CHARLES UNIVERSITY

Faculty of Science

Department of Cell Biology

Lukáš Kolumpek

Control of DNA replication mediated by the ubiquitin-proteasome system

Regulace replikace DNA pomocí ubikvitin proteazomového systému

Bachelor's thesis

Supervisor: Mgr. Lukáš Čermák, Ph.D.

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Poděkování

Rád bych poděkoval své rodině, přátelům a kolegům z fakulty Přírodovědecké a Filosofické za jejich podporu v posledních měsících. Také bych chtěl vřele poděkovat všem členům Laboratoře nádorové biologie z ÚMG a především svému školiteli Mgr. Lukáši Čermákovi Ph.D. za jeho čas, spolupráci a cenné připomínky k mé práci.

Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

Abstract

The ubiquitin-proteasome system is an essential cellular instrument that provides timely degradation of specific protein substrates. This thesis examines its role in the regulation of DNA replication with emphasis on human cells, while thoroughly exploring DNA replication, with respect to its position in the cell cycle, and the ubiquitin-proteasome system.

Keywords: Ubiquitin, proteasome, ubiquitin ligase, DNA replication, cell cycle

Abstrakt

Ubikvitin-proteazomový systém je důležitý buněčný systém, který zajišťuje včasnou degradaci specifických proteinů. Tato práce se zaměřuje na jeho roli při regulaci replikace DNA s důrazem na lidské buňky a detailní výklad replikace DNA a ubikvitin-proteazomového systému.

Klíčová slova: Ubikvitin, proteazom, ubikvitin ligáza, DNA replikace, buněčný cyklus

List of abbreviations

AAA^+	ATPase associated with diverse cellular activities
APC/C	Anaphase promoting complex/cyclosome
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
ATR	Ataxia telangiectasia and Rad3-related protein
C	Carbon
CAK	Cyclin-dependent kinase activating kinase
CCNF	Cyclin F
CDC6	Cell division cycle 6
CDC20	Cell division cycle 20
CDC45	Cell division cycle 45
Cdc48	Cell division cycle 48
CDH1	Cadherin-1
CDK	Cyclin-dependent kinase
CDT1	Chromatin licensing and DNA replication factor 1
CDT2	Chromatin licensing and DNA replication factor 2
CHK1	Checkpoint complex 1
CMG	CDC45-MCM-GINS complex
CP	20S core particle
CRL	Cullin-RING ligase
CRL4	Cullin-RING ligase 4
CRYAB	Alphas-crystallin B chain
CUL	Cullin
CUL3	Cullin 3
CUL4A	Cullin 4A

Ddi1	DNA damage-inducible 1 homolog 1
DNA	Deoxyribonucleic acid
Dpb11	DNA replication regulator DPB11
DBF4	Dumbbell former 4 protein
Ddb1	Damage-specific DNA binding protein 1
Dbf4p	Dumbbell former 4 protein
DDK	Dbf4-dependent kinase
DDR	DNA damage response
DDT	DNA damage tolerance
DPC	DNA-protein crosslinks
dsDNA	Double-strand deoxyribonucleic acid
Dsk2	DSK2 ubiquitin domain-containing protein
DUB3	Deubiquitinase 3
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
E2F	E2F family of transcription factors
EMI1	Early meiotic induction protein 1
FA	Fanconi anemia
FEN1	Flap endonuclease 1
FBW7	F-box/WD repeat-containing protein 7
FBX4	F-box only 4
FBXL2	F-box and leucine-rich repeat protein 2
FBXO31	F-box protein 31
FBXW8	F-box/WD repeat-containing protein 8
G0	Gap 0 phase

G1	Gap 1 phase
G2	Gap 2 phase
GINS	Go, Ichi, Nii and San complex
GSK-3β	Glycogen synthase kinase-3β
HECT	Homologous to E6-AP Carboxyl Terminus
hspa6	Heat shock protein family A member 6
HR	Homologous recombination
IBR	In-between-RING domain
ICL	Interstrand crosslinks
K	Lysine
KEAP1	Kelch-like ECH-associated protein 1
KPC	Klebsiella pneumoniae carbapenemase
LRR1	Leucine-rich repeat protein 1
M	Mitosis or Methionine
MAPK	Mitogen-activated protein kinase
MCC	Mitotic checkpoint complex
MCM	Minichromosome maintenance complex
mESC	Mouse embryonic stem cell
mRNA	Messenger ribonucleic acid
N	Nitrogen
NEIL3	Nei endonuclease VIII-like 3
NER	Nucleotide excision repair
NSD	N-terminal serine/threonine-rich domain
O	Oxygen
OBI1	ORC ubiquitin ligase 1
ORC	Origin recognition complex

ORCA	Origin recognition complex
PCNA	Proliferating cell nuclear antigen
PDIP	Pol δ interacting protein
PIP box	PCNA interaction protein box
Pirh2	p53-induced protein with a RING-H2 domain
Pol2	DNA polymerase epsilon catalytic subunit
Pol3	DNA polymerase delta catalytic subunit
Pol α	DNA dependent DNA polymerase α
Pol γ	DNA dependent DNA polymerase γ
Pol δ	DNA dependent DNA polymerase δ
Pol δ3	Trimeric DNA dependent DNA polymerase δ
Pol δ4	Tetrameric DNA dependent DNA polymerase δ
Pol ε	DNA dependent DNA polymerase ε
Pol η	DNA dependent DNA polymerase η
POLA/PRIM	DNA polymerase α/Primase complex
POLE1	DNA polymerase epsilon catalytic subunit
POLD1	DNA polymerase delta catalytic subunit
PP1	Protein phosphatase 1
pRb	Retinoblastoma protein
Pre-RC	Pre-replication complex
Rad23	Nucleotide Excision Repair Protein
RBR	RING-between-RING
RBX1	Ring-box 1
RFC	Replication factor C
RPC	Replisome progression complex
RFWD3	Ring Finger And WD Repeat Domain 3

RING	Really Interesting New Gene
RNA	Ribonucleic acid
RNF8	Ring Finger Protein 8
RP	19S regulatory particle
RPA	Replication protein A
Rpn	Ribophorin
Rpt1-6	Regulatory Particle Triphosphatase 1-6
S	Synthesis phase or Svedberg
SAC	Spindle assembly checkpoint
SCF	Skp, Cullin, F-box containing complex
Sem1	26S proteasome complex subunit SEM1
SKP2	S-phase kinase-associated protein 2
Sld2	Synthetically Lethal with Dpb11 2
Sld3	Synthetically Lethal with Dpb11 3
ssDNA	Single strand deoxyribonucleic acid
SUMO	Small Ubiquitin-like Modifier
SUMO3	Small Ubiquitin-like Modifier 3
Thr	Threonine
TLS	Translesion synthesis
TRAIP	TRAD Interacting Protein
TS	Template switching
Ub	Ubiquitin
UBD	Ubiquitin-binding domain
Ufd1	Ubiquitin fusion degradation protein 1
UPS	Ubiquitin-proteasome system
USP7	Ubiquitin-specific protease 7

Usp37	Ubiquitin-specific protease 37
UV radiation	Ultra-violet radiation
VCP	valosin-containing protein
VprBP	Vpr (HIV-1) Binding Protein

Contents

1. Introduction

The ubiquitin-proteasome system is an intricate degradation machinery that plays an essential role in many cellular processes, importantly including DNA replication. This thesis aims to explore the complexity of this regulation in human cells, while shedding light on the fundamental processes involved. The loss of function of the ubiquitin-proteasome pathway has been shown to lead to pathogenesis and is currently thoroughly studied with the aspiration to medical advances and future opportunities in targeting cancer.

2. Eukaryotic DNA replication

2.1. A brief history of DNA

From the discoveries in the late 19th century made by J.F. Miescher, who unveiled an unusual ratio of distinct elements, particularly phosphorus, in the content of the cell nucleus, and two decades later by R. Altmann, who successfully separated nucleic acid from proteins, to the transforming principle which was revealed during experiments with *S. pneumoniae* by F. Griffith, a novel breakthrough arose - there truly might be a specific molecule standing behind one of the prime principles of life: replication[1, 2]. This presumption was finally confirmed by the team of O.T. Avery, C.M. McLeod, and M. McCarthy, who discovered that the molecule behind the transforming principle is DNA, thus revealing the genetic material for bacteria (later it was discovered that DNA serves as the genetic material also for archaea, eukaryotes, and some viruses)[3, 4].

Watson and Crick later famously proposed the first highly accurate model of the structure of DNA and gave solid arguments for the semiconservative model of replication (the two others being the conservative and the dispersive model)[5]. The semiconservative model of replication was finally confirmed in 1958 by Meselson and Stahl, who discovered that the content of N^{15} in the DNA of E. coli grown in N^{15} containing medium halves in the next generation if grown on N^{14} and then continues to correspond to the semiconservative model in every subsequent generation[6].

2.2. The mechanism of DNA replication

The semiconservative model states that during DNA replication, both strands of the parental dsDNA are used as templates for creating nascent dsDNAs, which, therefore, contain one newly synthesized and one parental strand. The underlying mechanism is that the sequence of bases incorporated in the structure of ssDNA is read base by base by DNA dependent DNA

polymerases that continuously assign corresponding bases to the ones in the template (Figure 2). Human cells contain many different families and types of DNA-dependent DNA polymerases depending on the location and situation (mitochondrial Pol γ belongs to the A-family of DNA polymerases, whereas nuclear polymerases α, δ and ε are all classified into the B-family – polymerases from this family typically have the catalytic activity and the proofreading activity, the latter is, however, inactive in Pol α)[7, 8]. The ligation of a base to the previous one is facilitated by the catalytic activity of distinct subunits of polymerases – Pol2/POLE1 subunit in Pol ε and Pol3/POLD1 in Pol δ[8].

Since DNA polymerases ε and δ require a free hydroxyl group for DNA synthesis, the launch of their activity depends on a preexisting DNA segment, which is initially represented by a primer composed of DNA and a short segment of RNA (Figure 2)[9]. The primer is synthesized by DNA polymerase α/Primase complex, comprising two subunits of the polymerase and two primase subunits. At first, the primase synthesizes a short RNA oligomer, which provides the free 3' end with a hydroxyl group. This fragment is then extended by Pol α, which, however, has a relatively low processivity and, as such, synthesizes only the first fraction of the nascent DNA strand[10, 11]. Upon the essential creation of the primer, replication is facilitated by Pol ε and Pol δ.

The highly required precision of DNA replication is controlled by a complex DNA repair system on which Pol δ and Pol ε participate by their proofreading activity, which is facilitated by their 3'-5' exonuclease activity (this is triggered if the nascent sequence does not pair properly with the bases in the template.)[12] It was also suggested that Pol δ participates on proofreading of both the lagging strand and the leading strand[13].

As mentioned above, Pol ε and Pol δ require a free hydroxyl group for their catalytic activity. However, as dsDNA consists of two antiparallel strands, only the one with the 5'-3' orientation, the leading strand, can be synthetised continuously. The other strand, also called the lagging strand, must thus be synthesized in a discontinuous manner, which utilizes the employment of multiple primers that are continuously added further from the nascent strand, thus allowing the 5'-3' catalytic activity of Pol δ to take place (Figure 2). Consequently, a series of fragments called the Okazaki fragments is formed[14]. These fragments are later ligated into an uninterrupted DNA strand during the Okazaki fragment maturation (Figure 1).

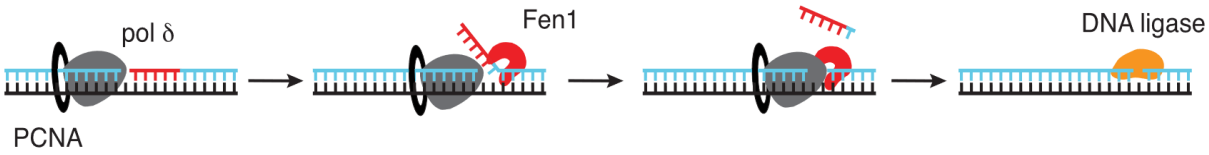

Figure 1: A simplified model of Okazaki fragment maturation. Taken from Greenough et al[17].

The progression of Pol δ upstream of an Okazaki fragment leads the polymerase directly into the downstream fragment, creating an overlapping 5' flap segment. This flap is cleaved by flap endonuclease 1 (FEN1)[15]. The nicks in between individual Okazaki fragments are consequently filled by the activity of DNA ligase I, thus connecting all the Okazaki fragments and forming two complete dsDNA molecules[16].

Although the central part of DNA replication occurs during the S phase of the cell cycle, the entire process is strictly temporally segregated, and a crucial part, licensing, takes place from metaphase on and through the G1 phase[17]. During licensing, several initiation factors bind to distinct parts of DNA called replication origins. The eukaryotic replication origins in higher eukaryotes, unlike the bacterial ones, usually do not contain consensus DNA sequence elements, instead, they rely on a combination of differing factors – mainly the local structure of chromatin and epigenetics[18 19].

The process of licensing is launched by the association of the Origin Recognition Complex (ORC) with a replication origin[20]. This allows the Cell Division Cycle 6 (CDC6) and Chromatin licensing and DNA replication factor 1 (CDT1) to bind to the origin of the replication site and recruit the minichromosome maintenance (MCM) complex (a ring-like heterohexamer composed of MCM2-7 subunits), which concludes the formation of the pre-replication complex (pre-RC)[21, 22]. In the S phase, the MCM complex is complemented by CDC45 and GINS, forming two head-to-head CMG complexes on the leading strand template. After the origin firing, CMGs start translocating in the 3'-5' direction (Figure 2)[23].

The helicase activation and, consequently, replication are controlled by multiple activating and inhibiting factors, which ensure the temporal segregation of origin licensing and firing. The formation of the CMG helicase is allowed by the activity of CDK2 complexes that phosphorylate Sld2 and Sld3. This allows them to create a complex with Dpb11 and consequently recruit Cdc45 to Mcm2-7[24, 25, 26, 27]. The dissociation of this complex subsequently allows the binding of GINS[28].

The Dbf4-dependent kinase (DDK) subsequently phosphorylates the MCM complex, promoting replication[26]. Interestingly, in *Saccharomyces cerevisiae*, it was shown that

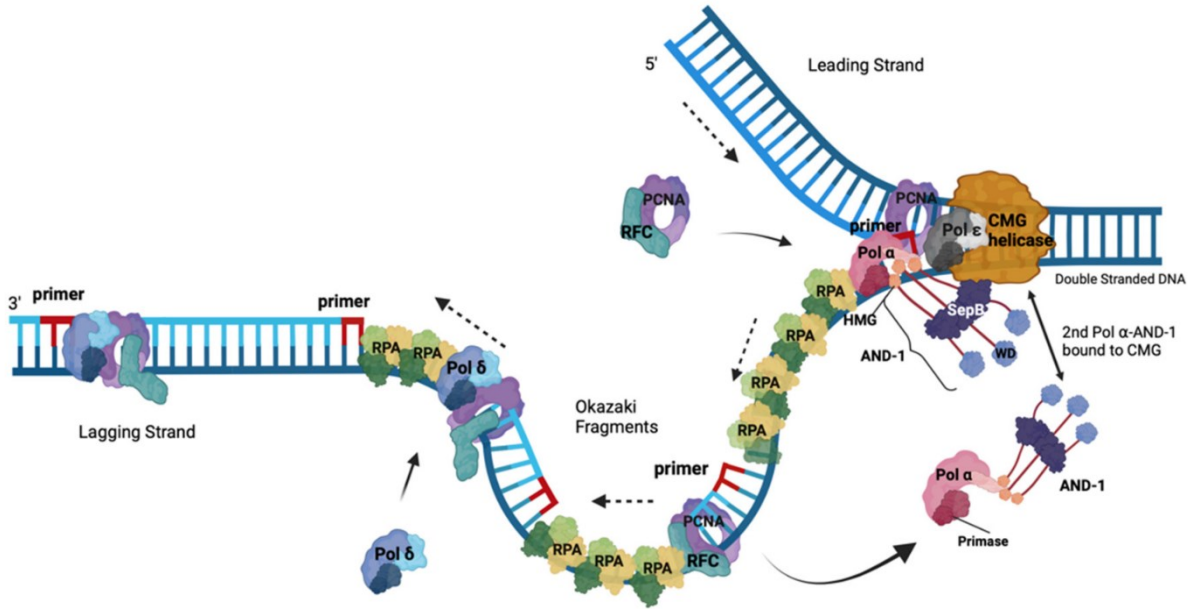

Figure 2: A schematic model of the eukaryotic replication fork. Taken from Nasheuer et al[30].

the impact of this modification is caused by discharging the inhibitory activity of the N-terminal serine/threonine-rich domain (NSD) of Mcm4[29].

Although DNA polymerases are the critical actors of DNA synthesis, there are several other factors whose presence is essential for their activity. After the replication bubble forms, segments of ssDNA form along. These segments could easily reassociate, which is prevented by the binding of replication protein A (RPA) (Figure 2)[33].

DNA polymerases ε and δ periodically dissociate from chromatin and must be replaced with new ones. To prolong the duration of their association with chromatin, they are bound by proliferating cell nuclear antigens (PCNAs) – homotrimeric ring-shaped clamp proteins that are continuously loaded onto chromatin by replication factor C (RFC) in an ATP-hydrolysis dependent manner (Figure 2)[30 31, 32]. Importantly, PCNA also functions as an organising centre for many vital processes, as explored further.

The helicase activity of CMG complexes creates positive supercoils in front of the helicase and negative supercoils behind it. This helical stress can be released either by rotation of the replication fork or by the activity of topoisomerases, which can cut DNA strands, rotate them, and reconnect them, thus lowering the linking number and releasing the helical stress[34].

2.3. Termination of DNA replication

The termination of an unperturbed DNA replication occurs when two replication forks meet. Since CMG helicases appear to associate with leading strand templates, two approaching CMGs will meet on different strands. Moreover, because there is not much evidence for stable

associations between the replicative machinery on leading and lagging strands, the CMG would proceed onto the lagging strand template after the fusion of the two converging replication forks. It is speculated that the CMGs continue their way on the lagging strand template until they reach the closest Okazaki fragment. After that, the CMG proceeds, however, without unwinding dsDNA, which is allowed by a 5' flap[35].

As discussed later, the CMG helicase is subsequently disassembled in a ubiquitin-dependent manner, together with components of the replisome progression complex that are bound to CMG. Other proteins that do not interact with the CMG complex but are used during the DNA replication probably dissociate independently of the DNA replication termination[35].

3. Eukaryotic cell cycle

The mechanistic and functional changes occurring during the life of a proliferating cell can be artificially divided into four distinct phases – G1, S, G2, and M phase. The M phase, itself composed of four main phases, allows cell division, while the former three phases, altogether called the interphase, prepare the cell for division. Nevertheless, most cells of an adult mammalian organism do not enter the cell cycle and instead exit to quiescence (G0), a reversible cell cycle arrest.

Each of the three phases of interphase encompasses several essential events, such as growth during G1, DNA replication during the S phase, and control of the replicated strands during G2. Moreover, the whole cell cycle is intertwined by several checkpoints that ensure accurate progress to the next phase.

The chief regulators of cell cycle progression are cyclin-dependent kinases (CDKs), whose activities are controlled by oscillating levels of specific cyclins and CDK-activating kinases (CAKs)[164].

4. The ubiquitin-proteasome system

4.1. The ubiquitin-proteasome system in the context of cellular life

The sustainment of life depends on the precise cooperation of temporally and spatially coordinated molecules, of which a fundamental fraction is represented by proteins. Consequently, an accurate apparatus controlling each protein throughout its existence – its synthesis, regulation of its function, and its degradation – is required.

The synthesis of proteins is mediated by transcription of a DNA sequence into mRNA and subsequently by translation of mRNA into a sequence of amino acids according to the genetic

code[36, 37]. However, a plain sequence of amino acids itself is not sufficient for the function of a protein. It is the proper 3D structure of proteins that usually allows their function. This native structure of a protein is achieved in an endoergic manner, according to the entropic principle, which proposes that the spontaneous folding is allowed by distinct properties of amino acids that may cause, depending on their characteristics, a structural change in the surrounding molecules of the water crystal, which would increase their organization. Since this would violate the second law of thermodynamics, the peptide is instead forced to fold in a manner that allows for the lowest organization of water molecules, while inner hydrogen bonds drive the formation of secondary structures[38, 39].

Nevertheless, this automatic process may lead to stable structures that are not native and thus must be refolded (which is allowed by chaperones) to their functioning conformation[40]. The other option is protein degradation. Many new proteins must be degraded for their improper folding, which is also facilitated by chaperones[41]. Moreover, proteins that are no longer needed must be disassembled too – firstly, it allows for the homeostasis of amino acid levels; secondly, such a protein may be pathogenic for the cell or cause behaviour pathogenic for the whole organism as discussed later[42, 43].

In eukaryotic cells, two systems are responsible for protein degradation – the autophagy-lysosome and the ubiquitin-proteosome systems. While the main focus was initially put on lysosomes, recent decades of research have highlighted the importance of the ubiquitin-proteasome system[44].

4.2. The ubiquitin system cascade

The ubiquitin-proteasome system serves as the central cellular protein-specific proteolysis system, the fundamental principle being that proteins ubiquitylated by the ubiquitin system cascade (Figure 3) are recognized by proteasome, which disassembles them[45].

The ubiquitin system cascade is composed of three main enzymes – the ubiquitin-activating enzyme (usually abbreviated as E1), the ubiquitin-conjugating enzyme (E2), and the ubiquitin ligase (E3) – the coordinated engagement of the three enzymes leads to the ligation of ubiquitin (a highly stable and among eukaryotes conserved protein) to the target substrate[46,47]. E1s, ATPases which of the three enzymes are the least specific ones, being able to interact with numerous E2s, bond ubiquitin due to hydrolysis of ATP, thus creating a macroergic thiolester (Figure 3)[48]. The activated ubiquitin is then reconnected to an E2, directly associating with an E3-substrate complex. The association of E2s and E3s catalyses the transfer of ubiquitin to the substrate. Consequently, the ubiquitylated substrate is released from the E3. Ultimately,

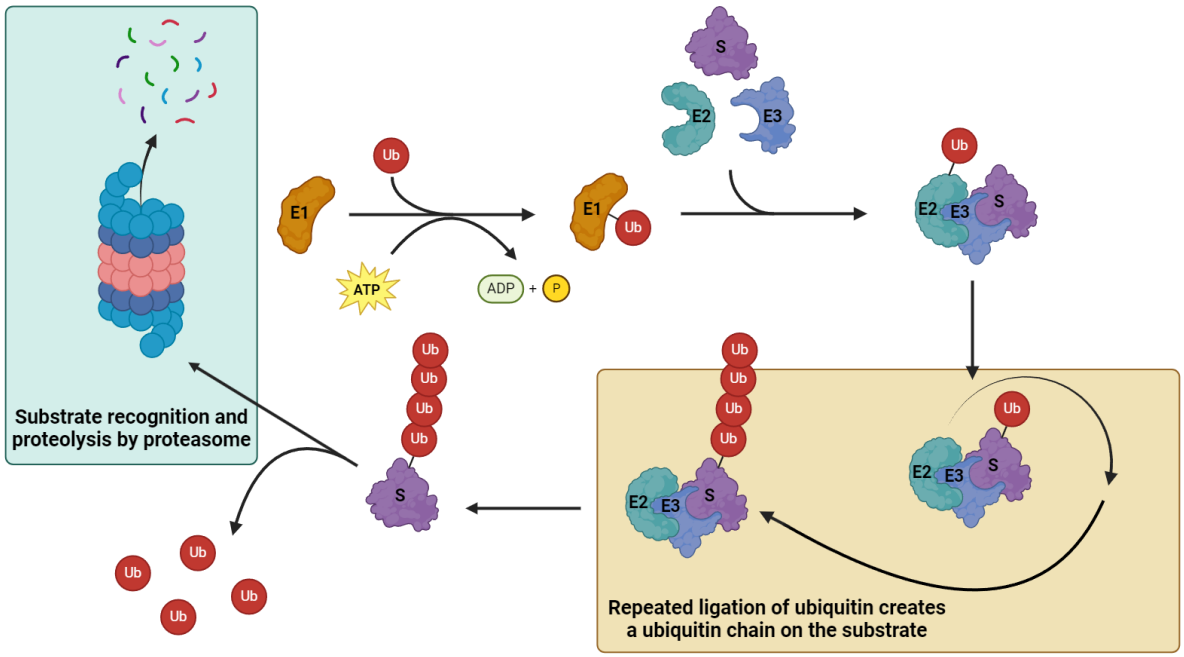

Figure 3: A simplified model of the ubiquitin system cascade. Created with BioRender.com

the substrate specificity of E3s side by side with the lower specificity of E2s and E1s contributes to a complex hierarchical system of enzymes that, with increasing specificity, carry ubiquitin to the target substrate while providing an effective system that does not require high variability of all involved types of enzymes[49, 50, 51].

Ubiquitin ligases are divided into three structurally and functionally distinct classes[52]. The largest one, the RING family, is defined by the presence of a Really Interesting New Gene (RING) domain that directly binds E2-Ub. This allows for the proximity of the activated ubiquitin and the target substrate while also allosterically catalysing the transfer[53]. Very frequent RING ligases are Cullin-RING ligases (CRLs), in which RING is accompanied by a cullin scaffold protein (CUL) and a substrate adaptor[54, 55].

Another E3 class is the Homologous to E6-AP Carboxyl Terminus (HECT) family. The N-terminal lobe of HECT ligases contains an E2 binding domain, while the C-terminal lobe contains a catalytic cysteine. A flexible hinge region connects both lobes[53]. Unlike RING E3s, HECT ligases initially need to accept the activated Ub from the E2, forming a thioester on their catalytic cysteine, and only then can they ubiquitylate the substrate[53].

The smallest class, called the RING-between-RING (RBR) family, is characterised by the presence of a RING1 domain, an in-between-RING domain (IBR), and a catalytic RING2 domain. Mechanistically, RBR ligases are hybrids of RING and HECT ligases. Although initially their RING1 domain associates with the E2-Ub, the rest of the interaction is more

similar to the one of HECT ligases, as the ubiquitin must be at first transferred to the E3 itself (to the catalytic cysteine of the RING2 domain) and only then can it be attached to the substrate[56].

4.3. The ubiquitin code

A single activity of the ubiquitin system cascade results in an isopeptide bond between the C-terminus of ubiquitin and, usually, a lysine from the substrate, that is – monoubiquitylation. However, the process of ubiquitylation may continue, leading either to the assignment of multiple moieties of ubiquitin, each to a distinct residue of the substrate (multimonoubiquitylation), or the creation of a ubiquitin chain (as short as two ubiquitins or longer than ten moieties) of a differing topology on an already substrate-linked ubiquitin (polyubiquitylation) (Figure 3). Accordingly, the variability of ubiquitylation enciphers a diverse system called the ubiquitin code, which, by employing specific proteins bearing ubiquitin-binding domains (UBDs), allows for leading proteins to their distinct fates[46].

Polyubiquitylation is allowed by modifying one of the seven lysine residues (K6, K11, K27, K29, K33, K48, K63) or the N-terminus of the already connected ubiquitin. This, combined with other factors (including topology**,** localization of the enzyme or substrate, or reversibility and timing of the reaction), contributes to the variability of the ubiquitin code. Ubiquitin chains promote proteolysis by the proteasome, often when K11- or K48-linked chains are used. This modification, although irreversible upon engagement of the substrate in proteasome, can be reversed by the activity of deubiquitinating enzymes (DUBs), which can remove ubiquitin moieties from the substrate[46].

Monoubiquitylation or K63-linked chains may lead to a non-proteasome mediated protein degradation. Many ubiquitylations also result in a completely non-proteolytic pathway. This is often connected with monoubiquitylation or K63-/M1-linked ubiquitin chains[46].

4.4. The structure of the 26S proteasome

Substrates ubiquitylated in a proteasome degradation-promoting manner are subsequently recognized by the proteasome (Figure 4), a multisubunit complex whose catalytic function is in eukaryotes facilitated by the 20S core particle (CP)[57]. Although the 20S proteasome can degrade ubiquitin-tagged substrates on its own, it is usually accompanied by other subunits, proteasomal activators[58]. The degrading activity itself is mediated by the proteolytic chamber of the β rings

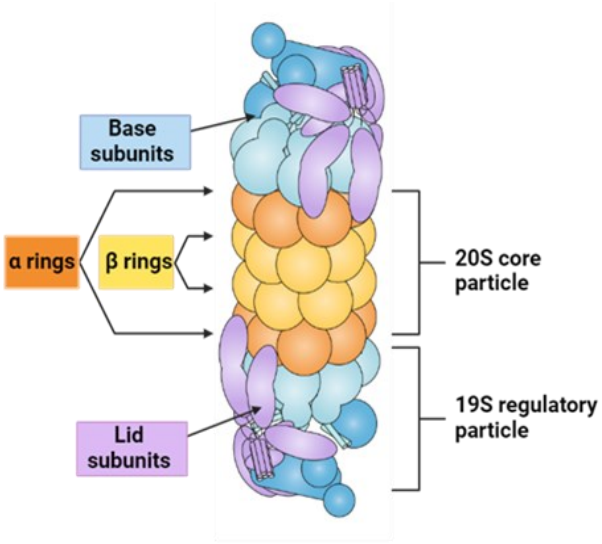

Figure 4: Schematic structure of the 26S proteasome. Taken from Rousseau and Bertolotti and modified with BioRender.com[61].
The 20S core particle consists of hexameric alpha and beta rings. The 19S regulatory particle consists of the base and the lid. The base is composed of four non-ATPase subunits (Rpn1, Rpn2, Rpn10 and Rpn13) and a heterohexameric ring that is composed of six AAA$^+$-ATPase subunits (Rpt1-Rpt6). The lid consists of nine non-ATPase subunits (Rpn3, Rpn5-9, Rpn11, Rpn12 and Sem1)[59].

(specifically by β1, β2, and β5 subunits) of CP (Figure 4), while proteasomal activators allow for the entrance of the protein into CP (by recognition, unfolding and translocation of the protein). The most thoroughly studied of these activators is the 19S regulatory particle (RP), which in a complex with 20S core particle forms the 26S proteasome[59, 60].
When the substrate is irreversibly engaged into the entry channel of the proteasome, which is mediated by the activity of the ubiquitin receptor subunits, Rpn1, Rpn10 and Rpn13 (Figure 4) and (in yeast) by shuttling factors Rad23, Dsk2 and Ddi1 that bring the ubiquitylated substrate to the proteasome, Rpn11 (or other distinct deubiquitinating enzymes) cleaves the ubiquitin chain[59, 60]. The inserted protein is then proteolyzed into small peptides (Figure 3)[42].

5. Ubiquitin-mediated control of DNA replication

The DNA replication is precisely controlled by a complex system of regulators. Factors that control replication, among others, include ubiquitin ligases and proteolytic or non-proteolytic ubiquitylation. Importantly, a set of ubiquitin ligases is focused on the regulation of the periodical transitions between distinct cell cycle phases.

5.1. The ubiquitin-proteasome system in the cell cycle context of replication

5.1.1. The ubiquitin-proteasome system during the G1/S transition

The principal control machinery behind the cell cycle progression is conservatively composed of cyclin-dependent kinases (CDKs) and oscillating levels of their regulators. Their activity is accompanied by several checkpoints, providing a system in which each step of the cell cycle must be finished before the initiation of the next one. A vital component of this mechanism during G1 is the inhibition of genes essential for S phase entry. Their transcription is controlled by members of the E2F family of transcription factors and is inhibited by the principal regulator

of E2F proteins, the retinoblastoma protein (pRb) (and the related p107 and p130, which target some of the E2F repressors) (Figure 5)[61, 62].

Binding of mitogens (growth factors and growth inhibitory factors) to a newly formed cell leads to a response that sets the subsequent fate of the cell (Figure 5). This event occurs during the restriction point in late G1 and leads either to entry to the next phase or cell cycle arrest[63, 64]. Positive signals from growth factors promote the S phase entry and progress through the rest of the cell cycle (Figure 5). This is facilitated by increased transcription of D-type cyclins, the binding partners of CDK4 and CDK6[65, 66]. The resulting complexes phosphorylate pRb, which together with the subsequent phosphorylation mediated by CDK2 (in complex with cyclin E in G1 and cyclin A in S phase) leads to the inactivation of pRb. This allows for the expression of E2F-controlled genes[67,68]. The inhibitory activity of pRb is then reestablished in late mitosis by PP1-mediated dephosphorylation[69].

On the other hand, the binding of growth inhibitory factors or depletion of growth factors activates pathways promoting cell cycle arrest, such as p21 transcription (Figure 5)[70, 71]. The activity of p21 leads to the inhibition of G1 cyclin-CDKs and consequently to obstruction of the aforementioned pathway[72]. Importantly, p21 can be also transcribed in response to DNA damage via activation of p53[73]. The activity of p21 is accompanied by p27, another prominent inhibitor of G1 cyclin-CDKs (Figure 5)[74].

The ubiquitin-proteasome system plays a crucial part in these processes, as proteolysis of distinct factors involved in the G1/S transition ensures the strict separation of cell cycle phases and protection from re-replication.

The E2F family comprises of multiple different factors, including both transcriptional activators and repressors. Members of both have been shown to be degraded by the ubiquitin-proteasome system (Figure 5), which restricts transcription of S phase-promoting genes to the appropriate phase of the cell cycle. All three activators (E2F1-3a) are degraded during S and G2 phases by SCF^{CCNF} [81]. This ubiquitin ligase also targets two repressors, E2F7 and E2F8, during G2[82, 83]. Similarly, APC/C^{CDH1} also targets activators and repressors – E2F8, E2F3 and E2F1[82, 84, 85]. Additionally, E2F1 has been shown to be ubiquitylated by SCF^{SKP2} and APC/C^{CDC20} during the S/G2 phase and mitosis respectively[86, 87].

While the ubiquitin-proteasome system does not target CDKs directly, it can modify their activity through ubiquitin-mediated degradation of cyclins (Figure 5). The ubiquitylation of cyclin D1 requires its previous transfer to cytoplasm, which is facilitated by phosphorylation of

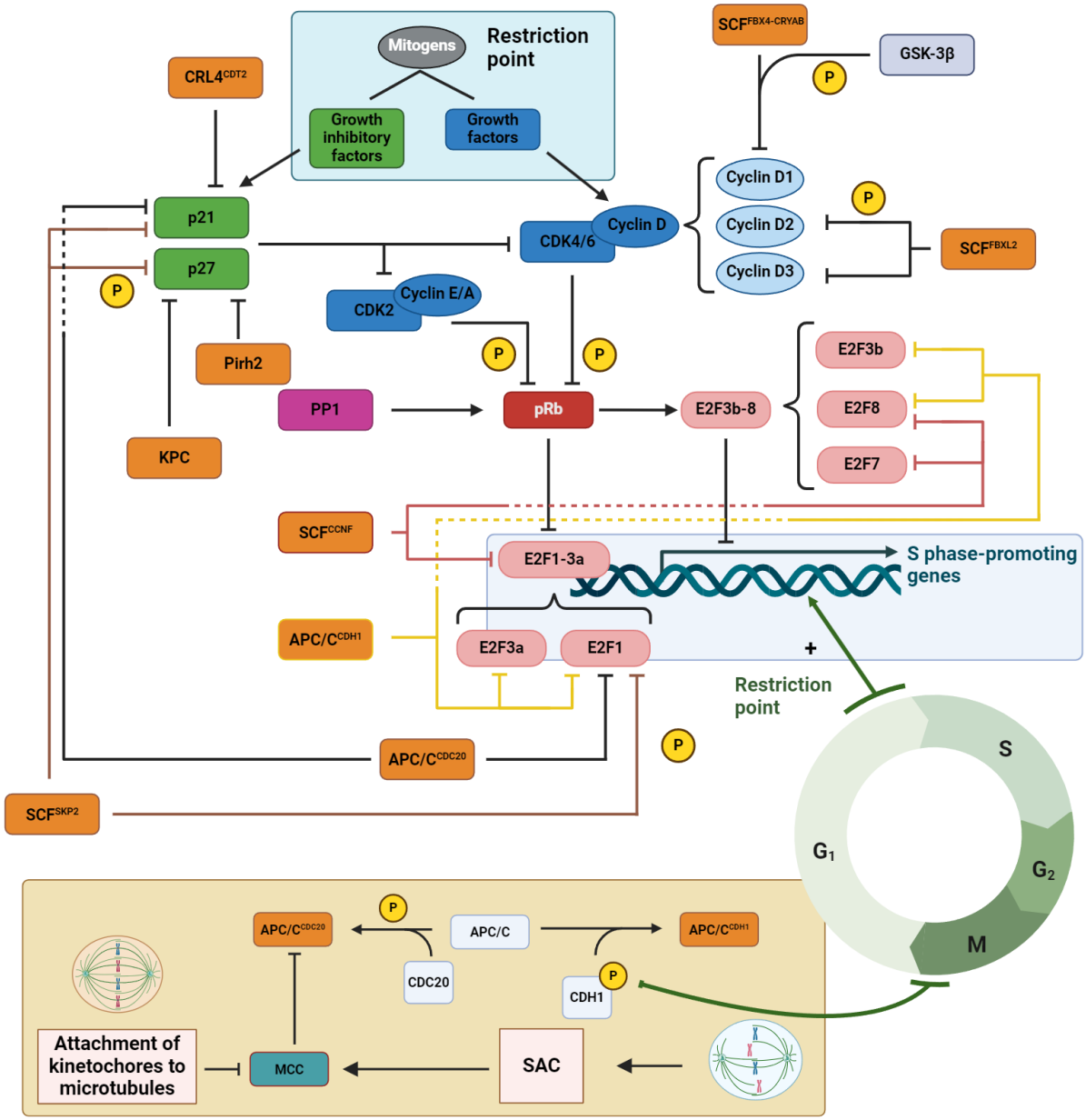

Figure 5: Diagram of the role of UPS at the G1/S transition. Created with BioRender.com.

its Thr286 during the S phase. This has been shown to be mediated by glycogen synthase kinase-3β (GSK-3β) and the E3 ligase $SCF^{FBX4\text{-}CRYAB}$ in unperturbed cells or alternatively by MAPK cascade and the E3 ligase FBXW8 in cancer cell lines[75, 76, 77]. A similar pathway has been discovered for cyclin D2. Its ubiquitylation, and the ubiquitylation of cyclin D3, is mediated by SCF^{FBXL2}, leading to cell cycle arrest[78, 79, 80].

The progression through the cell cycle also requires timely degradation of CDK inhibitors. The principal inhibitors of G1 cyclin-CKDs, p21 and p27, are polyubiquitylated by SCF^{SKP2} during the G1/S transition and later during the S phase[78, 79]. Although modification of both proceeds similarly, the ubiquitylation of p27 by SCF^{SKP2} requires previous phosphorylation by

CDK2 (in complex with cyclin E or cyclin A), while phosphorylation of p21 is not necessary[78,79,94]. The degradation of both p21 and p27 does not depend solely on the activity of SCF^{SKP2}. p21 is additionally ubiquitylated by $CRL4^{CDT2}$ during the S phase and later during prometaphase by APC/C^{CDC20} [90,91]. The ubiquitylation of p27 is also mediated by Pirh2 at G1/S transition and by KPC after translocation of p27 out of the nucleus during G1[92,93].

After the decrease of levels of CDK inhibitors and the phosphorylation of pRb, the cell can enter the S phase, which requires the previous assembly of the replication machinery and the origin firing which is controlled by DDK and CDK2 complexes[26]. To prevent re-replication, these enzymes are subsequently inactivated in a UPS-dependent manner, as distinct ubiquitin ligases target binding partners necessary for their activity.

Cyclin E and cyclin A, the activator of CDK2, are conservatively ubiquitylated by SCF^{FBW7} and APC/C^{CDC20} respectively[98,99]. Dbf4p, the activator of DDK, was shown to be ubiquitylated by APC/C upon anaphase in yeast extracts[100]. Moreover, as transcription of all these binding partners is mediated by E2F1, their accumulation is inhibited by the previously explored ubiquitin-dependent degradation of this transcription factor[65,86,97].

5.1.2. Introduction of APC/C, a pivotal ubiquitin ligase of mitosis and G1

The cyclosome/anaphase-promoting complex (APC/C) is essential for the degradation of many proteins and the onset of anaphase. Inhibition of APC/C is controlled by the spindle assembly checkpoint (SAC), a mitotic event that protects key anaphase inhibitors from degradation before the attachment of all chromosomes to the mitotic spindles is secured. This is ensured by the assembly of the mitotic checkpoint complex (MCC) that binds and inhibits the APC/C^{CDC20} complex[101]. The tension generated upon attachment of kinetochores to microtubules later signals the completion of metaphase and leads to silencing of SAC and consequently releasing of APC/C^{CDC20} [102,103].

The substrate specificity of APC/C is allowed by two cofactors – CDC20 and CDH1 (Figure 5). This association is importantly regulated by the phosphorylation of both APC/C and CDH1, which secures the sequential binding of CDC20 and CDH1 respectively. CDC20 associates with phosphorylated APC/C, while binding of CDH1 is inhibited until its dephosphorylation upon mitotic exit[104,105]. APC/C^{CDH1} then promotes its own activity by targeting CDC20[66]. The activity of APC/C^{CDH1} is subsequently inhibited by EMI1 at the G1/S transition after its transcription mediated by E2F factors[124].

5.2. Control of licensing factors mediated by the ubiquitin-proteasome system

As described above, licensing is an essential process that takes place at the end of mitosis and during the G1 phase and results in forming the pre-RC (composed of ORC, Cdc6, Cdt1, and MCM2-7).

5.2.1. The degradation pathways of the Origin Recognition Complex

Uninhibited licensing factors support the danger of re-replication. To prevent it, cells have developed precise mechanisms that ensure the timely degradation of these proteins.

While levels of most subunits of the ORC complex remain stable throughout the cell cycle in human cells, the largest subunit (ORC1) is sequentially expressed and degraded. It is transcribed during the M/G1 transition and later proteolyzed in a ubiquitin-dependent manner during the S phase, which is facilitated by SCF^{SKP2} (Figure 6) [106]. Interestingly, it was suggested that this modification might play multiple roles in DNA replication regulation, as it could take part in the control of origin firing[106]. Importantly, SKP2 is degraded upon the initiation of the next cell cycle by APC/C^{CDH1}, which allows for the accumulation of ORC1 and licensing[95, 96].

Although there is not much more evidence of UPS-mediated control in the case of subunits of the human ORC complex, ubiquitin itself was shown to have a significant impact on the stability and regulation of the complex (Figure 6). Experiments in multiple cancer cell lines revealed that ORC3 and ORC5 are multimonoubiquitylated by OBI1 during the S phase, which likely takes part in origin firing and might be involved in selecting pre-RCs for firing[107]. Moreover, RFWD3-mediated ubiquitylation was shown to be required for the association of ORC with ORC-associated (ORCA), a protein essential for the recruitment of ORC to chromatin and possibly for DNA replication initiation (Figure 6)[108, 109, 110]. The activity of RFWD3 is targeted at ORCA, which is later also polyubiquitylated (likely for degradation since a K48 ubiquitin linkage is created) by Cul4A-Ddb1 (and other, to date unknown, ubiquitin ligases). The latter modification is prevented by ORC2, which binds ORCA and shields its ubiquitylation site until the G1/S transition when ORC2 dissociates from chromatin[111].

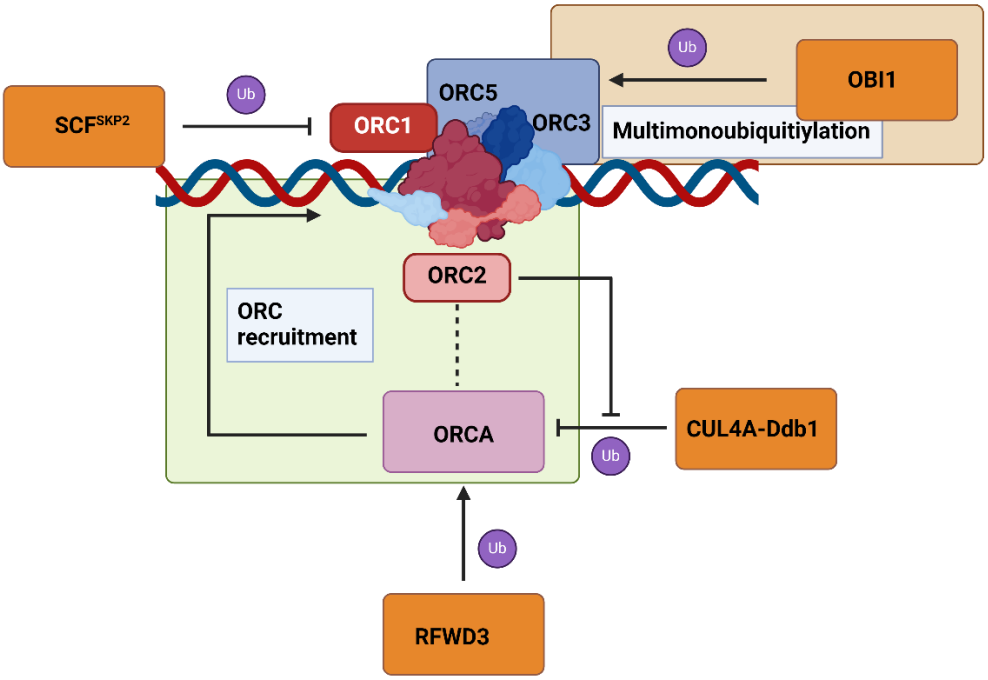

Figure 6: Ubiquitin-mediated control of origin recognition complex. Created with BioRender.com

5.2.2. The UPS and the Chromatin licensing and DNA replication factor 1

The employment of CDT1 in licensing relies on the inactivation of its inhibitor, geminin, which binds CDT1 and blocks its association with chromatin outside of a short time window during late mitosis and G1 (Figure 7). Geminin levels decrease upon metaphase-anaphase transition due to its polyubiquitylation by APC/C^{CDH1} [112, 113, 114]. It was hypothesised that this modification predominantly does not lead to proteasome-mediated degradation. Instead, while a minority of geminin is degraded, the rest is deubiquitylated by DUB3 and USP7, leaving geminin in an inactive state likely caused by structural changes brought by the previous ubiquitylation[113, 115].

Geminin also affects the stability of CDT1 by blocking its ubiquitylation site[114]. Interestingly, even though geminin is present during the S and G2 phases, it does not participate on the accumulation of CDT1 until mitosis, likely because geminin cannot bind it before the nuclear envelope breakdown event during prometaphase[116].

After the inactivation of geminin during the metaphase-anaphase transition, CDT1 can participate on licensing. Subsequently, it must be degraded to prevent re-licensing, which utilises several distinct ubiquitin ligases depending on the phase of the cell cycle (Figure 7). During the S phase, it is ubiquitylated by CRL4^{CDT2}, employing PCNA as an organizing platform. Both CDT1 and CDT2 contain a PCNA interaction protein box (PIP box), which

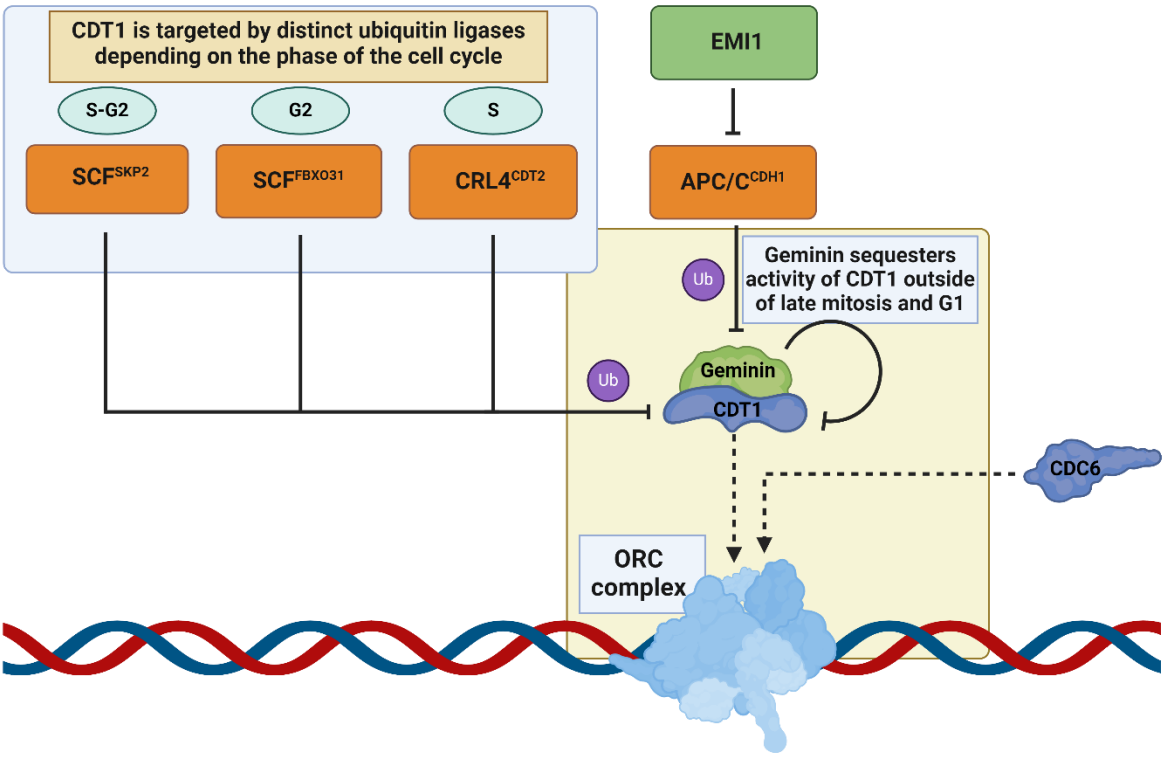

Figure 7: Diagram of UPS-mediated control of CDT1. Created with BioRender.com.

allows them to bind PCNA[117, 120]. Another ubiquitin ligase targeting CDT1 during S-G2 phases is SCF^{SKP2}. This modification requires the previous formation of a phosphodegron mediated by phosphorylation of CDT1 by cyclin E-CDK2 and cyclin A-CDK2[117, 119]. The remaining CDT1 is targeted by SCF^{FBXO31} during G2 in the cytoplasm, where it is transferred after its previous phosphorylation[118].

Interestingly, p97, a prominent factor frequently used for removing ubiquitylated substrates from an attached surface (as explored later), was shown to be involved in removing CDT1 from chromatin. This was demonstrated in *X. leavis* after exposure to UV irradiation, which led to ubiquitylation of CDT1 by $CRL4^{Cdt2}$ and a subsequent removal for proteasomal degradation by p97 and its cofactor Ufd1. A similar pathway was discovered in *Caenorhabditis elegans*, indicating the conservation of this process in higher eukaryotes. Moreover, it was suggested that this pathway might be employed even during an unperturbed S phase[121, 122].

5.2.3. The role of UPS in regulating the Cell Division Cycle 6

As with other licensing factors, the activity of CDC6 too must be restricted to a distinct phase of the cell cycle, after which it is degraded in a ubiquitin-dependent manner (Figure 8). After its association with chromatin at the M/G1 transition, the unbound CDC6 is targeted by

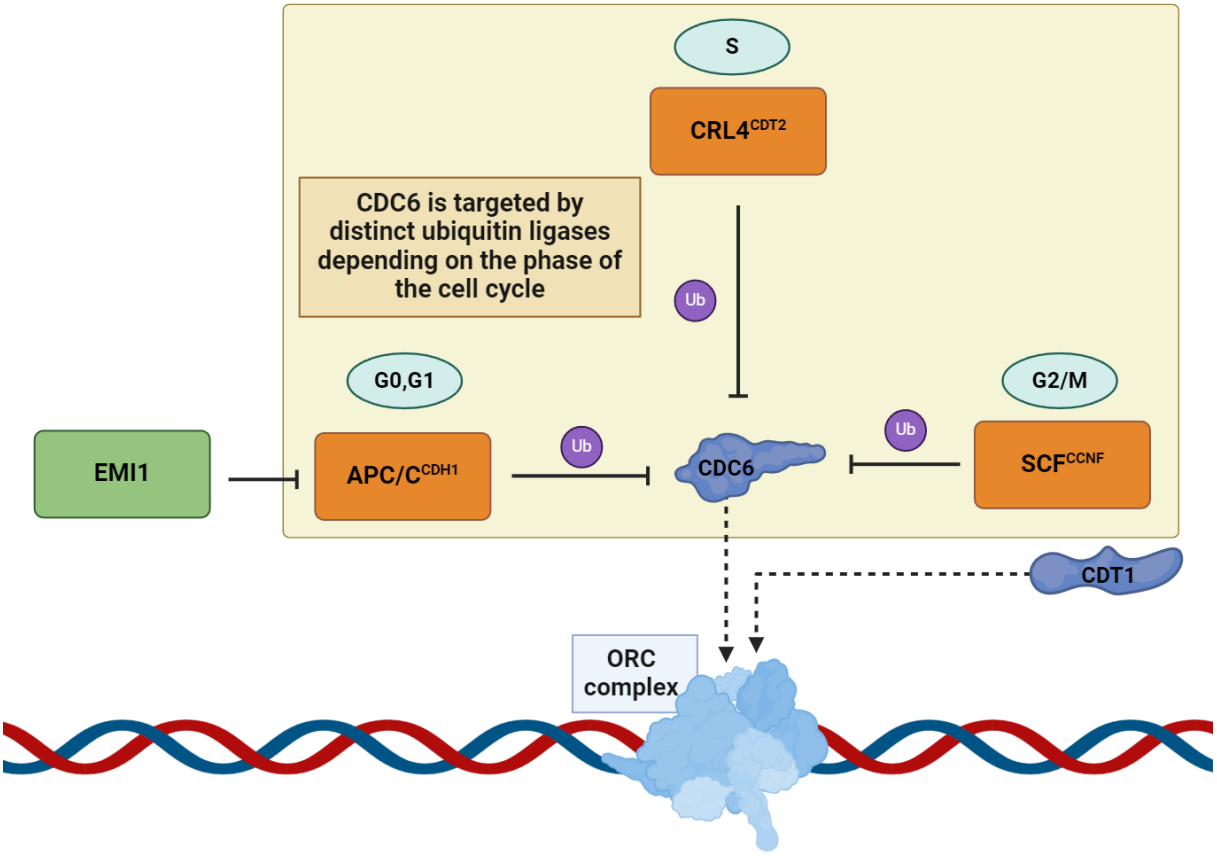

Figure 8: Diagram of UPS-mediated control of CDC6. Created with BioRender.com.

APC/C^{CDH1} during early G1[123]. Later, upon the inhibition of APC/C^{CDH1} by EMI1 at the G1-S transition, CDC6 levels could rise again, leading to the danger of re-replication. This is prevented by another ubiquitin ligase, CRL4^{CDT2}, that targets CDC6 for degradation upon S phase entry. This ubiquitylation utilises the PIP-box-like motif found in CDC6, allowing PCNA to be established as a platform for the process[125]. Later, mainly during late G2 and early mitosis, CDC6 is polyubiquitylated by SCF^{CCNF} [126, 127].

5.2.4. CDT1 and CDC6 in the context of quiescence exit and rapid proliferation

Interestingly, the regulation of CDC6 noticeably differs between rapidly proliferating cells and quiescent cells.

The cell cycle arrest of quiescent cells is a result of activity of a multitude of factors, importantly including the activity of APC/C, which leads to decreased levels of CDC6 and many other proteins. When quiescent cells aim to re-enter the cell cycle, accumulation of cyclin E (a result of E2F-dependent transcription) leads to the formation of cyclin E-CDK2 complexes, which can phosphorylate CDC6, thus masking its degron from APC/C. This process, together with

APC/C mediated degradation of geminin and cyclin A (inhibitor of CDC6), allows for the accumulation of CDC6 and CDT1. APC/C is then inactivated in late G1, leading to the accumulation of geminin and cyclin A[128, 129].

On the other hand, the control of licensing in proliferating cells of metazoa is independent of cyclin E levels. While CDT1 is degraded later during the S phase, CDC6 is degraded upon mitotic exit[130]. This sequential degradation of distinct licensing factors provides a sufficient system for the prevention of re-licensing [116].

5.3. The DNA dependent DNA polymerases and the UPS

While ubiquitylation plays a crucial role in regulating licensing factors to prevent the risk of re-replication, its function in the context of DNA polymerases revolves mainly around the DNA damage response. This has been demonstrated in both Pol α and Pol δ. The importance of ubiquitylation for Pol ε in human cells is unclear.

5.3.1. The DNA dependent DNA polymerase δ

DNA synthesis of the lagging strand during replication in mammals is mainly provided by Pol δ3[144], which comprises three subunits - p50, p68 (p66 in humans), and p125. Nevertheless, the activity of this heterotrimeric polymerase is accompanied by a second, less abundant, form of the polymerase, which comprises one more subunit – p12[148].

The prevailing Pol δ3 was shown to be a more vigilant tool for DNA synthesis, being less prone to performing incorrect synthesis past lesions in DNA – such as an abasic site, a thymine-thymine dimer, 8-oxoguanin or O^6-methylguanine[145]. Moreover, it was shown that Pol δ3 is more effective in processing Okazaki fragments[146].

On the other hand, Pol δ4 has a higher processivity and can associate with distinct Pol δ interacting proteins (PDIPs) that do not associate with Pol δ3[147]. Accordingly, it is utilised during DNA replication, although in a minor degree, because of its higher error production[148]. Accordingly, since the properties of Pol δ3 are ideal for executing DNA synthesis upon DNA damage, the existing Pol δ4s can be converted to Pol δ3s in response to mutagenic factors. This has been shown in experiments with UV, methyl methanesulfonate, hydroxyurea, and aphidicolin treatment, upon which p12 was targeted by proteolysis promoting polyubiquitylation mediated by RNF8, which is controlled by ATR, the apical kinase of DNA damage response regulation during S phase[149, 150]. Interestingly, it was proposed that the RNF8-mediated ubiquitylation of p12 might be involved in the unperturbed cellular turnover of this

subunit[150]. p12 is also targeted by $CRL4^{CDT2}$ in a PCNA-dependent manner upon both DNA damage and S phase entry[151].

In human cells, it was shown that the p66 subunit is also ubiquitylated (mainly monoubiquitylated) and SUMOylated by SUMO3. However, these modifications do not seem to promote proteolysis[152].

5.3.2. The DNA dependent DNA polymerase α

It was proposed that the activation of ATR during the unperturbed S phase is promoted by loading of POLA/PRIM (DNA polymerase α/Primase complex) on chromatin upon the origin firing. This process is limited by the activity of VCP/p97, which removes POLA/PRIM from chromatin, thus preventing excessive ATR activity. It was suggested that the cumulative activation of ATR (and subsequently CHK1) could explain how cells count the number of active DNA replication forks, which helps to prevent excessive origin firing. Extraction of POLA/PRIM by VCP/p97 could also promote DNA polymerases switching from Pol α to elongation polymerases Pol δ and Pol ε[153].

5.3.2.1 The role of MCM10 in regulating the DNA dependent DNA polymerase α

Mcm10 is an essential part of the eukaryotic replisome, bearing several functions as a scaffold protein vital for proper replication. By interacting with DNA and all three main components of CMG helicase, Mcm10 promotes DNA replication initiation. Moreover, Mcm10 also participates in replication fork stability control and recruits PCNA and polymerase α/primase complex, thus mediating the synthesis of Okazaki fragments. In the absence of MCM10, polymerase α/primase complex dissociates from chromatin[154, 155]. Interestingly, it was shown that upon UV irradiation, Mcm10 is promoted for degradation by $CRL4^{VprBP}$-mediated ubiquitylation, which could have an essential role during loading of translesion synthesis mediating polymerases (such as Pol η) during DNA damage response by facilitating dissociation of POLA/PRIM[156].

5.4. The role of the ubiquitin-proteasome system during DNA replication termination

5.4.1. The CDC45-MCM2-7-GINS helicase complex

5.4.1.1 The minichromosome maintenance (MCM) complex

While ubiquitylation has been suggested to be involved in the regulation of the MCM complex in many organisms, little is known about its role in human cells. However, as discussed further, it has been shown that ubiquitylation plays a pivotal role in mediating CMG disassembly through targeting the MCM7 subunit. Moreover, MCM3 has been proposed to be ubiquitylated

by KEAP1 in metazoa as a part of the KEAP1-CUL3-RBX1 complex, nevertheless, the specific role of this modification is unclear[140, 141].

The disassembly of CMG appears to be conservatively driven by ubiquitylation of the MCM7 subunit throughout metazoa[131, 132, 136]. The precise mechanism of CMG disassembly regulation in human cells is unknown since studying CMG helicase in diploid human cells bears many obstructions (the ideal model for studying mammalian CMG helicases was proposed to be mouse embryonic stem cells, mESCs)[131].

In mESCs, the CMG-MCM7 subunit is ubiquitylated by $CUL2^{LRR1}$, which leads to the activation of CMG disassembly during the termination of DNA replication (Figure 8). As a negative regulator of this modification, the deubiquitylating enzyme Usp37 has been suggested[131]. During the mitotic pathway of CMG disassembly, ubiquitylation is provided by TRAIP instead of $CUL2^{LRR1}$ (as also observed in *Xenopus laevis* egg extracts and *Caenorhabditis elegans*, suggesting conservativeness of this modification)[131, 133, 136]. Reasons for restricting $CRL2^{LRR1}$-mediated ubiquitylation to DNA replication termination are unclear. However, it has been proposed that this specificity might be caused by repression of its activity by DNA replication fork during elongation[139].

Interestingly, in *Xenopus* egg extracts, TRAIP has been shown to be the master regulator of DNA lesion repair. Two pathways can be employed during a collision of replication fork with interstrand crosslinks (ICLs, which block the progress through DNA replication) - Fanconi anemia (FA) pathway or a simpler NEIL3-mediated cleavage of the crosslink. Both pathways appear to be regulated by TRAIP-mediated ubiquitylation. TRAIP is associated with replisome, and while it cannot ubiquitylate the replisome to which it is attached during the S phase (a conformational change occurring during mitosis releases this block), it can ubiquitylate factors that would obstruct the course of the replisome, such as DNA-protein crosslinks (DPCs) or other CMGs *in trans* at interstrand DNA crosslinks (ICLs). In this manner, two different types of ubiquitin chains can arise - short chains, which lead to the recruitment of NEIL3, and long chains, which promote the p97-dependent unloading of CMG, which allows for the FA pathway[136].

Both ubiquitylations (S phase-specific and M phase-specific) appear to conservatively lead to a p97-mediated disassociation of MCM7, which, as has been shown in budding yeast, leads to disassembly of CMG into CDC45, GINS, and MCM2-7 (Figure 8), followed by disassembly of the whole replisome progression complex (RPC) during S phase (nevertheless, it is unsure whether other ubiquitin ligases are involved in the process or not)[137, 131].

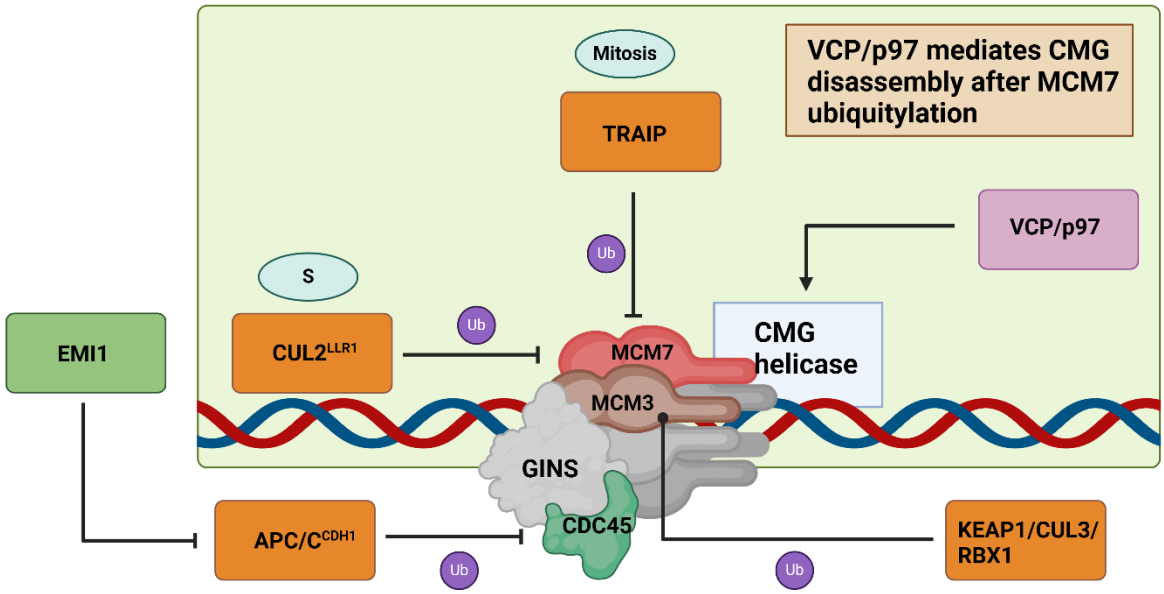

Figure 8: Diagram of UPS-mediated control of CDC6. Created with BioRender.com

p97 (also known as VCP in humans or Cdc48 in *Saccharomyces cerevisiae*) is a hexameric AAA^+ protein that bears several vital functions that are modulated by distinct cofactors. One of its pivotal functions is the remodeling of ubiquitylated proteins that releases targeted proteins from complexes, allowing for proteasomal degradation. p97 is also involved in the coordination of ubiquitylation and ubiquitin editing and might also participate in substrate unfolding by proteasome[138].

5.4.2. GINS and CDC45:

To date, there is little information on ubiquitin-mediated control of the other two subunits of the CMG helicase – GINS and CDC45. While experiments in recent years showed the role of UPS in control of CDC45, to which extent ubiquitin controls GINS remains to be elucidated. CDC45 in human cells is accumulated during late G1, employed in CMG during the S phase, and then promoted for degradation during the subsequent G1 phase by APC/C^{CDH1} [143]. It was demonstrated that high levels of CDC45 block S phase entry, probably due to enhanced expression of *hspa6* (heat shock protein family A (Hsp70) member 6), showing the importance of this ubiquitylation[142].

5.5. The role of ubiquitin in DNA damage response

There is substantial evidence for the role of ubiquitin in the DNA damage response (DDR) during the DNA replication. However, to properly explore the entire role of ubiquitylation in DDR is beyond the extent of this thesis, which will thus encompass only a brief introduction of some of the roles of ubiquitylation in this cellular event.

While PCNA plays an essential role during unperturbed DNA replication, serving as a processing enhancer and an organizing platform, it is also a key factor of the cellular response to DNA damage.

When the moving replication fork encounters an obstacle, such as DNA damage, the progression of the replication fork is obstructed[157]. To ensure the continuation of DNA replication (DNA damage can be repaired during later stages of the cell cycle), cells can turn on DNA damage tolerance (DDT) pathways, which ensure the bypassing of lesions. DDT can be mediated by error-prone translesion synthesis (TLS) or error-free template switching (TS). The pathway employed is chosen by ubiquitylation (or SUMOylation) of PCNA on K164 – monoubiquitylation signals for TLS, whereas polyubiquitylation for TS[158, 159, 160, 161].

Interestingly, ubiquitylation of many histones also plays a vital role in DDR as a part of the nucleotide excision repair (NER) response or homologous recombination (HR) response[162, 163].

6. Conclusion

Throughout this thesis, it was demonstrated that the ubiquitin-proteasome system plays a pivotal role in regulating the intricate enzymatic processes involved in orchestrating cell cycle and replicating DNA, which is principal in preserving genome stability and allowing proper transitions between distinct cell cycle phases. The primary emphasis of this thesis was a detailed description of pathways controlling the degradation of proteins involved in replication. However, it has also been shown that non-proteolytic modifications mediated by ubiquitin binding are an essential part of the proper progress of both DNA replication and cell cycle.

Although this thesis has presented many essential degradation pathways that affect the progress of DNA replication, it has also pointed out that there are a number of cell cycle regulation areas whose molecular mechanisms are still unknown, and thus, research focused on targeted protein degradation is a viable and important direction of the molecular biology studies. This is especially clear by comparison of our understanding of these processes in human cells with knowledge gained from studies in classical laboratory model organisms. This knowledge gap is an opportunity for future research focused on developing novel therapeutic approaches towards cancerous malignancies.

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