Post-translational modifications impart complexity to the eukaryotic proteomes that is several orders of magnitude greater than the coding capacity of the genome. Although protein folding and refolding play a critical role in protein function, the modification of amino acid side chains by various post-translational modifications, including phosphorylation, acetylation, glycosylation, ubiquitination, acetylation and hydroxylation, contribute significantly to the structural and functional diversity of the proteins. Post-translational modifications of proteins influence the enzyme activity, protein turnover and localization, protein-protein interactions, modulation for various signalling cascades, DNA repair and cell cycle.

Different phases of cell division cycle in eukaryotes require tight regulation which is provided, in majority, by various types of post-translational modifications. Among different kinds of post-translational modifications, acetylation, ubiquitination and most importantly phosphorylation are vital for critical regulation of cell cycle. Evidently, the major class of protein kinases that carry out the phosphorylation of different proteins to control cell cycle progression and cell division in eukaryotes comprises of periodically activated cyclin dependent kinases (Cdks) [15]. Phosphorylation of retinoblastoma protein (pRB) by cyclin D-Cdk4/6 is the key initial event for cell cycle progression in higher eukaryotes followed by subsequent phosphorylation of several proteins like Cdc6, ORC proteins, MCM by cyclin E/A-Cdk2 in mammals. On the other hand, dephosphorylation of Cdk2/1 by a
family of Cdc25 phosphatases also plays crucial role to maintain regulated progression of cell cycle.

The critical role of Ku protein in formation of pre-replication complex (pre-RC) in mammalian cell is well established. However, the mechanism of cell cycle dependent periodic regulation of Ku protein activity related to replication initiation has not been addressed before. In this context, prior identification of a Ku70 related protein as a substrate of the S-phase cell cycle kinase from *L. donovani* in our laboratory [174] and the presence of putative Cy-motifs and Cdk target sites, along with the report of uncharacterized phosphorylation events of human Ku70 by S-phase Cdk2 [122], have made it important to explore the contribution of the post-translational modification by cell cycle kinases on the regulation of replication initiation related function of Ku. In the present studies, it has been shown that cyclin B1-Cdk1, along with cyclin E1-Cdk2 and cyclin A2-Cdk2, phosphorylates Ku70 subunit of the Ku dimer inhibiting its interaction with replication origin (figure 6.1). The results confirm that the phosphorylation of Ku by cell cycle kinases during high kinase phases prevents its interaction with the origin of replication. Due to the low kinase activity at the end of mitosis, the non-phosphorylated state of Ku allows its interaction with origin DNA facilitating the role in pre-replication formation in G1-phase (Figure 6.2).

The phosphorylation of Ku during S, G2 and M phases is extremely critical to avoid untimely replication initiation during cell cycle progression as it has been observed that the overexpression of non-phosphorylable mutant of Ku70 subunit induces re-replication in HeLa cells. The critical balance between Cdt1 and Geminin is extremely important for avoiding re-replication. Loss of Geminin and over-expression of Cdt1 induces re-replication.

![Figure 6.1](image.png)

**Figure 6.1:** Periodic phosphorylation of Ku70 subunits of Ku heterodimer by cyclin-Cdk5 inhibit its interaction with origin during S, G2 and M phases.
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in mammals and results in giant nuclei formation [175]. The Cdt1 level is high and Geminin level is low in cells over-expressing the non-phosphorylatable mutant of Ku70 subunit as compared to control. Also, microscopic analysis of cells over-expressing mutant Ku70 shows giant nuclei with nuclear sizes 1.5-4 times greater than the average nuclear size of the control cells. Therefore, phosphorylation of Ku70 is extremely important for preventing re-replication and cell cycle kinases regulate the periodic origin association activities of Ku protein like other players of replication licensing system including Cdc6, cdt1 and MCMs [172].

Ku70 contains one well-conserved canonical Cdk phosphorylation site (401TPRR) with two nearby minimal sites (428TP and 455TP). In addition, it also contains another minimal site (58TP) on the N-terminal region. The study presented here using site directed mutagenesis has confirmed that Thr residues at 401 and 428 positions are the major target phosphorylation sites of cyclin B1-Cdk1. On the other hand, cyclin A2-Cdk2 phosphorylates Thr residues at 401, 428 and 455 positions apparently with equal specificity. However, cyclin E1-Cdk2 also phosphorylates some other target residue – probably Thr-58, which could have additional functional implication. Again, like other Cdk targets, the phosphorylation of Ku70 depends on its interaction with cyclin B1 through the RxL type

Figure 6.2: Modulation of replication initiation related function of Ku through phosphorylation by cyclin-Cdks. Ku70 is phosphorylated by cyclin-Cdks during high Cdk phases - S, G2 and M. The phosphorylation of Ku70 by cyclin B1-Cdk1 disrupts its association with Ku80 subunit, abrogating the interaction of Ku with replication origin. With the removal of cyclin B1 at the end of mitosis, Ku70 is dephosphorylated and as a result the functional Ku heterodimer can be formed for the assembly of origin recognition complex at replication origin in early G1-phase.
Cy-motif located at amino acid positions 516-519. It is established that the region spanning amino acids 378-482 of Ku70 is involved in heterodimerization with Ku80 [176]. Interestingly, the identified phosphorylation sites of cyclin B1-Cdk2 are present in the region of Ku70 that is responsible for dimerization with Ku80. Also, the identified functional RxL type Cy-motif of Ku70 is located very close to the dimerization domain on the C-terminal. This explains the findings of the disruption Ku heterodimerization due to the interaction of Ku70 with cyclin B1-Cdk1 and the incorporation of phosphates. The study has established by co-immunoprecipitation that in mitotic cells, there are two pools of Ku70 - one is in complex with cyclin B1 and another with Ku80. This supports the observation in several previous studies indicating independent regulation of Ku70 and Ku80 in mitosis [135, 177].

As discussed before, licensing in eukaryotes starts in late mitosis/early G1-phase through sequential assembly of a multiprotein complex - pre-RC [178]. Cdk activity is then required for the onset of S-phase [179, 180]. After firing, origins are kept in an unlicensed state until the completion of mitosis in the cell cycle. High Cdk activity inhibits pre-RC assembly and licensing during S, G2 and M phases in eukaryotes [116, 181]. Ku as an origin binding protein, associates maximally with chromosomal replication origins in G1-phase. The binding of Ku to chromosomal origins decreases as cells enter S-phase. Ku acts in the initiation of DNA replication and dissociates after origin firing [157]. Although C-terminal of Ku70 shows similarity with the SAP domain that is involved in DNA interaction [145, 182], binding of Ku to origin of DNA replication requires heterodimerization [164]. The results presented here confirm that the increased phosphorylation of Ku70 by Cdks from S-phase onwards till M-phase is responsible for its non-interaction with origin DNA during these phases. This is further supported by the observed binding of non-phosphorylatable variant of Ku to origin DNA even in G2/M phase cells. Therefore, the prevention of the interaction of the Ku with origin DNA in S, G2, and M phases is also critical for replication licensing mechanism. The conclusion is supported by the observed rereplication in the cells overexpressing the non-phosphorylatable variant of Ku70. Taken together, the results described here provide an insight how the replication related function of Ku can be periodically regulated in cells. Due to the formation of the complexes of Ku70 with cyclin-Cdks and its phosphorylation, the Ku dimer is disrupted. This results in the decrease of the amount of functional Ku dimer available for binding to replication origins during the high Cdk activity phases, thus preventing premature assembly.
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of origin recognition complex till the end of mitosis (Figure 6.2). After the degradation of cyclin B1, Ku70 can be converted to dephosphorylated state and is able to form heterodimer with ku80 for taking part in origin licensing during G1-phase.

**Concluding remarks:** In eukaryotic cells, various types of post-translational modifications, particularly protein phosphorylation by cyclin dependent kinases, are critically important for regulation of cell cycle progression including DNA replication. One of the major activities of cell cycle machineries is to ensure the accurate replication of DNA in eukaryotic cells via a periodic licensing mechanism that allows formation of a competent pre-replication complex only after mitotic segregation of sister chromatids into daughter cells. To form a functional pre-replication complex at replicator, at first a complete origin recognition complex comprising of six Orc subunits is assembled during G1 phase followed by loading of other licensing factors including Cdt1, Cdc6 and MCM. Intriguingly, Ku – a DNA repair protein has been shown to be involved in the process but its regulation remains long unknown. Therefore, in order to elucidate further molecular insights of the most vital process of replication initiation during cell cycle progression regulated by post-translational modifications, in the present study detailed characterization of the replication related activities of the Ku protein have been carried out. The results prove that the periodic modulation of replication related function of the multi-functional Ku protein is dependent on reversible phosphorylation of its Ku70 subunit by cell cycle kinases. However, the characterization of additional target sites of cyclin E-Cdk2 in Ku70 along with its functional consequences may provide further molecular insights of the cell cycle related activities of Ku protein. Also, it would be interesting to study the role of Ku protein in spatiotemporal regulation of replication origin uses in a genome wide scale in future. Nevertheless, the significance of cell cycle dependent periodic phosphorylation of Ku70 subunit of Ku protein by cyclin-Cdks in regulation of its critical role in replication licensing has been firmly established in the present study.