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REVIEW

The CRL4^{Cdt2} E3 ubiquitin ligase: A comprehensive review of its substrates, mechanisms, and roles in genomic stability

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Abstract

The CRL4^{Cdt2} ubiquitin ligase complex plays a pivotal role in maintaining genomic integrity and regulating the cell cycle, specifically during the S phase. By targeting key proteins such as Cdt1, p21, and Set8 for ubiquitination and degradation, CRL4^{Cdt2} ensures proper DNA replication and cell cycle progression. Dysregulation of this complex has been implicated in various cancers, including melanoma, breast cancer, gliomas, and ovarian cancer, where elevated levels of Cdc10-dependent transcript 2 (Cdt2) expression is often associated with poor prognosis and increased tumor aggressiveness. CRL4^{Cdt2} role extends beyond cell cycle regulation, as it also participates in the DNA damage response by degrading proteins like XPG, which is essential for nucleotide excision repair. Impaired CRL4^{Cdt2} function leads to DNA re-replication, genomic instability, and cancer progression. Recent studies have highlighted the therapeutic potential of targeting CRL4^{Cdt2} in cancer treatment, with inhibitors like pevonedistat showing promise in preclinical models. However, challenges remain, including the lack of a three-dimensional structure for Cdt2, which limits our understanding of its substrate recognition and degradation mechanisms. This review revisits the role of CRL4^{Cdt2} in regulating its cellular substrates, updated substrates targeted by CRL4^{Cdt2}, explores its pathological consequences in cancer, and discusses potential therapeutic strategies to target this complex, offering new insights into its function and clinical relevance.

Keywords: cell cycle, CRL4^{Cdt2}, PCNA-dependent degradation, ubiquitination

INTRODUCTION

The third principle of cell theory emerges that a cell arises from a pre-existing living cell through a process called cell division (Müller-Wille, 2010). Cell division is a process where the parent cell splits into two daughter cells (Bai et al., 2017). The process of splitting those cells occurs in the mitosis phase in eukaryotic and prokaryotic cells to conserve cells generation and replace the dead cell in a mannered and complex system. Each cell divides by passing through a cell cycle where it comprises four distinct stages known as G1, S, G2 and M (Hunt et al., 2011). The cell cycle consists of four main phases: G1 (cell growth and preparation for deoxyribonucleic acid (DNA) replication), S (DNA synthesis), G2 (further growth and preparation for division), and M (mitosis and cytokinesis) (Ingham & Schwartz, 2017).

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Daughter cells are genetically identical to the parent cell because they inherit the same DNA sequence (Niu et al., 2016). The regulation of genetic information transmission must be well controlled to make sure the DNA is inherited genuinely; thus, the cells can function properly and survive. It is fundamental for the cell cycle system to be strictly regulated as cell growth can be mutated due to chromosomal instability, DNA damage and cells re-replication. Cyclin-dependent kinases, CDKs, is a family of serine/threonine protein kinases that are responsible for strictly controlling each cell cycle stage with the aid of cyclins, and CDK inhibitors (CDKIs), its regulatory companions in suppressing the cell cycle. CDKs promotes protein phosphorylation and conversely dephosphorylation by phosphatases. The phosphorylation and dephosphorylation of essential proteins assist cells in regulating the cell cycle and biological processes. The fluctuation of CDK concentrations typically governs the entire progression of the cell cycle, whereas $CRL4^{Cdt2}$, sometimes referred to as Cullin RING E3 ligase, particularly regulates the S phase (Wang, 2021).

$CRL4^{Cdt2}$ facilitates in controlling important cellular substrates throughout the cell cycle in order to maintain genome integration (Garcia-Barcena et al., 2020). $CRL4^{Cdt2}$ is responsible for maintaining genome integrity, specifically during the S phase in a cell cycle and after DNA damage, by regulating multiple substrates (Havens & Walter, 2011). The process involves proteolysis of essential cellular proteins to facilitate the timely degradation of various cell cycle and DNA replication regulatory proteins through the proteasome. This includes Cdt1, a replication licensing factor, which is targeted for ubiquitin-mediated degradation by the $CRL4^{Cdt2}$ during S phase and following DNA damage to avert genomic instability (Dang et al., 2020). The deregulation of $CRL4^{Cdt2}$ in a cell cycle correlates with tumours such as melanoma and gastric cancer as the protein affects the overexpression of substrates involved in DNA replication (Panagopoulos et al., 2020). The licensing factor, Cdt1 plays an significant roles in the late M and early G1 phases for DNA replication (Panagopoulos et al., 2020). In the G1 phase, Cdt1 cooperates with Cdc6 to help in the loading of the hexameric mini chromosome maintenance (MCM2-7) helicase complex at Origin Recognition Complex (ORC) bound sites. This recruitment facilitates in the formation of pre-replicative complex (pre-RC) on origins which is required to initiate the DNA replication (Nukina et al., 2018). Certain substrates like Cdt1, Set8, and p21 are found to be vitally degraded by $CRL4^{Cdt2}$ through ubiquitin-mediated proteolysis so that the DNA replication only occurs once-per-cell-cycle (Panagopoulos et al., 2020).

Research on $CRL4^{Cdt2}$ and its ubiquitination mechanism for substrate degradation during the S phase of the cell cycle and following DNA damage has been inadequate to date. The challenge in comprehending the mechanism of $CRL4^{Cdt2}$ ubiquitin substrates arises from the absence of the three-dimensional protein structure of the Cdt2 the substrate receptor of CRL4. Currently, a comprehensive review of $CRL4^{Cdt2}$ substrates is lacking, which limits detailed insights into the degradation mechanisms of each substrate and their roles in the cell cycle. This review seeks to consolidate information on the reported $CRL4^{Cdt2}$ substrates to expand current research efforts.

Survey methodology

We conducted searches through various databases, including Google Scholar, Scopus, PubMed, and Web of Science, to obtain accurate information about $CRL4^{Cdt2}$. Scopus AI was used to screen all substrates for $CRL4^{Cdt2}$. This study employed searches that are related to the $CRL4^{Cdt2}$ ubiquitination mechanism and its potential as a therapeutic target treatment. Keywords used included $CRL4^{Cdt2}$ substrates, PIP degron, $CRL4^{Cdt2}$ ubiquitination mechanism, $CRL4^{Cdt2}$ potential drug target, specific drug name, and concept that is related to our study. As a result of the findings, we reorganized all data according to the scientific topic. Importantly, the data obtained specifically for the substrate and function of $CRL4^{Cdt2}$ in human cells were also filtered. We excluded studies of $CRL4^{Cdt2}$ that did not specifically relate to human cells including those in chicken, zebrafish, mouse, *Xenopus* and *Schizosaccharomyces pombe*.

$CRL4^{Cdt2}$ overview and mechanism of action

$CRL4^{Cdt2}$

E3 ubiquitin ligase facilitates the degradation of specific target proteins through the process of ubiquitination (Callis, 2014). Ubiquitination functions as a significant signalling marker due to its ability to modify various targets. Ubiquitination functions by attaching ubiquitin to substrate proteins, facilitating their proteolytic degradation via the proteasome system. The process of ubiquitination can take place via two separate reactions (Garcia-Barcena et al., 2020; Komander & Rape, 2012). For instance, mono-ubiquitination refers to a single ubiquitin molecule and is primarily associated with post-translation modification signal. In contrast, the combination of ubiquitin molecules is referred to as poly-ubiquitination, which initiates ubiquitin functionalization through three different and

consecutive enzymatic steps (Abbas & Dutta, 2011). Poly-ubiquitination acts as an indicator for protein destruction through a proteasome system. The E1 ubiquitin-activating enzyme leads to ubiquitin activation, followed by the transfer of active ubiquitin from the E1 enzyme to a conjugating enzyme of E2 and subsequently to the substrate through the action of an E3 ubiquitin ligase (Havens & Walter, 2011; Panagopoulos et al., 2020).

$CRL4^{Cdt2}$ functions as a crucial E3 ubiquitin ligases that act as the primary and final enzyme responsible transferring ubiquitin molecules to substrates (Garcia-Barcena et al., 2020). The $CRL4^{Cdt2}$ complex is composed of two major subunits, the backbone, cullin4A-RING E3 ubiquitin ligase (CRL4) and the receptor, Cdc10-dependent transcript 2 (Cdt2). CRL4 is one of the seven members of CRL1-7 identified in higher eukaryotes. This functional protein is mainly based on the Cullin 4 subfamilies, specifically CUL4A and CUL4B. The activation of CUL4 depends on the NEDD8 protein via the process of neddylation. Inhibition of NEDD8 conjugation prevents neddylation process, thereby suppressing the activity of CRLs function (Jackson & Xiong, 2010; Jang et al., 2018). Similar to other CUL1-CUL7 proteins found in vertebrates, CUL4A/CUL4B operate as scaffold proteins to engage with specific substrate adaptors (Moreno & Gambus, 2015). DNA damage binding protein 1 (DDB1) serves as a substrate adaptor and interacts with the CUL4 N-terminal region. DDB1 contains three β-propeller characterized by WD-40 repeat domains: BPA, BPB, and BPC. The BPB domain is associated with CUL4 N-terminal. Meanwhile, the substrate receptor, Cdt2 N-terminal, is linked to the remaining domains, BPA and BPC. (Nukina et al., 2018; Panagopoulos et al., 2020). The C-terminal region of CUL4 is linked to RBX1, the RING domain protein, facilitating the recruitment of E2 enzyme (Panagopoulos et al., 2020).

Cdt2, on the other hand, serves as the substrate receptor for CRL4 ubiquitin ligase. Cdt2 is also referred to as DCAF2, DTL, RAMP, and L2DTL (Dar et al., 2014; Abbas et al., 2013). CRL4 activation in the S phase and following DNA damage involves the recruitment of Cdt2 to form $CRL4^{Cdt2}$ complex. This complex serves as a significant component engaged in the ubiquitin-proteosome system (Panagopoulos et al., 2020; Huh & Piwnica-Worms, 2012; Havens & Walter, 2011). Human Cdt2 encodes polypeptides consisting of 730 amino acids, featuring seven WD-40 repeats. The conserved N-terminal segment comprises approximately 400 amino acids, while the less conserved C-terminal segment contains 330 amino acids. Additionally, the conserved N-terminal segment interacts with DDB1, forming a seven-propeller structure. The less conserved C-terminus encompasses several crucial motifs that activate $CRL4^{Cdt2}$ activity, including the DNA Binding Domain (DBD), phosphorylation sites, and a PCNA Interacting Protein-box (PIP box) (Panagopoulos et al., 2020; Mazian et al., 2019, Nukina et al.,2018; Hayashi et al., 2018).

Substrate recognition of $CRL4^{Cdt2}$

The activation of $CRL4^{Cdt2}$ ubiquitin ligase requires a proliferating cell nuclear antigen (PCNA) loaded on DNA ($PCNA^{DNA}$), to recognize its substrates (Havens & Walter, 2011; Shibata et al., 2014; Mazian et al., 2019, Ponogopoulus et al., 2020). As illustrated in Figure 1, PCNA is a ring-shaped replication clamp encircling DNA as a homotrimer to aid in the initiation of DNA replication and DNA damage repair (Panagopoulos et al., 2020). Upon DNA damage and during the S phase, PCNA is loaded on chromatin as a platform for interaction of various proteins involved in DNA replication, DNA repair and chromatin metabolism (Shiomi & Nishitani, 2017; Nukina et al., 2018). Interacting proteins typically contain a PIP box (Panagopoulos et al., 2020), which is associated with the interdomain connection loop (IDL) of PCNA. The $CRL4^{Cdt2}$ target substrates necessitate a PIP degron for effective ubiquitination. A PIP degron is a standard PIP box characterized by a TD motif, consisting of threonine and aspartate residues at positions 5 and 6 of the PIP box, respectively, together with basic amino acids downstream of the PIP box to facilitate high-affinity binding to the PCNA. (Havens & Walter, 2009; Michishita et al., 2011; Slenn et al., 2014). Upon the stabilization of the substrate PIP degron-PCNADNA complex, $CRL4^{Cdt2}$ is autonomously recruited to PCNA via its PIP box at the C-terminal end of Cdt2, where it identifies substrates through the Cdt2 substrate receptor domain for ubiquitination (Mazian et al.,2019).

Overview of $CRL4^{Cdt2}$ Substrates and Their Functional Roles

A diverse range of $CRL4^{Cdt2}$ Ubiquitin Ligase target substrates

$CRL4^{Cdt2}$ is recognized as a master regulator of the cell cycle due to its ability to regulate numerous essential proteins during the S phase and in response to DNA damage. Wide ranges of substrates targeted by the ligase have been studied including Cdt1, Set8, p21, TDG, CHK1, CRY 1, E2F1, HBV Polymerase, Polymerase δ (p12), XPG, SDE2, Polymerase η, and FBH1 (Table 1 and Figure 1).

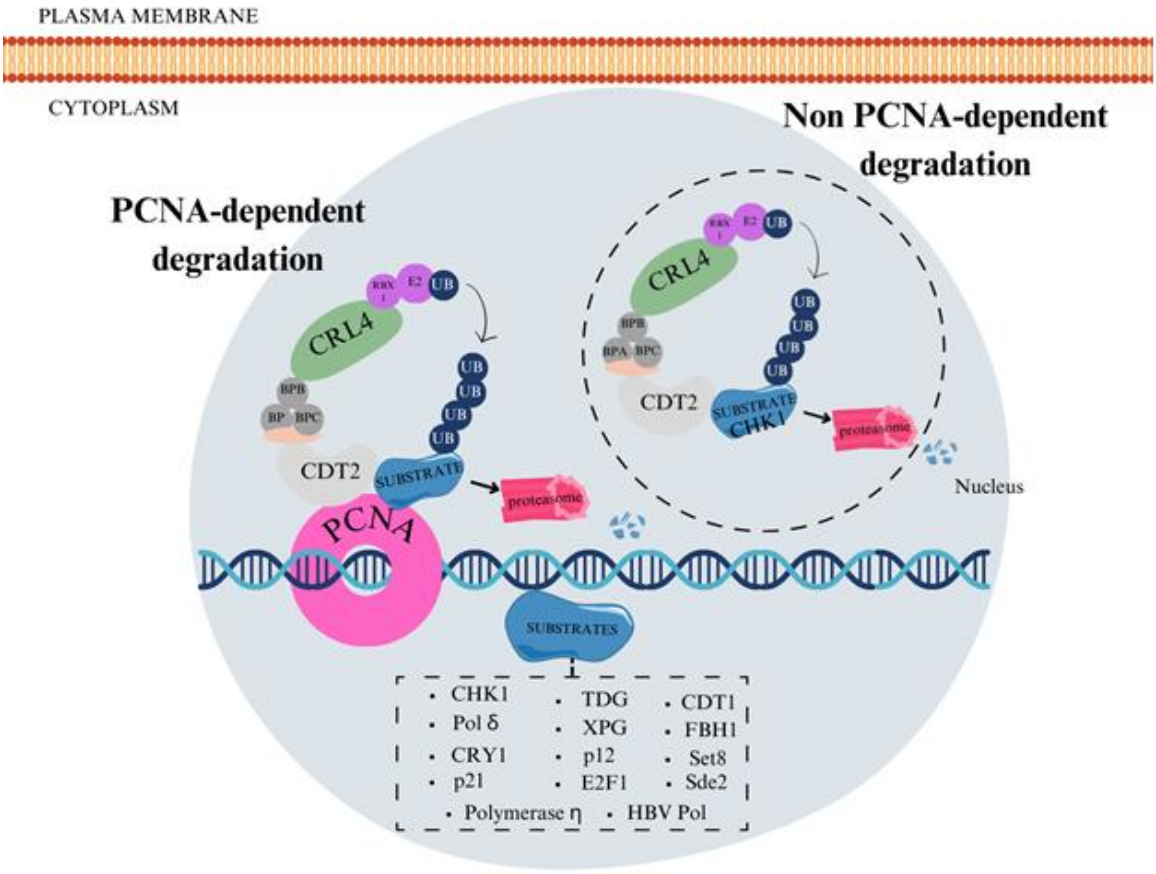

Figure 1. The overview of CRL4^{Cdt2} complex structure and polyubiquitination mechanism. The E2 ubiquitin-conjugating enzyme, loaded with ubiquitin, transfers the ubiquitin molecule to a lysine residue on target protein. This process is facilitated by RING domain RBX1. Multiple rounds of ubiquitination occur, leading to the formation of a polyubiquitin chain on the substrate. This polyubiquitin chain serves as a degradation signal. The substrates include Cdt1, Set8, p21, TDG, CHK1, CRY1, E2F1, HBV Pol, p12, XPG, Sde2, Polymerase η, Pol δ and FBH1. All the substrates except CHK1 are polyubiquitinated on PCNA^{DNA}. CHK1 is polyubiquitinated at nucleoplasm and not PCNA^{DNA}-dependent.

(a) Cdt1

Cdc10-dependent transcript-1 (Cdt1) is a key protein that establishes a license for DNA replication in the late M phase and G1 phase (Panagopoulos et al., 2020). The formation of the pre-replicative complex (pre-RC) on the DNA origin initiates DNA replication. The pre-RC which also acts as a DNA replication licensing factor, requires Cdc6 and Cdt1 to load mini chromosome maintenance (MCM2-7) helicase complex onto the Origin recognition complex (ORC) binding site (Mazian et al., 2019). Cdt1 levels are elevated during the G1 phase to facilitate the establishment of the pre-replicative complex but sharply decline following the initiation of the S phase (Ballabeni et al., 2013; Pozo & Cook, 2017). Once DNA replication begins, Cdt1 is rapidly destructed to prevent relicense, re-replication, and checkpoint activation (Cheng et al., 2013; Panagopoulos et al., 2020). The inactivation of Cdt1 is due to regulation driven by Geminin, CRL1^{Skp2} and CRL4^{Cdt2} (Havens & Walter, 2011; Pozo & Cook, 2017). Cdt1 is regulated through a multi-layered system to prevent re-replication. In S phase, CRL4^{Cdt2} degrades it in a PCNA-dependent manner. Concurrently and into G2, the CRL1^{Skp2} complex (dependent on Cyclin A) also targets Cdt1 for ubiquitination. Finally, from late S through M phase, Geminin provides a third layer of control by directly binding to and inhibiting Cdt1 function (Pozo and Cook, 2017; Jang et al., 2020 and Zhou et al., 2020).

The poly-ubiquitination-driven degradation of Cdt1 by CRL4^{Cdt2} (Nukina et al., 2018) happens on PCNA^{DNA} during the S phase following DNA damage (Cheng et al., 2013). This happens when Cdt1

is no longer necessary following the loading of MCM2-7 complexes onto chromatin (Havens & Walter, 2011). The PCNA binds to the chromatin once the cell enters the S phase. This enables Cdt1 to bind to $PCNA^{DNA}$ through its N-terminal PIP degron (Havens & Walter, 2011; Nukina et al., 2018). The binding of Cdt1 PIP degron to PCNA is synergically operated with the Cdt2 PIP box that binds to a different IDL site of $PCNA^{DNA}$ (Hayashi et al., 2018). The N-terminal domain of Cdt2 then recognizes the Cdt1 PIP degron on PCNA and starts the poly-ubiquitination and degradation (Panagopoulos et al., 2020).

Table 1. List of known $CRL4^{Cdt2}$ substrates including their functions, the nature of the degron motif that targets them for ubiquitylation and the conditions under which degradation occurs.

Substrates	Primary Function	Degradation Time	PIP Degron (TD motif)	Reference
Cdt1 (DNA replication factor 1)	DNA replication licensing	S phase and after DNA damage	Yes	Arias and Walter et al., 2007; Nishitani et al., 2004; Zhang 2021
Set 8 (SET Domain Containing 8)	Chromatin compaction, DNA replication and DNA damage response	S phase and after DNA damage	Yes	Jørgensen et al., 2013; Jørgensen et al., 2007; Abbas and Dutta et al., 2011
p21 (Cyclin-dependent kinase inhibitor 1)	Inhibits cyclin-dependent kinase activity, promotes cellular senescence and apoptosis	S phase and after DNA damage	Yes	Harper et al., 2007; Abbas and Duta., 2009; Karimian et al., 2016
TDG (Thymine-DNA glycosylase)	Base excision repair (BER) – removes mispaired thymine (T)	S phase and after DNA damage	Yes	Slenn et al., 2014; Shibata et al., 2014
CHK1 (Checkpoint kinase 1)	Regulates the cell's response to DNA damage or replication stress by acting as a checkpoint regulator	After DNA damage	No	Huh et al.,2013; Zhang et al., 2014
CRY1 (Cryptochrome 1)	Regulates the timing of human biological rhythms	S phase	Unknown	Parico et al., 2020; Mekbib et al., 2019; Tong et al., 2015
E2F1 (E2F transcription factor 1)	Regulates cell cycle progression, cell division and apoptosis	S phase	No	Denechaud et al., 2017; Wang et al., 2024; Davidson et ai., 2012; Zielke et al., 2011
HBV polymerase	Regulates Hepatiti B virus replication	Unclear	No	Hou et al., 2019
p12 (DNA polymerase delta subunit p12)	p12 is DNA polymerase δ subunit that helps in DNA replication and repair	S phase and after DNA damage	Yes	Lee et al., 2019; Zhang et al., 2013; Lee et al., 2014; Terai et al., 2013; Gonzalez-Magaña et al., 2019
XPG (Xeroderma pigmentosum group G protein)	Endonuclease involves in nucleotide excision repair (NER)	After DNA damage	No	Han et al., 2015; He et al., 2012; Borsos et al., 202
Sde2 (Suppressor of Defective Silencing 2)	Multifunctional protein involves in DNA damage respond, RNA splicing regulation, and genomic stability maintenance	S phase and after DNA damage	Canonical PIP Box	Jo et al., 2016
Polymerase η	Facilitates translesion DNA synthesis (TLS) after DNA damage	After DNA damage	No	Ma et al.,2017; Tsanov et al., 2014; Oda et al., 2010
FBH1(F-box DNA Helicase 1)	Regulates Homologous recombinant (HR) mechanism	After DNA damage	Yes	Fugger et al., 2009; Bacquin et al., 2013

(b) Set8

Histone methyltransferase, Set8 is responsible for mono-methylating of histone H4 on Lysine 20 (H4K20) during the late S phase and G2 phase. The mono-methylation of H4K20 (H4K20me1) by Set8 is extensive for ensuring a proper chromatin structure, essentially in gene expression and chromatin condensation (Abbas et al., 2010; Jørgensen et al., 2011). Since newly incorporated histone H4 is not methylated at K20, it must be methylated to monomethylated state, and then to the di- and trimethylated states at a proper time point. The expression level of Set8 rises throughout the late S phase and G2 phase, peaking in the M phase, as H4K20me1 is essential for chromatin compaction into heterochromatin, which regulates pre-RC component loading during the licensing of DNA replication origins (Jang et al., 2018; Jørgensen et al., 2011; Shoaib et al., 2018). However, the level of Set8 starts to cease during G1 and is absent in the S phase, leading to a low H4K20me1 level during the S phase (Havens & Walter, 2011).

The Set8 expression pattern is regulated by four types of E3 ubiquitin ligase, $CRL4^{Cdt2}$, $CRL1^{Skp2}$, APC/C^{Cdh1} and $SCF^{\beta\text{-}TRCP}$. In the G1 phase, $CRL1^{Skp2}$, APC/C^{Cdh1} and $SCF^{\beta\text{-}TRCP}$ maintain the low expression level of Set8 to avoid re-replication, G2/M arrest and control cell cycle progression (Havens & Walter, 2011; Pet al., 2015; Wu et al., 2010; Zheng et al., 2016). In the S phase, $CRL4^{Cdt2}$ is responsible for the total absence of Set8 by targeting the substrate for degradation (Jang et al., 2018; Jørgensen et al., 2011). The efficient degradation of Set8 during the onset of the S phase ensures a low level of H4K20me1 is maintained and inhibits over-recruitment of MCM2-7 (Shoaib et al., 2018). This is because the elevation of Set8 and H4K20me1 induces re-replication, premature and unmannered chromatin compaction, H4 methylation and G2 arrest (Benamar et al., 2016; Havens & Walter, 2011; Jørgensen et al., 2011; Rizzardi et al., 2015). $CRL4^{Cdt2}$ is also responsible for the degradation of Set8 following DNA damage caused by UV irradiation (Jørgensen et al. 2011). The degradation of Set8 upon DNA damage is essential to inhibit the replication of unrepaired DNA resulting from mutations. The Set8 degradation during the S phase and upon DNA damage is influenced by its specialized PIP box interaction with $PCNA^{DNA}$. The Set8 is associated with two types of PIP box: PIP1 and PIP2. PIP1 allows the process of H4K20me1, while PIP2 is used for $PCNA^{DNA}$ binding for degradation (Abbas et al., 2010; Jørgensen et al., 2011). The PIP2 is essential for degradation as it contains unique elements: Thr182 (position 5), Asp183 (position 6), Arg188 (position +3) and Arg189 (position +4) (Abbas et al., 2010; Jørgensen et al., 2007). Similar to Cdt1, Set8 is polyubiquitinated by the $CRL4^{Cdt2}$ ligase. This process is initiated when Set8 associates with PCNA on DNA ($PCNA^{DNA}$), an event that promotes the recognition and subsequent targeting of Set8 by the ligase (Abbas et al., 2010; Rizzardi et al., 2015; Shibata et al., 2014).

(c) p21

Cyclin-dependent kinase inhibitor 1, p21, is a protein composed of 165 amino acids and belongs to the family of CDK inhibitors (Gartel, 2006; Karimian et al., 2016). p21 functions as a CDK inhibitor, halting cell cycle transitions by activating the G1/S and G2/M checkpoints through the tumour suppressor, p53. DNA damage activates p53, which increases the transcription of the p21 gene, leading to the synthesis of p21 protein (Barr et al., 2017). p21 plays a vital role in inhibiting all cyclin-dependent CDK complexes, CDK4, CDK6/cyclin-D and CDK2/cyclin-E in G1 checkpoint and CDK1 in G2 checkpoint (Barr et al., 2017; Jang et al., 2018; Sheng et al., 2019). The inhibition of CDKs stimulates retinoblastoma protein (Rb) to bind to E2F transcription factors and prevents progression from the G1 phase to the S phase (Suryadinata et al., 2011; Wagner and Gil, 2020).

p21 levels are elevated throughout the G1 and G2 phases, but diminish during the S phase (Sheng et al., 2019). During the S phase, the concentration of p21 diminishes due to degradation facilitated by $CRL4^{Cdt2}$ (Nishitani et al, 2007; Abbas & Dutta, 2009; Sheng et al., 2019). The level of p21 is maintained low in the S phase to prevent p21 accumulation. Accumulation of p21, in turn, will add more damage and reduce the repair efficiency. Hence, the malfunction replication will increase genomic instability and induce cell fates or senescence (Sheng et al., 2019). Furthermore, the inability to control the p21 level results in p21 binding to $PCNA^{DNA}$ during the S phase, hence disrupting polymerase activity and displacing other DNA replication factors. This leads to the inhibition of unperturbed DNA replication and modulates $PCNA^{DNA}$ repair (Abbas & Dutta, 2009; Cazzalini et al., 2003; Mansilla et al., 2020).

The ubiquitination of p21 by $CRL4^{Cdt2}$ in S phase and after DNA damage is also PCNA-dependent where, PCNA need to be loaded on DNA (Barr et al., 2017; Sheng et al., 2019). Binding of p21 to PCNA through its PIP degron stimulates the recruitment of $CRL4^{Cdt2}$ for ubiquitination (Abbas & Duta 2009).

(d) TDG

Thymine DNA glycosylase (TDG) is a uracil DNA glycosylase enzyme that is involved in the base excision repair (BER) pathway and DNA demethylation (Shibata et al., 2014; Slenn et al., 2014).

The BER pathway plays a crucial role in DNA repair, addressing mismatches caused by oxidation or deamination, specifically targeting G:U and G:T lesions (Coey et al., 2016). The epigenetic repair by TDG significantly impacts genome stability from mutagenesis and propounds in embryonic development (Da et al., 2018; Nakamura et al., 2017). The TDG initiates BER pathway by removing the uracil and thymine bases from the guanine base mismatch. It also involves cytosine demethylation by removing 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) from DNA, which are oxidized products of 5-methylcytosine (5mC) produced by TET enzymes (Da et al., 2018; Shibata et al., 2014). These removals generate an AP (apurinic/ apyrimidinic) site to restore cytosine through AP endonuclease and further BER enzyme processing (Slenn et al., 2014). The interaction between TDG-DNA (AP site) plays a vital role in the repair of mismatched bases and the ubiquitination of TDG by $CRL4^{Cdt2}$. Observations indicate that the active TDG reaches its peak during the transition from the G2 to M phase, as well as in the G1 phase for the BER pathway. Nonetheless, the accumulation of TDG is hindered during the S phase due to the PCNA-facilitated degradation through $CRL4^{Cdt2}$ (Moreno & Gambus, 2015; Slenn et al., 2014). The TDG degradation mediated by $CRL4^{Cdt2}$ in S phase prevents TDG overexpression, causing cell accumulation and prohibiting collision between DNA replication forks and TDG- AP site (Slenn et al., 2014).

Like Cdt1, Set8 and p21, TDG also requires $PCNA^{DNA}$ recruitment to be poly-ubiquitinated by $CRL4^{Cdt2}$ (Shibata et al., 2014). The recruitment of PCNA onto chromatin is triggered by the DNA repair or DNA replication (Shibata et al., 2014). Mutation of TDG PIP and knockdown of proteasome lead to inhibition of TDG degradation and accumulation of poly-ubiquitinated TDG, confirm that TDG is a substrate of $CRL4^{Cdt2}$ (Wei et al., 2024; Slenn et al., 2014).

(e) CHK1

CHK1 or checkpoint kinase 1, is a serine or threonine kinase that acts as a master regulator of DNA damage response (DDR) and cell cycle checkpoints pathway in vertebrate cells (Cassidy et al., 2020; Smits & Gillespie, 2015). In a normal and undisturbed cell cycle, CHK1 plays a vital role in preventing premature entry of the mitotic phase (Smits & Gillespie, 2015) and regulating spindle assembly checkpoints (Patil et al., 2013).

CHK1 regulates S phase progression in response to DNA damage by engaging the checkpoint for DNA repair and replication fork stabilization (González Besteiro et al., 2019; Huh & Piwnica-Worms, 2013; Zhang & Hunter, 2014). The DNA damage sensing complex, Ataxia telangiectasia and Rad3-related protein (ATR) and ATR-interacting protein (ATRIP) are recruited upon replication stress. The ATR, ATRIP, RAD17 and 9-1-1 complex are attracted to the single-strand DNA (ssDNA) that is stabilized by replication protein A (RPA) (Huh & Piwnica-Worms, 2013; Zhang & Hunter, 2014). The formation thereby stimulates the activation of CHK1 through phosphorylation on Ser317 and Ser345 in a ATR claspin-dependent manner (Zhang & Hunter, 2014). The ATR-CHK1 phosphorylation is essential in DNA damage repair (DDR) because the activated CHK1 will inhibit both phosphatases Cdc25A, hence stimulating the S and G2 checkpoints in the cell cycle (Moses et al., 2020). The cell cycle will then temporarily stalled for replication fork stabilization, thus, DNA repair can be regulated (Huh & Piwnica-Worms, 2013). The CHK1-mediated phosphorylation of Cdc25A results in its proteasomal degradation, hence inhibiting the early activation of CDKs and ensuring that cells do not proceed to mitosis with unresolved DNA damage (Goto et al., 2019). This function is essential because it enables cells to keep a correct cell cycle arrest, which provides them with enough time to repair the DNA damages before moving on to the mitotic phase (Yarden et al., 2012).

CHK1 is unique and classified as a non-canonical substrate where its PIP boxes are not required to recruit $CRL4^{Cdt2}$ to PCNA (Huh & Piwnica-Worms, 2013). This is because the CHK1 PIP box does not contain TD motif at both C-terminal and N-terminal. Thus, the degradation of CHK1 is not $PCNA^{DNA}$-dependent. Rather than targeted on chromatin like other $CRL4^{Cdt2}$ substrates, active CHK1 is targeted in nucleoplasm by $CRL4^{Cdt2}$ (Huh & Piwnica-Worms, 2013). However, the specific recruitment of CHK1 to Cdt2 is still unclear as the PIP boxes and $PCNA^{DNA}$ are not required to link between them.

The CHK1 ubiquitination is regulated by two different E3 ligases, which are $CRL1^{FBX6}$ and $CRL4^{Cdt2,}$ through distinct substrate receptors and subcellular locations (Smits & Gillespie, 2015). The released CHK1 is targeted and ubiquitinated by both $CRL4^{Cdt2}$ and $CRL1^{FBX6}$ in nucleoplasm and cytoplasm, respectively, for CHK1 PCNA-independent destruction (Huh & Piwnica-Worms, 2013; Smits & Gillespie, 2015). The poly-ubiquitination of CHK1 by $CRL4^{Cdt2}$ is essential for signalling the termination of the checkpoint. If CHK1 is stabilized, a termination signal will not be transmitted. Consequently, the subsequent phase of the cell cycle cannot advance, resulting in an extended cell cycle arrest (Smits & Gillespie, 2015).

(f) CRY1

In most eukaryotic organisms, the daily physiological rhythms are regulated by the circadian clock that consists of CRY1 (Liu et al., 2016). The circadian clock is biological time-keeping mechanism that controls energy metabolism, hormonal secretion and sleep cycle. In recent studies, CRY1 was discovered to be the key glucose metabolism regulator and affects glucocorticoid receptor activity during gluconeogenesis induction (Tong et al., 2017; Chen et al., 2021). Ordinarily, CRY1 is a transcription factor repressor that binds to CLOCK: BMAL1 to limit its function and terminate the central feedback mechanism (Parico, 2020). BMAL1 and CLOCK are positive regulators that cause the production of negative regulators, including Rev-erbα, group of Period 1-3 (PER1, PER2, and PER3) and two types of Cryptochrome (CRY1 and CRY2) (Mekbib et al., 2019). The inactivation of the CLOCK:BMAL1 complex is achieved through the ubiquitin-proteasome-mediated degradation of PER and CRY proteins. This degradation event provides the essential negative feedback that drives circadian rhythmicity (Tong et al., 2017; Mekbib et al., 2019). CRY1 degradation must occur continuously to regulate the abundance of critical clock proteins (Mekbib et al., 2019). $CRL4^{Cdt2}$ ubiquitinates CRY1, stimulating its degradation both in vitro and in vivo. Consequently, CRY1 stability increases in cells lacking essential components of this ligase, such as Ddb1 or Cdt2 (Tong et al., 2015). The exact regulation of $CRL4^{Cdt2}$ over the CRY1 proteins' stability and rhythmic behaviour remains uncertain and is still an area of active research. However, conclusive evidence shows that the Cdt2 targets the CRY1 ubiquitination site, lysine 585 (K585), for destruction (Tong et al., 2015). For validation, the degradation of mutated CRY1-585KA was suppressed as Cdt2 cannot recognize alanine as a ubiquitination site (Tong et al., 2015). Furthermore, depletion of PCNA in cell blocks CRY1 protein degradation, demonstrating that the CRY1 degradation is PCNA-dependent (Tong et al. 2015). Although the degradation is driven by PCNA-dependent, there is no evidence yet on the present of PIP box nor PIP degron in the CRY1. It is noteworthy that existing research indicates that CRY1 degradation occurs just during S phase where PCNA is associated with DNA and does not follow DNA damage. Additional study is required to address the concerns.

(g) E2F1

E2F1 (E2 promoter binding factor 1) is a transcription factor recognized for its function as either an activator or repressor of transcription (Denechaud et al., 2017). This activator is essential for S phase entry by promoting the expression of replication-related proteins in G1 while also regulating proliferation (Denechaud et al., 2017). E2F1 is a cell cycle progression factor in response to DNA damage and apoptosis, particularly in metazoans (Dubrez, 2017). Furthermore, Denechaud et al. (2017) research provides evidence that E2F1 regulates metabolism in non-proliferating conditions, and the deregulation of the E2F1 process disrupts the hepatic metabolic homeostasis. E2F1 also plays a part in metabolism regulation, and its dysregulation will result in most metabolic diseases that eventually lead to cancer cells (Mandigo et al., 2021). However, the role of $CRL4^{Cdt2}$ in this mechanism remains unknown. Recently, it has been reported that the E3 ubiquitin ligase $CRL4^{Cdt2}$ contributes to the degradation of E2F1 in Drosophila. The ubiquitin-dependent degradation of E2F1 occurs during the S phase when the Cyclin E is at a high level and is performed by $CRL4^{cdt2}$ via the PIP degron located at the C-terminal of E2F1 (Kim et al., 2018; Kim et al., 2020). The retinoblastoma gene product Rb inhibits activator E2F in the early G1 phase (Havens & Walter, 2011). The ubiquitin-proteasome system (UPS) typically targets E2F1 for degradation. However, the binding of Rb protein sterically shields E2F1 from this fate. This protection facilitates the accumulation of the E2F1-Rb complex, thereby ensuring that a sufficient E2F1 is available to drive the transcription of genes critical for the G1/S phase transition. (Havens & Walter, 2011; Kim et al., 2018). E2F1 ubiquitination is prevented by deleting the carboxyl-terminal of E2F1 that coincides with the Rb binding region. Because ubiquitin acceptor lysine residue is absent in this area, it may include an E3-ubiquitin ligase binding domain that is capable of beating the E2F1 binding with Rb (Dubrez, 2017).

Studies in Drosophila have helped clarify key cancer pathways like Jak/STAT, EGFR-Ras, PI3K/AKT, and p53, all of which play central roles in human cancer (Capuozzo et al., 2022). For instance, Drosophila models have revealed how tumor suppressors and oncogenes interact, enabled the development of living tumor models, and identified cancer-related genes such as salvador, which is also mutated in human cancers (Read et al., 2009). Drosophila undergoes endocycles, a cell cycle that includes DNA synthesis and the S and G phases but does not include mitosis (Zielke et al., 2011). CycE/CDK2 (Cyclin E Dependent Kinase 2) is responsible for activating the DNA replication of Drosophila cells at endocycle, where the kinase will be rendered inactive first in G phase prior to entering S phase to enable the creation of pre-RCs (Kim & Moon, 2019). The expression of the CycE and S phase cycle are fostered by E2F1 transcription factor

(Kim & Moon, 2019; Kim et al., 2018). In different circumstances, $CRL4^{Cdt2}$ is activated and subsequently targets E2F1 for degradation (Zielke et al., 2011; Glorian et al., 2018). E2F1 is targeted for proteasomal degradation via a PIP-box motif. This motif mediates its recruitment to PCNA-loaded DNA ($PCNA^{DNA}$), which in turn facilitates its recognition and ubiquitination by the $CRL4^{Cdt2}$ ubiquitin ligase (Zielke et al., 2011). This causes Cdt2 to detect E2F1 and proceed with the E2F1 poly-ubiquitination (Havens & Walter, 2011; Dubrez, 2017; Denechaud et al., 2017). Cells with high PCNA levels have low E2F1 expression. Conversely, low PCNA levels lead to high-level E2F1 expression (Havens & Walter, 2011; Kim et al., 2018). Kim and Moon (2019) revealed that PCNA depletion resulted in the lack of oscillations between E2F1 and CycE. To summarize, chromatin-bound PCNA-dependent activity of $CRL4^{Cdt2}$ is required during the S phase for E2F1 degradation (Havens & Walter, 2011; Davidson & Duronio, 2011; Kim & Moon, 2019).

(h) HBV Pol

The Hepatitis B virus (HBV) is a diminutive, encapsulated human pathogen that generates both infectious and non-infectious particles. Consequently, its infection would result in chronic liver inflammation, ultimately leading to organ failure and hepatocellular carcinoma mortality (Kong et al., 2019). The HBV polymerase plays an important role in viral life cycle, especially in the replication of the viral genome. This enzyme is categorized as a DNA-directed DNA polymerase and is crucial for synthesizing viral DNA from its RNA precursor, termed pregenomic RNA (pgRNA) that is catalysed by reverse transcriptase of the viral polymerase. The polymerase starts this process by attaching to certain RNA sequences, notably the epsilon (ε) element, which is essential for the encapsidation of pgRNA and the commencement of reverse transcription (Huang et al., 2016). The HBV polymerase has multiple different domains: the terminal protein (TP), spacer, reverse transcriptase (RT), and RNase H domains, each playing a role in its function in viral replication and pathogenesis (Clark and Hu 2015; Pley et al.,2022). Encapsidation of the pgRNA is critical for HBV replication mediated by the polymerase of virus reverse transcriptase activity. Virus-encoded proteins are well-known to be required for viral replication (Hou et al., 2019). Several studies, however, have revealed that the ubiquitin-proteosome system (UPS) acts as an anti-viral mechanism by selectively degrading viral proteins (Kong et al., 2019). The CRL4 ubiquitin ligase complex regulates both viral and host protein degradation during HBV infection. $CRL4^{Cdt2}$ restricts viral replication by targeting HBV polymerase for destruction (Kar et al., 2024). Conversely, the viral HBx protein suppressed CRL4 activity to degrade host antiviral proteins, promoting viral persistence and potentially contributing to cancer development (Xie et al., 2025). The non-receptor tyrosine kinase, c-Ab1, regulates the $CRL4^{Cdt2}$ ubiquitination activity to target the HBV polymerase ubiquitin-proteasome degradation (Hou et al., 2019). Specifically, c-Abl phosphorylation of DDB1 causes the tiny regulatory protein DDA1 to be recruited and stimulate the ubiquitin ligase activity. The ubiquitination of HBV polymerase by $CRL4^{Cdt2}$ is driven by the engagement of the regulatory protein DDA1 after DDB1 has been phosphorylated by c-Ab1 (Gao et al., 2017). Overexpression of DDA1 enhanced the degradation of HBV polymerase due to the massive activation of ubiquitination activity by $CRL4^{Cdt2}$ (Hou et al., 2019; Gao et al., 2017).

(i) Pol δ (p12)

Replicative B-family polymerases such as pols α, δ, and ε play a vital role in replicating the human genome. Human DNA Pol δ serves primarily as the lagging strand polymerase during DNA synthesis and in the DNA repair mechanism (Lee et al., 2017). DNA Polymerase δ (Pol δ) is essential for the high-fidelity replication of the nuclear genome. It also plays a critical role in several DNA repair pathways. Furthermore, during translesion synthesis, Pol δ helps to manage and bypass DNA lesions that would otherwise cause replication to stall (Prindle & Loeb, 2012). The architecture of human Pol δ is a heterotetramer composed of four subunits: p125, p50, p68, and p12 (Zhao et al., 2014). p12 is the smallest subuit and is crucial for efficient DNA replication and cellular proliferation. p12 has facilitated interaction of PIP box with PCNA to enhance the accuracy of polymerase delta activity (Meng et al., 2010; Huang et al., 2010). As a direct consequence of DNA damage that occurs during the beginning of the S phase, p12 is promptly degraded by the process of ubiquitination. To ensure proper DNA repair, S phase progression must be inhibited through early degradation. This mechanism prevents the cell from entering mitosis until the damage is resolved (Terai et al., 2013). There are two significant implications that are instantly activated when DNA damage occurs during the process of DNA replication. These consequences are a reduction in the progression rate of replication fork and an inhibition of new origin firing (Terai et al., 2013). Therefore, the degradation of p12 is a reaction to immediate consequence following DNA damage, where the absence of p12 weakens the binding of Pol δ to PCNA, which in turn causes progression to be

slowed down. p12 contains a PIP degron motif at the N-terminal, indicating its role as a marker for $CRL4^{Cdt2}$ substrates (Gozalez-Magaña et al., 2019). The ubiquitination and degradation of p12 occur in a PCNA-dependent manner, with p12 being ubiquitinated by $CRL4^{Cdt2}$ on PCNA and subsequently degraded by the proteasome. Failure to degrade p12 following DNA damage will restore halted DNA replication, even in the absence of repair, resulting in the replication of damaged DNA (Terai et al., 2013).

(j) XPG

Xeroderma pigmentosum group G (XPG) is an endonuclease crucial for DNA damage response (DDR). It partners with other repair proteins to address damage caused by environmental factors like ultraviolet radiation. In nucleotide excision repair (NER), XPG acts not only as an enzyme but also as a structural scaffold that helps direct the repair pathway (Tsutakawa et al., 2020). The total residue for the XPG nuclease domain is 1186 amino acids. XPG is separated into four regions, which are N- subdomain, spacer, I- subdomain and C tail, with 112, 637, 241 and 196 residues, respectively (González-Corrochano et al., 2020). XPG have a helix-turn-helix (HLH) motif in a part of their structure that is shared by all endonuclease family members (Park et al., 1997). The HLH motif gives XPG its flexibility and ability to bind to DNA and search for damaged DNA sites (González-Corrochano et al., 2020). Ultimately, XPG identifies and attaches to damaged DNA regions, facilitating the formation of a gap by cleaving the DNA strand at the 3' terminus of the lesion. This procedure eliminates the impaired area, facilitating future DNA synthesis to reinstate the sequence (Tsutakawa et al., 2020).

Following the excision of the damaged region, the removal of XPG from the DNA is essential for subsequent DNA repair activities, and the concentration level of XPG needs to be regulated (Park & Kang, 2016). $CRL4^{Cdt2}$ seems to localize at the site of injury where XPG binds to DNA and resulted in degradation of XPG (Han et al., 2015; Park & Kang, 2016). Although the XPG PIP box lacks TD motif and B+4 residues downstream, XPG is still ubiquitinated and degraded by $CRL4^{Cdt2}$ (Han et al., 2015). The PIP box in the C-terminal of the XPG protein engages with PCNA on DNA (Han et al., 2015). The interaction of XPG and PCNA subsequently attracts $CRL4^{Cdt2}$ to the damage site, promoting the ubiquitination and degradation of XPG via the proteasome (Borsos et al., 2020). Consequently, PCNA is essential for facilitating Cdt2 interaction with XPG, thereby enhancing the synthesis of DNA during nucleotide excision repair to fill the gaps (Tanaka et al., 2017; Han et al., 2015).

(k) Sde2

Suppressor of Defective Silencing 2, Sde2 is an essential protein in human cells that plays a significant role in various cellular processes, particularly in the context of DNA damage response (DDR) and RNA processing. It is a homolog of the yeast Sde2 and is highly conserved across species, suggesting its fundamental biological importance (Luo et al., 2020). The human Sde2 protein features a ubiquitin-like (UBL) domain at its N-terminal, which is subjected to enzymatically cleavage by deubiquitinating enzyme, resulting in distinct functional fragments that participate in cellular regulation (Floro et al., 2021). Sde2 is critically involved in the DNA damage response (DDR). The depletion of Sde2 leads to a significant increase in γH2AX levels upon UVC irradiation, indicating an accumulation of DNA damage (Jo et al., 2016). In addition to its role in DNA damage response, Sde2 has been implicated in alternative splicing regulation. It acts as a trans-acting factor that influences RNA processing, and its depletion has been associated with widespread changes in splicing patterns in human cell line (Floro et al., 2021). This suggests that Sde2 not only plays a role in DNA repair but also in the regulation of gene expression at the post-transcription level.

Sde2 has been shown to be regulated by $CRL4^{Cdt2}$, which targeted for ubiquitination and subsequent degradation by 26S proteasome in respond to DNA damage and replication stress (Jo et al., 2016). This regulation is essential for maintaining genomic stability, as the timely degradation of Sde2 allows the proper progression of the cell cycle and the activation of DNA repair mechanisms. The degradation of Sde2 is necessary for cellular survival under of replication stress at the replication fork (Rageul et al., 2019). Furthermore, the cleavage of Sde2 UBL domain in response to DNA damage results in the generation of both an N-terminal UBL fragment and C-terminal fragment, which may have distinct regulatory functions within the cell (Floro et al., 2021). The cleavage of UBL domain is prerequisite before the C- terminal of Sde2 subjected to ubiquitination by $CRL4^{Cdt2}$ (Jo et al., 2016).

(l) Polymerase η

Polymerase η (Pol η) is essential for translesion synthesis (TLS), enabling DNA replication to continue beyond lesions that would otherwise impede replication forks. The $CRL4^{Cdt2}$ ubiquitin ligase complex preserves genomic integrity by regulating DNA repair. In response to DNA damage, the Cdt2

subunit recruits DNA polymerase η (Pol η) to the complex, mediating its ubiquitination and subsequent proteasomal degradation. This process is crucial for controlling translesion synthesis and maintaining genomic stability (Oda et al., 2010; Tsanov et al., 2014). The process of ubiquitination is essential for modulating the amounts of Pol η, guaranteeing its availability for translesion synthesis, especially in the context of DNA damage (Hou et al., 2019; Bacquin et al., 2013). Furthermore, the interaction with Pol η and the PCNA is crucial for its localization to DNA damage sites. Pol η O-GlcNAcylation at threonine 457 induces the recognition by Cdt2 on PCNA and subsequent polyubiquitination of Pol η at lysine 462 (Ma et al., 2017). Inactivation of Pol η O-GlcNAcylation at threonine 457 impairs its polyubiquitination degradation, resulting in accumulation of Pol η level. Mono-ubiquitination of PCNA at lysine 164 functions as a signal for the recruitment of TLS polymerases, such as Pol η, to the replication fork (Nishimoto et al., 2016). The presence of ubiquitin-binding domains in Pol η enables this connection, allowing the polymerase to efficiently interface with damaged DNA and execute its role in lesion bypass (Freudenthal et al., 2010; Pozhidaeva et al., 2012). Studies indicate that the ubiquitination of Pol η transpires at several lysine residues, hence affecting its interaction with PCNA and impacting its function during translesion synthesis (Powers et al., 2018; McIntyre & Woodgate, 2015). This post-translational alteration is crucial for the recruitment of Pol η to damaged sites and regulates its activity and stability within the cell (Ma et al., 2017; Woodruff et al., 2010). The interaction between ubiquitination and the recruitment of Pol η to PCNA highlights the intricacy of the cellular response to DNA damage and the necessity of meticulous management in preserving genomic integrity.

(m) FBH1

FBH1, or F-box DNA Helicase 1, is a multifunctional protein and essential for DNA repair, especially in homologous recombinant (HR) and the cellular response to replication stress. It is classified as a member of the UvrD helicase family and is distinguished by its F-box pattern. One of the primary functions of FBH1 is to modulate a key protein that is involved in HR, Rad51. FBH1 has been shown to disrupt RAD51 filaments, thereby preventing excessive recombination that could lead to genomic instability (Chu et al., 2015). This negative regulation is essential for maintaining the balance between DNA repair and replication fork stability, especially under conditions of stress such as UV irradiation or hydroxyurea treatment (Jennings et al., 2023). FBH1 helicase activity is required for the regression of stalled replication forks, which is a critical step in the DNA damage response (Fugger et al., 2015). Recent study has highlighted the role of $CRL4^{Cdt2}$ ubiquitin ligase complex in regulating the FBH1 activity through ubiquitination which is essential in controlling the stability or various protein involved in DNA repair (Bacquin et al., 2013; Zhang et al., 2020; Watson et al., 2023). The interaction between FBH1 and $CRL4^{Cdt2}$ is mediated through a PCNA-dependent mechanism, where FBH1 possesses a PIP degron motif that allows it to be recognized by the E3 ubiquitin ligase while tethering at the PCNA (Bacquin et al., 2013). The interaction between FBH1 and $CRL4^{Cdt2}$ is vital during the S phase of the cell cycle when DNA replication stress is most prevalent. Furthermore, FBH1 degradation by $CRL4^{Cdt2}$ is important for maintaining a balance between DNA repair pathways, particularly under conditions of replication stress, where the activity of FBH1 must be finely tuned to prevent detrimental effects on genomic integrity (Sommers et al., 2015; Mankouri et al., 2012). In addition, the degradation of FBH1 itself is crucial for the proper functioning of TLS and HR pathways at the blocked replication fork.

Regulation of $CRL4^{Cdt2}$ activity

Ubiquitination enhancer

c-Abl

c-Abl or known as cellular Abelson tyrosine kinase is a non-receptor tyrosine kinase encoded by the ABL1 gene in humans. In contrast to receptor tyrosine kinases, which are triggered by extracellular signals through ligand binding to membrane-bound receptors, c-Abl is a cytoplasmic and nuclear protein that plays numerous roles in the regulation of cellular activities including DNA damage response, timely cell cycle progression, signal transduction, and cell apoptosis (Bohio et al., 2020).

During the infection of hepatitis B virus (HBV) in human, c-Abl activates the $CRL4^{Cdt2}$ through kinase-dependent manner to suppress HBV polymerase to undergo replication (Hou et al., 2019). The mechanism somehow is still ambiguous, however there is a notion that asserts that c-Abl phosphorylates DDB1 at residue Tyr-316 to recruit a small regulatory protein, DDA1, resulting in enhanced $CRL4^{Cdt2}$ substrates ubiquitination (Figure 2) (Gao et al., 2017). A DDB1 triple mutant (Y126F, Y718F, and Y316F), which abolishes phosphorylation by c-Abl and its association with DDA1, was seen to impede c-Abl-induced HBV polymerase degradation when overexpressed in HEK293 cells (Hou et al., 2020).

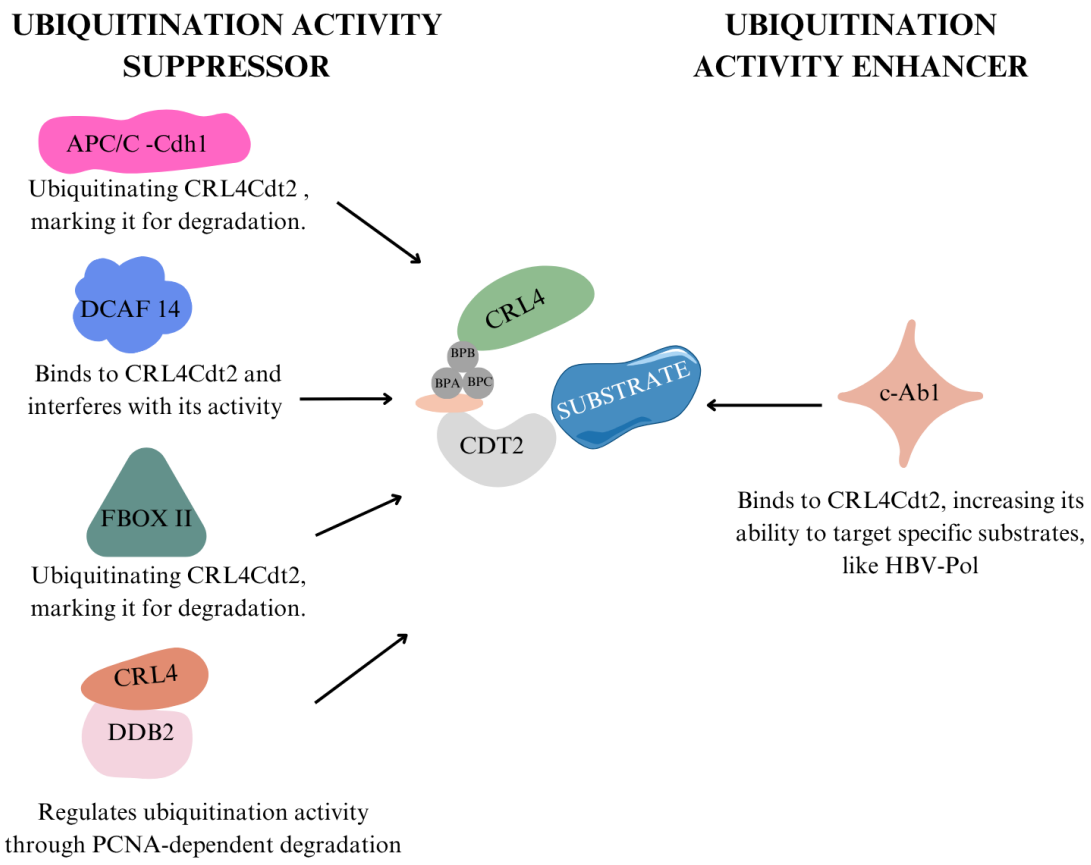

Figure 2. The ubiquitination activity of CRL4^{Cdt2} is facilitated by specific protein complexes. Ubiquitination activity suppressor on right side includes APC/C-Cdh1, DCAF 14, FBOX 11, and CRL4^{DDB2} whereas c-Ab1 enhance the ubiquitination activity.

Ubiquitination suppressor

(a) APC/C-Cdh1

The APC/C-Cdh1 (Anaphase-Promoting Complex/Cyclosome-Cdh1) is an essential protein complex that governs cell cycle progression by directing particular substrates for destruction through the ubiquitin-proteasome pathway. APC/C-Cdh1 assures the accurate timing of cellular cycle event, prominently during late mitotic phase where anaphase spindle dynamic and cytokinesis occur. Proteins targeted for proteasomal degradation by the APC/C-Cdh1 complex possess specific degradation motifs, primarily the D-box and the KEN box (Pfleger et al., 2000; Kramer et al., 2000). APC/C-Cdh1 recognizes these two motifs for the ubiquitination process. Cdt2 on the other hand, contains two D-box motifs and one KEN box motif, which are recognized by the APC complex as their substrate (Pan et al., 2006). Regulation of Cdt2 in M phase by APC/C-Cdh1 is crucial as a green light for the mitotic exit. The continuous presence of Cdt2 during M-phase may result in the unregulated activity of CRL4^{Cdt2} (Figure 2), which could lead to the degradation of essential protein outside their designated phases. Misregulation may disrupt mitotic exit, hinder cell cycle progression, or result in genomic instability, frequently linked to cancer diseases.

(b) CRL1F^{BOX11}

CRL1 (Cullin-RING Ligase 1) F-Box protein 11, is a part of the F-box protein family, which is integral to the ubiquitin-proteasome system. F-box proteins are defined by the presence of an F-Box motif, which is crucial for their interaction with the Skp1 adaptor protein. This interaction facilitates their incorporation into the SCF (Skp1-Cullin1-F-box) Cullin-RING ligase complex (Deshaies and Joazeiro, 2009; Yumito et al., 2020). The ubiquitination of Cdt2 is facilitated by CRL1^{Fbox11}, which is significant because Cdt2 is responsible for targeting proteins for destruction, particularly those that inhibit DNA replication, such as Cdt1 (Abbas et al., 2013; Rossi et al., 2013). CRL1^{Fbox11} modulates the activity of the CRL4^{Cdt2} complex by promoting the polyubiquitination of Cdt2

via a conserved 9 amino acids (456-464) located in the C-terminal of Cdt2 for the proteasome-degradation dependent (Figure 2) (Abbas et al., 2013; Rossi et al., 2013). This Cdt2 ubiquitination destruction mechanism is essential for sustaining appropriate Cdt2 levels, thereby affecting the overall ubiquitination function of its substrates degradation. The degradation interplays between $CRL1^{Fbox11}$ and $CRL4^{Cdt2}$ is essential for averting the early degradation of $CRL4^{Cdt2}$ substrates for DNA replication and for assuring the proper progression of the cell cycle (Abbas et al., 2013; Rossi et al., 2013).

(c) $CRL4^{DCAF14}$

DDB1 and Cul4 associated factor 14, $CRL4^{DCAF14}$ is a distinct variant of the CRL4 E3 ubiquitin ligase complex, which is pivotal in modulating key cellular activities. DCAF14 functions as a substrate receptor in the CRL4 complex, enabling the identification and ubiquitination of certain target proteins, therefore affecting their demise. $CRL4^{DCAF14}$ is localized at stalled replication forks to promote genomic stability through an unclear mechanism, where DCAF14 protects the halted replication fork by modulating the ubiquitination activity of $CRL4^{Cdt2}$ (Figure 2) (Tirado-Class et al., 2023). The interaction is essential for preventing the excessive degradation of Set8, which aids in preventing nascent strand degradation (Tirado-Class et al., 2023). The regulatory mechanism of $CRL4^{DCAF14}$ on $CRL4^{Cdt2}$ is not yet understood. Understanding the $CRL4^{DCAF14}$ and $CRL4^{Cdt2}$ interaction could shed light on the impact towards cellular regulation level.

(d) $CRL4^{DDB2}$

DNA damage binding protein 2 (DDB2) is another receptor for CRL4 E3 ubiquitin ligase. DDB2 previously was reported involved in nucleotide excision repair (NER) where it helps in recognizing the damage DNA induced by UV radiation (Dualan et al., 1995; Ray et al., 2013). $CRL4^{DDB2}$ also known as versatile E3 ubiquitin ligase that degraded multiple substrates including H2A, XPC and p27 (Yan et al., 2011; El-Mahdy et al., 2006). Recruitment of ATM and ATR, DNA damage response kinases to the damage site was not observed in the DDB2 defective cell resulted in reduced phosphorylation form of checkpoint protein such as Chk1, Chk2, H2AX and BRCA1 (Ray et al., 2013). Recently it has been reported that possible $CRL4^{DDB2}$ E3 ligase regulates the activity of Cdt2 through PCNA-dependent degradation (Figure 2) (Wu at el., 2021). Cdt2 is highly expressed in cancer cells including breast cancer, colon cancer and lung cancer (Baraniskin et al., 2012; Pan et al., 2006; Ueki et al., 2008). The aggressive overexpression of Cdt2 in cancer cells following DDB2 knockdown indicates that DDB2 regulates Cdt2 protein stability at the post-translational level (Wu et al., 2021). However, the detail regulatory mechanism is still unclear.

Pathological consequences of CRL^{Cdt2} dys-regulation

Overview of diseases linked to $CRL4^{Cdt2}$ substrate dysregulation, especially cancers

$CRL4^{Cdt2}$ contributes a significant role in regulating the cell cycle particularly in the S phase, to ensure a proper progression of DNA replication and maintaining genomic integrity. Development of cancer hallmarks, such as DNA re-replication and genomic instability, due to uncontrolled DNA propagation potentially emerges when $CRL4^{Cdt2}$ ubiquitin ligase activity is suppressed (Benamar et al., 2016; Mazian et al., 2022). Cdt2 and cancer development are strongly associated, where elevated expression of Cdt2 has been observed in several cancer types, including gastric carcinoma, hepatocellular carcinoma, melanoma and breast cancer. Unregulated expression of Cdt2 is often correlated with poor prognosis and increased tumour aggressiveness (Benamar et al., 2016). In melanoma, for instance, researchers have proposed that inhibiting $CRL4^{Cdt2}$ activity could be a promising therapeutic strategy, highlighting its critical role in tumor growth (Benamar et al., 2016). Similarly, in breast cancer, the dysregulation of Cdt1 has been linked to gene amplification, highlights the critical importance of stringent regulation of $CRL4^{Cdt2}$ stability for accurate cell cycle progression. (Mahadevappa et al., 2017). The impact of $CRL4^{Cdt2}$ dysregulation extends beyond melanoma and breast cancer. In gliomas, for example, reduced levels of DNA repair proteins like O6-methylguanine-DNA methyltransferase (MGMT) have been correlated with $CRL4^{Cdt2}$ activity, suggesting that its malfunction may contribute to the aggressiveness of these tumors (Mostofa et al., 2018). $CRL4^{Cdt2}$ also plays a role in an ovarian cancer, where suppressing $CRL4^{Cdt2}$ has been shown to induce cell death, offering potential therapeutic opportunities (Pan et al., 2013). The therapeutic targeting of the $CRL4^{Cdt2}$ ubiquitin ligase complex represents a promising strategy in oncology, as its inhibition induces selective oncogenic cytotoxicity. Inhibiting the $CRL4^{Cdt2}$ complex kills cancer cells by preventing the breakdown of proteins like CDT1, p21, and SET8. This causes the proteins to build up, leading to DNA damage, halted cell division, and cell death. This effect can be achieved either by using a drug (like MLN4924) or by genetically disabling the $CRL4^{Cdt2}$, both of which have been shown to suppress tumors. (Pan et al., 2013). Additionally, by degrading p21, the $CRL4^{Cdt2}$ complex

promotes cell cycle progression. Preventing this degradation leads to cell cycle arrest or chemoresistance, underscoring the complex's critical role in cancer (Benamar et al., 2016).

In addition to $CRL4^{Cdt2}$ role in regulating cell cycle progression, $CRL4^{Cdt2}$ also involves in regulation and facilitation of DNA damaged repair. For example, $CRL4^{Cdt2}$ regulates the degradation of XPG protein that functions in nucleotide excision repair. Elevated XPG protein levels due to suppression of $CRL4^{Cdt2}$ activity may lead to replication fork collapse and replication of damaged DNA thus increased susceptibility to genomic instability. Consequently, it is crucial to ensure the timely degradation of XPG for maintaining genomic integrity, and any disturbance can initiate the cancer progression and DNA mutation (Han et al., 2014). The complexity of $CRL4^{Cdt2}$ regulation is further highlighted by its interactions with other cellular pathways, such as those involving deubiquitinases like USP46 (Kiran et al., 2018). These interactions underscore the intricate balance required to maintain genomic stability and prevent cancer. In summary, the deregulation of the $CRL4^{Cdt2}$ ubiquitin ligase complex is closely linked to the development and progression of numerous cancers. Its role in controlling key cell cycle proteins and DNA repair mechanisms makes it a central player in preserving genomic integrity. Targeting this complex with therapeutic strategies could open new avenues for cancer treatment, particularly in cancers where $CRL4^{Cdt2}$ activity is abnormally high.

Potential and gap for targeting $CRL4^{Cdt2}$ in therapeutic interventions

The ability of $CRL4^{Cdt2}$ to become a master cell cycle regulator in conserving genomic integrity by controlling the cell cycles progression has emerged as a potential target treatment in several malignancies. Targeting $CRL4^{Cdt2}$ by inhibiting the expression of Cdt2 will result in the accumulation of DNA replication key proteins including Cdt1 and Set8, hence triggering massive DNA re-replication and cancer cell death, and finally reduce tumor development. Significant evidence for targeting $CRL4^{Cdt2}$ is derived from research on malignant melanoma.

The evidence demonstrated that the targeted reduction of Cdt2 using pevonedistat (MLN4924), a selective NEDD8-activating enzyme inhibitor, effectively suppressed melanoma cell proliferation in vitro and inhibited tumor development in human melanoma xenografts in murine models (Figure 3) (Benamar et al., 2016). The proliferation of melanoma cells is contingent upon Cdt2, while pevonedistat impedes the formation of active $CRL4^{Cdt2}$ complex, hence reducing melanoma both in vitro and in vivo by stabilizing the $CRL4^{Cdt2}$ substrates p21 and SET8, and promoting DNA re-replication and senescence (Benamar et al., 2016). This indicates that $CRL4^{Cdt2}$ is a viable therapeutic target for cutaneous melanoma, with pevonedistat demonstrating potential as an anti-melanoma drug (Wong et al., 2017). Pevonedistat also shows a synergistic effect to inhibit vemurafenib-resistant melanoma cells when works in concert with vemurafenib, a BRAF inhibitor (Rughani et al., 2013; Pastwińska et al., 2022). This combination therapy enhances the effectiveness of existing treatments and provides a strategy to address drug resistance in melanoma (Benamar et al., 2016). These findings highlight the potential of $CRL4^{Cdt2}$ as a therapeutic target in many cancer scenarios. However, inhibiting the synthesis of $CRL4^{Cdt2}$ through Cdt2 knockdown is more efficacious in destroying cutaneous squamous cell cancer than targeting CRL4 via DDB1 RBX1 suppression by pevonedistat (McHugh et al., 2020).

It has been demonstrated that the reduction of Cdt2 in 12 distinct human cancer cell lines from various tissues (U2OS, A549, DLD1, EBC1, HCT116, HeLa, HOS, HS764T, MG63, SK-OV-3, SUIT2, and TOV-21G) triggered apoptotic cell death (Olivero et al., 2014). Unexpectedly, the decrease of Cdt2 did not induce cell death in non-transformed human cells, encompassing immortalized kidney, lung, and breast cell lines, as well as primary cultures of endothelial cells. Therefore, elucidating the characteristics of the specific Cdt2 functional domains could represent a pivotal discovery in the development of a medication aimed at this specific domain in Cdt2. The structural attributes of Cdt2 present additional avenues for targeted therapy. Nevertheless, the absence of a three-dimensional Cdt2 protein structure, makes it impossible to fully comprehend the process underlying $CRL4^{Cdt2}$ activation. Research has delineated critical regions within Cdt2 that govern its ubiquitination activity, encompassing the substrate recognition domain, phosphorylation sites, DNA-binding domain and the PCNA-interacting protein-box (PIP) motif. The function of $CRL4^{Cdt2}$ can be inhibited by drugs targeting specific domains, leading to the accumulation of its substrates and subsequent apoptosis in cancer cells. This highlight how $CRL4^{Cdt2}$ can be targeted by pharmacological inhibitors and small molecule drugs that can hinder Cdt2 ability to identify its substrates. Additionally, another Cdt2-associated mechanism that has the potential to be used as a therapeutic target is related to the USP46 deubiquitinase protein, where it functions as a protein stabilizer and is closely linked to the carcinogenic pathway. In HPV-related cancer cells, tumour proliferation depends much on stabilization of Cdt2

and Set 8 via DNA replication. The stabilization of these two key proteins UPS46 relies on the formation of UPS46 and E6 complex. Therefore, targeting the UPS46-E6-Cdt2-Set8 pathway may offer a novel therapeutic strategy for HPV-related tumours (Kiran et al., 2018). Consequently, targeting the $CRL4^{Cdt2}$ pathway for therapeutic intervention appears to be a more advantageous strategy, as it may also impact other cancer types. Ongoing investigation of $CRL4^{Cdt2}$ mechanism and its linkages with other cellular pathway is vital for designing feasible therapies to improve outcomes in malignancies defined by its dysregulation. The example of pathway linkages that may serve as potential therapeutic therapies is the interaction between XPG and $CRL4^{Cdt2}$. The cellular concentration of XPG will be sustained at a normal level when $CRL4^{Cdt2}$ activation is suppressed, hence enhancing DNA repair in cancer cells and potentially increasing the effectiveness of DNA-damaging chemotherapeutics (Han et al., 2015). This strategy may be especially advantageous in malignancies with impaired NER pathways, as reinstating XPG activity might enhance tumor sensitivity to therapy. The $CRL4^{Cdt2}$-mediated degradation of XPG is a vital regulating mechanism in DNA repair processes. Dysregulation of this system correlates with several malignancies and is a possible therapeutic target for augmenting DNA repair and boosting cancer therapy efficacy. Ongoing investigation into the processes regulating XPG degradation and its ramifications for cancer biology will be crucial for formulating successful treatment approaches.

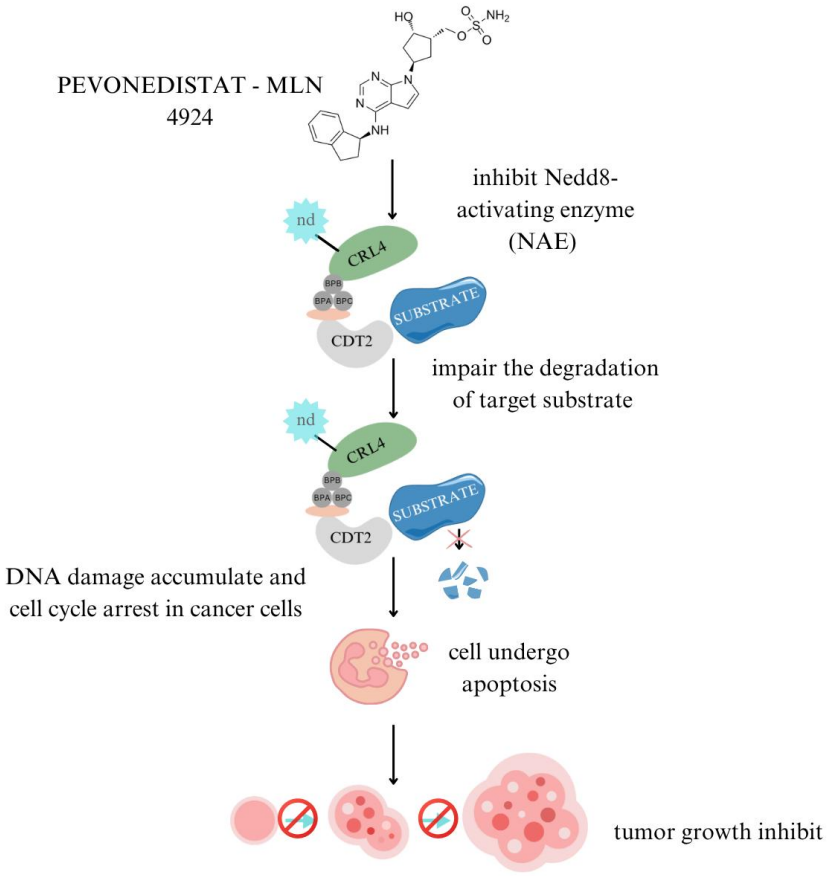

Figure 3. Pevonedistant (MLN4294) inhibits neddylation of CRL4 and suppresses $CRL4^{Cdt2}$ ubiquitination activity. The accumulation of $CRL4^{Cdt2}$ substrates leads to DNA damage, induces DNA re-replication, and promotes cell apoptosis.

CONCLUSION

In conclusion, the ubiquitination activity of $CRL4^{Cdt2}$ is indispensable in preserving genomic integrity. Inhibition of $CRL4^{Cdt2}$ activity can cause accumulation of its substrates and have a high impact on the existence of cancer cells and aggressive tumour growth. However, the inhibition may lead to excessive DNA re-replication, and the cell will eventually activate the cell death program, where the cell will undergo apoptosis. Therefore, by inactivating $CRL4^{Cdt2}$, cancer cell growth can be controlled. Cdt2, the substrate recognition may serve as a target for the development of antidotes for cancer drugs. As reported, the C-terminal of Cdt2 has several domains that regulate ubiquitination activity, including phosphorylation sites, DNA binding domain and PIP box motif. These important domains can be used as specific targets to prevent the formation of $CRL4^{Cdt2}$ complexes. Nonetheless, significant gaps remain in our understanding of $CRL4^{Cdt2}$ regulatory mechanisms, particularly the structural basis of its substrate recognition and ubiquitination. The absence of a three-dimensional structure of Cdt2 makes it difficult to understand more precisely and in detail to design target therapies that can inhibit its function. Future research should focus on elucidating the structural details of $CRL4^{Cdt2}$ and its interactions with substrates, as well as exploring the complex's role in different cancer types. Additionally, the development of more specific inhibitors that target $CRL4^{Cdt2}$ without disrupting other cullin-RING ligase complexes could provide more effective and less toxic therapeutic options.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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