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ARTEMIS INTERACTS WITH THE CUL4A UBIQUITIN E3 LIGASE
COMPLEX AND REGULATES THE CELL CYCLE PROGRESSION

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ARTEMIS INTERACTS WITH THE CUL4A UBIQUITIN E3 LIGASE
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A
DISSERTATION

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The University of Texas
Health Science Center at Houston
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M. D. Anderson Cancer Center
Graduate School of Biomedical Science
in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

by
Yiyi Yan
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Houston, Texas
August, 2010

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ARTEMIS INTERACTS WITH THE CUL4A UBIQUITIN E3 LIGASE COMPLEX AND REGULATES THE CELL CYCLE PROGRESSION

Publication No.

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Artemis, a member of the *SNM1* gene family, is one of the six known components of the non-homologous end joining pathway. It is a multifunctional phospho-protein that has been shown to be modified by the phosphatidylinositol 3-kinases (PIKs) DNA-PKcs, ATM and ATR in response to a variety of cellular stresses. Artemis has important roles in V(D)J recombination, DNA double strand breaks repair and damage-induced cell-cycle checkpoint regulation. The detailed mechanism by which Artemis mediates its functions in these cellular pathways needs to be further elucidated. My work presented here demonstrates a new function for Artemis in cell cycle regulation as a component of Cullin-based E3 ligase complex. I show that Artemis interacts with Cul4A-DDB1 ligase complex via a direct interaction with the substrate-specific receptor DDB2, and deletion mapping analysis shows that part of the *Snm1* domain of Artemis is responsible for this interaction. Additionally, Artemis also interacts with p27, a substrate of Cul4A-DDB1 complex, and both DDB2 and Artemis are required for the degradation of p27 mediated by this complex. Furthermore, I show that the regulation of p27 by Artemis and DDB2 is

critical for cell cycle progression in normally proliferating cells and in response to serum withdrawal. Finally, I provide evidence showing that Artemis may be also a part of other Cullin-based E3 ligase complexes, and it has a role in controlling p27 levels in response to different cellular stress, such as UV irradiation. These findings suggest a novel pathway to regulate p27 protein level and define a new function for Artemis as an effector of Cullin-based E3-ligase mediated ubiquitylation, and thus, a cell cycle regulator in proliferating cells.

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Chapter I. Introduction

Severe Combined Immunodeficiency (SCID)

Severe combined Immunodeficiency is a group of heritable disorders in which both T cell and B cell maturation are severely affected (1, 2). $T^{-}B^{-}NK^{+}$ phenotype can be found in about 20% of SCID patients. This is an autosomal recessive condition that is characterized by a complete absence of both circulating T and B lymphocytes, while natural killer (NK) cells are at normal level and are functional (3). Such patients usually die within the first year of life and stem cell transplantation is the only definite cure. The phenotype is associated with a defect in the V(D)J recombination pathway and mutations of several responsible genes have been identified. For example, mutations in either RAG or LIG4 gene account for a subset of patients with $T^{-}B^{-}NK^{+}$ SCID (4-7). Two groups of $T^{-}B^{-}NK^{+}$ with normal RAG and LIG4 genes have also been reported. Patients from one of these groups have increased sensitivity to ionizing radiation of both bone marrow cells and primary skin fibroblasts (RS-SCID) (8, 9). Another group of patients with a similar SCID condition was found in Athabascan-speaking individuals of Navajo and Apache descent (SCIDA) (10-12). Cells from RS-SCID and SCIDA patients showed impairment in coding joint (CJ) formation in the extra-chromosomal V(D)J recombination assay (13, 14). The gene defective in these two groups of SCIDs, Artemis, was identified a few years later, and the impaired CJ formation in both RS-SCID and SCIDA cells could be effectively complemented by Artemis constructs (15, 16). Thus, Artemis, a novel protein playing an essential role in V(D)J recombination and NHEJ, is responsible for a subset of SCID.

V(D)J recombination and Non-homologous end joining (NHEJ)

V(D)J recombination is a process that randomly rearranges the germline components of the variable (V), diversity (D), and joining (J) gene segments of the T-cell receptor (TCR) and Immunoglobulin (Ig). The completion of this process is indispensable for the survival and maturation of T- and B- precursors, and it is responsible for the recognition of diverse antigens by TCR and Ig (17, 18). Each V, D, and J segment is flanked by recombination signal sequences (RSSs). The recombination is initiated by the lymphoid-specific recombinase activating proteins RAG1 and RAG2, which form a complex that recognizes RSSs and introduces a DNA double strand break (DSBs) at the border of the heptamer. There are two types of DNA ends generated during this process, one is called the signal end and the other the coding end. The blunt signal end can be directly ligated without further processing, while the covalently closed hairpin coding end requires processing (including hairpin opening) before rejoining (19, 20). The rejoining of these two types of ends are both mediated by the non-homologous end joining (NHEJ) machinery, a pathway used to repair DSBs, and a signal joint (SJ) and a coding joint (CJ) are generated, respectively. While the SJs are precise, CJs are much more diverse, including variable nucleotide loss or addition (21). Deficiency in the V(D)J recombination can result in failure of T- and B- lymphocytes maturation, and is responsible for the SCID condition with a profoundly defective immune system.

In mammalian cells, DSBs can either be induced by different DNA-damaging agents or stresses (for example, ionizing irradiation) or be a biological intermediate during the

recombination of TCR and Ig genes. Unrepaired or misrepaired DSBs can lead to cellular senescence, programmed cell death, gross chromosomal rearrangements, and the development of different diseases, such as cancer. There are two mechanisms facilitating DSBs repair, namely homologous recombination (HR) and non-homologous end joining (NHEJ). And the NHEJ is the predominant DSB-repairing pathway in human cells (22, 23). The process of NHEJ can be described as 3 consecutive steps, end recognition, termini trimming, and ligation. First, the Ku70/80 heterodimer recognizes and binds to the DSB ends, forming a scaffold that holds the DNA ends together and recruits the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) to the damaged sites (24, 25). The damaged DNA ends activate DNA-PKcs, which is then able to phosphorylate itself as well as other substrates. One of these substrates is Artemis. When it is complexed with and activated by DNA-PKcs, Artemis acquires an endonucleolytic activity that may be involved in the termini processing, as well as an ability to open hairpins (26). Subsequently, the DNA ends are ligated by DNA Ligase IV and its interacting protein XRCC4, which is also a phosphorylation substrate of DNA-PKcs (27). Since it is an important pathway to maintain genomic integrity, deficiency in any of the NHEJ components can lead to increased cellular sensitivity to DSB-inducing agents and disrupted lymphocyte development.

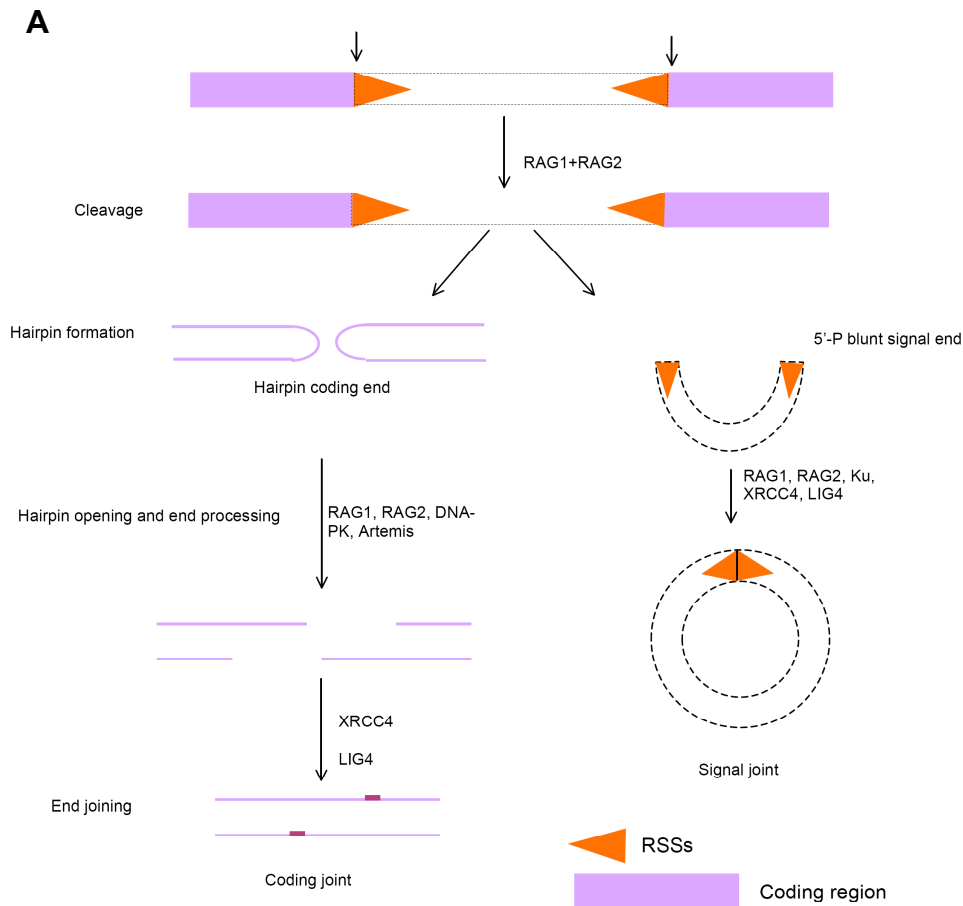


Figure A. Mechanism of V(D)J recombination. V(D)J recombination is initiated by the lymphoid-specific recombinase activating proteins RAG1 and RAG2. RAG1 and RAG2 form a multimeric complex that recognizes RSSs and introduces a DNA double strand break (DSB) at the border of the heptamer. Two DNA ends are generated during this process, a blunt and 5' phosphorylated signal end which can be directly ligated, and a covalently closed hairpin coding end, which requires processing (including hairpin opening) before rejoining. The signal sequence and the coding sequence are both then rejoined by a process involving the non-homologous end joining (NHEJ) machinery, a pathway to repair DSBs, to generate a signal joint (SJ) and a coding joint (CJ) respectively. (*Adapted from Yiyi Yan's Master's thesis)

Artemis

Artemis is a member of the *SNM1/PSO2* (Sensitive to Nitrogen Mustard) gene family, which includes four other known mammalian homologs: SNM1, SNM1B, CPSF73, and ELAC2 (28-31). Members of this family share a region of homology termed the SNM1 domain, which contains a metallo- β -lactamase fold and a β -CASP motif (metallo- β -lactamase-associated CPSF Artemis SNM1/PSO2) (32). This conserved domain is on the amino terminal half of Artemis, spanning amino acids 1-385. The metallo- β -lactamase fold was first described for the *Bacillus cereus* β -lactamase, which is an enzyme that cleaves β -lactam ring, a structure common to many antibiotics. This motif is adopted by various metallo-enzymes with diverse distribution and substrate specificity, and its catalytic activity may be preserved in the SNM1 homologs. The β -CASP motif appended to the metallo- β -lactamase fold has been shown to target the SNM1 proteins to binding of nucleic acids and functions as a nuclease together with the metallo- β -lactamase motif (reviewed in (33)) .

Artemis was originally identified molecularly as deficient in RS-SCID and SCIDA cells, which are characterized by defects in V(D)J recombination. Later, a role of Artemis in V(D)J recombination was verified by showing that Artemis possesses a 5'-3' exonuclease activity on single-stranded DNA, and when activated by DNA-PKcs it gains an endonuclease activity on 5'-3' overhangs and the ability to open hairpins (26, 34-36). This hairpin-opening ability is consistent with the defective coding joint formation observed in Artemis-deficient cells. Since these cells also show a hypersensitivity to IR, those findings suggested a role for Artemis in the NHEJ pathway of DSB repair. In fact,

studies have showed that Artemis, as a substrate of ATM, is required for the repair of approximately 10-15% of all DSBs caused by IR, and that the nuclease activity of Artemis is necessary for the damage processing prior to NHEJ rejoining (37).

Artemis is a phosphor-protein that is extensively phosphorylated both in vitro and in vivo by the three phosphatidylinositol 3-kinases (PIKs) ATM, ATR, and DNA-PKcs depending on the type of DNA damage and cellular stress (37-46). At low to moderate doses of IR (2-3 Gy), Artemis is phosphorylated by ATM. However, at a substantially higher dose (10 Gy), DNA-PKcs also contributes to this modification (37, 39-42). Interestingly, Artemis has also been shown to be phosphorylated by the ATR kinase in vivo in response to other forms of stress such as UV irradiation, MMC, and hydroxyurea (39, 41-43). Artemis is unlikely to have a direct role in repair of these lesions since Artemis-deficient cells are not hypersensitive to UV or MMC (47). All these findings indicate a wider role of Artemis in DNA damage response than just acting as a nuclease in the NHEJ or V(D)J recombination.

Studies conducted by our lab and other groups have showed that Artemis plays important roles in the cell cycle regulation in response to DNA damage. In Artemis depleted humans cells, the initial imposition of the G2/M checkpoint after IR is normal, however, the recovery from this checkpoint is defected, which is due to a delayed activation of Cdk1-cyclin B complex. Mutation of two serine sites on Artemis, S516 and S645, which are rapidly phosphorylated by ATM after IR, can prevent this activation, indicating that the phosphorylation of Artemis is essential for cell cycle checkpoint recovery after IR (41, 42).

As mentioned above, ATR is responsible for the phosphorylation of Artemis after UV and MMC. Damage induced by these agents can result in a block of DNA replication fork progression, and therefore trigger an S-phase cell cycle checkpoint. Artemis mutations on S516 and S645 cause a delayed recovery from this checkpoint due to an impaired degradation of cyclin E. This is explained by the finding that the phosphorylation of these two sites can enhance its binding to the SCF^{Fbw7}, which is the ligase responsible for the ubiquitylation of Cyclin E (48).

As a multifunctional protein, Artemis can also act as a negative regulator of p53 in response to oxidative stress produced by mitochondrial respiration in both primary cells and cancer cell lines (49). p53 is phosphorylated, which is then stabilized and activated, by DNA-PKcs upon such stress. Artemis can inhibit this phosphorylation and stabilization, and therefore control the degree of activation of p53 in response to oxidative stress.

Cells from Artemis-deficient SCID patients exhibit an impaired V(D)J recombination as well as hypersensitivity to IR. Disruption of *Artemis* in mice leads to a phenotype that is very similar to the human syndrome (47). In addition, *Artemis* null mouse embryonic fibroblasts (MEFs) show an increased chromosomal abnormality suggesting a role of Artemis in maintenance of genomic stability. While *Artemis* null mice do not show increased tumorigenesis, *Artemis/p53* double knockout mice develop progenitor B lymphomas, indicating its potential function as a tumor suppressor (50).

All these recent findings suggest that Artemis is a multifunctional protein with important roles in regulating different biological pathways. And some of these functions

cannot be simply explained by its nuclease activity. However, the exact and detailed mechanisms of how Artemis is involved in these regulations are yet to be elucidated.

B

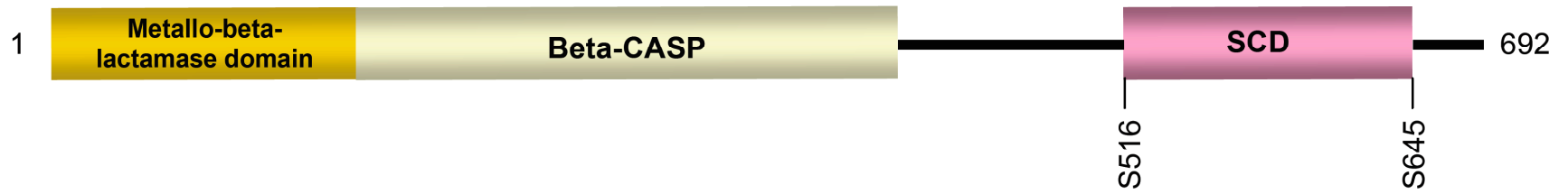


Figure B. Schematic of Artemis showing conserved SNM1 domain and (S/T)Q cluster on the C-termini.

Nucleotide Excision Repair (NER) and Xeroderma Pigmentosum (XP)

DNA damaging agents from the environment such as ultraviolet (UV) irradiation constantly attacks mammalian DNA. The major lesions caused by UV light are cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4 PPs). These helix-distorting lesions, as well as bulky chemical adducts produced by psoralenes and cisplatin, can be eliminated from the genomic DNA by a repair pathway known as NER (51). NER is composed of two sub-pathways: global genome repair (GGR), which repairs lesions over the entire genome, and transcription-coupled repair (TCR), which removes damage in the template strand of transcriptionally active DNA. Approximately 30 genes are known to be involved in the NER pathway, and execute this multi-step repair process in a sequential order: damage recognition (XPC, XPE, RNAPII), unwinding of the helix (TFIIH), validation and stabilization of damage (XPA/RPA), incision of the damaged DNA strand on both sides of the lesion (XPG and XPF/ERCC1), excision of a 22-31-mer oligonucleotide containing the damage, and DNA synthesis followed by strand ligation (DNA polymerase and DNA Ligase I) (reviewed in (52)).

NER is important for cells to withstand potentially lethal and mutagenic DNA damage, and to maintain the integrity of the human genome. A defective NER pathway can result in heritable diseases known as Xeroderma Pigmentosum (XP) and Cockayne Syndrome (CS) (53).

XP is a rare autosomal recessive disease that is characterized by hypersensitivity to UV light. Patients with XP have pigmentation changes in the sun-exposed skin area and

an increased incidence (1000-fold) of all types of skin cancer (basal cell carcinoma, squamous cell carcinoma and melanoma) (53-55). XP has been classified into seven complementation groups (XPA-XPG) by cell fusion analysis (53). The corresponding genes of these groups are all involved in the NER pathway. Among these seven groups, patients with XP-E disease have the mildest degree of clinical phenotype. Compared to the defects in both GGR and TCR found in XP-A, -B, -D, -F, and -G patients, cells from XP-E patients are only defective in the GGR sub-pathway of NER, which is caused by the mutations of the damaged-DNA binding (DDB) gene (56).

C

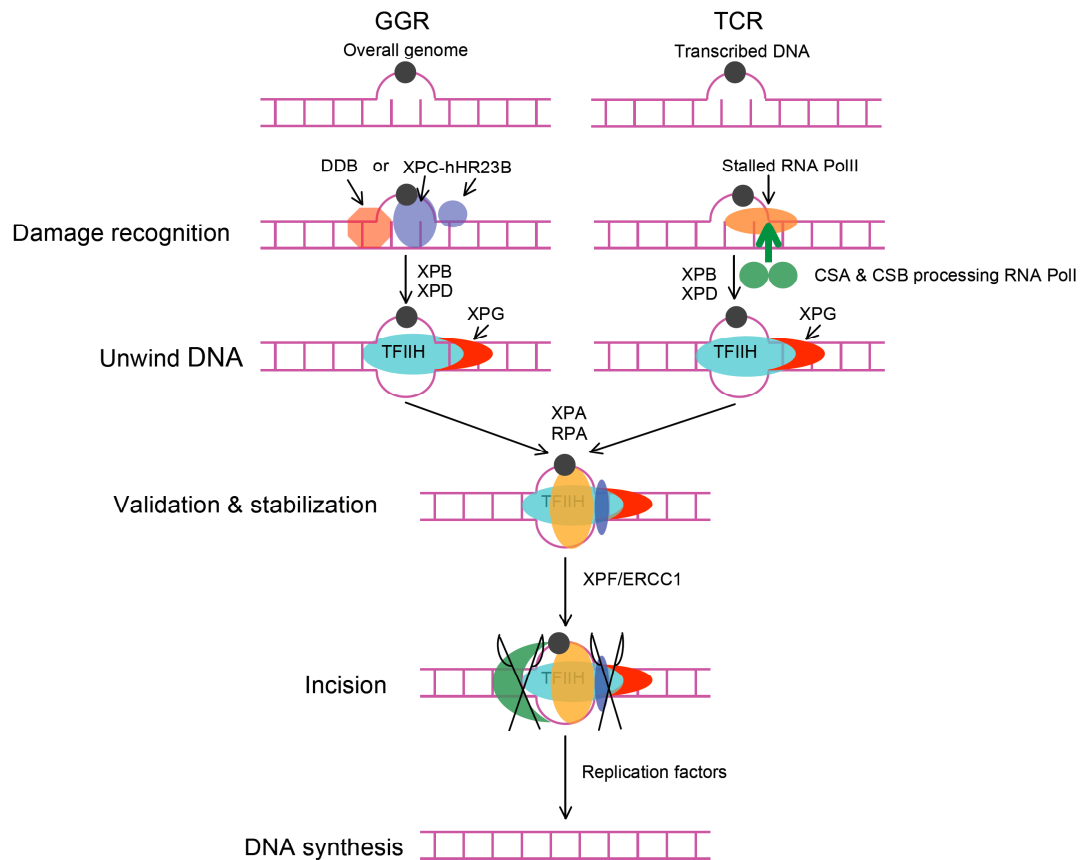


Figure C. Mechanism of Nucleotide Excision Repair. NER is a multi-step process. Approximately 30 proteins sequentially execute damage recognition, unwinding the helix, validation and stabilization, incision, and DNA synthesis. (*Adapted from Yiyi Yan's Master's thesis)

Damaged-DNA Binding (DDB) Protein

DDB was first identified by testing the UV-induced damaged-DNA binding activity of the cells from XP-E patients. DDB was purified as a heterodimer containing two subunits, DDB1 and DDB2, and both of them are required for its damaged-DNA binding activity (57, 58).

DDB1 is strongly conserved during evolution (59, 60). It localized in both the cytoplasm and nucleus and binds tightly to damaged-DNA on chromatin upon UV in a complex with DDB2 (61-63). Deletion of *Ddb1* in fission yeast results in increased spontaneous mutation rates as well as an impaired degradation of Spd1, a replication inhibitor. The latter leads to a failed progression into meiosis (64, 65). Loss of *Ddb1* in fruit flies induces melanotic tumors and causes complete lethality, suggesting a critical role of *Ddb1* in development (66). Consistent with these results, a null mutation of *Ddb1* has been found to cause early embryonic lethality in mice (67).

DDB2 homologs are only found in mammals. While no mutations of DDB1 have been found in mammalian cells, several DDB2 mutations have been reported in XP-E patient cells, which proved to be the cause of XP-E (68, 69). The transcription of DDB2 can be regulated by p53, E2F1, and BRAC1 (70-73). Interestingly, DDB2 protein is rapidly ubiquitinated and degraded after UV, and the removal of DDB2 upon damage is thought to be necessary for the later recruitment of other repair factors such as XPC (74, 75). Compared to *Ddb1* null mice, depletion of *Ddb2* is not embryonic lethal. Instead, these animals exhibit an increased skin carcinogenesis (while enhanced *Ddb2* expression

made them more resistant to UV-induced skin cancer) and decreased apoptosis after UV-irradiation, in line with the phenotype observed in XP-E patients (76-78).

As implied by its name, DDB has an important function in UV-induced DNA damage repair. While it has a higher affinity to 6-4PPs compared to CPD, DDB function is mainly connected with the efficient GGR of CPD rather 6-4PPs. Recently, the damage-bound (either 6-4PPs or an abasic site) structure of DDB1-DDB2 complex has been described (79). DDB1 contains three WD40 seven-bladed β -propeller domains designated BPA, BPB, and BPC in the order from N- to C- terminus, respectively. DDB2 also has a β -propeller (BP) domain. In addition to that, DDB2 contains a helix-loop-helix (HLH) segment on its N-termini. The BPA and BPC domain on DDB1 tightly bind with each other, and this cluster interacts with the HLH segment on DDB2. On the other hand, the BPB domain of DDB1 binds to the N-termini of Cul4A (80). The interaction between damaged-DNA and the complex is mediated by the BP domain on DDB2. This finding is consistent with the observation that mutations in DDB2 compromise the damaged-DNA binding activity.

Accumulated results have shown that the DDB complex is essential for the GGR and functions at the very first step, damage recognition. Actually, it is now thought to be the first factor that detects and binds to the damaged site in GGR. It seems to be required for the accumulation of XPC and the recruitment of other NER factors onto the sub-nuclear DNA damaged site, which may depend on an interaction between DDB and XPC (75, 81-83).

As mentioned above, genetic analyses of DDB1 disruptions have been performed in different species, and results revealed broad functions for DDB1 beyond DNA repair.

Lately, a new function of DDB complex in the protein ubiquitylation pathway has been established. When ectopically expressed in cells, DDB can be purified as a part of Cul4A-Roc1 E3 ligase complex, and several substrates of the Cul4A-DDB-Roc1 complex have been reported, which suggests a new function of DDB in regulating cell cycle progression and DNA damage response through this new role (84). (This detail will be described in the following section).

The Ubiquitin/Proteasome Pathway of Protein Degradation and Cul4A-Based Complex

Protein degradation can be mediated by two different systems named lysosome and ubiquitin-proteasome system (UPS), respectively. While the former mainly degrades extracellular proteins by digestive enzymes, the latter plays an important role in degradation of cellular regulatory proteins. The UPS pathway is utilized to modulate a wide range of diverse protein functions and cellular processes, and its deregulation has been reported in various cancer cells. The ubiquitin-proteasome mediated degradation pathway comprises two steps: the covalent attachment of ubiquitin to the substrate, known as ubiquitylation, and the degradation of the substrate by the 26S proteasome (reviewed in (85, 86)). Ubiquitin, a 76 amino acid highly conserved polypeptide, is attached to substrate proteins through three sequential steps in the presence of ATP. First, it is activated by E1 (activating) enzyme, and then is transferred to an E2 ubiquitin-conjugating enzyme. Finally, an E3 ubiquitin ligase recognizes a specific target protein

and promotes the ligation of ubiquitin to the substrate. Specificity of ubiquitylation for a huge variety of cellular targets is achieved by the presence of a large pool of E3 ubiquitin ligases. In fact, there are more than 1000 distinct E3 ligases in higher eukaryotes, which can be categorized into four major classes based of their specific structural motif: HECT-type, RING-type, U-box type and PHD-finger type.

Cullin-RING E3 ligase (CRL) family, a subgroup of RING-type ligases, which recruit E2s via a RING domain motif, is one of the largest classes of E3s (reviewed in (87, 88)). There are 6 cullin family members (Cul1, 2, 3, 4A, 4B, and 5) in humans, which are the scaffolds responsible for the assembling of the CRL (89). A small ubiquitin-like protein Nedd8 can modify all of the cullins. The E3-ligase activity of Cullin-based RING complexes is up-regulated when Nedd8 is covalently bound to the cullin protein (90). A distinct character of CRLs is that Cullins do not directly bind to the substrates, but rather recruit the substrates by a receptor and /or an adaptor protein. One example of the well-characterized CRLs is the prototypical Skp1-Cul1-F box protein (SCF) complex (91, 92). It is involved in the degradation of many cell cycle regulators such as p27. The SCF complex consists of three invariable components: Roc1 (RING-finger protein), which binds to E2s, Cul1 (Cullin1), providing a platform for complex assembly, and Skp1, acting as an adaptor. Additionally, there is one variable component, an F-box protein, which binds to Skp1 via its F-box and is responsible for substrate recognition. For example, the F-box protein Skp2 binds to Skp1. In conjugation with Skp2, the SCF complex (termed as SCF^{skp2}) targets p27 for degradation(93, 94). In addition to p27, p21, E2F1 and p57 have also been reported as targets of SCF^{skp2} (95-

97). Increased Skp2 expression level, which is inversely correlated with p27, has been detected in many human cancers, indicating the oncogenic function of Skp2.

Currently, another group of CRLs, known as DDB1-Cul4A-Roc1 complex, has been undergoing extensive investigation. Cul4A is amplified and overexpressed in breast cancer and hepatocellular carcinomas (98, 99). The association between Cul4A, DDB1 and Roc1 was first identified during immune-purification of ectopical DDB2 and recently the crystal structure of this complex was resolved (80, 84). The globular C-terminal domain of Cul4A binds to Roc1, and the N-terminal arc-shaped domain of Cul4A binds to the BPB domain on DDB1. This BPB domain is flexible, which allows a rotation and tilt in a certain degree, thus providing space for the catalytic center of the E2-Roc1 to move around. This observation is consistent with DDB1's function in this complex as an adaptor for various substrates.

Similar to the SCF complex, which utilizes a F-box protein to recruit a specific substrate, the DDB1-Cul4A-Roc1 complex employs the interaction between DDB1 and a group of substrate-recognizing receptor proteins to target different substrates. More than 50 different DDB1-binding receptor proteins, one of which is DDB2, have been identified. They are referred to as DCAFs (DDB1-Cul4A-associated factors), and contain WD40 repeats, a protein motif that is responsible for protein-protein interaction (100-103). DCAFs share a common motif defined as WDXR that is characterized by an Arginine residue at position 16 following the WD dipeptide. This Arg residue is essential, and its point mutation in some of the DCAFs can abolish their interaction with DDB1. Interestingly, such a mutation in DDB2 can be found in several XP-E patient cell lines (102).

Recent studies have shown that the Cul4A-DDB1 ligase is responsible for the degradation of more than twenty proteins that have diverse functions in DNA repair, cell cycle regulation, and cellular DNA damage responses. For example, in association with the adaptor protein CDT2, Cul4A-DDB1-CDT2 complex (Cul4A-DDB1^{CDT2}) can target Cdt1 for degradation during the cell cycle and in response to DNA damage (102, 104, 105). Cdt1 is a DNA replication licensing factor, and the interaction between Cdt1 and PCNA is essential for its Cul4A-mediated degradation. The Cul4A-DDB1^{CDT2} can also promote the degradation of p21 after UV-irradiation, which abolishes its inhibitory effect on PCNA and therefore facilitates NER (106-108). Another ligase complex, Cul4A-DDB1^{DDB2}, promotes the polyubiquitylation on DDB2 and XPC (74, 75). While this modification on DDB2 targets itself for degradation rapidly after UV, polyubiquitylated XPC is stable and able to bind damaged DNA. These observations support the model that DDB2 is removed from the lesion after binding to the damaged site and promoting the XPC ubiquitylation, which is critical for the recruitment of XPC and other repair factors. Cul4A-DDB1^{DDB2} also catalyzes the monoubiquitylation of Histone H2A, H3 and H4, suggesting a role of Cul4A-DDB1 complex in regulating chromatin remodeling upon DNA damage (109, 110). In association with CSA (Cul4A-DDB1^{CSA}), this E3 complex can target CSB for ubiquitylation (111). Both CSA and CSB are involved in the TCR-NER, and the mutation of CSA is the cause of Cockayne Syndrome (112). Several recent studies provided evidence showing that Cul4A-DDB1 complex is also responsible for the ubiquitylation and degradation of the cell cycle inhibitor p27 (113, 114). The identification of its multiple substrates reveals the diverse role of Cul4A-DDB1 complex plays in various cellular processes.

In addition to Cul4A, DDB1 can also bind to Cul4B, a Cul4A-related Cullin found only in mammals, and which shares significant sequence homology with Cul4A (101). Although The Cul4B-DDB1 complex is less well understood, it has been shown that this complex also has E3 ligase activity (115).

Genetic analyses of Cul4A deletion have been performed in different species. *cul4a* deletion leads to a growth retardation in yeast as well as an impaired development in *C. elegans* and *Arabidopsis*, consistent with its multiple functions in cells (116-118). In mice, the phenotype of *Cul4a* differs from early embryonic lethal to mild or no apparent phenotype, depending on the exact exon(s) being deleted in the experiment, and the protein levels of its substrate such as DDB2, p27 are deregulated in the MEFs (119-121).

D

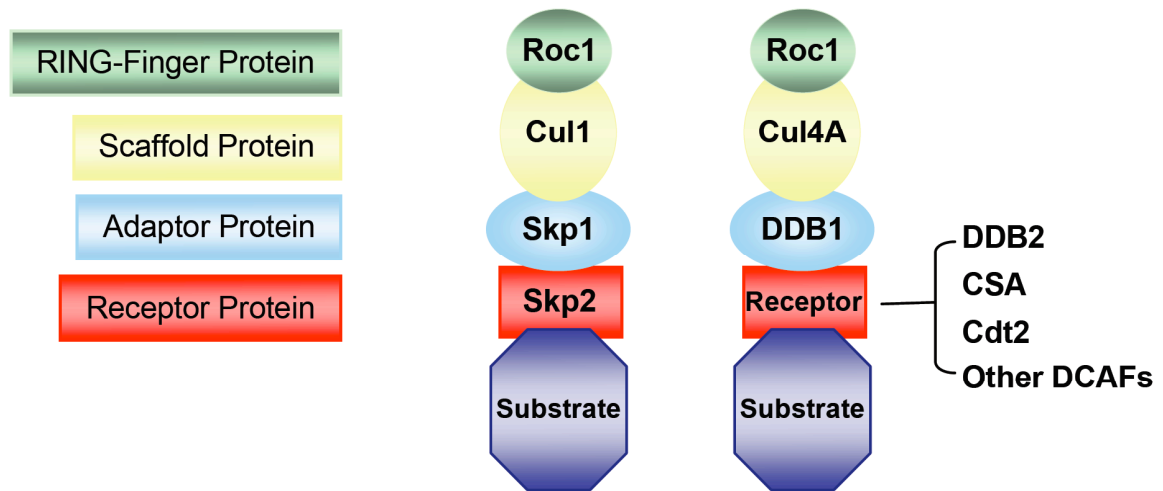


Figure D. Schematic of SCFSkp2 complex (left) and Cul4-DDB1 complex (right).

p27^{kip}

p27^{kip} (hereafter p27) is a member of the kip family of cyclin-dependent kinase (Cdk) inhibitors. Loss of a single allele of p27 confers increased susceptibility to carcinogen-induced tumors in mice and low levels of p27 protein have been associated with poor prognosis in several human cancers. p27 expression level peaks in quiescent cells and drops upon mitogen stimulation (122, 123). It has a crucial role in the G1-S transition by interacting with and inhibiting cyclin E-CDK2 activity (124). In early G1, p27 increases cyclin D-CDK4/6 stability through promoting the complex assembly and nuclear import (125). The cyclin D bound p27 serves as a reservoir for p27 and release of p27 allows it to inhibit cyclin E /CDK2 complex through association with CDK2, thus arresting the cell cycle at the G1 phase (126, 127).

p27 is regulated predominantly at the post-translational level through the proteasome-mediated degradation pathway. However it is also regulated at the level of transcription and sub-cellular localization. Transcription of p27 is induced by FOXO family of forkhead transcription factors. Negative regulation of p27 promoter is accomplished by Akt mediated inhibition of the FOXO transcription factor (128).

p27 function is also governed by its subcellular localization since to act as cell cycle inhibitor it must be localized in the nucleus. This localization of p27^{kip} is tightly regulated by its phosphorylation. For example, Akt phosphorylates p27^{kip} at Thr 157 and Thr 198, which impairs the nuclear import of p27 (129, 130). Additionally, hKIS/MAPK phosphorylates p27 at Ser10 (131, 132). This phosphorylation is necessary for p27^{kip} binding to CRM1 (a nuclear export carrier protein) and JAB-1 (Jun activation-domain

binding, also known as CSN5), which allows its nuclear export. The cytoplasmic localization of p27 serves two purposes. First, it favors cell cycle progression and may facilitate cellular transformation. Secondly, it facilitates p27 a target for the E3-ubiquitin ligase complex (133-135).

Several E3 ligase complexes responsible for p27 ubiquitylation and degradation have been identified. SCF^{skp2} is the most well understood one, it is responsible for the degradation of p27 in the nucleus during S-G2 phase. Skp2 is the substrate binding protein in the SCF complex; it binds to p27 and promotes its ubiquitylation and degradation within the nucleus only when p27 is phosphorylated at Thr187 by CyclinE/Cdk2 complex (136-138). In addition to SCF^{skp2}, KPC (Kip1 ubiquitination-Promoting Complex) has been reported to mediate ubiquitylation of p27^{kip}. It targets p27^{kip} in the cytoplasm, in G1 phase, allowing its cytoplasmic destruction following nuclear export (139). Recently, several groups reported that DDB1-Cul4A is also responsible for the ubiquitylation of p27. A physical association between DDB1, Cul4A and Skp2 has been shown. Furthermore, overexpression of DDB1 or Cul4A can promote p27 proteolysis, indicating a role of DDB1-Cul4A in p27 ubiquitylation (113, 114, 140). However, the regulation of this process is yet to be established.

Chapter II. Materials And Methods

Cell Culture

HeLa, HEK293, HEK293T cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). hTert-RPE1 (hereafter RPE1) cells were cultured in DMEM-F12 containing 10%FBS. *Artemis* mice were provided by Frederick Alt. Preparation of MEF cells and genotyping was carried out as described (47). *Artemis* $+/+$ and $-/-$ MEFs were grown in DMEM containing 10%FBS, 1x nonessential amino acids, and 55mM 2-mercaptoethanol.

Inhibition of Protein Expression by siRNA

Small interfering RNA oligonucleotides (siRNAs) were synthesized with 3' dTdT overhangs by Dharmacon (Lafayette, CO) in a purified and annealed duplex form. The sequence of *Artemis* siRNA was 5'-UUAGGAGUCCAGGUUCAUG; The sequence of the DDB2-1 siRNA was 5'-CCAGAAGACCUCCGAGA; and the sequence of DDB1 siRNA was 5'-GCAGAAUCGACUCAAUAAA. The following commercially available siRNAs from Sigma were used: DDB2-2 (00101645) and p27 (00113637). siRNAs for Cul4A (sc-44355) and Skp2 (sc-36499) were purchased from Santa Cruz Biotechnology. SiRNAs were transfected into proliferating cells using oligofectamine RNA transfection reagent (Invitrogen) in accordance with the manufacturer's instructions. Three hundred and eighty μ l Opti-MEM was incubated with 8 μ l oligofectamine for 5 minutes and then 20 μ l (20 μ M) siRNA was added to the mixture and incubated for another 20 minutes at

room temperature. Medium was removed from cells cultured on a 60-mm plate at 30-40% confluency, and the entire mixture together with 1.6 ml Opti-MEM was added to the plates. Cells were cultured for 4 hours before 2 ml DMEM with 20% FBS was added. Transfected cells were grown for 36-48 hours, after which cells were harvested. In some cases, siRNAs were transfected by electroporation (Nucleofector Amaxa GmbH, Cologne, Germany) according to the manufacturer's instruction. siRNA at 1.5 μ g was used for each $1-5 \times 10^6$ cells. Cells were harvested 36 hours after transfection. Knockdown efficiency was determined by immunoblotting.

DNA constructs and DNA Transfection

A construct that expresses myc-tagged mouse Cul4A was obtained from Dr. Pengbo Zhou (141). A plasmid containing the hDDB2 cDNA was a gift from Yoshihiro Nakatani (84). The DDB2 open reading frame was removed by restriction enzyme digestion and religated into the pENTR3C vector for use in the Gateway system (Invitrogen). Human DDB1, Skp2 and Cul1 full-length cDNAs in pCMVSPORT6 vector were purchased from Open Biosystems. The cDNAs were amplified by PCR and were ligated into the pENTR11 vector. Human Artemis cDNAs and Artemis phosphorylation mutants were prepared as previously described (41, 42). Artemis deletion constructs were generated using restriction enzyme digestion on pENTR11-Artemis plasmid. DNAs in the pENTR11 vectors were transferred via site-specific recombination (LR reaction, Invitrogen) to various destination vectors with a CMV or

T7 promoter for either in vivo or in vitro expression. The Flag-p27 was provided by Dr. Mong-Hong Lee (M.D. Anderson Cancer Center), and Flag-p27 T157A, T157D, T187A and T187D were generated by PCR-based mutagenesis (Stratagene). All plasmid constructs were confirmed by DNA sequencing. A construct that expresses GST-tagged human Cdt2 was obtained from Dr. Jianpin Jin (University of Texas Medical School at Houston).

Both transient and stable DNA transfections were performed using FuGENE6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's recommendations. Selective medium for stable transfections with the plasmids derived from pDEST27 contained G418 at a concentration of 400 µg/ml (Sigma).

Immunoblotting

Whole-cell extracts were prepared as follows. Cells were grown in monolayer culture in DMEM with 10% FBS to 80% confluency. Medium was removed and 2X SDS loading buffer was added to the dish to lyse the cells. After the proteins were denatured at 100°C for 3 minutes, the sample was passed through a 27 1/2 gauge needle for several times. Protein samples were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a nitrocellulose membrane (Millipore), and the membrane was blocked with 5% dry milk in 1X phosphate-buffer saline (PBS). Immunoblotting was performed using standard protocols with antibodies diluted in binding solutions (5% BSA in 1X PBS with 0.05% sodium azide, or 5% dry

milk in 1X PBS with 0.1% Tween-20). Protein detection was performed using the enhanced chemiluminescent assay (Amersham Pharmacia Biotech). Secondary antibodies conjugated with horseradish peroxidase specific for mice, rabbit or goat IgG were obtained from Amersham-Pharmacia or Santa-Cruz Biotechnology.

Antibodies

Artemis polyclonal antibodies and Artemis phosphor-specific antibodies were generated as previously described. The following commercially available antibodies were used: goat anti-DDB1 (abCAM), rabbit anti-DDB2, mouse and rabbit anti-GFP, rabbit anti-Skp2, rabbit anti-p27 (Santa Cruz Biotechnology), monoclonal antibody for bromodeoxyuridine (BrdU) (Becton Dickinson), mouse anti-HA (Roche), mouse anti-Flag (Sigma), mouse anti-MBP (New England Biolab), and mouse anti-GST (Cell Signaling). Rabbit anti-Cul4A, anti-DDB1 and anti-Cdt1 antibody was kindly provided by Dr. Yue Xiong (University of North Carolina).

Protein Expression and In Vitro Translation

The in vitro protein translation was performed as previously described in my master thesis (142). In Brief, Artemis, DDB1, DDB2 and Cul4A cDNAs were cloned into

pDEST vectors under the T7 promoter. Proteins labeled with ^{35}S -methionine were produced in vitro using the T7 TNT Coupled Reticulocyte Lysate System (Promega).

Bacterially expressed glutathione *S*-transferase (GST)-Artemis (full length and truncated), MBP-DDB2, MBP-Cul1, MBP-Skp2 were purified using GST beads (Amersham) or amylose resin (New England Biolabs), respectively, according to the manufacturer's protocols.

Immunoprecipitation, GST Pull-down and Mass Spectrometric Analyses

Co-immunoprecipitation (co-IP) experiments were performed as previously described (42). In brief, cells were lysed with EBC buffer (50mM Tris pH 8.0, 120mM NaCl and 0.5% Nonidet P-40). Indicated antibodies were incubated with the cell lysates before the protein A-Sepharose CL-4B (Amersham Pharmacia biotech) were added.

For in vitro IP experiments, ^{35}S -methionine-labeled proteins were mixed and were incubated on ice for 1 hour. Two hundred ml 1X PBS together with the indicated antibody was added, and the reactions were incubated at 4°C for 1 hour. Ten ml of protein A-Sepharose CL-4B beads were added, and the reactions were rotated for another hour at 4°C. After the supernatants were removed, beads were washed. Bound proteins were eluted by 2X SDS sample loading buffer and separated on SDS-PAGE for autoradiography.

The in vitro GST pull-down assay was performed as previously described in my master thesis. Briefly, purified proteins were mixed and incubated at 4°C overnight in

the presence of BSA and then GST beads were added to the mixture. After a 1-hour incubation, beads were washed, and bound proteins were eluted with 2X SDS loading buffer and separated by SDS-PAGE.

The purification of Artemis complexes for mass spectrometric analysis was previously described in my master thesis. HEK293 cells were transfected with a GST-tagged Artemis expressing plasmid. Fifty x 10⁶ transfected or control cells were lysed with Net-N buffer and the lysates were incubated with GST beads. Immunocomplexes were resolved by SDS-PAGE, stained with Coomassie blue, and protein bands were digested with trypsin and subjected to mass spectrometric analysis for identification (M.D. Anderson Cancer Center proteomics core facility).

In Vivo Ubiquitylation Assay

HeLa cells were transfected with HA-ubiquitin, GST-Artemis, and Flag-p27. Forty-eight hours after transfection, 20 μ M MG-132 was added into the media. Cells were harvested 5 hours after MG-132 treatment in lysis buffer (50 mM Tris PH 7.5, 0.5 mM EDTA, 1% SDS and 1 mM DTT). Lysates were boiled for 10 minutes and then diluted 10 times in Net-N buffer containing protease inhibitors (Roche). Immunoprecipitations were performed using anti-Flag M2-conjugated agarose (Sigma).

Serum Starvation and Cell Cycle Analyses

For serum starvation experiments, RPE1 cells were grown in DMEM-F12 media containing 10% FBS. Twenty-four hours after transfection with indicated DNA constructs or siRNAs, media was removed and dishes were washed with 1XPBS twice. Then DMEM-F12 with 0.1% FBS was added, 24 hours later (or at different time points as indicated in the figure), cells were harvested and fixed in 75% ethanol, and DNA was labeled by propidium iodide (PI) for flow cytometry analysis.

For BrdU labeling, cells were labeled with BrdU at a concentration of 10 μ M for 30 min prior to harvesting and fixation. Cells were then incubated in PBS containing 4% BSA and 0.2% Triton X-100 for 1 hour. After the DNA was denatured by 2N HCl for 30 minutes, cells were incubated with anti-BrdU antibody for 1 hour, and then fluorescein isothiocyanate-conjugated goat antibodies to mouse IgG were added. After washing with PBS, cells were resuspended in PBS with 50 μ g/ml PI and 20 μ g/ml DNase-free RNase. Fluorescence was measured on FACS Calibur flow cytometer (BD Biosciences) using 488 nm laser excitation by the cytometry and cellular imaging core facility in M.D. Anderson Cancer Center.

RNA Purification and RT-PCR

Total cellular RNA was extracted using the RNAqueous-4PCR kit (Ambion, TX). One μ g of RNA was reverse-transcribed into cDNA. The cDNA was subjected to real-

time quantitative PCR using the SYBR green supermix and iCycler iQ Real-time PCR detection system (Bio-Rad). All results were normalized to the amount of GAPDH.

Chapter III. Results

Artemis interacts with the DDB heterodimer via DDB2

It is known that Artemis is required for V(D)J recombination (15, 26), has a minor role in the repair of DBSs (37), and is involved in cell cycle checkpoint regulation (41, 42, 48). However, the precise mechanism of how Artemis controls these biological functions needs to be further elucidated. In order to investigate the function of Artemis, we initiated a search for novel cellular proteins that interact with human Artemis. And the following results presented in this section has been previously described in my master thesis (142). In summary, a construct expressing GST-Artemis was transfected into HEK293 cells, and proteins in cell lysates were pulled-down using glutathione sepharose beads, and separated by SDS-PAGE. Coomassie blue staining of the resulting gel is shown in Fig. 1A. A mock purification from non-transfected cells was performed as a negative control. Polypeptides with the molecular mass of ~ 125 KD were specifically detected in the transfected cell population. This band was analyzed by mass spectrometric analysis, and shown to contain GST-Artemis and DDB1, suggesting that DDB1 might be an Artemis interacting protein.

To confirm this interaction, HEK293 cells were treated with UV, IR, or mock treated, and 2 hours later co-IPs were carried out using Artemis-specific antiserum. As shown (Fig. 1B), DDB1 and Artemis co-IPed with each other, suggesting that Artemis interacts with DDB1 both before and after DNA damage. A reciprocal IP was also performed using DDB1 antibody, and Artemis was detected in the precipitates both with and without UV treatment (Fig. 1C). Because DDB1 can rapidly translocate from the cytoplasm to the nucleus and tightly bind to chromatin after UV irradiation (61), we

examined whether its interaction with Artemis would be affected by UV irradiation as a function of time. HEK293 cells were UV irradiated and harvested at different time points following treatment. Upon IP using anti-Artemis serum, we found that DDB1 interacted with Artemis with or without UV treatment, and the amount of DDB1 precipitated by Artemis specific serum was not affected in response to UV up to 2 hours after irradiation (Fig. 1D). Taken together, these experiments demonstrate that Artemis and DDB1 form a complex, and this interaction is not affected by the DNA damage induced by UV or IR irradiation. These findings are also interesting from the standpoint that Artemis is rapidly phosphorylated in response to DNA damage by PIKKs at multiple sites (41), but this phosphorylation apparently does not affect its interaction with DDB1.

DDB was originally purified as a DDB1-DDB2 heterodimer that tightly binds to chromatin after UV-inflicted DNA damage. Since Artemis interacts with DDB1 *in vivo*, I examined whether Artemis also interacts with DDB2. GST-DDB2 was expressed in HEK293 cells, and the result of a co-IP experiment performed using Artemis anti-serum indicated that Artemis interacts with DDB2 (Fig. 1E). Also, an interaction between ectopically expressed GFP-Artemis and GST-DDB2 was observed in HEK293 cells (Fig. 1F). From these results, I conclude that Artemis interacts with both DDB1 and DDB2 *in vivo*, presumably as components of the DDB complex. In order to determine which subunit of the DDB complex directly interacts with Artemis, I conducted the following *in vitro* assays. ³⁵S-methionine-labeled Artemis, DDB1, and GST-DDB2 were translated *in vitro* using rabbit reticulocyte lysate, and subsequently mixed together for co-IP assays. Although Artemis and DDB1 interact with each other *in vivo*, Artemis was

not co-IPed by DDB1 antibody in vitro, nor was DDB1 co-IPed by Artemis antiserum (Fig. 2A,B). However, GST-DDB2 was able to be co-IPed by Artemis antibody (Fig. 2C), indicating a direct interaction between Artemis and DDB2. This direct interaction was confirmed by a GST-pull down assay using recombinant GST-Artemis and MBP-DDB2 expressed and purified from *E. coli* (Fig. 2D). Taken together, these results demonstrated a direct association between Artemis and DDB2, but not with DDB1, indicating that DDB2 is the subunit that mediates the Artemis interaction with the DDB complex.

To determine the region of Artemis that mediates the interaction with DDB, I generated a number of Artemis deletion constructs, and examined their ability to interact with DDB1 and DDB2. GST-tagged truncated and full-length Artemis constructs were transfected into HEK293 cells, and GST pull-down assays were performed. As shown (Fig. 3A,B), both DDB1 and DDB2 were pulled-down with full-length Artemis consistent with the results shown above. Among the deletion mutants, only mutant B, in which amino acids 71-292 were deleted, lost its ability to interact with DDB1 or DDB2 (Fig. 3C). This region is located within the conserved SNM1 domain, suggesting that an intact conserved domain on Artemis is essential for its interaction with DDB complex.

Figure 1. Artemis interacts with DDB1 and DDB2 in vivo.

Figure 1A*. Gel stained with Coomassie blue showing proteins from a GST-Artemis pull-down experiment. GST-Artemis expressed in HEK293 cells was pulled-down by glutathione sepharose beads (left lane). A pull-down from untransfected cells was used as a control (middle lane). Labeled band indicates proteins identified by mass spectrometric analysis.

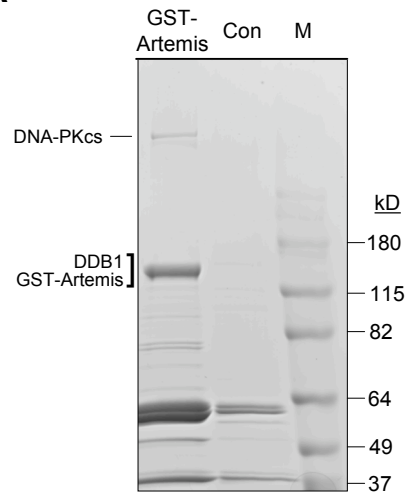
Figure 1B*. Immunoblot showing that endogenous DDB1 interacts with Artemis in vivo before and after UV or IR irradiation. HEK293 cells were treated with UV (25 J/m²), IR (10 Gy), or mock treated (UT). Cells were then harvested for co-IP analysis by Artemis antiserum two hours post-irradiation.

Figure 1C. Reciprocal co-IP experiment showing that endogenous Artemis interacts with DDB1 in vivo before and after UV damage. HEK293 cells were treated with UV (25 J/m²) or left untreated. Cells were incubated for two hours after treatment, and then harvested for analysis. IP was performed using rabbit polyclonal anti-DDB1 antibody.

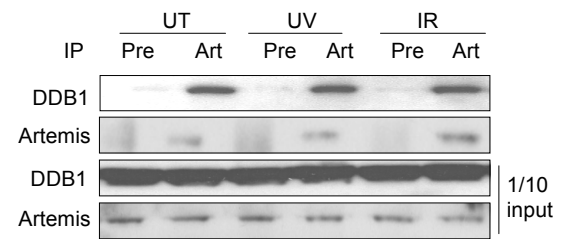
Figure 1D*. Artemis interacts with DDB1 after UV irradiation. HEK293 cells were irradiated with UV (25 J/m²). After irradiation, cells were incubated for the indicated time prior to harvesting. IP was carried out using Artemis-specific antiserum (Art) or pre-immune serum (Pre) as control.

** indicates adapted from my master thesis.*

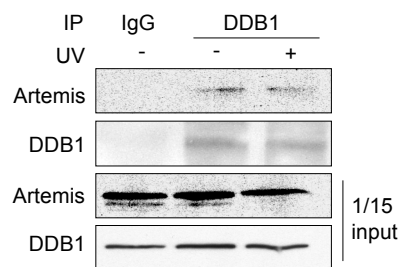
1A*



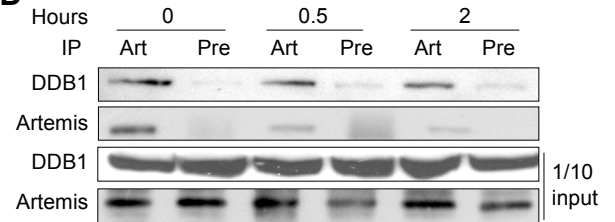
1B*



1C



1D*

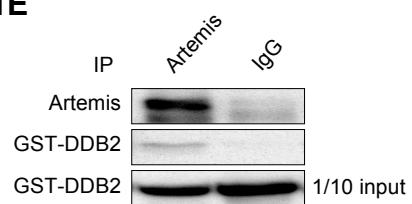


**Adapted from my master thesis*

Figure 1E. Immunoblot showing that endogenous Artemis interacts with ectopically expressed DDB2. HEK293 cells were transfected with GST-DDB2, and 48 hours later cells were harvested for co-IP experiments using Artemis antiserum.

Figure 1F*. Co-IP showing the interaction between Artemis and DDB2. GST-DDB2 and GFP-Artemis were co-transfected into HEK293 cells, and 48 hours later cells were harvested for co-IP experiments using rabbit polyclonal antibody against GFP or rabbit IgG for control.

1E



1F*

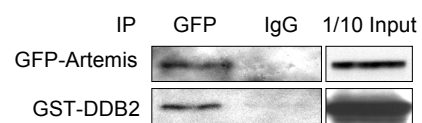


Figure 2. Artemis directly interacts with DDB2

Figure 2A*, 2B*. Artemis does not interact with DDB1 in vitro. Artemis and DDB1 labeled with ^{35}S -methionine were expressed by in vitro transcription coupled translation. Artemis and DDB1 were mixed and IPed with DDB1 antibody (Fig. 2A) or Artemis antiserum (Fig. 2B) as indicated. Bound proteins were eluted, separated by SDS-PAGE, and detected by autoradiography.

Figure 2C*. Artemis directly interacts with GST-DDB2 in vitro. Artemis and GST-DDB2 were in vitro translated and labeled with ^{35}S -methionine. Synthesized proteins were mixed and incubated as indicated. The panels show the autoradiography of labeled proteins. Immunoprecipitation was performed by Artemis serum or pre-immune serum.

Figure 2D*. Purified recombinant Artemis and DDB2 directly interact with each other. GST-Artemis, GST, and MBP-DDB2 proteins were expressed and purified from *E. coli*. Purified proteins were mixed as indicated, and pull-down assays were performed and analyzed by immunoblotting.

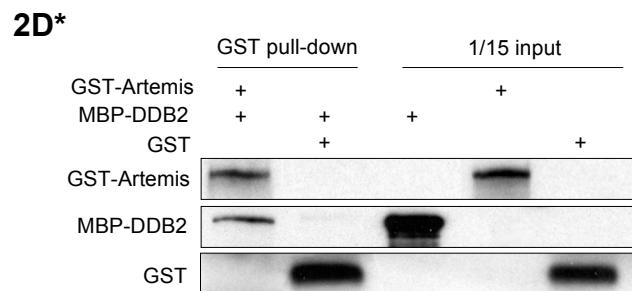
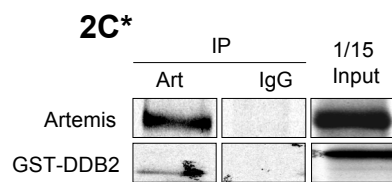
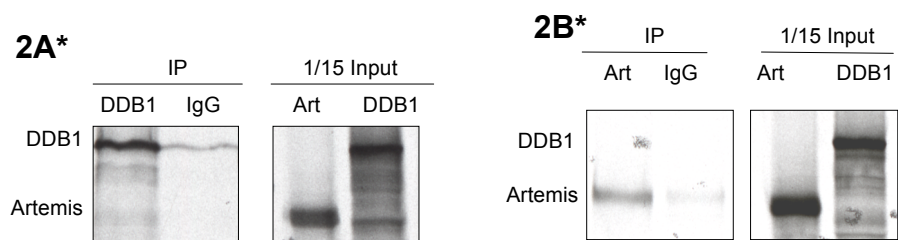


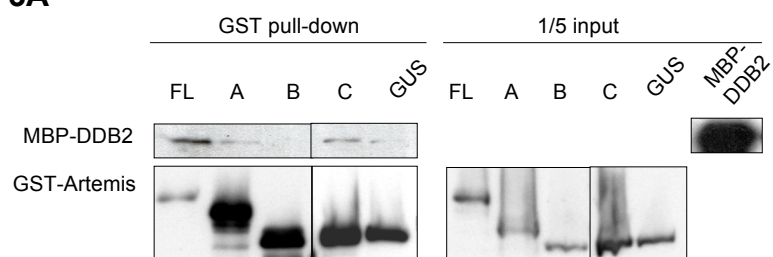
Figure 3. DDB interacts within the conserved SNM1 domain of Artemis.

Figure 3A*. Mapping of Artemis region that is required for interaction with DDB2. GST-Artemis constructs and GST-GUS (negative control) were expressed in HEK293 cells, and fusion proteins were purified using GST beads. Beads with bound proteins were incubated with bacterially expressed and purified MBP-DDB2. Beads were then washed and bound proteins were subsequently eluted and separated by SDS-PAGE for immunoblotting using GST or MBP antibody.

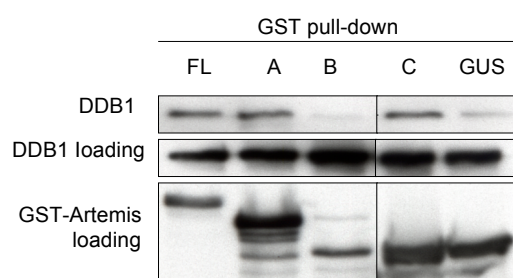
Figure 3B*. The DDB2 interaction region on Artemis is also required for its interaction with DDB1 in vivo. Indicated GST-Artemis constructs and GST-GUS were expressed in HEK293 cells, and pull-down assays were performed using GST beads. Bound proteins were analyzed by Western blot using DDB1 or GST antibody.

Figure 3C*. Schematic showing interaction of DDB with Artemis deletion mutants derived from data shown in A and B.

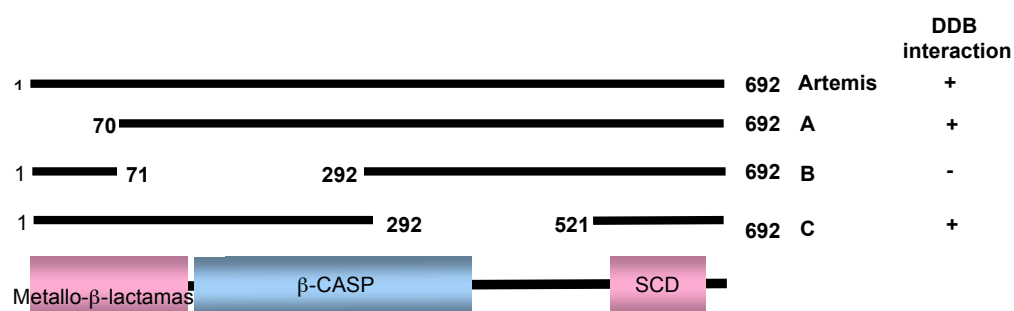
3A*



3B*



3C*



siRNA mediated down-regulation of Artemis or DDB2 stabilizes p27

DDB1 and DDB2 are known components of Cul4A-based E3 ligase complexes (84). Substrates of these complexes are involved in many important cellular pathways such as cell cycle regulation and the DNA damage response. Because Artemis interacts with both DDB1 and DDB2, I next investigated whether Artemis also plays a role in regulating the stability of Cul4A-DDB1 substrates. DDB1-Cul4A has been shown to promote the degradation of the Cdk inhibitor, p27 (114). However, the substrate receptor remained unknown and neither DDB2 nor Artemis had been implicated in this regulation. We depleted expression of Artemis or DDB2 in HeLa cells using specific siRNAs, and examined p27 protein levels by immunoblot analysis (Fig. 4A, lanes 1-4 and 8-11). Interestingly, p27 levels were significantly increased in cells with decreased Artemis or DDB2 expression compared to control cells treated with a non-specific siRNA. This increase in p27 protein levels was reduced when an Artemis construct resistant to the Artemis specific siRNA was expressed in the cells depleted of endogenous Artemis (Fig. 4A, lane 5-7). Additionally, p27 levels were also found to be lower in *Artemis*^{-/-} mouse embryonic fibroblasts (MEFs) compared to wild-type MEFs (Fig. 4B). Moreover, when Artemis or DDB2 was overexpressed in HeLa cells, the p27 level was significantly decreased (Fig. 4C).

p27 is regulated at both transcriptional and post-translational levels (143-146). Thus, to rule out the possibility that p27 accumulation in the absence of Artemis or DDB2 was a result of increased transcription, real-time PCR was performed in HeLa cells depleted of Artemis or DDB2 by siRNA transfection. As shown (Fig. 4D), p27 mRNA levels

remained stable in transfected cells with or without UV treatment, indicating that the observed accumulation of p27 is regulated at a post-transcriptional level. In addition, upon treatment with the protease inhibitor MG-132, the differential expression of p27 protein levels between control and Artemis or DDB2 overexpression was abolished (Fig. 4E). I conclude from these results that alterations in expression of Artemis or DDB2 affect the stability of p27 in the cell, and this due to the protein degradation mediated by the ubiquitin-proteasome pathway.

Artemis and DDB2 promote the ubiquitylation of p27 in vivo

So far, I have shown that Artemis and DDB interact with each other and regulate p27's stability. Next, I investigated the direct involvement of Artemis and DDB2 in p27's ubiquitylation.

As shown in Fig. 4F, overexpression of Artemis or DDB2 promotes the ubiquitylation of p27 in the presence of MG132 (compare lanes 2 and 4, and lanes 2 and 5). These findings are consistent with the observation that overexpression of Artemis or DDB2 reduces p27 protein level in vivo (Fig. 4C), and indicate that these two interacting proteins plays a significant role in p27 ubiquitylation and hence degradation in vivo.

It has been shown that p27 can be phosphorylated at different sites, and this modification is important for regulation of its stability, cellular localization and biological function. For example, as a Cdk2 kinase substrate, p27 is phosphorylated at Thr 187, which is required for SCF^{Skp2} mediated ubiquitylation. In addition, Akt can

phosphorylate p27 at Thr 157 and this phosphorylation is responsible for its cytoplasmic retention. To test if phosphorylations of these sites are required for Artemis mediated p27 degradation, wild-type, T157A or T187A mutant p27 was overexpressed in HeLa cells together with GST-Artemis or GST-GUS. Then, the Flag-p27 level was detected. As shown in Figure 4G, both wild-type and mutant p27 were degraded when GST-Artemis was co-expressed in the cell. This is also supported by the following experiment. I expressed wild-type or mutant p27 with or without overexpression of Artemis and then the in vivo ubiquitylation assay was performed. As shown in Figure 4H, Artemis can promote the ubiquitylation of both wild-type and mutant p27 (compare lane 1-3). This result suggests that Artemis can promote the degradation of p27 in the nucleus and that p27's phosphorylation by Cdk2/Cyc E is not required for this activity.

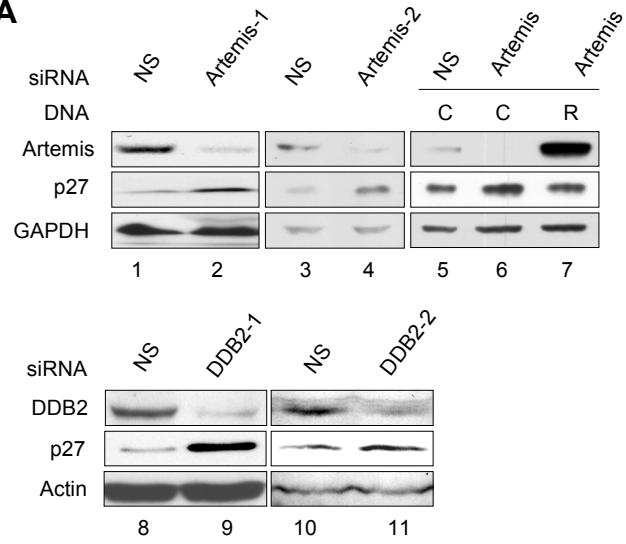
Figure 4. Artemis and DDB2 regulate p27 protein levels via ubiquitin-mediated pathway.

Figure 4A. p27 accumulates in Artemis or DDB2 depleted cells. HeLa cells were transfected with control (NS), Artemis and/or DDB2 siRNAs. Forty-eight hours after transfection, cells were harvested and cell lysates were subjected to immunoblot analysis. Artemis-1 and Artemis-2, DDB2-1 and DDB2-2 indicate distinct siRNAs. In lanes 5-7, 24 hours after siRNA transfection, cells were transfected with control plasmid DNA (C) or an Artemis construct refractory to Artemis siRNA (R). Cells were then incubated for an additional 24 hours before harvesting. GAPDH and Actin indicate loading controls.

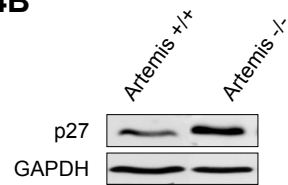
Figure 4B. p27 accumulates in *Artemis* null MEF cells. Lysates prepared from *Artemis*^{+/+} and *Artemis*^{-/-} MEF cells were subjected to immunoblot analysis for p27.

Figure 4C. Overexpression of Artemis or DDB2 reduces p27 levels. HeLa cells were transfected with GST-Artemis (left panel) or GST-DDB2 (right panel) plasmid DNAs or treated with mock transfections. Forty-eight hours after transfection, cells were harvested and cell lysates were subjected to immunoblot analysis.

4A



4B



4C

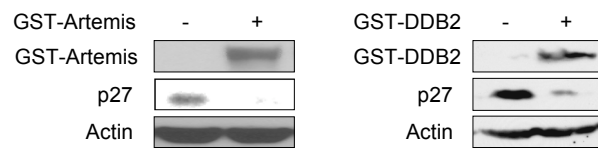
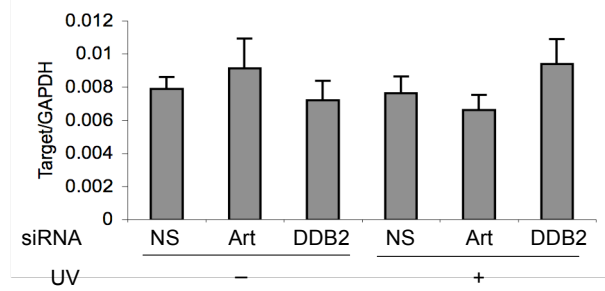


Figure 4D. p27 mRNA is stable in Artemis or DDB2 depleted cells. mRNA was isolated from HeLa cells 48 hours after transfection with control (NS), Artemis or DDB2 siRNAs. Cells were exposed to UV or mock treated and incubated for 2 hours prior to harvesting. p27 mRNA levels were determined by real-time PCR. Results were normalized using GAPDH as an internal control.

Figure 4E. Artemis and DDB2 regulate p27 protein levels through proteasome mediated degradation. HeLa cells were transfected with GST-Artemis or GST-DDB2 DNAs. After 48 hours cells were treated with 10 μ M MG-132, and 5 hours later cells were harvested and lysates subjected to immunoblot analysis.

4D



4E

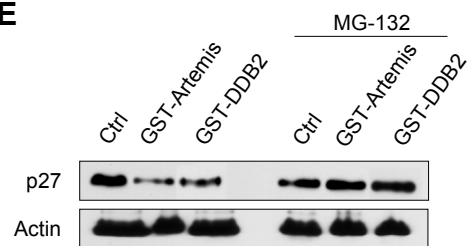
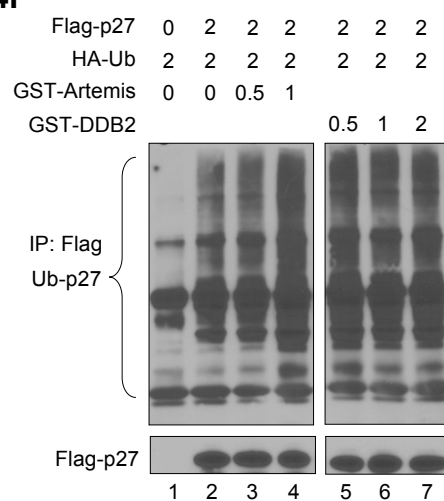


Figure 4F. Artemis and DDB2 promote ubiquitylation of p27 in vivo. HeLa cells were transfected with indicated amount of Flag-p27, HA-Ub, GST-Artemis, and GST-DDB2 DNAs. Cells were harvested 48 hours after transfection, and an in vivo ubiquitination assay was performed using anti-Flag M2 agarose as described in materials and methods. Ubiquitinated proteins were detected using an HA antibody. Flag-p27 indicates a loading control at 10% of input.

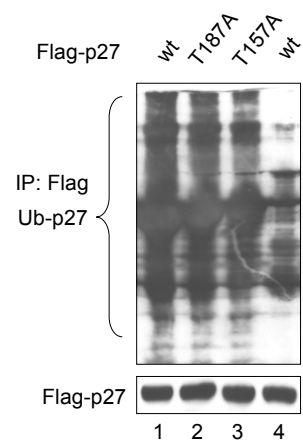
Figure 4G. Artemis promotes the degradation of p27 independent of its phosphorylation on T187 or T157. Flag-tagged wild-type, T187A, or T157A p27 were expressed in the HeLa cells together with either GST-Artemis or GST-GUS as indicated. Cells were harvested 48 hours post-transfection. The amount of p27 protein was determined by SDS-PAGE followed by immunoblot using monoclonal Flag antibody.

Figure 4H. Artemis promotes ubiquitylation of p27 independent of its phosphorylation on T187 or T157. HeLa cells were transfected with Flag-tagged wild-type (lane 1 and 4), T187A (lane 2), or T157A (lane3) p27, HA-Ub, GST-Artemis (lane1-3), and GST-GUS (lane 4). Forty-eight hours after transfection, an in vivo ubiquitylation assay was carried out as indicated in Figure 4F.

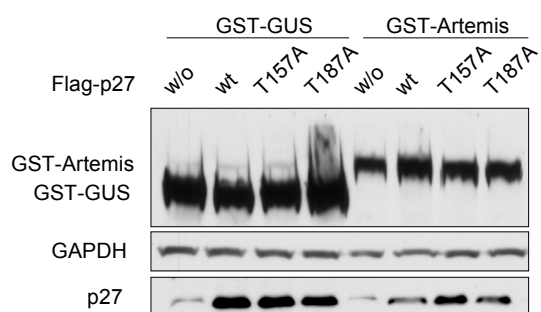
4F



4H



4G



Artemis regulates p27 protein level through Cul4A-based E3 ligase complex

Artemis interacts with DDB1 and DDB2, which are both components of the Cul4A-based E3 ligase complex. Recently, this E3 complex has been shown to target p27 for its degradation (114). Here, I showed that Artemis and DDB2 could promote p27's ubiquitylation. Based on this evidence, I hypothesized that Artemis might regulate p27's stability through a Cul4A-DDB1^{DDB2} mediated pathway. Several studies have shown that overexpression of Cul4A or DDB1 promotes p27 degradation (113, 114, 140); therefore, I examined the effect of Cul4A or DDB1 overexpression on p27 protein levels in cells treated with Artemis or DDB2 siRNAs. As shown in Fig. 5A (lanes 1-5), when cells were treated with control siRNA, transfection of Cul4A or DDB1 destabilized p27 as did transfection of GST-Artemis or GST-DDB2. However, p27 protein levels remained largely unchanged when either Artemis or DDB2 specific siRNA was used to treat these DNA transfected cells (Fig. 5A, lane 7, 8, 11, 12). In addition, GST-DDB2 did not promote p27's degradation when it was overexpressed in Artemis depleted cells, nor did GST-Artemis overexpression in the DDB2 depleted cells (Fig. 5A, lanes 9, 13), suggesting that they are involved in a common pathway. Furthermore, in a reciprocal experiment I depleted Cul4A and transfected GST-DDB2 or GST-Artemis DNA at the same time. The overexpression of either Artemis or DDB2 did not cause reduction of p27 protein levels in Cul4A depleted cells (Fig. 5B, lane 4-6)). Taken together, these results suggest that acting together, Artemis and DDB2 are both required for Cul4A-mediated p27 degradation pathway.

It has been reported that the Cul4A-DDB1 complex may utilize the F-box protein Skp2, which normally associates with SCF^{Skp1}, as a substrate receptor to target p27 for ubiquitylation (114). Therefore, I examined whether Skp2 was also required for Artemis or DDB2 promoted p27 degradation. As shown (Fig. 5B, lanes 7-9), when cells were depleted of Skp2, overexpression of Artemis or DDB2 did not destabilize p27, indicating that Skp2 may also be involved in the Artemis-DDB2 mediated p27 degradation pathway. In the cells treated with the Skp2 siRNA, we observed a more dramatic stabilization of p27. This is consistent with the proposed possibility that Skp2 can be utilized by two p27 targeting E3 ligase complexes, namely SCF^{Skp2} and Cul4A-DDB1.

The activity of Cullin-based E3 ligase activity can be regulated by a small ubiquitin like molecule, Nedd8. The attachment of Nedd8 to the Cullins leads to an increased ligase activity. Next, I examined if Artemis or DDB2 can change the neddylation level of Cul4A and hence the p27 degradation. As shown in Figure 5C, depletion of Artemis or DDB2 did not affect the level of neddylation of Cul4A (indicated by the upper band detected by Cul4A antibody). Additionally, unlike overexpression of DDB1, which can increase Cul4A neddylation (114), its level remained unchanged in cells transfected with Artemis or DDB2 constructs (data not shown), indicating that Artemis and DDB2 do not regulate Cul4A-DDB1 complex through the neddylation modification.

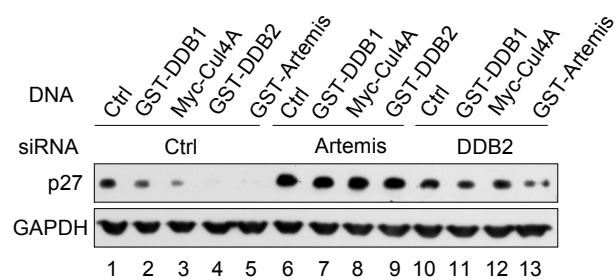
Figure 5. Artemis and DDB2 regulate p27 protein levels through a Cul4A-based E3 ligase complex.

Figure 5A. Artemis and DDB2 are required for p27 degradation by the Cul4A-based E3 ligase complex. HeLa cells were transfected with control, Artemis or DDB2 siRNAs. After 24 hours, cells were transfected with the indicated DNA, and 24 hours later cells were harvested for immunoblot analysis.

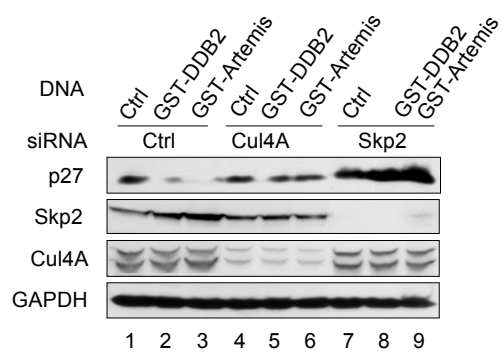
Figure 5B. Cul4A is required for Artemis or DDB2-mediated p27 degradation. HeLa cells were transfected with control, Cul4A or Skp2 siRNAs, and 24 hours later, cells were transfected with the indicated DNA. Cells were harvested after an additional 24 hours for immunoblot analysis.

Figure 5C. Cul4A neddylation is not regulated by Artemis depletion. HeLa cells were transfected with control, Artemis and Cul4A siRNA as indicated. Forty-eight hours after transfection, cells were harvested and the indicated proteins were detected by immunoblot analysis. The upper band detected in the Cul4A row is the nedd8-modified form of Cul4A.

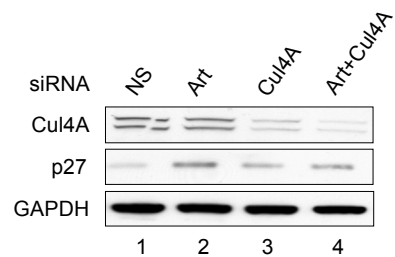
5A



5B



5C



Artemis interacts with Cul4A, p27, Skp2 and Cul1

My results demonstrate an interaction between Artemis and the DDB complex, and that Artemis is involved in the Cul4A-DDB1-mediated degradation of p27. Since DDB1 was shown to be associated with Cul4A and control the degradation of p27, I examined whether Cul4A and p27 are also Artemis interacting partners. Immunoprecipitation experiments performed in Figure 6A shows that Cul4A and p27 specific antibodies can both pull down endogenous Artemis, indicating an association between Artemis and these proteins in vivo. Next, a direct interaction between purified GST-Artemis and MBP-p27 proteins was confirmed by the in vitro GST-pull down assays (Fig. 6B). In the previous section, I have shown that Skp2 is also involved in the Artemis-mediated p27 degradation. To understand the function of Skp2 in the Artemis-dependent pathway, we examined the physical interaction between Artemis and Skp2. As shown in Figure 6C, Artemis directly associates with Skp2 in vitro. The F-box protein Skp2 acts as a receptor recruiting p27 in the SCF^{Skp2} complex, and it also has a role in Cul4A-DDB1 mediated p27 degradation. As a component of Cul4A-DDB1 complex, Artemis not only interacts with p27, but also directly interacts with Skp2 and DDB2, both of which are substrate-specific receptors for Cullin-based ligase. These results suggest that Artemis is functioning in substrate recruitment in the Cul4A-DDB1 ligase complex.

The published work by our lab has shown that Artemis can regulate the stability of Cyclin E through its interaction with Fbw7, another F-box protein utilized by SCF complex. Therefore, I next investigated the possibility that Artemis may interact with Cul1. GST-Artemis and MBP-Cul1 were expressed in *E. Coli*, and a direct interaction

was observed in the in vitro assay as shown in Figure 6B. This result indicates that the function of Artemis in the E3 ligase complex is not limited to the Cul4A-DDB1 complex, but in the Cul1-based ligase as well.

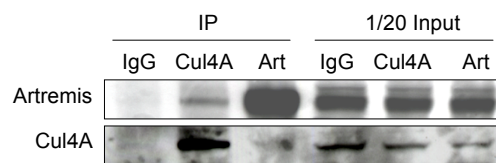
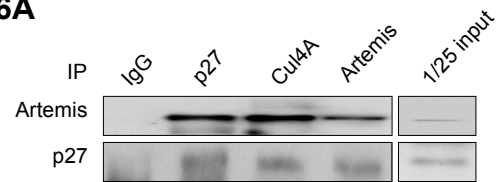
About two dozens of substrates have been identified for the Cul4A-DDB1 ligase complex (147, 148). Here I showed an involvement of Artemis in this complex to promote the ubiquitylation of p27. To determine if this role of Artemis is substrate specific, I examined the protein level of Cdt1, a known substrate of the Cul4A-DDB1^{Cdt2} complex (104), in Artemis depleted cells. It has been shown that Cdt1 can be degraded by Cul4A-DDB1^{Cdt2} both during S phase and after UV irradiation. As indicated in Figure 6D, Cdt1 levels in cells with and without UV treatment were not affected by Artemis knockdown. Moreover, no interaction between Artemis and Cdt2, the receptor protein for Cdt1 in the Cul4A-DDB1 complex, was detected. Taken together, these results suggest that Artemis is specifically involved in the degradation of certain substrates such as p27 as opposed to playing a general role in Cul4A-DDB1-mediated substrates ubiquitylation.

Figure 6. Artemis interacts with Cul4A, p27, Skp2 and Cul1.

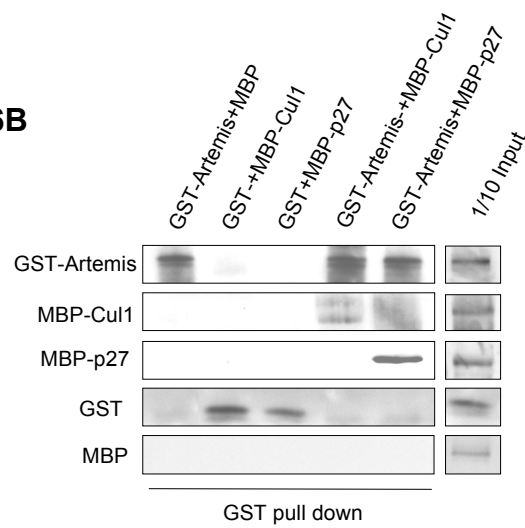
Figure 6A. Artemis interacts with Cul4A and p27 in vivo. Immunoblot analysis showing co-IP experiments performed in HEK293 cells using the indicated antibodies.

Figure 6B, C. Artemis directly interacts with p27, Skp2 and Cul1 in vitro. GST, GST-Artemis, MBP, MBP-p27, MBP-Skp2, and MCP-Cul1 were expressed in *E. Coli*. Purified proteins (as indicated) were mixed and subjected to in vitro GST pull-down assays. Anti-GST and anti-MBP antibodies were used for immunoblotting.

6A



6B



6C

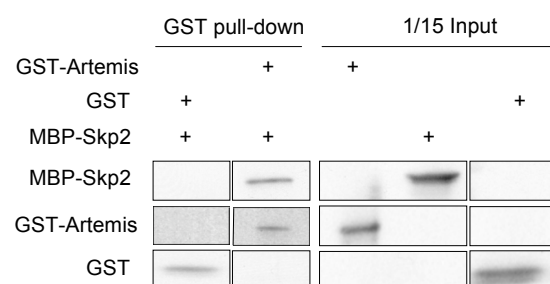
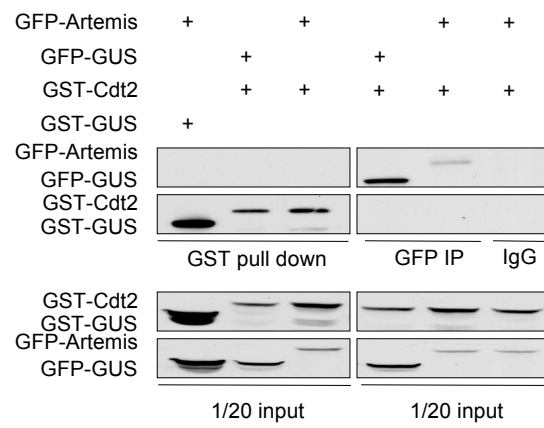
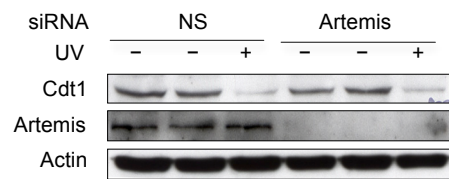


Figure 6D. Artemis is not a regulator of the Cul4A-DDB1 substrate Cdt1. Upper panel: UV-induced Cdt1 is not affected by Artemis depletion. HeLa cells were transfected with control (NS) or Artemis siRNAs, and 48 hours later cells were treated with or without 20 J/m². After a 2-hour incubation cells were harvested for immunoblot analysis. Actin is shown as a loading control. Lower panel: Artemis does not interact with Cdt2. The indicated constructs were transfected into HeLa cells, and 48 hours later cells were harvested for GST pull-down and GFP co-IP assays. Anti-GFP and anti-GST antibodies were used to detect the indicated proteins.

6D



Artemis and DDB2 regulate cell cycle progression

p27 is a well known cell cycle inhibitor. It has a crucial role in the G1-S transition by interacting with and inhibiting cyclin E-CDK2 complex. Here I showed that the stability of p27 is regulated by Artemis and DDB2 through Cul4A-DDB1 E3 ligase complex. These results led me to investigate the possibility that Artemis and DDB2 are involved in controlling cell cycle progression through p27. Htert-RPE1 cells, a telomerase-immortalized primary human cell line, were employed for cell cycle analysis. First, I depleted the expression of Artemis and/or p27 by specific siRNAs. Cells treated with p27 siRNA exhibited a higher level of BrdU incorporation, indicating a less inhibited G1/S transition, while Artemis depleted cells demonstrated a smaller S phase population. However, when Artemis and p27 expression were depleted together, these differences in S phase population were no longer detectable compared to the control group (Fig. 7A). This observation suggests that by affecting the p27 protein stability, Artemis can control G1/S transition in normally proliferating cells.

In response to the absence of mitogenic signal, p27 protein is accumulated and cells are arrested in a quiescent state. It has been well established that a down-regulation of p27 protein level is required for these cells to re-enter the cell cycle. Based on my results, I hypothesized that Artemis and DDB2 may control the cell cycle progression upon serum starvation through p27. Again, Htert-RPE1 cells were used for the serum starvation experiments. As indicated, these cells were well arrested in G0 after 0.1% serum treatment for 24 hours (Fig. 7B, lane 1 and 2 in vector transfection). Consistent with my previous observations, overexpression of Artemis or DDB2 destabilized p27,

and therefore resulted in a higher S phase population in normally growing cells (Fig. 7B, lane 1). After serum starvation for 24 hours, these transfected cells had almost 20 times more S phase population compared to the vector control group, which was reflected by the decreased amount of p27 protein (Fig 7B, lane 2).

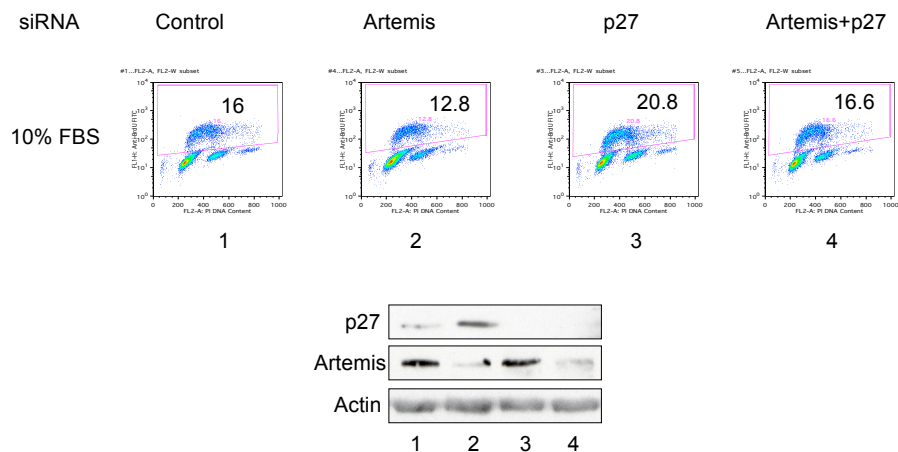
In order to examine their response to serum withdrawal, RPE cells were kept in the 0.1% serum media for more than 24 hours as a part of my preliminary experiments. An interesting observation is that when these cells were kept under low serum condition for a prolonged period, they tend to adapt and re-enter the cell cycle (indicated by an increased BrdU-incorporated population). This cell cycle reentry was paralleled with a gradually decreased p27 protein level (Fig 7B, lane 3-5). This behavior gave us another useful tool to examine my hypothesis since an up-regulated p27 level in these cells should be able to block this re-entry. I treated the RPE1 cells with non-specific, Artemis or DDB2 specific siRNAs. Next, cells were put into media containing 0.1% serum for different periods of time. Cells in the control group demonstrated the ability to re-enter into S phase after 36 hours of serum starvation, however in the Artemis or DDB2 siRNA treated cells, this reentry was abolished and cells were kept arrested in G0 phase (Fig. 7C lane 2-3). This observation appears to be correlated with an induction of p27 protein level due to the Artemis or DDB2 depletion (Fig. 7C, lower panel). Taken together, these results provide strong evidence to support the conclusion that p27 degradation promoted by Artemis and DDB2 is involved in cell cycle regulation in normally growing cells as well as in cells upon withdrawal of mitogenic signal.

Figure 7. Artemis and DDB2 regulate cell cycle progression

Figure 7A. Depletion of Artemis causes a p27 dependent G1 arrest. hTert-RPE1 (hereafter RPE1) cells were transfected with Artemis and/or p27 specific siRNA or non-specific (NS) siRNA as control. Forty-eight hours after transfection, BrdU was added to label S-phase cells. Thirty minutes after incubation with BrdU, cells were harvested and fixed. Upper panel: Cell cycle analysis was performed as stated in materials and methods. Lower panel: Samples collected in the cell cycle analysis were subjected to Western blot analysis to determine the levels of p27 and Artemis. Numbers in the panels indicate the percentage of S phase cells.

Figure 7B. Artemis and DDB2 affect the G0-G1 transition via regulation of p27. RPE1 cells were transfected with GST-Artemis or GST-DDB2 expressing constructs. Thirty hours after transfection, cells were placed into DMEM F-12 media containing 0.1% FBS. Cells were harvested after the indicated incubation time. And 30 minutes prior to harvest, BrdU was added into the media. Cell cycle profiles were collected by flow cytometry after anti-BrdU and PI labeling. Cells stained positive for BrdU are shown in the upper panel. Lower panel: Samples collected in the cell cycle analysis were subjected to Western blot analysis to determine the level of p27.

7A



7B

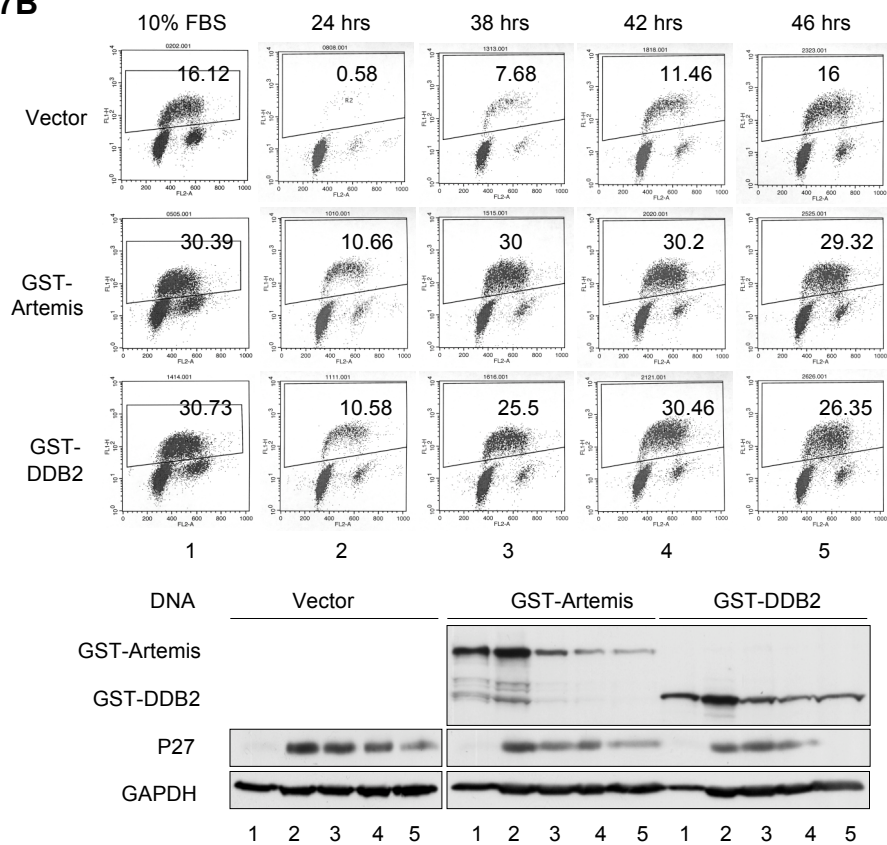
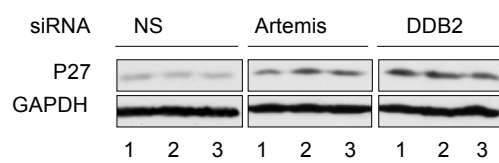
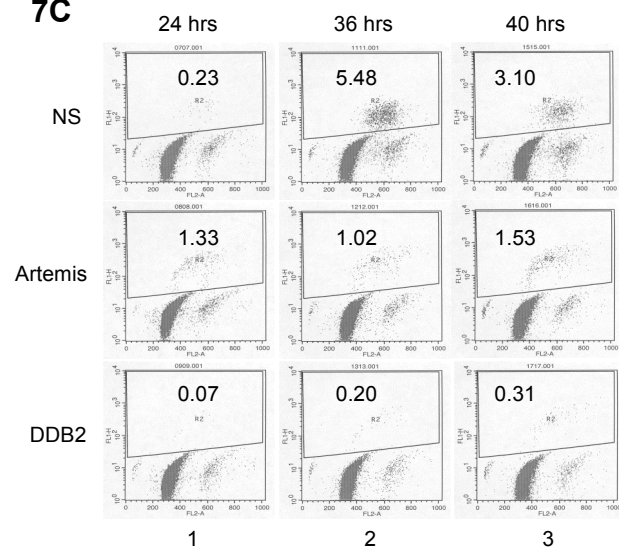


Figure 7C. Artemis and DDB2 affect the G0-G1 transition via regulation of p27.

Upper panel: RPE1 cells were transfected with Artemis or DDB2 specific siRNA, and non-specific siRNA (NS) was used as control. Twenty-four hours after transfection cells were washed with PBS and put into media containing 0.1% FBS for the indicated time. BrdU was added into the culture 30 minutes before harvest. Cell cycle analysis was performed as indicated in Fig. 7B. Lower panel: Samples collected in the cell cycle analysis were subjected to the Western blot analysis to determine the level of p27.

7C



Artemis is regulated by its phosphorylation upon serum starvation

Artemis is involved in different cellular process such as NHEJ, V(D)J recombination, and DNA damage-induced checkpoint regulation. These functions have shown to be largely regulated by its phosphorylation by kinases such as ATM, ATR and DNA-PKcs in response to different types of stress. My results here indicated that Artemis has a role in controlling p27 degradation and cell cycle progression upon serum starvation, I therefore speculated that this activity of Artemis could also be regulated by its phosphorylation.

Different types of cellular stress, such as IR, UV, and MMC, have been shown to induce phosphorylation of Artemis at various sites, however, it is still unknown if its phosphorylation can also be triggered by serum starvation. Therefore, I tested Artemis phosphorylation by gel shift mobility assay under this condition. As shown in Figure 8A, Artemis was phosphorylated after cells were treated with 0.1% serum for 24 hours as indicated by the its decreased mobility on the western blot. Pre-treatment with protein phosphatase eliminated this mobility shift. Several serine sites on Artemis have been identified to be phosphorylated, and phosphorylation specific antibodies toward some of these sites were generated by our laboratoty. These reagents allowed me to search for the site(s) that was phosphorylated in response to low serum condition. I examined phosphorylation levels on 5 serine sites (S645, S516, S518, S534, S538) on Artemis using the phospho-specific antibodies, and only phosphorylation at serine 645 was increased upon serum withdrawal (Fig. 8B). S-to-A and S-to-D mutant of S645 were generated and transfected into HeLa cells. In contrast to wild-type Artemis, an increased

protein level of p27 was observed in cells overexpressing S645D, but not other mutants of Artemis (Fig. 8C). Taken together, these results suggest that Artemis is phosphorylated after serum starvation, and this modification impairs Artemis's ability to down-regulate p27, thereby allowing p27 protein to accumulate and cells to arrest in the G0 phase. To test the latter statement, p27 protein levels were determined and cell cycle profiles were analyzed in cells expressing either wild-type, S516/645A, or S516/645D mutant. The double mutants were used here since no significant difference in cell cycle profile could be detected by using single mutants. However, previous research in our lab has shown that these two sites may be functionally related, and double mutants had an enhanced effect in phenotype under different conditions (41, 48). A decreased p27 protein level was observed in wild-type or S516/645A Artemis transfected RPE1 cells, resulting in an increased S phase population. Meanwhile, the p27 level was much higher in S516/645D Artemis-expressing cells, and a larger G1 phase population was detected compared to the wild-type and S516/645A cells (Fig. 8D). These results provide direct evidence that the phosphorylation of Artemis has an inhibitory effect on its activity to promote p27's degradation, and thus leads to a p27-induced cell cycle arrest.

Figure 8. Artemis is phosphorylated upon serum starvation.

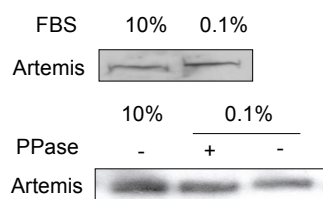
Figure 8A. Artemis is phosphorylated upon serum starvation. RPE1 cells were grown in media containing either 10% or 0.1% FBS for 24 hours prior to harvest. Upper panel: Cell lysates were subjected to SDS-PAGE followed by Western blot using affinity purified Artemis antibody. Artemis phosphorylation is indicated by a reduced mobility band detected by the antibody. Lower panel: Artemis protein was immunoprecipitated from cell lysates. Protein A-Sepharose beads with bound protein were incubated with 20 U of alkaline phosphatase at 30°C for 30 min. After washing, the proteins were eluted in SDS sample buffer and separated by SDS-PAGE for immunoblotting using purified Artemis antibody.

Figure 8B. Artemis serine 645 site is phosphorylated after serum withdrawal. RPE1 cells were transfected with GST-Artemis constructs. Twenty-four hours after transfection cells were placed into DMEM-F12 containing either 10% or 0.1% FBS and cultured for an additional 24 hours prior to harvesting in SDS sample buffer. Cell lysates were subjected to immunoblot analysis using the indicated site-specific phospho-Artemis antibodies.

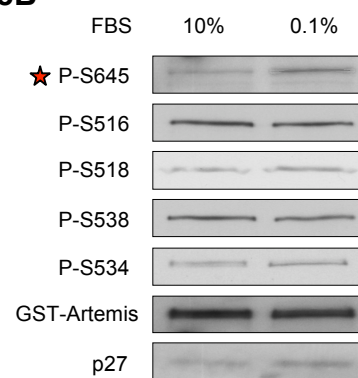
Figure 8C. Artemis phosphorylation on Ser 645 negatively regulates p27 degradation. GST-tagged wild-type Artemis construct or Artemis constructs with indicated mutations were expressed in HeLa cells. Forty-eight hours post-transfection, cells were harvested and the amount of p27 was detected by immunoblot.

Figure 8D. Artemis phosphorylation on Ser 645 affects cell cycle progression via regulation of p27. RPE1 cells were transfected with GST-tagged wild type, S516/645A or S516/645D Artemis constructs. Forty-eight hours after transfection, cells were harvested and DNA was labeled by PI staining. Upper panel: Cell cycle profile was obtained by flowcytometry analysis. Lower panel: Samples collected in the cell cycle analysis were subjected to the Western blot to determine the protein level of p27.

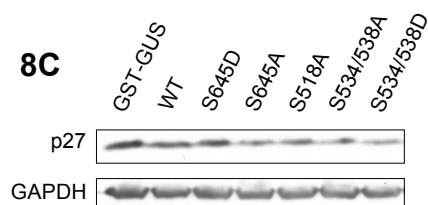
8A



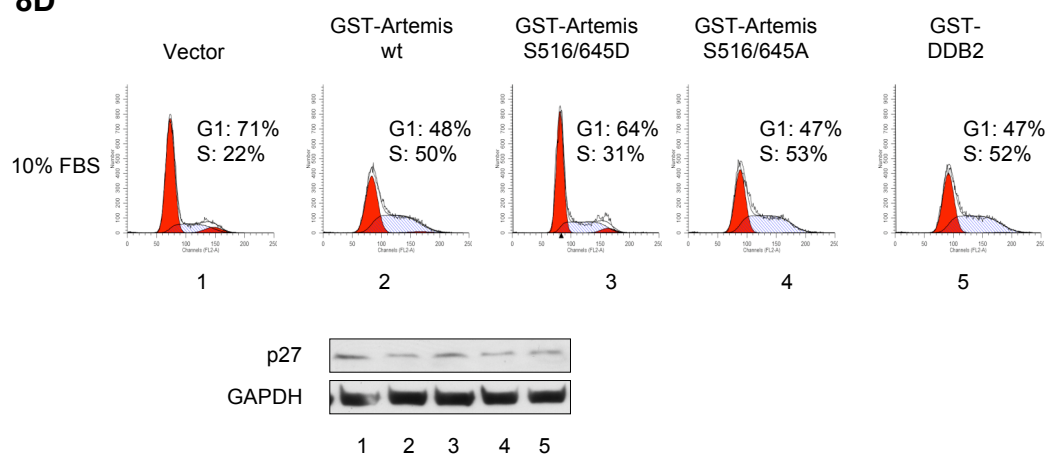
8B



8C



8D



Artemis and DDB2 regulate p27 level in response to UV-irradiation

As a negative cell cycle regulator, the role of p27 in G1/S transition and cell cycle re-entry has been well established. However, its regulation and function after DNA damage is less understood.

It has been reported that Artemis can be phosphorylated on serine 645 and 516 by ATR kinase in response to UV. In addition, my results showed that Artemis phosphorylation can regulate its function in promoting p27 degradation. Therefore, I next investigated the effect of UV-irradiation on p27 protein level. In Figure 9A, 293T cells were treated with UV-C irradiation. Soon after the irradiation (1 hour), p27 protein level was elevated, and this increase lasted for up to 4 hours post-UV. The UV-induced accumulation of p27 protein was also observed in HeLa and U2OS cells (data not shown here). In undamaged cells, ectopically expressed Artemis or DDB2 can promote p27 degradation, therefore, I examined if these proteins can also downregulate p27 level in UV-damaged cells. As shown in Figure 9B and 9C, overexpression of Artemis or DDB2 not only caused a decrease of p27 level in untreated cells but also resulted in a decreased amount of p27 upon UV irradiation. Moreover, consistent with the finding shown in Figure 8, p27 was stabilized in both treated and untreated cells when the S645/516D mutant of Artemis was overexpressed (Fig. 9C). Since these two serine sites on Artemis are phosphorylated after UV, this observation suggests decreased Artemis function in promoting p27's degradation after UV. It is well known that p27 is one of the negative regulators of the G1/S transition. The results shown here indicate that Artemis phosphorylation after UV is responsible for a decreased E3 ligase activity targeting p27,

and therefore, the stabilization of p27, which can contribute to UV-induced cell cycle arrest.

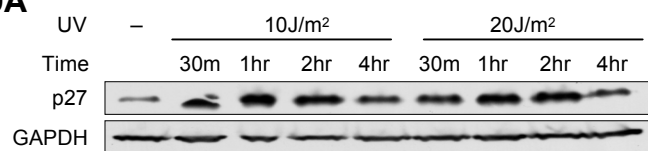
Figure 9. Artemis and DDB2 regulate p27 upon UV-irradiation.

Figure 9A. p27 protein level increases after UV exposure. 293T cells were treated with 10 J/m² or 20 J/m² UV-C. After the indicated incubation time, cells were harvested and p27 levels were detected by Western blot analysis.

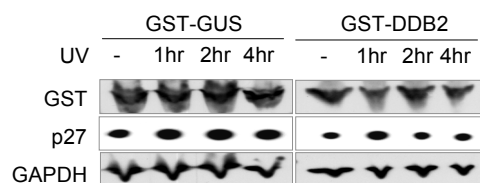
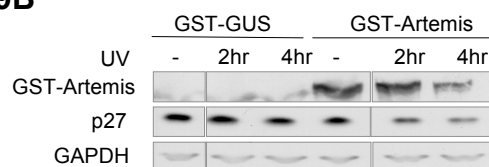
Figure 9B. Artemis and DDB2 decrease p27 protein level in UV-irradiated cells. 293T cells were transfected with GST-Artemis, GST-DDB2 or GST-GUS constructs as indicated. Forty-eight hours after transfection, cells were exposed to 10 J/m² UV radiation. After the indicated incubation time, cells were harvested and p27 levels were determined by Western blot analysis.

Figure 9C. Phosphorylation of Artemis decreases its ability to promote the degradation of p27 both before and after UV. 293T cells were transfected with wild type, S516/645A or S516/645D Artemis constructs. Forty-eight hours after transfection, cells were exposed to 10J/m² UV-C. Cells were subsequently harvested in SDS sample buffer after the indicated incubation time, and p27 levels were determined by Western blot analysis.

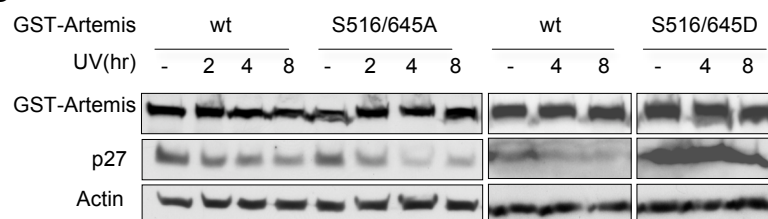
9A



9B



9C



Chapter III. Discussion

Artemis is a component of Cul4A-DDB1 complex

Artemis has previously been shown to interact with PIKKs such as DNA-PKcs, ATM, and ATR, and to participate in multiple pathways including V(D)J recombination, NHEJ, and cell cycle checkpoint responses. In this study, I have defined an entirely novel function for Artemis as a component of the Cul4A-DDB1 E3 ligase complex.

Using mass spectrometry, an Artemis interacting-protein, DDB1 was identified. Later, I confirmed this interaction in vivo. However, Artemis does not interact with DDB1 directly, but rather with DDB2. This observation indicates that the interaction between Artemis and the DDB heterodimer is mediated by DDB2.

The DDB complex was first identified as damaged DNA binding proteins with crucial functions in GGR-NER of UV-induced damage. However, UV or IR does not affect the interaction between Artemis and DDB1. This observation is interesting for few reasons. First, because Artemis has been shown to be extensively phosphorylated at various sites after UV, my result indicates these phosphorylations are not essential for this interaction, meaning that the Cul4A-DDB1-DDB2-Artemis complex assembly does not require Artemis phosphorylation. Secondly, DDB2 protein is rapidly degraded after UV; meanwhile, the interaction between Artemis and DDB1 remains unchanged. Moreover, this unchanged interaction was also previously observed when DDB2 expression is depleted using specific siRNA (142). This raises the possibility that DDB2 is not the only factor mediating this interaction. One possible candidate is CSA. CSA is a repair protein involved in TCR-NER pathway of UV-induced lesion. Similar to DDB2, it directly associates with and acts as a substrate receptor in the Cul4A-DDB1 ligase. In

contrast to DDB2, CSA does not undergo degradation after UV. A direct interaction between Artemis and CSA was reported previously in my master thesis. In addition to CSA, another Cul4A-DDB1 interacting protein Skp2 has been shown to interact with Artemis as well in this study. These results indicate that besides DDB2, both CSA and Skp2 can bridge the interaction between Artemis and DDB1, and therefore, provide a possible explanation for the unchanged interaction between Artemis and DDB1 after UV, when DDB2 is degraded. Thirdly, DDB1 and Cul4A localize in both the cytoplasm and the nucleus, and their association is unchanged regardless of DDB2 level (115). The nuclear localization of both DDB1 and Cul4A depends on, but not solely, DDB2 (69, 115, 149). Upon UV irradiation, they translocate into the nucleus and bind tightly to the damaged sites on chromatin (84). On the other hand, I previously observed that Artemis localizes in both cytoplasm and nucleus, which is independent of UV damage. Since an interaction between Artemis and DDB1 unaffected by UV is detected here, a further investigation of the sub-cellular localization of this interaction before and after UV will be necessary to explain this observation. It is possible that Artemis interacts with both cytoplasmic and nuclear Cul4A-DDB1, in order to keep a stable association regardless of UV. It is also a possibility that Artemis interacts with several different DDB1-containing complexes, and these associations can be regulated differently by UV treatment, which leads to an unchanged overall interaction with DDB1. As mentioned above, DDB2 is required for the chromatin binding of DDB1 and Cul4A upon UV, it will be interesting to examine if Artemis can also associate with these lesions as part of the complex in a DDB2 dependent manner. Moreover, since it has been reported that the nuclear localization of DDB1 in undamaged cells is dependent on DDB2 and Cul4B

(84), I will like to investigate if these proteins can affect the Artemis subcellular localization as well. The results of these future studies will provide important information to further understand the importance and function of the interaction between Artemis and DDB1. Since Artemis not only physically interacts with the DDB1 complex, but also regulates its associated E3 ligase activity, further localization studies of Artemis can also be helpful in understanding its role in protein ubiquitylation pathways and identification of more substrates.

Artemis was first identified as a putative endonuclease that in association with DNA-PKcs cleaves DNA hairpin structures, a required step during the V(D)J recombination. As a member of the SNM1 family, Artemis contains a conserved SNM1 domain, which includes a metallo- β -lactamase fold and an appended β -CASP motif. The SNM1 domain is thought to be essential for the nuclease activity of Artemis in association with DNA-PKcs. However, in this study, the SNM1 conserved domain on Artemis is demonstrated to serve as an interface to mediate the interaction with DDB. This finding is consistent with our recently published results that hSnm1B/Apollo, another member of SNM1 gene family, interacts with the endonuclease Mus81-Eme1, repair protein Mre11-Rad50-Nbs1 complex and microtubule binding protein Astrin via the SNM1 domain (150, 151). Therefore, in addition to a hydrolase activity, the SNM1 conserved domain is also essential for the ability of its family members to interact with different proteins, and therefore, participate in various cellular pathways.

Besides DDB2, Artemis can also directly interact with p27, whose stability is regulated by Artemis. A deletion mapping analysis on Artemis will be performed in order to determine the required domain/sequence for this interaction. Even though the

phosphorylation of Artemis has no effect on its interaction with the DDB complex, this modification regulates its ability to promote the degradation of p27. Based on this evidence, I speculate that the p27-interacting domain on Artemis is likely to be located on C-terminal half that contains multiple (S/T)Q sites phosphorylated in response to various types of stress, and that these phosphorylations can weaken its ability to interact with p27. If the results of the GST pull-down assays using Artemis deletion constructs and phospho-serine mutants support this speculation, then the interaction between Artemis and the Cul4A-DDB1 complex can be described as follows. The N-terminal conserved domain of Artemis is mediating its interaction with DDB2, hence the interaction with the Cul4A-DDB1^{DDB2}, meanwhile, the C-terminal phospho-sites are required for its interaction with the substrate(s), which may be negatively inhibited by Artemis phosphorylation. Although this model needs to be further investigated, my results here strongly support a potential substrate-recruiting function of Artemis in the Cul4A-DDB1^{DDB2} complex.

Like the SCF complex, the Cul4A-DDB1 E3 ligase also utilizes multiple substrate-specific receptors to recruit its substrates. These receptors are DDB1 interacting proteins (known as DCAF) that bridge the association between DDB1 and substrates. More than 50 different DCAFs have been identified, and majority of them are WD 40 proteins containing a conserved WDXR motif. Although Artemis interacts with Cul4A-DDB1 and controls its activity toward p27, it is unlikely that Artemis has a function parallel to DCAFs for the following reasons. First of all, Artemis does not directly interact with DDB1, which is proved to be the case for all the known DCAFs. In addition, a motif search of Artemis did not reveal any WD40 repeats or WDXR motifs that are found in

the DCAFs. Moreover, in my study Artemis is found to interact with two of the DCAFs, DDB2 and CSA, as well as the substrate p27; and both Artemis and DDB2 are required for p27 degradation. Taken together, this evidence suggests that Artemis is more likely acting as an additional factor for p27 degradation by Cul4A-DDB1^{DDB2}. Actually, several published results support the concept that additional factor is needed for the recruitment of substrates by CRL. For example, it has been established that SCF^{Skp2} requires the presence of Cks1 to target the ubiquitylation of p27(152, 153). Cks1 interacts with the T187 phosphorylated p27, which is essential for the F-box protein Skp2 to interact with p27. Another example of such a factor has been reported for Cul4A-DDB1^{Cdt2} complex, a ligase responsible for the degradation of both Cdt1 and p21. In both cases, direct interactions between the substrates and PCNA are needed for the Cul4A-DDB1^{Cdt2} to interact and ubiquitylate p21 and Cdt1 (102, 108). In line with these results is my observation that Artemis interacts with both DDB2 and p27, which leads me to the speculation that Artemis is functioning in the Cul4A-DDB1^{DDB2} like Cks1 and PCNA in SCF^{Skp2} and Cul4A-DDB1^{Cdt2}, respectively. Nevertheless, in order to confirm this hypothesis an Artemis-dependent interaction between DDB2 and p27 needs to be examined.

Multiple phosphorylation sites on Artemis have been identified, however, the role of these modification in Artemis function remains largely undefined. Although earlier studies suggest that phosphorylation of Artemis, especially by DNA-PKcs, is important for its endonuclease activity and V(D)J recombination (154-156), Artemis mutants defective in DNA-PKcs interaction are capable of rescuing V(D)J defect in Artemis deficient cells (38). Moreover, reports from our laboratory and several other groups

showed that mutations at multiple phosphorylation sites of Artemis do not affect its endonuclease activity and ability to rescue V(D)J recombination; instead, they cause defects in the recovery from damage-induced cell cycle checkpoints (38, 41-43, 48, 157). These observations imply that the phosphorylation of Artemis does not play a major role, if any, in regulating its nuclease function, but rather in other cellular pathways. My results here show that its phosphorylation can affect the stability of p27 protein, a substrate of Cul4A-DDB1^{DDB2}, suggesting a possible function of Artemis phosphorylation in controlling E3 ligase activity. This notion is also supported by a recent finding from our laboratory that the phosphorylation of Artemis regulates S phase checkpoint recovery by promoting the degradation of cyclin E (48). As mentioned above, it is possible that Artemis phosphorylation modulates its interaction with different substrates, or it may be a possibility that this modification can regulate the ligase activity without affecting its ability to interact with the substrates. The mechanism of how the phosphorylation regulates Artemis function in protein's ubiquitylation is currently under investigation in our laboratory.

Artemis and DDB2 regulate the degradation of p27 through the Cul4A-DDB1 complex

As a CDK inhibitor, p27's protein level, localization and activity are mainly regulated through post-translational modification. SCF^{Skp2} and KPC1/2 are two well-established E3 ligases promoting the degradation of p27. These two complexes are

distinct, with SCF^{Skp2} functioning in the nucleus and targeting p27 phosphorylated by Cdk2/Cyclin E at Thr187 during S/G2 phase, while the cytoplasmic KPC1/2 ubiquitylates p27 that is exported from the nucleus upon serine 10 phosphorylation during the early G1 phase. Recent studies indicate a possible involvement of Cul4A-DDB1 in p27 degradation. My results presented here not only confirm these previous findings, but also reveal that both Artemis and DDB2 are part of the complex, and that they are required for p27 degradation via the Cul4A-DDB1 complex. These observations provide direct evidence to support the hypothesis that in complex with Artemis Cul4A-DDB1^{DDB2} (Cul4A-DDB1^{DDB2-Artemis}) can function as the third E3 ligase responsible for p27 ubiquitylation. As shown in my in vivo ubiquitylation assay, overexpression of Artemis or DDB2 can promote the ubiquitylation of p27. Interestingly, Thr to Ala mutations of p27 on Thr187 and Thr157 do not affect the level of ubiquitylation compared to wild-type p27. Several conclusions can be drawn from this result. i) Thr187 on p27 can be phosphorylated by Cdk2/Cyc E, and this modification is a pre-requirement for its degradation by SCF^{Skp2}. However, this is not true for Artemis promoted p27 ubiquitylation, implying that an Artemis-containing Cul4A-DDB1 complex can target p27 for degradation during early G1 phase even when Cdk2/Cyclin E is not yet activated. ii) Thr157 is located within the NLS (nuclear localization signal) of p27, and phosphorylation of Thr157 by AKT results in cytoplasmic sequestration of p27. Artemis can effectively promote the ubiquitylation of nuclear localized T157A mutant of p27, suggesting that Cul4A-DDB1^{DDB2-Artemis} has activity toward nuclear p27. In contrast to KPC1/2, which ubiquitylates cytoplasmic p27 during G1 phase, Cul4A-DDB1^{DDB2-Artemis} can target p27 in the nucleus during this same phase of cell cycle. Furthermore, the

protein level of DDB2 is cell cycle regulated, and is high during early G1 to the G1/S boundary (74). This finding also supports a DDB2-required ligase activity toward p27 during G1 phase.

Why do cells need a third ligase to degrade p27? As a member of Cip/Kip family member, p27 can bind and inhibit Cdk2. p27 level is high in G0 phase, which is concomitant with low Cdk activity. p27 protein level decreases through the ubiquitin-proteasome pathway when cells re-enter the cell cycle and progress to S phase, thereby allowing the activation of Cdk kinase. Cdk2 can phosphorylate p27 at Thr 187, which is then recognized and ubiquitylated by SCF^{Skp2}. Nevertheless, p27 inhibits the activity of the very kinase that activates it, and p27 degradation precedes the activation of Cdk2. Moreover, Skp2 is not expressed until late G1 phase, and Skp2^{-/-} cells exhibit normal G0-G1 progression and p27 degradation (139, 158). All this evidence indicates that a SCF^{Skp2}-independent p27 degradation pathway(s) is employed by cells, and this speculation led to the later discovery of the KPC1/2 complex (139). Upon cell cycle re-entry, p27 can be phosphorylated on serine 10 by KIS kinase (131). This phosphorylation signals the nuclear export of p27, and then cytoplasmic p27 is ubiquitylated and degraded by KPC1/2. The subcellular redistribution and the subsequent degradation decrease p27 level in the nucleus, thereby relieving the inhibitory effect on the Cdk2 to permit its activation, which facilitates SCF^{Skp2} mediated p27 degradation. However, only a fraction of p27 is phosphorylated on serine10 and translocated into the cytoplasm in the early G1 phase. In addition, nuclear retained p27 can still be degraded independent of serine 10 phosphorylation (158). Therefore, the existence of another E3 ligase in the nucleus to downregulate p27 level is not unlikely.

This new pathway may have an additive yet not redundant function compared to KPC1/2, and thus keep p27 levels low enough in the nucleus for the activation of Cdk2 and the subsequent degradation by SCF^{Skp2}. My research here provides important information to support that Cul4A-DDB1^{DDB2-Artemis} is one of the candidates functioning to fulfill this role. It works as a Thr187-independent pathway in the nucleus to decrease p27 protein level, thereby triggering subsequent biological events and promoting cell cycle progression.

It has been reported that Cul4A-DDB1^{Cdt2} is responsible for the degradation of Cdt1 and p21 during S phase and after UV irradiation. Meanwhile, these proteins also undergo SCF^{Skp2}-mediated degradation in undamaged cells, suggesting overlapped but not totally redundant functions between SCF^{Skp2} and Cul4A-DDB1. This statement is further supported by the results shown in this work. I found that in addition to SCF^{Skp2}, p27 can also be degraded by Cul4A-DDB1^{DDB2-Artemis}, and this pathway appears to function in early G1 phase in the nucleus as well as after UV irradiation.

However, there are still several questions that remain to be examined. The stability of p27 is mainly regulated by ubiquitin-mediated degradation, and its phosphorylations play an important role in this pathway. Here, I revealed that such modifications of Thr157 and Thr 187 are not required for its degradation by Cul4A-DDB1^{DDB2-Artemis}. However, there are multiple other sites on p27 that can be phosphorylated by various kinases, some of which control subcellular redistribution (e.g. Thr 198, Ser 10), while others are of undetermined function (e.g. Try 88). Therefore, additional studies will be needed to determine if any of these sites are required for Artemis-promoted p27 proteolysis. One of the sites that will be included in our future study is Y88. This site can

be phosphorylated by nonreceptor tyrosine kinase Lyn and Abl rapidly after mitogen stimulation, and this modification can decrease the protein stability of p27 and increase its phosphorylation on Thr187 (159, 160). Although degradation by SCF^{Skp2} due to increased T187 phosphorylation is thought to be responsible for the decreased protein stability in this case, as I mentioned above, SCF^{Skp2} works at a later cell cycle stage, indicating the possibility that another ligase (maybe Cul4A-DDB1^{DDB2-Artemis}) has a function in this tyrosine phosphorylation-regulated pathway. p27 can bind with Cdk2/Cyclin E to form an inhibitory complex during G0/G1. It will be interesting to understand how Cul4A-DDB1^{DDB2-Artemis} physically targets p27 in this inhibitory complex. There are several possibilities. For example, the interaction between p27 and Artemis can compete with its association with Cdk2; or modifications such as phosphorylation of p27 results in a conformational change, making it a better substrate for the Cul4A-DDB1 ligase. Further structural and phosphorylation sites studies will provide information to define the exact mechanism of how Cul4A-DDB1^{DDB2-Artemis} targets p27 for degradation.

Artemis plays a role in cell cycle regulation through p27

p27 degradation is essential for mitogen-induced cell cycle re-entry and G1/S transition. The findings that Artemis and DDB2 regulate p27 stability led us to examine the function of these two proteins in controlling cell cycle progression.

G0 phase is characterized by a higher p27 level and a lower Cdk activity. RPE1 cells were employed in my cell cycle studies. This is an untransformed diploid and nontumorigenic primary human epithelial cell line, and as shown in my results were well arrested at G0 phase within 24 hour upon serum withdrawal. However, an impaired arrest was observed in cells with overexpression of Artemis and DDB2, consistent with the concomitant lower p27 levels. Although Artemis has been shown to control p53 stability (49), p27 is the major effector for G0 arrest upon serum withdrawal (124). Thus, based on these results we speculated that the Artemis-mediated p27 degradation pathway plays a role in cellular responses upon serum starvation. p27 is stabilized during G0 phase, and its stabilization is controlled by its phosphorylation on serine 10 by the Mirk kinase involved in the MAP kinase pathway (161). In contrast to the serine 10 phosphorylation by KIS that targets p27 for nuclear export during the G1 phase, this phosphorylation in G0 phase is responsible for its stabilization in the nucleus. Interestingly, Artemis is also phosphorylated at the same time. Moreover, Artemis phosphorylation leads to an increased p27 protein level as well. These observations help us to further understand the mechanism of p27 stabilization in the G0 phase. Cul4A-DDB1^{DDB2-Artemis} can target p27 for degradation in normally progressing cells. Upon serum withdrawal, a series of signal transduction events trigger the phosphorylation of Artemis on serine 645 site. This modification has an inhibitory effect on Artemis's ability to promote the degradation of p27 by Cul4A-DDB1^{DDB2-Artemis}, and as a result, p27 protein levels increase. In addition, p27 is also phosphorylated at its serine 10 site, which further stabilizes its protein level. Hyperphosphorylation on serine 645 on Artemis has been reported after UV or IR treatment by ATR and ATM, respectively, yet

this is the first report showing that this phosphorylation can also be induced by serum withdrawal. The kinase responsible for this modification needs to be defined in the future. Nevertheless, the Mirk kinase will make a good candidate to be tested, since it can also phosphorylate p27 on serine 10. And this would be a more efficient mechanism for cells to regulate two events that have the same consequence under the same condition (serum starvation) by one kinase. We are currently planning on testing this hypothesis by checking Artemis phosphorylation and its ability to downregulate p27 by using specific inhibitors of kinases involved in the Mirk pathway. Another possibility we would like to investigate is whether the phosphorylation on p27 serine 10 can affect its degradation by Cul4A-DDB1^{DDB2-Artemis}. The reason for this hypothesis is that p27 phosphorylated at serine 10 is not only stabilized during G0 phase, but is also stabilized during G1 phase in the nucleus (132).

Upon mitogen stimulation, p27 protein level is downregulated and therefore cells are able to re-enter the cell cycle. Although RPE1 cells can be well arrested after 24-hours of serum starvation, these cells tend to re-progress into the next cell cycle after prolonged time. Although we do not understand how these cells become adapted to the low serum stress, this phenotype gave us a tool to examine the effect of Artemis or DDB2-mediated p27 degradation on cell cycle reentry, an event associated with a decreased p27 level. Without Artemis or DDB2 an impaired cell cycle re-entry was observed, indicating that the degradation of p27 mediated by Cul4A-DDB1^{DDB2-Artemis} is required for this progression. Taken together with the phosphorylation of Artemis triggered by low serum, I hypothesized that a kinase activity specific to serum withdrawal phosphorylates Artemis, which leads to a decreased Cul4A-DDB1^{DDB2-Artemis}

ligase activity and a stabilized p27 level. However, after mitogen stimulation or in normally growing cells, Artemis is not phosphorylated or its phosphorylation pattern changes, allowing a re-activation of Cul4A-DDB1^{DDB2-Artemis} complex to maintain a low p27 protein level that is essential for the cell cycle re-entry and progression. In order to confirm this hypothesis, the phosphorylation and dephosphorylation kinetics of Artemis in response to serum withdrawal and mitogen stimulation will be examined. Artemis may be dephosphorylated when the signal triggered by the growth factor depletion is removed, however, as I mentioned above, de-phosphorylation is not the only possibility, Artemis may also be phosphorylated at different sites in response to mitogen stimulation, which in turn upregulate the ligase activity. Phospho-specific Artemis antibodies on multiple sites have been developed by our laboratory, I will use these reagents to investigate the cell cycle specific phosphorylation pattern of Artemis at multiple sites. If the any of these phosphorylations are found to be cell cycle regulated or mitogen dependent, then mutations at these sites will be generated. These Artemis mutants will be further tested for their abilities to promote p27 degradation. Because mitogen exposure can activate various kinases to phosphorylate p27, I will exam whether newly identified phosphorylation sites on Artemis are substrates for these kinases.

A decreased p27 is essential for the activation of Cdk2/Cyclin E complex, and therefore the G1/S transition. Depletion of Artemis expression in normally growing RPE1 cells resulted in a G1 arrest, indicating its involvement in this transition. Although Artemis also has a role in regulating p53 stability, this G1 arrest has also been observed in p53 null cells previously by our laboratory (data not shown). In addition, when p27 is depleted together with Artemis, similar cell cycle profile is detected compared to normal

cells, suggesting that the observed G1 arrest in Artemis depleted cells was due to the resultant increased p27 levels in these cells. These findings not only confirm the function of Cul4A-DDB1^{DDB2-Artemis} in promoting p27 degradation that is essential for cell cycle progression in normally proliferating cells, but also provide information to link this ligase activity toward p27 to the previously reported function of DDB in controlling the G1/S transition. In addition to participating in the NER pathway for UV-induced DNA damage, a transactivation regulatory activity of DDB has also been reported (162). DDB2 can bind to transcription factor E2F1, which induces the expression of genes such as cyclin E that are responsible for progression into S phase. In association with DDB1, DDB2 can stimulate the E2F1-dependent transcription, therefore facilitating the G1/S transition (63, 74). My results here provide an additional explanation to this previous observation. p27 can inhibit the E2F1 transactivation activity by inhibiting Cdk2 activity and increasing the inhibitory E2F1-p130 complex, thereby negatively regulating the G1/S transition. In complex with Cul4A and Roc1, DDB1 and DDB2 form an active ligase complex that utilizes Artemis as a substrate-specific receptor to target p27 for degradation, which results in increased Cdk2 and E2F1 activity. Furthermore, the DDB heterodimer can directly upregulate the E2F1-dependent transcription of genes required for S phase entry. These two pathways positively enhance each other, and together with the Skp2-dependent p27 proteolysis, facilitate the G1/S transition.

Artemis regulates the stability of p27 in response to UV-irradiation

The roles of p27 in controlling G0-G1 and G1-S transitions have been extensively studied. However, as a Cdk inhibitor, p27's function in cellular response to DNA damage is largely undefined. Several reports have shown a different response in p27 protein level upon UV-irradiation varying from increased to unchanged to decreased protein levels, depending on the cell type, UV wave length, and UV dose used in the individual study (67, 163-167). In my study here, an increased p27 protein level has been detected in 3 different cell lines after UVC treatment. Although these results are still preliminary, they provide important information for us to understand the cell cycle regulation after UV-induced damage.

After IR irradiation, an upregulated p53 level and the subsequent increased p21 level is involved in cell cycle arrest. Nevertheless, accumulated evidence suggests that p21 is degraded by a PCNA-dependent Cul4A-DDB1^{Cdt2} mediated pathway rapidly after low dose UV irradiation. This degradation is thought to facilitate repair of the UV-induced DNA damage, since p21 binds to PCNA and inhibits its activity in gap-filling DNA synthesis. Nevertheless, cell cycle progression is arrested upon UV treatment to allow the efficient repair of DNA damage even though p21 has undergone proteolysis. Based on the findings in this study, I speculate that the regulation of p27 stability by Cul4A-DDB1^{DDB2-Artemis} is involved the control of cell cycle arrest after UV. Upon UV treatment, Artemis is phosphorylated at multiple sites, including serine 645, by the ATR kinase. The phosphorylation on serine 645 of Artemis has a negative effect on its activity to promote p27 degradation, which in turn stabilizes p27 in response to UV damage. Moreover, Cul4A-DDB1^{DDB2} can also target DDB2 for degradation after UV, which will further arrest the cell cycle progression by two means. First, DDB2 is required for the

Cul4A-DDB1 mediated p27 ubiquitylation, therefore, the removal of DDB2 from the nucleus after damage will stabilize p27 in order to inhibit Cdk1/2 activity. Secondly, the degradation of DDB2 will prevent its enhancement of E2F1 transcriptional activity, which can also contribute to the cell cycle arrest. The mechanism of how p27 stability regulated by Cul4A-DDB1^{DDB2-Artemis} controls the UV-induced cellular response is currently under investigation in our laboratory.

Artemis as a component of Cullin-based E3 ligases

As discussed above, I have found that Artemis is a component of the Cul4A-DDB1^{DDB2} ubiquitin ligase complex and regulates the degradation of p27. In addition, my results also demonstrate that Artemis can directly interact with Skp2 and Cul1, which are components of the Cul1-based SCF ligase complex. Although it was suggested that Skp2 could be utilized by Cul4A-DDB1 complex for p27 ubiquitylation, no association between Cul1 and DDB1 was reported, therefore, the association with Cul1 more likely indicates Artemis's involvement in the Cul1-based complex. A recent report from our laboratory also showed a direct interaction between Artemis and Fbw7, another substrate recruiting F-box protein for the SCF complex (48). Taken together, this evidence suggests that Artemis has a generalized function involved in Cullin-based ligase activity regulation that is not limited to Cul4A-DDB1. This role of Artemis may be one of its essential functions, and may explain the multiple pathways in which this

protein appears to participate. One of our current research focuses is to further explore the mechanism of how Artemis functions in Cullin-based E3 ligases.

As mentioned above, Artemis directly interacts with 2 DCAFs proteins, DDB2 and CSA, and 2 F-box proteins, Skp2 and Fbw7. The substrates they target include Cdt1, DDB2, CSB, XPC, H2A, H3, H4, p21, c-myc, and many others. Although I showed that Artemis does not affect the degradation of Cdt1 after UV, its ability to regulate the stabilities of the other substrates is worth investigating. In addition, I will also evaluate the interaction between Artemis and other DCAFs or F-box proteins. DDB1 is known to interact with both Cul4A and Cul4B. While the Cul4B-DDB1 complex is less understood, it appears to have some overlapped yet not redundant functions compared to Cul4A-DDB1 (115, 168). It will be interesting to determine if Artemis has any involvement in the Cul4B-DDB1 ligase complex. These results will lead us to the identification of additional substrates that are regulated by Artemis, and provide us information to further understand its function in the Cullin-based ligase.

Significance

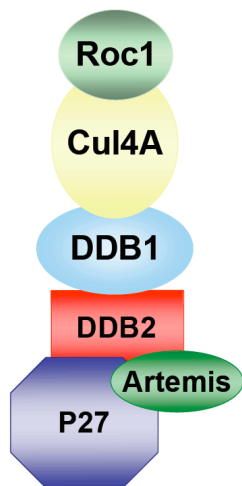
Artemis was first identified as the causative gene for a subset of SCID patients with defective V(D)J recombination. Earlier studies have shown that its major function is to act a nuclease in V(D)J recombination and NHEJ repair. Recent findings from our laboratory have demonstrated new functions for Artemis that is beyond a nuclease, including controlling DNA damage checkpoint responses, and regulating cellular

response to oxidative stress through p53. My research here defines a novel function of Artemis as a component of Cul4A-DDB1^{DDB2} to regulate p27 stability. It is also the first report showing the involvement of DDB2 as the linker in Cul4-DDB1 mediated p27 degradation. In addition, a role for Artemis in cell cycle regulation in normally proliferating cells and serum-starved cells is also demonstrated. These observations suggest that by regulating Cullin-based ubiquitin ligase activities, and thereby affecting the stabilities of various substrates, is one of the common mechanisms by which Artemis participates in various biological pathways.

The negative cell cycle regulator p27 is an atypical tumor suppressor that regulates cell proliferation, apoptosis and cell motility. It has been reported that inactivation of Skp2 in mice, a negative regulator of p27, can suppress tumorigenesis by inducing senescence specifically in cancer cells but not normal cells (169). Taken together with my results showing that Artemis can downregulate p27 and interact with Skp2, I speculate that Artemis can be used as a potential target for cancer treatment. Moreover, since Artemis is also a negative regulator of p53, targeting Artemis may be more efficient than Skp2. Although Artemis has been shown to be a repair factor involved in the NHEJ, that is unlikely to conflict with its potential effects on tumorigenesis. In normal cells, Artemis is required for cell cycle progression through degradation of p27. Upon DNA damage, it has functions in cell cycle arrest, maintenance of DNA repair checkpoints and participating in NHEJ. However, under the specific genetic backgrounds of cancer cells, Artemis's ability to downregulate proteins such as p27 and p53 may contribute to tumor development. Furthermore, it is likely that Artemis is deregulated in cancer cells, and either the overexpression or mutations on the

phosphorylation sites of Artemis can largely affect the protein stabilities of its downstream targets such as p27 and p53. p27 is downregulated in many cancers and its low level is usually associated with a poor prognosis. In order to explore the effects of Artemis's disruption on tumorigenesis, we would like to check the expression levels of Artemis in different types of cancer samples, and later we will also establish mouse models to further investigate this hypothesis.

10A



10B

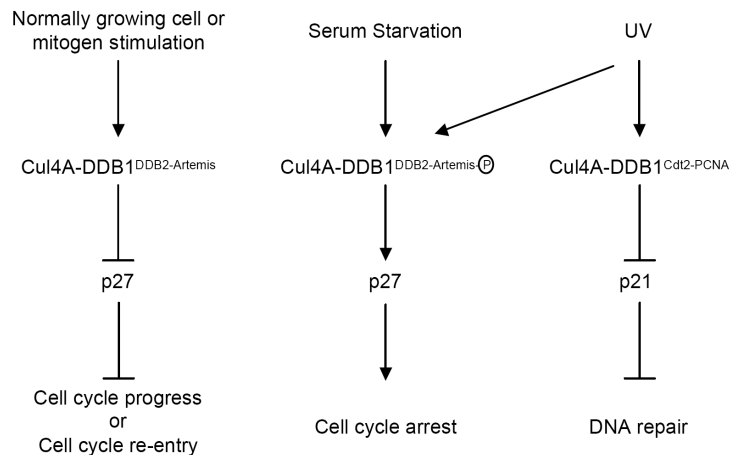


Figure 10A. Model depicting Artemis and DDB2 as components of the Cul4A-DDB1 E3 ligase complex that targets p27 for degradation. DDB2, a DCAF protein, functions as a receptor in this ligase complex. Artemis directly interacts with DDB2 and p27, and is an additional factor required for the ubiquitylation of p27 by Cul4A-DDB1^{DDB2}.

Figure 10B. Schematic depicting the roles of Artemis in cell cycle control via regulation of Cul4A-DDB1-mediated p27 degradation. In normally growing or mitogen stimulated cells, Artemis, as a component of Cul4A-DDB1^{DDB2-Artemis}, promotes p27 ubiquitylation and degradation, which facilitates cell cycle re-entry and progression. Upon serum starvation or UV-irradiation Artemis is phosphorylated, which impairs its activity to promote the degradation of p27, which results in p27 stabilization and cell cycle arrest. Cul4A-DDB1^{Cdt2-PCNA} targets p21 for degradation after UV, thereby relieving its inhibitory effect on PCNA and facilitate the NER.

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