Cyclin A–Cdk1 regulates the origin firing program in mammalian cells

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Somatic mammalian cells possess well-established S-phase programs with specific regions of the genome replicated at precise times. The ATR–Chk1 pathway plays a central role in these programs, but the mechanism for how Chk1 regulates origin firing remains unknown. We demonstrate here the essential role of cyclin A2–Cdk1 in the regulation of late origin firing. Activity of cyclin A2–Cdk1 was hardly detected at the onset of S phase, but it was obvious at middle to late S phase under unperturbed condition. Chk1 depletion resulted in increased expression of Cdc25A, subsequent hyperactivation of cyclin A2–Cdk1, and abnormal replication at early S phase. Hence, the ectopic expression of cyclin A2–Cdk1AF (constitutively active mutant) fusion constructs resulted in abnormal origin firing, causing the premature appearance of DNA replication at late origins at early S phase. Intriguingly, inactivation of Cdk1 in temperature-sensitive Cdk1 mutant cell lines (FT210) resulted in a prolonged S phase and inefficient activation of late origin firing even at late S phase. Our results thus suggest that cyclin A2–Cdk1 is a key regulator of S-phase programs.

Chk1 | DNA replication | molecular combing | ATR protein | checkpoint

Duplication of the eukaryotic genome is regulated by multiple elements including initiation of DNA replication, rate of fork progression, stability of replication forks, and the origin firing program (1). Replication origins are fired in small groups that are activated together within individual replication factories and thus can be visualized as foci (2). Replication origins in a single replication factory are actually comprised of several candidate origins, most of which are not normally used through the mechanism by which firing of 1 origin inhibits activation of any other Mcm2–7 complexes within that factory (3). Thus, S-phase programs appear to be regulated by 2 distinct levels of origin firing; one is the sequential activation of replicon clusters characterized as visible replication foci, and the other is the selection of 1 Mcm2–7 complex around the ORC within a single replication factory.

The DNA replication checkpoint system was reported to be involved in the origin firing program in vertebrate cells (4). In analysis using *Xenopus* egg extract, ATR/Chk1 was shown to regulate the sequential activation of early and late replication origins (5). Chk1 also regulates the density of active replication origins during S phase of avian cells (6). Therefore, ATR/Chk1 may be involved in the regulation of sequential activation of replicon clusters and selection of origins within a single replication factory. Chk1 has been shown to regulate the physiological turnover of Cdc25A and its phosphatase activity, which in turn regulates several cyclin–Cdk activities (7) that are prerequisite for origin firing throughout S phase.

In budding yeast, Clb5-dependent Cdk activity is indispensable for activation of late replication origins (8), suggesting the existence of a specific transfactors for late origin activation in other eukaryotes. In fission yeast, however, clear late origins have not been characterized (9) and replication origins fire stochastically (10, 11). As for mammals, although almost half of origins are activated equally throughout S-phase progression (12), stable subunits of chromosomes equivalent to replication foci maintain their replication timing from S phase to S phase (13).

In this article, we demonstrate that Chk1 depletion resulted in an aberrant origin firing and a hyperactivation of cyclin A2–Cdk1 at early S phase. Ectopic expression of cyclin A2–Cdk1AF induced late origin firing at early S phase, and a loss of Cdk1 activity compromised activation of late origins at late S phase. Our results thus suggested that cyclin A2–Cdk1 might function as a transregulator of late origin firing in mammals.

Results

Chk1 Depletion Results in an Aberrant Origin Firing and a Hyperactivation of Cyclin A2–Cdk1 at Early S Phase. $Chk1^{lox/-}$ mouse embryonic fibroblasts (MEFs) were infected with adenoviruses expressing either LacZ or Cre and synchronized into G_0 phase by serum starvation (14). $Chk1^{lox/-}$ and $Chk1^{del/-}$ MEFs were then stimulated by 15% serum and double-labeled with iododeoxyuridine (IdU) and chlorodeoxyuridine (CIdU) at the indicated times, and their spatiotemporal patterns of replication sites were examined. The mammalian S phase is structured so that the sequential activation of replicon clusters occurs at spatially adjacent sites (15). This spatial relationship is maintained in $Chk1^{lox/-}$ MEFs (Fig. 1*A*), where 86.6 ± 4.4 of foci showed colocalization visualized as yellow color. In contrast, colocalization was detected only at 53.9 ± 4.8 of foci in $Chk1^{del/-}$ MEFs (Fig. 1*B*), indicating that Chk1 depletion in mammals resulted in the aberrant origin firing as observed in avian cells (6). Molecular combing of single DNA molecules was performed to visualize individual origin activation, measure the fork elongation, and define replication structures (Fig. 1*C* and Fig. S1). In asynchronized $Chk1^{lox/-}$ MEFs infected with control LacZ adenoviruses interorigin spacing (90.4 kb on average) was similar to that in mock-infected cells. Chk1 depletion resulted in a clear reduction in origin spacing (34.8 kb on average)(Fig. 1*C Top*). Spatiotemporal pattern of replication sites could also be affected by fork elongation. Chk1 depletion reduced the rate of fork elongation throughout the labeling period (Fig. 1*C Middle*).

Double-labeling protocol also defines 5 classes of replication structure as described (6). Chk1 depletion resulted in a significant reduction in a proportion of consecutively elongating forks (class 1) and an increase in number of new firing initiation during the first (class 2) and second (class 4) labeling period (Fig. 1*C Bottom*). A dramatic increase in the frequency of closely-spaced active origins

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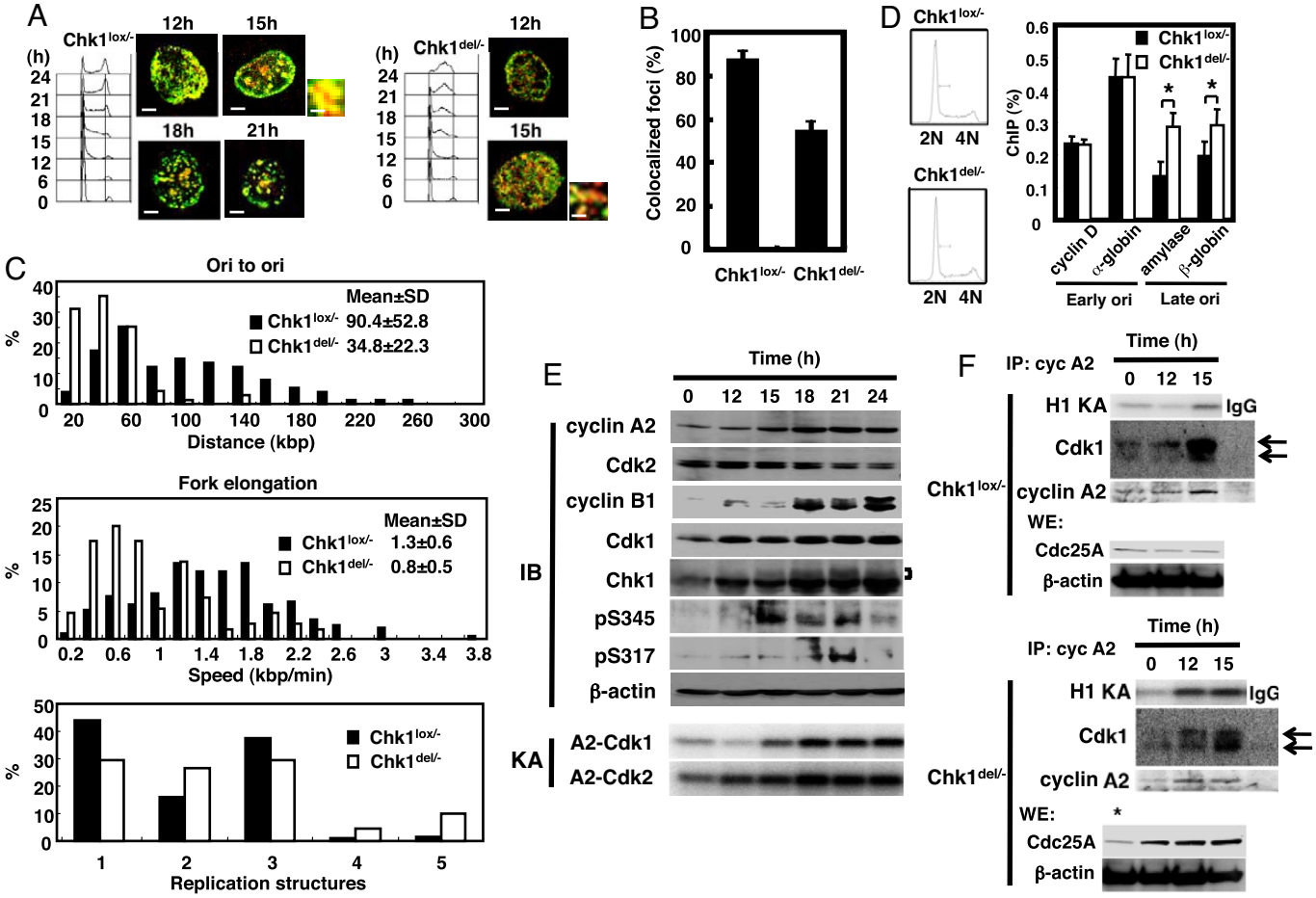

Fig. 1. Chk1 depletion results in an aberrant S-phase program and an activation of cyclin A2–Cdk1 at early S phase. (*A*) Chk1$^{lox/-}$ and Chk1$^{del/-}$ MEFs were synchronized at quiescence by serum starvation and then released by the addition of 15% serum. Cells were harvested at the indicated times, and their cell cycle distributions were analyzed by FACS. Replication sites were pulse-labeled for 15 min with 100 μM IdU and then for 15 min with 100 μM CldU, and analyzed with a Zeiss LSM5 confocal fluorescence microscope. Typical patterns of replication sites at the indicated times are presented. High-power details are from the boxed areas shown. (Scale bars: 5 and 0.5 μm in detail.) (*B*) Colocalization of IdU and CldU foci in Chk1$^{lox/-}$ and Chk1$^{del/-}$ MEFs. Relative colocalization of IdU and CldU foci was determined as a percentage of total foci in both cells ($n > 30$). Data are means ± SD of at least 3 independent experiments. (*C*) Asynchronized Chk1$^{lox/-}$ MEFs were infected with the indicated adenoviruses and double-labeled with IdU and CldU before harvesting at 28 h after infection. Replication structures were visualized by means of dynamic molecular combing. Adjacent origins in replicon clusters (Ori to ori), fork elongation, and replication structure defined by ref. 6 were determined ($n > 100$). Frequency histograms show the distribution of separation in distance (kbp), speed (kbp/min), and replication structure (1, elongating fork; 2, fork growing from 1 ori; 3, terminal fusions; 4, isolated; 5, interspersed). (*D*) Asynchronized Chk1$^{lox/-}$ MEFs were infected with adenoviruses expressing either LacZ or Cre. Cells were labeled with BrdU for 1 h before harvesting at 28 h after infection, and the cell cycle profiles were then analyzed by FACS. Early S-phase fraction indicated by bars was sorted, and nascent DNA was enriched by immunoprecipitation using α-BrdU. The indicated genes were amplified by quantitative PCR, and the results are presented as a percentage of mtDNA. Data are means ± SD of at least 3 independent experiments. Statistical significance was assessed by Student's *t* test (*, $P < 0.01$). (*E*) Synchronized Chk1$^{lox/-}$ MEFs as in *A* were harvested at the indicated times, and the lysates were subjected to immunoblotting by using the indicated antibodies or to an in vitro kinase assay (KA) for cyclin A2–Cdk1 and cyclin A2–Cdk2 with HH1 (2 μg) as a substrate. (*F*) Synchronized Chk1$^{lox/-}$ and Chk1$^{del/-}$ MEFs as in *A* were harvested at the indicated times, and the lysates were immunoprecipitated by using α-cyclin A2 antibodies after 3 times preabsorbance with α-Cdk2. The resultant immunoprecipitates (IP: cyclin A2) or whole-cell extracts (WE) were subjected to immunoblotting or in vitro kinase assay as in *E*. Arrows indicate the fast (active) or slow (inactive) migrated bands of Cdk1. An asterisk represents cell lysates from Chk1$^{lox/-}$ MEFs at 15 h.

(class 5) was observed. These observations suggest that loss of Chk1 frequently stalls and collapses active forks.

We next examined whether Chk1 regulates global sites of DNA synthesis by quantitative ChIP using FACS-based cell sorting (16). To avoid unexpected effects from gross changes in cell cycle profile on this analysis, we analyzed Chk1$^{del/-}$ MEFs 28 h after adenoviral infection, the time at which Chk1 was completely depleted, but the cell cycle profile was almost the same as that of Chk1$^{lox/-}$ cells (Fig. 1*D*). Cells from the first third of S phase were collected (Fig. 1*D Left*). Nascent (BrdU-containing) DNA was enriched by immunoprecipitation using α-BrdU antibodies and amplified by quantitative PCR with specific primers for cyclin D and α-globin for early-replicating DNA and primers for amylase and β-globin for late-replicating DNA. We also monitored amplification of mtDNA as a control, which replicates throughout the cell cycle and is equally represented in nascent DNA preparations (16, 17). The relative amounts of early replication (cyclin D and α-globin) in Chk1$^{del/-}$ MEFs were almost the same as those in Chk1$^{lox/-}$ cells, whereas those of late replication (amylase and β-globin) in Chk1$^{del/-}$ MEFs were significantly higher than those in Chk1$^{lox/-}$ cells (Fig. 1*D Right*). Given that 1 cell possesses ≈1,000 copies of mitochondrial genome, but they replicate throughout the cell cycle, relative amplification of nascent DNA (≈0.3) for early and late origins appeared consistent.

Chk1 is phosphorylated during unperturbed S phase (18, 19), which regulates the activity and stability of Cdc25 phosphatases, leading to the inactivation of Cdks through increased phosphorylation of their Y15 residues (20). Thus, we speculated that Chk1

regulates origin firing program through affecting certain cyclin–Cdks activities. The band corresponding to Chk1 was shifted upward at 15 h and thereafter. This band shift was reversed by phosphatase treatment, indicating that the modification was caused by phosphorylation. Chk1 phosphorylation was also confirmed by using phospho-specific antibodies to Chk1 at Ser-317 and Ser-345 (Fig. 1*E*).

Cyclin A2–Cdk1 activity was first detected at 15 h (middle S phase) and increases thereafter. Cyclin A2–Cdk2 was detected at 6 h (early S phase) and reached maximum at 18 h (Fig. 1*E* and Fig. S2*A*). These results are consistent with the recent report that cyclin A2 starts to form a complex with Cdk1 at mid-S phase (21). Cyclin A2–Cdk1 activity was detected earlier and enhanced in $Chk1^{del/-}$ MEFs when compared with $Chk1^{lox/-}$ MEFs (Fig. 1*F*), where immunodepletion of Cdk2 was equally achieved in both cyclin A2 immunoprecipitates (Fig. S2*B*). Cyclin A2–Cdk2 activity was not apparently affected by Chk1 depletion (Fig. S2*A*). Intriguingly, the amount of Cdc25A was highly elevated in $Chk1^{del/-}$ MEFs. Consistent with this increase in amount of Cdc25A, fast mobility band (active; Y15 dephosphorylation) and slow band (inactive; Y15 phosphorylation) of Cdk1 protein were dominant in those from $Chk1^{del/-}$ MEFs and $Chk1^{lox/-}$ MEFs, respectively (Fig. 1*F*). Specificity of cyclin A2–Cdk1 activity was confirmed by Cdk1 knockdown experiment, where cyclin A2–Cdk1 activities in both MEFs were significantly reduced after Cdk1 depletion (Fig. S3). To further confirm the functional interaction between Chk1 and cyclin A2–Cdk1, $Chk1^{lox/-}$ MEFs were treated with UV light, which phosphorylated Chk1 in an ATR-dependent manner. Chk1 phosphorylation was correlated with the reduction of Cdc25A, the appearance of slow mobility band of Cdk1 protein, and inhibition of cyclin A2–Cdk1 activity (Fig. S4). Taken together, cyclin A2–Cdk1 is likely to be a target of Chk1 through regulation of Cdc25A.

Aberrant Origin Firing in Cells Expressing Cyclin A2–Cdk1AF Fusion Protein. To examine the role of each cyclin–Cdk complex in the origin firing program, we generated a cyclin A2–Cdk1 fusion construct. Because cyclin–Cdk activities are regulated mainly by the phosphorylation of Y15, we generated a constitutively active mutant (CdkAF) in which residues at inhibitory phosphorylation sites were replaced with alanine and phenylalanine and therefore the mutant was not affected by the Chk1–Cdc25 pathway. Recombinant cyclin A2–Cdk2AF, cyclin A2–Cdk1AF, and cyclin B1–Cdk1AF complexes and the fusion proteins were examined for their enzymatic kinetics by using histone H1 (HH1) and lamin B as substrates. Dose-dependent increases in activities of both cyclin–Cdks complex and their fusion proteins were observed (Fig. 2*A*). The kinetic values of these complexes were the same as those of the fusion proteins (Table S1).

Expression of cyclin B1–Cdk1AF, but not cyclin A2–Cdk2AF or cyclin A2–Cdk1AF, induced γH2AX foci in HeLa cells (Fig. 2*B*). Amounts of cyclin B1–Cdk1AF, cyclin A2–Cdk1, and cyclin A2–Cdk2 fusion proteins expressed at 24 h after infection were almost equal to endogenous Cdk1 and Cdk2 proteins, respectively (Fig. 2*C* and Fig. S5). Again, γH2AX was not detected by immunoblotting in cells expressing cyclin A2–Cdk1 or cyclin A2–Cdk2 fusion protein.

Expression of cyclin A2–Cdk1AF and cyclin A2–Cdk2AF fusion protein at the endogenous level did not appear to affect the gross progression of S phase (Fig. 3*A*) although they arrested the cell cycle at M phase because of their inability to be degraded by APC-C at mitosis and thus mitotic exit was inhibited. The expression of cyclin A2–Cdk1AF fusion protein caused the appearance of late replication sites during early S phase when cells were double-labeled with IdU and CIdU (Fig. 3*B*). Dynamic molecular combing revealed that expression of cyclin A2–Cdk1AF fusion protein reduced origin spacing (75.0 kb on average), whereas that of cyclin A2–Cdk2AF did not (113.0 kb on average)(Fig. 3*C Top* and Fig. S6). Unlike Chk1 depletion, expression of cyclin A2–Cdk2AF did not cause significant changes in the proportion of abnormal replication structures (Fig. 3*C Bottom*). Taken together, these results suggested that cyclin A2–Cdk1 had a specific role in the origin firing program.

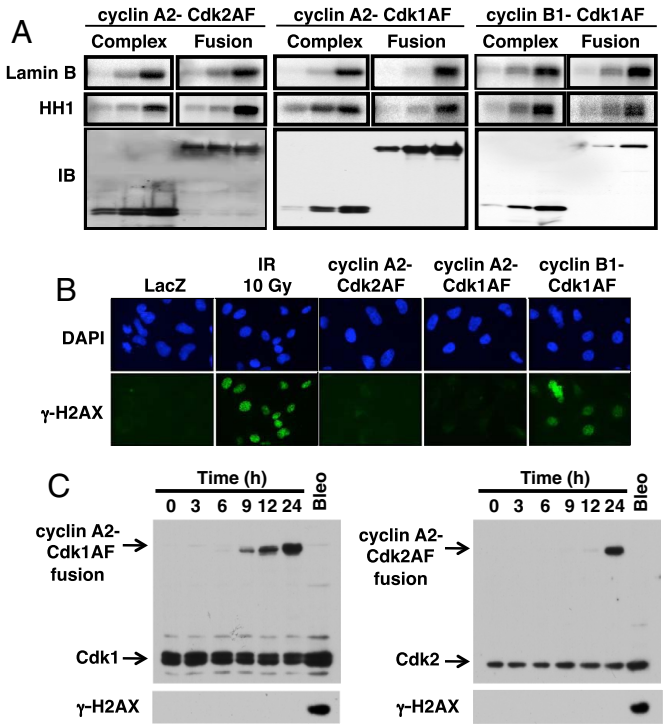

Fig. 2. Enzymatic kinetics of cyclin–Cdk fusion proteins. (*A*) Baculoviruses expressing cyclin A2 together with those expressing Cdk2 or Cdk1 (Complex) or those expressing cyclin A2–Cdk2, cyclin A2–Cdk1, or cyclin B1–Cdk1 fusion protein (Fusion) were used to infect insect cells. Their complexes or fusion proteins were purified and subjected to an in vitro kinase assay using lamin B (2 μg) or HH1 (2 μg) as a substrate or subjected to immunoblotting by using Cdk2 or Cdk1 antibodies (IB). (*B*) HeLa cells were infected with adenoviruses expressing the indicated proteins 24 h before fixing and immunostained with α-γH2AX antibodies. Their nuclei were counterstained with DAPI. As a positive control, cells infected with adenoviruses expressing LacZ were treated with IR (10 Gy). (Magnifications: 100×.) (*C*) HeLa cells were infected with adenoviruses expressing either cyclin A2-Cdk1AF or cyclin A2-Cdk2AF fusion proteins. Cells were harvested at the indicated times, and the lysates were subjected to immunoblotting using α-Cdk1 (*Upper Left*), α-Cdk2 (*Upper Right*), or α-γH2AX antibodies (*Lower*). As a control, HeLa cells were treated with bleomycin for 24 h (20 μg/mL).

ChIP analysis revealed that considerable enrichment of early- and late-replicating DNA was specifically observed in the early and late S-phase fractions of control LacZ cells, respectively (Fig. 3*D*). Ectopic expression of cyclin A2–Cdk1AF resulted in the dramatic increase in replication of late origins in early S-phase fractions, but that of cyclin A2–Cdk2AF did not apparently affect it.

Cdk1 Is Required for Proper Timing of Origin Firing. FT210 cells possess a temperature-sensitive Cdk1 gene product (22). FACS analysis revealed a 2-h-longer S phase in FT210 cells compared with the parental FM3A cells (Fig. 4*A*). S-phase progression of FT210 cells at a permissive temperature was almost the same as that of FM3A cells. The progression of the spatiotemporal pattern of DNA replication sites in FM3A at the nonpermissive temperature was almost the same as in HeLa cells or MEFs. In contrast, the specific pattern of DNA replication sites observed in late S phase showing a few large internal foci was hardly detected in FT210 cells even at late S phase at nonpermissive temperature (Fig. 4*A*). Loss of Cdk1

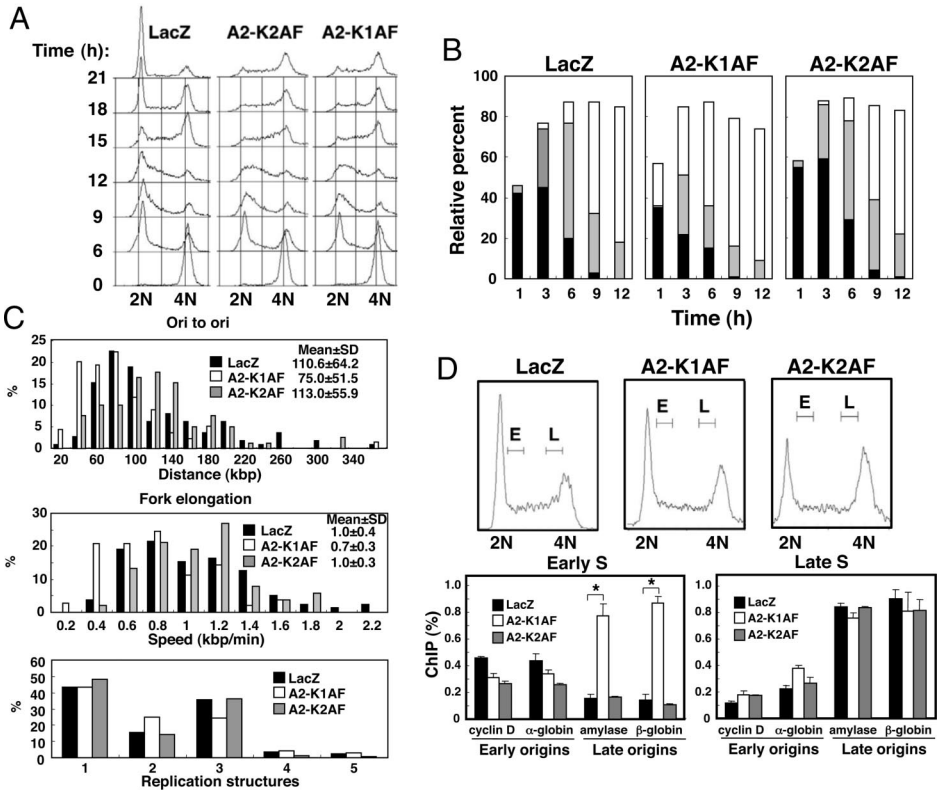

Fig. 3. Ectopic expression of cyclin A2-Cdk1AF, but not cyclin A2–Cdk2AF resulted in an aberrant temporal regulation of origin firing. (*A*) HeLa cells were synchronized by thymidine (2 mM, 24 h)/release period (6 h)/nocodazole (0.1 μg/mL, 12 h) and infected with adenoviruses expressing either cyclin A2–Cdk2AF, cyclin A2–Cdk1AF, or LacZ (control) 12 h before nocodazole washout (time 0). Cells were harvested at the indicated times, and their cell cycle profiles were analyzed by FACS. (*B*) HeLa cells were infected with the indicated adenoviruses 24 h before mimosine (0.6 mM, 16 h) washout (time 0). Replication sites were pulse-labeled for 15 min with IdU (100 μM) and then for 15 min with CIdU (100 μM) before harvesting at the indicated times. The number of replication sites of each pattern was counted, and the relative percentage of the total number of cells ($n > 300$) is indicated. Black bars represent the early pattern, gray bars represent the middle pattern, and white bars represent the late pattern. Data are means of at least 3 independent experiments. (*C*) Asynchronous HeLa cells were infected with the indicated adenoviruses. Cells were harvested 24 h after infection and subjected to molecular combing. Adjacent origins in replicon clusters (Ori to ori), fork elongation, and replication structure were determined ($n > 100$) as in Fig. 1*C*. (*D*) Asynchronous HeLa cells infected with the indicated adenoviruses were pulse-labeled with BrdU before harvesting cells 24 h after infection. S-phase cells were then sorted into early (E) or late (L) fractions (*Upper*). The sorted cells were collected, and replication firing at the indicated origins was analyzed by ChIP analysis (*Lower*) as in Fig. 1*D*. Data are means ± SD of at least 3 independent experiments. Statistical significance was assessed by Student's *t* test (*, $P < 0.01$).

resulted in a significant increase in origin spacing (104.7 kb on average) when compared with control cells (78.6 kb on average) (Fig. 4*B Top* and Fig. S6). Loss of Cdk1 did not cause changes in the proportion of replication structures, further supporting the notion that Cdk1 is not involved in the stabilization of replication forks.

ChIP analysis revealed that replication patterns of early S-phase fractions in both cells were very similar, whereas replication of late origins in late S-phase fraction from FT210 cells was specifically impaired (Fig. 5*A Right*). Cdk2 activity during S phase in FT210 cells appeared the same as that in FM3A cells (Fig. S7). Collectively, these results suggested that Cdk1 activity is involved in the proper timing of late origin firing.

Finally, we attempted to determine the molecular basis by which cyclin A2–Cdk1 regulates origin firing program. In *Xenopus* and yeast systems, it was reported that cyclins, Cdk1 specifically, interact with the origin recognition complexes (ORCs) (23, 24). To examine whether the specific interaction of Cdk1 to ORCs is conserved among mammals, we performed ChIP analysis with α-Cdk1 and α-Cdk2 antibodies. Both Cdk1 and Cdk2 were detected at genes replicating early, whereas Cdk1 was specifically detected at genes replicating late (Fig. 5*B*). Relative binding of Cdk1 and Cdk2 appeared somewhat low, presumably because of an asynchronous cell cycle. These results suggested that the specific binding of Cdk1 to late origins may also be involved in the regulation of origin firing programs.

Discussion

Conditional Chk1 knockout MEFs revealed that Chk1 plays an important role in the regulation of origin firing at 2 distinct levels in mammals, namely activation of origins within a single replication factory and activation of replicon clusters (Fig. 1 *A–D*). Consistent with our observations, it was very recently proposed that Chk1 suppresses initiation in both inactive, later-firing clusters and active clusters, and the former is more strongly repressed (25). We then successfully showed that expression of cyclinA2–Cdk1AF fusion proteins activated origin firing at both levels as Chk1 depletion did (Fig. 3 *B–D*). The expression patterns of Cdk1 and cyclins during S phase and the enhancement of their activities in response to Chk1 depletion are also consistent with our conclusions (Fig. 1 *E* and *F*). The most striking evidence for the involvement of Cdk1 in DNA replication is the fact that inactivation of Cdk1 in mammalian cells resulted in a prolonged S phase accompanied by ineffective firing of late replicon clusters and reduced the density of active origins (Figs. 4 and 5*A*). Although our present results clearly demonstrate that cyclin A2–Cdk1 is involved in the regulation of late origin firing, functioning downstream of Chk1, we cannot rule out the possibility that cyclin A2–Cdk2 has a redundant function. Hochegger *et al.* (26) reported that Cdk1 activity was essential for DNA replication initiation when Cdk2 was depleted in chicken DT40 cells. When Cdk2 was present, Cdk1 inhibition did not delay S phase or block centrosome duplication. In this regard, DNA replication in DT40 cells appears complete within a shorter period (8 h) when compared with mammalian cells (10 h at 37 °C). Therefore, it is possible that DT40 cells possess a strong Cdk2 activity, presumably because of a loss of functional p53 that reduces the level of p21 Cdk inhibitor, and the high Cdk2 activity may compensate for the loss of Cdk1 activity in the context of S-phase control. In agreement with this notion, both Cdk1 and Cdk2 were recently reported to be involved in the control of DNA replication and replication origin firing under unperturbed S phase in the *Xenopus* system (27). It was also suggested that Cdk1 and Cdk2 must have different activities toward the genuine substrates involved in DNA replication although one kinase alone is minimally sufficient to promote substantial DNA replication.

Neither cyclin A2–Cdk1 nor cyclin A2–Cdk2 appeared to be involved in the stabilization of replication forks during S phase

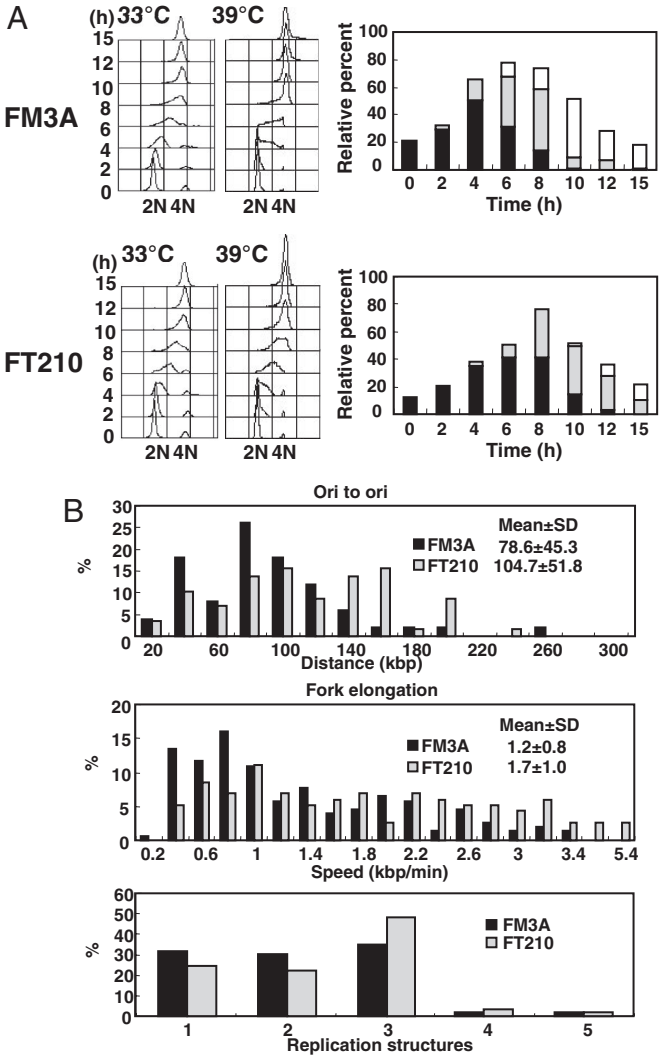

Fig. 4. Prolonged S phase in temperature-sensitive Cdk1 mutant FT210 cells. (*A*) FT210 and the parental FM3A cells were synchronized at M phase by nocodazole (0.5 μg/mL, 16 h) and then released at an either permissive (33 °C) or nonpermissive (39 °C) temperatures. Cells were then harvested 3 h after release (time 0) and at various times thereafter. Their replication sites were analyzed ($n > 300$) as in Fig. 3*B*. Data are means of at least 3 independent experiments. (*B*) Asynchronized FM3A and FT210 cells were shifted at 39 °C for 4 h. Cells were then harvested and subjected to molecular combing. Adjacent origins in replicon clusters (Ori to ori), fork elongation, and replication structure were determined ($n > 100$) as in Fig. 1C.

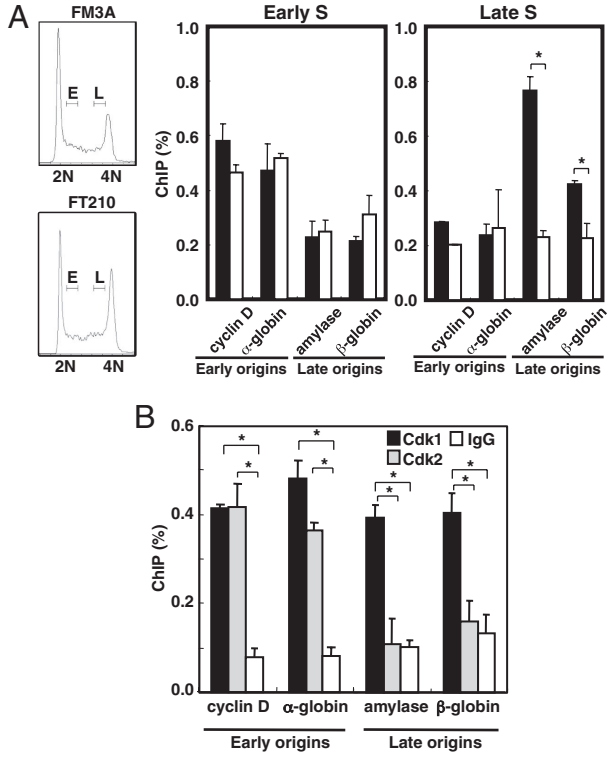

Fig. 5. Impaired late origin firing in temperature-sensitive Cdk1 mutant FT210 cells. (*A*) Asynchronous FM3A and FT210 cells were shifted at 39 °C for 4 h. Cells were pulse-labeled with BrdU (25 μM) for 1 h and sorted into early (E) or late (L) fractions. Replication firing at the indicated origins was analyzed by ChIP analysis as in Fig. 3*D*. Data are means ± SD of at least 3 independent experiments. Statistical significance was assessed by Student's *t* test (*, $P < 0.01$). Filled bars indicate FM3A cells; empty bars indicate FT210 cells. (*B*) Asynchronous FM3A cells were cultured at 33 °C and harvested. Cell lysates were subjected to ChIP analysis as described in *Materials and Methods*. Data are means ± SD of at least 3 independent experiments. Statistical significance was assessed by Student's *t* test (*, $P < 0.01$).

when replication structures were assessed by dynamic molecular combing technology (Fig. 3*C*). This idea was further supported by the observations that ectopic expression of cyclin A2–Cdk1AF and cyclin A2–Cdk2AF failed to induce DNA damage (Fig. 2 *B* and *C*). These findings present a clear contrast to the case with Chk1 depletion in which stability of replication forks during S phase was strikingly reduced. Therefore, Chk1 likely regulates the fork stability in a manner independent of cyclin–Cdk activities.

Cdk activities have both positive and negative roles during S phase, namely to initiate DNA synthesis and prevent rereplication. A quantitative model has proposed for explain the biphasic effects of Cdks (28). In addition to a quantitative model, the accessibility of Cdk to substrates could play a role in the regulation of the S-phase program. Studies in *Xenopus* and yeast systems suggested that Cdk1 specifically interacts with ORC and phosphorylates the components more efficiently than Cdk2 although this interaction is proposed to be involved in prevention of rereplication (23, 24). We found that Cdk1 could bind to both early and late origins but Cdk2 failed to bind to late origins (Fig. 5*B*). Thus, Cdk1 could potentially activate early origin firing. This notion is supported by the fact that Cdk1 could complement the Cdk2 function of S-phase initiation in Cdk2-depleted cells (26). However, because neither ectopic expression of cyclin A2–Cdk2AF nor cyclin A2–Cdk1AF resulted in the further enhancement of early origin activation (Fig. 3*D*), activation of endogenous cyclin A/E–Cdk2 at the S-phase onset appeared to be sufficient for early origin firing. Furthermore, given that the majority of endogenous Cdk1 and Cdk2 existed in soluble fractions (Fig. S8), the origin activation program appeared to be regulated not only by induction of Cdks and their binding to prereplicative complex components, but also by alternative ways such as complex formation with cyclins or regulation of inhibitory phosphorylation of Cdks. In this regard, it was very recently reported that Cdk1 started to form a complex with cyclin A2 after cyclin A2–Cdk2 complexes reached a plateau in mid S phase (21). Taken together, our results suggest that cyclin A2–Cdk1 may regulate origin firing program through both its specific accessibility to late origins and regulation of Cdk1 activity at late S phase.

In conclusion, the present results indicate that ATR/Chk1–cyclin A2–Cdk1 controls the activation of late replication origins and the density of active origins in mammals. Similar regulation was reported in a budding yeast system in which Clb5–Cdk1 was required for late origin firing (8). Taken together, these results suggest the existence of conserved mechanisms for the temporal program of origin activation among a number of eukaryotes.

Materials and Methods

Antibodies. Antibodies used in this study were as follows: α-CDK2 (sc-748; Santa Cruz), α-Cyclin A2 (sc-751; Santa Cruz), α-Cdc2 (sc-54; Santa Cruz), α-Cyclin B1 (sc-245; Santa Cruz), rat α-BrdU (ab6326; Abcam), mouse α-BrdU (347580; BD), α-γH2AX (05-636; Upstate), α-rabbit IgG HRP (NA934; GE Healthcare), α-mouse IgG HRP (NA931; GE Healthcare), Alexa Fluor 555-conjugated goat α-mouse IgG (A-21422; Invitrogen), and Alexa Fluor 488-conjugated rabbit α-rat IgG (A-21210; Invitrogen).

Cell Culture and Double Labeling with IdU and CldU. HeLa cells, Chk1$^{lox/-}$ MEFs, Chk1$^{del/-}$ MEFs, FM3A, and FT210 cells were cultured as described (14, 29). For analyses of origin firing programs, cells were incubated with 100 μM IdU for 15 min, then 100 μM CldU for 15 min, fixed with 4% paraformaldehyde, and permabilized. Cellular DNA was denatured in 1.5 M HCl and stained as reported (6). The spatiotemporal patterns of replication were analyzed by counting at least 300 cells by 2 individuals under blinded conditions.

Dynamic Molecular Combing and Immunofluorescent Detection. Genomic DNA was prepared and combed onto the silanated cover slips as described (30) with modifications as detailed (31). A total of 2×10^6 cells were pulse-labeled for 20 min with 100 μM IdU, washed with PBS twice, and pulse-labeled for 20 min with 100 μM CldU. For preparation of genomic DNA, to remove the mitochondrial genome the nuclei were extracted with buffer A [250 mM sucrose, 20 mM Hepes (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA (pH 8.0), 1 mM EGTA (pH 6.8), 1 mM DTT, 0.1 mM PMSF] before resuspension into low-melting point agarose. Combed DNA molecules were heat-denatured in 50% formamide, 2 × SSC at 72 °C for 12 min. For immunodetection of labeled DNA, denatured DNA molecules were incubated with mouse α-BrdU mAb (1:5) and rat α-BrdU mAb (1:25) for 1 h at 37°C. After washing with PBS and 0.05% Tween 20 for 5 min 3 times, DNA molecules were incubated with Alexa Fluor 555-conjugated goat α-mouse IgG (1:500) and Alexa Fluor 488-conjugated rabbit α-rat IgG (1:500) for 30 min at 37°C. All antibodies were diluted in blocking solution [1% (wt/vol) blocking reagent in PBS, 0.05% Tween 20]. After washing with PBS and 0.05% Tween 20 for 5 min 3 times, coverslips were mounted in VECTASHIELD (Vector Laboratories). To estimate the extension of DNA molecules, coverslips were prepared with λ-DNA, and then the DNA molecules were stained with 6.7 mM YOYO-1 at 25°C for 1 h. YOYO-1-stained DNA molecules measured 21 ± 0.9 μm. As the virus genome is 48.5 kbp, the extension of DNA molecules is 2.32 ± 0.11 kbp/μm. DNA fibers were examined with a Zeiss Axioplan 2 MOT with a 63X Plan-APOCHROMAT (NA 1.4) objective lens, equipped with MicroMAX CCD camera (Princeton Instruments). Fluorescent signals were measured by using MetaMorph version 6.1 software (Universal Imaging).

Construction of Cyclin A2–Cdk1, Cyclin A2–Cdk2, and Cyclin B1–Cdk1 Fusion Vectors. For subcloning of full-length mouse Cdk1 and Cdk1AF, either cDNAs from mouse MEFs or pcDNA3.1Cdk1AF were used as a template. The PCR products were digested with EcoRI and NotI and subcloned into pcDNA3.1Myc/HisA vector (Invitrogen). For subcloning of full-length mouse Cdk2 and Cdk2AF, either cDNAs from mouse MEFs or pcDNA3.1Cdk2AF were used as a template. For preparation of cyclin A2–Cdk1, cyclin A2–Cdk2, and cyclin B1–Cdk1 fusion constructs, sets of primers and mouse cDNA derived from MEFs as a template were used. The PCR products were digested with BamHI and EcoRI and subcloned into pcDNA3.1Cdk1Myc/HisA or pcDNA3.1Cdk2Myc/HisA vectors. The primer sets used are listed in Table S1.

Purification of Recombinant Cyclin–Cdk Fusion Proteins. pcDNA3.1cyclin A2–Cdk1, pcDNA3.1cyclin A2–Cdk2, pcDNA3.1cyclin B1–Cdk1, and their AF mutants were digested with BamHI and PmeI. The fragments were subcloned into pVL1392 vector and transfected into Sf9 cells. Sf9 cells infected with baculoviruses expressing cyclin–Cdk fusion proteins or coinfected with the individual cyclins and Cdks were lysed with immunoprecipitation kinase buffer (7) containing a mixture of protease inhibitors. The fusion proteins and cyclin–Cdk complexes were purified by ProBond Resin (Invitrogen) and used for the in vitro kinase assay.

Preparation of Adenoviruses Expressing Cyclin–Cdk Fusion Proteins. The BamHI–PmeI fragments of cyclin–Cdk fusion constructs were subcloned into pENTER vector (Invitrogen) predigested with BamHI and EcoRV. pENTERcyclin–Cdks and pENTERcyclin–CdkAFs were then subcloned into pAdCMV vectors according to the manufacturer's instructions (Invitrogen). pAdcyclin–Cdks and pAdcyclin–CdkAFs were transfected into 293A cells (Invitrogen).

ChIP Assay. Asynchronized Chk1$^{lox/-}$ MEFs, Chk1$^{del/-}$ MEFs, HeLa cells infected with adenoviruses expressing cyclin A2–Cdk1AF or cyclin A2–Cdk2AF, and mouse FM3A or FT210 cells were labeled with 25 μM BrdU before cell sorting. Cells were then sorted into early and late S-phase fractions by using a cell sorter (BD). At least 60,000 cells were collected during each phase and used for the chromatin preparation. Nascent DNA was enriched by immunoprecipitation using α-BrdU antibodies as reported (16) and subjected to quantitative PCR with the ABI PRISM7000 system using Power SYBR Green PCR Master Mix (Applied Biosystems). Primers used for PCR are listed in Table S1. As a control, mtDNA in BrdU-containing DNA was also amplified, and the results were presented as a percentage of mtDNA. For Cdk1 and Cdk2 bindings to origins, FM3A cells were cultured at 33 °C, and ChIP analysis was performed with α-Cdk1 and α-Cdk2 antibodies as described (14). The results were presented as a percentage of input.

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