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Aurora-A controls pre-replicative complex assembly and DNA replication by stabilizing geminin in mitosis

Takaaki Tsunematsu¹, Yoshihiro Takihara², Naozumi Ishimaru³, Michele Pagano^{4,5}, Takashi Takata¹, Yasusei Kudo^{1,3,6}

¹Department of Oral and Maxillofacial Pathobiology, Graduate School of Biomedical & Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan.

²Department of Stem Cell Biology, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan.

³Department of Oral Molecular Pathology, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto, Tokushima 770-8504, Japan.

⁴Department of Pathology, New York University Cancer Institute, New York University School of Medicine, 522 First Ave, Smilow Research Building 1107, New York, NY 10016, USA.

⁵Howard Hughes Medical Institute.

⁶Correspondence should be addressed to Y.K. (e-mail: yasusei@tokushima-u.ac.jp)

Geminin, an essential factor for DNA replication, directly binds to the licensing factor Cdt1 and inhibits pre-replicative complex (pre-RC) formation to prevent re-replication. In G₁, geminin levels are controlled by the anaphase-promoting complex/cyclosome ubiquitin ligase complex (APC/C), which targets geminin for proteasomal degradation to allow pre-RC formation. Conversely, from S to G₂, geminin is stabilized thanks to APC/C inhibition to ensure inhibition of pre-RC formation. However, mitotic regulation of geminin has hitherto not been described. Here we show that Aurora-A phosphorylates geminin on Thr25 during M phase, and this event induces geminin stabilization by preventing its APC/C-mediated degradation during mitosis. In turn, stabilized geminin inhibits SCF^{Skp2}-mediated degradation of Cdt1 to ensure pre-RC formation in the ensuing S phase. The Aurora-A-geminin-Cdt1 axis therefore represents a critical regulator of proper DNA replication.

In eukaryotes, DNA replication is restricted to occur only once per cell cycle. Licensing is mediated by the assembly of the pre-RC on the origins. The assembly of the essential pre-RC at replication origins can only occur between late mitosis and early G₁ when cyclin-dependent kinase (CDK) activity is low and APC/C activity is high. Pre-RC is composed of the origin recognition complex (ORC), Cdc6, Cdt1 and the mini-chromosome maintenance (MCM) proteins¹. During late M and early G₁, Cdc6 and Cdt1 are loaded onto replication origins in an ORC-dependent manner and subsequently, recruit MCM proteins to origins. Once pre-RC are assembled, origins are licensed for replication in the subsequent S phase and ready to fire. Geminin, a known repressor of re-replication, directly binds to Cdt1 and inhibits its function². Furthermore, geminin controls the basal level of Cdt1 and induces its accumulation during mitosis by inhibiting its ubiquitin-dependent proteolysis². Thereby, the depletion of geminin during mitosis causes the de-stabilization of Cdt1 and subsequently prevents MCM loading in the subsequent G₁ (ref. 3). Thus, it is proposed that geminin plays both negative and positive roles in pre-RC formation, indicating that protein level of geminin must be appropriately and strictly controlled to ensure proper DNA replication.

The protein level of geminin is regulated by the ubiquitin-proteasome system; geminin is degraded at the exit of mitosis by the APC/C complex in *Xenopus* egg extract⁴, whereas it is a target of the APC/C^{Cdh1} ubiquitin ligase complex in mammalian cells^{5,6}. The APC/C activity is dependent on its co-activators, Cdc20 and Cdh1, which associate with APC/C during specific periods in the mammalian cell cycle⁷. APC/C^{Cdc20} is active early in mitosis, and APC/C^{Cdh1} plays an essential role from late M to the G₁/S transition⁷. Cdh1 can only activate APC/C once APC/C^{Cdc20} has decreased the Cdk1 activity by initiating cyclin destruction, when Cdh1 has been dephosphorylated by protein phosphatases in late M (ref. 7). DNA replication depends on the APC/C-mediated degradation of geminin, which releases the inhibition of Cdt1 incorporation into pre-RCs and permits the proper assembly of these

complex components^{2,8}. Origin firing can only occur after the APC/C is inactivated and CDKs become active. To strictly inhibit the assembly of Pre-RC during S to G₂ phase, APC/C is inactivated by several mechanisms: binding to its inhibitor Emi1, CDK-mediated phosphorylation of Cdh1 (which blocks its ability to activate APC/C), and degradation of both Cdh1 (ref. 9) and its major UBC, namely UbcH10 (ref. 10). In this study, we found that Aurora-A phosphorylates geminin to protect it from APC/C during mitosis. In turn, Aurora-A-mediated stabilization of geminin inhibits SCF^{Skp2}-mediated degradation of Cdt1 to ensure pre-RC formation in the ensuing S phase.

RESULTS

Phosphorylation of geminin prevents its degradation

Geminin protein levels oscillate during the cell cycle, i.e. accumulating in S and disappearing in early G₁ (Supplementary Fig. S1a). The half-life of geminin in mitosis (mitotic geminin) was elongated compared with that of either asynchronous or G₁-synchronized cells (Supplementary Fig. S1b and S1c) and treatment with the proteasome inhibitor MG132 resulted in geminin protein accumulation in G₁ (Supplementary Fig. S1d). Geminin is known to be ubiquitylated via APC/C in G₁ phase⁴. Recent analyses at the single cell level by immunocytochemistry have shown the timing of geminin degradation in mitosis, which takes place following cyclin B degradation, in late anaphase¹¹. We confirmed that the depletion of Cdh1 stabilized geminin protein (Supplementary Fig. S1e). Emi1 is induced by E2F to inactivate APC/C^{Cdh1} at the G₁/S transition¹². At the onset of mitosis, Emi1 is degraded following the phosphorylation by Plk1 and ubiquitylation by SCF^{β-TrCP}, and this allows the activation of APC/C^{13,14}. Although geminin is absent during G₁ and accumulates during S, G₂ and M, consistent with the idea that it is an APC/C substrate, geminin is stable even in mitosis after APC/C is activated. These findings led us to hypothesize that geminin may be stabilized during mitosis by a hitherto unknown

pathway.

We firstly noted a band shift of geminin during mitosis by immunoblotting (Supplementary Fig. S1a). The band shift was eliminated by lambda-phosphatase (λ -PPase) treatment (Fig. 1a), suggesting that geminin is phosphorylated during mitosis. Therefore, we assumed that phosphorylation is important for geminin stabilization during mitosis. Geminin is a highly conserved protein and has three conserved putative phosphorylation serine/threonine sites, Thr25, Ser32 and Ser60 surrounding the destruction box (D-box) motif that is recognized by APC/C (Fig. 1b). To identify the phosphorylation site and further clarify the implication in geminin protein stability, we generated three phospho-mimicking mutants, geminin^{T25D}, geminin^{S32D} and geminin^{S60D}, in which serine/threonine was replaced with aspartic acid. Cells overexpressing either of these mutants were synchronized in M (noc 0 h) and G₁ (noc 4 h). These mutants were highly expressed in M, but only geminin^{T25D} was abundant in G₁ (Fig. 1c). Interestingly, Thr25 is a component of the D-box in geminin (Fig. 1b). We also generated a phospho-defective mutant (T25A) by replacing threonine with alanine. We examined the stability of these mutant molecules after release from nocodazole arrest. Geminin^{T25A} was already unstable during M, whereas geminin^{T25D} remained stable during G₁ (Fig. 2a). We further confirmed this phenomenon through double thymidine block release (Supplementary Fig. S2a). In G₁ extracts, *in vitro*-translated geminin^{T25A} degraded faster than wild-type geminin, whereas geminin^{T25D} was stable (Fig. 2b). MG132 treatment increased wild-type geminin and geminin^{T25A} but not geminin^{T25D} protein levels (Supplementary Fig. S2b). These findings suggest that geminin is phosphorylated on Thr25 during mitosis and is degraded via APC/C^{Cdh1} in G₁ phase after dephosphorylation. To demonstrate this hypothesis, we examined the effect of Thr25 phosphorylation on geminin degradation by APC/C^{Cdh1}. Cdh1 depletion increased wild-type geminin and geminin^{T25A} protein levels, but not geminin^{T25D} protein levels (Supplementary Fig. S2c). As Emi1 depletion is known to give rise to ectopic APC/C^{Cdh1}

activation², we attempted to deplete Emi1, resulting in the reduction of wild-type geminin and geminin^{T25A} but not geminin^{T25D} protein levels (Supplementary Fig. S2d). Moreover, the *in vitro* ubiquitylation of geminin^{T25D} via APC/C^{Cdh1} was not detectable, whereas that of wild-type geminin and geminin^{T25A} was observed (Fig. 2c). Interestingly, immunoprecipitation analysis revealed that HA-tagged Cdh1 interacted with wild-type geminin and geminin^{T25A} but not with geminin^{T25D} (Fig. 2d), indicating that the inability of geminin^{T25D} to interact with APC/C^{Cdh1} may explain its resistance to APC/C^{Cdh1}-dependent proteolysis.

Geminin^{T25A} protein levels were lower than wild-type geminin protein levels at prometaphase (Fig. 2a), whereas *in vitro*-translated wild-type geminin, which was not phosphorylated on Thr25, and geminin^{T25A} were degraded in mitotic extracts (Fig. 2e), suggesting that phosphorylated geminin is protected from degradation via APC/C^{Cdc20}. As expected, the *in vitro* ubiquitylation of geminin^{T25D} via APC/C^{Cdc20} was not detectable (Fig. 2f). Moreover, HA-Cdc20 interacted with wild-type geminin and geminin^{T25A} but not with geminin^{T25D} (Fig. 2d). Thus, phosphorylation of geminin on Thr25 also protects it from APC/C^{Cdc20}-mediated proteolysis in mitosis.

Next, to elucidate the physiological role of geminin phosphorylation on Thr25, we generated an antibody against geminin phosphorylated on Thr25. λ -PPase treatment almost completely eliminated the recognition of geminin in mitotic extracts by this antibody (Supplementary Fig. S3a). Moreover, this antibody recognized wild-type geminin, but not geminin^{T25A} (Supplementary Fig. S3b). We then used this antibody to probe for geminin phosphorylated on Thr25 at M and G₁ phase. Thr25 phosphorylation of endogenous geminin was detectable exclusively in mitosis (Supplementary Fig. S3c).

Thr25 of geminin is phosphorylated by Aurora-A

To further deepen the understanding of geminin phosphorylation, we identified which kinase that

phosphorylates geminin on Thr25. We found that amino acids 23-26 (RRTL) within the D-box (which include Thr25) matched the consensus sequences (R-X-S/T-L/V) for phosphorylation by Aurora-A, a known mitotic kinase (Fig. 3a). The serine/threonine kinase Aurora-A is essential for mitotic entry, centrosome duplication, spindle formation, chromosome segregation and cytokinesis¹⁵. Geminin was co-localized with Aurora-A through mitotic progression (Fig. 3b and Supplementary Fig. S4a), but Aurora-A and phosphorylated geminin disappeared together in G₁ (Supplementary Fig. S4b). Interestingly, APC/C^{Cdh1} targets Aurora-A for destruction at mitotic exit¹⁶.

We then investigated the direct interaction between geminin and Aurora-A. FLAG-tagged geminin co-immunoprecipitated with Xpress-tagged Aurora-A (Fig. 3c). Moreover, the endogenous interaction between Aurora-A and geminin was observed in mitosis, but not in interphase (Fig. 3d). Thus, Aurora-A exhibited mitosis-specific interaction with geminin. To determine the ability of Aurora-A to phosphorylate geminin, we performed an *in vitro* kinase assay. Aurora-A strongly phosphorylated purified wild-type GST-geminin, but not GST-geminin^{T25A} (Fig. 3e). Moreover, a kinase-dead mutant (K162R) of Aurora-A displayed significantly reduced ability to phosphorylate geminin at Thr25, which further highlights the specificity of Aurora-A-mediated geminin phosphorylation (Fig. 3f). In fact, geminin phosphorylated on Thr25 co-localized with Aurora-A in mitotic cells (Supplementary Fig. S4b). Moreover, the depletion of Aurora-A impaired geminin phosphorylation (Fig. 3g). As shown in Fig. 2e, wild-type geminin and geminin^{T25A} were degraded in mitotic extracts, whereas wild-type geminin but not geminin^{T25A} was stabilized in mitotic extracts after *in vitro* phosphorylation by Aurora-A (Fig. 3h). Moreover, a shorter half-life of geminin was observed in nocodazole-arrested cells after Aurora-A depletion (Supplemental Fig. S5). Cdc20 depletion stabilized geminin in mitotic Aurora-A-depleted cells (Supplemental Fig. S5), indicating that Aurora-A-mediated geminin phosphorylation prevents geminin degradation by APC/C^{Cdc20} at prometaphase.

In mitosis, phosphorylated geminin stabilizes Cdt1

We then investigated the physiological role of geminin phosphorylation during mitosis. A previous report indicated that the inhibition of geminin synthesis during mitosis leads to impaired pre-RC formation and DNA replication in the ensuing cell cycle, and that geminin binds and protects Cdt1 from proteasome-dependent degradation to ensure the accumulation of Cdt1 in G₂ and M (ref. 5). Similarly, Cdt1 was reported to be rapidly down-regulated after geminin was silenced in *Drosophila* cells¹⁷. As shown by previous studies^{3,18}, we confirmed that Cdt1 was hyperphosphorylated in mitosis and did not bind to chromatin (Supplemental Fig. S6). Moreover, dephosphorylation of Cdt1 coincided with chromatin association (Supplemental Fig. S6). A previous report revealed that the inhibition of CDK1 in mitosis induced an increase in the chromatin-associated Cdt1 fraction to the expense of the soluble Cdt1, and that geminin bound with Cdt1 interfered with this binding to the chromatin³. These findings suggest that Cdt1 recruitment to chromatin may be regulated by dephosphorylation in late M. Interestingly, geminin downregulation and Cdt1 recruitment to chromatin occur simultaneously (Supplemental Fig. S6). To understand the role of geminin in Cdt1 recruitment to chromatin, we examined whether mitotic depletion of geminin affected Cdt1 recruitment to chromatin (Fig. 4a). Consistent with what published before⁵, mitotic depletion of geminin decreased the levels of both soluble and chromatin-associated Cdt1 and impaired MCM6 loading (Fig. 4b). Interestingly, mitotic depletion of Aurora-A induced a similar decrease in the level of both soluble and chromatin-associated Cdt1 (Fig. 4c). These findings suggest that geminin phosphorylated by Aurora-A stabilizes soluble Cdt1, which, in turn, is recruited to the chromatin. In contrast to what observed when geminin is depleted during interphase¹⁹, mitotic depletion of geminin or Aurora-A did not induce the DNA damage response, as shown by the lack of Chk2 phosphorylation (Supplemental Fig. S7). Moreover, protein level and kinase activity of Aurora-A

were not affected by the depletion of mitotic geminin (data not shown), and the phosphorylation of geminin on Thr25 did not affect its interaction with Cdt1 (Supplementary Fig. S8a).

Next, we examined whether introducing ectopic geminin^{T25D} rescued the downregulation of soluble Cdt1 protein at M phase in geminin- or Aurora-A-depleted cells. As we used siRNA targeting the 3'-UTR region to deplete geminin, exogenous geminin expression was not affected by this siRNA (Supplementary Fig. S8b). Interestingly, ectopic geminin^{T25D} in geminin- or Aurora-A-depleted cells induced a stabilization of soluble Cdt1 in M (Fig. 4b and 4c). As expected, the loading of Cdt1 and MCM in G₁ was suppressed by ectopic geminin^{T25D} (Fig. 4b and 4c), indicating that phosphorylated geminin which cannot be degraded via APC/C prevents Cdt1 from loading onto chromatin during M. Indeed, ectopic Cdt1 rescued the impairment of MCM loading in geminin- or Aurora-A- depleted cells (Fig. 5a and 5b). Moreover, the depletion of geminin or Aurora-A in mitosis suppressed BrdU incorporation in the ensuing S phase (Supplemental Fig. S9a, S9b, Fig. 5c and 5d), even though the kinetics of mitotic exit was not affected, as shown by the phosphorylation of histone H3 on Ser10 (Fig. 4b, 4c, 5a and 5b) and FACS analysis (Supplementary Fig. S10 and S11). Ectopic geminin^{T25D} in geminin or Aurora-A depleted cells could not rescue BrdU incorporation in the ensuing S phase (Supplemental Fig. S9a and S9b). This is likely due to the impairment of Cdt1 and MCM6 loading (Fig. 4b and 4c). Interestingly, ectopic Cdt1 in geminin or Aurora-A depleted cells rescued BrdU incorporation in the ensuing S phase (Fig. 5c and 5d). These findings suggest that strict regulation of geminin and Cdt1 protein levels depending on the cell cycle phase may be essential for DNA replication.

Phosphorylated geminin interferes with Cdt1 degradation

We investigated why soluble Cdt1 protein becomes unstable when geminin is depleted in mitosis. Treatment with MG132 or the neddylation inhibitor MLN4924 increased soluble Cdt1 levels in mitotic geminin- or Aurora-A-depleted cells (Fig. 6a and 6b), suggesting that soluble Cdt1

downregulation in response to the mitotic depletion of geminin or Aurora-A may be caused by ubiquitin-dependent proteolysis. During S and G₂, human Cdt1 is recognized for proteolysis by two distinct E3 ubiquitin ligases, SCF^{Skp2} and CRL4^{Cdt2} (ref. 20). It has been demonstrated that Cdt1 is degraded via CRL4^{Cdt2} in geminin-depleted cells because of the DNA damage response²¹. We also found that geminin depletion induced Thr68 phosphorylation of Chk2, which is phosphorylated by ATM/ATR after DNA damage (Supplementary Fig. S7). However, geminin depletion in mitosis did not induce DNA damage (Supplementary Fig. S7). These findings suggest that Cdt1 may not be ubiquitylated by CRL4^{Cdt2} in mitotic geminin depleted cells. Indeed, Skp2 depletion but not Cdt2 depletion remarkably increased soluble Cdt1 protein levels (Fig. 6c). Accordingly, Skp2 depletion rescued the levels of both soluble and chromatin-associated Cdt1 due to the mitotic depletion of geminin or Aurora-A (Fig. 7a and 7b). In mitotic geminin depleted cells, Cdt1 expression was increased by introduction of ectopic FLAG-geminin in an amount dependent manner (Fig. 7c). SCF^{Skp2}-dependent *in vitro* ubiquitylation of Cdt1 was inhibited by adding *in vitro*-translated geminin protein (Fig. 7d). Cdt1^{T29A}, in the presence of which CDK phosphorylation and Skp2 binding are impaired²², was not ubiquitylated by SCF^{Skp2} (Fig. 7d). These findings indicate that in mitosis geminin may protect soluble Cdt1 from SCF^{Skp2}-dependent degradation for pre-RC formation.

DISCUSSION

In this study, we describe a novel regulation of geminin during mitosis (Fig. 7e and Supplemental Fig. S12). Aurora-A phosphorylates geminin on Thr25 for it from protecting APC/C-dependent proteolysis. Importantly, stabilized mitotic geminin ensures pre-RC formation by protecting Cdt1 from SCF^{Skp2}-dependent proteolysis. The pre-RC formation regulated by Aurora-A-geminin-Cdt1 axis leads to proper DNA replication.

The APC/C complex distinctively targets specific substrates and it is tightly regulated

to adjust the critical timing of substrate destruction⁷. However, it is still unclear how activated APC/C specifically degrades each of a large number of substrates. In fact, all its substrates are not necessarily degraded simultaneously by APC/C even though APC/C becomes active during mitosis. The timing of the ubiquitylation of the substrates is tightly regulated by the following mechanisms: (i) protein modification such as phosphorylation or acetylation protects the substrates from APC/C^{16,23-26}, (ii) intrinsic regulation of APC/C by substrate ordering influences kinetic differences in the process of ubiquitylation²⁷ and (iii) the binding of proteins with APC/C substrates inhibits ubiquitylation²⁸. Phosphorylation protects Cdc6, Aurora-A, Skp2 and geminin from APC/C-mediated degradation^{16,23-25}. In particular, the inability of phosphorylated Cdc6, Skp2 or geminin to interact with APC/C explains its resistance to APC/C-mediated proteolysis^{23,25}. Recently, we demonstrated that the phosphorylation of human Aurora-A on Ser51 inhibits its APC^{Cdh1}-mediated ubiquitylation and that constitutive phosphorylation on Ser51 is observed in cancer cells with protein overexpression and stabilization¹⁶. As geminin is highly expressed in certain human cancers^{29,30}, it will be interesting to examine the involvement of geminin phosphorylation on Thr25 in cancer.

Geminin restricts nuclear DNA replication to one round per cell cycle, which ensures genome stability and prevents cells from becoming aneuploid¹. Re-replication may occur when the pathways controlling pre-RC formation are impaired. Indeed, geminin depletion in mammalian cells is sufficient to cause re-replication^{19,31}, indicating that geminin plays a critical role in replication control. The pre-RC assembly reaction, known as licensing, involves the loading of the Mcm2-7 complex in an ATP-dependent reaction that requires ORC and two essential factors, Cdc6 and Cdt1 (ref. 4). It is suggested that the binding of geminin to Cdt1 protects the latter from ubiquitylation and subsequent proteolysis⁵. In this study, we found that introduction of *in vitro*-translated geminin^{T25D} protein suppressed SCF^{Skp2}-dependent Cdt1 ubiquitylation (Fig. 7d), suggesting that geminin protects soluble Cdt1 from SCF^{Skp2}-dependent

proteolysis by interfering with the binding between Cdt1 and Skp2. However, as Cdt2 depletion slightly increased soluble Cdt1 protein levels (Fig. 6c), the possibility of Cdt1 degradation by CRL4^{Cdt2} in mitotic geminin depleted cells cannot be denied. Further detailed studies on the mechanism of inhibition of Cdt1 ubiquitylation may be required.

In *Xenopus* eggs, geminin is suggested to inhibit licensing from S phase to metaphase and appears to be inactivated in interphase through activation of the licensing process^{8,32-34}. However, a significant proportion of molecules appears to escape degradation at the metaphase-anaphase transition in *Xenopus* egg extracts³⁴, and the Cdt1-inhibiting activity of these remaining molecules must be suppressed through mechanisms other than by proteolysis. Indeed, exogenous geminin associates with and inhibits Cdt1 in interphase egg extracts^{6, 8,35}. Although geminin rapidly disappears at the exit of mitosis and accumulates during S phase in cultured cells^{2,4}, the remaining pool of geminin in egg extracts may be regulated by Aurora-A-mediated phosphorylation through escape from APC/C-mediated degradation. It is interesting to examine the precise molecular mechanism of this phenomenon in *Xenopus* eggs.

In S phase, chromatin-associated geminin presents even in the absence of Cdt1, possibly as a precaution to prevent Cdt1 from gaining access to chromatin if it accidentally accumulates during S phase³⁶. In this situation, geminin is phosphorylated at Ser45 and Ser49 in HeLa cells³⁷, although the physiological relevance of this modification remains to be clarified. Indeed, we identified geminin phosphorylation on Thr25 by Aurora-A kinase during mitosis. In Fig. 1c, the band of geminin^{T25D} was still shifted at G1 similar to that of wild-type geminin and other geminin mutants (geminin^{S32D} and geminin^{S60D}), suggesting that geminin is phosphorylated at multiple sites during mitosis. These findings indicate that phosphorylation may be important for the functional regulation of geminin in mammalian cells. Furthermore, we found that abundant soluble geminin and Cdt1 protein expression was observed at prometaphase and that abundant chromatin-associated Cdt1 expression was observed during

the G₁ phase. Although it is still unclear how the molecular interaction between Cdt1 and geminin can be regulated during M to G₁ in mammalian cells, recent studies revealed that (i) the geminin-Cdt1 complex can exist in two distinct forms, a licensing-permissive heterotrimer and a licensing-inhibitory heterohexamer, to regulate a molecular switch between licensing-competent and licensing-defective states in *Xenopus* and mammalian cells³⁸ and (ii) the two forms of the Cdt1-geminin complex act as active or inactive complexes for licensing in *Xenopus* egg extracts³⁹. In addition, geminin-mediated nuclear Cdt1 foci formation supports the presence of local interactions between geminin-Cdt1 complexes on chromatin in *Xenopus* egg extracts⁴⁰. Ode *et al.* suggested that the higher-order interactions of Cdt1-geminin complexes are cooperatively stabilized on chromatin⁴⁰. Overall, these findings suggest that licensing for proper DNA replication may be strictly regulated by the geminin protein level during cell cycle progression. The amount of geminin, which is determined by Emi1- and Aurora-A-mediated protection from APC/C-mediated proteolysis, may thus be essential for proper DNA replication.

METHODS

Reagents and antibodies. Reagents were from the following suppliers: MG132 (Z-Leu-Leu-Leu-CHO), Peptide Institute Inc. (Osaka, Japan); CHX, Sigma; λ-PPase, New England Bio Labs; MLN4924, Millennium Pharmaceuticals. The geminin phospho-Thr25-specific antibody was generated by immunizing rabbits with the synthetic peptide CVPRR*TLKMIQ, corresponding to amino acids 21-30 of the human geminin sequence with a phospho-threonine at position 25 (*T). The antibody was purified from serum by two rounds of affinity chromatography on a phospho-Thr25 peptide column followed by a non-phosphopeptide column. The anti-geminin polyclonal antibody was generated using GST-fusion recombinant molecules⁴¹, and the anti-Cdt1 monoclonal antibody was a gift from

Dr. Kristian Helin (University of Copenhagen). Commercial antibodies were from the following suppliers: anti-p27, anti-Aurora-A and anti-Aurora-B antibodies, Transduction Laboratories; anti-HA (Y-11), anti-cyclin A, anti-cyclin B, anti-MCM6, anti-Cdc25A, anti-Cdc6 and anti-GAPDH antibodies, Santa-Cruz Biotechnology; anti-phospho-Histone H3 (Ser10) antibody, Upstate; anti-Cul1, anti-Emi1, anti-Skp2 and anti-PLK1 antibodies, Zymed; anti-Xpress antibody, Invitrogen; anti-Cdh1 antibody, MBL; anti-geminin (2H7) antibody, Abnova; anti-histone H2A antibody, Abcam; anti-FLAG (M2) and anti- β -actin antibodies, Sigma; anti-phospho-tyrosine antibody, Merck Millipore; anti-Cdt1 and anti-Cdt2 antibodies, Bethyl.

Plasmids. pEF-FLAG-tagged geminin and pcDNA-HA-tagged Cdt1 were kind gifts from Dr. Anindya Dutta (University of Virginia). To produce GST-tagged recombinant proteins, geminin cDNA was subcloned into pGEX 4T-1 vectors (GE Healthcare). For *in vitro* translation, geminin cDNA was also subcloned into pcDNA3.1 His/myc-tagged vectors (Invitrogen). pcDNA3.1 His/Xpress-tagged Aurora-A was previously described¹⁵. pCMV HA-tagged Cdt1 and HA-tagged Cdh1 expression vectors were gifts from Dr. Kristian Helin (University of Copenhagen). The pEF, pGEX and pcDNA3.1 vectors encoding the FLAG-tagged geminin substitution mutants (T25A and T25D), the pcDNA3.1 vector encoding His/Xpress-tagged Aurora-A kinase dead mutant (K162R) and the pcDNA3 vector encoding HA-tagged Cdt1 substitution mutant (T29A) were generated using a KOD plus mutagenesis kit (Toyobo).

Cell culture and transient transfection. HeLa, U2OS and 293T cells were routinely maintained in Dulbecco's Modified Eagle Medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated foetal bovine serum (Gibco) and 100 U/ml

penicillin-streptomycin (Gibco) at 37°C and 5% CO₂ in air. For experiments, cells were grown to subconfluence in this medium. Transient transfections were performed using FuGENE HD (Roche) following the manufacturer's instructions. For synchronization, nocodazole (Sigma) and thymidine (Sigma) were used. HeLa cells were synchronized at prometaphase using 50 ng/ml nocodazole treatment followed by mitotic shake-off. Subsequently, synchronized cells were released from the mitotic arrest by washing out nocodazole. Adherent cells remaining after mitotic shake-off were also used. Adherent cells dominantly included the cells arrested at G₂, as confirmed by FACS analysis (data not shown). U2OS were synchronized at the G₁/S border by a double thymidine block. In brief, cells were incubated for 20 hr in 2 mM thymidine (Sigma), released for 8 h, and then incubated for another 20 hr in 2 mM thymidine.

Immunoprecipitation and immunoblot analysis. Cells were transfected with vectors by using FuGENE HD (Roche). Cell lysis and immunoprecipitation were performed as described¹³. Twenty micrograms of protein were subjected to 10% polyacrylamide gel electrophoresis followed by electroblotting onto a nitrocellulose membrane. To detect the immunocomplex, an ECL Western blotting detection system (Amersham) was used. The immunoprecipitates were subjected to immunoblot analysis. To detect phospho-Thr25 geminin, we performed immunoprecipitation with a phospho-specific antibody against Thr25 of geminin followed by immunoblotting analysis with a monoclonal antibody against endogenous geminin or an anti-FLAG antibody against FLAG-tagged exogenous geminin.

***In vitro* ubiquitylation assay.** Ubiquitylation assays were performed as previously described¹⁶. [³⁵S] methionine-labelled human geminin protein was prepared by coupled transcription-translation reactions in wheat germ extract (Promega). GST-tagged Cdh1 (Abnova) or cold *in vitro*-translated human Cdc20 protein was also used. The extracts from

HeLa cells were immunoprecipitated with an anti-Cdc27 antibody (Sigma). Immunoprecipitants were incubated with Cdh1 or Cdc20 for 1 hr at 37°C and washed four times in QA buffer (10 mM Tris-HCl pH7.5, 100 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 1 mM dithiothreitol (DTT)). The purified APC/C^{Cdh1} or APC/C^{Cdc20} complexes were incubated in reaction mixtures containing the following components in a volume of 7 µl: 40 mM Tris-HCl (pH7.6), 1 mg/ml carboxymethyl bovine serum albumin, 1 mM DTT, 5 mM MgCl₂, 10 mM phosphocreatine, 50 µg/ml creatine phosphokinase, 0.5 mM ATP, 50 µM ubiquitin, 1 µM ubiquitin aldehyde, 1 pmol of E1, 5 pmol of E2-C, 1 µM okadaic acid and 1-2 pmol of [³⁵S]methionine-labelled human geminin protein. Following incubation at 30°C for 2 hr, samples were loaded for electrophoresis on an 8% polyacrylamide-SDS gel.

In vitro ubiquitylation assays for Cdt1 were performed in a volume of 30 µl containing 0.1 µM E1 (Boston Biochem), 10 ng/ml Ubch3, 10 ng/ml Ubch5c, 1 µM ubiquitin aldehyde, 2.5 µg/ml ubiquitin (Sigma), and SCF^{Skp2} complex in a ubiquitylation buffer (50 mM Tris [pH 7.6], 2 mM ATP, 5 mM MgCl₂, 0.6 mM DTT, 0.1 µM okadaic acid). *In vitro*-translated HA-tagged wild-type Cdt1 or Cdt1^{T29A} was used as the substrate. The reactions were incubated at 30°C for the indicated times and analysed by SDS-PAGE with an anti-HA antibody. The SCF^{Skp2} complex was obtained from the lysates of 293T cells after transfection with FLAG-tagged Skp2 together with other SCF components. After 24 hr of transfection, cells were incubated with MG132 for 3 hr, prior to lysis. Anti-FLAG antibody was used to immunoprecipitate the SCF^{Skp2} complex. The immunoprecipitants were washed four times in lysis buffer and two times in ubiquitylation reaction buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM MgCl₂, and 1 mM DTT). Finally, beads were then used for *in vitro* ubiquitylation assays.

Chromatin fractionation. For chromatin fractionation, we separated the soluble and insoluble

fractions as previously described⁴². Briefly, cells were lysed in CSK buffer and centrifuged for 4 min at 3200 rpm. After centrifugation, the supernatant contained the soluble fraction. The pellet was resuspended in 2×Laemmli buffer after washing with CSK buffer and then sonicated. This lysate contained the chromatin-enriched fraction.

RNA interference. The following siRNA oligonucleotides were obtained from Qiagen: geminin: CCUAUUGCAUUAAGUACA; Aurora-A: CACCUUCGGCAUCCUAAUA; Cdh1: UCUGGUGGACUGGUCGUCC. Emi1 siRNA was obtained from Dhamacon as the following sequence: GAUUGUGAUCUCUUAUUA. Cdt2 siRNA was obtained from Bonac (Kurume, Japan) as the following sequence: CAAUGGACACCAGAACUCUACCUUU. The negative control (siTrio negative control siRNA) was obtained from Cosmo Bio Inc. (Tokyo, Japan). Transfections were performed using 20 nM siRNA and Oligofectamine[®] RNAi MAX (Invitrogen), according to the manufacturer's instructions.

***In vitro* degradation assay.** For M or G₁ extracts, HeLa cells were synchronized in prometaphase by nocodazole treatment and collected at 0 or 5 hr after mitotic shake-off. The collected cells were lysed in hypotonic buffer (25 mM HEPES pH 7.5, 1.5 mM MgCl₂, 5 mM KCl, 1 mM DTT, 15 mM creatine phosphate, 2 mM ATP) by 10 freeze and thaw cycles and centrifuged for 5 min at 5000 rpm and subsequently for 60 min at 13200 rpm. As a substrate, cold IVT-myc-geminin (wild-type, T25A and T25D) was prepared by coupled transcription-translation reactions in wheat germ extracts (Promega). The substrate was incubated in 20 µl of cell extract and a degradation cocktail containing the following components in a volume of 20 µl: 1.5 mg/ml ubiquitin, 7.5 mM creatine phosphate, 1 mM ATP, 1 mM MgCl₂, 25µg/mL CHX. Following incubation at 30°C for 2, 4 and 6 hr, samples were

loaded for electrophoresis on a 12% polyacrylamide-SDS gel.

Immunofluorescence. Cells were grown on glass coverslips, fixed in 4% paraformaldehyde for 10 min and permeabilized with PBS containing 0.1% Triton X-100 for 20 min. After three rinses with PBS, the coverslips were incubated with the primary antibodies. Anti-geminin, anti-geminin phospho-Thr25 and anti-Aurora-A antibodies were used. Alexa Fluor 488-conjugated anti-mouse or rabbit IgG and Alexa Fluor 594-conjugated anti-mouse or rabbit IgG were used as secondary antibodies (Invitrogen). DNA was visualized by 4',6-diamidino-2-phenylindole (DAPI) staining. Immunostaining of the cell preparations was recorded by using an epifluorescence Zeiss Axioplan 2 microscope (Zeiss, Inc., Thornwood, NY) attached to a charge-coupled-device camera.

***In vitro* kinase assay.** GST-tagged wild-type and geminin^{T25A} mutant geminin were subcloned into pGEX, expressed in *E. coli* and purified. Aurora-A kinase was obtained from Millipore. Purified GST-tagged wild-type geminin or geminin^{T25A} was incubated with Aurora-A in a kinase reaction buffer (25 mM Tris-HCl pH7.5, 5 mM β -glycerophosphate, 2 mM DTT, 0.1 mM Na₃VO₄, 10 mM MgCl₂, 0.1 mM ATP) supplemented with 5 μ Ci of [γ -³²P] ATP at 30°C for 60 min. The reactions were stopped with Laemmli buffer for 5 min at 95°C. After the reaction mixtures were resolved by SDS-PAGE, the gels were stained with coomassie brilliant blue, dried, and subjected to autoradiography.

BrdU staining. Cells were treated with 33 μ M BrdU for 30 min at 10.5 h after double thymidine block release (Fig. 4b). Cells were then fixed with 70% ethanol containing 10 mM glycine for 10 min, stained with mouse anti-BrdU primary antibody (Roche) and fluorescein-conjugated anti-mouse IgG secondary antibody (Roche), and analysed by indirect

immunofluorescence. Nuclei were simultaneously counterstained with DAPI. The BrdU-positive nuclei were counted and plotted. Three independent counts (n=150 cells each count) were used to calculate means \pm S.D.

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AUTHOR CONTRIBUTIONS

T.Ts. performed most of the experiments. Y.K. and M.P. designed the experiments. Y.K., M.P. and Y.T. wrote the manuscript. All authors discussed the experiments and provided suggestions for the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

FIGURE LEGENDS**Figure 1 Geminin is stabilized during mitosis through the blockade of proteasomal**

degradation. (a) Nocodazole arrested-HeLa cells were used as arrested mitotic cells (at mitotic shake-off) (Noc) and adherent cells after mitotic shake-off (Ad). Synchronized cell extracts were treated with lambda phosphatase (λ -PPase) at 30°C for 30 min. Cdc25A and pHH3 was used as controls for phosphorylated proteins. (b) Candidates for conserved phosphorylation sites in geminin. Geminin phosphorylation sites in *Homo sapiens* (Thr25, Ser32 and Ser60) are conserved in other species. D-box indicates an APC/C^{Cdh1}-recognition site designated as a destruction box. (c) HeLa cells were transfected with either FLAG-tagged wild-type (WT) geminin, geminin^{T25D}, geminin^{S32D} or geminin^{S60D}. Cells were synchronized by nocodazole. Cells in M were collected after 0 hr from mitotic shake-off. Cells in G₁ were collected in G₁ after nocodazole release for 4 hr, FLAG-tagged geminin, phospho-histone H3 (pHH3) and Cull1 expression was examined by immunoblotting.

Figure 2 Phosphorylation of geminin on Thr25 during mitosis prevents its proteasomal**degradation by both APC/C^{Cdh1} and APC/C^{Cdc20}.**

(a) HeLa cells were transfected with either WT geminin, geminin^{T25A} or geminin^{T25D} and synchronized in M-G₁ by nocodazole. Cells were collected at 0, 2 and 4 hr after mitotic shake-off. FLAG-tagged geminin, pHH3 and Cull1 expression was examined by immunoblotting. (b) The half-life of *in vitro*-translated (IVT)-myc-tagged geminin proteins (WT, T25A and T25D) examined by the *in vitro* degradation assay. Cell extracts were obtained from synchronized HeLa cells in G₁ (5 hr after mitotic shake-off). Cell lysates with IVT-myc-tagged geminin protein were incubated for the indicated times. IVT-myc-tagged geminin protein expression was examined by immunoblotting with an anti-myc antibody. (c) An *in vitro* ubiquitylation assay was performed using ³⁵S-labeled IVT-geminin WT, geminin^{T25A} or geminin^{T25D} and immuno-purified

APC/C^{Cdh1} from HeLa cells. ³⁵S-labeled IVT-geminin protein was incubated for the indicated times at 30°C. The abundance of ³⁵S-labeled IVT-geminin proteins was detected by autoradiography. (d) FLAG-tagged geminin WT, geminin^{T25A} or geminin^{T25D} with or without HA-tagged Cdc20 or Cdh1 were transfected into 293T cells. After 24 hr, cells were treated with 25µM MG132 for 7 hr. Cell extracts were immunoprecipitated with an anti-HA antibody and blotted with an anti-FLAG antibody and an anti-HA antibody. IP: immunoprecipitation; WCE: whole cell extracts. (e) The half-life of IVT-myc-tagged geminin proteins (WT, T25A and T25D) was examined by the *in vitro* degradation assay. Cell extracts were obtained from synchronized HeLa cells in M (at mitotic shake-off). Cell lysates were incubated with IVT-myc-tagged geminin protein for the indicated times. IVT-myc-tagged geminin protein expression was examined by immunoblotting with an anti-myc antibody. (f) An *in vitro* ubiquitylation assay was performed using ³⁵S-labeled IVT-geminin WT, geminin^{T25A} or geminin^{T25D} and immuno-purified APC/C^{Cdc20} from HeLa cells. ³⁵S-labeled IVT-geminin proteins were incubated for the indicated times at 30°C. The abundance of these proteins was detected by autoradiography.

Figure 3 Phosphorylation of geminin on Thr25 by Aurora-A kinase during mitosis. (a) Comparison of the amino acid sequences of human geminin from 22 to 27 with those of phosphorylation sites of other known Aurora-A substrates. (b) Subcellular co-localization of geminin and Aurora-A during mitosis. Expression of geminin (green) and Aurora-A (Red) was visualized in HeLa cells by immunofluorescence analysis. (c) FLAG-tagged geminin WT or empty vector was transfected into 293T cells with or without Xpress-tagged Aurora A. Cell lysates were immunoprecipitated with an anti-Xpress antibody. IP: immunoprecipitation, WCE: whole cell extracts. (d) Nocodazole arrested-HeLa cells were used as arrested mitotic cells (at mitotic shake-off) and adherent cells after mitotic shake-off. Cell lysates were

immunoprecipitated with an anti-geminin polyclonal antibody. Noc: arrested mitotic cells (at mitotic shake-off), Ad: adherent cells after mitotic shake-off. (e) *In vitro* Aurora-A kinase assay using GST-tagged recombinant geminin protein (WT or T25A) and γ^{32} -ATP. The phosphorylation was detected by autoradiography. (f) *In vivo* Aurora-A kinase assay using 293T cells transfected with Xpress-tagged Aurora-A WT or the kinase-dead mutant (K/R). After 24 hr, cells were collected and lysed. Phosphorylation status was examined by immunoblotting with an anti-phosphorylated geminin Thr25-specific antibody (P-geminin^{Thr25}). (g) Levels of geminin phosphorylation on Thr25 in Aurora-A depleted cells. 293T cells were co-transfected with FLAG-tagged geminin WT and control or Aurora-A siRNA. Expression of phospho-geminin (P-geminin^{Thr25}), FLAG-tagged geminin, Aurora-A and Cull1 was examined by immunoblotting. (h) The half-life of IVT-myc-tagged geminin proteins (WT and T25A) after Aurora-A-mediated phosphorylation was examined by an *in vitro* degradation assay. Cell extracts were obtained from synchronized HeLa cells in M phase (at mitotic shake-off). IVT-myc-tagged geminin protein (WT or T25A) was incubated with recombinant Aurora-A protein in kinase buffer at 30°C for 1hr. Then, IVT-myc-tagged geminin protein was incubated with cell lysates for the indicated times. Expression of IVT-myc-tagged geminin protein was examined by immunoblotting with an anti-myc antibody.

Figure 4 Geminin stabilization during mitosis controls pre-RC formation. (a) Experimental outline for the synchronization of U2OS cells. Cells were synchronized with double thymidine block as indicated schedule. G₁/S arrested cells were released and transfected siRNA for 8 hr, followed by nocodazole treatment for 12 hr. Mitotic cells were collected by shake-off and released for indicated time. (b) Geminin depletion during mitosis impaired licensing at mitotic exit. U2OS cells were treated with geminin siRNA during release from the second thymidine block and collected at 0, 1 and 2 hr after release from the nocodazole block as

described in Fig. 4a. After release from the first thymidine block, cells were transfected with FLAG-tagged geminin^{T25D}. The soluble and chromatin fractions were separated and immunoblotted for the indicated proteins. (c) Aurora-A depletion during mitosis impaired licensing at mitotic exit. U2OS cells were treated with Aurora-A siRNA during release from the second thymidine block and collected at 0, 1 and 2 hr after release from the nocodazole block as described in Fig. 4a. After release from the first thymidine block, cells were transfected with FLAG-tagged geminin^{T25D}. The soluble and chromatin fractions were separated and immunoblotted for the indicated proteins.

Figure 5 Depletion of geminin or Aurora-A during mitosis impaired DNA replication. (a)

Geminin depletion during mitosis impaired licensing at mitotic exit. U2OS cells were treated with geminin siRNA during release from the second thymidine block and collected at 0, 1 and 2 hr after release from the nocodazole block as described in Fig. 4a. After release from the first thymidine block, cells were transfected with HA-tagged Cdt1. The soluble and chromatin fractions were separated and immunoblotted for the indicated proteins. (b) Aurora-A depletion during mitosis impaired licensing at mitotic exit. U2OS cells were treated with Aurora-A siRNA during release from the second thymidine block and collected at 0, 1 and 2 hr after release from the nocodazole block as described in Fig. 4a. After release from the first thymidine block, cells were transfected with HA-tagged Cdt1. The soluble and chromatin fractions were separated and immunoblotted for the indicated proteins. (c) U2OS cells plated on glass coverslips were synchronized as described in Fig. 4a and labelled with BrdU for 30 min at 10.5 hr after release from the nocodazole block. Cells were then fixed and stained for BrdU (green) by indirect immunofluorescence analysis. Figure shows representative images obtained by indirect immunofluorescence analysis of the cells. The cell nuclei were stained with DAPI (blue). (d) Graph shows the average number of BrdU-positive cells in geminin or Aurora-A

mitotic depleted cells with or without HA-tagged Cdt1.

Figure 6 Downregulation of Cdt1 by geminin or Aurora-A mitotic depletion is caused by SCF^{Skp2}. (a) U2OS cells were treated with geminin or Aurora-A siRNA during release from the second thymidine block and collected immediately after release from the nocodazole block as described in Fig. 4a. Cells were treated with 25 μ M MG132 for 5 hr before mitotic shake-off. The soluble fraction was separated and immunoblotted for the indicated proteins. (b) U2OS cells were treated with geminin or Aurora-A siRNA during release from the second thymidine block and collected immediately after release from the nocodazole block as described in Fig. 4a. Cells were treated with 1 μ M MLN4924 for 2 hr before mitotic shake-off. The soluble fraction was separated and immunoblotted for the indicated proteins. (c) U2OS cells were treated with geminin siRNA with or without Skp2 or Cdt2 siRNA during release from the second thymidine block and collected immediately after release from the nocodazole block as described in Fig. 4a. The soluble fraction was separated and immunoblotted for the indicated proteins.

Figure 7 Geminin or Aurora-A depletion during mitosis induces SCF^{Skp2}-dependent proteolysis of soluble Cdt1 protein. (a) Geminin depletion during mitosis impaired licensing at mitotic exit. U2OS cells were treated with geminin and/or Skp2 siRNA during release from the second thymidine block and collected at 0 and 2 hr after release from the nocodazole block as described in Fig. 4a. The soluble and chromatin fractions were separated and immunoblotted for the indicated proteins. (b) Aurora-A depletion during mitosis impaired licensing at mitotic exit. U2OS cells were treated with Aurora-A and/or Skp2 siRNA during release from the second thymidine block and collected at 0 and 2 hr after release from the nocodazole block. Soluble and chromatin fractions were separated and immunoblotted for the indicated proteins. (c) Cells were treated with the geminin siRNA during release from second thymidine block and

collected immediately after mitotic shake-off. After release from the first thymidine block, cells were transfected with 0.5, 1 or 2 μg of FLAG-tagged geminin^{T25D}. Cdt1, FLAG-tagged geminin, Skp2 and Cull1 expression was examined by immunoblotting. (d) An *in vitro* ubiquitylation assay was performed using wild-type or T29A mutant IVT-HA-tagged Cdt1 and immuno-purified SCF^{Skp2} from 293T cells. Wild-type IVT-HA-tagged Cdt1 protein was incubated with or without IVT-myc-tagged geminin^{T25D} protein for the indicated times. As a negative control, IVT-HA-tagged Cdt1^{T29A} protein known as a non-degradable mutant for SCF^{Skp2}-mediated degradation was also used. The abundance of IVT-HA-tagged Cdt1 was detected by SDS-PAGE with a HA antibody. (e) Schematic model for pre-RC formation regulated by the Aurora-A-geminin-Cdt1 axis. Aurora-A protects geminin from APC/C^{Cdc20}- and APC/C^{Cdh1}-mediated proteolysis through phosphorylation. Phosphorylated geminin may stabilize soluble Cdt1 protein by inhibiting SCF^{Skp2}-mediated proteolysis. Stabilized soluble Cdt1 may load onto chromatin after dephosphorylation and then form the pre-RC.

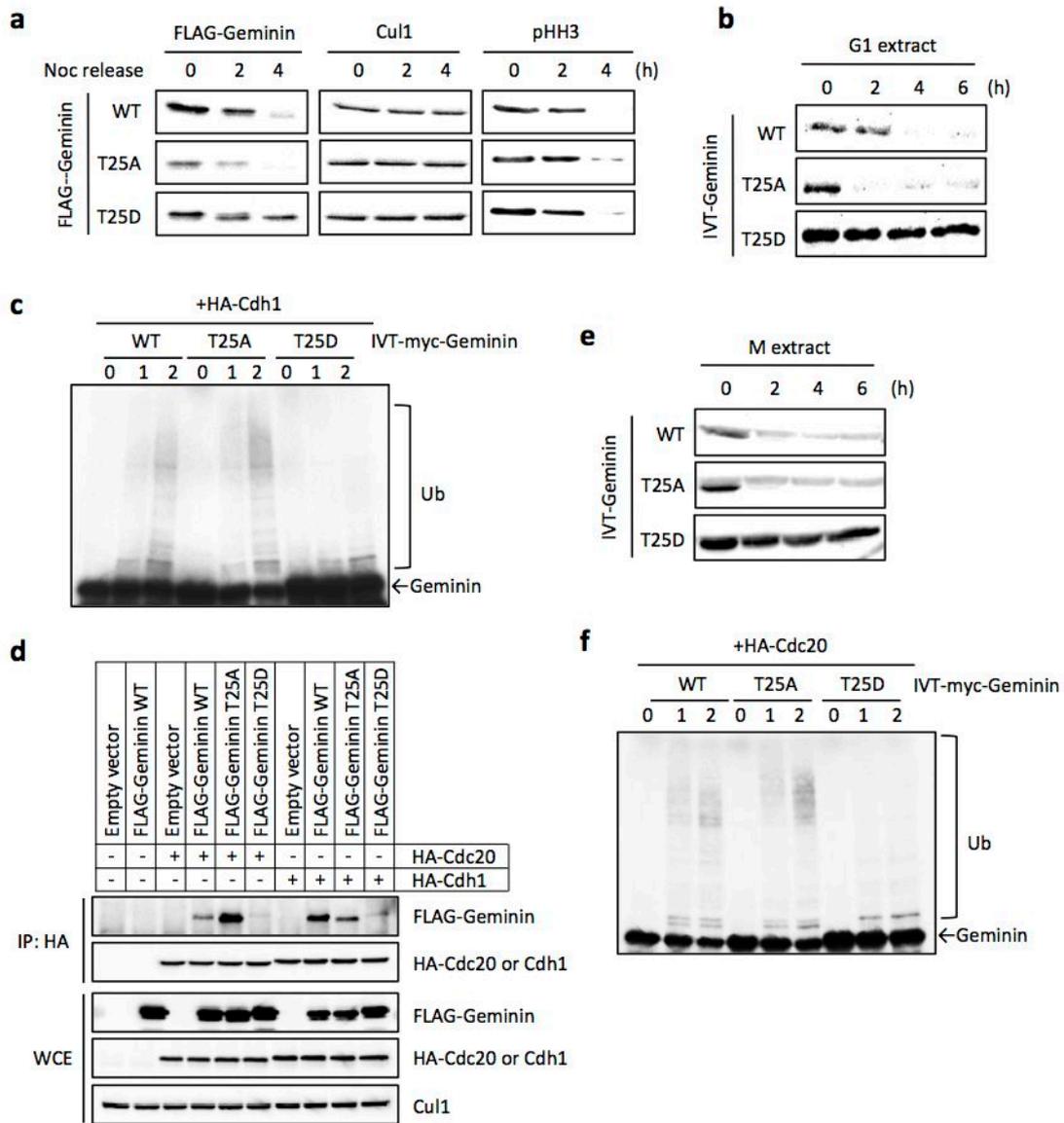


Figure 2. Tsunematsu *et al.*

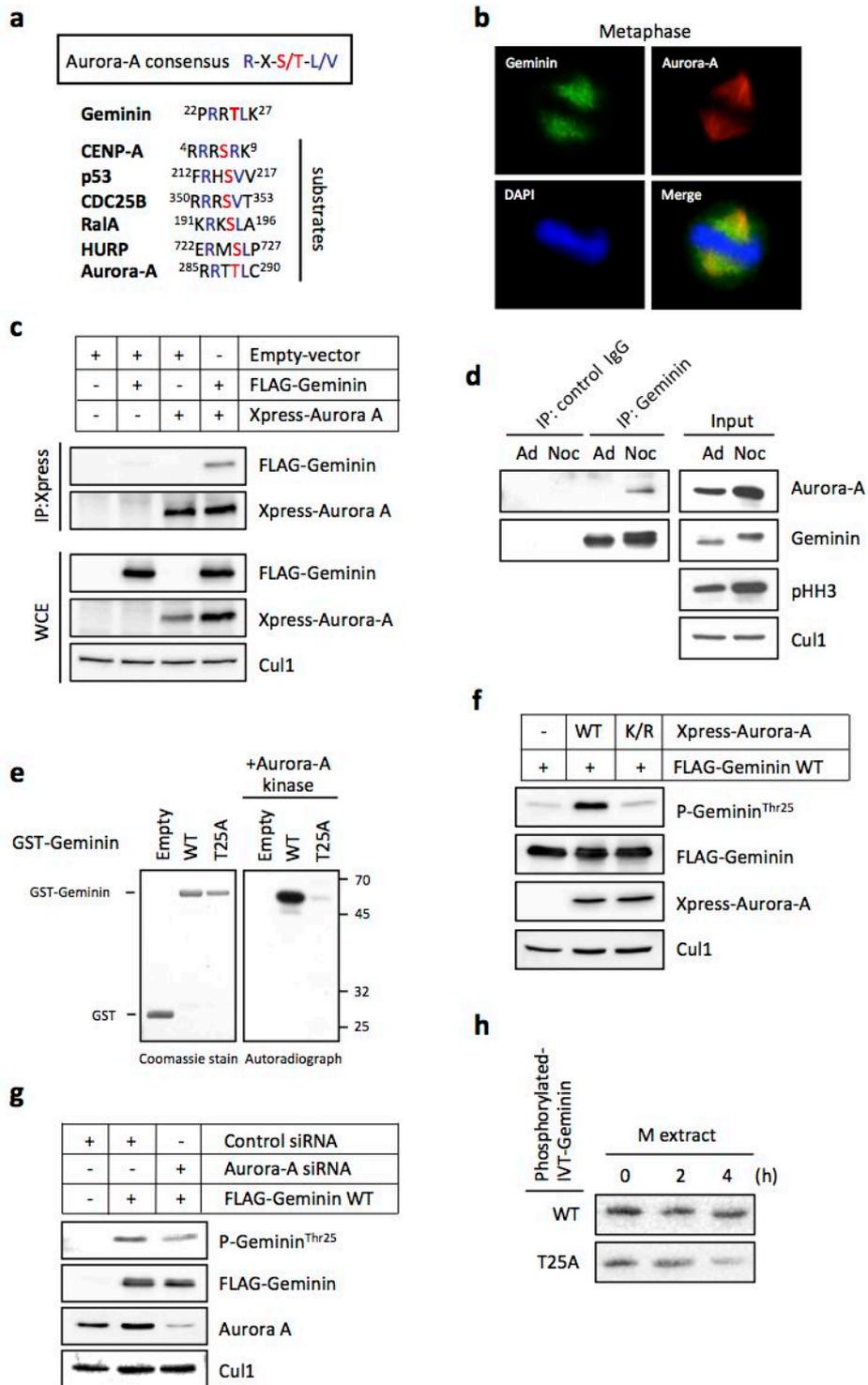


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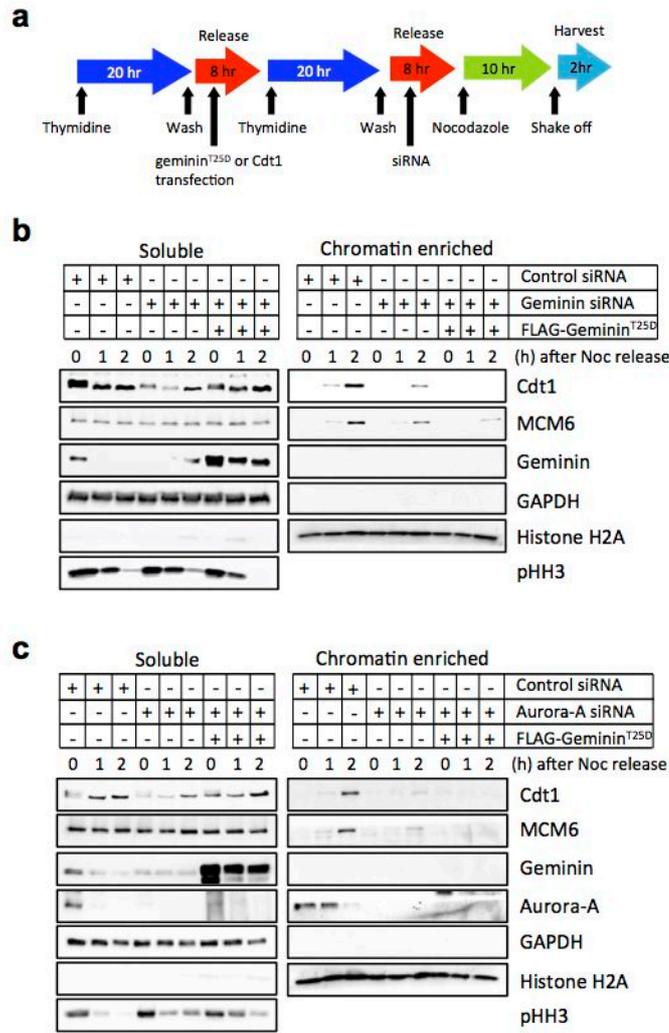


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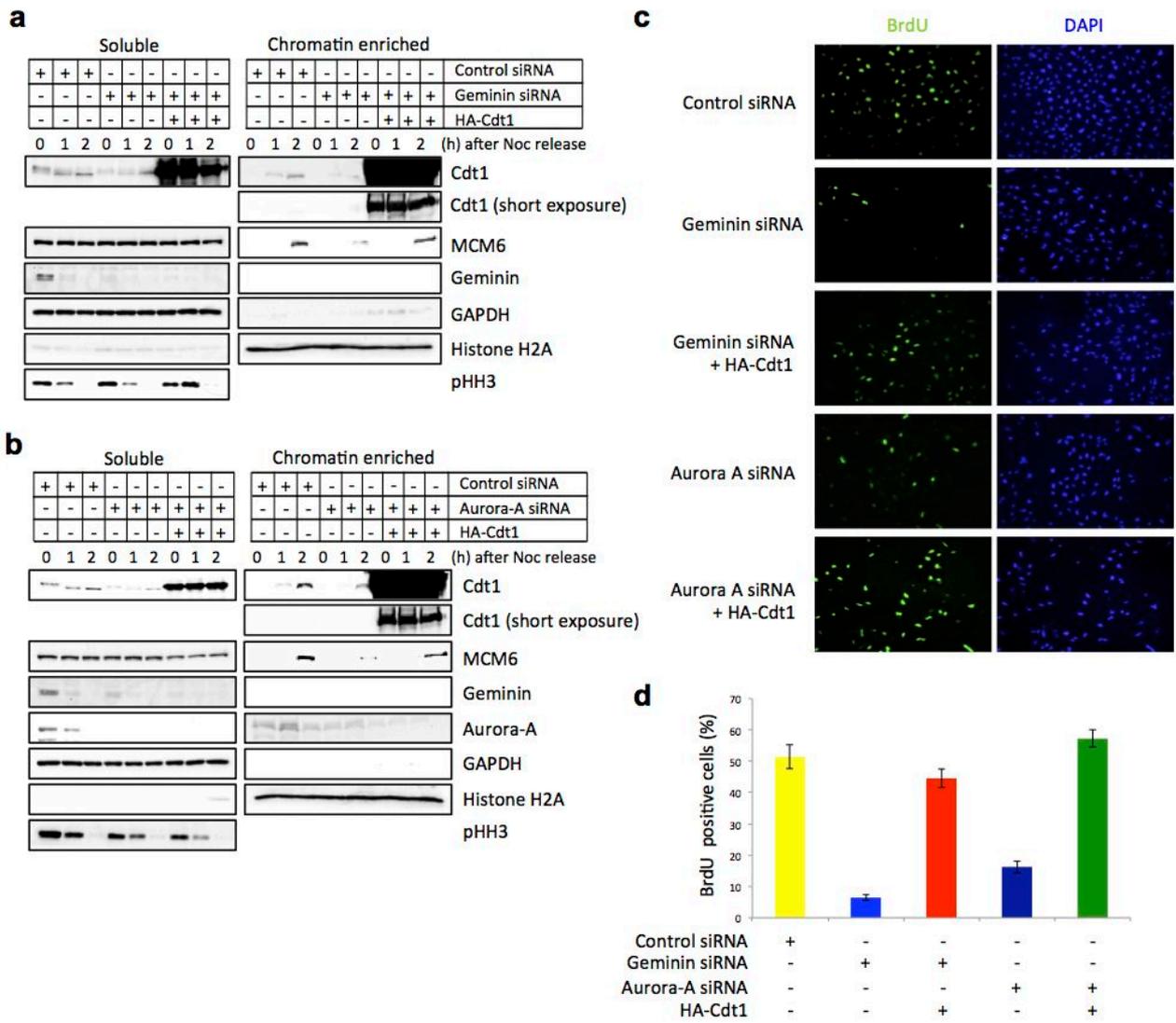


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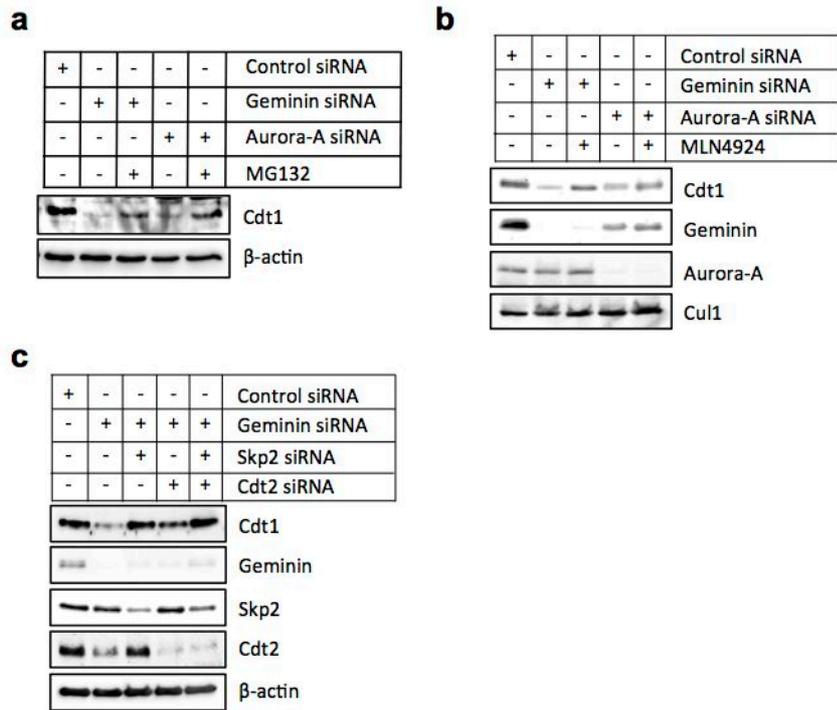


Figure 6. Tsunematsu *et al.*

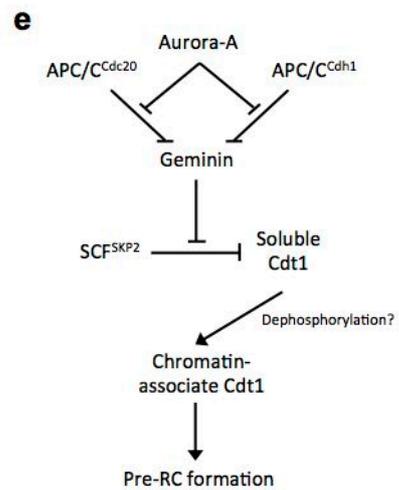
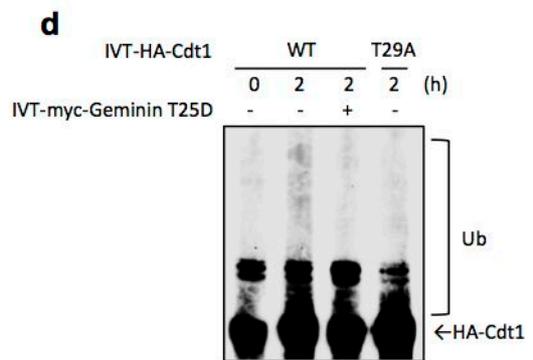
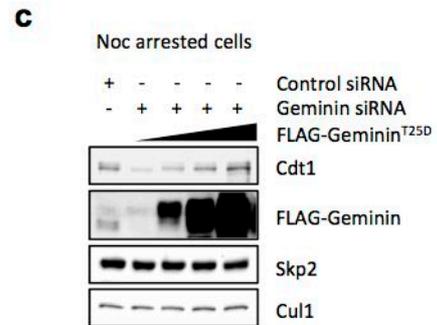
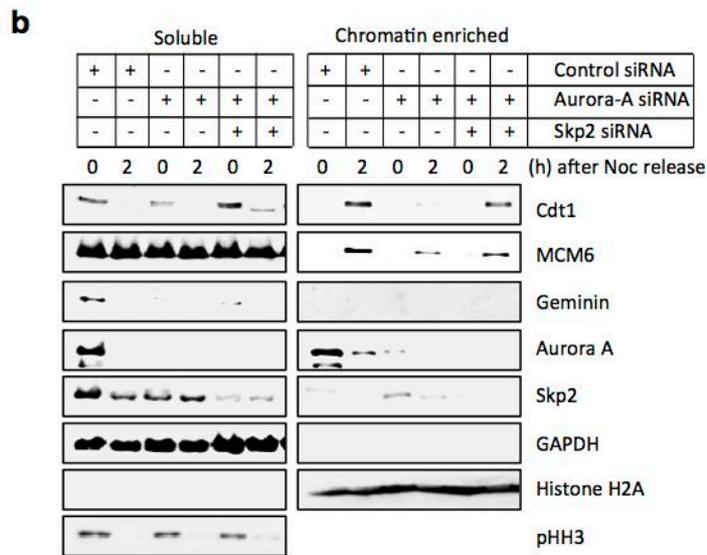
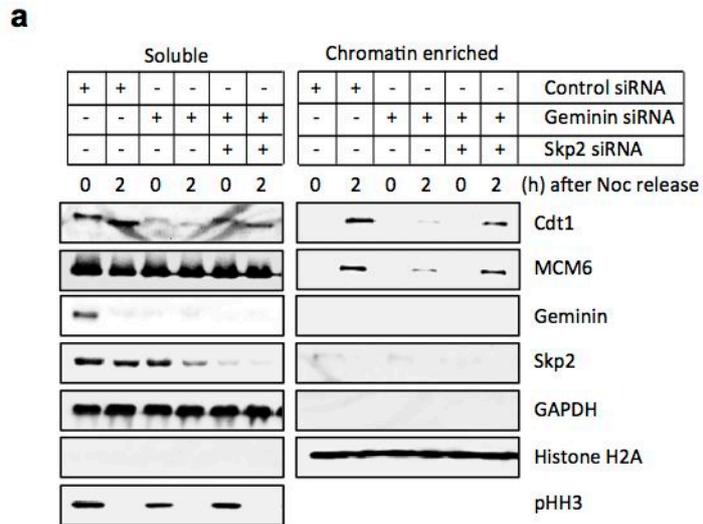
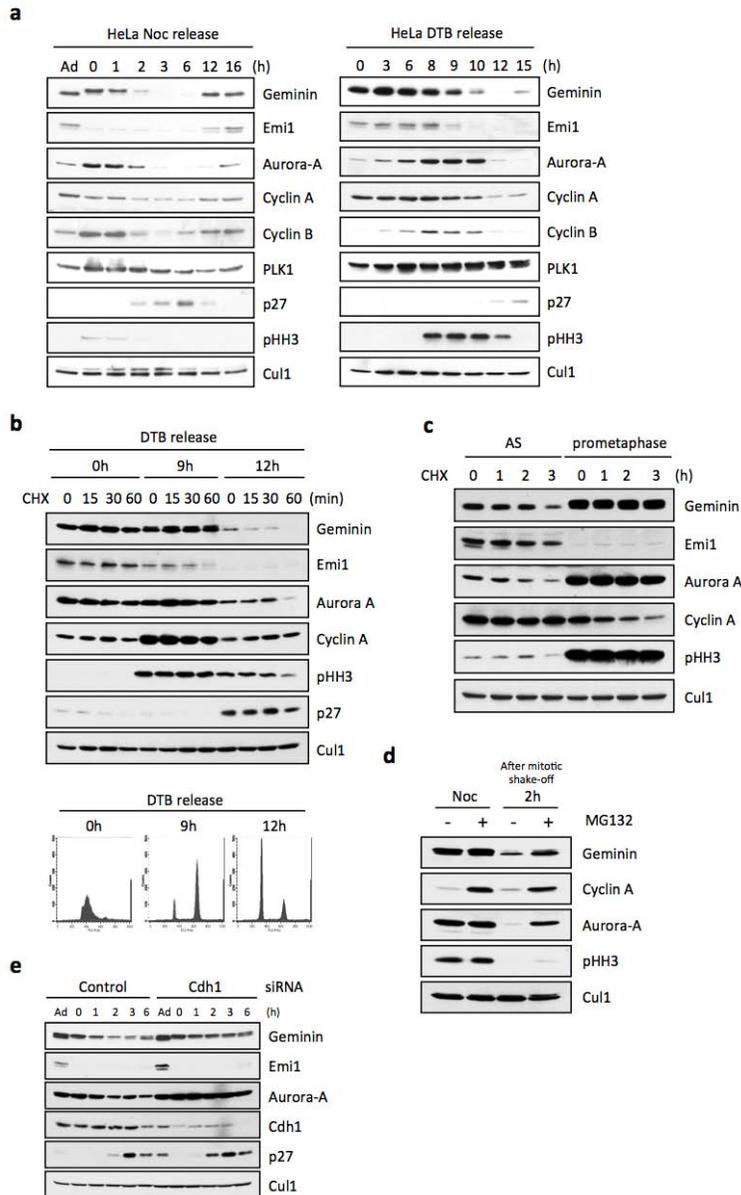


Figure 7. Tsunematsu *et al.*

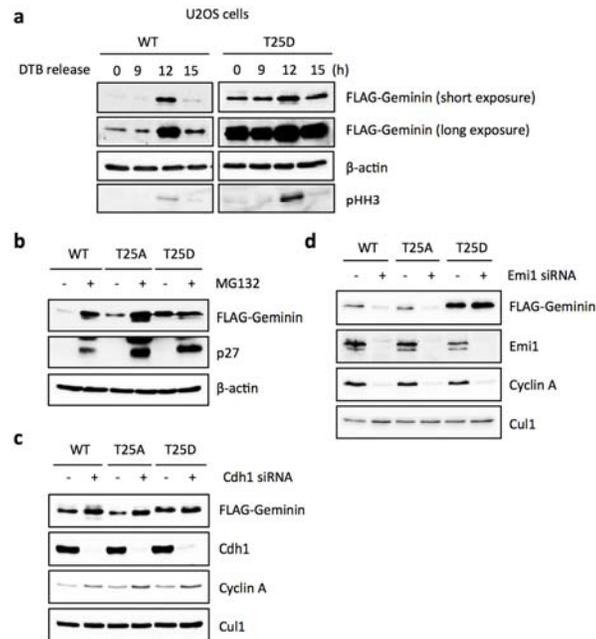
Supplementary Figure S1 Stabilization of geminin during mitosis.



(a) HeLa cells were released from a prometaphase arrest with 50 ng/ml nocodazole and collected at the indicated times (left panel). HeLa cells were synchronized at the G₁/S border by a double thymidine block (right panel). After release, cells were collected at the indicated times. The endogenous geminin protein level was analysed by immunoblotting. The expression of cell cycle regulatory proteins including Emi1, Aurora-A, Cyclin A, Cyclin B, PLK1, p27 and pHH3 was examined by immunoblotting. Cul1 expression was used as a loading control. 'Ad' indicates adherent cells after mitotic shake-off. These cells largely composed of G₂ phase cells by FACS analysis (Data not shown) (b) Half-life of endogenous geminin during cell cycle progression. HeLa cells were synchronized by a double thymidine block and released. Then, 25 µg/mL CHX was added at 0 hr (S phase), 9 hr (M phase) and 12 hr (G₁

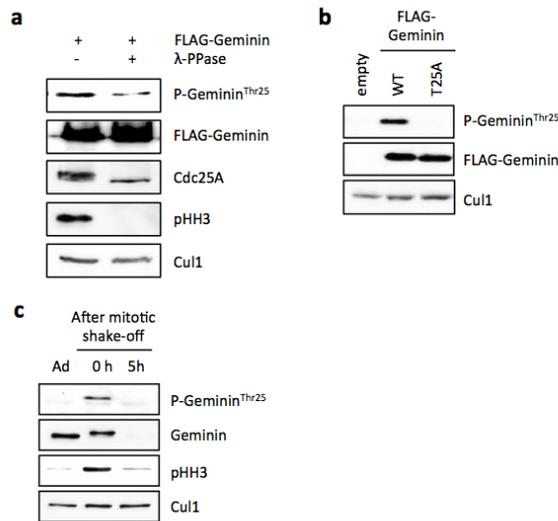
phase) and incubated as indicated times. The cell cycle distribution of each time point was confirmed by flow cytometric analysis. (c) Half-life of endogenous geminin during mitosis. Nocodazole-arrested HeLa cells were treated with 25 µg/mL CHX at the indicated times. (d) HeLa cells were synchronized at the indicated time by mitotic shake-off and were released. Cells were treated with or without 25 µM MG132 for 5 hr before mitotic shake-off (Noc). Moreover, cells were treated with or without 25 µM MG132 for 5 hr after 2 hr from mitotic shake-off. Geminin, cyclin A, Aurora-A and pHH3 expression was examined by immunoblotting. Cul1 expression was used as a loading control. (e) HeLa cells were transfected with control or Cdh1 siRNA and followed by nocodazole treatment for 12hr. After release from nocodazole block, cells were collected at the indicated times. Adherent cells after mitotic shake-off were also collected (Ad). Expression of geminin, Emi1, Aurora-A, Cdh1 and p27 was examined by immunoblotting. Cul1 expression was used as a loading control.

Supplementary Figure S2 Stabilization of geminin phosphorylated on Thr25 during mitosis.



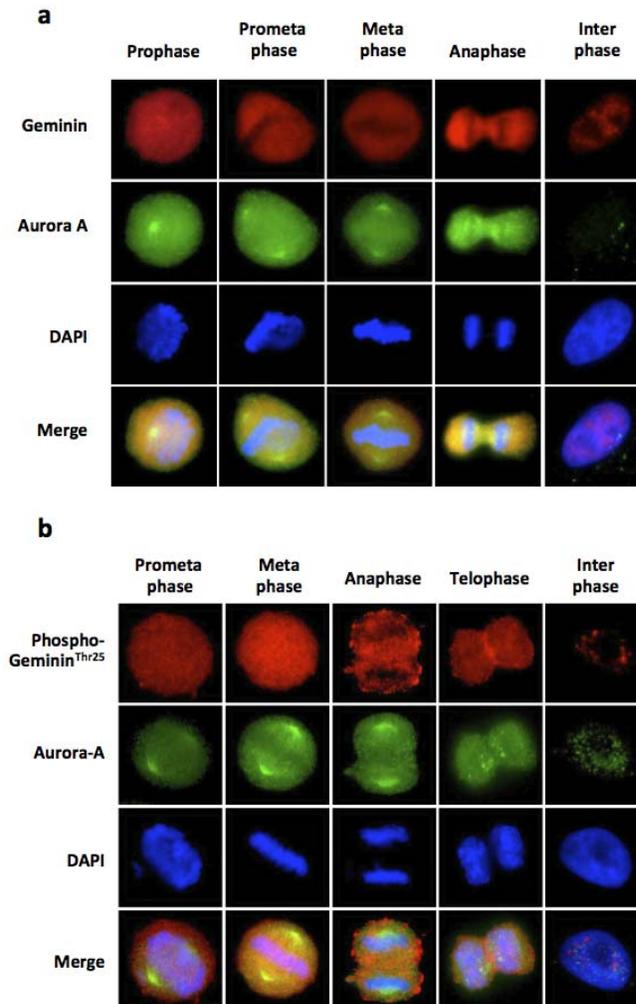
(a) U2OS cells were transfected with WT and geminin^{T25D}, synchronized at the G₁/S border by a double thymidine block and subsequently released at S to G₁ at the indicated times. FLAG-tagged geminin, pHH3 and β-actin expression was examined by immunoblotting. (b) FLAG-tagged geminin (WT, T25A and T25D)-transfected HeLa cells were treated with 25 μM MG132 for 5 hr, and FLAG-tagged geminin, p27 and β-actin expression was examined by immunoblotting. p27 was used as a control for MG132 treatment. (c) Depletion of Cdh1 was performed using siRNA in FLAG-tagged geminin (WT, T25A and T25D)-expressing HeLa cells. FLAG-tagged geminin, Cdh1, cyclin A and Cul1 expression was examined by immunoblotting. Cyclin A was used as a control for APC/C^{Cdh1} substrate. (d) Depletion of Emi1 was performed using siRNA in FLAG-tagged geminin-transfected HeLa cells. FLAG-tagged geminin, Emi1, cyclin A and Cul1 expression was examined by immunoblotting. Cyclin A was used as a control for APC/C^{Cdh1} substrate.

Supplementary Figure S3 Generation and characterization of an antibody against geminin phosphorylated on Thr25.



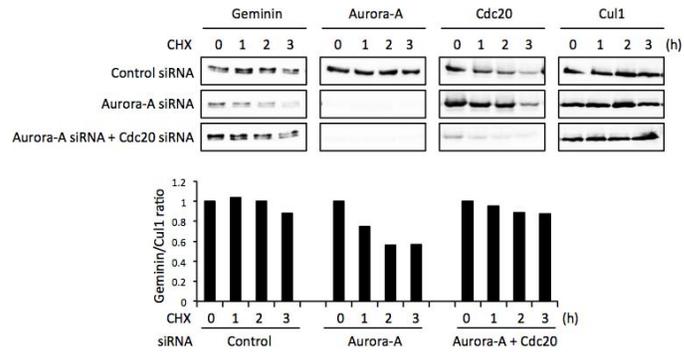
(a) Extracts from FLAG-tagged geminin-transfected HeLa cells were treated with λ -PPase for 1 hr at 30°C and analysed for geminin phosphorylation status by immunoblotting with an anti-phospho-geminin^{Thr25} antibody (P-geminin^{Thr25}). Cdc25A and pHH3 expression was used as a positive control, and Cul1 was used as a loading control. (b) Phosphorylation levels of geminin WT and geminin^{T25A} was examined in nocodazole-treated 293T cells by immunoblotting with an anti-phospho-geminin^{Thr25} antibody (P-geminin^{Thr25}). (c) Phosphorylation levels of endogenous geminin in synchronized HeLa cells was detected by immunoblotting with an anti-phospho-geminin^{Thr25} antibody. For synchronization, cells were treated with 50 ng/ml nocodazole for 12 hr. Adherent cells after mitotic shake-off (Ad) and cells obtained 0 and 5 hr after mitotic shake-off were used.

Supplementary Figure S4 Geminin phosphorylated on Thr25 co-localized with Aurora-A during mitosis.

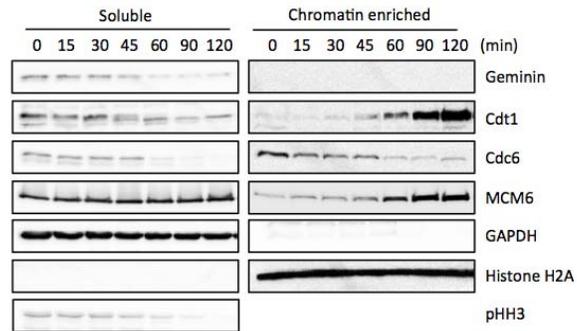


HeLa cells were synchronized at prometaphase by nocodazole treatment followed by mitotic shake-off. Subsequently, synchronized cells were released from mitotic shake-off. DNA was visualized by DAPI staining (blue). (a) Co-immunostaining of geminin (red) and Aurora-A (green). (b) Co-immunostaining of phosphorylated geminin at Thr25 (phospho-geminin^{Thr25}) (red) and Aurora-A (green).

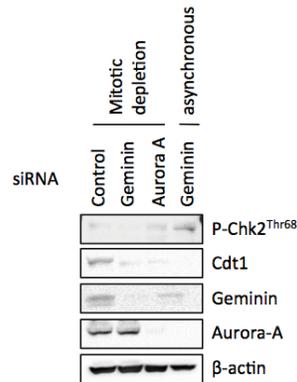
Supplementary Figure S5 Influence of Aurora-A and/or Cdc20 depletion on the half-life of endogenous geminin in arrested mitotic cells.



U2OS cells were treated with Aurora-A siRNA with or without Cdc20 siRNA during release from the second thymidine block and collected immediately after release from the nocodazole block as described in Fig. 4a. Nocodazole-arrested U2OS cells were treated with 25 $\mu\text{g}/\text{mL}$ CHX at the indicated times. Geminin, Cdc20, Aurora-A and Cul1 expression was examined by immunoblotting. Anti-Cdc20 antibody was obtained from Santa-Cruz Biotechnology.

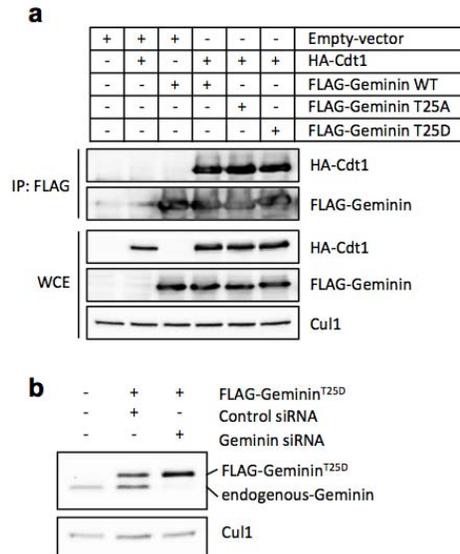
Supplementary Figure S6 Oscillations of soluble and chromatin-associated geminin, Cdt1, Cdc6 and MCM6 during mitosis and G₁.

After release from a double thymidine block, U2OS cells were treated with nocodazole. After release from the nocodazole block, cells were collected at 0, 15, 30, 45, 60, 90 and 120 min. The soluble and chromatin fractions were separated and immunoblotted for the indicated proteins.

Supplementary Figure S7 No DNA damage response induced after the mitotic depletion of geminin or Aurora-A.

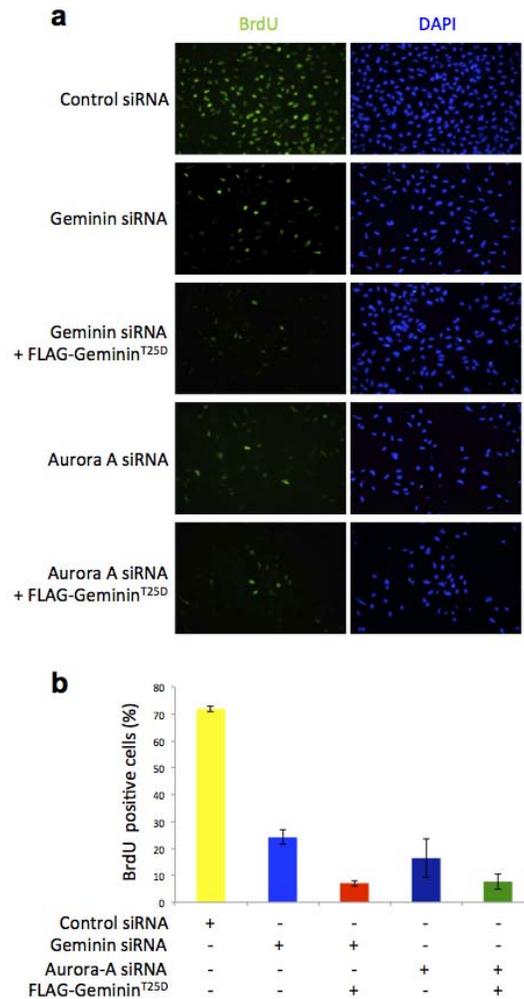
U2OS cells were treated with geminin or Aurora-A siRNA during release from the second thymidine block and were collected at 0 h after release from the nocodazole block as described in Fig. 4a. As a positive control of the DNA damage response, we used asynchronous U2OS cells after 48 hr of geminin siRNA treatment. Phospho-Chk2^{Thr68}, Cdt1, geminin, Aurora-A and β-actin expression was examined by immunoblotting.

Supplementary Figure S8 Aurora-A depletion during mitosis impaired licensing at mitotic exit.



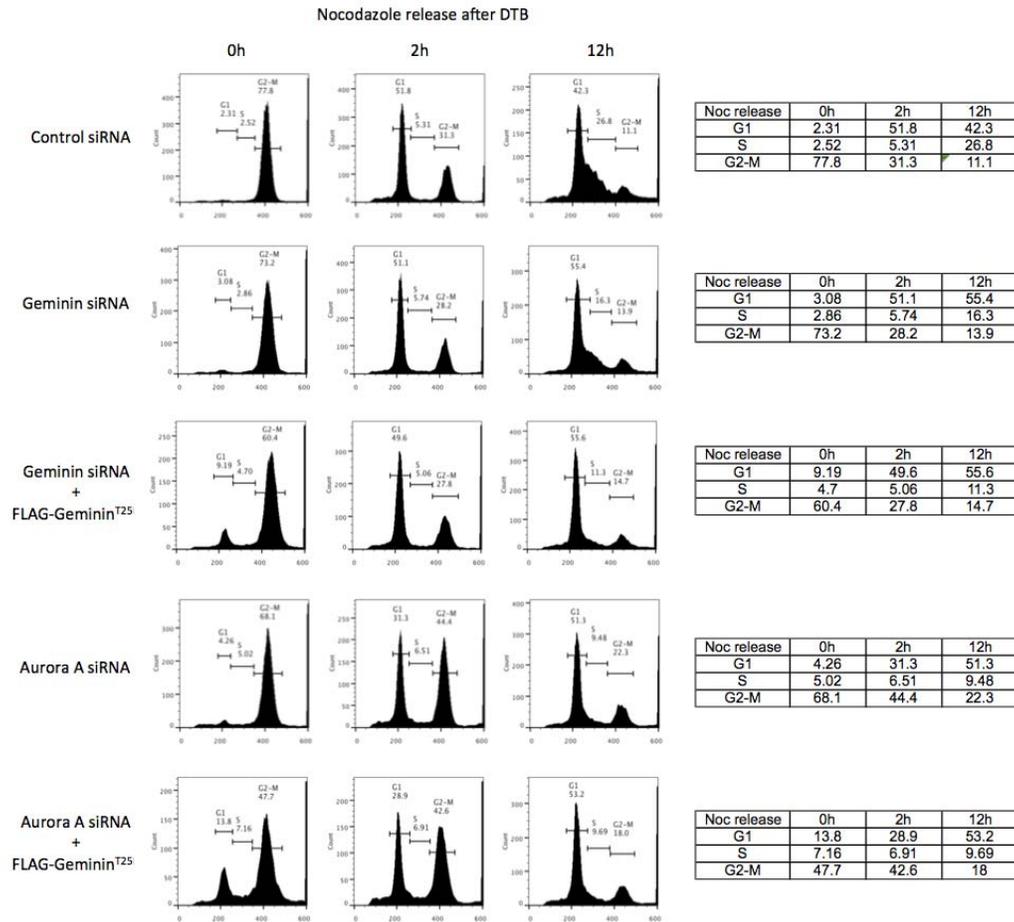
(a) Interaction between geminin and Cdt1. 293T cells were co-transfected with FLAG-tagged geminin (WT, T25A and T25D) and HA-tagged Cdt1. Transfected 293T cells were treated with nocodazole for 12 hr. Cell extracts were immunoprecipitated using an anti-FLAG antibody, and the precipitates were blotted with anti-FLAG and anti-HA antibodies. β -actin was used as a loading control. IP, immunoprecipitation; WCE, whole cell extracts. (b) Ectopic expression of FLAG-tagged geminin^{T25D} in geminin-depleted HeLa cells by the siRNA. Twenty-four hours before siRNA treatment, cells were transfected with FLAG-tagged geminin^{T25D}. Cells were collected and blotted with an anti-geminin antibody. The anti-geminin antibody can recognize exogenous (FLAG-geminin^{T25D}) and endogenous geminin. Cul1 was used as a loading control.

Supplementary Figure S9 Depletion of geminin or Aurora-A during mitosis impaired DNA replication.



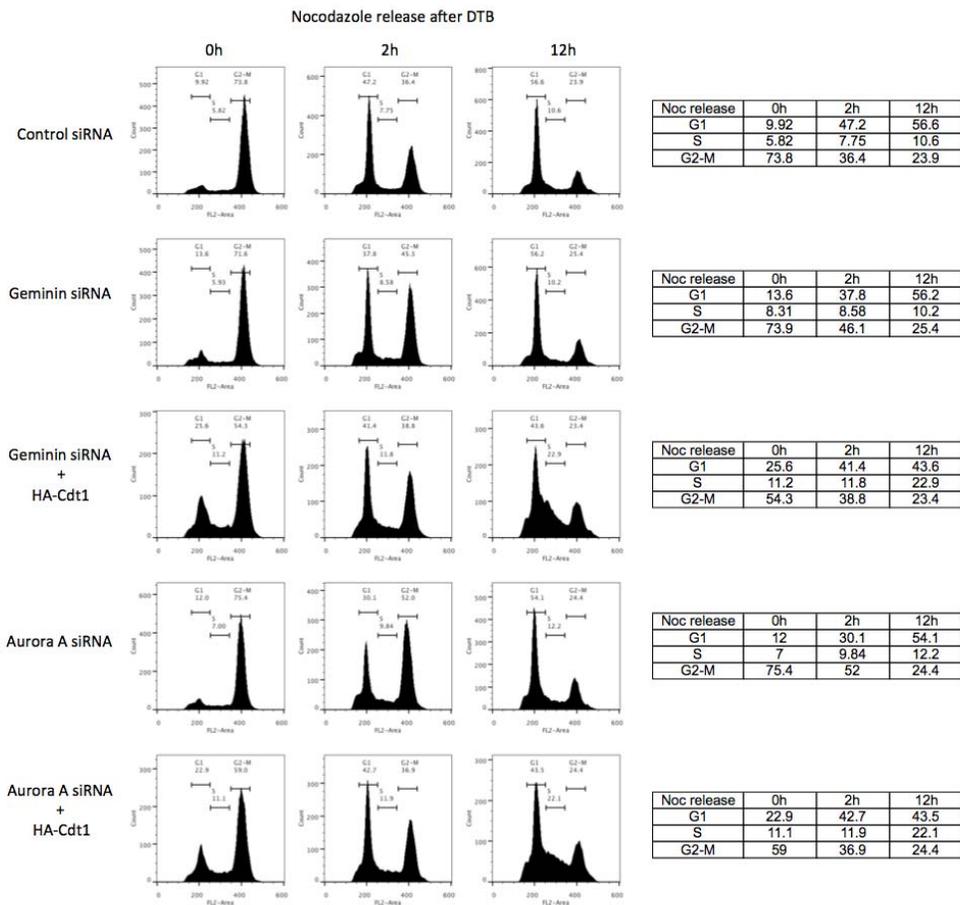
(a) U2OS cells plated on glass coverslips were synchronized as described in Fig. 4a and labeled with BrdU for 30 min at 10.5 hr after release from the nocodazole block. Cells were then fixed and stained for BrdU (green) by indirect immunofluorescence analysis. Figure shows representative images obtained by indirect immunofluorescence analysis of the cells. The cell nuclei were stained with DAPI (blue). (b) Graph shows the average number of BrdU-positive cells in geminin or Aurora-A mitotic depleted cells with or without FLAG-tagged geminin^{T25D}.

Supplementary Figure S10 FACS analysis of mitotic depletion of geminin or Aurora-A with or without ectopic geminin^{T25D} overexpression.



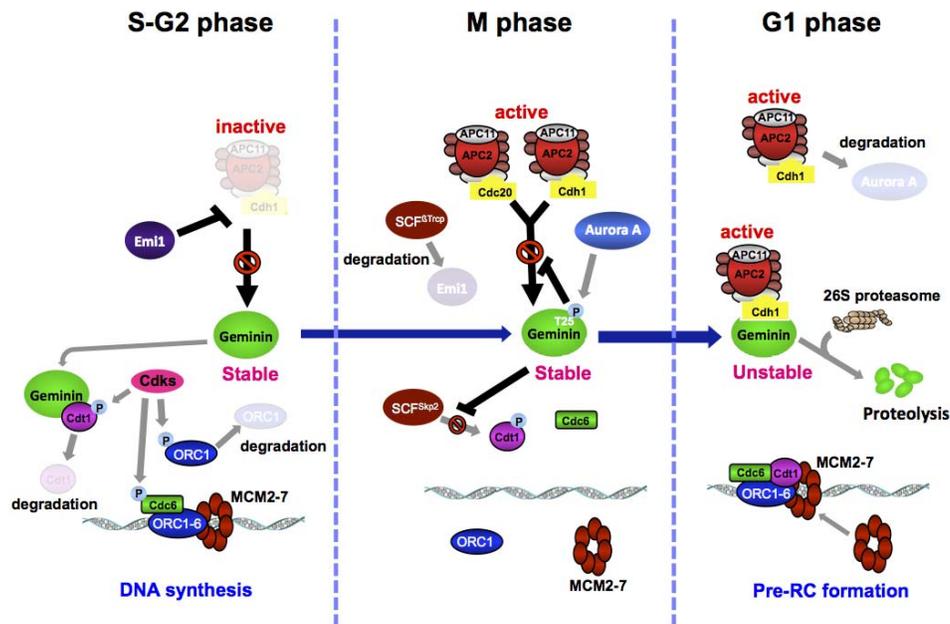
U2OS cells were synchronized as described in Fig. 4a. Cells were collected at the indicated times after nocodazole release as shown in Fig. 4b and 4c. Samples were fixed and stained as described in the Methods section, and then analysed by FACS.

Supplementary Figure S11 FACS analysis of mitotic depletion of geminin or Aurora-A with or without ectopic Cdt1 overexpression.



U2OS cells were synchronized as described in Fig. 4a. Cells were collected at the indicated times after nocodazole release as shown in Fig. 5a and 5b. Samples were fixed and stained as described in the Methods section, and then analysed by FACS.

Supplementary Figure S12 Schematic model for pre-RC formation regulated by the Aurora-A-geminin- Cdt1 axis.



In mitosis, Aurora-A phosphorylates geminin on Thr25. Thr25 phosphorylation protects it from APC/C^{Cdc20}- and APC/C^{Cdh1}-mediated proteolysis. Moreover, phosphorylated geminin on Thr25 stabilizes soluble Cdt1 protein by inhibiting its SCF^{Skp2}-mediated proteolysis. At late M, Aurora-A protein is degraded by APC/C^{Cdh1} and subsequently, geminin is degraded by APC/C^{Cdh1}. After geminin degradation, Cdt1 loads on chromatin and forms pre-RC in G₁.

Supplementary Methods

Flow cytometry. Flow cytometric analysis was previously described⁴³. Cell cycle distribution was determined by DNA content analysis after propidium iodide staining (Sigma). Cells were cultured as described above, fixed in 70% ethanol and stored at 4°C before analysis. Flow cytometric determination of DNA content was performed on a FACS Calibur (Becton-Dickinson, San Jose, CA) flow cytometer. For each sample, 10,000 events were stored.

Antibody. Anti-phospho-Chk2^{Thr68} antibody was obtained from Cell signaling technology.

Supplementary References

43. Tsunematsu T, *et al.* RUNX3 has an oncogenic role in head and neck cancer. *PLoS ONE* **4**, e5892 (2009).