CELL CYCLE AND APC/C UBIQUITYLATION REGULATE CHROMATIN DYNAMICS TO ACHIEVE TIMELY CELL PROLIFERATION

Jennifer L. Kernan

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Approved by:

Michael J. Emanuele

Lee M. Graves

J. Mauro Calabrese

Jeanette G. Cook

Brian D. Strahl

ABSTRACT

Jennifer L. Kernan: Cell Cycle and APC/C Ubiquitylation Regulate Chromatin Dynamics to Achieve Timely Cell Proliferation
(Under the direction of Michael J. Emanuele)

Cell cycle progression is an intricately controlled, temporally regulated biological process that involves multiple regulators, both transcriptionally and post-transcriptionally to integrate diverse signaling pathways to facilitate productive cell proliferation. A combination of oscillatory gene transcription, cyclin phosphorylation of downstream targets, and APC/C-mediated ubiquitylation governs the cell cycle program. Importantly, the epigenome also influences cell proliferation through structural compaction that controls transcription factor access to the DNA (for cell cycle gene expression and histone biogenesis), regulates DNA replication timing, and facilitates chromosomal condensation for mitotic segregation. Similarly, the cell cycle regulates the epigenetic environment through histone transcription, origin licensing and firing, chromosomal condensation and segregation, cell cycle regulated histone/DNA post-translational modifications, and manipulation of chromatin modifiers' expression and stability. Significantly, cell proliferation dynamics are aberrantly regulated in cancer and other genetic diseases.

The central finding of this research project shows that APC/C^{Cdh1} ubiquitylation controls many epigenetic regulators that are implicated in cell cycle progression. In particular, we characterize the KEN-dependent APC/C^{Cdh1}-mediated proteasomal degradation of UHRF1. UHRF1 is a critical epigenetic modifier that coordinates DNA methylation maintenance during S phase and is an oncogene that is overexpressed in many cancers. We discovered that in contrast to wild-type UHRF1, a non-degradable version of UHRF1 stably expressed in cells

binds less well to the Cdh1 co-activator and is not robustly ubiquitylated at mitotic exit. These cells also enter S phase more rapidly and have upregulated cyclin E levels. Furthermore, lack of UHRF1 degradation induces hypermethylation of early replicating regions and hypomethylation of late replicating sites of the genome. This work provides mechanistic insight into how UHRF1 itself is regulated during cell cycle progression, an area which remains largely understudied in the field.

For God and country.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

APC/C	Anaphase Promoting Complex/Cyclosome
ATR	Ataxia-Telangiectasia and Rad3-related protein
Cdc	Cell division cycle
Cdc6	Cell division cycle 6
Cdc20	Cell division cycle 20
Cdc25C	Cell division cycle 25C
Cdh1	Fizzy and cell division cycle 20 related 1
CDK(s)	Cyclin-Dependent Kinase(s)
CENPF	Centromere protein F
Chk1	Checkpoint Kinase 1
CKI	Cyclin Dependent Kinase Inhibitor
DNMT1	DNA Methyltransferase 1
DUBs	Deubiquitinases
E2F1	E2F transcription factor 1
EGF	Epidermal Growth Factor
Emi1	Early mitotic inhibitor I
Fbw7	F-box and WD repeat domain containing 7
FL	Full-Length
FoxM1	Forkhead box M1
G0	Quiescence
G1	Gap phase 1
G2	Gap phase 2
GO	Gene Ontology

GPS	Global Protein Stability Profiling
HAT	Histone acetyltransferase
HECT	Homologous to the E6AP Carboxyl Terminus
pH3-S10	Phosphorylation of Histone H3 on Serine 10
HR	Homologous Recombination
ICBP90	CCAAT box-binding protein of 90 kDa
IP/co-IP	Immunoprecipitations/co-immunoprecipitations
LIG1	DNA Ligase I
LINE-1	Long Interspersed Nucleotide Element-1
LPS	Linker, PHD, and SRA domains (UHRF1)
M	M phase, mitosis
5mC	5-methylcytosine
MAPK	Mitogen-Activated Protein Kinase
MCC	Mitotic Checkpoint Complex
MDS	Multidimensional scaling
monoUb	Monoubiquitylation
MPF	Maturation Promoting Factor
MTase	Methyltransferase domain
mTOR	mechanistic Target of Rapamycin kinase
NEB	Nuclear Envelope Breakdown
NHEJ	Non-Homologous End Joining
NPAT	Nuclear Protein, Coactivator of Histone Transcription
ORCs	Origin Recognition Complexes
PBR	Polybasic region
PCH	Pericentromeric heterochromatin

PCNA	Proliferating Cell Nuclear Antigen
PHD	Plant homeodomain domain
PI3K	Phosphoinositide 3-Kinase
PI5P	Phosphatidylinositol 5-phosphate
PKB/Akt	Protein Kinase B/Akt serine/threonine kinase
PLK1	Polo-like kinase 1
polyUb	Polyubiquitylation
PRC2	Polycomb Repressive Complex 2
PTM(s)	Post-Translational Modification(s)
RB	Retinoblastoma protein
RBR	RING-between-RINGS
RCA1	Regulator of Cyclin A
RFTS	Replication Foci Targeting Sequence
RING	Really Interesting New Gene
S	S phase
SAC	Spindle Assembly Checkpoint
SAM	*S*-adenyl methionine
SCC1	Sister Chromatid Cohesion protein 1
SCF	SKP(1/2)-Cullin 1- F-box protein
SKP2	S phase kinase associated protein 2
SLBP	Stem-Loop Binding Protein
SRA	SET- and RING-Associated
TF	Transcription Factor
TTD	Tandem Tudor Domain
Ub	Ubiquitin

UBL	Ubiquitin-like domain
UHRF1	Ubiquitin-like PHD and RING finger domain 1
USP7	Ubiquitin Specific Peptidase 7

CHAPTER 1: THE CELL CYCLE IS GOVERNED BY TWO KEY MECHANISMS AND CROSSTALKS WITH THE EPIGENETIC ENVIRONMENT FOR TIMELY CELL DIVISION

1.1). Cell proliferation is a vital process for normal growth and division

Cell proliferation is an essential biological process that is central to normal cell homeostasis and division and is often dysregulated in disease. This highly regulated molecular process is critical to various cellular pathways including injury/regeneration, tissue development and differentiation, cell migration, repair/stress response, and apoptosis. Cell proliferation is regulated through the cell cycle program that is evolutionarily concerned from yeast to humans(1). The cell cycle is divided into four stages: G1 (Gap phase 1), S (DNA replication), G2 (Gap phase 2), and M (mitosis), which in most cells occurs over 24 hours.

In G1 phase, cells must sense and integrate various adhesion, differentiation, and mitogenic molecular cues into the decision to proliferate(2) and physically grow larger in preparation for dividing into two daughter cells. When mitogens and/or other favorable conditions are absent, cells shift to an alternative, non-dividing, but metabolically active state called quiescence(3–5), termed “G0”, which is reversible(6) upon metabolite stimulation . Thus, this sensory input is critical to initiate cell division and overcome the restriction (“R”) point(7, 8) that once passed ensures cells finish their cycles regardless of any further changes in the environment.

Once cells are committed to dividing in G1, the DNA must be duplicated in S phase. Within S phase, cells may activate the DNA replication or intra-S checkpoint when DNA damage is induced by intrinsic or extrinsic replication stress or genotoxic insults(7). This checkpoint, which typically involves Ataxia-Telangiectasia and Rad3-related protein (ATR) kinase/ Checkpoint

Kinase 1 (Chk1) signaling to detect stalled or collapsed forks (that may further devolve into double-strand breaks) as well as errors in nucleotide excision(9, 10), is instigated to arrest proliferation and repair any damaged DNA before continuing the cell cycle.

Next, G2 phase provides a brief pause to check genome integrity, ensuring that all the DNA is replicated and no errors are passed onto the next generation. If double-strand breaks are detected, two main repair pathways are employed: high fidelity homologous recombination (HR) or error-prone non-homologous end joining (NHEJ) (the latter also occurs in the G1 DNA damage response, which is often bypassed in cancers due to the loss of p53)(11).

Assuming the G2 checkpoint is satisfied, cells proceed to mitosis. M phase, which is divided into 5 phases (prophase, prometaphase, metaphase, anaphase, and telophase) is followed by cytokinesis or the cleaving of the cytoplasm to form two new daughter cells. In prophase, centrosomes move to the opposite poles of the cell, the mitotic microtubules form, and the DNA is compacted into duplicate pairs of chromatids or chromosomes. In prometaphase, the nuclear envelope breaks down and the mitotic spindle assembles while the paired chromatids' kinetochores are amphitelically attached to microtubules(12). During this time, cells activate the spindle checkpoint (SAC) which involves a mitotic checkpoint complex of Mad2, BubR1, Bub3, and Cdc20 to sense the kinetochores under tension on the spindle fibers and inhibit Cdc20 from binding the anaphase-promoting complex/cyclosome (APC/C), thereby halting segregation until chromosome congression and formation of the metaphase plate is achieved(13). The SAC detects spindle attachment and chromosomal alignment errors that could result in aneuploidy unless promptly corrected(14). Once the SAC is satisfied, the cell commences the final stages of mitosis: segregation, cytokinesis, reformation of the nuclear envelope, and decompaction of chromosomes back into chromatin(12).

Should there be aberrant cell proliferation (e.g. which may also occur via DNA damage assaults and segregation errors as mentioned or the disruption of normal cyclin/CDK activity as will be discussed below(15)), retarded growth(16), cancer(17), aging disorders(18), and

neurological diseases(19) can result. Notably, the regulatory proteins that govern these various genome integrity checkpoints are often mutated in cancer(20), insinuating how unchecked growth and division in disease bypasses homeostatic control conditions. How a cell orchestrates its phase transitions to control proliferation dynamics is very tightly, temporally regulated. There are two primary mechanisms cells use to control growth, namely cell cycle gene transcriptional programs and ubiquitin-mediated proteolysis, which will be elaborated in detail below.

1.2). Cell cycle progression is controlled by oscillatory cyclin transcription leading to CDK phosphorylation of defined targets

At any given stage of the cell cycle, cell cycle genes are transcribed to produce proteins whose functions are required for a particular function in the immediate or following cell cycle phase (e.g. transcribed in G1 for an upcoming role in S phase)(7). One key set of target cell cycle genes are cyclins, which regulate cell cycle timing through the activation of Cyclin-Dependent Kinase (CDKs)(21, 22). Cyclins are temporally expressed regulatory subunits that bind and allosterically activate their cognate, catalytic, proline-directed, serine and threonine (S/T-P) CDKs(23). These CDKs, which were discovered in multiple organisms during the 1980s, including frogs(24, 25), yeast (which have only one Cdk1: Cdc28/Cdk1(23) or $p34^{cdc2}$(26)), sea urchins(27), and mammalian cells(28), are the primary drivers of cell cycle control(29), in addition to other key kinases such as Polo-like kinases(30). Higher eukaryotes have multiple cyclins and CDKs, indicative of additional layers of differential regulatory control, despite some overlapping functionality(23). These unique cyclin/CDK complexes phosphorylate numerous proteins to propel cell cycle progression in a tightly coordinated manner. Below will be discussed the key cyclin/CDK complexes and their well-defined targets.

In G1 phase, cyclin D/CDK4/6 phosphorylates(31) a major G1 transcriptional co-repressor protein called retinoblastoma (RB) to remove its inhibitory activity on the E2F transcription factor 1 (E3F1)(32–34), thus driving cells toward S phase. Cyclin D/CDK4/6(15) has also been reported to

target p107 and p130 co-repressors as well, both of which along with RB are referred to as pocket proteins present at G1 (p107(35)) and G0/G1 (p130(35, 36)) phases. RB inhibits E2F1(34, 37) as well as E2F2-3(38) in G1. p130 and p107 cooperate with co-repressor E2F4(5, 35, 36, 39, 40) and E2F5(41, 42) in G0 and G1, respectively, to restrain S phase entry. Importantly, while p130 is high in G0 but not in cycling cells, RB and p107 primarily regulate transcription in proliferating cells (7). Furthermore, RB also controls repression of G1/S genes in senescent cells(38).

Cyclin D is transcriptionally regulated through various pathways, such as Ras/mitogen-activated protein kinases (MAPK) and Phosphoinositide 3-kinase (PI3K)-Protein Kinase B(PKB)/Akt signaling(43–45) as well as growth factors such as Epidermal Growth Factor (EGF)(46). Moreover, cyclin D mRNA translation is regulated by mechanistic Target of Rapamycin kinase (mTOR)(47). Different degradation mechanisms have been reported via the SCF(FBX4-alphaB crystalline)(48) and FBXW8(49) E3 ligases, which occur as cells are transitioning into S phase. Two deubiquitinases have also been reported to antagonize cyclin D degradation, namely, USP2(50) and USP22(51). Other studies have identified cell proliferative roles of cyclin D/CDK4/6 that are independent of its phosphorylation activity, including facilitating transcription(52, 53) and interactions with various epigenetic regulators(54–56).

Following the removal of RB inhibition and eviction of repressor transcription factors E2F4-5 from chromatin in G1 (through CDK phosphorylation activity), G1/S promoters are derepressed. Then, activator transcription factors E2F1-3 initiate G1/S gene transcription(7, 57, 58). It is notable that RB has other non-canonical chromatin functions, but these are beyond the scope of this dissertation. One of the primary downstream targets of E2F1 is cyclin E (cyclin E is one of the earliest G1 transcribed genes(59)), which is evolutionarily conserved among species(60). Interestingly, as a G1 cyclin, cyclin E is transcribed preferentially before other E2F1 targets(59), thus enabling cells to commit to division. Cyclin E couples with CDK2 at the G1/S transition and has a very important role in hyperphosphorylation of RB to fully inactivate the transcription factor (TF) co-repressor(61–63). Importantly, the initial G1 cyclin transcription upregulates subsequent

rounds of G1 cyclin transcription, culminating in an accelerated spike in CDK activity that acts as a timely, positive feedback loop to ensure unrepealable, hysteretic commitment to S phase(64).

Cyclin E has numerous proliferative roles in the cell, including centrosomal duplication(65), nuclear protein, coactivator of histone transcription (NPAT) phosphorylation to facilitate histone transcription(66, 67), and origin licensing via cell division cycle 6 (Cdc6) phosphorylation (thereby protecting Cdc6 from degradation by the anaphase-promoting complex/cyclosome (APC/C^{Cdh1}))(68). Cyclin E/CDK2 also promotes proteolytic destruction of the cyclin dependent kinase inhibitor (CKI) p27 by the SCF^{Skp2} ligase (Skp2 is the substrate receptor, S phase kinase associated protein 2)(69, 70), although this switch of p27 from cyclin E/CDK2 inhibitor to substrate is not well understood. Cyclin E abundance is proteolytically controlled by two mechanisms. When unbound by CDK2, Cullin-3 ligase(71) degrades monomeric cyclin E. SCF^{Fbw7} (Fbw7 is the substrate receptor, F-box and WD repeat domain containing 7) degrades cyclin E when in association with CDK2(72), which is blocked when p21 or p27 are also present in the cyclin E/CDK2 complex(73) via the removal of essential phosphorylation marks. Excess cyclin E (as is the case in cancers when it is amplified) can lead to an early and extended S phase entry(74, 75), likely due to the lack of time to adequately prepare for replication through sufficient pre-replication assembly (76).

Besides cyclin E positive feedback, transcriptional repressors such as E2F6-7 are themselves G1/S target genes that act as negative feedback controls to bind and silence G1/S promoter gene transcription as cells pass into S phase(7, 77, 78). While E2F8 is less well known, it appears to have a similar role as E2F7(79–81).

Intriguingly, G1/S transcription is also regulated by the DNA replication checkpoint (mentioned in brief earlier), which involves a transcriptional response to replication stress. When fork stalls or collapses are encountered during S phase, CHK1, activated through ATR which senses the stress(82–86), adds an inhibitory phosphorylation onto E2F6(87) to promote its inactivation. As a result, E2F1-3 TFs are no longer repressed by E2F6 and the resulting G1/S

transcription ensures cell survival to cope with the replication stress. At the same time, the CKI p21 is activated(88) to arrest the cells in S phase to repair the damage(89). Also, the phosphatase CDC25 is degraded upon DNA damage by CHK1 phosphorylation to block CDC25 from removing the inhibitory phosphorylation marks on CDK1 and CDK2, thereby restraining G2/M progression(86, 90). Thus, these PTMs following replication stress direct transcriptional responses that ultimately influence cell cycle progression.

As cells progress into S phase(91), cyclin A2, whose transcriptional activation is cell-cycle regulated and is repressed in G1 phase by p107, switches binding partners with CDK2 (taking the place of cyclin E) (92–94). An E2F1 target whose transcriptional activation can be stimulated by cyclin D and cyclin E activity(94), cyclin A2 levels rise in early S phase and are stable until prometaphase when it is degraded in a Cdc20-independent anaphase promoting complex/cyclosome (APC/C) manner(95). Cyclin A2 has multiple roles in the cell cycle, including DNA replication initiation and control(96) (via RPA, Cdc6, MCM4, and DNA polymerase phosphorylation)(97), prevention of re-replication(98), regulation of nuclear envelope breakdown and chromosomal condensation(99, 100), kinetochore attachment(101), and mitotic entry (102, 103). Importantly, cyclin A/CDK2 adds an inhibitory phosphorylation mark onto Cdh1 to block the co-activator from binding and activating the APC/C, thus shutting off the complex during S/G2 phases(104).

Cyclin A/CDK2 also regulates transcriptional activity through inhibitory E2F1 phosphorylation (7, 105–107), thereby disengaging the activator TF from DNA(7) to extinguish G1/S transcriptional signaling as cells progress into S phase. Simultaneously, E2F1 is targeted for proteolytic destruction by the SKP2-Cullin 1-F-box protein (SCF) complex(108) and SKP2 is itself an E2F1 target gene(109), underling the importance of negative feedback loops to abolish G1/S transcription as cells prepare to enter S phase. Similarly, repressor E2F6-8 TFs are also transcriptional targets of E2F1-3 and accumulate on G1/S promoters to terminate transcription in S phase. Later in the cell cycle, cyclin A associates with CDK1 in late S/G2 phases(94, 110, 111)

and may facilitate cyclin B/CDK1 complex activity, which is aided by the dephosphorylation of CDK1 (by phosphatase Cdc25C) to promote G2 phase (102, 112).

Another S/G2 phase cyclin, cyclin F, is a non-canonical cyclin that does not bind a CDK, but is rather a member of the SCF (SKP2-Cullin 1-cyclin F) complex(113). The founding member of the 69 F-box proteins, cyclin F(114) acts as a substrate receptor that binds various target proteins to promote their proteolytic destruction. For example, cyclin F regulates dNTP availability during DNA replication (RRM2(115)), centrosome doubling (CP110(116)), and mitotic spindle dynamics (NUSAPI(117)) to promote genomic integrity(118). Notably, our laboratory showed that cyclin F also negatively regulates Cdh1 through ubiquitin-mediated degradation at the G1/S transition(119) to ensure timely termination of APC/C activity in S phase.

Then in M phase, CDK1 (also known as Cdc2p in *S. pombe* and Cdc28p in *Saccharomyces cerevisiae*) partners with cyclin B(120) to phosphorylate key mitotic proteins including Aurora B, lamins(121, 122), and other cytoskeleton proteins(123, 124) to promote timely nuclear envelope breakdown, chromosomal alignment and segregation, and mitotic division(125). Cyclin B is a G2 phase transcriptional target of the master transcription factor Forkhead box M1 (FoxM1), which transcribes numerous G2/M cell cycle genes (e.g. cyclin B, Aurora B, Polo-like kinase 1 (PLK1), CDK1) in preparation for cellular division(126). Significantly, in early mitosis, cyclin B/CDK1 phosphorylates APC/C subunits to activate the complex and initiate binding to Cdc20(127), which has been shown to be a cyclin A/CDK2 and a cyclin B/CDK1 substrate(128). Simultaneously, cyclin B/CDK1 also phosphorylates the other APC/C co-activator, Cdh1, thus inhibiting it from interacting with APC/C(129). Later in mitosis, CDK1 is inactivated (by APC/C^{Cdh1} degradation of cyclin B), and APC/C^{Cdh1} ubiquitylates Cdc20(130). Besides cyclin B, CDK1 also coordinates with Polo-like and Aurora kinases(131, 132) as well to facilitate G2/M cellular events.

It is important to note that the aforementioned cyclin/CDK phosphorylation events can be antagonized by another class of enzymes called protein phosphatases, including PP2A(133)

and Cdc25(134, 135) to negatively regulate these phosphorylation waves to ensure proper synchronization while integrating other internal and external signaling pathways. Similarly, CKIs also inhibit cyclin/CDKs to regulate phase transitions until some threshold of activity or protein abundance is achieved. For example, cyclin E/CDK2 is initially blocked by CKIs in early G1, but cyclin D/CDK4/6 acts as a "sink" to seclude p27 and p21(136) and abrogate their inhibition of cyclin E/CDK2 activation(15). Moreover, should the environment prove unfavorable (e.g. DNA damage or lack of nutrients), CKIs will halt the cell cycle to respond to the crises before re-initiating proliferation(137, 138).

In conclusion, these various temporal transcriptional responses and post-translational cyclin/CDK phosphorylation and dephosphorylation events coordinate the activity of numerous cell cycle target genes/proteins to exquisitely regulate the timing of each cell cycle phase.

1.3). Protein degradation also regulates cell cycle progression

A second mechanism by which cells regulate the irreversible progression of the cell cycle is achieved by turning off cyclin activity (among other proteins) through a protein degradation process called ubiquitylation, a well-studied post-translational modification that regulates a variety of cellular processes(139). The ubiquitin proteasome system is a tripartite enzymatic cascade that involves three core enzymes: an E1-ubiquitin activating enzyme (2 total), an E2-ubiquitin conjugating enzyme (38+), and an E3 ubiquitin ligase (600+)(140–142). The three enzymes coordinate to transfer ubiquitin (Ub), a small 76 amino acid, 8.6kDa peptide, and conjugate it to substrates through the formation of a isopeptide bond between the carboxyl (G76) of ubiquitin and the *epsilon* amino acid of lysine in target proteins(143). This process requires ATP and involves the formation of a thioester bond between the catalytic cysteine in the E1 and E2 enzymes with the C-terminus of Ub. E2s orchestrate ubiquitylation with select E3s which confer substrate specificity (141, 144, 145). E3s can be subdivided into either Homologous to the E6AP Carboxyl Terminus (HECT), Really Interesting New Gene (RING), or RING-between-RINGS (RBR) ligases,

where the type of ligase determines how Ub is conjugated (directly or first indirectly onto the E3) onto its substrates(146, 147).

Ub is unique in that is contains seven lysine residues (K6, K11, K27, K29, K33, K48, K63) that can form various chain topologies, including monoubiquitylation (monoUb) (addition of a single ubiquitin monomer), multi-monoubiquitylation (addition of several unique monomers), and polyubiquitylation (polyUb) (addition of many ubiquitin moieties linked through their C-termini) in either homotypic or mixed, branched or linear chains(148, 149). The different Ub signatures engender differential responses, the most well studied of which is in DNA repair (e.g. K6, K27, K33, K48, K63 Ub), endocytic trafficking and lysosomal degradation (e.g. K63 and K29 Ub), and protein degradation via the proteasome (K11 and K48 Ub) (150–152). For simplicity, all remaining Ub references refer specifically to proteasomal degradation only. While it is not relevant for this dissertation, it is important to note that the ubiquitin modifications can be removed by deubiquitinases (DUBs)(153, 154), such as USP37(155) and Cezanne(156), to halt protein degradation, which is important for synchronously integrating the various cell proliferation signaling mechanisms.

1.4). APC/C is a master E3 Ub ligase of the cell cycle

Among the E3 ligases, the anaphase-promoting complex or cyclosome (APC/C) is particularly essential for cell cycle control(157). Discovered in the mid-1990s using developmental models and mammalian cells, this enzyme is most well studied for its role in regulating cell division((158–160) and reviewed here (161)). APC/C controls the M/G1 and G1/S transitions by both promoting chromosomal segregation in M phase (via cyclin B and securin degradation)(162) and restraining S phase entry (via ubiquitylation of DNA replication promoting factors)(163).

An intricate, 1.2 MDa complex, recent advances in cryogenic electron microscopy studies have illuminated how the enzyme's structure contributes to its biochemical functions

(164, 165). Its large and flexible structure enables the APC/C to specifically target numerous substrates for ubiquitylation. APC/C accomplishes this feat with the aid of two, related co-activators called cell division cycle 20 (Cdc20)(166) and fizzy and cell division cycle 20 related 1 (FZR1/Cdh1)(167, 168). APC/C binds and is activated allosterically (169, 170) by these partners in different cell cycle phases. Cdc20 interacts with APC/C in metaphase to anaphase of mitosis and Cdh1 binds APC/C from anaphase through G1 phase(171, 172). Notably, these coactivators are also substrate receptors. They bind targets and recruit them to the APC/C holoenzyme core. Target proteins are identified through the presence of key consensus amino acid sequences called degrons to which the substrate receptors bind. The most well-described degrons are KEN boxes (amino acids “K-E-N”) and D-boxes (amino acids “R-X-X-L”, were “x” is any amino acid)(130, 173, 174). Other degrons exist(157), such as the TEK box(175) and the ABBA motif(176), but not all have been well characterized. Interestingly, Cdc20 and Cdh1 share a subset of targets (e.g. APC/C^{Cdc20} and APC/C^{Cdh1} both ubiquitylate cyclin B(177)), but APC/C^{Cdh1} has many more substrates compared to APC/C^{Cdc20} (168). The coactivators can bind KEN degrons(178), while both the substrate receptors and the D-box acceptor APC/C subunit, Apc10, are needed to coordinate binding to D-boxes in substrates(179–182). In summary, these coactivators are critical partners in defining the substrate targets of the APC/C.

APC/C is a unique E3 Ub ligase as it coordinates with two different E2s to ubiquitylate substrates (183). To initiate ubiquitin priming (addition of single Ub onto a protein), APC/C binds the initiating E2, UbcH10(165, 184–187). Then, to catalyze polyUb chains, APC/C switches partners and interacts with the chain-building E2, Ube2S(188). E2s are also important to the ubiquitylation of targets in that they also confer specificity in addition to the E3(189). Importantly, in mitosis, APC/C adds K11 specific Ub onto targets, with the aid of UBE2S(190–194). These Ub topologies are unique in that polyUb K11 and K48 chains are built to promote rapid degradation by the proteasome (195–197). Interestingly, the number of multiubiquitination

events (many monoUb or short polyUb chains) appears to increase the rate of degradation compared to canonical, long polyUb linkages(198, 199).

APC/C regulates mitotic exit via multiple targeted protein destruction events, including selected prometaphase destruction of Nek2A(200) and cyclin A(94). Later in metaphase, APC/C^{Cdc20} targets cyclin B(158, 159, 174, 201), which causes CDK1 inactivation, and securin for destruction. Securin degradation releases inhibition of separase which severs sister chromatid cohesion protein 1 (SCC1), a component of the cohesion assembly(202, 203), thus facilitating sister chromatid segregation(204)). Without CDK1 present to maintain the inhibitory phosphorylation of Cdh1(205), APC/C^{Cdh1} is activated and ubiquitylates the Cdc20 co-activator(206, 207). APC/C^{Cdh1} ubiquitylates several mitotic kinases (e.g. Aurora A, PLK1)(208, 209) and spindle factors (e.g. NUSAPI)(210) in late mitosis. Then in G1, this complex continues to ubiquitylate numerous proteins (e.g. Cdc6, Geminin, Orc1) to restrict S phase entry until after the DNA replication machinery is translated, DNA origins are licensed, and the replisome is assembled(168, 211).

APC/C is itself modified by various PTMs to promote its activity, particularly phosphorylation. Throughout the cell cycle, APC/C is progressively hyperphosphorylated and binds Cdc20 in mitosis(212, 213). Upon APC/C^{Cdc20} degradation of cyclin B in metaphase of mitosis, CDK1 is inactivated, thus removing the inhibitory phosphorylation signals that prevent Cdh1 from interacting with APC/C (as mentioned above). Later at the G1/S transition, Cdh1 is phosphorylated by cyclin A/CDK2 (211, 214), thereby preventing binding to APC/C until the next anaphase. However, APC/C^{Cdh1} can be re-activated following DNA damage in S phase. Phosphatase Cdc14B dephosphorylates Cdh1 to prompt binding to APC/C(215) and DNA repair signaling before continuing with division.

APC/C functional activity is also potently regulated by its biological antagonist, early mitotic inhibitor I (Emi1)(216, 217) (also reviewed in depth in Chapter 2), during G1/S through G2/M phases. Emi1 acts as pseudosubstrate and blocks APC/C function by binding both the

APC/C and Cdh1 to obstruct substrate access to the D-box receptor region(218). In G2/M, SCF ubiquitylates Emi1 to reactivate APC/C(219), following PLK1 and cyclin B/CDK1 phosphorylation(220). A few, specific pharmacological drugs, such as apcin and proTAME(221), have been developed to study APC/C function, but these have only been marginally useful. Thus, the lack of direct chemical inhibitors presents a challenge to studying APC/C biology. However, much success has been achieved with microtubule toxins (such as Taxol and Nocodazole) and other inhibitors such as Eg5 and p31comet(222) which have facilitated many studies probing APC/C function and targets in mitosis.

1.5). Cell cycle proteins and APC/C are dysregulated in cancer

Given their critical role in proliferation, many cell cycle proteins are altered in cancer, which is a disease of unrestricted proliferation that often develops dependencies upon particular regulators of growth and division, termed 'oncogene addiction'(223). Here will be highlighted in brief the major changes in the cell cycle oscillator found in tumors. Beginning with the cell cycle transcriptional program, RB is often mutated in cancers(224) and the loss of which correlates with unfavorable patient survival(225). RB loss upregulates the E2F pathway and given the crucial role of E2F1 in promoting proliferation through S phase entry, it is well established that many cancers have misregulated E2F1 function(58, 226–228). Importantly, cyclins can often be dysregulated in cancer(15, 229, 230). A known oncogene, cyclin D mRNA and protein levels are often amplified in many cancers including breast, lung, melanoma, and oral squamous tumors(231). Cyclin D1 amplification is one of the most frequent copy number changes found in cancers(232). Cyclin D overexpression also occurs as a consequence of mitogenic pathway upregulation, such as mutation in MAPK signaling(2). For example, in mice, breast cancers with overexpression or mutant ERBB2 or RAS signaling lead to high cyclin D1 expression(233, 234). Likewise, in human HER2/neu positive mammary cancers, cyclin D1 is robustly upregulated(235–237). Not

surprisingly, cyclin D overexpression corresponds to a poorer patient survival and augmented metastasis(238, 239).

Cyclin E is also deregulated in cancers. Cyclin E amplification occurs in many tumors (e.g. ovary, endometrium, breast, stomach, and colon) and is often associated with poorer patient survival(240). One mechanism aside from genetic overexpression that contributes to cyclin E upregulation in cancer is mutation of the tumor suppressor Fbw7 (241–243). Several mechanisms exist describing how cyclin E overexpression leads to aneuploidy in cancers as well(73). Additionally, cyclin A is upregulated in many cancers, particularly colorectal, liver, mammary, prostate, and leukemic cancers(94, 99). Cyclin A2 levels affects p53 function and the DNA damage response, and these cancers exhibit proliferative and mitotic exit defects(94). Cyclin B is also overexpressed in many cancers, including breast, colorectal, non-small lung, pancreatic, and head and neck carcinomas, and contributes to aneuploidy and dysregulated cell growth(244). This upregulation is often correlated with poorer patient outcomes, higher tumor grade, and increased presence of proliferative factors, especially in breast cancer(245). Additionally, cyclin F has been shown to have a role in DNA damage and is an important guardian of genomic integrity(246) that is mutated in certain cancers, such as breast tumors and lymphomas (247, 248).

Moreover, many of the CKIs are also mis-regulated in cancer as well. $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ are two such examples. $p16^{INK4a}$ primarily functions to inhibit cyclin D/CDK4/6 phosphorylation of RB, and among its pleotropic roles, $p21^{WAF1/Cip1}$ blocks the activity of CDK2 and CDK1 as well as proliferating cell nuclear antigen (PCNA). The $p16^{INK4a}$ tumor suppressor is often lost (through deletion, silencing, or mutations) in many cancers (e.g. bladder, brain, head and neck, breast, lung, pancreatic, and skin among others), and $p16^{INK4a}$ loss is known to be an early event in tumorigenesis (249). In fact, nearly all cancers have some disruption in the p16-cyclin D-Cdk4/6-RB axis that enables uncontrolled proliferation and cancer predisposition. Similarly, p21 expression is aberrantly regulated. Mitogenic regulators such as oncogenic Ras induce p21-mediated senescence pathways(250) to facilitate oncogenesis. Many cancers, including prostate,

breast, cervical, and skin, have high p21 expression which is associated with aggressive cancer characteristics and poor prognosis(250). Many mechanisms suggest how p21 aberrations may promote tumorigenesis, including stimulation of cyclin D/CDK4/6 assembly, downregulation of p21 signaling in cell cycle arrest and DNA repair, transcriptional repression of the p21 promoter, and dysregulated degradation by negative regulators such as SKP2 (via cyclin E/CDK2 phosphorylation), Cul4A, and CDT2 (which are overexpressed in cancers)(251).

In addition to these cyclin abnormalities, it is not surprising that APC/C activity is also often dysregulated in cancer and other genetic diseases. Several subunits have been shown to be upregulated(252) and APC/C^{Cdh1} malfunction induces genomic instability that is caused by precocious DNA replication, re-replication, and chromosomal abnormalities(253, 254). Moreover, Cdh1 is a haploinsufficient tumor suppressor in mice(255) such that Cdh1 loss promotes sporadic tumor formation. Many human cancers also have irregular Cdh1 expression(256, 257). Similarly, the APC/C E2 enzymes UbcH10(258, 259) and Ube2S (260, 261) are aberrantly expressed in several cancers, indicative of their functional importance in regulating normal cell proliferation, which is perturbed in disease.

Notably, many of the current inhibitors employed in clinical trials target known upregulated APC/C substrates in cancer(163), such as mitotic kinase inhibitors like PLK1(262) and Aurora kinases(263) as well as other cell cycle oscillator components such as overexpressed CDK4/6 kinases which are targeted with checkpoint inhibitors like Palbociclib(264). Thus, the levels of the cell cycle oscillator, including cyclins, CKIs, RB/E2F1, and APC/C^{Cdh1} (along with the stoichiometry of APC/C complex members), are critical to ensure proper cell proliferation dynamics. Furthermore, understanding the role of cell cycle proteins, APC/C, and ubiquitin-mediated proteolysis in normal cell physiology and disease is a significant area of research that has many far-reaching benefits for human health.

1.6). The epigenetic environment and the cell cycle machinery coordinate to facilitate cell proliferation

Another level of protein post-translational modifications (PTMs) that influences cell proliferation dynamics is the epigenetic signature that is faithfully duplicated each mitotic cycle. Chromatin, comprised of both the protein that compacts and organizes DNA and the DNA itself, is a critical layer of regulation that governs multiple cellular processes, especially cell growth, through controlled gene expression and is heritable through successive cell generations. This transcriptional regulation is highly coordinated and intricately connected to multifarious signaling pathways, including cell cycle progression.

With respect to cell cycle, the chromatin environment influences transcription and replication events that regulate the timing of growth and division. Chromatin must be modified and rendered accessible temporarily so that transcription factors can bind the DNA and transcribe specific gene products at particular cell cycle phases (e.g. cyclin D and cyclin E in early G1, but not cyclin A which occurs later at the G1/S border nor cyclin B which is not transcribed until S/G2 phases). “Euchromatin” is a more relaxed or open chromatin state (enables transcription) compared to “heterochromatin” which is tightly compacted (blocks transcription). In addition, this degree of compaction also influences when origins fire: the chromatin architecture (which particularly affects histone synthesis(265, 266)), corresponds to the timing of DNA replication such that euchromatin (which is centrally located in the nucleus) is replicated earlier and heterochromatin (which is associated with the nuclear periphery) is duplicated in late S/early G2 phases(267, 268). Moreover, the chromatin environment is dramatically altered during S phase as histones must be temporarily displaced, duplicated, and then re-assembled (using a combination of *de novo* histones and recycled parental histones(269)), around newly synthesized DNA for replication to occur smoothly. Finally, DNA must be compacted into chromosomes for segregation during mitotic division(270). Thus, the epigenetic state plays a critical role in the rate and order of events of cell cycle progression.

Conversely, just as the chromatin environment influences cell cycle dynamics, so the cell cycle impacts the epigenome(270). One way the cycle program influences epigenetics is by initiating chromatin gene transcription. For example, during S phase, cyclin E/CDK2 phosphorylates NPAT(271, 272) to promote histone biogenesis. Histone gene transcription is tightly coordinated within the cell cycle: histone synthesis occurs in early S phase, but is inhibited in G2 phase(273). In addition, cyclin E is degraded by Fbw7 which also serves to restrict NPAT transcription to S phase only. Similarly, histone mRNA half-life is controlled by the cell cycle regulated stem-loop binding protein (SLBP), which itself is regulated through cyclin A/CDK1(274, 275). Besides regulating histone transcription, cyclin E/CDK2 also phosphorylates origin proteins such as Cdc6 in early S phase and is thought to have other roles in pre-RC assembly, including MCM loading(276, 277). Additionally, cyclin A plays a distinct, follow-up role to cyclin E from late G1/S through S phase which involves initiating origin firing of assembled DNA replication complexes and blocking the formation of new complexes by restricting the window of pre-RC to early G1(96). The APC/C also contributes to the cell cycle regulation of chromatin dynamics by ubiquitylating and degrading the protein Geminin to promote Cdt1 activity in pre-RC assembly on chromatin in early G1(278).

Besides regulating the timing and process of duplicating histones and the epigenetic signature during S/G2 phases, the cell cycle also initiates chromatin remodeling to achieve timely division. In early phases of mitosis, the cell cycle (via cyclin B/CDK1) induces nuclear envelope breakdown (NEB)(279) (a consequence of nuclear pore complex destruction(280, 281)). Additionally, chromosome condensation is regulated by various phosphorylation events (e.g. CDK1 and Aurora B kinase phosphorylation of the Condensin II complex(282, 283)). During mitosis, the spindle microtubules attach to the chromosomes' kinetochores (which is a complex of proteins that bind the centromere or mid-body of each chromosome) and, after achieving bipolar amphitelic attachment at the metaphase plate, bi-directionally segregate one chromatid from each paired chromosome to each pole of the cell. Importantly, should delays in

each of these mitotic remodeling events (condensation, NEB, segregation, etc.) occur, cell cycle progression is impaired.

Some noteworthy epigenetic marks that are particularly important for cell proliferation are DNA methylation, histone methylation, histone acetylation, and histone phosphorylation. DNA methylation occurs each cell cycle during S/G2 phase immediately following passage of the replication fork and is a critical part of maintaining cell identity through successive divisions. This process involves modifying the fifth carbon of cytosine in a single DNA nucleotide with a methyl group to form 5-methylcytosine (5mC), transferred from *S*-adenyl methionine (SAM) with the aid of a methyltransferase. During semi-conservative DNA replication, the transitory formation of hemi-methylation DNA is used to copy the parental marks onto the newly synthesized daughter strand. The main methyltransferase in humans that catalyzes the 5mC maintenance methylation mark to achieve hereditary mitotic inheritance is DNA methyltransferase 1 (DNMT1). DNA methylation marks on CpG promoters are often categorized as repressive marks (because they block transcription factor association or recruit silencing complexes(284)) that ensure DNA compaction, which is an important structural feature of chromatin that restricts gene expression. Significantly, densely methylated CpG islands exist in gene promoter regions that maintain gene silencing(285), which is a pivotal aspect of developmental control and cell identity. In contrast, DNA methylation in gene bodies appears to occur alongside replication and facilitates transcription: SETD2, a histone methyltransferase, deposits methylation groups on H3K36 to form H3K36me3(285). In fact under certain conditions (in development, cancer), DNA methylation antagonizes H3K27me3- Polycomb repressive complex 2 (PRC2) silencing complexes to facilitate gene expression(285). Moreover, DNA methylation also plays a role in heterochromatin formation which is important for chromatin remodeling, particularly during cell cycle progression(285).

There are various forms of histone methylation (mono-, di-, and tri-methylation) that influence various outcomes and are deposited onto lysines or arginines of histones (e.g.

H3K9me, H3K27me). An example of a methylation mark that promotes licensing is H4K20 monomethylation, the levels of which oscillate throughout the cell cycle(286). This mark is also important for chromosome condensation during mitosis(287) and inhibits H4K16 acetylation(288), which promotes an open chromatin conformation(289) (H4K16Ac levels are high in S phase and diminishes in M phase and are anti-correlated with H4K20Me(290)). Also, genes undergoing transcription exhibit trimethylated histone H3 Lys4 (H3K4me3)(285) in CpG dense promoter regions, which prevents DNA methylation.

In contrast to histone methylation, histone acetylation is thought to promote a more open architecture which is important for gene transcription. This PTM involves the addition of an acetyl group (from Acetyl-Coenzyme A) onto the lysine resides of histones deposited by acetyltransferases. Like methylation, acetylation also influences cell cycle progression. For example, histone acetylation on H4 is augmented in G1 phase, is associated with pre-RC and early replicating regions at sites of active transcription, and is catalyzed by MYST-family histone acetyltransferase (HAT) HBO1, which is critical to establish origin licensing and pre-RC assembly(291–294).

Histone phosphorylation also impacts cell cycle dynamics. In M phase, Aurora B kinase phosphorylates Histone H3 on Serine 10 (pH3-S10) (among other residues—T3, T11, S28), which is thought to regulate chromosome condensation(295). When there is defective phosphorylation on these sites, insufficient compaction and the resulting segregation errors ensue as a consequence of poor engagement of the Condensin I complex(270). Intriguingly, pH3-S10 also regulates other PTMs such as preventing H3K9me3 (referred to as the “phospho-methyl switch” (296)), which hinders HP1 binding and heterochromatin formation on mitotic chromosomes(297).

In addition to histone PTMs, histone writers and erasers are also cell cycle regulated and their levels oscillate during cell cycle progression. For example, the protein and mRNA expression of methylase PR-Set7 climax in G2/M (concomitant with H4K20Me), are reduced in

G1, and are kept low in S phase (by CRL4/Cdt2 Ub(298–300)) to prevent premature chromatin compaction(290). Moreover, cyclin B/CDK1 phosphorylates the demethylase PHF8 to eject it from chromatin to enable H4K20Me accretion(301). Similarly, EZH2 (H3K27 methylase that is part of the PRC2 complex and has a role in transcriptional gene silencing) is also regulated by the cell cycle via E2F(302) and CDKs(303, 304).

Another aspect of the epigenome that is an important area of study is how it is vastly re-shaped during disease. In cancers, the chromatin environment is re-organized to facilitate oncogenic growth. In a healthy cell, the euchromatin is a permissive chromatin state that readily allows transcriptional activity and pre-RC assembly prior to DNA replication, whereas the non-permissive, restrictive heterochromatin associates with the nuclear lamina preferentially, is less accessible, and is generally duplicated later in the cell cycle. However, in diseased cells, the decision to aberrantly silence some genes while expressing others can be abnormally re-wired to aid the tumor's proliferation. In fact, many tumor suppressor gene promoters are hypermethylated and silenced (with dense nucleosomal compaction over transcription start sites) to facilitate overproliferation, bypass senescent and apoptotic signaling, upregulate DNA repair mechanisms to cope with genotoxic stress, and promote invasion and metastasis(305, 306). Moreover, in many cancers, there is also a general loss of DNA methylation or global hypomethylation that drives cancer's over-proliferative capacity(307) through impaired genomic stability and augmented aneuploidy (308). For example, in cancer cells with demethylation, normally silenced DNA regions become sites of active transcription, leading to depression of repeats (which causes chromosomal recombination events), proto-oncogenes, and transposons (308–310). These changes accompany altered histone methylation patterns (e.g. H3K4Me, H3K9Me, H3K27Me), increased histone acetylation marks (such as H3K9aAc and H3K14Ac), and more open nucleosomes (nucleosome-depleted DNA and lack of DNA methylation at promoters)(311–314). Finally, cancer cells also have accumulated mutated 5mC marks that result from endogenous deamination, mutating the cytosine base to thymine (which is harder for

DNA repair enzymes to correct in mismatch damage)(307). 5mC has also been shown to facilitate DNA adduct formation and pyrimidine dimers upon carcinogenic (e.g. toxins in cigarette smoke) and UV/sunlight exposure, respectively(307). Overall, these alterations in the chromatin structure and accessibility that are present in cancers influence gene expression and DNA replication timing, which impact global cell cycle progression(315).

Given that cancer is a disease of uncontrolled growth, it is not surprising that these aforementioned dysregulated chromatin dynamics (which affect proliferation) contribute to oncogenesis. In fact, disruption of the epigenetic environment, particularly DNA methylation(314), is a bonafide hallmark of cancer(229). Moreover, many of the chromatin-modifying enzymes (readers, writers, and erasers) that interface with the transcriptional machinery(316) are aberrantly regulated(314) as evidenced by the abnormal DNA and histone PTMs observed in tumors. Below will be described in detail one very fascinating chromatin regulator called Ubiquitin-like PHD and RING finger domain 1 (UHRF1), also known as CCAAT box-binding protein of 90 kDa (ICBP90) or Np95, an essential reader and writer of DNA and histone modifications, respectively.

1.7) UHRF1 is a cell cycle regulated epigenetic modifier with multiple biological functions

During semi-conservative DNA Replication, UHRF1 coordinates with DNMT1 and other replisome assembly factors to copy the hemi-methylation marks located at CpG nucleotides of the parental DNA onto the newly nascent daughter DNA(317, 318). UHRF1 binds hemi-methylated DNA through its SET- and RING-associated (SRA) domain(317–319) and binds histone tails combinatorially(320, 321) through its plant homeodomain domain (PHD) (e.g. H2R2(322–324)) and tandem tudor domain (TTD) (e.g. H3K9me2/3(325–327)). The DNA methyltransferases, Suv39H1 and G9a, generate the methylated H3K9 mark that UHRF1 recognizes and have been identified in protein complexes with UHRF1(328–330). Additionally, the RING domain of UHRF1 confers E3 Ub ligase(331, 332) activity, and with the aid of the

ubiquitin-like domain (UBL), UHRF1 preferentially interacts with E2 UbcH5a/UBE2D1(333, 334). UHRF1 catalyzes the monoUb (333, 334) of H3K23 and H3K18 among others(333, 335–337) to physically recruit DNMT1 to the DNA(338). In a still not fully-understood mechanism that involves allosteric activation, UHRF1 simultaneously binds DNMT1 (through the UHRF1 UBL domain(339)) and engages its SRA to flip out the hemi-methylated DNA and present it as a substrate to DNMT1(340–342). As part of the N-terminus, the replication foci targeting sequence (RFTS) domain of DNMT1 autoinhibits its methyltransferase (MTase) domain(343), which is relieved by binding UHRF1(317, 344). This RFTS domain pinpoints DNMT1 to replication forks and preferentially binds the monoUb of H3 tails(345, 346), post-translationally modified by UHRF1 (333, 334). Notably, before UHRF1 can engage with hemimethylated DNA, histones, and other proteins such as DNMT1, UHRF1, which exists in an intramolecularly inhibited, “closed” state (the TTD domain is bound to the polybasic region (PBR) and the SRA is bound to the PHD domain), must be allosterically activated towards its substrates and binding partners (321, 347, 348). Various proteins and substrates contribute to UHRF1 activation via conformational changes that allosterically regulate the enzyme’s activity(349), including phosphatidylinositol 5-phosphate (PI5P)(350) which binds to the UHRF1 PBR(351), hemi-methylated DNA(335, 352), the deubiquitinase ubiquitin specific peptidase 7 (USP7)(353), and even a region within DNA Ligase I (LIG1) that resembles methylated H3K9(354). Several of these modifications are crucial in regulating when UHRF1 is bound to chromatin. Importantly, UHRF1 preferentially binds pericentromeric heterochromatin (PCH) and is thought to have an important role in duplicating heterochromatin in S/G2(332, 355) as a member of the replisome(356–358). UHRF1 stability on DNA is thought to be influenced by USP7 which has been shown to regulate DNMT1(359–361) as well as UHRF1 stable association with DNA until M phase when UHRF1 is phosphorylated by cyclin B/CDK1 on S652, breaking the USP7-UHRF1 interaction(353, 362).

Besides it's well-documented role in DNA methylation, UHRF1 is a cell cycle regulated phosphoprotein(363) that has other diverse biological functions. For example, several researchers have described a role for UHRF1 in DNA repair(364–367) and the DNA damage response, with UHRF1 loss sensitizing cells to genotoxic stress(368–371). UHRF1 has also been shown to be important for regulatory T cell growth and development(372), tissue differentiation(373), stem cell proliferation following injury(358), and neurogenesis(374), all of which implicate UHRF1 in cell proliferation. In fact, UHRF1 levels have been shown to be correlated with cell proliferation(375). Moreover, UHRF1 loss or degradation results in cell cycle arrest(358, 369, 376) and senescence(377, 378), further demonstrating that UHRF1 levels are important for cell growth.

In fact, UHRF1 is an oncogene(379) that is frequently overexpressed in many cancers, most notably mammary, ovarian, pulmonary, gastric, hepatocellular, urinary, renal, prostate, and colorectal tumors(375, 380). Interestingly, the protein is not widely mutated, but mutations in the UBL domain have been observed in select cancers(334). One of the main mechanisms through which UHRF1 promotes tumorigenesis is by hypermethylating and silencing tumor suppressor genes such as $p16^{INK4A}$, $p14^{ARF}$, RB, p21, and BRCA1 among others (reviewed here(380, 381)). Additionally, several researchers have pointed to a role for UHRF1 in enabling resistance to chemotherapeutic drugs such as cisplatinum, etoposide, and camptothecin(380, 382), and UHRF1 loss re-sensitizes tumors to these and other radiotherapies and cross-linking agents (364, 365, 369, 383). While the exact mechanisms are still unknown, these data suggest a role of UHRF1 in bypassing DNA damage by promoting anti-apoptotic and anti-senescent programs(329, 384, 385). For example, UHRF1 oncogene activity necessitates circumventing the p53 senescence pathway(386). Moreover, several studies have described how negative regulators (such as miRNAs) are frequently down-regulated in cancers where UHRF1 is highly abundant(380, 387), suggesting that loss of negative feedback controls may have significant impacts on UHRF1 expression and activity. Also, researchers have demonstrated how high

UHRF1 expression induces hypomethylation(388) and re-expression of silent retrotransposons, such as long interspersed nucleotide element-1 (LINE-1)(389, 390), which may contribute to the genomic instability seen in cancers(391–394). While it is still unclear how UHRF1 achieves global hypomethylation, UHRF1 ubiquitylation of DNMT1 may play a role (346, 359, 395). Furthermore, upregulated UHRF1 levels are correlated with high tumor grade, poor prognosis, and increased invasiveness and metastatic potential in many tumors, particularly breast cancers(396–398), again indicating that as an epigenetic master regulator UHRF1 is a major contributor to proliferative capacity in disease. These studies of UHRF1 in cancer demonstrate how perturbing the normal epigenetic regulatory environment has significant consequences on proliferation and disease progression. This field remains an important area of ongoing study to further elucidate how UHRF1 overexpression facilitates oncogenesis.

CHAPTER 2[1]: WHO GUARDS THE GUARDIAN? MECHANISMS THAT RESTRAIN APC/C DURING THE CELL CYCLE

2.1). Overview

It is well established that cell cycle progression is driven by Cyclin-CDK kinase complexes. Cyclin expression is tightly controlled during cell cycle, with upregulation determined by transcriptional changes and downregulation triggered by proteolytic destruction. The destruction of multiple Cyclins, as well as dozens of other cell cycle regulated proteins, is controlled by the APC/C, a megacomplex E3 ligase and core component of the cell cycle oscillator. In this review, we will introduce the APC/C by providing a brief, historical overview related to the discovery of Cyclin proteolysis and how this precipitated the discovery of the enzyme controlling their degradation. This review differentiates itself from other excellent reviews on APC/C in that we focus on the growing body of evidence highlighting the role and regulation of APC/C outside of mitosis. We discuss the contribution of APC/C to G0/G1 phase and its potential role in tumor suppression. We highlight recent studies that suggest APC/C inactivation commits cells to cell cycle progression, that it could be inactivated or antagonized in cancer to promote proliferation, and the consequences of its activation state on cell cycle control, proliferative decision-making, and G1/S checkpoint function. Throughout the review, we often refer to APC/C as “active” or “inactive”, a clear over-simplification given the dynamic and complex enzymology of APC/C. Nevertheless, this captures the notion that at specific points in

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the cell cycle (e.g. late mitosis) its activity is very high, and at others (e.g. in S-phase) its activity is quite low or undetectable by conventional approaches.

2.2). Cyclin degradation and the discovery of APC/C

Cyclin-CDK complexes serve as signaling hubs that direct progression through the cell cycle. The activity and identity of Cyclin-CDK rose to prominence with the convergence of genetic screens in yeast and biochemical studies in human cells and amphibian oocytes. These latter studies uncovered diffusible factors, which could promote mitosis in cultured cells (399), and maturation in oocytes (400). The molecular identity of the maturation promoting factor (MPF) was pursued by several groups. Classic studies showed that MPF activity cycled as eggs moved through cell division and was precipitously lost at the end of mitosis (401). Then, leveraging a recently developed frog egg extract system, the Kirschner lab demonstrated that Cyclin synthesis, as well as Cyclin destruction, played an important role in the oscillating activity of MPF (402). Shortly thereafter, the identity of MPF became clear, along with the realization that it was composed of two components (24, 403, 404). One was Cyclin, which had been discovered years earlier as a protein whose abundance oscillates during early development in the embryos of sea urchins and clams (27, 405). The second was a kinase, later named CDK, that corresponded to the conserved Cdc2/Cdc28 kinase, which had been shown to be a key driver of cell cycle progression that was conserved across enormous evolutionary timescales (406, 407).

The embryonic Cyclins, Cyclin A and Cyclin B, first described by Hunt and colleagues, control MPF activity during early development (27, 405). While transcriptional changes could account for Cyclin accumulation and MPF activation, how MPF was inactivated at the end of mitosis remained elusive. The answer came with the discovery that Cyclin degradation is controlled by the ubiquitin pathway (174). It is notable that at the time that Cyclin was first observed, the ubiquitin system had just recently been discovered; however, Alexander

Varshavsky predicted the role of ubiquitination in controlling Cyclin abundance years before it was shown experimentally (408).

In 1995, companion papers from the Kirschner and Hershko labs reported the identification of a large, multi-subunit complex capable of promoting the ubiquitination of Cyclin B, which they independently named the Anaphase Promoting Complex and Cyclosome (APC/C), respectively (409, 410). Notably, many APC/C subunits had been identified decades earlier by Hartwell and colleagues as cell division cycle (*cdc*) mutants in budding yeast (411).

Since the discovery of APC/C, many labs have scrutinized when and how it is regulated and the mechanisms underlying the ubiquitination of its substrates. Most studies have focused on the essential role of the APC/C in mitotic exit. Importantly, APC/C is the key downstream effector of the mitotic spindle assembly checkpoint (SAC), which prevents exit from mitosis until all sister chromatid pairs achieve bipolar attachment to mitotic spindle microtubules. Interested readers are directed to reviews that describe the details of SAC signaling (14, 412). More recently, the role of mitotic post-translational modifications of APC/C in controlling its dynamic activity has furthered our understanding of the biochemical regulation of cell division and represents an important advance in our understanding of APC/C biology. Interested readers are pointed to papers detailing the role and mechanisms of phosphorylation (127, 410, 413–418), SUMOylation (419, 420), and acetylation (421).

In addition, while primarily studied in mitosis and G1 phase of actively proliferating cells, APC/C is re-activated after DNA damage. Specifically, DNA damage caused by doxorubicin during S/G2 leads to APC/C re-activation to avoid entry into mitosis while allowing cells to initiate repair (422). Furthermore, in the nervous system, APC/C restrains axonal growth to maintain neurons in a quiescent state (423, 424).

Over the last five years, advances in cryo-electron microscopy, coupled with the ability to purify the APC/C from baculovirus-infected insect cells, provided atomic level understanding of the APC/C structure and enzymology. Curious readers should refer to other, excellent reviews

discussing APC/C structure, enzymology, spatiotemporal regulation, and substrate recognition (162, 412, 425–427). Nevertheless, a brief introduction to APC/C structure and architecture follows in order to better understand its role and regulation in the cell cycle.

2.3). APC/C structure and function

The core of the APC/C is composed of at least fourteen different proteins, some in multiple copies, that assemble into a tremendous 1.2 MDa macro-molecular ubiquitinating machine. To our knowledge, APC/C represents the largest E3 ligase ever described. It is hitherto unknown why the APC/C is so large. To put its size in perspective, the APC/C is larger than the catalytic core of a ribosome. Many E3 ubiquitin ligases rely on substrate receptor subunits to designate targets for ubiquitination. Likewise, during somatic cell cycles, the APC/C uses either of two substrates receptors, Cdc20 and Cdh1/Fzr1 (hereafter referred to as Cdh1). The activity of APC/C depends on its association with these coactivators, whose primary role is to recruit substrates to the APC/C for ubiquitination.

The Cdc20-bound form of APC/C functions during the metaphase to anaphase transition where it catalyzes the degradation of Cyclin B and Securin to promote mitotic progression. Then in late mitosis, the Cdh1-bound form of APC/C is activated. Important for the discussion below, APC/C^{Cdh1} remains active throughout G1 as well as during quiescence (G0). The APC/C also relies on the sequential activity of a pair of E2 ubiquitin conjugating enzymes, UBCH10/UBE2C and UBE2S. First, UBE2C decorates substrates with a combination of very short ubiquitin chains and/or ubiquitin monomers. Then, UBE2S extends these chains, generating degradation signals for substrates (428, 429). Notably, UBE2S assembles non-canonical ubiquitin chains, linked through K11 in ubiquitin (430–433). In addition, more recent evidence points to an ability of APC/C to form branched or heterotypic chains, and these unique chain topologies could

provide strong signals for organizing the temporal ordering of substrate degradation at mitotic exit (148, 434, 435)

2.4). APC/C in G1 control

Since the APC/C literally “promotes anaphase,” it is essential for the exit from mitosis. However, the APC/C also plays an important and less well-understood role in G1. Similar to its role in mitosis, the function of APC/C during G1 is evolutionarily conserved and vital to homeostatic cell cycle dynamics.

Prior to the biochemical identification of APC/C, studies from Amon and Nasmyth demonstrated that proteolysis of the budding yeast B-type Cyclin Clb2 begins in mitosis but then continues into the ensuing G1. Therefore, the enzyme which catalyzes Cyclin destruction is active not just during mitosis but also in G1 phase (436). Cdh1 was subsequently identified as the APC/C substrate receptor that controls Clb2 degradation in G1 (437–439). Early experiments, foreshadowing the importance of APC/C^{Cdh1} in G1 maintenance, showed that the loss of Cdh1 could not be tolerated in budding yeast that had also lost the CDK inhibitor Sic1, which itself restrains S-phase entry (437, 438). This genetic relationship suggested that Cdh1 works in collaboration with other G1 restriction factors to restrain G1/S. Further studies in budding yeast highlighted the continued activity of APC/C^{Cdh1} up to the point of S phase entry (440). In the fission yeast *S. pombe*, Cdh1 promotes Cyclin B degradation in G1 phase (441). Similarly, Cdh1 prevents the unscheduled accumulation of Cyclin B during G1 in Drosophila (442). Likewise, Cyclin B is unstable when introduced into cell extracts produced from both quiescent and G1 phase cells but remains stable in extracts produced from cells in S phase (443). Thus, the activity of APC/C, which is initiated in mitosis to promote anaphase, continues throughout G1, and this functionality represents an evolutionarily conserved feature of the cell cycle.

These studies in diverse experimental systems pointed to a potential role for APC/C in G1 phase. The G1/S border represents a major barrier to proliferation in eukaryotes. During this time in the cell cycle, cells integrate diverse extracellular and intracellular signals to decide whether to enter the cell cycle. In support of a role for APC/C^{Cdh1} in G0/G1 maintenance and restraining DNA replication, Cdh1 depletion in budding yeast renders cells unable to arrest in G1 phase in response to the hormone alpha-factor, which induces a cell cycle arrest in wild-type cells (438). This important result pointed to a role for APC/C^{Cdh1} in preventing unscheduled proliferation. Consistently, Cdh1 loss in Drosophila leads to an extra cell cycle in the epidermis during development whereas its overexpression blocked cell division (442). Similarly, in chicken DT40 cells the loss of Cdh1 promoted the premature accumulation of Cyclins A and B in late G1 phase and rendered cells unable to arrest in G1 in response to pharmacological targeting of the mTOR pathway (444). Further confirmation that the premature accumulation of APC/C substrates led to an accelerated rate of G1 progression came from several groups using mouse embryonic fibroblasts that were deleted for Cdh1 (255, 445). These results have been further validated in diverse human cell systems using Cdh1 overexpression (446) and depletion (119, 445, 447–449), which altogether showed a critical role for Cdh1 in restraining progression through G1 and S phase entry. Finally, evidence that the role of Cdh1 in G1/S is directly related to the function of APC/C came from conditional knockout studies in mouse livers. The loss of APC/C activity, triggered by APC2 inactivation, caused cell cycle entry even in the absence of proliferative signals (450). Taken together, these studies demonstrated that the APC/C is active in G0 and G1 phase cells, where it acts as a critical restriction factor to prevent unscheduled proliferation. This places APC/C^{Cdh1} among a small group of key regulators known to restrain the entry of cells into S phase, including the retinoblastoma tumor suppressor (RB) and CDK inhibitory proteins (172, 451). In the next section, we describe emerging data highlighting the overlapping role of APC/C and RB in controlling the G1/S transition.

2.5). APC/C and RB coordinate G1/S progression

The G1/S boundary represents a major barrier to proliferation and oncogenesis. The G1/S border is controlled in part by CDK4/6 which binds to any of the three D-type Cyclins and phosphorylates the tumor suppressor protein RB (452). The hyper-phosphorylation of RB by Cyclin D-CDK4/6 and also by Cyclin E-CDK2 triggers its dissociation from the E2F transcription factor, initiating a feedback loop that promotes S phase entry (analogous proteins exist in yeast and their regulation of cell cycle entry is similar (453)). Due to its role in restraining G1/S, RB is a potent tumor suppressor in human cancers. RB is lost through mutation in a subset of malignancies, most notably in retinoblastoma and small cell lung cancer (454, 455). In addition, RB is functionally inactivated across diverse human cancers as evidenced by the aberrant expression of the E2F transcriptional program and resistance to CDK4/6 inhibition in specific, aggressive subtypes of cancers (456, 457).

Several studies have demonstrated a collaboration between APC/C^{Cdh1} and RB in restraining cell cycle entry. The first evidence came from a genetic screen in *C.elegans*, which identified Cdh1 as a synthetic genetic interactor with the sole worm RB orthologue, Lin-35 (458). Importantly, mutations in *Cdh1* and *Lin-35* led to profound hyperproliferative defects throughout the worm (458). Similarly, genetic studies in flies showed that forced E2F expression was insufficient to drive cell cycle entry, whereas concomitant activation of E2F together with loss of APC/C activity triggers proliferation (459). In human cells, there is evidence that their coordination could be direct: APC/C was identified in a proteomic screen for physical RB interactors. It was shown that Cdh1 is required for RB induced cell cycle arrest by triggering the degradation of the ubiquitin ligase that targets the CDK inhibitory (CKI) protein p27 (460). The molecular mechanisms of p27 regulation and activity are discussed later. Furthermore, Cdh1 is required for cell cycle arrest induced by CDK4/6 inhibition in both worms and human cells (461). Taken together, these studies point to an important role for APC/C function in restraining cell cycle entry in collaboration with RB. Consistent with the important tumor suppressor role for RB,

Cdh1 haploinsufficiency produces epithelial tumors in mice, suggesting a potential role in tumor suppression (255). However, Cdh1 is not recurrently mutated or transcriptionally silenced in human cancers. While this could be due to an essential function, consistent with bi-allelic loss being lethal in mice (255, 462), it raises the question as to whether Cdh1 is truly a tumor suppressor in human cancers.

Given its vital role in regulating the G1/S transition, it is essential to inactivate APC/C^{Cdh1} to execute timely S phase initiation. Several mechanisms of APC/C inactivation exist. The relationship and role of these pathways in potentially restraining APC/C in both normal and cancer cells is discussed below.

2.6). Mechanisms modulating APC/C inactivation

During progression through the cell cycle, APC/C is controlled by several overlapping and often interconnected mechanisms. The most well-studied mechanism is the control of APC/C activity by the mitotic spindle checkpoint, which keeps APC/C activity in check until metaphase, when all chromosome achieve bipolar microtubule attachments (412). However, numerous other pathways control APC/C activity in late G1, S, and G2 phases. Interestingly, the mechanisms governing APC/C, and the CDKs that it controls, are largely analogous. For example, as is detailed below, both APC/C and CDKs are controlled by coactivators that are essential for their activity and whose expression and degradation are tightly controlled throughout the cell cycle. Fig. 1 summarizes some of the mechanisms described below.

Gene expression: The expression of Cdc20 and Cdh1 is controlled at the mRNA level during the cell cycle. In systematic studies examining cell cycle transcriptional dynamics, both transcripts emerged in multiple studies displaying oscillatory expression. In addition, the APC/C utilizes two E2 ubiquitin conjugating enzymes, UBCH10/UBE2C and UBE2S, which are also controlled at the mRNA level throughout the cell cycle. In fact, in the five most well-validated

studies examining cell cycle transcriptional dynamics, UBE2S and UBCH10/UBE2C are among a small group of approximately 100 genes that show cell cycle regulation in all studies (463–467). Likewise, both Cdh1 and Cdc20 expression oscillated in four out of five of these studies. Much like the role of Cyclins expression in regulating CDK activity, the cyclical expression patterns of coactivator and E2 mRNAs suggest an important role for transcription in organizing the activity of APC/C during cell cycle.

Cdh1 phosphorylation: Cdh1 is a phosphoprotein with more than ten apparent CDK consensus motifs as well as other less well-studied phosphorylation sites (468). Phosphorylation of Cdh1 on these CDK sites plays an important role in preventing APC/C activation. In budding yeast, elegant gene replacement strategies have demonstrated the essentiality of Cdh1 phosphorylation at CDK consensus sites (469). Mechanistically, phosphorylation of Cdh1 regulates its function in at least two ways. First, Cdh1 phosphorylation regulates its binding to APC/C. A non-phosphorylatable version of Cdh1, harboring alanine mutations at 11 CDK phospho-consensus motifs, is constitutively bound to the APC/C and triggers the destruction of Clb2 (439). These sites can be dephosphorylated by the cell cycle regulated phosphatase Cdc14 (470). Consistent with the regulation of Cdh1 by CDK phosphorylation, the inactivation of APC/C requires the accumulation of S phase Cyclins, which are not under APC/C control in yeast. Accordingly, overexpression of Sic1, which inhibits both CDK activity and Cdh1 phosphorylation, reactivates the APC/C by promoting the association of Cdh1 with the APC/C complex (440). Cdh1 is also heavily phosphorylated during cell cycle progression in humans on its myriad of CDK consensus motifs and at other sites as well (363). Consistent with the aforementioned observations in yeast, a non-phosphorylatable mutant version of human Cdh1 constitutively binds and activates APC/C, impairing S phase entry (104, 171, 446).

In addition to controlling binding to the APC/C, Cdh1 phosphorylation also regulates its localization. It is important to note that the core APC/C complex is largely thought to localize to

the nucleus. In yeast, Cdh1 localization is cell cycle regulated and depends on CDK, with phosphorylation enhancing its nuclear export and contributing to APC/C inactivation (471). More recent studies showed that selected phosphorylation sites can control APC/C binding, whereas others are responsible for regulating Cdh1 localization (472). Likewise, in humans, a version of Cdh1 harboring alanine substitutions at CDK consensus motifs was exclusively nuclear, whereas a phospho-mimetic mutant version was cytoplasmic (473).

In human cells, the role of different Cyclin-CDK complexes in phosphorylating Cdh1 continues to expand, raising the important question as to the identity of the kinase(s) responsible for Cdh1 phosphorylation. Originally, Lukas et al. showed that Cdh1 co-precipitated with Cyclin A, but not Cyclin E, and that Cyclin A-Cdk2 could phosphorylate Cdh1 in an *in vitro* kinase assay (104), consistent with earlier findings in cell extracts (443). This evidence is corroborated by a recent study showing that Cdh1 is an excellent Cyclin A-CDK2 substrate (474). Perhaps most significantly, depletion of Cyclin A, but not Cyclin E, strongly increased the association of Cdh1 with the APC/C core complex (104). Since the substrate selectivity of Cyclin-CDK complexes most often relies on the Cyclin, these data indicate that in humans Cyclin A-Cdk2 or Cyclin A-Cdk1 phosphorylate Cdh1 to prevent its association with the APC/C complex. This suggests a positive feedforward loop wherein Cyclin A promotes its own stability by preventing activation of the ligase that catalyzes its degradation. Recent biochemical data showed that Cyclin B could also phosphorylate Cdh1 *in vitro*, adding an additional layer of regulation that likely accounts for the inability of Cdh1 to bind APC/C until late mitosis, only after Cyclin A and Cyclin B have been largely destroyed (475).

In contrast to these observations, more recent studies implicated additional Cyclin-CDK complexes in inhibiting APC/C. First, it was shown that the worm ortholog of Cdh1 could be phosphorylated by both human and worm Cyclin D-CDK4 complexes on several CDK consensus sites in the Cdh1 amino-terminus (461). These data were corroborated by a second study, which also showed that human Cdh1 was a substrate of Cyclin D-CDK4/6 (476). In a

third study, using single cell live imaging of APC/C biosensors, the authors showed that depletion of Cyclin E, but not Cyclin A, altered APC/C activation, although it is not known if this is due to direct phosphorylation of APC/C or Cdh1 by Cyclin E/CDK2 (449). At this time, it remains unknown whether Cdh1 is a promiscuous substrate that can be phosphorylated by these myriad kinases or if there is greater selectivity in vivo than what is currently appreciated. Resolving these differences and defining precisely when Cdh1 becomes phosphorylated, by which kinase, on which sites, and the importance of these modifications represents important future questions. Notably, in yeast, different phosphorylation sites on Cdh1 differentially contribute to changes in APC/C binding and localization (472).

Finally, it was recently shown that Cdh1 could be phosphorylated by a non-Cyclin-CDK entity, ERK, the downstream kinase in the MAPK cascade, and that phosphorylation also inhibits APC/C^{Cdh1} activity (476). It is notable that CDK and ERK are both proline directed kinases that phosphorylate serine and threonine residues that have a proline in the +1 position. Further, while most cell cycle focused research groups studying APC/C would envision these phosphorylation sites as being controlled by CDK, there is ample space to imagine the coordinate control by the MAPK pathway, which also plays an important role in controlling proliferation. Recent studies in yeast have highlighted cooperation between CDK and MAPK pathways in cell cycle associated signaling (477).

Emi1 binding: Early mitotic inhibitor 1 (Emi1) is a member of the F-box family, a set of substrate receptors proteins for the Skp1-Cul1-F-box (SCF) family of E3 ubiquitin ligases. In the SCF, F-box proteins, of which there are 69 in humans, designate substrates for degradation (478). However, while Emi1 can assemble into an SCF complex, it is unknown if it functions as a substrate receptor. Instead, Emi1 was identified and is known as an important inhibitor of APC/C (216, 479). In humans, Emi1 prevents the APC/C dependent degradation of Cyclin A and Cyclin B (480, 481). Furthermore, depletion of Emi1 causes cell cycle arrest in late S phase and G2 and re-replication that is due to the reactivation of APC/C (480, 481). The Emi1 ortholog

in Drosophila, Regulator of Cyclin A (RCA1), is so-named because it is also required to prevent Cyclin degradation in G2 phase (482). Moreover, *rca1* mutant fly imaginal cells have enlarged nuclei, consistent with re-replication due to endocycling (482). Emi1 is also reported to contribute to APC/C inactivation at the G1/S border. Emi1 overexpression can drive cell cycle entry and proliferation in cells overexpressing Cdh1 or RB (479). In addition, the rate of APC/C inactivation is slower in Emi1 depleted cells (449). However, few studies have reported a strong G1 arrest in Emi1 depleted cells. Thus, it is unknown to what extent Emi1 contributes to APC/C shut-off versus maintaining APC/C in an inactive state.

At the onset of mitosis, Emi1 becomes a substrate of another SCF ligase using the substrate receptor protein βTRCP1, and its destruction in early mitosis is dependent on the phosphorylation of Emi1 by PLK1 (220, 483–485). The degradation of Emi1 in early mitosis is vital to the subsequent activation of APC/C at the metaphase to anaphase transition.

Cdh1 and E2 degradation: Cdh1 protein levels oscillate during the cell cycle in both yeast and humans, lending credence to the notion that dynamics in Cdh1 abundance contribute to APC/C activity (171, 439). This phenomenon is analogous to the mechanism by which Cyclins control CDK. In human cells, Cdh1 levels are high in G1, decrease significantly in S phase, and then reappear in G2/M (119, 171, 474, 486, 487). Multiple E3 ligases have been implicated in Cdh1 destruction. First, APC/C was suggested to control its own degradation through auto-catalytic degradation (486). Later, the SCF family of E3 ubiquitin ligases were implicated in Cdh1 degradation (488). Two independent studies have recently implicated two different F-box substrate receptors in Cdh1 destruction.

First, the Wei laboratory showed that Cdh1 degradation could be triggered by SCF, in complex with its F-box protein, βTRCP (474). While βTRCP often binds to the degron sequence DSGxx(x)S, it recognizes a non-canonical D-box in Cdh1 (DDGxxxS). This recognition is controlled by sequential phosphorylation of the βTRCP degron in Cdh1, first by Cyclin A-CDK2 and then by PLK1. More recently, our laboratory showed that Cdh1 is also a substrate of Cyclin

F, the founding member of the SCF family of substrate receptors and a non-canonical Cyclin that neither binds nor activates a CDK (118, 119, 489). Notably, Cyclin F is among the most highly cell cycle regulated of all F-box proteins and is the *only* F-box containing protein whose mRNA emerged in all cell cycle transcriptional studies that have been performed to date (490). In a remarkable twist of fate, Cyclin F is itself a substrate of APC/C-mediated degradation in late mitosis and early G1 phase, suggesting a tightly coordinated, reciprocal relationship between Cyclin F and Cdh1 in regulating S phase entry. In addition, this latter study noted that the previously identified degron in Cdh1, which was thought to mediate its degradation by APC/C (486), overlaps with the Cyclin F binding sequence (119). Thus, the extent to which APC/C and $SCF^{Cyclin\ F}$ coordinate the destruction of Cdh1 at the G1/S border remains an open question and an important area of investigation. Furthermore, since PLK1 is only active in the hours preceding mitotic entry, it remains unknown how these convergent, SCF-related, proteolytic pathways coordinately control the abundance of Cdh1 throughout the cell cycle.

Finally, the two APC/C E2s, UBE2C and UBE2S, are both substrates of the APC/C (429, 491). The auto-catalytic degradation of its E2s suggests that the APC/C potentially functions as an “autonomous oscillator” that inactivates itself by destroying its own E2s after all of its substrates have been consumed (491). However, this interpretation is complicated by a conflicting study that analyzed UBE2C expression and role in cell cycle, and showed that it accumulates in late G1/early S phase before Cyclin A (492). Determining the mechanisms underlying degradation of the APC/C E2s and how their degradation contributes to S phase entry will be important to fully understand the nature of APC/C inhibition.

Deubiquitinating enzymes: Ubiquitin is conjugated to substrates through an isopeptide bond between its carboxyl-terminal amino acid (Glycine) and the epsilon amino group in substrate lysine residues. Similarly, the formation of polyubiquitin chains occurs by the addition of ubiquitin onto other ubiquitin molecules, either through any of the seven lysines in ubiquitin or through its amino terminus. These variations lead to different ubiquitin linkages with unique

physical topologies that promote diverse cellular outcomes. This is commonly referred to as the ubiquitin code (152). In the same way that phosphatases regulate kinase-signaling cascades by controlling the dephosphorylation of substrates, so-called deubiquitinating enzymes (DUBs) regulate ubiquitin signaling cascades to control the ubiquitination and degradation of substrates. There are approximately 100 DUBs in humans, which are implicated in various aspects of cellular physiology and disease (493). Because of its tremendous importance in cell physiology and disease, some studies suggest that the APC/C could be controlled by deubiquitination and several DUBs have been linked to APC/C and the degradation of its substrates.

In budding yeast, UBP15 deubiquitinates the Cyclin Clb5 and promoting S phase entry (494). In humans, USP37 was identified as an APC/C^{Cdh1}-associated deubiquitinating enzyme ((495), corroborated by our unpublished data) that promotes cell cycle progression via Cyclin A deubiquitination, and USP37 is also an APC/C substrate (495). Thus, USP37 and Cyclin F both negatively regulate APC/C and are also APC/C substrates, highlighting complex feedback mechanisms involved in APC/C control (495). In early mitosis, APC/C activation is prevented by the SAC, which sequesters Cdc20 away from APC/C in the mitotic checkpoint complex (MCC) (412). The inactivation of the SAC, and thus the full activation of APC/C, is driven by auto-ubiquitination of Cdc20 (496). The DUB USP44 emerged from an RNAi-based screen for regulators of the SAC (497). USP44 reversed Cdc20 ubiquitination, suggesting that USP44 is a SAC component. However, USP44 is unique among spindle checkpoint proteins in that it is non-essential, since USP44 knockout mice are viable and have a seemingly intact SAC (498).

Finally, we recently identified OTUD7B/Cezanne as a key DUB that antagonizes APC/C substrate ubiquitination. The specificity of Cezanne for specific ubiquitin chains has remained controversial, with the Komander group reporting specificity of Cezanne for K11-linked ubiquitin chains and others suggesting a role in K63 and K48-linked deubiquitination (499–502). Our results support an extraordinary specificity of Cezanne for K11-linkages and demonstrate that it is itself cell cycle regulated, and importantly, antagonizes APC/C substrate ubiquitination in

mitosis (156). Cezanne depletion induces defects in chromosome segregation and the formation of micronuclei, indicative of a role in cell division (156). In the future, it will be important to understand how Cezanne is regulated during cell cycle progression, how it binds substrates, and the mechanism by which it restrains APC/C-mediated substrate ubiquitination.

Together, these studies support the notion that the ubiquitinating activity of the APC/C is regulated and counteracted by DUBs. Since the APC/C is widely considered the master E3 ligase involved in cell division, it is highly possible that additional DUBs and new layers of regulation for the ones already described are yet to be discovered.

2.7). Convergent CDK-APC/C networks and commitment to S phase

In response to suboptimal proliferative conditions, cells arrest at a so-called “restriction point” prior to the start of S phase. These observations in human cultured cells date back several decades (503–505). The restriction point is controlled, in part, by the CDK-RB-E2F pathway. Despite the importance of CDK-RB in G1/S control, cells continue to proliferate in vivo and in vitro following pathway ablation (e.g. loss of CDK4 and CDK6) and, remarkably, have a functional G1/S checkpoint (506, 507). Therefore, additional pathways coordinate G1/S control and the commitment to cell cycle progression at the G1/S boundary.

A recent study from Cappell et al. shed light on the role of APC/C in the commitment to S phase. They demonstrated that APC/C^{Cdh1} inactivation at the G1/S boundary is rapid, exhibits characteristics of a bistable switch, and this switch to an “APC/C off” state is triggered by Cyclin E/CDK2 (449). Once inhibited, APC/C is irreversibly maintained in an inactive state by the inhibitory protein Emi1 (449). Together, these data suggest that Cyclin E initiates the inactivation of APC/C that is then locked down by Emi1. Using single cell fluorescent reporters combined with immunostaining, they also showed that RB phosphorylation precedes APC/C inactivation by several hours, and APC/C inactivation, not RB phosphorylation, temporally

coincided with S phase entry. The phosphorylation of RB has long been considered the point of no return in G1, after which cells are committed to starting S phase. Significantly, these recent studies showed that cells could still arrest in G1 following mitogen withdrawal or stress after RB had become phosphorylated, so long as APC/C had not yet been inactivated. These data led to the proposition that APC/C inactivation at G1/S represents a “commitment point” for cell cycle entry (449).

The above study places Cyclin E activation upstream of APC/C. In contrast, single cell analysis combined with molecular modeling had previously suggested that the key role of APC/C^{Cdh1} in controlling S phase entry was due to its role upstream of CDK2 activation (448). The activation of CDK2 is restrained by the CKI protein p27, whose degradation is controlled by an SCF ubiquitin ligase in combination with the adaptor protein SKP2. Importantly, SKP2 is a substrate for the APC/C (508, 509). Therefore, APC/C inactivation causes an increase in the abundance of SKP2. This allows SKP2 to assemble into SCF^{SKP2} ligase complexes and trigger p27 degradation. The degradation of CKI allows for full Cyclin E/CDK2 activation and entry into S phase. Thus, in cells lacking Cdh1, the amount of Cyclin E which is needed to drive S phase entry is lowered, explaining why cells without Cdh1 progress rapidly into S phase (448).

Notwithstanding the differences in mechanistic explanations between these studies, together, they highlight the central role of the APC/C in organizing regulatory networks involved in G1/S control. Modeling these systems and networks, incorporating additional regulators discussed above, and testing hypothesis derived from this modeling is an important area of future study. Doing so will further elucidate the intricate role of APC/C in cell cycle control and define the molecular features controlling the G1/S boundary, a major barrier to transformation which is perturbed almost universally in cancer.

2.8). Relevance of APC/C inactivation in cancer

APC/C^{Cdh1} is among a small group of key regulators implicated in restraining the start of DNA replication. The other key members of this group, RB (and its related pocket proteins) and CKIs, are definitively linked to cancer. As discussed above, RB is lost or functionally inactivated in many malignancies. In addition, the CKI $p16^{INK4A}$, which inhibits CDK4/6, is silenced and mutated in cancer, and the CKI p21 is a downstream transcriptional target of the tumor suppressor p53. However, it is largely unknown if Cdh1 is similarly inactivated in cancers.

The role of Cdh1 in restraining S phase is conserved throughout evolution and is evident in budding and fission yeast, flies, worms, chickens, mice, and humans. Moreover, single allelic loss of Cdh1 leads to the formation of epithelial tumors (255), pointing to a role in tumor suppression. APC/C^{Cdh1} assembly has been reported to be influenced by MAPK signaling, and Cdh1 loss cooperates with additional oncogenic lesions to promote transformation in melanocytes (476). However, Cdh1 is not recurrently mutated, deleted by copy-number loss, or transcriptionally silenced in human malignancies, suggesting that cancer cells might use alternative mechanisms to block APC/C^{Cdh1} function. Nevertheless, reductions in Cdh1 abundance have been noted in aggressive breast and colorectal cancers (510, 511). Since Cdh1 levels are reduced in S phase, these changes could be the indirect consequence of the start of S phase. Alternatively, transient reductions in Cdh1 levels could weaken the barrier to cell cycle entry, promoting S phase entry, similar to phosphorylation of RB, which drives and coincides with the start of S phase. Unlike RB, it is unknown if Cdh1 is repressed in cancer to lower the barrier to cell cycle entry. Moreover, and in contrast to RB, Cdh1 is essential for animal development and survival, with knockout mice embryos dying around day E9.5 (512).

The results above indicate that repression of Cdh1 dosage or activity could weaken the G1/S boundary and promote tumorigenesis. Importantly, changes in gene dosage play an important role in human cancers, highlighted by the fact that copy-number alterations represent the most pervasive recurrent changes across all human cancers. Moreover, in any given

cancer, copy-number changes are recurrent and not stochastic. For example, the landscape of copy-number changes observed in specific subtypes of breast cancer are distinct (e.g. amplification of 1q and 8q), not random, and point to a functional importance for these regions in disease (456, 513, 514). The enrichment of proliferative drivers in copy-number amplified regions (e.g. Myc on chromosome 8q), and loss of genes that impair growth in deletions, further supports this notion (515).

How then might APC/C^{Cdh1} be inactivated in human cancers? Studies to date highlight three potential mechanisms. The first comes from the analysis of the human papillomavirus (HPV) E7 oncoprotein. The E7 oncoprotein is well known as a suppressor of RB. A recent study showed that the presence of E7 leads to abnormally high-level expression of Emi1, the APC/C inhibitory factor (516). Elevated Emi1 in turn restrains the degradation of APC/C substrates and leads to mitotic abnormalities.

The next two studies come from our own laboratory. We previously showed that Cyclin F is targeted for degradation by APC/C in late mitosis and early G1, and subsequently, Cyclin F targets Cdh1 for degradation at the end of G1 and in S phase. Moreover, we demonstrated that Cyclin F is phosphorylated and activated by the oncogenic kinase AKT/PKB (517). AKT is a key downstream effector in the phosphoinositide 3-kinase (PI3K) signaling cascade (518). Importantly, we showed that interfering with AKT–mediated phosphorylation of Cyclin F altered the stability of both Cyclin F and Cdh1. Significantly, activating mutations in PI3K represent the most recurrent, activating mutations in all cancers (519). Thus, activation of PI3K-AKT could trigger the degradation of Cdh1 in human cancers, thereby lowering the barrier to cell cycle entry. These data also imply that APC/C is an information switchboard that integrates extracellular signaling through the PI3K-AKT cascade with the core of the cell cycle machinery. This is similar to that of CDK4/6, which integrates information on mitogen availability via the transcriptional induction of Cyclin D through the Ras-MAPK pathway (520).

The final mechanism relates to the aforementioned DUB Cezanne. As discussed above, changes in gene expression and gene copy number are hallmarks found in virtually all cancers. In many cases, including breast and ovarian cancers, amplifications on chromosome 1q represent a hallmark of disease. However, little is known about how 1q amplification drives oncogenesis. Interestingly, Cezanne is located at 1q22, the center of a genomic amplicon in breast cancer (521). In fact, Cezanne is one of three most amplified and over-expressed DUBs in all of breast cancers. Cezanne mRNA and gene copy number are increased in a remarkable 32% of breast cancers. By comparison, the Myc-oncogene is amplified or over-expressed in approximately 21% of breast tumors. Cezanne over-expression could have myriad effects on the cell cycle. By counter-balancing the APC/C (156), Cezanne could alter kinetics of mitosis that impact chromosome stability, as well as the G1/S commitment point, promoting aberrant cell cycle progression, a hallmark of cancer. Thus, Cezanne amplification/overexpression could contribute to tumorigenesis through various and yet to be characterized pathways, on top of its newly described role in restraining APC/C-mediated ubiquitination.

2.9). Future questions

There still remain many important questions to understand the role and regulation of APC/C in cell cycle entry at the G1/S boundary and how it coordinates with CDK-RB to facilitate proliferation. First, as discussed above, myriad mechanisms can contribute to APC/C inactivation, including Cdh1 phosphorylation and ubiquitination, E2 degradation, and Emi1 binding. However, it is unknown how these pathways interconnect and the relative utilization of each pathway in a specific cell line or system is unknown. It is also unknown if specific pathways are deployed to functionally inactivate distinct sub-populations of APC/C that in turn allow for the stabilization of specific substrates but not others. This is a particularly interesting idea given the localization of APC/C to distinct structures in the cell (162, 522–524). In

considering the role and potential importance of the diverse mechanisms which function to control APC/C, it is useful to consider the paradigm of Cyclin-CDK complexes, which are perceived as playing essential roles as core components of the cell cycle oscillator yet are individually dispensable for cell and animal growth and viability. Similarly, we imagine that each of the pathways described above cooperatively function to regulate APC/C but are likely individually dispensable, or play context dependent roles, and could be compensated for by other, redundant mechanisms.

Another open question relates to how APC/C substrates accumulate as cells enter S phase. When looking across various studies, it becomes clear that not all substrates accumulate with similar kinetics as cells enter S phase. This variability is likely controlled by substrate transcription, translation, and degradation by other E3s. In addition, emerging evidence has pointed to substrate level regulation of APC/C-mediated degradation, the paradigm being that of Cdc6, which becomes phosphorylated and protected from APC/C (525). The question of substrate accumulation kinetics is the mirror of the differential degradation kinetics observed at mitotic exit, where some proteins are degraded early and others much later. Although this notion is well documented in the case of mitotic substrates, where different binding affinities lead to different processivity rates, the mechanisms and enzymes underlying this regulation and the consequences on S phase entry and progression remain entirely unstudied.

Finally, like RB, APC/C represents a strong G1/S restriction factor and mounting evidence points towards its role in tumor suppression. In the same way that CDK4/6 inhibition “re-awakens” RB for therapeutic benefit, it will be interesting in the future to determine if APC/C can be similarly “re-awakened”. This could potentially be achieved by inactivating PI3K-AKT, since we have shown that this pathway activates Cyclin F to trigger Cdh1 degradation. Alternatively, one could imagine inhibiting the ligases that trigger Cdh1 degradation, despite the fact that such pharmacological tools are not yet available. Nevertheless, future studies are needed to understand how APC/C function changes and contributes to the process of tumorigenesis, and if

as we predict, APC/C activity is reduced in cancer to lower the barrier to cell cycle entry. Addressing these questions will provide vital insight regarding mechanisms that *guard* against the inappropriate or untimely inactivation of APC/C, a key *guardian* of cell cycle and genome integrity, and the consequences of this regulation in pathological settings.

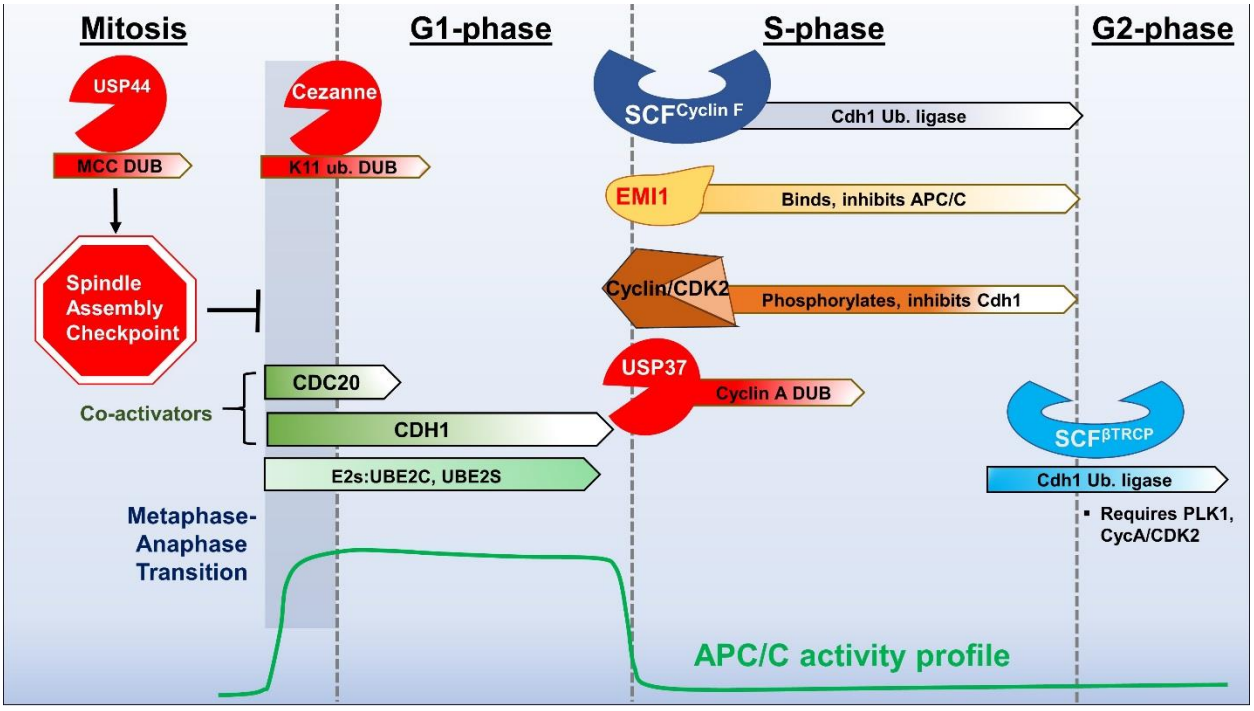

Figure 2.1. Schematic overview of the pathways that restrain APC/C activity post-translationally. The relative timing of their activation is generally shown.

The APC/C is most highly active from mid-mitosis and then throughout G1 phase. A schematic representation of the pathways that control APC/C and the degradation of its substrates during cell cycle progression is shown, with emphasis on those that operate outside of mitosis. Prior to metaphase, the activity of APC/C is prevented by the spindle assembly checkpoint, or SAC. The Cdc20-bound form is activated at metaphase (APC/C^{Cdc20}). In late mitosis, the Cdh1-bound form of APC/C becomes active (APC/C^{Cdh1}). The APC/C uses two E2 ubiquitin conjugating enzymes, UBE2C and UBE2S, which form K11-linked chains on substrates. The degradation of APC/C substrates is antagonized by the deubiquitinase Cezanne, which reverses K11-linked ubiquitin chains formed on APC/C substrates(156). The APC/C is then inactivated at G1/S through myriad mechanisms. The $SCF^{Cyclin\ F}$ E3 ubiquitin ligase triggers Cdh1 degradation(119). The APC/C inhibitory protein EMI1 accumulates and binds APC/C to block substrate ubiquitination(216, 479, 482). CDK2 phosphorylates Cdh1, preventing its binding to APC/C and promoting its cytoplasmic localization(104, 171, 439, 446). The APC/C E2s are also degraded(429, 491). A second ubiquitin ligase, $SCF^{\beta TRCP}$ controls the destruction of Cdh1 and Emi1 later, and the degradation of both via this mechanisms is dependent on PLK1, which becomes active just prior to mitotic entry(474, 483).

CHAPTER 3[2]: *IN SILICO* APC/C SUBSTRATE DISCOVERY REVEALS CELL CYCLE DEGRADATION OF CHROMATIN REGULATORS INCLUDING UHRF1

3.1). Introduction

Regulated protein degradation is central to cell and organismal physiology and plays a particularly important role in proliferation. In eukaryotes, protein degradation is controlled largely by the ubiquitin (Ub) system. E3 Ub ligases provide substrate specificity and facilitate the transfer of Ub onto substrates. The formation of poly-Ub chains on substrates provides a signal that often targets substrates to the proteasome for degradation (526).

The Anaphase-Promoting Complex/Cyclosome (APC/C) is a 1.2 megadalton, multi-subunit E3 ligase and essential cell cycle regulator. APC/C utilizes two coactivators, Cdc20 and Cdh1, which directly bind substrates, recruiting them to the E3 complex (425). APC/C^{Cdc20} becomes active in mid-mitosis and promotes the metaphase to anaphase transition. APC/C^{Cdh1} becomes active in late mitosis and remains active until the end of G1, during which time it prevents S-phase entry (162). Thus, APC/C^{Cdc20} and APC/C^{Cdh1} play opposing roles, the former promoting cell cycle progression in mitosis and the latter inhibiting cell cycle progression in G1.

In addition to its role in normal cell cycles, APC/C dysfunction has been implicated in disease. Cdh1 is a haploinsufficient tumor suppressor in mice and cooperates with the retinoblastoma protein to restrain proliferation (255, 449, 458, 459, 527). Several oncogenic

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kinase cascades impinge on Cdh1 function, further supporting a role for APC/C^{Cdh1} in tumor suppression (476, 517, 528). In addition, the APC/C subunit Cdc27 is mutated in cancer and associated with aneuploidy (529). APC/C is also linked to inherited disorders that give a range of disease phenotypes, including microcephaly, cancer predisposition, and skeletal abnormalities (530, 531).

Cdh1 and Cdc20 bind substrates through short, linear sequence motifs termed degrons. The most well-defined APC/C degron motifs are the KEN-box and D-box (532, 533). In addition, binding of Cdc20 and Cdh1 to APC/C promotes a conformational change in the E3 that stimulates ligase activity (164). This results in substrate poly-ubiquitylation by its two cognate E2 enzymes. UBE2C/UbcH10 deposits the first Ub monomers onto substrates and forms short Ub chains, whereas UBE2S elongates poly-Ub chains (148, 183, 190, 429).

Most known APC/C substrates are linked to cell cycle processes, including mitotic progression, spindle function and DNA replication. The paramount importance of APC/C in cell cycle and non-cell cycle processes, and its dysfunction in disease, highlight the importance of systematically defining substrates, whose regulation (or dysregulation), will likely contribute to proliferation and disease phenotypes. Nevertheless, barriers exist to the identification of APC/C substrates, as well as most other E3s. E3-substrate interactions are dynamic and binding often triggers substrate proteolysis. Additionally, the abundance of most substrates is low, and for APC/C, most targets are cell cycle regulated. Furthermore, since APC/C is a massive complex with many substrates, the relative binding stoichiometry to each individual substrate is low. Finally, degron sequences are short and occur vastly across proteomes, making it difficult to predict substrates.

We developed a simple *in silico* approach to identify potential APC/C targets. We took advantage of common features among known substrates, namely their transcriptional regulation during cell cycle and the presence of a degron motif. Super-imposing these features onto the proteome enriched for substrates and suggested previously undescribed targets.

This analysis revealed a role for APC/C in chromatin biology. We validate several substrates involved in chromatin dynamics, highlighting a previously underappreciated role for APC/C in chromatin regulation. We further define the mechanisms of ubiquitylation for UHRF1 (Ubiquitin-like with PHD and RING finger domains 1), a multivalent chromatin binding protein and itself an E3 ligase that can ubiquitylate histone H3 (321, 335–337). UHRF1 plays an important role in DNA methylation and has been implicated in other DNA templated processes, including DNA repair (317, 318, 365). Additionally, UHRF1 is suggested to be an oncogene, whose expression correlates with high tumor grade and poor prognosis (375, 379, 534). Altogether, these results reveal a role for APC/C-dependent UHRF1 degradation in cell cycle progression and shaping the DNA methylation landscape. More broadly, our data suggest that cell cycle regulated protein degradation helps organize the epigenetic landscape during proliferation. This suggests a potential mechanistic link contributing to changes in the chromatin landscape observed between proliferating and non-proliferating cells (535, 536). We predict that altering APC/C function could promote changes in the histone and DNA modification landscape, and that these effects could contribute to the biochemical and phenotypic features of diseases, including cancer and neurological disorders.

3.2). Results

Identification of APC/C substrates

To identify human APC/C substrates, we first performed FLAG immunoprecipitations (IP) from HEK-293T cells expressing amino-terminal tagged FLAG-Cdh1 or an empty vector and analyzed precipitated proteins by mass spectrometry (Table S1). Several APC/C complex components and known substrates, including Rrm2, Kif11, Claspin, and cyclin A were enriched in Cdh1 pulldowns. Compared to a previously established dataset (537), we identified 15 out of 53 known substrates. However, hundreds of proteins were enriched over controls and many

known substrates scored weakly, confounding our ability to prioritize candidates. For example, a single spectral count was observed for the substrate Kif22/KID (538, 539).

We considered computationally identifying substrates based on features common among substrates. APC/C binds substrates most often through D- and KEN-box degron motifs. The minimal D-box motif (R-x-x-L) is present in most human proteins and insufficient as a prediction tool. The KEN-motif is found in approximately 10% of human proteins (2,206; Table S2), and several D-box regulated substrates also contain a KEN-motif, including Securin and Cdc6 (68, 540). In addition, the gene expression of most APC/C substrates oscillates during the cell cycle (541). We cross-referenced the KEN-motif containing proteins against a set of 651 proteins whose mRNAs scored in at last two cell cycle mRNA profiling studies (463–465, 467). Overlapping the 2,206 KEN-motif containing proteins with 651 transcriptionally controlled genes produced a set of 145 proteins, which represent known and putative APC/C substrates (Fig. 1A, Table S2).

We compared our *in silico* analysis with two previously curated datasets, one containing 53 known APC/C targets (537), and a second containing 33 specifically KEN-dependent APC/C substrates (533). When compared to these lists of 53 and 33 substrates, our dataset captured 26 and 22 of them, respectively, the latter representing an enrichment of more than 140-fold, compared to what would be expected by chance (Fig. 1B). We also compared our data to other studies that identified APC/C substrates, interactors, proteins degraded at mitotic exit, or proteins ubiquitylated in mitosis (Table S3) (537, 542–547). Our *in silico* analysis identified the most KEN-dependent substrates relative to these studies (Figure 1C; Table S3). When compared to the set of 53 substrates, which includes both D- and KEN-box dependent substrates, our dataset captured 26 out of 53 known substrates, despite not focusing on D-box substrates. Combining the *in silico* predictions with our Cdh1-pulldown proteomics data, we captured 31 out of 53 substrates.

Among the 145 computationally identified known and potential substrates, gene ontology (GO) analysis showed a strong enrichment for processes linked to various aspects of cell division (Fig. 1D). Manual curation demonstrated that nearly half of the proteins we identified (70 of 145) have well-established roles in cell cycle. These were sub-classified into the sub-categories of cytoskeleton and motors, centromere-kinetochore, APC/C and spindle checkpoint, cytokinesis, mitotic entry, cell cycle transcription, cohesion and condensation, and DNA replication (Fig. 1E). Among these 70, 50% have literature evidence for regulation by APC/C, highlighting our enrichment for APC/C substrates (Fig. 1E; shown in magenta). All 145 proteins, their known function, sub-category, KEN-box sequence motif with flanking sequence, aliases, and citations describing regulation by APC/C are detailed in Table S2.

Regulated degradation of chromatin factors

Unexpectedly, our dataset revealed several proteins involved in chromatin regulation (Fig. 2A) and an enrichment for GO processes related to chromatin (Fig. 2B). The dataset includes readers and writers of histone post-translational modifications, including the lysine acetyltransferases, PCAF/KAT2B and NCOA3/KAT13B, the lysine methyl-transferase MLL2/KMT2D, the chromatin reader and histone Ub ligase UHRF1, and the mitotic histone H3 kinase Aurora B (Fig. 2A and 1E). We identified proteins involved in chromatin assembly and structure, including: CHAF1B, a component of the CAF-1 nucleosome assembly complex; TTF2, a Swi2/Snf2 family member and DNA-dependent ATPase; KI-67, which prevents chromosome aggregation in mitosis and regulates histone post-translational modifications; and proteins associated with cohesion and condensation, including SMC4 and NIPBL (Fig. 1E). We also identified proteins involved in DNA damage repair.

To validate potential substrates, we developed an *in vivo* APC/C activation assay that is amenable to analysis of endogenous or exogenously expressed proteins, and which is similar to approaches described elsewhere (548). U2OS cells were synchronized in mitosis with the microtubule poison nocodazole. After harvesting cells by mitotic shake-off, CDK1 was

inactivated with either the CDK1-specific inhibitor RO-3306 or pan-CDK inhibitor Roscovitine, driving cells out of mitosis and triggering APC/C activation and destruction of substrates, including FoxM1, NUSAP1, and Cyclin B (Fig. 2C, Fig. S1) (549).

Using a combination of exogenous expression and endogenous protein analysis, we examined the levels of chromatin related proteins not previously shown to be APC/C substrates. Using this assay, there was a decrease in the levels of several writers of histone modifications, including UHRF1, PCAF, TTF2, and NCOA3 (Fig. 2C, S1A, S1B). We observed a decrease in the levels of the chromatin assembly factors NASP and CHAF1B as well as the RNA processing proteins LARP1 and LARP7 (Fig. 2C, S1A, S1B). All of these have been previously identified as ubiquitylated in proteomics studies by an unknown E3 ligase (117, 550–553).

Since the role of APC/C in chromatin regulation is not well established, we focused our attention on the potential regulation of chromatin proteins by APC/C. We determined the ability of a subset to bind Cdh1 by coIP. CHAF1B, PCAF, NCOA3, and TTF2 interact with Cdh1 by coIP in 293T cells (Fig. 2D-2G). Accordingly, the levels of endogenous CHAF1B, TTF2, and NCOA3 oscillate during the cell cycle in U2OS, analyzed following a nocodazole-induced block in mitosis and then release into the cell cycle (Fig. 2H). PCAF levels did not decrease at mitotic exit in U2OS (Fig. S1C) but do decrease at mitotic exit in HeLa cells (Fig. S1C), suggesting a potentially complex regulation. Finally, we purified recombinant TTF2 and found that APC/C could trigger its ubiquitylation *in vitro* (Fig. S2). A table of all proteins tested in these assays and their validation is shown in Table S4. Taken together, this analysis uncovered new APC/C substrates and a role for APC/C in controlling chromatin regulators.

UHRF1 regulation by APC/C^{Cdh1}

To further understand the function of APC/C in chromatin biology, we pursued UHRF1, a key chromatin regulator that reads and writes histone modifications. UHRF1 associates with the DNA methyltransferase DNMT1 and is required for DNA methylation (317). UHRF1 has also

been implicated in replisome assembly (358, 554) and its phosphorylation oscillates during the cell cycle (363).

We examined UHRF1 protein levels following a mitotic block and release. Immunoblotting for UHRF1 and other cell cycle markers showed that UHRF1 protein levels decrease during mitotic exit in HeLa S3, HeLa, and U2OS cell lines (Fig. 3A, S3A-B). In each cell line, UHRF1 levels remain low in G1 and then re-accumulate starting around G1/S, based on the expression of other cell cycle markers, including cyclin E and cyclin A, and then further increasing throughout the subsequent G2/M phase.

We performed several assays to assess whether UHRF1 is regulated by APC/C. We analyzed UHRF1 in the aforementioned *in vivo* APC/C activation assay. U2OS cells were arrested in mitosis and then treated with RO-3306. We observed a decrease in UHRF1 that was partially mitigated by the proteasome inhibitor, MG-132, indicating that the reduction is dependent on the proteasome (Fig. 3B). In addition, transient siRNA depletion of Cdh1 (Fzr1 mRNA transcript) augmented UHRF1 protein levels (Fig. 3C). Conversely, ectopic expression of increasing concentrations of FLAG-Cdh1 led to a dose-dependent decrease in both exogenous and endogenous UHRF1 protein levels (Fig. 3D). We examined UHRF1 levels in cells that were first synchronized in G1 by a mitotic block and release, and then treated with the pharmacological APC/C inhibitor proTAME for 90 minutes (Fig. S3C). This led to an increase in UHRF1 levels. Together, these data suggest that APC/C controls UHRF1 *in vivo*.

UHRF1 ubiquitylation by APC/C^{Cdh1}

UHRF1 is a multi-domain protein (Fig. 4A) that exhibits multivalent binding with chromatin through histone and DNA binding domains (321, 346, 555). Additionally, UHRF1 is a RING domain E3 that ubiquitylates histone H3 (335–337). To determine whether UHRF1 is a direct APC/C^{Cdh1} substrate, we tested its binding to Cdh1 by expressing HA-Cdh1 and Myc-UHRF1 in 293T cells. Cells were treated with the proteasome inhibitor MG-132 prior to

harvesting to prevent UHRF1 degradation. Myc-UHRF1 was enriched in the HA-Cdh1 pull-down, and HA-Cdh1 was enriched in the Myc-UHRF1 pull-down (Fig. 4B, 4C).

Next, we purified and fluorescently labeled recombinant, bacterially expressed, full-length (FL) UHRF1 (FL-UHRF1*, where the * denotes fluorescently labelled protein). We found that FL-UHRF1* was ubiquitylated in an APC/C- and Cdh1-dependent manner using an entirely *in vitro* recombinant system (Fig. 4D). Multiple, high molecular weight ubiquitylated forms are observed using either wild-type Ub or methylated-Ub, the latter of which cannot form poly-Ub chains. This indicates that APC/C ubiquitylates multiple lysines in UHRF1 (Fig. 4D, S4A-B).

Since UHRF1 can auto-ubiquitylate itself through its RING domain, we confirmed that its ubiquitylation is APC/C dependent. First, we purified a version of APC/C selectively missing the APC2 WHB domain and the APC11 RING domain, which are required to recruit its initiating E2 UBE2C (designated ΔRINGΔWHB) (187, 556). This version of APC/C was unable to ubiquitylate UHRF1 (Fig. 4E).

Next, we purified and fluorescently labeled a truncated version of UHRF1 that contains the Linker, PHD, and SRA domains (termed LPS), spanning amino acids 287-715 (Fig. 4A). The LPS fragment omits three potential APC/C D-box degron motifs, as well as the RING domain, precluding auto-ubiquitylation. A D-box motif remains in the highly structured SRA domain but is unlikely to be accessible as a degron motif (557).

Significantly, LPS-UHRF1* is more robustly ubiquitylated in an APC/C- and Cdh1-dependent manner compared to FL-UHRF1* (Fig. 4D-E). Moreover, UHRF1 ubiquitylation is fully inhibited by the APC/C inhibitor Emi1 (Fig. 4F). Ubiquitylation of UHRF1 is initiated by APC/C^{Cdh1}-UBE2C while APC/C^{Cdh1}-UBE2S elongates Ub chains, indicating that UHRF1 ubiquitylation is similar to that of other substrates tested in this *in vitro* system (Fig. 4F). We conclude that UHRF1 is a *bona fide* APC/C substrate.

The ubiquitylation of truncated LPS-UHRF1* (Fig. 4D, 4E, 4F) strongly suggests the importance of the KEN-motif, located in an unstructured region at amino acids 622-624 (Fig.

4A). Alanine substitutions were introduced into the KEN sequence (UHRF1$^{KEN:AAA}$). The KEN mutant version (Myc-UHRF1$^{KEN:AAA}$) showed reduced, although not completely abolished, binding to HA-Cdh1 by coIP, compared to Myc-UHRF1^{WT} (Fig 4G). Additionally, the KEN mutant versions of FL-UHRF1* and LPS-UHRF1* were completely resistant to ubiquitylation by APC/C (Fig. 4H). We conclude that UHRF1 ubiquitylation by APC/C^{Cdh1} is dependent on its KEN-box motif.

APC/C substrates are recruited by Cdc20 and Cdh1, and many substrates can be controlled by both. To test if UHRF1 is controlled by APC/C^{Cdc20}, in addition to APC/C^{Cdh1}, we used a phosphomimetic version of APC/C (termed pE-APC/C) that can utilize either Cdc20 or Cdh1, since Cdc20 cannot bind to unphosphorylated APC/C (556). Surprisingly, unlike other, well-established APC/C substrates, including Cyclin B (CycB^{NTD}, amino acids 1-95) and Securin, the FL-UHRF1* and LPS-UHRF1* were ubiquitylated by APC/C^{Cdh1} but not by APC/C^{Cdc20} (Fig. 4I, S4C).

We transiently expressed FLAG-Cdh1 in HEK-293T cells in combination with either Myc-UHRF1^{WT} or mutant versions harboring alanine mutations in either the KEN-box (Myc-UHRF1$^{KEN:AAA}$) or the fourth D-box motif (Myc-UHRF1^{D4}). Ectopic FLAG-Cdh1 overexpression triggers the degradation of Myc-UHRF1^{WT} and Myc-UHRF1^{D4}, whereas Myc-UHRF1^{KEN} is resistant to degradation (Fig. 5A), further supporting the importance of the KEN-motif in UHRF1 degradation.

Next, we generated cell lines constitutively expressing GFP-tagged UHRF1^{WT} or UHRF1$^{KEN:AAA}$ using lentiviral transduction and examined UHRF1 stability upon mitotic exit. Exogenous UHRF1 levels were only moderately overexpressed compared to endogenous levels (Fig. 5B). Following synchronization with nocodazole, GFP-UHRF1^{WT} levels decrease at mitotic exit. Conversely, GFP-UHRF1$^{KEN:AAA}$ levels remain stable through mitotic exit and G1 phase (Fig. 5B). Cells expressing GFP-UHRF1$^{KEN:AAA}$ exit mitosis normally based on immunoblotting for the APC/C substrates cyclin A, cyclin B, cyclin F and Aurora A, which are degraded with

normal kinetics (Fig. 5B). Thus, the KEN box regulates UHRF1 ubiquitylation *in vitro* and degradation *in vivo*. In addition, the mild over-expression of UHRF1 in these cells does not affect overall APC/C activity.

UHRF1 degradation and cell cycle progression

Since many APC/C substrates are linked to proliferative control, we examined the contribution of UHRF1, and its degradation by APC/C, to cell cycle. Consistent with prior reports, UHRF1 depletion increased the fraction of cells in G1-phase ((374); data not shown). To further investigate the role of UHRF1 in cell cycle, we examined mitotic cells following UHRF1 depletion. We observed an approximately three-fold increase in cells with mis-aligned chromosomes in metaphase and anaphase in UHRF1 depleted cells using two independent siRNA oligonucleotides (Fig. S5A). Surprisingly, there was no statistically significant difference in the overall percent of mitotic cells.

To determine the role of UHRF1 degradation in cell cycle, we examined cell cycle markers in cells expressing UHRF1^{WT} or UHRF1$^{KEN:AAA}$. In Hela cells traversing the cell cycle after synchronization at G1/S, following a double thymidine block and release, we found that the GFP-UHRF1$^{KEN:AAA}$ cells contain more of the G1/S regulator cyclin E (Fig. S6A). This was also evident in cells that had been synchronized in mitosis and released into G1 (Fig. 5B). This suggested that an inability to degrade UHRF1 in G1 alters cyclin E expression, a key driver of S-phase entry.

These data suggested that UHRF1 might promote progression into S-phase and that a failure to degrade UHRF1 could shorten the duration of G1. To better address this possibility, we depleted endogenous UHRF1 with an shRNA targeting the UHRF1 3'UTR (327). Cells expressing GFP-UHRF1^{WT} or GFP-UHRF1$^{KEN:AAA}$ were synchronized in mitosis, released into the cell cycle, and analyzed by immunoblot. Several markers of S-phase entry accumulate early in cells expressing GFP-UHRF1$^{KEN:AAA}$ compared to GFP-UHRF1^{WT}. Both cyclin E and the G1/S

transcription factor E2F1 are elevated at early time points following release from mitosis (Fig. 6A). Elevated levels of cyclin E and E2F1 are evident in asynchronous RPE1-hTRET cells, and to a lesser extent in asynchronous HeLa S3 cells, where cell cycle transcription is perturbed due to HPV oncoproteins (Fig. S6B, S6C).

To analyze G1 duration, cells were release from a mitotic block and pulsed with EdU prior to harvesting for flow cytometry, to determine the percent of cells that were in S-phase. GFP-UHRF1$^{KEN:AAA}$ expressing cells begin S-phase earlier than control cells (Fig. 6B). Six hours after release into the cell cycle, 3.6% of control cells had entered S-phase, whereas 9.6% of GFP-UHRF1$^{KEN:AAA}$ expressing cells had started S-phase. Thus, a failure to degrade UHRF1 accelerates G1, indicating a key role for UHRF1 destruction in determining timing between the end of mitosis and start of DNA synthesis.

UHRF1 degradation and DNA methylation homeostasis

UHRF1 is required for DNA methylation maintenance (317). To determine if stabilizing UHRF1 in G1 affects DNA methylation, we performed base-resolution DNA methylation analysis at approximately 850,000 unique human CpG loci spanning all genomic annotations and regulatory regions using the Infinium Methylation EPIC BeadChip (EPIC arrays) (558, 559). We compared parental U2OS cells and those expressing GFP-UHRF1^{WT} or GFP-UHRF1$^{KEN:AAA}$. Considering all probes, DNA methylation changes between parental, GFP-UHRF1^{WT}, and GFP-UHRF1$^{KEN:AAA}$ were insignificant (Fig. 7A). However, multidimensional scaling (MDS) of the top 50,000 variable CpG probes among all samples/replicates (agnostic of sample group) clustered experimental conditions (Fig. 7B), indicating a unique and reproducible profile of methylation patterning.

We queried the GFP-UHRF1^{WT} and GFP- UHRF1$^{KEN:AAA}$ samples for differentially methylated CpGs relative to the parental controls. Consistent with a previous report (379), expression of GFP-UHRF1^{WT} and GFP-UHRF1$^{KEN:AAA}$ induced a comparable number of

hypomethylation events (Fig. 7C). Alternatively, GFP-UHRF1$^{KEN:AAA}$ induced approximately two-fold more hypermethylated CpGs compared to GFP-UHRF1^{WT} (Fig. 7C). Analysis of differentially methylated CpG probes between GFP-UHRF1^{WT} and GFP-UHRF1$^{KEN:AAA}$ revealed a 32% overlap in hypomethylated probes and a 17% overlap in hypermethylated probes (Fig. 7D). Significantly, hypermethylated CpG probes in the GFP-UHRF1$^{KEN:AAA}$ expressing cells were 2.5-fold more abundant compared to GFP-UHRF1^{WT}, despite no significant change in hypomethylated CpG probes. Thus, the non-degradable form of UHRF1 induces site-specific DNA hypermethylation (Fig. 7D).

The CpGs that were hypermethylated in GFP-UHRF1$^{KEN:AAA}$ -expressing cells started with a higher methylation level than other categories and gained methylation due to expression of non-degradable mutant (Fig. 7E). Enrichment analysis of the differentially methylated CpGs revealed that gene body annotations, including exons, introns, and transcription termination sites (TTS), were positively enriched for hypermethylation in GFP-UHRF1$^{KEN:AAA}$ -expressing cells (Fig. 7F, left panel). We next queried enrichment of differential methylation events in regions of early and late replication (560). Hypermethylation events in GFP-UHRF1$^{KEN:AAA}$, but not GFP-UHRF1^{WT}, were positively enriched in early replicating regions of the genome, while hypomethylation events by both GFP-UHRF1^{WT} and GFP-UHRF1$^{KEN:AAA}$ (alone or shared in common) were enriched in late replicating DNA (Fig. 7F). The enrichment of these hypermethylated features was consistent with known DNA methylation patterns that occur across gene bodies and early replicating DNA (Fig. 7E), as CpG loci in these regions typically demonstrate high levels of methylation (561, 562) . Taken together, these results demonstrate that expression of non-degradable UHRF1 enhances methylation at gene-rich, early replicating regions of the genome.

3.3). Discussion

Identification of new E3 ligase substrates

APC/C is a core component of the cell cycle oscillator and mounting evidence points to its dysfunction in cancer and neurological disease. Here we provide a comprehensive, unencumbered, annotated list of known and candidate APC/C substrates. Our data highlights the importance of APC/C in various aspects of proliferative control and points to its potentially broader impact on unanticipated cellular processes, including chromatin organization.

Identifying E3 substrates remains technically challenging. Since E3-substrate interactions exhibit low stoichiometry, mapping substrates by defining interactors is difficult. In addition, Ub ligase substrates are often in low abundance. APC/C is inhibited throughout the cell cycle by myriad mechanisms (563) and the time when it binds substrates coincides with when targets are being degraded and their abundance is lowest. This complicates many proteomics-based approaches. Alternative techniques for identifying E3 ligase substrates, including Global Protein Stability Profiling (GPS) and *in vitro* expression cloning, circumvent these challenges by measuring changes in substrate stability using fluorescent reporters or metabolic labeling with radioisotopes. These represent powerful tools for mapping E3 substrates (117, 564). However, both approaches are laborious and time intensive, require significant technical expertise, and depend on gene expression libraries, which are neither complete nor available to most laboratories.

We bypass these challenges using a simple *in silico* approach based on publicly available information, which is simple, inexpensive, and easily repeated with different variables. While our approach shares some similarities with previous approaches, it improves upon those in its simplicity, expanded use of multiple cell cycle mRNA datasets, and inclusion of a degron motif in the search criteria (538, 541, 565). Its success stems from the use of orthogonal filtering criteria, that is, unlinked features between mRNA and proteins. We predict that similar uses of unrelated properties could be leveraged for mapping targets of other enzymes, such as kinases,

where defining substrates has proven similarly challenging. It is notable that degron sequences remain unknown for most Ub ligases, highlighting the importance of mechanistic studies in enabling systems-level discoveries.

Involvement of APC/C in chromatin regulation

Determining the enzymes and substrates in kinase signaling cascades has been instrumental in determining proliferative controls in normal cells, their responses to stress and damage, and disease phenotypes and treatments. Relatedly, decoding Ub signaling pathways involved in proliferation is likely to provide insight into enzyme function in normal cell physiology as well as in disease.

A major finding of this work is that numerous chromatin regulators are controlled temporally during proliferation by APC/C. Impairing the degradation of one such substrate, UHRF1, altered the timing of cell cycle events and changed global patterns of DNA methylation. Since numerous chromatin regulators are controlled by APC/C, we anticipate widespread, pleiotropic effects on chromatin in cells where APC/C activity is impaired, either physiologically or pathologically.

Our observations raise the possibility that dysregulation of the cell cycle machinery, as is seen in diseases such as cancer, could alter the chromatin environment. The discovery that many chromatin regulators are mutated in cancer, a disease of uncontrolled proliferation, together with our data, imply a bidirectional relationship between the chromatin landscape and the cell cycle oscillator. Consistent with the notion that dysregulation of APC/C controlled proteins could play important roles in determining the chromatin environment in disease, the mRNA expression of our 145 known and putative substrates strongly predict breast cancer aneuploidies and copy number variations (Fig. S7). This observation is not due solely to the selection of specific breast cancer subtypes, since our gene signature is elevated in multiple breast cancer subtypes. Interestingly, the expression of this signature correlates with the CIN70

signature, which was previously developed based on gene expression in chromosomally unstable cancers (566). We observed an extraordinary correlation between the CIN70 and our 145 gene signature in breast cancer (Fig. S7). This is remarkable since our signature was generated completely independent of gene expression in cancer and was instead derived, in part, by short sequence motifs on proteins.

APC/C^{Cdh1}, but not APC/C^{Cdc20}, ubiquitylates UHRF1. This is notable because the Cdh1-bound form of APC/C is active both G1 and quiescent cells and is critical for restraining S-phase entry. Our findings suggest that impaired UHRF1 degradation promotes a premature G1/S transition. We propose that the proper degradation of UHRF1, and other chromatin regulators, serves to integrate growth factor dependent proliferative decisions with the chromatin regulatory environment. This could help explain the complex chromatin rearrangements observed in quiescent cells, where APC/C^{Cdh1} is active (270, 535, 536). Further, APC/C controls key cell cycle transcriptional regulators, including the G2/M transcription factor FoxM1 and the repressor E2F proteins, E2F7 and E2F8 (567, 568). Thus, our data point to a higher order role regulatory role for APC/C in gene regulation, by controlling transcription factors (i.e. FoxM1), transcriptional repressors (i.e. E2F7, E2F8,) and chromatin modifiers.

Aberrant DNA methylation is a hallmark of cancer (229). UHRF1 promotes DNA methylation maintenance, and too much or too little UHRF1 expression is detrimental to methylation stasis (317, 379). It is interesting to speculate that the redistribution of DNA methylation in disease could be caused, in part, by the aberrant stabilization of UHRF1, resulting from APC/C^{Cdh1} inactivation. It will be important, in the future, to determine if oncogene activation acts through the APC/C to re-organize the chromatin landscape. Furthermore, determining ubiquitin ligase substrates, like UHRF1, that might be dysregulated in pathological settings via altered degradative mechanisms could suggest therapeutic strategies to reverse their effects.

3.4). Materials and Methods

Computational identification of putative APC/C substrates

Human proteins containing a KEN-box sequence (amino acid sequence K-E-N) were identified using the "Find a Sequence Match" feature on the Scansite web search platform (currently https://scansite4.mit.edu/4.0/#home). Proteins with cell cycle regulated mRNA were curated from four independent cell cycle transcriptional studies (463, 465, 467, 569). The genes which scored in two or more of these screens was previously compiled in the supplemental data of Grant et al., 2013. Gene and protein name conversions were performed using the DAVID online tool (https://david.ncifcrf.gov/conversion.jsp). The overlapping set 145 proteins, which contain a KEN sequence and exhibit oscillating cell cycle regulated mRNA expression, were identified. For all 145 proteins, we manually curated information on their alias, function, sequence flanking the KEN motif, and evidence for regulation by APC/C from various online databases and repositories, including UNIPROT, PubMed, and Genecards.

The set of 33 well-validated, KEN-containing, human APC/C substrates was derived from (533). Our own FLAG-Cdh1 IPs were compared to other APC/C substrate discovery efforts (545, 546). Singh et al. identified "clusters" of proteins whose levels changed at mitotic exit. For each cluster, they reported a top percentile, and for the clusters that most accurately revealed APC/C substrates (1, 2, and 3), we compile their data in Supplemental Table 3 in terms of which KEN-dependent substrates were identified. Their data from Cluster 1, which identified the most KEN-containing APC/C substrates, is shown in Figure 1C. Lafranchi et al. rank ordered proteins based on the degree of change from mitosis to G1, analyzed by proteomics. We curated their data to identify the cut-off point where the last KEN-dependent APC/C substrate was identified among their rank ordered list. Since they provided no cut-off point, the data comparison in Figure 1C represents the best estimate of their ability to capture APC/C substrates.

Cell Culture

HeLa, HeLa S3, U2OS, RPE-1, and HCT116 cells were grown in 10% FBS with high glucose DMEM without antibiotics. Cell culturing utilized standard laboratory practices whereby cells were grown and incubated at 37°C containing 5% CO_2. Frozen cell stocks were stored under liquid nitrogen in 10% DMSO/90% FBS.

GFP-UHRF1^{WT} and GFP-UHRF1$^{KEN:AAA}$ stable overexpression cells were generated by transducing HeLa S3, U2OS, and RPE-1-hTERT cell lines with pHAGE-GFP lentivirus that had been produced in HEK293T cells. Infections were performed in the presence of 8µg/mL polybrene for 48 hours prior to antibiotic selection. Cells were selected for 5-7 days with 8ug/mL (HeLa S3 and U2OS) or 10ug/mL (RPE-1) Blasticidin. Lentiviral particles were produced by transfecting HEK293T cells with Tet, VSVg, Gag/pol, and Rev viral packaging vectors together with the pHAGE-GFP lentiviral vectors using *Trans*IT® MIRUS. Viral particles were collected 48 and 72 hours after transfection and stored at -80°C prior to transduction.

To generate the rescue cell lines, the U2OS and HeLa S3 stable GFP-UHRF1^{WT} and GFP-UHRF1$^{KEN:AAA}$ expression cell lines were transduced with previously described and validated pLKO.1 lentiviral vectors encoding either shControl or 3'UTR targeting shUHRF1 (570), using 8ug/mL polybrene to aid infection. After 48 hours, cells were selected with 2µg/mL Puromycin for 3-5 days. Viral particles were produced by transfecting HEK293T cells with the pLKO.1 constructs and psPAX2 and pMD2.G packaging vectors using *Trans*IT® MIRUS (cat no. MIR 2700), collecting after 48 and 72 hours as mentioned previously.

Mitotic block was induced by treating 25% confluent HeLa S3 cells with 2mM thymidine for 24 hours. After washing the plates three-four times with warm media and incubating in drug-free media for 3-4 hours, cells were treated with 100 ng/mL nocodazole for 10-11 hours prior to harvesting by mitotic shake-off. Samples were washed three or four times with warm media, counted, and re-plated for indicated timepoints.

To synchronize cells in G1/S, HeLa S3 were plated at 20% confluency prior to addition of 2mM thymidine. After 16 hours, cells were washed three times with warm media and left to incubate for 8 hours before the second block in 2mM thymidine for another 16 hours. Cells were washed three times in warm media and collected at specific timepoints as they progress through the cell cycle.

To transiently inactivate the APC/C, HCT116 or U2OS cells were treated with 15µM proTAME (Thermo Fisher cat no. I-440-01M), a pan-APC/C inhibitor (571), for 90 minutes prior to harvest and immunoblotting. Cells had been released from nocodazole-induced mitotic block for 90 minutes in drug-free media prior to addition of drug.

In vivo APC/C Activation assay

70-80% confluent U2OS cells were transfected with the indicated plasmids for 24 hours and then exchanged into fresh media. Alternatively, untransfected cells were used to analyze endogenous proteins. After an eight-hour incubation in fresh media following transfection, cells were treated with 250ng/mL nocodazole for 16 hours. Mitotic cells were isolated by shake-off, washed once in pre-warmed media, counted, and divided equally among 15mL conical tubes. Cells in suspension were treated with DMSO, RO-3306 (10 µM), Roscovitine (10 µM), or MG-132 (20µM) for the indicated amount of time at 37ºC. Identical volumes of cells were removed from cell suspensions by pipetting, isolated by centrifugation, and frozen at -20°C prior to processing for immunoblot.

Molecular Biology

Plasmid transfection of HEK293T, U2OS, and HCT116 was performed with either MIRUS or PolyJet (cat no. SL100688) at 1:3 or 1:4 DNA: plasmid ratio on cells with 50-60% confluency. After 24 hours, the media was changed, and cells were expanded to larger dishes as needed. Samples were collected 24-48 hours after siRNA transfection was performed using

a 1:3 ratio of RNAi oligonucleotide to RNAiMAX (cat no. 13778-030). UHRF1 was cloned into the indicated lentiviral vectors mentioned previously using standard gateway recombination cloning. Other APC/C substrates tested for binding to Cdh1 or degradation in the APC/C activation assay were obtained from either the ORFeome collection and cloned into the indicated vectors using gateway recombination cloning or from Addgene (see supplemental table) (572).

Cell lysis and immunoblotting

Cells were lysed on ice for 20 minutes in Phosphatase Lysis buffer (50 mM NaH_2PO_4, 150 mM NaCl, 1% Tween-20, 5% Glycerol, pH 8.0, filtered) or NETN (20 mM Tris pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 0.5% NP40) supplemented with 10µg/mL each of aprotonin, pepstatin A, and leupeptin, 1mM sodium orthovanadate, 1mM NaF, and 1mM AEBSF. Following incubation on ice, cell lysates were centrifuged at (20,000 x g) in a benchtop microcentrifuge at 4°C for 20 minutes. Protein concentration was estimated by BCA assay (Thermo Fisher cat no. PI-23227) according to manufacturer's protocol. Cell extracts were diluted with SDS-PAGE Gel Loading Buffer (Laemmli Buffer) prior to analysis by SDS-PAGE. Typically, 20-40 µg of protein were loaded on SDS gels (either BioRad 4-12% Bis-Tris or homemade SDS-PAGE gels) and separated at 140-200V for approximately 1 hour. Proteins were transferred by wet-transfer methods onto nitrocellulose membrane, typically at 100V for 1 hour or 10-17V overnight at 4°C. Nitrocellulose membranes were then incubated with TBST (137mM NaCl, 2.7mM KCl, 25mM Tris pH 7.4, 0.5% Tween-20) supplemented with either 5% bovine serum albumin or non-fat dry milk for at least one hour or overnight at 4°C. Blocked membranes were incubated overnight with primary antibodies at 4°C, washed in TBST, incubated in appropriate secondary antibodies for 1 hour at room temperature, and then developed by chemiluminescence using Pierce ECL (Thermo Fisher) or Clarity ECL (Bio-Rad). See reagent list in supplement for detailed primary and secondary antibody information.

Immunoprecipitation

For co-immunoprecipitation (coIP) experiments, cells were lysed in NETN for 20 minutes on ice and then centrifuged in a benchtop centrifuge on maximum speed (20,000 x g) for 20 minutes at 4°C, prior to determining protein concentration by either Bradford or BCA assay.

A master mix of 1-2 mg/mL protein concentration was calculated, 10% of which was retained as input while the remaining 90% was used for coIP. Prior to coIP, antibody coated beads were prewashed with 1X TBST three times prior to incubation with lysis buffer. Cell lysates were also pre-cleared by incubation with the same volume of empty Protein A/G agarose beads. Clarified cell lysates were immunoprecipitated for 2-4 hr at 4°C with 25-50uL of EzView M2- or Myc-antibody beads (F2426-1ML or E6654-1ML). After coIP, beads were pelleted at low speed centrifugation, washed twice with wash buffer, and one time with lysis buffer to remove unbound proteins. Buffers were removed from beads using a 27 gauge needle to avoid the aspiration of beads between washes. Washed beads were resuspended in 2X SDS-PAGE Gel Loading Buffer (Laemmli Buffer) and boiled 5-10 minutes at 95°C. Samples were removed from the beads using a 27-gauge needle to avoid the aspiration of beads after boiling. Typically, 20µL of coIP was loaded alongside 1% of the input volume. Samples were analyzed by immunoblotting as described.

Protein Purification

Substrates for *in vitro* ubiquitylation assays were expressed as N-terminal GST-TEV-fusion (TTF2) or His-MBP-TEV-fusions (FL-UHRF1^{WT}, LPS-UHRF1^{WT}, FL-UHRF1$^{KEN:AAA}$, LPS-UHRF1$^{KEN:AAA}$) in BL21 (DE3) codon plus RIL cells. TTF2 was purified by glutathione-affinity chromatography, treated with TEV protease to liberate GST, and further purified by ion exchange chromatography. UHRF1 wild-type and variants were purified by amylose-affinity chromatography, treated with TEV, and followed by ion exchange chromatography. Fluorescently labeled substrates were generated by incubating 1 µM Sortase, 20x 5-

carboxyfluorescein (5-FAM)-PEG-LPETGG peptide, and substrates in 10 mM HEPES pH 8, 50 mM NaCl, and 10 mM $CaCl_2$. After 2 hours of incubation at 4°C, reactions were stopped by removing the His_6-tagged Sortase by nickel affinity chromatography. Then, excess 5-FAM-LPETGG was removed by size exclusion chromatography.

Expression and purification of UBA1, UBE2C, UBE2S, recombinant APC/C and pE-APC/C, Cdh1, Cdc20, Emi1, ubiquitin, and methylated ubiquitin were performed as described previously in Brown et al. 2016 (188, 573–576).

APC/C Ubiquitylation assays

Qualitative assays to monitor APC/C-dependent ubiquitylation were performed as previously described (188). In brief, reactions were mixed on ice, equilibrated to room temperature before the reactions are initiated with Ub or meUb, and quenched at the indicated time points with SDS. TTF2 ubiquitylation was monitored by mixing 100 nM APC/C, 1 µM Cdh1, 5 µM UBE2C, 5 µM UBE2S (when indicated), 1 µM UBA1, 5 µM TTF2, 5 mM Mg-ATP, and 150 µM Ub or meUb (Fig. S2). Ubiquitylation of UHRF1 wild-type or its variants by APC/C were performed with 100 nM APC/C or pE-APC/C, 1 µM Cdh1 or Cdc20, 0.4 µM UBE2C, 0.4 µM UBE2S (when indicated), 1 µM UBA1, 0.4 µM UHRF1, 5 mM Mg-ATP, and Ub or meUb (Fig. 4 and Fig. S4). Following SDS-PAGE, ubiquitylation products of the fluorescently labeled substrates were resolved by SDS-PAGE and imaged with the Amersham Typhoon 5.

Flow cytometry cell cycle analysis

HeLa S3 GFP-UHRF1^{WT} and GFP-UHRF1$^{KEN:AAA}$ (shUHRF1) cells were synchronized in mitosis by sequential thymidine-nocodazole treatment as described above, using 2mM thymidine and 100ng/mL nocodazole. After release, cells were pulsed with 10µM EdU thirty minutes prior to collection at specific timepoints. After counting the cells, 2 million cells were retained for Western blotting (WB) analysis and 1 million cells were fixed for flow cytometry. For

WB, cells we pelleted and washed once with cold PBS prior to freezing at -20°C. For flow cytometry, cells were fixed in 4% formaldehyde/PBS for 15 minutes at room temperature. Cells were pelleted and resuspended in 1% BSA/PBS and stored overnight at 4°C. The next day, cells were pelleted and resuspended in 1% BSA/PBS/0.5% Triton X-100 for 15 minutes at room temperature. Cells were pelleted, resuspended with labelling solution (100mM ascorbic acid, 1mM $CuSO_4$, 2µM Alexa Fluor 488 azide in PBS), and incubated for thirty minutes in the dark at room temperature. After addition of 1% BSA/PBS/0.5% Triton X-100, cells were pelleted and stained with 1µg/mL DAPI in 1% BSA/PBS/0.5% Triton X-100 for one hour in the dark at room temperature. Flow cytometry was performed on an Attune™ Nxt Flow Cytometer (Thermo Fisher Scientific). Channel BL1 was used for Azide 488 dye. Channel VL1 was used for DAPI dye. Following acquisition, data were analyzed using FlowJo software.

Immunofluorescence imaging

HeLa cells were plated on poly-L-lysine-coated #1.5 coverslips. Next day, cells were treated with siRNA (control siFF and siUHRF1) and RNAi Max according to manufacturer's protocol (Invitrogen). After 48 hours of siRNA treatments, cells were fixed in 3% paraformaldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 7.0) for 15 minutes at 37 °C. Then, cells were washed with PHEM buffer and permeabilized using 0.5% of Nonidet P-40 in PHEM buffer for 15 minutes at room temperature. Cells were washed and then blocked with 5% BSA in PHEM. Primary antibodies used were: α-CENP-C (MBL:1:1000) as a kinetochore marker and α-tubulin (Sigma: 1:500). Samples were incubated in primary antibody solution for 1 hour at 37 °C. All fluorescently labeled secondary antibodies (anti-mouse Alexa 488, anti-guinea pig 564) were diluted 1:200 dilution, and cells were incubated for 1 hour at 37 °C. DNA was counterstained with DAPI for 15 minutes at room temperature after washing out secondary antibodies. All samples were mounted onto glass slides in Prolong Gold antifade (Invitrogen). For image acquisition, three-dimensional stacked

images were obtained sequentially at 200 nm steps along the z axis through the cell using MetaMorph 7.8 software (Molecular Devices) and a Nikon Ti-inverted microscope equipped with the spinning disc confocal head (Yokogawa), the Orca-ER cooled CCD camera (Nikon), and an ×100/1.4 NA PlanApo objective (Nikon).

Genomic DNA isolation for methylation analysis

Genomic DNA was isolated from Parental U2OS cells and U2OS cells overexpressing either GFP-UHRF1^{WT} or GFP-UHRF1$^{KEN:AAA}$. All samples groups were processed in biological triplicates. Briefly, cells were lysed overnight at 37°C in 2 mL of TE-SDS buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 0.5% SDS), supplemented with 100 µl of 20 mg/ml proteinase K. DNA was purified by phenol:chloroform extraction in three phases: (1) 100% phenol, (2) phenol:chloroform:isoamyl alcohol (25:24:1), and (3) chloroform:isoamyl alcohol (24:1). For each phase, the aqueous layer was combined with the organic layer in a 1:1 ratio. Samples were quickly shaken, allowed to sit on ice for approximately 5 minutes, and then separated by centrifugation at 1,693 RCF for 5 minutes at 4°C. The top aqueous layer was then transferred to a new tube for the next organic phase. Following extraction, DNA was precipitated with 1/10 volume 3M sodium acetate pH 4.8 and 2.5 volumes 100% ethanol and stored overnight at -20°C. Precipitated DNA was pelleted by centrifugation at 17,090 RCF for 30 minutes at 4°C. The pelleted DNA was washed twice with 70% ethanol, allowed to dry for 15 minutes, and resuspended in TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). Samples were then treated with 1 mg/ml RNAse A at 37°C for 30 minutes and then re-purified by ethanol precipitation as described above.

Infinium Methylation EPIC BeadChip (EPIC array)

Genomic DNA was quantified by High Sensitivity Qubit Fluorometric Quantification (Invitrogen), and 1.5 ug of genomic DNA was submitted to the Van Andel Institute Genomics

Core for quality control analysis, bisulfite conversion, and DNA methylation quantification using the Infinium Methylation EPIC BeadChIP (Illumina) processed on an Illumina iScan system following the manufacturer's standard protocol (558, 559).

EPIC array data processing

All analyses were conducted in the R statistical software (Version 3.6.1) (R Core Team). R script for data processing and analysis is available in Supplemental Code File 1.

Raw IDAT files for each sample were processed using the Bioconductor package "SeSAMe" (Version 1.2.0) for extraction of probe signal intensity values, normalization of probe signal intensity values, and calculation of β-values from the normalized probe signal intensity values (577–579). The β-value is the measure of DNA methylation for each individual CpG probe, where a minimum value of 0 indicates a fully unmethylated CpG and a maximum value of 1 indicates a fully methylated CpG in the population. CpG probes with a detection p-value > 0.05 in any one sample were excluded from the analysis.

Genomic and Replication Timing annotation

CpG probes were mapped to their genomic coordinate (hg38) and were then annotated to their genomic annotation relationship (promoter-TSS, exon, etc.) using HOMER (Version 4.10.3) (580).

Repli-seq data for U2OS cells used for determining CpG probe localization relative to replication timing was generated by Dr. David Gilbert's lab (Florida State University) as part of the 4D Nucleome project (Experiment #4DNEXWNB33S2)(560). Genomic regions were considered early-replicating if the replication timing value was > 0 and late-replicating if < 0. CpG probes were annotated for replication timing domains by intersecting the Repli-seq genomic coordinates with CpG probe coordinates using BEDTools (Version 2.16.2) (581).

Identification of differentially methylated CpG probes

The Bioconductor package "limma" (Version 3.40.6) was used to determine differential methylation among sample groups and perform multidimensional scaling (MDS) analysis (578, 579, 582). For statistical testing of significance, β-values were logit transformed to M-values: $M = log_2\left(\frac{\beta}{1-\beta}\right)$. M-values were then used for standard limma workflow contrasts to determine differential methylation of U2OS GFP-UHRF1^{WT} or GFP-UHRF1$^{KEN:AAA}$ overexpression to Parental U2OS cells (582, 583). CpG probes with an adjusted p-value ≤ 0.05 were considered significant, and log fold-change of M-value was used to determine hypermethylation (logFC > 0) or hypomethylation (logFC < 0) relative to U2OS parental cells.

Enrichment Bias Calculation and Hypergeometic Distribution Testing

Enrichment Bias Calculations were done by first determining the following values for each feature (e.g. Genomic Annotation, Replication Timing):

q = Number of CpGs that are differentially methylated in feature (e.g. exon)

m = Total number of CpGs on the EPIC array that match feature (e.g. exon)

n = Total number CpGs on the EPIC array that do not match feature (e.g. everything that is not an exon)

k = Total number of all differentially methylated CpGs

Next, the expected number of CpGs that would be differentially methylated in that feature by random chance was determined with the following equation:

$$e = \left(\frac{m}{m+n}\right)k$$

Finally, percent enrichment bias was calculated with the following equation:

$$\%\ enrichment\ bias = \left(\frac{q-e}{k}\right) \times 100$$

Where positive or negative enrichment values indicate more or less enrichment for a feature than would be expected by random chance, respectively.

Hypergeometric distribution testing for determining significance of enrichment bias was performed using the phyper() function in R with the following values: *q,m,n,k*.

Data access

EPIC array data can be found under GEO Accession # GSE137913.

To review GEO accession GSE137913:

Go to https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE137913

The following secure token has been created to allow review of record GSE137913 while it remains in private status: eletaomyfnqrlun

Signature evaluation in TCGA BRCA samples

Upper quartile normalized RSEM gene expression data for TCGA BRCA (n=1201) was downloaded from the GDC legacy archive (https://portal.gdc.cancer.gov). The data was log2 transformed and median centered. To determine the per sample UB signature score, the samples were ranked by the median expression of the 145 UB gene signature. Sample were then divided at the median and grouped as high or low based on rank. Copy number burden, aneuploidy, and homologous recombination deficiency data were extracted from Thorsoon et. al. (584) and plotted by UB signature group and PAM50 subtype (585). Significance was calculated by t-test. The CIN70 score was determined as previously described in Fan et. al. (586). The CIN70 was plotted against the UB, colored by PAM50 subtype, and r^2 and Pearson correlation were calculated. All analysis was performed in R (v3.5.2).

Cdh1 pulldown for analysis of interactors by mass spectrometry

FLAG-tagged Cdh1 was expressed in HEK293T cells for 24 hours by transient transfection. Transfections were performed on 150 mm dishes (8 per condition) using Mirus TransIT®-LT1 Transfection Reagent (Mirus Bio) and Lipofectamine 2000 (Life Technologies). Cells were treated with MG-132 (10 μM for 4 hours) in culture prior to lysis, dislodged by trypsinization, washed with PBS, and lysed in NETN supplemented with 2 μg/ml pepstatin, 2 μg/ml apoprotinin, 10 μg/ml leupeptin, 1 mM AEBSF (4-[2 Aminoethyl] benzenesulfonyl fluoride), 1 mM Na_3VO_4, and 1 mM NaF on ice for 20 minutes. Cell lysates were then clarified by centrifugation at 15,000 rpm for 15 minutes.

Anti-FLAG M2 agarose (Sigma, catalog no. F2426) was used for precipitation (6 hours at 4°C). The beads were washed with NETN three times and eluted twice with 150 μl of 0.1 M Glycine-HCl, pH 2.3 and then neutralized with Tris 1M (pH 10.0). The total eluted protein was reduced (5 mM DTT) and alkylated using iodoacetamide (1.25 mM) for 30 minutes in the dark. The resultant protein was then digested overnight with sequencing grade trypsin (Promega). The trypsin: protein ratio was maintained at 1:100. Total peptides were purified on Pierce C18 spin columns (Cat 89870) using the manufacturer's protocol. Peptides were eluted using 70% acetonitrile and 0.1% TFA solution in 50 μl volumes twice, dried on a SpeedVac at room temperature, and processed by mass spectrometry proteomic analysis.

Mass Spectrometry

Peptides were separated by reversed-phase nano-high-performance liquid chromatography using a nanoAquity UPLC system (Waters Corp.). Peptides were first trapped in a 2 cm trapping column (Acclaim® PepMap 100, C18 beads of 3.0 μm particle size, 100 Å pore size) and a 25 cm EASY-spray analytical column (75 μm inner diameter, C18 beads of 2.0 μm particle size, 100 Å pore size) at 35°C. The flow rate was 250 nL/minute over a gradient of 1% buffer B (0.1% formic acid in acetonitrile) to 30% buffer B in 150 minutes, and an in-line

Orbitrap Elite mass spectrometer (Thermo Scientific) performed mass spectral analysis. The ion source was operated at 2.6 kV with the ion transfer tube temperature set at 300°C. A full MS scan (300–2000 m/z) was acquired in Orbitrap with a 120,000 resolution setting, and data-dependent MS2 spectra were acquired in the linear ion trap by collision-induced dissociation using a 2.0 m/z wide isolation window on the 15 most intense ions. Precursor ions were selected based on charge states (+2, +3) and intensity thresholds (above 1e5) from the full scan; dynamic exclusion (one repeat during 30 seconds, a 60 seconds exclusion time window) was also used. The polysiloxane lock mass of 445.120030 was used throughout spectral acquisition.

Raw mass spectrometry data files were searched using SorcererTM-SEQUEST® (build 5.0.1, Sage N Research), the Transproteomic Pipeline (TPP v4.7.1), and Scaffold (v4.4.1.1) with the UniProtKB/Swiss-Prot human canonical sequence database (20,263 entries; release 07/2013). The search parameters used were a precursor mass between 400 and 4500 amu, zero missed cleavages, a precursor ion tolerance of 3 amu, accurate mass binning within PeptideProphet, fully tryptic digestion, a static carbamidomethyl cysteine modification (+57.021465), variable methionine oxidation (+15.99492), and variable serine, threonine and tyrosine (STY) phosphorylation (79.966331). A 1% protein-level FDR was determined by Scaffold.

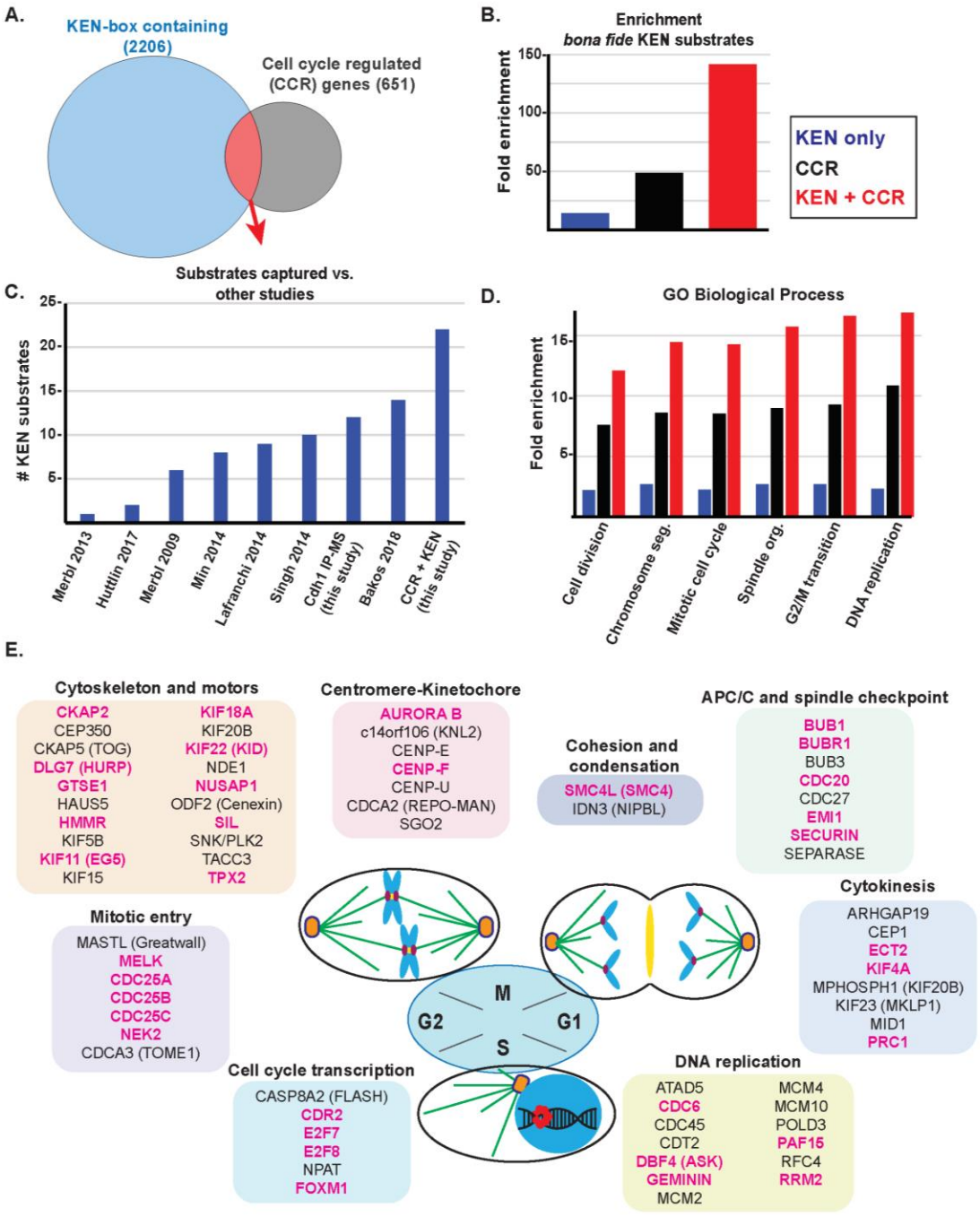

Figure 3.1. *In silico* analysis reveals a high confidence set of APC/C substrates involved in mitosis.

(A) KEN-box containing human proteins were identified and cross-referenced against a set of 651 genes whose expression is cell cycle regulated based on multiple, independent studies. This revealed a set of 145 KEN-box containing proteins whose mRNA expression is cell cycle regulated.

(B) Analysis of the enrichment of bona fide KEN-dependent substrates among these three datasets (blue- KEN box only set (2206); black- cell cycle regulated mRNAs (651); red-the overlapping set of 145 proteins) compared against a curated set of *bona fide*, KEN-dependent APC/C substrates (Davey and Morgan, Mol Cell, 2016). Enrichment was calculated based on the expected number of substrates which would be captured by chance based on the size of the dataset.

(C) Analysis of putative substrates recovered in the indicated studies.

(D) Gene ontology (GO) analysis for indicated studies (blue- KEN box only set (2206); black-cell cycle regulated mRNAs (651); red-the overlapping set of 145 proteins).

(E) The set of 145 putative substrates was manually curated and analyzed for roles in various aspects of cell cycle progression. Seventy proteins, involved in cell cycle activities, are shown. The ones labelled in magenta signify that there is evidence in the literature of their regulation by APC/C. (Note that AURORA B, a mitotic kinase that phosphorylates histone H3, is listed here and in Figure 2A)

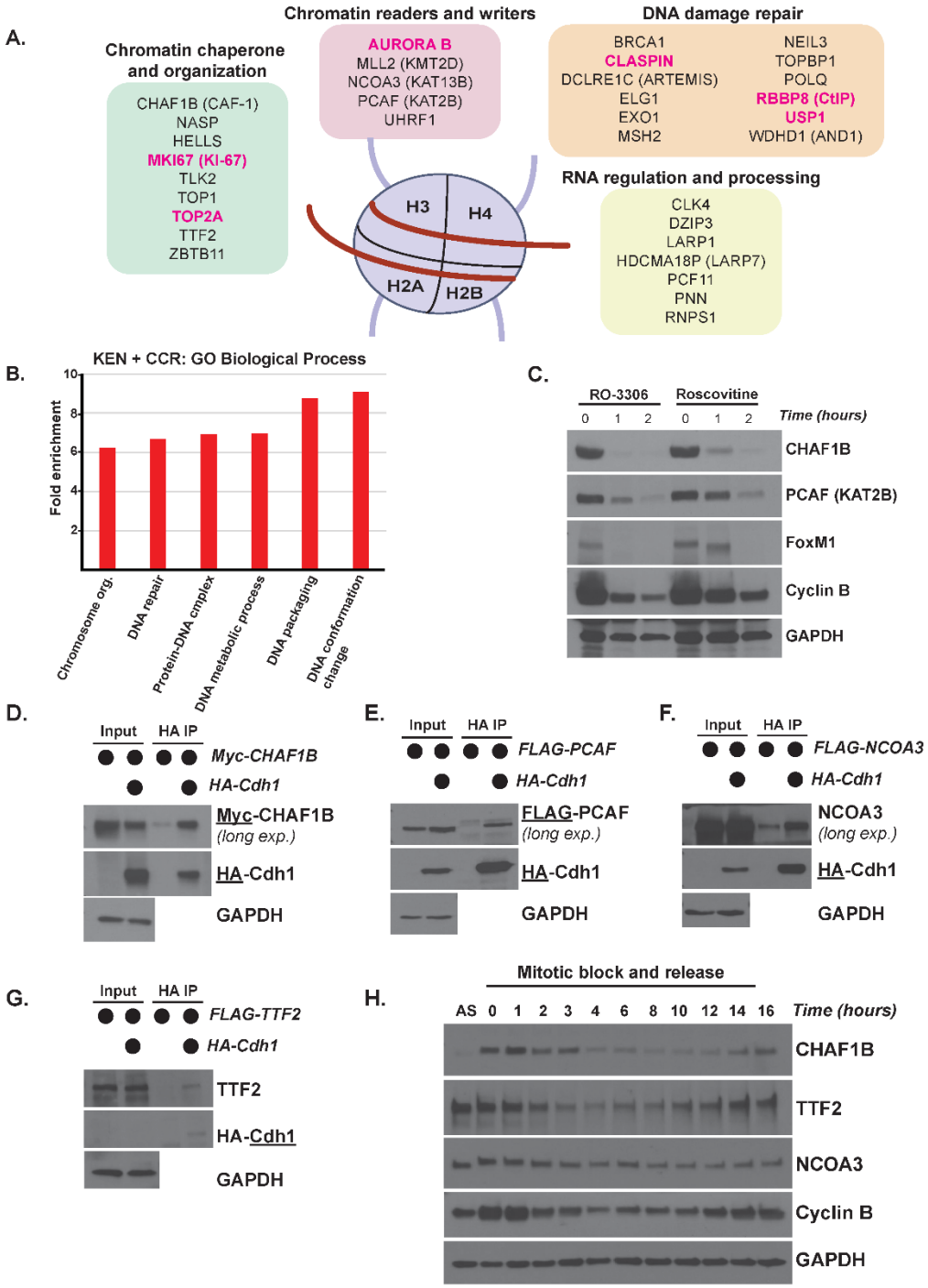

Figure 3.2. Putative APC/C substrates are enriched for roles in chromatin regulation.

(A) The set of 145 known and putative APC/C substrates is enriched for proteins involved in various chromatin related process. This includes chromatin readers and writers, chaperones, RNA regulation and processing, DNA damage repair, and others. (Note that AURORA B, a mitotic kinase that phosphorylates histone H3, is listed here and in Figure 1E)

(B) Gene ontology (GO) analysis of the overlapping KEN-box containing cell cycle regulated transcripts. This set is enriched for the indicated biological process, including DNA metabolism, protein-DNA complex assembly, DNA packaging, and DNA conformation.

(C) APC/C activation assay to monitor substrate degradation. Following synchronization in mitosis, cells were washed one time and treated with CDK inhibitors to remove inhibitory phosphorylation marks that hinder the formation of APC/C^{Cdh1} needed for the M/G1 phase transition. Protein degradation was monitored by immunoblot. CHAF1B and PCAF are putative APC/C substrates, and FoxM1 and Cyclin B are known targets.

(D) coIP of HA-Cdh1 with Myc-CHAF1B in transiently transfected 293T cells treated with proteasome inhibitors prior to harvesting. The underline indicates which protein or tag was blotted for in a particular panel (here and below). Input equal to 1% of IP, here and below.

(E) coIP of HA-Cdh1 with FLAG-PCAF in transiently transfected 293T cells treated with proteasome inhibitors prior to harvesting.

(F) coIP of HA-Cdh1 with FLAG-NCOA3 in transiently transfected 293T cells treated with proteasome inhibitors prior to harvesting.

(G) coIP of HA-Cdh1 with FLAG-TTF2 in transiently transfected 293T cells treated with proteasome inhibitors prior to harvesting.

(H) Mitotic shake-off of synchronized U2OS cells collected after release at the indicated timepoints. Immunoblotting for select endogenous proteins that are putative APC/C substrates or the positive control Cyclin B.

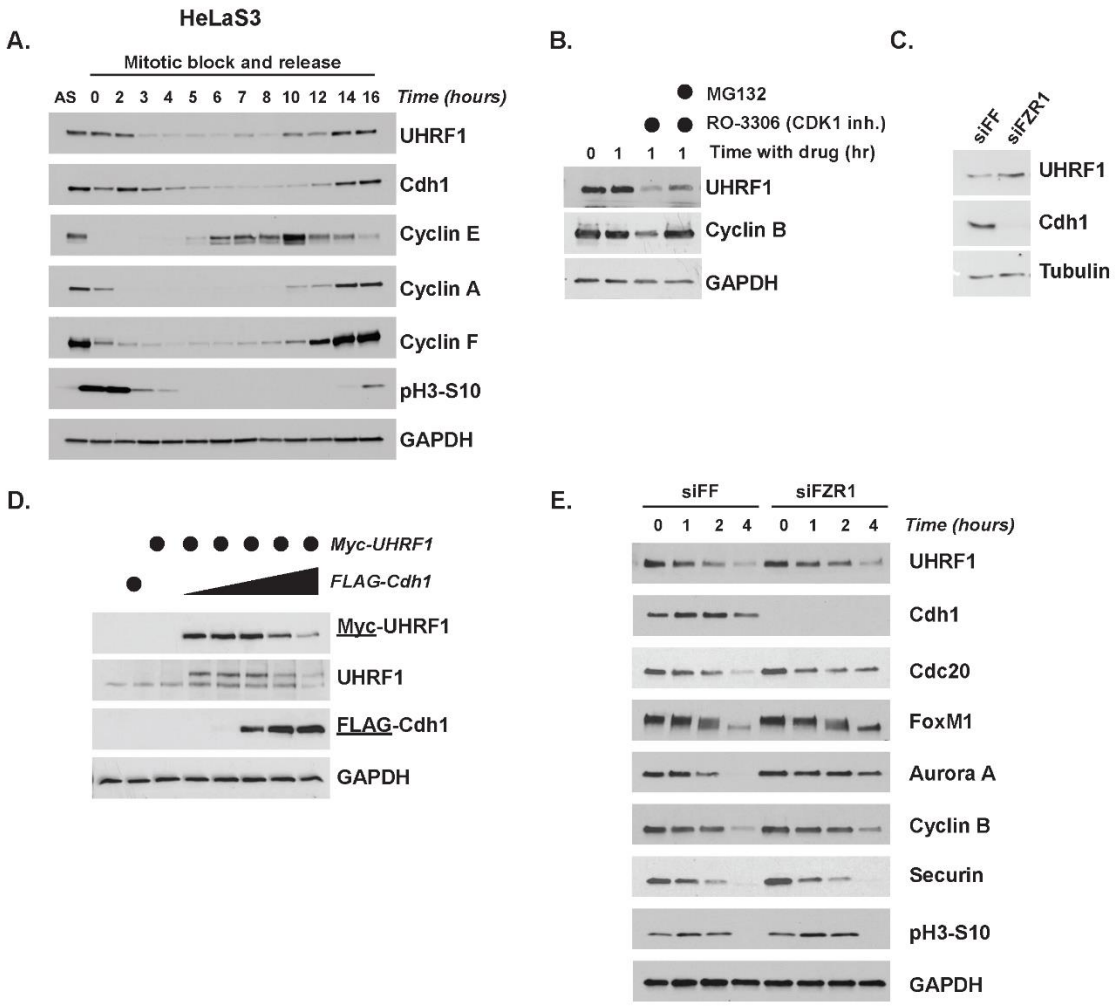

Figure 3.3. UHRF1 levels are controlled by APC/C^{Cdh1}.

(A) HeLa S3 cells were synchronized in mitosis and released into the cell cycle. Timepoints were taken at the indicated time points and analyzed by immunoblot.

(B) U2OS cells were synchronized in prometaphase with 250ng/mL nocodazole for 16hr prior to mitotic shake-off. Cells were released into fresh media containing 10µM RO-3306 CDK inhibitor (used as described in Fig. 2C) with or without addition of 20µM of proteasomal inhibitor MG-132 and harvested 1hr later. Cyclin B is a positive control for a known APC/C substrate that is degraded at mitotic exit.

(C) HCT116 cells were transfected with siRNA targeting Cdh1 (Fzr1 mRNA) or firefly luciferase as a control and harvested after 24 hr for immunoblotting.

(D) Myc-UHRF1 was transiently expressed in 293T cells with increasing concentrations of FLAG-Cdh1 for 24hr before analysis by immunoblot.

(E) HeLa S3 cells transfected with siRNA targeting FF or FZR1 at 50nM for 8hr prior to synchronization in mitosis for 14 hr and then released into the cell cycle. Timepoints were

taken at the indicated time points and analyzed by immunoblot.

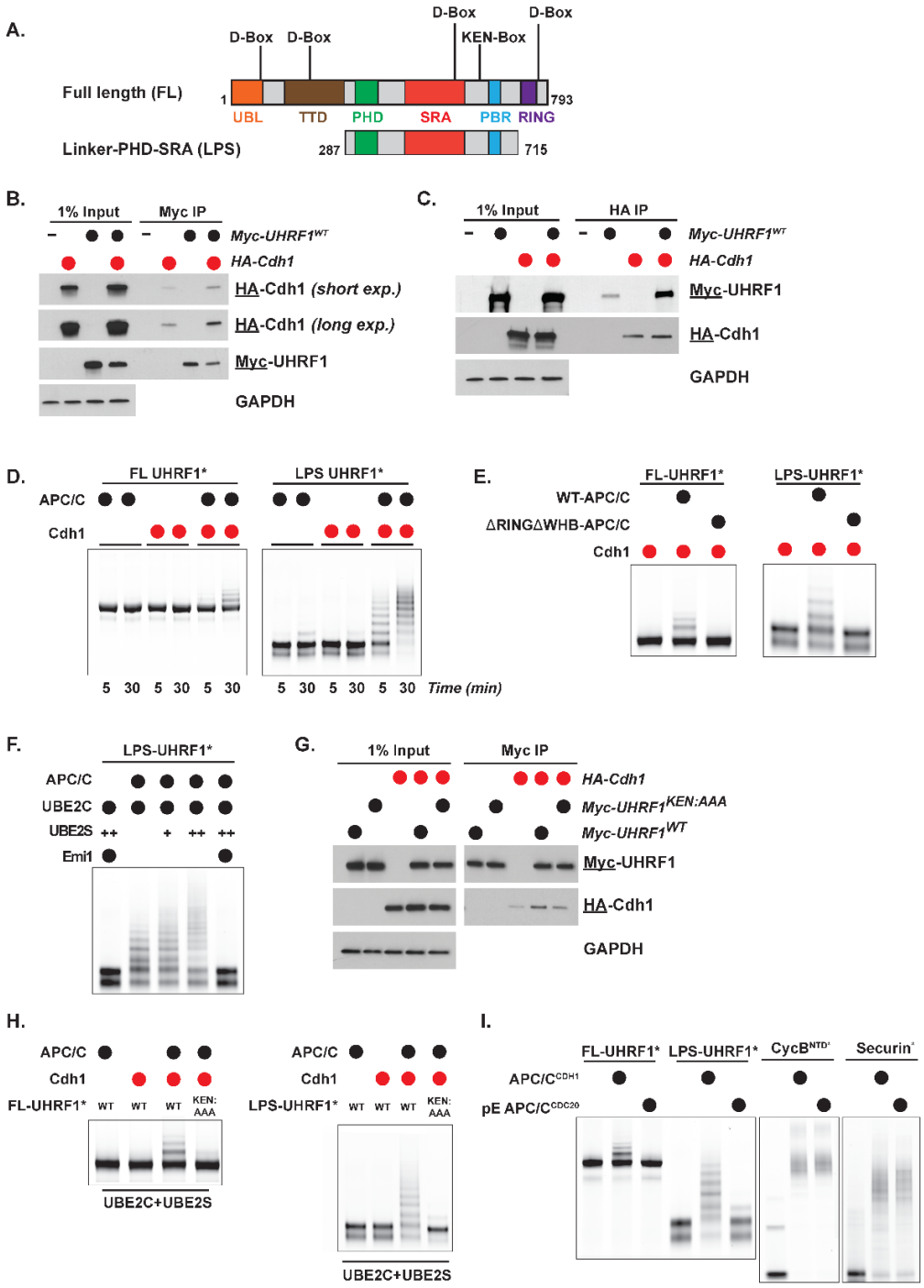

Figure 3.4. UHRF1 binding and ubiquitylation by APC/C^{Cdh1} depends on KEN degron.

(A) Schematic of UHRF1 domain structure with location of KEN degron in both full-length (FL) and truncated LPS UHRF1.

(B) coIP of HA-Cdh1 with Myc-UHRF1 in transiently transfected 293T cells treated with proteasome inhibitors prior to harvesting and α-Myc IP. Input equal to 1% of IP, here and below.

(C) coIP of Myc-UHRF1 with HA-Cdh1 in transiently transfected 293T cells treated with proteasome inhibitors prior to harvesting and α-HA IP.

(D) Ubiquitylation reactions with APC/C^{Cdh1}, UBE2C, FL UHRF1* or LPS UHRF1*, and wild-type ubiquitin. UHRF1 was detected by fluorescence scanning (* indicates fluorescently labeled protein).

(E) Ubiquitylation reactions similar as in (D) but using two variants of APC/C: WT and catalytically dead APC/$C^{\Delta RING\Delta WHB}$, a version of APC/C that can neither recruit nor activate its E2, UBE2C. UHRF1 was detected by fluorescence scanning. Samples were collected at 30 min.

(F) Representative *in vitro* ubiquitylation reactions showing UBE2S-dependent chain elongation reactions of LPS UHRF1*. Titration of UBE2S: 0 μM, 0.1 μM (+), 0.5 μM (++). The addition of Emi1 completely inhibited the reaction. UHRF1 was detected by fluorescence scanning. Samples were collected at 30 min.

(G) coIP of HA-Cdh1 with Myc-$UHRF1^{WT}$ or Myc-$UHRF1^{KEN:AAA}$ in transiently transfected 293T cells treated with proteasome inhibitors prior to harvesting and α-Myc IP.

(H) Polyubiquitylation reactions of FL-UHRF1* and LPS-UHRF1* by APC/C^{Cdh1}, UBE2C, and UBE2S. UHRF1 ubiquitylation by APC/C^{Cdh1} is dependent on the KEN degron motif (lane 4 in both gels). UHRF1 was detected by fluorescence scanning. Samples were collected at 30 min.

(I) Dependence of UHRF1 ubiquitylation on phosphorylation state of the APC/C (referred to as pE-APC/C) and subsequent coactivator recruitment. The well-established APC/C substrates, $CycB^{NTD}$* and Securin*, are ubiquitylated by either APC/C^{Cdc20} or APC/C^{Cdh1}, whereas UHRF1 is only ubiquitylated by APC/C^{Cdh1}. Reactions were run in parallel. Collections taken at 1hr (for FL and LPS UHRF1*) and 30 min (for $CycB^{NTD}$* and Securin*). Ubiquitylated proteins were detected by fluorescence scanning.

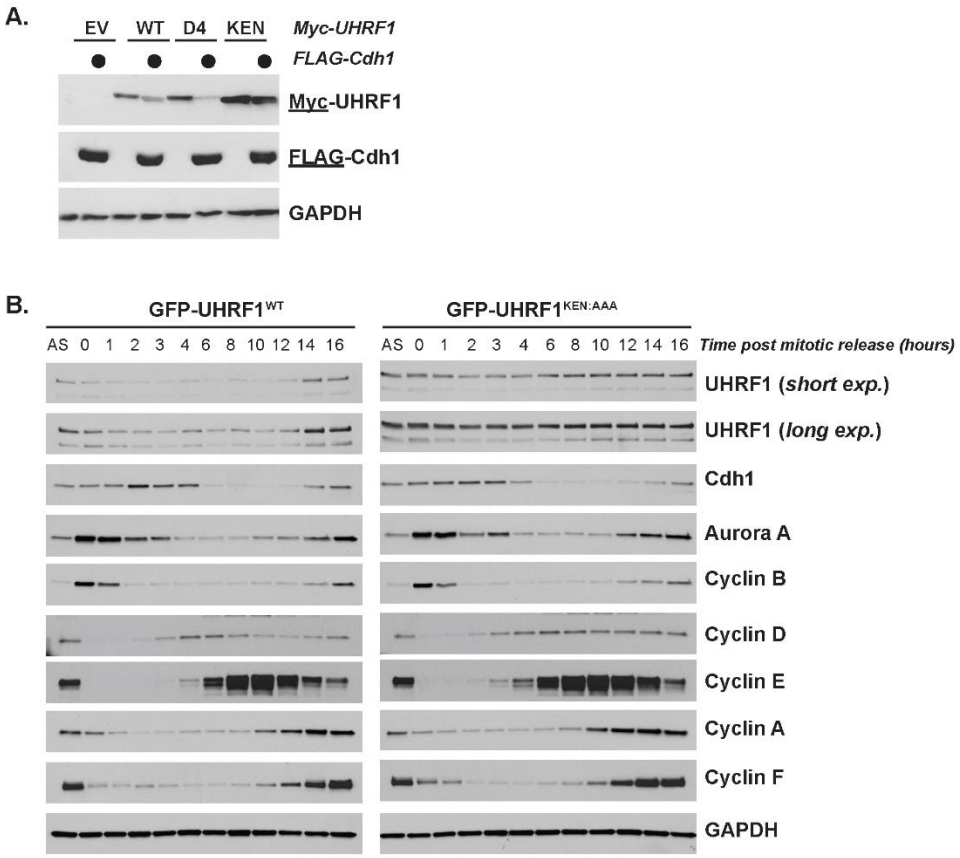

Figure 3.5. UHRF1 non-degradable mutant protein is stable at mitotic exit.

(A) Myc-UHRF1^{WT} or mutant versions harboring alanine substitutions in either its KEN-box (KEN) or the fourth putative D-box motif (D4) (see Fig 4A for location of sequences) were transiently expressed in 293T cells with or without FLAG-Cdh1 for 24hr before analysis by immunoblot.

(B) HeLa S3 stably expressing GFP-UHRF1^{WT} or GFP-UHRF1$^{KEN:AAA}$ were synchronized in mitosis, released into the cell cycle, and collected for immunoblot analysis at the indicated timepoints.

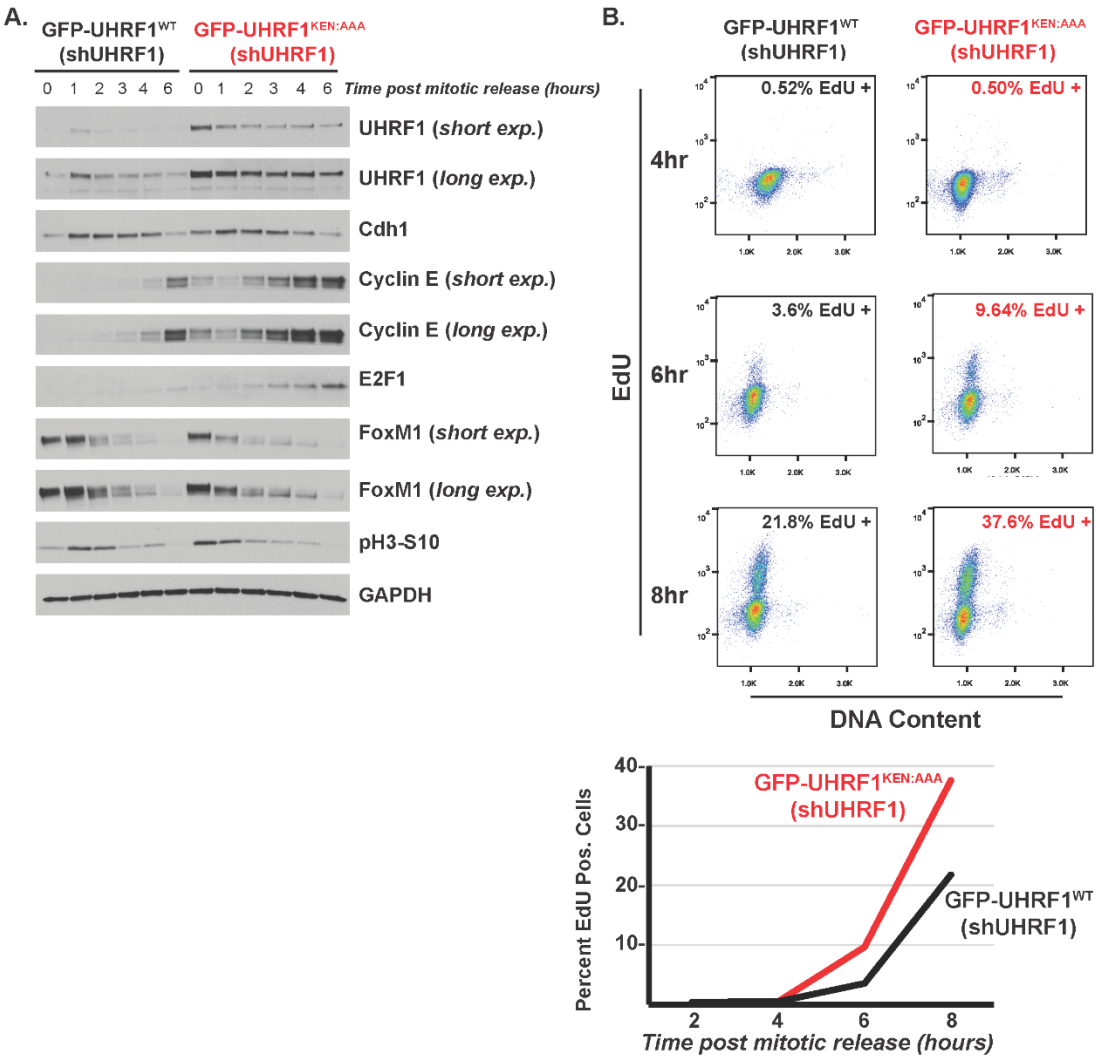

Figure 3.6. UHRF1 degradation restrains S phase entry.

(A) HeLa S3 stably expressing GFP-UHRF1^{WT} or GFP-UHRF1$^{KEN:AAA}$ along with 3'UTR targeting shUHRF1 were synchronized in mitosis as described previously, released into the cell cycle, and collected for immunoblot analysis at the indicated timepoints, probing for cell cycle proteins as shown.

(B) HeLa S3 stably expressing GFP-UHRF1^{WT} or GFP-UHRF1$^{KEN:AAA}$ along with 3'UTR targeting shUHRF1 were synchronized in mitosis, released into the cell cycle, and pulsed with 10μM EdU for thirty minutes prior to harvest and analysis by flow cytometry. A representative experiment (n=3) is shown.

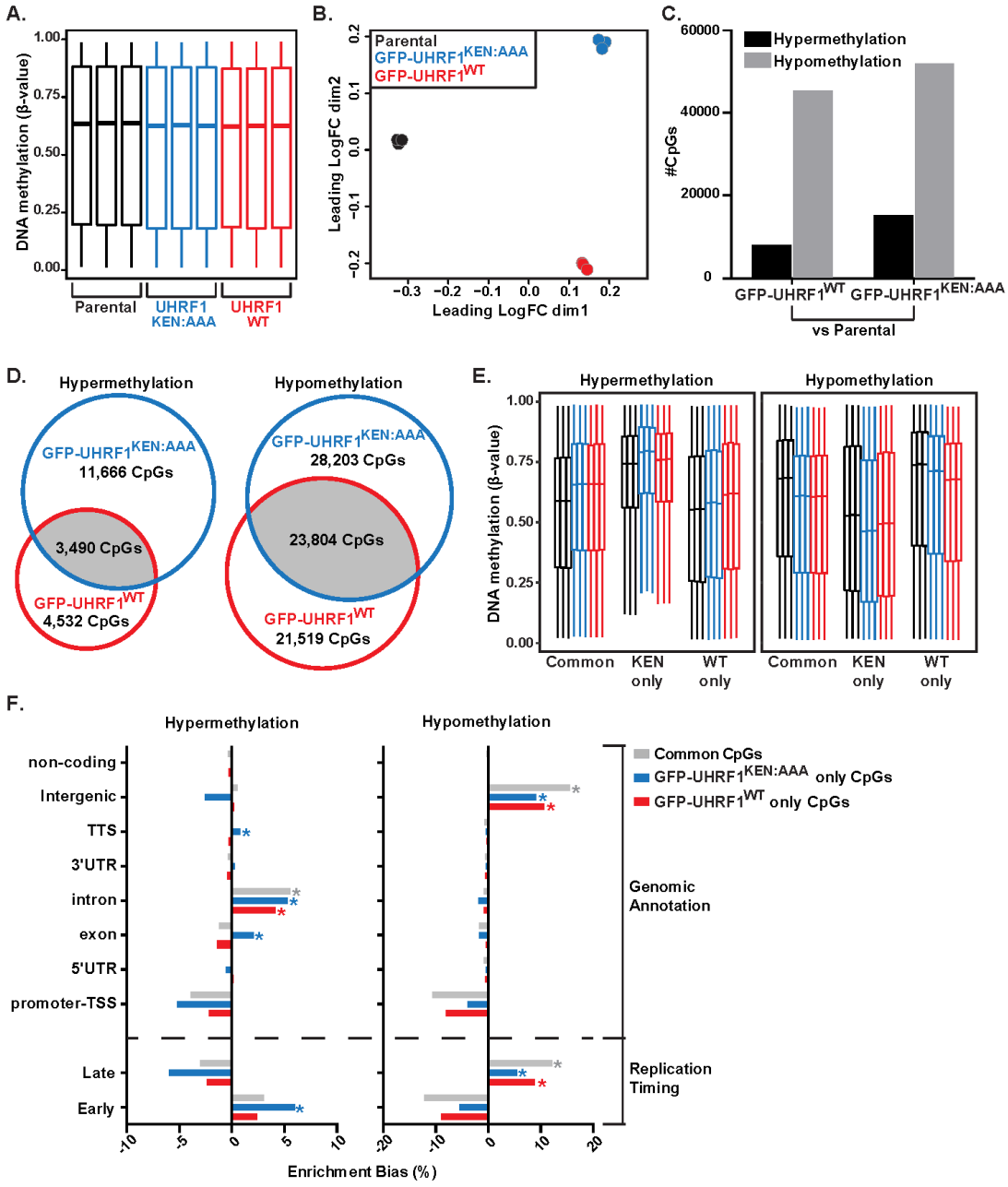

Figure 3.7 A non-degradable form of UHRF1 induces DNA hypermethylation of gene bodies and early replicating regions of the genome.

(A) Global DNA methylation analysis for Parental U2OS and U2OS cells overexpressing GFP-UHRF1^{WT} or GFP-UHRF1$^{KEN:AAA}$ with the Infinium Methylation EPIC BeadChip (Illumina) platform. Each sample group is represented in biological triplicate. All CpG probes that passed quality control analysis (n = 724,622 CpGs) are plotted as β-values population averages from 0 (fully unmethylated) to 1 (fully methylated). The midlines of each box plot represent the median DNA methylation value for all CpG probes in a sample.

(B) Multidimensional scaling (MDS) of the top 50,000 variable CpG probes among samples.

(C) Number of CpG probes that were differentially hypermethylated or hypomethylated in the GFP-UHRF1^{WT} and GFP-UHRF1$^{KEN:AAA}$ groups relative to the Parental samples adjusted p-value ≤ 0.05).

(D) Overlap analysis of significantly hypermethylated (left) or hypomethylated (right) CpG probes between GFP-UHRF1^{WT} and GFP-UHRF1$^{KEN:AAA}$ sample groups.

(E) DNA methylation levels of significantly hypermethylated (left) or hypomethylated (right) probes from (D) that are common between GFP-UHRF1^{WT} and GFP-UHRF1$^{KEN:AAA}$ sample groups, unique to GFP-UHRF1$^{KEN:AAA}$ (KEN only), or unique to GFP-UHRF1^{WT} (WT only). Color code from Fig. 7A applies. Outliers removed to simplify visualization.

(F) Enrichment bias analysis of significantly hypermethylated (left) or hypomethylated (right) CpG probes among genomic annotations and U2OS replication timing data. *p-value ≤ 1E-300 for positive enrichment of the feature by hypergeometric testing.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

4.1). Conclusions

Proper temporal coordination and regulatory control of the cell cycle and APC/C^{Cdh1} Ub ligase function is paramount to ensure successful, timely cell proliferation (Chapter 1). The cell cycle is primarily regulated through oscillatory transcriptional waves of specific cell cycle genes, whose protein products initiate phosphorylation (and transcriptional) events on downstream targets to commit to division. These cell cycle regulated proteins are antagonized by various interrelated Ub signaling pathways that result in their proteasomal destruction in the subsequent phase following their functional activity. When these tightly regulated mechanisms are perturbed, cancer and other genetic diseases develop, culminating in detrimental, uncontrolled growth. Hence, cells have developed many regulatory systems to protect viability. APC/C^{Cdh1}, a highly complex enzyme that coordinates with two different E2s to ubiquitylate its targets upon binding substrate KEN and D-box degrons, is itself regulated by PTMs, including phosphorylation and SUMOylation as well as negative regulators such as Emi1, E2 availability, and Cdh1 Ub (Chapter 2). These mechanisms ensure that this master regulator is active only from anaphase of mitosis through G1 (barring exceptions such as DNA damage) and its activity is extinguished at the G1/S transition through diverse methods. APC/C^{Cdh1} controls myriad aspects of cell proliferation from cell division (Ub of cyclin B and securin in mitosis to initiate chromosomal segregation) to restraining S phase (Ub of licensing and replicative factors until the cell is committed to division).

Importantly, the central finding of this research project (Chapter 3) connected APC/C^{Cdh1} Ub to the epigenetic environment, implicating a role for APC/C^{Cdh1} in integrating chromatin

dynamics alongside cell cycle progression. The chromatin environment is decorated with many cell cycle regulated PTMs (i.e. DNA methylation and histone methylation/phosphorylation/acetylation) and several epigenetic modifiers' activity and stability are controlled by cell cycle proteins. In the enclosed manuscript, we describe an ingenious bioinformatic approach to identify novel APC/C targets, based on independent variables that are common to known APC/C substrates (i.e. cell-cycle regulated mRNA expression, presence of the KEN-box degron, and evidence of ubiquitylation). We discovered that many chromatin modifying factors are putative APC/C substrates and validated several including UHRF1, whose expression oscillates throughout the cell cycle, peaking in mitosis but then rapidly quenched in early G1. We characterized the Cdh1-UHRF1 interaction using genetic and in vitro biochemical approaches. We demonstrated that loss of Cdh1 stabilizes UHRF1 and overexpression of Cdh1 ablates UHRF1 protein levels, which can be rescued with either MG-132, a proteasomal inhibitor, or proTAME, an APC/C prodrug. We found that mutating the KEN box in UHRF1, located in a flexible linker region between the SRA and RING domains, diminishes binding to Cdh1 and completely ablates APC/C^{Cdh1}-mediated Ub of both full-length and a truncated LPS version (lacking the UBL and RING domains) of UHRF1. Notably, this ubiquitylation is specifically attributed to Cdh1 as APC/C^{Cdc20} failed to Ub UHRF1. Upon generating UHRF1 wild-type and non-degradable mutant cell lines, we discovered that stable GFP-$UHRF1^{KEN/AAA}$ expression facilitates a premature S phase entry after mitotic exit and exhibits upregulated cyclin E levels. Furthermore, these cells also exhibit hypermethylation in early-replicating regions and hypomethylation in late-replicating genomic sites, suggesting how lack of UHRF1 degradation could promote a cancer-like DNA methylation phenotype. This work is particularly significant in that it connects two related fields in a new way, providing a definitive link between cell cycle progression and epigenetic regulation (via APC/C^{Cdh1} Ub of UHRF1) to ensure faithful DNA replication and methylation dynamics for a successful, productive cell division. Furthermore, this project offers novel insight into how UHRF1 is regulated temporally by another

E3 ligase, while much of the previous research has assessed the role of UHRF1 in controlling other cell biological and biochemical phenomena. Continued research is needed to elucidate the detailed mechanism how APC/C^{Cdh1} Ub of UHRF1 leads to precocious S phase entry and aberrant DNA methylation.

4.2). Future Directions

One future direction of this project would be to assess whether the UHRF1 stable cell lines can ever enter G0 (quiescence) or whether the inability to degrade UHRF1 constitutively keeps the cells in the cell cycle and mechanistically inspect the aberrant signaling pathways. It is possible that the GFP-$UHRF1^{KEN/AAA}$ cells have a weakened (or non-existent) G0. One way to evaluate this hypothesis would be to immunoblot for low p130 (marker for G0 that is high in G0 and diminishes during G1 entry) and rapidly decreasing p27 protein levels, which should be high in G0/early G1 (p27 is later degraded in S phase). If p27 levels are different in the non-degradable UHRF1 stable lines, it may be interesting to examine further whether GFP-$UHRF1^{KEN/AAA}$ hypermethylates the p27 promoter in early G0/G1 (post mitosis) to reduce protein expression by extinguishing the p27 mRNA levels. An earlier reduction of p27 mRNA expression may accelerate the molecular switch of p27 from inhibitor (in early G1) to being inhibited (at G1/S). This attenuation of p27 levels may facilitate activation of cyclin/CDK complexes to commit cells into S phase (particularly cyclin E/CDK4/6 as evidenced by the high cyclin E in these cells which also tips the balance against p27 inhibition(587)). However, if p27 level are not affected and the promoter is not a UHRF1 target, it would be intriguing to test for another negative regulator of cell proliferation that UHRF1 is turning off quickly in G0/G1 phase. For example, one could test whether GFP-$UHRF1^{KEN/AAA}$ cells induce abnormal silencing of Fbw7. Fbw7 negatively regulates cyclin E, and Fbw7 loss enables aberrant cyclin E dynamics(76). Thus, the increased and more rapid accumulation of cyclin E in the GFP-

UHRF1$^{KEN/AAA}$ cells could be due to loss of Fbw7 control, thereby facilitating precocious S phase entry. Or, UHRF1 may hypermethylate the E2F repressors, such as E2F6-8, to silence any restrictive signals on S phase entry. Epigenetically, it would be also very interesting to scrutinize the chromatin state of these cells and ask whether the GFP-UHRF1$^{KEN/AAA}$ cells exist in a more cell-proliferative or "permissive" chromatin state (e.g. H3K27ac, H3K4me3, H3K36me3) unlike the GFP-UHRF1^{WT} cells, which may have a more normal, "repressive" chromatin state (e.g. H3K9me3, H3K27me3, H4K20me1/3, H2AUb1)(588, 589). Therefore, the GFP-UHRF1^{WT} cells may readily undergo quiescence following serum withdrawal or contact inhibition in contrast to the GFP-UHRF1$^{KEN/AAA}$ cells. To better address this question, it may be necessary to generate alternate cell lines of UHRF1 in a background that still retains the capacity to enter G0 (unlike HeLa cancer cells which are not well inclined).

A second direction would involve delving more deeply into how the non-degradable UHRF1 is promoting S phase. For example, several papers have described a role of UHRF1 in promoting replisome assembly. It would be interesting to determine if GFP-UHRF1$^{KEN/AAA}$ cells interact with different protein complexes (i.e. to promote repressive silencing on TSG promoters—may be different from maintenance methylation), have different rates of complex assembly, or exist in different stoichiometry (as the KEN>AAA mutation may perturb and/or facilitate certain intra/inter-molecular interactions within UHRF1 and with its binding partners, respectively). This query would need to evaluate how GFP-UHRF1$^{KEN/AAA}$ may interact with chromatin and other proteins differentially compared to GFP-UHRF1^{WT} (perhaps through IP/MS analysis). Significantly, since the GFP-UHRF1$^{KEN/AAA}$ cells have augmented cyclin E, these cells can be evaluated for an prolonged S phase (which is also indicative of obstacles traversing through S phase)(73). As cyclin E is critical for origin licensing and firing among other DNA replication initiation functions, it would be intriguing to assess if a connection exists between UHRF1 overexpression and increased cyclin E activity to promote an earlier establishment and activation of origins (as assessed by early MCM loading, pCdc6, etc.). Moreover, it might be

interesting to examine whether the augmented cyclin E in the GFP-UHRF1$^{KEN/AAA}$ cells plays any role in phosphorylating and inhibiting Cdh1 to rapidly enter S phase (although most studies have studied the role of cyclin A in this inhibitory function(590)). The very subtle decrease in Cdh1 observed in the GFP-UHRF1$^{KEN/AAA}$ cells may also facilitate a shorter G1 phase(448), but at a consequence of increased genome instability (255, 445). SKP2 levels could be also assessed as well since it too is a cyclin E target and its formation into the SCF^{SK2} Ub ligase complex enables p27 and p21 degradation, which again commit the cells to enter S phase(587). It may also worth examining how UHRF1 overexpression may negatively regulate cyclin-dependent kinase inhibitors such as p21 to achieve a rapid S phase entry. p21 is paradoxically required for both cyclin D/CDK4/6 complex assembly to promote G1 phase as well as restraining DNA replication via CDK inhibition (which blocks E2F1 transcription) and PCNA inhibition(250) and has been well-studied for its role in cell cycle arrest, apoptosis, and senescence(591). Thus far, only one paper reported a role for UHRF1 in controlling p21 transcription(330), and given the pleiotropic oncogenic and suppressive functions of p21, it is likely that more complex interactions exist between overexpressed, stable UHRF1 and p21.

A third future direction of this work would be to assess the role of UHRF1 stable expression in the DNA damage response. Given that GFP-UHRF1$^{KEN/AAA}$ cells enter S phase faster, it would be important to assess whether these cells exhibit defects in MCM/pre-RC loading or have upregulated DNA damage signaling and genomic instability. Moreover, it is likely that when stressed these cells are more susceptible to DNA damage given that they enter S phase rapidly following mitosis and thus may exit G1 with under-licensed DNA(592). As a result, cells expressing stable UHRF1 overexpression may experience oncogene-induced replication stress(593, 594). It is possible that cells may cope by utilizing the excess UHRF1 to facilitate the DNA repair mechanism, as UHRF1 has been reported to localize to damaged sites(364, 365, 376, 595). Additional mechanistic studies are needed to decipher the exact role of UHRF1 in DNA repair in response to DNA damage.

A fourth direction would be to scrutinize the possible mitotic abnormalities that may arise from early S phase entry. Mitotic deficiencies can result from replication errors and/or DNA damage occurring in S/G2 phase. Aberrations can also arise from errors in chromosomal compaction, spindle attachment/alignment, and segregation. One could interrogate whether the UHRF1 hypo/hyper-methylation phenotype described in Chapter 3 causes chromosomal structural issues. Since UHRF1 is associated with DNA methylation and PCH replication, it is possible that when UHRF1 is overexpressed, global demethylation induces chromosomal compaction issues and disturbs proper centromeric/pericentromeric boundaries, leading to kinetochore and microtubule attachment abnormalities that ultimately result in misalignment and segregation errors (described in more detail below).

During the M/G1 transition, UHRF1 may also alter cell cycle transcription dynamics. Reported to bind Origin Recognition Complexes (ORCs) on methylated nucleosomes(596), particularly ORC1, UHRF1 loss perturbs ORCs association with DNA(597). This finding is particularly interesting given that ORC1 binds SUV39H1 (and its silencing mark H3K9me3) as cells are exiting mitosis to block cyclin E transcription, which is antagonized later in G1 by Cdc6(598). This research suggests that UHRF1 may be important for ORC1 binding to chromatin to restrain cyclin E transcription until later in G1 (presumably occurring briefly until UHRF1 degradation in late mitosis halts additional ORC1 association). However, stable UHRF1 expression and chromatin occupancy during mitotic exit may disrupt this relationship with ORC1 and facilitate an earlier (and augmented) cyclin E transcription. Additionally, given that UHRF1 silences the RB promoter(599), stable UHRF1 overexpression may induce high E2F gene transcription (both UHRF1 and cyclin E are target E2F genes), causing robust, positive feedforward signaling that enables a fast S phase entry. Moreover, since ORCs are important not only for pre-RC formation, but also in establishing a silent epigenetic environment(600), it is also possible that UHRF1 interfaces with ORCs for structural remodeling in M phase in addition to pre-RC and licensing prior to replication (in G1/S phase). Interestingly, in flies(601), APC/C

degrades ORC1 in mitosis and G1, suggesting that ORCs must be temporally regulated to control events in mitosis through G1/S phases, a similar pattern of which exists for UHRF1 destruction in human cells (Chapter 3).

Furthermore, regarding how the loss of UHRF1 results in DNA/kinetochore disorganization along the metaphase plate, anaphase bridges, and micronuclei (Chapter 3), it would be interesting to explore whether UHRF1 knockdown cells have increased rates of mitotic catastrophe(602) and conduct additional studies to characterize the mitotic defects in depth. Since UHRF1 has a well-defined role in DNA methylation, UHRF1 may be necessary to maintain PCH structural integrity (through interactions with H3K9me3, DNA methylation, and methyltransferase SUV39H(603)). Given that DNA methylation is associated with silencing of transposons and tandem satellite repeats in PCH to prevent chromosomal recombination events, it is possible that UHRF1 oncogenic overexpression results in the hypomethylation of these regions, leading to the genomic instability(285, 604). Moreover, the significant presence of micronuclei in the UHRF1 knockdown experiment is very illuminating as micronuclei are obvious signs of segregation abnormalities and damaged DNA that likely resulted from the lack of proper chromosome structure (due to PCH demethylation(605, 606) and centromeric dysregulation)(607). Furthermore, it has been reported that lack of sufficient methylation disturbs tension in kinetochores, impairs microtubule attachment, and perturbs segregation, resulting in micronuclei formation(608). Thus, in addition to its role in DNA methylation during replication, UHRF1 may be critical in mitosis to ensure PCH formation (these sites are often replicated later in S/G2 phases and localize to the nuclear periphery) to promote proper centromeric/pericentromeric structural boundaries for efficient kinetochore/spindle attachment and could potentially act as a hub protein for Condensin complexes to achieve chromosome condensation for successful segregation.

Another interesting mitotic connection to assess would be how the possible protein interaction (609) and downstream regulation of centromere protein F (CENPF) by UHRF1(387,

610) could interplay to affect chromosome/kinetochore attachment at the centromere (which is bordered by PCH where UHRF1 binds(338, 611)). Follow-up studies could be conducted to evaluate whether the UHRF1 knockdown mitotic defects observed in Chapter 3 phenocopies the CENP-F knockdown aberrant mitotic phenotypes, including prolonged mitosis, kinetochore attachment and spindle alignment errors, and activation of the spindle checkpoint(612–614).

Finally, it would be interesting to determine the relationship between upregulated UHRF1 expression, hypomethylation status, and late-replicating genomic regions as these factors interact and have important consequences for mitotic division(615). Evidence already exists that there is a correlation between DNA replication timing and methylation dynamics in cancers, underlining how UHRF1 may be critical to facilitate oncogenic growth(616). In tumors, late replication regions are comprised of hypomethylation, which this included manuscript in Chapter 3 also supports(616). Notably, partially methylated domains (PMDs) in late-replicating regions are known to contribute to genomic instability and the evolution of cancers (i.e. activation of oncogenes due to less insulation and increased mutations), and these regions often contain genes involved in cell cycle and proliferation(615). Intriguingly, high PMD-containing cancers also exhibited upregulated UHRF1 expression(615). Further studies are needed to better delineate the connection of UHRF1 with DNA methylation and replication dynamics and their combined influence on cell division.

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