified plasmid DNA was then cut by restriction digestion to release the inserted DNA from the plasmid vector. A 15 µl sample of the purified plasmid DNA was incubated with the restriction enzymes targeted to the restriction sites generated by the PCR primers, at a concentration of 10-12 units with 1x restriction enzyme buffer at 37°C for 3 hours. DNA loading dye was added before the digested DNA samples were run on an agarose gel electrophoresis, as above, to check for the presence of both the pGEM-T vector and the inserted construct DNA. The correct construct DNA was then cut from the gel and purified, as above, to give construct DNA with DNA overhangs at each end produced by the restriction enzymes. The construct DNA was then inserted into the plasmid pRN3 for expression as mRNA or the plasmid pET-32a(+) for expression as recombinant protein. The appropriate plasmid expression vectors were cut using the corresponding restriction enzymes to produce complementary ends for successful insertion of the purified construct DNA. The construct DNA was then ligated into the plasmid expression vector, transformed into JM109 (minus addition of IPTG and X-gal to the plates) and checked via restriction digestion and agarose gel electrophoresis as above. Any samples that appeared to show correct insertion of the construct DNA into the final expression vector underwent DNA sequencing for confirmation. For DNA sequencing the plasmid DNA samples were sent to DNA Sequencing and Services at the University of Dundee. The DNA sequencing result of the construct was then aligned against the known DNA sequence of the construct of interest using the EMBOSS needle alignment program. The alignment was checked to ensure there were no base pair errors or frame shift mutations.

For addition of a fluorescent protein tag to the Cdt1 construct DNA, the DNA for the fluorescent protein RFP was produced via PCR, inserted into the pGEM-T vector, purified and restriction enzyme digested as above. The fluorescent protein DNA fragment was then purified from the restriction digestion as above to produce a fluorescent protein DNA fragment with DNA overhangs at each end produced by the

restriction enzymes. The plasmid vector, either pRN3 or pET-32a(+), containing the correct Cdt1 construct was then opened using the restriction enzymes complementary to the overhangs to allow insertion of the fluorescent protein DNA fragment by overnight ligation. The final expression vector containing the Cdt1 DNA and fluorescent protein DNA was then ran on an agarose gel electrophoresis before being sent for DNA sequence for conformation as above. The final Cdt1 plasmid constructs are shown in Figure 2.1.
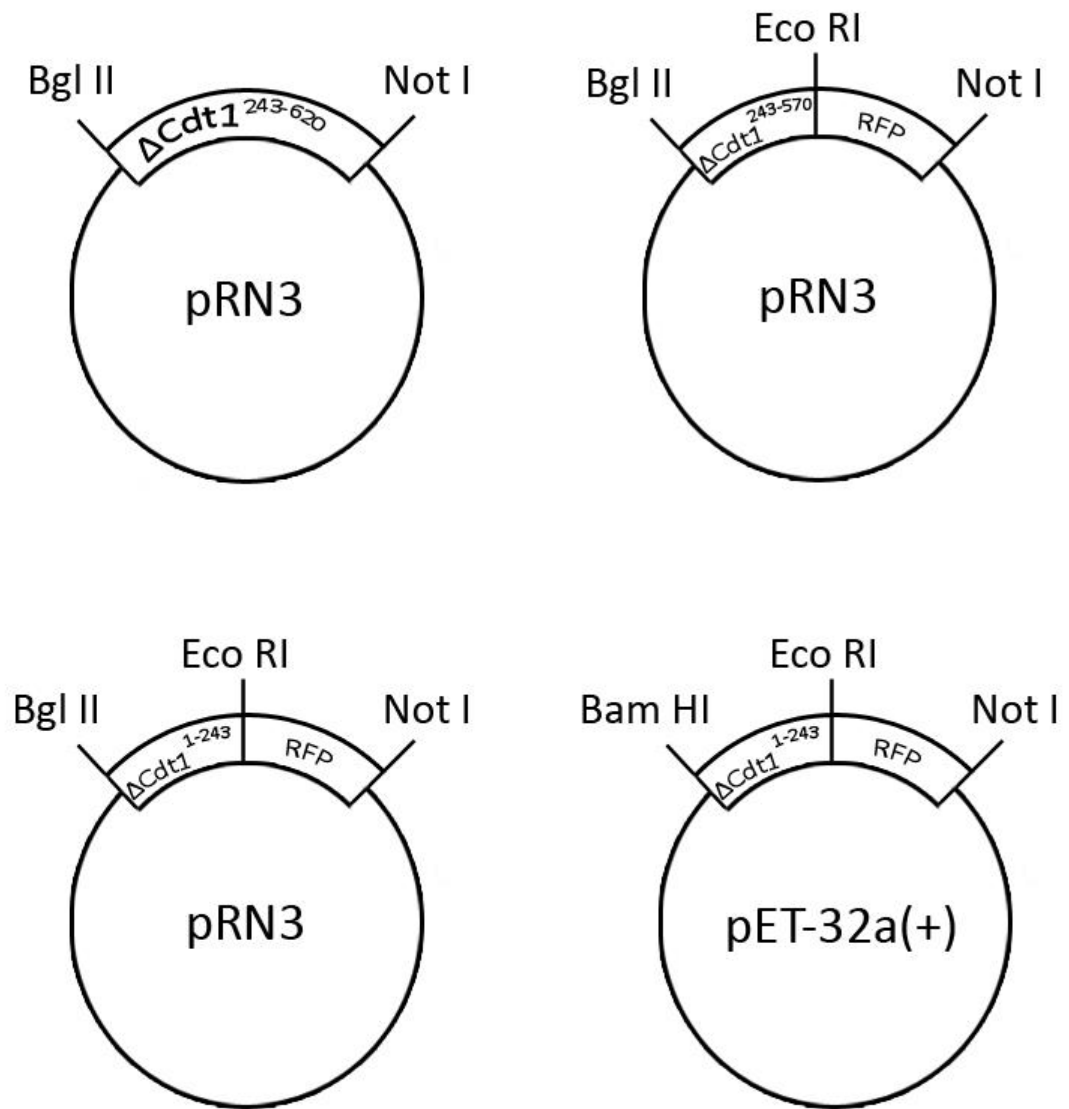
Figure 2.1. Completed Cdt1 constructs. The Cdt1 constructs that were inserted into the pRN3 plasmid are for mRNA expression. The Cdt1 construct that was inserted into the pET-32a(+) plasmid is for recombinant protein expression.

2.2 In vitro Transcription

The final pRN3 mRNA expression vector containing the verified construct DNA was linearised using the restriction enzyme Kpn I as this cuts the vector downstream of the construct DNA sequence and leaves the construct DNA within the vector intact. The linearised construct DNA was then purified using the Qiagen QIAquick PCR Purification Kit according to manufacturer's instructions. Ethanol precipitation was then used to ensure any salt carry over from the kit buffers were removed and to further purify the DNA as cleaner template DNA preparations produce greater yields of mRNA. For ethanol precipitation, a one-tenth volume of sodium acetate and three volumes of 100% ethanol were added to the DNA followed by incubation at -80°C for 10 minutes. The DNA was then centrifuged at 14,000 g, 4°C for 20 minutes before re-suspending the pellet in 100 µl of 70% ethanol. The DNA was centrifuged at maximum speed, 4°C for 10 minutes, after which the supernatant was discarded and the purified DNA pellet left to air dry before re-suspension in 6 µl DEPC-treated water. The linearised and purified DNA was then used as a template for synthesis of mRNA using the T3 mMESSAGE mMACHINE Kit according to manufacturer's instructions (Ambion, Austin, TX).

2.3 Protein Expression and Purification

The appropriate DNA sequences were sub-cloned from the pGEM-T vector into the pET-32a(+) expression vector, which also includes an N and C terminal His-Tag, as above. The correctly sequenced pET-32a(+) constructs were transformed into expression bacteria, the *E.coli* strain Tuner, and grown on ampicillin-containing (100 µg/ml) agar plates overnight at 37°C. Colonies were then removed from the plate and cultured small scale, overnight at 37°C in 10 ml 2xyt media containing ampicillin (100 µg/ml). The small scale cultures were used to inoculate 1-3 litres of 2xyt media + 100 µg/ml ampicillin. The large scale cultures were grown to OD_{600} 1-1.2 at 37°C before inducing with 0.2 mM IPTG overnight at 16°C, 150 rpm. The cultures were centrifuged at 8000 rpm for 8 minutes at 4°, the supernatant discarded and the bacterial pellets stored at -80°C. To purify the recombinant protein from the bacterial cells the Novagen His-bind purification kit was used according to manufacturer's instructions with minor modifications. The bacterial pellets were re-suspended in x1 binding buffer supplemented with protease inhibitors (2 µg/ml aprotinin, 2 µg/ml pepstatin A, 2

μg/ml leupeptin and 100 μM benzamidine) and 0.05% detergent (Triton-X100). Lysozyme (100 μg/ml) was then added to the resuspension before placing on a roller mixer for 15 minutes at room temperature. The bacterial cells were then lysed using pulsed sonication on ice. DNase (5 μg/ml) and RNase (10 μg/ml) was then added to the cell lysate before placing on a roller mixer for 15-30 minutes at room temperature. The cell lysate was then centrifuged at 10,000 rpm, 4°C for 40 minutes. The recombinant protein was purified from the supernatant by his-bind resin column chromatography using the Novagen His-bind purification kit according to manufacturer's instructions. The purified protein was eluted from the his-bind resin column in x1 elution buffer collected in 1 ml fractions.

To determine which fractions contained higher purity and recombinant protein levels 5 μl samples were collected, added to 15 μl Laemmli buffer (final concentrations of Laemmli buffer 3% SDS, 7.5% β-mercaptoethanol, 10.5% glycerol, 0.12 M TRIS pH 6.8 and 0.003% bromophenol blue), boiled for 10 minutes at 96°C and centrifuged at maximum speed for 1 minute before running on an SDS-PAGE gel (4-12% BIS-TRIS gradient gel, Invitrogen) at 180 V for 1 hour. The gel was then stained using coomassie blue stain (0.25% coomassie brilliant blue, 10% acetic acid, 45% methanol, 45% deionised water) and destained (5% acetic acid, 45% methanol, 50% deionised water) to reveal the protein bands. Fractions containing appropriate levels of purity and recombinant protein were then combined and the elution buffer exchanged for x0.5 PBS using GE Healthcare PD-10 desalting columns according to manufacturer's instructions. The PD column was equilibrated using x0.5 PBS. The recombinant protein was then concentrated using vivaspin 6 ultrafiltration spin columns with a molecular weight cut off of either 10,000 or 35,000 Daltons (Sartorius Stedim Biotech). The concentration of the recombinant protein was determined using the Thermo Scientific Pierce BCA Protein Assay Kit according to manufacturer's instructions.

2.4 Xenopus laevis

To induce egg laying, female Xenopus laevis frogs were injected subcutaneously with 150 units of PMSG to promote oocyte maturation before subcutaneous injection of 500 units of hCG 3 days later. Once injected with hCG the frogs were placed in individual tanks containing 1x MMR (1 M NaCl, 50 mM HEPES, 20 mM KCl, 20 mM CaCl$_2$, 10 mM MgCl$_2$, and 1 mM EDTA pH 7.8 in dH$_2$O) for laying. Both the frogs and the

eggs should be kept at 23°C to preserve egg quality. Once collected the eggs can be fertilised to produce embryos or instead used to produce *Xenopus* egg extract.

2.4.1   *Xenopus* Embryos

To fertilise the eggs, one testis from the male *Xenopus laevis* was macerated in 1x MMR to maintain in an inactive state. To fertilise, the inactive sperm was spread around a petri dish of eggs before being activated by 2 ml 0.1x MMR followed 5 minutes later by flooding with 0.1x MMR. The sperm is activated due to osmotic shock triggered by the reduction in salt content of the 0.1x MMR buffer which mimics the low salinity of the pond water environment to trigger sperm motility (Tholl *et al.*, 2011). This method of fertilisation allows all of the eggs to be fertilised at the same time point therefore producing a batch of synchronously dividing embryos. Figure 2.1 shows a batch of synchronously dividing *Xenopus* embryos in which every embryo in the batch divides at approximately the same time. After incubation at 23°C for 20 minutes the protective vitelline coat of the embryos was removed using 2% cystine (pH 7.8) for 5-8 minutes. The embryos were then washed with- and preserved in- 0.1x MMR. Embryo development was monitored using a Leica M205 FA macro imaging system set to take one image every 5 minutes.

Embryo samples taken for Western blot analysis were devoid of buffer and fixed using liquid nitrogen or dry ice before storage at -80°C. For immunofluorescence analysis embryos were fixed in 2 ml fix solution (10x MEMFA and 37% formaldehyde in ddH$_2$O) on a roller for 45 minutes before addition of 2 ml methanol. After a further 15 minutes on the roller the embryo sample was placed in 2 ml methanol for 5 minutes before storing in 2 ml of fresh methanol at -20°C.

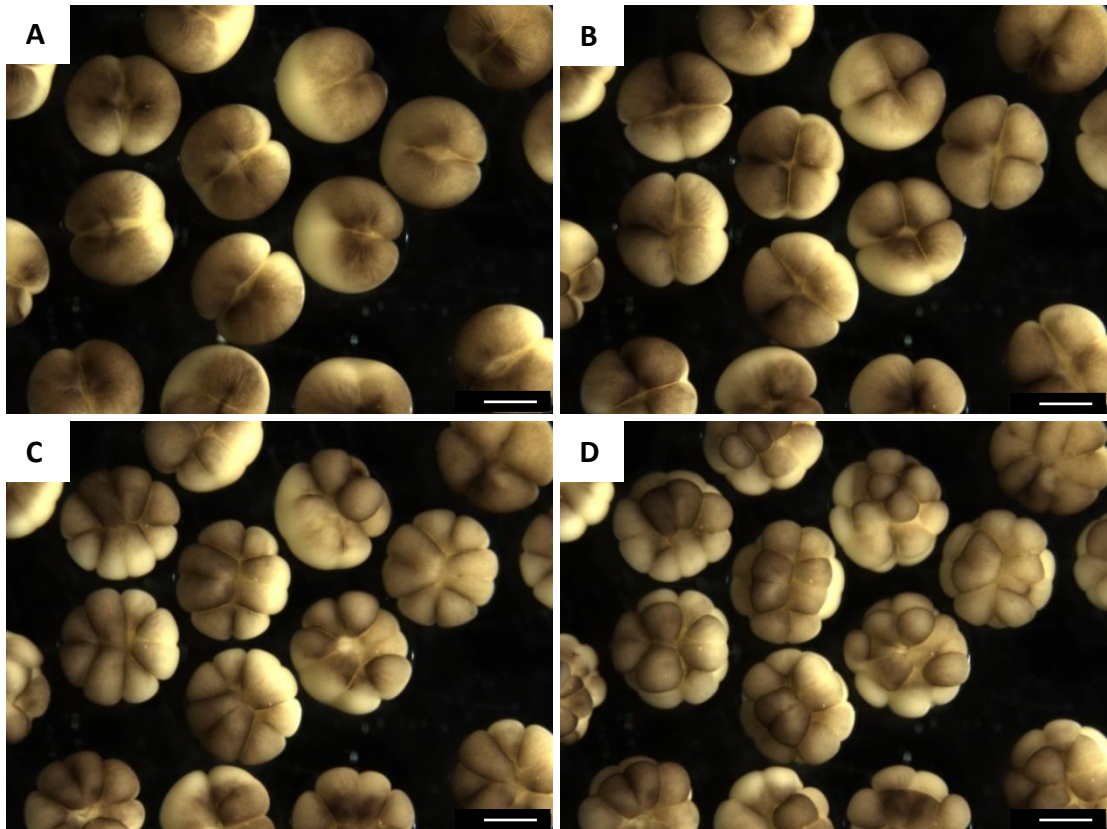**Figure 2.2. Synchronously dividing *Xenopus laevis* embryos.** All embryos in the batch were fertilised and divide at the same time. The scale bar is 500μm. **(A)** First embryonic cell division (developmental stage 2). **(B)** Second embryonic cell division (developmental stage 3). **(C)** Third embryonic cell division (developmental stage 4). **(D)** Fourth embryonic cell division into a 16 cell embryo (developmental stage 5).

2.4.2   *Xenopus* Egg Extract Production

The eggs of *Xenopus laevis* are arrested at metaphase of meiosis II. Upon fertilisation a calcium wave releases the egg from the metaphase arrest. By mimicking this calcium wave, the presence of exogenous calcium can activate the unfertilised eggs and egg extract, releasing them from metaphase arrest into interphase of the first mitotic cell cycle. The presence of the calcium chelator EGTA during production of the egg extract allows the metaphase arrest to be maintained and the resultant extract is therefore metaphase arrested *Xenopus* egg extract.

For production of the extract only the highest quality eggs were used. High quality eggs were those identified as having a clear distinction between the dark animal pole and light vegetal pole with a clean vitelline coat. The high quality eggs were pooled and washed with 1x MMR before removal of the vitelline coat in de-jellying solution (2% cysteine, 1 mM EGTA) for 5-10 minutes. Once the coat was completely removed the eggs were washed with XBE2 [1x αβ salts (100 mM KCl, 2 mM Mg_2Cl, 0.1 mM $CaCl_2$), 10 mM hepes KOH, 5 mM EGTA, 1.71% w/v sucrose]. Damaged or activated eggs, which appear white to grey in colour, were then removed and the remaining eggs washed with XBE2 containing 10 µg/ml protease inhibitors leupeptin, pepstatin A and aprotinin. The eggs were then centrifuged at 3000 rpm, 16°C for 1 minute in 1 ml of XBE2 buffer containing 10 µg/ml leupeptin, pepstatin A, aprotinin and 100 µg/ml cytochalasin D to pack. Once the eggs were packed down, excess buffer and any activated eggs were removed before centrifuging at 10,000 rpm, 16°C for 10 minutes. The middle layer of extract containing the cytoplasmic fraction was removed before addition of 10 µg/ml leupeptin, pepstatin A, aprotinin and cytochalasin D along with a one-twentieth volume of ER (energy regenerator, which consists of 25 mM phosphocreatine and 15 µg/ml creatine phosphokinase) and 15% LFB1/50 (50 mM KCl, 40 mM hepes KOH, 20 mM potassium phosphate, 2 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 10% w/v sucrose and 10 µg/ml leupeptin, pepstatin and aprotinin). The extract was then centrifuged at 30,000 rpm, 4°C for 17 minutes. Next the golden cytoplasmic layer was removed ensuring that the membrane layer below was not disturbed as this contains the mitochondria which lyse after freeze-thawing to promote apoptosis within the extract making it un-useable. Glycerol (2%) was then added to the extract for cryoprotection before snap freezing 20 µl drops in liquid nitrogen to form beads of

20 μl aliquots which were stored at -80°C. A schematic of *Xenopus* egg extract production is shown in Figure 2.3. This method of producing egg extract was developed in Professor Julian Blow's laboratory at the University of Dundee and was used in order to keep extracts consistent between laboratories (Gillespie *et al.*, 2012).
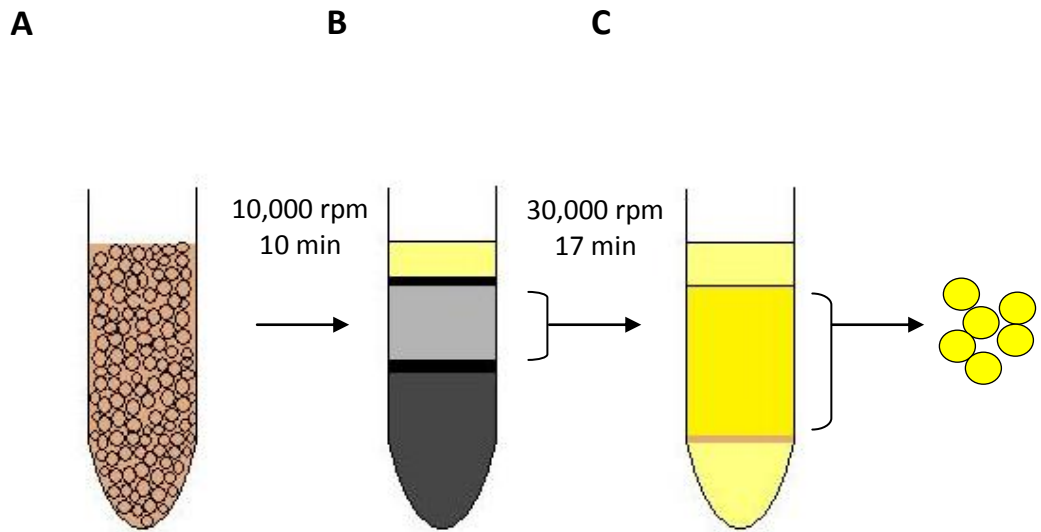
Figure 2.3. Production of *Xenopus* egg extract. (A) High quality *Xenopus* eggs were collected and centrifuged at 10,000 rpm for 10 minutes. **(B)** The grey fraction of crude cytoplasm was collected and centrifuged at 30,000 rpm for 17 minutes. **(C)** The purified cytoplasmic fraction was collected and frozen in 20 µl beads for storage at -80°C.

To ensure that the egg extract is metaphase arrested and competent for DNA replication, the ability of the extract to undergo nuclear assembly was confirmed (Figure 2.3). To check nuclear assembly, a sample of extract was supplemented with 10 ng/µl demembranated sperm DNA and a one-fortieth volume of ER (final concentrations of 25mM phosphocreatine and 15µg/ml creatine phosphokinase in the extract) and cycloheximide (final concentration on 250 µg/ml in the extract) before activation with 0.3 mM $CaCl_2$. At 10 minutes, 60 minutes and 120 minutes post-activation 1 µl extract samples were placed on microscope slides with 1 µl of extract fix solution (67% glycerol, 10% 10x MMR, 10% formaldehyde and 0.001% Hoechst) The Hoechst in the fix solution stained the DNA allowing the structure of the DNA to be observed using UV microscopy.

At 10 minutes after activation with $CaCl_2$ the sperm DNA appears linear and has begun to decondense. By 60 minutes the decondensed sperm DNA has completed nuclear assembly forming approximately circular nuclei. At 120 minutes DNA licensing and replication is complete and the sperm DNA is condensed (Figure 2.4).

10 min	60 min	120 min
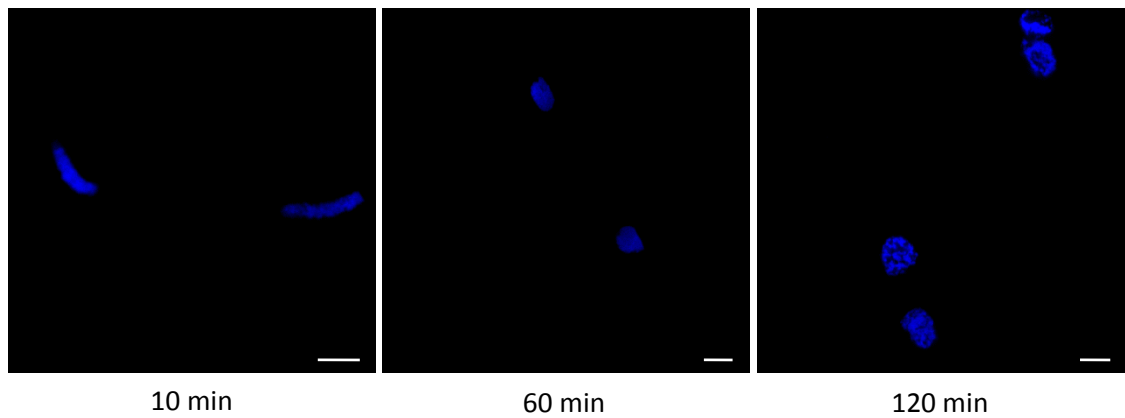
Figure 2.4. Nuclear assembly in *Xenopus* egg extract. The extract was supplemented with 10 ng/μl demembranated sperm DNA and released from metaphase arrest by addition of 0.3 mM $CaCl_2$. At 10, 60 and 120 minutes post-calcium addition 1 μl samples were fixed and the DNA stained with Hoechst before imaging using UV microscopy. Scale bar is 10 μM.

2.4.3 Use of *Xenopus* Egg Extract

Aliquots of *Xenopus* egg extract were thawed at room temperature. To maintain ATP levels and prevent progression into mitosis the extract was supplemented with one-fortieth volume of ER (final concentrations of 25mM phosphocreatine and 15μg/ml creatine phosphokinase in the extract) and cycloheximide (final concentration on 250 μg/ml in the extract) respectively. The extract was then released from the metaphase arrest into interphase by addition of 0.3 mM $CaCl_2$ and DNA added in the form of demembranated sperm nuclei at 20 ng/μl unless otherwise stated. The extract was incubated at 23°C until each experimental time point which was measured as the time elapsed following activation. Depending on the rationale of the experiment, the extract was optionally supplemented with inhibitors or recombinant protein constructs which are listed in Table 2.2.

Component	Description	Concentration
p27	Cyclin-dependant kinase inhibitor	100 nM
D-Box peptide	Competitive APC/C inhibitor	2 mM
ΔCdt1$^{1\text{-}243}$-RFP	Truncated recombinant Cdt1 consisting of the N-terminal 1-243 amino acids of Cdt1 with an RFP fluorescent tag	19 ng/µl
GemH-RFP	Non-degradable form of geminin with an RFP fluorescent tag	0.38 µg/µl

Table 2.2. Inhibitors and recombinant proteins used to optionally supplement *Xenopus* egg extract. Each component, which was optionally added to the *Xenopus* egg extract depending on the rational of the experiment, is listed with a description and the final working concentration used unless otherwise stated.

2.5 Chromatin Isolation

2.5.1 Isolation of Chromatin from *Xenopus laevis* Embryos

Healthy synchronously dividing embryos were collected at selected time points during the cell division cycle. A 0.5-1 ml volume of embryos was packed by gentle centrifugation for 2 seconds using a bench top centrifuge and devoid of buffer before fixing in liquid nitrogen and storage at -80°C. To isolate the chromatin the embryo samples were thawed and centrifuged at maximum speed, 4°C for 10 minutes. After the centrifuge the layer containing the chromatin was collected and supplemented with 10 µg/ml of the protease inhibitors pepstatin A, leupeptin and aprotinin and ten volumes of buffer A (10 mM HEPES pH 7.6, 15 mM KCl, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 0.5 mM DTT, 0.2% Triton-X100 and 10 µg/ml each of pepstatin A, leupeptin and aprotinin). The nuclear extract was then mixed and centrifuged at 500 g, 4°C for 20 minutes. The supernatant was discarded before addition of 10 µg/ml pepstatin A, leupeptin and aprotinin to the pellet and re-suspension in 10 ml buffer A. The re-suspended pellet was then layered over 4 ml of buffer A containing 15% glycerol and centrifuged at 500 g, 4°C for 20 minutes. The supernatant was discarded before a final centrifugation at maximum speed, 4°C for 1 minute. The remaining supernatant was removed and the pellet re-suspended in Laemmli buffer (10% SDS, 25% β-mercaptoethanol, 35% glycerol, 0.4 M TRIS pH 6.8 and 0.01% bromophenol blue) before boiling at 96°C for 10 minutes and centrifuging at maximum speed for 1 minute. The chromatin samples were then stored at -20°C.

2.5.2 Isolation of Chromatin from *Xenopus* Egg Extract

Xenopus egg extract was thawed and supplemented with a one-fortieth volume of ER and 250 µg/ml cycloheximide followed by activation with 0.3 mM $CaCl_2$ and incubation for 15 minutes at 23°C. Following incubation there was optional supplementation with 100 nmol p27 or $\Delta Cdt1^{1-243}$-RFP before addition of 20 ng/µl DNA. The extract was then incubated at 23°C. At selected time points the DNA was then isolated from 20 µl of extract. To the 20 µl extract samples 500 µl of NIB (50 mM KCl, 50 mM HEPES pH 7.6, 5 mM $MgCl_2$, 2 mM DTT, 0.5 mM Spermidine, 0.15 mM Spermine, 0.1% Triton-X100, and 1 µg/ml each of Leupeptin, Pepstatin A and Aprotinin) was added followed by addition of a 100 µl cushion of NIB containing 20% w/v sucrose to the bottom of the eppindorf tube and centrifugation at 5000 g, 4°C for 5 minutes. The supernatant was then

removed leaving approximately 15 µl to cover the chromatin pellet. The pellet was then centrifuged at maximum speed, 4°C for 2 minutes before complete removal of the supernatant. The chromatin pellet was then re-suspended in 10 µl Laemmli buffer (10% SDS, 25% β-mercaptoethanol, 35% glycerol, 0.4 M TRIS pH 6.8 and 0.01% bromophenol blue), boiled at 96°C for 10 minutes, centrifuged at maximum speed for 1 minute and stored at -20°C in preparation for western blot analysis.

2.6 Microinjection

A Narishige PC-10 micropipette puller was used to produce micropipettes for use with the World Precision Instruments Nanoliter 2000 oil-based microinjection system. Oil was loaded into the tip of the micropipette followed by protein or mRNA loading. The protein or mRNA was then injected into the embryos at various developmental stages. Following microinjection the embryos were maintained in 0.1x MMR and development was monitored using either a Motic stereomicroscope or a Leica M205 FA fluorescence macroimaging system.

2.7 Western Blot

2.7.1 Sample Preparation

Whole embryo samples stored at -80°C were thawed on ice to prevent protein degradation and homogenised in 12 µl of extraction buffer (20 mM EGTA, 20 mM HEPES pH 7.5, 15 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF and 3 µg/ml of the protease inhibitors leupeptin, pepstatin A and aprotinin). The extraction buffer was optionally supplemented with the deubiquitylase inhibitor NEM (20 mM) when the preservation of any ubiquitination was required. The samples were then centrifuged for 10 minutes at maximum speed, 4°C and the supernatant collected and added to 8 µl Laemmli buffer (final concentrations in Laemmli buffer are 4% SDS, 10% β-mercaptoethanol, 14% glycerol 0.16 M TRIS pH 6.8 and 0.004% bromophenol blue). The samples were boiled for 5-10 minutes at 96°C and centrifuged for 1 minute at maximum speed. Prepared samples in Laemmli buffer were then either used immediately or stored at -20°C.

Xenopus egg extract samples were fixed in Laemmli buffer at a ratio of 0.5-1 µl of extract per 10 µl of Laemmli buffer (final concentrations in Laemmli buffer are 9-9.5% SDS, 22.5-23.75% β-mercaptoethanol, 31.5-33.25% glycerol, 0.36-0.38 M TRIS pH 6.8

and 0.009-0.0095% bromophenol blue). The samples were then boiled for 5-10 minutes at 96°C and centrifuged for 1 minute at maximum speed before storage at -20°C.

2.7.2 SDS-PAGE and Western Blot

Samples stored at -20°C were thawed, boiled for 5-10 minutes at 96°C and centrifuged for 1 minute at maximum speed before gel loading. Prepared samples were run on a 4-12% BIS-TRIS gradient gel (Invirogen) at 180 V for approximately 1 hour 5 minutes in 1x MOPS buffer (Invitrogen). The gel was placed in a sandwich with the membrane, filter paper and filter pads and placed in a blotting tank (Biorad) in transfer buffer (25 mM TRIS base, 192 mM glycine and 20% methanol in dH_2O). Blotting was for 3 hours 30 minutes at 50 V onto a hydrophobic polyvinylidene difluoride (PVDF) membrane (Thermo Scientific) which was pre-activated by soaking in 100% methanol for 5 seconds. The membrane was blocked in PBST+5% milk (PBST is x1 PBS and 0.1% Triton-X100 in dH_2O) either overnight at 4°C or for 2 hours at room temperature. The membrane was probed with primary antibody overnight at 4°C followed by treatment with secondary antibody either overnight at 4°C or for 2 hours at room temperature. The primary and secondary antibodies and concentrations used are listed in Table 2.3. After each step the membrane was washed 3 times in PBST. The membrane was then treated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) before development on medical X-ray film (Kodak blue sensitive).

When analysing isolated chromatin samples, prior to protein transfer to the PVDF membrane, the bottom 1 cm of the SDS-PAGE gel was removed and placed in coomassie blue stain. The gel section was then destained to reveal the histone protein bands. For preservation the gel was placed between two deionised water-soaked cellulose sheets (Promega Gel Drying Film), clamped to seal and air dried for 24 hours.

		Antibody	Concentration	Company	Catalogue Number
Primary	**Rabbit**	Cdt1	1:1000	Prof. Julian Blow's Lab	
		Geminin	1:1000	Prof. Julian Blow's Lab	
		Orc1	1:2000	Prof. Julian Blow's Lab	
		Phospho-Ser	1:500	Cell Signaling	#2324S
	Mouse	RFP	1:1000	Abcam	ab65856
		PCNA	1:2000	Cell Signaling	#2586
		Mcm2	1:1000	Prof. Julian Blow's Lab	
		β-actin	1:500	Sigma	A2228-200UL
		His-tag	1:1000	Abcam	ab18184
		Phospho-Thr	1:1000	Cell Signaling	#93915
Secondary		Anti-mouse HRP conjugated	1:35000	Sigma	A5278-1ML
		Anti-rabbit HRP conjugated	1:35000	Sigma	A8275-1ML

Table 2.3. List of antibodies used for western blot analysis. All primary antibodies were diluted in 3% BSA with 0.02% sodium azide, stored at 4°C and re-used. All secondary antibodies were diluted in PBST buffer with 5% milk and used once. The working concentrations of each antibody are listed along with the company and catalogue number for each antibody. Antibodies obtained from Professor Julian Blow's lab were a kind gift and are not commercially available.

2.7.3 Image J Analysis

The developed films were scanned in greyscale using a HP Scanjet G3010 scanner. To analyse the densities of the bands on the blots Image J software was used. To select the first band on the blot the rectangular tool was used and through the analyse menu, gels and first lane were selected. After outlining all the bands with the rectangular tool, graphs of the densities within each rectangle were produced using the 'plot lane' function. Due to background signal on the blots the peaks of the graphs did not touch the baseline. The bottom of the peaks for each band was therefore closed using the straight line tool to allow the areas of the peaks to be selected using the wand tool. Once the areas of each peak were highlighted the 'label peaks' function was used to express each peak as a percentage of the total area of all the peaks combined. The area and percentage data for each band on the blot was then transferred to an excel spread sheet. The percentage data was used to calculate the relative densities for each of the bands on the blot. The relative densities were calculated relative to the band corresponding to time point zero of the experiment, unless otherwise stated.

2.7.4 Statistical Analysis

The relative density values calculated from the Image J data were analysed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego California, USA). The relative densities were entered into the Prism data table and plotted on an XY graph as mean ±SEM. The linear regression line of each data set was then calculated and plotted on the graph. The linear regression line was calculated using the 'least squares' method in which the sum of the squares of the distance vertically from the line of each point on the graph was minimised.

To determine whether or not there was a significant difference between protein degradation rates under different conditions the slopes of the linear regression lines for each condition were compared. The test, which is equivalent to an analysis of covariance (ANCOVA), was performed to test the null hypothesis that the slopes of the linear regression lines were identical (Zar, 1999). A P value of less than 0.05 indicated that the slopes of the lines were significantly different and thus there was a significant difference between the rates of protein degradation between the two conditions. In addition the slopes of the linear regression lines were also tested against the null hypothesis that the slope was equal to zero. A P value of less than 0.05 indicated that

the slopes of the lines significantly deviated from zero. A slope that does not significantly deviate from zero shows that there is no rate of change, the level stays constant and therefore there is no change in the protein level and thus no protein degradation within the sample.

2.8 Immunofluorescence

Embryo samples were placed in 2ml bleaching solution (10% hydrogen peroxide and 67% methanol) on a white light transluminator for 2 hours followed by rehydration in 1x PBS to limit background fluorescence. The embryos were then washed in TBS (155 mM NaCl, 10 mM Tris-Cl pH 7.4 and 0.1% Triton-X100) before blocking in TBS+5% milk for 2 hours at room temperature. The embryos were again washed in TBS before treatment with primary antibody overnight at 4°C. The embryo samples were then washed in TBS before treatment with secondary antibody in overnight at 4°C on the shaker. The primary and secondary antibodies and concentrations used are listed in Table 2.4. The embryos were then washed and stored in TBS. Once the embryo samples were placed on microscope slides Vectashield mounting medium for fluorescence with DAPI was added before placing the cover slip. The embryo slides were stored at 4°C before imaging using a Zeiss confocal/2-photon (model LSM 510) with numerical aperture 1.4, and 63x oil immersion objective.

		Antibody	Concentration	Company	Catalogue Number
Primary	**Rabbit**	Cdt1	1:50	Prof. Julian Blow's Lab	
		Rad 51	1:100	Abcam	ab31769-100
		p53	1:100	Abcam	ab38497-100
		IgG	1:100	Sigma	I5006-10mg
	Mouse	Mcm2	1:200	Prof. Julian Blow's Lab	
		RFP	1:200	Abcam	ab65856
Secondary		Alexa fluor 488 goat-anti-mouse	1:1000	Invitrogen	A31619
		Alexa fluor 555 goat-anti-rabbit	1:1000	Invitrogen	A31629

Table 2.4. List of antibodies used for immunofluorescence analysis. All primary and secondary antibodies were diluted in TBS buffer containing 3% BSA. The working concentrations of each antibody are listed along with the company and catalogue number for each antibody. Antibodies obtained from Professor Julian Blow's lab were a kind gift and are not commercially available.

2.9 Immunoprecipitation

Protein A sepharose beads were swollen in 1x PBS at 4°C. Once the beads were swollen 0.02% sodium azide was added to prevent bacterial contamination. To couple RFP antibody (Abcam, ab65856) to the protein A beads a one-tenth volume of antibody was added to the beads and made up to a 50% slurry with IP buffer (20 mM TRIS pH 8, 50 mM NaCl, 10 mM EGTA pH 8, 100 µM sodium fluoride, 100 µM sodium orthovanadate, 0.5 mM PMSF, 0.5% sodium deoxycholate, 1% Triton-X100 and 10 µg/ml each of leupeptin, pepstatin A and aprotinin). The antibody and bead slurry was then placed on a roller mixer for one hour at room temperature. The beads were then centrifuged for 1 minute at 800 rpm, 4°C before removal of the antibody solution and addition of 1 ml 0.2 M sodium borate pH 9. The beads were again centrifuged at 800 rpm, 4°C for 1 minute and the sodium borate treatment was repeated. After centrifuging at 800 rpm, 4°C for 1 minute the sodium borate was removed and 1 ml 0.2 M sodium borate containing 20 mM DMP (dimethylpimelimidate, an imidoester crosslinker) added before incubation at room temperature for 30 minutes on a roller mixer. The beads were then centrifuged at 800 rpm, 4°C for 1 minute before removal of the supernatant and addition of 1 ml 0.2 M ethanolamine pH 8. The beads were again centrifuged at 800 rpm, 4°C for 1 minute before removal of the supernatant. The beads were then incubated in 1 ml 0.2 M ethanolamine for 2 hours at room temperature on a roller mixer. Finally, the beads were washed by centrifugation at 800 rpm, 4°C for 1 minute followed by removal of the supernatant and addition of 1x PBS repeated twice. The RFP antibody was now cross-linked to the beads.

The antibody coupled beads were then separated into 20 µl aliquots and excess PBS removed. To each aliquot of beads 500 µl IP buffer was added before centrifuging at 800 rpm, 4°C for 1 minute. Excess IP buffer was then removed and 20µl extract samples were added with 150 µl IP buffer and incubated at 4°C overnight on a roller mixer. The beads were washed by centrifugation at 800 rpm, 4°C for 1 minute followed by removal of the supernatant and addition of 500 µl IP buffer. This wash step was then repeated three times and after the third wash step the supernatant was removed. The beads were then washed in high salt by addition of 500 µl IP buffer containing 200 mM NaCl, centrifugation at 800 rpm, 4°C for 1 minute and removal of the supernatant. The high salt wash step was then repeated three times and after the

third wash the supernatant was removed. The beads were then washed twice in 500 µl IP buffer, centrifuged at 800 rpm, 4°C for 1 minute followed by removal of the supernatant. Finally the beads were washed in 1x PBS twice before centrifugation at 800 rpm, 4°C for 1 minute followed by removal of the supernatant. To release the bound protein from the antibody beads and prepare the samples for western blot analysis, 15 µl of Laemmli buffer (10% SDS, 25% β-mercaptoethanol, 35% glycerol, 0.4 M TRIS pH 6.8 and 0.01% bromophenol blue) was added to each aliquot of beads before boiling at 96°C for 10 minutes and centrifuging at maximum speed for 1 minute. The supernatant was run on an SDS-PAGE gel and blotted onto hydrophobic polyvinylidene difluoride nitrocellulose membrane for western blot analysis as described in section 2.7.

2.10 Chemicals and Reagents

All chemicals were from Sigma-Aldrich or Melford Laboratories Ltd unless otherwise stated. Restriction enzymes, vectors and DNA ladders were from Promega unless otherwise stated. All the reagents used were of the highest quality available.

Chapter 3.

Results I. Cdt1 Construct Production

3.1 Introduction

In every organism it is imperative that the DNA is replicated accurately during each cell division in order to maintain genomic integrity and prevent potentially cancerous alterations to the genome. In the cells of the Metazoa the genome is large and consequently DNA replication is enlisted from multiple sites, known as the origins of replication. It is therefore essential that DNA replication is tightly coordinated and controlled to ensure complete and accurate replication of the entire genome only once per cell cycle. This tight control is achieved by the process of DNA licensing which primes the replication origins for DNA replication during the coming S-phase (Blow and Dutta, 2005).

Faithful DNA replication is in part ensured by regulation of the DNA licensing pathway. DNA licensing is sanctioned only during late M-phase to early S-phase thereby preventing re-licensing of replicated DNA and subsequent re-replication (Truong and Wu, 2011). Regulation of the DNA licensing protein Cdt1 is crucial in ensuring that DNA licensing and therefore replication occur only once in a single cell cycle. It has been shown that addition of recombinant Cdt1 to G2 nuclei in *Xenopus* egg extract causes re-licensing and subsequent re-replication of the DNA highlighting the importance of correct Cdt1 regulation (Maiorano *et al.*, 2005).

In Metazoans there are two mechanisms which regulate the activity of Cdt1 to prevent DNA re-replication: proteolysis and inhibition by the natural Cdt1 inhibitor protein geminin (Li and Blow, 2005). Geminin is active during S-phase, G2 and M-phase and degraded prior to the onset of G1 and therefore DNA licensing (McGarry and Kirschner, 1998). However a significant level of geminin escapes degradation during M-phase (Hodgson *et al.*, 2002) and it has been shown that a Cdt1-geminin complex is both capable of licensing the DNA and preventing DNA re-replication (Lutzmann *et al.*, 2006). This suggests that rather than simply blocking the activity of Cdt1, geminin may form a complex with Cdt1 and act as a molecular switch with the stoichiometry of the complex changing to allow a shift between licensing –active and –inactive states (Lutzmann *et al.*, 2006).

Degradation of Cdt1 is also a crucial regulatory mechanism in ensuring the restriction of DNA licensing to once per cell cycle. There are several distinct pathways for Cdt1

degradation involving different E3 ubiquitin ligases. In *Xenopus*, *Drosophila* and mammalian cell lines Cdt1 is ubiquitinated and targeted for degradation by the Cul4-DDB1 ubiquitin ligase complex in a replication-dependant manner requiring PCNA as a co-factor (Arias and Walter, 2005a; Senga *et al.*, 2006; Lee *et al.*, 2010). In human cells a further E3 ubiquitin ligase complex, SCF^{Skp2}, acts redundantly with the Cul4-DDB1 pathway to polyubiqutinate Cdt1 and target the protein for proteasome mediated degradation (Li *et al.*, 2003; Takeda *et al.*, 2005). In addition a further ubiquitin ligase, the APC/C, also plays a role in ubiquitin mediated proteolysis of Cdt1 in *Xenopus* egg extract (Li and Blow, 2005) and mammalian cells (Sugimoto *et al.*, 2008).

The early cleavage cell cycles of the *Xenopus* embryo differ considerably from the cell cycle in somatic cells. During the first 12 embryonic cleavages the cell divisions are rapid, synchronous, and occur in the absence of growth and transcription. The embryo instead relies on a maternally derived store of mRNA and proteins. The cleavage cell cycles also lack the distinct gap phases, G1 and G2, which are present in the somatic cell cycle. This pattern of cell division occurs until the onset of the Mid-Blastula Transition (MBT) which is triggered after cleavage cycle 12. The post-MBT cell cycles are asynchronous, contain gap phases and are transcriptionally active, closer resembling the somatic cell cycle (O'Farrell *et al.*, 2004).

The *Xenopus* embryo provides a unique opportunity to investigate the regulation of Cdt1 and effects of mis-regulation of Cdt1 not only on an individual cell cycle but also the consequences for embryo development. The embryos can be easily manipulated and protein function can be uniquely studied with the possibility to inject recombinant proteins or mRNA which can be efficiently translated by the developing embryo (Gurdon *et al.*, 1974). Combined with the abundance of published data on the structure of Cdt1 it is therefore possible to design mutated constructs of Cdt1 for expression in *Xenopus* embryos allowing Cdt1 structure and function to be investigated during the embryonic cell cycle *in vivo*.

3.2 Results

The organisation of the functional domains within the Cdt1 protein has been extensively investigated. It has been shown that a *Xenopus* Cdt1 construct consisting of amino acids 243 to 620 is the smallest section of Cdt1 capable of licensing DNA whilst

also possessing partial resistance to geminin. The N-terminal region of *Xenopus* Cdt1 shows weak geminin binding activity while the central region of the protein between amino acids 193-447 contains the main geminin-binding region. The 173 amino acids at the extreme C-terminal of the *Xenopus* Cdt1 protein binds to a complex of the Mcm subunits 2, 4, 6 and 7 and is therefore essential for the licensing activity of Cdt1 (Ferenbach *et al.*, 2005). A schematic representation of Cdt1 is shown in Figure 3.1A.

The N-terminal region of Cdt1 contains multiple sites which are crucial for regulation. Firstly a conserved PIP box, which is essential for interaction with PCNA and therefore PCNA-dependant degradation, is located within the first 21 amino acids of Cdt1 (Arias and Walter, 2005a; Senga *et al.*, 2006). Within the extreme N-terminal 100 amino acids of Cdt1 there is also a Cy motif which is essential for cyclin/Cdk dependant phosphorylation of Cdt1 required for SCF^{Skp2} dependant proteolysis (Li *et al.*, 2003; Liu *et al.*, 2004; Nishitani *et al.*, 2004; Nishitani *et al.*, 2006). Finally there are also several destruction boxes (D-box) located within the N-terminal which are recognised by the APC/C for ubiquitin mediated proteolysis of Cdt1 (Sugimoto *et al.*, 2008).

Taking into account the previously published Cdt1 structure and function data, three truncated Cdt1 constructs were designed. The ΔCdt1$^{243-620}$ construct, which is proficient at DNA licensing but partially resistant to geminin (Ferenbach *et al.*, 2005), lacks the N-terminal 242 amino acids important for Cdt1 regulation. This stabilised Cdt1 construct was designed to examine the effect of mis-regulation of Cdt1 on *Xenopus* embryonic development. The ΔCdt1$^{243-570}$-RFP construct, missing some of the C-terminal amino acids required for MCM binding and therefore DNA licensing (Ferenbach *et al.*, 2005), was designed as a licensing null control for the ΔCdt1$^{243-620}$ construct. Finally the ΔCdt1$^{1-243}$-RFP construct consists only of the N-terminal 243 amino acids and therefore lacks licensing activity and only weakly binds geminin (Ferenbach *et al.*, 2005). This construct was designed to specifically investigate the regulation of Cdt1. Schematic representations of each Cdt1 construct are shown in Figure 3.1.
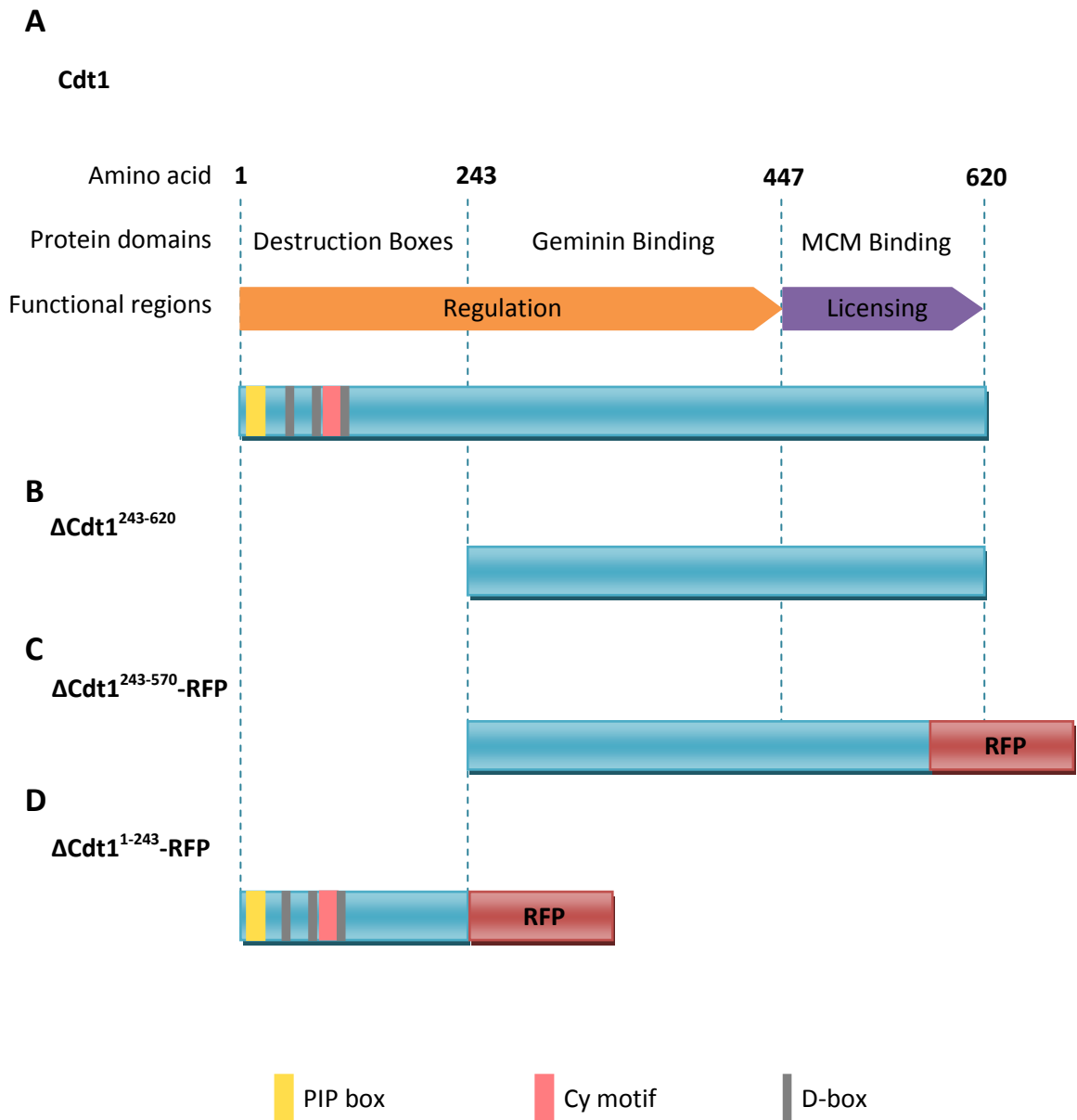
Figure 3.1. Cdt1 structure and construct design. (A) Cdt1 has a domain structure. The N-terminal 1-243 amino acids of Cdt1 contain sequences for ubiquitin mediated degradation and a PIP box for PCNA dependant degradation as well as sites for Cdt1 phosphorylation. The central region is essential for geminin binding. The C-terminal contains the MCM binding region which is necessary for the licensing activity of Cdt1. **(B)** Stabilised Cdt1 lacking the N-terminal region which contains sites for degradation of Cdt1. **(C)** Licensing null Cdt1 lacking part of the MCM binding domain (with an RFP tag). **(D)** Cdt1 N-terminal regulatory domain with an RFP tag.

The ΔCdt1$^{243-620}$ construct of Cdt1 was designed to lack the N-terminal 242 amino acids of Cdt1 which contains protein sequences and domains required for regulation of Cdt1. This truncated ΔCdt1$^{243-620}$ protein will therefore be resistant to degradation and partially resistant to inhibition by geminin (Li *et al.*, 2003; Liu *et al.*, 2004; Nishitani *et al.*, 2004; Arias and Walter, 2005a; Ferenbach *et al.*, 2005; Nishitani *et al.*, 2006; Senga *et al.*, 2006; Sugimoto *et al.*, 2008). By microinjection and expression of this ΔCdt1$^{243-620}$ construct in *Xenopus* embryos the effect of mis-regulation of Cdt1 on embryonic cell division and also embryo development can be investigated.

To produce the ΔCdt1$^{243-620}$ construct, PCR was used with primers designed to incorporate only the DNA bases which code for the amino acids 243 to 620 of Cdt1 from a plasmid template containing the DNA sequence for full length Cdt1. The PCR primers were also designed to incorporate restriction enzyme sites at the start and end of the sequence to allow sub-cloning of the Cdt1 fragment between plasmid vectors. An ATG start codon was also added to the 5' –end primer to ensure efficient translation of the mRNA sequence into protein.

The PCR product was run on an agarose gel and the ΔCdt1$^{243-620}$ DNA fragment produced is shown in Figure 3.2A. During the PCR reaction the DNA polymerase generates a single A base overhang at the 3' –end which allows efficient insertion of PCR products into the pGEM-T vector containing a complementary 3' –T overhang. Once the ΔCdt1$^{243-620}$ DNA fragment was successfully inserted into the pGEM-T vector (Figure 3.2B) the fragment could be removed using the restriction enzyme sites which were added during the PCR.

To allow production of mRNA from this ΔCdt1$^{243-620}$ DNA template for microinjection into *Xenopus* embryos, the DNA must first be transferred to a plasmid which is compatible for *in vitro* mRNA transcription. The pRN3 vector was chosen as the final mRNA expression vector as it was produced specifically for synthesis of mRNA to be microinjected into *Xenopus* embryos and as such has been used successfully for this purpose (Lemaire *et al.*, 1995; Kisielewska and Blow, 2012). The ΔCdt1$^{243-620}$ fragment was therefore cut from the pGEM-T vector using the restriction enzymes Bgl II and Not I as these restriction sites are also present in the pRN3 mRNA expression vector. To allow insertion of the ΔCdt1$^{243-620}$ DNA fragment the empty circular pRN3 plasmid was

opened using the Bgl II and Not I restriction enzymes producing ends complementary to the cut $\Delta Cdt1^{243-620}$ DNA fragment.

Once the $\Delta Cdt1^{243-620}$ DNA was inserted into the pRN3 vector (Figure 3.2C) the complete plasmid was sent for DNA sequencing to ensure that the DNA sequence of $\Delta Cdt1^{243-620}$ was in the correct reading frame with no base pair errors. The pRN3 vector containing verified $\Delta Cdt1^{243-620}$ DNA was then used as a template for synthesis of mRNA for microinjection and expression in *Xenopus* embryos.
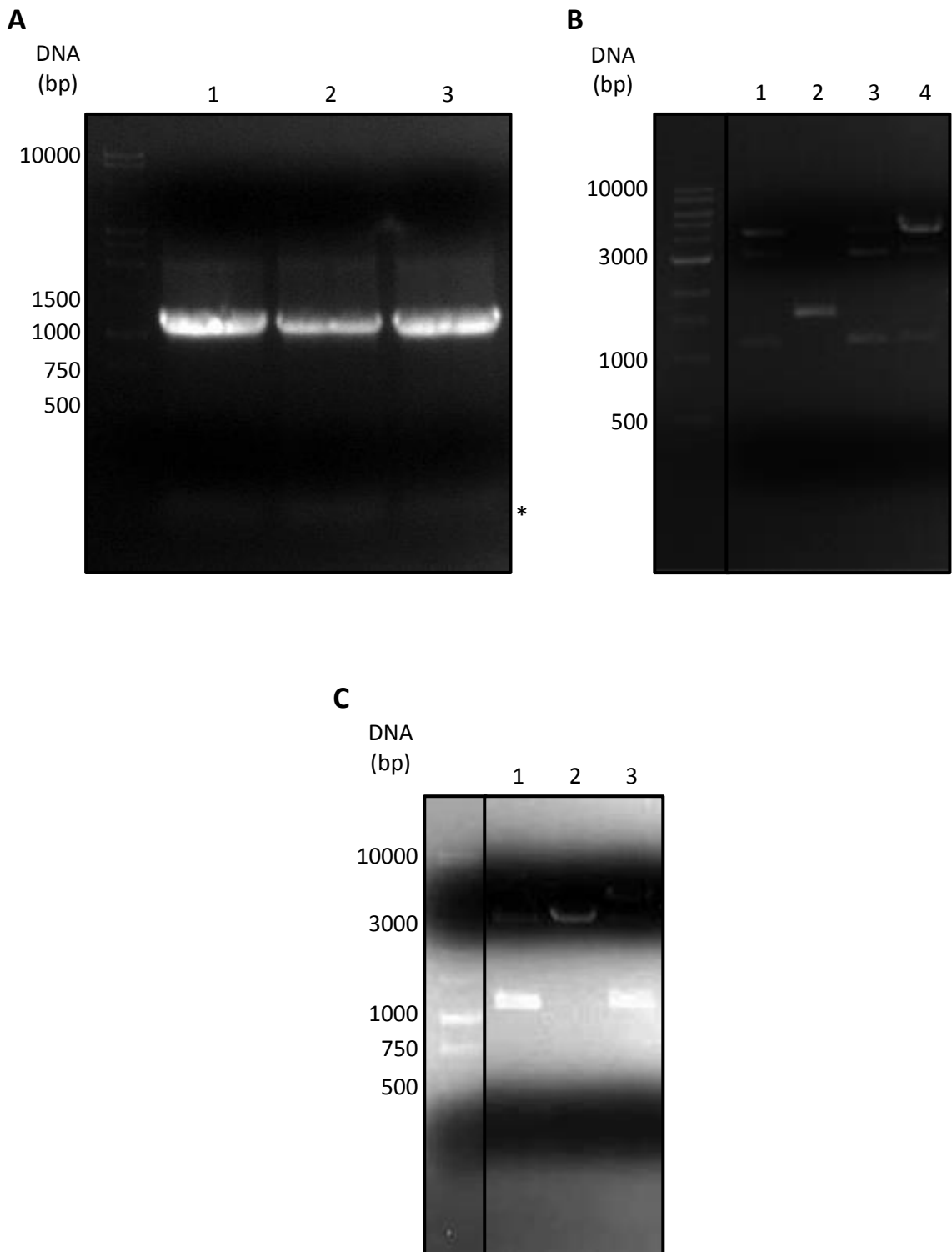
Figure 3.2. PCR of the ΔCdt1$^{243-620}$DNA fragment and sub-cloning into the pRN3 mRNA expression vector. (A) PCR to produce the ΔCdt1$^{243-620}$ DNA fragment. PCR reaction mix was run on an agarose gel. Unused primers are marked by asterisk. **(B)** pGEM-T plasmid containing ΔCdt1$^{243-620}$ DNA was purified from bacteria and digested with Bam HI and Not I before running on an agarose gel. Lanes 1, 3 and 4 show three bands corresponding to ΔCdt1$^{243-620}$ DNA (1.1kb), pGEM-T plasmid (3kb) and uncut pGEM-T containing ΔCdt1$^{243-620}$ DNA (4.1kb). **(C)** pRN3 plasmid containing ΔCdt1$^{243-620}$ DNA was purified from bacteria, digested with Bgl II and Not I and ran on an agarose gel. Lanes 1 and 3 show bands corresponding to ΔCdt1$^{243-620}$ DNA (1.1kb) and pRN3 (3.3kb). DNA ladder is 1kb.

The primary antibody to Cdt1 is polyclonal and therefore some cross-reactive bands are also visible on western blots in addition to the Cdt1 band. To confirm expression of Cdt1 construct mRNA within the *Xenopus* embryos the corresponding Cdt1 protein constructs need to be detectable via western blot analysis. Where the Cdt1 construct is difficult to detect due to non-specific bands a tagged version of the construct would allow simple detection. A fluorescent protein tag would also be useful for detection of the Cdt1 constructs by fluorescence microscopy. Since it has been shown that fluorescence in the red wavelength gives a higher signal-to-noise ratio and reduced autofluorescence, RFP rather than GFP would provide a practical tag with less detection of background fluorescence (Wildt and Deuschle, 1999). There are also monoclonal primary antibodies targeted to RFP readily available to allow specific detection of RFP tagged Cdt1 constructs via western blot.

To produce an RFP construct which could be inserted into the plasmid vectors containing Cdt1 construct DNA, PCR was used with primers designed to incorporate complementary restriction enzyme sites at the start and end of the sequence. The RFP DNA fragment produced by PCR was then inserted into the pGEM-T vector (Figure 3.3A and B). Since the pRN3 plasmid only has three restriction enzyme sites within its multiple cloning site, Bgl II, Eco RI and Not I, the RFP construct was designed to be inserted between the Eco RI and Not I restriction sites. With the Cdt1 constructs inserted into pRN3 between the Bgl II and Eco RI sites this allows insertion of RFP to produce a Cdt1 construct with a C-terminal RFP tag. The RFP DNA fragment was therefore cut using Eco RI and Not I restriction enzymes and inserted into the pRN3 vector which had also been opened using Eco RI and Not I. This produced a pRN3 plasmid containing RFP DNA (Figure 3.3C) from which the RFP fragment could be sub-cloned into separate plasmid vectors containing Cdt1 construct DNA or alternatively the Cdt1 construct DNA could be sub-cloned into the pRN3 vector containing RFP.
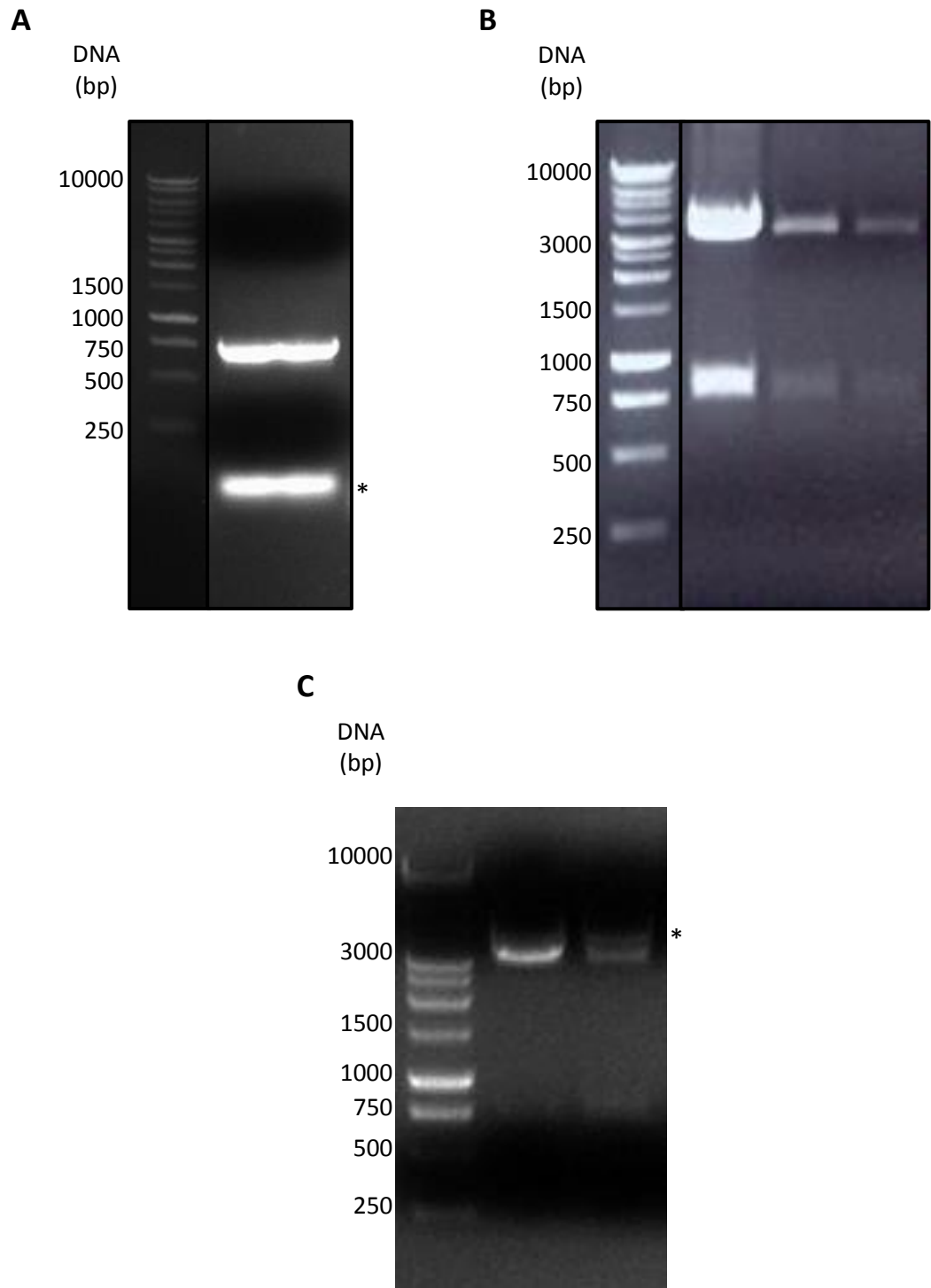
Figure 3.3. Cloning of RFP. (A) The total PCR reaction mix (50µl) was run on an agarose gel. RFP is approximately 720bp. *Unused PCR primers. **(B)** pGEM-T RFP plasmid DNA purified from bacteria, digested with EcoRI and NotI and ran on an agarose gel. pGEM-T is 3kb and RFP is approximately 720bp. **(C)** pRN3 RFP plasmid DNA purified from bacteria, digested with EcoRI and NotI and ran on an agarose gel. *uncut pRN3 RFP plasmid DNA. DNA ladder is 1kb.

The ΔCdt1$^{243-570}$-RFP construct was designed to lack not only the N-terminal 242 amino acids of Cdt1, which contain domains important for regulation, but also 50 amino acids at the extreme C-terminal which contains the MCM binding region (Ferenbach *et al.*, 2005). This ΔCdt1$^{243-570}$-RFP construct of Cdt1 will therefore be resistant to degradation and partially resistant to inhibition by geminin as with the ΔCdt1$^{243-620}$ construct, but will also be incapable of licensing the DNA due to the truncation of the MCM binding domain which is crucial for the licensing activity of Cdt1 (Ferenbach *et al.*, 2005). Due to the lack of licensing activity, the ΔCdt1$^{243-570}$-RFP construct will act as a licensing null control to the ΔCdt1$^{243-620}$ construct. This will allow confirmation of whether or not any effects on embryo development caused by ΔCdt1$^{243-620}$expression are mediated by the ability of ΔCdt1$^{243-620}$ to license the DNA.

To produce the ΔCdt1$^{243-570}$ construct, PCR was used with primers designed to incorporate only the DNA bases which code for the amino acids 243 to 570 of Cdt1 from a plasmid template containing the DNA sequence for full length Cdt1. The PCR primers were also designed to incorporate restriction enzyme sites Bam HI and Bgl II at the 5' –end and Eco RI at the 3' –end of the sequence. The ΔCdt1$^{243-570}$ DNA fragment produced by PCR was then inserted into the pGEM-T vector (Figure 3.4 A and B). To add an RFP tag, the ΔCdt1$^{243-570}$ DNA fragment was sub-cloned from the pGEM-t vector as a Bgl II-Eco RI fragment and inserted into the pRN3 vector containing the RFP DNA sequence shown in Figure 3.3C.

Once the ΔCdt1$^{243-570}$ DNA fragment was inserted into the pRN3 vector containing RFP to form ΔCdt1$^{243-570}$-RFP (Figure 3.4C) the complete plasmid was sent for DNA sequencing to ensure both the ΔCdt1$^{243-570}$ and RFP DNA sequences were present and correct. The pRN3 vector containing verified ΔCdt1$^{243-570}$-RFP DNA was then used as a template for synthesis of mRNA for expression in *Xenopus* embryos.
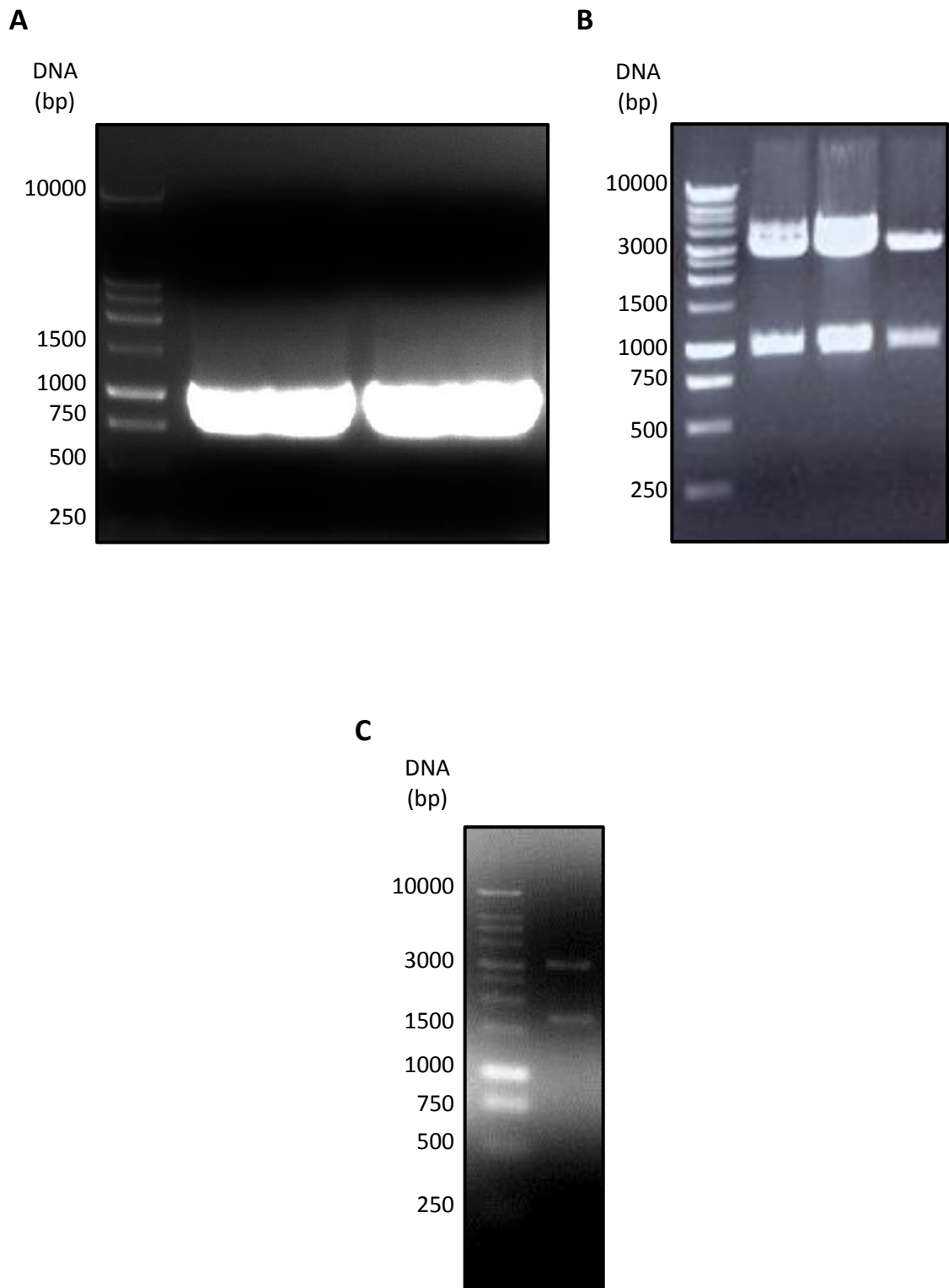
Figure 3.4. Cloning of ΔCdt1$^{243-570}$ into the pRN3 mRNA expression vector. (A) PCR reaction ran on an agarose gel. ΔCdt1$^{243-570}$ is 1.1kb in size**. (B)** pGEM-T ΔCdt1$^{243-570}$ plasmid DNA purified from bacteria, digested with Bgl II and Eco RI and ran on an agarose gel. pGEM-T is 3kb and ΔCdt1$^{243-570}$ is 1.1kb. **(C)** pRN3 ΔCdt1$^{243-570}$-RFP plasmid DNA purified from bacteria, digested with Bgl II and Not I and ran on an agarose gel. pRN3 is 3.3kb and the ΔCdt1$^{243-570}$-RFP fragment is approximately 1.8kb. DNA ladder is 1kb.

The ΔCdt1$^{1-243}$-RFP construct was designed to include only the N-terminal 243 amino acids of Cdt1. This N-terminal ΔCdt1$^{1-243}$-RFP construct therefore lacks the main geminin binding region and the C-terminal MCM binding site which is required for DNA licensing (Ferenbach *et al.*, 2005). The ΔCdt1$^{1-243}$-RFP construct represents the main regulatory region of Cdt1 which includes the PIP box, Cy motif and D-boxes required for several mechanisms of Cdt1 proteolysis (Li *et al.*, 2003; Liu *et al.*, 2004; Nishitani *et al.*, 2004; Arias and Walter, 2005a; Nishitani *et al.*, 2006; Senga *et al.*, 2006). This N-terminal Cdt1 construct will therefore be useful for investigating the mechanisms of Cdt1 regulation in the *Xenopus* embryonic cell cycle *in vivo*.

To produce the ΔCdt1$^{1-243}$ construct, PCR was used with primers designed to incorporate only the DNA bases which code for the amino acids 1 to 243 of Cdt1 from a plasmid template containing the DNA sequence for full length Cdt1. The PCR primers were also designed to incorporate restriction enzyme sites Bgl II at the 5' –end and Eco RI at the 3' –end of the sequence. The ΔCdt1$^{1-243}$DNA fragment produced by PCR was then inserted into the pGEM-T vector (Figure 3.5 A and B). To add an RFP tag to the C-terminal of the ΔCdt1$^{1-243}$ construct, the ΔCdt1$^{1-243}$DNA fragment was sub-cloned from the pGEM-t vector as a Bgl II-Eco RI fragment and inserted into the pRN3 vector containing the RFP DNA (Figure 3.3C) which was opened with Bgl II and Eco RI to allow insertion.

Once the ΔCdt1$^{1-243}$ DNA fragment was inserted into the pRN3 vector containing RFP to form ΔCdt1$^{1-243}$-RFP (Figure 3.5C) the complete plasmid was sent for DNA sequencing to ensure both the ΔCdt1$^{1-243}$ and RFP DNA sequences were present and correct. The pRN3 vector containing verified ΔCdt1$^{1-243}$-RFP DNA was then used as a template for synthesis of mRNA for expression in *Xenopus* embryos.
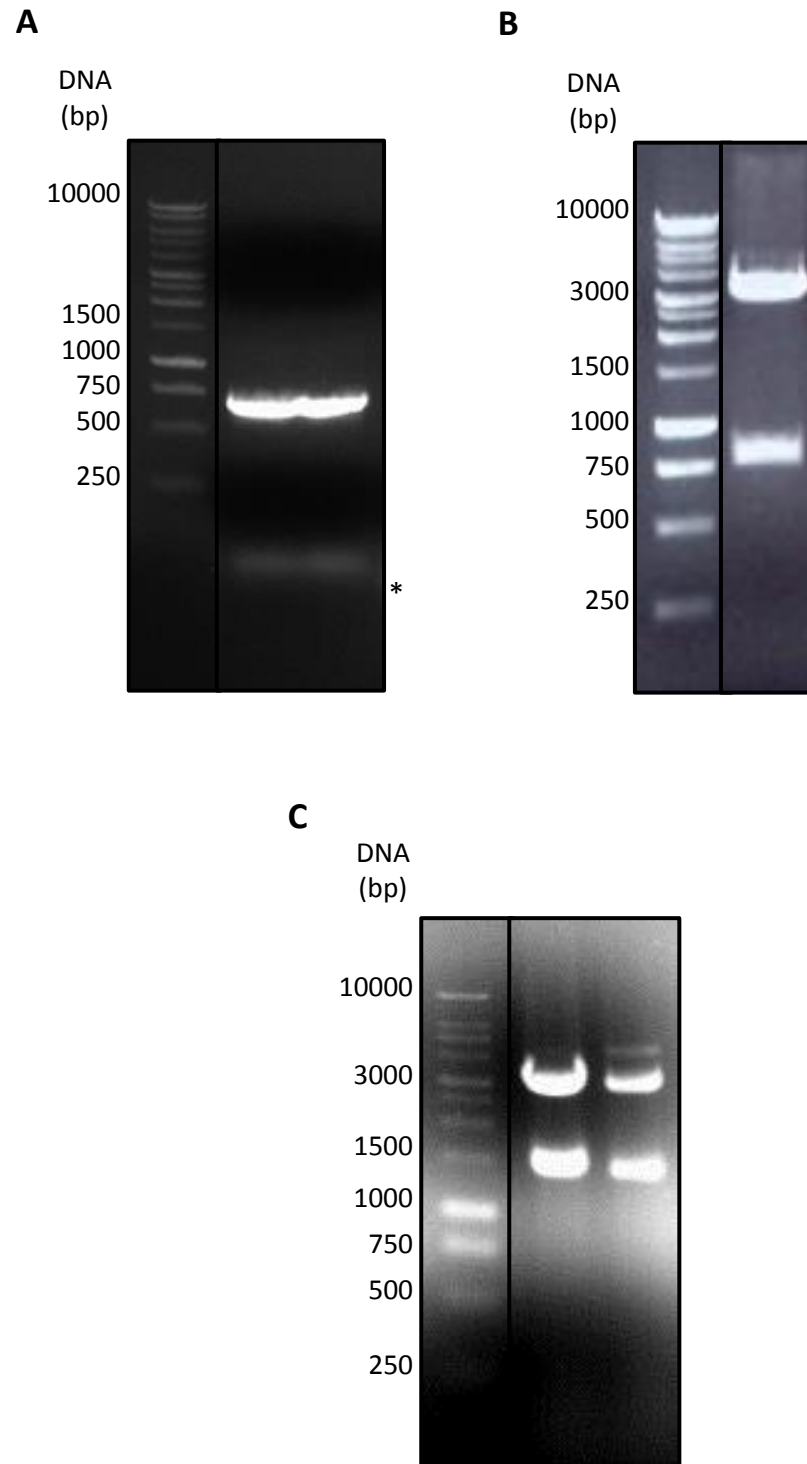
Figure 3.5. Cloning of ΔCdt1$^{1-243}$-RFP into the pRN3 mRNA expression vector. (A) The total PCR reaction mix (50μl) was ran on an agarose gel. The band at approximately 750bp is ΔCdt1$^{1-243}$. *Unused primers from PCR reaction. **(B)** pGEM-T ΔCdt1$^{1-243}$ plasmid DNA purified from bacteria, digested with Bgl II and Eco RI and ran on an agarose gel. pGEM-T is 3kb and ΔCdt1$^{1-243}$ is approximately 750bp. **(C)** pRN3 ΔCdt1$^{1-243}$-RFP plasmid DNA purified from bacteria, digested with Bgl II and Not I and ran on an agarose gel. pRN3 is 3.3kb and the ΔCdt1$^{1-243}$-RFP fragment is approximately 1.4kb. DNA ladder is 1kb.

The pRN3 vector in which the $\Delta Cdt1^{1-243}$-RFP construct was inserted is suitable for use as a template to produce mRNA through *in vitro* transcription. Once microinjected into *Xenopus* embryos the mRNA is translated to produce $\Delta Cdt1^{1-243}$-RFP protein and therefore allow investigation of Cdt1 regulation *in vivo*. However in order to complement the *in vivo* experiments using the *in vitro Xenopus* egg extract model system a recombinant protein construct of $\Delta Cdt1^{1-243}$-RFP would be more appropriate.

In order to be expressed as a recombinant protein, the $\Delta Cdt1^{1-243}$-RFP construct DNA must first be inserted into a suitable protein expression vector. The pET-32a(+) plasmid was chosen as this vector has previously been used successfully to produce functional recombinant protein constructs of *Xenopus* licensing proteins (Kisielewska and Blow, 2012). To produce $\Delta Cdt1^{1-243}$ DNA containing appropriate restriction enzyme sites for insertion into pET-32a(+), PCR was used with primers designed to include the first 243 amino acids of Cdt1 with a 5' –end Bam HI site and a 3' –end Eco RI site. The $\Delta Cdt1^{1-243}$ DNA fragment produced by PCR was then inserted into the pGEM-T vector (Figure 3.6A). The $\Delta Cdt1^{1-243}$ DNA fragment was then sub-cloned from the pGEM-T vector as a Bam HI-Eco RI fragment and inserted into a pET-32a(+) vector opened using Bam HI and Eco RI (Figure 3.6B). To add an RFP tag to the C-terminal of the $\Delta Cdt1^{1-243}$ construct, RFP was sub-cloned from the pRN3 vector as an Eco RI-Not I fragment and inserted into the pET-32a(+) vector containing the $\Delta Cdt1^{1-243}$ construct.

Once the $\Delta Cdt1^{1-243}$ DNA fragment was inserted into the pET32a(+) vector containing RFP to form $\Delta Cdt1^{1-243}$-RFP (Figure 3.6C) the complete plasmid was sent for DNA sequencing to ensure both the $\Delta Cdt1^{1-243}$ and RFP DNA sequences were present and correct. The pET-32a(+) vector containing the verified $\Delta Cdt1^{1-243}$-RFP DNA was then used to express $\Delta Cdt1^{1-243}$-RFP as a recombinant protein.
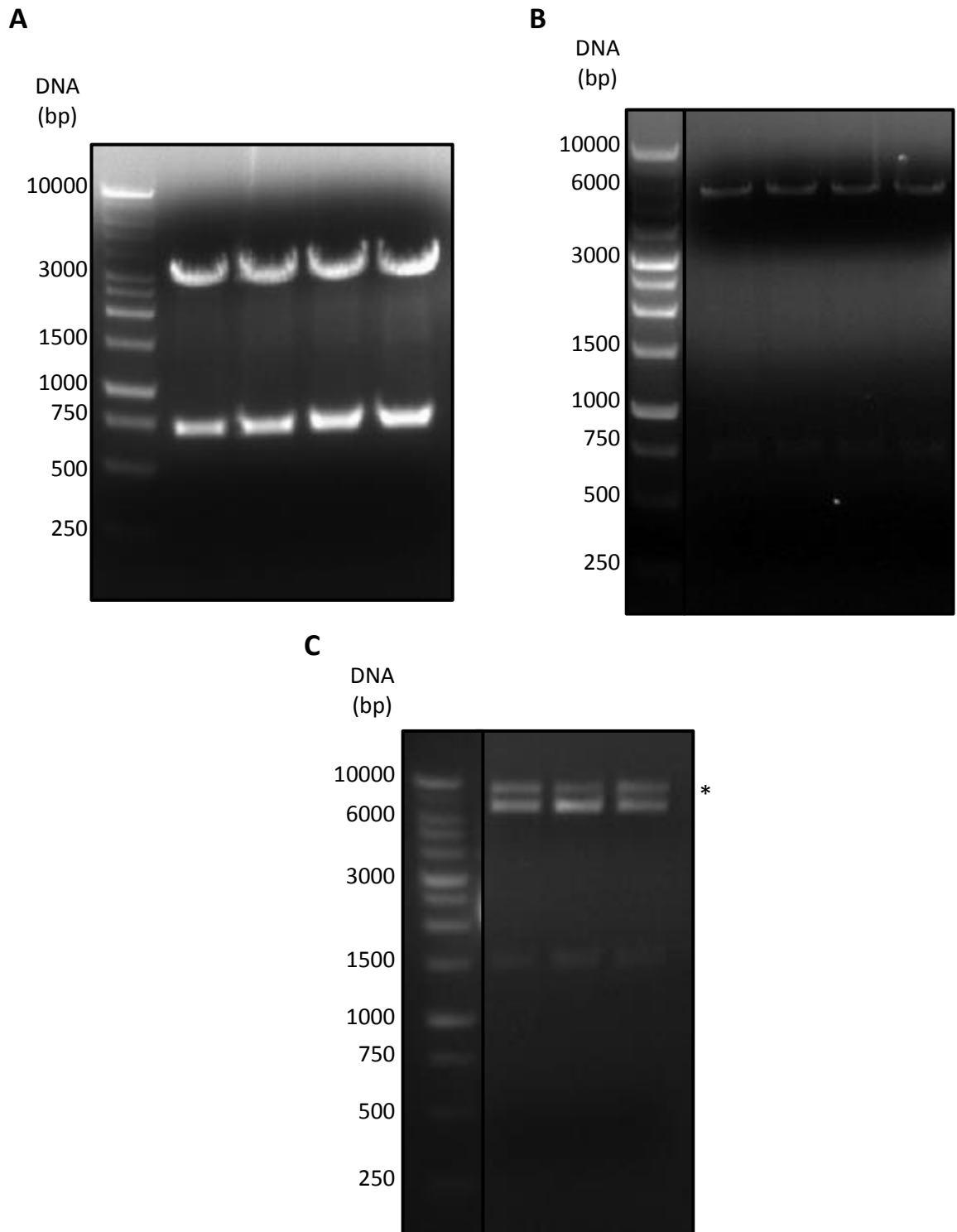
Figure 3.6. Cloning of ΔCdt1$^{1-243}$-RFP into the pET-32a(+) protein expression vector. (A) pGEM-T ΔCdt1$^{1-243}$ plasmid DNA purified from bacteria, digested with Bam HI and Eco RI and ran on an agarose gel. pGEM-T is 3kb and ΔCdt1$^{1-243}$ is approximately 729bp. **(B)** pET-32a(+) ΔCdt1$^{1-243}$ plasmid DNA purified from bacteria, digested with Bam HI and Eco RI and ran on an agarose gel. pET-32a(+) is 5.9kb and ΔCdt1$^{1-243}$ is approximately 729bp. **(C)** pET-32a(+) ΔCdt1$^{1-243}$-RFP plasmid DNA purified from bacteria, digested with Bam HI and Not I and ran on an agarose gel. pET-32a(+) is 5.9kb and ΔCdt1$^{1-243}$-RFP is approximately 1.44kb. *uncut plasmid DNA (containing pET-32a(+) and ΔCdt1$^{1-243}$-RFP DNA). DNA ladder is 1kb.

To produce soluble recombinant $\Delta Cdt1^{1-243}$-RFP protein, the *E. coli* bacterial strain Tuner was transformed with the pET-32a(+) plasmid containing the $\Delta Cdt1^{1-243}$-RFP DNA. Once a stably transformed large scale culture was established IPTG was used to induce expression of $\Delta Cdt1^{1-243}$-RFP. The IPTG activates expression from the T7 promoter of pET-32a(+) which lies downstream of the $\Delta Cdt1^{1-243}$-RFP DNA sequence. Expression from the pET-32a(+) plasmid produces recombinant proteins containing both an N-terminal and C-terminal His-Tag. The $\Delta Cdt1^{1-243}$-RFP protein was therefore purified from the induced Tuner bacterial culture by his-bind resin column chromatography. The recombinant $\Delta Cdt1^{1-243}$-RFP protein was eluted from the his-bind resin column in five fractions. Samples from each fraction were then ran on an SDS-PAGE gel and stained to detect $\Delta Cdt1^{1-243}$-RFP (Figure 3.7A). Fractions 1, 2 and 3 were combined and further purified using viva spin columns with a molecular weight cut off of 35,000 Daltons which would result in removal of the lower bands observed on the gel in Figure 3.7A. In addition the bands detected around the $\Delta Cdt1^{1-243}$-RFP construct from 35 kDa upwards were not detected by either the His-Tag or Cdt1 antibodies (Figure 3.7B). This suggests that those proteins are not truncated fragments of the $\Delta Cdt1^{1-243}$-RFP construct and therefore are unlikely to interfere with the function of the $\Delta Cdt1^{1-243}$-RFP protein.

It was important to verify that the purified protein band observed on the SDS-PAGE gel was indeed $\Delta Cdt1^{1-243}$-RFP. Samples from a serial dilution of $\Delta Cdt1^{1-243}$-RFP in non-activated *Xenopus* egg extract were therefore run on an SDS-PAGE gel and immunoblotted for His-Tag and Cdt1. Figure 3.7B shows that the band detected on the purification gel was also detected by both His-Tag and Cdt1 antibodies confirming successful purification of $\Delta Cdt1^{1-243}$-RFP recombinant protein.

The predicted molecular weight of the $\Delta Cdt1^{1-243}$-RFP protein construct is approximately 60 kDa including the His-Tags which were added due to the sequence from the pET-32a(+) vector. Although the $\Delta Cdt1^{1-243}$-RFP protein construct runs at approximately 80 kDa on the SDS-PAGE gel (Figure 3.7A) the protein is detected by both His-Tag and Cdt1 antibodies. This confirms that the purified protein contains the Cdt1 fragment and His-Tag as expected (Figure 3.7B). Since the RFP antibody is monoclonal and highly specific this was used to detect the $\Delta Cdt1^{1-243}$-RFP construct in further blots (Figure 5.2 onwards). This also confirms that in addition to the His-Tag

and Cdt1 fragment, the RFP protein is also present as expected in the purified ΔCdt1$^{1-243}$-RFP protein construct.

A

B
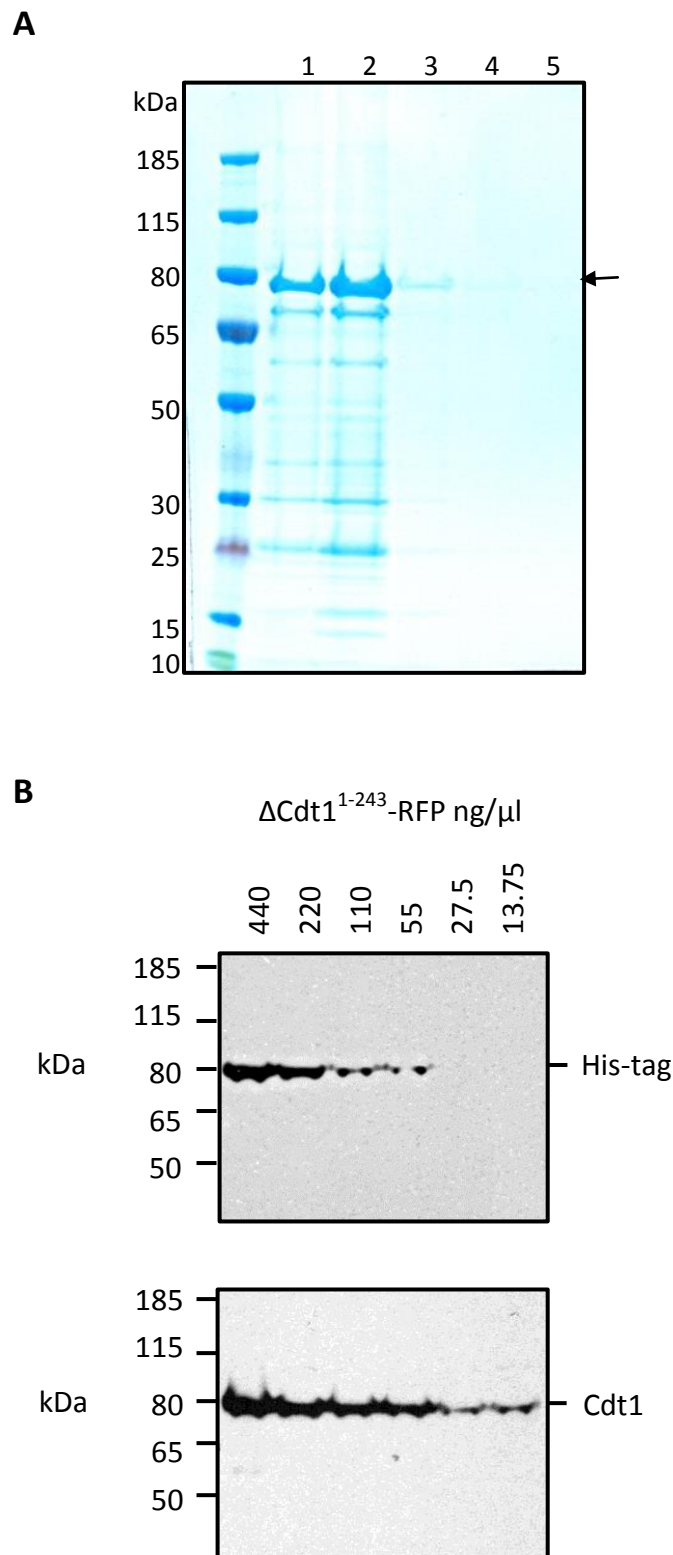
$\Delta Cdt1^{1-243}$-RFP ng/µl

Figure 3.7. Purification of ΔCdt1$^{1-243}$-RFP recombinant protein. (A) ΔCdt1$^{1-243}$-RFP protein was eluted from a his-tag purification column in 5x 1 ml aliquots. From each aliquot 5 µl samples were collected and ran on an SDS-PAGE gel. ΔCdt1$^{1-243}$-RFP is arrowed. **(B)** ΔCdt1$^{1-243}$-RFP protein was diluted in *Xenopus* egg extract. From each dilution, 1 µl samples were ran on an SDS-PAGE and blotted for His-Tag and Cdt1.

3.3 Discussion

One method which has been successfully used to investigate the regulation of the embryonic cell cycle is microinjection and expression of mRNA constructs in developing *Xenopus* embryos (Kisielewska and Blow, 2012). The benefit of this *in vivo* system is that the effect of the expressed constructs on not only one cell division cycle but also whole organism development can be determined. Also, mRNA expressed within the embryo itself consequently allows translation to protein under native conditions, increasing the prospect of correct protein folding to produce functional protein.

It has previously been shown that depletion of endogenous geminin mRNA has no effect of pre-MBT embryonic cell division with arrest of embryonic development only at MBT (McGarry, 2002). It was therefore suggested that geminin and Cdt1 are inconsequential during the pre-MBT cleavage cell cycles (McGarry, 2002; Kerns *et al.*, 2007). However it has since been shown that functional knockdown of geminin, and therefore deregulated Cdt1, arrested pre-MBT cell cycle progression (Kisielewska and Blow, 2012). With conflicting data on the importance of Cdt1 regulation for correct embryo development, mRNA expression in *Xenopus* embryos provides the ideal method for clarification of the role of Cdt1 during the pre-MBT embryonic cell cycle.

The functional domains of Cdt1 were previously elucidated using the *in vitro Xenopus* egg extract system supplemented with truncated Cdt1 protein constructs (Ferenbach *et al.*, 2005). With much of the data on DNA licensing to date carried out using *Xenopus* egg extract, the use of this system is well characterised. *Xenopus* egg extract therefore provides an ideal *in vitro* system for use to complement *in vivo* experiments utilising *Xenopus* embryos.

With $\Delta Cdt1^{243-620}$, $\Delta Cdt1^{243-570}$-RFP and $\Delta Cdt1^{1-243}$-RFP constructs in the pRN3 mRNA expression vectors, *in vitro* transcription can be utilised to produce mRNA for microinjection into *Xenopus* embryos. The purified and characterised $\Delta Cdt1^{1-243}$-RFP protein construct is suitable for both microinjection into *Xenopus* embryos and supplementation into *Xenopus* egg extract. These Cdt1 constructs therefore provide the tools to allow investigation of the mechanisms of Cdt1 regulation both *in vivo* and *in vitro*.

Chapter 4.

Results II. Stabilised Cdt1 Causes Cell Cycle Arrest in *Xenopus* embryos

4.1 Introduction

In somatic cells, regulation of Cdt1 activity is critical in ensuring DNA licensing and therefore replication occur only once per cell cycle. It has been shown that when recombinant Cdt1 is added to G2 nuclei in which the DNA has already undergone DNA licensing and replication in G1 and S-phase, re-licensing and re-replication of the DNA occurs (Maiorano *et al.*, 2005). In addition when the two main mechanisms of Cdt1 regulation: proteolysis and inhibition by geminin are abrogated, uncontrolled re-licensing and re-replication of the DNA occurs. This demonstrates the importance of Cdt1 regulation for proper DNA licensing and replication during the somatic cell cycle (Li and Blow, 2005).

Although the importance of Cdt1 regulation during the somatic cell cycle is clear, the role and mechanisms of Cdt1 regulation during the embryonic cell cycle is not well characterised. In *Drosophila* embryos, PIP box-mediated degradation of Cdt1 is important for normal embryonic cell division. A mutant Cdt1 lacking the N-terminal PIP box expressed in *Drosophila* embryonic cells was stable during S-phase and lead to tissue malformation and developmental defects (Lee *et al.*, 2010). However there have been conflicting reports on the importance of Cdt1 regulation for embryonic cell division in the early pre-MBT *Xenopus* embryo. Depletion of endogenous geminin mRNA in pre-MBT embryos had no effect on cell division with embryonic cell cycle arrest only at the onset of MBT suggesting that regulation of both geminin and Cdt1 is inconsequential prior to MBT (McGarry, 2002; Kerns *et al.*, 2007). It has since been shown that functional knockdown of the geminin protein causes arrest of pre-MBT embryonic cell cycle progression. This suggests that geminin and therefore Cdt1 regulation are essential for proper pre-MBT embryonic development (Kisielewska and Blow, 2012).

With conflicting data, it is important to clarify the role of Cdt1 regulation on *Xenopus* pre-MBT embryonic cell division and development. Since the truncated ΔCdt1$^{243-620}$ construct produced in chapter 3 lacks the N-terminal regulatory region and is partially resistant to geminin, expression in *Xenopus* embryos provides the ideal tool to clarify the importance of Cdt1 regulation for pre-MBT cell division and embryonic development.

4.2 Results

Microinjection the mRNA for the truncated de-regulated $\Delta Cdt1^{243-620}$ construct of Cdt1 into *Xenopus* embryos provides the tool with which to study the effect of miss-regulation of Cdt1 on the pre-MBT embryonic cell cycle *in vivo*. To first ensure that any effects of $\Delta Cdt1^{243-620}$ mRNA injection on the cell cycle and embryonic development were due to activity of the $\Delta Cdt1^{243-620}$ protein rather than as a consequence of the microinjection itself, microinjection controls were carried out.

The mRNA for the truncated Cdt1 constructs $\Delta Cdt1^{243-620}$, $\Delta Cdt1^{243-570}$-RFP and $\Delta Cdt1^{1-243}$-RFP was solubilised in DEPC-treated water. It was therefore important to establish that neither the DEPC-treated water nor the microinjection procedure, induced cell cycle arrest or developmental abnormalities in *Xenopus* embryos. A 30 nl volume of DEPC-treated water was injected into 1 cell of a 4 cell embryo at 2 hours post-fertilisation. The embryos were then maintained and monitored over a 48 hour period. Figure 4.1 A and B shows that embryos injected with DEPC-treated water progress normally through development as with non-injected control embryos. This shows that neither the DEPC-treated water nor the microinjection procedures are capable of causing the cell cycle arrest and developmental abnormalities associated with microinjection of $\Delta Cdt1^{243-620}$ mRNA.

The recombinant protein $\Delta Cdt1^{1-243}$-RFP produced in chapter 3 for microinjection into *Xenopus* embryos was diluted in x0.5 PBS. To ensure that the x0.5 PBS would not affect the cell cycle or embryo development a 32.2 nl volume was injected into embryos at 1 hour 15 minutes post-fertilisation before the first cell division. Figure 4.1C shows that the embryos injected with x0.5 PBS progress normally through development as with the non-injected control shown in Figure 4.1A. This shows that x0.5 PBS has no effect on the embryonic cell cycle and embryo development. Even with damage to the embryo at the injection site (Figure 4.1, arrowed), the embryos still progress through development with no sign of abnormalities 48 hours post-fertilisation. This shows that even with some damage to the embryo at the site of injection, the embryo develops normally. Therefore the microinjection procedure itself does not cause cell cycle arrest or developmental abnormalities.

Time post-fertilisation/hr:min
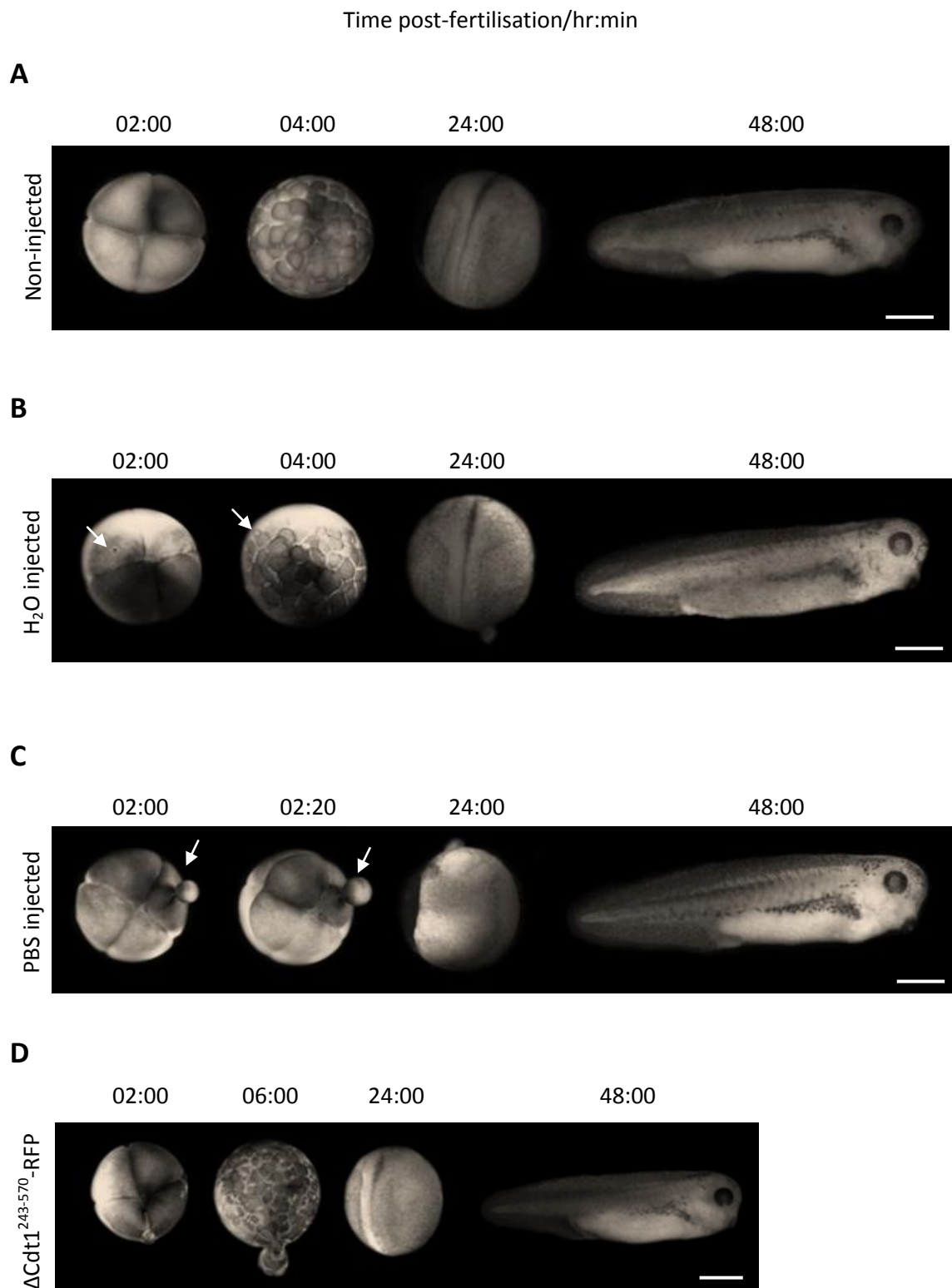
Figure 4.1. Microinjection controls. (A) The non-injected embryo shows normal embryonic development from 2 hours to 48 hours post-fertilisation. **(B)** 30nl of DEPC-treated water was injected into one cell of a four cell embryo at 2 hours post fertilisation (blastula stage 3). **(C)** 32.2nl of x0.5 PBS was injected into the embryo at 1 hour 15 minutes post-fertilisation before the first cell division (developmental stage 1). Injection site is arrowed. Scale bar is 500μm. **(D)** 18 ng of licensing null ΔCdt1$^{243-570}$-RFP mRNA was injected into 1 cell of a 2 cell embryo (developmental stage 2) and embryo development monitored until 72 hours after fertilisation. The control shows development of a non-injected embryo monitored over a 72 hour period following fertilisation. Scale bar is 500μm.

Figure 4.1 shows that the microinjection procedure its self has no effect on embryonic development. As an additional control a licensing null construct of Cdt1 was also microinjected into developing *Xenopus* embryos. The pRN3 vector containing $\Delta Cdt1^{243-570}$-RFP construct DNA (developed in Chapter 3) was used to produce $\Delta Cdt1^{243-570}$-RFP mRNA by *in vitro* transcription. To determine the effect of $\Delta Cdt1^{243-570}$-RFP on the pre-MBT cell cycle 18ng of $\Delta Cdt1^{243-570}$-RFP mRNA was injected into 1 cell of a 2 cell embryo and embryo development monitored over a 72 hour period. Figure 4.1D shows that following injection of $\Delta Cdt1^{243-570}$-RFP mRNA into pre-MBT embryonic cells the cells continue to divide and embryo development progresses normally as with non-injected control embryos. This further confirms that the microinjection procedure or materials injected have no effect on embryo development themselves and therefore any effect observed is due solely to the Cdt1 construct itself.

The *Xenopus* embryos were maintained at 23°C during the course of the microinjection experiments. Under these conditions each cell cycle takes approximately 25-30 minutes to complete. Due to the time restriction when injecting the embryos at a particular developmental stage and the necessity to inject as many embryos as possible with the Cdt1 constructs, non-injected embryos were used as controls during subsequent experiments.

To investigate the effect of mis-regulation of Cdt1 a truncated de-regulated construct of Cdt1, $\Delta Cdt1^{243-620}$, was used. The pRN3 vector containing $\Delta Cdt1^{243-620}$ construct DNA (Chapter 3) was used to produce $\Delta Cdt1^{243-620}$ mRNA by *in vitro* transcription. Firstly, 11 ng of $\Delta Cdt1^{243-620}$ mRNA was injected into fertilised *Xenopus* eggs one hour post-fertilisation, before the first cell division. The microinjection was embryonic lethal with none of the injected embryos undergoing the first cell division. Instead each embryo became apoptotic before rupturing. The failure of the embryos to progress after injection could suggest that expression of the$\Delta Cdt1^{243-620}$ construct causes cell cycle arrest. Alternatively the embryonic death could have been caused by a problem with fertilisation of the injected embryos.

To eliminate the risk of injecting non-fertilised or unhealthy embryos, the stage of embryo development at microinjection was changed to one cell of a two cell embryo. Microinjection at this stage of development allows the non-injected half of the embryo

to act as a natural control of how the embryo would have divided without injection. Next, 11 ng of ΔCdt1$^{243-620}$ mRNA was therefore microinjected into one cell of a two cell embryo at 1 hour 30 minutes post-fertilisation and the embryo development monitored. As shown in Figure 4.2A the cell injected with ΔCdt1$^{243-620}$ mRNA arrests in development at the stage of injection while the non-injected cell of the embryo continues to divide. The control embryo shows normal *Xenopus* embryonic cell division of a non-injected embryo (Figure 4.2B).

Time post-fertilisation/hr:min

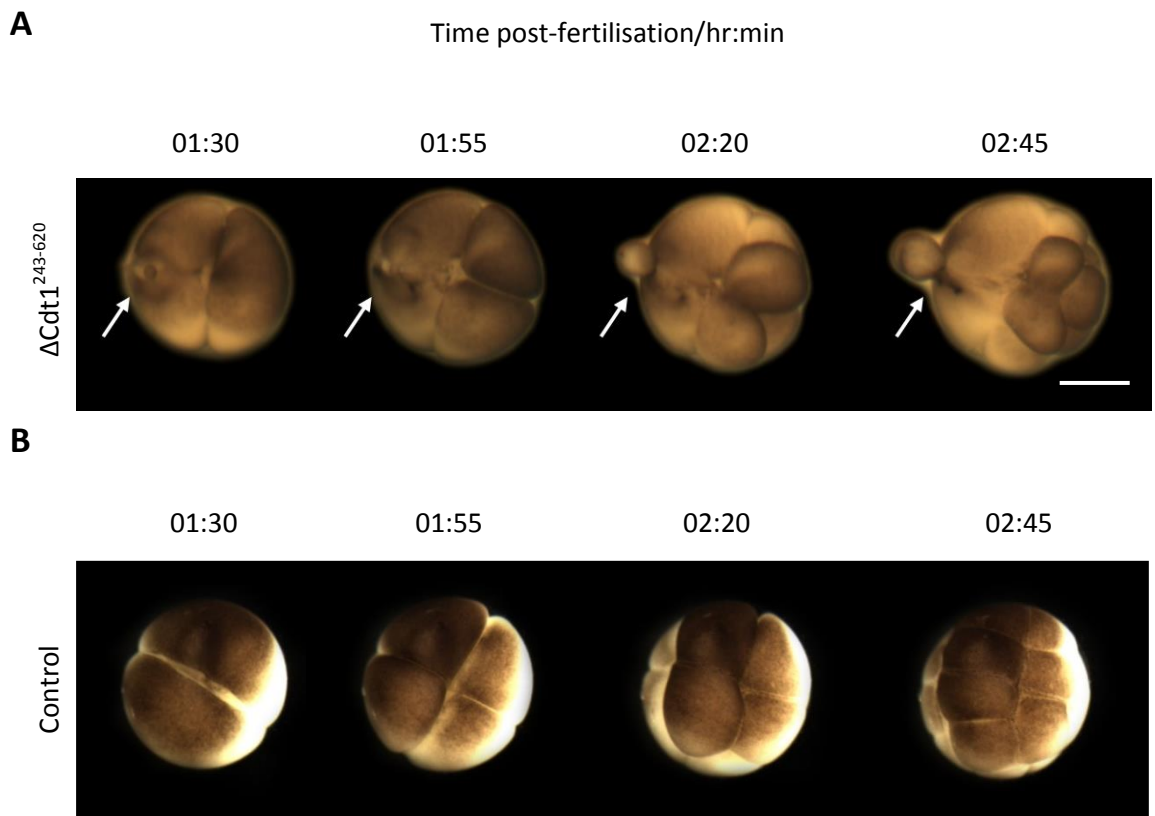**Figure 4.2.Microinjection of ΔCdt1$^{243-620}$ mRNA into *Xenopus* embryos. (A)** 11ng of ΔCdt1$^{243-620}$ mRNA injected into one cell of a 2 cell embryo (stage 2, 1 hour 30 minutes post-fertilisation). Injection site is arrowed. **(B)** Control non-injected embryo development from 1 hour 30 minutes to 2 hours 45 mınutes post-fertilisation. Scale bar is 500µm.

To confirm that the cell cycle arrest observed was due to expression of the $\Delta Cdt1^{243-620}$ mRNA and therefore unregulated $\Delta Cdt1^{243-620}$ activity, injected embryos were immunoblotted and probed with Cdt1 antibody. From 1 hour following injection single embryo samples were collected every 30 minutes between 2 hours 30 minutes and 4 hours post-fertilisation. Single non-injected embryo samples were also collected as controls. Figure 4.3 shows that a band was present at the correct molecular weight for $\Delta Cdt1^{243-620}$ (approximately 43 kDa) in the injected embryo samples. In contrast in the non-injected controls the band at that molecular weight was absent. This confirms that the injected $\Delta Cdt1^{243-620}$ mRNA was expressed to produce $\Delta Cdt1^{243-620}$ protein within the *Xenopus* embryos.

The embryonic cells injected with and expressing truncated $\Delta Cdt1^{243-620}$ show rapid cell cycle arrest within one cell division of injection. With unregulated Cdt1 activity causing such rapid arrest of embryonic cell division this suggests that correct Cdt1 activity is essential during the pre-MBT embryonic cell cycle.
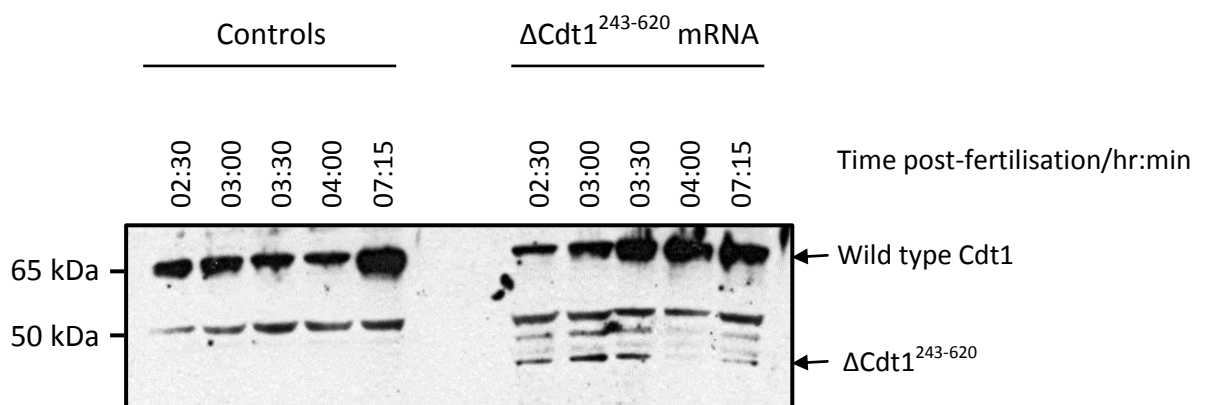
Figure 4.3. Expression of ΔCdt1$^{243\text{-}620}$ mRNA in *Xenopus* embryos. Embryos were injected with 11ng ΔCdt1$^{243\text{-}620}$ mRNA into 1 cell of a 2 cell embryo at 1 hour 30 minutes post-fertilisation (blastula stage 2). One embryo was collected every 30 minutes from 2 hours 30 minutes post-fertilisation to 4 hours post-fertilisation, which covers 3 complete cell cycles, and blotted with Cdt1 antibody. Control embryos are non-injected *Xenopus* embryos collected in parallel to ΔCdt1$^{243\text{-}620}$ mRNA injected embryos.

It was next important to determine the consequences of the cell cycle arrest on embryonic development. To determine the effect of arrest of one embryonic cell during development, embryos were injected with 6.12 ng of ΔCdt1$^{243\text{-}620}$ mRNA into 1 cell of a 2 cell embryo at 1 hour 30 minutes post fertilisation. The embryos were then maintained in optimal conditions and their development monitored. To determine whether or not cell cycle arrest of one cell at different stages of early embryonic development would cause similar abnormalities the microinjection was repeated into 1 cell of a 2 cell embryo, 1 cell of an 8 cell embryo and 1 cell of a 16 cell embryo. Figure 4.4 shows that regardless of the developmental stage of the embryo at injection of ΔCdt1$^{243\text{-}620}$ mRNA, the embryos progress although with severe developmental abnormalities. The developmental abnormality is characteristic between each injected embryo producing a phenotype of curvature of the spine. This shows that although the loss of one cell of the embryo is not embryonic lethal, even at the 2 cell stage where this constitutes a loss of half of the embryo, subsequent development is impaired.

A further embryo injected with 6.12 ng of ΔCdt1$^{243\text{-}620}$ mRNA into the animal pole later in development at 4 hours 30 minutes post-fertilisation, also develops an abnormal curvature of the spine (Figure 4.4). The cells of the animal pole of the *Xenopus* embryo give rise to several structures within the embryo including the mesoderm, nervous system and epidermis. The mesoderm of the embryo gives rise to several different tissue types including bone and muscle, malformation of which could potentially produce the curved embryo phonotype observed (Gilbert, 2000b).

Time post-fertilisation/hr

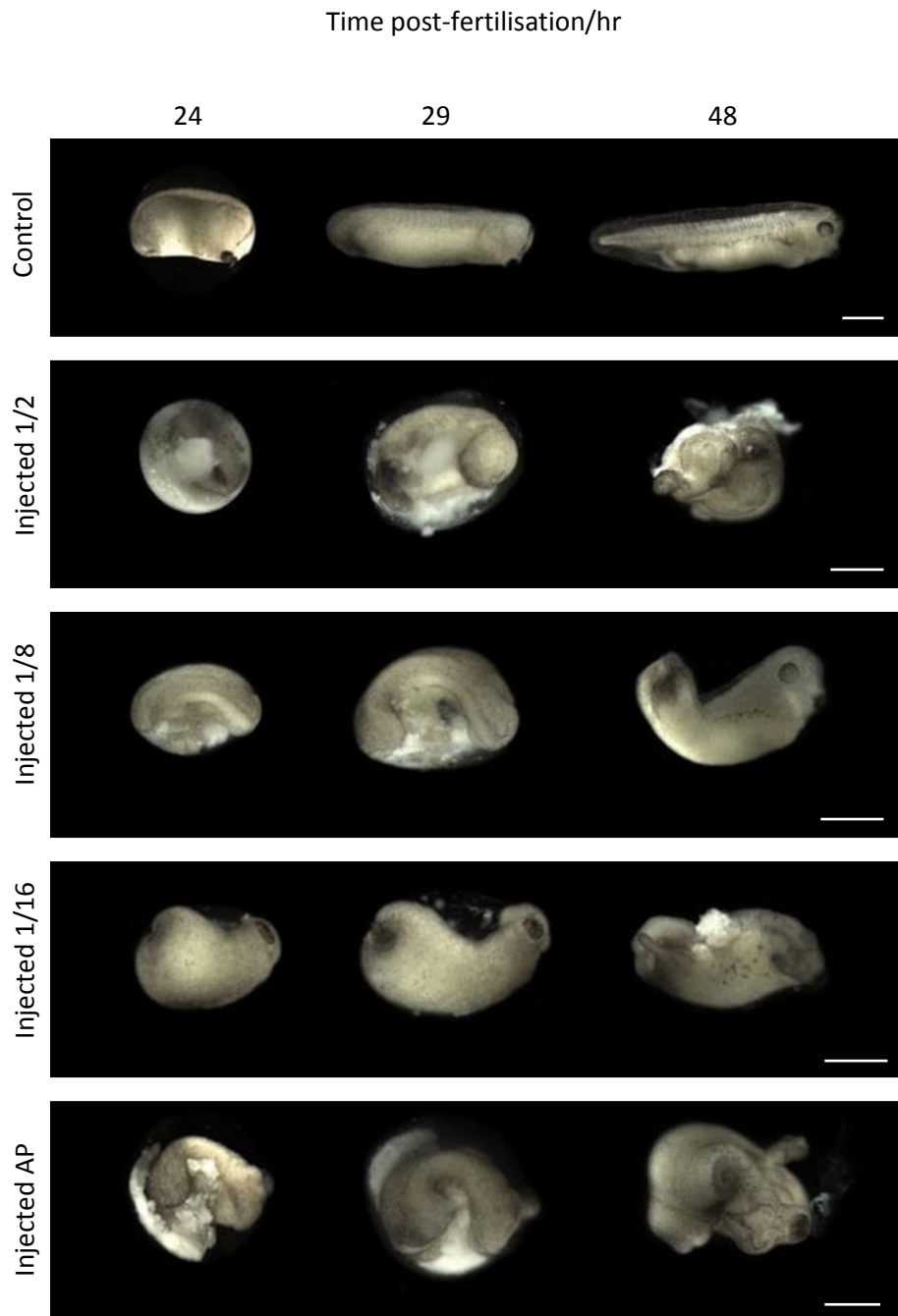**Figure 4.4. Development of *Xenopus* embryos injected with ΔCdt1$^{243\text{-}620}$mRNA at different developmental stages.** Embryos were injected with 6.12ng ΔCdt1$^{243\text{-}620}$ mRNA into one cell of either 2, 8 or 16 cells (blastula stages 2-4) or into the animal pole (AP) at 4 hours 30 minutes post-fertilisation (blastula stage 7.5). Controls are non-injected embryos. Scale bars are 500μm.

Next, it was important to determine the mechanisms behind the cell cycle arrest induced by the truncated ΔCdt1$^{243-620}$ construct. Since the ΔCdt1$^{243-620}$ construct lacks the N-terminal regulatory region but possesses an intact MCM domain essential for DNA licensing, it is likely that the cell cycle arrest observed was triggered by uncontrolled DNA re-licensing. Immunofluorescence was therefore used to investigate the licensing state of the arrested cells.

For immunofluorescence analysis, the cells of the embryo must be small enough to allow imaging using confocal/multiphoton 2P microscopy. The embryos were therefore maintained to allow development until 4 hours 30 minutes post-fertilisation before microinjection of ΔCdt1$^{243-620}$ mRNA into the animal pole. Figure 4.5A shows an embryo injected with 11 ng of ΔCdt1$^{243-620}$ mRNA imaged at 7 hours 50 minutes post-fertilisation, 3 hours 20 minutes following injection. A patch of arrested cells at the injection site are outlined. The control embryo shows normal embryonic development of a non-injected *Xenopus* embryo at 7 hours 50 minutes post-fertilisation. Figure 4.5 shows that when the embryo is injected into the animal pole at 4 hours 30 minutes post-fertilisation a group of arrested cells results rather than just one arrested cell. At this stage in development the animal pole of the embryo consists of an outer wall of cells only 2-3 layers thick covering a cavity known as the blastocoel (Nieuwkoop and Faber, 1967b; Gilbert, 2000b). It is therefore possible that the ΔCdt1$^{243-620}$ mRNA was microinjected into this cavity from which it was up taken by multiple cells resulting in an area of arrested cells.

The control and the ΔCdt1$^{243-620}$ injected embryo shown in Figure 4.5A were then fixed for immunofluorescence and probed with Mcm2 and Cdt1 to determine the licensing state of the cells. Figure 4.5B shows that in the arrested cells of the embryo injected with ΔCdt1$^{243-620}$ mRNA both Mcm2 and Cdt1 are arrested on chromatin. In contrast in the control embryo, which is at the post-MBT stage of development, the cells are not arrested and therefore can be observed at different cell cycle stages with differing levels and localisation patterns of Mcm2 and Cdt1 accordingly. In prophase and S-phase the DNA is licensed with both Mcm2 and Cdt1 associated to chromatin. In metaphase the Mcm2 level is greatly reduced and Cdt1 localises around the mitotic spindle as S-phase is complete and the DNA is no longer licensed (Figure 4.5B). This shows that the ΔCdt1$^{243-620}$ construct caused cell cycle arrested with the DNA in a

licensed state. This suggests that ΔCdt1$^{243-620}$ caused re-licensing of already replicated DNA leading to re-replication which then triggered the cell cycle arrest.
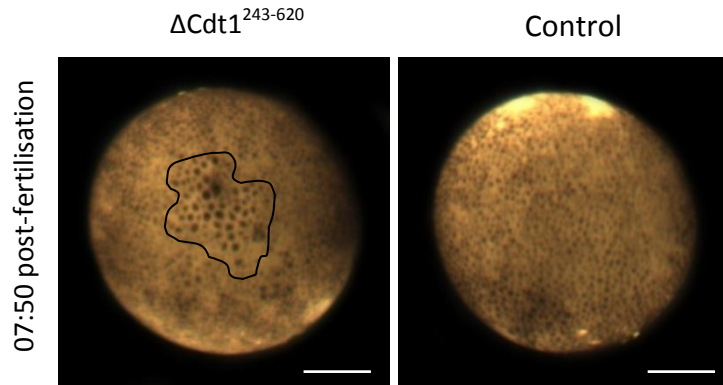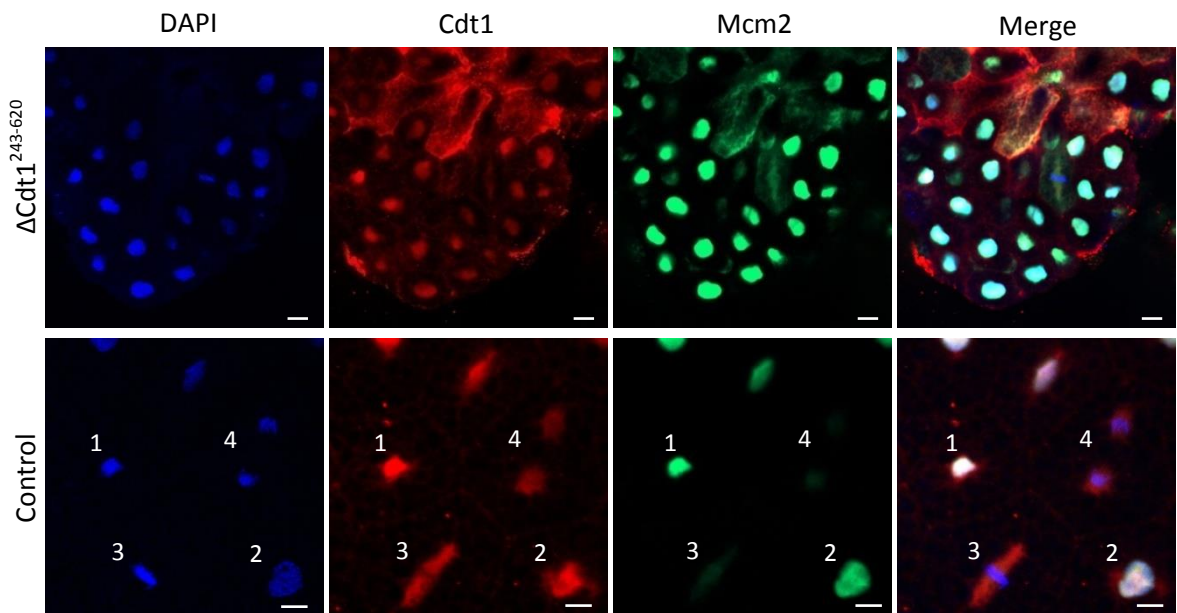
Figure 4.5. Cell cycle state of embryonic cells exposed to ΔCdt1$^{243-620}$ mRNA compared to controls. (A) Embryos were injected with 11ng ΔCdt1$^{243-620}$ mRNA into the animal pole at 4 hours 30 minutes post-fertilisation (blastula stage 7.5). The embryos were then imaged at 7 hours 50 minutes post-fertilisation (3 hours 20 minutes after injection). A section of arrested cells of the ΔCdt1$^{243-620}$ mRNA -injected embryo are outlined. The control embryo shows normal development at 7 hours 50 minutes post-fertilisation (blastula stage 9.5). The scale bars are 250μm. **(B)** The embryo samples from (A) were fixed at 7 hours 50 minutes post-fertilisation (blastula stage 9.5) for immunofluorescence. The DNA was stained using DAPI to show the cell cycle stage and Mcm2 and Ctd1 were used to determine the licensing state of the cells. The arrested cells of the embryo injected with ΔCdt1$^{243-620}$ mRNA are arrested in S-phase. In the control embryo, cells are shown at different stages of the cell cycle which are numbered: 1. S-phase, 2. Prophase, 3. Metaphase and 4. Anaphase. The scale bars are 20μm.

The cells of the embryo injected with ΔCdt1$^{243-620}$ mRNA were arrested in S-phase with both Mcm2 and Cdt1 bound to chromatin. In contrast the cells of the control non-injected embryo were cycling the cell cycle and therefore were in different cell cycle stages with Mcm2 and Cdt1 bound accordingly (Figure 4.5B). In addition, the cells of the embryo injected with ΔCdt1$^{243-620}$ mRNA which were not expressing the mRNA and therefore not arrested, were also observed to be cycling the cell cycle. In order to quantify this effect the cell cycle stage of the cells within a 200 micrometer squared region of the embryo injected with ΔCdt1$^{243-620}$ mRNA at both the effected and non-effected regions was analysed.

Figure 4.6 shows that the arrested cells of the embryo injected with ΔCdt1$^{243-620}$ mRNA were predominantly arrested in S-phase with both Mcm2 and Cdt1 arrested on chromatin. This shows that the cells arrested at S-phase with licensed chromatin confirming that the cells arrested in a licensed state. The one cell observed in metaphase at the arrested cell region was located towards the edge of the arrested region and likely represents a non-affected cell. In contrast the non-affected cells of the embryo were observed to be in the different cell cycle stages S-phase, prophase, metaphase and anaphase with lower levels of chromatin bound Mcm2 and Cdt1 during prophase, metaphase and anaphase (Figure 4.5B and Figure 4.6).
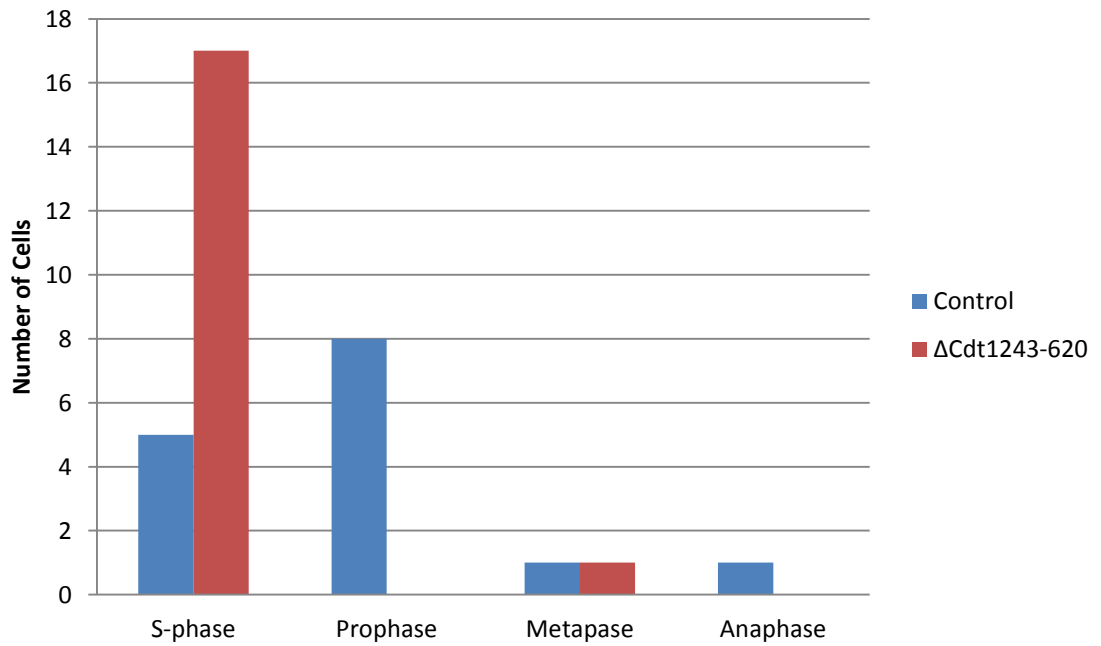
Figure 4.6. Quantification of cell cycle stage of arrested and non-arrested cells of an embryo injected with ΔCdt1$^{243-620}$ mRNA. A 200 micrometer squared region of both the arrested and non-arrested region of the embryo injected with ΔCdt1$^{243-620}$ mRNA shown in figure 4.5A was selected. The cell cycle stage of each cell within the selected region, as determined by the state of the chromatin and binding of Mcm2 and Cdt1, was recorded and plotted.

The results so far show that expression of ΔCdt1$^{243\text{-}620}$ mRNA in *Xenopus* embryos causes cell cycle arrest with the cells arrested in a licensed state. This suggests that the cell cycle arrest is mediated by re-licensing and re-replication of the DNA. It was therefore likely that DNA re-replication induced DNA damage had occurred triggering cell cycle arrest. The presence of DNA damage in the arrested cells was therefore investigated.

Immunofluorescence was used to detect Rad51 bound to DNA as a marker for DNA damage. Rad51 binds to DNA during the process of homologous recombination, a DNA damage repair pathway. Therefore Rad51 is only present at sites of DNA damage (Balakrishnan *et al.*, 2009). Figure 4.7A shows an embryo injected with 11 ng of ΔCdt1$^{243\text{-}620}$ mRNA into 1 cell of 16 at 2 hours 30 minutes post-fertilisation. The embryo was then maintained for 4hours 20 minutes post-injection before fixing for immunofluorescence. Figure 4.7B shows an embryo injected with 11 ng ΔCdt1$^{243\text{-}620}$ mRNA into the animal pole at 4 hours 40 minutes post-fertilisation. The embryo was then fixed for immunofluorescence at 1 hour 10 minutes post-injection. Chromatin bound Rad 51 was detected in both ΔCdt1$^{243\text{-}620}$ injected embryos. In contrast in the control non-injected embryos there was no evidence of chromatin bound Rad 51. This shows that there is DNA damage in the cells expressing ΔCdt1$^{243\text{-}620}$ mRNA.

Figure 4.7A shows that although the embryo was injected with ΔCdt1$^{243\text{-}620}$ mRNA into 1 cell of 16 there are multiple nuclei containing chromatin bound Rad51. Although at this stage in development the blastocoel has not formed, the precursor to the blastocoel known as the cleavage cavity is present beneath the cells of the animal pole. The cleavage cavity is present from the four cell stage onwards and increases in size with each cleavage to form the blastocoel by stage 7 of development (Nieuwkoop and Faber, 1967b). It is again possible that the microinjection needle pierced through the injected animal pole cell to release the ΔCdt1$^{243\text{-}620}$ mRNA into the cleavage cavity. Up take of ΔCdt1$^{243\text{-}620}$ mRNA from the cleavage cavity by multiple animal pole cells would explain the presence of multiple nuclei showing chromatin bound Rad 51 and DNA damage.

The presence of DNA damage provides further evidence that the unregulated $\Delta Cdt1^{243-620}$ construct induces cell cycle arrest through uncontrolled re-licensing and re-replication leading to the DNA damage observed.

Figure 4.7. Immunofluorescence showing the presence of Rad 51 in arrested embryonic cells following ΔCdt1^243-620 mRNA injection. *Xenopus* embryos were fixed and probed for the presence of Rad 51, a marker of DNA damage. DAPI was used to stain the DNA before the embryo samples were whole mounted and imaged using a Zeiss confocal/2-photon microscope. Scale bars are 20μm. **(A)** *Xenopus* embryos were injected with 11ng ΔCdt1^243-620 mRNA into 1 cell of a 16 cell embryo at 2 hours 30 minutes post-fertilisation (blastula stage 5). Injected embryos and non-injected controls were then fixed at 7 hours 10 minutes post-fertilisation (blastula stage 9) to ensure that the nuclei of the cells were small enough for confocal imaging. Rad 51 was localised to the DNA of arrested cells of injected embryos but not controls. **(B)** *Xenopus* embryos were injected with 11ng ΔCdt1^243-620 mRNA into the animal pole at 4 hours 40 minutes post-fertilisation (blastula stage7. 5). Injected embryos and non-injected controls were then fixed at 5 hours 50 minutes post-fertilisation (blastula stage 8.5) to ensure that the nuclei of the cells were small enough for confocal imaging. Again Rad 51 localised to the DNA of cells of the injected embryo but not controls.

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Since ΔCdt1$^{243\text{-}620}$ mRNA expression causes cell cycle arrest mediated by DNA damage, the next step was to investigate whether or not the DNA damage lead to checkpoint activation therefore causing the cell cycle to arrest. One cell cycle checkpoint known to be activated by DNA damage is the p53 checkpoint pathway. Here, DNA damage leads to activation of protein kinases which phosphorylate p53 thereby reducing degradation and subsequently increasing p53 levels within the cell. The p53 protein then induces transcription of p21 which inhibits S-phase CDK activity to arrest the cell cycle (Alberts *et al.*, 2002c). To determine whether or not the DNA damage observed in Figure 4.7 triggered the p53 checkpoint, immunofluorescence was used probing for p53 in cells expressing ΔCdt1$^{243\text{-}620}$ mRNA.

Figure 4.8A shows an embryo injected with 11 ng ΔCdt1$^{243\text{-}620}$ mRNA into 1 cell of 8 at 2 hours 10 minutes post-fertilisation. The embryo was then maintained for 3 hours 35 minutes post-injection before fixing for immunofluorescence. In the nuclei of the injected embryo chromatin bound p53 was present. Again in the embryo injected with ΔCdt1$^{243\text{-}620}$ mRNA, there are multiple nuclei arrested with chromatin bound p53 likely due to injection into the cleavage cavity. In contrast the control non-injected embryo shows a barely detectable level of p53. Since there is known to be a stockpile of maternal p53 in the cytoplasm of early *Xenopus* embryonic cells (Tchang *et al.*, 1993) and the presence of p53 is essential for development (Wallingford *et al.*, 1997), a small background level is to be expected.

Figure 4.8B shows an embryo injected with 11 ng ΔCdt1$^{243\text{-}620}$ mRNA into the animal pole at 4 hours 40 minutes post-fertilisation. The microinjection was aimed at the blastocoel cavity below the animal cap cells in order to allow uptake of ΔCdt1$^{243\text{-}620}$ mRNA into multiple cells. The embryo was then fixed for immunofluorescence 1 hour 50 minutes post-injection. Chromatin bound p53 was again detected in the embryo injected with ΔCdt1$^{243\text{-}620}$. In contrast in the control non-injected embryo there was little to no p53 detected either bound to DNA or in the cytoplasm. This provides evidence that the deregulated ΔCdt1$^{243\text{-}620}$ construct causes DNA damage leading to cell cycle arrest mediated by the p53 DNA damage checkpoint.

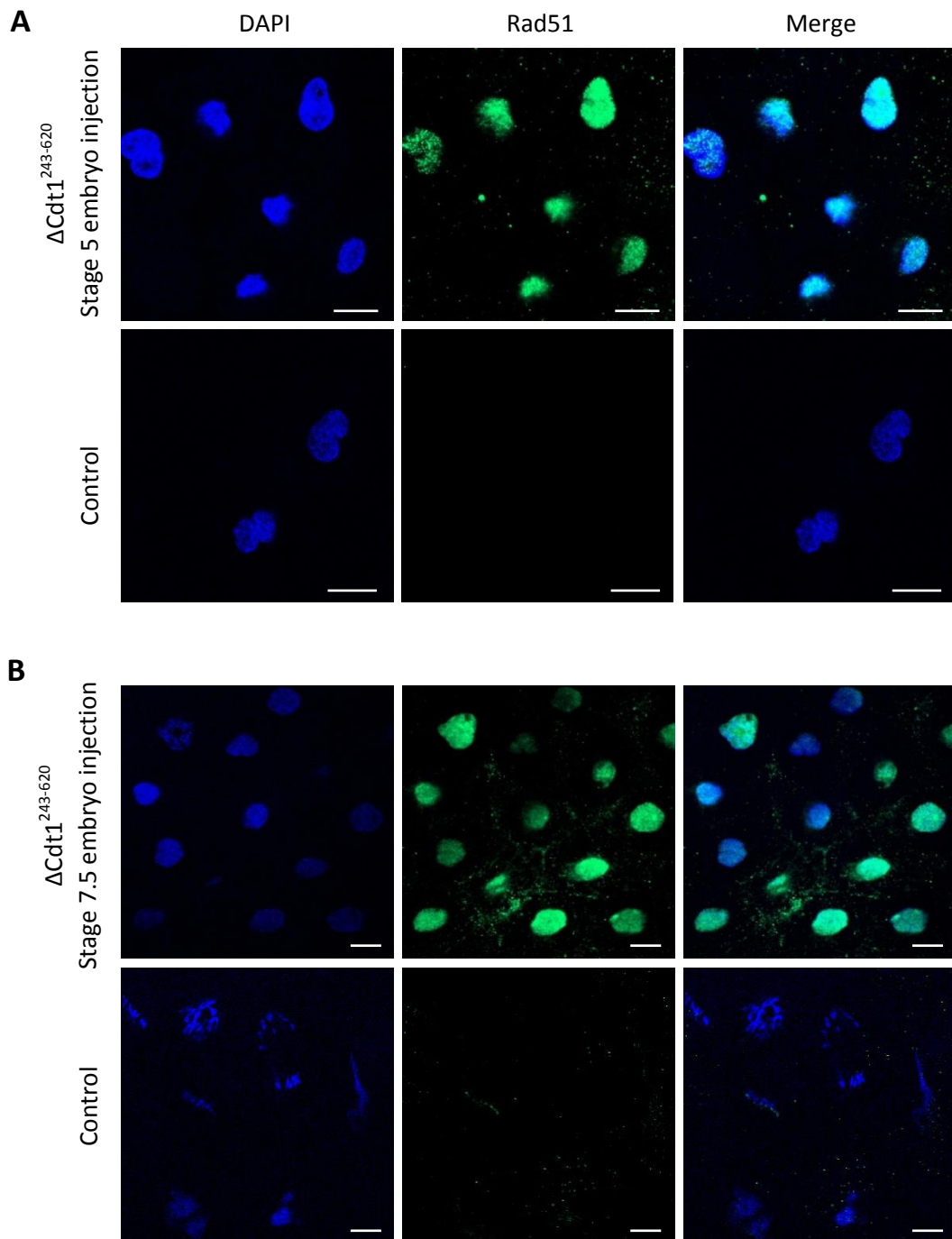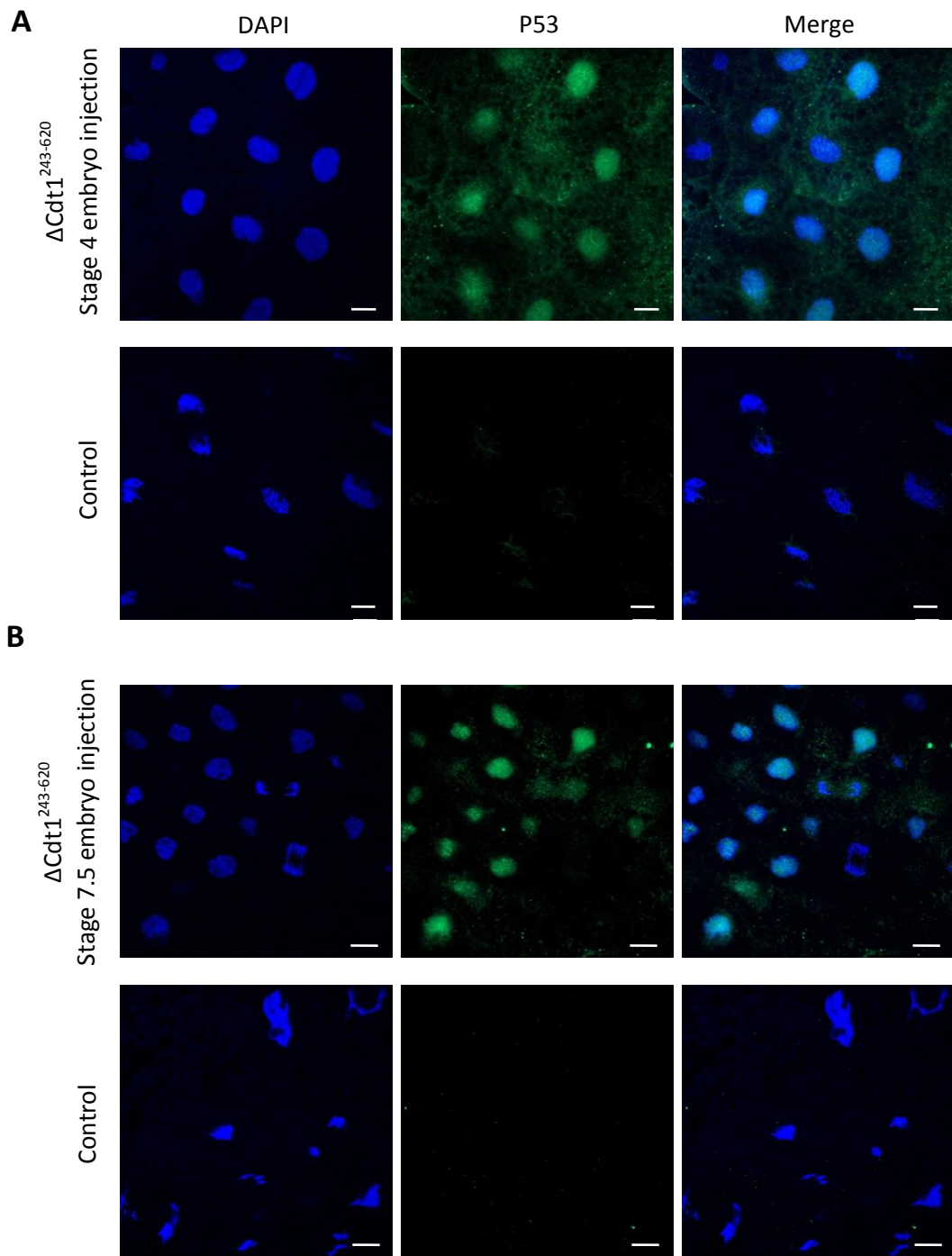**Figure 4.8. Immunofluorescence showing the presence of p53 in arrested embryonic cells following ΔCdt1$^{243-620}$ mRNA injection.** *Xenopus* embryos were fixed and probed for the presence of p53 to detect checkpoint activation. DAPI was used to stain the DNA before the embryo samples were whole mounted and imaged using a Zeiss confocal/2-photon microscope. Scale bars are 20 μm. **(A)** *Xenopus* embryos were injected with 11 ng ΔCdt1$^{243-620}$ mRNA into 1 cell of an 8 cell embryo at 2 hours 10 minutes post-fertilisation (blastula stage 4). Injected embryos and non-injected control embryos were then fixed at 5 hr 45 min post-fertilisation (blastula stage 8.5). p53 was detected localised to the DNA in the injected embryo cells but not controls. **(B)** *Xenopus* embryos were injected with 11 ng ΔCdt1$^{243-620}$ mRNA into the animal pole at 4 hours 40 minutes post-fertilisation (blastula stage 7.5). Injected embryos and non-injected controls were then fixed at 6 hours 30 minutes post-fertilisation (blastula stage 8.5). p53 was detected on the DNA of injected embryo cells but not controls.

In order to quantify the expression of Rad51 and p53 in the embryos injected with $\Delta Cdt1^{243-620}$ mRNA image J, was used to measure the average fluorescence intensity of the Rad51 and p53 signals in injected versus non-injected embryos.

The average fluorescence intensity of the Rad51 signal was measured in a minimum of 5 nuclei in each of 2 embryos injected with 11 ng $\Delta Cdt1^{243-620}$ mRNA into the animal pole at 4 hours 40 minutes post-fertilisation (blastula stage7. 5). Following injection the embryos were fixed for immunofluorescence between 5 hours 30 minutes and 7 hours 30 minutes post-fertilisation. Non-injected embryos fixed for immunofluorescence in parallel to the injected embryos were used as controls. Figure 4.9A shows that the nuclear Rad51 signal was higher in the $\Delta Cdt1^{243-620}$ mRNA injected embryos compared to non-injected controls. This confirms that expression of $\Delta Cdt1^{243-620}$ mRNA in *Xenopus* embryos causes DNA damage.

The average fluorescence intensity of the p53 signal was also measured in a minimum of 5 nuclei in each of 2 embryos injected with 11 ng $\Delta Cdt1^{243-620}$ mRNA into 1 cell of an 8 cell embryo at 2 hours 10 minutes post-fertilisation (blastula stage 4). Following injection the embryos were fixed for immunofluorescence between 5 hours 45 minutes and 7 hours post-fertilisation. Non-injected embryos fixed for immunofluorescence in parallel to the injected embryos were used as controls. Figure 4.9B shows that the nuclear p53 signal was higher in the $\Delta Cdt1^{243-620}$ mRNA injected embryos compared to non-injected controls. This confirms that expression of $\Delta Cdt1^{243-620}$ mRNA in *Xenopus* embryos triggers p53 expression suggesting that the DNA damage causes cell cycle arrest via the p53 checkpoint.

A
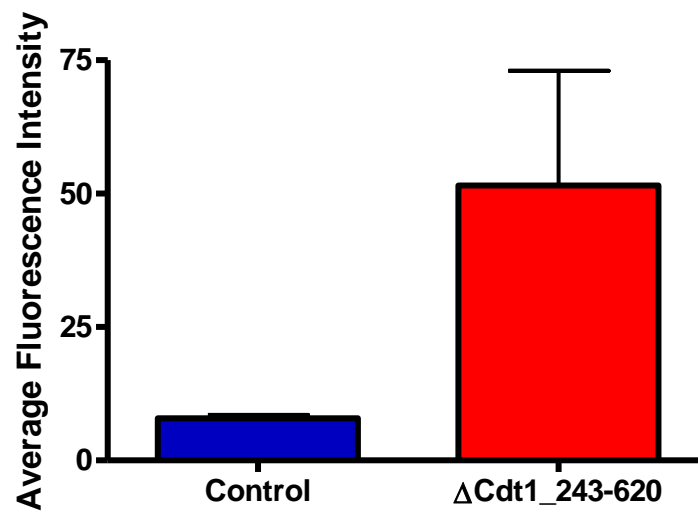
B
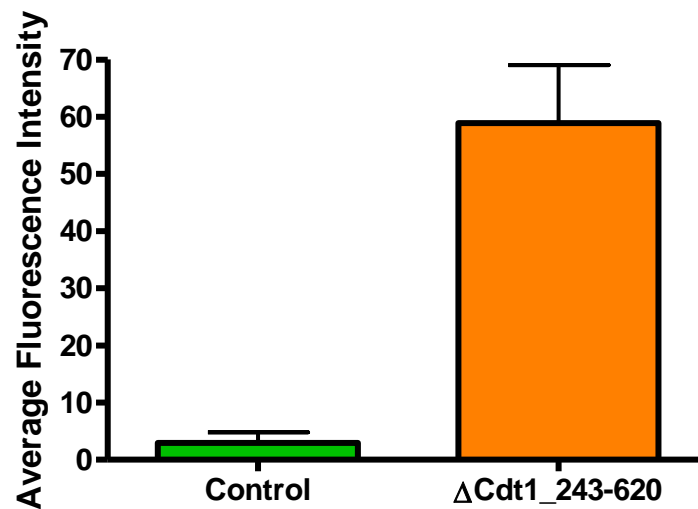
Figure 4.9. Quantification of the average fluorescence intensity of the Rad51 and p53 signals in embryos injected with ΔCdt1[243-620] mRNA compared to non-injected controls. (A) The average fluorescence intensity of the Rad51 signal was measured in a minimum of 5 nuclei in each of 2 embryos injected with 11 ng ΔCdt1[243-620] mRNA into the animal pole at 4 hours 40 minutes post-fertilisation (blastula stage7. 5). The signal from the 5 nuclei was averaged and the averages for each of the 2 embryos plotted as a bar chart. The error bars show the SEM for n=2. **(B)** The average fluorescence intensity of the p53 signal was measured in a minimum of 5 nuclei in each of 2 embryos injected with 11 ng ΔCdt1[243-620] mRNA into 1 cell of an 8 cell embryo at 2 hours 10 minutes post-fertilisation (blastula stage 4). The signal from the 5 nuclei was averaged and the averages for each of the 2 embryos plotted as a bar chart. The error bars show the SEM for n=2.

The results so far suggest that the deregulated $\Delta Cdt1^{243-620}$ construct causes cell cycle arrest through uncontrolled DNA licensing leading to re-licensing and re-replication of the DNA. To further confirm this, a licensing null Cdt1 construct ($\Delta Cdt1^{243-570}$-RFP) was designed to consist of the same amino acids of Cdt1 as $\Delta Cdt1^{243-620}$ but lacking the extreme C-terminal 50 amino acids. The loss of the C-terminal 50 amino acids destroys the MCM binding domain which is necessary for DNA licensing. Therefore $\Delta Cdt1^{243-570}$-RFP constitutes a Cdt1 construct that is deregulated as with $\Delta Cdt1^{243-620}$ but is also incapable of licensing the DNA.

The pRN3 vector containing $\Delta Cdt1^{243-570}$-RFP construct DNA (Chapter 3) was used to produce $\Delta Cdt1^{243-570}$-RFP mRNA by *in vitro* transcription. To determine the effect of $\Delta Cdt1^{243-570}$-RFP on the pre-MBT cell cycle 18ng of $\Delta Cdt1^{243-570}$-RFP mRNA was injected into 1 cell of a 2 cell embryo and embryo development monitored over a 72 hour period. Figure 4.10A shows that following injection of $\Delta Cdt1^{243-570}$-RFP mRNA into pre-MBT embryonic cells the cells continue to divide and embryo development progresses normally as with non-injected control embryos. Western blot was used to confirm expression of the $\Delta Cdt1^{243-570}$-RFP mRNA within the embryos to produce $\Delta Cdt1^{243-570}$-RFP protein. Embryos were injected with 18 ng of $\Delta Cdt1^{243-570}$-RFP mRNA either before the first cell division at 1 hour 10 minutes post-fertilisation or into one cell of two at 1 hour 30 minutes post-fertilisation. Between 2 and 3 hours following injection with $\Delta Cdt1^{243-570}$-RFP mRNA, whole embryos were fixed and their extracts blotted for RFP to detect $\Delta Cdt1^{243-570}$-RFP. Figure 4.10B shows a band detected by RFP antibody confirming expression of the $\Delta Cdt1^{243-570}$-RFP mRNA.

This shows that when the licensing activity of deregulated $\Delta Cdt1^{243-620}$ is abolished, the pre-MBT cells are able to divide and the embryo develop normally. This further confirms that it is the uncontrolled licensing activity of deregulated $\Delta Cdt1^{243-620}$ causing the DNA damage and cell cycle arrest through DNA re-licensing and re-replication.
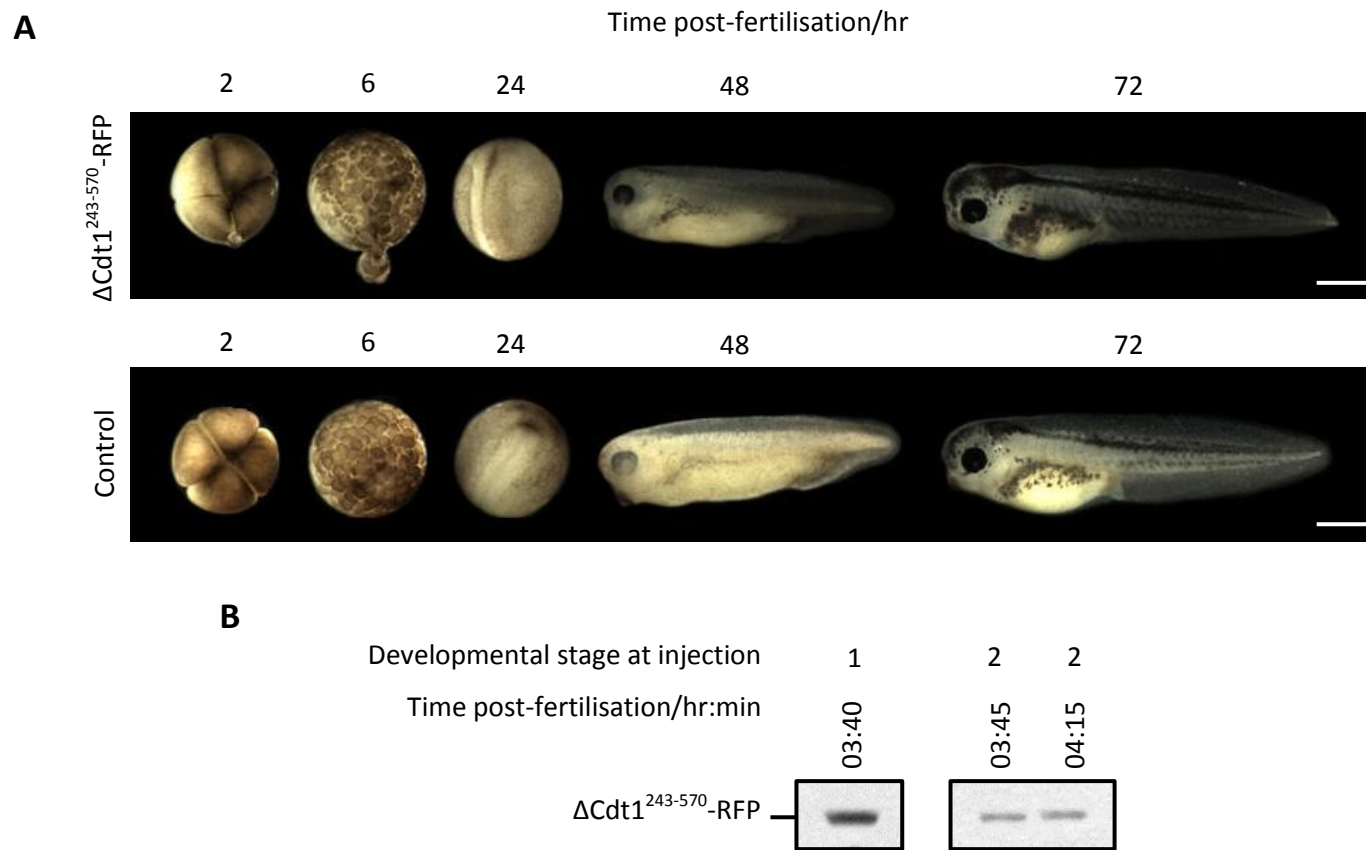
Figure 4.10. Microinjection and expression of ΔCdt1$^{243-570}$-RFP mRNA in *Xenopus* embryos. (A) 18 ng of ΔCdt1$^{243-570}$-RFP mRNA was injected into 1 cell of a 2 cell embryo (developmental stage 2) and embryo development monitored until 72 hours after fertilisation. The control shows development of a non-injected embryo monitored over a 72 hour period following fertilisation. Scale bar is 500μm. **(B)** Embryos were injected with 18ng ΔCdt1$^{243-570}$-RFP mRNA at either the one cell stage (developmental stage 1) or into 1 cell of 2 (developmental stage 2). Single embryos were fixed at various time points post fertilisation, their extracts ran on an SDS-PAGE gel and immunoblotted for RFP.

Since deregulated ΔCdt1$^{243-620}$ causes cell cycle arrest and developmental abnormalities in pre-MBT embryos, this suggests that proper regulation of Cdt1 is critical for correct pre-MBT cell division.

4.3 Discussion

With conflicting data on the requirement of correct Cdt1 regulation for maintenance of proper DNA replication and cell division within pre-MBT *Xenopus* embryonic cells it was important to clarify the importance of Cdt1 regulation for embryonic development. A deregulated truncated Cdt1 construct, ΔCdt1$^{243-620}$, was designed to lack the N-terminal region which contains domains required for the regulation of Cdt1. Upon expression in *Xenopus* embryos, ΔCdt1$^{243-620}$ caused the cell to arrest in development. In addition the cells were arrested in a licensed state suggesting that re-licensing and re-replication occurred. The presence of chromatin bound Rad 51 and p53 show that DNA damage was present and suggests that checkpoint activation lead to the cell cycle arrest observed. The presence of DNA damage further confirms the likelihood of re-licensing and re-replication as the primary cause of the cell cycle arrest. Since a licensing null Cdt1 mutant (ΔCdt1$^{243-570}$-RFP) did not cause cell cycle arrest, this suggests that the arrest triggered by the ΔCdt1$^{243-620}$ construct was due to aberrant DNA licensing. This provides strong evidence that Cdt1 must be carefully regulated to allow correct DNA replication and embryonic cell cycle progression.

In a study by McGarry, morpholinos was used to deplete geminin from early developing *Xenopus* embryos in order to determine the importance of geminin-dependant regulatory mechanisms on progression of the embryonic cell cycle. In the geminin morpholino experiment the affected cells arrested only at the onset of the MBT in a Chk1 dependant manner once the G2 phase was introduced to the cell cycle. This lead to the suggestion that geminin and therefore regulation of Cdt1, the protein it inhibits, are dispensable for preventing re-replication in early pre-MBT *Xenopus* embryos (McGarry, 2002). However the geminin depletion was not complete until just prior to the MBT which possibly explains the later cell cycle arrest. There is also evidence that siRNAs are unable to support RNA interference in early *Xenopus* embryos as they instead bind to maternal Ago proteins in a sequence independent manner. The Ago proteins which are inactivated by the siRNA are required for pre-

miRNA processing at MBT and the result is embryonic defects at later developmental stages (Lund *et al.*, 2011).

In a further study by Kerns *et al.*, when both non-geminin binding and non-degradable Cdt1 mutants were injected separately into early *Xenopus* embryos the cells arrested only at the onset of the MBT again suggesting that Cdt1 regulation is dispensable for pre-MBT cell cycle progression (Kerns *et al.*, 2007). However, it has since been shown that functional knock down of geminin causes cell cycle arrest in pre-MBT *Xenpous* embryonic cells suggesting that regulation of Cdt1 via geminin binding is critical for cell division in pre-MBT embryonic cells (Kisielewska and Blow, 2012). This is in agreement with the results presented here in which deregulated Cdt1 activity causes pre-MBT cell cycle arrest with evidence of re-licensing and re-replication. One of the possibilities for the discrepancy is that in the study by Kerns *et al.*, the concentration of Cdt1 mutant mRNA injected into the *Xenopus* embryos was in the picogram region (300 pg) whereas both in this chapter and in the study by Kisielewska and Blow, the mRNA injected was in the nanogram region (6-12 ng). It is therefore possible that when low pg levels of mRNA are injected it takes time for the accumulation of translated protein constructs to a level sufficient to exert effects on DNA replication and the cell cycle. Slow accumulation of protein constructs as the embryo progresses through pre-MBT cell divisions could explain the later arrest at the onset of MBT.

It was initially believed that early pre-MBT embryos did not possess checkpoints to allow cell cycle arrest in response to DNA damage (Finkielstein *et al.*, 2001). In *Drosophila* embryos, the early cleavage cell divisions continue even in the presence of aphidicolin-induced DNA damage (Raff and Glover, 1988). Similarly in *Xenopus* embryos the pre-MBT cleavage cell cycles also continued despite DNA damage with cell cycle arrest occurring only at the MBT. However the arrest was independent of transcription suggesting that the checkpoint may be present but inhibited prior to the onset of MBT (Newport and Dasso, 1989). Evidence has since emerged that, under certain conditions, the DNA damage checkpoint can be activated in pre-MBT *Xenopus* embryonic cells. It has been shown that when the DNA to cytoplasm ratio is increased, DNA double strand breaks lead to activation of the Chk1 checkpoint in pre-MBT cells. This indicates the presence of a maternal DNA damage response in pre-MBT *Xenopus* embryos. It is therefore possible that ΔCdt1$^{243-620}$-induced re-replication altered the

DNA to cytoplasm ratio facilitating checkpoint activation and cell cycle arrest (Conn *et al.*, 2004). In addition it has been previously shown that excess p53 expression in pre-MBT embryonic cells leads to cell cycle arrest (Hoever *et al.*, 1994). This demonstrates that the p53 checkpoint machinery is present in pre-MBT cells. Furthermore it has been shown that in pre-MBT cells p53 is imported into the nucleus in a manner linked to S-phase of the cell cycle with accumulation following DNA damage. This suggests that p53 may play a role in S-phase regulation during the cleavage cycles of the *Xenopus* embryo (Tchang and Mechali, 1999). Combined with the presence of p53 in $\Delta Cdt1^{243-620}$ expressing cells, this suggests that p53 may also contribute to the cell cycle arrest observed.

In mammalian somatic cells Cdt1 is degraded during S-phase and is present at almost undetectable levels during G2 (Nishitani *et al.*, 2001). In contrast the Cdt1 natural inhibitor protein geminin accumulates during S-phase and G2 and is absent during G1 following degradation at the metaphase-anaphase transition (McGarry and Kirschner, 1998). However in pre-MBT *Xenopus* embryonic cells both Cdt1 and geminin levels remain persistently high throughout the cell cycle. This suggests that in pre-MBT cells Cdt1 is regulated by mechanisms other than degradation. One possibility is that in pre-MBT cells DNA licensing is regulated instead through dynamic Cdt1 and geminin interactions (Kisielewska and Blow, 2012). In agreement with this it has previously been shown that a Cdt1-geminin complex is capable of both competent DNA licensing and prevention of DNA re-replication. This suggests that a Cdt1-geminin complex may act as a molecular switch to shift between licensing active- and inactive- states (Lutzmann *et al.*, 2006). It is likely that the switch between licensing states of the Cdt1-geminin complex is induced by changing stoichiometry of the complex. Indeed a heterohexamer consisting of a 2:4 ratio of Cdt1:geminin is unable to license the DNA due to an inability to engage the MCM complex (De Marco *et al.*, 2009).

Although the N-terminal region of Cdt1 is necessary for its degradation in mammalian somatic cells (Nishitani *et al.*, 2004), the lack of Cdt1 degradation in pre-MBT cells (Kisielewska and Blow, 2012) suggests that $\Delta Cdt1^{243-620}$ perturbs licensing regulation via a separate mechanism. Given that the stoichiometry of the Cdt1:geminin complex may be important for the licensing activity of Cdt1 (Lutzmann *et al.*, 2006; De Marco *et al.*, 2009), it is possible that $\Delta Cdt1^{243-620}$ disturbs the balance between Cdt1 and

geminin leading to the Cdt1:geminin complex conforming to the licensing active conformation. Since the Cdt1:geminin complex is unable to license DNA when in a 2:4 ratio complex (De Marco *et al.*, 2009) it is possible that the presence of $\Delta Cdt1^{243-620}$ lead to the levels of Cdt1 outweighing geminin and preventing formation of the licensing inactive 2:4 ratio complex. This would allow licensing active Cdt1:geminin complexes or Cdt1 alone to re-license the DNA.

The main geminin binding domain of Cdt1 lies in the central region of the protein. However there is evidence of a second region for geminin binding towards the N-terminal of Cdt1 (Saxena *et al.*, 2004; Ferenbach *et al.*, 2005). It is therefore possible that the $\Delta Cdt1^{243-620}$ construct is at least partially resistant to geminin inhibition (Ferenbach *et al.*, 2005). The DNA damage and cell cycle arrest triggered by the $\Delta Cdt1^{243-620}$ construct may therefore be due to the ability to avoid regulation via geminin binding thereby allowing $\Delta Cdt1^{243-620}$ to re-license already replicated DNA.

It has been widely documented that there several regulatory domains present at the N-terminal region of Cdt1 including a PIP box for PCNA interaction, destruction boxes, ubiquitination sites and a cy motif for phosphorylation by CDKs (reviewed in (Caillat and Perrakis, 2012)). Human Cdt1 is phosphorylated at the cy motif by cyclin A-Cdk complexes which then targets Cdt1 for degradation by the SCF^{Skp2} E3 ubiquitin ligase complex. However the phosphorylation at the cy motif also reduces the DNA binding activity of Cdt1. This suggests that Cdt1 phosphorylation in the absence of degradation may also have an inhibitory effect on Cdt1 licensing activity (Sugimoto *et al.*, 2004).

Chapter 5.

Results III. Cdt1 Regulation via Mechanisms Mediated at the N-terminus

Region

5.1 Introduction

The results presented in Chapter 4 show that when a truncated, mutant form of Cdt1 which lacks the N-terminal region (ΔCdt1$^{243-620}$) is expressed in pre-MBT *Xenopus* embryonic cells, deregulated Cdt1 activity leads to re-licensing, re-replication, DNA damage and ultimately cell cycle arrest. There are two possible mechanisms by which the ΔCdt1$^{243-620}$ construct avoids regulation. Firstly, ΔCdt1$^{243-620}$ may override regulation by geminin either by disruption of the ratio of Cdt1 to geminin within the cell (Lutzmann *et al.*, 2006; De Marco *et al.*, 2009; Kisielewska and Blow, 2012) or due to a partial resistance to inhibition by geminin (Ferenbach *et al.*, 2005). Secondly, although Cdt1 levels remain stable within the pre-MBT embryo, there may be post-translational modifications directed at the N-terminal region which inhibit Cdt1 licensing activity in the absence of degradation. Indeed there is evidence that in human cells phosphorylation of Cdt1 at the N-terminal cy motif reduces the DNA binding activity of Cdt1 as well as targeting the protein for degradation (Sugimoto *et al.*, 2004). Reduced DNA binding activity of Cdt1 could potentially reduce the licensing activity of Cdt1 since DNA binding is required for the loading of the MCM complex, the final step in producing a licensed DNA origin (Takara and Bell, 2011).

It was next important to determine which of the two possible mechanisms were involved in the mis-regulation of Cdt1 activity which lead to the cell cycle arrest observed with the ΔCdt1$^{243-620}$ construct. A construct consisting of only the N-terminal region of Cdt1 provides a tool to investigate the possibility that Cdt1 is regulated by post-translational modifications such as ubiquitination or phosphorylation in pre-MBT cells. The N-terminal ΔCdt1$^{1-243}$-RFP construct produced in Chapter 3 is contained within both the mRNA and protein expression vectors pRN3 and pET-32a(+) respectfully. Expression of ΔCdt1$^{1-243}$-RFP mRNA within *Xenopus* embryos would allow the regulation of this region of Cdt1 to be investigated *in vivo* while the ΔCdt1$^{1-243}$-RFP recombinant protein provides a tool for investigation of regulation in the *in vitro Xenopus* egg extract system.

5.2 Results

The N-terminal region of Cdt1 contains sites for both phosphorylation and ubiquitination. To examine the regulatory mechanisms targeted to the N-terminal region of Cdt1 in *Xenopus* pre-MBT embryos, mRNA for the ΔCdt1$^{1\text{-}243}$-RFP construct was produced for microinjection into *Xenopus* embryos. The pRN3 vector containing ΔCdt1$^{1\text{-}243}$-RFP construct DNA which was developed in Chapter 3 was used to produce ΔCdt1$^{1\text{-}243}$-RFP mRNA by *in vitro* transcription. An 11 ng amount of ΔCdt1$^{1\text{-}243}$-RFP mRNA was then microinjected into one cell of a two cell embryo at 1 hour 30 min post-fertilisation. The embryos were then maintained in optimal conditions for growth and their development monitored.

Figure 5.1 shows development of a ΔCdt1$^{1\text{-}243}$-RFP injected embryo compared to a non-injected control between 2 hours and 48 hours post-fertilisation. The cell injected with ΔCdt1$^{1\text{-}243}$-RFP continues to divide as normal with no evidence of cell cycle arrest or slowing of cell division. At 48 hours post-fertilisation the embryo has developed normally into a tadpole as with the non-injected control embryo. The absence of cell cycle arrest in the presence of the ΔCdt1$^{1\text{-}243}$-RFP construct is to be expected since this N-terminal construct lacks the MCM binding domain which is essential for the licensing activity of Cdt1. The ΔCdt1$^{1\text{-}243}$-RFP construct is therefore unable to induce re-licensing and re-replication as observed with the ΔCdt1$^{243\text{-}620}$ construct in Chapter 4.

Time post-fertilisation/hr
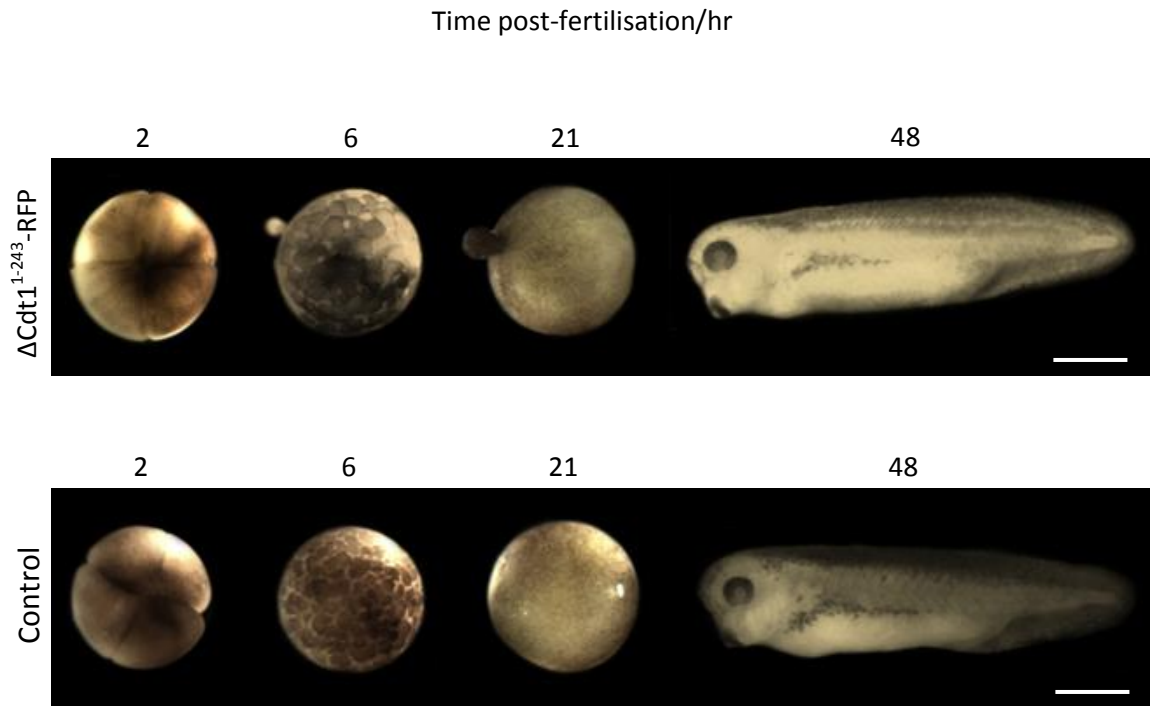
Figure 5.1. Microinjection of ΔCdt1$^{1\text{-}243}$-RFP mRNA in *Xenopus* embryos. 11 ng of ΔCdt1$^{1\text{-}243}$-RFP mRNA was injected into 1 cell of a 2 cell embryo (developmental stage 2) and embryo development monitored until 48 hours after fertilisation. The control shows non-injected embryo development monitored over a 48 hour period following fertilisation. Scale bar is 500 μm.

It was next important to establish whether or not the $\Delta Cdt1^{1-243}$-RFP mRNA was expressed within the injected embryos. Single embryo samples were collected every 10 minutes from 50 minutes post-injection over a 2 hour 40 minute period. The extracts from each embryo sample containing the total protein from the embryo were run on single lanes on an SDS-PAGE gel and immunoblotted for RFP to detect the presence of expressed $\Delta Cdt1^{1-243}$-RFP. Figure 5.1A shows that a band is detected by RFP antibody confirming expression of the $\Delta Cdt1^{1-243}$-RFP mRNA. In comparison no band was detected by RFP antibody in the control non-injected embryo samples (Figure 5.2B). Geminin was used as a loading control since geminin levels remain stable throughout the cell cycle in pre-MBT embryos (Kisielewska and Blow, 2012).

Although $\Delta Cdt1^{1-243}$-RFP expression was detected, the embryos at time points 02:40, 02:50 and 03:10 do not show a band detected by RFP. Also the embryos at time points 02:20, 03:00, 03:20, 03:40 show a much smaller band than time points 02:30, 03:30, 03:50 and 04:00. The differing densities of the bands are not due to sample loading as the loading control geminin remains constant (Figure 5.2A). There are two possibilities for the differing band densities of $\Delta Cdt1^{1-243}$-RFP. Firstly, it could be that the $\Delta Cdt1^{1-243}$-RFP mRNA was expressed and then subsequently degraded within the cell. Since Cdt1 is not significantly degraded during the pre-MBT cell cycle (Kisielewska and Blow, 2012) this would mean that the full length Cdt1 protein is somehow protected from degradation while the N-terminal $\Delta Cdt1^{1-243}$-RFP construct is not. This could possibly be achieved by binding to other proteins with the complex being resistant to degradation. One potential interaction partner which could protect Cdt1 from degradation is geminin. With the main geminin binding site on Cdt1 located at the central region of the protein, the $\Delta Cdt1^{1-243}$-RFP has only weak geminin binding activity (Ferenbach et al., 2005). The weak geminin binding activity could therefore decrease the interaction between geminin and $\Delta Cdt1^{1-243}$-RFP allowing $\Delta Cdt1^{1-243}$-RFP to escape the complex to be degraded. Secondly, it is also possible that since separate single embryo samples are used for each time point it could be that there are differing levels of expression or a lack of expression within different embryos.

To determine whether or not the N-terminal region of Cdt1 is targeted for regulation by mechanisms involving ubiquitination in pre-MBT embryos the ubiquitin state of $\Delta Cdt1^{1-243}$-RFP was examined. The embryo samples expressing $\Delta Cdt1^{1-243}$-RFP were

immunoblotted for ubiquitin at the site on the blot of the ΔCdt1$^{1-243}$-RFP construct. Figure 5.2A shows two bands just above the ΔCdt1$^{1-243}$-RFP construct detected by ubiquitin antibody. However, the bands were also present in the embryo samples at time points 02:40, 02:50 and 03:10 which did not show a band for ΔCdt1$^{1-243}$-RFP mRNA expression (Figure 5.2A). This suggests that the ubiquitin bands may be background unspecific binding or ubiquitination at a separate protein of similar size within the embryo rather than ubiquitination of the ΔCdt1$^{1-243}$-RFP construct.

A

Time post-fertilisation/hr:min

Ubiquitin

ΔCdt1$^{1-243}$-RFP

Geminin

B

Time post-fertilisation/hr:min

ΔCdt1$^{1-243}$-RFP

Geminin
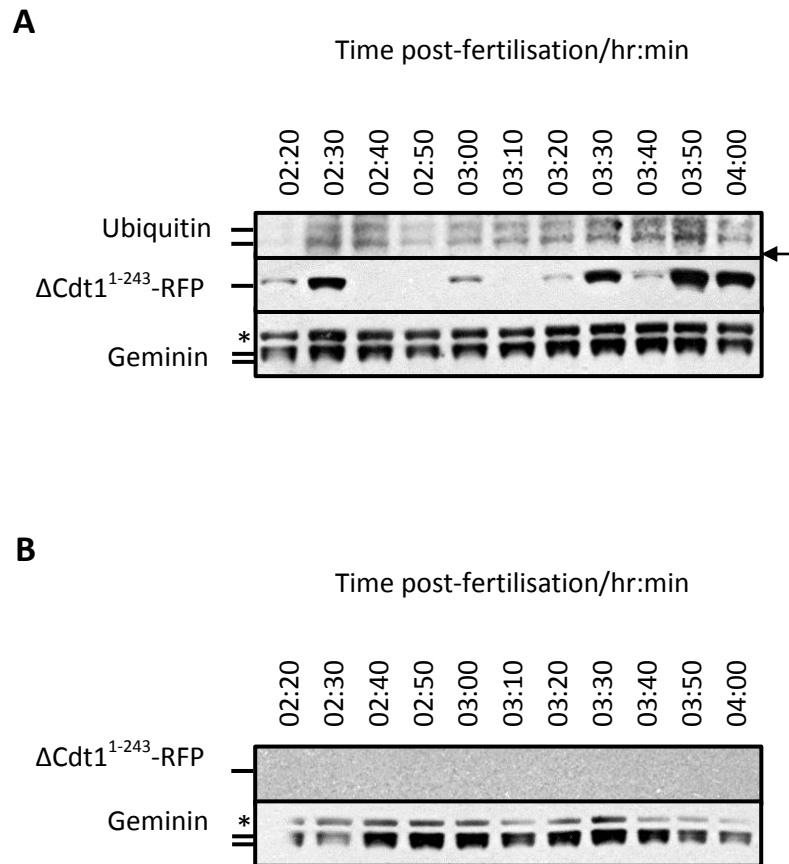
Figure 5.2. ΔCdt1$^{1-243}$-RFP mRNA expression in early *Xenopus* embryos. (A) Embryos were injected with 11 ng ΔCdt1$^{1-243}$-RFP mRNA into one cell of a two cell embryo (developmental stage 2). Single embryo samples were collected every 10 minutes from 2 hours 20 minutes to 4 hours after fertilisation, ran on an SDS-PAGE gel and blotted for RFP to detect the presence of ΔCdt1$^{1-243}$-RFP. Ubiquitin antibody was used to detect ubiquitination of ΔCdt1$^{1-243}$-RFP. The arrow marks the location of ΔCdt1$^{1-243}$-RFP on the blot. Geminin was used as a loading control. Asterisk indicates unspecific binding. **(B)** Control non-injected embryos were also collected every 10 minutes from 2 hours 20 minutes to 4 hours post-fertilisation and blotted for RFP and geminin. Asterisk indicates unspecific binding.

Since it appears that the ΔCdt1$^{1-243}$-RFP construct may not be ubiquitinated in pre-MBT embryos, it was next important to examine the ubiquitin state of wild type full length Cdt1 to confirm whether or not ubiquitination plays a role in Cdt1 regulation in early embryos. To investigate the ubiquitin state of full length Cdt1, *Xenopus* embryos were placed in buffer containing the proteasome inhibitor MG132. MG132 inhibits the 26S proteasome complex and therefore prevents degradation of ubiquitinated proteins (Lee and Goldberg, 1998). Consequently there is an accumulation of ubiquitinated proteins within the cell. Although Cdt1 is not regulated by degradation (Kisielewska and Blow, 2012), if there is even a small amount of ubiquitin mediated degradation in pre-MBT embryos the MG132 would cause an accumulation of ubiquitinated Cdt1 over time.

Xenopus embryos were placed in 0.1x MMR buffer supplemented with 0.4 mM MG132 at 2 hours post-fertilisation. The MG132 stock was diluted in DMSO, therefore the control embryos were placed in 0.1x MMR buffer supplemented with an equal volume of DMSO as added to the MG132 treated embryos. Figure 5.3A shows that the embryo continues to divide and progress normally through the early cleavage cell cycles until 6 hours post-fertilisation at which point the embryo undergoes the MBT. Following the MBT the cells of the embryo become less defined and begin to apoptose up until 10 hours post-fertilisation. By 24 hours all MG132 treated embryos had died and ruptured. In contrast the DMSO treated embryos continued to divide normally through the MBT to develop into normal tadpoles at 48 and up to 96 hours post-fertilisation (Figure 5.3B). This suggests that the MG132 was responsible for the cell death within the embryos which occurred at approximately MBT. There are two possible explanations for embryo death only after the MBT following treatment with MG132. Firstly, the MG132 may be slow to permeate the cells of the embryo leading to cell death occurring 4 to 5 hours following supplementation of the media with MG132. However, MG132 is cell permeable and a highly potent inhibitor of the proteasome (Lee and Goldberg, 1998). The second possibility is that protein degradation is not a main mechanism of regulation during the pre-MBT embryonic cell cycles. This is in line with data showing that in pre-MBT embryos, Cdt1 and geminin levels remain stable (Kisielewska and Blow, 2012). Once the embryo goes through the MBT the cell cycles become somatic like as zygotic transcription begins and the cell cycles become longer

and asynchronous with distinct gap phases G1 and G2 (Newport and Kirschner, 1982; O'Farrell *et al.*, 2004). In somatic cells and *Xenopus* egg extract, both Cdt1 and geminin are degraded (McGarry and Kirschner, 1998; Li and Blow, 2005). This would explain the onset of MG132 induced cell and embryo death only after the MBT.

A

Time post-fertilisation/hr:min

02:30   06:00   08:00   10:00

MG132

B

Time post-fertilisation/hr

26   48   96

DMSO Control

C

Time post-fertilisation/hr:min

04:00 04:10 04:20 04:30 04:40 04:50 05:00

Ubiquitin

Cdt1

β-actin

D

Time post-fertilisation/hr:min

04:00 04:10 04:20 04:30 04:40 04:50 05:00

Cdt1
Geminin
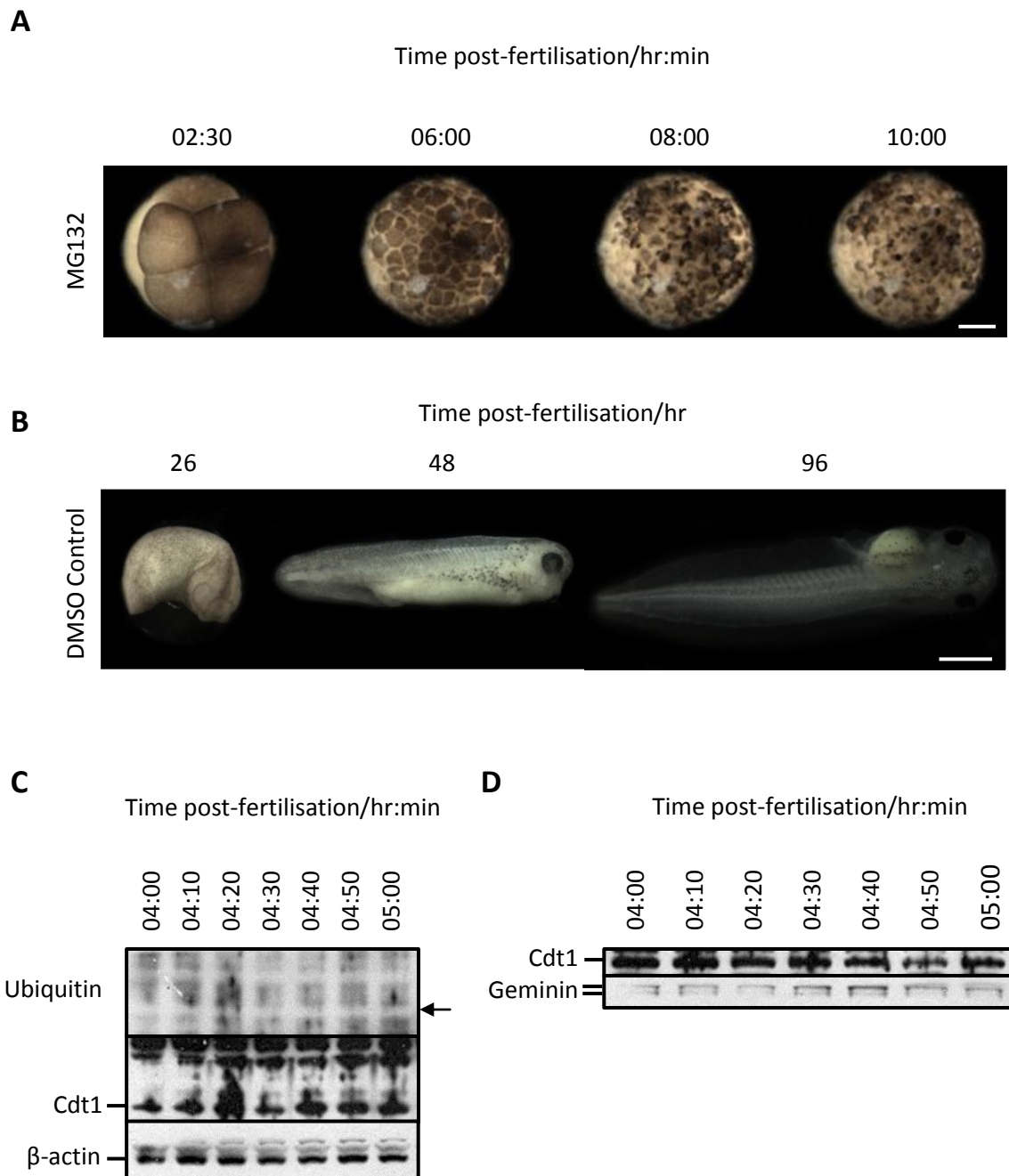
Figure 5.3. *Xenopus laevis* **embryos treated with the proteasome inhibitor MG132. (A)** Embryos were placed in 0.1x MMR buffer containing 0.4 mM MG132 (solubilised in DMSO) and their development monitored. Scale bar is 250 μM **(B)** Control embryos were placed in 0.1x MMR buffer containing DMSO. Scale bar is 500 μM. **(C)** Single MG132 treated embryo samples were collected every 10 min from 4 hours after fertilization, ran on an SDS-PAGE gel and blotted for Cdt1, ubiquitin and β-actin. The arrow marks the location of Cdt1 on the blot. β-actin was used as a loading control. **(D)** Single DMSO treated embryo samples were collected every 10 min from 4 hours after fertilization, ran on an SDS-PAGE gel and blotted for Cdt1 and geminin. Geminin was used as a loading control.

To detect ubiqutination of Cdt1, single MG132 treated embryos and DMSO controls were collected every 10 minutes from 4-5 hours post-fertilisation (2-3 hours post-treatment). Since the pre-MBT cell cycle length is approximately 25 minutes, this range of time points encompasses at least one whole cell cycle. The whole cell extract of the embryos were then run, in single lanes for each time point, on an SDS-PAGE gel. Figure 5.3C shows the MG132 treated embryo samples immunoblotted for Cdt1 and ubiquitin. As a loading control the samples were also immunoblotted for β-actin. The western blot shows that there is no accumulation of Cdt1 over the 1 hour time period in the presence of MG132. The Cdt1 levels do not show any accumulation in comparison to the Cdt1 levels in the control DMSO treated embryos (Figure 5.3D). This provides further evidence that Cdt1 is not regulated by proteolysis in pre-MBT *Xenopus* embryos. There is also neither a clear ubiquitin chain present at wild type Cdt1 nor an accumulation in the ubiquitin signal over time which would be expected if Cdt1 was regulated by ubiquitination (Figure 5.3C). This suggests that in pre-MBT embryos Cdt1 may not be regulated by ubiquitination. Another possibility is that the ubiquitin antibody is inadequate for detection of ubiquitinated Cdt1. Combined with the lack of detectable ubiquitination of the $\Delta Cdt1^{1-243}$-RFP construct (Figure 5.2) this provides evidence that Cdt1 may not be ubiquitinated during the pre-MBT cell cycle.

It has been shown previously that the N-terminal region of Cdt1 is necessary to allow degradation and that without this region Cdt1 becomes stabilised (Nishitani *et al.*, 2004). Since there was varying levels of $\Delta Cdt1^{1-243}$-RFP protein in the embryos injected with $\Delta Cdt1^{1-243}$-RFP mRNA which could be due to degradation or differing mRNA expression levels (Figure 5.2) it was next important to characterise the degradation of the $\Delta Cdt1^{1-243}$-RFP construct. It was also important to confirm that the RFP tag does not interfere with the degradation and that the $\Delta Cdt1^{1-243}$-RFP construct is capable of undergoing degradation.

In *Xenopus* egg extract endogenous Cdt1 levels decrease upon release from metaphase by calcium addition. In line with the N-terminal region being necessary for Cdt1 degradation, a mutant Cdt1 construct lacking the N-terminal 242 amino acids was stable in *Xenopus* egg extract upon release from metaphase (Li and Blow, 2005). Since the N-terminal region of Cdt1 is required for degradation it was assumed that the $\Delta Cdt1^{1-243}$-RFP construct would follow a similar pattern of degradation as with

endogenous Cdt1 in *Xenopus* egg extract upon release from metaphase. To test this theory, *Xenopus* egg extract was supplemented with 20 ng/μl sperm DNA and 19 ng/μl ΔCdt1$^{1-243}$-RFP recombinant protein, released from metaphase with 0.3 mM CaCl$_2$ and the levels of ΔCdt1$^{1-243}$-RFP monitored. At 10 to 20 minute intervals following activation 1 μl samples were collected and blotted for RFP to detect ΔCdt1$^{1-243}$-RFP levels. The final extract sample was collected at 120 minutes following activation because by this time both DNA licensing and replication are complete.

Figure 5.4 shows that in activated egg extract supplemented with 19 ng/μl ΔCdt1$^{1-243}$-RFP, the level of ΔCdt1$^{1-243}$-RFP decreases over time. As a control *Xenopus* egg extract was supplemented with 20 ng/μl sperm DNA and 19 ng/μl ΔCdt1$^{1-243}$-RFP in the absence of CaCl$_2$ addition and therefore absence of activation. In the non-activated egg extract ΔCdt1$^{1-243}$-RFP levels remain stable as is the case with endogenous Cdt1. This again suggests that in activated *Xenopus* egg extract which has been released from metaphase arrest, ΔCdt1$^{1-243}$-RFP is degraded over time. In addition when the egg extract is not activated and held under metaphase arrest, ΔCdt1$^{1-243}$-RFP is not degraded as is also the case with endogenous Cdt1.
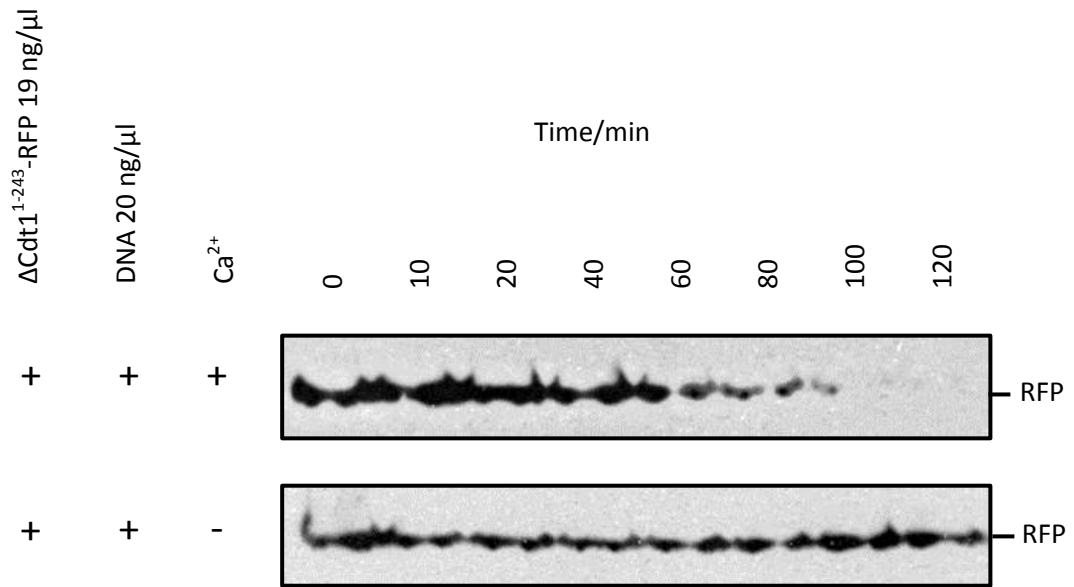
Figure 5.4. Levels of ΔCdt1$^{1-243}$-RFP over time in activated and non-activated *Xenopus* egg extract. *Xenopus* extract was supplemented with 19 ng/µl ΔCdt1$^{1-243}$-RFP protein, 20 ng/µl DNA and +/- 0.3 mM CaCl$_2$. 1µl samples were collected and blotted for RFP to detect ΔCdt1$^{1-243}$-RFP protein at time points selected to cover DNA licensing and replication. The blot labelled '+' Ca^{2+} shows samples from active extract and the '-' Ca^{2+} blot shows samples from non-active (metaphase arrested) extract.

On the western blot Figure 5.4 the bands of $\Delta Cdt1^{1-243}$-RFP appear to decrease in intensity over time following activation of the extract. To quantify the western blots and confirm the decrease in band intensity densitometry analysis was used. For the densitometry analysis Image J was used to calculate the densities of each band on each of the western blots. The density of each band was then divided by the density of the first band, corresponding to time point 0 min, to give the relative densities. Therefore the density of the band for time point 0 is always 1 and a decrease in relative density of each band over time is indicative of a decrease in band density and therefore a decrease in $\Delta Cdt1^{1-243}$-RFP levels. Each density was made relative to the density of the band for time point 0 because as the first time point this corresponds to the starting level of $\Delta Cdt1^{1-243}$-RFP within the extract.

To ensure the degradation of $\Delta Cdt1^{1-243}$-RFP in activated *Xenopus* egg extract was reproducible the experiment was repeated twice more under the same conditions. Again *Xenopus* egg extract was supplemented with 20 ng/µl sperm DNA, 19 ng/µl $\Delta Cdt1^{1-243}$-RFP and +/- 0.3 mM $CaCl_2$. Every 10 to 20 minutes between 0 and 120 following +/- calcium addition, a 1 µl sample was collected, ran on an SDS-PAGE gel and blotted for RFP to detect $\Delta Cdt1^{1-243}$-RFP. The band intensities of each $\Delta Cdt1^{1-243}$-RFP band on the western blots, including those shown in Figure 5.4, were calculated using image J. Using the band intensities the relative density of each band was calculated by dividing the density of each band by the density of the band corresponding to time point 0 as the band at 0 minutes represents the starting level of $\Delta Cdt1^{1-243}$-RFP within the extract. The relative densities for each experiment (n=3) were then entered into the statistical software program GraphPad Prism and plotted on a graph as mean ±SEM (Figure 5.5). The linear regression lines for the relative densities of the $\Delta Cdt1^{1-243}$-RFP bands were then plotted for $\Delta Cdt1^{1-243}$-RFP levels in active ($+Ca^{2+}$) and inactive ($-Ca^{2+}$) *Xenopus* egg extract. Figure 5.5 shows that there is a decrease in $\Delta Cdt1^{1-243}$-RFP band density over time in active but not inactive egg extract.

To determine whether the difference in $\Delta Cdt1^{1-243}$-RFP levels between active and inactive egg extract is significantly different an analysis of covariance (ANCOVA) was carried out using GraphPad Prism. The ANCOVA tested the null hypothesis that the slopes of the linear regression lines for $\Delta Cdt1^{1-243}$-RFP levels in active and inactive egg

extract are identical. The ANCOVA analysis gave a p value of $p < 0.0001$ thus there is a statistically significant difference between $\Delta Cdt1^{1-243}$-RFP levels in active extract compared to inactive extract. In addition the slops of the linear regression lines were also tested against the null hypothesis that the slope was equal to zero. The analysis of the linear regression line slopes gave a p value of $p < 0.0001$ for $\Delta Cdt1^{1-243}$-RFP levels in active extract and a p value of $p = 0.6528$ for $\Delta Cdt1^{1-243}$-RFP levels in inactive extract. This shows that the slope of the linear regression line of $\Delta Cdt1^{1-243}$-RFP levels in active extract is significantly non-zero. This therefore further confirms that in active *Xenopus* egg extract $\Delta Cdt1^{1-243}$-RFP is degraded over time following activation and release from metaphase.
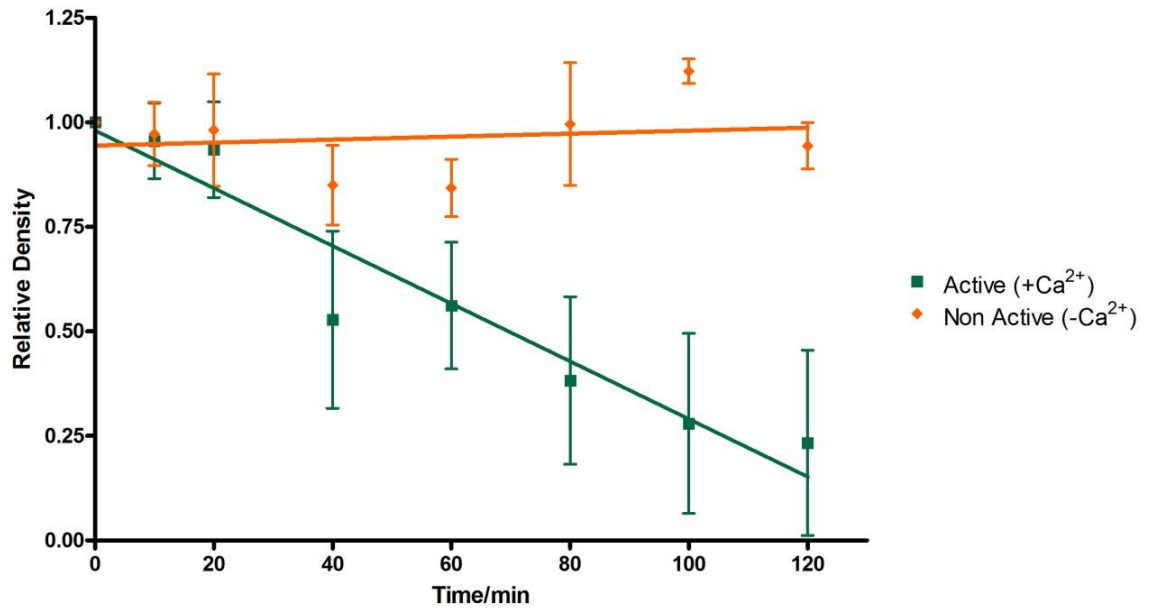
Figure 5.5. Graph showing ΔCdt1$^{1-243}$-RFP band intensities on western blots of Ca^{2+} activated (+Ca^{2+}) and non-activated (-Ca^{2+}) *Xenopus* egg extract. *Xenopus* egg extract was supplemented with 20 ng/μl DNA, 19 ng/μl ΔCdt1$^{1-243}$-RFP recombinant protein and +/- 0.3 mM CaCl$_2$. 1μl samples were taken every 10 to 20 minutes, ran on an SDS-PAGE gel and blotted for RFP to detect ΔCdt1$^{1-243}$-RFP. The band intensities of ΔCdt1$^{1-243}$-RFP bands were measured using Image J software and the relative densities calculated (relative to time point 0) and analysed using Prism software (n=3, p<0.0001).

As shown in Figure 5.4 and Figure 5.5 following release from metaphase, $\Delta Cdt1^{1-243}$-RFP is degraded in *Xenopus* egg extract. It was therefore next important to establish the mechanism behind the degradation. The degradation of endogenous Cdt1 in *Xenopus* egg extract upon release from metaphase is accomplished through ubiquitination mediated by the APC/C (Li and Blow, 2005). It is therefore possible that the $\Delta Cdt1^{1-243}$-RFP construct is also degraded through APC/C-mediated ubiquitination.

To investigate whether or not $\Delta Cdt1^{1-243}$-RFP is degraded by an APC/C mediated mechanism the degradation of $\Delta Cdt1^{1-243}$-RFP in the presence of an APC/C inhibitor was monitored. Proteins which are targeted for degradation by the APC/C contain a conserved destruction box motif of nine amino acids which is necessary for proteolysis. A peptide inhibitor consisting of only these nine amino acids (Dbox) competes for binding to the APC/C and therefore inhibits degradation of APC/C substrates (Peter *et al.*, 2001). *Xenopus* egg extract was therefore supplemented with 20 ng/µl sperm DNA, 19 ng/µl $\Delta Cdt1^{1-243}$-RFP and +/- 2 mM of the APC/C inhibitor Dbox and activated by 0.3 mM $CaCl_2$. At 10 to 20 minute intervals following activation 1 µl samples were collected, run on an SDS-PAGE gel and blotted for RFP to detect $\Delta Cdt1^{1-243}$-RFP levels. Figure 5.6A shows that in the presence of Dbox $\Delta Cdt1^{1-243}$-RFP levels appear to remain relatively stable. In contrast with the control blot $\Delta Cdt1^{1-243}$-RFP levels appear to reduce over time in activated egg extract in the absence of Dbox. This suggests that Dbox inhibits $\Delta Cdt1^{1-243}$-RFP degradation and therefore the degradation is mediated by the APC/C.

The experiment shown in Figure 5.6A was then repeated before performing densitometry analysis on both western blots. For the densitometry analysis Image J was used to calculate the intensities of each band. The band intensities were then used to calculate the relative densities relative to time point 0 as the starting level of $\Delta Cdt1^{1-243}$-RFP. The relative densities were then plotted as mean ±SEM against time and the linear regression lines calculated using GraphPad Prism statistical software (Figure 5.6B). The slops of the linear regression lines were then tested against the null hypothesis that the slope was equal to zero. The analysis of the linear regression line slopes gave a p value of p=0.0043 for $\Delta Cdt1^{1-243}$-RFP levels in the absence of Dbox and a p value of p=0.9499 for $\Delta Cdt1^{1-243}$-RFP levels in the presence of Dbox. This shows that the slope of the linear regression line of $\Delta Cdt1^{1-243}$-RFP levels in the presence of Dbox

is not significantly non-zero and therefore $\Delta Cdt1^{1-243}$-RFP is not degraded in the presence of the APC/C inhibitor Dbox. This is further evidence that $\Delta Cdt1^{1-243}$-RFP is degraded via mechanisms mediated by the APC/C.

The level of degradation of $\Delta Cdt1^{1-243}$-RFP in the absence of Dbox (Figure 5.6) is small and incomplete compared with the level of degradation of $\Delta Cdt1^{1-243}$-RFP in active extract in Figure 5.4 and Figure 5.5. However the levels of $\Delta Cdt1^{1-243}$-RFP degradation were variable between experiments as shown by the error bars for active extract on Figure 5.5. Due to this variability further repeats of the experiment with the Dbox inhibitor would have provided a higher n number and therefore more robust statistics.

A
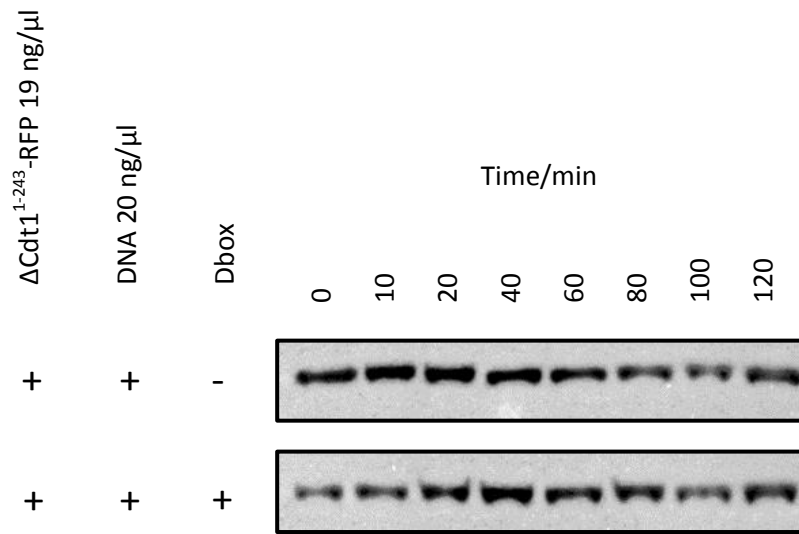
B
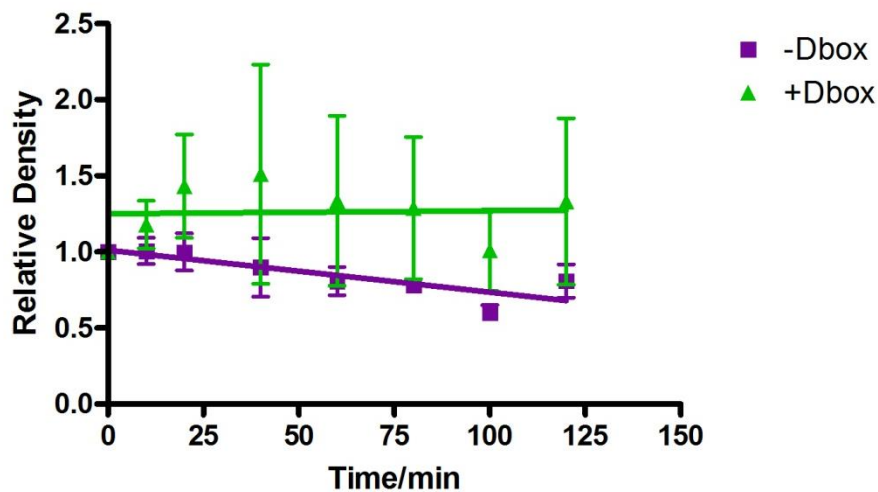
Figure 5.6. Levels of ΔCdt1$^{1-243}$-RFP over time in activated *Xenopus* egg extract in the presence of the APC/C inhibitor Dbox. (A) *Xenopus* egg extract was supplemented with 20 ng/μl DNA, 19 ng/μl ΔCdt1$^{1-243}$-RFP recombinant protein and +/- 2 mM Dbox. 1 μl samples were taken every 10 to 20 minutes, ran on an SDS-PAGE gel and blotted for RFP to detect ΔCdt1$^{1-243}$-RFP. **(B)** The band intensities of ΔCdt1$^{1-243}$-RFP bands were measured using Image J software and the relative densities calculated (relative to time point 0) and analysed using Prism software (n=2).

The N-terminal region of Cdt1 also contains a PIP box motif of eight amino acids which is required for the interaction of Cdt1 with PCNA. The interaction between Cdt1 and PCNA is required for the replication-dependant degradation of Cdt1 during S-phase. PCNA loading at S-phase triggers degradation of Cdt1 via the Cul4 E3 ubiquitin ligase (Arias and Walter, 2005a). Since the PIP box is located within the N-terminal region included in the ΔCdt1$^{1-243}$-RFP construct it is possible that this PCNA dependant mechanism of degradation also plays a role in ΔCdt1$^{1-243}$-RFP degradation.

To investigate whether or not ΔCdt1$^{1-243}$-RFP is degraded by PCNA mediated replication-dependant proteolysis p27 was used to block PCNA loading to chromatin. By blocking PCNA loading initiation of DNA replication is inhibited and therefore replication-dependant mechanisms of degradation are also inhibited. The protein p27 is a CDK inhibitor which inhibits cyclin E-Cdk2 and cyclin A-Cdk2 leading to G1 cell cycle arrest and thus prevents entry into S-phase (Toyoshima and Hunter, 1994). *Xenopus* egg extract was therefore supplemented with 20 ng/µl sperm DNA, 19 ng/µl ΔCdt1$^{1-243}$-RFP plus increasing concentrations of p27 from 26-364 µM and activated by 0.3 mM CaCl$_2$. At 120 minutes post-activation once DNA licensing, and in extract not supplemented with p27, S-phase was complete, 1 µl samples were taken, run on an SDS-PAGE gel and blotted for RFP to detect ΔCdt1$^{1-243}$-RFP. As a control non-activated, metaphase arrested extract was used to show the level of ΔCdt1$^{1-243}$-RFP at 120 minutes when there is no degradation. At the lowest concentration of p27 (26 µM) there is still degradation of ΔCdt1$^{1-243}$-RFP compared to the non-activated control. However, at higher p27 concentrations, from 52-364 µM, the ΔCdt1$^{1-243}$-RFP degradation appears reduced (Figure 5.7A). This suggests that some of the ΔCdt1$^{1-243}$-RFP degradation may be replication-dependant degradation during S-phase.

A

B
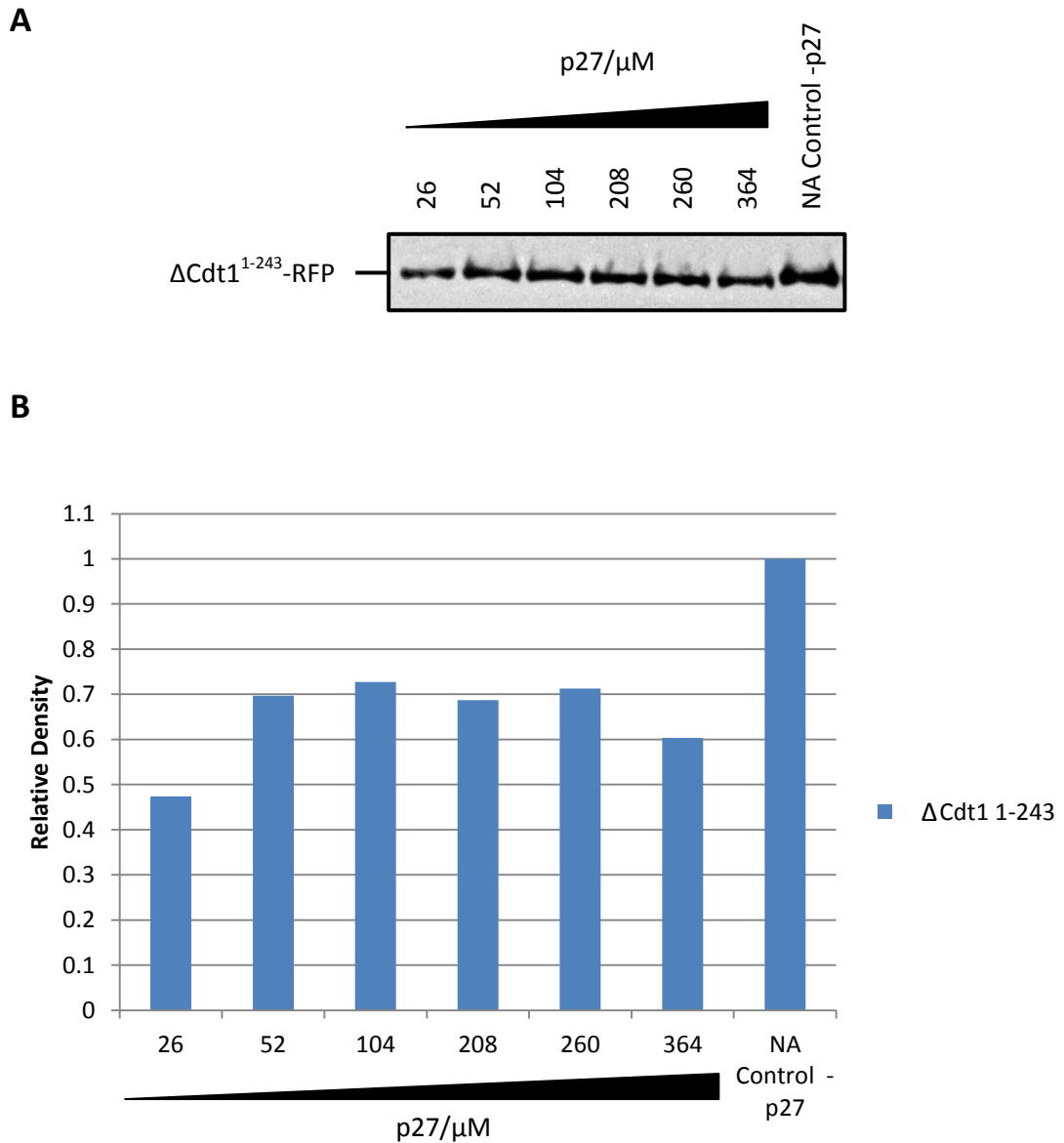
Figure 5.7. Effect of differing concentrations of p27 on ΔCdt1$^{1-243}$-RFP degradation. (A) *Xenopus* egg extract was supplemented with increasing concentrations of p27, 20 ng/µl DNA, 19 ng/µl ΔCdt1$^{1-243}$-RFP and activated with 0.3 mM CaCl$_2$ to release the cell cycle. At 120 minutes post-activation once DNA licensing and S-phase was complete, 1 µl samples were taken and blotted for RFP. The NA control minus p27 shows the level of ΔCdt1$^{1-243}$-RFP in non-activated extract at 120 minutes. **(B)** The relative densities (relative to NA control) of the bands from (A).

To quantify the degradation of $\Delta Cdt1^{1-243}$-RFP in the presence of p27 (Figure 5.7A), Image J was used to measure the band intensities of $\Delta Cdt1^{1-243}$-RFP. The band intensities were then used to calculate the relative densities relative to the non-activated control in which there is no degradation of $\Delta Cdt1^{1-243}$-RFP. The relative density of $\Delta Cdt1^{1-243}$-RFP at 26 µM p27 is just under half (at approximately 0.5) compared to control. The relative density of $\Delta Cdt1^{1-243}$-RFP at each concentration of p27 between 52 and 364 µM is between approximately 0.6-0.7. This suggests that at higher concentrations p27 prevents some of the degradation of $\Delta Cdt1^{1-243}$-RFP. However, the relationship between p27 concentration and prevention of degradation of $\Delta Cdt1^{1-243}$-RFP is not reciprocal suggesting that beyond a concentration of 52 µM p27 the inhibition of $\Delta Cdt1^{1-243}$-RFP degradation plateaus (Figure 5.7B). The densitometry analysis confirms that at higher concentrations p27 reduces the degradation of $\Delta Cdt1^{1-243}$-RFP and therefore PCNA mediated replication-dependant mechanisms at least partially mediate $\Delta Cdt1^{1-243}$-RFP degradation.

In *Xenopus* egg extract $\Delta Cdt1^{1-243}$-RFP is degraded upon release from metaphase following activation with calcium Figure 5.4. However, the extract was also supplemented with 20 ng/µl sperm DNA. It has previously been reported that in *Xenopus* egg extract when the DNA concentration is low, endogenous Cdt1 levels remain stable following release from metaphase (Kisielewska and Blow, 2012).

The next step was therefore to investigate whether or not the $\Delta Cdt1^{1-243}$-RFP construct also stabilised in *Xenopus* egg extract under low DNA conditions upon release from metaphase. *Xenopus* egg extract was therefore supplemented with lower concentrations of 2.5- or 0.15- ng/µl DNA plus 19 ng/µl $\Delta Cdt1^{1-243}$-RFP and released from metaphase arrest using 0.3 mM $CaCl_2$. At 10 to 20 minute intervals following activation 0.5 µl samples were collected, run on an SDS-PAGE gel and blotted for RFP to detect $\Delta Cdt1^{1-243}$-RFP levels. Figure 5.8 shows that at both 2.5- and 0.15- ng/µl DNA the level of $\Delta Cdt1^{1-243}$-RFP remains constant over a 120 minute time period following release from metaphase. The 120 minute time period covers both DNA licensing and DNA replication within the extract. As a control, the level of $\Delta Cdt1^{1-243}$-RFP in non-activated *Xenopus* egg extract in which there is no $\Delta Cdt1^{1-243}$-RFP degradation was sampled following a 120 minute incubation period (sample NA 120). This suggests that

as with endogenous Cdt1, ΔCdt1$^{1-243}$-RFP also stabilises upon release from metaphase in *Xenopus* egg extract supplemented with low DNA concentrations.
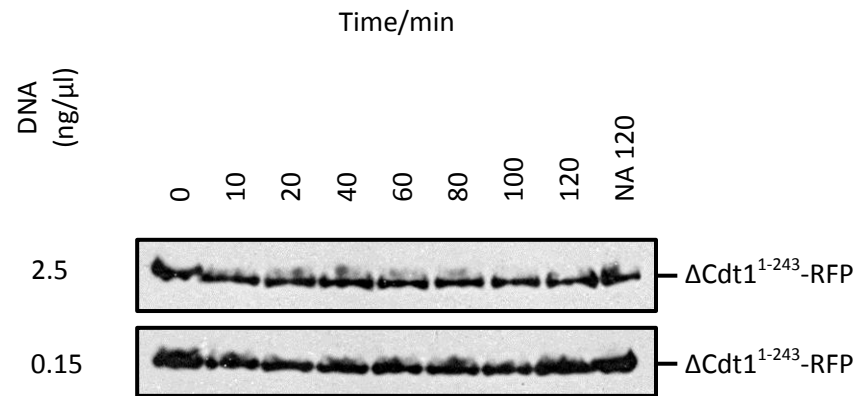
Figure 5.8. ΔCdt1$^{1-243}$-RFP protein degradation in *Xenopus* egg extract under low DNA conditions. *Xenopus* extract was supplemented with 19 ng/µl ΔCdt1$^{1-243}$-RFP protein and either 2.5- or 0.15- ng/µl DNA then activated with 0.3 mM CaCl$_2$ to undergo one cell cycle. At time points chosen to cover DNA licensing and replication, 0.5µl samples were taken and blotted for RFP to detect ΔCdt1$^{1-243}$-RFP protein. The 'NA 120' sample is a non-activated control sample at 120 minutes post-activation to show ΔCdt1$^{1-243}$-RFP level when the cell cycle is not triggered and so no degradation should occur.

To ensure the stabilisation of $\Delta Cdt1^{1-243}$-RFP in activated *Xenopus* egg extract supplemented with lower concentrations of DNA was reproducible the experiment was repeated twice more under the same conditions. Again *Xenopus* egg extract was supplemented with 2.5- or 0.15 ng/μl sperm DNA plus 19 ng/μl $\Delta Cdt1^{1-243}$-RFP and activated with 0.3 mM $CaCl_2$ to release from metaphase arrest. Every 10 to 20 minutes between 0 and 120 following calcium addition and release from metaphase, a 0.5 μl sample was collected, ran on an SDS-PAGE gel and blotted for RFP to detect $\Delta Cdt1^{1-243}$-RFP. Image J was then used to calculate the band intensities of the $\Delta Cdt1^{1-243}$-RFP band from Figure 5.8 and the subsequent repeats. Using the band intensities the relative density of each band was calculated relative to time point 0 as this band represents the starting level of $\Delta Cdt1^{1-243}$-RFP. The relative densities for each experiment (n=3) were then plotted as mean ±SEM against time and the linear regression lines calculated using GraphPad Prism statistical software (Figure 5.9). The slops of the linear regression lines were then tested against the null hypothesis that the slope was equal to zero. If the slope is equal to zero there is no rate of change over time and therefore no degradation of $\Delta Cdt1^{1-243}$-RFP. The analysis of the linear regression line slopes gave a p value of p=0.2218 for $\Delta Cdt1^{1-243}$-RFP levels in extract supplemented with 2.5 ng/μl DNA and a p value of p=0.5598 for $\Delta Cdt1^{1-243}$-RFP levels in extract supplemented with 0.15 ng/μl DNA. These p values indicate that the slopes of the linear regression lines do not significantly deviate from zero. This therefore confirms that as with endogenous Cdt1, in egg extract supplemented with low concentrations of DNA, $\Delta Cdt1^{1-243}$-RFP is not degraded following release from metaphase arrest.
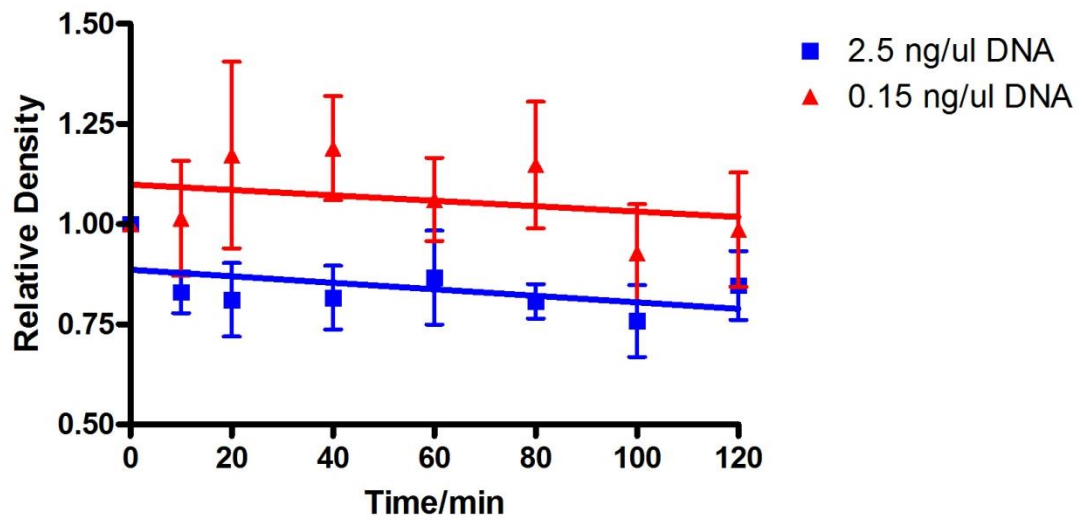
Figure 5.9. ΔCdt1$^{1-243}$-RFP levels in activated *Xenopus* egg extract with low DNA concentrations. *Xenopus* egg extract was supplemented with 19 ng/μl ΔCdt1$^{1-243}$-RFP recombinant protein, either 2.5- or 0.15- ng/μl DNA and activated with 0.3 mM CaCl$_2$. 1 μl samples were taken at various time points post-activation, ran on an SDS-PAGE gel and immunoblotted for RFP. The RFP band intensities were measured using Image J software and relative densities calculated and analysed using Prism statistical software (n=3).

Although the results shown in Figure 5.2 and Figure 5.3 provide evidence that Cdt1 may not be regulated by ubiquitination in pre-MBT embryos, there may be regulation via other mechanisms of post-translational modification such as phosphorylation. To investigate this possibility the phosphorylation state of wild type Cdt1 was investigated. The Cdt1 antibody is polyclonal and as such also recognises unspecific bands on western blots as well as Cdt1. One of the ways in which the embryo samples can be purified to produce an extract containing less non-specific proteins and therefore reduce unspecific binding is to isolate the chromatin and analyse only chromatin bound proteins. Since Cdt1 is a DNA licensing protein it will be present in the isolated chromatin samples when it is DNA bound. It is also reasonable to assume that the nuclear and DNA bound pools of Cdt1 within the embryonic cells would need to be tightly regulated in order to prevent DNA re-licensing. Therefore analysis of the phosphorylation state of chromatin bound Cdt1 would be appropriate.

To examine the phosphorylation state of wild type Cdt1 the chromatin was therefore isolated from synchronously dividing early embryos. Prior to the MBT the cell cycles of each cell within the embryo are synchronous. By also synchronising fertilisation and therefore cell division of each embryo within the entire batch, the isolated chromatin from 0.5 ml of embryos represents one time point of the cell cycle specifically (Kisielewska and Blow, 2012). The chromatin was isolated from 0.5 ml of synchronous embryos every 5 minutes from 4 hours to 4 hours 50 minutes post-fertilisation. Since one complete pre-MBT cell cycle takes approximately 25 minutes these time points were chosen to encompass at least one whole cell cycle. Also the fertilised egg contains one compete copy of the genome which is then duplicated and transferred to the daughter cell during each cell division. Therefore the total level of DNA within the embryo starts low and grows exponentially with each division. The chromatin was isolated form embryos from 4 hours post-fertilisation to allow sufficient quantities of DNA to allow reliable isolation.

Figure 5.10A shows a band detected by phosphoserine antibodies which overlaps with the location of the Cdt1 band on the blot. The chromatin samples were immunoblotted using the phosphorylation antibodies before immunoblotting for Cdt1 to ensure that the Cdt1 signal did not interfere with the phosphorylation signal. The histones were stained as a loading control and to ensure uncontaminated isolation of

the chromatin. This could suggest that in pre-MBT embryos Cdt1 may be at least partially regulated by phosphorylation of serine residues. However it is also possible that the phosposerine band detected represents phosphorylation of a separate protein, distinct from Cdt1, which is of the same molecular weight as Cdt1.

Since phosphorylation of Cdt1 could potentially occur at threonine residues as well as serine residues the Cdt1 section of the blot was also immunoblotted for phosphothreonine. Figure 5.10A shows that no phosphothreonine band was detected at Cdt1 which could suggest that chromatin bound Cdt1 is not phosphorylated at threonine. However since there is no positive control for phosphothreonine it as also possible that the phosphothreonine antibody did not work.

To attempt to determine whether or not the serine phosphorlation detected in Figure 5.10A was Cdt1 phosphorylation, the phosphorylation state of the ΔCdt1$^{1\text{-}243}$-RFP construct was investigated. If Cdt1 undergoes phosphorylation at serine residues as a mechanism of regulation it is likely that the phosphorylation occurs at the N-terminal regulatory region which is known to be phosphorylated in mammalian cells. To investigate this possibility the ability of the ΔCdt1$^{1\text{-}243}$-RFP protein construct to undergo phosphorylation in *Xenopus* egg extract was investigated. One method which would allow isolation and purification of the ΔCdt1$^{1\text{-}243}$-RFP construct from the extract in order to check the phosphorylation state would be immunoprecipitation (IP). As the Cdt1 antibody is polyclonal and also recognises unspecific bands, RFP antibody has so far been used to detect the RFP tagged constructs as it is monoclonal and therefore highly specific. Since the RFP antibodies used to detect the ΔCdt1$^{1\text{-}243}$-RFP construct have not been tested for use in IP experiments by the manufacturer (Abcam, product number ab65856), it was first important to establish whether or not IP using these RFP antibodies would successfully isolate the ΔCdt1$^{1\text{-}243}$-RFP construct from the extract. To test the RFP antibodies for use in IP experiments the RFP antibodies were coupled to protein A sepharose beads. *Xenopus* egg extract containing sperm DNA was supplemented +/- 19ng/µl ΔCdt1$^{1\text{-}243}$-RFP protein and activated with 0.3 mM CaCl$_2$ as positive and negative controls. The extract samples were then added to the RFP antibody coupled beads overnight. Figure 5.10B shows that the ΔCdt1$^{1\text{-}243}$-RFP construct was successfully isolated using the RFP beads. This confirms that the RFP

antibodies are suitable for use in IP experiments to isolate the ΔCdt1$^{1-243}$-RFP construct under the IP conditions used.

For the IP, *Xenopus* egg extract was supplemented with 20 ng/μl sperm DNA and +/- 19 ng/μl ΔCdt1$^{1-243}$-RFP before activation with 0.3 mM CaCl$_2$. At the time points 0, 60, 90 and 120 minutes following activation, 150 μl of IP buffer was added to the extract sample before incubation with the RFP antibody beads. These time points were chosen to encompass the stages of DNA licensing and replication within the extract with DNA licensing complete by 60 minutes and DNA replication complete by 120 minutes (Gillespie *et al.*, 2012).

A

Time post-fertilisation/hr:min

| | 04:00 | 04:05 | 04:10 | 04:15 | 04:20 | 04:25 | 04:30 | 04:35 | 04:40 | 04:45 | 04:50 |

Cdt1

Phospho-Ser

Phospho-Thr

Histones

B

ΔCdt1$^{1-243}$-RFP   +   −

RFP

C

| Time/min | 0 | 60 | 90 | 120 | 120 |

ΔCdt1$^{1-243}$-RFP   +   +   +   +   −

RFP
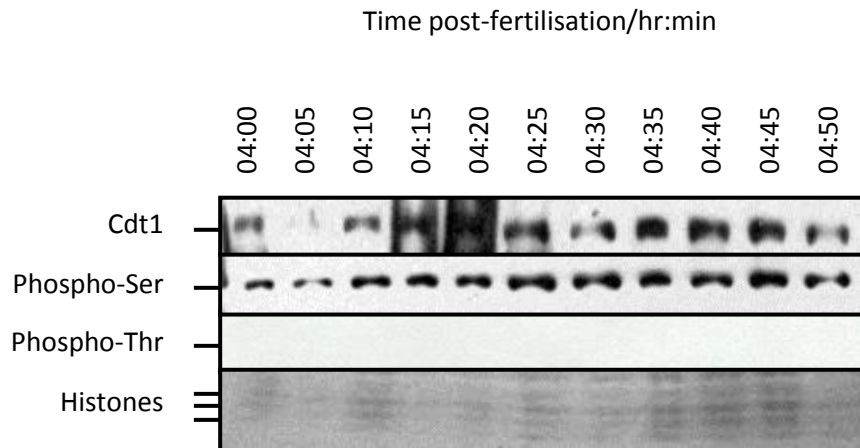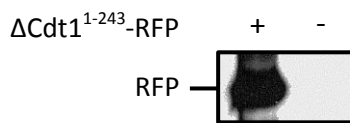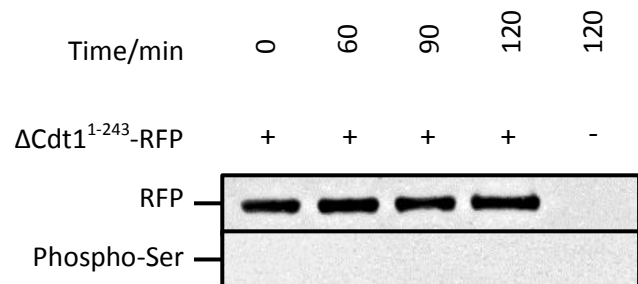
Phospho-Ser

Figure 5.10. Phosphorylation of Cdt1 in pre-MBT *Xenopus laevis* embryos and *Xenopus* egg extract. (A) Chromatin was isolated from 0.5 ml of synchronized embryos every 5 minutes from 4 hours post-fertilisation. Samples were collected for 50 minutes to encompass one complete cell cycle. 10 µl samples were run on an SDS-PAGE gel and blotted for Cdt1 and phosphorylation at Serine and Threonine residues of Cdt1. Histones were used as a loading control and to confirm chromatin isolation. **(B)** *Xenopus* egg extract was supplemented with 20 ng/µl sperm DNA and +/- 19 ng/µl ΔCdt1$^{1-243}$-RFP before activation with 0.3 mM CaCl$_2$. The ΔCdt1$^{1-243}$-RFP was isolated by IP using RFP antibody beads, ran on an SDS-PAGE gel and immunoblotted for RFP to detect ΔCdt1$^{1-243}$-RFP. **(C)** *Xenopus* egg extract was supplemented with 20 ng/µl sperm DNA and +/- 19 ng/µl ΔCdt1$^{1-243}$-RFP before activation with 0.3 mM CaCl$_2$. The time is in minutes post-activation. At each time point ΔCdt1$^{1-243}$-RFP was isolated by IP using RFP antibody beads. The bound protein was then released from the RFP antibody beads, ran on an SDS-PAGE gel and immunoblotted for phosphor-ser and RFP.

Figure 5.10C shows the isolation of the $\Delta Cdt1^{1-243}$-RFP construct from the extract at each time point. For the negative control egg extract minus $\Delta Cdt1^{1-243}$-RFP was added to the RFP antibody beads at 120 minutes following activation. The negative control confirms that there is no band detected by the RFP antibody in the absence of $\Delta Cdt1^{1-243}$-RFP. Therefore the band detected by the RFP antibody is indeed the $\Delta Cdt1^{1-243}$-RFP construct. In addition there was no phosphorylation of serine residues of the $\Delta Cdt1^{1-243}$-RFP construct detected. This could suggest that the N-terminal 1-243 amino acid region of Cdt1 is not regulated by phosphorylation of serine. However, since there is no positive control of a phosphoserine phosphorlated protein included it as also possible that the phosphothreonine antibody did not work.

5.3   Discussion

The results presented in Chapter 4 show that when a truncated, mutant form of Cdt1 which lacks the N-terminal region ($\Delta Cdt1^{243-620}$) is expressed in pre-MBT *Xenopus* embryonic cells, deregulated Cdt1 activity causes cell cycle arrest induced by DNA re-licensing and re-replication. This shows that correct regulation of Cdt1 is essential for normal pre-MBT *Xenopus* embryo progression. It was therefore important to establish the mechanisms involved in Cdt1 regulation during the pre-MBT cell cycle.

There are two possible mechanisms by which the $\Delta Cdt1^{243-620}$ construct abrogates regulation resulting in uncontrolled re-licensing. The first possibility is that $\Delta Cdt1^{243-620}$ overrides the inhibitory effects of geminin. This could be caused by disruption of the ratio of Cdt1 to geminin within the cell preventing formation of an inactive Cdt1:geminin complex stoichiometry (Lutzmann *et al.*, 2006; De Marco *et al.*, 2009; Kisielewska and Blow, 2012), or due to a partial resistance to inhibition by geminin (Ferenbach *et al.*, 2005). The second possibility is that, since the N-terminal region contains multiple sites for Cdt1 regulation (reviewed in (Caillat and Perrakis, 2012)), there may be post-translational modifications of Cdt1 targeted to this region but in the absence of degradation since Cdt1 levels remain stable in pre-MBT embryos (Kisielewska and Blow, 2012). A truncated Cdt1 construct containing only the N-terminal region, $\Delta Cdt1^{1-243}$-RFP, provides the ideal tool with which to investigate the mechanisms of Cdt1 regulation in *Xenopus* embryos that were potentially abrogated by the $\Delta Cdt1^{243-620}$ construct.

The N-terminal region of Cdt1 contains sites targeted for phosphorylation and ubiquitination. In human cells there are three separate E3 ubiquitin ligase complexes which recognise the N-terminal domain of Cdt1 and mediate Cdt1 proteolysis (reviewed in (Caillat and Perrakis, 2012)). Although Cdt1 levels remain stable in pre-MBT cells (Kisielewska and Blow, 2012) there may be inhibitory post-translational modification of Cdt1 in the absence of degradation. Indeed in human cells phosphorylation at the N-terminal cy motif of Cdt1 reduces the DNA binding activity of Cdt1 which in turn could reduce the licensing activity of Cdt1 (Sugimoto *et al.*, 2004). To investigate this possibility both the phosphorylation and ubiquitination status in MBT cells or $\Delta Cdt1^{1-243}$-RFP in *Xenopus* egg extract was investigated (Figure 5.2 and Figure 5.3). This suggests that Cdt1 activity is not regulated by ubiquitination during

the pre-MBT cell cycle. There was also no evidence of endogenous Cdt1 accumulation in pre-MBT cells in the presence of the proteasome inhibitor MG132 (Figure 5.3) which further confirms that the stable Cdt1 levels are due to a lack of Cdt1 degradation in pre-MBT embryos. Taken together this suggests that the mechanisms of ubiquitination which mediate Cdt1 degradation in somatic cells may be absent or inactive in pre-MBT embryos or the full length endogenous Cdt1 is somehow protected from degradation. One possibility is that geminin binding to Cdt1 which inhibits the licensing activity of Cdt1 also protects Cdt1 from degradation (Lutzmann *et al.*, 2006). Indeed it has been shown that in human cells geminin is capable of stabilising Cdt1 by preventing ubiquitination and proteasome mediated degradation (Ballabeni *et al.*, 2004).

Phosphorylation of serine but not threonine residues of chromatin was detected at the molecular weight of chromatin bound Cdt1 in stage 7 pre-MBT embryos (Figure 5.10). It is possible that Cdt1 may be regulated by mechanisms involving serine phosphorylation during the pre-MBT cell cycle. However at the N-terminal $\Delta Cdt1^{1-243}$-RFP region there was no serine phosphorylation detected (Figure 5.10). It is possible that the serine phosphorylation detected at chromatin bound endogenous Cdt1 was not located to the N-terminal region although regulatory phosphorylation occurs at the N-terminal region in somatic cells (Sugimoto *et al.*, 2004). However it is possible that the serine phosphorylation detected was of another protein distinct from Cdt1 but with the same molecular weight. In addition it is possible that the lack of detection of serine phosphorylation at $\Delta Cdt1^{1-243}$-RFP was due to a failure of the antibody rather than a true lack of phosphorylation.

Another potential limitation is that the method of IP used to detect phosphorylation of $\Delta Cdt1^{1-243}$-RFP was insufficient. However steps were taken to ensure the preservation of any phosphorylation present at $\Delta Cdt1^{1-243}$-RFP. To preserve $\Delta Cdt1^{1-243}$-RFP phosphorylation the IP buffer was supplemented with the phosphatase inhibitors sodium orthovanadate and sodium fluoride which inhibit tyrosine and serine/threonine phosphatases respectively. The extract samples were also only incubated with the RFP antibody beads for one hour rather than overnight in order to preserve any phosphorylation. However there was also no gel shift of $\Delta Cdt1^{1-243}$-RFP detected which would occur in the presence of $\Delta Cdt1^{1-243}$-RFP phosphorylation. This

therefore further suggests that $\Delta Cdt1^{1-243}$-RFP is not phosphorylated in *Xenopus* egg extract *in vitro*.

Since there is no evidence of ubiquitination or phosphorylation of the N-terminal, $\Delta Cdt1^{1-243}$-RFP construct this suggests that the $\Delta Cdt1^{243-620}$ construct does not abrogate regulation through avoidance of inhibitory post-translational modification. It is therefore likely that the $\Delta Cdt1^{243-620}$ construct exerts its effects on the pre-MBT cell cycle and cell division by disrupting the ratio of Cdt1 to geminin within the cell and therefore avoiding geminin inhibition.

In *Xenopus* egg extract, endogenous Cdt1 is degraded in an APC/C mediated manner upon release of the extract from metaphase arrest. It has previously been shown that a truncated Cdt1 mutant lacking the first 243 amino acids is stable in *Xenopus* egg extract. This shows that it is the N-terminal regulatory region of Cdt1 which is targeted for degradation upon release of the extract from metaphase (Li and Blow, 2005). In agreement with this, the results presented here show that the N-terminal $\Delta Cdt1^{1-243}$-RFP construct is degraded upon release from metaphase arrest (Figure 5.4 andFigure 5.5). In addition the levels of $\Delta Cdt1^{1-243}$-RFP stabilise in the presence of the APC/C inhibitor Dbox. This shows that like with endogenous Cdt1, degradation of $\Delta Cdt1^{1-243}$-RFP is also mediated by the APC/C. It has also been shown previously that when *Xenopus* egg extract contains a lower DNA concentration, endogenous Cdt1 remains stable and is not degraded upon release from metaphase (Kisielewska and Blow, 2012). The $\Delta Cdt1^{1-243}$-RFP construct also remains stable in extract at lower DNA concentrations (Figure 5.8 and Figure 5.9). Taken together the results presented here show that the degradation patterns of $\Delta Cdt1^{1-243}$-RFP in *Xenopus* egg extract are analogous to the degradation patterns of endogenous Cdt1. This further confirms that the N-terminal region, containing protein regulatory domains, is targeted to allow degradation of Cdt1. It also shows that the RFP tag of $\Delta Cdt1^{1-243}$-RFP does not interfere with the function of the Cdt1 1-243 amino acid region. The $\Delta Cdt1^{1-243}$-RFP construct therefore provides a functional tool with which to investigate other Cdt1 functions such as DNA and protein binding.

It has been shown that in *Xenopus* egg extract supplemented with a low concentration of DNA, endogenous Cdt1 is not degraded and instead remains stable (Kisielewska and

Blow, 2012). One of the main mechanisms of Cdt1 degradation is DNA replication dependant in which chromatin bound Cdt1 is targeted for degradation following interaction with PCNA (Arias and Walter, 2005a). It therefore follows that with lower levels of DNA there is less DNA dependant degradation and so Cdt1 levels stabilise. The interaction of Cdt1 with PCNA required for DNA dependant degradation is mediated by a PCNA interaction domain, the PIP box, located at the N-terminal region of Cdt1 (Arias and Walter, 2005a). In line with this, the N-terminal ΔCdt1$^{1-243}$-RFP construct is also stabilised at lower concentrations of DNA which suggests that ΔCdt1$^{1-243}$-RFP also undergoes DNA replication dependant degradation. Also, the CDK inhibitor p27 which inhibits DNA replication initiation and PCNA loading (Toyoshima and Hunter, 1994), blocks some of the degradation of ΔCdt1$^{1-243}$-RFP at p27 concentrations above 52 μM (Figure 5.8). This provides further evidence that some of the Cdt1 degradation is replication dependant and mediated via the N-terminal region of Cdt1.

While Figures 5.6 to 5.10 show evidence of degradation of the N-terminal ΔCdt1$^{1-243}$-RFP construct in both an APC/C and replication dependant manner they only present limited evidence of degradation. In order to be fully conclusive some of the experiments require additional controls. The western blots shown in Figures 5.4, 5.6, 5.7 and 5.8 require loading controls. However since the extract is a cell free system the standard loading controls of actin or tubulin are not present therefore a membrane or gel stain for total protein loading such as Ponceau S stain or Coomassie stain would be more appropriate. Additional controls to show that the Ca^{2+} activation of the extract was achieved and that the DBox inhibitor was active in this experimental set up would strengthen the results presented here. Therefore the conclusions drawn here are preliminary and would require further experimental confirmation to be conclusive.

Chapter 6.

Results IV. The Cdt1 N-terminal Region is Recruited to Chromatin

6.1 Introduction

It has been well documented that the N-terminal region of Cdt1 contains multiple domains which are necessary for regulation of Cdt1 activity. There is a PIP box which is required for interaction with PCNA and replication dependant degradation (Arias and Walter, 2005a; Senga *et al.*, 2006) as well as a cy motif which is required for cyclin/Cdk phosphorylation dependant SCF^{Skp2} mediated degradation (Li *et al.*, 2003; Liu *et al.*, 2004; Nishitani *et al.*, 2004; Nishitani *et al.*, 2006). In addition there are several N-terminal D-box motifs which are targeted for APC/C mediated Cdt1 degradation (Sugimoto *et al.*, 2008).

In addition to the regulatory domains there is also a bipartite nuclear localisation sequence (NLS) within the N-terminal region of Cdt1 (Nishitani *et al.*, 2004). A bipartite NLS is a classical NLS sequence consisting of two clusters of basic amino acids separated by a linker region of amino acids. The classical NLS binds to an NLS receptor on the surface of the nuclear envelope to allow transport of the protein into the nucleus (Lange *et al.*, 2010). The presence of an NLS in the N-terminal region suggests that this region of Cdt1 may be important for nuclear localisation.

The results presented in Chapter 5 show that the $\Delta Cdt1^{1-243}$-RFP construct follows the same patterns and mechanisms of degradation as with endogenous Cdt1 in line with the N-terminal region containing multiple domains to target Cdt1 for regulation and degradation. This shows that the Cdt1 region ($\Delta Cdt1^{1-243}$) of the construct is functional and that the RFP tag does not interfere with this functionality. The $\Delta Cdt1^{1-243}$-RFP construct therefore provides a functional tool to allow investigation of the role of the N-terminal region of Cdt1 in nuclear entry and DNA binding.

6.2 Results

To investigate the localisation of the $\Delta Cdt1^{1-243}$-RFP construct during the pre-MBT cell cycle *in vivo*, the mRNA for the $\Delta Cdt1^{1-243}$-RFP was produced for microinjection and expression in *Xenopus* embryos. The pRN3 vector containing $\Delta Cdt1^{1-243}$-RFP construct DNA produced in Chapter 3 was used to produce $\Delta Cdt1^{1-243}$-RFP mRNA by *in vitro* transcription.

A 12 ng amount of $\Delta Cdt1^{1-243}$-RFP mRNA was then microinjected into one cell of a two cell embryo at 1 hour 30 min post-fertilisation. The embryos were then maintained in optimal conditions for growth. To confirm expression of the $\Delta Cdt1^{1-243}$-RFP mRNA and investigate the localisation of the $\Delta Cdt1^{1-243}$-RFP protein within the cell immunofluorescence was used. For immunofluorescence the embryos injected with $\Delta Cdt1^{1-243}$-RFP mRNA and the non-injected controls were maintained in development until 6 hours 45 minutes post-fertilisation. At this point in development the cells of the embryo are small enough to allow imaging using confocal/multiphoton 2P microscopy. Figure 6.1 shows immunofluorescence of a whole mount embryo injected with $\Delta Cdt1^{1-243}$-RFP mRNA and a non-injected control fixed at 6 hours 45 minutes post-fertilisation which is 5 hours 15 minutes following injection. RFP was used to detect $\Delta Cdt1^{1-243}$-RFP and DAPI was used to stain the DNA. The RFP signal was high in the embryo injected with $\Delta Cdt1^{1-243}$-RFP mRNA compared to the control non-injected embryo therefore confirming expression of the mRNA.

The immunofluorescence shows two nuclei in prophase and two nuclei in S-phase of the cell cycle. The cell cycle stage was determined due to the appearance of the DNA which is made visible by staining with DAPI. During prophase the DNA is condensed whereas in S-phase the DNA is decondensed to allow access of the replication fork proteins to DNA. Therefore the DAPI staining is smoother and more consistent in S-phase when the DNA is decondensed (Alexandrow and Hamlin, 2005). Figure 6.1A shows a higher signal intensity of RFP in the nucleus during S-phase (cells 3 and 4) compared to prophase (cells 1 and 2). This suggests that during S-phase $\Delta Cdt1^{1-243}$-RFP is localised to the nucleus and possibly bound to the DNA.

In order to quantify the expression and nuclear localisation of $\Delta Cdt1^{1-243}$-RFP in the embryos injected with $\Delta Cdt1^{1-243}$-RFP mRNA, image J was used to measure the average

fluorescence intensity of the nuclear RFP signal in injected versus non-injected embryos at different cell cycle stages.

The average fluorescence intensity of the RFP signal was measured in a minimum of 2-3 nuclei and averaged for different cell cycle stages in each of 2 embryos injected with 12 ng ΔCdt1$^{1-243}$-RFP mRNA into one cell of two at 1 hours 30 minutes post-fertilisation (developmental stage 2). Following injection the embryos were fixed for immunofluorescence at 6 hours 45 minutes post-fertilisation as at this stage of development the cells are small enough to be imaged by confocal microscopy. Non-injected embryos fixed for immunofluorescence in parallel to the injected embryos were used as controls. Figure 6.1C shows that the nuclear RFP signal was higher during S-phase in the ΔCdt1$^{1-243}$-RFP mRNA injected embryos compared to non-injected controls. However, during prophase and metaphase of the cell cycle the nuclear RFP signal was comparative to that of the controls. This further suggests that ΔCdt1$^{1-243}$-RFP localises to the DNA during S-phase of the cell cycle.
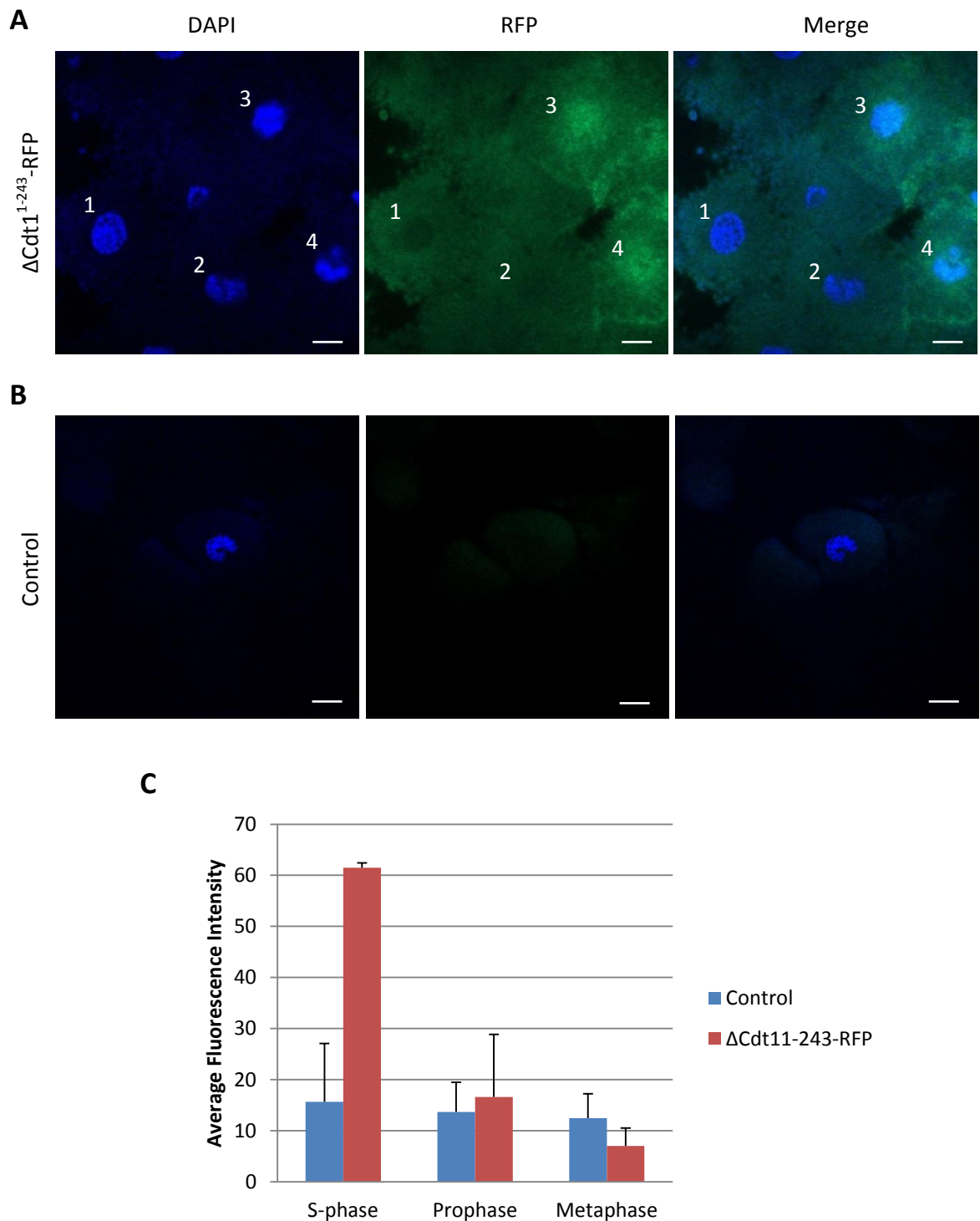
Figure 6.1. Immunofluorescence showing ΔCdt1$^{1\text{-}243}$-RFP mRNA expression in *Xenopus* embryos. (A)
12ng ΔCdt1$^{1\text{-}243}$-RFP mRNA was injected into one cell of a two cell embryo (developmental stage 2). The embryo was fixed at 6 hours 45 minutes post-fertilisation and probed using RFP antibody. The cell cycle stages of each cell are numbered: 1. and 2. Prophase, 3. and 4. S-phase. **(B)** Control non-injected embryo fixed at 6 hours 45 minutes post-fertilisation and probed using RFP antibody. Scale bar is 10μm. **(C)** Quantification of the average fluorescence intensity of the nuclear RFP signal in at difference cell cycle stages in embryos injected with 12ng ΔCdt1$^{1\text{-}243}$-RFP mRNA and control non-injected embryos. The signal was measured in a minimum of 2-3 nuclei and averaged in each of 2 separate embryos. The error bars represent the standard deviation, n=2.

Figure 6.1 shows that the RFP signal of ΔCdt1$^{1-243}$-RFP overlaps with the DNA signal during S-phase *in vivo* in *Xenopus* embryos. This shows that ΔCdt1$^{1-243}$-RFP is localised to the nucleus and suggests that ΔCdt1$^{1-243}$-RFP may also be chromatin bound during S-phase. To confirm the localisation of ΔCdt1$^{1-243}$-RFP to the nucleus the *in vitro Xenopus* egg extract system was used.

Xenopus egg extract was supplemented with 20 ng/μl demembranated sperm DNA plus +/- 200 ng/μl ΔCdt1$^{1-243}$-RFP and released from metaphase arrest using 0.3 mM CaCl$_2$. At 40 minutes following activation with calcium, at which point DNA licensing is complete, 1 μl samples were fixed and the DNA stained using Hoechst. The extract samples were then imaged using a Leica Confocal with UV filter to detect Hoechst stained DNA and a red filter to detect RFP and therefore ΔCdt1$^{1-243}$-RFP. Figure 6.2A shows that as expected, upon activation of the extract the sperm DNA has decondensed and completed nuclear assembly forming circular nuclei (Gillespie *et al.*, 2012). The ΔCdt1$^{1-243}$-RFP construct is again localised to the nucleus. Figure 6.2B shows multiple nuclei showing the localisation of ΔCdt1$^{1-243}$-RFP to the DNA (arrowed). In contrast in the control sample only a background RFP signal is detected with no RFP signal located to the nuclei or DNA (Figure 6.2C).

This further confirms that the ΔCdt1$^{1-243}$-RFP construct localises to the nucleus. This suggests that the N-terminal region of Cdt1 contains domains required to cross the nuclear envelope and localise within the nucleus.
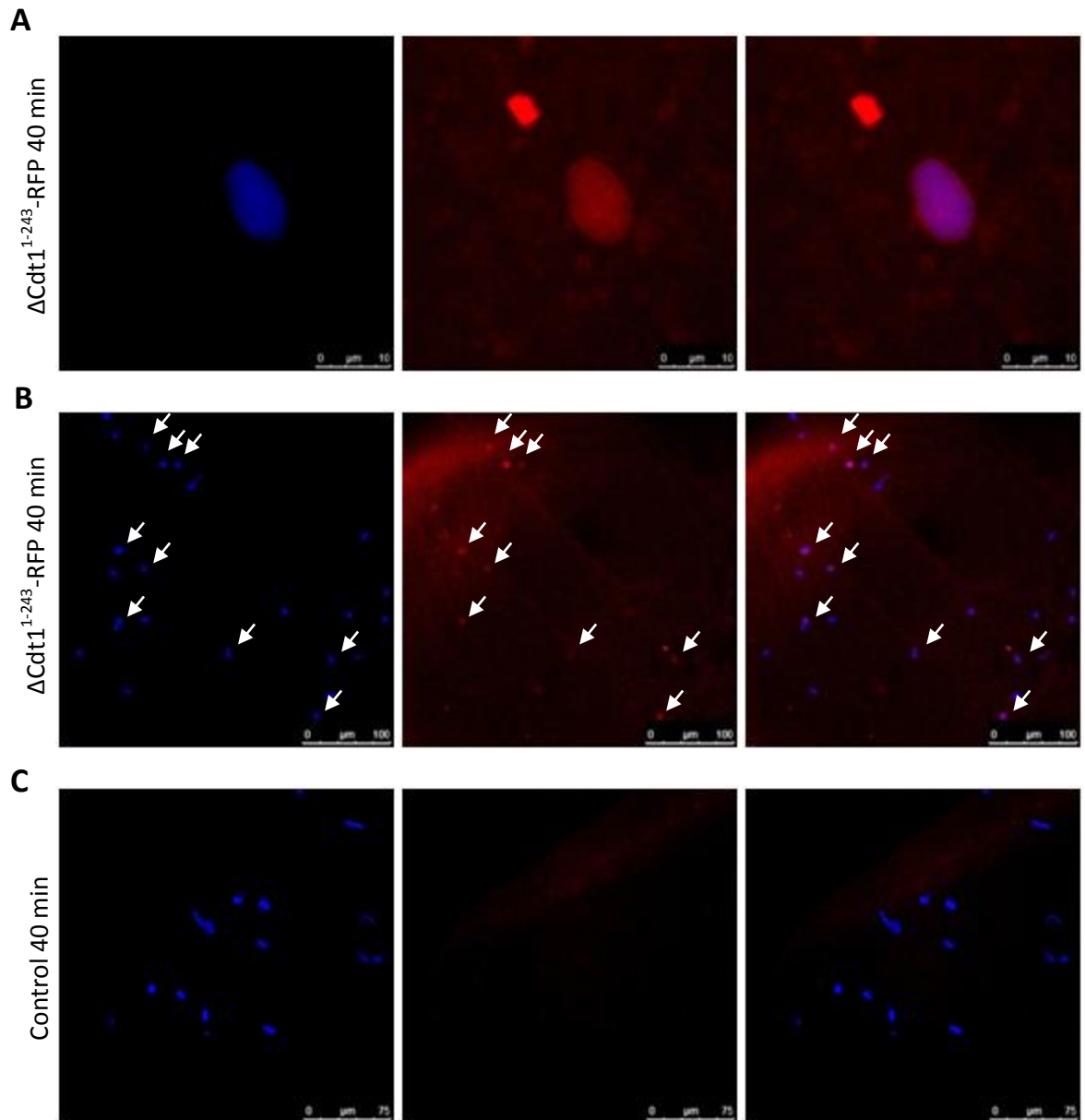
Figure 6.2. Chromatin binding of ΔCdt1$^{1-243}$-RFP recombinant protein. *Xenopus* egg extract was activated with 0.3 mM CaCl$_2$ and supplemented with 20 ng/µl DNA and +/- 200 ng/µl ΔCdt1$^{1-243}$-RFP. 1 µl samples were fixed at 40 minutes post-activation, at which point DNA licensing is complete. The DNA within the extract was stained using Hoechst. Samples were imaged using a Leica Confocal with UV filter for Hoechst and red filter for RFP. **(A)** Single S-phase nuclei showing the presence of ΔCdt1$^{1-243}$-RFP. Scale bar 10 µm. **(B)** Multiple nuclei. Arrows highlight S-phase nuclei with ΔCdt1$^{1-243}$-RFP. Scale bar 100 µm. **(C)** Control sample. *Xenopus* extract was activated and supplemented with 20 ng/µl DNA but no ΔCdt1$^{1-243}$-RFP. 1 µl samples were fixed after 40 minutes and the DNA stained using Hoechst. Samples were imaged using Leica Confocal with 405 mm UV laser for Hoechst and 485 mm red Argon-Krypton laser to detect RPF. Scale bar 75µm.

To quantify the number of cells with nuclear $\Delta Cdt1^{1-243}$-RFP the percentage of cells with nuclear RFP signal was calculated from Figure 6.2. As shown in Figure 6.3, in excess of 60% of nuclei showed RFP staining in the extract supplemented with 20 ng/μl DNA and +/- 200 ng/μl $\Delta Cdt1^{1-243}$-RFP. In contrast in the control embryo which was supplemented with 20 ng/μl DNA only none of the nuclei showed RFP staining. This further confirms that the $\Delta Cdt1^{1-243}$-RFP construct of Cdt1 is capable of localising to the nucleus.
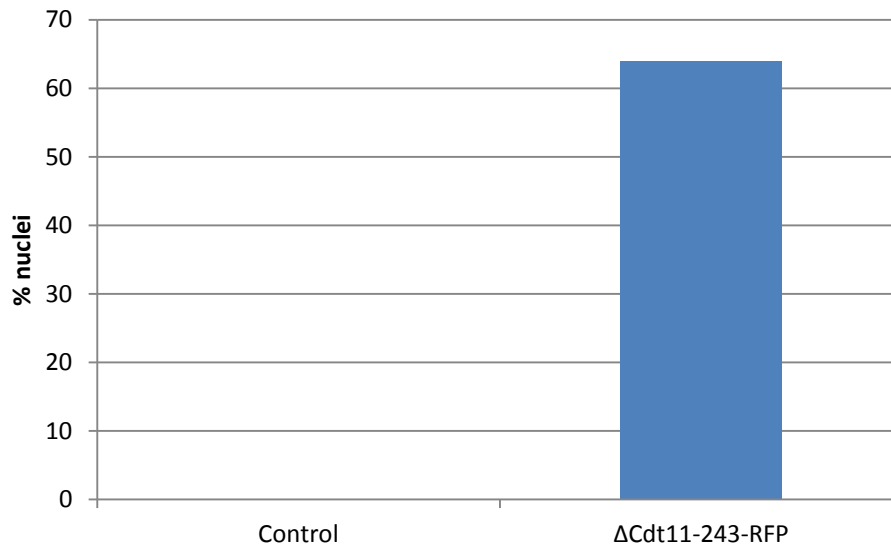
Figure 6.3. Quantification of nuclear ΔCdt1$^{1-243}$-RFP in activated *Xenopus* egg extract. *Xenopus* egg extract was activated with 0.3 mM CaCl$_2$ and supplemented with 20 ng/μl DNA and +/- 200 ng/μl ΔCdt1$^{1-243}$-RFP. 1 μl samples were fixed at 40 minutes post-activation, at which point DNA licensing is complete. The DNA within the extract was stained using Hoechst. Samples were imaged using a Leica Confocal with UV filter for Hoechst and red filter for RFP. The percentage of nuclei showing RFP staining was then calculated from the images shown in Figure 6.2 B and C.

It was next important to confirm whether or not $\Delta Cdt1^{1-243}$-RFP is also capable of binding to the DNA once localised to the nuclear space. To isolate chromatin from *Xenopus* embryos, a 0.5 ml volume of embryos is required. Since this is too high a number to successfully inject with mRNA at the same point in development, chromatin isolation from *Xenopus* egg extract was instead utilised.

Xenopus egg extract was activated to release from metaphase with 0.3 mM $CaCl_2$ before supplementing +/- 20 ng/μl sperm DNA and -/+ 44 ng/μl $\Delta Cdt1^{1-243}$-RFP. At 70 minutes following activation, at which point DNA licensing is complete, the reaction was stopped and the chromatin isolated. To detect chromatin bound proteins the isolated chromatin samples were run on an SDS-PAGE gel and blotted for RFP to detect $\Delta Cdt1^{1-243}$-RFP. The samples were also blotted for Mcm2 and PCNA to detect successful DNA licensing. The histones were stained to confirm isolation of the chromatin and to act as a loading control. A sample of extract activated with calcium but not supplemented with either DNA or $\Delta Cdt1^{1-243}$-RFP was used as a negative control to show the pattern of bands when there is no DNA and therefore no DNA licensing or isolated histones.

The extract sample containing only DNA and no $\Delta Cdt1^{1-243}$-RFP shows licensed DNA. In the extract containing both DNA and $\Delta Cdt1^{1-243}$-RFP the Mcm2 and PCNA bands also show licensed DNA. This shows that $\Delta Cdt1^{1-243}$-RFP does not affect DNA licensing which is to be expected since the $\Delta Cdt1^{1-243}$ region does not contain an MCM binding domain and so is not licensing active. Figure 6.3 shows that the $\Delta Cdt1^{1-243}$-RFP protein is isolated with the chromatin. The $\Delta Cdt1^{1-243}$-RFP construct therefore was chromatin bound in activated *Xenopus* egg extract following DNA licensing. This confirms that the $\Delta Cdt1^{1-243}$-RFP construct is capable of binding to DNA as well as localising to the nucleus.
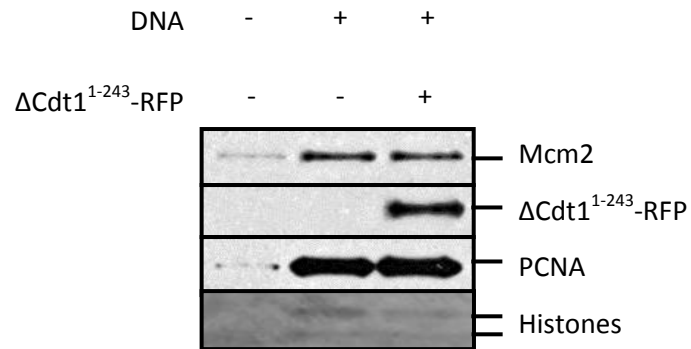
Figure 6.4. Chromatin binding of ΔCdt1$^{1-243}$-RFP recombinant protein. *Xenopus* extract was activated with 0.3 mM CaCl$_2$ and supplemented with 20 ng/µl DNA and -/+ 44 ng/µl ΔCdt1$^{1-243}$-RFP. The reaction was stopped 70 minutes after activation, at which point DNA licensing should be complete within the extract, and the chromatin isolated, ran on an SDS-PAGE gel and blotted for Mcm2, RFP and PCNA. The - DNA sample was used as a negative control and the + DNA sample was used as a positive control to show isolation of licensed chromatin. Histones were used as a loading control and to confirm chromatin isolation.

The results presented in Figure 6.4 confirm that the $\Delta Cdt1^{1-243}$-RFP construct is able to bind to chromatin. The chromatin was isolated from the extract at 70 minutes following activation following DNA licensing. This shows that $\Delta Cdt1^{1-243}$-RFP was bound to chromatin once licensing was complete but does not show at which point during DNA licensing at which the $\Delta Cdt1^{1-243}$-RFP construct was recruited.

To determine the point during the cell cycle at which $\Delta Cdt1^{1-243}$-RFP is recruited to chromatin and whether or not $\Delta Cdt1^{1-243}$-RFP is subsequently released from chromatin, a time course chromatin isolation was used. *Xenopus* egg extract was activated to release from metaphase with 0.3 mM $CaCl_2$ before supplementing with 20 ng/µl sperm DNA and 22 ng/µl $\Delta Cdt1^{1-243}$-RFP. Samples of the extract were then fixed and the chromatin isolated at selected time points between 0 and 160 minutes post-activation to encompass one whole cycle of DNA licensing and replication within the extract. The isolated chromatin samples were run on an SDS-PAGE gel and immunoblotted to detect chromatin bound proteins. The chromatin samples were blotted for Mcm2, PCNA and geminin to determine the licensing and replication state of the chromatin and blotted for RFP to detect $\Delta Cdt1^{1-243}$-RFP.

Figure 6.5 shows that at 0 minutes following activation and supplementation of the extract with DNA and $\Delta Cdt1^{1-243}$-RFP there are no licensing proteins bound to DNA and therefore DNA licensing has not begun. At 10 minutes Mcm2 is loaded as DNA licensing has begun. Some geminin is also present at 10 minutes which was likely recruited with endogenous Cdt1 since Cdt1 is required for MCM loading and geminin and Cdt1 have previously been shown to co-localise on DNA (Gillespie *et al.*, 2001). At 90 minutes PCNA is bound to the DNA as DNA licensing is now complete and S-phase is underway. There is also an increase in chromatin bound geminin at 90 minutes which will likely be recruited to inhibit endogenous Cdt1 activity and prevent re-licensing of the DNA during S-phase which would result in re-replication. At 60 minutes a small amount of $\Delta Cdt1^{1-243}$-RFP is loaded to the DNA with more loaded at 90 minutes. This suggests that $\Delta Cdt1^{1-243}$-RFP was loaded on chromatin at the end of DNA licensing and start of S-phase.

A

Time post-activation/min

B

Time post-activation/min
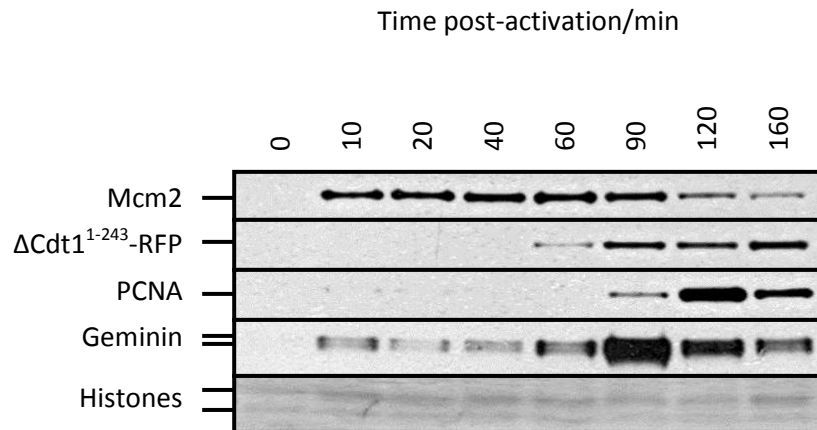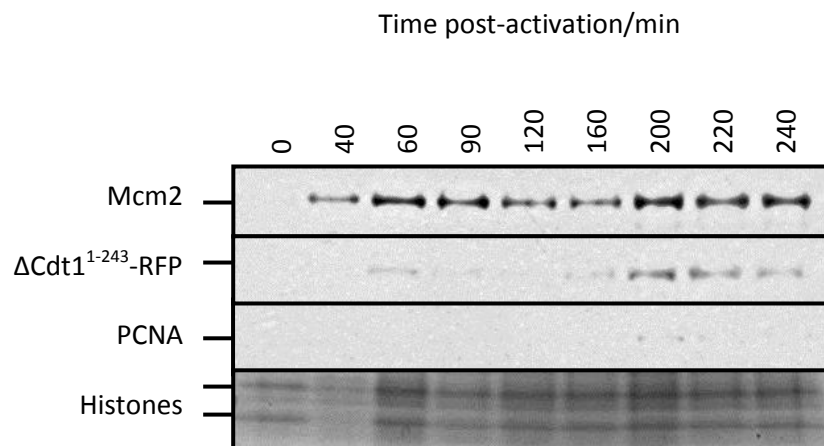
Figure 6.5. Chromatin binding of ΔCdt1$^{1-243}$-RFP recombinant protein during one cell cycle in *Xenopus* egg extract in the presence and absence of p27. (A) *Xenopus* extract was activated with 0.3 mM CaCl$_2$ and supplemented with 20 ng/μl DNA and 22 ng/μl ΔCdt1$^{1-243}$-RFP. The reaction was stopped and the chromatin isolated at selected time points after activation to encompass DNA licensing and replication within the extract. The chromatin samples were ran on an SDS-PAGE gel and blotted for Mcm2, RFP, PCNA and geminin to determine the licensing and replication state of the chromatin. The samples were also blotted for RFP to detect ΔCdt1$^{1-243}$-RFP. Histones were used as a loading control and to confirm chromatin isolation. **(B)** *Xenopus* extract was activated with 0.3 mM CaCl$_2$ and supplemented with 20 ng/μl DNA, 19 ng/μl ΔCdt1$^{1-243}$-RFP and 100 nmol p27. The reaction was stopped and the chromatin isolated at selected time points after activation to encompass DNA licensing and replication within the extract. The chromatin samples were ran on an SDS-PAGE gel and blotted for Mcm2 to determine the licensing state of the chromatin and PCNA to confirm p27 inhibition of PCNA loading and DNA replication initiation. The samples were also blotted for RFP to detect ΔCdt1$^{1-243}$-RFP. Histones were used as a loading control and to confirm chromatin isolation.

It was next important to determine how the ΔCdt1$^{1-243}$-RFP construct is loaded onto chromatin. Although ORC, Cdc6 and Cdt1 are all required to load the MCM complex onto DNA and thereby license the DNA for replication (Gillespie *et al.*, 2001) the sequence of events towards chromatin loading are unclear. In the yeast *S.cerevisiae* Orc6 is required for the interaction of the ORC complex with Cdt1 to facilitate Mcm2-7 loading and therefore DNA licensing (Chen *et al.*, 2007). In contrast in *Xenopus* egg extract it has been suggested that the ORC protein Orc6 is not required for DNA licensing (Gillespie *et al.*, 2001). In fission yeast it has been shown that Cdt1 recruitment to chromatin is independent of Cdc6 (Nishitani *et al.*, 2000). Although it has been shown that Cdt1 can bind DNA independently of DNA strand, sequence or conformation (Yanagi *et al.*, 2002), in *Xenpous* egg extract prior binding of Cdc6 to chromatin is essential for chromatin bound Cdt1 to function in DNA licensing. In this system Cdt1 recruited to chromatin prior to Cdc6 is not functional in licensing and suggests that a strict order of recruitment of the licensing proteins to chromatin is essential for licensing activity (Tsuyama *et al.*, 2005).

It has since been shown in *S.cerevisiae* that a chromatin bound ORC-Cdc6 complex recruits multiple molecules of Cdt1 which in turn initiate formation of an MCM complex double hexamer for loading to the DNA to complete licensing (Takara and Bell, 2011). It has also been shown that the ORC-Cdc6 complex recruits a Cdt1-Mcm2-7 complex with an ORC-Cdc6-Cdt1-Mcm2-7 complex forming as an intermediate in DNA licensing (Sun *et al.*, 2013). It is thought that following formation of the ORC-Cdc6-Cdt1-Mcm2-7 complex ATP hydrolysis of Orc1 and Cdc6 leads to release of Cdt1 from the DNA to leave an ORC-Cdc6-Mcm2-7 complex bound to DNA. Since licensing requires the loading of a double Mcm2-7 hexamer, the second Mcm2-7 hexamer is then loaded to the DNA in a Cdt1 dependant manner although the exact mechanism of formation of the Mcm2-7 double hexamer remains unknown (Fernandez-Cid *et al.*, 2013).

Given that Cdt1 is recruited to DNA following ORC and Cdc6 loading, it is therefore possible that the ΔCdt1$^{1-243}$-RFP construct is loaded to chromatin by forming a complex with other licensing proteins such as interaction with an ORC-Cdc6 complex. Due to the loading of ΔCdt1$^{1-243}$-RFP onto chromatin towards the end of licensing and start of

S-phase (Figure 6.5A) it is also possible that the loading is PCNA dependant and mediated through the PIP box of ΔCdt1$^{1-243}$-RFP (Arias and Walter, 2005a).

In *Xenopus* egg extract Mcm2 loading occurs at 10 minutes post-activation whereas ΔCdt1$^{1-243}$-RFP loading only began to appear at 60 minutes post-activation (Figure 6.5A). Since ΔCdt1$^{1-243}$-RFP loading to chromatin occurs towards the end of DNA licensing and start of S-phase it is unlikely that it is recruited by an Orc1 interaction as ORC loading is one of the first steps in DNA licensing. It is therefore plausible that the ΔCdt1$^{1-243}$-RFP protein is loaded to chromatin in a PCNA dependant manner. PCNA is only chromatin bound during DNA replication.

To investigate the possibility that ΔCdt1$^{1-243}$-RFP is loaded to DNA in a PCNA dependant manner, the CDK inhibitor p27 was used to block PCNA loading and initiation of DNA replication. *Xenopus* egg extract was activated with 0.3 mM CaCl$_2$ before supplementing with 20 ng/µl sperm DNA, 19 ng/µl ΔCdt1$^{1-243}$-RFP and 100 nmol p27. Samples of the extract were then fixed and the chromatin isolated at selected time points between 0 and 240 minutes post-activation to encompass one whole cycle of DNA licensing and what would be DNA replication in the absence of p27. The isolated chromatin samples were run on an SDS-PAGE gel and immunoblotted to detect chromatin bound proteins. The chromatin samples were blotted for Mcm2 to determine the licensing state of the chromatin and blotted for RFP to detect ΔCdt1$^{1-243}$-RFP. The samples were also blotted for PCNA to confirm the inhibition of DNA replication initiation by p27 as in the absence of initiation PCNA is not loaded onto chromatin.

Figure 6.5B shows that p27 has inhibited the loading of PCNA onto chromatin. In addition, at the time points 60 to 160 minutes following activation, ΔCdt1$^{1-243}$-RFP chromatin loading is also inhibited. In extract minus p27, ΔCdt1$^{1-243}$-RFP was chromatin bound between 60 and 160 minutes post activation. This shows that when PCNA chromatin loading is inhibited, ΔCdt1$^{1-243}$-RFP loading is also inhibited suggesting that ΔCdt1$^{1-243}$-RFP DNA loading is via a PCNA dependant mechanism. Although some ΔCdt1$^{1-243}$-RFP was detected between 200 And 240 minutes post-activation this is past the time at which DNA replication was blocked and is likely due to sample contamination.

The loading of the $\Delta Cdt1^{1-243}$-RFP construct to DNA at the end of licensing in a PCNA dependant manner suggests that the interaction between the N-terminal of Cdt1 and DNA is not important for DNA licensing. Since one of the main mechanisms of Cdt1 regulation is via DNA replication and PCNA dependant degradation mediated at the N-terminal PIP box of Cdt1 (Arias and Walter, 2005a), this suggests that the $\Delta Cdt1^{1-243}$-RFP construct binding could be important for Cdt1 regulation rather than Cdt1 licensing activity.

In order for $\Delta Cdt1^{1-243}$-RFP to be loaded to DNA in either an ORC-Cdc6 or PCNA dependant manner the $\Delta Cdt1^{1-243}$-RFP protein must have the capacity to interact with these licensing proteins. However, the interactions between the Cdt1 protein and other licensing components such as Orc1-6 and Cdc6 have not been identified (Caillat and Perrakis, 2012). The binding sites of Cdt1 to licensing components other than MCM and geminin (Ferenbach *et al.*, 2005) have also not been mapped.

Since the interaction domains of Cdt1 to the other licensing proteins including the ORC complex proteins are unknown it was important to determine whether or not the $\Delta Cdt1^{1-243}$-RFP construct is capable of binding to ORC. To investigate the interacting proteins of $\Delta Cdt1^{1-243}$-RFP IP was used. *Xenopus* egg extract was activated with 0.3 mM $CaCl_2$ before supplementing with 20 ng/µl sperm DNA and 19 ng/µl $\Delta Cdt1^{1-243}$-RFP. At 60 minutes and 90 minutes post-activation which represent the end of licensing and mid-point of S-phase, the licensing reaction was stopped and the extract added to RFP antibody beads overnight. During the IP the $\Delta Cdt1^{1-243}$-RFP protein is isolated using the RFP antibody and any proteins that co-precipitate were therefore bound to $\Delta Cdt1^{1-243}$-RFP. The isolated $\Delta Cdt1^{1-243}$-RFP samples from the IP were run on an SDS-PAGE gel and blotted for the Orc1 protein. Since Figure 6.5B suggests that $\Delta Cdt1^{1-243}$-RFP is recruited to chromatin in a PCNA dependant manner the samples were also blotted for PCNA. To confirm isolation of $\Delta Cdt1^{1-243}$-RFP the samples were blotted for RFP. For the negative control activated *Xenopus* egg extract supplemented with 20 ng/µl DNA but minus $\Delta Cdt1^{1-243}$-RFP was added to the RFP antibody beads.

Figure 6.6 shows that at both 60 minutes and 90 minutes in activated egg extract, both Orc1 and PCNA co-precipitate with $\Delta Cdt1^{1-243}$-RFP. The negative control confirms that without the presence of the $\Delta Cdt1^{1-243}$-RFP construct the RFP antibody is unable to

isolate Orc1. This verifies that Orc1 was isolated as a result of binding to ΔCdt1$^{1-243}$-RFP rather than indirectly by the RFP antibody itself. Therefore this shows that both Orc1 and PCNA are able to bind to the ΔCdt1$^{1-243}$-RFP construct.

A PIP box for interaction of Cdt1 with PCNA has previously been mapped to the first 13 amino acids of the N-terminus of Cdt1 (Arias and Walter, 2005a) which explains the binding of PCNA to ΔCdt1$^{1-243}$-RFP. The binding of Orc1 to ΔCdt1$^{1-243}$-RFP (Figure 6.5) suggests that there is an Orc1 binding region located within the N-terminal 243 amino acids of Cdt1. As shown in Figure 6.5, ΔCdt1$^{1-243}$-RFP interacts with both Orc1 and PCNA. Since Figure 6.5 shows greater binding of PCNA to ΔCdt1$^{1-243}$-RFP at 90 minutes post-activation, once S-phase is underway, this further suggests that ΔCdt1$^{1-243}$-RFP is recruited to chromatin by PCNA rather than via an Orc1-Cdc6 interaction.
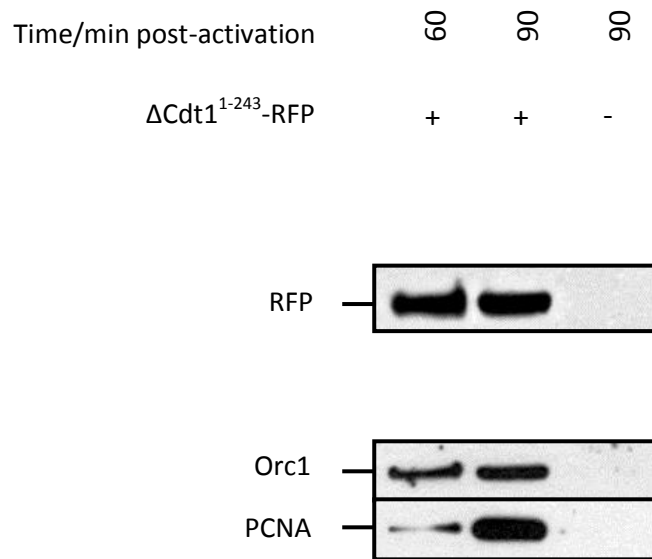
Figure 6.6. Protein binding of ΔCdt1$^{1-243}$-RFP to other DNA licensing proteins in *Xenopus* egg extract. *Xenopus* egg extract was activated with 0.3 mM CaCl$_2$ and supplemented with 20 ng/µl demembranated sperm DNA and 19 ng/µl ΔCdt1$^{1-243}$-RFP. At 60 minutes and 90 minutes post-activation to represent the end of licensing and mid-point of S-phase, IP with RFP antibody beads was used to isolate the ΔCdt1$^{1-243}$-RFP protein. The isolated ΔCdt1$^{1-243}$-RFP samples were then ran on an SDS-PAGE gel and blotted for the licensing and replication proteins Orc1 and PCNA. The samples were also blotted for RFP to detect ΔCdt1$^{1-243}$-RFP. As a negative control active extract minus ΔCdt1$^{1-243}$-RFP was added to RFP antibody beads and purified by IP at 90 minutes post-activation.

6.3 Discussion

The results presented here show that the Cdt1 N-terminal $\Delta Cdt1^{1-243}$-RFP construct is localised to the nucleus during S-phase in *Xenopus* embryos. In agreement with this the $\Delta Cdt1^{1-243}$-RFP construct is also localised to the nucleus *in vitro* in *Xenopus* egg extract. Once in the nucleus the $\Delta Cdt1^{1-243}$-RFP protein is recruited to DNA towards the end of DNA licensing and at the start of S-phase. Although the $\Delta Cdt1^{1-243}$-RFP protein binds to both Orc1 and PCNA in *Xenopus* egg extract, when PCNA DNA loading is inhibited $\Delta Cdt1^{1-243}$-RFP loading is also abolished suggesting that PCNA rather than Orc1 is involved in the chromatin loading of $\Delta Cdt1^{1-243}$-RFP.

It has previously been shown that in human cells a Cdt1 mutant lacking the first 161 amino acids localises to the cytoplasm and does not enter the nucleus. This was attributed to the absence of an NLS which is located at the N-terminal region of Cdt1 between amino acids 48-71 (Nishitani *et al.*, 2004). In agreement, the results presented here show that the N-terminal 1-243 amino acid region of Cdt1 has the capacity to localise to the nucleus. However, the results presented in Chapter 4 show that a $\Delta Cdt1^{243-620}$ construct is capable of inducing re-licensing and re-replication of DNA leading to DNA damage and cell cycle arrest. The $\Delta Cdt1^{243-620}$ construct must therefore also have the ability to cross the nuclear envelope into the nucleus. One possibility for the discrepancy is that there are further domains away from the N-terminal region which are also capable of targeting Cdt1 to the nucleus. It is also possible that the $\Delta Cdt1^{243-620}$ construct binds to other licensing proteins such as Cdc6 or one of the ORC or MCM complex proteins and is then transported into the nucleus as part of the protein complex. Indeed it has been shown that in budding yeast Cdt1 is recruited to the nucleus as part of a Cdt1-Mcm2-7 protein complex (Tanaka and Diffley, 2002). Since the $\Delta Cdt1^{243-620}$ construct contains an MCM binding domain it is therefore possible that $\Delta Cdt1^{243-620}$ was able to localise to the nucleus through forming a complex with Mcm2-7.

In the study showing that truncated Cdt1 mutants lacking the N-terminal domain are localised to the cytoplasm rather than the DNA, the cell cycle stage of the localisation of the Cdt1 mutant was not stated (Nishitani *et al.*, 2004). It is therefore possible that the different mechanisms of Cdt1 nuclear import occur at different cell cycle stages.

Indeed the N-terminal $\Delta Cdt1^{1-243}$-RFP construct was shown to localise to the nucleus during S-phase but not prophase in *Xenopus* embryonic cells (Figure 6.1).

Since both the $\Delta Cdt1^{1-243}$-RFP and $\Delta Cdt1^{243-620}$ constructs are capable of localising to the nucleus via separate mechanisms this suggests that there are multiple redundant mechanisms for Cdt1 nuclear import. Due to the importance of Cdt1 activity for correct DNA licensing and replication, this would not be the first instance of redundancy in Cdt1 regulation. In *Xenopus* egg extract both geminin inhibition and Cdt1 proteolysis must be abolished before there is DNA re-replication due to uncontrolled Cdt1 activity (Li and Blow, 2005).

Data regarding the interactions between Cdt1 and the other licensing proteins Orc1-6 or Cdc6 are currently lacking (Caillat and Perrakis, 2012). The co-precipitation of Orc1 with $\Delta Cdt1^{1-243}$-RFP suggests that there is an Orc1 binding site in the N-terminal region of Cdt1 (Figure 6.6). However only 60 and 90 minutes following extract activation were sampled which represent the end of licensing and mid-point of S-phase. Since Orc1 binding is one of the earlier time points in DNA licensing, the role of Orc1 binding the N-terminal of Cdt1 during later licensing and S-phase remains unclear and therefore requires further investigation.

Chapter 7.

Discussion

7.1 Summary and Conclusions

7.1.1 Introduction

In order to maintain genetic integrity it is essential that the DNA is replicated completely and accurately during each cell cycle. To ensure faithful duplication of the genome, DNA replication is tightly controlled. This tight control is achieved through first licensing the DNA for replication which primes the DNA to sanction replication from the licensed sites during S-phase. The process of DNA licensing must also be tightly controlled to allow licensing during late M-phase to G1 only. Re-licensing of the already replicated DNA can lead to re-replication and genomic instability which is a hallmark of cancer (Blow and Dutta, 2005; Blow and Gillespie, 2008).

One of the main mechanisms by which DNA licensing is limited to once per cell cycle is through regulation of the activity of the DNA licensing protein Cdt1. In Metazoan somatic cells Cdt1 is regulated via two mechanisms: degradation and inhibition by geminin (Arias and Walter, 2005b; Li and Blow, 2005; Caillat and Perrakis, 2012). However, the importance of Cdt1 regulation for correct cell cycle progression in early embryonic cells has been a point of contention. Depletion of geminin mRNA from *Xenopus* embryos using morpholinos had no effect on cell cycle progression until the onset of the MBT. This suggested that regulation of geminin activity, and by association Cdt1 activity, was redundant during the pre-MBT embryonic cell cycle (McGarry, 2002). In contrast it has also been shown that functional knockdown of geminin activity causes arrest of the pre-MBT cell cycle suggesting that regulation of geminin and therefore Cdt1 activity is crucial during the pre-MBT cell cycle (Kisielewska and Blow, 2012). The role of Cdt1 regulation for proper embryonic development therefore required further clarification.

The structure of Cdt1 can be organised into three functional regions. The N-terminal region of the protein contains many of the regulatory regions including destruction boxes, a PIP box and cy motif which are each involved in Cdt1 regulation via degradation (Li *et al.*, 2003; Liu *et al.*, 2004; Nishitani *et al.*, 2004; Arias and Walter, 2005a; Nishitani *et al.*, 2006; Senga *et al.*, 2006). The central region of Cdt1 contains sites for binding to the Cdt1 inhibitor protein geminin while the C-terminal region contains the MCM binding domain which is essential for the licensing activity of Cdt1

(Ferenbach *et al.*, 2005). The highly characterised structure of the Cdt1 protein allowed truncated Cdt1 constructs to be designed which were deregulated but licensing active (ΔCdt1$^{243-620}$) and regulated but licensing inactive (ΔCdt1$^{1-243}$-RFP). The constructs were therefore designed to allow the effect of mis-regulation of Cdt1 to be investigated along with the mechanisms of Cdt1 regulation.

Upon microinjection into developing *Xenopus* embryos, mRNA is efficiently translated into protein (Gurdon *et al.*, 1974). This technique therefore provides the opportunity to investigate Cdt1 regulation during the pre-MBT cell cycle *in vivo* using the truncated Cdt1 constructs. The Cdt1 constructs were inserted into the pRN3 vector which is compatible with *in vitro* transcription to produce mRNA for expression in *Xenopus* embryos. In addition the ΔCdt1$^{1-243}$-RFP construct was also inserted into the pET-32a(+) vector to allow expression as recombinant protein to investigate Cdt1 regulation using the *in vitro Xenopus* egg extract system.

7.1.2 Correct Cdt1 Regulation is Crucial for pre-MBT Cell Division

Expression of the mRNA of the deregulated ΔCdt1$^{243-620}$ construct caused cell cycle arrest in pre-MBT *Xenopus* embryonic cells. The ΔCdt1$^{243-620}$-expressing cells were arrested with both Mcm2 and Cdt1 bound to the DNA which shows that the cells were arrested in a licensed state and suggests that re-licensing and subsequent re-replication of the DNA occurred. In addition chromatin bound Rad 51 and p53 were detected in the arrested cells showing the presence of DNA damage and suggesting activation of cell cycle checkpoints. The presence of DNA damage further substantiates the occurrence of DNA re-replication in the arrested cells. The cell cycle arrest also lead to severe developmental abnormalities in the effected *Xenopus* embryos. This provides strong evidence that Cdt1 activity must be tightly regulated for normal pre-MBT embryonic cell cycle progression.

It has previously been shown that depletion of geminin mRNA from pre-MBT *Xenopus* embryos using morpholinos has no effect on the embryonic cell cycle until the onset of the MBT at which point cell cycle arrest occurred. This lead to the suggestion that geminin and therefore Cdt1 activity were inconsequential for preventing re-replication in pre-MBT *Xenopus* embryos (McGarry, 2002). However it has since been shown that siRNA is unsuited to the *Xenopus* embryonic model and as such does not induce RNAi.

The siRNA in fact binds non-specifically to Ago proteins resulting in embryonic defects during later development (Lund *et al.*, 2011). This suggests that in the study by McGarry the effects observed were due to the geminin morpholinos binding to Ago proteins rather than depletion of geminin. It has also been shown that functional knockdown of the geminin protein in pre-MBT cells causes arrest of the cell cycle (Kisielewska and Blow, 2012). This is in agreement with the results presented here and therefore provides extensive evidence that Cdt1 regulation by geminin is essential to prevent re-replication and allow cell cycle progression in pre-MBT *Xenopus* embryos.

7.1.3   Cdt1 is Regulated by Changing Interactions with Geminin in pre-MBT Cells

Since Cdt1 regulation is critical to prevent re-licensing and re-replication to allow normal pre-MBT cell cycle progression it was important to determine the mechanisms of Cdt1 regulation in this system. The ΔCdt1$^{1-243}$-RFP construct consisting of only the N-terminal regulatory region of Cdt1 was used to investigate Cdt1 regulation during the embryonic cell cycle both *in vivo* and *in vitro*.

The N-terminal region of Cdt1 contains sites necessary to target the protein for ubiquitination during the somatic cell cycle. However there was no evidence of ubiquitination of the ΔCdt1$^{1-243}$-RFP construct either in *Xenopus* embryos or in the *Xenopus* egg extract system. In addition, when *Xenopus* embryos were treated with the proteasome inhibitor MG132 there was no accumulation of wild type Cdt1 and no evidence of accumulation of an ubiquitin ladder or chain. Taken together this suggests that Cdt1 is not regulated by ubiquitination during the pre-MBT cell cycle.

In addition to sites targeted for ubiquitination there are also sites for phosphorylation of Cdt1 located at the N-terminal region. In pre-MBT *Xenopus* embryos serine phosphorylation was detected at the molecular weight of chromatin bound Cdt1 and could indicate that phosphorylation may play a role in Cdt1 regulation. It is also possible that the serine phosphorylation detected was of a protein of the same molecular weight as Cdt1. There was also no detection of serine phosphorylation of the N-terminal ΔCdt1$^{1-243}$-RFP construct. It could be that the potential serine phosphorylation of chromatin bound Cdt1 was localised to a different area of the Cdt1 protein away from the N-terminal domain. However there is a PEST domain, which has recently been characterised in mouse Cdt1 but is conserved across different species,

located at the N-terminal of Cdt1 which is rich in serine and threonine residues. In human cells the PEST domain is phosphorylated to prevent Cdt1 recruitment to chromatin during M-phase (Coulombe *et al.*, 2013). Therefore it may be that this region is not phosphorylated in the *in vitro* system or it is also possible that the methods used were insufficient to detect the phosphorylation.

In *Xenopus* egg extract Cdt1 is degraded upon release from metaphase through APC/C mediated proteolysis (Li and Blow, 2005). The ΔCdt1$^{1-243}$-RFP construct was also degraded in *Xenopus* egg extract upon release from metaphase arrest. The degradation of ΔCdt1$^{1-243}$-RFP was inhibited by addition of the APC/C inhibitor Dbox while some of the degradation of ΔCdt1$^{1-243}$-RFP was also inhibited by p27, a CDK inhibitor which blocks initiation of DNA replication. This shows that as with endogenous Cdt1 the ΔCdt1$^{1-243}$-RFP construct is degraded by two different pathways: APC/C mediated degradation and replication dependant degradation. As is the case with endogenous Cdt1, the degradation of ΔCdt1$^{1-243}$-RFP in activated egg extract was abolished in extract supplemented with lower DNA concentrations (Kisielewska and Blow, 2012). This can be attributed to less DNA content resulting in less replication dependant degradation of ΔCdt1$^{1-243}$-RFP.

Although the N-terminal ΔCdt1$^{1-243}$-RFP construct of Cdt1 is targeted for degradation as with endogenous Cdt1, there was a lack of accumulation of Cdt1 in pre-MBT embryos treated with the proteasome inhibitor MG132. This suggests that Cdt1 is not degraded during the pre-MBT cell cycle. This is in line with recently published data showing that Cdt1 and geminin levels remain persistently high during the pre-MBT cell cycle (Kisielewska and Blow, 2012) and therefore confirms that degradation of Cdt1 is not enlisted to regulate Cdt1 activity in pre-MBT *Xenopus* embryos. This suggests that during the pre-MBT cell cycle either the mechanisms for targeting Cdt1 for proteolysis are absent or inactive; or alternatively Cdt1 is protected from degradation. One potential candidate protein for protecting Cdt1 from proteolysis is geminin which has been shown to stabilise Cdt1 in human cells by preventing ubiquitination and subsequent degradation (Ballabeni *et al.*, 2004).

It has previously been suggested that a complex between Cdt1 and geminin may act as a switch to flip between licensing active and inactive states with a Cdt1:geminin

complex shown to possess licensing activity (Lutzmann *et al.*, 2006). In agreement with this it has been shown that the Cdt1:geminin complex can form two stoichiometries of 2:4 and 1:2 with the 2:4 Cdt1:geminin complex incapable of licensing the DNA (De Marco *et al.*, 2009). The results presented here show that Cdt1 regulation is crucial during the pre-MBT cell cycles. However Cdt1 is not degraded nor is there evidence of Cdt1 ubiquitination, although there may be phosphorylation specific to chromatin bound Cdt1. Combined with the persistently high levels of both Cdt1 and geminin throughout the pre-MBT cell cycle (Kisielewska and Blow, 2012) this suggests that Cdt1 is regulated through changing interactions with geminin. This mechanism would allow Cdt1 to bind geminin in a licensing active stoichiometry during DNA licensing and in a licensing inactive stoichiometry during S-phase to prevent re-licensing and re-replication. The proposed model for regulation of Cdt1 in the pre-MBT cell cycle is shown in Figure 7.1.

The model of Cdt1 regulation through dynamic complex formation with geminin (Figure 7.1) would allow regulation of Cdt1 in the absence of degradation. It would also explain how $\Delta Cdt1^{243-620}$ was able to cause the cell cycle arrest by disrupting the Cdt1:geminin ratio and escaping inactive complex formation.
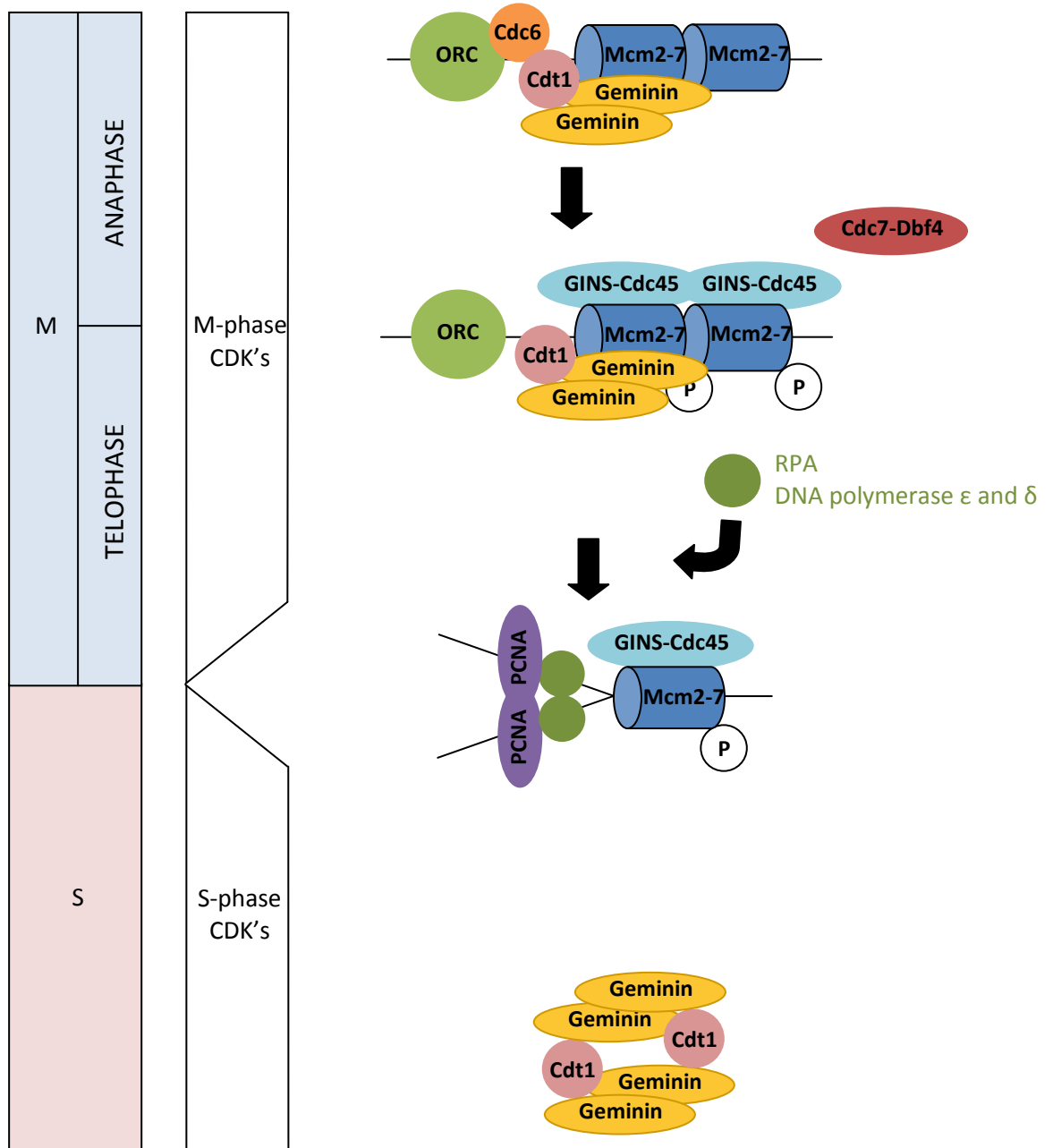
Figure 7.1. Proposed model for DNA licensing and Cdt1 regulation in pre-MBT *Xenopus* embryos. The pre-MBT embryonic cell cycle consists only of alternating S- and M- phases. DNA licensing therefore occurs during anaphase of M-phase with PCNA detected on chromatin during telophase suggesting initiation of DNA replication. Regulation of Cdt1 is predominantly through forming a licensing active complex with geminin during M-phase and a licensing inactive complex during S-phase with some regulation of chromatin bound Cdt1 via serine residue phosphorylation. Based on the Cdt1:geminin complex structures characterised by De Marco *et al.*, the active complex is shown in a 1:2 ratio of Cdt1:geminin and the inactive complex is shown in a 2:4 ratio.

7.1.4   The Cdt1 N-terminal Region Participates in DNA and Protein Interactions

The DNA and protein interactions of the N-terminal ΔCdt1$^{1\text{-}243}$-RFP construct were also investigated. The ΔCdt1$^{1\text{-}243}$-RFP construct is capable of both crossing the nuclear envelope, which is in agreement with previous work showing a nuclear localisation signal located to the Cdt1 N-terminal (Nishitani *et al.*, 2004), and binding DNA. In addition the ΔCdt1$^{1\text{-}243}$-RFP construct was able to bind DNA during S-phase in *Xenopus* embryonic cells and at the onset of S-phase in *Xenopus* egg extract. The binding of the N-terminal region to DNA was via a mechanism dependant on PCNA. However since the ΔCdt1$^{243\text{-}620}$ construct which lacks the N-terminal region is capable of DNA licensing (Ferenbach *et al.*, 2005) this suggests that there are other mechanisms for Cdt1 DNA binding targeted away from the N-terminal region. There may therefore be redundancy in mechanisms for DNA loading of Cdt1.

The results presented here also show that the ΔCdt1$^{1\text{-}243}$-RFP construct binds to Orc1. The interaction domains of Cdt1 to other licensing proteins have not been previously mapped (Caillat and Perrakis, 2012) and this data suggests that there is an Orc1 binding site located at the N-terminal region of Cdt1. It is possible that an interaction between Cdt1 and Orc1 through the N-terminal binding site plays a role during DNA licensing. However the ΔCdt1$^{243\text{-}620}$ construct, which lacks the N-terminal region and therefore the N-terminal Orc1 binding site, is still capable of licensing the DNA (Ferenbach *et al.*, 2005). This suggests that the interaction between the Cdt1 N-terminal and Orc1 is not essential for DNA licensing. It is possible that this interaction acts redundantly to interactions with other ORC proteins. It is therefore important for further work to map the other protein-protein interaction domains of Cdt1.

It has previously been shown that mechanisms of regulation of Cdt1 act redundantly in *Xenopus* egg extract with re-replication of DNA occurring only when both geminin inhibition and Cdt1 proteolysis are abolished (Li and Blow, 2005). This redundancy is unsurprising given the importance of correct regulation of Cdt1 activity for proper DNA licensing. The potential redundancy observed in the Cdt1 interactions with DNA and Orc1 suggests that there may be multiple layers of redundancy across many aspects of Cdt1 function in order to ensure correct activity of Cdt1.

7.2 Future Work

In Chapter 5 the regulation of the N-terminal $\Delta Cdt1^{1-243}$-RFP construct was investigated. It was shown that concentrations of the p27 inhibitor above 52 µM partially prevented the degradation of $\Delta Cdt1^{1-243}$-RFP. This suggests that degradation of $\Delta Cdt1^{1-243}$-RFP is at least in part dependant on replication and interaction with PCNA since p27 blocks PCNA loading and initiation of DNA replication. This is consistent with reports showing that full length Cdt1 is degraded in a replication dependant manner through interaction with PCNA via a PIP box located at the N-terminal region of Cdt1 (Arias and Walter, 2005a). The stabilisation of $\Delta Cdt1^{1-243}$-RFP levels in *Xenopus* egg extract under low DNA conditions in which lower DNA content would lead to lower levels of replication-dependant degradation also provides evidence that the $\Delta Cdt1^{1-243}$-RFP construct is degraded via this mechanism. However, it would also be appropriate to repeat the experiment at a single concentration of p27 to observe the stabilisation of $\Delta Cdt1^{1-243}$-RFP in the presence of p27 over time from activation of the extract in comparison to the degradation of $\Delta Cdt1^{1-243}$-RFP in the absence of p27. Repeating this experiment in duplicate would also allow statistical analysis of the data and robust confirmation of the role of PCNA-mediated replication-dependant degradation of the $\Delta Cdt1^{1-243}$-RFP construct.

It has been shown that in *Xenopus* egg extract endogenous Cdt1 is degraded through mechanisms mediated by the APC/C (Li and Blow, 2005). Consistent with this in Chapter 5 it was shown that the APC/C inhibitor Dbox prevents degradation of $\Delta Cdt1^{1-243}$-RFP. The N-terminal $\Delta Cdt1^{1-243}$-RFP construct is therefore also targeted for degradation mediated by the APC/C. This experiment was repeated twice and statistical analysis of the results confirmed that Dbox inhibited degradation of $\Delta Cdt1^{1-243}$-RFP. However it would of provided a more robust statistical analysis if the n number of the experiments was increased to at least n=3 which was used to show the degradation of $\Delta Cdt1^{1-243}$-RFP in activated egg extract.

Although it has been shown previously that there are sites in the N-terminal region of Cdt1 which are targeted for ubiquitination there was no evidence of ubiquitination of either $\Delta Cdt1^{1-243}$-RFP or wild type Cdt1 in *Xenopus* embryos *in vivo*. This suggests that Cdt1 is not regulated by ubiquitin mediated mechanisms during the embryonic cell cycle. However, ubiquitination is difficult to detect using ub antibodies and western

blotting as there is the possibility that the antibodies may be unreliable. In an attempt to preserve any ubiquitination present and aid detection, a deubiquitylase inhibitor was used in the buffer when producing the embryo extract which was blotted for uniquitin (Figure 5.3C). An alternative technique which could be used to confirm that there is no ubiquitination of either $\Delta Cdt1^{1-243}$-RFP or endogenous Cdt1 in embryos would be to express a tagged version of ubiquitin and then immunoblot for the tag rather than the ubiquitin itself (Choo and Zhang, 2009). The tagged ubiquitin could also be added to egg extract along with $\Delta Cdt1^{1-243}$-RFP before co-immunoprecipitation to determine whether or not the two interact.

To determine whether or not the $\Delta Cdt1^{1-243}$-RFP construct was able to bind to other licensing proteins IP was used. The results presented in Chapter 6 show that $\Delta Cdt1^{1-243}$-RFP binds to Orc1 and PCNA. However, only two time points of the cell cycle in the egg extract were sampled and therefore it cannot be ruled out that there are other protein-protein interactions involving Cdt1 and the licensing proteins at other time points during DNA licensing. It would therefore be useful to repeat the experiments and sample more time points from earlier in the cell cycle during the time of active DNA licensing and investigate binding of other licensing proteins such as Cdc6. Since the interaction domains of the Cdt1 protein with the other licensing proteins other than the MCM complex and geminin have not been mapped (Caillat and Perrakis, 2012), IP using $\Delta Cdt1^{1-243}$-RFP would be a useful technique to map any binding sites present in the N-terminal region. Given that Orc1 binds to $\Delta Cdt1^{1-243}$-RFP it is also possible that $\Delta Cdt1^{1-243}$-RFP interacts with one of the other ORC complex proteins Orc2, Orc3, Orc4, Orc5 or Orc6.

Until recently the interactions and mechanisms of Mcm2-7 loading via the licensing proteins ORC, Cdc6 and Cdt1 was not known. Recent studies in yeast have shown that there are several steps during the process of DNA licensing in which multi-protein complexes between ORC, Cdc6, Cdt1 and Mcm2-7 are formed in order to load the Mcm2-7 complex and form a licensed DNA origin (Takara and Bell, 2011, Sun *et al.*, 2013)). The mechanism behind the loading of the second Mcm2-7 complex to form the Mcm2-7 heterohexamer is still unclear (Fernandez-Cid *et al.*, 2013). It is therefore important for future work to establish the mechanisms behind the loading of the second Mcm2-7 complex in this yeast model system. In addition given the differences

between the yeast system and the *Xenopus* embryo it is also important to establish whether or not the same interactions and complexes exist between the ORC, Cdc6 and Cdt1 proteins in loading the Mcm2-7 complex in both *Xenopus* embryos *in vivo* and in *Xenopus* egg extract *in vitro*.

Chapter 8.

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