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Periodic Ubiquitination of Ku70 subunit regulates the cell cycle related activity of Ku protein

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Ku, a heterodimer of Ku70 and Ku80 subunits, is well-known for its role in DNA repair as the DNA-binding component of DNA-PKcs. In addition, it is involved in other important cellular processes including replication initiation. Periodic pphosphorylation of Ku70 by cyclin dependent kinases (Cdk) inhibits its interaction with replication origin, resulting in prevention of re-replication during S, G2 and M-phases. Interestingly, Ku70 is also ubiquitinated at K299 and K605 residues that are located on its dimerization and DNA-binding interfaces, respectively, suggesting crucial effect on its function by the posttranslational modification. Significantly, when the target lysine residues are mutated, Ku70 is ubiquitinated on alternative sites which are also located in the DNA-binding domain. Moreover, the identification of Cdc20 as the responsible ubiquitin ligase implicates periodicity in Ku70 ubiquitination. Expectedly, Ku70 ubiquitination occurs once at G1-phase and again at early S-phase, affecting its dimerization with Ku80. Overall, the earlier observation of Ku binding to replication origin during early G1 and S-phases along with detailed time-point experiments in the present study establish that, in the absence of Cdk activity, the windows of reversible Ku70 ubiquitination during G1 and early S-phases remove Ku from replication origin after its replication initiation related function.

Keywords: Eukaryotic cell cycle, Ku protein, Ku70 ubiquitination, Posttranslational modification, Pre-replication complex, Replication initiation, Ubiquitination

Post-translational modifications (PTMs) regulate major cellular processes like cell cycle transitions, protein degradation, replication initiation and many others. The heterodimeric Ku protein, consisting of Ku70 (XRCC6) and Ku80 (XRCC5) subunits, is well-known for its major role in non-homologous end-joining (NHEJ) pathway[1]. Ku acts as a sensor of DNA double strand break (DSB) and facilitates the recruitment of other factors including DNA-PKcs and DNA Ligase for NHEJ to take place[2]. However, in addition to its role in NHEJ, the Ku heterodimer plays vital role in multiple other cellular processes such as V(D)J recombination, telomere maintenance, transcriptional regulation, apoptosis, and also in replication initiation[2]. Notably, periodic phosphorylation of Ku70 subunit by cyclin-Cdks during S, G2 and M-phases has been found to regulate the replication licensing related activity of Ku[3].

Replication initiation in eukaryotes is carried out in two temporally distinct steps to prevent re-replication – "Origin Licensing" through the formation of a pre-Replication Complex (pre-RC) at G1 phase followed by Initiation of DNA synthesis *via* "Firing" in S phase[4]. The association of Ku in sequence specific and cell cycle dependent manner with mammalian replication origins Ors8 and Ors12 has implicated its involvement in replication initiation. Expectedly, the association has been shown to be five-fold higher at G1/S phase in dividing cells, compared to that in G0 cells[5]. Interaction of Ku has also been reported with several other replication origins, including c-myc, Lamin B2[6], adenovirus type 2[7], A3/4 sequence[8], Chinese hamster dihydrofolate reductase oriβ[8], human dnmt1[5]. Besides this, a whole range of DNA replication proteins including Orc2, Orc3, Orc4, Orc6, DNA polymerase α, δ, ε, PCNA, RFC, RPA, topoisomerase II, has been found to interact with Ku[9]. More intriguingly, after initial binding of Orc2 to

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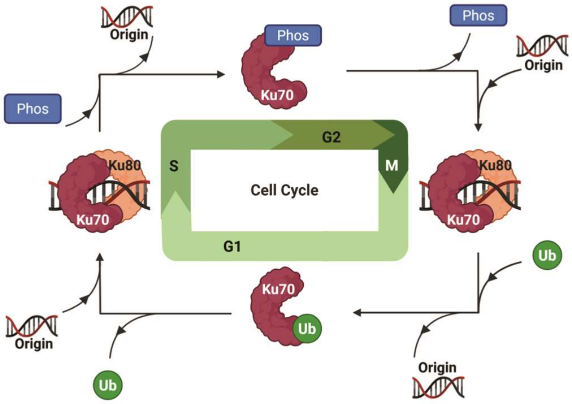

Graphical abstract

origin, subsequent loading of Orc3, Orc4 and Orc6 necessitates the binding of Ku-heterodimer with the origin indicating its critical role in pre-RC formation[10]. However, the removal Ku from origin sequence during pre-RC formation is crucial though the mechanism remains unexplored. Therefore, it will be interesting to investigate whether any posttranslational modification (PTM) of Ku is responsible for its removal from the origin.

Unlike the replication clamp protein PCNA and many other ring-shaped DNA-binding complexes that can be released from DNA through conformational changes separating the subunits, both subunits of Ku encircle the DNA and form an extended protein-protein inter-digitation[11,12]. Thus, the two subunits cannot be separated by a simple conformational change, and a distinct removal mechanism is likely necessary. Previous work of Postow et al. has shown that the ubiquitination of Ku80 subunit is responsible for its removal from DNA after DSB repair[11]. Thus, it would be intriguing to investigate the ubiquitination of Ku protein during cell cycle progression and the effects of this PTM on replication initiation.

Through the coordinated actions of activating (E1), conjugating (E2) and ligating (E3) enzymes, ubiquitination occurs on ε-amino group of specific lysine (K) residues in the target proteins. Although it is well-known that ubiquitination is a signal for protein degradation via proteasome mediated mechanism ensuring ordered and well-timed cell cycle transitions, in recent years an increasing number of non-proteolytic outcomes of protein ubiquitination involving several critical cellular processes have been reported[13]. A protein can be monoubiquitinated, multi-monoubiquitinated, or polyubiquitinated. Poly-ubiquitination of a substrate happens via one or more of the seven lysine residues of ubiquitin itself, resulting in variable chain lengths and different topologies targeting distinct cellular processes[14]. Earlier, the effect of cell cycle dependent periodic phosphorylation of Ku protein on replication initiation has been elucidated in our laboratory. It has been shown that Ku70 is maintained in a phosphorylated state during S, G2 and M-phases by cyclin E1-Cdk2, cyclin A2-Cdk2 and cyclin B1-Cdk1. Thus the association of Ku with replication origin is prevented in those phases of the cell cycle, and on exit from mitosis, Ku is readily dephosphorylated resulting its interaction with origin sequences in G1-phase for replication licensing related function[3]. Since, Cdks remain inactive throughout G1-phase till START or Restriction point, the role of ubiquitination can be crucial for the regulation of reversible origin binding Ku-protein during pre-RC formation.

Therefore, a detailed characterization of periodic ubiquitination of Ku-protein during cell cycle has been carried out in the present study in order to elucidate its possible role in replication initiation. The study shows that Ku70 subunit is reversibly ubiquitinated during G1-phase indicating its possible role during pre-RC formation. The responsible E3 ligase and the specific lysine residues that are ubiquitinated in Ku70 are also characterized.

Materials and Methods

Protein expression and purification

Human Ku70 Open Reading Frame (ORF) was amplified from the cDNA clone MHS1010-74186 (Open Biosystem) and inserted into pET28a (bacterial expression vector) and pEBG (mammalian expression vector). The protein induction in *Escherichia coli* BL21-DE3 strain was carried out overnight at 18°C with 1 mM IPTG. The 6×His-tagged Ku70 (His-

Ku70) protein was purified using Ni-NTA agarose beads (Qiagen).

Preparation of S-100 fraction

2×10^9 HeLa cells were suspended in 1.3 ml chilled hypotonic lysis buffer of 10 mM HEPES-KOH (pH 7.8), containing 5 mM $MgCl_2$, 10 mM KCl and protease inhibitor cocktail and subjected to three rapid freeze-thaw cycle using liquid nitrogen for quick freezing. The resulting suspension was then homogenized with 40 strokes in Dounce homogenizer and centrifuged at 15,000×g at 4°C. The supernatant was then centrifuged again at 100,000×g for 4h to obtain the S-100 fraction[15].

In vitro Ubiquitination

For *in vitro* ubiquitination, the S-100 extract of HeLa cells and the energy regeneration buffer (EBR) were prepared first. The energy regeneration buffer was prepared in 20mM Tris-HCl (pH 7.5) containing 5 mM ATP, 5 mM $MgCl_2$, 20 mM creatine phosphate and creatine kinase.

At first, the suspension of His-Ku70 protein beads was equilibrated with 20 mM Tris-HCl (pH 7.5). Then S-100 extract, MG132 and ubiquitin-aldehyde were pre-incubated together for 15 min at 30°C, followed by the addition of HA-tagged Ubiquitin, ERB and His-Ku70 protein to the pre-incubated mix. The reaction was allowed to continue for 2h at 30°C. For the negative control reaction, no ubiquitin, ERB and ubiquitin-aldehyde were added. The reaction mix was then washed thrice with 25 mM Tris-HCl (pH 7.5) and the samples were analyzed by immunoblotting using anti-HA antibody (sc-7392; Santa Cruz Biotechnology) as well as anti-ubiquitin antibody (MMS-258R; Covance) for the detection of ubiquitination. The blot was probed again with anti-Ku70 antibody (sc-9033; Santa Cruz Biotechnology) to ensure that all the reaction mixes contained the comparable amounts of Ku70 protein.

Cell culture and synchronization

The human cell line HeLa was cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were arrested and synchronized at early S-phase using double thymidine block. The cells were first incubated with 2 mM Thymidine for 18 h, then allowed to grow for 9h without thymidine and finally, incubated again in the presence of 2 mM thymidine for 17 h. Finally, the cells were harvested at different time-points.

Flow cytometry

The adherent cells were first washed with phosphate buffered saline (PBS), followed by harvesting by enzymatic release using accutase solution. The harvested cells were pelleted down by centrifugation and fixed in 70% chilled ethanol for at least 30 min on ice. Then the cells were washed thrice with PBS, followed by resuspension in RNase A (200 µg/µL) containing PBS solution and incubation for 1h at 37°C. Propidium Iodide (10 µg/mL) was added and the cells were kept at dark for 15 min, followed by the analysis of the samples in Beckman-Coulter EPICS XL flow cytometer.

Immunoprecipitation

For determination of ubiquitination status of Ku70 in different phases of the mammalian cell cycle, the protein extracts from the synchronized HeLa cells at various time-points were subjected to immunoprecipitation with anti-ubiquitin antibody, followed by anti-Ku70 immunoblotting. The cell cycle progression was ascertained by differential expression of Cyclin E and Cyclin B using immunoblotting with anti-Cyclin E1 (05-363; Millipore) and anti-Cyclin B (05-373; Millipore) antibodies. The synchronicity of cells was also confirmed by the analysis in a flow cytometer.

After cell synchronization, HeLa cells were detached by gentle scrapping; and the cell pellets were resuspended in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 0.5% Triton X-100, 5 mM NaF, 1 mM Na_3Vo_4, 5% glycerol, 1 mM EDTA, 1 mM PMSF, protease inhibitor cocktail; and extracted by vortexing occasionally, followed by centrifugation at 15,600×g for 30 min at 4°C. The protein concentration was estimated by Bradford assay. A fraction of the total protein was precleared with Protein-G agarose beads for 1 h at 4°C. The precleared lysate was subjected to immunoprecipitation by incubating overnight with anti-ubiquitin antibody (sc-8017; Santa Cruz Biotechnology) and Protein-G agarose beads at 4°C. When incubation time was over, the samples were centrifuged and the beads were washed three times. Finally, the precipitated complexes were analysed by immunoblotting with antibody against Ku70 (sc-9033, Santa Cruz Biotechnology).

Kinase assay

The kinase assay was carried out at 30°C in 50 mM Tris–HCl (pH 8.0) containing 10 mM $MgCl_2$, 50 µM ATP, with bead suspension of His-Ku70 in the presence of Cyclin E1/Cdk2, Cyclin A2/Cdk2 or Cyclin B1/Cdk1 for 30 min. The products were analysed finally by immunoblotting using anti-Phospho-Threonine-Proline antibody (#9391; Cell Signalling).

The kinase assay was followed by ubiquitination of the phosphorylated proteins to investigate the effects of phosphorylation of Ku70 on the ubiquitination. After kinase reactions with Cyclin E1/Cdk2, Cyclin A2/Cdk2 or Cyclin B1/Cdk1, parts of the phosphorylated samples were analysed by immunoblotting using anti-phospho-threonine-proline antibody. The remaining portions of phosphorylated protein were used for ubiquitination. Two additional control reactions were carried out – a negative control (no phosphorylation, no ubiquitination) and a positive control (no phosphorylation but ubiquitination). The samples from all the reactions were analysed by immunoblotting using anti-HA antibody (sc-7392; Santa Cruz Biotechnology) and anti-Ku70 antibody (sc-9033; Santa Cruz Biotechnology).

Ubiquitin ligase competition assay

Four separate ubiquitination reactions of His-Ku70 were conducted, of which one was negative control and one positive control. The positive control was a normal *in vitro* ubiquitination reaction. Anti-Cdc20 antibody (sc-13162; Santa Cruz Biotechnology) or anti-Cdh1 antibody (34-200; Thermo Fisher Scientific) was added into each of the other two reactions; which otherwise contained all other reagents as present in positive control including S100, MG-132, HA-tagged ubiquitin and energy regeneration buffer (ERB). Before performing the ubiquitination, anti-Cdc20 primary antibody (400 ng) or anti-Cdh1 primary antibody (400 ng) was incubated at 30°C for 15 min with S100.

Co-immunoprecipitation study

For determining when Cdc20 was involved in ubiquitination of Ku70 protein in different phases of the mammalian cell cycle, the protein extracts from the synchronized HeLa cells at various time-points were subjected to co-immunoprecipitation with anti-Cdc20 antibody (sc-13162; Santa Cruz Biotechnology), followed by anti-Ku70 immunoblotting (sc-9033, Santa Cruz Biotechnology).

To find out when Ku80 interacts and form heterodimer with Ku70 in different phases of the mammalian cell cycle, the protein extracts from the synchronized HeLa cells at different time-points were subjected to immunoprecipitation with anti-Ku70 antibody (sc-9033; Santa Cruz Biotechnology), followed by immunoblotting with anti-Ku80 antibody (sc-5280; Santa Cruz Biotechnology).

In-gel tryptic digestion

In-gel tryptic digestion of Ku70 protein was performed using the Thermo Scientific In-Gel Tryptic Digestion Kit (Cat. no. 89871) with minor standard laboratory adaptations. Briefly, the His-Ku70 protein band was excised from a Coomassie-stained SDS–polyacrylamide gel and cut into small pieces, followed by complete removal of the stain using a destaining solution. The gel pieces were then treated with TCEP for the reduction of di-sulphide bonds at an elevated temperature. The alkylation of cysteine residues was carried out with iodoacetamide (IAA) in dark at room temperature to prevent reformation of di-sulphide linkages. Following sequential washing and dehydration steps, the gel pieces were rehydrated with the sequencing-grade modified trypsin supplied with the kit and incubated briefly on ice to facilitate enzyme penetration. The proteolytic digestion was subsequently carried out at 37°C overnight, allowing efficient cleavage of Ku70 into tryptic peptides. The resulting peptides were extracted from the gel matrix using the extraction buffer (1% TFA), pooled, and concentrated using a vacuum concentrator. The dried peptide samples were finally reconstituted in the reconstitution solution (0.1% TFA and 50% acetonitrile) for further analysis by mass spectrometry.

Liquid chromatography-tandem mass spectrometry

The resulting tryptic peptides were subsequently analysed by LC-ESI-MS (Waters Xevo-G2-XS Q-TOF* at Bose Institute, Kolkata, India) which facilitated the precise identification of ubiquitination sites on specific lysine residues of the Ku70 protein.

Results and Discussion

The interaction of Ku with replication origin and its involvement in pre-replication complex (pre-RC) formation during G1-phase are well documented[5,10,16,17]. However, the timely removal of protein factors from a functional complex is equally important for the completion of a cellular process, and in this regard, mechanism of Ku removal from replication origin at G1-phase after its role in pre-RC formation remains unexplored. Periodic phosphorylation of the Ku70 subunit by cyclin-Cdks inhibits the interaction of Ku with origin sequences in S, G2 and M-phases, and plays a critical role in preventing re-initiation of replication[3]. Since Cdk activities are absent during G1-phase, Ku subunits can be target of other forms of posttranslational modification (PTM) including ubiquitination. Notably, Ku80 has been shown to be ubiquitinated for its efficient removal from DNA following completion of a repair process[11,18]. Since Ku70 has already been shown to be a target of cell cycle kinases and important for periodic activities of Ku heterodimer, it will be interesting to investigate the possibility of Ku70 being the target of ubiquitination for reversible functional role of the heterodimer in G1-phase. Moreover, Ku70 contains 59 lysine residues and several of them can be targets of ubiquitination as predicted by various online tools (Suppl. Table S1). Therefore, the ubiquitination of Ku70 subunit and its possible role in periodic activity of Ku protein are investigated in the present study.

Ubiquitination of Ku70

In order to explore the possibility of Ku70 ubiquitination *in vitro*, the protein was expressed with a 6×His tag (His-Ku70) in *E. coli* and purified using Ni-agarose affinity chromatography (Suppl. Fig. S1). The purified protein was analysed by SDS-PAGE followed by Coomassie blue staining and confirmed by anti-Ku70 immunoblotting (Suppl. Fig. S1). The Ni-agarose bead suspension of the purified His-Ku70 was used for subsequent experiments.

His-Ku70 bead suspension was subjected to ubiquitination using HA-ubiquitin and the products were analysed by immunoblotting with anti-HA as well as anti-ubiquitin antibodies. A single band at 70 kDa position in the anti-HA immunoblot (Fig. 1a, lane 2) with no band in the negative control reaction without HA-ubiquitin (lane 1) confirmed mono-ubiquitination of Ku70. The result was further ascertained with anti-ubiquitin immunoblot where a specific band of significantly higher intensity was observed in the ubiquitination lane (lane 2) compared to that in the negative control lane. The presence of comparable levels of Ku70 protein in both the reactions was confirmed by anti-Ku70 immunoblotting. Collectively, the results demonstrated that Ku70 could be modified by ubiquitination *in vitro*.

Since phosphorylation by Cdks regulates activities and fates of their targets[21] including their ubiquitination status[22], and Ku70 is phosphorylated by multiple Cdks[3], it would be interesting to explore the effect of Cdk phosphorylation on its ubiquitination. Therefore, to determine the effect of phosphorylation on Ku70 ubiquitination, His-Ku70 was phosphorylated by cyclin E1/Cdk2, cyclin B1/Cdk1 or cyclin A2/Cdk2 (Suppl. Fig. S2) and then subjected to ubiquitination *in vitro*. However, Ku70 phosphorylated by different cyclin-Cdks displayed comparable levels of ubiquitination, as evidenced by anti-ubiquitin immunoblotting showing bands of similar intensities across all lanes (Fig. 1b), confirming that Ku70 ubiquitination was not significantly affected by its phosphorylation status. Overall, the data suggest that

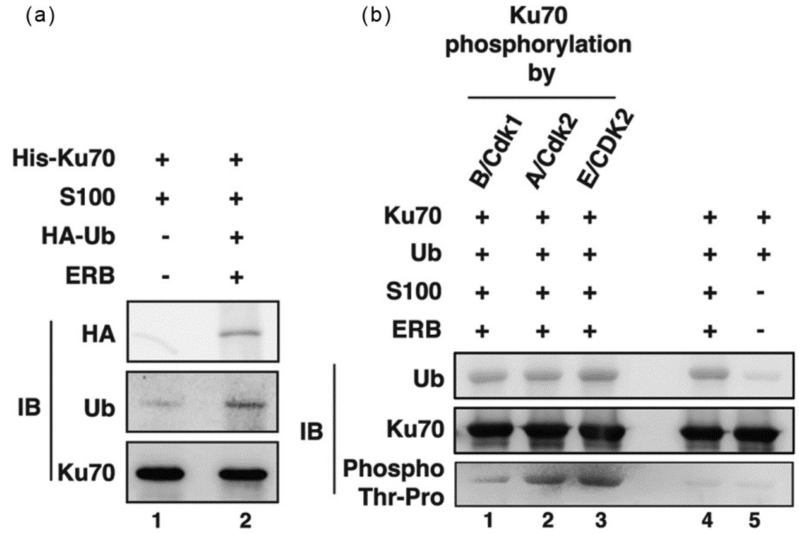

Fig. 1 — (a) *In vitro* ubiquitination of Ku70. Ubiquitination of His-Ku70 was done with HA-ubiquitin and checked by immunoblotting (IB) with anti-ubiquitin as well as anti-HA tag antibodies. A control reaction was carried out without HA-Ub and energy regeneration buffer (ERB) (lane 1). The presence of equal amount of His-Ku70 protein was verified by anti-Ku70 immunoblotting; and (b) Effect of phosphorylation on ubiquitination of Ku70. His-Ku70 was phosphorylated by cyclin E-Cdk2, cyclin A-Cdk2 or cyclin B-Cdk1 (confirmed by anti-phospho-threonine-proline immunoblotting) and the ubiquitination of the phosphorylated proteins was carried out

Ku70 phosphorylation and ubiquitination are independent regulatory events that occur, most likely, at distinct cell cycle phases.

Sites of Ubiquitination on Ku70

As mentioned before, number lysine residues in Ku70 were predicted to be the possible ubiquitination targets (Suppl. Table S1), and so, the identification of the actual sites of ubiquitination would be useful for functional characterization of the protein. To determine the sites of ubiquitination in Ku70, two *in vitro* reactions were performed: one with ubiquitin and another with no ubiquitin. The corresponding SDS-PAGE bands were analysed by LC-ESI-MS, which identified K299 of the DNA binding domain and K605 of the SAP domain of Ku70 protein as the ubiquitination sites (Suppl. Table S2 and Fig. 2a). Out of these two residues, K605 was predicted to be present on the surface of the Ku70 protein (Suppl. Table S1). The incorporation of a ubiquitin moiety at any one of the identified targets could potentially jeopardise the DNA binding activity of Ku70. The modification with an 8 KDa ubiquitin moiety might also affect the subunit interaction as the modified residues, particularly K605, would be quite close to the dimerization domain.

In order to validate the sites of ubiquitination in Ku70, two variants were prepared by mutating K299 or K605 residues to arginine (Ku70-K299R or Ku70-K605R). A double mutant was also generated where both K299 and K605 were mutated to arginine (Ku70-K299R-K605R). When the mutants were subjected to ubiquitination, it was observed that the signals were reduced for K605R as well as K299R mutants, but the reduction was significantly higher for K605R variant (Fig. 2b), suggesting it being more probable target site for ubiquitination. Intriguingly, the ubiquitination signal for double mutant was almost similar as the wild-type, indicating that the mutations might induce some changes in Ku70 protein exposing non-canonical sites for the modification. To determine the sites of ubiquitination in the double mutant of Ku70, LC-ESI-MS analysis of the ubiquitinated protein was carried out and K94, K543, K461/463 were found to be the sites of ubiquitination (Suppl. Table 3). Interestingly, the non-canonical sites of ubiquitination are also located in DNA binding interface of Ku70 (Fig. 2a).

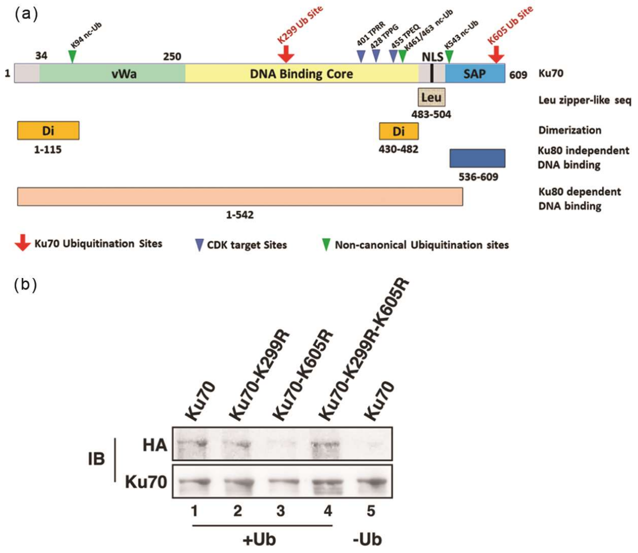

Fig. 2 — Ubiquitination of wild type and mutant Ku70. (a) The different functional domains of Ku70 protein are shown in the schematic diagram indicating the Cdk phosphorylation sites and the ubiquitination sites. The lysine residues that were ubiquitinated in the double-mutant where the primary ubiquitination sites were changed, are also indicated (non-canonical ubiquitination sites); and (b) 6×His tagged wild type Ku70 and the mutant versions (the indicated lysine residues were mutated to arginine) were subjected to ubiquitination with HA-ubiquitin and confirmed by anti-HA tag immunoblotting (IB)

Periodic ubiquitination of Ku70 during cell cycle and its effect on heterodimerization

To investigate the Ku70 ubiquitination status in mammalian cells, the level of the PTM in Ku70 was analysed in synchronized HeLa cells. The cells were arrested at the early S-phase using a double thymidine block and subsequently the block was released allowing the cells to continue the cycle synchronously. The samples of the synchronously growing cells were collected for flow cytometry and immunoprecipitation at the indicated time-points following the release (Fig. 3a). The immunoprecipitation was carried out with anti-ubiquitin antibody and the presence of Ku70 in the precipitates, indicating its ubiquitination, was analysed by anti-Ku70 immunoblotting.

Interestingly, the analysis revealed that Ku70 ubiquitination occurred in a cell cycle dependent manner, with prominent ubiquitination detected at 0 h and again at 18 h post-release, corresponding to early S-phase (Fig. 3a). As observed, the total duration of the HeLa cell cycle was approximately 18 h in the present study, as the cells were re-entering S-phase around 18 h after release. Notably, an additional ubiquitination event was observed at 10 h point, corresponding to the middle third of G1 phase (Fig. 3a). The synchronicity of the culture and cell cycle phases were validated by the periodic expression patterns of cyclin E for S-phase and cyclin

B for M-phase, as well as by flow cytometric analysis. The findings clearly demonstrated that Ku70 was differentially ubiquitinated at multiple phases of the cell cycle, specifically during early S-phase and middle third of G1-phase. In order to characterize the ubiquitination event in more details during G1-phase, the samples were collected at shorter intervals during M and G1-phase as indicated, and as before, anti-ubiquitin immunoprecipitation was carried out followed by detection of Ku70 in the precipitates. As shown in Figure 3b, Ku70 could be detected in anti-ubiquitin immunoprecipitates, indicating its ubiquitination during 9-10.15 h after the release from double thymidine block. No ubiquitination of Ku70 could be detected during 7.5 h and 8.15 h time-points after the release from the block. As per the cyclin B immunoblot and flow cytometer analysis (Fig. 3a and 3b), the 8 h time-point corresponded to late M-phase after which transition to G1-phase occurred. As shown previously[3], Ku70 remained phosphorylated till 7.5 h (M-phase) time point, and the present study showed that the protein was ubiquitinated during 9-10h period (G1-phase). Taken together, it was clearly established that Ku70 briefly remained unmodified during late M-phase to early G1-phase, when Ku might interact with the origin DNA to carry out its role in pre-RC formation.

In this regard, it will be interesting to see the effect of ubiquitination on interaction between the Ku subunits during cell cycle. For this, anti-Ku70 immunoprecipitation from synchronously growing HeLa cells at the indicated time-points (Fig. 3c) were carried out and the presence of Ku80 subunit in the precipitates were analysed by immunoblotting. As shown in Figure 3c, no heterodimer could be detected during 0 h and 10 h time-points, corresponding to

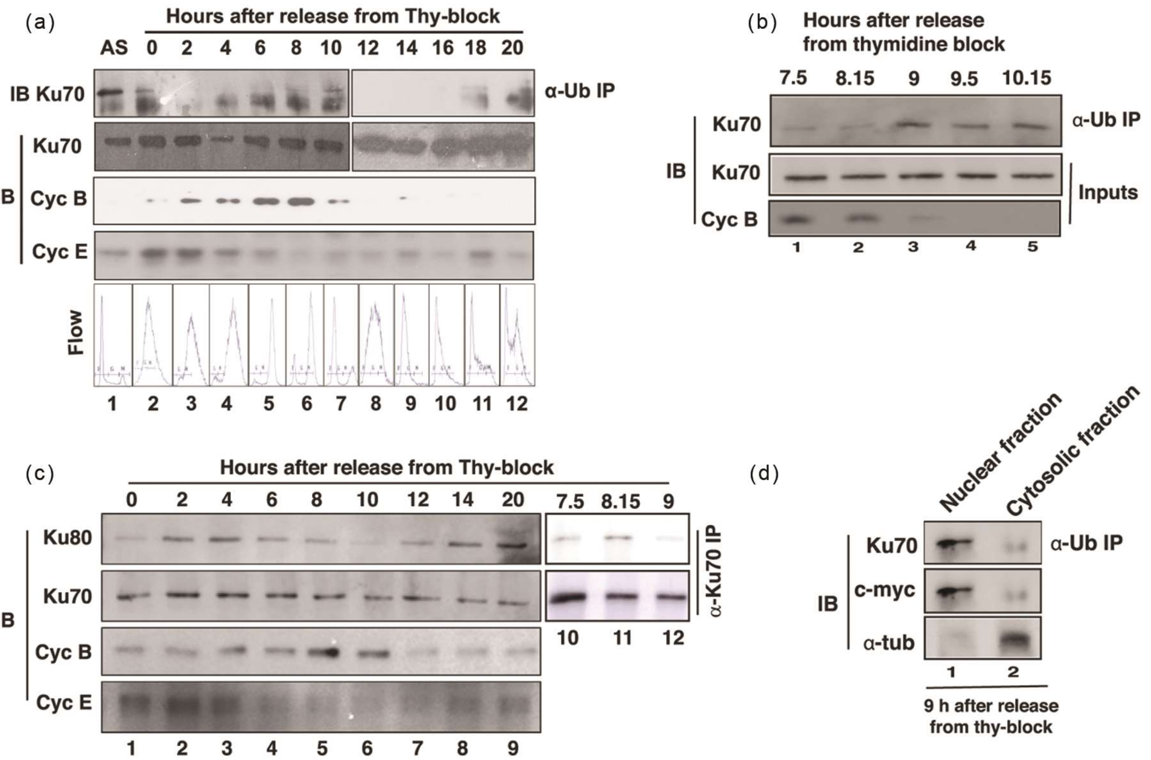

Fig. 3 — (a) Cell cycle dependent ubiquitination of Ku70. The presence of Ku70 in the anti-ubiquitin immunoprecipitates (IP) from the extracts of synchronized HeLa cells were checked by anti-Ku70 immunoblotting (IB). The cell cycle status of the HeLa cells were checked by anti-cyclin E and anti-cyclin B immunoblotting and further confirmed by flow cytometric analysis. (b) Ubiquitination of Ku70 in G1-phase. The ubiquitination status of Ku70 was analyzed in the part of G1 phase in further details by detecting the presence of Ku70 in anti-ubiquitin immunoprecipitates. (c) Effect of ubiquitination on heterodimerization of Ku during cell cycle. The presence of Ku80 was analyzed by immunoblotting in the anti-Ku70 immunoprecipitates from the extracts of synchronized HeLa cells; and (d) Presence of ubiquitinated Ku70 in nuclear fraction of HeLa cells in G1-phase. Anti-ubiquitin immunoprecipitations were carried out from nuclear and cytosolic fractions of HeLa cells collected at 9 h time-point after release from double thymidine block and the presence of Ku70 was analyzed by immunoblotting. The nuclear and cytosolic fractions were characterized by anti-c-myc and anti-α-tubulin immunoblotting

early S-phase and middle third of G1-phase. This was consistent with the maximum Ku70 ubiquitination profiling at those time-points (Figs. 3a and 3b), indicating the disruption of the Ku heterodimer by the PTM. The dimerization pattern during 7.5-9 h window was analysed in more details (Fig. 3c, lanes 10-12), and expectedly, a dimerization peak was observed at 8.15 h (Fig. 3c, lane 11), when ubiquitination was minimal (Fig. 3b, lane 2). No dimerization was observed at 9 h time point (Fig. 3c, lane 12), since ubiquitination was high at that time point (Fig. 3b, lane 3). The cell fractionation at 9 h time point after release from double thymidine block (G1-phase) showed ubiquitination of Ku70 at nucleus (Fig. 3d), which further substantiated the absence of Ku in heterodimeric form at this time point.

Possible ubiquitin ligase for Ku70 ubiquitination

Two ubiquitin ligases – APC/C^{Cdc20} and APC/C^{Cdh1}, are primarily active during M and G1-phases. APC/C^{cdc20} is active from prophase to early G1-phase and responsible mainly for proteasomal degradation of cyclin A/B and Securin ensuring efficient metaphase to anaphase transition. APC/C^{Cdh1} functions from Anaphase to G1-phase primarily modulating degradation of cyclin A/B, PLK1 and Aurora kinase for smooth M/G1 transition[19,20]. To check if one of these E3 ubiquitin ligases was responsible for Ku70 ubiquitination, anti-Cdc20 or anti-Cdh1 antibodies was added to the reaction. If either Cdc20 or Cdh1 was involved as a component of the responsible E3 ubiquitin ligase for Ku70, the antibody-mediated inhibition of the respective ligase in the S100 fraction would lead to reduced Ku70 ubiquitination. As shown in Figure 4a, notable reduction in band intensity was observed in the reaction containing anti-Cdc20 antibody (lane 3) compared to the positive control lacking any of the antibodies (lane 4). On the other hand, minimal inhibition was detected upon addition of anti-Cdh1 antibody (Fig. 4a, lane 2), suggesting that Cdc20 associated APC/C could be responsible for Ku70 ubiquitination.

In order to confirm the possible correlation of Cdc20 with Ku70 ubiquitination in cell, HeLa cells were synchronized at the G1/S boundary using double thymidine block and subsequently, the cells were harvested in S-phase (0 h and 4 h) and G1-phase (10 h) following release from the block. As shown in Figure 4b, Ku70 was specifically detected in anti-Cdc20 immunoprecipitate from the cells collected at 10h time-point post-release, confirming a physical interaction between the two proteins during G1. The findings further ascertain that Ku70 can be a substrate of Cdc20 at this stage of the cell cycle.

Conclusion

In summary, Ku70 subunit of Ku heterodimer is ubiquitinated periodically during early S-phase and middle third of G1-phase disrupting its interaction with Ku80. During a brief period in early G1-phase, when Ku70 is not modified either with phosphorylation or ubiquitination, Ku can play its role in pre-RC formation at replication origin (Fig. 5). However, the exact role of Ku-binding to origin in early S-phase is yet to be determined. As shown before[3], Ku70 phosphorylation by cyclin dependent kinases inhibits efficient dimerization of Ku subunits preventing the interaction with replication origin during late S-phase to M-phase. In the absence of

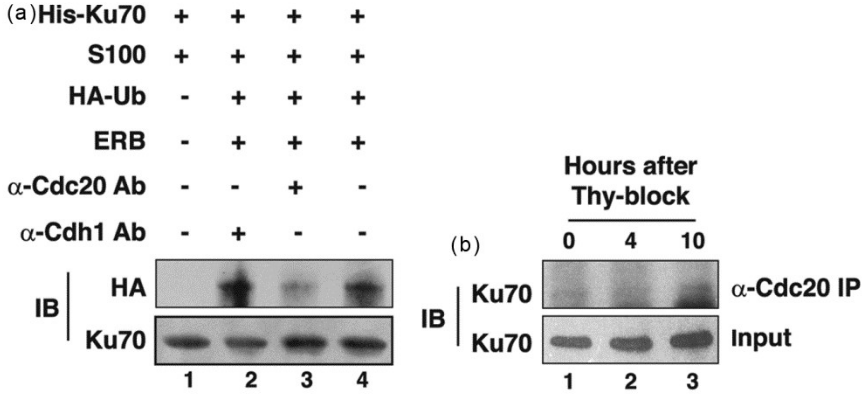

Fig 4 — (a) Identification of the ubiquitin ligase. Ubiquitination of His-Ku70 was carried out with HA-ubiquitin in presence or absence of anti-Cdc20 or anti-Cdh1 antibody and followed by anti-HA immunoblotting (IB); and (b) Interaction of Ku70 with the ubiquitin ligase in G1-phase. The presence of Ku70 in the anti-Cdc20 immunoprecipitates (IP) from the extracts of synchronously growing HeLa cells at 0 h, 4 h and 10 h time-points after the release from double thymidine block was detected by anti-Ku70 immunoblotting

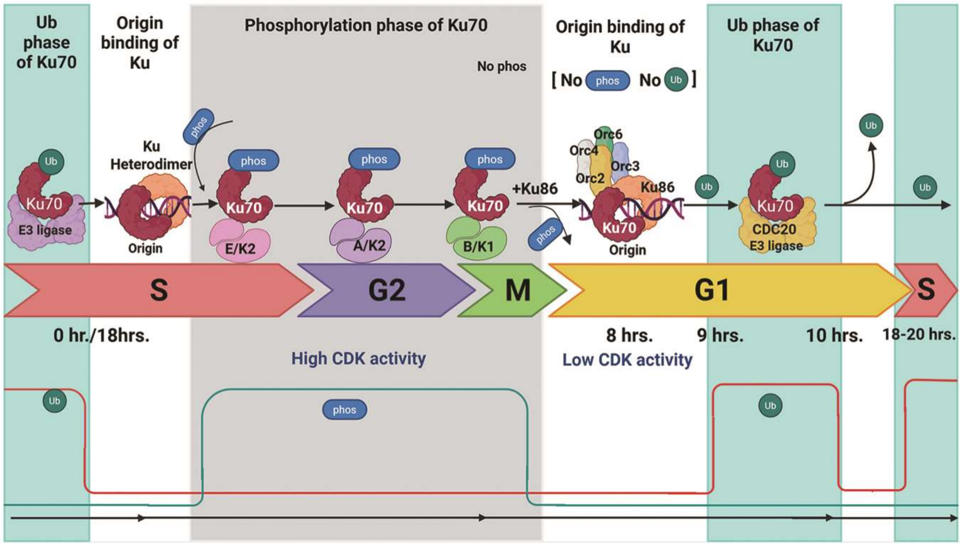

Fig. 5 — Periodic phosphorylation and ubiquitination of Ku70 regulate the interaction of Ku heterodimer with replication origin. Created in BioRender. Debnath, S. (2026) https://BioRender.com/a7hcxqr

cyclin dependent kinase activity in G1-phase, a brief window of Ku70 ubiquitination in the middle third of G1-phase is likely to remove Ku from replication origin after its role in pre-RC formation. Therefore, it can be concluded that alternating periods of phosphorylation and ubiquitination of Ku70 subunit regulate the replication related function of Ku heterodimer during cell cycle.

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Conflict of interest

All authors declare no conflict of interest.

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