Mechanistic Insights into the Role of Hematopoietic PBX Interacting Protein in Cell Cycle Regulation

A Thesis

Submitted for the Degree of

Doctor of Philosophy

By

Anita Kumari

(Regd. No. 15LBPH11)

Department of Biochemistry

School of Life Sciences, University of Hyderabad

Hyderabad-500046

Telangana, India

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University of Hyderabad
Hyderabad -500046, India

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DECLARATION

I hereby declare that the work presented in this thesis is entirely original and was carried out by me in the Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad under the supervision of **Prof. Bramanandam Manavathi, Ph.D.** I further declare that this work has yet to be submitted earlier for the award of a degree or diploma from any other University or Institution.

25/04/24

Prof. Bramanandam Manavathi, Ph.D.,

Research supervisor

Anita
26/04/2024

Anita Kumari

Reg. No:15LBPH11

Date: 25/04/24

Place: Hyderabad

University of Hyderabad

Hyderabad -500046, India

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26/04/24

Research Supervisor
Prof. Bramanandam Manavathi, PhD
Department of Biochemistry
School of Life Sciences
University of Hyderabad
Hyderabad-500 046. (TS), INDIA

26/04/24

Head,
Dept. of Biochemistry

अध्यक्ष / Head
जीवरसायनविभाग / Department of Biochemistry
जीवविज्ञान संकाय / School of Life Sciences
हैदराबाद विश्वविद्यालय / University of Hyderabad
हैदराबाद / Hyderabad-500046. INDIA

26/4/24

Dean,
School of Life Sciences

संकाय अध्यक्ष / Dean
जीव विज्ञान संकाय / School of Life Sciences
हैदराबाद विश्वविद्यालय / University of Hyderabad
हैदराबाद / Hyderabad-500 046.

University of Hyderabad

Hyderabad -500046, India

प्रतिष्ठित संस्थान
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Conferences attended:

1. Participated in a workshop and titled cancer drug Discovery and development held on 22-28th 2016 at the University of Hyderabad, India.
2. Participated in an international conference on "Radiation research: impact on human and environment" held on 1-4th February 2018 at the University of Hyderabad, India.

Poster presentations:

1. "To investigate the role of HPIP phosphorylation in cell cycle regulation" in 90th Annual Meeting of SBC(I) "Metabolism to Drug Discovery: Where Chemistry and Biology Unite" organized in virtual mode during 16-19 December 2021 by Amity Institute of Biotechnology and Amity institute of Integrative Science and Health. Amity University Haryana (AUH) Gurugram.
2. "Functional characterization of Hematopoietic PBX Interacting Protein (HPIP) phosphorylation in control of cell division" in 92th Annual Meeting of SBC(I) organized during 18-20 December 2023 by BITS PILANI, K K BIRLA GOA Institute, Goa.

Details of research articles published in refereed journals:

1. Khumukcham SS, Samanthapudi VSK, Penugurti V, **Kumari A**, Kesavan PS, Velatooru LR, Kotla SR, Mazumder A, Manavathi B. Hematopoietic PBX-interacting protein is a substrate and an inhibitor of the APC/C-Cdc20 complex and regulates mitosis by stabilizing cyclin B1. ***J Biol Chem***. 2019 Jun 28;294(26):10236-10252.

2. Dwivedi A, Padala C, **Kumari A**, Khumukcham SS, Penugurti V, Ghosh S, Mazumder A, Goffin V, Manavathi B. Hematopoietic PBX-interacting protein is a novel regulator of mammary epithelial cell differentiation. ***FEBS J***. 2022 Mar;289(6):1575-1590.

3. Khumukcham SS, Penugurti V, Bugide S, Dwivedi A, **Kumari A**, Kesavan PS, Kalali S, Mishra YG, Ramesh VA, Nagarajaram HA, Mazumder A, Manavathi B. HPIP and RUFY3 are noncanonical guanine nucleotide exchange factors of Rab5 to regulate endocytosis-coupled focal adhesion turnover. ***J Biol Chem***. 2023 Nov;299(11):105311.

Further, the student has passed the following courses towards the fulfilment of the coursework requirement for a Ph.D.

Course code	Name	Credits	Pass/Fail
BC 801	Analytical Techniques	4	Passed
BC 802	Research ethics, Data analysis and Biostatistics	3	Passed
BC 803	Lab Seminar and Records	5	Passed

26/04/24

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Prof. Bramanandam Manavathi, PhD
Department of Biochemistry
School of Life Sciences
University of Hyderabad
Hyderabad-500 046. (TS), INDIA

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हैदराबाद विश्वविद्यालय / University of Hyderabad
हैदराबाद / Hyderabad-500046. INDIA

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हैदराबाद विश्वविद्यालय / University of Hyderabad
हैदराबाद / Hyderabad-500 046.

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List of Abbreviations

APC/C- Anaphase promoting complex/ Cyclosome

CAK- CDK activating kinase

CAK- cyclin-dependent kinase activating kinase

CDC6- Cell division cycle 6

CDKs- cyclin-dependent kinases

CKIs- cyclin-dependent kinase inhibitors

Dbf- Dumbbell former

DNA- Deoxyribonucleic acid

E2F- Transcription Factor

Eg5- kinesin-5 or kinesin spindle protein

FBW7- F-box/WD repeat-containing protein 7

G1 or G2 phase- Gap1 or Gap2 phase

HAT- Histone acetyltransferase

HDAC- Histone deacetylase

HPIP/PBXIP1-Hematopoietic PBX interacting protein or pre-B-cell leukaemia homeobox transcription factor (PBX)-interacting protein 1

KAT- Lysine acetyltransferase

KT- Kinetochore

M- Mitosis

MAP- Microtubule-associated protein

MCM2- Minichromosome maintenance 2

MT- Microtubule

NEB- Nuclear envelope breakdown

NER- nuclear envelop reformation

NLS- Nuclear localization signals

Nϵ/Nα- N-terminal Epsilon, Alpha

ORC- Origin of recognition complex

PCAF- p300/CBP-associated factor

Plk1- Polo-like kinase 1

pRB- Retinoblastoma pocket

Pre-RC/ (Cdt1)- Pre-Replicative complex/ (Cdc10-dependent transcript 1 protein)

PTMs- post-translational modifications

RanGAP- Ran GTPase-Activating Protein

SAC/MCC- Spindle assembly checkpoint or Mitotic checkpoint complex

Skp1-Cul1-F box (SCF)- S-Phase Kinase Associated Protein 1 Cullin 1- F box

S-Synthetic phase

SUMO- Small ubiquitin-related modifier

Ub- Ubiquitin

γ-TuRC- γ-tubulin ring complex

SAC/MCC- Spindle assembly checkpoint or Mitotic checkpoint complex

Table of Contents

Abstract

Pre-B cell leukaemia homeobox interacting protein (PBXIP1) also known as Hematopoietic PBX-interacting protein (HPIP), is a protooncogene that is notably elevated in various cancer types, playing a crucial part in the initiation and progression of cancer. It involves multifaceted functions such as cell migration, proliferation, and differentiation. Despite the mechanistic details of HPIP-mediated cell migration and differentiation functions are unveiled partially; how it controls cell proliferation is largely unknown. We followed both gain-of function and loss-of function and gain of function approaches to unravel the role of HPIP in cell division using HeLa cells. The study uncovered that HPIP is essential for proper cell proliferation as reduced expression of HPIP expression leads to G2/M arrest and delayed mitosis. Mechanistic studies additionally revealed that HPIP causes cyclin B1 stabilization by alleviating APC/C-Cdc20 action towards cyclin B1 to ensure G2/M transition and timely mitosis to occur. Interestingly, HPIP is proteolyzed via APC/C-Cdc20-facilitated ubiquitination in mitosis. Further investigation revealed that HPIP is subjected to phosphorylation at serine 43 by CDK1-cyclin B1 complex during mitosis. Loss of phosphorylation of HPIP at serine 43 severely hampered HPIP-mediated cell division due to delayed mitotic entry and exit in HeLa cells. Additionally, loss of serine 43 phosphorylation in HPIP results in improper chromosomal segregation and the formation of abnormal spindle poles. Further mechanistic studies revealed that mtHPIP-S43A failed to stabilize cyclin B1 and timely G2/M transition because loss of HPIP phosphorylation by CDK1-cyclin B1 complex impairs its proteolysis by APC/C-Cdc20. Collectively, these findings indicated an essential role for HPIP and its phosphorylation at serine 43 by CDK1 in cell division.

Chapter 1

Introduction

1.1. Cell cycle

The cell is the structural and functional unit of a living being. In prokaryotic organisms, the cell acts as an organism, whereas in multicellular organisms, several millions of cells constitute an organism. In antiquity, Paracelsus and Aristotle stated that “all living organisms, including plants and animals, however complex they may be, constitute a few elements that are repeated in them.” This statement inferred that multicellular organisms might constitute several millions of cells. Cell division involves growth, development, reproduction, and repair and replacement of dead cells. The cell division occurs in a series of growth and division in a cyclic fashion.

According to the concept of cell cycle theory, to produce genetically identical cells require coordination between chromosomal replication and cell division (1). Therefore, replication of DNA occurs before cells divide, then divide once more prior replicating their DNA (2). If cells' primary function were to proliferate, the cell cycle apparatus's role would be most likely simplified to that of a basic inner oscillator that regulates DNA duplication during cell division. The cell cycle is a complicated and ubiquitous process of successive steps regulated by several proteins. Ultimately, this process leads to a mother cell divides to producing two daughter cells.

The cell cycle is defined as the series of processes of cell’s maturation and division. The eukaryotic cell cycle is typically divided into two primary stages: the interphase and mitosis (M) phase. Interphase, which comprises of the G1, S, and G2 phases, is when the cell prepares for division by growing and replicating DNA. It comprises for nearly 95% of the total cycle time; the remaining time is accountable for mitosis. Hence, the G1, S, G2, and M stages make up the normal cell cycle in eukaryotes. M phase is including prophase, prometaphase, metaphase, anaphase, telophase, and cytokinesis (Figure 1).

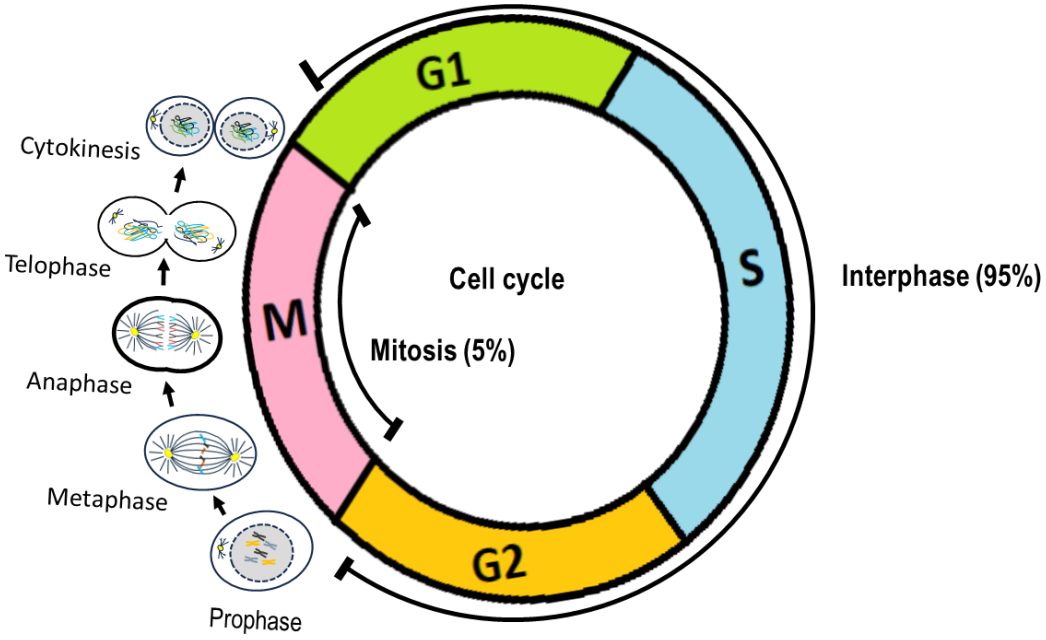

Figure 1. Schematic diagram representing cell cycle and mitosis (M phase). G1, S, and G2 represents interphase followed by mitosis (M phase). Mitosis is separated into four stages: prophase, where condensation of chromosomes and the nuclear envelop breakdown takes placed. In metaphase, completely folded chromosomes align together at the metaphase plate. During anaphase, the chromosome divides into two chromatids, which move towards opposite poles. Telophase occurs when chromosomes arrive at their poles and the nuclear envelope is reformed, followed by cytokinesis, where the mother cells split into two daughter cells.

i. Interphase- Interphase is a period that produces no visible alterations under a microscope, which comprises G1, S, and G2 phases, is a period that produces no visible alterations under a microscope. Throughout the period of interphase, the cell grows (G1), double its chromosome (S), and makes ready for mitosis (G2).

a. Gap1 (G1) phase- The cell's G1 phase begins before mitosis and the onset of DNA replication. During this phase, the cell remains active in metabolism and increases without duplicating its DNA. Passage via G1 phase is governed by cyclin-dependent kinases (CDKs)

4, 6, and 2, along with their related cyclin-dependent kinase inhibitors (CKIs) and cyclins (3-5). In addition to these proteins, the G1 is profoundly controlled by retinoblastoma protein (RB). Cyclin D1 expression is directed by external growth-regulating molecules in the G1 phase that activate CDKs (6). Phosphorylation at the carboxy-terminal domain of the retinoblastoma protein (RB) by CDK4/6 -cyclin D1, blocks the expression of genes by interacting directly with the transactivation domain of E2F transcription factors (Figure 2). The retinoblastoma (Rb) gene is an important tumor restrainer found in retinoblastoma, a kind of retinal tumor. Rb2/p130, Rb/p105, and p107 constitute the RB lineage of genes, called "pocket" proteins (7). Pocket retinoblastoma (pRB) proteins potentially influence the early G0 to G1 transition (8). Several human cancers, like small-cell lung carcinoma, osteosarcoma and, retinoblastoma have *Rb* gene mutations and deletions, and inherited *Rb* allelic loss or indirectly through changed activity or expression of upstream regulators provides greater vulnerability to cancer formation (9, 10). pRB/p107 phosphorylated by CDK4/CDK6-cyclin D1 complex, whereas CDK4-cyclin D3 complex phosphorylates Rb2/p130. The cyclin E-CDK2 complex phosphorylates three pocket proteins p130 p107, and p105, and CDK2-cyclin A activates by phosphorylating Rb2/p130 and p107 but not Rb/p105.

E2F family members are members of a broad family of E2F transcription factors which usually belong to either transcriptional suppression (E2F3B, E2F4, E2F5, E2F6, E2F7, and E2F8) or activation (E2F1, E2F2, E2F3, E2F4, and E2F5) (11). E2F1, E2F2, E2F3, E2F4, and E2F5 can be bound by pRb and activate E2F-responsive genes when dissociating from pRB (12). E2F family members regulate expression of target genes in different ways for example, over-expression of E2F1 selectively induces cyclin D and E, whereas E2F3 preferentially activates CDK2 (13, 14). CDC6 is required for DNA replication and is activated by E2F1, E2F2, and, to a lesser extent, E2F3 (15).

b. S-phase: The ‘S’ is a phase defined as the “synthesis phase “where DNA replication occurs. Also, centriole divides into two pairs in this phase. Chromosomal DNA must be accurately copied before segregation to transmit genetic information through generations. To do this, the eukaryotic cell exhibits regulatory systems that restrict DNA duplication once in each cell cycle. DNA duplication occurs in two steps (15, 16): licensing is the first reaction, where a particular DNA-protein complex identified as the pre-replicative complex or pre-RC is produced at G1. Several components, including cell division cycle 6 (CDC6), chromatin licensing and DNA replication factor 1 (Cdt1), mini-chromosome maintenance 2-7 (MCM2-7), and origin recognition complex (ORC) comprise the pre-RC. Pre-RC assembly helps in depositing the replicative helicase MCM2-7 complex onto origins. Formation of Pre-RC is the most crucial step before actual DNA synthesis occurs. The pre-RC initiates a second reaction, called initiation, is followed by replication forks that move in both directions form to polymerize DNA (17, 18). S phase kinases CDC7-Dbf4 (Dbf4-dependent) and CDK2-cyclin A are conserved protein kinases that regulate pre-replicative complex formation and initiation separately to enable faithful DNA duplication.

c. G2-phase- G2 phase is the period of synthesis of RNA, proteins, and other macromolecules necessary for cell organelle growth, spindle formation, and growth of cells. The cell cycle's Gap phases are critical for perceiving internal and external cues and controlling cell division and differentiation (Figure 2). Many signals converge on the cell cycle machinery in multicellular organisms, controlling organ growth and development. These signals encourage, hinder, or stop cell cycle progression, altering the rate of cell division. Cyclin A retains to the cytoplasm at time of S phase to G2 phase transition and governs the G2 phase by shuttling between the cytoplasm and nucleus despite lacking a traditional nuclear localization signal

(NLS) (19). The Bora protein is phosphorylated by the CDK1-cyclin A complex and stimulates Aurora A-directed PLK1 phosphorylation and activation. Activated PLK1 phosphorylates Bora, leading to SCF-β-TrCP-mediated proteolysis of Bora and thus mitotic entry (20, 21). CDK2-cyclin A phosphorylates several S-phase essential proteins molecules such as CDC6, pre-replication complexes, and replication machinery components (22-25).

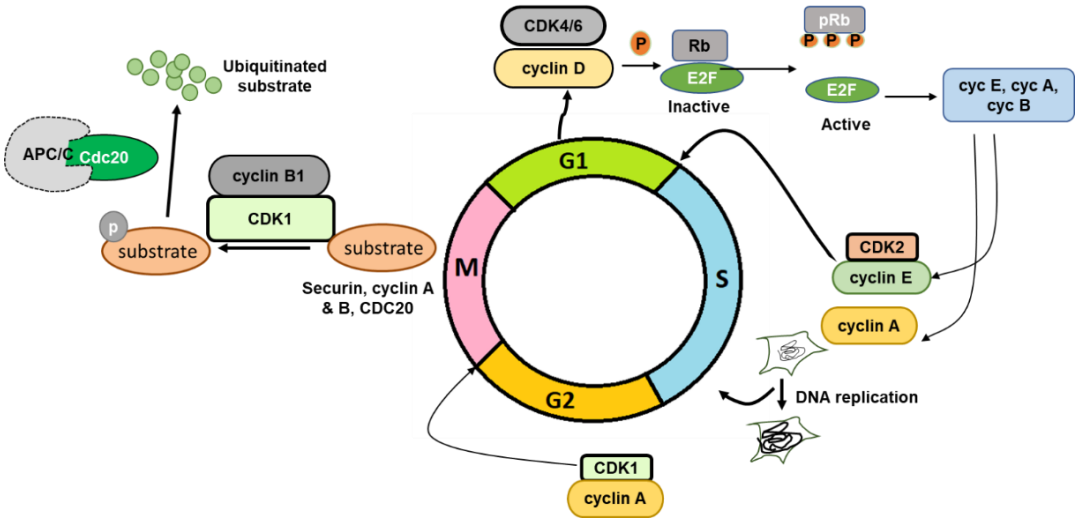

Figure 2. A diagrammatic view of cell cycle regulation by CDKs, cyclins, and other proteins. Cell cycle comprise of four different phases G1, S, G2, and M. The cell cycle's G1 phase is controlled by CDK4/6- cyclin D, which phosphorylates RB protein to relieve E2F, which regulates the transcription of cyclin E, A, and B. Cyclin E, in turn associates with CDK2 to further trigger G1/S transition. CDK1-cyclin A regulates S-G2 transition and G2/M phase progression. During M,-phase, CDK1-cyclin B1 phosphorylates their target proteins (cyclin B, Securin, Cdc20, etc.), which led to their APC/C-mediated ubiquitination and proteolysis.

i. Mitosis- Walther Flemming discovered the process of mitosis in 1882. Equal distribution of DNA into two newly formed cells occur in mitosis, creating two identical nuclei to prepare cells to divide, and thus sustain genomic integrity which is critical for viability of all species (26) . Altered chromosome segregation leads to various diseases like cancers.

Four phases of mitosis are further divided as follows: Prophase, Metaphase, Anaphase, and Telophase.

a. Prophase- Chromosome condensation is the major cytological hallmark that usually indicates the start of prophase (Figure 1A). Phosphorylation by CDK1-cyclin B1 activates condensin, which is considered an important process in the initial transition phase (27). Besides phosphorylating condensin, CDK1-cyclin B appears to be involved in chromosomal condensation (28). The collective action of both condensin I and II leads in helically structured nested loop arrays during prometaphase and cause chromosome condensation. Centromeres are aligned back-to-back, allowing each half-spindle to collect only one chromatid.

b. Metaphase- The end of prophase is indicated by nuclear envelope breakdown (NEB), and the accomplishment of chromosomal arrangement at the mid of metaphase plate suggests the initiation of metaphase (Figure 1). Spindle fibre become more active and cross the nuclear space after nuclear envelop breakdown. This allows spindle fibre to connect kinetochores (KTs), thereby starting chromosome movement (i.e., congression) to the cells' equator. The mitotic checkpoint or spindle-assembly checkpoint (SAC) controls anaphase initiation and inhibits chromosome mis-segregation, which could result in chromosomal instability or cell death a common feature of cancer (29). These mechanisms ensure that MTs attach to chromosomes after NEB, that an active bipolar mitotic spindle is built, and that incorrect attachments are fixed (29, 30). During interphase, microtubule-associated protein 7 (MAP7)-Ensconsin complex and MAP4 associate with microtubules and stabilize them. Phosphorylation of MAP7-Ensconsin complex and MAP4 by CDK1-cyclin B1 leads to their dissociation from MTs (31, 32).

c. Anaphase- Precise alignment of the chromosome at the equatorial plane of spindle is critical for the migration of sister chromatids toward the poles and mitotic fidelity (Figure 1) (33, 34).

Microtubules are polar polymeric structures with more dynamic plus ends undergo extended periods of expansion and contraction are crucial for chromosomal placement within the spindle (35, 36). *In vitro* research has shown that the contraction and expansion of microtubules can cause pulling or pushing forces with the help of kinesins (37, 38), implying that both the forces influence migration of chromosome within cells, such as chromosomal positioning on the spindle (39).

d. Telophase- The final purpose of mitosis is to actual separation of copied chromosomes so that genetic information can be propagated properly during cell division (Figure 1). Aurora B activation governs the spatial regulation and is important for the anaphase-telophase transition. Transportation of Aurora B to the equatorial plane of spindle causes gradient phosphorylation of its substrates while the DNA remains compacted. The pattern of gradient phosphorylation forms an "area of exclusion" which inhibits nuclear envelope reformation (NER) (40). Substrates of Aurora B kinase is no more affected by the gradient phosphorylation after DNA segregation and phosphatase activity takes over, allowing NER and the subsequent mitotic exit.

1.2. Role of post-translational modifications in cell cycle regulation

Post-translational modifications (PTMs) of protein are covalent modifications that change protein's characteristics by the linking a small modifying groups such as phosphates, acetyl groups, SUMO, ubiquitin (Ub), etc. (41). A significant portion of proteins produced by eukaryotes are covalently modified either during or after protein synthesis. These modifications required in many biological processes by altering the conformation and activity of proteins that can be either reversible or irreversible in general (42-44). Covalent alterations are present in reversible reactions, while proteolytic changes are present in irreversible reactions in just one direction. Amongst post-translational modifications, ubiquitination and phosphorylation play vital roles in cell fate decisions during cell cycle progression.

i. Phosphorylation- Phoebus Levene, in 1906, found phosphorylated vitellin (phosvitin), leading to the finding of protein phosphorylation, but this change occurs primarily on amino acids Serine, Threonine, Tyrosine, and Histidine (45, 46). Phosphorylation is the well-known and is one of the critical kinds of PTM that happens on target proteins in the cytosol or nucleus (46). Kinase enzymes use adenosine triphosphate (ATP) as a donor of a phosphate group to receptor residues Thr, Ser, Tyr, and His residues (47). This is a crucial reversible control process in prokaryotes and eukaryotes that controls the activities of several proteins, enzymes, membrane channels, etc. (48, 49). Phosphorylation is necessary for several biological activities, including stress response, replication, transcription, cell motility, cell metabolism, immunological reactivity**,** and death (50-52). Abnormalities in the phosphorylation signaling have been shown to cause diseases such as cancer, Parkinson's disease, Alzheimer's disease, etc. (53-55).

Cyclin-dependent kinases (CDKs) predominantly control protein phosphorylation during the cell cycle. Diverse CDK subunit complexes are involved in various phases of cell cycle, including the G1/S and G2/M transitions. It is widely known that CDKs, which are made up of a protein kinase catalytic subunit and an activating cyclin component, are necessary for various cell cycle stages (56, 57). Controlling CDK1 activity by multiple complicated mechanisms safeguard the correct events of the cell cycle's timing and coordination. CDKs are similar in size (35-40 kDa) and sequence (more than 40% identity). These CDKs interact with regulatory subunit of cyclin and become activated (58). In a typical cell cycle, cellular CDK levels remains unchanged however, their activities are altered. Their catalytic activity is mainly regulated by post-translational modification, such as threonine a conserved residue for CDK-activating kinase (CAK) and cyclin binding. The most recent human and mouse genome versions reveal that 21 genes code for CDKs, and five more genes produce CDK-like proteins with conserved primary structure (59). Each cyclin exhibits a distinct pattern of expression across the cell cycle, and CDKs associate to their respective cyclin protein. Cyclins A-H are a vast family of cyclins

that bind to and activate several CDKs (58). Cyclin turnover and transcriptional regulation control the rate at which the cell cycle can progress through its various stages. The CDK subunit's phosphorylation and dephosphorylation events then influence the CDK-cyclin complex's activity. Each phase of cell cycle in eukaryotes is precisely regulated by distinct CDK-cyclins and respective inhibitors in distinct phases. Additionally, the levels of their partner cyclins, CDKs, and CDK kinase inhibitors (CKIs) are regulated by ubiquitin. In eukaryotic cells, several of these CDK regulators are ubiquitinated and proteolyzed by proteasomes by two families of E3 ubiquitin ligases: anaphase-promoting complex/cyclosome (APC/C), and Skp1-CUL1-F-box (SCF) which make sure proper cell cycle progression (60).

ii. Ubiquitination- Ubiquitination is one of the most vital reversible PTMs first investigated by Gideon Goldstein in 1975. It may occur on all twenty amino acids, but it happens more typically in lysine (61). Ubiquitination is a covalent linkage between the lysine ε-NH2 and the C-terminal of an active ubiquitin polypeptide (62). Ubiquitination play crucial title role in the destruction of proteins within cells by the ubiquitin (Ub)-proteolysis pathway (63). Conversely, deubiquitination is the specialized modification process of removing the ubiquitin group from protein by deubiquitinases (64). Many cell activities are regulated by ubiquitination, such as transcription, proliferation, DNA repair, intracellular trafficking protein degradation, replication, etc. (50, 65).

1.3. Role of phosphorylation and Ubiquitination in cell cycle regulation

CDK activation is primarily determined by cyclin binding and phosphorylation. Phosphorylation regulates both cyclins and CDKs post-translationally (66, 67). The interaction between CDK and its respective cyclin results in a limited extend of activation., But complete activation required phosphorylation of CDK4/6 on Thr 172, CDK2 on Thr 160, and CDK1 on

Thr 161, which is conserved near the ATP-binding groove (68-70). Structure of CDK2-cyclin A complex exhibit that the CDK-cyclin interaction alters the CDK's conformation, rendering the T-loop more approachable for triggering phosphorylation (71, 72). Phosphorylation influences the T-loop's shape in CDK, make the catalytic cleft totally available to ATP. Cyclin H-CDK7 and MAT1 the assembly factor, constitute CDK activating kinase (CAK) that triggers CDKs by phosphorylating them (73).

Not only activation but CDKs phosphorylation can also impinge on their kinase activity (66, 67). All CDKs undergo inhibitory phosphorylation at their N-termini of CDK2 and CDK1, more especially on Tyr 17 of CDK4 and CDK6. Another repressive phosphorylation involving Thr 14 in the case of CDK2 and CDK1. Bifunctional kinases MYT1 and WEE1 are capable of phosphorylating both (74) threonine and tyrosine residues, while WEE1 prefers Tyr 15, whereas MYT1 prefers Thr 14, for phosphorylating on inhibitory sites of CDK2 and CDK1 (75). CDC25 family of protein phosphatases eliminate CDK inhibitory phosphorylations (76). Interestingly, CDK-cyclin phosphorylates CDC25 protein, and phosphorylation increases their phosphatase activity (77). CDK2-cyclin E phosphorylates CDC25A, which in turn activates CDK2-cyclin E complex, while cells progress through G1/S phase, resulting in a positive feedback loop. CDC25B dephosphorylates CDK1-cyclin B1 in the cytoplasm before transporting it to the nucleus (78). CDC25C regulates mitosis further by activating nuclear CDK1-cyclin B1 via dephosphorylation (79).

1.4. Anaphase Promoting Complex/Cyclosome (APC/C)/ Skp1-Cullin1-F-box (SCF)/ Cullin4-RING-E3 ubiquitin Ligase (CRL4)/ Complexes mediates ubiquitination of cell cycle regulators

Many cyclins are destroyed via ubiquitination/proteasome processes involving the anaphase-promoting complex/cyclosome (APC/C) and Skp1-Cullin1-F-box (SCF) complex, and these degradations usually depend on cyclin-dependent kinase (CDK) phosphorylation, providing a self-limiting way to regulate CDK's activity. Cyclins are ubiquitinated through their phosphorylation-dependent interaction with SCF complexes (80, 81). GSK-3β phosphorylates cyclin D1 in the S phase at Thr 286 (82) that enhances its interaction with the nuclear export complex CRM1 for nuclear export of cyclin D1 where SCF E3 ligase FBX4 binds to Thr 286-phosphorylated cyclin D1 for ubiquitination and degradation (83). Similarly, CDK2-cyclin E dependent autophosphorylation of cyclin E Thr 380 triggers its own proteasome-dependent proteolysis by SCF-Fbw7 (84).

The anaphase-promoting complex/Cyclosome (APC/C) is a big 14 subunits multimeric complex including APC1, APC4 and APC5 (scaffold platform), APC2 (the Cullin subunit), APC3, APC6, APC7, APC8 (tetratricopeptide repeat (TPR) proteins), APC10 (the substrate recognition module), APC11 (the RING domain subunit), APC12, APC13, APC15, APC16 (TPR-accessory subunits) and co-activators Cdc20 or Cdh1 acts as substrate receptors (85-87). Global evaluation of cell cycle-associated proteins and transcripts revealed that the proteome is highly diverse than the transcriptome (88). Ubiquitination of proteins such as cyclin and securin, which induces separation of sister chromatid and mitotic exit (87, 89) is important for proper cell division.

Anaphase Promoting Complex/Cyclosome (APC/C): APC/C is large E3 RING ubiquitin ligase, switch on from anaphase to the G1 phase. The ubiquitin ligase RING E3 serves as a base, bringing close an E2-Ub and substrate (Figure 3A), transferring ubiquitin from E2 to the

substrate. Furthermore, the APC/C utilizes co-activators, for example Cdh1 or Cdc20, as both are APC/C activators and substrate recruitment adaptors (Figure 3B). It utilizes two E2 enzymes, such as Ube2C and Ube2S, to perform timed ubiquitination processes (Figure 3C). Components of APC/C (E3 ligase, Cdc20, substrate and ubiquitin) alter their conformation and activity by inhibitor/pseudo-substrate binding and phosphorylation which results in activation of APC/C.

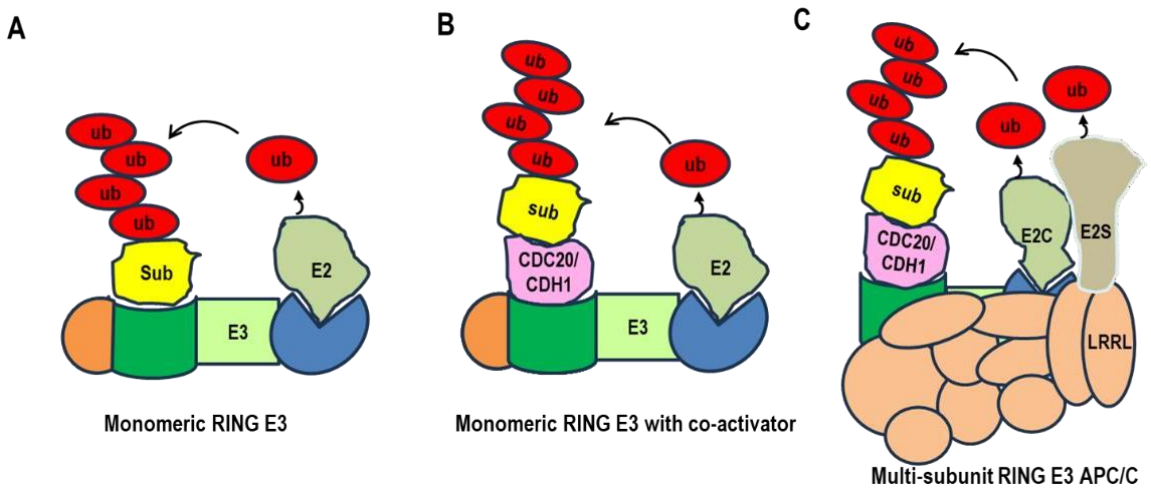

Figure 3. Cartoon diagram representing subunit composition of three RING E3 ubiquitin ligases. RING-type E3 ligases act as scaffolds, bringing the E2Ub conjugate and the substrate together. E3s stimulate Ub transport from the E2Ub conjugate to the substrate. The E2-binding RING domain is highlighted in light blue. **(A)** Monomeric RING E3 ubiquitin ligases, such as c-Cbl. **(B)** APC/C RING E3 ubiquitin ligase and its co-activators like Cdc20 or Cdh1. **(C)** The APC/C ubiquitin ligase is a multi-subunit Cullin-RING E3 ubiquitin ligase that uses two E2s. APC/C; anaphase-promoting complex/cyclosome.

i. Regulation of the APC/C activity- Cdc20/Fizzy, and Cdh1 are the two coactivators of APC/C. APC/C-Cdh1 acts as the late M phase and G1 phase while APC/C-Cdc20 become active in the early M phase (90, 91). Co-activators and Apc10 play a vital role in substrate

recognition, which is required for ubiquitination (92, 93). Substrate bears either the destruction box (D-box) or KEN box with a conserved sequence of RxxLxxxxN or KENxxxN, respectively, which is recognized by co-activators (94, 95), are evolutionarily conserved. CDK1-mediated phosphorylation of APC3 and APC1 of APC/C, initiates APC/C interaction with Cdc20, leading to APC/C activation and anaphase initiation (96, 97). But CDK1-mediated phosphorylation of Cdc20, like Cdh1, inhibits APC/C activity (91, 98).

Mitotic checkpoint or spindle-assembly checkpoint (SAC), checks for disengaged or tensionless kinetochores and pauses anaphase till all kinetochores associates to form a functional bipolar mitotic spindle structure (99). SAC complex, which includes Cdc20, BuBR1, Mad2, and Bub3, is an effective APC/C inhibitor. MCC has recently been demonstrated to impedes Cdc20 that is required for APC/C activity, indicating that MCC can inhibit APC/C rather than only taking away Cdc20 (100). The central cavity of the APC/C occupied by the mitotic checkpoint complex (MCC) which impedes the Ube2C recruitment, blocking most of the substrate ubiquitination. Surprisingly, cyclin A and Nek2A can undergo degradation despite SAC being active because these proteins are capable of binding to the APC/C without the WD40 of co-activators (101). When spindle microtubule binds properly to all kinetochores then only SAC must be turned off to allow anaphase to begin.

ii. Skp1-Cullin1-F-box (SCF) ubiquitin ligase- Unlike the APC/C complex, the SCF-Skp2 is activated later during the G1/S boundary and stays effective until the end of the S phase. Partners of SCF complex include Roc1 (RING-domain protein), Cul1 (Cullin scaffold), and Skp1 (confer substrate selectivity) serves as one of several substrate adaptors for F-box proteins. CDC14B dephosphorylates Skp2 during the M/G1 progress causes Skp2 ubiquitination by APC/C-Cdh1(102). As cell cycle progress towards mid G1 phase, cyclin E expression increases results in CDK2-cyclin E activation, it in turn phosphorylates Skp2 at

Serine 64/72 in G1 and during S phase, cyclin A-CDK2 maintains its phosphorylation at Serine 64, and to a lesser extent at Serine 72 (102). Dephosphorylation of Skp2 by CDC14B during the M/G1 transition causes Skp2 ubiquitination by APC/C-Cdh1(102). Skp2 phosphorylation impedes its ubiquitination by APC/C-Cdh1, and SCF-Skp2 increases S phase progression by reducing origin licensing during the S phase.

1.5. Cell cycle dysregulation as a hallmark of cancer

Defective checkpoint or block of cell cycle progress at various checkpoints in the S, G2, or M phases after significant cellular injuries such as chromosomal aberration or metabolic disorder comprise cell cycle dysregulation. Dysregulation of cell cycle machinery can induce abnormal cell behaviour, leading to cancer initiation and progression (103). Two types of dysregulations linked to the cell cycle are alterations in the constitutive cell cycle machinery and alterations in checkpoint signaling proteins.

i. Alterations in the constitutive cell cycle machinery- Genetic alterations such as deletion mutations at the pRB locus frequently occur in many human malignancies, including breast cancer, retinoblastoma, and osteosarcoma. Tumor suppressor protein pRB a gatekeeper, that regulates G1/S transition by controlling E2F transcription factor activity (104). CDK4-cyclin D1 complex is activated in response to external growth signals and drives G1/S transition by phosphorylating pRB to restore E2F activation. Protein molecules of the signaling pathways that control pRB function are regularly modified and observed in cancers. Increased cyclin D and cyclin E levels or genes amplification that encode CDK4 and CDK6 or loss of p16 are found in various cancers (104). Cyclin D is over-expressed in around 50% of invasive breast tumors. Transgenic mice implanted with mammary cells exhibit elevated expression of human cyclins E or D1 form mammary adenocarcinomas (105). Similarly, gliomas, sarcomas,

melanomas, breast malignancies, etc. display amplified expression of CDK4 and CDK6 gene (106).

ii. Alterations in checkpoint signaling proteins- Checkpoints are the procedures that track the series, integrity, and accuracy of the cell cycle's basic activities. These involve optimal growth of cells, DNA doubling and integrity, and proper segregation during mitosis (107). Mutations in checkpoint protein genes occur frequently in all form of cancer due to faulty control in cell cycle, which promote genetic instability (108). Disruptions in biochemical path can results in biological diseases such as cancer (109-111).

Phosphorylation of pRB protein controls the G1-S checkpoint (restriction point) (112). pRB inhibits S phase initiation of the cell cycle restricts cell growth while enhancing final differentiation by activating gene expression responsible for quitting from cell cycle (7). Loss of pocket protein (Rb/p105, p107, and Rb2/p130) functions by point mutations or deletions are linked to a broad spectrum of human malignancies, most notably retinoblastomas and sarcomas (7). Pocket retinoblastoma (pRB) protein can be turned into non-functional via mutations, viral oncoprotein binding, or phosphorylation. When RB is inactive, E2F transcription factors become unbound, leading to disrupted cell cycle control and the development of cancer.

The DNA damage response (DDR) or S-G2 checkpoint is critical for maintaining genome integrity when encountering an assault of internal and external DNA damage (113, 114). Many target proteins such as CHK2, p53, and H2AX governing cell cycle arrest, DNA repair, and apoptosis, are phosphorylated by active ATM (115). ATM performs a vital function in G1/S checkpoint, mainly via its influence on p53 function. CHK2, a kinase that regulates DNA repair apoptosis, and cell cycle arrest, is the most important ATM signal transducer. CHK2 threonine 68 phosphorylation by ATM, leading to the formation of dimers and the self-phosphorylation

of the kinase domain which is necessary for complete activation (116). Cancer frequently exhibits ATM gene mutations. Mutations in both copies of the ATM gene in the germline lead to ataxia-telangiectasia (A-T), a widely recognized recessive genetic illness associated with an increased risk of developing cancer. (117). ATM somatic mutations have been found in various cancers, most notably lymphoid cancer, implying that loss of ATM facilitates carcinogenesis (118). ATM expression has been lost in various cancer, including pancreatic cancer, lung adenocarcinoma, colorectal cancer, breast cancer, etc. (119, 120). The G2-M phase checkpoint pauses progression in the cell cycle to rectify DNA damage. WEE1, an intranuclear serine/threonine protein kinase, specifically adds phosphate groups to tyrosine residues on CDK1, resulting to a pause in the cell cycle during the G2-M transition (121).

The spindle is a highly ordered and regulated structure that separates chromosomes during cell division. The SAC keep track on mitotic spindle attachment and traction on kinetochores, by pausing transition from metaphase-anaphase until all defects are rectified (122, 123). The mitotic checkpoint or SAC regulates the function of APC/C, a multi-subunit complex, that ubiquitinates regulators of sister-chromatid segregation. Spindle perturbations activate MAD proteins attach to Cdc20 subunit and blocks APC/C activity, causing a cell cycle halt in metaphase. Spindle checkpoint defects frequently exhibited chromosome segregation abnormalities, resulting in aneuploidy, which could be attributed to BuB1 and BuB1R kinase mutations (124, 125).

1.6. Hematopoietic PBX interaction protein (HPIP)

HPIP, commonly referred to as Pre-B-cell leukemia homeobox interacting protein (PBXIP1), was originally found as a corepressor of PBX1 interacting protein. HPIP has demonstrative role in primitive stages of hematopoiesis (126, 127). Chromosome 1 harbours *HPIP* gene located

at position q21.3, and it bears 731 amino acid-containing protein without any homology with known protein. HPIP can interact with PBX family members like PBX2, PBX3, and PBX1. It also inhibits the E2A-PBX transcriptional activity by preventing the interaction of DNA to HOX-PBX complexes (126). As a scaffold protein, it fosters interactions with many molecular signals such as ER, Src, and microtubules governs many cellular functions such as differentiation, cell proliferation, migration, etc. (128, 129).

i. Role of HPIP in cancer - The involvement of HPIP in cancinogenesis was initially discovered in breast cancer by Manavathi *et al.* in 2006 (129). Further research has established that HPIP expression is raised in several cancers and promotes tumorigenesis. HPIP inhibits the transcriptional activity of estrogen receptor α (ERα) in breast cancer (129). Further, studies have also shown that estrogen-activated kinase TBK1 adds phosphate groups to HPIP, resulting in its degradation through a process that relies on MDM2 (130). HPIP primarily resides in the cytosol but possesses the capability to move between the cytosol and nucleus. Amino acid from 443-731 of HPIP contains a nuclear export signal that enables it to enter the nucleus (131). It impacts adhesion and migration of cells by stimulating the FAK/ERK/Calpain2 signaling leads to focal adhesion turnover, to enhance breast cancer migration (132). A recent finding suggests that HPIP can interact with RUFY3 and RAB5 to regulate cell migration (133). RUFY3 and HPIP act as guanine nucleotide exchange factors (GEFs) for RAB5, which regulates focal adhesion turnover regulated by endocytosis, thereby controlling cell migration.

Many studies have found that HPIP is heavily expressed in a various type of cancers, such as leiomyosarcomas, hepatocellular carcinoma (HCC), gastric cancer, colorectal cancer, glioma, and oral carcinoma pancreatic cancer, renal cell carcinoma (RCC), epithelial

ovarian cancer (EOC), lung cancer, cervical cancer, and endometrial cancer (EC) (129, 134-137). HPIP serves as a predictive indicator for ovarian cancer as its expression is correlated with advance-grade ovarian tumors. Moreover, HPIP can also be a possible target for cisplatin-resistant ovarian cancers (137). In the case of breast cancer cells, HPIP sensitized them to paclitaxel in a microtubule-dependent manner (138). Recent studies revealed that CSR1 (cellular stress response), a tumor inhibitor gene, reduces cell migration, growth, and invasion by deactivating HPIP and subsequently diminished PI3K/AKT signaling (139).

Interestingly, HPIP promotes cell invasion while reducing oral squamous cell carcinoma (OSCC) cell proliferation (135). However, normal human epidermal keratinocytes (NHEK) invasion is inhibited by HPIP, while NHEK proliferation is enhanced (135). All increasing proof shows that HPIP enhances migration, proliferation, anchorage-independent growth, and invasion of tumor cells by activating the AKT/MAP Kinase signaling (134, 140). It promotes EMT by stimulating the TGF-β/Smad2 and PI3K/AKT/GSK3β/SNAIL circuits in lung cancer, and ovarian respectively (141).

ii. Cell cycle regulation by HPIP- Recent studies established that HPIP regulates the cell cycle. According to research by the Qinong Ye group, HPIP promotes the development of liver cancer cells by stimulating the G2/M transitions of the cell cycle (142). They have also shown that in colorectal cancer, HPIP controls the G1/S progress as well as facilitates G2/M phase of the cell cycle. It regulates the expression of cyclin D1 and cyclin A during the G1/S phase, and cyclin B1 during the G2/M phase to encourages tumor growth (143). However, the molecular mechanism of HPIP regulating cyclin B1 expression remains elusive.

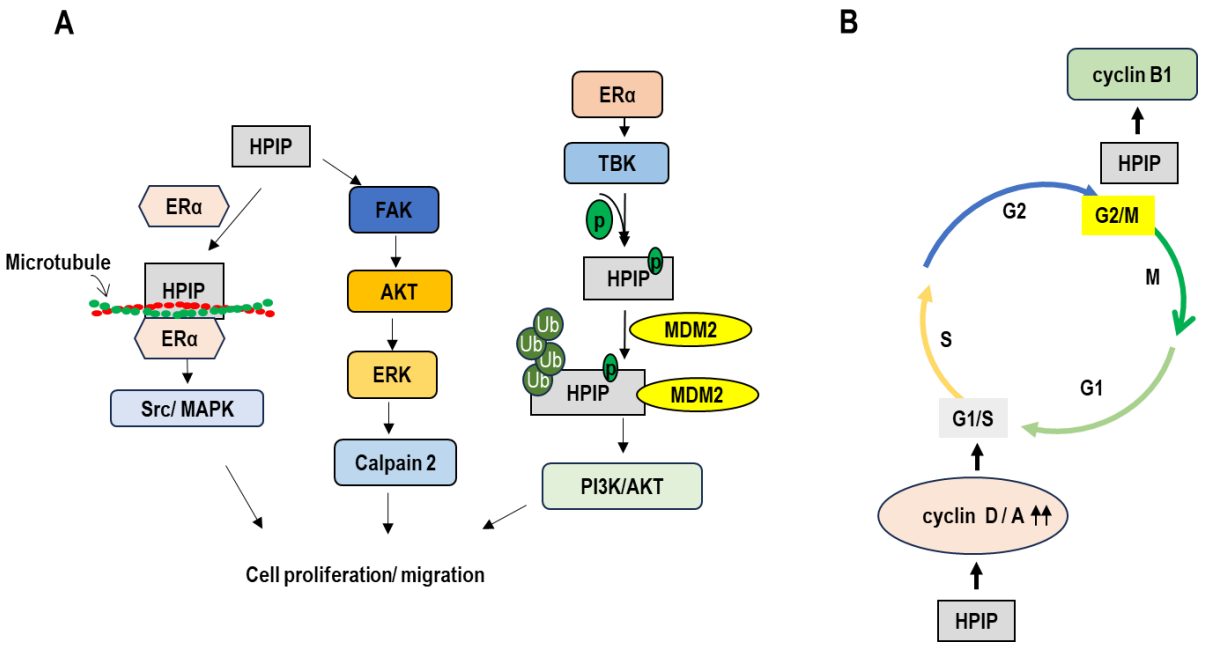

Figure 4. **Role of HPIP in tumor progression and cell cycle. (A**) The ERα-HPIP signaling cascade triggers Src/MAPK signaling circuit helping cell proliferation. HPIP activates FAK and calpain2, leading to focal adhesion turnover and cancer cell migration. HPIP undergo degraded when phosphorylated by TBK1, activating the MDM2-mediated AKT pathway and promoting cell proliferation. **(B)** HPIP governs the cell cycle's G1/S and G2/M phase transitions. It stabilizes cyclin B during the G2/M phase and promotes tumor growth.

Additionally, in U87 and U251 (glioblastoma cells), HPIP controls the G1/S transition (144) and promotes cell division by unknown mechanisms in ductal adenocarcinoma of pancreas cells (145). HPIP stimulates proliferation and G1/S transition in gastric cancer cells by activating cap-dependent translation of cyclin D1 via the AKT/mTORC1 pathway (146).

1.7. Rationale and Objectives

Dysregulated cell division is a defining characteristic of cancer. Therefore, understanding the molecular mechanism underlying cell division and molecular regulators of the cell cycle is paramount. HPIP is a proto-oncogene that is over-expressed in a plethora of cancers, such as breast infiltrative ductal carcinoma, hepatocellular carcinoma, glioma, etc. (147). Recent studies on its role in cell division provided a few clues into its role in cell division. However, mechanistic insights into HPIP's role in cell division remain largely unknown. Given this background, the present study aims to address HPI–P's role in cell cycle control and its phosphorylation by CDK1-cyclin B1 during mitosis with the following tangible objectives:

Objectives

1. To understand the function of HPIP in cell division
2. To study the functional significance of HPIP phosphorylation in the regulation of cell cycle
3. To assess the impact of phosphorylation on the stability of HPIP during cell cycle progression

Chapter 2

Materials and methods

2.1. Cell lines and cell culture

HeLa and HEK293T cells were obtained from the National Centre for Cell Science (NCCS), Pune, India. These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) (Invitrogen, New York, USA) supplemented with 100 U/ml penicillin, 2 mM L-glutamine, and 100 µg/ml streptomycin at 37ºC in 5% CO_2. Media and antibiotics details are mentioned in table 3.

2.2. Transfection

A day before transfection, approximately 1x 10^6 cells were cultured in a 60 mm petri plates. After 24 hr, replaced with fresh serum and antibiotic-free media. Lipofectamine (Invitrogen™ Lipofectamine™ 2000 Transfection Reagent, USA)-DNA complexes were prepared in Opti-MEM (Gipco, Thermofisher scientific, USA) at the recommended ratio of 1:1 (1 µl Lipofectamine per 1 µg of DNA). Cells were then incubated with Lipofectamine - DNA complexes for 6 h before being replaced with DMEM containing 10% fetal bovine serum medium when necessary 24 hr of post-transfection, cells were subjected to drug treatment or harvested for further analysis.

2.3. Generation of stable cell lines

Lentiviruses were generated using Lipofectamine2000 for transfection mentioned in the manufacturer's protocol in HEK293T cells by co-transfection of the lentiviral plasmid of interest pMNDUS control, pMNDUS-HPIP, pMNDUS-mtHPIP-S43A or pMNDUS-mtHPIP-S43E (Flag-tagged) together with packaging plasmids, pREV, VSV-G and pΔR (1:0.4:0.5 ratio). Six hr post-transfection, media was replaced with DMEM, and ∼24 hr later, the viral soup was collected, centrifuged for 3 min at 500x g, and sieved through a 0.45 µm filter. HeLa cells were trypsinized and collected, centrifuged, and the supernatant was discarded. An equal

volume of viral soup and fresh DMEM were added to cells and incubated for 24 hr. This process was repeated 4-5 times to enrich the transduced cell population, and then Western blotting verified protein expression.

2.4. Clonogenic assay

HeLa cells stably expressing empty vector (control), wtHPIP, mtHPIP-S43A, or mtHPIP-S43E were collected after trypsinization followed by centrifugation at 1200 rpm and counted using Cell Counter (Luna Dual Fluorescence cell counter, USA). Approximately 300 cells/well were added to a 6-well plate. Plates were taken out after 15 days, rinsed with PBS, and stained for 10-15 min with Crystal Violet and then excess dye washed out with PBS (Phosphate buffer saline) to visualize the colony. Colonies were counted, and results were represented as dot plot graphs using GraphPad Prism.

2.5. Cell proliferation assay (WST -1 Assay)

Proliferation of cells was measured using the WST-1 assay as described earlier (148). Cells were collected after trypsinization followed by centrifugation at 1200 rpm. The cell pellet was dissolved in 1 ml of new medium and counted using the Luna cell counter. Approximately $5x10^3$ cells/well were added in 96 well plates and incubated for various time points. WST-1 reagent was added at each time point, (Roche Diagnostics, Indianapolis, IN, USA) to all well, and incubated at 37^{o}C for for 3 hr. Absorbance at 455 nm was assessed with a reference background at 655 nm, and the result was represented in a line graph using a GraphPad prism.

2.6. Western blotting

PBS washed cells were lysed in pre-cooled Nonidet P-40 (NP-40) buffer (1% NP-40, 50 mM Tris-Hcl (pH 7.4), 5 mM EDTA, 150 mM NaCl, protease inhibitor mixture). Cell lysates were

spun at 13000 rpm at 4°C for 15 min. The supernatant was then taken out to a fresh microcentrifuge tube. The reducing agent and detergent compatible (RC-DC) assay determined protein concentration following the manufacture protocol (Bio-Rad, USA). Approximately 100 µg of protein lysates were loaded, resolved on SDS-PAGE, then transferred to nitrocellulose (NC) membrane (Pall Corporation, USA) employing Bio-Rad electrophoresis units, and probed with the protein-specific antibodies for 16 hr at 4^{o}C. After incubation with HRP conjugated secondary antibodies for 1 hr, enhanced chemiluminescence (ECL) detection reagents (GE HealthCare, USA) used to develop the blots by Chemidoc imaging system (Vilber Fusion Solo 6S, France). The antibodies used are listed in Table 1.

2.7. Molecular Cloning

Site-directed mutagenesis using site-specific mutations in primers was followed to introduce mutation at serine 43 locus in the *HPIP* gene using pMNDUS-HPIP (127) (Manavathi *et al.*, 2012), pcDNA3.1c-HPIP or pET28a-HPIP as templates. PCR amplicons were digested with *DpnI* enzyme at 37^{o}C overnight to degrade parental plasmid DNA. The digested PCR amplicons were used to transform into *E. coli* (DH5α), spreaded on LB (Luria Bertani) agar plates with Ampicilin (100 µg/ml). Two colonies were inoculated in LB broth and incubated in a bacterial shaker at 37^{o}C for overnight. Plasmids were isolated using the *QIAprep Spin Miniprep Kit*. Clones were confirmed by gene sequencing and were named mtHPIP-S43A or mtHPIP-S43E. For protein expression and purification, pET28a-HPIP was used.

2.8. Recombinant protein expression and purification

Competent *E. coli* (Rosetta DE3) cells were prepared by following the $CaCl_2$ method. Then, competent cells were transformed with pET28a-HPIP or pET28a-mtHPIP-S43A constructs and spread on LB agar plates containing Kanamycin. Following this picked up a single colony with

a toothpick and inoculated in LB broth containing Kanamycin incubated overnight (~16 hr) at 37°C. Next, a 2% overnight culture (primary culture) was utilized for secondary inoculation and cultivated until the optical density (OD) reached 0.6 (measured at 600 nm). Then, 0.5 mM of Isopropyl β- D-1-thiogalactopyranoside (IPTG) was used for protein expression of protein at 37°C for 2 hr. After induction, cells were then spun down, and the bacterial pellet was resuspended to lysed with the lysis buffer, as mentioned in Table 4. The bacterial lysate was sonicated at 40 amplitudes with 20 secs ON and 40 secs OFF cycle for 20-25 sonic bursts. After sonication, the sample was spun for 20 min at 11000 rpm at 4^{o}C to separate the supernatant from the pellet. The supernatant containing the protein was then incubated with Ni Sepharose beads (washed priorly with the same lysis buffer) for 2 hr at 4^{o}C. This was followed by washing the beads with wash buffer (5 mM Tris pH 8.3, 25 mM NaCl, 0.05 % Triton X-100, and 2 mM Imidazole) to remove the non-specific binding of other proteins. SDS-PAGE followed by Coomassie staining for overnight and then destained using destaining was used to verify the protein's purity. After purification, beads were used for CDK1 kinase assay.

2.9. Cell synchronisation

Double thymidine treatment was followed for cell synchronization, as described earlier (149). HeLa cells were plated at 20-30% density in a 60 mm petri plates incubated overnight. Thymidine (2 mM final concentration) was added and then incubated for 18 hr in CO_2 incubator at 37°C. By washing cells thrice with 2 ml of pre-warmed PBS, thymidine was removed. Fresh medium was added and incubated for 9 hr. Second round of thymidine (2 mM) was added and cultured at 37°C for 18 hr. Cells were now in the G1/S transition. After thorough washing, fresh medium was added to the plates and taken out at various intervals of time for multiple analyses.

Nocodazole (100 ng/ml working concentration) was used to treat HeLa cells (40% density) and incubated for 12 hr at 37°C in cell culture incubator to synchronize them at the mitosis phase. After three washes with PBS, cells were allowed to release into fresh DMEM for various time points before harvesting for further analysis.

2.10. Co-Immunoprecipitation (Co-IP)

NP-40 lysis buffer (NP-40) buffer was used to prepare cell lysates. Approximately 1 mg of the protein was incubated with protein-specific primary antibodies (MPM2, HPIP, CDK1, or cyclin B1) according to the mentioned dilution (1 µg of antibody for 1 mg of protein) for overnight period under rotation at 4^{o}C. Protein A/G agarose beads slurry (30 µl) were added to the protein-antibody complex then incubated at 4^{o}C at 10 rpm for 1 hr. Afterward, the protein mixture was spun at 1200 rpm for 5 min at 4^{o}C to collect agarose beads bound to antigen-antibody complex. After thorough washing, A/G beads containing protein-antibody complex were resuspended in 20 µl sample buffer before being subjected to Western blotting.

2.11. CDK1 kinase assay

Mitosis-synchronized HeLa cells were harvested, and cell lysate was prepared in NP-40 lysis buffer. Approximately 250 µg of cell lysate was incubated with either anti-CDK1 antibody or normal rabbit IgG overnight at 4^{o}C under rotation on a roto-spin. Protein A/G agarose slurry was added to this antigen-antibody mixture and incubated for 3 hr to immunoprecipitated CDK1-cyclin B1 complex. The immunoprecipitated CDK1 was washed thrice with lysis buffer. His-wtHPIP or His-mtHPIP-S43A (40 µl each) beads after three washes with lysis buffer mixed with CDK1 precipitated beads then after reaction was initiated by adding CDK1 kinase buffer and incubated for 15 min at 30^{o}C for 25 min. Laemmli buffer (40 µl, 2x) were added to cease the reaction, followed by boiling for 8 min. Subsequently, the protein mixture was separated on SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). Then, the

protein was transferred onto the nitrocellulose membrane, and Ponceau's was stained and photographed. The dried membrane was subjected to autoradiography using the GE HealthCare Typhoon Trio, USA variable mode imager system.

2.12. Time-lapse microscopy and cell cycle progression

Double thymidine approach used to block cells at G1/S boundary allowed to release into fresh phenol red free DMEM medium (FluoroBrite DMEM Thermo Fisher Scientific, USA) and then imaged at regular intervals of 20 min for cell division analysis using a fluorescence microscope (Olympus IX83 inverted microscope with Andor Zyla 4.2 sCMOS camera, U-FGW for mCherry, F-UBW for GFP, Oko lab Uno live cell chamber, and Retiga 6000 monochrome detector, Singapore. PBS wash given to mitosis arrested cells and liberated into fresh fluoroBrite DMEM, and images were captured at every 5 min interval for mitotic exit analysis. Using ImageJ software (Wayne Rasband, USA) Videos were generated, and the results were quantified.

2.13. Cell imaging by immunofluorescence (IF)

Nocodazole arrested or double thymidine (DT) synchronized cells were fixed for 20 min at room temperature using 4% paraformaldehyde (PFA), then 0.25% Triton X-100 was used for permeabilization for 15 min at room temperature. Afterwards, cells were blocked with 1% BSA (Bovine serum albumin) in PBS at room temperature for 1 hr. Protein-specific antibodies were added to the cells, including HPIP, CDK1, cyclin B1, MPM2, or β-Tubulin at 4^{o}C for overnight and then washed with TBS, TBST, and TBS for 5 min each. Afterwards, secondary antibody (Alexa Fluor 546 or 488) was added to the cells and incubated for 1 hr in the dark at room temperature. Cells were washed for 5 min each with TBS, followed by TBST and then TBS. 4,6-diamidino-2-phenylindole (DAPI) was used for DNA staining, and edges were sealed with

nail polish to avoid air contact, and then images were captured by microscope (Olympus IX83 microscope, Singapore). The data was quantified using GraphPad prism.

2.14. RO-3306 treatment

HeLa cells were plated in 60 mm culture patri plate at 60-70 % density. Twenty-four hr of post seeding, cells were treated with RO-3306 (625 nM) or DMSO (vehicle). After washing NP-40 buffer used to lysed the cells after 24 hr of treatment. For the RO-3306 release assay, following 24 hr of RO-3306 treatment, three times PBS washed cells were released into fresh medium for 4 hr and lysed in NP40 lysis buffer. Protein extracts were then analyzed using Western blotting.

For IF analysis, cells were synchronized with double thymidine (DT) treatment followed by the addition of 625 nM RO-3306 for 12 hr. After drug treatment, cells were processed for IF imaging.

2.15. Pulse-chase assay

Cycloheximide (CHX) (final concentration 25 µM) was used to treat the cells for the specified time before the lysis. Western blotting was used to analyze the cell lysates, probing protein-specific antibodies. Using Fiji-ImageJ, the band intensities were measured and quantified. The quantified bands were normalized to protein bands of 0 hr CHX treated samples, graphed as (log normalized) relative protein.

2.16. Mitotic Spindle Enrichment

Enrichment of the mitotic spindle was done by following the protocol described earlier (150). HeLa cells were exposed to a concentration of 1.6 µg/ml aphidicolin. for 16 hr to synchronize

HeLa cells at the G1/S phase. Cells were then placed for 14 hr in fresh media with 40 ng/ml nocodazole to prevent mitosis. Shake-off technique was used to collect mitotic cells. These mitotic cells were then centrifuged at 300xg and put into regular media for 30-40 min after twice with PBS wash until the majority of them had reached metaphase (For metaphase plate formation, immunofluorescence examination of cells stained with DAPI was used). Microtubules were then stabilized in the media for 3 min by adding 5 g/ml taxol. After that, the cells were harvested, rinsed with PBS mixed with 1 mM PMSF, 5 g/ml taxol, and 2 g/ml latrunculin B and then incubated at 37°C for 15 min in mitotic spindle enrichment (MSE) lysis buffer. Then after, centrifuged for 2 min at 700x g to obtained lysed cells. pellets were dissolved in the MSE buffer and allowed to incubate for 5 min, subsequently centrifuged, and the supernatant was discarded. Lysed cell "ghosts" were treated in an extraction buffer for 5-10 min until differential interference contrast (DIC) microscopy displayed spindles devoid of intermediate filaments, and then the spindle was sedimented for 3 min at 1,500x g. This process was performed again as needed. The spindle mixture was sonicated after resuspension in 0.1 M glycine, pH 2.8 buffer, in an ultrasonic water bath for 30 seconds before being precipitated in acetone. Proteins were resuspended in NP-40 buffer and subjected to Co-IP using anti-HPIP antibody. Approximately 10% lysate was used for Western blotting to check the HPIP pulldown, and the rest of the lysate was subjected to Nano-LC-MS (151).

2.17. Ubiquitination assay

In order to analyze the *in vivo* ubiquitination of HPIP, ectopically expression was carried out in HeLa cells with the pMNDUS-wtHPIP or pMNDUS-mtHPIP-S43A plasmid construct (Flag tag) along with hemagglutinin tagged-ubiquitin (HA-Ub) construct using Lipofectamine2000. After 48 hr of transfection, cells were rinsed with pre-cooled PBS and lysed in NP-40 buffer (250 µl/ $2x10^6$ cells). Lysates were spun at 14000 rpm for 15 min at 4ºC to pellet down cell

debris. After protein estimation, approximately 1 mg of protein lysate was used and rotated with Flag beads or mouse IgG for overnight for immunoprecipitation (Thermo Fisher Scientific, USA) at 4^{o}C. Then, approximately 30 µl protein A/G slurry was added to the mouse IgG sample and kept for rotation at 4^{o}C for another 1 hr to pull down the immune complex. After a brief centrifugation, the supernatant was discarded to collect beads containing antigen-antibody complex. Resuspended in 30 µl Laemmli buffer after washing the beads with lysis buffer before being subjected to Western blotting.

2.18. Mitotic index and cell cycle analysis by flow cytometry (FACS)

After 36 hr of transfection, cells were harvested using trypsin and collected by spinning for 5 min at 1200 rpm. PFA (1%) was used for fixing the cells for 20 min at room temperature. After PBS wash permeabilization was carried out in 0.01% triton-x100 for 10 min after PBS wash, then after 1% BSA used for blocking. Cells were then incubated with primary anti-rabbit phospho-Histone3-ser10 overnight at 4^{o}C. Later, FITC-conjugated anti-rabbit secondary antibody was used to treat the cells for 1 hr. After thorough washing, the cell population was acquired using the Aria Fortesa (BD Bioscience, USA).

For cell cycle analysis, 1% PFA was used for 20 min to fix the cells and permeabilized for 20 min in 0.01% triton-x100 at RT. The cells were rinsed with PBS, followed by the addition of 100 µg/ml of RNase, and stained with DAPI (1 µg/ml) overnight before cell populations were captured by Aria Fortesa Flow cytometer (BD Bioscience). FlowJo software used to analyse data. The mitotic index and percentage distribution of cells' population in various phase was calculated and represented as a dot plot and histogram respectively using GraphPad prism.

2.19. Real-time Quantitative Reverse Transcriptase PCR (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA) was used to isolate RNA from HeLa cells. The RNA was converted into cDNA utilizing an cDNA synthesis kit (iScript from Bio-Rad) per the supplier's instructions. Diluted (1:10) cDNA was used to perform the quantitative real-time PCR employing FastStart SYBR Green Master mixture (Roche Applied Science, Mannheim, Germany) and the LightCycler®96 Real-Time PCR System machine. The Primer sequence details are in Table 2.

2.20. Statistical analysis

Every experiment was carried out at least three times. Data is displayed as a standard deviation of the mean (SDM±), and differences between groups were evaluated using a two-tailed unpaired Student's t-test and multiple comparisons using the ANOVA test applying a GraphPad prism to determine statistical significance. ***, P<0.0001, **, p<0.001, *, p<0.01. ns, non-significant.

Buffers and reagents used in this study are mentioned in the tables.

Sl. no.	Name of Antibody	Catalogue Number	Company Name
1	HPIP	HPA006949	Sigma Aldrich, USA
2	PBXIPI	A301-629A	Bethyl Laboratories, USA
3	Flag tag	8146S	Cell Signalling Technology, USA
4	β-Actin	A3854	Sigma Aldrich, USA

5	GAPDH	2118S	Cell Signalling Technology, USA
6	Phospho-Histone3 ser10	3377	Cell Signalling Technology, USA
7	cyclin B1	4138	Cell Signalling Technology, USA
8	cyclin A1	sc-751	Sancta Cruz Biotechnology, USA
9	MPM2	05-368	Merck Millipore, Germany
10	CDK1	NBP2-34331	Novus Biological, USA
11	cyclin B1	sc-245	Sancta Cruz Biotechnology, USA
12	Cdh1	cc43	Calbiochem, USA
13	Cdc20	14866	Cell Signalling Technology, USA
14	HA	sc-7392	Sancta Cruz Biotechnology, USA
15	T7	69522	Novagen, Germany
16	GFP	G-1544	Sigma Aldrich, USA
17	Beta-Tubulin	6323	Sigma Aldrich, USA
18	mouse IgG	sc-2025	Sancta Cruz Biotechnology, USA
19	rabbit IgG	2729	Cell Signalling Technology, USA

20	Alexa fluor 488	A11008	Invitrogen, USA
21	Alexa fluor 546	A11003	Life Technologies, USA

Table 2

Sl. no.	Primer Name	Site-directed mutation	Restriction site	Clone name	Expression tag
1	pMNDUS-HPIP FP	5'-TGGCCAATTGCCACCATGGACTACAAAGAC-3'	*NsiI*	pMNDUS-wtHPIP	Flag
	pMNDUS-HPIP RP	5'-AGTCATGCATTCAGCCCCGGTGGTGGTG-3'	*MfeI*		
2	mtHPIP-S43A FP	5'-GCCCCTCACGCCCCCTCCAAG-3'	None	pMNDUS-mtHPIP-S43A	Flag
	mtHPIP(S43A) RP	5'-CTTGGAGGGGGCGTGAGGGGC-3'			
3	mtHPIP-S43E FP	5'-GCCCCTCACGAGCCCTCCAAG-3'	None	pMNDUS-mtHPIP-S43E	Flag
	mtHPIP-S43E RP	5'-CTTGGAGGGCTCGTGAGGGGC-3'			
4	HPIP- FP	5'-GATCCG**GGATCC**ATGGCCTCCTGCCCAGA-3'	*BamHI*	pET28a-wtHPIP, pET28a-mtHPIP-S43A	His
	HPIP- RP	5'-TTGCCG**CTCGAG**TCAGCCCCGGTGGTGGT-3'	*XhoI*		
5	HPIP-FP	5'-GATCCGGGATCCATGGCCTCCTGCCCAGA-3'	*BamHI*	pcDNA3.1c-mtHPIP-S43A,	T7
	HPIP-RP	5'-TTGCCGCTCGAGTCAGCCCCGGTGGTGGT-3'	*XhoI*	pcDNA3.1c-mtHPIP-S43E	

For RT-PCR

Sl. no	Name	Sequence
1	HPIP-RP	5'-ATGGGTCTTCTGCTGGACAA-3'
	HPIP-FP	5'-CAGGCTCTGAAGCTCTTCCTT-3'
2	β - Actin	5'-AGCCATGTACGTAGCCATCC-3'
	β - Actin	5'-CTCTCAGCTGTGGTGGTGAA-3'
3	cyclin B1 F.P	5'-GTCGGCCTCTACCTTTGCACTTCCTTC-3'
	cyclin B1 RP	5'-GAGTTGGTGTCCATTCACCATTATCCAG-3'

shRNA

Sl.no	Name	Sequence

1	pGIPZ-shHPIP #1	5’-ATGTTCTTAGCAGAGAGGC-3’
	pGIPZ-shHPIP #2	5’-AATTCTTTCCCATCTGTC-3’

Table 3

Sl. No.	Reagents	Catalogue no.	Company
1	Fluoro Brite DMEM	A1896701	Thermo Fisher Scientific, USA
2	Dulbecco's Modified Eagle's Medium (DMEM)	11965	Thermo Fisher Scientific, USA
3	Phosphate buffer saline (PBS)	10010-023	Thermo Fisher Scientific, USA
4	Fetal bovine serum (FBS)	10270106	Thermo Fisher Scientific, USA
5	Antibiotic-Antimycotic (100X)	15240-062	Thermo Fisher Scientific, USA
6	Trypsin	25200-072	Thermo Fisher Scientific, USA
7	Lipofectamine2000	11668019	Thermo Fisher Scientific, USA
8	Optimal-Minimal Essential Media (Oti-MEM)	31985070	Thermo Fisher Scientific, USA
9	WST-1	10008883	Roche Diagnostics, Indianapolis, IN, USA
10	Crystal violet	17610	Sisco Research Laboratories (SRL), India
11	Coomassie blue R250	1.12553	Sigma Aldrich, USA
12	IGEPAL CA630 (Nonidet P40)	I8896	Sigma Aldrich, USA
13	Ethylenediaminetetraacetic acid sodium (EDTA)	E7889	Sigma Aldrich, USA
14	Sodium Chloride (NaCl)	41721	Sisco Research Laboratories (SRL), India
15	*Dpn1*	R0176S	New England Biolabs, USA
16	Luria Bertani (LB) broth	M1245	Himedia, India
17	Luria Bertani (LB) Agar	M1151	Himedia, India
18	Ampicilin	470024-776	Avantor VWR Life Science, USA
19	Kanamycin	97062-956	Avantor VWR Life Science, USA
20	Calcium Chloride (CaCl2)	MB034	Himedia, India
21	Magnesium Chloride (MgCl2)	M2670	Sigma Aldrich, USA

22	Isopropyl-β-D-thiogalactopyranoside (IPTG)	97061-778	Avantor VWR Life Science, USA
23	Triton-X100	T8787	Sigma Aldrich, USA
24	Imidazole	5710-OP	Merck Millipore, Germany
25	Protease inhibitor (PI)	4511697498001	Merck Millipore, Germany
26	Nickel Sepharose beads	GE17-5268-01	Merck Millipore, Germany
27	Thymidine	T9250	Sigma Aldrich, USA
28	Nocodazole	M1404	Sigma Aldrich, USA
29	Protein A/G beads	sc-2003	Sancta Cruz Biotechnology, USA
30	RO-3306	SML0569	Sigma Aldrich, USA
31	Cycloheximide	239763	Merck Millipore, Germany
32	Aphidicolin	A4487	Sigma Aldrich, USA
33	4',6-Diamidino-2-phenylindole (DAPI)	ab104139	Abcam, USA
34	Taxol	T7191	Merck Millipore, Germany
35	Latrunculin	428021	Sigma Aldrich, USA
36	Sodium Flouride (NaF)	29821	Sisco Research Laboratories (SRL), India
37	Paraformaldehyde (PFA)	TCL119	Himedia, India
38	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT)	M6494	Thermo Fisher Scientific, USA

Table 4

Sl. No.	**Buffers**	**Compositions**
1	Nonidet P-40 (NP-40) buffer	(1% NP-40, 50 mM Tris-Hcl (pH 7.4), 5 mM EDTA, 150 mM NaCl, protease inhibitor mixture.
2	Crystal violet staining solution	(0.05 % w/v in 10 % ethanol and 40 % methanol).
3	Destaining	Methanol 40%, Acetic acid 10% and 50% water

4	Bacterial lysis buffer	50 mM Tris pH 8.3, 150 mM NaCl, 10% glycerol and 0.1% Triton X-100, and 10 mM Imidazole supplemented with protease inhibitor.
5	Wash buffer	5 mM Tris pH 8.3, 25 mM NaCl, 0.05 % Triton X-100, and 2 mM Imidazole
6	CDK1 kinase buffer	500 µM ATP containing of 100 µCi/µl of [γ^{32}P] ATP and 5 mM MgCl2,
7	Mitotic spindle enrichment (MSE) lysis buffer	100 mM piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES), pH 6.9, 2 mM EGTA, 1 mM MgSO4, 0.5% Nonidet P-40, 2 µg/ml latrunculin B, 5 µg/ml taxol, including nucleases (200 µg/ml DNAseI, 10 µg/ml RNase A, 1 U/ml micrococcal nuclease, 20 U/ml benzoase), protease inhibitors (1 µg/ml pepstatin, 1 µl/ml leupeptin, 1 µg/ml aprotinin, 1 mM PMSF), and 20 mM β-glycerolphosphate.
8	Spindle isolation buffer	pH 6.9, 5 µg/ml taxol and 1 mM PIPES.
9	Mitotic spindles resuspension buffer	0.1 M glycine, pH 2.8.

Chapter 3

Results

Objective 1: To study the role of HPIP in cell division

3.1. HPIP knockdown delays cell division and arrests cells in the G2/M phase

To investigate the role of HPIP in cell proliferation, we utilized lentiviral-based shRNA silencing to attenuate intrinsic HPIP expression in HeLa cells. The silencing of HPIP in HeLa cells was verified through Western blot analysis (Figure 5A). Reducing HPIP levels ensured a notable decline in the cell proliferation rate of HeLa cells (Figure 5B). The kinetics of cell division were then observed using time-lapse microscopy. The imaging results demonstrated that the depletion of HPIP caused a significant delay of 3.4 hr in cell division compared to control shCtrl (14.2 ± 0.6 hr vs shHPIP 17.6 ± 1.0 hr) (Figure 5, C and D). Flow cytometry (FACS) was subsequently employed to identify the specific phase of the cell cycle affected by HPIP. Silencing of HPIP led to a notable increase in the gathering of cells in the G2/M phase when compared to control cells. (Figure 5E). To gain a better understanding of HPIP's role in cell-cycle progression, we first decreased its levels by shRNA-mediated silencing approach in HeLa cells. Later, we ectopically expressed α-tubulin-mCherry and H2B-eGFP, then synchronized the cells at the G1/S boundary using a double thymidine (DT) block. We then monitored the duration taken from the S to M phase in HeLa cells expressing H2B/tubulin using time-lapse microscopy after release from the DT block. Our observations revealed a notable delay in the initiation of mitosis in HPIP diminished cells compared to cells treated with control shRNA. (shCtrl vs shHPIP 12.9 ± 1.9 vs 18.7 ± 2.5 h) (Figure 5F and G).

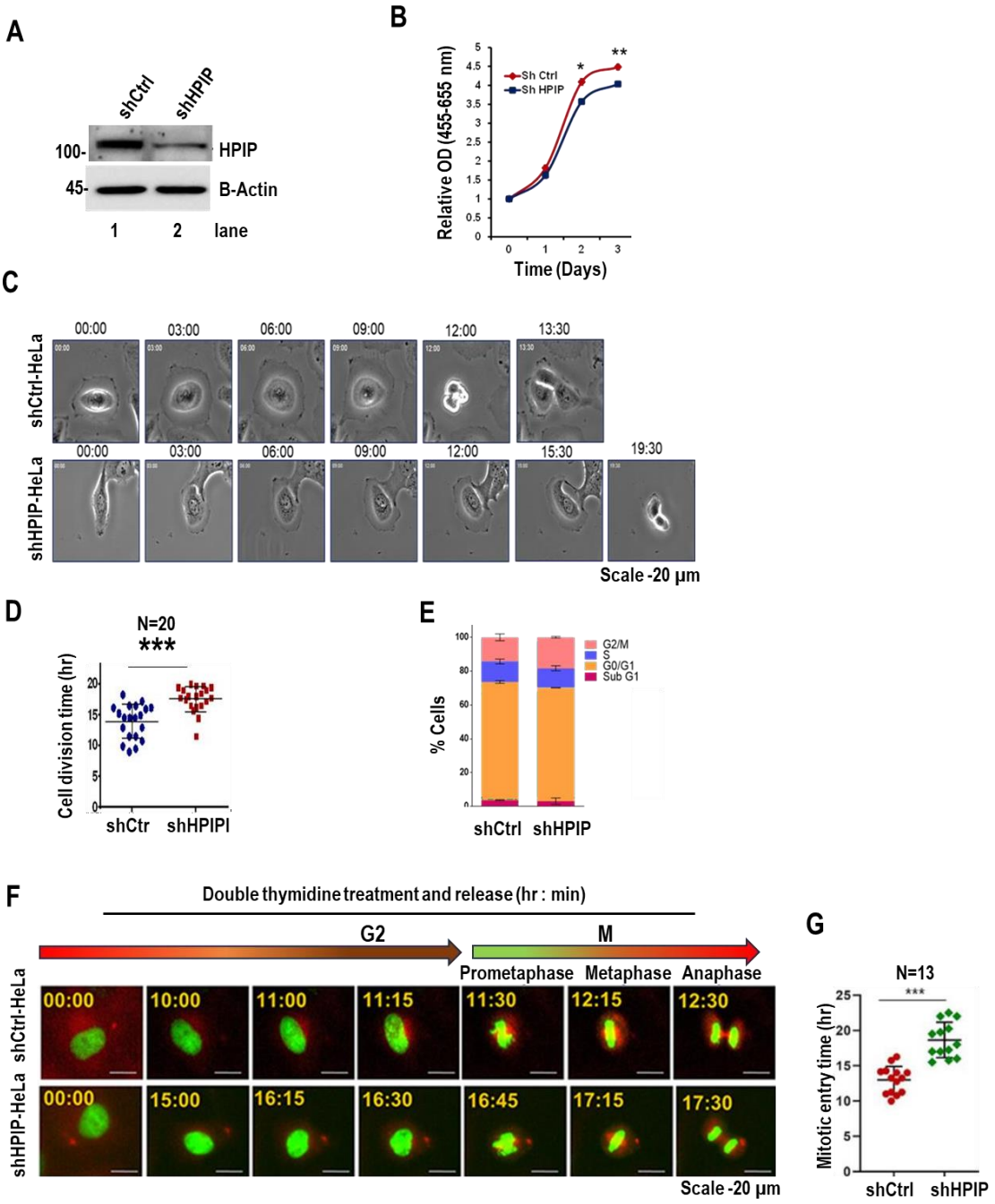

Figure 5. Loss of HPIP expression delays cell division. (A) Western blotting was used to examine HPIP knockdown in HeLa cells using HPIP-specific shRNAs. **(B)** WST-1 assay measured cell proliferation in HeLa cells following HPIP knockdown. **(C)** Representative time-lapse live cell photos of HPIP-depleted HeLa cells (magnification, 20x). Numbers above the images represents time in hours. **(D)** Quantification data from panel C, a total of 20 cells (n = 20) were examined. **(E)** Flow cytometry

(FACS) study of HeLa cells at various stages of the cell cycle (%) after HPIP knockdown. (**F**) Representative time-lapse live cell fluorescent images of either siCtrl or siHPIP-treated HeLa–H2B/tubulin cells that are synchronized by DT block at the S phase followed by release into fresh medium and captured at indicated time points. *Green*, H2B–EGFP; *red*, α-Tubulin–mCherry (magnification, 20×). *EGFP*, enhanced green fluorescence protein. (**G)** Quantification data of *F*. A total of 13 cells were analyzed for each sample (n = 13) Using the Student's t-test, the quantified outcomes are shown as means ± S.D. *, $p< 0.01$; **, $p< 0.001$; ***, $p< 0.0001$ were considered significant. Ctrl stands for control; sh stands for short hairpin; and Rel. OD stands for relative optical density.

3.2. HPIP protein levels fluctuate throughout the cell cycle

To determine the fundamental mechanisms of HPIP regulated cell division, we first studied HPIP expression patterns at various phases of the cell cycle. Using double thymidine approach, G1/S synchronized HeLa cells were allowed to release at various time intervals (Figure 6A), and levels of HPIP mRNA was evaluated using quantitative real-time polymerase chain reaction (qRT-PCR) and protein by Western blotting. FACS analysis exhibits cell populations in various phases of the cell cycle. HPIP protein levels fluctuated to varied degrees during the cell cycle, with a dramatic drop between hours 9 and 10 when mitosis occurred (Figure 6B). HPIP expression dynamics mimicked cyclin A, a ubiquitin target of APC/C-Cdc20. However, the levels of HPIP mRNA remain unaltered (Figure 6C). Flow cytometry analysis (Figure 6C bottom panel) indicated the cell cycle phase and percentage population of HeLa cells after release from the DT block. Cell progression from G2 to the M phase is stimulated by activated CDK1-cyclin B1 during the normal cell cycle, whereas its decreased activity causes G2 phase

arrest (152). In line with earlier findings, it is plausible that HPIP increases the proliferation of cells by promoting the transition from the G2/M phase.

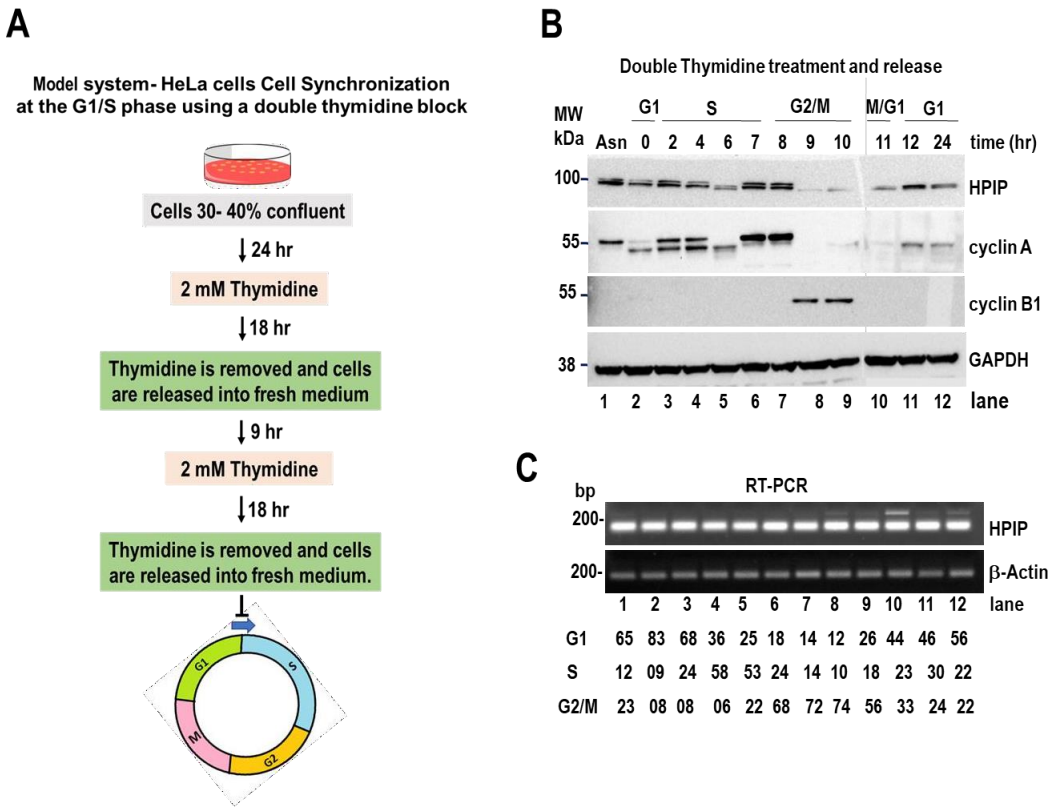

Figure 6. HPIP protein levels oscillate across the cell cycle progression and stabilize cyclin B1 in HeLa cells. **(A)** The model depicts the method for cell synchronization by double thymidine treatment**.** Cells were seeded at a density of 30-40%. Following 24 hr of seeding, 2 mM thymidine was added and incubated for 18 hr. Cells were then washed three times with PBS to remove thymidine. Cells were then cultured in fresh media for 9 hr and then treated again with thymidine for 18 hr. After thorough washing with PBS, cells were released into fresh medium and harvested at various time points for further analysis. **(B)** HeLa cells synchronized by double thymidine block were released into fresh media. Western blotting was performed to assess the various protein expressions at the respective time points. (Note: Separate gels were run for protein samples from lanes 1-9 and 10-12). **(C)** Real-time analysis (upper panel) was used to observe the change in HPIP gene expression. Flow cytometry data (lower

panel) represents the percentage of cell's population various stages of the cell cycle at respective time points.

3.3. HPIP stabilizes cyclin B1 and blocks APC/C-Cdc20-mediated proteolysis

Next, we investigated the mechanistic pathway by which HPIP increases the cell cycle's G2/M phase. HPIP silencing led to a significant decrease in cyclin B1 expression when compared to control HeLa cells (Figure 7A, *lanes 1* and *2*), however, it recovered after MG132 treatment, indicating that cyclin B1 may be degraded by proteasome pathway (Figure 7A, *lane 3*). The APC/C-Cdc20 complex degrades cyclin B1 during metaphase (153). Elevated expression of T7-tagged HPIP resulted in an increase in cyclin B1 levels in HEK293T cells. (Figure 7B, *lane 2*). However, the co-expression of HA-Cdc20, a co- activator of the APC/C complex, significantly decreased it, suggesting the idea that HPIP is involved in inhibiting Cdc20-mediated cyclin B1 degradation (Figure 7B, *lane 3*). To supplement these findings, a chase experiment was performed in synchronized HeLa cells utilizing cycloheximide, an inhibitor of *de novo* protein synthesis. HPIP silencing dramatically reduced cyclin B1 stability ($t_{1/2}$ = 41±10 min) compared to control cells ($t_{1/2}$ = 72±15 min) (Figure 7, C and D). We anticipated that an increase in cyclin B1 after ectopic expression of HPIP could be attributable to reduced cyclin B1 degradation via APC/C-Cdc20. To ascertain this, we ectopically expressed either T7-HPIP or control along with GFP-cyclin B1 and HA- Cdc20 into HEK293T cells, then after we assessed cyclin B1 proteasomal degradation. As previously reported, cyclin B1 proteolyzed upon ectopic expression of HA-Cdc20 (154) (Figure 7E, lane 2), while T7-HPIP co-expression significantly diminished it (Figure 7E, lane 3).

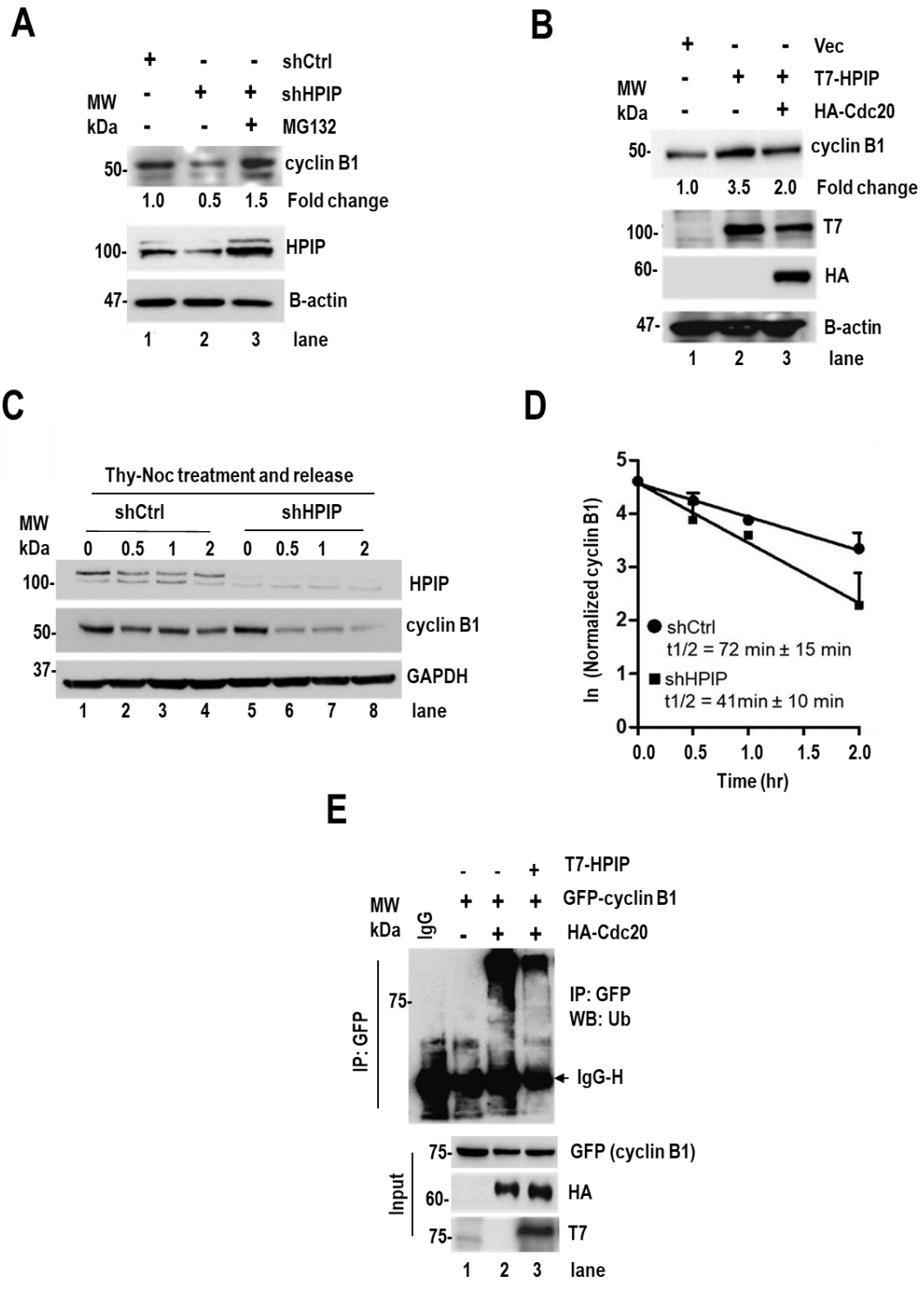

Figure 7. HPIP diminishes cyclin B1 degradation. (A) Control shRNA or HPIP shRNA transfected in HeLa cells and cell lysates were blotted as indicated after 8 hr of MG132 treatment. **(B**) HeLa cells were transfected with empty vector, T7-HPIP, or HA-Cdc20, and cell lysates were analyzed as noted. **(C)** HeLa cells harboring shRNAs were synchronized using thymidine-nocodazole block and treated

with cycloheximide (20 μg/ml) at the specified time points. The cell lysates were then examined using Western blotting. **(D)** The line graph depicts the quantification of the cyclin B1 protein bands from panel C. **(E)** HEK293T cells were ectopically expressed GFP-cyclin B1 or HA-Cdc20, along with or without T7-HPIP. Cell lysates were immunoprecipitated with T7 and blotted as indicated 48 hr after transfection.

3.4. HPIP is a substrate of APC/C-Cdc20 but not APC/C-Cdh1

Data from Figure 6 indicated that HPIP expression dynamics mimics cyclin A and is subjected to APC/C-Cdc20-regulated proteolysis. We hypothesized whether HPIP is also targeted as a substrate by APC/C-Cdc20 during cell cycle progression like cyclin A. Given that, we looked at if HPIP is destroyed by a ubiquitin based proteasomal degradation by APC/C mechanism. During cell cycle progression, the APC/C complex utilizes Cdh1 or Cdc20 as co-activators to proteolyze its substrates (155). Together, we ectopically expressed T7-HPIP with either HA-Cdh1 or HA-Cdc20 to establish which co-activator most likely participated in HPIP protein breakdown during mitosis. HPIP levels were assessed using Western blotting. T7-HPIP protein levels were reduced in HA-Cdc20 co-transfected cells (Figure 8A, *lane 5*) but restored by MG132 treatment (Figure 8A, *lane 6*). T7-HPIP levels, however, did not change when HA-Cdh1 was overexpressed (Figure 8A, *lane 3*). We then examined the effect of increasing the concentration of Cdh1 or Cdc20 expression on HPIP in HEK293T cells. As shown in Figure 8B, a dose-dependent rise in HA-Cdc20 resulted in a progressive decrease in T7-HPIP but not in HA-Cdh1 (Figure 8C). This result indicates that APC/C-Cdc20 may be involved in HPIP proteolysis by proteasome pathway.

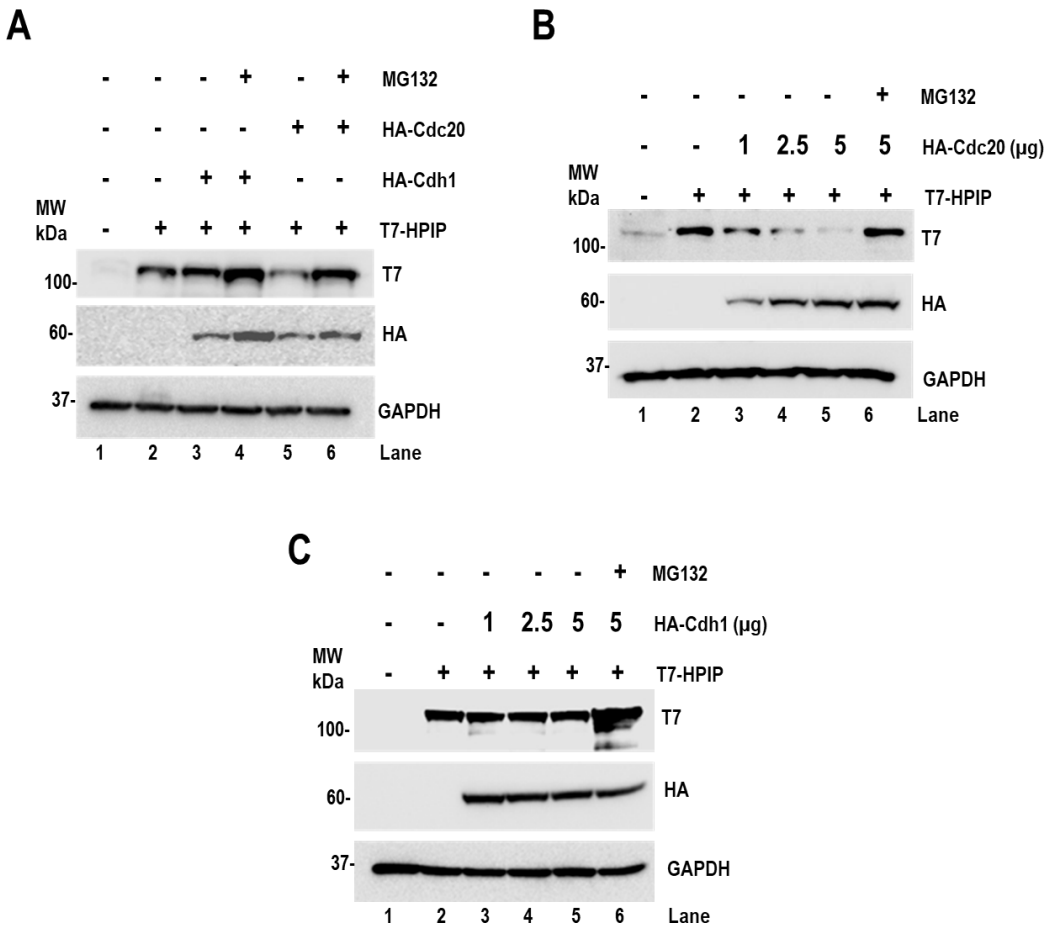

Figure 8. Cdc20 but not Cdh1 mediates HPIP degradation. (A) HEK293T cells were co-transfected with T7-HPIP and either HA-Cdc20 or HA-Cdh1 plasmid constructs. Following MG132 (10 µM) treatment, cell lysates were blotted as indicated. (**B and C)** T7-HPIP with escalating concentrations of either HA-Cdc20 (B) or HA-Cdh1 (C) constructs (1-5 µg) were co-transfected into HEK293T cells and blotted as indicated.

3.5. APC/C-Cdc20 interacts and mediates HPIP ubiquitination *in vitro*

We predicted that HPIP protein breakdown is dependent on its association with Cdc20. HA-Cdc20 and T7-HPIP were co-transfected into HEK293T cells. Afterwards 36 hr of post transfection protein lysate were subjected to co-immunoprecipitation (Co-IP) using T7 antibody. The data revealed that T7-HPIP was precipitated with HA-Cdc20 (Figure 9A) but not in control IgG-treated cells. Following this, we performed Co-IP using protein extracts from

HEK293T cells ectopically expressing T7-HPIP, HA-Ub, or GFP-Cdc20 to assess proteasomal breakdown of HPIP via APC/C-Cdc20 (Figure 9B). As shown in Figure 9B, HPIP was readily subjected to ubiquitination in Cdc20 transfected cells but not in control cells (Figure 9B, *lane 3*), indicating that Cdc20 mediates HPIP ubiquitination.

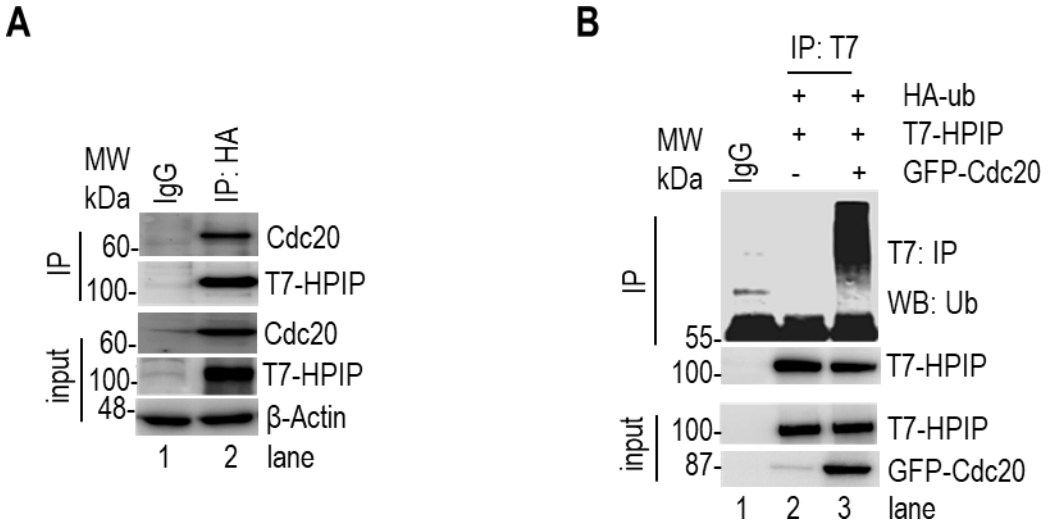

Figure 9. Cdc20 interacts and mediates HPIP ubiquitination. (A) T7-vector or T7-HPIP (T7 tag) and HA-Cdc20 plasmid constructs were co-transfected into HEK293T cells. Cell lysates were immunoprecipitated with HPIP antibody 48 hr after transfection and blotted as mentioned. **(C)** T7-HPIP was transfected into HEK293T cells with or without GFP-Cdc20 and HA-Ub constructs. T7-immunoprecipitated cell lysates were blotted with the indicated antibodies after 48 hr of transfection.

3.6. Cdc20-mediated HPIP degradation is cell cycle-specific

APC/C-Cdc20 activity start from mitosis until the onset of anaphase, whereas APC/C-Cdh1 activated from the beginning of anaphase until beginning of S phase. Cdc20 becomes active in early mitosis and interacts with phosphorylated APC/C become active, leading to the breakdown of prometaphase substrates such as NeK2 and cyclin A (97, 156). Securin and cyclin B1 are degraded by APC/C-Cdc20 to facilitate the metaphase-anaphase transition of the cell immediately after all chromosomes attain appropriate microtubule biorientation in metaphase.

Cyclin B degradation prevents CDK1 activity during anaphase and permits APC/C-Cdh1 activation, which kills the remaining mitotic regulators, including Cdc20 (157). Given their phase-specific roles during the cell cycle, we next examined whether HPIP degradation by Cdc20 is cell cycle-dependent or not. To address this, we knocked down Cdc20/Cdh1 in HeLa cells. Cdc20 knockdown rise HPIP protein in HeLa cells considerably (Figure 10A, *lane 2*), while Cdh1 knockdown had no effect on it (Figure 10A, *lane 3*). To assess if HPIP degradation by Cdc20 is cell cycle specific, HeLa cells were synchronized and released into various time points and performed Western blotting. We could get a 50% knockdown of Cdc20 in HeLa cells as its complete knockdown was lethal, and cells got arrested in mitosis. HPIP levels were decreased when cells entered mitosis (~ 8^{th} hr) in control cells. Whereas in Cdc20 knockdown cells, HPIP levels were elevated at the same time point and further it maintained until the 12^{th} hr. This data supports that the loss of HPIP in early mitosis was due to Cdc20-mediated degradation.

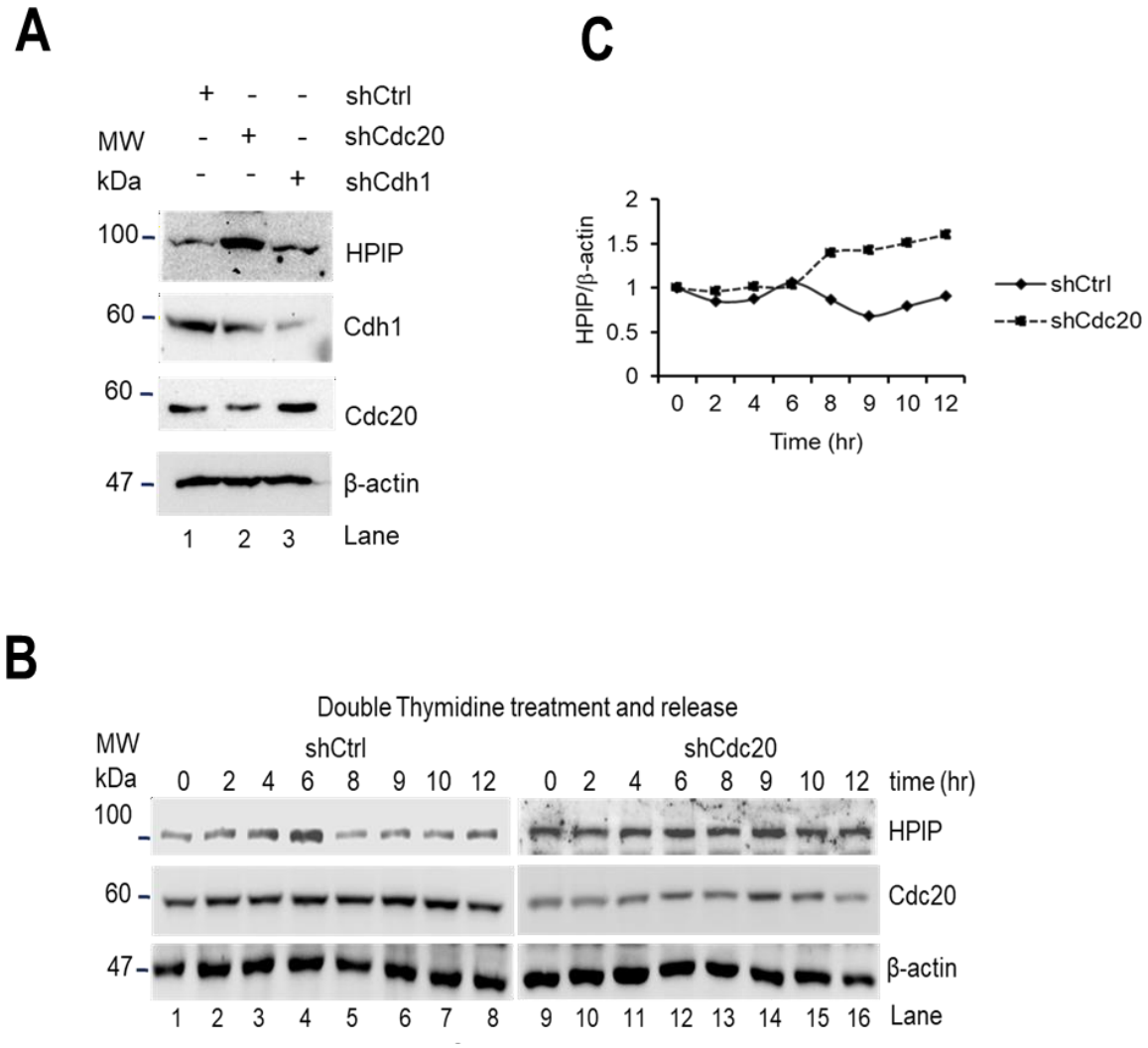

Figure 10. Cdc20-mediated degradation of HPIP is cell cycle-specific. (A) Control shRNA, Cdc20 shRNA, or Cdh1shRNA were transfected into HeLa cells, and cell lysates were examined by Western blotting as indicated. **(B)** Cdc20 shRNA-treated cells were synchronized, released, and blotted at the specified time points (top panel). **(C)** The line graph shows the quantification of HPIP protein band intensity from panel B. β-Actin is used as internal loading control; sh stands for short hairpin; and MW stands for molecular weight.

Together, the results from Objective 1 demonstrated that HPIP is needed for proper cell division, and it is specifically required for G2/M transition. Mechanistic studies further revealed that HPIP expression fluctuates during cell cycle progression, being low in mitosis.

HPIP antagonizes APC/C-Cdc20- mediated degradation of cyclin B1. Hence, HPIP possibly promotes G2/M transition and cell division by controlling cyclin B1 expression.

Objective 2) To study the functional significance of HPIP phosphorylation in cell cycle regulation

3.7. HPIP localizes to the mitotic spindle during mitosis

Proper segregation of chromosome is critical for forming two cells with identical genetic information. The spindle is a non-permanent structure, it is formed during cell division, serve to pull apart metaphase chromosome towards opposite pole of the cell, and eventually disintegrates after mitosis. Biochemical isolation of spindles from cultured mammalian cells opens novel possibilities for the biochemical investigation of spindle components. The latest proteomic investigations have been initiated to study the makeup of the mammalian cell centrosome (158), the hamster spindle midbody (159), and *in vitro*-assembled spindle structures (160, 161). Double thymidine synchronised HeLa cells were followed treatment with nocodazole to produce mitogenic cells. Mitotic shake-off cells were collected, and the microtubules were stabilized using Taxol, followed by Taxol treatment, cells were resuspended in an isolation buffer for 5-10 min, and then spindles were collected by sedimentation at 1500x g (figure 11A). Co-immunoprecipitation was carried out using spindle isolate by incubating with HPIP antibody followed by SDS-PAGE to separate the proteins that could potentially interact with HPIP from the spindle isolate. Trypsin digested protein mixtures were subjected to nano-LC-MS/MS analysis. A small portion (5%) of the spindle isolate after Co-IP was used to analyze HPIP by Western blotting. We observed a significant amount of HPIP associated with the spindle, suggesting HPIP is a component of the mitotic spindle (Fig 11B, *lane 2*). Proteome analysis revealed the presence of several proteins that are associated with HPIP,

including cyclin B1, tubulin, etc. (150) (Figure 11C). Immunofluorescence analysis further supported the co-localization of HPIP with β-tubulin in HeLa cells (Figure 11D).

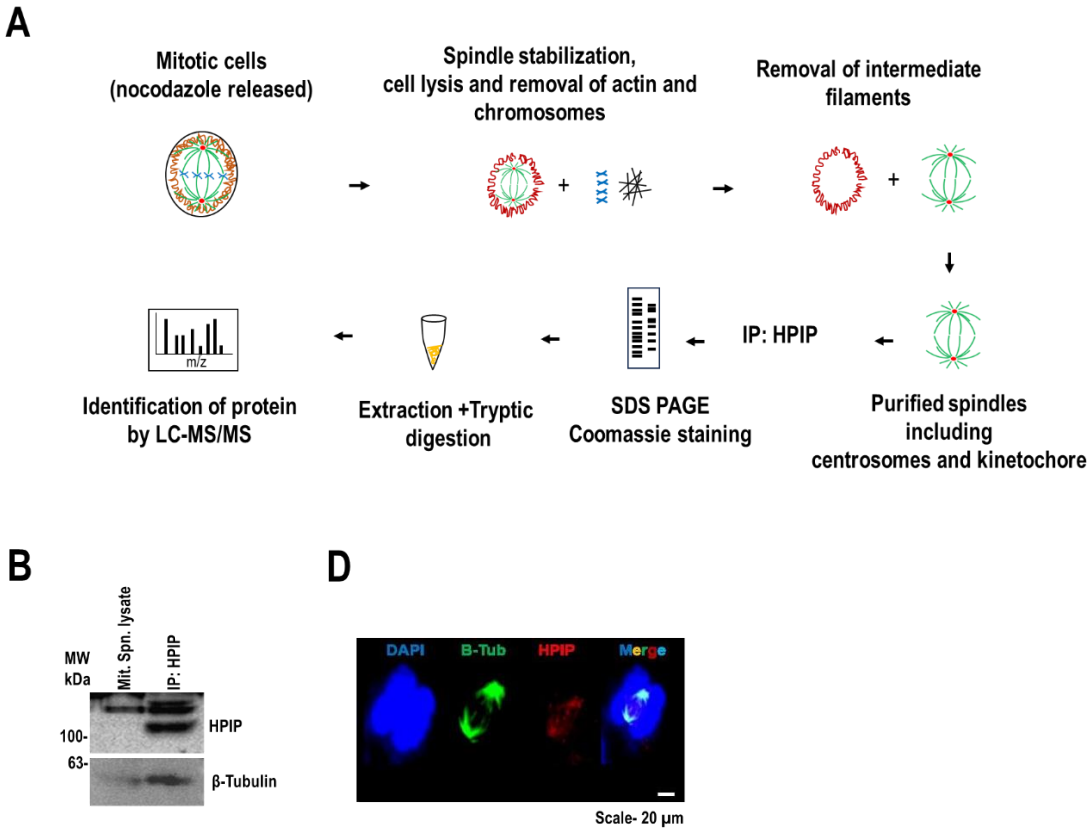

Figure 11. HPIP is identified as a spindle-associated protein. (A) Schematic representation of mitotic spindle isolation**. (B)** Western blot analysis of spindle-isolated lysates was pulled with HPIP. **(C)** Nano-LC/MS analysis revealed cyclin B1 as one of the HPIP-interacting protein from mitotic spindle complex**. (D)** Representative fluorescence images showing HPIP (red), spindle pole protein β-tubulin (green), and DNA are shown by DAPI. Scale bar; 20 µm.

3.8. CDK1- cyclin B1 interacts and phosphorylates HPIP at serine 43

It is an overwhelming fact that the regulation of the eukaryotic cell cycle is governed by cyclin-dependent kinases (CDKs) (162). CDK1-cyclin B is critical for G2/M transition and mitosis as

CDK1 kinase activity rises during G2/M transition, during which several key regulators of mitosis undergo phosphorylation for fine-tuning their activation/inactivation (163). Previously, we have demonstrated that HPIP expression oscillates across various cell cycle phases, which could be due to its post-translational regulation (143). To gain further molecular insights into HPIP regulation of mitosis, we prepared protein extracts from HeLa cells, and HPIP interacting proteins were pulled down using HPIP-specific antisera, and samples were analyzed by nano-LC/MS analysis. Of several interacting partners of cell cycle, cyclin B1 attracted our attention to pursue further for the following reasons: 1) HPIP regulates cyclin B1 levels during mitosis (143), 2) CDK1 and cyclin B1 are critical regulators of mitosis, 3) HPIP possesses CDK1 conserved phosphorylation site, S/T*-P-x-K/R at serine 43 position (Figure 12A), which was earlier detected by a global mitotic phosphorylation screening approach (88, 162) (Figure 12B). Given these points, we hypothesized that HPIP could be phosphorylated by CDK1-cyclin B1 complex and argued whether HPIP phosphorylation has functional relevance during cell cycle progression. To ascertain this, first, we performed a Co-IP analysis to confirm their interaction in HeLa cells. The data revealed a moderately weak interaction of HPIP with cyclin B1 and CDK1 in HeLa cells (Figure 12C and D). Since CDK1-cyclin B1 plays an active role during G2/M and mitosis, we synchronized the cells in mitosis using nocodazole and then released to progress them to next phases of mitosis and then analyzed their association by confocal imaging. The data revealed a marked co-localization of HPIP with CDK1 and cyclin B1 in the mitotic spindles during mitosis (Fig 12E-F).

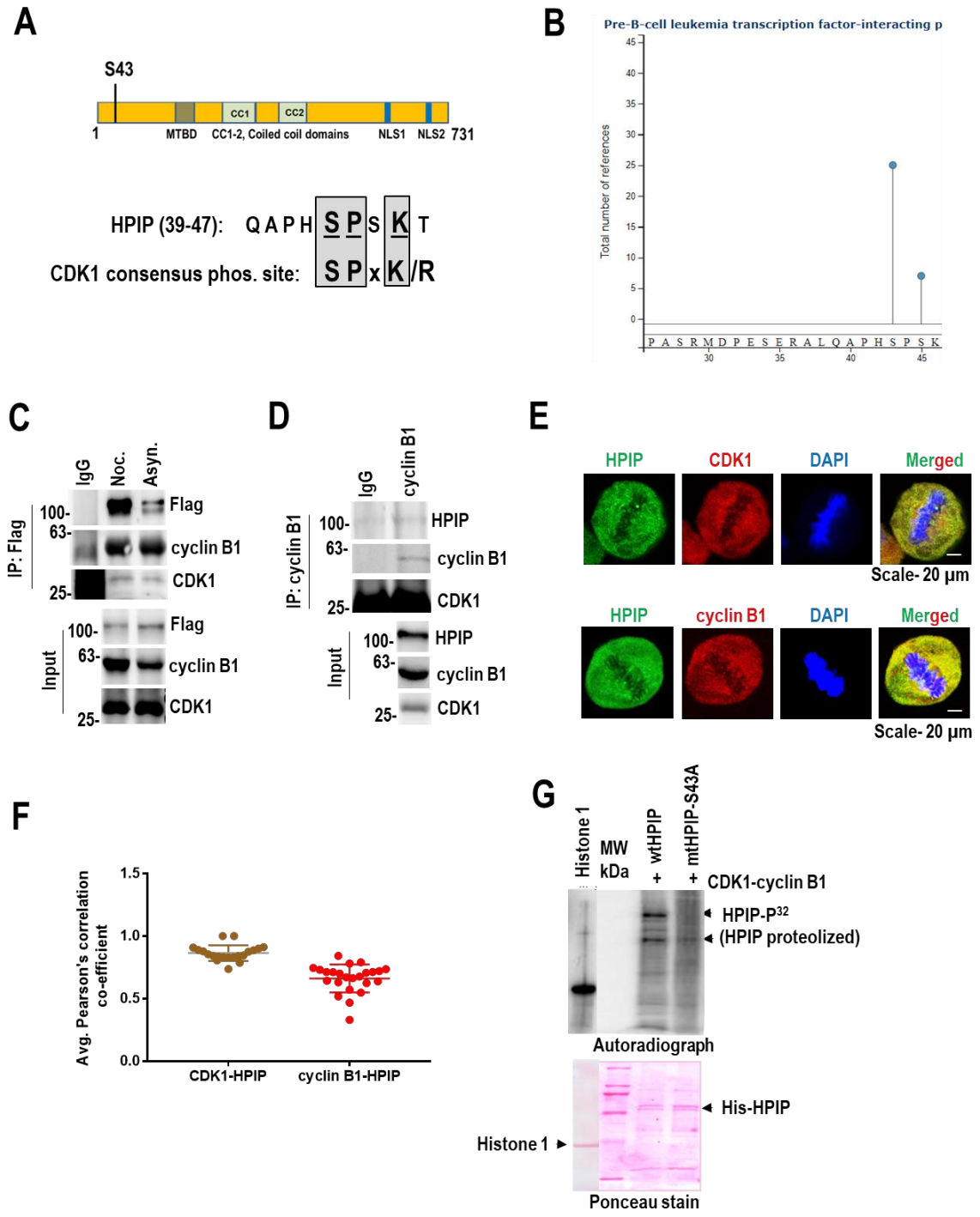

Figure 12. CDK1 interacts and phosphorylates HPIP at serine 43 (A) Schematic representation of HPIP protein domains showing amino acid sequence of CDK1 consensus phosphorylation sequence and CDK1 phosphorylation site in HPIP at serine 43, MTBD (microtubule binding domain) (brown) 190 aa -218 aa, CC (Coil-coiled domains) CC1 270 aa -341 aa, CC2, 370 aa -415 aa, NLS (nuclear localization sequence) NLS1 485 aa -505 aa, NLS2 695 aa -720 aa. **(B)** Global mitotic phosphorylation screening data showing phosphorylation signals at serine 43 (164-166). **(C**

and D) Immunoprecipitation (IP) with Flag beads or cyclin B1 antibody followed by western blotting with CDK1, cyclin B1 or Flag antibodies were utilised to confirm the interaction of HPIP with CDK1 or Cyclin B1. (**E)** Immunofluorescent images representing the colocalization of HPIP (Alexa Flour 488, green) with CDK1(top) or cyclin B1 (bottom) (Alexa Flour 546, red) in HeLa cells arrested with nocodazole. DAPI (blue) was used as the nuclear stain. Scale bar, 20 μm. **(F)** Quantification of colocalization data using Pearson's correlation coefficient from panel E. (**G**) Top: CDK1 in-vitro kinase assay. Histone 1 used as a positive control. Protein substrates are indicated at the bottom by Ponceau's staining. Molecular weight in kilodaltons (kDa). Arrowheads point to recombinant proteins His-HPIP, and His-mtHPIP-S43A at the appropriate molecular mass.

Since CDK1 is a kinase that regulates the cell cycle by phosphorylating its partners, we checked if CDK1 phosphorylates HPIP or not. To check this, we mutated serine at 43 to alanine (non-phosphorylatable) (HPIP-S43A) or to glutamate (phospho-mimetic), which mimics phosphorylation status by imparting a negative charge to the protein at this site (HPIP-S43E). We expressed wtHPIP or phospho-mutant of HPIP in *E. coli* (Rosetta-DE3) as His-tagged proteins. The purified proteins were subjected to CDK1 kinase assay using (γ^{32}P)-labelled ATP and purified CDK1-cyclin B1 co-immunoprecipitated mixture from HeLa cells. The samples were subjected to SDS-PAGE, transferred to an NC membrane, and stained using Ponceau's solution (Figure 12G bottom panel). The degree of phosphorylation was measured by autoradiography. The data revealed that wtHPIP is being readily phosphorylated by CDK1-cyclin B1 complex but not mutant HPIP-S43A, suggesting wtHPIP is indeed a phosphorylation target of CDK1 at serine 43 (Figure 12G top panel). Together, the data indicates that the CDK1-cyclin B1 complex could interact with and phosphorylate HPIP at serine 43.

3.9. Phosphorylation of HPIP at serine 43 is required for proper cell division and G2/M transition

To ascertain if HPIP phosphorylation at serine 43 by CDK1 is necessary for cell division, we generated stable cells expressing Flag-tagged HPIP, mtHPIP-S43A or mtHPIP-S43E in Hela cells (Figure 13A). GFP is also co-expressed as a separate tracker in these clones. Next, cell growth of HeLa cells expressing Flag-tagged wtHPIP, mtHPIP-S43A, or mtHPIP-S43E was analyzed by colony formation assay (Figure 13B). While wtHPIP and mtHPIP-S43E show a significant increase in colony formation, inhibition of wtHPIP phosphorylation at serine 43 abrogated colony-forming efficiency in HeLa cells (Figure. 13B-C). Whether the differences in their colony-forming efficiency were due to altered cell division dynamics, we next measured cell division time by live cell imaging with a fluorescence microscope. The data revealed that wtHPIP cells took ~ 3 hr shorter for cell division than control cells (control vs wtHPIP: 20.1 ± 0.31 hr vs. 17.36 ± 0.69 hr). Interestingly, non-phosphorylatable mtHPIP-S43A cells exhibited approximately 3.54 hr delay in cell division as compared to wtHPIP cells (Figure. 13D-E) (mtHPIP-S43A vs. HPIP: 20.9 ± 0.41 hr vs. 17.36 ± 0.69 hr). Whereas mtHPIP-S43E cells divided approximately 5.4 hr faster than mtHPIP-S43A HeLa cells (mtHPIP-S43E vs mtHPIP-S43A: 15.5 ± 0.64 h vs 20.9 ± 0.41 h, Figure. 13D-E).

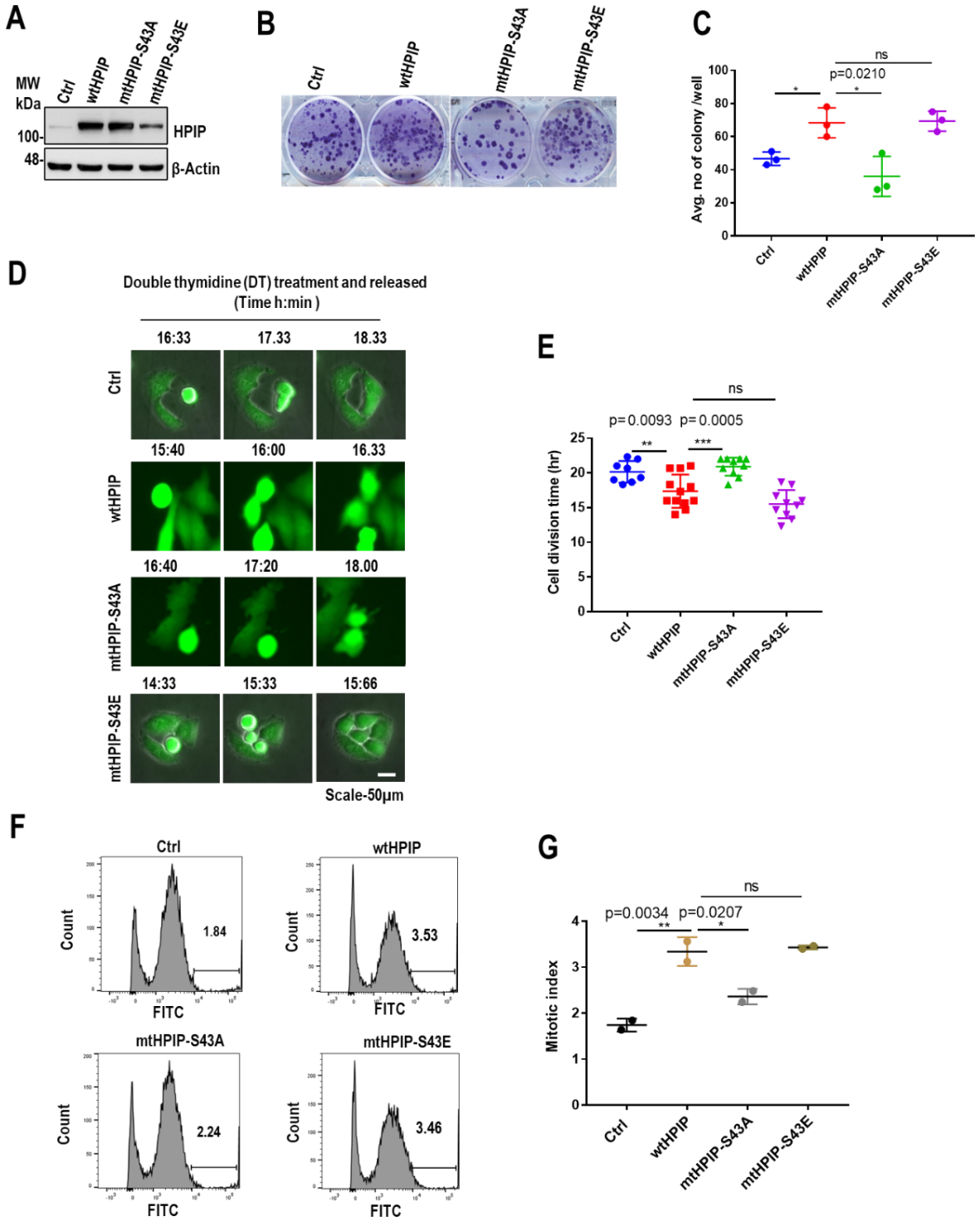

Figure 13. Loss of phosphorylation of HPIP at serine 43 affects proper cell division and G2/M transition- (A) Stable expression of wtHPIP, mtHPIP-S43A, or mtHPIP-S43E in HeLa cells. **(B)** Representative crystal violet-stained image of clonogenic assay exhibits colony formation efficiency of wtHPIP, mtHPIP-S43A, or mtHPIP-S43E compared to control. **(C)** Data quantification from panel B.

(D) Representative time-lapse fluorescent images of either control, wtHPIP, mtHPIP-S43A, or mtHPIP-S43E HeLa cells that are synchronized with double thymidine (DT) at G1/S phase, released into fresh medium and imaged at indicated time points. **(E)** Data quantification from panel D, presented as mean ± SD., a total number of 8 to 10 cells analyzed for each set of samples (magnification 20x). **(F)** Representative flow cytometry histogram showing phospho-Histone3 ser10 staining. **(G)** Data from the F panel is quantified and represented as dot plot. Statistical significance was determined using the **s**tudent t- test is shown as means ± S.D. *p < 0.05; **p < 0.01; ***p < 0.001; was regarded as significant. Ctrl, control, ns, non-significant.

Since HPIP regulates G2/M transition and loss of its expression affects mitotic index, next employed FACS-based phospho-Histone3 ser10 staining to evaluate mitotic index. The data revealed that wtHPIP and mtHPIP-S43E displayed higher mitotic index than control as well as mtHPIP-S43A (HPIP vs. mtHPIP-S43A: 3.34 ± 0.311 vs. 2.36 ± 0.169%) (Figure 13F-G). Together, the data indicates that the loss of HPIP phosphorylation at serine 43 affects proper cell division and G2/M transition.

3.10. Phosphorylation of HPIP at serine 43 is required for timely entry and exit from mitosis

We further examined the role of HPIP phosphorylation in the entry of mitosis in HeLa cells. We performed time-lapse microscopy using ectopic expression of mCherry-H2B, which was used as a DNA condensation marker during mitotic entry. HeLa cells expressing empty vector, wtHPIP, mtHPIP-S43A, or mtHPIP-S43E, were synchronized in the G1/S boundary using double thymidine procedure and then evaluated the duration between the S to M phase. We observed mtHPIP-S43A HeLa cells showed an average of 1.64 hr delayed S/M transition as compared to wtHPIP (Figure 14A-B; wtHPIP vs mtHPIP-S43A; 16.83 ± 0.44 hr vs 18.46 ± 0.45 hr). Whereas mtHPIP-S43E cells showed significantly quicker S/M transition compared to wtHPIP (Figure 14A-B, wtHPIP vs mtHPIP-S43E; 16.83 ± 0.44 vs 15 ± 0.33 hr).

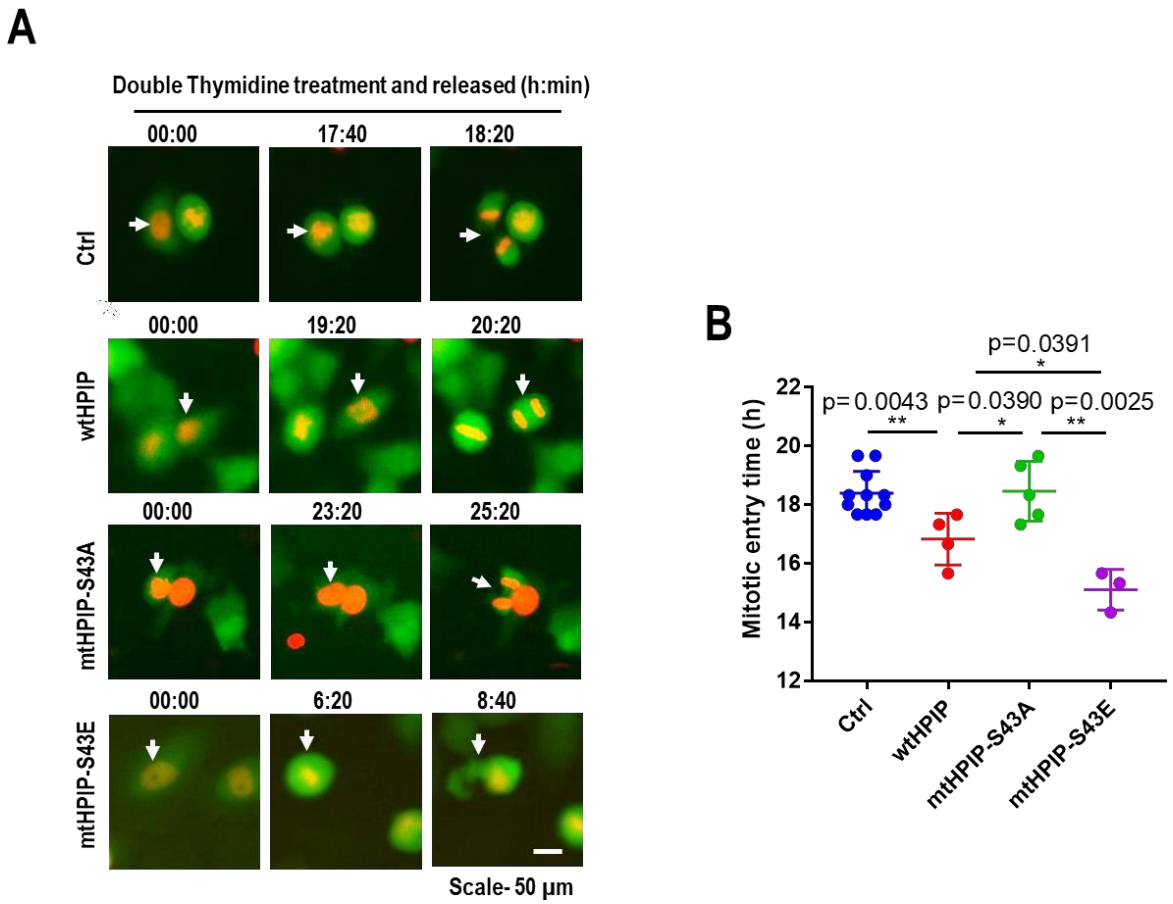

Figure 14. Phosphorylation at serine 43 is required for the timely entry of mitosis. (A) Representative time-lapse live cell fluorescence images of DT synchronized stably expressing control, wtHPIP, mtHPIP-S43A, or mtHPIP-S43E transfected with H2B-mCherry (red) HeLa cells were captured at the specified time points (magnification, 20x). **(B)** Time for mitotic entry is represented as mean ± SD from panel A. Results are displayed as scatter plot mean ± SD., unpaired Student's t-test was used to determine statistical significance *$p < 0.05$; **$p < 0.01$; were regarded as significant. Ctrl, control, ns, non-significant.

3.11. Phosphorylation at serine 43 is required for timely exit from mitosis

Next, we evaluated the mitotic exit or duration of mitosis taken by these mutants along with control and wtHPIP HeLa cell clones were evaluated. About 12 hr nocodazole treated control vector, wtHPIP, mtHPIP-S43A, or mtHPIP-S43E HeLa cells were liberated into the fresh DMEM until the cells divided into two daughter cells to measure the mitotic duration (time

taken to exit) employing time-lapse imaging. We noticed the control cells exited the mitosis in approximately 25 min. Interestingly, mtHPIP-S43A took longer duration for mitotic exit than wtHPIP or HPIP-S43E. (Figure 15A-B; wtHPIP vs mtHPIP-S43A; 17 ± 1.2 vs 33.75 ± 1.2 min; wtHPIP vs mtPIP-S43E; 17 ± 1.2 vs 20.5 ± 1.6 min).

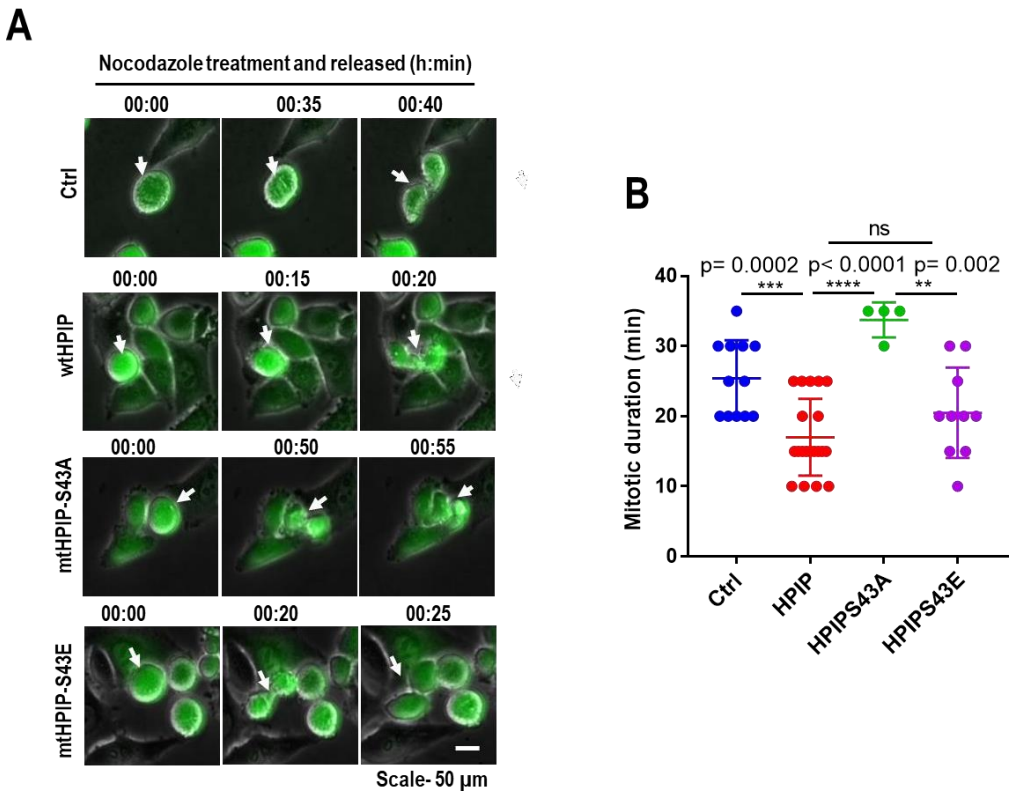

Figure 15. Phosphorylation of HPIP at serine 43 is required for timely exit from mitosis. (A) Representative time-lapse fluorescence images of nocodazole arrested and released for 12 hr, Ctrl, HeLa wtHPIP, mtHPIP-S43A, or mtHPIP-S43E cells were captured at the specified time points (magnification, 20x). **(B)** Elapsed time from prometaphase to cytokinesis onset from panel A. The results are presented as scatter plot mean ± SD, to evaluate statistical significance, the unpaired Student's t-test was used **, p< 0.01; ***, p= 0.001; ****, p< 0.0001 were regarded as significant. Ctrl, control, ns, non-significant.

3.12. Phosphorylation of HPIP at serine 43 influences cyclin B1 expression dynamics and G2/M progression

The CDK1-cyclin B1 activity is crucial for both the entry and exit of mitosis (167). Aligning these studies, we recently demonstrated that HPIP protein levels are elevated during the G2/M transition required for cyclin B1 stabilization, which is implicated in mitosis regulation (143) (Figure 6A). We reasoned that inhibition of HPIP phosphorylation may affect cyclin B1 expression dynamics across the cell cycle and mitosis. To ascertain this, we utilized synchronized Ctrl, wtHPIP, mtHPIP-S43A, or mtHPIP-S43E clones by double thymidine at G1/S boundary, harvested at specified time points, and Western blotting was used to analyze protein levels of HPIP, cyclin B1 and phospho-Histone3 ser10. The data revealed that wtHPIP protein levels gradually increased from 0 hr to 8^{th} hr and sharply declined thereafter (Figure 16A, wtHPIP panel, lanes *6 and 7*). In the case of mtHPIP-S43A cells, mtHPIP protein started accumulating from the 7^{th} hr, maintaining its levels up to the 11^{th} hr, and started declining from the 11^{th} hr onward (Figure 16A, mtHPIP-S43A panel, *lane 5, 6, 7, 8 and 9*). Meanwhile, in mtHPIP-S43E cells, mtHPIP protein did not change throughout the cell cycle phases (Figure 16A, mtHPIP S43E panel). Comparative analysis suggests that phosphorylation of HPIP at serine 43 is crucial for cyclin B1 stability to reach its threshold level of protein around 3 hr faster in wtHPIP or mtHPIP-S43E than mtHPIP-S43A cells (Figure 16 B-C, wtHPIP or mtHPIP-S43E vs. mtHPIP-S43A; 8 hr vs. 11 hr) that is required for CDK1 catalytic activity. Relatively low levels of phospho-Histone H3 ser10 phosphorylation in mtHPIP-S43A than in wtHPIP or mtHPIP-S43E clones. Altogether, this result suggests that specific regulation of cyclin B1 by wtHPIP is directly related to CDK1-cyclin B1 activation that triggers G2/M transition and its nuclear import culminating in nuclear envelop breakdown (NEBD) during prophase (168, 169). These results imply that altered cyclin B1 expression dynamics in mtHPIP cells impinge on its G2/M phase of the cell cycle.

Next, we assessed cell cycle's which phase might affected by HPIP phosphorylation at serine 43 by FACS analysis. This analysis revealed that an average proportion of cells were significantly accumulated at G2/M in mtHPIP-S43A cells (11.85%) as compared to wtHPIP (14.55%) and mtHPIP-S43E (9.435%). Control had 11.55% cells in the G2/M phase. (Figure 16D-E).

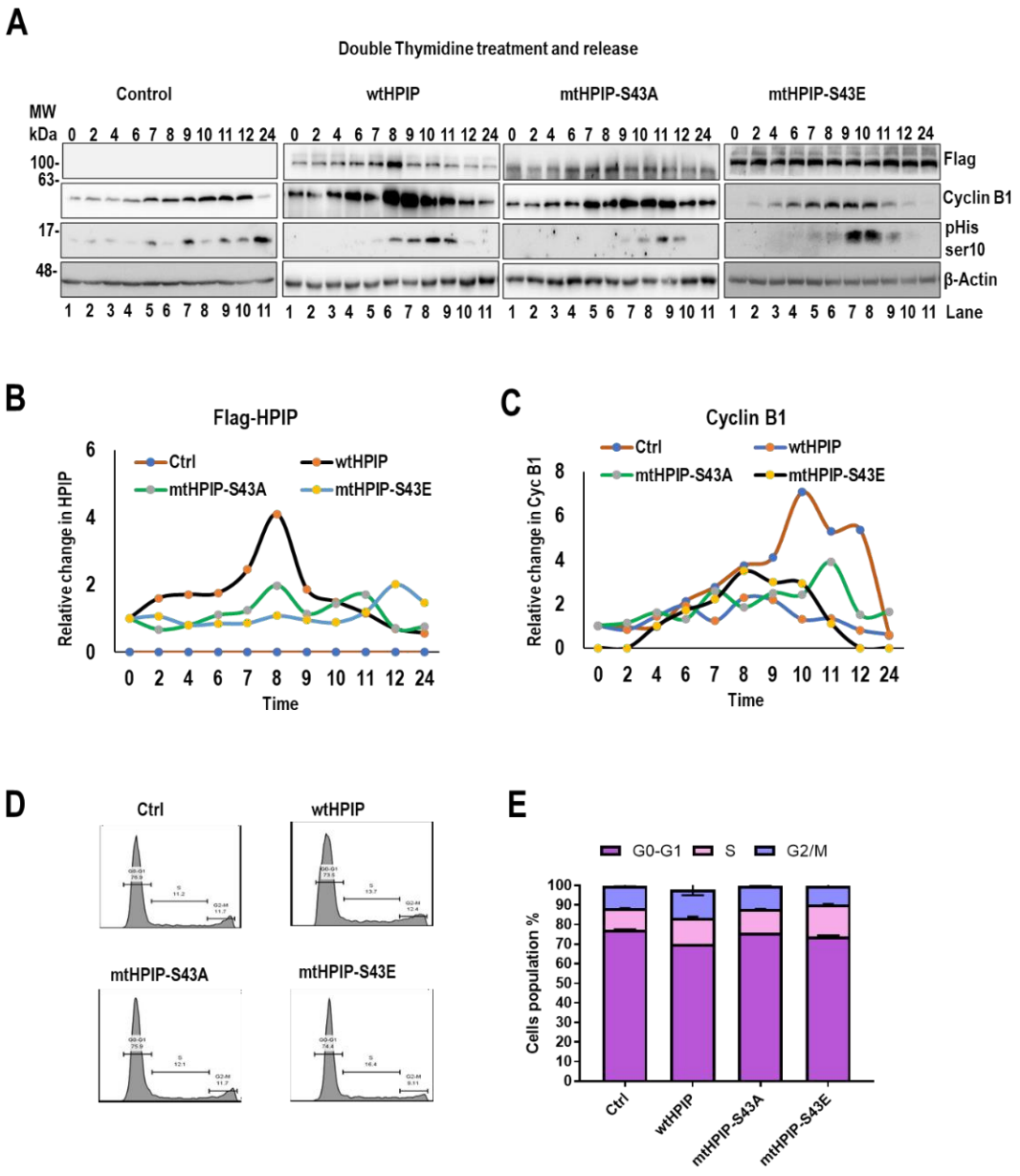

Figure 16. Loss of phosphorylation at serine 43 in HPIP affects cyclin B1 expression dynamics and G2/M transition- (A) Western blotting analysis demonstrating expression dynamics of HPIP,

mtHPIP-S43A, mtHPIP-S43E, cyclin B1 or phospho-Histone3 ser10 in HeLa cells that are subjected to double thymidine synchronization followed by release into the fresh medium at various time points. **(B-C)** Line graph showing quantification of Flag-HPIP and cyclin B1 from panel A. **(D)** FACS analysis showing the percentage of cells in different phases of the cell cycle in Ctrl, wtHPIP, mtHPIP-S43A or mtHPIP-S43E HeLa clones. **(E)** Quantification of data from panel D. Ctrl, control.

3.13. Loss of HPIP phosphorylation at serine 43 results in abnormal spindle poles and chromosomal segregation during mitosis

Chromosome segregation is facilitated by microtubules, a crucial component of the mitotic spindle (170). Prior research has shown that microtubule-binding protein HPIP (129, 131), also involve in mitotic spindle function has not been completely studied. Therefore, we analyzed spindle morphology by fluorescence microscope upon serine 43 mutation in HPIP. The data revealed that overexpression of wtHPIP in HeLa cells induced more monopolar and multipolar spindles compared to control, mtHPIP-S43A, or mtHPIP-S43E (Figure 17 A-D). Interestingly, inhibition of phosphorylation resulted in a high rate of chromosome misalignment and congression in mtHPIP-S43A cells (Figure 17 E-F) during mitosis. This result shows that HPIP phosphorylation at serine 43 is required for spindle maintenance and chromosomal segregation during mitosis.

Altogether, the compelling evidence from objective 2 implies that CDK1-cyclin B1 interacts and phosphorylates HPIP at serine 43, which is required for proper cell division. Furthermore, HPIP phosphorylation at serine 43 plays crucial role in timely mitotic entry and exit, which is attributable to the altered cell cycle dynamics in mutant clones, i.e., mtHPIP-S43A. In addition, loss of HPIP phosphorylation at serine 43 leads to chromosome misalignment and congression. Mechanistically, the loss of serine 43 phosphorylation in HPIP leads to altered cyclin B1

stability, which is attributable to the altered cell cycle dynamics in mutant clones, i.e., mtHPIP-S43A.

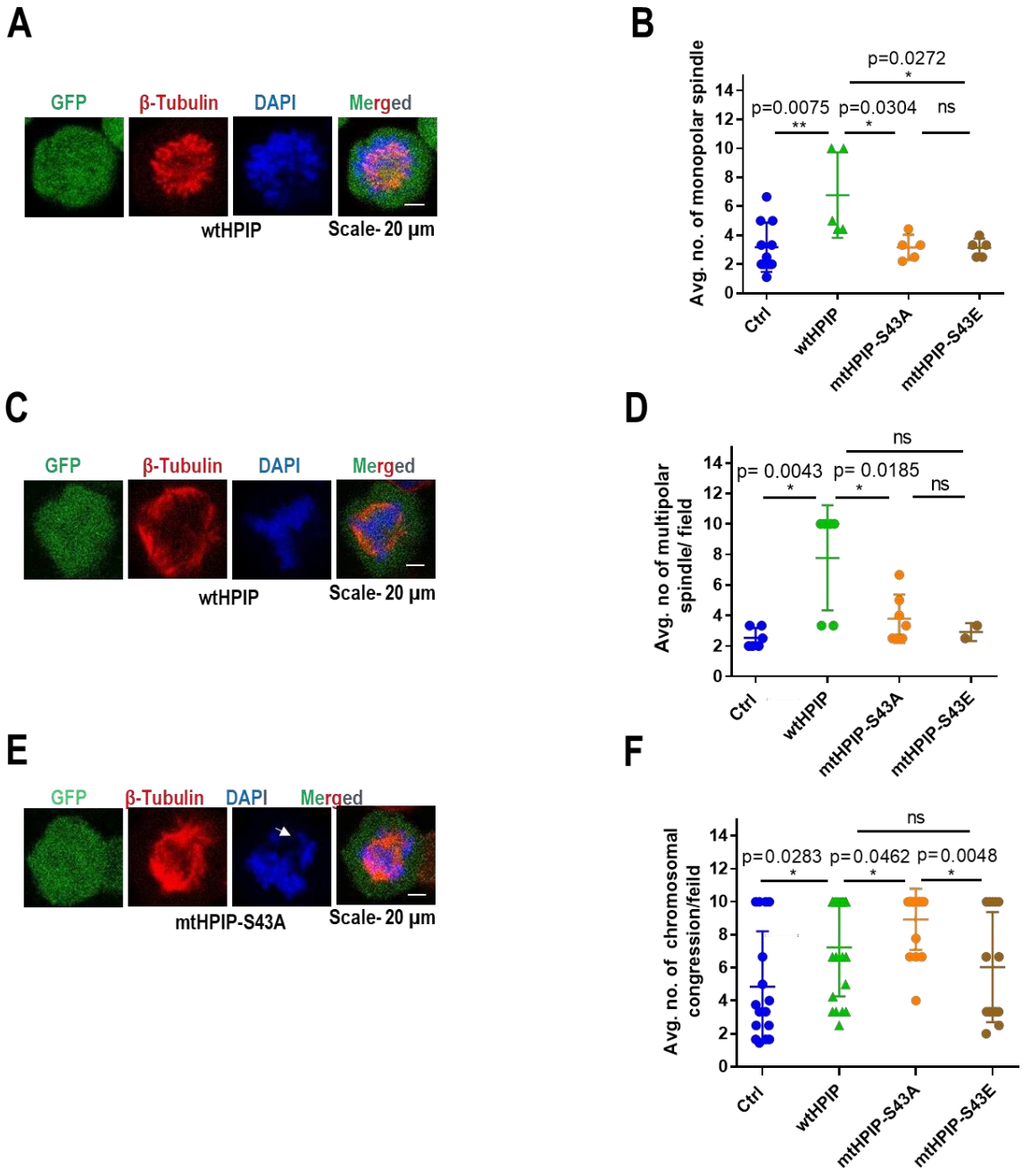

Figure 17. Loss of HPIP phosphorylation at serine 43 results in abnormal spindle poles and chromosomal segregation. (A and **C)** Representative fluorescence images of stably expressing wtHPIP, mtHPIP-S43A, or mtHPIP-S43E HeLa clones. Spindle poles were stained with β-Tubulin, and DAPI

was used for DNA staining. **(B** and **D)** Dot plot quantification from panels A and C shows an average number of monopolar and multipolar spindles. **(E)** Representative fluorescence images of mtHPIP-S43A HeLa cells. The arrow denotes a misoriented chromosome in mtHPIP-S43A clones. **(F)** Dot plot quantification from panel E. The results are presented as mean ± SD, unpaired Student's t-test was used to evaluate statistical significance. *$p < 0.05$; **$p < 0.01$; were regarded as significant. Ctrl, control, ns non-significant.

Objective 3) To assess the impact of phosphorylation on the stability of HPIP during cell cycle progression

3.14. mtHPIP-S43A is resistant to APC/C-Cdc20 mediated degradation

E3 ubiquitin ligase APC/C, comprising at least 14 core subunits, has paramount importance and involve in cell cycle regulation (171, 172). One of the two activator subunits, Cdh1 or Cdc20, regulates the APC/C functions (173, 174). Early in mitosis, Cdc20 binds with the APC/C and initiates the onset of anaphase by facilitating the destruction of a subset of mitotic cyclins and securin (175). It is well established that CDK1 phosphorylated proteins are subjected to APC/C-Cdc20 ubiquitination and destruction during early mitosis (176). Whereas in late mitosis, Cdh1 activates APC/C to ensure timely mitotic exit by targeting its substrates (177, 178). Based on the previous reports that HPIP interacts with Cdc20, we assumed if HPIP phosphorylation by CDK1-cyclin B1 primes its degradation by APC/C-Cdc20. To ascertain this assumption, we co-expressed ectopically either wtHPIP or mtHPIP-S43A and GFP-Cdc20 in HEK293T cells. On ectopic expression of HA-Cdc20, wtHPIP expression was markedly decreased, while mtHPIP-S43A was unaffected (Figure 18A). We next examined the effects of CDK1 phosphorylation on HPIP ubiquitination by APC/C-Cdc20. We ectopically expressed either Flag-tagged wtHPIP or non-phosphorylatable mutant mtHPIP-S43A and HA-Ub into HEK293T cells with or without GFP-Cdc20 expression and co-immunoprecipitation (Co-IP)

was conducted utilizing Flag beads. As anticipated, mtHPIP-S43A showed reduced Cdc20-mediated ubiquitination, while Flag-wtHPIP readily ubiquitinated in similar conditions (Figure 18B; *lane 6* vs *4*).

We next measured the half-life ($t_{1/2}$) of wtHPIP, mtHPIP-S43A, or mtHPIP-S43E in HeLa cells by cycloheximide (CHX) chasing experiment. HeLa cells expressing either wtHPIP, mtHPIP-S43A, or mtHPIP-S43E were treated with 20 μM CHX, a protein synthesis inhibitor, for the specified period, and protein expression have been assessed by Western blotting. The half-life ($t_{1/2}$) of wtHPIP was ~1.84 hr. The half-life of mtHPIP-S43A or mtHPIP-S43E was dramatically increased (Figure 18C-D; wtHPIP vs mtHPIP-S43A: 1.82 ± 0.3 hr vs 11.299 ± 3.66 hr or wtHPIP vs mtHPIP-S43E: 1.82 ± 0.3 hr vs 2.59 ± 0.5 hr) in mtHPIP-S43A or mtHPIP-S43E HeLa cells compared to wtHPIP cells after treatment with CHX. Together, this data indicates that HPIP phosphorylation at serine 43 may prime Cdc20-mediated ubiquitination and degradation.

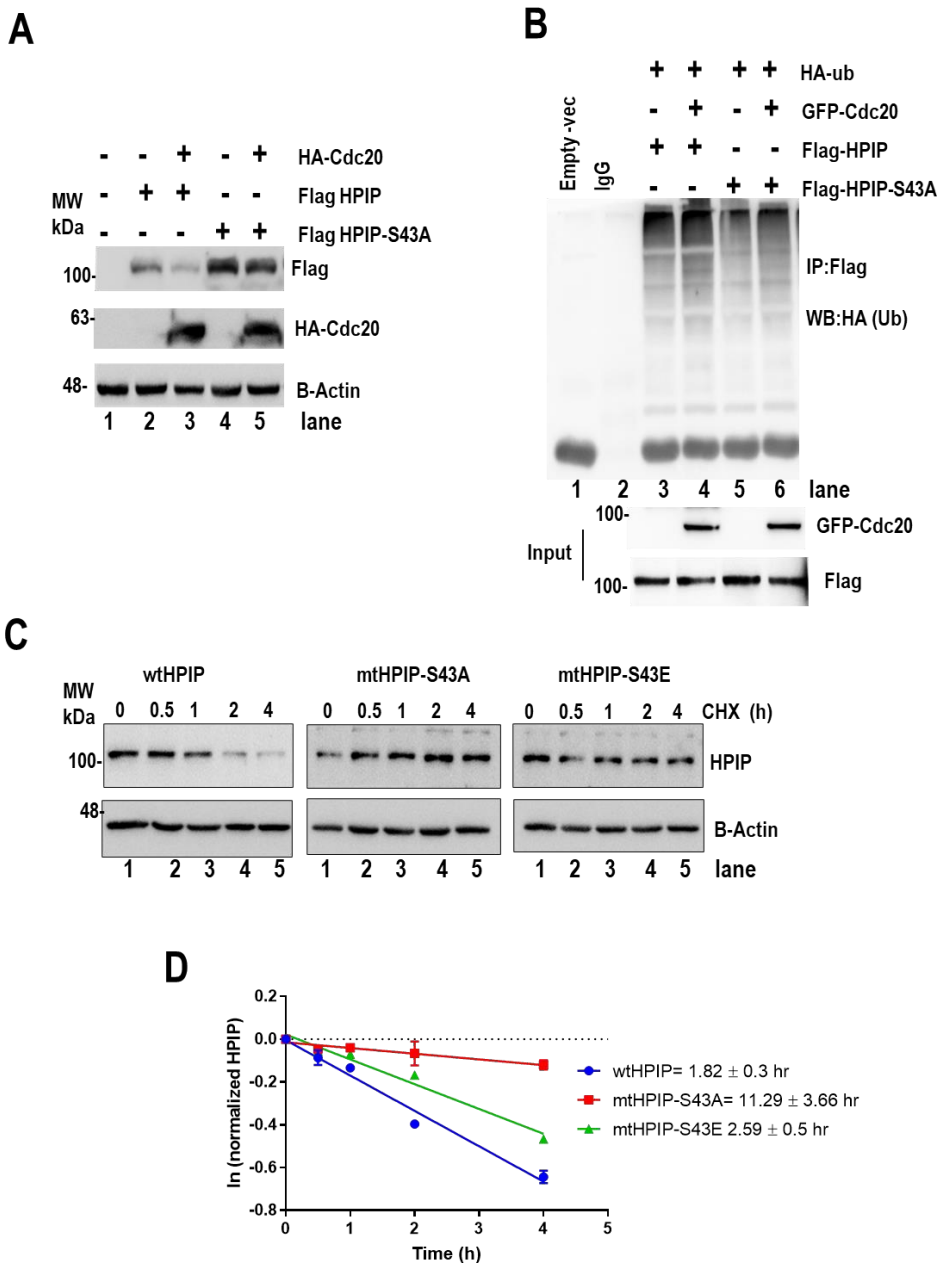

Figure 18. mtHPIP-S43A is resistant to APC/C-Cdc20 mediated degradation. (A) Western blotting analysis showing the expression of Flag-tagged wtHPIP or mtHPIP-S43A with or without expression of HA-Cdc20 in HEK293T cells, β-Actin serves as loading control. **(B)** In HEK293T cells, HA-ubiquitin is co-transfected with the plasmid constructs, wtHPIP or mtHPIP-S43A (Flag-tag), with or without GFP-Cdc20 constructs. Forty-eight hours post-transfection, Flag immunoprecipitates were blotted, as shown. **(C)** HeLa cells transfected with wtHPIP, mtHPIP-S43A, or mtHPIP-S43E (T7-tag) were treated with CHX for indicated time points and cell lysates were analyzed by Western blotting as indicated. **(D)** The quantification of HPIP protein bands is represented by the line graph from panel C. Ctrl, control.

3.15. CDK1 phosphorylation primed degradation of wtHPIP

We reasoned that if CDK1 activity is inhibited, it can no longer phosphorylate its target proteins, and thus, APC/C-Cdc20 would not be able to degrade them. CDK1 activity can be blocked with Quinolinyl thiazolinone derivative, RO-3306. It is a small molecule that is an ATP-competitive inhibitor that binds at the ATP pocket of CDK1 (179). To ascertain this, HeLa cells were subjected to 625 nM of RO-3306 for 24 hr, and HPIP expression was subsequently analyzed (Figure 19A). As anticipated, HPIP protein levels were significantly increased upon being treated with RO-3306, similar to cyclin B1 (Figure 19B). In support of this data, we did not notice any significant upregulation in HPIP mRNA levels (Figure 19C). Next, we surmised that HPIP accumulation must decline if CDK1 is liberated from RO-3306 inhibition in G2/M. To address this, RO-3306 treated HeLa cells (24 hr), after through washing, were then liberated into fresh DMEM for 4 hr to observe the changes in HPIP expression. As we anticipated, HPIP protein levels declined after the RO-3306 release (Figure 19D, *lane 2 vs 3*). Similarly, we used RO-3306 to treat Hela cells expressing either wtHPIP, mtHPIP-S43A, or mtHPIP-S43E to assess the impact of CDK1 inhibition. Increased levels of cyclin B1 and phospho-Histone H3 ser10 is observed under similar conditions. In wtHPIP or mtHPIP-S43E clones, we observed. HPIP protein elevation upon CDK1 inhibition (Figure 19D, *lane 1* and *2*; *lane 5* and *6*), but not in mtHPIP-S43A clones (Fig 19D, *lane 3* and *4*).

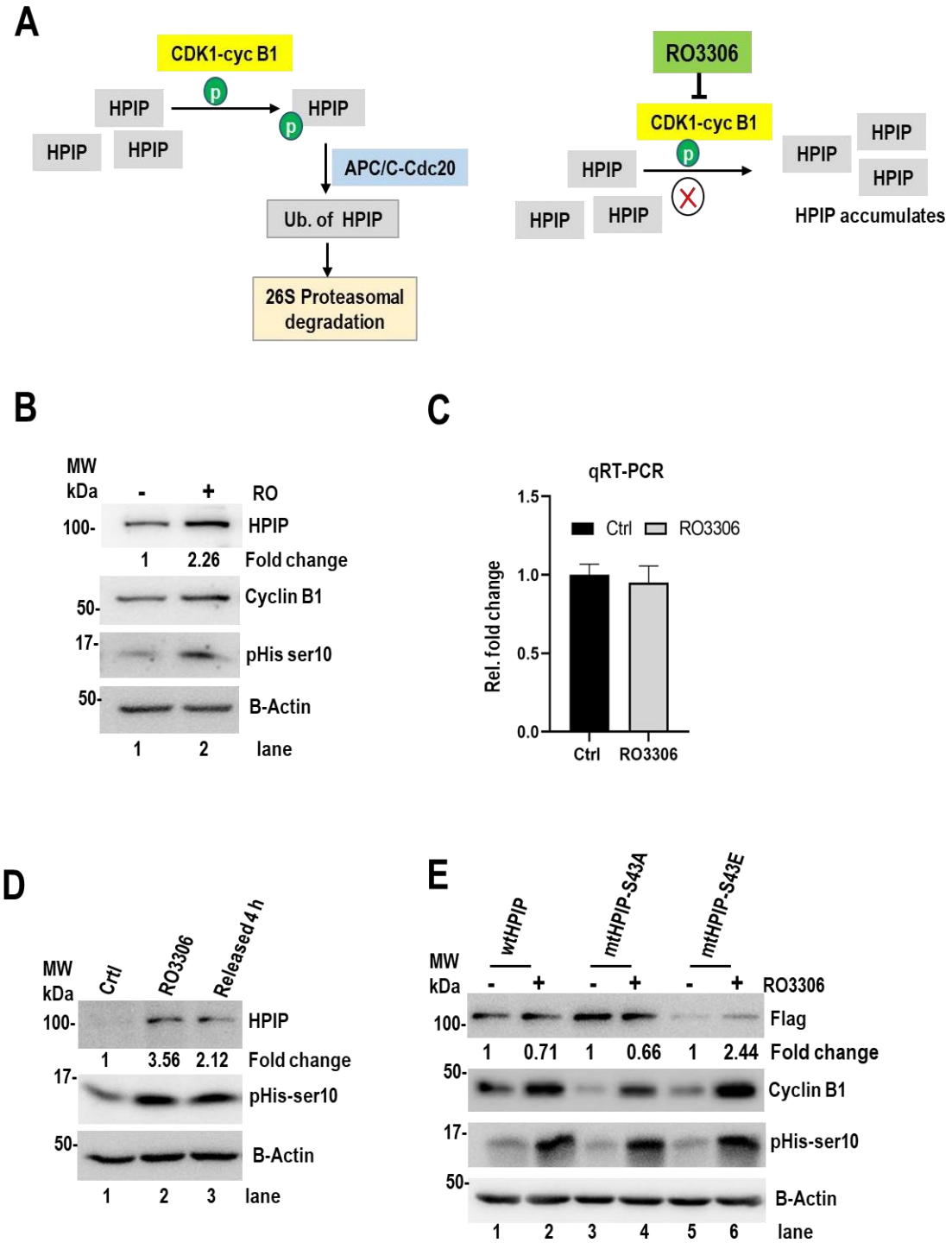

Figure 19. Inhibition of CDK1 phosphorylation by RO-3306 increases HPIP expression in HeLa cells. (A) The schematic representation of inhibitory action of RO-3306 on CDK1 and HPIP degradation

by APC/C-Cdc20. **(B)** HeLa cells were treated with DMSO (vehicle ctrl) or RO-3306 (625 nM) for 24 hr, cell lysates were analysed by immunoblotting. Fold change was calculated by taking the ratio between the protein band intensities of HPIP and β-actin. **(C)** qRT-PCR data from the same experiment shown in panel B. **(D)** HeLa cells were incubated with RO-3306 for 24 hr, released into fresh medium for 4 hr, and immunoblotted with HPIP, phospho-Histone3-ser10 or cyclin B1 antibodies. β-Actin used as loading control. **(E)** Immunoblotting analysis demonstrating effect of RO-3306 treatment (625 nM for 24 hr) on the expression of various proteins in wtHPIP, mtHPIP-S43A, or mtHPIP-S43E HeLa cells.

3.16. Interaction of MPM2 antibody with HPIP implying its spindle localization

Although we have established that HPIP is phosphorylated by CDK1 *in vitro* but, it lacks identification of *in vivo* phosphorylation of HPIP. Identifying *in vivo* phosphorylation of HPIP requires a photo-specific antibody that can detect HPIP-S43 phospho-form. Since we could not raise this antibody, we sought an alternative approach. MPM2 is a monoclonal antibody that recognizes a cell cycle-specific phospho-Ser/Thr-Pro epitope (180-182).

Since HPIP protein harbors a CDK1 phosphorylation site at serine 43, we assumed that MPM2 can detect the phosphorylation form of HPIP. To address this, co-immunoprecipitation (Co-IP) was performed using MPM2 antibody followed by Western blotting with HPIP antibody. As anticipated, HPIP was readily precipitated with MPM2 from cells of asynchronous population and mitosis (9^{th} and 10^{th} hr) and, barely detected at later time points (11 and 14^{th} hr) (Figure 20B, *lane 3*). Next, we utilized G1/S synchronized HeLa cells, treated with RO-3306, and then cells were subjected to immunofluorescence assay to analyse the colocalization of HPIP with

MPM2. As anticipated, we observed a significant reduction of MPM2 staining and its colocalization with HPIP upon CDK1 inhibition in HeLa cells (Figure 20C-D).

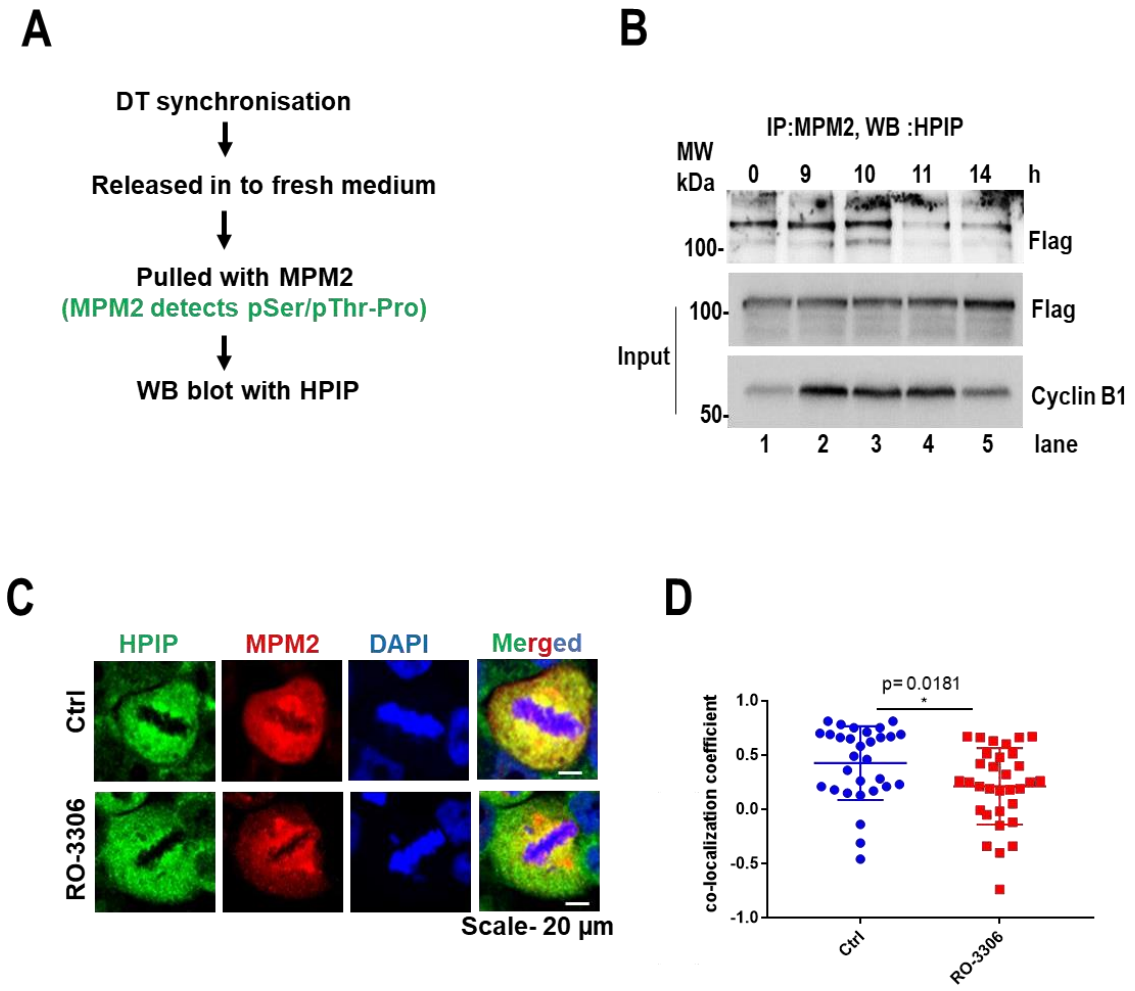

Figure 20. Detection of pSer-Pro (S43) of HPIP with MPM2 antibody. (A) Schematic illustration represents HeLa cells synchronization with DT method followed by immunoprecipitation (IP) of the cell lysate using MPM2 antibody to detect phospho-form of HPIP. **(B)** Representative immunofluorescence images showing the effect of RO-3306 on the co-localization of HPIP with MPM2 antibody in HeLa cells. MPM2 (Red) and HPIP (Green) (Magnification 60x). **(C)** Pearson's correlation co-efficient to measure protein colocalization from panel B. Student's t-test to calculate statistical significance *, $p < 0.05$. Ctrl, control.

Chapter 4

Discussion

4.1. HPIP acts as a G2/M transition regulator

Previous studies revealed the crucial role of HPIP in cell proliferation (128, 146). However, molecular mechanisms underlying this function are mainly unknown. This study provides mechanistic evidence for HPIP's role in regulating the cell cycle. We explore various state-of-the-art biochemical and cell biology assays combined with time-lapse cell imaging assay. We followed both loss of function by shRNA-mediated HPIP gene silencing by lentiviral-mediated transduction and gain of function approach by overexpression of HPIP. We demonstrate that HPIP is needed for proper cell division. Loss of HPIP expression results in assemblage of cells in G2/M phase which dwindle the cell division rate. It is well established that the G2/M transition is a decisive point of the cell cycle at which, after successively completing the G2 phase following DNA replication, it begins the mitosis phase, the phase during which cells physically separate into two daughter cells. Accumulating evidence supports that the CDK1-cyclin B1 complex modulates G2/M transition, as its reduced function arrest cells at the G2 phase (183). A recent study further supported the statement of cyclin B1 gene knockout embryo of mouse arrest in the G2 phase (184). Not only the entry and exit of mitosis can be regulated by the rise and decay in cyclin B1 level, but it can also potentially regulate the G2/M transition (185). In support of these arguments, our gain and loss of function studies demonstrate that HPIP indeed impedes cyclin B1 destruction by APC/C-Cdc20-mediated 26S proteasome. We arrive at this conclusion based on the experimental observation that loss of HPIP expression leads to diminished cyclin B1 stability.

It is interesting to note that both cyclin B1 and HPIP are overexpressed in human cancers (132, 186). We surmise that the elevated cyclin B1 expression in human cancer could be partly due to the over expression of HPIP. The increased ploidy observed in human cancer is associated

with cyclin B1 elevation, which could be partly attributable to a rise in HPIP level in those cancers. Hence it is worth investigating whether inhibition of HPIP expression by small molecule inhibitors to curb the cyclin B driven cancers.

4.2. APC/C-Cdc20 complex degrades HPIP

Another intriguing observation from this study is that APC/C-Cdc20 degrades HPIP during mitosis. We first observed that the HPIP level oscillates throughout the cell cycle phases, which mimicked the cyclin A expression pattern. Previous studies demonstrated that the destruction of cyclin A occurs just before the mitotic entry (187, 188). E3 ubiquitin ligase APC/C-Cdc20 is an essential regulator of mitosis and regulates metaphase-anaphase transition during mitosis by targeting several mitotic substrates. It utilizes the co-activators, either Cdh1 or Cdc20. Cdc20 keeps the APC/C complex active at the beginning of mitosis, whereas Cdh1 triggers the APC/C complex during late mitosis and the G1 phase (90, 91). We found that HPIP destruction occurs during the initial phase of mitosis by the action of APC/C-Cdc20 complex. Further, we demonstrate that Cdc20 interacts with and recruits HPIP to APC/C complex for its ubiquitination followed by proteolysis by 26S proteasome. Collectively this study supports that HPIP could inhibit and serves as a substrate for the APC/C-Cdc20 complex. HPIP facilitates G2/M transition by promoting cyclin B1 stability via impeding APC/C-Cdc20 activity. On the other hand, HPIP itself serves as a substrate for APC/C-Cdc20 during early mitosis. It is plausible that HPIP stabilizes cyclin B1 to drive the CDK1 activity, which in turn phosphorylates and stimulates APC/C activity. The activated APC/C-Cdc20 in mitosis will degrade HPIP and other substrates, such as securin cyclin A, etc. to ensure the metaphase-anaphase transition. Another possible reason for HPIP proteolysis during mitosis via APC/C-Cdc20 is to ensure cyclin B1 proteolysis, which triggers mitotic exit.

4.3. CDK1-cyclin B1 phosphorylates HPIP during mitosis

Phosphorylation is a fundamental regulatory mechanism of controlling cyclin dependent kinase activity and the cell cycle regulation. Among CDKs, CDK1 is one of the essential regulators for driving mitosis during the cell cycle. CDK1 is a proline directed kinase that preferentially phosphorylates the target proteins at the consensus sequence S/T-P x K/R (where x is any amino acid) (189). CDK1 is known to directly phosphorylate approximately 200 proteins, as listed in the SIGNOR database (190). By phosphorylating several cell cycle dependent proteins, CDK1 drives G2/M transition and mitosis. A few CDK1 substrates of cell cycle includes CDC25, CDC27, BuBR1, WEE1, KIF2A, NuMA, etc. CDK1 dependent phosphorylation of CDC25 involves amplification of CDK1 activity as WEE1 mediated inhibition of CDK1 is reverted, by CDC25-mediated dephosphorylation mechanism. During mitosis, BuBR1 phosphorylation by the CDK1 at Thr 620 triggers PLK1 recruitment to ensure kinetochore-microtubule interaction and timely mitotic progression (191). Another important substate for CDK1-cyclin B1 complex is CDC27, an essential subunit of APC/C complex whose activation during G2/M transition allows chromatid segregation and metaphase-anaphase transition (192).

Herein, we report that HPIP is subjected to phosphorylation mediated by CDK1-cyclin B1 complex that is important for mitotic function. We mapped CDK1 phosphorylation site in HPIP at serine 43 which align with the CDK1 consensus phosphorylation sequence. We make a note that serine 43 in HPIP is not conserved across the species. This is in line with the earlier report that position of the most CDK1 phosphorylation site in its substrate is not conserved in evolution (193). To gain the functional insight about the phosphorylation at this site we generated phosphorylation-inactive and phospho-mimetic mutants of HPIP in HeLa cells. mtHPIP-S43A clones formed significantly smallernumber of colony than wtHPIP or mtHPIP-

S43E. This could be due to delayed cell division in mtHPIP-S43A. Mechanistically we further show that mtHPIP-S43A had delayed cyclin B1 accumulation. It is well established that the accumulation of cyclin B1 during the G2/M transition is essential for the proper mitotic entry of eukaryotic cells. Based on our results we argue that the altered cyclin B1 dynamics during G2/M transition in HPIP-S43A mutant is due to loss of HPIP phosphorylation by CDK1 that retendered accumulation of cells at G2/M phase.

Another important observation from this objective is that the loss of HPIP phosphorylation at serine 43 results in the formation of abnormal spindle poles and improper chromosomal segregation during mitosis. Also, loss of HPIP phosphorylation at serine 43 results in chromosome miss-alignment and congression. Furthermore, mutant clones had less monopolar and multipolar spindles. Further studies are warranted to understand molecular mechanism underlying the role of HPIP phosphorylation in spindle function.

Since we could not generate phospho-specific antibody that can detect phosphorylated HPIP at serine 43, we utilised MPM2 antibody. MPM2 is a monoclonal antibody that recognizes phospho-Ser/Thr-Pro epitopes (180). MPM2 readily detected phospho-form of HPIP from both asynchronous and mitosis cells, where HPIP is subjected to CDK1-cyclin B1 mediated phosphorylation. HPIP is a microtubule interacting protein that can associate with mitotic spindle during mitosis (143). Whether phosphorylation at serine 43 is required for HPIP localisation to mitotic spindle is obscure. This study demonstrates that HPIP phosphorylation is important for its localisation to the mitotic spindle as CDK1 inhibition by RO3306 abrogates its localisation to mitotic spindle. We also noticed that loss of HPIP phosphorylation disrupts HPIP proteolysis by APC/C-Cdc20 complex. Cycloheximide chasing experiment along with Cdc20-overexpression studies indeed supported that mtHPIP-S43A, but not wtHPIP, was

resistant to degradation by APC/C-Cdc20 suggesting that HPIP phosphorylation may prime its ubiquitination followed by destruction via 26S proteasome.

The possible scenarios for HPIP phosphorylation by CDK1 are as follows: One of the important essential functions of HPIP in cell cycle progression is to drive the G2/M transition by stabilizing cyclin B1 by precluding APC/C-Cdc20 mediated destruction. HPIP prevent cyclin B1 destruction to facilitate APC/C-Cdc20 activation during G2/M transition phase. Cyclin B1 destruction however occurs in mid-mitosis to ensure the timely mitosis exit. Once this function is accomplished, cells must trigger HPIP phosphorylation by CDK1 during early mitosis for its degradation, otherwise it may ensure cyclin B1 stability that blocks mitotic exit. Since phospho-HPIP is localised to mitotic spindle, it is unclear from this study whether phosphorylated HPIP is necessary for its mitotic spindle localisation and its destruction.

Faithful chromosome segregation is crucial for maintaining genomic integrity in the daughter cells as genomic abnormalities due to mis-segregation of chromosomes leads to cancer (194). Chromosome segregation is facilitated by microtubules which are critical component of mitotic spindle (195). Earlier reports suggest that HPIP is microtubule-associated protein and loss of HPIP phosphorylation results in the formation of multiple spindles. However, the role of HPIP phosphorylation in mitotic spindle assembly was not pursued. We found that wild type HPIP induced more monopolar and multipolar spindles than either mtHPIP-S43A or mtHPIP-S43E. Furthermore, we also observed high rate of chromosome mis-segregation and congression in mtHPIP-S43A than wtHPIP and mtHPIP-S43E clones. Several microtubules associated proteins (MAPs) undergo phosphorylation during cell cycle progression. The phosphorylation state of MAPs is known to influence the microtubule dynamics and thus spindle function. For instance, MAP9 is subjected to phosphorylation at serine 625 and serine 289 respectively by

Aurora A and PLK1 and regulates its localization to mitotic spindle and controls their integrity (196, 197). Phosphorylated MAP9 localized to centrosome when cells switched from G2 to M phase and mid-body during cytokinesis (196). Phosphorylation at this site in MAP9 results in abnormal spindle formation and delayed mitotic progression. Another example is CDK1-mediated phosphorylation of EML3 regulating spindle assembly during mitosis. CDK1 phosphorylated EML3 (Thr881) can binds to Augmin/γ-TuRC and its subsequent spindle localization (198). Loss of EML3 phosphorylation can affect spindle assembly checkpoint, delayed chromosome congression and cell division. Yet another example is CDK1-cyclin B1 phosphorylated WEE1 is targeted for degradation by CRL1 β-TrCP leading to CDK1-cyclin B1 dependent APC/C activation (199). Align to these earlier reports, our result suggests that HPIP phosphorylation by CDK1 can impact mitotic spindle function as we observed elevation of monopolar and multipolar spindles upon loss of HPIP phosphorylation at serine 43. Furthermore, we also observed high rate of chromosome mis-segregation and congression in mtHPIP-S43A than wtHPIP and mtHPIP-S43E clones indicating that HPIP phosphorylation may be essential for normal spindle function.

4.4. CDK1-mediated phosphorylation primes HPIP destruction during mitosis

Both phosphorylation and ubiquitination are the two important post-translational modifications that regulate cell cycle progression by dynamically altering levels of various cell cycle regulators (200, 201). APC/C is E3 ubiquitin ligase complex play vital role in cell cycle regulation by the orderly degradation of its substrates during mitosis and G1 phase (202, 203). APC/C-Cdc20 is activated by CDK1-cyclin B1 dependent phosphorylation during late G2 phase. Once APC/C is fully activated during early mitosis, it ubiquitinates several substrates to ensure metaphase-anaphase transition. For instance, APC/C ubiquitinates securin which renders separase activation that leads to the proteolysis of cohesin and subsequent separation

of sister chromatids to the daughter cells (89, 204). Also, it has been shown that APC/C dependent ubiquitination precedes substrate phosphorylation for timely degradation (86). For instance, phosphorylation of Mcl-1 by CDK1-cyclin B1 primes it's ubiquitination by APC/C-Cdc20 and subsequent destruction by 26s proteosome (205). CDK1 phosphorylation influences securin destruction by APC/C Cdc20 (206). Securin phosphorylation at Thr27 and Ser71 by CDK1 blocks its degradation by APC/C Cdc20 (206). Similarly, Dbf4 phosphorylation at Ser 11 by CDK1 inhibits APC/C-Cdc20-mediated proteolysis of Dbf4 (89, 193). We observed that HPIP phosphorylation by CDK1 primes its degradation during mitosis as CDK1 inhibition accumulated HPIP in Hela cells and HPIP-S43A mutant was resistant for degradation by APC/C-Cdc20. However, it is unclear whether mitotic spindle association could have any possible influence on its binding and destruction by APC/C-Cdc20. Khumukcham *et al.,* has shown that Lys 274 is subjected to ubiquitination by APC/C-Cdc20 (143). Hence, whether CDK1 phosphorylation at Ser 43 will have any influence on Lys 274 ubiquitination to be investigated in the future.

Chapter 5

Conclusions/Summary

Summary

Despite HPIP is characterised as a protooncogene in diverse cancers, its role in cell division remains elusive. This study demonstrated that HPIP expression is needed for proper cell division, and it is specifically required for G2/M transition during cell cycle progression. HPIP possibly promotes G2/M transition and cell division by controlling cyclin B1 expression. Mechanistic studies further revealed that HPIP antagonizes APC/C-Cdc20-mediated degradation of cyclin B1 to ensure timely G2/M transition.

Another intriguing observation in this study is HPIP phosphorylation at serine 43 by CDK1-cyclin B1 complex during mitosis. Loss of phosphorylation of HPIP at serine 43 severely hampered HPIP-mediated cell division due to delayed mitotic entry and exit in HeLa cells. Additionally, loss of serine 43 phosphorylation in HPIP results in improper chromosomal segregation and the formation of abnormal spindle poles. Further mechanistic studies revealed that mtHPIP-S43A failed to stabilize cyclin B1 and timely G2/M transition because loss of HPIP phosphorylation by CDK1 impairs its proteolysis by APC/C-Cdc20. Collectively, these findings indicated an essential role for HPIP and its phosphorylation at serine 43 by CDK1 in cell cycle regulation.

Our model illustrated in Figure 21 represents that in the late G2 phase HPIP accumulates and prevents early activation of the APC/C complex, which helps in cyclin B1 stability, G2/M transition, spindle pole maintenance, and timely mitotic entry. In mitosis, HPIP is phosphorylated by CDK1-cyclin B1 complex at serine 43, leading to its ubiquitination by APC/C-Cdc20 and subsequently by proteasomal degradation.

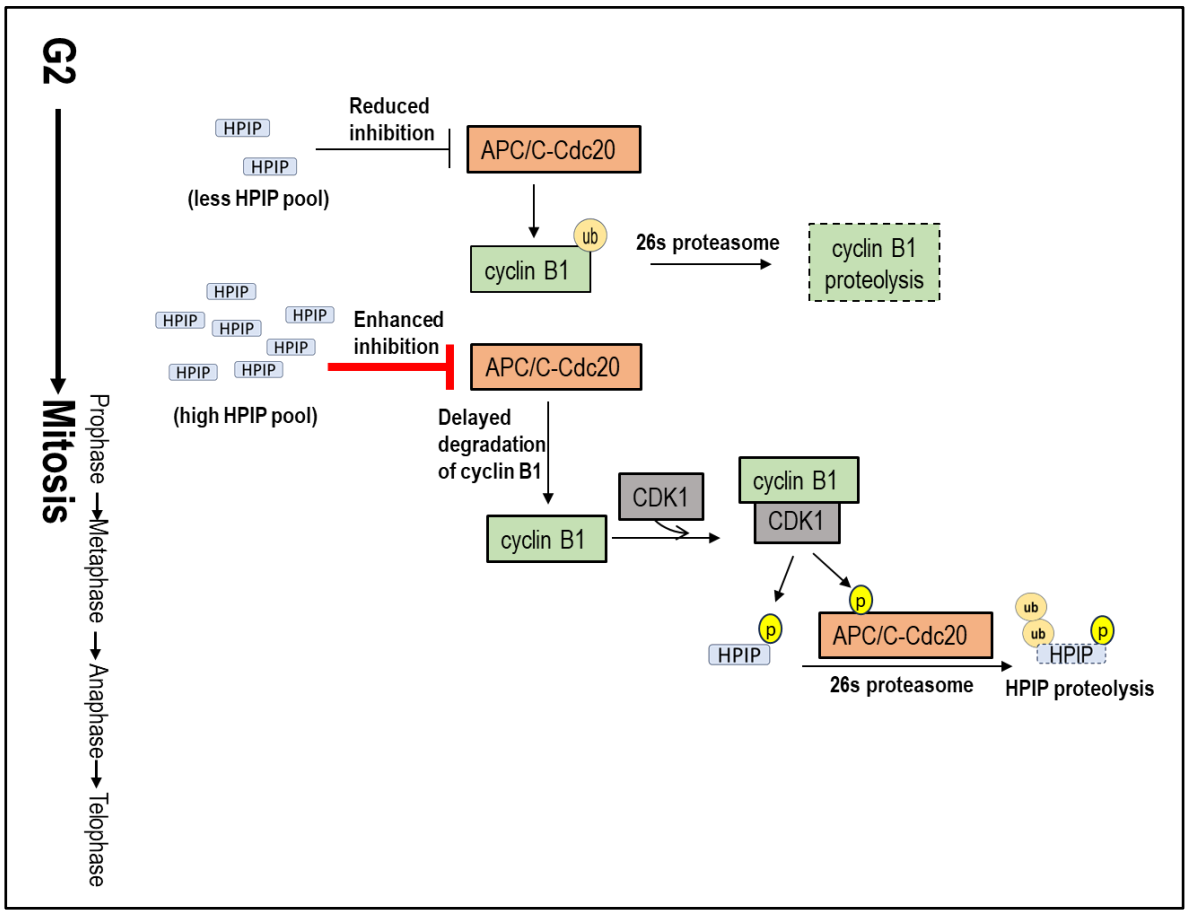

Figure 21 Summary illustrating the mechanism of CDK1-cyclin B1 mediated phosphorylation of HPIP and its ubiquitination by APC/C complex. In the late G2 phase, HPIP accumulates and prevents early activation of the APC/C complex, which helps in cyclin B1 stability, G2/M transition, spindle pole maintenance, and timely mitosis. In late mitosis, HPIP is phosphorylated by CDK1-cyclin B1 complex at serine 43, leading to its ubiquitination by APC/C-Cdc20 and subsequently by proteasomal degradation.

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Publications

JBC ARTICLE

Hematopoietic PBX-interacting protein is a substrate and an inhibitor of the APC/C–Cdc20 complex and regulates mitosis by stabilizing cyclin B1

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Saratchandra Singh Khumukcham[‡1], Venkata Subramanyam Kumar Samanthapudi[‡1], Vasudevarao Penugurti[‡1], Anita Kumari[‡], P. S. Kesavan[§], Loka Reddy Velatooru[‡], Siva Reddy Kotla[‡], Aprotim Mazumder[§], and Bramanandam Manavathi[‡2]

From the [‡]Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India and the [§]Centre for Interdisciplinary Sciences, Tata Institute of Fundamental Research Hyderabad, Hyderabad 500107, Telangana, India

Edited by Xiao-Fan Wang

Proper cell division relies on the coordinated regulation between a structural component, the mitotic spindle, and a regulatory component, anaphase-promoting complex/cyclosome (APC/C). Hematopoietic PBX-interacting protein (HPIP) is a microtubule-associated protein that plays a pivotal role in cell proliferation, cell migration, and tumor metastasis. Here, using HEK293T and HeLa cells, along with immunoprecipitation and immunoblotting, live-cell imaging, and protein-stability assays, we report that HPIP expression oscillates throughout the cell cycle and that its depletion delays cell division. We noted that by utilizing its D box and IR domain, HPIP plays a dual role both as a substrate and inhibitor, respectively, of the APC/C complex. We observed that HPIP enhances the G_2/M transition of the cell cycle by transiently stabilizing cyclin B1 by preventing APC/C–Cdc20–mediated degradation, thereby ensuring timely mitotic entry. We also uncovered that HPIP associates with the mitotic spindle and that its depletion leads to the formation of multiple mitotic spindles and chromosomal abnormalities, results in defects in cytokinesis, and delays mitotic exit. Our findings uncover HPIP as both a substrate and an inhibitor of APC/C–Cdc20 that maintains the temporal stability of cyclin B1 during the G_2/M transition and thereby controls mitosis and cell division.

Accurate chromosome segregation is essential for proper cell division in normal cells. Two important post-translational modifications, phosphorylation, and ubiquitin-mediated proteolysis during mitosis, play crucial roles in this process (1). The anaphase promoting complex/cyclosome (APC/C),[3] a ubiquitin ligase, controls the cell division by regulating mitosis through ubiquitin-directed proteolysis of key substrates in an ordered fashion to direct progression through the mitotic exit, chromosome segregation, and cytokinesis (2–4). The activity of APC/C is coordinated by two regulatory proteins, Cdc20 and Cdh1, through phase-specific interactions during the cell cycle and promotes cell division with precision and accuracy (5). For example, APC/C–Cdc20 degrades Securin and cyclin B1 at anaphase onset. This ensures Separase activation and proteolysis of Cohesin, which holds pair of sister chromatids together during early mitosis. The spindle assembly checkpoint, which depends on multiprotein complexes including Mad2, BubR1, and Bub3, delays APC/C–Cdc20 activation until all chromosomes are properly aligned at the metaphase plate (6, 7). Perturbation of this checkpoint results in chromosomal abnormality (8).

Entry into mitosis is coordinated by cyclin B1–dependent activation of cyclin-dependent kinase 1 (CDK1) during G_2 phase and form a Cdk1–cyclin B1 complex also known as maturation-promoting factor, which is crucial for G_2/M transition (9). Cyclin B1 accumulates in the nucleus as the cells progress to mitosis, although the activation of CDK1–cyclin B1 is initiated at the cytoplasm (10). Cyclin B1 binding triggers a conformational change in Wee1 phosphorylated and inactive CDK1, restoring the activity in CDK1 (11). The activated CDK1–cyclin B1 complex triggers initiation of chromosome condensation, nuclear envelope breakdown, and mitotic spindle assembly through phosphorylation of its substrates (10). It also phosphorylates APC/C–Cdc20 for its complete activation, but later during mitosis cyclin B1 is degraded by APC/C–Cdc20 (12, 13). Abolishing the degradation of cyclin B1 leads to arrest of cells in mitosis, suggesting timely degradation of cyclin B1 by APC/C, is important for proper cell cycle progression (14, 15). Although transcriptional up-regulation of cyclin B1 and its increased stability of mRNA during the G_2 phase has been described before (16), the role of proteasomal pathway in increased cyclin B1 levels is largely unknown.

Hematopoietic PBX-interacting protein (HPIP, also known as PBXIP1) is a protooncoprotein that has been shown to be overexpressed in several cancer types including infiltrative ductal carcinoma (17), hepatocellular carcinoma (18), glioma (19), and ovarian cancer (20). Previous reports have shown that HPIP promotes cell proliferation by modulating the expression of cyclins during the

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This article contains supporting "Materials and methods," Tables S1 and S2, Figs. S1 and S2, and Videos S1–S13.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed. E-mail: manavbrahma@gmail.com.

[3] The abbreviations used are: APC/C, anaphase-promoting complex/cyclosome; HPIP, hematopoietic PBX-interacting protein; CDK, cyclin-dependent kinase; DT, double thymidine; DMEM, Dulbecco's modified Eagle's medium; DAPI, 4′,6′-diamino-2-phenylindole; SAC, spindle assembly checkpoint; MCC, mitotic checkpoint complex.

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Hematopoietic PBX-interacting protein is a novel regulator of mammary epithelial cell differentiation

Anju Dwivedi[1], Chiranjeevi Padala[1], Anita Kumari[1], Saratchandra Singh Khumukcham[1], Vasudevarao Penugurti[1], Sinjini Ghosh[2], Aprotim Mazumder[2], Vincent Goffin[3] and Bramanandam Manavathi[1]

1 Department of Biochemistry, School of Life Sciences, University of Hyderabad, India
2 TIFR Centre for Interdisciplinary Sciences, Tata Institute of Fundamental Research Hyderabad, India
3 Institut Necker Enfants Malades (INEM), Inserm U1151/CNRS 8253, Université de Paris, France

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Correspondence
B. Manavathi, Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad-500046, India
Tel: +914023134546
E-mails: manavathibsl@uohyd.ac.in; manavathi.hcu@gmail.com

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Hematopoietic PBX-interacting protein (HPIP, also known as PBXIP1) is an estrogen receptor (ER) interacting protein that regulates estrogen-mediated breast cancer cell proliferation and tumorigenesis. However, its functional significance in the context of mammary gland development is unexplored. Here, we report that HPIP is required for prolactin (PRL)-induced lactogenic differentiation *in vitro*. Molecular analysis of HPIP expression in mice revealed its induced expression at pregnancy and lactation stages of mammary gland. Moreover, PRL is a lactogenic hormone that controls pregnancy as well as lactation and induces *Hpip*/*Pbxip1* expression in a signal transducer and activator of transcription 5a-dependent manner. Using mammary epithelial and lactogenic-competent cell lines, we further show that HPIP plays a regulatory role in PRL-mediated mammary epithelial cell differentiation, which is measured by acini formation, β-casein synthesis, and lipid droplet formation. Further mechanistic studies using pharmacological inhibitors revealed that HPIP modulates PRL-induced β-casein synthesis via phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) activation. This study also identified HPIP as a critical regulator of autocrine PRL signaling as treatment with the PRL receptor antagonist Δ1-9-G129R-hPRL restrained HPIP-mediated PRL synthesis, AKT activation, and β-casein synthesis in cultured HC11 cells. Interestingly, we also uncovered that microRNA-148a (miR-148a) antagonizes HPIP-mediated mammary epithelial cell differentiation. Together, our study identified HPIP as a critical regulator of PRL signaling and revealed a novel molecular circuitry involving PRL, HPIP, PI3K/AKT, and miR-148a that controls mammary epithelial cell differentiation *in vitro*.

Introduction

Mammary glands are highly evolved, specialized exocrine glands made up of lobes, and ducts [1]. The alveoli (hollow cavities) in mammary glands are lined with milk-secreting cuboidal cells and are surrounded by myoepithelial cells. The alveolus undergoes development and differentiation under the control of

Abbreviations
AKT, protein kinase B; bPRL, bovine prolactin; HPIP, hematopoietic PBX-interacting protein; JAK2, Janus kinase 2; miR-148a, microRNA-148a; PI3K, phosphoinositide 3-kinase; PRL, Prolactin; PRLR, prolactin receptor; PTEN, phosphatase and tensin homolog deleted on chromosome 10; STAT5, signal transducer and activator of transcription 5.

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HPIP and RUFY3 are noncanonical guanine nucleotide exchange factors of Rab5 to regulate endocytosis-coupled focal adhesion turnover

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Saratchandra Singh Khumukcham[1,‡], Vasudevarao Penugurti[1,‡], Suresh Bugide[1], Anju Dwivedi[1], Anita Kumari[1], P. S. Kesavan[2], Sruchytha Kalali[1], Yasaswi Gayatri Mishra[1], Vakkalagadda A. Ramesh[3,4], Hampapathalu A. Nagarajaram[5], Aprotim Mazumder[2], and Bramanandam Manavathi[1,*]

From the [1]Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad, Telangana, India; [2]Department of Biological Sciences, Tata Institute of Fundamental Research (TIFR), Hyderabad, Telangana, India; [3]Laboratory of Computational Biology, Centre for DNA Finger Printing and Diagnostics (CDFD), Hyderabad, Telangana, India; [4]Laboratory of Computational Biology, Manipal Academy of Higher Education, Manipal, Karnataka, India; [5]Department of Systems and Computational Biology, University of Hyderabad, Hyderabad, Telangana, India

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While the role of endocytosis in focal adhesion turnover-coupled cell migration has been established in addition to its conventional role in cellular functions, the molecular regulators and precise molecular mechanisms that underlie this process remain largely unknown. In this study, we report that proto-oncoprotein hematopoietic PBX–interacting protein (HPIP) localizes to focal adhesions as well as endosomal compartments along with RUN FYVE domain–containing protein 3 (RUFY3) and Rab5, an early endosomal protein. HPIP contains two coiled-coil domains (CC1 and CC2) that are necessary for its association with Rab5 and RUFY3 as CC domain double mutant, that is, mtHPIPΔCC1-2 failed to support it. Furthermore, we show that HPIP and RUFY3 activate Rab5 by serving as noncanonical guanine nucleotide exchange factors of Rab5. In support of this, either deletion of coiled-coil domains or silencing of HPIP or RUFY3 impairs Rab5 activation and Rab5-dependent cell migration. Mechanistic studies further revealed that loss of HPIP or RUFY3 expression severely impairs Rab5-mediated focal adhesion disassembly, FAK activation, fibronectin-associated-β1 integrin trafficking, and thus cell migration. Together, this study underscores the importance of HPIP and RUFY3 as noncanonical guanine nucleotide exchange factors of Rab5 and in integrin trafficking and focal adhesion turnover, which implicates in cell migration.

Endocytosis is an essential cellular process that regulates numerous signaling pathways by controlling cell surface receptors. It is increasingly evident that endocytosis modulates cell adhesion signaling *via* integrins, receptors for extracellular matrix proteins, to control cell migration (1, 2). During cell migration, integrins are internalized from the cell surface *via* endocytosis and recycled back to the plasma membrane to redistribute the pools of integrin from one part of the cell to another (3–5). It has been known that the internalization of integrins *via* clathrin-mediated endocytosis regulates the turnover of focal adhesions (FAs) (6, 7). In this context, focal adhesion kinase (FAK) has been recognized as a key player in FA signaling/turnover and clathrin-mediated endocytosis as FAK-null cells display impaired FA turnover and decreased cell migration (6, 8, 9). While FAK activation is a prerequisite for endosome-mediated FA turnover and cell migration, the molecular regulators of FAK activation and coordinators of this process remain elusive.

Endosomal proteins, such as Rab5 and early endosome antigen 1, are essential players in the generation and propagation of early endosomes during endocytosis (10, 11). While endocytosis is associated with cell migration, in particular, the role of Rab5 in cell adhesion and migration has been pertinent. Several studies revealed that Rab5 regulates cell migration *via* cytoskeletal remodeling that results in Rac1 activation (12–14), β1 integrin internalization and recycling (13), or microtubule (MT)-dependent adhesion disassembly (15). While the current concepts envisage the crucial role of Rab5 in endocytosis-mediated adhesion signaling *via* integrins, the molecular regulators of Rab5 activity and the detailed mechanisms that underlie this process are not fully understood.

Hematopoietic PBX–interacting protein (HPIP) is an oncoprotein with an adaptor function that can integrate various signaling proteins in the regulation of cell migration (16–24). Its overexpression has been reported in various cancers (25). Not only that its overexpression is implicated in the development of osteoarthritis (26). Recent studies revealed a novel signaling axis involving HPIP and Talin in FA turnover and cell migration (27). While there is increasing evidence regarding its role in cell migration, its precise molecular mechanism in FA signaling and cell migration is elusive (27).

In this report, we have characterized HPIP and RUN FYVE domain–containing protein 3 (RUFY3) as noncanonical guanine nucleotide exchange factors (GEFs) of Rab5 that regulate

‡ These authors contributed equally to this work.
* For correspondence: Bramanandam Manavathi, manavathibsl@uohyd.ac.in.

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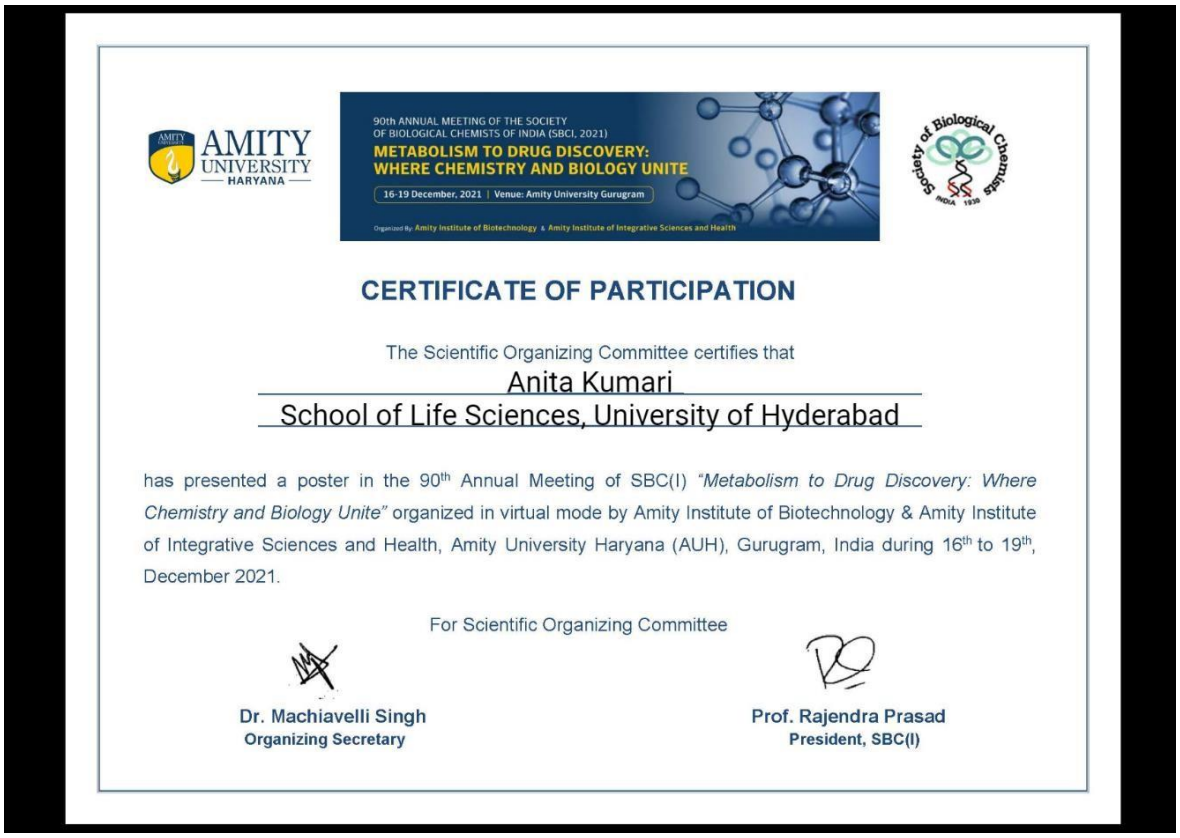

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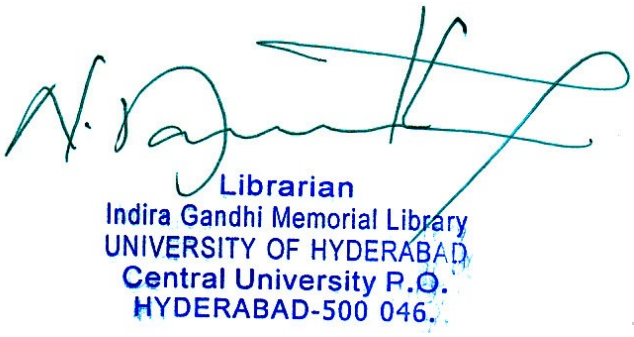

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