Ku is involved in cell growth, DNA replication and G1-S transition

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Summary

The Ku protein (Ku70-Ku80) is involved in various genome-maintenance processes such as DNA replication and repair, telomere maintenance, and chromosomal stability. We previously found that Ku80 is implicated in the loading of members of the pre-replicative complex (pre-RC) onto replication origins. Here, we report that acute reduction of Ku80 to 10% of its normal levels leads to impaired DNA replication and activation of a replication stress checkpoint. In the absence of Ku80, decreased levels of the initiator proteins Orc1 and Orc6 as well as reduced chromatin binding of Orc1, Orc4 and Cdc45 were observed, leading to decreased origin firing, whereas Orc2 and Orc3 were unaffected. Prolonged perturbation of DNA replication caused the block of cell-cycle progression in late G1 phase with low Cdk2 activity due to increased p21 expression and decreased Cdc25A and Cdk2 levels. The data suggest the interplay between the DNA-replication and cell-cycle machineries and shed light on a new role of Ku in G1-S transition.

Key words: DNA replication, Ku, Cell growth, Nascent DNA, Replication stress checkpoint

Introduction

Ku is a heterodimeric protein (Ku70-Ku80) that was first identified as an autoantigen in the sera of patients with autoimmune diseases (Mimori et al., 1981). It is a versatile guard of the genome that participates in its duplication, repair and telomeric maintenance, as well as the suppression of chromosomal rearrangements (reviewed in Tuteja and Tuteja, 2000; Zannis-Hadjopoulos et al., 2004; Gullo et al., 2006). Ku is the DNA-binding subunit and allosteric activator of DNA protein kinase (DNA-PK) (West et al., 1998), but DNA-PK-independent roles have also been ascribed to it, such as a role in cell proliferation, as indicated by the phenotypes of knockout mice (Nussenzweig et al., 2000; Gao et al., 1998; Kurimasa et al., 1999). Increasing evidence has implicated Ku in DNA replication, expanding its repertoire of functions (Park et al., 2003; Zannis-Hadjopoulos et al., 2004; Sibani et al., 2005a; Sibani et al., 2005b; Bürckstümmer et al., 2006; Shimura et al., 2007).

DNA replication initiates at specific chromosomal regions termed origins of replication. The ordered assembly of a pre-replicative complex (pre-RC) followed by its activation marks the origins for firing during S phase (Bell and Dutta, 2002). In human cells, the ORC hexamer (Orc1-Orc6) binds to chromatin in late mitosis, followed by Cdc6 and Cdt1, which recruit the putative replicative helicase MCM (MCM2-MCM7 complex), forming the pre-RC (Méndez and Stillman, 2000). Subsequent activation of the pre-RC then loads Cdc45 and the ring-shaped GINS complex, which target the replicative polymerases on the DNA, leading to the onset of S phase (Coverley et al., 2002; Sclafani, 2000; Sato et al., 1997; Woo and Poon, 2003). After replication initiation, the pre-RCs are inactivated, ensuring that the chromosomes are replicated only once per cell cycle and that the DNA content of the cell remains constant (Machida et al., 2005b).

Ku has been shown to associate with a number of human replication origins, such as those associated with the lamin B2, *c-myc*, β-globin (Sibani et al., 2005a; Sibani et al., 2005b) and *DNMT-1* (Araujo et al., 1999) loci, in a cell-cycle-dependent manner (Novac et al., 2001), as well as to co-fractionate with complexes competent for DNA synthesis (Vishwanatha and Baril, 1990; Ruiz et al., 1995). Recently, Ku was shown to form a complex in vivo with the MCM hexamer (Bürckstümmer et al., 2006) and to also participate in the loading of ORC onto origins of replication (Sibani et al., 2005a), implicating it in pre-RC formation.

The DNA replication and Cdk cycles are in direct communication, with both the activation and inactivation of the pre-RCs relying on the activity of cyclin-dependent kinases (Cdks). Low levels of cyclin E and Cdk2 activity are required for proper loading of the MCM proteins (Ekholm-Reed et al., 2004), whereas Cdk2 and Cdc7/Dbf4 (Dbf4-dependent kinase, DDK) kinase activities are required for the activation of the pre-RCs (Takeda and Dutta, 2005) and G1-S transition in mammalian cells (Pagano et al., 1993; Tsai et al., 1993; van den Heuvel and Harlow, 1993). Cyclin E was recently shown to also have a Cdk-independent function in the loading of the MCM helicase during G0-S transition (Geng et al., 2007). Conversely, replication errors trigger changes in the string of events in the Cdk cycle, either blocking the cells in specific stages or causing them to succumb to apoptosis in the case of extensive damage. Inhibition of fork progression by topoisomerase inhibitors (Downes et al., 1994; Clifford et al., 2003; Mikhailov et al., 2004) or double-strand breaks (Kastan and Bartek, 2004) leads to the activation of a G2-M checkpoint before mitotic entry, whereas low levels of replication initiator proteins block cells to late G1 phase (Machida et al., 2005a) or promote apoptosis (Feng et al., 2003), protecting cells from DNA replication crisis and possible aberrant genome duplication.

In the present study, we examined the effect of Ku80 knockdown in DNA replication, cell growth and cell-cycle progression. Reduction of Ku80 expression to 10% of its normal levels by RNAi resulted in impaired cell growth with the doubling time increasing

from 30 hours to 58.8 hours. This growth defect was independent of DNA damage and apoptosis, but reliant on impaired chromatin loading of replication factors and origin activation. Ku80-deficient cells were blocked in late G1 phase and had increased levels of cyclin E but decreased Cdk2 activity, suggesting a new role of Ku80 in G1-S progression.

Results

Knockdown of Ku80 leads to reduced cell proliferation

Initial characterization of human Ku80-haploinsufficient cells (HCT116) revealed a defect in cell proliferation (doubling time 20.5 hours compared with 17.7 hours for the Ku80 wild-type cells) (Li et al., 2002). A second round of gene targeting generated Ku80-null cells that had a more severe growth defect and underwent massive apoptosis, signifying differences between the human and the murine knockout cells, which are viable. To verify the validity of these results in different cell types, we knocked-down Ku80 levels in HeLa cells, using an RNAi approach, and analyzed the effect on cellular proliferation. At 96 hours post-transfection of an siRNA targeting *Ku80* mRNA, the protein levels decreased to approximately 10% of normal levels, as measured both by western blot analysis and immunofluorescence (Fig. 1A,B). The decrease in the levels of Ku protein was gradual over time (Fig. 1A,C), thus providing us with a system to study Ku80 deficiency mimicking the generation of Ku80-knockout cells. Consistent with the growth defect observed in the human heterozygous colorectal cancer cells (HCT116 *Ku80*$^{+/-}$), the cervical cancer cells (HeLa) depleted of Ku80 also displayed a proliferation defect (Fig. 1D, compare panels vii, viii, ix with i, ii, iii and iv, v, vi), with their doubling time increasing twofold compared with cells transfected with a dsRNA with a scrambled sequence (hereafter referred to as scr-siRNA) or cells treated with the transfection agent (hereafter referred to as control) (doubling time 58.8 hours compared with 32.7 and 29.5 hours, respectively) (Fig. 1E). The results suggest that the role of

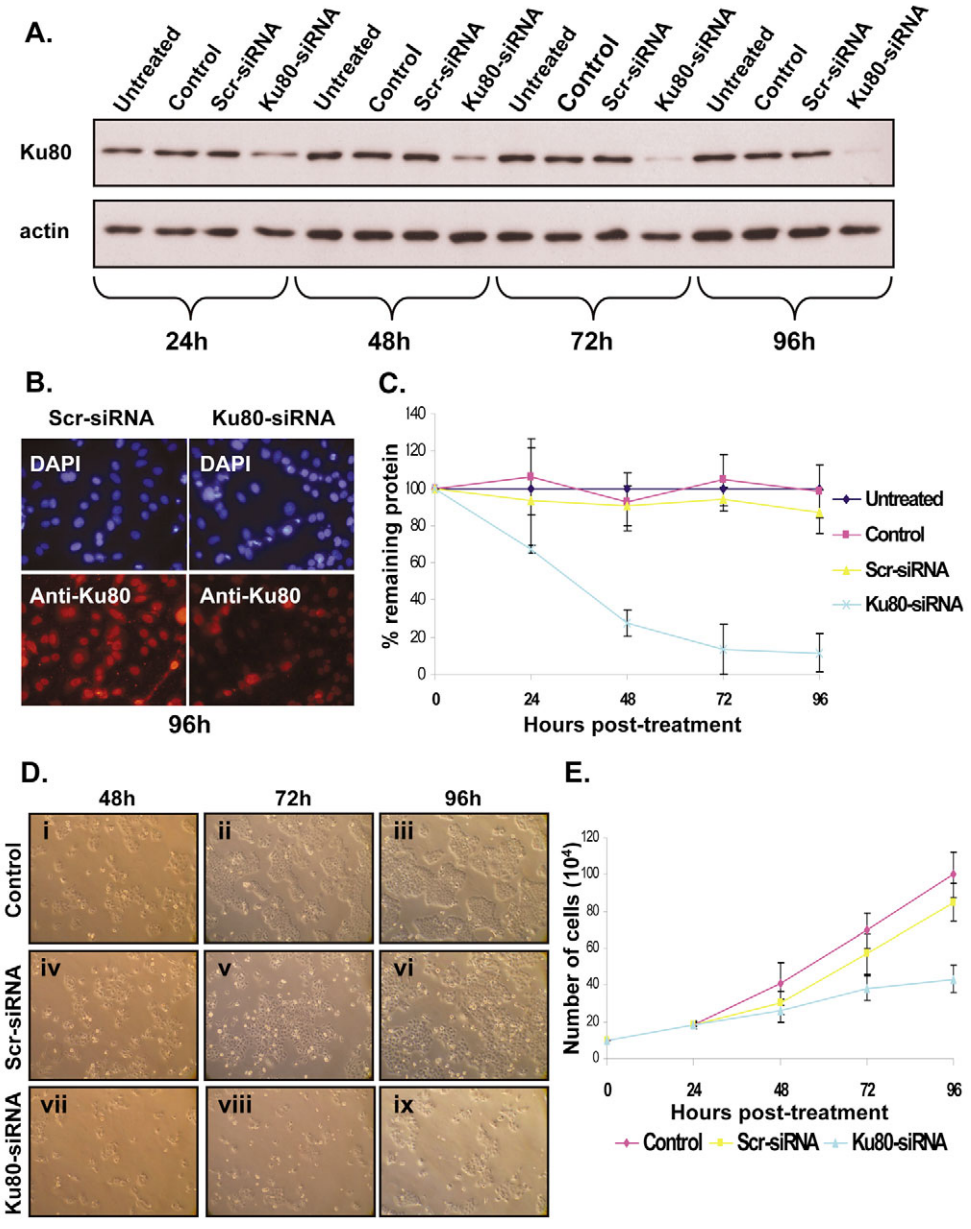

Fig. 1. Reduced cell growth upon RNAi knockdown of Ku80. (A) HeLa cells treated with either transfection agent (control), a *Ku80*-targeting siRNA (*Ku80*-siRNA) or an siRNA with scrambled sequence (scr-siRNA) were grown for 24, 48, 72 and 96 hours. Untreated cells were also included as a control. Whole cell extracts were prepared at each time point and immunoblotted for Ku80. Actin was used as a loading control. (B) Staining of DNA with DAPI (blue) and anti-Ku80 (red) by immunofluorescence 96 hours after transfection with scr-siRNA or *Ku80*-siRNA. (C) Quantification of Ku80 levels in each sample shown in A. Values are expressed as relative optical density (ROD) compared to the untreated cells and represent the average of three experiments and one standard deviation (s.d). (D) Representative phase-contrast microscopic images of control, scr-siRNA-transfected and *Ku80*-siRNA-transfected cells at the indicated times after transfection. (E) Growth curves of the cells shown in D during the time-course of the *Ku80* RNAi knockdown. Values represent the mean number of cells and the error bars are equivalent to 1 s.d.

Ku80 in cell proliferation is not cell-type specific and is reminiscent of other DNA-replication proteins such as Orc2 (Dhar et al., 2001), Orc6 (Prasanth et al., 2002), Cdc6, MCM2 and Cdc45 (Feng et al., 2003).

Decreased DNA replication in Ku80-knockdown cells

Deregulated expression of Ku80 has been reported to affect both the initiation and progression of S phase, either dependently or independently of DNA-PK activity. On one hand, Ku80-haploinsufficient cells display decreased origin activation and delayed G1-S transition after synchronization in late G1 phase (Sibani et al., 2005b), suggesting a role of Ku80 in replication initiation. On the other hand, application of genotoxic stress to cells revealed a role of Ku in S-phase progression that seems to be different from its initiation role in the absence of cellular stress; Ku was implicated in the maintenance of the proliferating cell nuclear antigen (PCNA) on chromatin following ionizing radiation (IR) and chromosomal double-strand break (DSB) induction, and this role was independent of the DNA-PK kinase activity (Park et al., 2003). Similarly, deceleration of DNA replication caused by the polymerase inhibitor aphidicolin induced transient DNA breaks, which were shown to be repaired with the action of DNA-PK (Shimura et al., 2007).

To determine whether the deregulated expression of Ku80 affected the efficiency of DNA replication, cells were pulse-labeled with the nucleotide analog bromodeoxyuridine (BrdU) and its incorporation was measured by FACS analysis, using an anti-BrdU antibody. The total rate of DNA replication was found to be similar for control, scr-siRNA-transfected and *Ku80*-siRNA-transfected cells at 24 hours post-transfection, at which point the protein levels were not significantly changed, whereas, at 72 hours, the replication efficiency of the *Ku80*-siRNA-transfected cells was approximately 41% of the control levels and dropped to background levels at 96 hours (Fig. 2A). A quantification of the FACS profiles is shown in Fig. 2B.

The observed reduction of BrdU incorporation indicates a decreased replication rate but does not distinguish between an impairment in the initiation of replication or in fork progression. To distinguish between these two possibilities, we analyzed the activities of two well-characterized replication origins, namely the lamin B2 (Giacca et al., 1994; Abdurashidova et al., 2000) and c-myc (Tao et al., 2000) (Fig. 3A), by measuring the nascent-strand abundance at these chromosomal regions, as previously described (Giacca et al., 1997; Tao et al., 2000). Similarly to the total replication rate, origin firing from both the lamin B2 (Fig. 3B) and c-myc (Fig. 3C) origins was reduced by approximately half at 72 hours post-siRNA transfection compared with scr-siRNA-treated cells (nascent-DNA-strand abundance in *Ku80*-siRNA-treated cells 46% and 41% of scr-siRNA-treated cells in the two origins, respectively), whereas it approached background levels at 96 hours (twofold enrichment of the origin-containing over the origin-lacking regions compared with a 16.5-fold enrichment in *Ku80*-siRNA-treated vs scr-siRNA-treated cells). The similarity in the patterns of origin firing and total replication rate suggests that it is the initiation step that is affected by Ku80 knockdown. This was further confirmed by two lines of evidence: (a) the total level of PCNA, the processivity factor for the replicative polymerase δ, was decreased in Ku80-knocked-down cells (Fig. 3D) in comparison to the control cells, suggesting the existence of a smaller number of active replication forks instead of impaired fork progression; and (b), the total level of Ser-139-phosphorylated H2AX histone variant

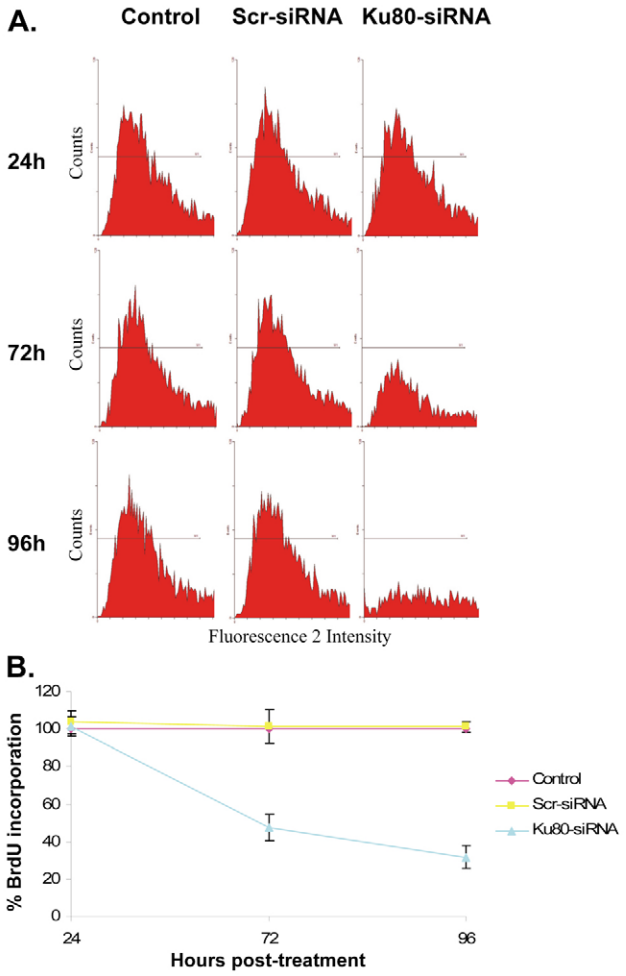

Fig. 2. Time-course of BrdU incorporation as a function of Ku80 knockdown. (A) Representative FACS analysis of BrdU incorporation of logarithmically growing control, scr-siRNA-transfected and *Ku80*-siRNA-transfected cells upon Ku80 silencing for 24, 72 and 96 hours. (B) Quantification of BrdU incorporation by each sample shown in A at the indicated times after transfection. Values are expressed as BrdU incorporation per 10,000 cells relative to control cells and are means of three individual experiments ± 1 s.d.

(γH2AX), one of the first molecules to appear following DSB formation (Rogakou et al., 1998), related to the role of Ku80 in replication elongation (Park et al., 2003; Shimura et al., 2007), was undetectable even at 96 hours post-siRNA-transfection, whereas it was present in hydroxyurea- and nocodazole-treated cells (Fig. 3E). Altogether, these data indicate that low levels of Ku80 are responsible for the observed impaired origin firing and decreased replication rate.

Reduced chromatin loading of DNA replication licensing factors

Previous studies have implicated Ku80 in the assembly and/or stabilization of the pre-RC onto origins of DNA replication. Ku was found to form a complex with the MCM helicase in vivo (Bürckstümmer et al., 2006) as well as to interact with the ORC subunits 3, 4 and 6 on the lamin B2, β-globin and c-myc origins of replication (Sibani et al., 2005a). Based on these observations, we analyzed whether the improper expression or loading of DNA replication licensing factors at 72 hours post-siRNA-treatment might

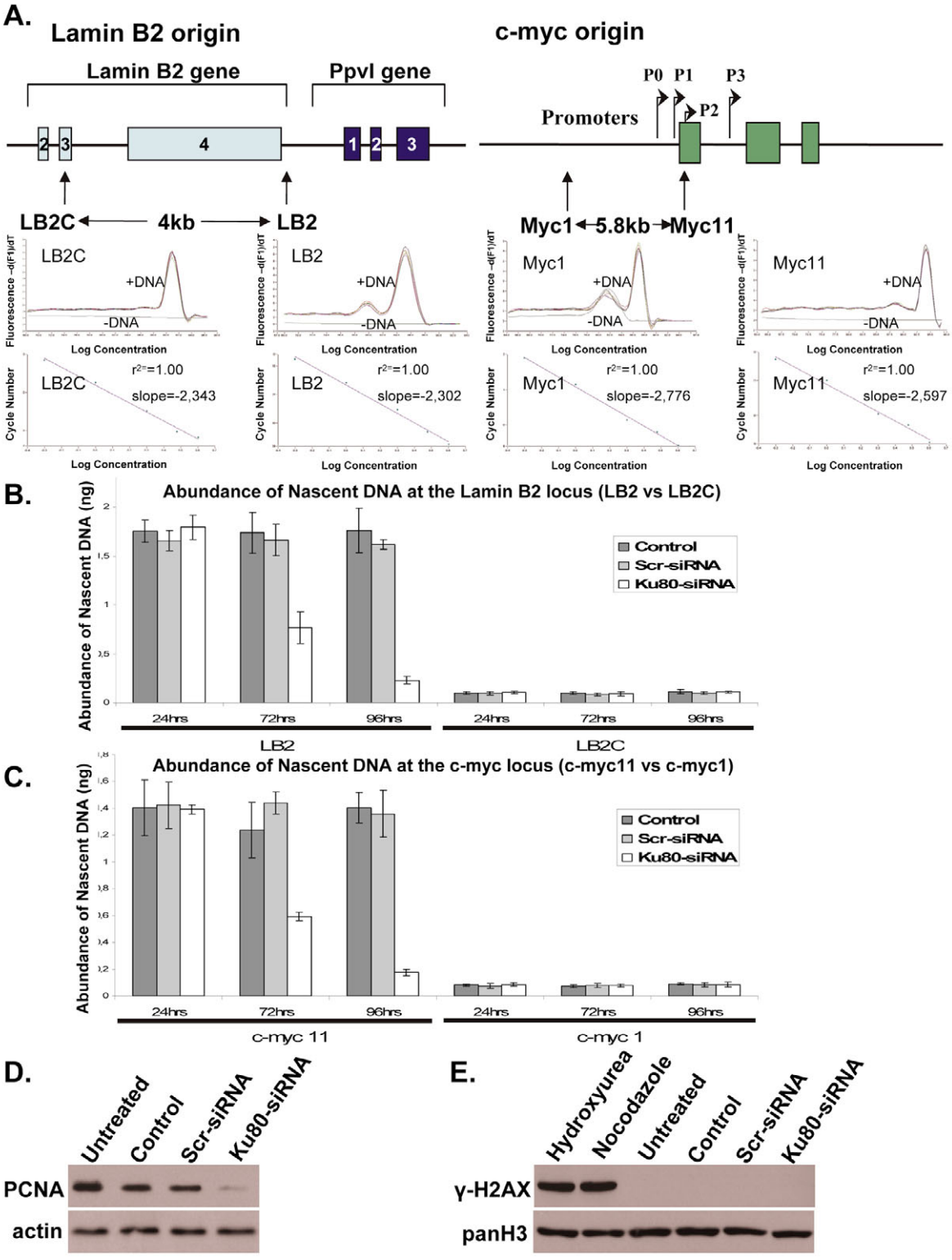

Fig. 3. Origin activation and DNA synthesis as a function of Ku80 knockdown. (A) Maps of the lamin B2 (left) and *c-myc* (right) origin loci, and melting curves of the primers corresponding to the origin-containing (LB2 and Myc11, respectively) and origin-lacking (LB2C and Myc1, respectively) amplicons. The location of the amplicons relative to the gene exons as well as their distance in kb is indicated on the maps. (B,C) Histogram plots of the lamin B2 and c-myc origin activities throughout the silencing time-course, as measured by nascent-DNA-strand abundance. The LB2 and Myc11 regions lie within the origins, whereas LB2C and Myc1 are distal origin-lacking regions. Values are expressed as ng of nascent DNA and represent three experiments ± 1 s.d. (D) Representative western blot analysis of PCNA protein expression at 72 hours post-transfection with *Ku80* siRNA. Actin was used as a loading control. (E) Representative western blot analysis of the phosphorylation levels of the H2AX histone variant at 96 hours of *Ku80* RNAi. As positive controls, extracts from cells treated with the drugs hydroxyurea (inhibitor of ribonucleotide reductase) and nocodazole (inhibitor of microtubule formation) were used. Immunostaining with a PanH3 antibody was used as a loading control.

be responsible for the observed decrease in origin activation. Neither the protein levels nor the chromatin loading of the Orc2 and Orc3 subunits – which were previously shown to bind to the origins, one independently and the other dependently of Ku80, respectively – were altered (Fig. 4A-D). By contrast, the levels of Orc1 and Orc6 were decreased in the absence of Ku80 (Fig. 4A); Orc1 by 2.7-fold in the *Ku80*-siRNA- vs scr-siRNA-treated cells and Orc6 by 4.7-fold (Fig. 4B). Furthermore, the chromatin association of Orc1 and Orc4 were both decreased by approximately half in the absence of Ku80 (Fig. 4C): 2- and 2.4-fold lower, respectively (Fig. 4D). The loading of Orc6 onto chromatin was unaffected (Fig. 4C,D), possibly because Orc6 is implicated in multiple roles besides DNA replication, such as chromosome segregation and cytokinesis (Prasanth et al., 2002).

We also examined the levels of MCM7 after Ku80 knockdown. The MCM7 protein appeared as a triplet in our western blots (Fig. 4A) when using extracts from control or scr-siRNA-transfected cells, whereas the *Ku80*-siRNA-transfected cells consistently exhibited reduced levels of the two lower bands. Because MCM4 and the MCM4-MCM6-MCM7 complex have been shown to be phosphorylated (Ishimi et al., 2000), the triplet bands might correspond to differentially phosphorylated forms of MCM7. The phosphorylation status of the MCM helicase is thought to change its conformation and facilitate the loading of subsequent factors, such as Cdc45 (Masai et al., 2006), required for origin activation (Pacek and Walter, 2004). As seen in Fig. 4, upon treatment with *Ku80*-siRNA, the cells exhibit a decrease in the loading of Cdc45 onto chromatin by 3.3-fold compared with scr-siRNA-treated cells (Fig. 4C,D), providing an explanation for the observed decrease in origin activation.

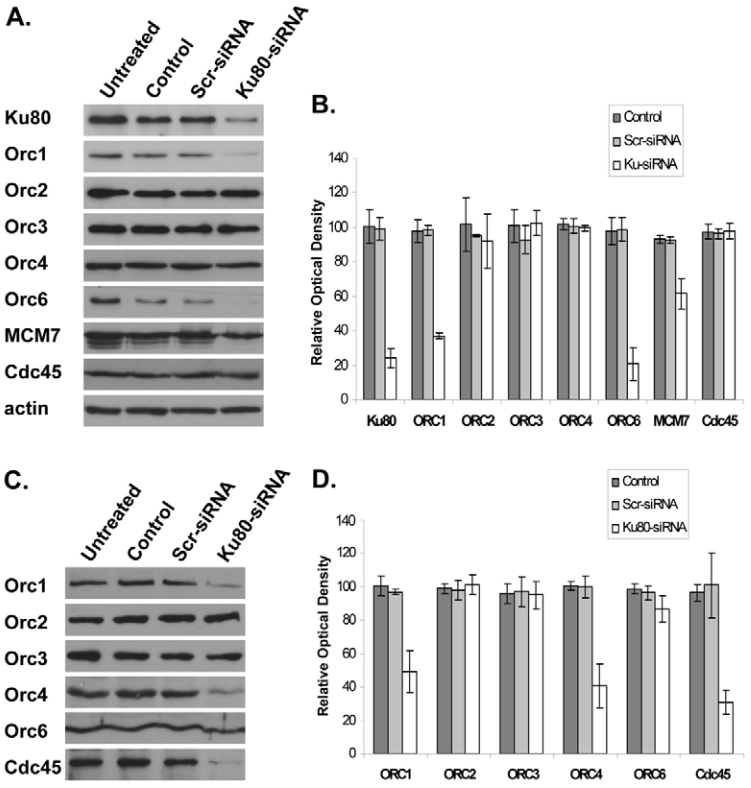

Fig. 4. Expression and chromatin loading of DNA replication licensing factors. (A) Representative western blot analyses of Ku80, Orc1, Orc2, Orc3, Orc4, Orc6, MCM7 and Cdc45 protein levels in whole cell extracts prepared from untreated, control, scr-siRNA-transfected and *Ku80*-siRNA-transfected HeLa cells at 72 hours post-transfection. Actin was used as a loading control. (B) Relative optical densities (ROD) of Ku80, Orc1, Orc2, Orc3, Orc4, Orc6, MCM7 and Cdc45 immunoreactivities. Values are expressed as ratios of the individual RODs to the one corresponding to untreated cells, and the error bars represent 1 s.d. of three experiments. (C) Representative western blot analyses of Ku80, Orc1, Orc2, Orc3, Orc4, Orc6, MCM7 and Cdc45 protein levels in the chromatin-bound fraction prepared from untreated, control, scr-siRNA-transfected and *Ku80*-siRNA-transfected HeLa cells 72 hours post-transfection. Orc2, the association of which with chromatin is essentially the same throughout the cell cycle, was used as loading control. (D) Quantification of the immunoreactivity of the proteins shown in C after Ku80 knockdown for 72 hours. Signal intensities (RODs) are means ± 1 s.d.

Cell-cycle analysis

We next examined whether the cell-cycle distribution of the cells was affected by the Ku80 deficiency and whether such a change might account for the reduced chromatin loading of the licensing factors and decline in origin activation. Interestingly, 72 hours after anti-*Ku80*-siRNA treatment, the cell-cycle distribution of the cells remained unaffected (data not shown), whereas, at 96 hours, a significant accumulation of cells at the G1 phase was observed, with 62.4% of the total cell population in *Ku80*-siRNA-treated cells having a G1-phase DNA content compared to 50.9% in scr-siRNA-treated and control cells ($P<0.05$, Student's *t*-test; $n=4$) (Fig. 5A). This suggests that the cell-cycle block did not play a causative role but was rather a consequence of the DNA-replication defect. The accumulation of cells at the G1 phase was also confirmed at the molecular level, as depicted by the cyclin A, cyclin D1 and cyclin E levels (Fig. 5B), which were appropriately decreased by threefold and 1.5-fold or increased by ninefold, respectively (Fig. 5C). In mammalian cells, two major types of cyclins, the D-type and E-type cyclins, are required for progression through G1 phase. D-type cyclins are active at mid-G1 and regulate the activity of Cdk4 and Cdk6 in response to mitogens, mainly through regulating the retinoblastoma (Rb) pathway (reviewed in Sherr, 1994), whereas E-type cyclins are accumulated later, just prior to G1-S transition, controlling it through the regulation of Cdk2 activity (reviewed in Hwang and Clurman, 2005). Cyclin A, by contrast, is expressed at the onset of S phase and participates in the activation of DNA synthesis, as well as S-phase and G2-phase progression (Pagano et al., 1992; Coverley et al., 2002). Taken together, these results suggest that Ku80-depleted cells were blocked after the G1 restriction point, at the G1-S border immediately before the activation of DNA synthesis (Fig. 5D).

Activation of a DNA replication stress checkpoint

In view of the observed accumulation of cells in late G1, we examined the underlying mechanism that prevents cells from entering S phase. Because the cyclin-E–Cdk2 complex is the major regulator of the G1-S transition (Sherr, 1994), we checked its activation status. Cdk2 activity is regulated by the periodic expression of cyclin E, the presence of the cyclin-dependent kinase inhibitors (CKIs) of the CIP/KIP family, which includes p21, p27 and p57 (Morgan, 1995), as well as an inhibitory phosphorylation at Tyr-15 that is removed by the action of Cdc25A phosphatase (Costanzo et al., 2000; Falck et al., 2001). Surprisingly, we observed a triple block of Cdk2 activity (Fig. 6A); namely, decreased levels

of Cdc25A phosphatase and Cdk2 kinase (2.5-fold and 4.7-fold decrease, respectively, Fig. 6B) and increased expression of p21 (3.6-fold increase, Fig. 6B). By contrast, the level of Cdc25B was not affected (data not shown). Similar results were also obtained with p53-containing HCT-116 *Ku80*$^{+/-}$cells (Fig. 6C), excluding the possibility of a cell-type-specific mechanism. Altogether, the data suggest a potent inactivation of Cdk2 kinase activity, which ensures that the cells do not proceed to S phase. Cyclin D1 degradation (Fig. 5B,C) also contributes to the p21-mediated inhibition of cyclin-E–Cdk2 through a mechanism termed CKI exchange, whereby less p21 is sequestered away from the cyclin-E–Cdk2 complex. Indeed, this has been shown to be the initiatory fast step of G1 arrest, which is followed by the more time-consuming transcriptional activation of p21 (Agami and Bernards, 2000).

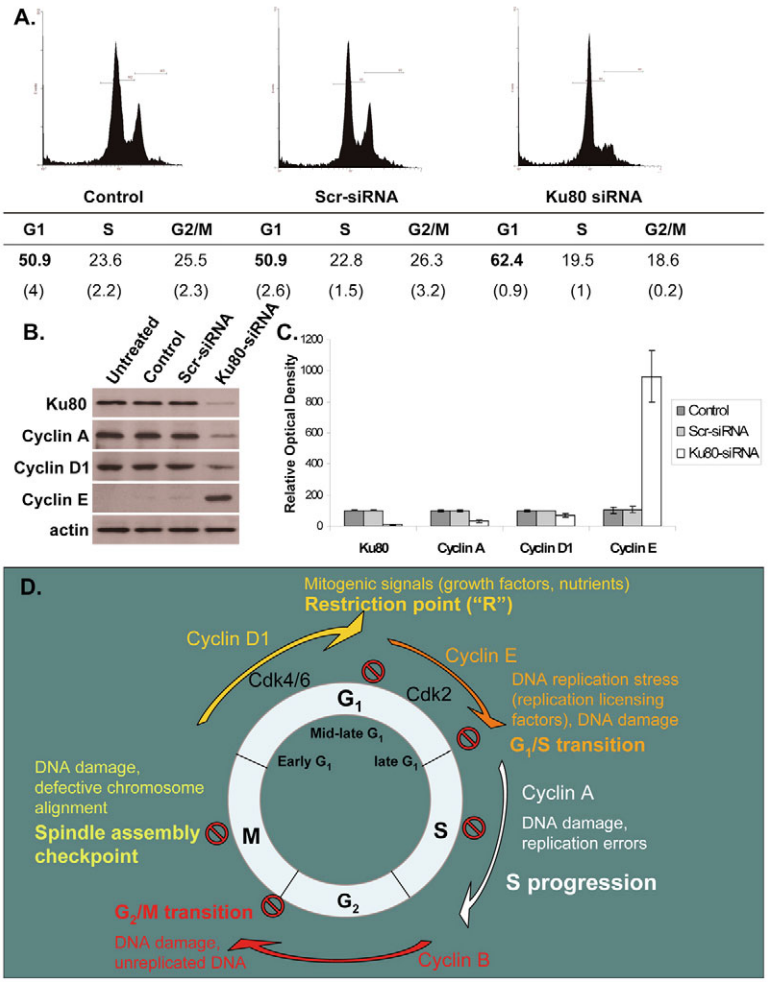

Fig. 5. Cell-cycle analysis of Ku80-knockdown cells. (A) Asynchronous cultures of control, scr-siRNA-transfected and *Ku80*-siRNA-transfected HeLa cells were harvested after 96 hours of Ku80 silencing and stained with propidium iodide for monitoring of their cell-cycle distribution. Representative flow cytometric analysis is shown (top). (Bottom) The table shows the means (n=3) of the quantification of the percentage of cells present in each phase of the cell cycle. In brackets, 1 s.d. of three individual experiments is shown. (B) Western blot analysis of the cyclin A, cyclin D1 and cyclin E protein levels in whole cell extracts prepared from untreated, control, scr-siRNA-transfected and *Ku80*-siRNA-transfected HeLa cells. (C) Quantification of the relative optical densities of cyclin A, cyclin D1 and cyclin E. Values are expressed as the average of three experiments ± 1 s.d. (D) Schematic presentation of the cyclin/Cdk cycle and the various checkpoints. Silencing of Ku80 blocks the cells at the G1-S transition point, as evidenced by high levels of cyclin E (dark-orange arrow).

p53 is a well-known inducer of p21 expression upon DNA damage; thus, we tested for its involvement in the p21 induction. HeLa cells contain no detectable p53 protein, although they contain *p53* mRNA that is translationally active, because they are HPV-18 positive and their p53 is highly unstable because of the presence of the E6 oncoprotein (Scheffner et al., 1991). Upon Ku80 silencing, no stabilization of p53 was observed in these p53-devoid cells (data not shown), suggesting that p21 expression is induced by a p53-independent mechanism. p21 expression was previously shown to be induced via a DNA-damage- and p53-dependent-manner, as well as by a DNA-damage- and p53-independent-manner (Macleod et al., 1995).

The ATM-Chk2 pathway is considered the predominant pathway that is activated upon DNA damage, leading to phosphorylation of Cdc25A and subsequent degradation (Costanzo et al., 2000; Falck et al., 2001). Again, no activation of Chk2 was observed after Ku80 knockdown (Fig. 6A,B), suggesting a DNA-damage-independent mechanism. These results are in agreement with the findings of another study, in which silencing of Orc2 also led to p21 induction independently of p53 stabilization and Chk2 activation (Machida et al., 2005a), suggesting that defects in the loading of initiator proteins and DNA damage act upon the cyclin/Cdk machinery via different pathways.

DNA damage and apoptosis are not induced in the absence of genotoxic stress

Ku has been extensively shown to be implicated in DSB repair by non-homologous end joining (NHEJ) as well as in telomere protection and maintenance (reviewed in Collis et al., 2005). In response to these stimuli, cells have evolved cell-cycle checkpoints that are activated in order for DNA repair mechanisms to take place or, in the case of extensive damage, drive the cells to apoptosis (Zhou et al., 2002; de Lange, 2002). In view of the multifunctional nature of Ku80, we examined whether silencing of its expression gave rise to apoptotic stimuli or damaged DNA, which might be responsible for the reduced growth rate and cell-cycle checkpoint activation. As can be seen in Fig. 6, apoptosis – as measured by genomic-DNA fragmentation (Fig. 6D), PARP-1 cleavage and caspase 3 activation (Fig. 6E) – was not induced after Ku80 knockdown. Because human Ku80-null cells are not viable, the survival of the Ku80 knockdown cells can be explained either by the presence of residual Ku80 protein (10% remaining protein at 96 hours) or by the unstable nature of p53 in HeLa cells (Scheffner et al., 1991). Alternatively, apoptosis might be induced at a later stage as a result of the G1 cell-arrest, beyond the 96 hours examined in this study.

Furthermore, no activation of the DNA-damage repair machinery, which detects both DSBs and short telomeres (reviewed in Verdun and Karlseder, 2007; Downs et al., 2007) was observed even after 96 hours of Ku silencing; phosphorylation of the H2AX histone variant (γH2AX), p53 stabilization and activation of Chk2 (Chk2-*P*) represent some of the primary molecular events upon DNA damage (Rogakou et al., 1998; Matsuoka et al., 1998; Chaturvedi et al., 1999; de Lange, 2002), none of which were observed here (Fig. 3E, Fig. 6A).

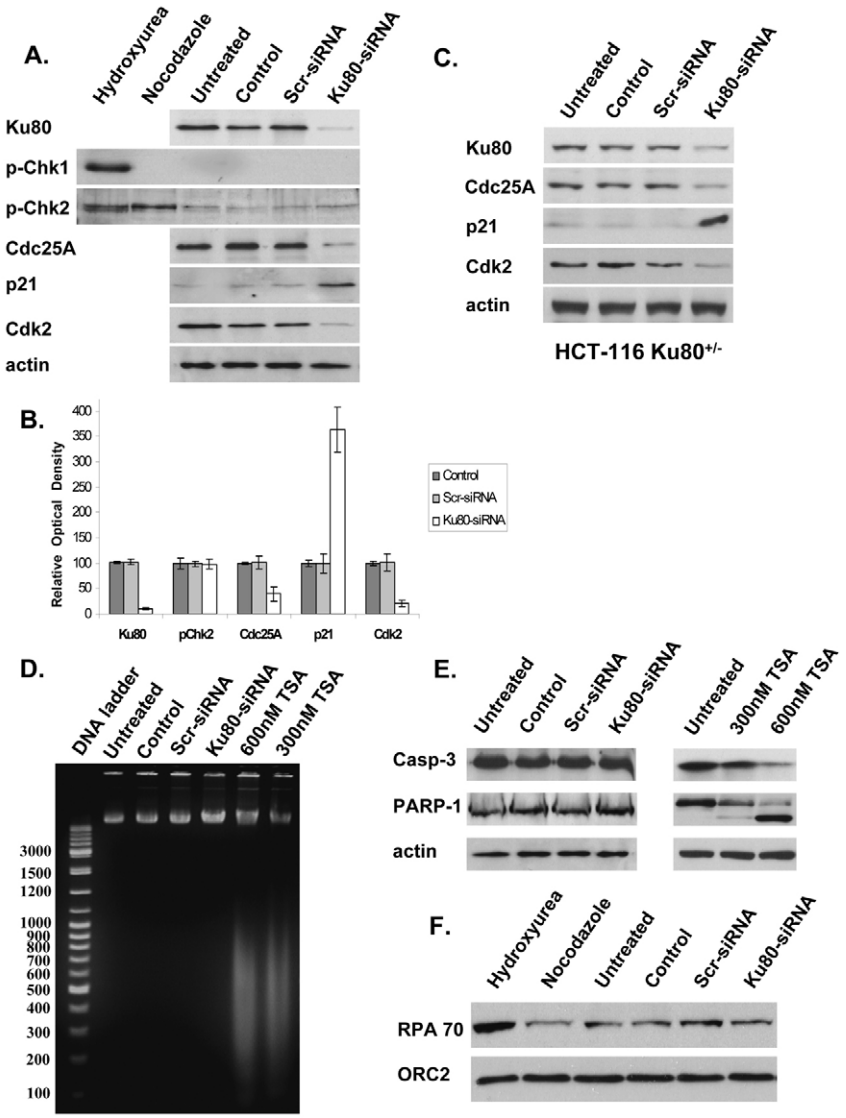

Fig. 6. Activation of a replication-stress checkpoint and analysis of apoptosis and ssDNA levels. (A) Protein expression of the cell-cycle regulators Chk1-*P*, Chk2-*P*, Cdc25A, p21 and Cdk2 in whole cell extracts prepared at 96 hours from untreated, control, scr-siRNA-transfected and *Ku80*-siRNA-transfected HeLa cells. Actin was used as loading control. Extracts from cells treated with the drugs hydroxyurea (10 mM) and nocodazole (100 ng/μl) were used as positive controls for the activational phosphorylation of Chk1 and Chk2. (B) Quantification of the protein levels of the cell-cycle regulators shown in A. Values represent the average of three experiments and the error bars correspond to 1 s.d. Chk1-*P* levels were not included in the bar graph because no detectable levels were observed. (C) Protein expression of the Cdk2 regulators Cdc25A, p21 and Cdk2 in whole cell extracts prepared after *Ku80*-siRNA transfection in HCT-116 *Ku80*$^{+/-}$ cells. (D) Genomic DNA was isolated from untreated, control, scr-siRNA-transfected and *Ku80*-siRNA-transfected HeLa cells after 96 hours of Ku80 silencing and was subjected to electrophoresis in order to determine its integrity. Extracts from cells treated with the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA), which is known to induce apoptosis, were used as positive controls. (E) Representative western blot analysis of the protein levels of full-length PARP-1 and the full-length precursor form of caspase 3, which are cleaved during apoptosis. (F) Chromatin association of the ssDNA-binding protein RPA70 at 96 hours post-transfection.

Similarly, no activation of the Chk1 kinase (Chk1-*P*) (Fig. 6A) or increase in the levels of the chromatin-bound single-stranded DNA (ssDNA)-binding protein RPA70 was detected, indicating the absence of DNA single-strand break (SSB) formation (Fig. 6F). Collectively, these results support our previous findings that impaired loading of licensing factors, and not DNA damage, is responsible for the inactivation of Cdk2 and the block of the cells at the G1-S border.

Discussion

In this study we have shown that depletion of Ku80 protein by RNAi severely impairs the growth rate of HeLa cells, with their doubling time increasing from 30 hours to 58.8 hours. This is in agreement with previous studies showing that human HCT-116 *Ku80*$^{+/-}$ cells have a growth defect (Li et al., 2002; Sadji et al., 2000) and that the proliferative state of established cell lines compared to normal tissues is correlated with increased levels of Ku expression (Cai et al., 1994). We found that this growth defect was not associated with DNA damage caused by the lack of the repair activity of Ku, but was due to decreased DNA synthesis. Because Ku has been previously implicated in the repair of DSBs caused either by IR, radiomimetic drugs, such as bleomycin, or replication-fork blockage (Collis et al., 2005; Shimura et al., 2007), this finding was surprising and suggests that, in the absence of genotoxic stress, no significant DNA damage was induced and thus the action of DNA-PK was not needed.

The knockdown of Ku80 was followed by a 59% decrease in the total rate of DNA replication at 72 hours post-treatment with the siRNA oligonucleotides, further decreasing to background levels at 96 hours. We also observed a similar pattern in the frequency of initiation events at replication origins, indicating that the Ku deficiency affected the initiation step of DNA synthesis. In eukaryotic cells, initiation of DNA replication relies on a two-step process: (1) the ordered assembly of a pre-RC onto the origin of DNA replication during late M and G1 phase, consisting of the ORC hexamer (Orc1-Orc6), Cdc6, Cdt1 and the putative replicative helicase MCM (MCM2-MCM7); and (2) the activation of the pre-RC with the action of two kinases, cyclin-E–Cdk2 and Cdc7-Dbf4, which leads to the loading of additional factors such as Cdc45, MCM10 and the GINS complex (Bell and Dutta, 2002). Subsequent recruitment of DNA primase and the replicative DNA polymerases results in origin firing. We therefore analyzed whether the expression and chromatin loading of replication licensing factors were responsible for the DNA-replication defect. In the absence of Ku80, Orc2 and Orc3 were loaded normally onto chromatin, but Orc1 and Orc4 were not, resulting in impaired formation of the ORC complex. Among the six human ORC subunits (Orc1-Orc6), Orc2, 3, 4 and 5 form a stable core complex, whereas Orc1 and Orc6 are weakly bound (Vashee et al., 2001; Dhar and Dutta, 2000) and are thought to regulate the function of the ORC hexamer. We found that the knockdown of Ku80 affected the chromatin association both of core and regulatory subunits, but the protein levels of only the latter. It

might be possible that, in the absence of a fully formed ORC core, these subunits are unstable and, therefore, targeted for proteolysis, although a transcriptional regulation cannot be excluded. Another interesting observation was the presence of MCM7 as a triplet throughout our study and the disappearance of the two lower bands upon Ku80 silencing. Although a post-translational modification of MCM7 has not been previously reported, MCM2, MCM4 and MCM5, as well as the MCM4-MCM6-MCM7 complex, have been shown to be phosphorylated (Ishimi et al., 2000), and this modification is thought to change the MCM-complex conformation, leading to the recruitment of additional factors such as Cdc45 (Masai et al., 2006). Use of the ELM server (Puntervoll et al., 2003) predicted five PIKK phosphorylation sites, suggesting that a post-translational modification of MCM7 could occur and thus the triplet might represent differentially phosphorylated forms of MCM7. As might be expected, low levels of Cdc45, a downstream licensing protein necessary for origin firing (Pacek and Walter, 2004), were also detected on total chromatin, further supporting the hypothesis that impaired loading of DNA replication licensing factors is responsible for the observed decreased growth rate and replication initiation.

Although the cell-cycle distribution of Ku80-deficient and control cells was the same at 72 hours when DNA replication was found to be impaired, these cells accumulated in G1 phase 24 hours later. Ku-knockdown cells were blocked very late in G1 phase, close to the G1-S transition point, but before origin activation, as evidenced by the high levels of cyclin E and low levels of cyclin A. Both cyclin E and cyclin A are co-factors of Cdk2, but they have distinct roles: cyclin E opens a 'window of opportunity' for replication-complex assembly, whereas cyclin A is implicated in the activation of the assembled replication complexes and the inhibition of the assembly of new complexes after origin firing (Coverley et al., 2002). Thus, in agreement with this statement and as we also observed, in the absence of sufficient replication complexes, Ku80-silenced cells would be blocked in G1 phase with the 'window' open in order for more complexes to be assembled onto the origins. Progression into S phase without the appropriate number of activated replication origins would lead to an increase in the average replicon size, resulting in stalled replication forks and chromosomal instability (Tanaka and Diffley, 2002; Ekholm-Reed et al., 2004). Our findings are in agreement with another recent study, in which the levels of Orc2 were knocked down and the cells also arrested in late G1 phase (Machida et al., 2005a), suggesting the existence of a mechanism that senses pre-RC levels and controls S-phase entry.

In response to genotoxic stress that produces damaged or unreplicated DNA, eukaryotic cells have evolved checkpoint responses, which lead to DNA repair, cell-cycle arrest or apoptosis (Sagata, 2002; Massagué, 2004). Two large protein kinases, the ataxia telangiecstasia-mutated (ATM) and the ATM and Rad3-related protein (ATR), are responsible for the induction of such checkpoints in response to DNA damage. ATM is activated by stimuli that induce DSBs, such as ionizing radiation, and acts through the ATM-Chk2-p53 pathway, whereas ATR is activated by the presence of ssDNA, which is induced by UV radiation or stalled DNA replication forks and acts through the Chk1/Chk2 downstream kinases (Fisher and Méchali, 2004). When we examined the activation status of these checkpoints in Ku80-silenced cells, we observed that neither of them was induced; no phosphorylation of the Chk1 or Chk2 kinases was detected and the highly unstable p53 protein of the HeLa cells was not stabilized. Interestingly, *Orc2* RNAi also did not activate these DNA-damage checkpoints (Machida et al., 2005a), in agreement with our findings. Instead, what we observed was a triple block acting upon Cdk2 activity. High levels of the Cdk2 inhibitor p21 in combination with low levels of the Cdk2-activating Cdc25A phosphatase and low Cdk2 levels provided a potent barrier against Cdk2 activity and G1-S progression.

Because Cdk2 has been implicated in the activation of the pre-RC and entry into S phase, it might be argued that the decreased levels of DNA replication might simply reflect low levels of Cdk2 activity. However, at 72 hours, when initiation of DNA replication was already impaired, none of the blocks in Cdk2 activity were observed, negating that possibility. Alternatively, our data support another model that was recently proposed; namely, the existence of a new type of checkpoint, which prevents cell-cycle progression into S-phase in the absence of sufficient initiator proteins by blocking the activity of the Cdks responsible for G1-S transition (Machida and Dutta, 2004). Ku binds onto origins in proximity to Orc2 and aids the ORC assembly, either by modifying the DNA environment or by stabilizing the interactions between the different subunits. Depletion of Ku, similar to depletion of other initiator proteins, leads to incomplete ORC formation and defective recruitment of downstream licensing factors onto chromatin, thus activating this checkpoint. Additional studies are necessary to substantiate the validity of this pathway, which represents an interesting perspective.

In conclusion, the results presented here further support the interplay between the DNA replication and Cdk-cycle machineries. This is the first study implicating Ku in G1-S progression via regulation of Cdk2 activity, providing a mechanism whereby the appropriate amount of pre-RCs is loaded onto chromatin before entering the S phase, thus preventing genomic instability.

Materials and Methods

Cell culture, cell-cycle analysis and drug treatments

HeLa and HCT-116 *Ku80*$^{+/-}$ cells were cultured in αMEM (minimum essential medium) (Invitrogen, Carlsad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), penicillin (100 μg/ml), streptomycin (100 μg/ml) and 1 mmol/l L-glutamine at 37°C and 5% CO_2. Cell-cycle progression was monitored by FACS analysis; cells were washed twice in ice-cold phosphate buffered saline (PBS), resuspended in Vindelov's solution [3.4 mM Tris, 75 μM propidium iodide, 0.1% NP-40, 700 U/L RNase A (Roche), 0.01 M NaCl] (Vindelov, 1977) overnight at 4°C and then analyzed using a Beckman flow cytometer and the WinMDI program. For hydroxyurea (HU) and nocodazole (NOC) treatment, cells were grown in complete medium in the presence of 10 mM HU or 100 ng/μl NOC, concentrations that are known to induce ssDNA and DSBs, respectively.

RNAi knockdown

The oligonucleotides (dsRNA) used in this study were previously described in Belenkov et al. (Belenkov et al., 2002). The targeting (anti-*Ku86*) siRNA (5′-CAGAGAAGAUUCUUCAUGGGTT-3′) and its mismatch control (5′-CAC-AGGAGCUUAUUGAUAGGTT-3′) were custom synthesized by Invitrogen. For RNAi knockdown, equal numbers of HeLa cells were seeded in medium lacking antibiotics 24 hours prior to transfection with 200 nM siRNA using Lipofectamine 2000 (Invitrogen) in serum-free Opti-MEM (Invitrogen). The oligonucleotide-containing Opti-MEM was removed from the cells and replaced with complete growth medium 4 hours post-transfection. The cells were grown at 37°C and 5% CO_2 in complete medium and harvested at 24, 72 and 96 hours for use in the appropriate experiments.

Immunofluorescence

For immunostaining with anti-Ku antibody, HeLa cells growing on coverslips were rinsed with PBS, treated with 0.5% Triton X-100 in PBS for 1 minute at room temperature (RT) and then fixed with 4% paraformaldehyde. After treatment with blocking buffer (1% BSA and 0.5% Triton X-100 in PBS) for 1 hour at RT, the cells were incubated with a mouse monoclonal anti-Ku antibody (NeoMarkers) and then with a Texas-red-conjugated goat anti-mouse IgG (Invitrogen). Co-staining with 0.1 mg/ml DAPI for 1 minute at RT was also performed to allow visualization of nuclear DNA. Fluorescence data were collected using a Nikon Eclipse E800 microscope and analyzed with the Nikon ACT-1 software.

Table 1. Sequences and amplification conditions of primers used for real-time PCR quantification of DNA with the LightCycler

Primer name	Sequence (5′-3′)	Amplicon size (bp)	$T_{Annealing}$ (°C)
LB2-F	GGCTGGCATGGACTTTCATTTCAG	232	66
LB2-R	GTGGAGGGATCTTTCTTAGACATC		
LB2C-F	GTTACCAGTCAGGCGCATGGGCC	240	66
LB2C-R	CCATCAGGGTCACCTCTGGTTCC		
Myc11-F	TATCTACACTAACATCCCACGCTCTG	192	62
Myc11-R	CATCCTTGTCCTGTGAGTATAAATCATCG		
Myc1-F	TCTCAACCTCAGCACTGGTGACA	248	60
Myc1-R	GACTTTGCTGTTTGCTGTCAGGCT		

Names and sequences of primers used for real-time quantification of DNA with the LightCycler (Roche Diagnostics). The primer sets correspond to the amplification regions shown at the maps of the lamin B2 and c-myc origins (see Fig. 3A). 'F' and 'R' designate the forward and reverse primers, respectively. The size of the PCR products in base pairs (bp) and the annealing temperature ($T_{Annealing}$) used in the PCR cycling conditions in °C is also indicated.

Growth curves

Equal densities of HeLa cells were seeded in complete medium and, at the indicated time-points after siRNA treatment, were harvested and their numbers determined using a Beckman Coulter counter.

BrdU incorporation

Cells were pulse-labeled with 50 μM 5-bromo-2′-deoxyuridine (BrdU) (Sigma) for 1 hour, harvested, washed with PBS containing 1% BSA at 4°C and fixed in cold (–20°C) 70% ethanol for 1 hour. Cells were subsequently pelleted, resuspended in a 2 M HCl solution containing 0.5% Triton X-100 and incubated at RT for 30 minutes. The acid was then neutralized with 0.1 M $Na_2B_4O_7$ (pH 8.5). Following centrifugation, the cells were washed with PBS + 0.1% BSA + 0.5% Tween 20 containing a fluorescein-conjugated anti-BrdU antibody (Roche) at a concentration of 0.5 μg/10^6 cells and incubated at RT for 30 minutes. After antibody incubation, cells were washed once with PBS and analyzed by FACS using a Beckman flow cytometer and the WinMDI program.

Extract preparation, immunoblot analysis and quantification

For the preparation of whole cell extracts (WCE), the cells were harvested, washed twice with ice-cold phosphate buffered saline (PBS) and resuspended in 2× packed cell volume (pcv) hypotonic buffer [20 mM HEPES-KOH, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 20 mM EDTA, 50 mM DTT and Complete Protease Inhibitor tablet (Roche Molecular Biochemicals, Indianapolis, IN)]. Following 1-hour incubation at 4°C in constant agitation, the cells were centrifuged at 14,000 ***g***, the supernatant harvested and its protein concentration determined using the Bradford Protein Assay (Bio-Rad, Hercules, CA). Western blot analysis was carried out according to standard protocols (Sambrook et al., 1989). Briefly, the indicated amounts of WCE were resuspended in SDS loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 10 minutes and loaded on an 8% SDS-PAGE gel. Following electrophoresis, the proteins were transferred onto a PVDF membrane and the membrane was immunoblotted with the indicated primary and corresponding HRP-conjugated secondary antibodies. The following antibodies were used: anti-Ku86 (sc-9034), anti-Orc1 (sc-23887), anti-Orc2 (sc-28742), anti-Orc3 (sc-21862), anti-Orc6 (sc-32735), anti-MCM7 (sc-9966), anti-Cdc45 (sc-20685), anti-PCNA (sc-56), anti-cyclin-E (sc-481), anti-cyclin-D1 (sc-753), anti-cyclin-A (H-432), anti-p53 (sc-6243), anti-p21 (sc-397), anti-Cdk2 (sc-6248), anti-Cdc25A (sc-7389), anti-phospho-Chk2 (Thr68) (sc-16297-R), anti-caspase-3 (sc-7148), anti-PARP-1 (sc-8007), anti-RPA70 (sc-25376) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Orc4 (Serotec), anti-actin (A 2066; Sigma), anti-phospho-Chk1 (2341; Cell Signaling), anti-phospho-Histone H2A.X (Ser139) (05-636; Upstate) and anti-panH3 (30374; Upstate). Proteins were visualized using the enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions (Amersham Biosciences, Arlington Heights, IL) and the signals were quantified using the ImageJ program.

Chromatin loading

Cell fractionation and preparation of the chromatin-enriched fractions was performed as described in Tatsumi et al. (Tatsumi et al., 2000). Untreated or transfected cells were harvested from 10-cm dishes into ice-cold PBS, centrifuged, resuspended in 1 ml of lysis buffer A [10 mM HEPES-KOH, pH 7.9, 100 mM NaCl, 300 mM sucrose, 0.1% Triton X-100 and Complete Protease Inhibitor tablet (Roche Molecular Biochemicals)], and lysed on ice for 10 minutes. After centrifugation at 2000 ***g*** for 3 minutes at 4°C, pellets were washed once more with ice-cold lysis buffer A and resuspended in lysis buffer B [10 mM HEPES-KOH, pH 7.9, 200 mM NaCl, 300 mM sucrose, 0.1% Triton X-100, 5 mM $MgCl_2$ and Complete Protease Inhibitor tablet (Roche Molecular Biochemicals)] containing 1000 U of DNase I (Invitrogen). Following incubation at 25°C for 30 minutes, the chromatin-enriched fraction was isolated in the supernatant after centrifugation at 2500 ***g*** for 5 minutes at 4°C.

Isolation of genomic and nascent-strand DNA

Isolation of nascent-strand DNA was performed using the λ exonuclease method as previously described (Giacca et al., 1997; Tao et al., 2000). Briefly, the cells were washed twice with PBS and lysed in Hirt's lysis buffer (50 mM Tris-HCl, pH 8.0, 0.6 M NaCl, 1 mM EDTA and 0.5% SDS) (Hirt, 1967). Following a 10-minute incubation at RT, the lysate was digested overnight with 0.1 mg/ml Proteinase K at 65°C. The lysate was extracted twice with phenol/chloroform (1:1) and the DNA was ethanol precipitated and sheared by passage through a 26G3/8 needle. 20 μg of DNA were denatured at 100°C for 5 minutes, phosphorylated with 10 U of T4-polynucleotide kinase (New England Biolabs) for 30 minutes at 37°C and the enzyme was then heat-inactivated at 100°C. Subsequently, the samples were subjected to λ exonuclease (NEB) digestion at 37°C for 12 hours. In order to separate nascent DNA from Okazaki fragments, the samples were subjected to electrophoresis on a 2% agarose gel. The DNA was visualized by staining with 0.01% (w/v) methylene blue (Sigma) and the origin-containing nascent DNA ranging between 350 and 1000 bp in size was excised from the gel and purified using the QiaExII extraction kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. DNA was eluted with dH_2O and quantified using real-time PCR.

For the genomic-fragmentation assay, total genomic DNA from cells at the indicated time points was prepared after ethanol precipitation as described above. 10 μg of DNA were subjected to electrophoresis on a 2% agarose gel and stained with ethidium bromide. As a positive control for apoptosis, DNA isolated from cells treated with the histone deacetylase inhibitor (HDAC) Trichostatin A (Sigma; T 8552) for 24 hours was used, which is known to stimulate cell death (Finzer et al., 2001).

Real-time PCR quantification of DNA

Nascent-strand DNA was quantified using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. PCR reactions were carried out in a total volume of 20 μl, as previously described (Sibani et al., 2005b). The sequences and amplification conditions for all primer sets are listed in Table 1. Non-replicating genomic DNA from serum-starved HeLa cells was included in each run to create a standard curve necessary for the quantification of the PCR products. A negative control without template DNA was also included with each set of reactions. The PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and visualized with an Eagle Eye apparatus (Speed Light/BT Sciencetech-LT1000).

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