Regulation of replication licensing by geminin and Cdt1 in *Xenopus* embryonic cell cycle

アフリカツメガエル初期胚型細胞周期における geminとCdt1による複製ライセンス化制御

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General introduction

This study focuses on replication licensing, an essential step in chromosomal replication. In the general introduction, I begin with a general view of DNA replication and the cell cycle (section I). In section II, I describe how the mechanism and components of replication licensing are identified. In sections III and IV, I discuss the findings of previous studies as well as unsolved questions specifically related to this study. Section III focuses on the mechanism of replication licensing, and section IV focuses on the inhibition of licensing.

I. REPLICATOR AND INITIATOR FOR SEMI-CONSERVATIVE REPLICATION

Semi-conservative DNA replication

Self-reproduction is a fundamental process by which cells proliferate, both create and maintain a multicellular organism from a single egg, and propagate throughout the world. During this process, genetic information must be precisely duplicated in order to generate two genetically identical daughter cells. Most of the genetic information is encoded as a sequence of deoxyribonucleic acids (DNA) in the cellular nucleus. In 1944, Avery et al. (Avery *et al.* 1944) demonstrated that DNA is a chemical entity that causes a genetic transformation of a pneumococcal bacteria reported by Griffith (Griffith 1928), thus showing that DNA is a carrier of genetic information. This notion about the nature of genetic information was supported by the work of Hershey and Chase (Hershey & Chase 1952), in which a chemical entity that infects *E. coli* from a bacteriophase T2 is DNA and not protein. The mechanism for encoding genetic information into DNA lies in the structure of DNA. In 1953, based on the X-ray diffraction study of DNA (Franklin & Gosling 1953; Wilkins *et al.* 1953) and Chargaff's rule about the ratio of four bases--adenine, thymine, guanine, and cytosine--in DNA (Chargaff *et al.* 1951), Watson and Click proposed the double-helical structure of DNA (Watson & Crick 1953). This structure suggests that the source of genetic information is the sequence of four bases aligned in DNA. Furthermore, complementary pairing of two strands of DNA implies a mechanism by which genetic information is precisely duplicated. This mechanism is called semi-conservative replication, in which a new DNA strand is synthesized using one of the original double strands as a template and the resulting duplicated double strands contain both an original and a new strand. The semi-conservative theory was experimentally confirmed by the work of Meselson and Stahl (Meselson & Stahl 1958). Using radio-labeled nitrogen and density-gradient centrifugation, they chased the radio-labeled DNA of one generation of *E.coli* down to several generations of progenitors. They found that the labeled strands of DNA are received equally by the next generation of bacteria, and subsequently the labeled strands are conserved through many successive generations, not diluted with non-labeled newly synthesized strands. These and many other studies make it clear that genetic information is carried by DNA and duplicated in a semi-conservative manner.

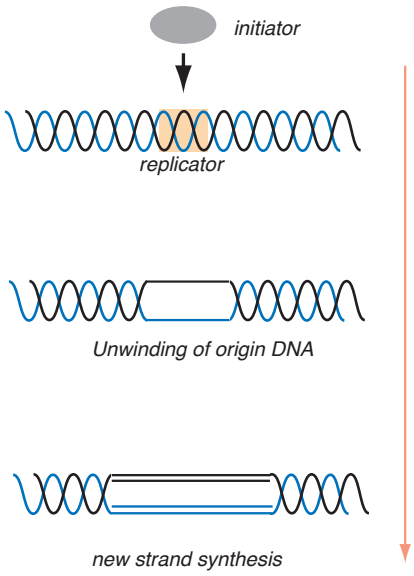
Replicon hypothesis

At the initiation step of semiconservative replication, DNA requires other factors that recognize the original DNA template and recruit DNA polymerases that synthesize new complementary DNA strands. In 1963, Jacob, Brenner, and Cuzin (Jacob *et al.* 1963) launched a replicon hypothesis as a first attempt at a basic concept of the relationship between template DNA and factors that replicate the template. In the replicon hypothesis, they claimed that regulation of DNA synthesis involves two elements. The first one is an initiator that is a diffusive *trans* element and activates DNA replication. The second one is a *replicator* that is a specific *cis* element on DNA upon which the initiator acts to initiate DNA replication. The replicator is assumed to function specifically in the initial step of DNA replication. Once replication is initiated, any DNA sequence attached to the replicator is subsequently replicated. In addition, the initiator is assumed to act on its corresponding replicator. For example, the bacterial initiator triggers replication from the bacterial replicator but not from the phage replicator. Therefore, a pair consisting of an initiator and a replicator is the minimum element needed for a unit of DNA to be autonomously replicated in a cell, namely a replicon. The replicon hypothesis was applied initially to explain the replication mechanisms of bacteria and phages, but later it became clear that the hypothesis could be applied to other organisms, from archaea to eukaryotes.

The molecular nature of replicator and initiator

The required functions of an initiator are to 1) recognize the replicator element, 2) unwind the double-helical DNA template, and 3) synthesize a primer for DNA polymerase. Then, DNA polymerase comes to extend the primer. Studies on bacterial DNA replication have revealed the molecular entities responsible for these functions (Fig. 1). The *E. coli* genome typically contains a single site where replication initiates. Regions of chromosomes where replication initiates are called origins of replication. In 1977, Yasuda and Hirota isolated the region of replicative origin as a DNA element, by which otherwise non-replicating plasmids can be replicated (Yasuda & Hirota 1977). The element, called *OriC*, serves as a replicator of the *E. coli* genome. The molecular nature of the initiator has been revealed through an *in vitro* reconstitution system of *OriC*-dependent DNA replication. *OriC* is recognized by DnaA protein, which binds to several consensus sequences within the *OriC* element (Fuller *et al.* 1984; Matsui *et al.* 1985). The loaded DnaA facilitates the melting of a specific site within the *OriC* element (Bramhill & Kornberg 1988), and loads the DnaB-DnaC complex onto the emerged single-strand DNA (Wahle *et al.* 1989). Then DnaG primase interacts with DnaB, and DnaC is released from DnaB. Upon the release of DnaC, DnaB is activated as a replicative helicase (Makowska-Grzyska & Kaguni 2010). Thus, DnaC is specifically required to recruit DnaB onto the DNA opened by DnaA. The resultant helicase-primase complex subsequently synthesizes the primer as the DnaB helicase unwinds DNA to generate a template for the primase. The primer is extended by PolII DNA polymerase holoenzyme. DnaB helicase proceeds to separate the DNA strands in front of the DNA polymerase to

maintain the polymerase progression. The single-strand regions that emerge are covered and stabilized with single-strand binding protein (SSB). Hence, in this scenario the anticipated functions of the initiator lie in a concerted function of several proteins.



	E. Coli	SV40	eukaryote
Replicator (Origin)	OriC	SV40 ori	ARS (yeast)
Initiator			
Replicator recognition	DnaA	T antigen	ORC
Helicase loader	DnaC	T antigen	Cdc6, Cdt1
Helicase	DnaB	T antigen	Mcm2-7
Primase	DnaG	Pol α	Pol α
Clamp loader	Pol III β subunit	RFC	RFC
Sliding clamp	Pol III γ subunit	PCNA	PCNA
DNA polymerase	Pol III	Pol δ	Pol δ, Pol ε

Fig. 1 Replicator and initiator in various organisms.

SV40 DNA replication *in vitro*

The mechanism underlying the replication of eukaryotic chromosomes was initially investigated through an *in vitro* system for replicating a viral DNA template in cell extracts. Simian virus 40 (SV40), an animal virus, is a useful system for screening eukaryotic factors required for DNA replication, since the viral "initiator" protein, large tumor antigen (T-antigen), has a characteristic property. T-antigen is a viral-coded protein that recognizes a viral origin of replication (SV40 ori). T-antigen binds to the SV40 ori, melting the origin and initiating replication. It then serves as a replicative helicase, whereas it utilizes cell-coded proteins involved in chromosomal replication machinery such as DNA polymerases and single-strand binding proteins. Thus, T-antigen initiates the replication of the DNA template with SV40 ori in cell extracts by recruiting cellular replication factors (Li & Kelly 1984, 1985; Wobbe *et al.* 1985). This cell-free system of SV40 DNA replication made a great contribution to the identification of cellular replication machinery. Finally, Waga and Stillman succeeded in reconstructing SV40 DNA replication using purified proteins instead of cell extracts (Waga & Stillman 1994). The reconstructed system, as well as yeast genetic studies, revealed a stepwise mechanism to recruit polymerases to the replication initiation site. When the T-antigen helicase unwinds the SV40 ori, Polα/primase and the single-strand DNA binding protein RPA (replication protein A) are loaded by virtue of protein-protein interaction among T-antigen, Polα, and RPA. Polα then produces an RNA primer, and the 3´-terminus of the primer/template junction is recognized by RFC (replication factor C). RFC is a loader for

clamp protein PCNA (proliferating cell nuclear antigen), which functions as a processivity factor for Polδ, a replicative polymerase. Thus, RFC on the primer/template junction recruits PCNA and Polδ, so that Polδ extends the primer along with long stretches of template DNA. Although Polδ appeared to be the only processive polymerase in this system, it is currently understood that Polδ participates mainly in lagging-strand synthesis and that another processive polymerase, Polε, participates in leading-strand synthesis *in vivo* (Karthikeyan *et al.* 2000; Pursell *et al.* 2007).

As T-antigen bypasses the initial step of eukaryotic DNA replication, the SV40 system cannot reveal cellular mechanisms for origin selection, helicase loading, or recruitment of DNA polymerases. These mechanisms for origin selection/activation were revealed through the understanding of how the initiation of replication is coupled with cell-cycle progression.

II. REPLICATOR AND INITIATOR IN EUKARYOTES

In contrast to *E.coli*, eukaryotic chromosomes contain multiple origins of replication. Using an autoradiography technique against radio-labeled replicating DNA of mammalian cells, Huberman and Riggs demonstrated that chromosomal DNA is made up of tandemly joined sections that are replicated by bidirectional replication "forks" originating from the single site of origin (Huberman & Riggs 1968). At present, it is known that numerous origins--e.g., tens to hundreds of thousands of origins in the entire chromosomes of metazoans--are involved in eukaryotic DNA replication. In order to precisely duplicate the whole chromosome, each origin should initiate DNA replication once, but only once, in a single round of the cell cycle. In other words, origins prior to replication can be replicated, but replicated origins must not be replicated (Fig. 2). This fundamental view of replication progression will lead to the following questions: what is the difference between pre-replicated and post-replicated origins? Why can't the initiator induce replication from post-replicated origins? The answers to these questions lie in the molecular nature of the initiator in eukaryotes. The function of the initiator in metazoans is carried out by four kinds of proteins: ORC, Cdc6, Cdt1, and Mcm2-7. In this section, I review how these factors are identified as the initiator in eukaryotes.

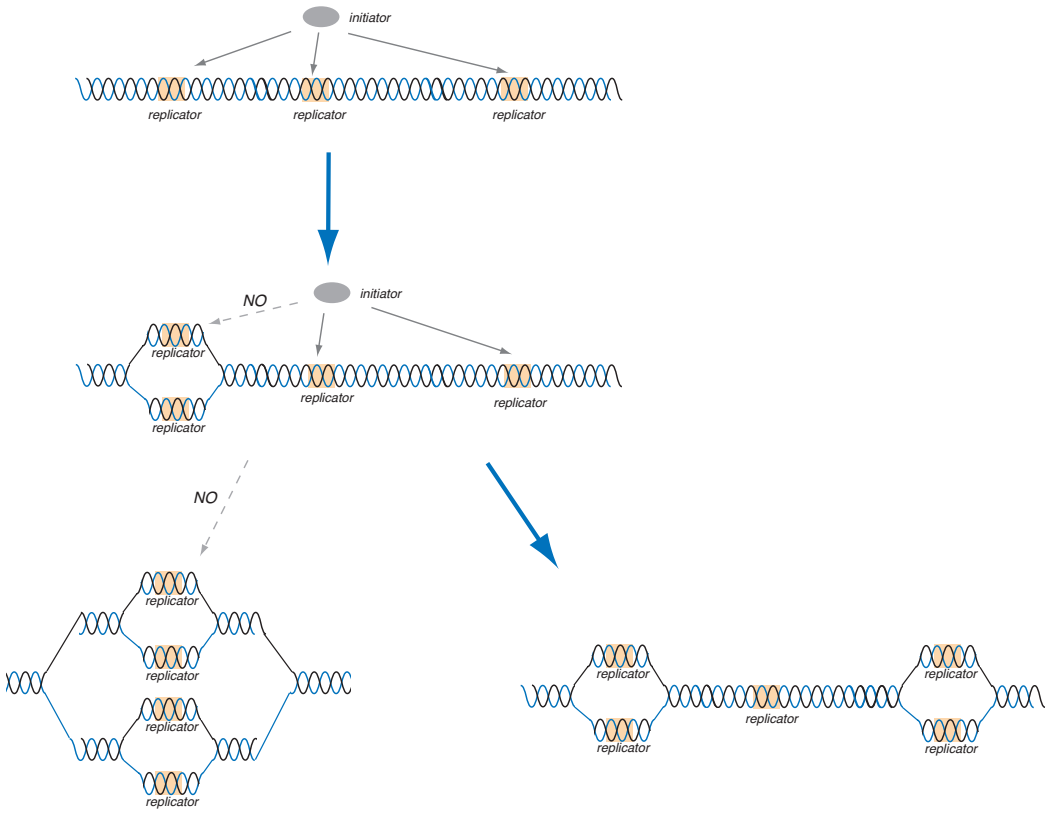


Fig. 2 Eukaryotic chromosomes involve multiple sites for replication initiation.
Replication initiates only from un-replicated regions of chromosome. Over-replication of replicated regions leads to an abnormal structure of chromosome resulting in chromosomal instability (left bottom). To avoid such over-replication, cells should distinguish un-replicated regions from replicated regions (middle part).

Cell-cycle control of DNA replication

In 1970, Rao and Johnson performed a cell fusion analysis that provides several clues to understanding the cell-cycle control of DNA replication (Rao & Johnson 1970) (Fig. 3). They fused cells in different stages of the cell cycle. When G1 cells were fused with S cells, nuclei derived from the G1 cells immediately initiated replication. In contrast, when G2 cells were fused with S cells, nuclei derived from G2 cells did not initiate replication. The outcome of these results is that 1) S-phase cells provide a factor, the so-called S-phase promoting factor (SPF), that drives the initiation of replication in the G1 nucleus; and that 2) G2 cells lose sensitivity to SPF. This indicates that DNA replication is initiated once and only once per round of cell cycle. The activity of SPF mainly lies in the protein complex of cyclin-dependent kinase (CDK) and its regulatory subunit, cyclin.

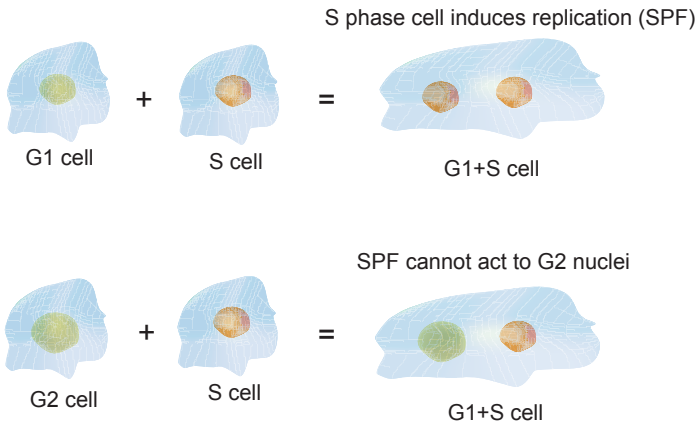


Fig. 3 Cell fusion experiments.
The cell fusion experiment performed by Rao and Johnson. S phase cell induces the initiation of replication in G1-derived nuclei but not in G2-derived nuclei.

The activity of cyclin-CDK was initially identified as a cytosolic activity of matured amphibian eggs. The cell cycles of immature eggs are arrested at meiosis I. The hormone progesterone triggers the maturation of eggs, and the eggs proceed into meiosis II. In 1971, Masui and Market showed that injection of cytosol from a mature frog egg into an immature frog egg induces the maturation of the injected immature eggs (Masui & Markert 1971). This activity was called the maturation-promoting factor (MPF), and further study revealed that MPF induces the initiation of mitotic events (thus the maturation-promoting factor was later realized as the M-phase promoting factor). Several studies suggested that MPF activity involves protein kinase activity. In 1988, MPF was eventually purified as a complex of two proteins (Lohka *et al.* 1988). One protein corresponds to cyclin. Cyclin was initially found in sea urchin whose protein expression synchronously oscillates with cell divisions (Evans *et al.* 1983). The other protein that retained kinase activity turned out to correspond to the Cdc2 protein, which had been identified as a gene essential to cell cycle progression in fission yeast (Hartwell 1973; Hartwell *et al.* 1974). Then it became clear that cyclin binds to Cdc2, and through this binding the cyclin-Cdc2 complex exhibits protein kinase activity. Thus, Cdc2 is categorized as cyclin-dependent kinase (CDK). CDK activates the initiation step of the M phase. In addition, CDK activity is essential for initiating DNA replication.

Engines for driving cell cycle: CDK, APC/C, and SCF

As the expression of cyclin oscillates during the cell cycle, CDK activity also oscillates along with the cell cycle. CDK exhibits its highest activity in the M phase. At the end of the M phase, CDK activity falls because its regulatory subunit, cyclin, is degraded at this time. Efforts to identify factors responsible for the cyclin degradation led to the finding of APC/C, a protein complex composed of tens of subunits (for review (Zachariae & Nasmyth 1999)). The APC/C complex is a ubiquitin ligase that leads its substrates, including cyclin, to a proteasome-mediated proteolysis (for review (Nakayama & Nakayama 2006)). CDK activates APC/C at the initial stage of the M phase; in turn, activated APC/C induces the destruction of cyclin at the M phase exit. A decrease in CDK activity results in a decrease in APC/C activity, and thus cyclin begins to re-accumulate. This reciprocal cycle of CDK and APC/C activation is called the "embryonic" cell cycle. The embryonic cell cycle is a simple one in which CDK activity begins to increase soon after the exit from the M phase. This type of cell cycle is seen in initial cleavage cycles after the fertilization of amphibian eggs such as those of *Xenopus* frogs.

In a somatic or "normal" cell cycle, there is a G1 phase between the M and S phases, in which CDK activity is stably low. The G1 phase is achieved, at least in part, by the action of the CDK inhibitor, which appears at the G1 phase in the somatic cell cycle. At the end of the G1 phase, the CDK inhibitor is destroyed, allowing the re-accumulation of CDK activity. SCF is found in studies focusing on the factors responsible for the degradation of the CDK inhibitor and other proteins at the G1/S transition (Feldman *et al.* 1997; Skowyra *et al.* 1997). SCF is a ubiquitin ligase that promotes the proteolysis of its substrates, as does APC/C (for review (Nakayama & Nakayama 2006)). SCF is activated by CDK at the end of the G1 phase and stimulates the further activation of CDK by degrading the CDK inhibitor. The activated

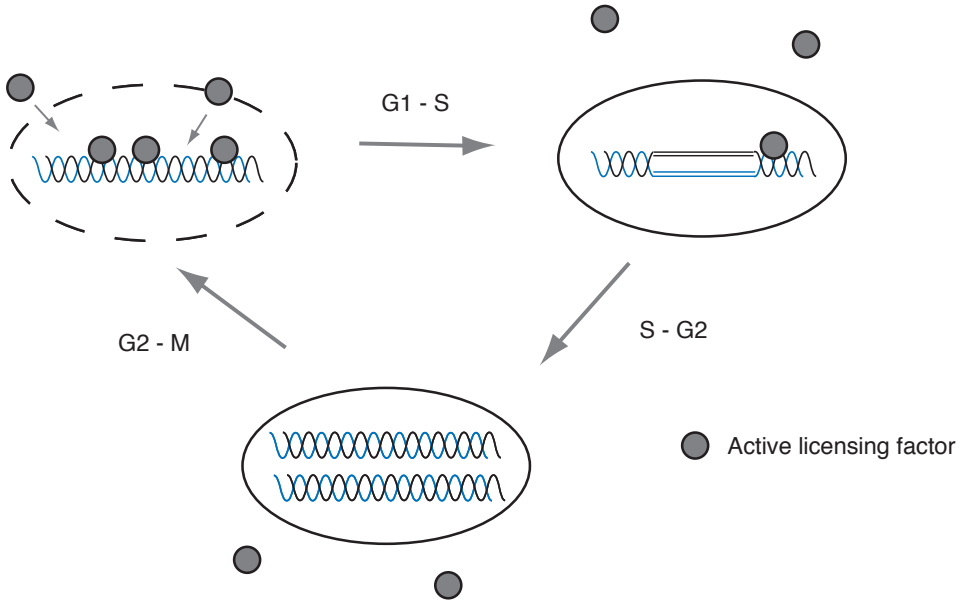


Fig. 4 Licensing factor hypothesis.
Licensing factor associates with chromatin when nuclear membrane breaks down. Formation of nuclear membrane allows the licensing factor to induce replication initiation. Subsequently, licensing factor on chromatin is inactivated by replication process. As licensing factor cannot pass through the nuclei, replication initiation can occur only once in a single round of the cell cycle.

CDK then initiates DNA replication, and cells enter the S phase. In contrast, in the embryonic cell cycle, there is no G1 phase with lower CDK activity. Thus, DNA replication is initiated soon after the exit from the M phase.

Xenopus egg extract and licensing hypothesis

Given that S-phase cells exhibit SPF activity, why do only G1 cells, and not G2 cells, initiate DNA replication in response to SPF? An important idea leading to the mechanism underlying this difference was provided by studies using extracts of African clawed frog *Xenopus laevis* eggs. *Xenopus* eggs are arrested in the metaphase of meiosis II. The artificial "activation" of the eggs, which mimics their fertilization through the entry of Ca^{2+} ion into them, releases the eggs from meiosis to the interphase. Extracts made from the activated eggs maintain the activity of initiating DNA replication (Lohka & Masui 1983). When chromatin from *Xenopus* sperm is added to the extract, a nuclear envelope is formed around the chromatin. Then several proteins, including cyclin-CDK and other proteins that are needed for the initiation of replication, are imported and accumulated into the formed nucleus through nuclear import. Hence, elevated SPF activity initiates the replication of sperm chromatin in the formed nucleus. The chromatin is replicated in a complete semi-conservative manner only once per cell cycle, and no replication is initiated after the completion of the first round of replication (Blow & Laskey 1986). In 1989, Blow and Laskey asked what kinds of changes in nuclei that have passed through DNA replication allow the initiation of the second round of replication, namely re-replication (Blow & Laskey 1988). They found that the only requirement for inducing re-replication to replicated nuclei was to permeabilize the nuclear envelop. This finding suggested that the breakdown of the nuclear envelope during mitosis allows the chromatin to be replicated in the next round of the S phase. Based on this result, they proposed the presence of a licensing factor (Fig. 4); the factor binds to DNA and is essential for initiating replication, thereby giving chromatin a "license" for initiating replication. The licensing factor was assumed to be inactivated by the initiation or passage of a replication fork. It was also assumed to be impossible to be imported into the nucleus. Therefore, a new licensing factor would be able to access chromatin only when the nuclear envelope breaks down at mitosis. Before mitosis, re-replication cannot occur because of the lack of a license on chromatin.

Egg extract also contributed to the identification of the licensing factor. Kubota et al. compared chromatin binding proteins in nuclei formed in two distinct conditions. In one condition, the nuclei are formed in the interphase extract, which could initiate replication. In the other condition, the nuclei are formed in the M-phase extract with kinase inhibitor, which cannot initiate replication. They found that the Mcm3 protein is one of the proteins that bind dominantly on chromatin formed in the interphase extract (Kubota *et al.* 1995). Mcm3 was identified as a member of the Mcm family by the genetic screening of factors required to maintain mini-chromosomes in successive yeast generations (Maine *et al.* 1984; Yan *et al.* 1991). Several studies in yeast, egg extract, and human cells identified Mcm3 and some other

Mcm proteins as factors showing the anticipated behavior of the licensing factor; the proteins bind to chromatin, are essential for initiating replication, and are displaced from chromatin after the initiation of replication (Chong *et al.* 1995; Kimura *et al.* 1995; Kubota *et al.* 1995; Madine *et al.* 1995; Todorov *et al.* 1995; Donovan *et al.* 1997). Thereafter, intensive studies have made it clear that six proteins in the Mcm family, Mcm2-7, form a hetero-hexameric complex that is essential for the initiation of DNA replication. It is now widely accepted that the binding of Mcm2-7 is the molecular nature of the license that allows origins to initiate replication. Mcm2-7 binds to chromatin, but this binding requires additional factors that directly recognize the replicator sequence and thus select the region of replication initiation.

Eukaryotic origins of replication

The eukaryotic DNA sequence responsible for replication origins was initially identified by using the yeast of *Saccharomyces cerevisiae*. Based on a similar strategy used for the identification of *OriC*, the origins of yeast replication, namely ARS (autonomous replicating sequence), were identified as the DNA element that gives a plasmid the potential to be replicated in the yeast (Struhl *et al.* 1979). The identified ARS was later confirmed to actually serve as a replicative origin in the yeast chromosomes (Brewer & Fangman 1987; Huberman *et al.* 1987). Thereafter, numerous origins containing conserved ARS elements were found in the yeast. In contrast to the case of budding yeast, the sequence-specificity of origins in other organisms is much more relaxed. Actually, apparent consensus sequences were not found in any organism other than budding yeast (Segurado *et al.* 2003; MacAlpine *et al.* 2004; Feng *et al.* 2006; Tanny *et al.* 2006; Hayashi *et al.* 2007; Cadoret *et al.* 2008; Sequeira-Mendes *et al.* 2009). Nevertheless, the sites for replication initiation are not random in most cell types. Factors that may affect the origin selection involve chromatin structure, transcription, and associated changes in DNA topology. In *Xenopus* egg extract, however, it was shown that DNA replication can initiate at random from any sequence (Mahbubani *et al.* 1992; Hyrien & Mechali 1993), whereas the intervals between adjacent replication initiation sites are almost constant at 10 kb (Herrick *et al.* 2000; Blow *et al.* 2001; Jun *et al.* 2004). The mechanisms for the selection of origins in metazoans are elusive. Hence, factors that directly recognize origin sites were found in budding yeast, which is an advantageous model organism by virtue of its clear sequence-dependency in origin selection.

Factors required for the recruitment of Mcm2-7 onto origins

The origin-recognition complex (ORC) was found as a protein complex that binds to the ARS in budding yeast. Through the fractionation of nuclear extract of the yeast, a protein complex composed of six subunits, Orc1-6, was identified as a factor that binds several regions of ARS1 origin of DNA replication (Bell & Stillman 1992). Mutation on the ARS sequence that abolishes the origin activity of ARS also causes a defect in the interaction between ORC and ARS. In addition, ORC was found to be essential for DNA replication (Bell *et al.* 1993; Micklem *et al.* 1993; Fox *et al.* 1995; Loo *et al.* 1995). A genomic footprinting assay revealed

that ORC is bound onto ARS throughout the cell cycle, but some additional proteins bind to ARS at the end of mitosis to the G1 phase (Diffley *et al.* 1994). The complex formed on ARS specifically before S phase was called pre-replicative complex (pre-RC). Then the Cdc6 protein, which had been identified in a pioneering screening for a budding yeast mutant that has a cell-cycle defect (Hartwell 1973), was identified as a factor required for the establishment of pre-RC (Cocker *et al.* 1996). Studies in Xenopus egg extract and yeast further revealed that both ORC and Cdc6 are required for recruiting Mcm2-7 on chromatin (Carpenter *et al.* 1996; Coleman *et al.* 1996; Romanowski *et al.* 1996; Rowles *et al.* 1996; Donovan *et al.* 1997). In higher eukaryotes, ORC binds to DNA without apparent sequence dependency, resulting in a sequence-independent origin selection. Thereafter, Cdt1 was identified as an essential factor for ORC-dependent Mcm2-7 binding on origins. Cdt1 was originally identified in fission yeast *Saccharomyces pombe* as a protein whose transcription is dependent on Cdc10 (Hofmann & Beach 1994). Subsequently, Cdt1 turned out to be an essential factor for Mcm2-7 binding on chromatin (Maiorano *et al.* 2000b; Nishitani *et al.* 2000). Cdt1 bound to chromatin depending on ORC, and depletion of Cdt1 disrupted the binding of Mcm2-7 on chromatin.

In summary, Mcm2-7 recruitment on origin is dependent on all three kinds of proteins: ORC, Cdc6, and Cdt1. Thus, essential components of pre-RC are Mcm2-7, ORC, Cdc6 and Cdt1. The sufficiency of ORC, Cdc6, and Cdt1 for Mcm2-7 recruitment was confirmed through a re-constructive approach. Initially, Mcm2-7 loading was re-constructed with purified proteins of *Xenopus* eggs (Gillespie *et al.* 2001). Recently, the loading was also re-constructed using purified ORC, Cdc6, Cdt1, and Mcm2-7 proteins from yeast extracts (Remus *et al.* 2009). Furthermore, studies with *Xenopus* egg extract and budding yeast have shown that ORC and Cdc6 are dispensable for the initiation of replication after Mcm2-7 is loaded onto chromatin (Donovan *et al.* 1997; Hua & Newport 1998; Rowles *et al.* 1999). Therefore, the essential function of ORC, Cdc6, and Cdt1 in replication initiation is to recruit Mcm2-7 on origins, and the molecular nature of the replicative license is Mcm2-7 recruited on chromatin.

III. MECHANISMS FOR REPLICATION LICENSING

The assembly of Mcm2-7 onto replicative origin is achieved by the coordinated function of ORC, Cdc6, and Cdt1. Previous studies revealed the dynamic behavior of each component to recruit the Mcm2-7 complex (Fig.5).

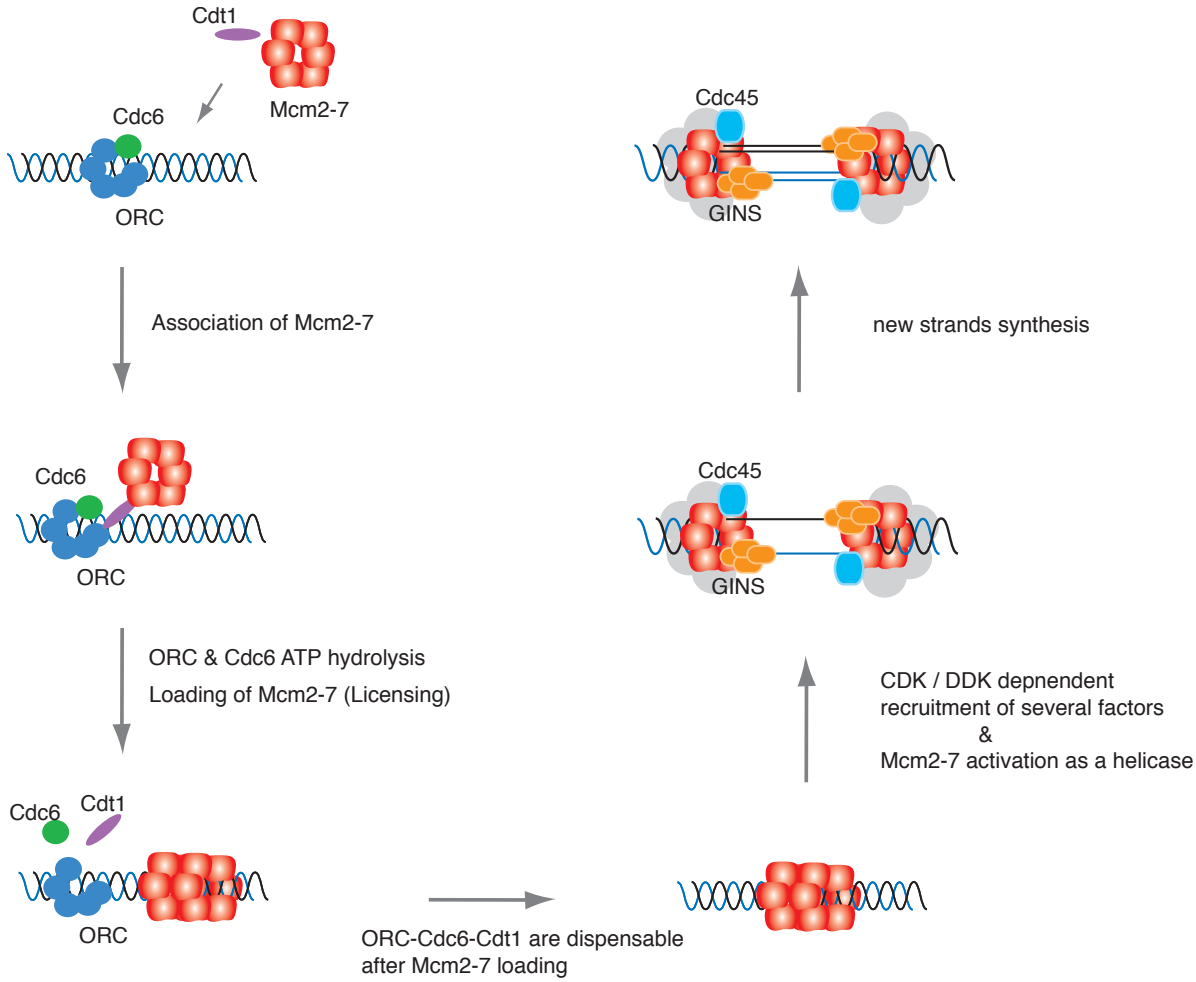


Fig. 5 Licensing and initiation of replication.

ORC, Cdc6 and Cdt1 recruit Mcm2-7 onto origins. Subsequently, CDK and DDK activate the loaded Mcm2-7 as a replicative helicase.

ATP-dependent regulation of ORC and Cdc6

Among six ORC subunits, ORC1-5 are members of the AAA$^+$ ATPase superfamily (Bell *et al.* 1995; Tugal *et al.* 1998; Speck *et al.* 2005). AAA$^+$ ATPase contains a conserved motif for ATP binding called the Walker A motif and another motif for ATP hydrolysis called Walker B (for reviews (Hanson & Whiteheart 2005; Erzberger & Berger 2006)). AAA$^+$ATPase proteins often form homo- or hetero-hexameric complexes, resulting in a closed-ring-like complex. All structurally characterized AAA$^+$ oligomers seem to exhibit a common mode of complex assembly, where a conserved arginine residue called an "arginine finger" is inserted into the ATP binding pocket of the adjacent subunit. Walker A and B and the arginine finger from the adjacent subunit cooperatively promote the binding and hydrolysis of ATP, which in turn

induces inter- and/or intra-subunit conformational change. Indeed, ORC requires ATP or ADP to bind with origins (Klemm *et al.* 1997; Gillespie *et al.* 2001; Takenaka *et al.* 2004). ATP hydrolysis seems not to be required for origin binding, whereas ATP hydrolysis of ORC1 is essential for the recruitment of the Mcm2-7 complex (Bowers *et al.* 2004).

Cdc6 has sequence similarity with Orc1 and is also a member of the AAA^+ATPase superfamily (Neuwald *et al.* 1999). ATP, and not ADP, is required for chromatin binding of Cdc6, depending on ORC (Gillespie *et al.* 2001; Frolova *et al.* 2002). As with ORC, ATP hydrolysis of Cdc6 is required for the recruitment of the Mcm2-7 complex (Frolova *et al.* 2002; Randell *et al.* 2006). A structural study showed that five subunits of ORC (except one of its subunits, ORC6, which is not required for the binding of ORC onto chromatin) are assembled into a hetero-pentameric structure that forms an open-ring structure (Speck *et al.* 2005). This study also implies that the disconnected ring is filled by the binding of Cdc6, resulting in a closed-ring structure. This and another biochemical study suggested that ORC and Cdc6 cooperatively stimulate their ATP hydrolysis activity, which in turn provides free energy to induce Mcm2-7 recruitment. Randel et al. showed that ATP hydrolysis of Cdc6 requires the presence of both ORC and origin DNA (Randell *et al.* 2006). In the absence of ATP hydrolysis by either ORC or Cdc6, the amount of Mcm2-7 recruited to chromatin is reduced. In addition, the remaining association of Mcm2-7 without ATP hydrolysis is salt-sensitive, whereas Mcm2-7 properly recruited with the ATP hydrolysis binds to the origin tightly even in the high-salt-buffer condition. These different styles of Mcm2-7 recruitment are distinguished as either “loading” for tight recruitment or “association” for weak recruitment. Cooperative ATP hydrolysis of ORC and Cdc6 would be required to convert “associated” into “loaded” Mcm2-7. After the loading of Mcm2-7, Cdc6 is dissociated from chromatin. This dissociation may reflect changes in the structure of ORC and/or Cdc6 due to the hydrolysis of ATP.

Cdt1, on the other hand, doesn’t contain apparent motifs of AAA^+ATPase. Although ATP binding activity may be involved in Cdt1 (personal communication with Drs. You and Masai), neither ATP hydrolysis nor any other enzymatic activity has been found in Cdt1. Cdt1 binds to chromatin depending on ORC, but this binding is independent of Cdc6 (Maiorano *et al.* 2000b; Tsuyama *et al.* 2005). Nevertheless, there is a strict requirement for the order of the action of Cdc6 and Cdt1. Tsuyama et al. showed that Cdt1 could support Mcm2-7 recruitment when it binds to chromatin that is already associated with Cdc6 (Tsuyama *et al.* 2005). The mechanism underlying Cdt1 in Mcm2-7 recruitment is unclear, but Cdt1 appears to physically link ORC, Mcm2-7, and possibly Cdc6 (Nishitani *et al.* 2000; Tanaka & Diffley 2002; Yanagi *et al.* 2002; Cook *et al.* 2004; Ferenbach *et al.* 2005; Chen *et al.* 2007; You & Masai 2008). In budding yeast, Cdt1 and Mcm2-7 form a stable complex. Randell et al. showed that Cdt1 can be detected on chromatin only when ATP hydrolysis of Cdc6 is disrupted and Mcm2-7 is “associated” on chromatin (Randell *et al.* 2006). Based on this observation, they proposed that Cdt1 dissociation from Mcm2-7 is stimulated by ATP hydrolysis of ORC and Cdc6. This dissociation of Cdt1 may contribute to the tight loading of Mcm2-7, similar to the case of

DnaB/DnaC origin loading. The behavior of Cdt1 in *Xenopus* eggs, and possibly that in metazoan cells, is somewhat different. In *Xenopus* egg extract, Cdt1 and Mcm2-7 appeared not to form a stable complex, and Cdt1 binds to chromatin independent of Mcm2-7 (Maiorano *et al.* 2000b). This difference may lead to the different regulatory mechanisms of Cdt1 activity between budding yeasts and metazoans.

ATP-dependent regulation of Mcm2-7

All subunits of Mcm2-7 are similar to each other and are members of the AAA^{+}ATPase superfamily (Neuwald *et al.* 1999; Tye & Sawyer 2000). The six subunits are very likely to exist as a hetero-hexameric complex and to form a closed-ring structure (Adachi *et al.* 1997; Remus *et al.* 2009). Although several forms of subcomplexes composed of different sets of subunits, such as Mcm2-7, Mcm4/6/7, Mcm2/4/6/7, Mcm3/5, and Mcm4/7 (Ishimi *et al.* 1996; Ishimi 1997; You *et al.* 1999; Lee & Hurwitz 2000; Maiorano *et al.* 2000a; Prokhorova & Blow 2000; Kanter *et al.* 2008), are identified in the course of cellular fractionation or biochemical purification/reconstruction, it is likely that all six subunits form a hetero-hexamer with an equal stoichiometry *in vivo* (Maiorano *et al.* 2000a; Prokhorova & Blow 2000; Remus *et al.* 2009). The Mcm2-7 complex shows ATPase activity (Schwacha & Bell 2001). A mutations in the Walker A motif of any one of its subunits affects the ATPase activity. In addition, there are specific pairings of Mcm2-7 subunits that act together to stimulate ATPase activity; that is, the ATPase activity of Mcm7 is activated by Mcm3 but not by Mcm6. This result suggests that the ATPase activity of the Mcm2-7 complex occurs upon a coordinated activation of each subunit organized in a particular pairing. The ATPase activity of Mcm2-7 is essental for cellular viability, but this activity is not neccessary for the chromatin loading of Mcm2-7 (Ying & Gautier 2005). Instead, the ATPase activity of Mcm2-7 is required for origin unwinding during DNA replication. Actually, Mcm2-7 has been implicated as a replicative helicase. Although the biochemically purified Mcm2-7 complex is inactive for a DNA helicase, it was found that the Mcm4/6/7 subcomplex shows helicase activity (You *et al.* 1999). A recent study also indicates that Mcm2-7 indeed acts as helicase in a particular buffer condition (Bochman & Schwacha 2008) or in the form of the complex with Cdc45 and GINS (Moyer *et al.* 2006), both of which are essential factors for the initiation of replication. Therefore, the ATPase activity of Mcm2-7 is required for its activity of melting the DNA double strand.

How is Mcm2-7 loaded onto chromatin?

Given that Mcm2-7 serves as a helicase, Mcm2-7 is likely to be loaded onto origins by encircling double- or single-strand DNA in the central pore of its closed-ring structure. Recently, Mcm2-7 loaded on origins was analyzed by electron microscopy (Remus *et al.* 2009), and it became clear that Mcm2-7 is loaded onto chromatin as a head-to-head double hexamer, in which double-strand DNA seems to run through the central channel of the double hexamer. In this case, ORC, Cdc6, and Cdt1 would function as the “loader” of the Mcm2-7

ring by opening the ring, taking it on the origin, and closing it. A plausible mechanism for this type of ring-loading on chromatin can be elucidated from the well-understood relationship between sliding-clamp PCNA and its loader, RFC. RFC is also a member of AAA^+ATPase (for review (Bowman *et al.* 2005)), and both ORC and Cdc6 have sequence similarity with sliding DNA clamp loaders (Iyer *et al.* 2004). RFC consists of five subunits that form an open-spiral ring structure, and PCNA consists of six domains that form a closed-plane ring structure. When PCNA, which normally forms a closed-ring structure, contacts the open-spiral RFC, PCNA cannot retain its planar structure, so that the closed ring is opened and then loaded to the DNA (Miyata *et al.* 2005). According to this hypothesis, the open-ring structure of ORC might serve to open the closed ring of Mcm2-7. Alternatively, ORC-Cdc6 may have functional similarity to DnaA. DnaA is also a member of the AAA^+ATPase superfamily, and the ATP binding of DnaA is essential for the initiation of bacterial DNA replication (for review (Mott & Berger 2007)). ATP-DnaA forms a helical filament, and origin DNA wraps around the DnaA filament, leading to the melting of DNA where helicase is loaded. Thus, it is possible that ORC-Cdc6 bound to chromatin by interacting with DNA through the outside of the ring. Although an atomic view of the mechanism for Mcm2-7 loading is not well understood, Mcm2-7 loading is a one-way reaction. ORC, Cdc6, and Cdt1 are not required to maintain loaded Mcm2-7, and so far, no physiological mechanism for destabilizing the loaded Mcm2-7 has been found.

The initiation steps of DNA replication

Cell fusion experiments by Johnson and Rao have revealed that S-phase cells can induce replication, and that G1-phase cells can initiate replication in response to a signal from S-phase cells. These abilities now turn out to be the activity of CDK and of loaded Mcm2-7. Then, how does CDK act on Mcm2-7 to initiate DNA replication?

The loading of Mcm2-7 onto chromatin is not sufficient to initiate replication. The initiation of replication requires the activities of two kinases, CDK and Dbf4/Drf1-dependent protein kinase (DDK). DDK consists of a Cdc7 catalytic subunit and a Dbf4/Drf1 regulatory subunit. Similar to CDK, Cdc7 exhibits its kinase activity by binding with Dbf4/Drf1. Both CDK and DDK kinase activities are elevated at the onset of the S-phase, then CDK and DDK stimulate the recruitment of several proteins on Mcm2-7-loaded origins (Bell & Dutta 2002; Masai *et al.* 2010). During the past few decades, several proteins have been identified as essential factors for the initiation of replication, including Cdc45 (Mimura & Takisawa 1998), GINS (the Sld5-Psf1-Psf2-Psf3 complex) (Kanemaki *et al.* 2003; Kubota *et al.* 2003; Takayama *et al.* 2003), Dpb11/Cut5 (Araki *et al.* 1995; Masumoto *et al.* 2000), Sld2 (Kamimura *et al.* 1998), and Sld3 (Kamimura *et al.* 2001). These proteins coordinately activate the helicase activity of Mcm2-7 and convert the loaded Mcm2-7 into replication machinery called replisome.

Mcm4 is one of the targets of DDK for initiating replication. The phosphorylation of Mcm4 by DDK stimulates the binding of Cdc45 to Mcm2-7 (Sheu & Stillman 2006). Mutation of

Mcm5 (Hardy *et al.* 1997) or deletion of the N-terminus of Mcm4 (Sheu & Stillman 2010) can bypass the requirement of DDK in the initiation of replication. Thus, DDK will stimulate the initiation of replication by removing the inhibitory action of Mcm2-7.

Recently, essential targets of CDK in replication initiation were identified in budding yeast. Zegerman and Diffley, as well as Tanaka et al., showed that the simultaneous introduction of mutations in Sld2 that mimic the phosphorylated form of CDK-targeted residue and covalent fusion of Sld3 to Dpb11 (or stimulate their interaction) can bypass the requirement of CDK for replication initiation (Tanaka *et al.* 2007; Zegerman & Diffley 2007). Sld3 phosphorylated by CDK interacts with Dpb11, and Dpb11 interacts with phosphorylated Sld2, Polε, and GINS depending on CDK (Muramatsu *et al.* 2010). Therefore, CDK and DDK will initiate DNA replication by evoking interaction among these factors. Finally, the double heterohexamer of Mcm2-7 would be separated, and the two hexamers would move in opposite directions from each other at the front of the replication machinery (Yardimci *et al.* 2010).

GINS, Polε, and Cdc45 are well conserved across species, while other factors are not. TopBP1 was identified as a homolog of Dpb11 (Hashimoto & Takisawa 2003), and RecQL4 was identified as a functional homolog of Sld2 (Matsuno *et al.* 2006). Treslin was identified as a candidate Sld3 homolog (Kumagai *et al.* 2010). Nevertheless, the sequence similarities between homologous partners are not high. Moreover, the behaviors of TopBP1, RecQL4, and Treslin on chromatin are not identical to those of Dpb11, Sld2, and Sld3, respectively, though all of them are essential for initiating replication. Further study will be required to understand the detailed mechanism of replication initiation in metazoans.

IV. ONCE AND ONLY ONCE INITIATION OF REPLICATION

The remaining but important lesson from the cell fusion assay is that the G2 nucleus cannot initiate replication, so that each origin initiates replication once and only once per round of the cell cycle. As proposed in the licensing hypothesis and following studies, this regulation is carried out by inhibiting the loading of Mcm2-7 after the onset of the S phase (Fig. 6).

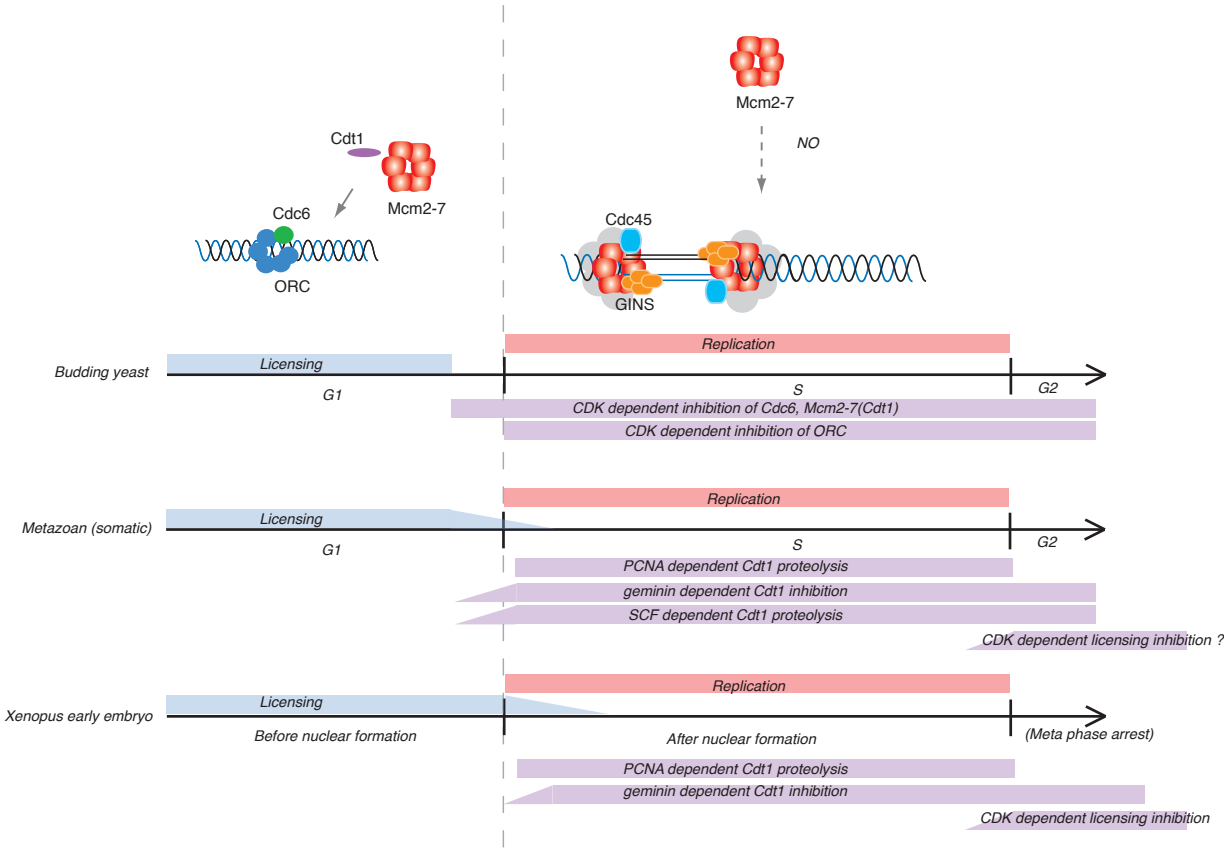


Fig. 6 Mechanisms for licensing inhibition.
CDK-dependent and -independent mechanisms for licensing inhibition are shown. In budding yeast, G1-Cyclins which operate at the end of G1 phase can inhibit Mcm2-7(Cdt1) and Cdc6. However, in other systems, it is unclear what mechanism can inhibit the licensing prior to the initiation of replication.

Point of no return; when licensing is terminated

Since Mcm2-7 moves along with the replication machinery as a replicative helicase, the replicated regions of chromosomes are left behind the Mcm2-7 as an unlicensed state. By inhibiting the loading of Mcm2-7 after the onset of the S phase, the replicated regions of chromosomes have no chance to be re-licensed, thus preventing the initiation of the second round of replication. To inhibit the Mcm2-7 loading, it is impossible to induce the destruction of Mcm2-7 or remove the loaded Mcm2-7, because the loss of Mcm2-7 from chromatin dismisses the helicase from replisomes. Alternatively, since Mcm2-7 is irreversibly loaded on chromatin, the inhibition of the loading steps of Mcm2-7 can be applied to prevent re-

replication without affecting loaded Mcm2-7. Thus, cells prohibit Mcm2-7 loading after the onset of the S phase. This mechanism establishes a point of no return at the boundary between the pre- and post-replication initiation phases. Indeed, transient destruction of Mcm2-7 causes the irreversible arrest of DNA replication (Labib *et al.* 2000), indicating that licensing is no longer reversibly given to origins after the onset of the S phase.

Because loaded Mcm2-7 cannot be removed, loss of the mechanism for licensing inhibition definitely leads to re-replication. The structure of a re-replicated chromosome, which would be a nested set of partially replicated bubbles on a replicated chromosome, has been proposed as a potential source of gene amplification and chromosomal aberration (Schimke *et al.* 1986; Stark *et al.* 1989). Recently, this model was experimentally confirmed by Green et al. (Green *et al.* 2010). They showed that a single site of re-replication can cause gene amplification at a rate 10^4 fold or higher than that observed in the normal replication process. Although gene amplification will promote the evolution of species from the standpoint of long-term life history, chromosomal instability may be fatal to individual organisms. In addition, a higher rate of re-replication directly causes fork collapse and double strand breaks (Davidson *et al.* 2006). Given that metazoan chromosomes involve 10^5 origins that are used for a single round of the S phase, 99.999% accurate control of each origin results in only $0.99999^{100000} = 0.37$ total accuracy of origin firing. To ensure more than 99% total accuracy, the regulatory mechanism at each origin is required to be higher than 99.99999%. It seems impossible to ensure such extremely high accuracy by a single biochemical reaction, such as the binding of one kind of inhibitory protein to one kind of licensing factor. Hence, cells apparently involve multiple mechanisms to prevent re-licensing of replicated chromosomes.

Preventing re-replication - Strategiesof yeasts

Mechanisms for licensing inhibition have been intensively studied in yeasts, *Xenopus* eggs, and mammalian cells, providing a comparative view of the mechanisms among species.

In budding yeasts, CDK plays a central role in preventing re-replication. Temporal inactivation of CDK during the G2/M phase induces an efficient, almost full cycle of re-replication (Dahmann *et al.* 1995; Piatti *et al.* 1996), showing that the central mechanisms for licensing inhibition exist under the control of CDK. In this mechanism, CDK inhibits every component of pre-RC. CDK inhibits Cdc6 in three different modes. First, phosphorylation of the N terminus of Cdc6 by CDK destabilizes Cdc6 (Drury *et al.* 1997). Through the phosphorylation, Cdc6 is recognized by SCF^{Cdc4} ubiquitin ligase, leading to its degradation through the proteasome-mediated pathway. Second, the phosphorylation of Cdc6 also promotes its interaction with CDK, by which the licensing function of Cdc6 is inhibited (Mimura *et al.* 2004). Finally, CDK inhibits the transcription of Cdc6 by acting on the transcription factor Swi5 (Moll *et al.* 1991). Orc2 and Orc6 are the second group of the CDK target, leading to the inactivation of ORC (Nguyen *et al.* 2001; Wilmes *et al.* 2004). Mcm2-7 itself is also sequestrated from licensing reaction by CDK. Phosphorylation of Mcm2-7 promotes the nuclear export of Mcm2-7 (Labib *et al.* 1999; Nguyen *et al.* 2000; Tanaka &

Diffley 2002; Liku *et al.* 2005). Cdt1 is also simultaneously exported from the nucleus due to its stable association with Mcm2-7. Thus, CDK controls all pre-RC factors. Inactivation of all of these pathways results in efficient re-replication (Nguyen *et al.* 2001). In addition, the analysis of re-replication using a genomic microarray revealed that only combinatorial disruption of several pathways can induce re-replication of several regions of chromosomes (Green *et al.* 2006; Green *et al.* 2010). Therefore, multiple pathways for licensing inhibition would not exist as redundant backups, but would operate with each other in order to meet the extreme accuracy of licensing inhibition.

Fission yeast also involves multiple pathways to prevent re-replication under the control of CDK, and transient inhibition of CDK induces efficient re-replication (Broek *et al.* 1991; Hayles *et al.* 1994; Moreno & Nurse 1994). CDK inhibits Cdc18, a fission yeast ortholog of Cdc6, by inducing proteasome-mediated destruction of Cdc18 (Jallepalli *et al.* 1997). In fission yeast, only the overexpression of Cdc18 is sufficient to induce re-replication (Nishitani & Nurse 1995; Muzi Falconi *et al.* 1996; Jallepalli *et al.* 1997). However, the expression of non-degradable Cdc18 to a physiological level does not induce detectable re-replication, suggesting the presence of another mechanism for re-replication inhibition. The secondary pathway seems to be the degradation of Cdt1 via $DDB1^{Cdt2}$ ubiquitin ligase (Nishitani *et al.* 2000). This mechanism for Cdt1 degradation is also conserved in higher eukaryotes (see below), and several studies in metazoan cells show that $DDB1^{Cdt2}$ specifically ubiquitinates Cdt1, which associates with PCNA on chromatin. Thus, the degradation is replisome-dependent but not directly regulated by CDK. Over-expression of Cdt1 stimulates re-replication in the presence of non-degradable Cdc18 (Nishitani *et al.* 2000; Gopalakrishnan *et al.* 2001; Yanow *et al.* 2001), showing the contributions of both pathways to preventing re-replication. In addition, there is a third pathway for licensing inhibition in the G2 and M phases. During these phases, CDK associates with Orc2. This association seems to prevent re-licensing during the G2/M phase but not the S phase, since disruption of the CDK-Orc2 interaction induces endoreduplication (Wuarin *et al.* 2002). In summary, fission yeast achieves only a single replication initiation through two pathways in the S phase--Cdc18 and Cdt1 destruction--and through ORC inhibition in the G2/M phase.

Preventing re-replication - Metazoans

In contrast to the case with yeasts, licensing inhibition in metazoans appears to center upon Cdt1, rather than being assigned to each licensing factor. Geminin, a metazoan-specific inhibitor of licensing, was found to be a factor that is mitotically degraded by APC/C and inhibits replication by preventing Mcm2-7 loading (McGarry & Kirschner 1998). Subsequently, it became clear that the target of geminin is Cdt1 (Wohlschlegel *et al.* 2000; Tada *et al.* 2001). Geminin stably binds to Cdt1 and inhibits the licensing activity of Cdt1. In HeLa and other cells, geminin is degraded at the M-phase exit and re-accumulates at the S phase (McGarry & Kirschner 1998; Wohlschlegel *et al.* 2000; Sakaue-Sawano *et al.* 2008). Thus the expression pattern of geminin will restrict the time range for licensing within the late

M to the G1 phase. In addition, studies of *Xenopus* egg extract showed a post-translational regulation of geminin. In *Xenopus* egg extract, a significant amount of geminin remains in the interphase extract. This geminin, however, is inactive for licensing inhibition. Blow and colleagues showed that the inactive geminin is activated through nuclear import (Hodgson *et al.* 2002). They further showed that geminin is inactivated, but not degraded, at the exit of the M phase (Li & Blow 2004). Strikingly, this inactivation requires ubiquitination by APC/C at the M phase but does not require the subsequent proteolysis of ubiquitylated geminin. It has not been clear what molecular mechanism accounts for the post-translational regulation and whether or not similar regulation of geminin occurs in other organisms and systems. Nevertheless, it is clear that geminin-directed licensing inhibition does not directly involve CDK activity.

The second mechanism of Cdt1 inhibition is PCNA-dependent proteolysis of Cdt1. During the S phase, the level of Cdt1 declines in metazoan cells as well as in fission yeast. Studies of *Xenopus* egg extract showed that Cdt1 is ubiquitylated on chromatin, leading to the degradation of Cdt1. This ubiquitylation is replication-dependent (Arias & Walter 2005). Cdt1 specifically interacts with PCNA on chromatin through a conserved PIP motif, and the Cdt1-PCNA complex on chromatin is then recognized by $DDB1^{Cdt2}$ ubiquitin ligase. $DDB1^{Cdt2}$ ubiquitin ligase ubiquitylates Cdt1 on chromatin, leading to proteasome-mediated degradation (Arias & Walter 2006; Havens & Walter 2009). Thus, Cdt1 is maintained at a low level during the time when replication machinery exists on chromatin.

Cdt1 is also shown to be a substrate of APC/C-dependent proteolysis in *Xenopus* eggs (Li & Blow 2005). In addition, Cdt1 is destroyed through the SCF^{skp2}-mediated ubiquitin-proteasome pathway in mammalian cells (Liu *et al.* 2004; Sugimoto *et al.* 2004; Takeda *et al.* 2005). The association of SCF^{skp2} with Cdt1 is mediated by CDK-dependent phosphorylation of the Cy-motif on Cdt1. Consistently, in some mammalian cells, Cdt1 is ubiquitylated and destroyed even in the G2 and M phases (Nishitani *et al.* 2006). During these phases, geminin inhibits the ubiquitylation of Cdt1 (Ballabeni *et al.* 2004). This positive action of geminin to Cdt1 stabilizes the level of Cdt1 at the M phase, allowing effective licensing in the subsequent G1 phase. Because both APC/C and SCF^{skp2} activities are periodically controlled in a cell-cycle-dependent manner, the level of Cdt1 would be regulated not only in the S phase but also throughout the cell cycle. Cdt1 is also reported to inhibit the progression of replication (Tsuyama *et al.* 2009). This mechanism might help reduce the chance of re-replication by suppressing the emergence of replicated regions of chromosomes in the presence of Cdt1.

Several studies have suggested cell-cycle-dependent changes in the activity of ORC and/or of Cdt1 in mammalian cells (reviewed in (Arias & Walter 2007)). The inhibition of CDK in the G2/M phase induces re-replication in some cases. In *Xenopus* egg extract, CDK affects the chromatin association of ORC (Hua *et al.* 1997; Mahbubani *et al.* 1997; Findeisen *et al.* 1999). In these systems, CDK activity may have a critical role in licensing inhibition, especially at the G2 and M phases (see below). Though the contribution of these pathways to the prevention of re-replication during the S phase is unclear, the simultaneous expression of

Cdc6 and ORC together with Cdt1 stimulates re-replication in human cells (Sugimoto *et al.* 2009), and cyclin was shown to be essential for preventing re-replication in human cells and *Drosophila* embryos (Mihaylov *et al.* 2002; Machida & Dutta 2007).

In summary, licensing inhibition in metazoans mainly involves the regulation of Cdt1 activity independent from CDK, but other mechanisms would regulate Cdt1 and other licensing factors.

Different contributions of geminin and Cdt1 proteolysis to the prevention of re-replication

How do the several mechanisms for licensing inhibition in metazoans cooperatively prevent re-replication? What is the hierarchy of priorities among these mechanisms?

In *Xenopus* egg extract, the central mechanisms for licensing inhibition lie in both geminin and Cdt1 proteolysis. Depletion of geminin from the extracts induces relatively little, if any, re-replication (McGarry 2002; Arias & Walter 2005; Li & Blow 2005; Yoshida *et al.* 2005; Kerns *et al.* 2007). Also, the inhibition of Cdt1 proteolysis itself causes no detectable re-replication (Arias & Walter 2005; Li & Blow 2005; Maiorano *et al.* 2005; Yoshida *et al.* 2005; Arias & Walter 2006). However, when both pathways are attenuated, substantial amounts of re-replication are observed (Li & Blow 2005; Maiorano *et al.* 2005; Yoshida *et al.* 2005; Arias & Walter 2006). Since the deletion of the PIP domain from Cdt1 could induce re-replication in the absence of geminin (Arias & Walter 2006), $DDB1^{Cdt2}$-mediated proteolysis is the main cause of the degradation of Cdt1 to prevent re-replication. The contribution of geminin and $DDB1^{Cdt2}$-mediated Cdt1 proteolysis for cell-cycle progression may be more serious *in vivo.* Either anti-sense RNA knockdown of geminin or injection of non-degradable Cdt1 into *Xenopus* embryo causes cell-cycle arrest and checkpoint activation (Kerns *et al.* 2007). It is possible that the deregulation of either pathway causes small and ineffective re-replication that is hard to detected in the extracts. Nevertheless, that small re-replication can induce chromosomal damage resulting in the stalling of the developmental process.

In metaphase-arrested extract from *Xenopus* eggs, CDK and geminin appear to be major inhibitors of licensing, because the depletion of geminin restores licensing activity only in the presence of CDK inhibitor (Tada *et al.* 2001). Therefore, geminin and CDK-dependent licensing inhibition operate predominantly to inhibit licensing after the completion of replication in *Xenopus* egg extract.

Geminin and $DDB1^{Cdt2}$-mediated Cdt1 proteolysis also play major roles in preventing re-replication in mammalian cells. Interestingly, the impact of the depletion of each pathway seems to be context-dependent. In HeLa cells, the siRNA knockdown of Cdt2 or DDB1 results in an efficient re-replication (Jin *et al.* 2006; Lovejoy *et al.* 2006), whereas the knockdown of geminin does not cause detectable re-replication (Kulartz & Knippers 2004; Nishitani *et al.* 2004; Machida & Dutta 2007). In contrast, the siRNA knockdown of geminin causes substantial re-replication in several types of transformed cell lines, primary cells, and

possibly mouse embryos (Mihaylov *et al.* 2002; Melixetian *et al.* 2004; Zhu *et al.* 2004; Gonzalez *et al.* 2006; Hara *et al.* 2006; Zhu & Dutta 2006; Zhu & Depamphilis 2009).

The differences in the effects of Cdt1 proteolysis and geminin are also highlighted in other model organisms. Geminin knockout causes re-replication in *Drosophila* tissue culture cells as well as in its embryos (Quinn *et al.* 2001; Mihaylov *et al.* 2002; Higa *et al.* 2003). Deletion of the PIP motif from Cdt1 also causes abnormal S-phase progression, but the effect appears to depend on the tissue or the developmental stage (Lee *et al.* 2010). In *C. elegans*, the knockdown of Cul4, a subunit of $DDB1^{Cdt2}$ ubiquitin ligase, induces massive re-replication (Zhong *et al.* 2003; Kim & Kipreos 2007). Nevertheless, the knockdown of GMN-1, an ortholog of geminin in *C. elegans*, causes no detectable re-replication (Yanagi *et al.* 2005). These results further suggest that the differences in the importance of these two pathways are cell-type specific.

In summary, while PCNA-dependent Cdt1 proteolysis and geminin are equivalently effective at preventing re-replication in *Xenopus* egg extract, other model systems illustrate different situations, where one of these inhibitory pathways dominantly leads to licensing inhibition in a context-dependent manner. It is still unclear what context determines the dominance of each pathway.

Determinants of the importance of geminin and Cdt1 proteolysis

Given that Cdt1 proteolysis and geminin have different effects even among genetically equivalent cell types, the determinant of the requirements of these pathways should, at least in part, lie in the conditions of cells.

What conditions would be involved in the impact determinant of Cdt1 regulation? The first candidate will be the phase of the cell cycle. Apparently, PCNA-$DDB1^{Cdt2}$-dependent Cdt1 proteolysis can operate only in the S phase, because PCNA dissociates from chromatin after the completion of replication. In contrast, geminin and CDK-dependent licensing inhibition (including SCF^{skp2}-mediated Cdt1 proteolysis and CDK-mediated ORC inhibition) can operate in the G2 and M phases as well as in the S phase. Therefore, the cells under the cell cycle that show relatively long G2 phases should have greater reliance on geminin-dependent licensing inhibition. This is supported by situations in metaphase-arrested egg extract and G2/M-arrested human cells.

The second aspect is the balance between the amount of geminin and that of Cdt1. Geminin would contribute greatly to licensing inhibition in cells expressing higher levels of Cdt1 and geminin. Thus, these cells would be sensitive to the depletion of geminin and/or resistant to Cdt1 overexpression. Indeed, the effect of Cdt1 overexpression differs depending on cell type (Vaziri *et al.* 2003; Melixetian *et al.* 2004; Tatsumi *et al.* 2006; Liu *et al.* 2007). Both Cdt1 and geminin expression are elevated in some types of cancer cells (for review (Blow & Gillespie 2008; Petropoulou *et al.* 2008)). In addition, disruption of Cdt1-geminin interaction causes abnormal cell-cycle progression in cancer cells but not in normal cells (Zhu & Depamphilis 2009). Therefore, the balance between Cdt1 and geminin would be important to

understanding the differences in their sensitivities to licensing defects. The quantitative analysis of the balance between Cdt1, geminin, and licensing activity is an open challenge.

The most pivotal point for licensing inhibition will be at the G1/S transition, where the licensing phase turns into a licensing-deficient phase. During that time, licensing should be inhibited prior to the initiation of replication, so that the licensing phase is separated from the replication phase. One solution for this separation would be a different specificity/sensitivity of licensing inhibition from replication initiation. In budding yeast, G1cyclin-CDK induces the inactivation of Cdc6 and Mcm2-7/Cdt1 (Labib *et al.* 1999; Drury *et al.* 2000; Perkins *et al.* 2001). This action of G1-CDK is prior to and not accompanied by the initiation of replication, which is stimulated by S-CDK and DDK. Thus, licensing activity would be reduced before the initiation of replication. In other words, there is a "blank" time window at the G1/S boundary during which neither licensing nor replication takes place on chromatin. Does a similar blank time exist in metazoan systems? This has not been clarified yet. Apparently it is difficult to inhibit the licensing of PCNA-dependent Cdt1 proteolysis prior to replication initiation, as this mechanism itself requires the initiation of replication. On the other hand, geminin and SCF^{skp2} may provide blank time. Geminin destruction is carried out by APC/C, whose activity declines at the end of the G1 phase. Therefore, geminin may re-accumulate faster than cyclin accumulates. In addition, SCF^{skp2} is activated at the boundary of the G1/S phase. Thus, the degradation of Cdt1 through this pathway might contribute to establishing the separation of licensing from replication initiation.

In the case of *Xenopus* egg extract showing the embryonic cell cycle, however, there are essentially no apparent mechanisms for licensing inhibition that can operate prior to replication initiation. In this system, geminin is activated through nuclear import, but the import also triggers the initiation of replication through the accumulation of CDK. Considering that the deletion of geminin and PCNA-dependent Cdt1 destruction causes extensive re-replication, there may be a fatal time point at the beginning of the S phase, when neither geminin activation nor Cdt1 destruction can catch up with the appearance of the replicated region of chromosomes. In order to overcome this situation, geminin should be activated quickly, but experimentation on this point remains to be done.

How does geminin inhibit Cdt1? It's not just the binding.

Since geminin inhibits licensing through its interaction with Cdt1, initial studies on the molecular mechanism of geminin's action focused on the interaction between Cdt1 and geminin. Geminin forms a homo-dimer through its coiled-coil region (Benjamin *et al.* 2004; Saxena *et al.* 2004). Geminin also forms a homo-tetramer (Okorokov *et al.* 2004) and a dimer-of-dimer (Thepaut *et al.* 2004). Through the coiled-coil region and the adjacent distinct region, the geminin dimer associates with Cdt1 monomer (Lee *et al.* 2004; Saxena *et al.* 2004). Cdt1 associates with geminin through its middle part, whereas the C-terminus region of Cdt1 interacts with Mcm2-7 and is essential for Mcm2-7 loading (Ferenbach *et al.* 2005). A structural study suggests that, by taking the Cdt1-geminin complex, the C-terminal region

of geminin is directed to the C-terminal region of Cdt1, thereby interfering with the interaction between Cdt1 and Mcm2-7 (Lee *et al.* 2004). Indeed, the C-terminal region of geminin is required for licensing inhibition by geminin. Actually, it was reported that geminin inhibits the interaction between Cdt1 and Mcm6 (Yanagi *et al.* 2002). In *Xenopus* egg extract, geminin does not inhibit the chromatin binding of Cdt1 and geminin itself binds to chromatin depending on Cdt1 (Gillespie *et al.* 2001; Lutzmann *et al.* 2006). Consistently, Cdt1 recruits geminin on chromatin in human cells (Xouri *et al.* 2007). Taken together, these results suggest that geminin provides steric interference of Cdt1-Mcm2-7 interaction on chromatin.

Recent progress in biochemical and structural studies took this idea one step further. Lutzmann et al. showed that different stoichiometric complexes of Cdt1-geminin show opposite licensing activity (Lutzmann *et al.* 2006). They identified the Cdt1-3×geminin (Cdt1:geminin = 1:3) complex as an active complex that can induce licensing as well as geminin-free Cdt1, and the Cdt1-4×geminin (1:4) complex as an inactive complex that cannot induce licensing. Similarly but not consistently, DeMarco et al. showed that Cdt1-geminin complex takes a quaternary structure, in which the dimer of Cdt1-2×geminin is formed through a previously unidentified "third" interface between Cdt1 and geminin (De Marco *et al.* 2009). Mutant geminin defective in forming the quaternary structure no longer inhibits licensing as efficiently as wild-type geminin. Both of these studies suggest that the simple binding of the geminin dimer to Cdt1 is not sufficient to inhibit licensing. However, it is not clear which type(s) of complex are formed on chromatin or, more basically, whether the inhibition of licensing by geminin occurs on chromatin or not.

In addition to the complex formation of Cdt1 and geminin, accumulative evidence presents the participation of chromatin status in licensing activity. Hbo1, a MYST family histone acetyltransferase, was shown to stimulate licensing and to associate with Cdt1 (Iizuka *et al.* 2006; Miotto & Struhl 2008). Further study showed that its acetylase activity is required for effective licensing (Miotto & Struhl 2010). Furthermore, geminin inhibits the acetylase activity of the Cdt1-HBO1 complex. A recent study also indicates that geminin interacts with a histone deacetylase, HDAC11, and antagonizes HBO1 in licensing (Wong *et al.* 2010). Cdt1 also interacts with several chromatin remodeling factors (Sugimoto *et al.* 2008). In *Drosophila*, depletion of geminin induces re-replication preferentially from origins in heterochromatin regions (Ding & MacAlpine 2010). These results suggest that both geminin and Cdt1 activity affect the chromatin status and *vice versa.* These regulations, however, would not be essential for licensing and its inhibition in *Xenopus* egg extract, because purified proteins that apparently do not involve these associated histone-modifying enzymes can re-construct licensing as well as licensing inhibition by geminin (Gillespie *et al.* 2001). Nevertheless, it is possible that licensing activity and inhibition by geminin are determined according to the chromatin context.

In summary, geminin action is likely to involve factors other than simple Cdt1-geminin complex formation. Higher-order complex formation of Cdt1-geminin, as well as changes in

the activities of Cdt1 and geminin depending on chromatin status, would be involved in geminin function, though the exact actions of geminin and Cdt1 on chromatin remain unclear.

Aim of this study: Robustness and sensitivity of geminin system

I aimed to discover the mechanisms of geminin-induced licensing inhibition in *Xenopus* egg extract. As discussed above, the expected situation of *Xenopus* egg extract, particularly at the early S phase, requires two prerequisite properties in geminin functions. The first property is the quick establishment of licensing inhibition so that geminin can inhibit the licensing prior to replication initiation after nuclear formation. The second is the robust licensing inhibition so that licensing can be strictly inhibited only by geminin at the early replication phase, where PCNA-dependent Cdt1 proteolysis will not be effective. Although these prerequisites have a basis in a particular situation seen in the early embryonic cell cycle without the G1 phase, they would represent a characteristic modality of geminin action. Moreover, the embryonic cell cycle is important for the developmental process of multi-cellular organisms, and geminin seems to exist only in metazoans, suggesting it has a major role in multicellular development. Therefore, discovery of the mode of geminin-induced licensing inhibition may answer a general biochemical question: How does a rather simple Cdt1-geminin binding mechanism establish robust and quick licensing inhibition? Discovery of the mode may also answer a developmental question: How does the geminin system engage with and contribute to the embryonic cell cycle?

Previous studies have shown multimodal complex formation of Cdt1 and geminin, as well as the involvement of chromatin status in geminin function. However, these approaches focusing on "component identification" are not enough to understand the geminin system's robustness (how stably can geminin inhibit the licensing against noisy fluctuation of the system?) and sensitivity (how quickly can licensing be inhibited in response to the elevation of geminin activity?). Those features of the system lie in system dynamics. If we increase the effect of geminin to 1.5-fold, what happens to the licensing? What if we increase that to 2.0-fold? In other words, it is important to know the quantitative relationship between a stimulus (= geminin, Cdt1, and other possible components) and the response (= Mcm2-7 loading).

Studies with *Xenopus* egg extract have a great advantage in the analysis of the quantitative stimulus-response (S/R) relationship, because this system allows us to manipulate the amounts of proteins in the system by adding proteins into or depleting proteins from the extract. A mathematical model is a complementary tool with quantitative biochemistry to relate the dynamics to their relevant reaction pathways (for review (Bintu *et al.* 2005)). When the mathematical models are built based on identified reaction schemes, the models can predict possible dynamics from the scheme and describe the contribution of each involved pathway in determining the behavior of the overall dynamics. Moreover, if the model requires some additional pathways to explain the experimental data, the assumed pathways lead to novel molecular mechanisms or novel interpretations of identified reactions. Thus, mathematical modeling, which can describe S/R relationships without the need to identify

every component in the system, can abstract essential components/interpretations of the reaction framework out of the complex nature of biological systems.

Although the combinational approach to the quantitative identification of the S/R relationship with its mathematical modeling has not been applied to licensing control, this approach has provided a nice characterization and understanding of several biological systems. The outstanding examples include: oscillatory systems such as the cell cycle and circadian clock ((Csikasz-Nagy *et al.* 2006), for review (Goldbeter 2002)), ultrasensitive response in the phosphorylation (Kim & Ferrell 2007) and developmental processes (Melen *et al.* 2005), replication kinetics (Yang *et al.* 2010) and distribution (Jun *et al.* 2004), microtubule dynamics around chromosomes (Athale *et al.* 2008), and transcriptional response to signals (Choi *et al.* 2008; Giorgetti *et al.* 2010).

In this study:

All-or-none licensing inhibition by geminin - mechanism and significance -

In this study, to investigate the robustness and sensitivity of geminin-induced licensing inhibition, I focused on the quantitative relationship between concentrations of geminin and Cdt1, as well as on licensing activity in *Xenopus* egg extract. Previous studies show that recombinant geminin induces all-or-none style licensing inhibition, i.e., when the concentration of geminin exceeds a critical level, licensing is abruptly inhibited (McGarry & Kirschner 1998; Gillespie *et al.* 2001; Waga & Zembutsu 2006). The all-or-none kinetics is hard to explained by a simple binding scheme of two components, but is often explained by a mechanism involving cooperative and/or feedback-driven pathways. In addition, an all-or-nothing response is useful for clarifying the switch from one state to the next. Therefore, an understanding of an all-or-none licensing switch by geminin will shed light on both the molecular mechanism of geminin action and the biological significance of geminin function in making licensing on and off states.

In part I of this thesis, I extended the notion of an all-or-none licensing inhibition by making a mutant geminin that shows rather relaxed inhibition instead of strict all-or-none inhibition. I found that the latter is highly correlated with geminin's ability to induce focal clustering of Cdt1 on chromatin, and to bridge the distinct Cdt1 molecules. I then investigated the actions of Cdt1 and geminin on chromatin, and found that the ORC-Cdc6-Cdt1 complex on chromatin is inactivated by geminin and re-activated by Cdt1. Based on this finding, I described a mathematical model to predict the S/R relationship between geminin, Cdt1, and licensing activity. Through the model-based prediction and its experimental verification, I proposed a novel modality of licensing control by geminin, where geminin induces coherent licensing inhibition among multiple origins, resulting in the concerted all-or-none style of inhibition.

In part II of this thesis, I described how this all-or-nothing system is implemented in the embryonic cell cycle of *Xenopus* embryo and the possible significance of this style of licensing inhibition. I show that geminin activity rises so swiftly that geminin can inhibit

licensing just after the formation of a nuclear envelope, whereas neither Cdt1 proteolysis nor expression level control can operate effectively in this time range. In addition, I found that proteolysis of Cdt1 does not operate effectively in the early embryonic situation, because of the extensive high ratio between the amount of cytosol and nucleus. These results suggest that geminin is a dominant inhibitor of licensing in *Xenopus* embryo, and that one factor that determines the importance of geminin would be the ratio between cytosol and nucleus. Finally I discuss the significance of feedback-based all-or-none licensing inhibition in terms of 1) its signal sensitivity to swiftly inhibit licensing and 2) its robustness to achieve stable licensing inhibition and to avoid premature licensing inhibition.

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Part I

Inter-origin cooperativity of geminin action establishes an all-or-none switch for replication origin licensing

Abstract

In metazoans, geminin functions as a molecular switch for preventing re-replication of chromosomal DNA. Geminin binds to and inhibits Cdt1, which is required for replication origin licensing, but little is known about the mechanisms underlying geminin's all-or-none action in licensing inhibition. Using *Xenopus* egg extract, I found that the all-or-none activity correlated with the formation of Cdt1 foci on chromatin, suggesting that multiple Cdt1-geminin complexes on origins cooperatively inhibit licensing. Based on experimental identification of licensing intermediates targeted by geminin and Cdt1, I developed a mathematical model of the licensing process. The model involves positive feedback owing to the cooperative action of geminin at neighboring origins and accurately accounts for the licensing activity mediated by geminin and Cdt1 in the extracts. The model also predicts that such cooperativity leads to clustering of licensing-inhibited origins, an idea that is supported by the experimentally measured distribution of inter-origin distances. I propose that geminin inhibits licensing through an inter-origin interaction, ensuring strict and coordinated control of multiple replication origins on chromosomes.

Introduction

In higher eukaryotes, DNA replication is initiated from thousands of origins distributed on chromosomes (Masai *et al.* 2010). In the late M to G1 phase, origins are licensed for replication by the formation of pre-replication complexes on chromatin where Mcm2-7 is loaded in ORC, Cdc6, and Cdt1 dependent manner. To ensure that initiation of replication occurs only once in a cell cycle, inhibition of the loading of Mcm2-7 after the onset of DNA replication is essential (Blow & Dutta 2005; Arias & Walter 2007; Masai *et al.* 2010). De-regulation of licensing inhibition at a single origin could induce genomic instability. Thus, the mechanism of licensing inhibition requires coordinated and tight switching activity of origin licensing.

Geminin was identified as a metazoan-specific inhibitor of licensing (McGarry & Kirschner 1998), and it is widely accepted that inhibition of Cdt1 by geminin plays a crucial role in preventing chromosome re-replication (Quinn *et al.* 2001; Mihaylov *et al.* 2002; Melixetian *et al.* 2004; Zhu *et al.* 2004; Li & Blow 2005; Yoshida *et al.* 2005; Kerns *et al.* 2007; Dorn *et al.* 2009). Numerous studies have established that geminin binds directly to and inhibits Cdt1 function in licensing (Wohlschlegel *et al.* 2000; Hodgson *et al.* 2002). Based on the structure of the Cdt1-geminin complex using truncated forms of Cdt1 and geminin, geminin was proposed to inhibit licensing by sterically interfering with Cdt1 to prevent the loading of Mcm2-7 at origins (Lee *et al.* 2004). The exact nature of the steric hindrance remains unknown, however, because the solved structure does not include the C-terminus of Cdt1 that is involved in licensing and neutralizing geminin function (Ferenbach *et al.* 2005).

Recent progress in determining the structural and biochemical properties of the Cdt1-geminin interaction has shed new light on the action of geminin in licensing inhibition. De Marco et al. identified two distinct forms of the Cdt1-geminin complex, a licensing-permissive heterotrimer and a licensing-inhibitory heterohexamer, and proposed that the transition between the two forms represents the geminin switch for licensing inhibition (De Marco *et al.* 2009). A biochemical study on recombinant complex of Cdt1-geminin also revealed that the two forms of the Cdt1-geminin complex act as active and inactive complexes for licensing (Lutzmann *et al.* 2006). Recent studies have also indicated the importance of geminin as a regulator of histone acetylase activity during licensing inhibition (Iizuka *et al.* 2006; Miotto & Struhl 2008, 2010). In addition to these complex geminin actions, geminin has another intriguing property—its ability to inhibit licensing manifests as an all-or-none type of switch, and geminin inhibits licensing only above a threshold concentration (McGarry & Kirschner 1998; Gillespie *et al.* 2001; Waga & Zembutsu 2006). Such a characteristic action of geminin can be explained by neither a straightforward consequence of the simple Cdt1-geminin binding scheme nor the above-mentioned structural and biochemical properties of geminin. Thus, the intermolecular interactions between Cdt1 and geminin that are needed to generate all-or-none licensing inhibition remains unknown.

Here I investigated the mechanism underlying the all-or-none type of inhibition by geminin. I employed mathematical modeling combined with quantitative measurements. Modeling of

molecular interactions in biochemical reactions is a powerful tool for estimating dose-response relationships, as in allosteric regulation of an enzyme by binding an effector and transcriptional regulation of a promoter by a transcription factor (Bintu *et al.* 2005). This approach helps us to predict plausible reaction steps behind the dynamics even if the components have not been experimentally identified. The predicted reaction steps can be tested experimentally, leading to the elucidation of novel mechanisms for understanding biological systems. I found that the all-or-none action of geminin correlated closely with the properties of geminin, namely induction of Cdt1 foci formation on chromatin and tethering of distinct Cdt1 molecules. These and other results led us to hypothesize that geminin cooperatively inhibits licensing on chromatin. I tested this idea through mathematical modeling of geminin action based on the identification of intermediates of the licensing process. Simulations and experimental verification revealed that a model with a positive feedback of geminin action can precisely account for the experimentally observed effect of Cdt1 concentration on the licensing inhibition by geminin. Furthermore, origin distributions estimated by both DNA combing and mathematical modeling suggest inter-origin cooperativity (IOC) evoked by geminin as a mechanism underlying the positive-feedback effect. These results provide us a novel possibility of licensing control in metazoans in which geminin controls the licensing status of thousands of origins in a concerted manner by its cooperative binding to chromatin.

Results

Switch-type vs. gradual-type inhibition of origin licensing by geminin

Previous studies have shown that recombinant geminin inhibits the origin licensing activity in *Xenopus* interphase egg extracts in a highly cooperative manner (McGarry & Kirschner 1998; Gillespie *et al.* 2001; Waga & Zembutsu 2006), i.e., the loading of Mcm2-7 onto chromatin is abruptly inhibited above a critical concentration of geminin when added to extract. I re-examined the inhibition around the threshold level of geminin. Fig. 1A shows that geminin inhibits origin licensing, monitored as the chromatin binding of Mcm2, in an all-or-none manner at a threshold concentration of 30–40 nM (Fig. 1A and see also Fig. 6A, WT). Binding of recombinant geminin to chromatin was reciprocal to binding of Mcm2, geminin binding abruptly increased at and above the threshold concentration of geminin. Although the binding of geminin to chromatin depends on Cdt1 (Lutzmann *et al.* 2006), the absence of geminin binding below the threshold is not due to decreased binding of Cdt1. A similar switch-like characteristic of geminin binding was observed in the absence of Mcm2-7 loading, which stabilizes Cdt1 binding to chromatin (Fig. 1B).

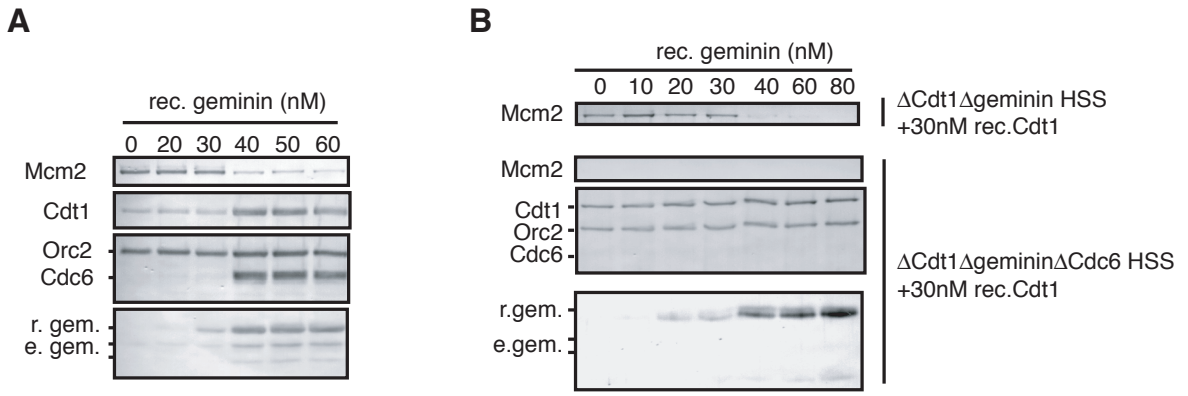

Fig. 1 All-or-none licensing inhibition by recombinant geminin.

(A) Switch-like licensing inhibition by recombinant geminin. Sperm chromatin was incubated in extract with the indicated amount of recombinant geminin for 20 min at 23°C. Chromatin fractions were isolated and subjected to SDS-PAGE and western blotting. rec., recombinant; r. gem; recombinant geminin. e. gem; endogenous geminin.

(B) Binding of geminin in the absence of Mcm2-7 loading.

Sperm chromatin was incubated in HSS double-depleted for Cdt1 and geminin in the presence of various concentrations of geminin and 30 nM recombinant Cdt1 for 15 min at 23ºC. Alternatively, sperm chromatin was incubated in HSS triple-depleted for Cdt1, geminin, and Cdc6 with 30 nM recombinant Cdt1 for 15 min at 23ºC. Chromatin fractions were collected and analyzed by Western blotting. The amounts of geminin bound to the chromatin were abruptly increased over the threshold concentration even in the absence of Mcm2-7 loading.

Endogenous geminin in the interphase extract is inactivated and then reactivated upon nuclear import (Hodgson *et al.* 2002; Li & Blow 2004). I therefore used nucleoplasmic extracts to confirm the all-or-none action of endogenous geminin in its active form. After incubating chromatin in the interphase extract with various amounts of nucleoplasmic extract (Fig. 2A), licensing was inhibited at a critical nucleoplasmic/cytoplasmic ratio. Above the

threshold ratio, the amount of geminin, Cdc6, and Cdt1 bound to chromatin increased abruptly. I quantified the concentration of geminin in the cytoplasm, which is thought to be inactive for licensing, as 50 nM, and that of nucleoplasm, which is thought to be the active form, as 1 μM (Figs. 2B and C). The concentration of active endogenous geminin at the threshold is estimated to be approximately 50 nM, which is close to the threshold concentration for inhibition by recombinant geminin.

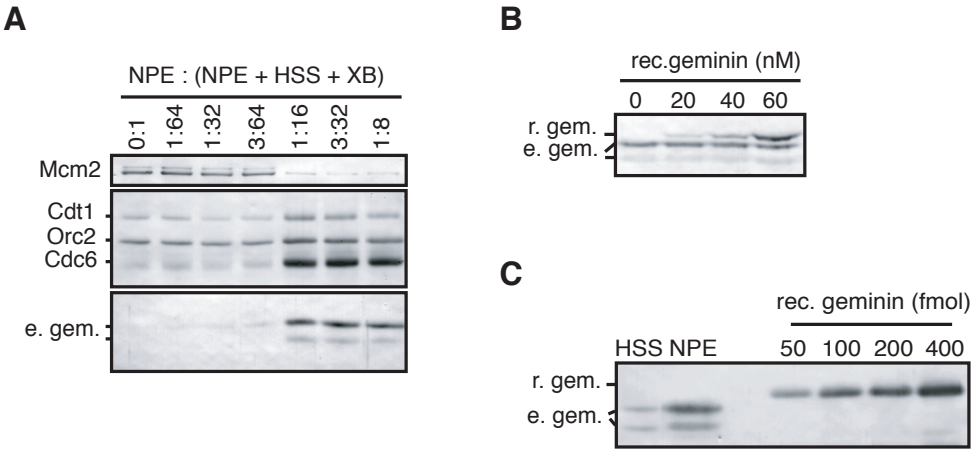

Fig. 2 All-or-none licensing inhibition by endogenous geminin.

(A) Licensing inhibition by endogenous geminin. Nucleoplasmic extract (NPE) was serially diluted with XB buffer, and then membrane-free extract (HSS) was mixed with diluted NPE at a ratio of 1:7. The final proportion of NPE to the total incubation mixture (NPE + HSS + XB) is indicated at the top of each lane. Sperm chromatin was incubated with this mixed extract for 15 min at 23°C, and the isolated chromatin fractions were analyzed by western blotting.

(B) Quantification of the concentration of endogenous geminin and Cdt1 in cytosolic and nucleoplasmic extract. Egg extract (0.75 μl) with indicated amount of recombinant geminin was analyzed by Western blotting. The concentration of endogenous geminin in the extract was estimated to be about 50 nM.

(C) Estimation of the concentration of endogenous geminin in nucleoplasmic extract (NPE). HSS and NPE (0.2 μl each) were analyzed by western blotting. The concentration of endogenous geminin in NPE was estimated to be 0.5–1.0 μM by comparison with known amounts of recombinant geminin.

If Cdt1 activates licensing in an all-or-none manner, then conversely the simple sequestration of Cdt1 by geminin in the extract may facilitate all-or-none inhibition of licensing. To test this possibility, I examined the dose-response relationship between Cdt1 and licensing activity. Endogenous Cdt1 was depleted from the extract using anti-Cdt1 antibody, and various concentrations of recombinant Cdt1 were added back to the extract. The amount of Mcm2 loaded on chromatin increased in a hyperbolic manner with the increase in recombinant Cdt1 (Fig. 3A, see also Fig. 12A). I also investigated the activity of endogenous Cdt1. The concentration of endogenous Cdt1 was manipulated by mixing Cdt1-depleted extracts with mock-depleted extracts at various ratios. Again, I found that endogenous Cdt1 supported Mcm2 loading in a hyperbolic manner (Figs. 3B and C). Thus, all-or none

inhibition of licensing by geminin relies on a unique feature of geminin activity but not the licensing activity of Cdt1.

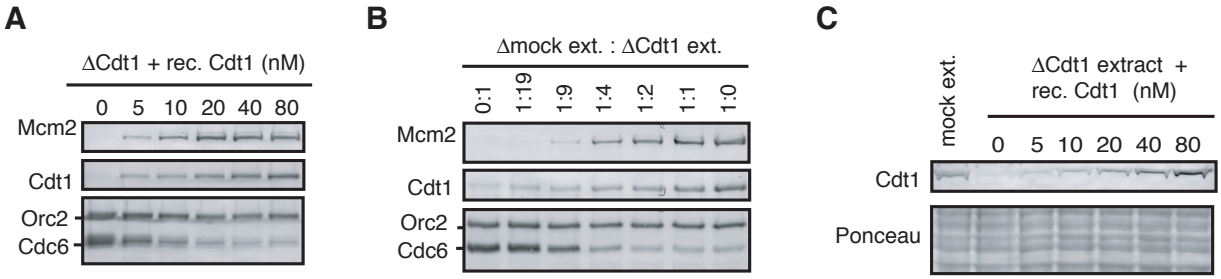

Fig. 3 Licensing activity of Cdt1.

(A) Licensing activity of recombinant Cdt1. Sperm chromatin was incubated with Cdt1-depleted egg extract supplemented with various concentrations of recombinant Cdt1 for 20 min at 23°C. Isolated chromatin fractions were analyzed by western blotting.

(B) Licensing activity of endogenous Cdt1. Mock-depleted and Cdt1-depleted extracts were mixed to give the final proportions indicated. Sperm chromatin was incubated in the mixed extract for 20 min at 23°C, and chromatin fractions were collected and analyzed by western blotting. The concentration of endogenous Cdt1 was ~30 nM by comparison with known amounts of recombinant protein (Fig. 3C).

(C) Estimation of endogenous Cdt1 concentration. Mock-depleted extract (mock ext.) and Cdt1-depleted extract (ΔCdt ext.) with indicated concentrations of recombinant Cdt1 were analyzed by western blotting. The concentration of endogenous Cdt1 was estimated to be about 30 nM by comparison with known amounts of recombinant protein. The tracks were loaded evenly, as shown by Ponceau S protein staining.

I next determined the region of the geminin polypeptide that is responsible for the all-or-none activity. Previously, McGarry and co-workers reported that the geminin mutant KKFEV (E100K, T101K, C104F, A111E, and A117V) shows reduced replication inhibition without affecting binding to Cdt1 (Benjamin *et al.* 2004). I found that the KKFEV mutant and the wild-type protein had similar switch-like ability to inhibit licensing (Fig. 4). The different responses to the mutant geminin may be because a small amount of Mcm2-7 on chromatin in a minimally licensed state can support replication. Thus, to obtain a mutant showing a clear defect in the all-or-none inhibition, I created mutants combining KKFEV with the geminin mutant, PTC (P102A, T103A, C104F), which shows similar characteristics as KKFEV (Benjamin *et al.* 2004). The resulting mutant, KKAAFEV (Fig. 5A), containing seven amino acid substitutions (E100K,

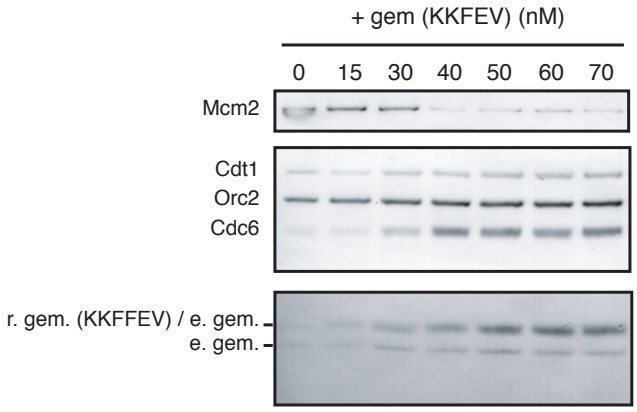

Fig. 4 Switch-like licensing inhibition by geminin mutant KKFEV.

Sperm chromatin was incubated in the extract with the indicated amount of recombinant geminin KKFEV for 20 min at 23ºC. The chromatin fractions were isolated and analyzed by western blotting.

T101K, P102A, T103A, C104F, A111E, and A117V) that are located near the "second" interface between Cdt1 and geminin (Fig. 5B) failed to inhibit licensing in an all-or-none manner but still retained weak inhibitory activity (Fig. 6A). The cooperative effect of wild-type geminin can be characterized by an apparent Hill coefficient of ≥8 (Fig. 6B), and that of the mutant is characterized by an apparent Hill coefficient of ~2 (Fig. 6C). Thus, mutant geminin still retains cooperative activity, but it is significantly reduced compared to wild-type geminin.

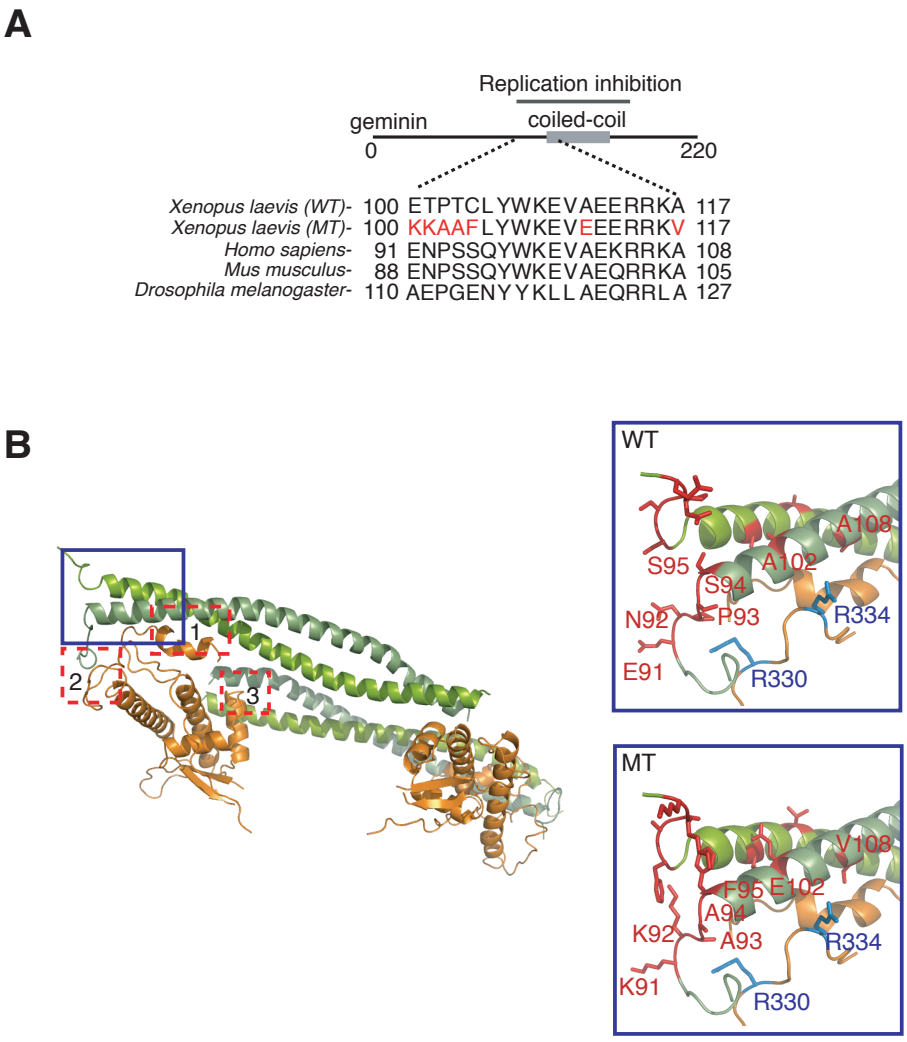

Fig. 5 Characterization of mutant geminin.

(A) Sequence alignment of the mutated region of *Xenopus* geminin compared with other metazoan geminins. The mutated residues are shown in red. WT, wild type; MT, mutant.

(B) Comparison of 3D structures of wild type and mutant geminin. (Left) Human truncated (2x [Cdt1:2xgeminin]) heterohexamer is shown as a cartoon representation (PDB:2WVR); Cdt1 molecules in orange, Geminin molecules in green. The primary, secondary, and tertiary interface regions reported in (De Marco *et al.* 2009) are boxed with red dotted lines and the region mutated in this work is boxed with blue line. (Right) The enlarged 3D view of the mutated region of wild-type and mutant geminin. (WT) Human geminin residues corresponding to the mutated residues of *Xenopus* geminin are shown in red-stick model. R330 and R334 residues of Cdt1 are shown in blue (see discussion). (MT) The mutated residues are represented depending on the backbone structure using MacPyMol software. Structural images were created by MacPymol software.

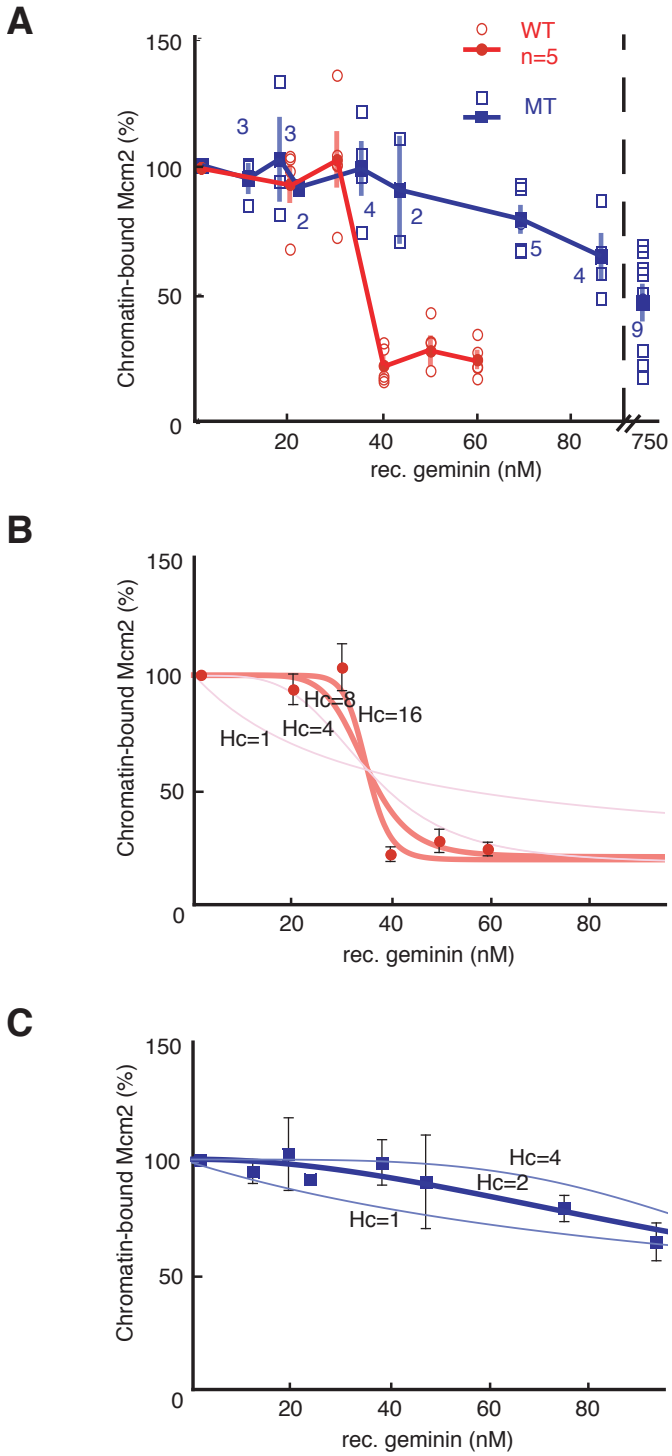

Fig. 6 Licensing inhibitory activities of wild-type and mutant geminin.
(A) Sperm chromatin was incubated with extract containing the indicated amount of wild-type or mutant geminin. Isolated chromatin fractions were analyzed by western blotting, and the amount of Mcm2 was quantified. Open circles (wild-type) and open squares (mutant) represent individual data points, and filled symbols and bars represent the average ± SEM (number of samples are indicated in the graph) at defined concentrations of geminin.
(B-C) Estimation of cooperativity of licensing inhibition by wild-type and mutant geminin. Licensing inhibition by wild-type (A) and mutant geminin (B) shown in Fig. 6A were fitted using Hill equation with various Hill constant (H_c). The apparent cooperativity of H_c>8 for wild-type action and H_c~2 for mutant geminin were obtained. Note that 20 % of licensing activity was assumed as a base line of the inhibition.

The new geminin mutant retained the ability to bind to Cdt1 in extracts, similar to the wild type. By immunoprecipitating Cdt1 from extracts in the presence of different concentrations of wild-type or mutant geminin, I found that the amount of geminin co-precipitated with Cdt1 increased gradually rather than showing a threshold effect with respect to total geminin concentration (Fig. 7). I did not detect all-or-none binding of either wild-type or mutant geminin to Cdt1 in extracts around the threshold concentration of geminin. These results

suggest that the source of the all-or-none action of geminin is not its interaction with Cdt1 in solution but rather the behavior of geminin in the licensing reaction that occurs on chromatin.

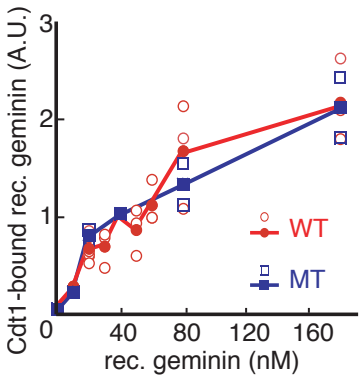

Fig. 7 Co-precipitation of geminin with Cdt1 in extract.
Cdt1 was immunoprecipitated from egg extract using anti-Cdt1 in the presence of various concentrations of recombinant wild-type or mutant geminin. Precipitated proteins were analyzed by western blotting, and the amount of geminin that co-precipitated with Cdt1 was quantified. Open symbols: individual data points. Filled symbols with bars: average intensity.

Geminin induces the formation of nuclear Cdt1 foci

To investigate the different actions of wild-type and mutant geminin on chromatin, I analyzed the nuclear localization of Cdt1 in the presence of geminin. Nuclei were fixed in the presence of detergent to remove proteins not bound to chromatin and nuclear structures. Cdt1 localization on chromatin was visualized by immunofluorescence microscopy. Cdt1 foci formed in the presence of wild-type geminin when licensing was inhibited and Cdt1 was stabilized on chromatin (Fig. 8A and B, WT, see also Fig. 8E). The Cdt1 foci closely co-localized with wild-type geminin, which also formed foci on chromatin (Fig. 8C). In the absence of geminin, most Cdt1 was dissociated from chromatin, and the number of foci was markedly decreased (Fig. 8A and B, control, see also Fig. 8F). Importantly, foci formation increased abruptly above the threshold concentration of geminin that inhibited replication (Fig. 8D). Addition of excess recombinant Cdt1 or CDK inhibitor, which counteracts or inhibits replication-coupled Cdt1 degradation, did not stabilize Cdt1 on chromatin (Fig. 8A and B, +Cdt1 and +p21). In comparison, in the presence of mutant geminin, Cdt1 bound stably to chromatin but the number of foci decreased compared with wild-type geminin (Fig. 8A and B, MT). In line with the fact that chromatin binding of geminin sharply increased above the threshold concentration for licensing inhibition (Figs. 8A and B), Cdt1 foci formation and licensing inhibition occurred in a highly cooperative manner near the same threshold level of geminin.

Geminin and Cdt1 counteract the formation of intermediate licensing complexes on chromatin

Close correlation of the formation of geminin-Cdt1 foci on chromatin with all-or-none licensing inhibition suggests that multiple Cdt1-geminin molecules on chromatin cooperatively inhibit licensing. To investigate the contribution of cooperative activity of geminin with the all-or-none inhibitory activity, I needed to identify the target of geminin in the inhibitory pathway on chromatin. I first examined chromatin binding of pre-replication

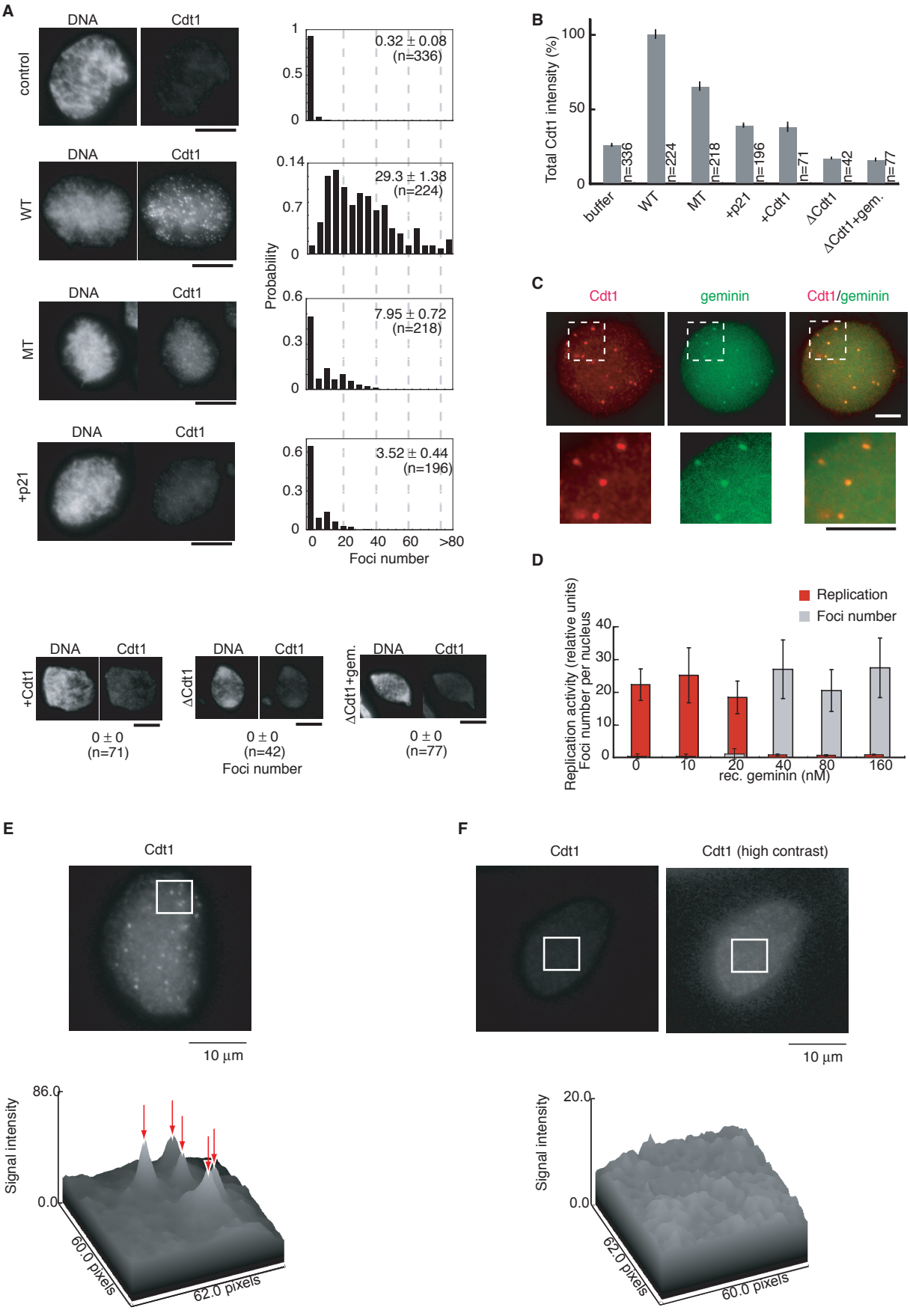

Fig. 8 Nuclear Cdt1 foci formation induced by geminin.

(A) Nuclear localization of Cdt1. Sperm chromatin was incubated with egg extract containing control (control), 80 nM recombinant wild-type geminin (WT), 600 nM mutant geminin (MT), 50 μg/ml p21 (+p21), 40 nM recombinant Cdt1 (+Cdt1), or with Cdt1-depleted extract in the absence (ΔCdt1) or presence of 80 nM recombinant geminin (ΔCdt1 + gem.) for 35 min at 23°C. Nuclei were fixed on coverslips in the presence of 0.25% NP40, and Cdt1 was immunostained with anti-Cdt1 and visualized with Alexa488-conjugated secondary antibody. DNA was visualized with Hoechst 33342. The number of foci and total fluorescence intensity of nuclear Cdt1 in each nucleus were quantified. The average number of foci in one nucleus ± SEM and the number of counted nuclei are shown in the upper-right part of the distributions of foci number (control, WT, MT, +p21) or below the images of nuclei (+Cdt1, ΔCdt1, ΔCdt1 + gem). Foci were not detectable under the conditions indicated by +Cdt1, ΔCdt1, and ΔCdt1 + gem. The identification of foci is described in Experimental Procedures (see also Fig. 8E and F). Scale bar = 10 μm.
(B) The amount of Cdt1 in each nucleus. The signal intensity of Cdt1 fluorescence of each nucleus was normalized to the average intensity observed in the presence of 80 nM geminin (i.e., the bar labeled WT).
(C) Colocalization of geminin and Cdt1 in nuclear foci. Sperm chromatin was incubated with egg extract containing 150 nM GFP-geminin for 30 min at 23°C. Nuclei were fixed on coverslips in the presence of 0.25% NP40, and Cdt1 was immunostained with anti-Cdt1 and visualized with Alexa555-conjugated secondary antibody. GFP-geminin was visualized by its endogenous fluorescence, and DNA was visualized by staining with Hoechst 33342. Areas indicated with dotted white lines are shown in higher magnification below the images. Scale bar = 5 μm.
(D) Cdt1 foci formation over the threshold concentration of geminin. Sperm chromatin was incubated with egg extract containing Cy3-labeled dCTP and various amounts of recombinant geminin. Nuclei were fixed, and the number of Cdt1 foci was counted as described in (A). DNA replication activity of each nucleus is represented by the integrated intensity of the nuclear Cy3 signal. The number of foci and the Cy3 intensity are shown as the means ± SD (n = 13~16 nuclei for each geminin concentration).
(E) A representative data of Cdt1 distribution in a nucleus containing Cdt1 foci. Upper panel shows Cdt1 localization of a nucleus prepared in the presence of wild type geminin (see the legend for Fig. 8A). Right panel shows the 3D distribution of signal intensity of Cdt1 shown in the left panel encircled with square. The signal intensity was measured by ImageJ software. Red arrows indicate the peaks with signal intensity higher than 1.3-fold of that found in the surrounding area.
(F) A representative data of Cdt1 distribution in a nucleus without Cdt1 foci. Upper-left panels show Cdt1 localization of a nucleus prepared in the absence of geminin, where the upper-right panel is highly contrasted. Lower panel shows the 3D distribution of the Cdt1 signal. The absence of red arrows indicates that there was no peaks with signal intensities higher than 1.3-fold of that found in the surrounding area.

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complex components and geminin under various conditions that affect licensing activity. In the presence of geminin, the binding of Cdc6 and Cdt1 to unlicensed chromatin increased (Fig. 9A, lane 3) compared with binding to licensed chromatin (lane 1; also see Figs. 1A and 2A). Results similar to those obtained in the presence of geminin were obtained when the licensing reaction was inhibited by incubating the reaction at 0ºC (Fig. 9A, lane 2) or Mcm2 was depleted from the extract (Fig. 9B). I also found that dissociation of Cdc6 from chromatin followed a reciprocal time course for the loading of Mcm2-7 when the incubation temperature was raised from 0ºC to 23ºC (Fig. 9C). These results suggested that a ternary ORC-Cdc6-Cdt1 complex is assembled on chromatin to enable the loading of Mcm2-7. Indeed, the amounts of Cdc6 and Cdt1 bound to chromatin were linearly dependent on that of Orc2 bound to

chromatin at 0ºC, and they correlated closely with the amount of Mcm2 loaded at 23ºC (Fig. 9D).

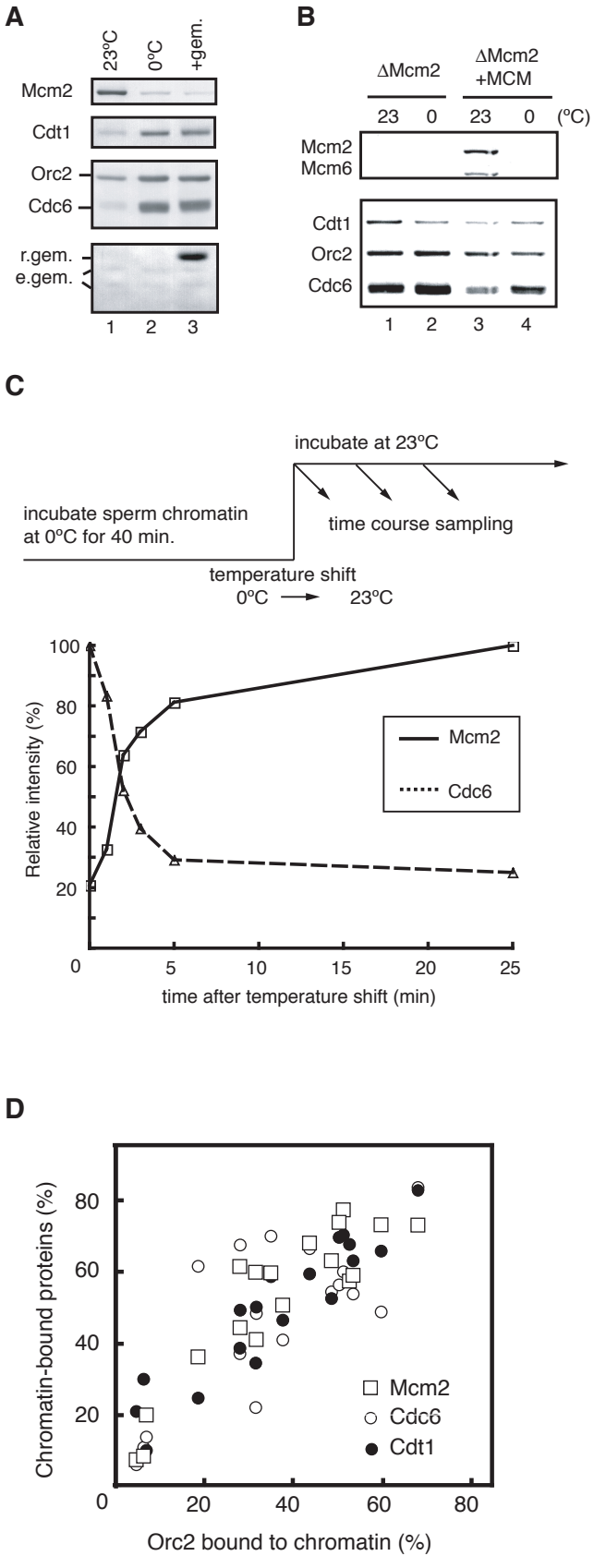


Fig. 9 The ORC-Cdc6 Cdt1 complex is formed on chromatin.

(A) Stabilization of Cdc6 and Cdt1 on chromatin in the absence of Mcm2-7 loading. Sperm chromatin was incubated with egg extract for 20 min at 23°C without or with 60 nM recombinant geminin, or for 40 min at 0°C without recombinant geminin. Chromatin fractions were analyzed by western blotting with the antibodies indicated.

(B) Formation of the ORC-Cdc6-Cdt1complex in the absence of Mcm2-7 loading. Sperm chromatin was incubated in the Mcm2- and Cdt1- depleted extracts supplemented with 20nM recombinant Cdt1 and without (ΔMCM) or with 1/3 volume of Cdc6- and Cdt1- double-depleted extract (ΔMCM +MCM) for 20 min at 23ºC or for 40 min at 0ºC, and chromatin binding of various factors were analyzed.

(C) Comparison of time courses of Cdc6 dissociation and Mcm2-7 loading following a temperature shift. Sperm chromatin was incubated in egg extract for 40 min at 0ºC. The incubation temperature was then shifted to 23ºC. Chromatin fractions were collected at the indicated times after the temperature shift and were analyzed by Western blotting. Signal intensity of Mcm2 and Cdc6 bound to chromatin were quantified. The relative intensity of the Cdc6 signal was measured by setting the intensity obtained at 0 min as 100%. By comparison, the relative intensity of the Mcm2 signal was measured by setting the intensity obtained after 25 min as 100%.

(D) Correlation between the chromatin binding of Orc2 and that of Cdc6, Cdt1, and Mcm2. Sperm chromatin was incubated with Orc2-depleted extracts mixed with mock-depleted extract in various proportions for 20 min at 23°C or for 40 min at 0°C. The amount of Cdc6 and Cdt1 bound to chromatin at 0°C and the amount of Mcm2 bound to the chromatin at 23°C were plotted against the amount of Orc2 bound to chromatin at 0°C. The values were normalized by taking the amount of protein bound to chromatin in mock-depleted extract as 100%.

I found that ORC, Cdc6, and Cdt1, when assembled on sperm chromatin, remained stable in chromatin fractions for more than 10 min after transfer to chromatin isolation buffer at 0ºC (data not shown). The stable association of the ORC-Cdc6-Cdt1 complex with chromatin allowed us to examine whether the ternary complex is the intermediate for chromatin loading of Mcm2-7 and for inhibition by geminin. Fig. 10A illustrates the experimental design. Chromatin was incubated with egg extract at 0ºC and then transferred to Cdc6 and Cdt1 double-depleted extract, which by itself cannot induce licensing (Fig. 10B). I found that

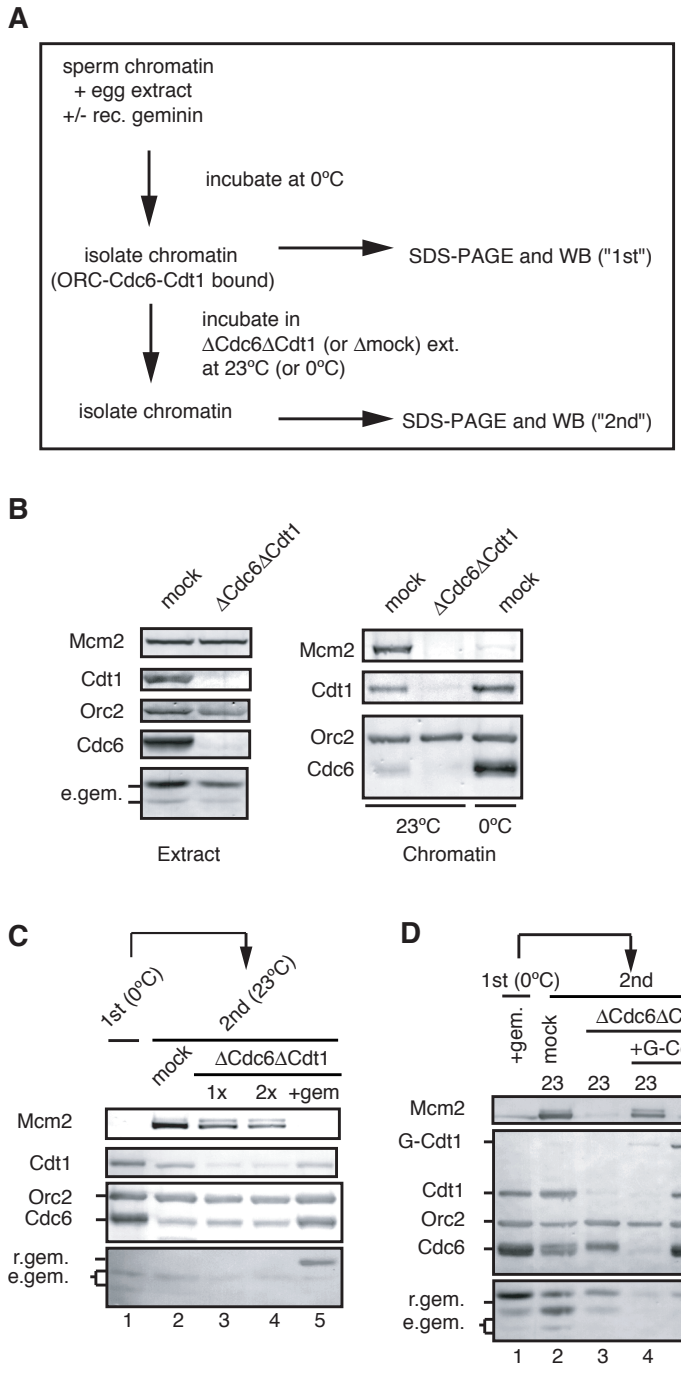


Fig. 10 Geminin targets an intermediate for origin licensing.

(A) Diagram of the chromatin transfer assay. WB, western blotting.

(B) Chromatin binding proteins in the absence of Cdc6 and Cdt1. Left panel shows extracts mock-depleted and double-depleted for Cdc6 and Cdt1 analyzed by western blotting. Right panel shows the chromatin binding of various proteins incubated in extracts double-depleted for Cdc6 and Cdt1 for 20 min at 23ºC, or in the mock-depleted extracts for 20 min at 23ºC or 40 min at 0ºC.

(C) Licensing activity of chromatin bound with ORC, Cdc6, and Cdt1. Sperm chromatin was incubated in egg extract for 40 min at 0°C, and the isolated chromatin fraction (1st) was suspended in various extracts (lane 2, mock-depleted extract; lane 3, extract double-depleted of Cdc6 and Cdt1; lane 4, 2-fold amount of double-depleted extract; lane 5, double-depleted extract with 60 nM recombinant geminin) and further incubated for 20 min at 23°C (2nd). The isolated chromatin fractions were analyzed by western blotting.

(D) Reversal of geminin-induced licensing inhibition by free Cdt1 in the extracts. Sperm chromatin was incubated with egg extract for 30 min at 0°C and then for another 10 min at 0°C with 60 nM recombinant geminin. The isolated chromatin fraction (1st) was suspended and further incubated in extract mock-depleted or double-depleted of Cdc6 and Cdt1 for 20 min at 23°C or for 40 min at 0°C. Recombinant GST-Cdt1 (G-Cdt1) (20 nM) was included in the double-depleted extracts as indicated. The isolated chromatin fractions were analyzed by western blotting.

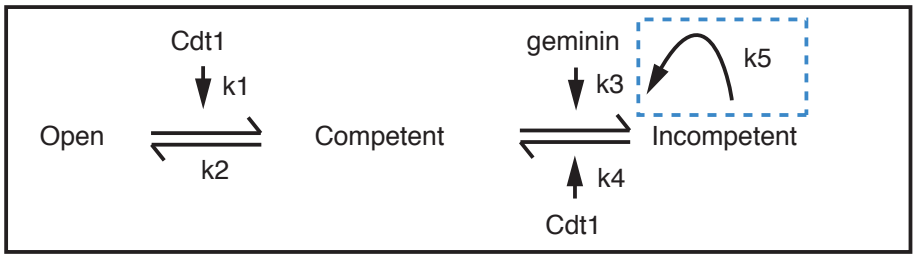
chromatin bound with the ternary complex prepared in the first incubation (Fig. 10C, lane 1) efficiently recruited Mcm2-7 in the absence of Cdc6 and Cdt1 at 23ºC (lane 3). Geminin added to the second extract completely inhibited the loading of Mcm2-7 onto the transferred chromatin (lane 5). These results indicated that Mcm2-7 loading is catalyzed by the ternary complex on the chromatin and that geminin in the extract inhibits the activity of the ternary complex. Thus, the inhibition by geminin in the second extract occurred faster than the loading of Mcm2-7 by the ternary complex because geminin could not remove Mcm2-7 from the chromatin (Oehlmann *et al.* 2004). Cdt1 released from the chromatin, if any, would not play a major role in the Mcm2-7 loading reaction because 1) the total concentration of Cdt1 in the double-depleted extract after the transfer was estimated to be less than 10 nM, which is suboptimal for the loading of Mcm2-7 in the extract (Figs. 3A and B), and 2) two-fold dilution of chromatin fractions with the double-depleted extract did not significantly affect the amount of Mcm2 loaded onto chromatin (Fig. 10C, compare lanes 3 and 4). Therefore, geminin in the extracts likely targets Cdt1 in the ternary complex formed on chromatin.

Inactivation of the ORC-Cdc6-Cdt1 complex by geminin binding was clearly demonstrated by preparing chromatin at 0ºC. When geminin was added after assembly of the ORC-Cdc6-Cdt1 complex at 0ºC, geminin bound to chromatin without affecting the binding of ORC, Cdc6, or Cdt1 (Fig. 10D, lane 1). When this geminin-bound chromatin was transferred to egg extract double-depleted of Cdt1 and Cdc6 and incubated at 23ºC, the ORC-Cdc6-Cdt1 complex failed to recruit Mcm2-7 (Fig. 10D, lane 3). Thus, geminin inhibited the loading of Mcm2-7 onto chromatin by binding to the ORC-Cdc6-Cdt1 complex. The inactive geminin-bound ORC-Cdc6-Cdt1 complex could be re-activated by free Cdt1 in the second extract. Mcm2 was similarly loaded onto chromatin when chromatin bound with ORC, Cdc6, Cdt1, and geminin was transferred to a mock-depleted extract or an extract double-depleted of Cdc6 and Cdt1 that was supplemented with recombinant Cdt1 and incubated at 23ºC (Fig. 10D, lanes 2 and 4). Cdt1 in the second extract bound to geminin-inhibited chromatin at 0ºC (Fig. 10D, lane 5), suggesting that re-activation was mediated by binding of Cdt1 to the inhibited complex. Taken together, these chromatin transfer experiments suggested that the licensing reaction is controlled at three distinct steps: 1) formation of the ORC-Cdc6-Cdt1 ternary complex capable of loading Mcm2-7 on chromatin, 2) inhibition of the complex by geminin binding, and 3) re-activation of the geminin-bound complex by Cdt1.

Three-state model for licensing control by geminin and Cdt1

My findings thus far suggested that two intermediate complexes are formed on chromatin during inhibition of licensing by geminin. One is the ORC-Cdc6-Cdt1 complex, which I term the “Competent complex”, that is competent for loading of Mcm2-7. The other is the ORC-Cdc6-Cdt1-geminin complex, which I term the “Incompetent complex”, that is not competent for loading of Mcm2-7. I refer to the precursor of the Competent complex (the ORC-Cdc6 complex) as the “Open complex”, the existence of which is based on a previous finding that Cdt1 is capable of licensing only after Cdc6 binding to chromatin pre-bound with ORC,

although the binding of Cdt1 itself is dependent on ORC but not Cdc6 (Tsuyama *et al.* 2005). I include the Open complex in the model to describe dose-dependent stimulation of Mcm2-7 loading by Cdt1 in the absence of geminin (see Fig. 3A and 12A, less than 20 nM Cdt1). Almost all complexes are of the Competent form, and the contribution of Open complexes becomes negligible in the presence of physiological concentrations of Cdt1 (estimated to be about 20 nM; Fig. 3C). The overall reaction is illustrated in Fig. 11. The binding of Cdt1 to an Open or Incompetent complex leads to the formation of the Competent complex, whereas binding of geminin to the Competent complex results in an Incompetent complex.



$$\frac{d[Competent]}{dt} = k_1[Cdt1]^a[Open] + k_4[Cdt1]^c[Incompetent] - k_2[Competent] - k_3[geminin]^b[Competent] - \frac{k_5[Incompetent]^d}{l+[Incompetent]^d}[Competent]$$

feedback model

Fig. 11 Three state model of licensing control by geminin and Cdt1.
(Upper diagram**)** Schematic representation of the conversion of three intermediates by geminin and Cdt1. (Lower equation) Ordinary differential equation of the conversion between the three states shown in the diagram. The equation for the rate of change in Competent complexes is shown. [Cdt1] and [geminin] denote the total concentration of Cdt1 and geminin. [Open], [Competent], and [Incompetent] denote the proportion of complexes in each state. k_1~k_5, a~d, and l are constants. The fifth term in the dashes box is used in the Feedback model shown in (C). For details, see Appendix, Model Construction section 1~3.

I hypothesized that the conversion rates between the three complexes were much faster than the loading of Mcm2-7 onto chromatin for the following reasons: 1) My current study showed that inhibition of the Competent complex by geminin is faster than the loading of Mcm2-7 onto chromatin (Fig. 10D), and 2) binding of Cdt1 to the Open or Incompetent complex is also considered to be faster than the Mcm2-7 loading reaction, which involves enzymatic hydrolysis of ATP by ORC and Cdc6 (Gillespie *et al.* 2001; Frolova *et al.* 2002; Bowers *et al.* 2004; Randell *et al.* 2006). Thus, I applied quasi-steady-state approximation to the conversion of intermediates, and the steady-state level of the Competent complex was considered as the licensing efficiency, which can be experimentally estimated as the amount of Mcm2-7 on chromatin. To calculate the steady-state level of the Competent complex, I formulated the conversion between the three states using ordinary differential equations based on the law of

mass action (Fig. 11, equation; section 1 of Model Construction in Appendix). For simplicity I considered the concentrations of the components affecting the conversion rates as the total amount of Cdt1 and geminin in the system, because I did not know the exact concentrations and activities of the various Cdt1-geminin complexes in egg extract.

The cooperativity of a component can be represented as a particular power, "*n*", where [component]^n is proportional to rate. The stimulation of licensing by Cdt1 in the absence or presence of mutant geminin could be reasonably predicted without assuming cooperativity of Cdt1 (Fig. 12A and B). Thus, I assumed "*n*" = 1 for the term involving Cdt1 concentration (*a* = *c* = 1 in the equation in Fig. 11; section 2 of Model Construction in Appendix). In contrast to the hyperbolic curve representing Cdt1 action in licensing, geminin action apparently involves cooperativity. Without assuming cooperativity in the action of geminin, the model cannot simulate the experimentally observed all-or-none behavior (taking *b* = 1 in the equation in Fig. 11; simulated result is shown in Fig. 13A).

Geminin cooperativity can be implemented into the scheme by the following two models. One model assumes geminin cooperativity at each individual origin, such as formation of higher-order Cdt1-geminin complexes or any other possible reactions involving geminin. Then, the cooperativity can be represented by taking the power "*b*" of the geminin concentration as the high value. I named this assumption the "Ultrasensitive model". By assuming extremely high geminin cooperativity (taking *b* = 8), the model exhibits all-or-none

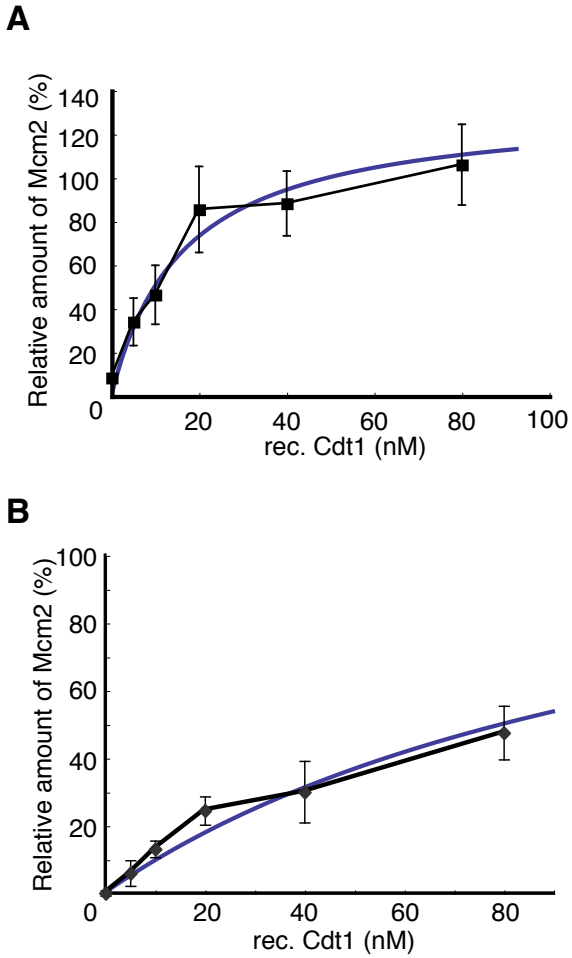


Fig. 12 Simulation of licensing activity of Cdt1. **(A)** Mcm2 signal shown in Fig. 3A was quantified. Data were taken from five independent experiments and are shown as mean ± SD. The amount of Mcm2 was quantified by setting the loaded amount in mock-depleted extract as 100%. Experimental data were fitted by the equation (14) in Appendix without assuming cooperativity in Cdt1 action (*a*=1). **(B)** Activation of origin licensing by Cdt1 in the presence of mutant geminin. The experiments were carried out under similar conditions to (A), except for the presence of 750 nM MT geminin in the extracts. Data were taken from three independent experiments and are shown as mean ± SD. Experimental data were fitted by the equation (15) without assuming cooperativity in Cdt1 action (*a* = *c* = 1) even in the presence of mutant geminin.

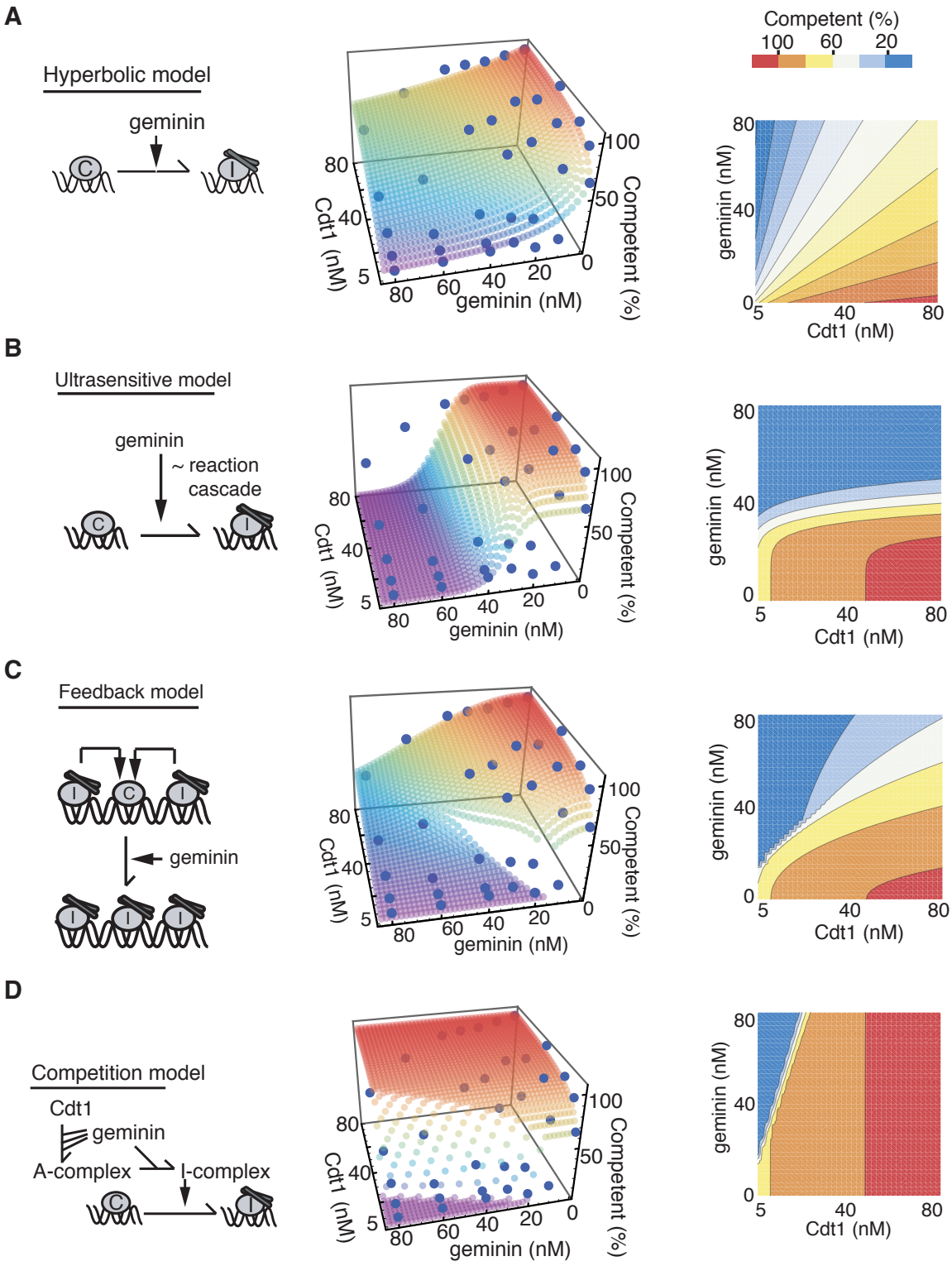


Fig. 13 Two-dimensional input function of licensing activity for Cdt1 and geminin concentrations.
(A-D) Simulation of the two-dimensional input functions by Hyperbolic (A), Ultrasensitive (B), Feedback (C) and Competition models (D). Parameters were selected to fit the threshold response to geminin concentrations at a Cdt1 concentration of 20 nM, and the level of Mcm2-7 loading was estimated from the level of Competent complexes. The simulated steady-state values of [Competent] at various amounts of Cdt1 and geminin are shown in the 3D diagram and 2D contour map. In the 3D diagram, these estimated values (rainbow-colored dots) were superimposed onto the experimental data (dark-blue dots) shown in Fig. 15. See Appendix, Model Construction section 3.1 to 3.4 for detailed description of the models. Parameters used in the simulation are summarized in Table S1.

inhibition of licensing, and it can account for the inhibition observed experimentally. The model predicts that the threshold geminin concentration remains at almost the same level regardless of Cdt1 concentration (Fig. 13B).

An alternative possibility is that geminin cooperativity operates on neighboring origins. Indeed, I found that geminin induced the formation of Cdt1 foci possibly by tethering multiple Cdt1 molecules onto individual origins. Consistently, I found that wild-type geminin tethered multiple Cdt1 molecules in extract, whereas mutant geminin had greatly reduced activity (Fig. 14). These results suggested that geminin cooperatively tethers neighboring origins to induce licensing inhibition. This model is therefore qualitatively different from the Ultrasensitive model. Formation of Incompetent complexes is stimulated by Incompetent complexes themselves. I named this the "Feedback model". In this model, the feedback effect is represented by an additional term (boxed term in the equation in Fig. 11). With this term, the rate of reaction from a Competent to an Incompetent state depends not only on the geminin concentration but also on the amount of Incompetent complexes; an increase in Incompetent complexes stimulates the conversion of Competent to Incompetent complexes. The Feedback model also accounts for all-or-none inhibition (Fig. 13C for Cdt1 <30 nM). This model further predicts the characteristic behavior of the threshold level of geminin—the threshold concentration of geminin initially increases upon increasing Cdt1 concentration, and at higher Cdt1 concentrations geminin no longer inhibits licensing in an all-or-none manner. This damping effect of Cdt1 reflects the higher rate of Competent–Incompetent complex

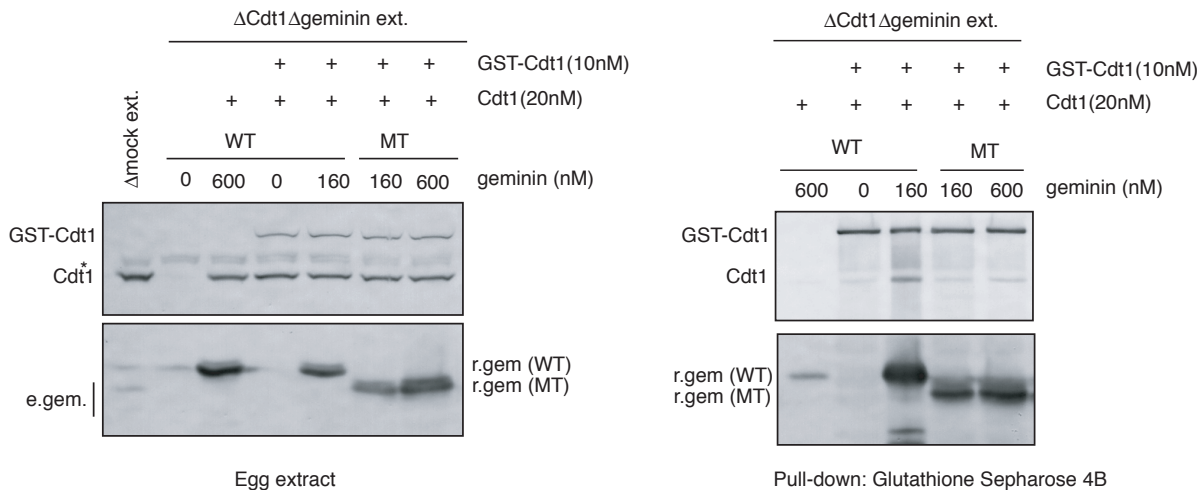
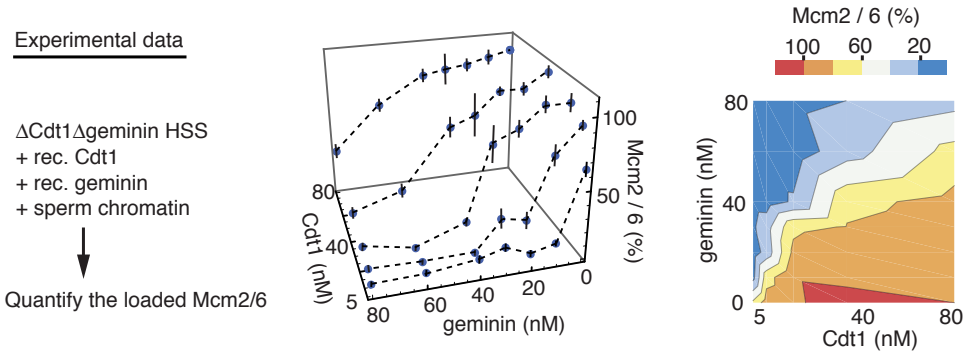


Fig. 14 Co-precipitation of non-tagged and GST-tagged Cdt1 by geminin.
Egg extract was depleted for both Cdt1 and geminin. The double-depleted extract was supplemented with 20 nM recombinant Cdt1 and/or 10 nM recombinant GST-Cdt1, and indicated amounts of recombinant wild-type (WT) or mutant (MT) geminin (left panel). After incubating for 20 min at 23ºC, GST-Cdt1 was precipitated using Glutathione Sepharose 4B beads (right panel). Various extracts and precipitates were analyzed by western blotting. If geminin has the ability to tether multiple Cdt1 molecules, non-tagged Cdt1 should be found in the precipitates of GST-Cdt1. In the presence of wild type geminin, non-tagged Cdt1 is co-precipitated with tagged Cdt1. In the presence of mutant geminin, the amount of non-tagged Cdt1 co-precipitated with GST-Cdt1 was markedly decreased.

conversion at higher Cdt1 and geminin concentrations, which masks the feedback reaction mediated by Incompetent complexes.

To quantitatively evaluate the Ultrasensitive and Feedback models, I examined whether one of the models could account for the experimental data obtained under various concentrations of Cdt1 and geminin. I measured licensing activity using Cdt1-geminin double-depleted membrane-free extract (HSS) to avoid potential effects of endogenous inactive geminin and nuclear formation. Fig. 15 shows the licensing efficiency measured as the average amounts of Mcm2 and Mcm6 loaded on chromatin in the presence of various amounts of recombinant

A



B

Average Mcm2 / 6 (%) ± SEM (sample number)

geminin (nM) \ Cdt1 (nM)	5	10	20	40	80
0	64.1±4.7 (14)	89.9±3.0 (8)	98.1±6.4 (9)	106.4±4.0 (10)	100
10	13.6±0.9 (16)	71.9±7.2 (10)	98.7±5.8 (11)	96.1±4.4 (12)	95.6±5.8 (12)
20	8.7±1.0 (16)	28.3±5.7 (10)	84.5±5.5 (11)	96.1±2.8 (12)	91.1±4.4 (16)
30	16.8±1.4 (16)	32.4±7.0 (10)	75.8±12.9 (11)	81.0±15.3 (12)	89.4±11.7(16)
40	11.2±1.7 (16)	10.4±0.9 (10)	22.4±2.6 (11)	74.5±7.0 (12)	86.3±4.6 (16)
60	7.9±0.8 (8)	9.9±1.0 (4)	8.8±1.0 (4)	31.7±4.6 (4)	66.4±4.5 (8)
80	6.2±0.5 (8)	11.4±1.3 (4)	16.1±1.9 (4)	20.1±3.7 (4)	32.9±4.5 (8)

Fig. 15 Experimental analysis of two-dimensional input function of licensing activity for Cdt1 and geminin concentrations.

(A) Licensing activity of egg extract in the presence of various concentrations of Cdt1 and geminin. Sperm chromatin was suspended in HSS double-depleted of Cdt1 and geminin and supplemented with various concentrations of recombinant Cdt1 and recombinant geminin, and then incubated for 15 min at 23°C. Isolated chromatin fractions were analyzed by western blotting, and Mcm2 and Mcm6 signals were quantified. Data were taken from three to eight independent experiments for each Cdt1 and geminin concentration. Average values ± SEM are shown in a 3D diagram and represent the Mcm2 and Mcm6 signals with Cdt1 = 80 nM, geminin = 0 nM as 100%.

(B) Individual data of the results of Fig. 15A. The average amounts of chromatin loaded Mcm2/6 ± SEM were shown.

Table 1 Summary of simulation and experimental results shown in Figs. 13 and 14.

	geminin action in lower concentration of Cdt1	geminin action in higher concentration of Cdt1	Threshold shifting along with Cdt1 increase
Experiment	all-or-none	hyperbolic	non-proportional increase
Hyperbolic	hyperbolic	hyperbolic	—
Competition	all-or-none	all-or-none	proportional increase
Ultrasensitive	all-or-none	all-or-none	modest shift
Feedback	all-or-none	hyperbolic	non-proportional increase

Cdt1 and geminin. Geminin inhibited licensing in an all-or-none manner for Cdt1 <40 nM. In the presence of 20 nM Cdt1 (considered the physiological concentration in *Xenopus* egg extract; Fig. 3C), the threshold concentration of geminin was 30–40 nM, which is consistent with the results obtained for interphase extract (Fig. 1A). The threshold concentration of geminin increased with Cdt1 concentration. Notably, at higher concentrations of Cdt1, such as 80 nM, inhibition of licensing by geminin followed a gradual curve. Such a decrease in geminin sensitivity with increasing Cdt1 concentration is consistent with the Feedback model (Fig. 13C) but not the Ultrasensitive model (Fig. 13B). I also considered another model that assumes multi-step formation of Cdt1-geminin complexes in solution, but the model failed to explain the experimental results (Fig. 13D). Regarding these models, I therefore concluded that the Feedback model is the most appropriate for describing the licensing control by Cdt1 and geminin in extracts (Table 1 summarizes each model's behavior). I could not exclude the possibility that other models, not considered here, may account for these results equally well or even better, but my current modeling and verification approach suggests a novel modality of geminin action that coordinately regulates the licensing of multiple origins.

Inter-origin cooperativity (IOC) of licensing inhibition as the source of feedback

(Note; computational simulation shown in this section; Fig. 16A-D was carried out through the collaboration with Dr. Koichi Fujimoto, Osaka university. I and Dr. Fujimoto set up the fundamental setting of the model. Writing a program for simulation, parameters setting and computational simulations were carried out by Dr. Fujimoto. We discussed about and interpreted the obtained data.)

My Feedback model involves cooperative interaction of the complexes formed on origins, here termed IOC. Such an interaction could be readily correlated with the formation of Cdt1 foci formed on chromatin by tethering Cdt1 by geminin, further predicting that the IOC operates locally on chromatin. If so, the local effect of IOC may change the distribution of unlicensed origins and, conversely, the distribution of licensed origins. To estimate the effect

of IOC on the distribution of licensed origins, we employed a minimal model assuming a spatial interaction between adjacent origins (i.e., the "IOC model"). For simplicity, we assumed that each origin is distributed evenly over chromatin fibers and considered the physical constraints of the conversion of each origin between competent and incompetent states (Fig. 16A). The IOC is represented by the rate constant, k_n, of geminin action to the n^{th} origin depending on the competency status of adjacent origins. When both adjacent origins are competent, the rate constant is $k_{autonomous}$. When one of the adjacent origins is incompetent, the additional rate constant $k_{cooperative}$ is introduced in k_n, and two-fold of $k_{cooperative}$ is included when both adjacent origins are incompetent. The strength of IOC is therefore represented by the value of $k_{cooperative}$.

The IOC model predicts the switch-like inhibition by geminin (Fig. 16B, high IOC). This switch-like inhibition is roughly fitted to the Hill equation with an apparent Hill constant of ≥8, which accounts for the experimental results of licensing inhibition by wild-type geminin (Figs. 6A and B; see also lower concentration range of Cdt1 in Fig. 15A). By decreasing the effect of IOC (taking a smaller value for $k_{cooperative}$ than that of high IOC), we could simulate the mode of inhibition by mutant geminin (Fig. 16B, low IOC), which has a Hill constant of ~2 (Figs. 6A and C). For comparison, we examined two additional types of inhibition in the absence of IOC by taking $k_{cooperative} = 0$. One type without geminin cooperativity follows a gradual curve (Fig. 16B, without IOC). The other assumes highly nonlinear geminin activity at each individual origin and corresponds to the Ultrasensitive model described above (Fig. 16B, ultrasensitivity without IOC).

Inhibition of competent origins by geminin with IOC leads to an increased probability that adjacent origins will be incompetent because an incompetent origin stimulates inhibition of licensing at adjacent origins. As a result, competent origins also tend to join to each other on chromatin (Fig. 16C). In contrast, inhibition without IOC would result in a more random distribution of competent as well as incompetent origins. To predict the probability distribution of competent origins on chromatin, we measured the distance between each pair of closest competent origins, called d_{comp} (see Fig. 16C for schematic representation of d_{comp} = 1, 2, 3, and 4). The distance d_{comp} takes a minimum value of 1 when neighboring origins are both competent, and the probability of $d_{comp} = 1$ is 100% when all origins are competent (Fig. 16C, without licensing inhibition). Fig. 16D shows the predicted distribution of d_{comp} when the competent state is inhibited by two-thirds relative to the fully competent state (Fig. 16B, dotted line). In the presence of IOC, more than 50% of the distances are $d_{comp} = 1$, verifying that competent origins tend to be adjoined, whereas a much smaller probability for $d_{comp} > 1$ indicates that incompetent origins tend not to form smaller numbers of clusters (Fig. 16C, lower diagram) but rather a larger one (Fig. 16C, middle diagram). These results depend little on the strength of IOC $k_{cooperative}$ (Fig. 16D, high and low IOC). In the absence of IOC, however, a broader distribution of d_{comp} than that in the presence of IOC was obtained from binominal probability distribution, indicating independent state determination for each origin even when the competent state is inhibited in an ultrasensitive manner (Fig. 16D, without IOC

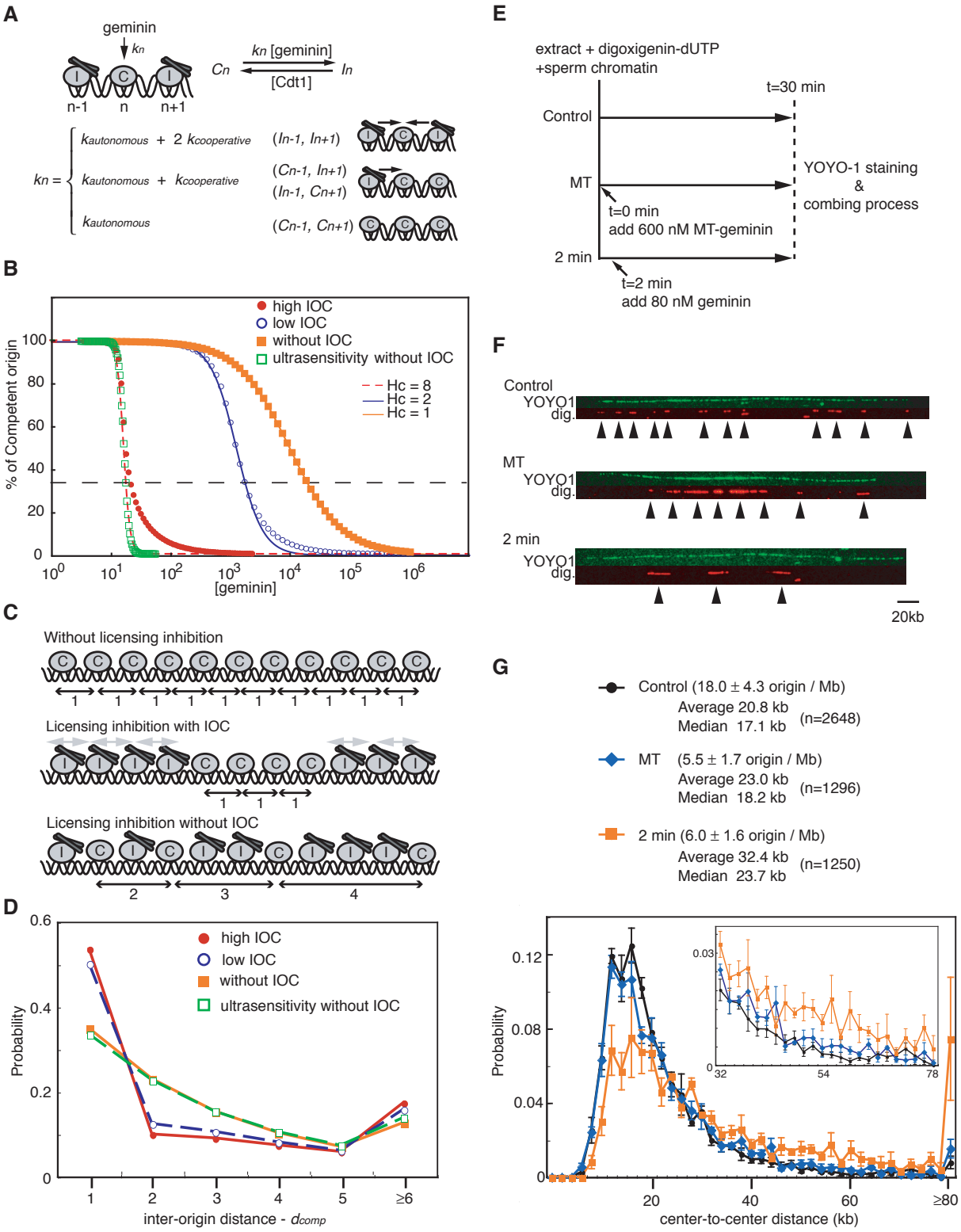


Fig. 16 Distribution of competent origins distinguishes the existence of IOC.

(A) Inter-Origin Cooperativity (IOC) model. Three possible states of adjacent origins including kinetic parameters are shown. See Appendix, Model Construction section 4 for detailed description of the model and simulation. Parameters used in the simulation are summarized in Table S1.

(B) Simulated licensing activity in the presence of geminin without or with IOC. Colored circles represent the steady-state level of Competent complexes at various concentrations of geminin in the absence or presence of IOC. Each smooth line represents the fitted curves of the Hill equation $[\text{geminin}]^{Hc}/([\text{geminin}]^{Hc}+K^{Hc})$ with the various Hill constants, *Hc*, indicated in the key.
(C) Schematic illustrations of geminin inhibition on chromatin without or with IOC compared with the control without inhibition. Numbers under the double-headed arrows represent distances between competent origins, d_{comp}.
(D) Simulated distribution of inter-origin distances based on the model without or with IOC when the steady-state levels of competent origins were decreased to 34%, as indicated by the horizontal dashed line in (B).
(E) Diagram showing the DNA combing assay. Sperm chromatin was incubated in the absence (control) or presence of 600 nM mutant geminin (MT). For sub-minimal licensing (2 min), 60 nM wild-type geminin was added to the extract 2 min after the addition of sperm chromatin. The extracts were incubated for 30 min at 23°C, and the replication tracks were then labeled with digoxigenin-dUTP. DNA fibers were stretched on a cover slip and stained with YOYO-1. The distances between neighboring centers of replication tracks (center-to-center distances) were quantified.
(F) Representative images of the combing assay. Arrowheads show the identified center of replication tracks.
(G) Distribution of distances between replicating origins. Data were taken from three to five independent experiments. For data acquisition, DNA fibers that retained a total length of 30 μm or longer were randomly selected. The average number of tracks per total DNA length ± SD, distribution of center-to-center distances ± SEM (n = 5 for control, n = 3 for MT, and 2 min), and the average and median of the distance (n: number of quantified center-to-center distances) are shown.

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and ultrasensitivity without IOC). This simulation indicated that different distribution patterns can be indicative of the existence of IOC for the inhibition of competent origins by geminin.

The distribution of competent origins may be directly correlated to that of licensed regions on chromatin. I therefore tried to experimentally evaluate the distribution of licensed region on chromatin in the presence of geminin. To this end, I carried out DNA combing analysis (Figs. 16E and F), which visualizes the replicated region of the stretched DNA fiber. The distribution of replicated areas definitely reflects the distribution of licensed regions on chromosomes, although DNA replication is not initiated from all licensed regions (Woodward *et al.* 2006). In my current assay, sperm chromatin was incubated in *Xenopus* egg extract with digoxigenin-dUTP to label the replicated region. Entire DNA fibers were stained with YOYO-1. To identify the position of replicated origins, DNA fibers over 30 μm (~75 kb) in length were selected randomly, and the center of continuously digoxigenin-dUTP-labeled DNA was identified (Fig. 16F). The digoxigenin-labeled DNA located at the end of the fiber was excluded. I then measured the distance between the neighboring centers of the DNA tracts (center-to-center distance). If each replicated tract originated from a distinct origin, this center-to-center distance would correspond to the distance between replicated origins. To minimize the fusion of replication forks, I isolated the chromatin early in the replication phase. When the chromatin was isolated 20 min after the start of incubation, only a few tracts were detected, and I could not obtain a sufficient number of center-to-center distances. Thus, I isolated the chromatin after 30 min and measured the distribution of the center-to-center-

distances (Fig. 16G, control). The distances were distributed with a peak around 10–15 kb, which is quite similar to reported values (Herrick *et al.* 2000; Blow *et al.* 2001; Jun *et al.* 2004). I therefore concluded that my method was adequate for estimating inter-origin distances, and the fusion of neighboring forks, if any, would not largely affect the results. Notably, the distribution of inter-origin distances was obtained early after replication initiation. Nevertheless, I believe that the origin distances obtained here represent the origin distance at steady state because previous studies have shown that the distribution of fired origins as well as the average of inter-origin distances change little throughout S-phase (Herrick *et al.* 2000; Jun *et al.* 2004).

To detect the effect of IOC, I needed to prepare partially licensed chromatin. For this purpose, wild-type geminin was not appropriate because it induces all-or-none licensing inhibition. The simulated distribution of licensed origins based on the IOC model predicts that the effect of IOC on the distribution is less sensitive to the strength of IOC and that similar distributions of the origins would be obtained in the presence of low and high IOC. My results already showed that mutant geminin inhibits licensing in a cooperative manner and induces Cdt1 foci formation, although these effects were greatly reduced compared with wild type. Therefore, I speculated that mutant geminin retains the effect observed in the presence of low IOC and that mutant geminin would allow us to measure the distribution of inter-origin distances in a partially inhibited licensing condition with IOC (Figs. 16E and F, MT). For comparison, I needed to prepare chromatin with partially inhibited licensing without IOC. For this purpose, I prepared "sub-minimally licensed" chromatin, which was obtained by restricting the licensing reaction to a very short period (Figs. 16E and F, 2 min). When sperm chromatin was incubated in the absence of geminin for a short period, licensed regions would be generated depending on Cdt1 that stimulates the licensing reaction essentially in a non-cooperative manner (Fig. 12A). After 2 min, the licensing reaction was terminated by adding wild-type geminin, which completely inhibited further licensing reactions but did not affect previously licensed origins. Accordingly, the non-cooperative action of Cdt1 should determine the licensed regions during the 2-min incubation without geminin. It should be stressed that the aim of using wild-type geminin in this experiment was not to compare its inhibitory effect with mutant geminin but solely to restrict the time for licensing mediated by Cdt1.

The concentration of mutant geminin and incubation time for “sub-minimal” licensing were adjusted to create the same degree of licensing inhibition as the IOC model in Fig. 16D; the average density of replicated regions was decreased to one-third of that of fully licensed chromatin. Because geminin does not affect the activation of licensed origins (McGarry & Kirschner 1998), the different density of replicated regions would be due to changes in the licensed regions of chromosomes. Fig. 16G shows quantification of the distribution of inter-origin distances under these conditions compared with the control obtained with fully licensed chromatin. In the absence of geminin, the center-to-center distances were distributed around 10–20 kb (Fig. 16G, control). The distribution was not substantially affected by mutant geminin (Fig. 16G, MT). With sub-minimally licensed chromatin, the distribution became

broader compared with mutant geminin (Fig. 16G, compare MT and 2 min). The peak of the probability around 10–20 kb was lower than that obtained with mutant geminin, whereas the probability around 30–80 kb, which corresponds to a two- to four-times greater center-to-center distance at the peak, was higher. Such differences in the distribution between the mutant (MT in Fig. 16) and 2-min conditions agree well with the differences predicted by the IOC model; the probability increased for $d_{comp} = 1$ but decreased for $d_{comp} = 3–4$ in the presence of IOC compared with its absence (Fig. 16D). These results therefore suggested that the IOC occurs in the presence of mutant geminin.

Discussion

Previous studies have established the importance of the geminin switch in the control of origin licensing. Recent studies have further proposed the importance of quaternary structures of geminin or geminin-associated activity such as histone modification in licensing inhibition (Iizuka *et al.* 2006; Lutzmann *et al.* 2006; Miotto & Struhl 2008; De Marco *et al.* 2009; Miotto & Struhl 2010). However, molecular mechanisms of the all-or-none geminin action have not been explored. In this study, I propose for the first time a feedback model of geminin switching. The model accurately describes the all-or-none behavior of geminin for licensing inhibition. My model proposes the involvement of IOC in establishing the feedback action of geminin. The analysis of distribution of inter-origin distances supports the idea that such an inter-origin interaction occurs in the presence of geminin. Nuclear Cdt1 foci formation by geminin further supports the presence of local interactions between geminin-Cdt1 complexes on chromatin. In addition, I found that wild-type geminin can tether multiple Cdt1 molecules in *Xenopus* egg extract, whereas mutant geminin has greatly reduced activity by comparison. The decreased ability of mutant geminin to induce foci formation and to tether Cdt1 molecules is consistent with the decreased strength of IOC predicted by the gradual-type inhibition of origin licensing.

Geminin has been reported to form different types of oligomers including dimers (Benjamin *et al.* 2004), dimer-dimers (Thepaut *et al.* 2004), and tetramers (Okorokov *et al.* 2004). Geminin also forms different stoichiometric complexes with Cdt1 (Lee *et al.* 2004; Lutzmann *et al.* 2006; De Marco *et al.* 2009). These biochemical and structural characteristics of geminin complexes suggest that each complex contains multiple interfaces for interacting with other Cdt1-geminin complexes. Such multiple interfaces between molecules for interaction would dramatically stabilize higher-order complexes. My study further suggests that the higher-order interactions of Cdt1-geminin complexes are cooperatively stabilized on chromatin.

The following scenario may explain the cooperative interaction of geminin-Cdt1 complexes on chromatin as propagating licensing inhibition over the entire chromosome (Fig. 17). Geminin is initially loaded at distinct origins through its binding to Cdt1, and Cdt1-geminin complexes at different origins interact with each other to form higher-order complexes that stably tether multiple origins. The resulting complexes connect adjacent origins, thereby further stabilizing and propagating inhibition. The chromatin itself plays an important role as a scaffold for the propagation reaction that increases the accessibility of geminin to the complex and provides physical linkage between each Cdt1-geminin complex. Multiple modes of geminin self-association and geminin binding to Cdt1 may also contribute to the effective interaction between origins. Here, I have shown a close correlation between the formation of Cdt1-geminin foci and the switch-like activity of geminin. A previous study also reported massive recruitment of geminin on chromatin after S phase onset, suggesting chromatin-dependent oligomerization of geminin (Lutzmann *et al.* 2006). My analysis further suggests the presence of local interactions between adjacent origins. I could not rule out the possibility

that geminin affects not only licensing distribution but also chromatin status, which could alter the accessibility of replication-initiation factors such as CDK and thus may account for the observed inter-origin distances. Nevertheless, mechanisms operating at a distance (or propagating through the chromosome) play important roles in various biological functions that regulate the global status of chromatin; e.g., X-chromosome inactivation, heterochromatin formation, etc. Licensing inhibition in S phase also needs to act on thousands of origins on chromosomes. Thus, it is reasonable that the geminin effect propagates on chromosomes.

I inferred the molecular nature of the interaction of geminin-Cdt1 complexes from the mutated geminin residues. I identified a region of geminin that is responsible for the all-or-none inhibition. Notably, all seven residues mutated in My current study are localized at the interface of geminin homodimers (Fig. 5B). Dimerization of geminin through its coiled-coil domain is essential for binding with Cdt1 and for licensing inhibition. Two mutated sites in the KKAAFEV mutant, A111E and A117V, may directly interfere with the dimerization of the coiled-coil domain, but the mutant protein binds to Cdt1 with a similar affinity as wild type. The other five mutated residues are located in the loop near the N terminus and are adjacent to the Cdt1-geminin "secondary" interface. I found that mouse Cdt1 R342 is involved in the secondary interface, and R346 is important for geminin inhibition but not critical for Cdt1-geminin interaction in egg extract (unpublished observation in collaboration with Drs. Z. You and H. Masai). Thus, the secondary interface of geminin is likely essential for regulating Cdt1 activity on chromatin rather than for the affinity between geminin and Cdt1 in solution. The decrease in nuclear Cdt1 foci formation with the KKAAFEV mutant suggests that the N-terminal loop of geminin serves as a novel component of the Cdt1-geminin interface when this complex is bound to chromatin. Alternatively, the N-terminal loop may affect the function of Mcm9 or HBO1, which regulate Cdt1 and geminin functions (Iizuka *et al.* 2006; Lutzmann & Mechali 2008; Miotto & Struhl 2008, 2010).

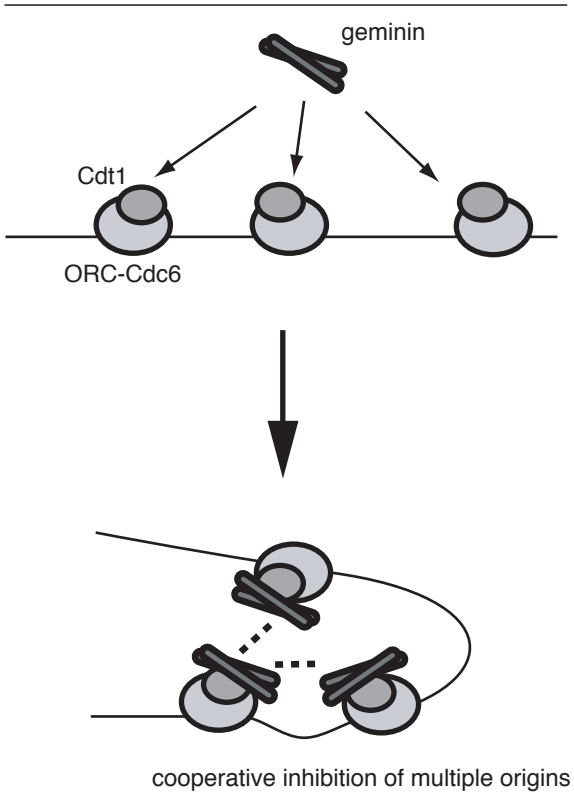

Figure 17. Model of cooperative licensing inhibition by geminin.
A proposed model of geminin action in all-or-none style licensing inhibition. Initially, geminin binds to Cdt1 on chromatin. Alternatively, Cdt1-geminin complexes may bind to ORC-Cdc6 complexes on chromatin. Next, geminin bound to chromatin interacts with adjacent origins bound with Cdt1-geminin or Cdt1, and the inhibition can be stabilized or stimulated. The interaction may be mediated by the ability of geminin to form higher-order Cdt1-geminin complexes.

In summary, combining theoretical and experimental approaches, I showed for the first time that IOC mediated by geminin precisely accounts for the all-or-none licensing inhibition by geminin. Further studies of Cdt1 and geminin function in the context of chromatin will be required to confirm the presence of inter-origin interactions and to clarify the molecular mechanism of IOC.

Experimental procedures

Note that all the extracts used in this study were interphase egg extracts unless stated as membrane-free (HSS) or nucleoplasmic extract (NPE).

Construction of mutant geminin

I generated mutant geminin by introducing point mutations in the wild-type geminin gene with the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Geminin-EV (A111E and A117V) was constructed by using pGEX6p-2 carrying wild-type geminin cDNA as the template and the following primers: 5′-GGTTGAAGAGGAACGAAGAAAGGTCCTCTATGAAGC- 3′ and 5′-GCTTCATAGAGGACCTTTCTTCGTTCCTCTTCAACC-3′. Geminin-KKFEV (E100K, T101K, C104F, A111E, and A117V) was constructed by using pGEX6p-2 geminin-EV as a template and the following primers: 5′-GGTGAAAAAAAAACCAACTTTCCTTTACTGG-3′ and 5′-CCAGTAAAGGAAAGTTGGTTTTTTTTTCACC-3′. Geminin-KKAAFEV (E100K, T101K, P102A, T103A, C104F, A111E, and A117V) was constructed by using pGEX6p-2 geminin-KKFEV as a template and the following primers: 5′-GGTGAAAAAAAAAGCAGCTTTCCTTTACTGG-3′ and 5′-CCAGTAAAGGAAAGCTGCTTTTTTTTTCACC-3′.

Expression and purification of recombinant proteins

Xenopus Cdc6, Orc2, Cdt1, and gemininH (wild type, KKFEV and KKAAFEV mutant) were expressed in the BL21-codonplus expression strain (Stratagene) transformed with pGEX 6p (Amersham) carrying the corresponding cDNAs. Expressed proteins were purified using Glutathione Sepharose^{TM} 4B beads (GE Healthcare). The GST tag was digested using PreScission protease (Amersham) and purified proteins were eluted according to the manufacturer's protocol, with the exception that Cdt1 and Cdc6 were eluted with GST-Elution buffer (600 mM NaCl, 1 mM dithiothreitol and 50 mM Tris-HCl at pH 8.7). GST-Cdt1 was eluted from the beads using Cdt1-elution buffer (10 mM reduced glutathione, 1mM dithiothreitol, 50 mM Tris-HCl at pH 8.0) without digestion of the GST tag. GFP-geminin was characterized in (Yoshida *et al.* 2005).

Antibodies

Polyclonal rabbit antisera were raised against purified recombinant *Xenopus* Cdc6, Cdt1, Orc2, and geminin H proteins (Hokudo Inc., Japan). The anti-*Xenopus* Mcm2 and Mcm6 antibodies used here have been described previously (Kubota. *et al.* 1997).

***Xenopus* egg extracts and sperm nuclei**

I induced ovulation in mature female *Xenopus* by an injection of human gonadotropin (700 IU). Eggs were collected in 0.1 M NaCl, and those eggs that appeared to have degenerated were discarded. Unfertilized eggs were dejellied in a solution consisting of 5 mM

dithiothreitol, 110 mM NaCl and 20 mM Tris-HCl at pH 8.5, then washed in 0.25× MMR (100 mM NaCl, 2 mM KCl, 0.5 mM $MgSO_4$, 2.5 mM $CaCl_2$, 0.1 mM EDTA, 5 mM Hepes-NaOH at pH 7.8), and activated with 0.5 μg/ml calcium ionophore A23187 in 0.25× MMR. Activated eggs were washed with 0.25× MMR and then with ice-chilled S-buffer (0.25 M sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 15 μg/ml leupeptin, and 50 mM HEPES-KOH at pH 7.5). The washed eggs were packed into tubes by brief centrifugation for several seconds at 3000*g*. All excess buffer was removed and the eggs were ruptured by centrifugation at 18 800*g* for 10 min. The resulting supernatant between the lipid cap and pellet was collected and mixed with 10 μg/ml cytochalasin B and then centrifuged again at 265 000*g* for 10 min. Both the cytosolic and membranous fractions were collected and combined as the interphase egg extract. The extracts were supplemented with 40 μg/ml cycloheximide, 60 mM creatine phosphate, 150 μg/ml creatine phosphokinase and 4% glycerol and were then frozen and stored under liquid nitrogen. To prepare HSS, the stored extract was thawed, centrifuged at 265 000*g* for 10 min and the cytosolic fraction was collected.

To prepare demembranated sperm nuclei, mature male *Xenopus* were anesthetized and their testes were collected and immersed in buffer C-0.2 (80 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.2 M sucrose, and 10 mM Hepes-KOH at pH 7.5). The testes were then minced with a loose-fit homogenizer and debris was removed by centrifugation at 180*g* for 2 min followed by centrifugation twice at 260*g* for 2 min each. Sperm were collected by centrifugation at 2900*g* for 10 min. The sperm pellet was suspended in buffer C-2.0 (buffer C-0.2 with 2 M sucrose) and underlayed with buffer C-2.3 (buffer C-0.2 with 2.3 M sucrose) and buffer C-2.5 (buffer C-0.2 with 2.5 M sucrose). Lipids and red blood cells were removed by spinning the sperm suspension through the underlayers by centrifugation at 89 000*g* for 20 min. The resultant sperm pellet was then suspended in buffer C-0.2, centrifuged at 2600*g* for 15 min, and then suspended in buffer S (1 mM EDTA, 250 mM sucrose, 75 mM NaCl, 0.5 mM spermidine, 0.15 mM spermin, and 10 mM Hepes-KOH at pH 7.5). Sperm was demembranated by incubation in buffer S containing 0.5 mg/ml lysolecithine at 20ºC for 5–10 min. The reaction was then stopped by placing the samples on ice and adding an equal volume of buffer S containing 3% BSA. The sperm chromatin was collected by centrifugation at 1,400*g* for 5 min and then washed twice with buffer S containing 0.3% BSA. The sperm chromatin was suspended in buffer S containing 30% glycerol, 1 mM dithiothreitol, 10 μg/ml aprotinine and 15 μg/ml leupeptine, and was then frozen by liquid nitrogen and stored at −80ºC.

NPE was prepared as described previously (Walter *et al.* 1998).

Isolation of chromatin and the chromatin transfer assay

To isolate the chromatin and nuclear fractions, sperm chromatin (2500 nuclei/μl) was incubated with extracts of *Xenopus* eggs for the times and temperatures indicated in the figure legends. Reactions were stopped by diluting the samples with a 10-fold volume of ice-chilled

XB (100 mM KCl, 2.5 mM $MgCl_2$ and 50 mM HEPES-KOH, pH 7.5) containing 0.25% **(v/v)** Nonidet-P 40 (NP40) and then centrifuging them through dilution buffer containing 10% sucrose at 2200 × *g* for 5 min at 4°C. The pellets were washed with XB and subjected to SDS-PAGE. For the chromatin transfer assay, the chromatin fractions were isolated as above except the buffers contained 0.01% NP40, and the pellets were suspended in the appropriate egg extract. To isolate chromatin fractions assembled with the HSS, chromatin fractions were pelleted by centrifugation at 8700 × *g* for 5 min at 4°C.

Immunodepletion and immunoprecipitation

For immunodepletion, the appropriate volume of antiserum was incubated with rProtein A Sepharose Fast Flow (Amersham Bioscience) beads with constant rotation (~ 9 rpm) for 60 min at 4ºC. Antibodies bound to the beads were recovered and washed three times with XB. Egg extract was treated several times at 4ºC with 1/4 volume of the beads bound to the specific antibodies as follows: (1) For depletion of Cdt1, the egg extract was treated three times for 30 min with anti-Cdt1 antibody; (2) For depletion of both Cdt1 and geminin, the egg extract was treated four times for 20 min each with anti-Cdt1 antibody (1^{st}), anti-Cdt1 and anti-geminin antibodies (2^{nd} and 3^{rd}), and anti-geminin antibody (4^{th}); (3) For depletion of both Cdt1 and Cdc6, the egg extract was treated three times each for 30 min with anti-Cdt1 and anti-Cdc6 antibodies; (4) For depletion of Orc2 the egg extract was treated four times for 20 min with anti-Orc2 antibody; (5) For depletion of Mcm2 and Cdt1, the egg extract was treated five times each for 20 min with anti-Mcm2 antibody (1^{st}, 2^{nd} and 3^{rd}), and anti-Cdt1 and anti-Mcm2 antibodies (4^{th} and 5^{th}).

For immunoprecipitation, antibodies were chemically coupled to rProtein A Sepharose Fast Flow (Amersham Bioscience) beads. The antibody-bound beads were washed with 0.2 M sodium borate at pH 9.0, mixed with 10-fold the bead volume of 5 mg/ml DMP in 0.2 M sodium borate at pH 9.0, and then incubated for 30 min at 20ºC. The coupling reaction was stopped by adding an equal volume of 0.2 M ethanolamine, and the beads were washed once with 0.2 M ethanolamine. The beads were washed a further three times with XB. For each immunoprecipitaion experiment, 5 µl of the beads were mixed with 20 µl of egg extract and incubated for 30 min at 4ºC. Proteins bound to the beads were recovered and washed three times with XB containing 0.25% Nonidet P-40 (NP40) and then washed once with XB. The washed beads were suspended in SDS-PAGE sample buffer. Samples were filtered through PVDF membrane (pore size 0.45 µm) to remove the beads. The filtered samples were supplemented with 2-mercaptoethanol (final concentration 4%), heated at 100ºC for 4 min and then subjected to SDS-PAGE.

Immunoblotting and Signal Quantification

After SDS-PAGE, proteins were electrophoretically transferred from a polyacrylamide gel to a nitrocellulose membrane. A gel sandwich was prepared with an anode metal plate at the bottom followed by two sheets of No. 1 filter paper soaked with transfer buffer A (0.3 M Tris,

10% methanol), a sheet of filter paper soaked with transfer buffer B (25 mM Tris, 10% methanol), and nitrocellulose membrane soaked with the transfer buffer B. The PAGE gel was washed once with transfer buffer B and then laid on top of the nitrocellulose membrane followed by three sheets of filter paper soaked with transfer buffer C (25 mM Tris, 60 mM 6-aminicaprone acid, and 0.01% SDS), and the cathode metal plate was placed on top. Electrophoretic transfer was carried out at 4.5 mA/cm^2 for 60 min. The membrane was then washed with TTBS (0.9% NaCl, 0.1% Tween-20, and 100 mM Tris-HCl at pH 7.5) and nonspecific binding sites were blocked by a 1 h incubation in TTBS containing 7.5% skim milk. The membrane was then incubated overnight with the primary antibodies at the appropriate dilutions in TTBS containing 7.5% skim milk at 4ºC. After washing the membrane with TTBS, the membrane was incubated with the peroxidase-conjugated secondary antibody in TTBS containing 7.5% skim milk for 1 h at room temperature. Immunoreactivities of blotted proteins were detected with Konica Immunostain HRP (Konica Minolta, Japan), and the development of each reaction was stopped before reaching saturation. Immunostained membranes were scanned and the images were analyzed by using ImageJ software (NIH). Care was taken to ensure that the signals obtained were within the linear range of detection.

Immunofluorescence

Sperm chromatin (2500 nuclei/µl) was incubated in 10 µl of egg extract for indicated times at 23°C. The reactions were stopped and chromatin was fixed by adding 100 µl of 3.7% formaldehyde in XB containing 0.25% NP40 and incubating for 10 min at 20°C. Fixation was stopped by adding 1 ml XB, and the fixed nuclei or chromatin fractions were attached to a poly-lysine coated coverslip by centrifugation at 1500 rpm for 5 min (RS-4 rotor, KUBOTA, Tokyo, Japan) through a layer of 30% sucrose in XB. The amounts of Cdt1 in nuclei were quantified as fluorescence intensities collected at identical settings on a cooled CCD camera (DP71, OLYMPUS, Tokyo, Japan). Fluorescence images of Hoechst staining were taken to determine the area of each nucleus. The average intensity per pixel of each nucleus was subtracted from the background level, and total pixel intensities of Cdt1 fluorescence signals per nucleus were estimated with ImageJ software. To count the number of Cdt1 foci, I selected foci with signal intensities at peak positions higher than 1.3-fold of those obtained for surrounding regions. A typical image of selected foci is shown in Figs. 8E and F.

Pull-down assay

Fourty micro litter of egg extract was mixed with 10 µl Glutathione Sepharose^{TM} 4B beads (GE healthcare) and incubated for 30 min at 4 ºC. Proteins bound to the beads were recovered and washed three times with XB containing 0.25% Nonidet P-40 (NP40) and then washed once with XB. The washed beads were suspended in SDS-PAGE sample buffer with 2-mercaptoethanol (final concentration 4%) and then subjected to SDS-PAGE.

Dynamic molecular combing

Dynamic molecular combing was performed as described previously (Sugimura *et al.* 2008), with several modifications as follows. Sperm chromatin was (2500 nuclei/μl) incubated in the extract containing 20 μM Digoxigenin-11-dUTP (Roche). Reactions were stopped by diluting the samples with a 10-fold volume of ice-chilled XB, and then centrifuging them through XB containing 10% sucrose 2000*g* for 5 min at 4ºC. The pellets were washed with XB and suspended into 0.7% low melting point agarose (Sigma) in XB. DNA was stained with 3.3 mM YOYO-1 (Invitrogen) at 25°C for 1 h and combed onto the silanized coverslips (Matsunami Glass). Combed DNA molecules were incubated with 4μg/ml Anti-Digoxigenin-Rhodamine Fab fragments (Roche). After washing with PBS containing 0.05% Tween 20 for 5 min three times, coverslips were mounted in VECTASHIELD (Vector Laboratories). To estimate the extension of DNA molecules, λ-DNA was stained with YOYO-1 and combed onto the coverslip. The length of extended λ-DNA (48.5 kbp) was measured as 19.4 ± 0.8 μm (average ± SD). The extension of DNA molecules is 2.52 ± 0.10 kbp/μm

Computational simulation of models

All computational simulations and data fitting (except for Fig. 16) were carried out using Mathematica software (Wolfram Research Inc., IL). The model for IOC in Fig. 16 was implemented (using C language) based on mass-action law–dependent stochastic kinetics of licensing for 10^4 origins. The detailed derivation of these models is described in Appendix.

Appendix: Model Construction

1. Basic Assumptions

The following section describes a simplified model for licensing and its inhibition by geminin. This model illustrates some of basic principles that govern the behavior of the overall system. I define the three states of the complex for licensing that forms on the chromatin (Fig. 11). The first is the "Open" complex that corresponds to the ORC-Cdc6 complex, the second is the "Competent" complex that corresponds to the ORC-Cdc6-Cdt1 complex, and the third is the "Incompetent" complex that corresponds to the ORC-Cdc6-Cdt1-geminin complex. The two assumptions I make for this model are as follows: 1) the licensing efficiency measured as the amount of Mcm2-7 loaded on chromatin increases proportionally to the increase in the amount of "Competent" complex on the chromatin, 2) the conversion rate between the states is faster than the rate of Mcm2-7 loading. These assumptions permit us to simplify the scheme without considering the Mcm2-7 loading process and take the steady-state amount of the Competent complex as an index of licensing efficiency. The total number of complexes is constant and each complex takes one of three states, therefore,

$$Open + Competent + Incompetent = total\ number\ of\ complexes \quad (1)$$

$$Competent_{ss} \sim the\ amount\ of\ Mcm2\text{-}7\ on\ chromatin \quad (2)$$

where *Open*, *Competent,* and *Incompetent* represent the number of complexes in each state and $Competent_{ss}$ is the steady state amount of the Competent complex. I consider the proportion of complexes at each state in the total number of complexes, which can be written as

$$[Open] = Open \,/\, total\ number\ of\ complexes \quad (3)$$

$$[Competent] = Competent \,/\, total\ number\ of\ complex \quad (4)$$

$$[Incompetent] = Incompetent \,/\, total\ number\ of\ complexes \quad (5)$$

and equation (1) is rewritten as

$$[Open] + [Competent] + [Incompetent] = 1 \quad (6)$$

The conversion rate of each process in the scheme follows the law of mass action and can be expressed as the following ordinary differential equation,

$$\frac{d[Open]}{dt} = -k_1[Cdt1]^a[Open] + k_2[Competent] \quad (7)$$

$$\frac{d[Competent]}{dt} = k_1[Cdt1]^a[Open] + k_4[Cdt1]^c[Incompetent] - k_2[Competent] - k_3[geminin]^b[Competent] \quad (8)$$

$$\frac{d[Incompetent]}{dt} = k_3[geminin]^b[Competent] - k_4[Cdt1]^c[Incompetent] \quad (9)$$

where k_1–k_4 are the rate constants, and *a, b,* and *c* are the Hill constants for the action of Cdt1 and geminin at each reaction step. [*Cdt1*] and [*geminin*] are taken as the total concentration of each protein in the nM scale. Under the experimental conditions used here the amount of Cdt1 or geminin bound to chromatin is negligible. This is supported by my finding that a five-fold dilution of sperm in the extracts did not alter the switch-like inhibition of licensing by geminin or any other reaction dynamics (data not shown). In the present and the following sections 2 and 3, every term is dimensionless.

2. Determination of the cooperativity of Cdt1 activity

In this section, I will determine the apparent cooperativity of Cdt1 action

In the absence of geminin the reaction kinetics can be represented by the following three equations,

$$[Open] + [Competent] = 1 \quad (10)$$

$$\frac{d[Open]}{dt} = -k_1[Cdt1]^a[Open] + k_2[Competent] \quad (11)$$

$$\frac{d[Competent]}{dt} = k_1[Cdt1]^a[Open] - k_2[Competent] \quad (12)$$

Using equation (10), equation (12) is rewritten as,

$$\frac{d[Competent]}{dt} = k_1[Cdt1]^a(1 - [Competent]) - k_2[Competent] \quad (13)$$

At steady state, [*Competent*] was solved by setting $d[Competent]/dt = 0$, resulting in

$$[Competent]_{ss} = \frac{[Cdt1]^a}{K_1 + [Cdt1]^a} \quad (14)$$

where $K_1 = k_2/k_1$. To evaluate K_1 and a, the licensing activity of Cdt1 was examined by adding various concentrations of recombinant Cdt1 back to Cdt1-depleted extracts (Fig. 12A). I found no remarkable cooperativity of Cdt1 action and the experimental results followed a simple hyperbolic function with $a = 1$ and $K_1 = 16.28$. It should be noted that licensing efficiency was shown as an arbitrary percentage by taking the value obtained with mock-depleted extracts as 100%; the mock-depleted extracts contain a finite concentration of Cdt1 (30–40nM). Thus, I designated licensing efficiency as 129% to be used as the scaling factor for data fitting at an infinite concentration of Cdt1.

Next, I considered reactivation of the incompetent complex by Cdt1. My major concern was the cooperation by Cdt1 in reactivating the incompetent complex (the value of c in the equation (8) and (9)). However, if licensing inhibition by wild-type geminin includes a feedback reaction, which will be discussed later, then, reactivation by Cdt1 will inevitably involve a nonlinear response. To avoid such complication, I used the mutant geminin lacking the switch-like activity for licensing inhibition. Though the mutant geminin retains more or less cooperative inhibitiory activity, I assume here that licensing inhibition by the mutant geminin follows a linear hyperbolic curve. Thus, the overall reaction kinetics can be expressed with equations (7) – (9) assuming $a = 1$, $b = 1$, $k_2/k_1 = 16.28$, and a constant concentration of geminin (750 nM). The steady-state concentration of the Competent complex is

$$[Competent]_{ss} = \frac{1}{1 + \frac{K_2[geminin]\big|_{=750}}{[Cdt1]^c} + \frac{16.28}{[Cdt1]}} \qquad (15)$$

where $K_2 = k_3/k_4$.

To evaluate the cooperative activity of Cdt1 in the reactivation of the Incompetent complex, I examined licensing activity in the presence of various concentrations of recombinant Cdt1 and 750 nM mutant geminin in Cdt1-depleted egg extracts (Fig. 12B). The experimental data is readily simulated by assuming $c = 1$ and $k_3/k_4 = 0.143$, but it is difficult to fit data assuming $c = 2$. Hence, for Cdt1, it is not necessary to assume any cooperative activity for simulating the experimental data. Ignoring the cooperative effect of Cdt1 action, I can simplify the kinetic reactions as follows,

$$\frac{d[Open]}{dt} = -k_1[Cdt1][Open] + k_2[Competent] \qquad (16)$$

$$\frac{d[Competent]}{dt} = k_1[Cdt1][Open] + k_4[Cdt1][Incompetent] - k_2[Competent] - k_3[geminin]^b[Competent] \qquad (17)$$

$$\frac{d[Incompetent]}{dt} = k_3[geminin]^b[Competent] - k_4[Cdt1][Incompetent] \quad (18)$$

3. Building a switch-like response of licensing activity to geminin

In this section, I explore what types of geminin action could generate the all-or-none inhibition of licensing. Then I compare experimentally-obtained feature of licensing control by Cdt1 and geminin with that obtained from each model. I consider the following four situations 1) without assuming non-linear activity of geminin action (Hyperbolic model), 2) assuming multi-step association of Cdt1 and geminin in solution (Competition model), 3) assuming highly cooperative effect of geminin action at individual origins (Ultrasensitive model) and 4) assuming positive-feedback effect of geminin action (Feedback model). Parameters and initial conditions used for simulations show in this section are summarized in Table S1.

3.1 Hyperbolic model

I began by analyzing the behavior of the model describing above eqs. (16)-(18) without assuming non-linear activity of Cdt1 and geminin (b=1). Under this condition, a hyperbolic response curve is obtained to a graded increase in both geminin and Cdt1 (Fig. 13A), and thus all-or-none response curve is not generated with the increase of geminin stimulus.

For simulating the two dimensional input function of licensing activity according to the Hyperbolic model, I used equations (16) – (18) with following parameters and initial conditions:

(parameters) k_1=1, k_2=3.4, k_3=1, k_4=1.5, b=1,

(initial conditions) $[Open]_{t=0}$=1, $[Competent]_{t=0}$=0, $[Incompetent]_{t=0}$=0.

The parameters are selected to fit the Cdt1 activity of the experimental results shown in Fig. 15A in the absence of geminin, and to simulate approximate 50% inhibition of licensing at 30-40 nM geminin in the presence of 20 nM Cdt1 (see Table S1). In this section and following section 3.2-3.4, I designated the maximum licensing efficiency as 107% in order to fit the obtained data at an infinite concentration of Cdt1 without geminin.

3.2 Competition model

To explain the all-or-none licensing inhibition as observed in Fig. 1A, I next assume multi-steps association of geminin to Cdt1 based on the finding reported by Mechali and coworkers (Lutzmann *et al.* 2006). I apply the following assumptions:

1. Cdt1 forms Cdt1-1×geminin (Cdt1:geminin ratio is 1:1), Cdt1-2×geminin (1:2), Cdt1-3×geminin (1:3) and Cdt1-4×geminin (1:4) complexes.
2. Only Cdt1-4×geminin complex can convert Competent state into Incompetent state.
3. The other forms of geminin can stimulate the formation of Competent state complex as well as free Cdt1.

Thus, the equations of this model are represented as follows:

$$\frac{d[Cdt1]}{dt} = -k_1[Cdt1][geminin] + k_2[Cdt1\text{–}_{1\times}geminin] \quad (19)$$

$$\begin{aligned}\frac{d[geminin]}{dt} &= -k_1[Cdt1][geminin] + k_2[Cdt1\text{–}_{1\times}geminin] \\ &- k_3[Cdt1\text{–}_{1\times}geminin][geminin] + k_4[Cdt1\text{–}_{2\times}geminin] \\ &- k_5[Cdt1\text{–}_{2\times}geminin][geminin] + k_6[Cdt1\text{–}_{3\times}geminin] \\ &- k_7[Cdt1\text{–}_{3\times}geminin][geminin] + k_8[Cdt1\text{–}_{4\times}geminin]\end{aligned} \quad (20)$$

$$\begin{aligned}\frac{d[Cdt1\text{–}_{1\times}geminin]}{dt} &= k_1[Cdt1][geminin] - k_2[Cdt1\text{–}_{1\times}geminin] \\ &- k_3[Cdt1\text{–}_{1\times}geminin][geminin] + k_4[Cdt1\text{–}_{2\times}geminin]\end{aligned} \quad (21)$$

$$\begin{aligned}\frac{d[Cdt1\text{–}_{2\times}geminin]}{dt} &= k_3[Cdt1\text{–}_{1\times}geminin][geminin] - k_4[Cdt1\text{–}_{2\times}geminin] \\ &- k_5[Cdt1\text{–}_{2\times}geminin][geminin] + k_6[Cdt1\text{–}_{3\times}geminin]\end{aligned} \quad (22)$$

$$\begin{aligned}\frac{d[Cdt1\text{–}_{3\times}geminin]}{dt} &= k_5[Cdt1\text{–}_{2\times}geminin][geminin] - k_6[Cdt1\text{–}_{3\times}geminin] \\ &- k_7[Cdt1\text{–}_{3\times}geminin][geminin] + k_8[Cdt1\text{–}_{4\times}geminin]\end{aligned} \quad (23)$$

$$\frac{d[Cdt1\text{–}_{4\times}geminin]}{dt} = k_7[Cdt1\text{–}_{3\times}geminin][geminin] - k_8[Cdt1\text{–}_{4\times}geminin] \quad (24)$$

$$\begin{aligned}\frac{d[Open]}{dt} &= -k_9([Cdt1] + [Cdt1\text{–}_{1\times}geminin] + [Cdt1\text{–}_{2\times}geminin] + [Cdt1\text{–}_{3\times}geminin])[Open] \\ &+ k_{10}[Competent]\end{aligned} \quad (25)$$

$$\begin{aligned}\frac{d[Competent]}{dt} &= k_9([Cdt1] + [Cdt1\text{–}_{1\times}geminin] + [Cdt1\text{–}_{2\times}geminin] + [Cdt1\text{–}_{3\times}geminin])[Open] \\ &+ k_{12}([Cdt1] + [Cdt1\text{–}_{1\times}geminin] + [Cdt1\text{–}_{2\times}geminin] + [Cdt1\text{–}_{3\times}geminin])[Incompetent] \\ &- k_{10}[Competent] - k_{11}[Cdt1\text{–}_{4\times}geminin]^b[Competent]\end{aligned} \quad (26)$$

$$\begin{aligned}\frac{d[Incompetent]}{dt} &= k_{11}[Cdt1\text{–}_{4\times}geminin]^b[Competent] \\ &- k_{12}([Cdt1] + [Cdt1\text{–}_{1\times}geminin] + [Cdt1\text{–}_{2\times}geminin] + [Cdt1\text{–}_{3\times}geminin])[Incompetent]\end{aligned} \quad (27)$$

where k_1–k_{12} take constant values.

As shown in Fig. 13D, assuming multiple forms of Cdt1-geminin complex that are permissive for licensing, competition model could generate an all-or-none licensing inhibition. For simulating the 2D-input function of licensing activity according to the Competition model, I used equations (19) – (27) with following parameters and initial

conditions:

(parameters) k_1=1×10^5, k_2=1, k_3=1×10^5, k_4=1, k_5=1×10^5, k_6=1, k_7=1×10^2, k_8=1, k_9=1, k_{10}=3.4, k_{11}=1, k_{12}=1.5, b=1

(initial conditions) [*Open*]$_{t=0}$=1, [*Competent*]$_{t=0}$=0, [*Incompetent*]$_{t=0}$=0.

The condition for generating the all-or-none response is that Cdt1-1×geminin, Cdt1-2×geminin and Cdt1-3×geminin are stably formed ($k_1/k_2 >> 1$, $k_3/k_4 >> 1$, $k_5/k_6 >> 1$), and Cdt1-4×geminin is also stably formed but less than the other three forms (k_1/k_2, k_3/k_4, $k_5/k_6 > k_7/k_8 >> 1$). Under these condition, majority of Cdt1-4×geminin complexes are only formed after when most of the Cdt1 are converted into Cdt1-3×geminin complex. Therefore, if there are 10 nM Cdt1 in the egg extract, Cdt1 can neutralize the inhibitory effect of upto 30 nM geminin. When geminin concentration exceeds over 30 nM, the inhibitory effect of geminin appears. In this case, the threshold concentration will change almost proportionally with Cdt1 concentrations.

3.3 Ultrasensitive model

In this and next subsections, I introduce the non-linear functions to the geminin-dependent pathway in order to simulate the switch-like inhibition of licensing.

In the Ultrasensitive model (Fig. 13B), Hill constant b higher than unity is introduced. To simulate the steepness of response as a function of [*geminin*] a high value of b would be required (b > ~8, see Fig. 6B). In the ordinary interaction between Cdt1 and geminin, a highly cooperative interaction is hard to be considered, thus, I assume that additional as-yet-unidentified mechanisms, such as coupled enzymatic reactions, are involved in the switch-like inhibition of licensing by geminin.

For simulating the 2D-input function of licensing activity according to the cooperative model, I used equations (16) – (18) with following parameters and initial conditions:

(parameters) k_1=1, k_2=3.4, k_3=6x10^{-12}, k_4=1, b=8,

(initial conditions) [*Open*]$_{t=0}$=1, [*Competent*]$_{t=0}$=0, [*Incompetent*]$_{t=0}$=0.

Those parameters were selected to simulate the experimental data of licensing activity in the absence of geminin (geminin: 0 nM, Cdt1: 5–80 nM) as well as licensing inhibition in the presence of a physiological concentration of Cdt1 (geminin: 0–80 nM, Cdt1: 20nM) (see Table S1). Fig. 13B shows that the Ultrasensitive model failed to generate a 2D-input function similar to the experimental results. Instead, this model predicts switch-like inhibition at essentially the same threshold concentration of geminin regardless of the concentration of Cdt1.

3.4 Feedback model

In the Feedback model (Fig. 13C), an all-or-none response is generated by a feedback pathway where the Incompetent complex stimulates the inhibitory reaction by converting the complex from a Competent state into an Incompetent state. In order to simulate a switch-like response by geminin, I introduce a new term to include positive feedback with cooperative

inhibition. Therefore, the equations can be rewritten as follows,

$$\frac{d[Open]}{dt} = -k_1[Cdt1][Open] + k_2[Competent] \tag{16}$$

$$\frac{d[Competent]}{dt} = k_1[Cdt1][Open] + k_4[Cdt1][Incompetent] - k_2[Competent] - k_3[geminin]^b[Competent] - \frac{k_5[Incompetent]^d}{l + [Incompetent]^d}[Competent] \tag{28}$$

$$\frac{d[Incompetent]}{dt} = k_3[geminin]^b[Competent] - k_4[Cdt1][Incompetent] \tag{18}$$

where the feedback function is given by the fifth term of equation (28) where k_5, l and d are constant values. For simulation of experimental data, I select the following parameters and initial conditions:

(parameters) k_1=1, k_2=3.4, k_3=0.006, k_4=1, k_5=250, d=4.9, b=2.2, l=0.65,

(initial conditions) $[Open]_{t=0}$=1, $[Competent]_{t=0}$=0, $[Incompetent]_{t=0}$=0.

To precisely fit the simulation of the 2D-input function over the entire range of given Cdt1 and geminin concentrations, I should introduce relatively low cooperativity for inhibitory geminin binding (b = 2.2).

Since the term for positive feedback in equation (28) depends only on the ratio of Competent to Incompetent complexes, and not on the concentration of Cdt1 and geminin, the overall contribution of this term to the behavior of geminin inhibition becomes relatively small in the presence of higher concentrations of Cdt1 and geminin. Thus, a switch-like response becomes gradual in the presence of higher concentrations of Cdt1 and geminin (Fig. 13C).

By comparing the Feedback, Ultrasenstive and the Competition models, I find that the feedback-based model explains the salient features of the 2D-input function for licensing in egg extracts (summarized in Table 1).

4. Inter-Origin Cooperativity (IOC) model

To simulate licensing of multiple origins on chromatin with the cooperative interaction that was predicted by Fig. 13C, here we represent each origin by two states, Incompetent "*I*" and Competent "*C*", respectively. The Open state is not considered because its fraction is negligibly small at endogenous Cdt1 concentration (>20nM; see Figs. 3A and B). As shown in Fig. 16A, switch kinetics between the two states is given by

$$C_n \underset{[Cdt1]}{\overset{k_n[\text{geminin}]}{\rightleftarrows}} I_n, \tag{29}$$

where n denotes index of each origin $\left(1 \le n \le N,\ N = 10^4\right)$. Probability of the state transitions from I to C and from C to I are proportional to Cdt1 and geminin concentration, respectively.

In addition, the reaction rate k_n depends on states of the neighboring origins by

$$k_n = \begin{cases} k_{autonomous} + 2k_{cooperative} & (I_{n-1}, I_{n+1}) \\ k_{autonomous} + k_{cooperative} & (I_{n-1}, C_{n+1}),\ (C_{n-1}, I_{n+1}). \\ k_{autonomous} & (C_{n-1}, C_{n+1}) \end{cases} \quad (30)$$

Here the IOC is introduced by $k_{cooperative}$ = 100 (min^{-1}) for high IOC and $k_{cooperative}$ = 1 (min^{-1}) for low IOC, respectively, as increase of its rate when either of neighboring origins takes *I* state, whereas $k_{autonomous}$ = 0.1 (min^{-1}) is identical among the three models. For ultrasentivity without IOC, the switch from *C* to *I* depends nonlinearly on geminin concentration as

$$C_n \underset{[Cdt1]}{\overset{k_n[\text{geminin}]^b}{\rightleftarrows}} I_n, \quad (31)$$

where *b*=8 denotes Hill constants and $k_{cooperative}$ = 0 (min^{-1}). $k_{autonomous}$ = 1 (min^{-1}) was introduced to take the identical threshold geminin concentration with that of high IOC models, where fraction of competent origin reaches 50%. The transition parameters form *I* to *C* state are identical between the above four models, i.e., its reaction rate = 2 (min^{-1}) and [*Cdt1*] = 20 (nM). For simplicity, circular shape of chromatin was considered by periodic boundary condition $(C_0, I_0) = (C_N, I_N)$ and $(C_{N+1}, I_{N+1}) = (C_1, I_1)$. The presented results of the simulation in Fig. 16 were irrelevant to the boundary condition such as linear chromatin. Parameters and initial conditions used for IOC model are summarized in Table S1.

The time evolution of chemical reactions are generally calculated by stochastic processes depending on reaction probabilities (Morton-Firth & Bray 1998), known as StochSim method. Here we numerically carried out the transition probabilities dependent stochastic process in C language as follows: For each time step and each origin, the random number is generated in an interval [0,1] with homogeneous probability distribution. The switch from *C* to *I* states occurs, only when the origin took *C* state at the previous time step and the switch probability in equation (29) to *I*, i.e., k_n [*geminin*] is larger than the random number. Likewise, the switch from *I* to *C* occurs. The geminin concentration dependence of fraction of *C* states (Fig. 16B), and the inter-origin distances (Fig. 16D) are measured numerically using equations (29) and (30) after the systems has reached steady state.

Table S1 Parameters and initial conditions used for computational simulation

Section 3.1 Hyperbolic Model (equations 16 - 18)

Parameters		Values	Equation No.
k_1	Rate constant of transition from Open complex into Competent complex depending on Cdt1	$1^{\#1}$	(16), (17)
k_2	Transition rate from Competent complex into Open complex	$3.4^{\#1}$	(16), (17)
k_3	Transition rate from Competent complex into Incompetent complex depending on geminin	$1^{\#2}$	(17), (18)
k_4	Transition rate from Incompetent complex into Competent complex depending on Cdt1	$1.5^{\#2}$	(17), (18)
b	Hill constant of cooperativity of geminin action	1	(17), (18)
Initial conditions			
$[Open]_{t=0}$		1	
$[Competent]_{t=0}$		0	
$[Incompetent]_{t=0}$		0	

#1 k_2/k_1 has the relation of $k_2/k_1=(1-[Competent])[Cdt1]/[Competent]$ in the steady state of equations (16)-(18) in the absence of geminin. k_2/k_1 is estimated from Fig. 15A where $[Cdt1]$~4 nM gives approximate 50% licensing at $[geminin]$=0 nM.

#2 k_3/k_4 has the relation of $k_3/k_4=([Cdt1]-k_2/k_1[Competent]-[Competent][Cdt1])/([geminin]^b[Competent])$ in the steady state of equations (16)-(18). Taking k_2/k_1=3.4 and b=1, k_3/k_4 is estimated from Fig. 15A where $[geminin]$~ 30-40nM gives approximate 50% licensing at $[Cdt1]$=20 nM.

Section 3.2 Competition Model (equations 19 - 27)

Parameters		Values	Equation No.
k_1	Association rate of Cdt1 and geminin	$1x10^5$	(19), (20), (21)
k_2	Dissociation rate of Cdt1-$_{1x}$geminin complex into Cdt1 and geminin	1	(19), (20), (21)
k_3	Association rate of geminin and Cdt1-$_{1x}$geminin	$1x10^5$	(20), (21), (22)
k_4	Dissociation rate of Cdt1-$_{2x}$geminin complex into geminin and Cdt1-$_{1x}$geminin	1	(20), (21), (22)
k_5	Association rate of geminin and Cdt1-$_{2x}$geminin	$1x10^5$	(20), (22), (23)
k_6	Dissociation rate of Cdt1-$_{3x}$geminin complex into geminin and Cdt1-$_{2x}$geminin	1	(20), (22), (23)
k_7	Association rate of geminin and Cdt1-$_{3x}$geminin	$1x10^2$	(20), (23), (24)
k_8	Dissociation rate of Cdt1-$_{4x}$geminin complex into geminin and Cdt1-$_{3x}$geminin	1	(20), (23), (24)
k_9	Transition rate from Open complex into Competent complex depending on Active complex*1	$1^{\#3}$	(25), (26)
k_{10}	Transition rate from Competent complex into Open complex	$3.4^{\#3}$	(25), (26)
k_{11}	Transition rate from Competent complex into Incompetent complex depending on Inactive complex*2	1	(26), (27)
k_{12}	Transition rate from Incompetent complex into Competent complex depending on Active complex*1	1.5	(26), (27)
b	Hill constant of geminin action	1	(26), (27)
Initial conditions			
$[Cdt1]_{t=0}$		Cdt1	
$[geminin]_{t=0}$		geminin	
$[Cdt1\text{-}_{1x}geminin]_{t=0}$		0	
$[Cdt1\text{-}_{2x}geminin]_{t=0}$		0	
$[Cdt1\text{-}_{3x}geminin]_{t=0}$		0	
$[Cdt1\text{-}_{4x}geminin]_{t=0}$		0	
$[Open]_{t=0}$		1	
$[Competent]_{t=0}$		0	
$[Incompetent]_{t=0}$		0	

#3 k_2/k_1 has the relation of $k_2/k_1=(1-[Competent])[Cdt1]/[Competent]$ in the steady state of equations (19)-(27) in the absence of geminin. k_2/k_1 is estimated from Fig. 15A where $[Cdt1]$~4 nM gives approximate 50% licensing at $[geminin]$=0 nM.

*1 Active complex: Cdt1, Cdt1-$_{1x}$geminin, Cdt1-$_{2x}$geminin, Cdt1-$_{3x}$geminin

*2 Inactive complex: Cdt1-$_{4x}$geminin

Table S1 (continued) Parameters and initial conditions used for computational simulation

Section 3.3 Ultrasensitive Model (equations 16 - 18)

Parameters		Values	Equation No.
k_1	Transition rate from Open complex into Competent complex depending on Cdt1	$1^{\#4}$	(16), (17)
k_2	Transition rate from Competent complex into Open complex	$3.4^{\#4}$	(16), (17)
k_3	Transition rate from Competent complex into Incompetent complex depending on geminin	$6\times10^{-12\ \#5}$	(17), (18)
k_4	Transition rate from Incompetent complex into Competent complex depending on Cdt1	$1^{\#5}$	(17), (18)
b	Hill constant of geminin action	$8^{\#6}$	(17), (18)
Initial conditions			
$[Open]_{t=0}$		1	
$[Competent]_{t=0}$		0	
$[Incompetent]_{t=0}$		0	

#4 k_2/k_1 has the relation of $k_2/k_1=(1-[Competent])[Cdt1]/[Competent]$ in the steady state of equations (16)-(18) in the absence of geminin. k_2/k_1 is estimated from Fig. 15A where $[Cdt1]$~4 nM gives approximate 50% licensing at $[geminin]=0$ nM.

#5 k_3/k_4 has the relation of $k_3/k_4=([Cdt1]-k_2/k_1[Competent]-[Competent][Cdt1])/([geminin]^b[Competent])$ in the steady state of equations (16)-(18). Taking $k_2/k_1=3.4$ and $b=8$, k_3/k_4 is estimated from Fig. 15A where $[geminin]$~ 30-40nM gives approximate 50% licensing at $[Cdt1]=20$ nM.

#6 b is estimated from the Hill constant of the all-or-none licensing inhibition of geminin (Fig. 6A WT, 6B, Fig. 15A Cdt1=20 nM)

Section 3.4 Feedback Model (equations 16, 18, 28)

Parameters		Values	Equation No.
k_1	Transition rate from Open complex into Competent complex depending on Cdt1	$1^{\#7}$	(16), (28)
k_2	Transition rate from Competent complex into Open complex	$3.4^{\#7}$	(16), (28)
k_3	Transition rate from Competent complex into Incompetent complex depending on geminin	0.006	(18), (28)
k_4	Transition rate from Incompetent complex into Competent complex depending on Cdt1	1	(18), (28)
k_5	Rate constant of feedback effect	250	(28)
b	Hill constant of geminin action	2.2	(18), (28)
d	Cooperativity of feedback effect	4.9	(28)
l	Michaelis constant of feedback effect	0.65	(28)
Initial conditions			
$[Open]_{t=0}$		1	
$[Competent]_{t=0}$		0	
$[Incompetent]_{t=0}$		0	

#7 k_2/k_1 has the relation of $k_2/k_1=(1-[Competent])[Cdt1]/[Competent]$ in the steady state of equations (16), (18), (28) in the absence of geminin. k_2/k_1 is estimated from Fig. 15A where $[Cdt1]$~4 nM gives approximate 50% licensing in $[geminin]=0$ nM.

Table S1 (continued) Parameters and initial conditions used for computational simulation

Section 4 IOC Model (equations 29 - 31)

Parameters		Values	Equation No.
high IOC			
$k_{autonomous}$	Transition rate from Competent state to Incompetent state independing from the state of neighboring origins	0.1	(29), (30)
$k_{cooperative}$	Transition rate from Competent state to Incompetent state depending on the state of neighboring origins	100	(29), (30)
low IOC			
$k_{autonomous}$	Transition rate from Competent state to Incompetent state independing from the state of neighboring origins	0.1	(29), (30)
$k_{cooperative}$	Transition rate from Competent state to Incompetent state depending on the state of neighboring origins	1	(29), (30)
without IOC			
$k_{autonomous}$	Transition rate from Competent state to Incompetent state independing from the state of neighboring origins	0.1	(29), (30)
$k_{cooperative}$	Transition rate from Competent state to Incompetent state depending on the state of neighboring origins	0	(29), (30)
ultrasensitivity without IOC			
$k_{autonomous}$	Transition rate from Competent state to Incompetent state independing from the state of neighboring origins	1	(30), (31)
$k_{cooperative}$	Transition rate from Competent state to Incompetent state depending on the state of neighboring origins	0	(30), (31)
b	Hill constant of geminin action	$8^{\#8}$	(30), (31)

Initial conditions

For high IOC, low IOC, without IOC and ultrasensitivity without IOC conditions, all 10^4 origins take Competent "C".

#8 b is estimated from the Hill constant of the all-or-none licensing inhibition of geminin (Fig. 6A WT, 6B, Fig. 15A Cdt1=20 nM)

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Part II

Geminin establishes the separation of origin licensing and replication in the embryonic cell cycle

Abstract

The embryonic cell cycle, with its alternate S- and M-phases without a gap period, challenges the origin licensing system to provide strict insulation of origin licensing from initiation of DNA replication, at the M/S boundary. Here I report that geminin, but not Cdt1 proteolysis, is responsible for the insulation of licensing at the M/S boundary. I found the early emergence of licensing inhibition by geminin soon after nuclear formation, which is the earliest time at which DNA replication occurs. On the other hand, replication-coupled proteolysis in nuclei could not complete the destruction of Cdt1 prior to the initiation of replication. In addition, the expression-level of Cdt1 was almost constant in *Xenopus* early cleavage cycles, suggesting that Cdt1 proteolysis in nuclei is not efficient enough to reduce the amount of total Cdt1 in the embryo, where cytosol/nuclei ratio is high. Therefore, I propose that geminin is the dominant inhibitor of licensing both at the M/S boundary and after the completion of replication in *Xenopus* early embryo.

Introduction

Eukaryotic DNA replication initiates from numerous replication origins, and each origin can be fired only once in a single round of the cell cycle. To ensure the accuracy of this process, eukaryotic cells employ a two-step mechanism for the control of chromosomal replication (Bell and Dutta, 2002; Blow and Dutta, 2005; Diffley, 2004). The first step, which occurs at the end of the M to G1 phase, is the licensing of replication origins where the pre-replication complex (pre-RC) is assembled by the loading of Mcm2-7 onto the DNA in the presence of the origin recognition complex (ORC), Cdc6, and Cdt1. The second step is the activation of the pre-RC by the S-phase promoting kinases S-CDK and DDK at the onset of S-phase. Once the origin has been licensed, Mcm2-7 is stably associated with the origin, even in the absence of ORC, Cdc6, and Cdt1, until replication is initiated (Jares and Blow, 2000; Rowles et al., 1999). Licensing and initiation are considered irreversible processes because no mechanism can reverse DNA replication. Therefore, the mechanisms underlying the licensing of replication origins should be separated from those underlying the initiation of replication. Otherwise, any small overlap of licensing and replication would lead to relicensing of replicated regions of the chromosome and fatal rereplication of chromosomes.

The separation of licensing of replication origins from DNA replication is a critical requirement of the licensing control system (Arias and Walter, 2007; Bell and Dutta, 2002; Blow and Dutta, 2005; Diffley, 2004). In budding yeast, this separation is thought to be established by the temporal separation of licensing inhibition by G1-CDK and replication initiation by S-CDK. In this case, a definite time window insulates the end of the licensing phase from the start of the replication phase, thus safeguarding against the overlapping of the two events. By comparison, the embryonic cell cycle of metazoans exhibits a rapid oscillation of S and M phase without a gap phase. Two independent mechanisms have been identified for licensing inhibition in cell-free extracts of *Xenopus* laevis eggs, a model system of embryonic cell cycle. One inhibitory mechanism, Cdt1 degradation, depends on the formation of replication machinery. Replication-coupled degradation of Cdt1 has been intensively investigated and involves multi-step reactions triggered by the interaction of Cdt1 and PCNA on chromatin. In these reactions, Cul4-DDB1-Cdt2 ubiquitin ligase polyubiquinates Cdt1, which then undergoes proteolysis by the proteasome (Arias and Walter, 2005, 2006; Jin et al., 2006; Nishitani et al., 2004; Nishitani et al., 2006). Introducing mutations to Cdt1 that disrupt the PCNA-Cdt1 interaction is sufficient to stabilize the Cdt1 in the egg extracts (Arias and Walter, 2006). Thus, Cdt1 degradation is triggered only after the onset of the replication phase. The other inhibitory mechanism involves geminin, which directly binds to Cdt1 and inhibits its function in licensing. *Xenopus* geminin is inactivated at the exit of M-phase, and reactivated through its import into the reformed nucleus after mitotic exit (Hodgson et al., 2002; Li and Blow, 2004). Nuclear formation also triggers the initiation of replication via the accumulation of S-CDK inside the nucleus. In this scenario, geminin activation and replication initiation would show similar timing. Thus, both mechanisms would be difficult to provide an apparent temporal separation of licensing and initiation in the embryonic cell

cycle.

Altough the mechanism underlying the strict insulation of licensing from replication in embryonic cells is unclear, previous studies indicates that both of Cdt1 proteolysis and geminin systems play a major role for preventing re-replication. In *Xenopus* egg extracts, blocking Cdt1 proteolysis results in no detectable rereplication, whereas the depletion of geminin leads to inefficient but distinct rereplication (Kerns et al., 2007; Li and Blow, 2005; Yoshida et al., 2005). Simultaneous deregulation of both pathways leads to substantial rereplication, indicating a synergistic function of the two pathways in preventing rereplication (Arias and Walter, 2006; Li and Blow, 2005; Yoshida et al., 2005). The contribution of these pathways to the prevention of rereplication appears to depend on the experimental system. In C. elegans, knockdown of the Cul4 ortholog but not of geminin, leads to substantial rereplication (Kim and Kipreos, 2007; Yanagi et al., 2005; Zhong et al., 2003), whereas in Drosophila elimination of geminin is sufficient for robust rereplication (Mihaylov et al., 2002; Quinn et al., 2001). Interestingly, in mammalian cells, elimination of geminin leads to substantial rereplication depending on cell type. In HeLa cell, siRNA knockdown of Cdt2 or DDB1 results in an efficient re-replication (Jin et al., 2006; Lovejoy et al., 2006), whereas knockdown of geminin does not cause detectable re-replication (Kulartz and Knippers, 2004; Machida and Dutta, 2007; Nishitani et al., 2004). In contrast, siRNA knockdown of geminin cause substantial re-replication in several types of transformed cell lines and primary cells (Gonzalez et al., 2006; Melixetian et al., 2004; Mihaylov et al., 2002; Zhu et al., 2004; Zhu and Depamphilis, 2009; Zhu and Dutta, 2006), suggesting that the contribution of each pathway depends not only on genetic disparity but also on the distinct properties of different cell types in the same species. The reasons for such varied contributions of the two pathways in the prevention of rereplication are not clear, but they likely are related to differences in the mechanisms underlying Cdt1 inhibition.

Here I analyzed the contribution of replication-coupled Cdt1 proteolysis and geminin system for licensing inhibition at early S phase of *Xenopus* egg extract. The results suggest that not replication-dependent proteolysis but geminin accounts for the licensing inhibition at this phase. Interestingly, I also found that nuclei in the extract at the low nuclei/cytosol ratio similar to that in early embryo cannot promote efficient Cdt1 degradation enough to reduce the total amount of Cdt1 in the extract. These results suggest that geminin is a dominant inhibitor of licensing not only at the early replication phase but also after the completion of replication phase when replication-coupled Cdt1 proteolysis no longer operates. Therefore, the role of geminin system for preventing re-replication would be more important than that of Cdt1 proteolysis in early embryonic cell cycle, especially at the M/S boundary and after the completion of replication.

Results

To investigate the contribution of multiple mechanisms for preventing re-licensing at the early timing of replication phase in *Xenopus* embryonic cell cycle, I examined the contribution of known regulations of Cdt1 activity; 1) expression-level of Cdt1 and geminin, 2) proteolysis of Cdt1 in replicating nuclei and 3) licensing inhibition by geminin.

The expression levels of Cdt1 and geminin are not oscillates during initial cleavage cycles of *Xenopus* embryo

In human and other metazoan somatic cells, the expression of Cdt1 and geminin oscillates along with the cell cycle progression (McGarry and Kirschner, 1998; Nishitani et al., 2004; Sakaue-Sawano et al., 2008). However, those in *Xenopus* early embryo have not been well investigated. To clarify the expression Cdt1 and geminin during initial cleavage cycles of *Xenopus* embryos, I artificially fertilized the eggs of *Xenopus* and examined the changes in geminin and Cdt1 level during initial cleavage cycles. The fertilized embryos underwent well synchronized cleavage cycles, and most of the embryos were cleaved at similar timing. The embryos after the fertilization were collected at every five minutes and subjected to western blotting to analyze the amount of Cdt1 and geminin expressed in the embryos. In the embryos, the amounts of Cdt1 and geminin were almost constant during the initial cleavage cycles (Fig. 1A). This result shows that the control of expression-level of these proteins does not largely contributed to licensing transition in the frog embryo.

Cdt1 is degraded depending on replication in *Xenopus* egg extracts. Early embryo contains maternal mRNA which can be translated, whereas the transcription of embryonic mRNA is prohibited at the initial several cleavage cycles (Veenstra et al., 1999). Thus, the constant expression of Cdt1 in the embryo may be due to the translation of Cdt1, which compensates the degraded Cdt1. To test whether the synthesis of Cdt1 can counteract the degradation of Cdt1, I analyzed the amount of Cdt1 in cycling egg extracts (Fig. 1B). The cycling extracts do not contain nuclei, but are able to translate mRNA existing in the extracts. If the synthesis of Cdt1 compensates the replication-dependent Cdt1 degradation, Cdt1 should be accumulated in the cycling extract without nuclei. The activity of translation was confirmed by the oscillating activity of CDK, that is driven by the translation of cyclin. CDK activity elevated and reached its peak level at 90 min after the start of incubation (Fig. 1B upper panel). Then the activity became low and re-elevated, though the second round of CDK activation was weak. During this incubation, the amount of Cdt1 as well as geminin was almost constant in the extracts (Fig. 1B lower panel), though geminin seems to be degraded 4 hour after the incubation. Thus, translation of Cdt1, if any, would not be enough to compensate the replication-dependent degradation of Cdt1. Based on this result, the constant expression of Cdt1 in embryo suggests that replication-dependent degradation of Cdt1 will not be effective *in vivo* embryo.

Previous studies using egg extracts showed that the total amount of Cdt1 in the extracts is rapidly decreased in response to the replication of sperm nuclei. One significant difference

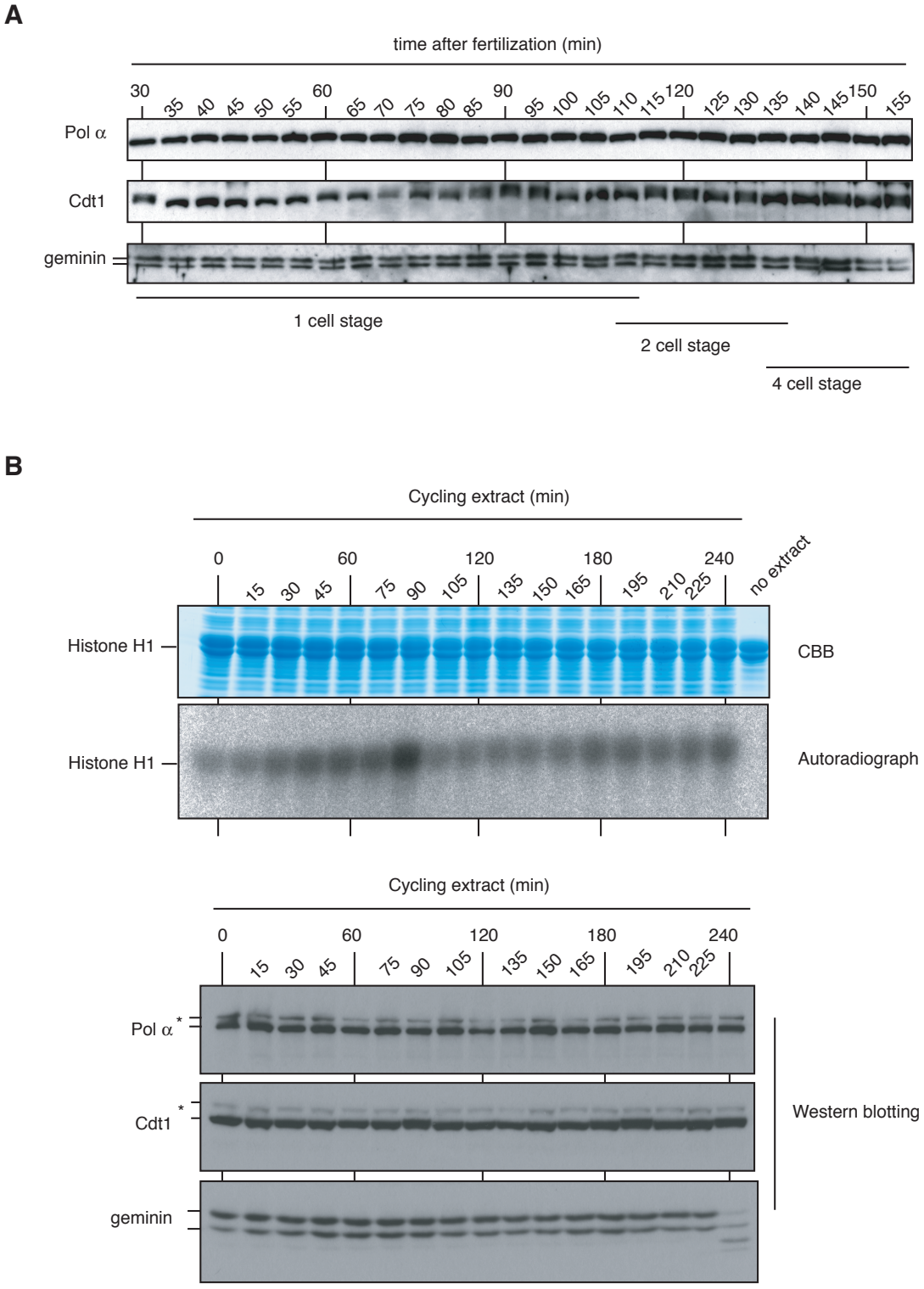

Fig. 1 Stability of Cdt1 and geminin proteins during early embryogenesis in *Xenopus* and cycling egg extract.

(A) In vitro fertilized eggs were collected at the indicated times and analyzed by Western blotting. Each lane contains a sample from one individual embryo. One representative result is shown from time course experiments that were repeated 10 times using eggs and sperm from five *Xenopus* males and five *Xenopus* females, respectively.

(B) Egg extracts without cycloheximide (cycling extract) were incubated at 23ºC and collected at indicated time. The collected extracts were divided into two, and subjected to histone H1 kinase assay (upper half) or western blotting (lower half). An asterisk indicates a non-specific band.

between typical experiments in the extracts and the situation in embryo is the ratio of the volume of nucleus to that of cytosol. To ask whether the different nucleus/cytosol ratio can account the observed constant level of Cdt1 in the embryo, I investigated the amount of Cdt1 in the egg extract, which was incubated in the presence of sperm nuclei at various nuclei/ cytosol ratios. In the presence of higher concentration of sperm nuclei, the amount of Cdt1 in the extract was decreased and this decrease was inhibited by the addition of CDK inhibitor p21 (Fig. 2). In contrast, nucleus supplied in the extracts at lower ratios including the ratio corresponding to the situation in the early embryo did not induce the effective reduction of Cdt1. This result indicates the minor contribution of Cdt1 degradation to control the total amount of Cdt1 in *Xenopus* early embryo.

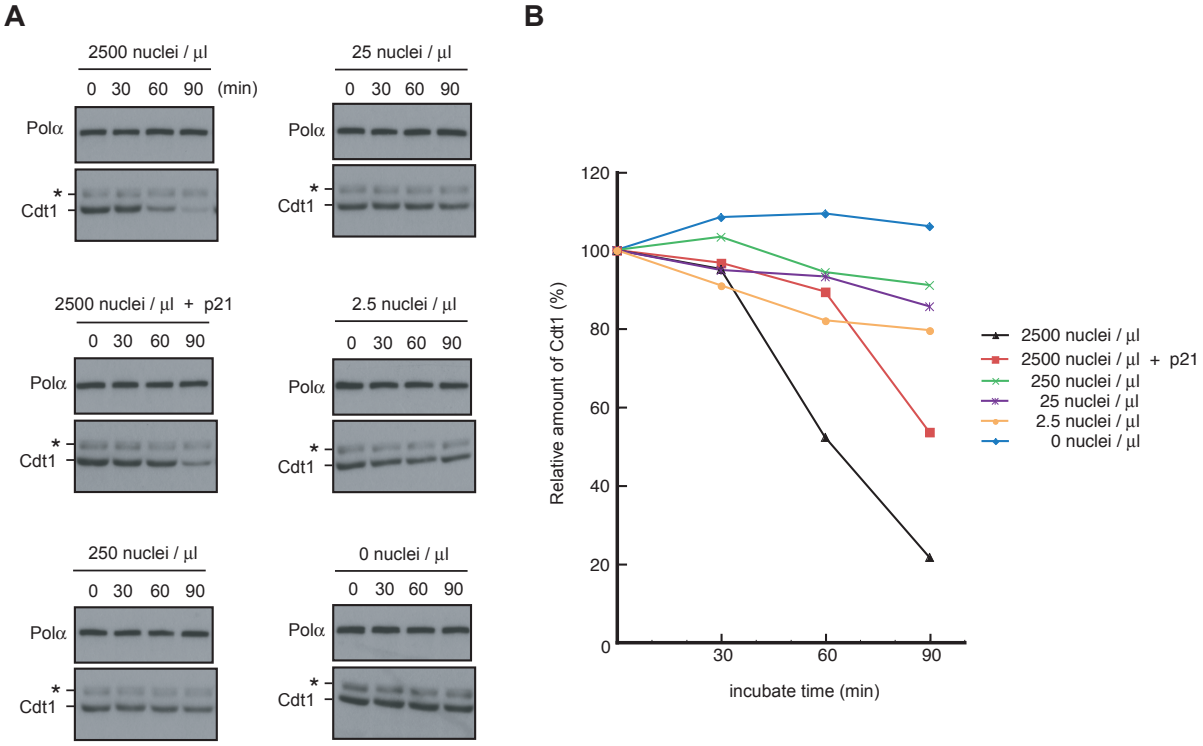

Figure 2. Nucleus/cytosol ratio-dependent decreasing of Cdt1 in the egg extract.
(A) Egg extracts were incubated at 23ºC with indicated amount of sperm chromatin at indicated concentrations in the absence or presence of 75 µg/ml p21. The extracts were collected at indicated time point. The extracts were then subjected to SDS-PAGE and western blotting. An asterisk indicates a non-specific band. Nucleus/ cytosol ratio at 2.5 nuclei / µl is assumed to correspond to the situation in early embryo, because one egg yields about 0.5 µl of egg extract.
(B) Cdt1 signals shown in (A) were quantified and normalized by taking the value obtained at 0 min as 100%.

Cdt1 proteolysis is not effective to inhibit the licensing at the early S phase.

Because replication-coupled Cdt1 degradation is occurred in nuclei, I re-evaluated the contribution of Cdt1 degradation to the insulation of licensing from the initiation of DNA replication by examining the amounts of Cdt1 in nuclei assembled in *Xenopus* egg extracts

(LSS). The nuclear envelope is formed around sperm chromatin within 20 min of the start of incubation, and DNA replication is initiated 20 to 30 min thereafter (Fig. 3A, left panel). In the extracts, CDK inhibitors suppressed replication activity (Fig. 3A, right panel) as well as the binding of DNA polymerase α and PCNA to the chromatin (Fig. 3B, right panel), whereas in the nuclear fractions they had little effect on the level of DNA polymerase α and PCNA (Fig. 3B, left panel). In the absence of CDK inhibitors, the amount of Cdt1 in isolated nuclei rapidly decreased with progression of DNA replication. In contrast, CDK inhibitors stabilized Cdt1 in isolated nuclei. By comparing the time-course of Cdt1 degradation and replication progression, I found that a significant amount of Cdt1 remained in the nuclear fractions when replication activity reached its maximum level, which was approximately 30 to 40 min after the start of chromatin incubation (Fig. 3C). Since the amount of Cdt1 detected by Western blot analysis of nuclear fractions represents the average amount of Cdt1 in the nuclei, I further examined the amount of Cdt1 in each individual nucleus compared with its replication activity (Fig. 3D). Again, I found that Cdt1 remains in almost all replicating nuclei 30 and 45 min after the start of incubation, though the average amount of Cdt1 in the nuclei decreased over the incubation period. These results confirm that the degradation of Cdt1, which depends on the initiation of DNA replication, would not be sufficiently fast to prevent relicensing at the early stage of DNA replication.

Geminin inhibits licensing at the onset of DNA replication

Endogenous geminin might inhibit relicensing at the early stage of DNA replication if the reactivation of geminin by nuclear import is sufficiently faster than the initiation of DNA replication. I therefore examined the timing of geminin activation in Cdt1-depleted LSS, which are incapable of supporting the licensing reaction and can become fully reactivated by the addition of recombinant Cdt1 (Fig. 4A and B). To detect early geminin activation, I compared the licensing activity of Cdt1-depleted and Cdt1-geminin double-depleted extracts upon addition of recombinant Cdt1. When Cdt1 was added at the start of incubation (0 min), the licensing activity, measured as the amount of Mcm2 bound to chromatin, was essentially the same in the presence and absence of endogenous geminin (Fig. 4C lanes 1 and 3). CDK inhibitors had no obvious effect (lane 2). On the other hand, Mcm2 loading onto chromatin was severely diminished when Cdt1 was added to the Cdt1-depleted extracts 20 min after the start of incubation, which is when nuclear formation is almost complete (Fig. 4C lane 5). The diminished Mcm2 loading was not rescued by inhibiting CDK activity but was fully recovered by depleting endogenous geminin (Fig. 4C lanes 5–7). When nuclear formation was prevented by using membrane-free extracts (HSS), the timing of addition of recombinant Cdt1 to the depleted extract did not affect licensing activity (Fig. 4C lanes 4 and 8). These results show that Cdt1 activity is suppressed soon after nuclear formation in the presence of endogenous geminin. In other words, endogenous geminin inhibited licensing activity soon after nuclear formation (at 20 min), which is the earliest time at which DNA replication occurs.

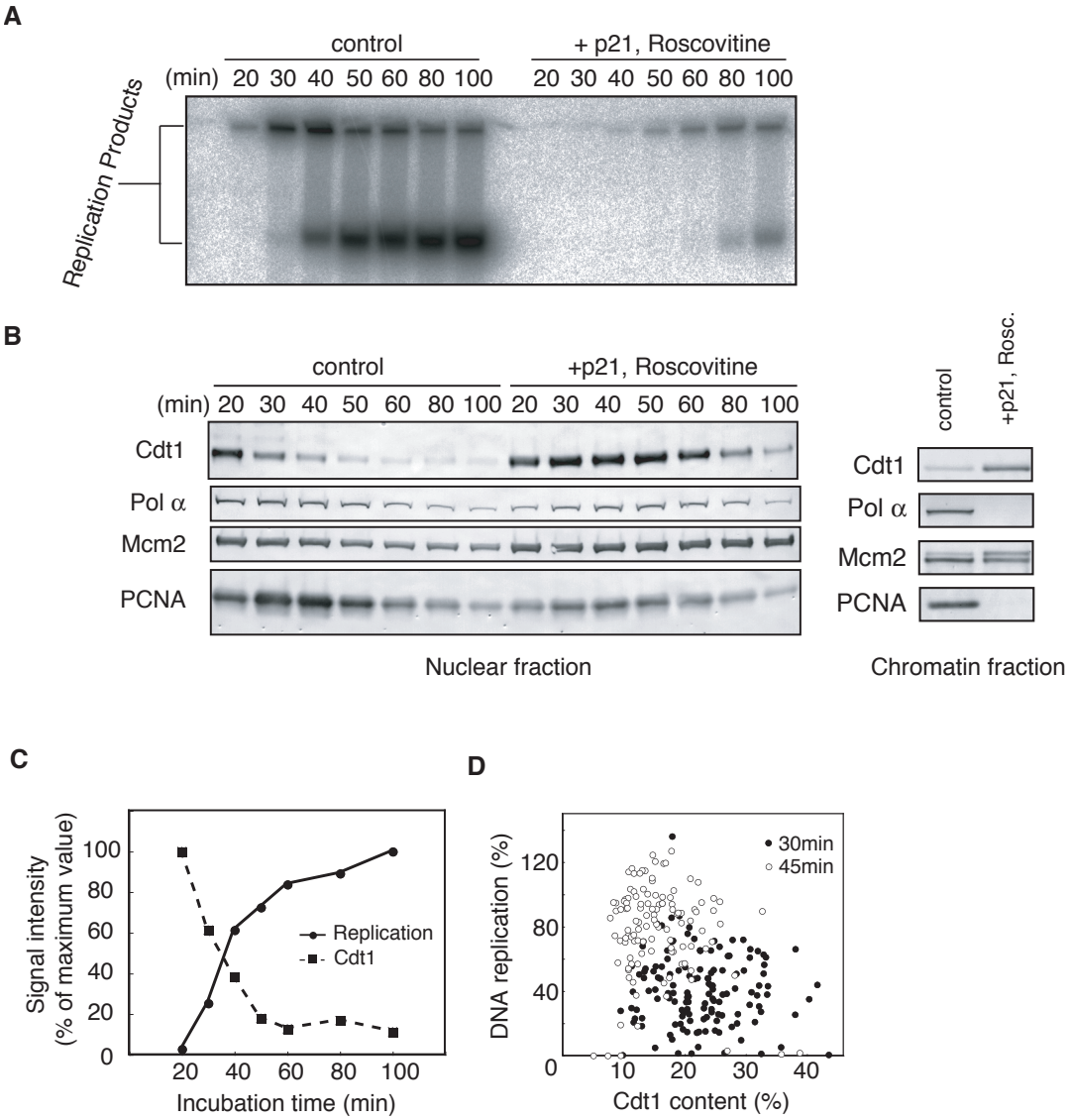

Figure 3. Cdt1 degradation at the onset of DNA replication.

(A) DNA replication in *Xenopus* egg extracts. Sperm chromatin was incubated in egg extracts containing [α-^{32}P] dATP with or without CDK inhibitors (50 μg/ml p21 and 100 μM roscovitine) for the indicated times. Replication products were separated by agarose gel electrophoresis and visualized by autoradiography.

(B) Analysis of Cdt1 in nuclear fractions and chromatin fractions. Nuclear fractions were isolated at the indicated times and analyzed by Western blotting. Chromatin fractions were isolated after 50 minutes incubation.

(C) Comparison of the time course for DNA replication activity with the Cdt1 content of nuclear fractions. Amounts of replication products shown in (A, +control) and Cdt1 in (B, +control) were quantified and plotted as a percentage of the maximum values versus the incubation time.

(D) Replication activity and Cdt1 degradation in individual nuclei. Sperm chromatin was incubated in egg extract with Cy3-labeled dCTP. Nuclei were fixed and immunostained with anti-Cdt1 antibody for the primary antibody and Alexa488-conjugated antibody for the secondary antibody. DNA was visualized by Hoechst 33342 staining to measure the area of nucleus, and the mean fluorescence intensity of Cy3 and Alexa488 signals for each nucleus were quantified. Data were taken from a representative experiment. DNA replication activity of each nucleus was represented by the integrated intensity of the Cy3 signal, which was calculated by setting as 100% the average value obtained after 60 min incubation. The concentration of Cdt1 in each nucleus was represented as the mean Cdt1 signal intensity, which was normalized by setting as 100% the average value after 30 min incubation in the presence of 50 μg/ml p21.

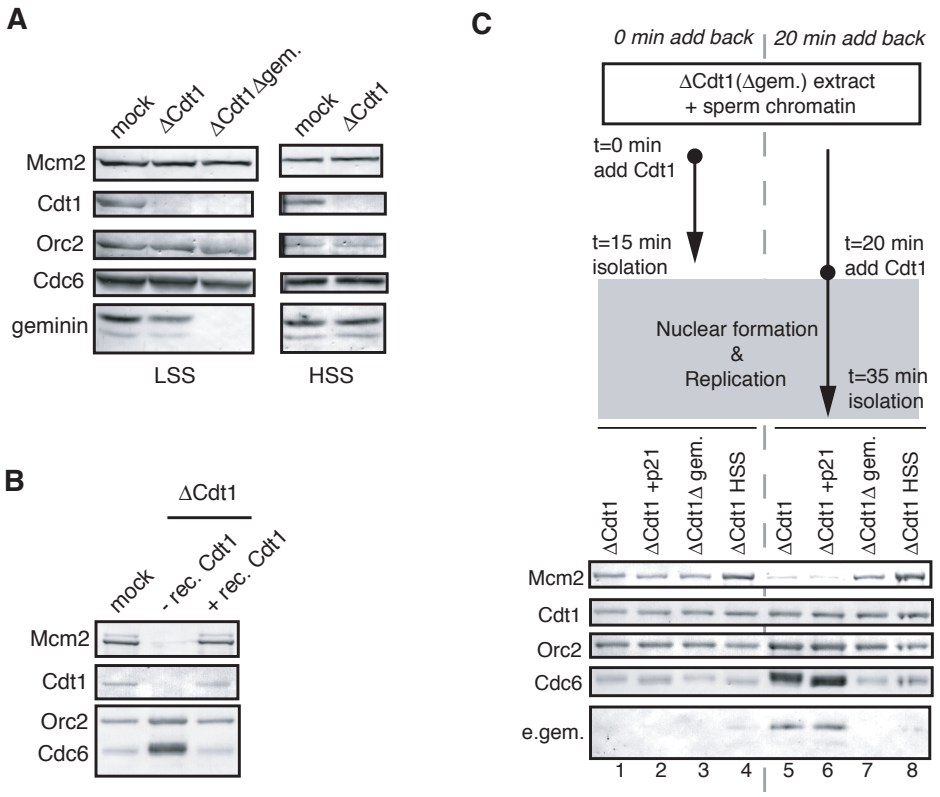

Fig. 4 Licensing inhibition by geminin at the earliest timing of replication phase.

(A) Immuno-depletion of Cdt1 and geminin. The low speed supernatant (LSS) and membrane-free high speed supernatant (HSS) of various depleted *Xenopus* egg extracts were analyzed by Western blotting with the antibodies indicated in the figure.

(B) Activity of recombinant Cdt1. Sperm chromatin was incubated in the mock-depleted or Cdt1-depleted extract with or without 20 nM recombinant Cdt1 (rec. Cdt1) for 20 min at 23ºC. Chromatin fractions were isolated and analyzed by Western blotting.

(C) Origin licensing in the presence of recombinant Cdt1 added before or after nuclear formation. The upper diagram shows the experimental design. Sperm chromatin was incubated in various extracts at 23ºC. All the extracts were interphase extract unless marked as HSS. Recombinant Cdt1 (5 nM) was added to the extract at 0 min (before nuclear formation) or 20 min (after nuclear formation) after the addition of sperm chromatin. Chromatin fractions were isolated 15 min after the addition of recombinant Cdt1 and were analyzed by Western blotting.

Cdt1 proteolysis is not effective after the completion of replication.

I next asked the efficiency of Cdt1 proteolysis and the behavior of geminin at later timing of the S phase. I speculated that replication-coupled Cdt1 proteolysis ends after replication was completed and replication machineries were dissociated from chromatin. Fig. 5A shows changes in the amount of Cdt1 in the egg extract incubated with sperm chromatin. Endogenous Cdt1 was decreased along with the incubation (Fig. 5A Control, 0-150 min). To monitor the activity of Cdt1 proteolysis at later timing of replication phase, I added recombinant Cdt1 at 150 min after the start of incubation, up until which timing DNA

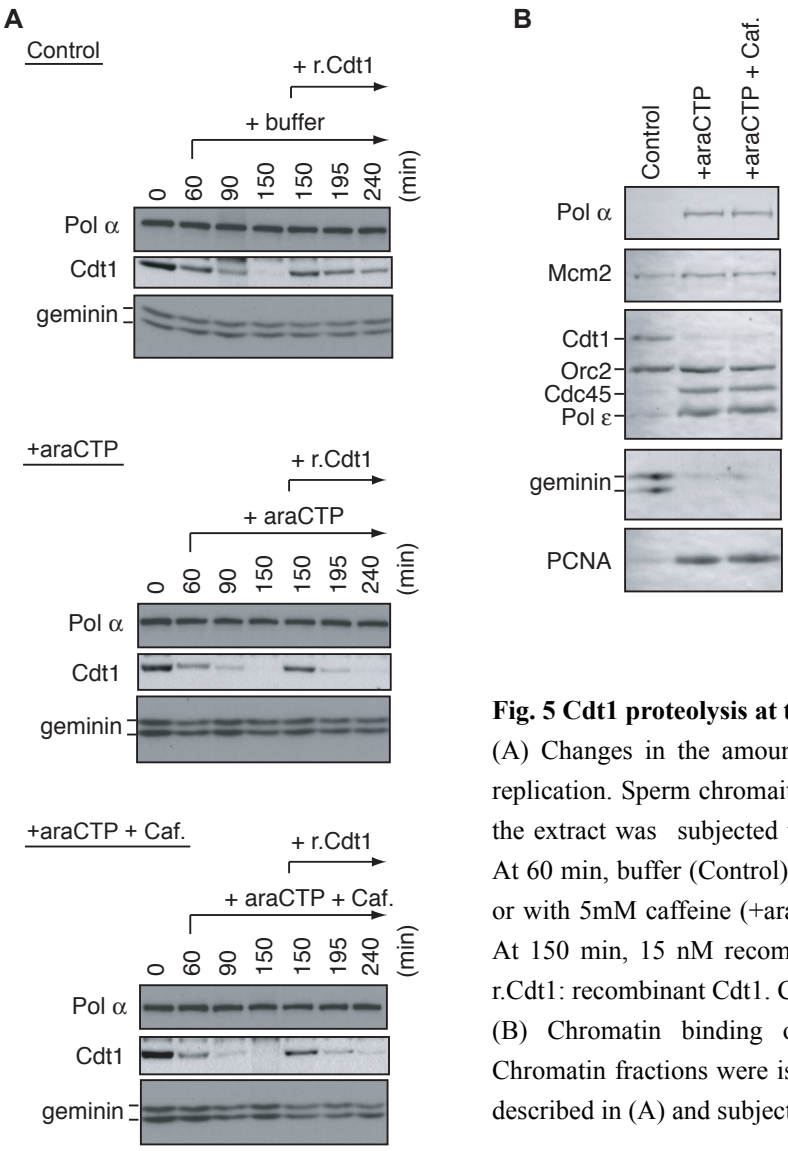

Fig. 5 Cdt1 proteolysis at the later replication phase.

(A) Changes in the amount of Cdt1 in the extract during DNA replication. Sperm chromaitn was incubated in the egg extract and the extract was subjected to western blotting at indicated timing. At 60 min, buffer (Control) or 200 μM araCTP without (+araCTP) or with 5mM caffeine (+araCTP + Caf.) was added to the extract. At 150 min, 15 nM recombinant Cdt1 was added to the extract. r.Cdt1: recombinant Cdt1. Caf.: caffeine.

(B) Chromatin binding of licensing and replication factors. Chromatin fractions were isolated at 240 min in each condition as described in (A) and subjected to western blotting.

replication had been almost completed. A CDK inhibitor was simultaneously added with Cdt1 to prevent the formation of replication machinery due to the re-licensing of chromatin by the added Cdt1. While the added Cdt1 was slightly decreased, a significant amount of the Cdt1 remained in the extract after further 90 min incubation (Fig. 5A Control, 150-240 min). At this time point, Cdt1 associated on chromatin (Fig. 5B Control). Interestingly, geminin was also recruited on chromatin. To confirm that this reduced efficiency of Cdt1 proteolysis is due to the completion of replication, I tested whether the reduced activity can be up-regulated by forced retention of replication machinery on chromatin. Arabinofuranosylcytosine triphosphate (araCTP), which inhibits DNA polymerases and thereby prevents the completion of replication, was added to the extract at 60 min after the start of the incubation. In this case, added recombinant Cdt1 was efficiently decreased compared to that in the absence of araCTP (Fig. 5A +araCTP, 150-240 min). Consistently, the amounts of Cdt1 and geminin on chromatin were markedly reduced, and the amount of replication machinery on chromatin involving PCNA was increased compared to those in the absence of araCTP (Fig. 5B +araCTP). Addition of caffeine, which inhibits ATR/ATM mediated checkpoint activation had

no effect on Cdt1 proteolysis (Fig. 5A +araCTP+Caf., 150-240 min), confirming that the effect of araCTP on Cdt1-proteolysis efficiency is not mediated by the activation of S phase checkpoint caused by stalled replication forks. Therefore, these results confirmed the idea that replication-coupled Cdt1 proteolysis is terminated after the completion of S phase, and further suggest that geminin plays an important role in preventing re-replication after replication is completed.

Discussion

The importance of geminin for preventing re-replication in embryonic cell cycle.

For preventing re-replication, it is important to insulate the licensing from replication phases so that these phases never overlap. In budding yeast, this separation is thought to be established by the temporal separation of licensing inhibition by G1-CDK and replication initiation by S-CDK (Arias and Walter, 2007). In higher eukaryotes, known mechanisms for licensing inhibition are initiated only after the onset of S phase. Proteolysis of Cdt1 is triggered only after the initiation of DNA replication. Nuclear formation after mitotic exit not only activates endogenous geminin but also initiates DNA replication in embryonic systems. One solution to avoid the overlap is to inhibit licensing quickly at the beginning of S phase. In this study, I found that geminin but not replication coupled Cdt1 degradation is responsible for such inhibition at the earliest replication phase. Geminin inhibits licensing in an all-or-none style (see Fig. 1A in part I of this thesis). Interestingly, the threshold geminin concentration for licensing inhibition is close to the geminin concentration in cytosol (see Fig. 2B in part I of this thesis). Thus geminin activity in nuclei would be elevated to the threshold level within a short time period provided that geminin is quickly reactivated upon nuclear import. Hence, the risk of rereplication at the earliest replication phase would be prevented by the rapid inhibition of licensing by geminin. Previous studies showing that depletion of geminin leads to inefficient but distinct rereplication may reflect the re-relicensing occurred at the earliest replication phase.

In addition, I showed that the amount of Cdt1 in *Xenopus* embryo at initial cleavage cycles is almost constant. This would be because cytosol is much more abundant than nuclei in the early embryo, and Cdt1 proteolysis in nuclei cannot efficiently reduce the total amount of Cdt1 in cytosol. Remained Cdt1 in cytosol may induce re-replication when it is imported into nuclei. Therefore, geminin will be particularly important not only at the earliest replication phase but also after the completion of replication, during which replication-coupled Cdt1 proteolysis no longer operates. I expect that nucleus/cytosol ratio is one of a important factor that determine the importance of geminin. Previous studies using egg extracts indicate that Cdt1 proteolysis can prevent the majority of re-replication without the contribution of geminin (Arias and Walter, 2005; Kerns et al., 2007; Li and Blow, 2005; McGarry, 2002; Yoshida et al., 2005). However, in embryo, this might not be the case because of the low nucleus/cytosol ratio. In contrast, HeLa cell in which Cdt1 proteolysis seems to play a more important role than geminin has large nucleus and nucleus/cytosol ratio is high in this cell. Further study concerning the amount of nucleus in experimental system will be required to clarify the relative importance of geminin and replication-dependent Cdt1 proteolysis in embryonic cell cycle, and possibly in other metazoan cells.

In summary, my finding underscores the importance of geminin for preventing re-replication especially at the M/S boundary and after the completion of replication in *Xenopus* early

embryo, and further propose nuclei/cytosol ratio as the determinant of relative importance between geminin system and replication-coupled Cdt1 proteolysis.

Experimental Procedures

Note that all the extracts used in this study were interphase egg extracts unless stated as membrane-free (HSS) or nucleoplasmic extract (NPE).

Expression and purification of recombinant proteins

Xenopus Cdc6, Orc2, Cdt1, and gemininH (wild type, KKFEV and KKAAFEV mutant) were expressed in the BL21-codonplus expression strain (Stratagene) transformed with pGEX 6p (Amersham) carrying the corresponding cDNAs. Expressed proteins were purified using Glutathione Sepharose^{TM} 4B beads (GE Healthcare). The GST tag was digested using PreScission protease (Amersham) and purified proteins were eluted according to the manufacturer's protocol, with the exception that Cdt1 and Cdc6 were eluted with GST-Elution buffer (600 mM NaCl, 1 mM dithiothreitol and 50 mM Tris-HCl at pH 8.7).

Antibodies

Polyclonal rabbit antisera were raised against purified recombinant *Xenopus* Cdc6, Cdt1, Orc2 and geminin H proteins (Hokudo Inc., Japan). The anti-*Xenopus* Mcm2, Cdc45, Polε and Polα antibodies used here have been described previously in (Kubota et al., 1997; Matsuno et al., 2006; Mimura et al., 2000).

In vitro fertilization

I induced ovulation in mature female *Xenopus* by an injection of human gonadotropin (700 IU). Eggs were collected in 0.1 M NaCl and were then mixed with the stripped testes from male *Xenopus* in 1× MMR (100 mM NaCl, 2 mM KCl, 0.5 mM $MgSO_4$, 2.5 mM $CaCl_2$, 0.1 mM EDTA, 5 mM Hepes-NaOH at pH 7.8) and incubated in 0.1× MMR. The fertilized eggs were then dejellied with 2% l-cysteine hydrochloride monohydrate in H_2O at pH 7.8 and then incubated in 0.1× MMR at 22ºC.

***Xenopus* egg extracts and sperm nuclei**

I induced ovulation in mature female *Xenopus* by an injection of human gonadotropin (700 IU). Eggs were collected in 0.1 M NaCl, and those eggs that appeared to have degenerated were discarded. Unfertilized eggs were dejellied in a solution consisting of 5 mM dithiothreitol, 110 mM NaCl and 20 mM Tris-HCl at pH 8.5, then washed in 0.25× MMR (100 mM NaCl, 2 mM KCl, 0.5 mM $MgSO_4$, 2.5 mM $CaCl_2$, 0.1 mM EDTA, 5 mM Hepes-NaOH at pH 7.8), and activated with 0.5 µg/ml calcium ionophore A23187 in 0.25× MMR. Activated eggs were washed with 0.25× MMR and then with ice-chilled S-buffer (0.25 M sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 15 µg/ml leupeptin, and 50 mM HEPES-KOH at pH 7.5). The washed eggs were packed into tubes by brief centrifugation for several seconds at 3000*g*. All excess buffer was removed and the eggs were ruptured by centrifugation at 18 800*g* for 10 min. The resulting supernatant between the lipid

cap and pellet was collected and mixed with 10 μg/ml cytochalasin B and then centrifuged again at 265 000*g* for 10 min. Both the cytosolic and membranous fractions were collected and combined as the interphase egg extract. The extracts were supplemented with 40 μg/ml cycloheximide, 60 mM creatine phosphate, 150 μg/ml creatine phosphokinase and 4% glycerol and were then frozen and stored under liquid nitrogen. To prepare cycling extract, all procedure is same as above except for without adding cycloheximide. To prepare HSS, the stored extract was thawed, centrifuged at 265 000*g* for 10 min and the cytosolic fraction was collected.

To prepare demembranated sperm nuclei, mature male *Xenopus* were anesthetized and their testes were collected and immersed in buffer C-0.2 (80 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.2 M sucrose, and 10 mM Hepes-KOH at pH 7.5). The testes were then minced with a loose-fit homogenizer and debris was removed by centrifugation at 180*g* for 2 min followed by centrifugation twice at 260*g* for 2 min each. Sperm were collected by centrifugation at 2900*g* for 10 min. The sperm pellet was suspended in buffer C-2.0 (buffer C-0.2 with 2 M sucrose) and underlayed with buffer C-2.3 (buffer C-0.2 with 2.3 M sucrose) and buffer C-2.5 (buffer C-0.2 with 2.5 M sucrose). Lipids and red blood cells were removed by spinning the sperm suspension through the underlayers by centrifugation at 89 000*g* for 20 min. The resultant sperm pellet was then suspended in buffer C-0.2, centrifuged at 2600*g* for 15 min, and then suspended in buffer S (1 mM EDTA, 250 mM sucrose, 75 mM NaCl, 0.5 mM spermidine, 0.15 mM spermin, and 10 mM Hepes-KOH at pH 7.5). Sperm was demembranated by incubation in buffer S containing 0.5 mg/ml lysolecithine at 20ºC for 5–10 min. The reaction was then stopped by placing the samples on ice and adding an equal volume of buffer S containing 3% BSA. The sperm chromatin was collected by centrifugation at 1,400*g* for 5 min and then washed twice with buffer S containing 0.3% BSA. The sperm chromatin was suspended in buffer S containing 30% glycerol, 1 mM dithiothreitol, 10 μg/ml aprotinine and 15 μg/ml leupeptine, and was then frozen by liquid nitrogen and stored at −80ºC.

Isolation of chromatin and nuclear fractions.

To isolate the chromatin and nuclear fractions, sperm chromatin (2500 nuclei/μl or indicated amount) was incubated with the extracts for the times and temperatures indicated in the figure legends. Reaction were stopped by diluting the samples with a 10-fold volume of ice-chilled XB (100 mM KCl, 2.5 mM MgCl2 and 50 mM Hepes-KOH at pH 7.5) (nuclear fractions) or XB containing 0.25% Nonidet-P 40 (NP40) (chromatin fractions), and then centrifuging them through the dilution buffer containing 10% (chromatin) or 30% (nuclear) sucrose at 2200g for 5 min (chromatin) or 8700g for 2min (nuclear) at 4ºC. The pellets were washed with XB and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To isolate chromatin fractions assembled with the HSS, chromatin fractions were pelleted by centrifugation at 8700g for 5 min at 4ºC.

Immunodepletion

For immunodepletion, the appropriate volume of antiserum was incubated with rProtein A Sepharose Fast Flow (Amersham Bioscience) beads with constant rotation (~ 9 rpm) for 60 min at 4ºC. Antibodies bound to the beads were recovered and washed three times with XB. Egg extract was treated several times at 4ºC with 1/4 volume of the beads bound to the specific antibodies as follows: (1) For depletion of Cdt1, the egg extract was treated three times for 30 min with anti-Cdt1 antibody; (2) For depletion of both Cdt1 and geminin, the egg extract was treated four times for 20 min each with anti-Cdt1 antibody (1^{st}), anti-Cdt1 and anti-geminin antibodies (2^{nd} and 3^{rd}), and anti-geminin antibody (4^{th}).

Immunofluorescence

Sperm chromatin (2500 nuclei/µl) was incubated in 10 µl of egg extracts for required times at 23ºC. For observation of nuclear proteins, the reactions were stopped and the nuclei were fixed by adding 100 µl of 3.7% formaldehyde in XB and incubating for 10 min at 20ºC. For observing proteins bound to chromatin, the reactions were stopped and chromatin was fixed by adding 100 µl of 3.7% formaldehyde in XB containing 0.25% NP40 and incubating for 10 min at 20ºC. The fixation reactions were stopped by adding 1 ml XB and the fixed nuclei or chromatin fractions were attached to a poly-lysine coated coverslip by centrifugation at 1500 rpm for 5 min (RS-4 rotor, KUBOTA, Japan) through a layer of 30% sucrose in XB. Immunofluorescence imaging was carried out as previously described (Kubota et al., 1997).

Immunoblotting and Signal Quantification

After SDS-PAGE, proteins were electrophoretically transferred from a polyacrylamide gel to a nitrocellulose membrane. A gel sandwich was prepared with an anode metal plate at the bottom followed by two sheets of No. 1 filter paper soaked with transfer buffer A (0.3 M Tris, 10% methanol), a sheet of filter paper soaked with transfer buffer B (25 mM Tris, 10% methanol), and nitrocellulose membrane soaked with the transfer buffer B. The PAGE gel was washed once with transfer buffer B and then laid on top of the nitrocellulose membrane followed by three sheets of filter paper soaked with transfer buffer C (25 mM Tris, 60 mM 6-aminicaprone acid, and 0.01% SDS), and the cathode metal plate was placed on top. Electrophoretic transfer was carried out at 4.5 mA/cm^2 for 60 min. The membrane was then washed with TTBS (0.9% NaCl, 0.1% Tween-20, and 100 mM Tris-HCl at pH 7.5) and nonspecific binding sites were blocked by a 1 h incubation in TTBS containing 7.5% skim milk. The membrane was then incubated overnight with the primary antibodies at the appropriate dilutions in TTBS containing 7.5% skim milk at 4ºC. After washing the membrane with TTBS, the membrane was incubated with the peroxidase-conjugated secondary antibody in TTBS containing 7.5% skim milk for 1 h at room temperature. Immunoreactivities of blotted proteins were detected with Konica Immunostain HRP (Konica Minolta, Japan), and the development of each reaction was stopped before reaching saturation. Data shown in Fig. 1 and Fig. 5A were obtained using Immobilon™ Western

Chemiluminescent HRP substrate (Milipore) and FUJI MEDICAL X-RAY FILM RX-U (Fjifilm, Japan). Immunostained membranes were scanned and the images were analyzed by using ImageJ software (NIH). Care was taken to ensure that the signals obtained were within the linear range of detection.

Histone H1 kinase assay

Two micro liter of extract at each time point was frozen in liquid nitrogen and store at -80ºC. The extract was thawed by adding 18µl of Kinase Buffer (80 mM beta-glycerophosphate, 20 mM EGTA, 5 mM $MgCl_2$ and 20 mM HEPES-KOH at pH 7.5). Ten micro liter of the mixture was then mixed with 10 µl of Reaction Buffer (80 mM beta-glycerophosphate, 20 mM $MgCl_2$, 0.6 mM ATP, 30 ug/ml Leupeptin, 30 ug/ml Aprotinin, 0.6 mg/ml Histone H1 and 1 uCi [gamma-32P] ATP) and incubated for 30 min at 23 ºC. The reaction was stopped by adding 20 µl of SDS-PAGE sample buffer. The sample was subjected SDS-PAGE and stained with coomassie brilliant blue (CBB). The incorporated radio-labeled signal in the gel was detected by autoradiography.

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General discussion and conclusion

Geminin provides both robust licensing before S phase and efficient licensing inhibition after the initiation of replication

Xenopus early embryo seems to offer a particularly challenging situation for licensing control. Firstly, the amount of cytosol is much abundant. Thus, there are large quantities of licensing factors to be controlled. Secondly, the cell cycle oscillates rapidly without the gap phases. Thus, licensing activity should be quickly turned on and off. Finally, high efficiency of DNA replication also leads high risk of the initiation of re-replication. How does geminin overcome these points and provide robust licensing control? Here I will discuss about this topic based on the results shown in both part I and II of this thesis.

In this study I found that

1) The all-or-none licensing inhibition is correlated with foci formation of Cdt1-geminin on chromatin,
2) geminin inhibits the ORC-Cdc6-Cdt1 complex on chromatin,
3) Feedback/IOC models could account for the kinetic/spatial dynamics of licensing control by geminin and Cdt1,
4) geminin inhibits licensing at the initial timing when replication initiates,
5) replication-coupled proteolysis is not effective at the initial timing,
6) the amounts of Cdt1 and geminin in *Xenopus* embryo are almost constant during initial several cleavege cycles.

The efficiency of replication-coupled Cdt1 proteolysis is highly depending on the nuclei/ cytosol ratio of the system. In contrast, geminin system, which targets chromatin to inhibit licensing, can operates in cell-size independent manner. Thus, geminin will allow embryos to switch licensing activity without the need of controlling the amount of licensing factors in embryo with large amount of cytosol through continuous protein degradation and synthesis. In order to account for the rapid cell cycle, it would be also reasonable to avoid time- and energy-consuming turning over of abundant licensing factors.

Nevertheless, it is a big challenge for geminin function to instantly turn licensing from “on” to “off” state immediately after the S phase entry. The challenge is to keep the sensitivity of geminin activity just enough for instant inhibition of licensing but not too much as to avoid premature inhibition of licensing by fluctuating activation of the inactive geminin. The risk of fluctuating activity of geminin would be particularly high in *Xenopus* embryonic cell cycle with constant amount of geminin throughout the cell cycle. Threshold type of licensing inhibition by geminin will be important for avoiding vulnerability of the system to such noisy changes in geminin activity: a small amount of active geminin that escapes from the geminin-inactivation process at the exit of M-phase is not able to inhibit licensing below the threshold level (Fig.1 region 1). When the amount of active geminin reaches the threshold level, licensing is abruptly inhibited (Fig.1 region 2 and 3). Therefore, all-or-none style inhibition

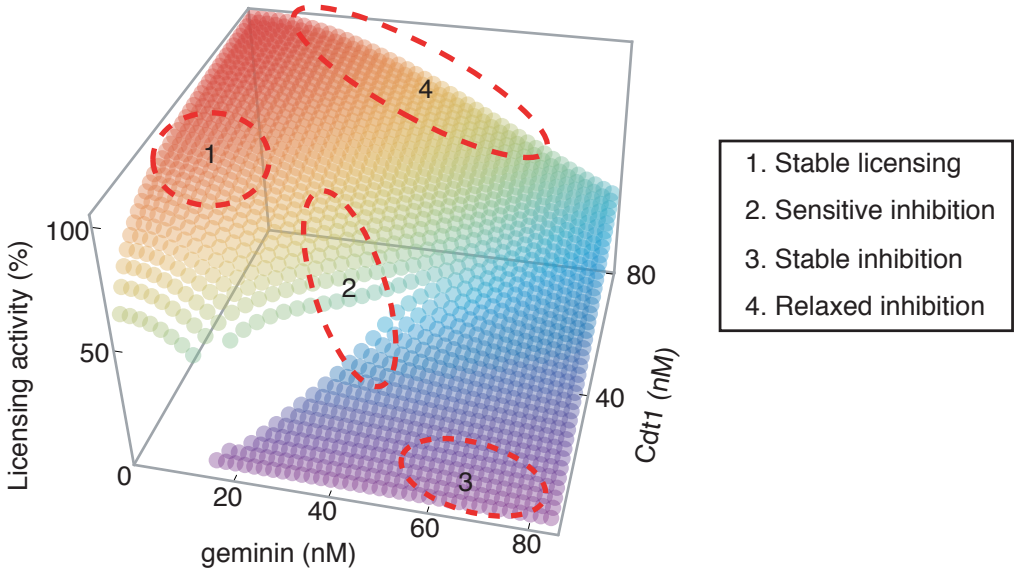

Fig. 1 geminin establishes robust and quick inhibition of licensing.
The switch-like action ensures 1) robust licensing below the threshold (region 1 in the 3D-plot) before the onset of S phase, 2) instant inhibition of licensing when geminin activity exceeds the threshold (region 2) upon nuclear formation and 3) robust inhibition over the threshold (region 3) during S phase. Deregulated over-expression of Cdt1 leads to blunting the geminin inhibition (region 4). For the switch-like inhibition, inter-origin interaction of Incompetent complex with the Competent one leads to create a positive-feedback loop in geminin action, making the inhibition in all-or-none style. 3D diagram is generated with the feedback model shown in Fig. 13C (part I).

by geminin will be important to guarantee both robust licensing before S phase and quick licensing inhibition at the S phase entry.

Cdt1 could counteract the licensing inhibition by geminin and the threshold point shifts with the amount of Cdt1. Therefore, during S phase, it is important to keep the amount of Cdt1 relatively lower than the amount of geminin. In embyo, replication-coupled Cdt1 degradation might important not only degrading Cdt1 in the embyo but also prohibiting the accumulation of Cdt1 in the nucleus during S phase. I also found that in the presence of excess Cdt1, geminin could no longer function as an all-or-none switch (Fig.1 region 4). This indicates that not only the balance between Cdt1 and geminin concentrations but also Cdt1 concentration itself is a crucial factor for ensuring the strict duplication of DNA. Transformed cells over-expressing both Cdt1 and geminin (Blow & Gillespie 2008; Petropoulou *et al.* 2008) might loose the sensitivity and robustness of the licensing switch, thereby amplifying the genomic instability, a major cause of cancer development.

In the embryonic cell cycle, geminin seems to be the sole inhibitor of licensing at two phases; one phase is at the earliest timing of S phase, where replication-coupled Cdt1 proteolysis is not effective, and the other phase is the intermediate phase after the completion of replication and before the initiation of the M phase, where replication-coupled Cdt1 proteolysis no longer operates but Cdt1 remains to exist in cytosol. During these phases, stable licensing inhibition should be ensured solely through geminin-pathway. Proposed inter-origin-cooperativity (IOC) of geminin action could be the basis for the robustness of

licensing inhibition. In the presence of IOC, by which multiple origins are cooperatively inhibited, each origin is difficult to escape from the inhibition. Even if one origin occasionally escapes from the inhibition, the origin will be immediately re-inhibited by the IOC effect of surrounding inhibited-origins. This system appears to be an alternative strategy for ensuring stable licensing inhibition compared with the strategy in budding yeast, where CDK inhibits every licensing factor through multiple pathways.

In order to provide effective IOC, multiple activated origins (i.e. replicated regions of chromosome) should be located close to each other. In metazoan cells, DNA replication synchronously initiates within spatially clustered domains of chromosome (Jackson & Pombo 1998; Berezney *et al.* 2000). In *Xenopus* egg extract, replication initiates within a cluster of chromosomal region and the efficiency of the initiation is even higher than the somatic cells (Blow *et al.* 2001). Therefore, IOC will be effectively operated on clustered replicated regions (Fig. 2).

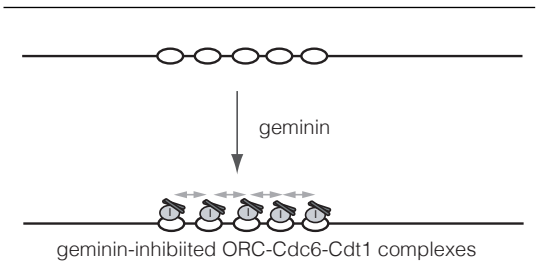

Fig. 2 Efficient licensing inhibition by geminin. IOC effect induced by geminin cooperatively inhibits licensing on synchronously emerged replicated origins on chromatin.

In conclusion, chromatin-targeted, all-or-none style licensing inhibition by geminin with IOC will be important to account for the sensitiveness and robustness of licensing inhibition. As this mechanism works cell-size independent and relatively quicker than the mechanisms based on proteolysis or modification of entire component in the cell, geminin system will be particularly useful in embryonic cell cycle. I believe these features of geminin are important to safely drive the metazoan cell cycle including embryonic cell cycle without the menace of re-replication.

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