Cell Cycle and Cell Growth Regulation by the
CUL4-DDB1-ROC1 Ubiquitin Ligases

by
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ABSTRACT

JIAN HU: Cell Cycle and Cell Growth Regulation by the CUL4-DDB1-ROC1 Ubiquitin Ligases
(Under the direction of Dr. Yue Xiong)

Timely and efficient destruction of proteins in the cell is critical for its normal function. The ubiquitin-proteasome system is the major pathway by which the cell targets proteins for degradation in a specific manner. Ubiquitination is a process in which ubiquitin is covalently conjugated to proteins via an enzymatic cascade composed of an E1 activating enzyme, an E2 conjugating enzyme and an E3 ubiquitin ligase. The cullins are a family of evolutionarily conserved proteins that assemble a large family of cullin-dependent E3 ligases (CDLs). A unique feature of CDLs is that the cullins, through a conserved N-terminal domain interact with a specificity factor - either directly or through an adaptor - to recruit specific substrates. This dissertation is directed toward understanding the mechanism by which CUL4 targets substrates. I show here that CUL4 utilizes an adaptor DDB1 (damaged DNA binding protein 1) and the specificity factor DWD (DDB1 binding and WD40 repeat) proteins to target various substrates. Specifically, a DNA pre-replicative licensing factor CDT1 is ubiquitinated and degraded by CUL4-DDB1-CDT2 ubiquitin ligase in response to DNA damage, thereby constituting a novel cell cycle G1-S checkpoint. Although the mechanistic role is not known, PCNA (proliferating cell nuclear antigen) is
required for the ubiquitination of CDT1 catalyzed by CUL4-DDB1-CDT2. TSC2 (Tuberous Sclerosis complex) is an important tumor suppressor which plays an essential role inhibiting cell growth. The majority of disease-associated mutations targeting TSC2 results in a substantial decrease in protein level, suggesting that protein turnover plays a critical role in TSC regulation. In this study, I present the evidence indicating that CUL4-DDB1-FBW5 is an E3 ubiquitin ligase regulating TSC2 protein stability.
To my family
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<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BTB</td>
<td>broad-complex C, Tramtrack (Ttk) and Bric-a-brac</td>
</tr>
<tr>
<td>CDL</td>
<td>cullin-dependent E3 ligases</td>
</tr>
<tr>
<td>CSN</td>
<td>COP9 signalosome</td>
</tr>
<tr>
<td>DMEM</td>
<td>DDelbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DWD</td>
<td>DDB1 binding and WD40 repeat</td>
</tr>
<tr>
<td>E1</td>
<td>ubiquitin activating enzyme</td>
</tr>
<tr>
<td>E2</td>
<td>ubiquitin conjugating enzyme</td>
</tr>
<tr>
<td>E3</td>
<td>ubiquitin ligase</td>
</tr>
<tr>
<td>E6AP</td>
<td>E6-associated protein</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>G1</td>
<td>gap 1 phase</td>
</tr>
<tr>
<td>G2</td>
<td>gap 2 phase</td>
</tr>
<tr>
<td>HA</td>
<td>haemagglutinin</td>
</tr>
<tr>
<td>HECT</td>
<td>homologous to E6-associated protein C-terminal domain</td>
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<tr>
<td>IP</td>
<td>immunoprecipitate</td>
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<tr>
<td>MCM</td>
<td>mini-chromosome maintenance</td>
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<tr>
<td>M</td>
<td>mitosis</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>NP-40</td>
<td>Nonidet P40</td>
</tr>
<tr>
<td>ORC</td>
<td>origin recognition complex</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>pre-RC</td>
<td>pre-replicative complex</td>
</tr>
<tr>
<td>RING</td>
<td>really interesting new gene</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROC</td>
<td>ring of cullin</td>
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<tr>
<td>S</td>
<td>synthesis phase</td>
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<tr>
<td>SCF</td>
<td>Skp1-Cdc53/Cullin 1-F box protein</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>siRNA</td>
<td>small interference RNAi</td>
</tr>
<tr>
<td>SOCS</td>
<td>suppressor of cytokine signalling</td>
</tr>
<tr>
<td>TAP</td>
<td>tandem affinity protein tag</td>
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<tr>
<td>TSC</td>
<td>tuberous sclerosis complex</td>
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<tr>
<td>Ub</td>
<td>ubiquitin</td>
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<tr>
<td>UBP</td>
<td>ubiquitin protease</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VHL</td>
<td>von Hippel-Lindau</td>
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<td>XPC</td>
<td>xeroderma pigmentosum group C</td>
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Chapter I
INTRODUCTION
**Ubiquitin-mediated proteolysis**

Timely and efficient destruction of proteins in the cell is critical for its normal function. The ubiquitin-proteasome system is the major pathway by which the cell targets proteins for degradation in a specific manner. That the modification of ubiquitin serves a proteolytic signal was first discovered in rabbit reticulocyte lysates while studying the degradation of misfolded proteins (Hershko and Ciechanover, 1982). Ubiquitin is an abundant, 76 amino acid protein highly conserved between species, differing only three amino acids between yeast and mammals (Ozkaynak et al., 1984). Ubiquitin gene usually contains several identical coding repeats and transcribes a precursor containing several ubiquitin repeats. Ubiquitin only becomes mature after the cleavage of the first 5 amino acids following a Glycine-Glycine dipeptide repeat (amino acid 75 and 76) at the C-terminal of the ubiquitin repeat (Ozkaynak et al., 1984). Deubiquitinating enzymes or ubiquitin proteases (UBP) are required for the maturation of ubiquitin as well as the regeneration of ubiquitin pool from conjugated ubiquitin chains (Jonnaalagadda et al., 1989). Ubiquitination is a process in which ubiquitin is covalently conjugated to proteins via an enzymatic cascade composed of an E1 activating enzyme, an E2 conjugating enzyme and an E3 ubiquitin ligase. There are several critical residues within the ubiquitin protein that are required for its tagging activity. The C-terminal Glycine is the site which is covalently conjugated on substrates or another ubiquitin. Ubiquitin contains seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) which can be conjugated by the C-terminal Glycine residue from another ubiquitin to form diverse types of chain linkage. Ubiquitin chains linked through K48-Glycine and
K63-Glycine are the best characterized, which signal proteasome-mediated degradation and non-proteolitical functions, respectively (Chen et al., 2006; Hofmann and Pickart, 1999; Mastrandrea et al., 1999; Pickart and Raasi, 2005; Raasi and Pickart, 2005; Raasi et al., 2005).

Ubiquitination requires a cascade of three enzymatic reactions. This series of reactions begins with activation of ubiquitin by an ubiquitin activating enzyme, or E1, in an ATP dependent manner. The C-terminal glycine of ubiquitin is covalently linked to an internal Cysteine residue within the E1 through a thiol ester bond, utilizing the energy released from hydrolysis of ATP to AMP and PPi (Ciechanover et al., 1982; Pickart and Vella, 1988). The charged ubiquitin is then transferred to an ubiquitin conjugating enzyme (Ubc), or E2, forming another thiol ester bond (Haas and Bright, 1988; Pickart and Rose, 1985). From here, the ubiquitin can be transferred to an ε-amino group of a lysine or the α-amino group of substrates directly, or indirectly through the facilitation by an E3 ubiquitin ligase (Hershko, 1991; King et al., 1996). Subsequent ubiquitin can be ligated to a lysine residue (K48 in most cases) within the previous ubiquitin repeatedly. The series of ligation of ubiquitin results in a polyubiquitin chain. The polyubiquitinated substrates can be rapidly detected and degraded by 26S proteasome (Figure 1.1).

The ubiquitin enzymatic system is in a hierarchical structure: there is only one E1 and several E2s in all species. Hundreds of E3s have been identified and the number is still
increasing. E4 has been discovered recently and it is proposed to be involved in ubiquitin chain elongation. However, its activity appears only be restricted in a limited number of substrates (Koegl et al., 1999).

During the past two decades, numerous studies have unraveled the role of ubiquitin-mediated proteolysis in an extremely broad array of cellular processes, among which are cell cycle, cell division, cell growth, DNA repair, differentiation, development, the cellular response to extracellular effectors and stress, modulation of cell surface receptors and ion channels, regulation of immune and inflammatory responses and biogenesis of organelles (Ciechanover et al., 2000a). Considering these numerous processes regulated by ubiquitin-mediated proteolysis, it is not surprising that the malfunction of this system has been implicated in pathogenesis of many diseases, such as cancer, neurodegenerative disease, immune disease, Cystic Fibrosis, Angelman's syndrome and Liddle's syndrome (Ciechanover et al., 2000b).
Figure 1.1 The ubiquitin-mediated proteolysis pathway.

Ubiquitin is a 76 amino acid protein which is highly conserved between species. The ubiquitination requires an enzymatic cascade composed of an ubiquitin activating enzyme E1, an ubiquitin conjugating enzyme E2 and an ubiquitin ligase E3. Activation of ubiquitin by E1 requires the energy provided by ATP hydrolysis, resulting in a high energy thiol ester linkage, indicated by a letter “S”. Subsequent degradation of polyubiquitinated proteins is processed by the 26S proteasome in an ATP dependent manner. The ubiquitin-mediated proteolysis pathway has been reported to regulate numerous cellular processes.
E3 ligases can be divided into two major families. The HECT family of E3s contains a domain homologous to the E6-associated protein (E6AP) carboxyl terminus that can form thiol ester linkage with ubiquitin. Multiple cellular proteins with diverse structures contain the HECT domain (five in budding yeast and around 30 in humans), implicating many substrates and physiological functions for the HECT family of E3 ligases (Pickart, 2001). The RING family of E3s contains either an intrinsic RING finger domain or an associated RING subunit essential for the ubiquitin ligase activity. RING finger domain was initially identified by a database search using an N-terminal sequence of a protein called RING1 (Really Interesting New Gene 1), and then refined by a sequence pattern matching program PROMOT (Sternberg, 1991). The RING finger sequence is defined as Cys-X2-Cys-X0.39-Cys-X1.3-His-X2.3-Cys-X2-Cys-X4.48-Cys-X2-Cys, in which X is any amino acid. The RING finger domain contains eight potential metal ligands (seven Cystein residues and one Histidine residue) and binds to two zinc atoms tetrahedrally by either four Cysteins or three Cysteins and one Histidine. In this system, the first pair of ligands share a zinc (Cys 1 and Cys 2) atom with the third pair (Cys 4 and Cys 5), whereas the second pair (Cys 3 and His 1) share the other zinc atom with the fourth pair (Cys 6 and Cys 7) (Figure 1.2)
Figure 1.2 Cross-braced structure of RING finger domain.

Eight Cysteins and Histidines within RING finger domain bind to two zinc atoms tetrahedrally, forming a cross-braced structure. Adapted from Borden & Freemont, *Curr Opin Struc Biol* 6:395-401, 1996

E3 ubiquitin ligases contain two distinct activities: catalysis of isopeptide bond formation and recruitment of substrates to this catalytic activity (Hershko and Ciechanover, 1998). The cullins are a family of evolutionarily conserved proteins that assemble a large family of cullin-dependent E3 ligases (CDL). The human cullin family includes six closely related proteins (CUL1, CUL2, CUL3, CUL4A, CUL4B and CUL5) and three distantly related proteins (CUL7, PARC and APC2). All cullins contain a conserved carboxy-terminal domain of approximately 100 amino acids, which binds to a small RING
finger protein: ROC1 (RING of Cullins, also known as Hrt1 and Rbx1), ROC2 or APC11 (Deshaies, 1999; Jackson et al., 2000). ROC proteins activate an E2 ubiquitin-conjugating enzyme to catalyze polyubiquitination through their RING finger, and bind with cullins through an N-terminal sequence flanking the RING domain to recruit the catalytic function of the E2 to cullins (Furukawa et al., 2000). A unique feature of CDLs is that the cullins, through a conserved N-terminal domain, interact with a specificity factor (substrate targeting protein)— either directly or through an adaptor protein or adaptor complex — to recruit specific substrates, rather than binding to substrates directly as most other ligases do. The SKP1 adaptor bridges an F-box protein to CUL1-dependent ligases (Bai et al., 1996; Feldman et al., 1997; Skowyra et al., 1997; Zheng et al., 2002a), a heterodimeric elongins B and C complex brings VHL (von Hippel-Lindau) box proteins and SOCS (suppressor of cytokine signaling) box proteins to CUL2-dependent and CUL5-dependent ligases, respectively (Kamura et al., 2001; Kamura et al., 2004; Kamura et al., 1998; Stebbins et al., 1999; Zhang et al., 1999), whereas CUL3 binds directly to the BTB domain (Broad-Complex C (BR-C), Tramtrack (Ttk) and Bric-a-brac) (Furukawa et al., 2003; Geyer et al., 2003; Pintard et al., 2003; Xu et al., 2003). The presence of numerous substrate specificity factors — mammals express more than 60 F-box, 40 SOCS and 200 BTB proteins — suggests that individual cullins may assemble into multiple E3 ligase complexes. (Figure 1.3 and Figure 1.4)

The assembly of these multi-subunit cullin-dependent ubiquitin ligase complexes, and thus the ubiquitination of substrates, is tightly regulated: all cullins are negatively regulated
by CAND1 (cullin-associated and neddylation-dissociated 1), a 120 kDa protein that consists of 27 tandem HEAT (huntingtin-elongation-A subunit-TOR) repeats and prevents binding of the adaptor protein and substrates to the complex. To assemble active ligase-substrate complexes, cullins are covalently modified by a small ubiquitin-like modifier, Nedd8, to dissociate CAND1 (Goldenberg et al., 2004; Liu et al., 2002; Zheng et al., 2002a). Dissociation of CAND1 from cullins exposes the site required for the binding with adaptors and hence recruits substrates. To inactive cullins, the COP9 signalosome removes the conjugated Nedd8, allowing CAND1 re-associates with cullins and resumes the inhibition (Lyapina et al., 2001). This seemingly complex regulation is essential to ensure the assembly of individual cullin-ROC cores, which are likely present in the cell as rate-limiting factor, into multiple distinct substrate-cullin-ROC complexes.

**DNA replication initiation is tightly regulated**

DNA replication in eukaryotic cells starts from the concerted formation of a multiprotein complex called the pre-replicative complex (pre-RC) in late M and early G1 phases of the cell cycle. The assembly of pre-RC begins with the loading of a six-component complex ORC (origin recognition complex) which binds to replication origins (Gilbert, 2001). The loading of ORC in metazoan appears to be less strict than single cell organisms, which might explain the broad initiation zones of many metazoan replication origins (Machida et al., 2005). ORC on the chromatin recruits two replication licensing factors, Cdc6 and CDT1, both of which are required to the loading of a replicative helicase MCM2-7 (Figure 1.5).
Figure 1.3 Substrate-targeting mechanisms of cullin family of E3 ligases.

CUL1 utilizes SKP1-F box proteins, CUL2 utilizes Elongin B/C-VHL box proteins, CUL5 utilizes Elongin B/C-SOCS box proteins, and CUL3 utilize BTB proteins to target various substrates. The substrate-targeting mechanism of CUL4 was solved by my colleagues and me, which will be presented in the thesis.
Figure 1.4 The structure of SCF\(^{SKP2}\).

The crystal structure of SCF\(^{SKP2}\) highlights the extended modular nature of cullin–RING ligases (Protein Data Bank file 1LDK). The F-box portion and the substrate-binding region of the substrate receptor SKP2 are shown — the former binds to the adaptor protein SKP1 and the latter extends towards the ubiquitin-conjugating enzyme (E2), which is bound to the E2-docking site on the RING protein. The substrate-specificity module of SCF is separated from the E2-docking site by a series of three cullin repeats that form the curved N-terminal stalk of cullin-1 (CUL1). The distance between a bound substrate and the active-site cysteine of an E2 that is docked on the RING protein has been estimated by computational methods to be \(-50\) Å. Yellow spheres represent zinc molecules, and the NEDD8 attachment site is highlighted. SCF, SKP1, CUL1, F-box protein. From Petroski, M.D., and Deshaies, R.J. *Nature reviews* 6, 9-20, 2005
Cdc6 belongs to the AAA+ family of ATPase (Harvey and Newport, 2003), while CDT1 does not have any defined motif or domain. The replicative helicase is a ring-shaped hexamer composed of Mcm2 to Mcm7. Mcm2 to Mcm7 are structurally related proteins containing AAA+ ATPase domain, which is required for unwinding the DNA double helix of the replication origin (Pacek and Walter, 2004). Pre-RC is activated by CDKs (cyclin dependent kinases), DDKs (Dbf4 dependent kinases) and the assembly of a second set of factors including Mcm10, GINS, Sld2, Cdc45, Sld3, Dpb11, RPA and replicative polymerases upon the entry of S phase (Araki et al., 1995; Brown and Kelly, 1999; Lee et al., 2003; Masumoto et al., 2002; Takayama et al., 2003; Takeda and Dutta, 2005; Wang and Elledge, 1999; Wohlschlegel et al., 2002).

Maintaining genome integrity requires precise regulation of DNA replication to ensure that the genome is replicated once and only once per cell cycle. The key mechanism is to inactive the pre-RC once DNA replication initiated. There are three mechanisms reported to inhibit pre-RC formation after the onset of S phase: (1) inhibition of pre-RC by binding between CDT1 and geminin, (2) degradation of CDT1 by ubiquitin-mediated proteolysis, and (3) phosphorylation of pre-RC components by Cdk in G2 phase (Figure 1.6). Geminin is conserved from the worm Caenorhabditis elegans to human, but has not been found in yeast. Geminin is present in S, M and G2 phases, but absent in G1 phase. At the metaphase-to-anaphase transition, geminin is ubiquitinated by APC and degraded by the 26S proteasome to allow the formation of pre-RC (McGarry and Kirschner, 1998).
Pre-RC is loaded onto chromatin in a concerted order which begins with loading of ORC. ORC recruits Cdc6 and CDT1, which are both required for the loading of the replicative helicase Mcm2-7. Pre-RC formation occurs in late M and early G1 phases of the cell cycle and activated upon the entry of S phase. Adapted from Takeda, D.Y., and Dutta, A. *Oncogene* **24**, 2827-2843, 2005.
Geminin prevents the formation of pre-RC by binding to CDT1 and inhibiting Mcm2-7 loading (Wohlschlegel et al., 2000). Opposite to geminin, CDT1 accumulates in late M and G1 phases, but is absent in S and G2 phases. The degradation of CDT1 by ubiquitin-proteasome pathway in S and G2 phases has been reported in *C. elegans* and human (Higa et al., 2003; Li et al., 2003; Liu et al., 2004; Nishitani et al., 2004; Zhong et al., 2003).

*Figure 1.6 Three possible mechanisms for the inhibition of the second-round pre-RC formation*

Rereplication is prevented by inhibition of pre-RC formation by three mechanisms: (A) An inhibitory complex between geminin and CDT1; (B) CDT1 degradation in S phase by an ubiquitin-mediated proteolysis; (C) High activity of cyclin dependent kinase (Cdk) in G2 and M phases inactivates (or destabilizes) pre-RC components Cdc6, CDT1, MCM2-7, and/or ORC. From Machida, Y.J., Hamlin, J.L., and Dutta, A. *Cell* **123**, 13-24, 2005.
TSC-mTOR pathway has a central role in regulation of cell growth

The Tor (target of rapamycin) genes were first identified in yeast as the targets of the drug rapamycin (Heitman et al., 1991), and the mammalian counterpart mTOR was discovered later (Brown et al., 1994; Chiu et al., 1994; Sabatini et al., 1994). TOR belongs to the phosphoinositide kinase-related kinase family, but it phosphorylate proteins instead of phosphoinositides (Harris and Lawrence, 2003). Genetic and biochemical studies in the past decade have shown that TOR plays a central role in controlling cell growth, proliferation and metabolism, and regulates numerous cellular processes, including translation, transcription, mRNA turnover, protein stability, actin cytoskeletal organization and autophagy (Inoki et al., 2005; Jacinto and Hall, 2003). Ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4E binding protein 1 (4EBP1), two essential regulators of protein translation, are best-characterized substrates of mTOR (Harris and Lawrence, 2003) (Figure 1.7). S6K is the major kinase for the ribosomal protein S6, phosphorylation of which selectively increases the translation of mRNAs containing a tract of pyrimidines motif. These mRNAs usually translate ribosomal proteins and other translation regulators, through which S6K enhances overall protein synthesis of the cell (Avruch et al., 2001; Meyuhas, 2000). 4EBP1, another target of mTOR, plays a role as a translation inhibitor by binding eukaryotic initiation factor 4E (eIF4E), which recognizes the 5' end cap of eukaryotic mRNAs (Gingras et al., 1999). Phosphorylation of 4EBP1 by mTOR dissociates the interaction between eIF4E and 4EBP1, thereby relieving the inhibition of 4EBP1 on eIF4E (Gingras et al., 1999). eIF4E increases the translation of a subset of growth stimulating
factors, including Cylin D1, e-Myc and vascular endothelial growth factor (VEGF) (Mamane et al., 2004).

mTOR links the cell growth to multiple extracellular and intracellular signals, including growth factors, nutrients and energy (Harris and Lawrence, 2003; Inoki et al., 2003b; Jacinto and Hall, 2003). All these signals act on mTOR through the tuberous sclerosis complex (TSC) gene products TSC1 and TSC2 and a small G protein Rheb (Figure 1.7). Tuberous sclerosis is an autosomal dominant disease causes benign tumors (hamartomas) in a variety of organs with an incidence estimated to be 1 in 6000 live births. TSC1 (also known as hamartin) and TSC2 (also known as tuberin) are two genes whose mutations are believed to cause this disease (Young and Povey, 1998). TSC2 gene encodes a 200 kDa protein containing a coiled-coil domain and a GAP (GTPase activating protein) domain, which inhibits the small G protein Rheb, an activator of mTOR (Inoki et al., 2003a). PI3K (phosphoinositide kinase-3) is a prosurvival and pro-oncogenic protein which is activated by growth factors such as insulin (Cantley, 2002). Activated PI3K phosphorylates di-phosphorylated phosphoinositide (PtdInsP2) on the third carbon of the inositol ring, resulting in tri-phosphorylated phosphoinositide (PtdInsP3) which activates Akt (also known as PKB), another pro-oncogenic protein (Brunet et al., 1999; Cantley, 2002). Biochemical studies in both D. melanogaster and mammalian cells showed that Akt phosphorylates TSC2 and inhibits its function (Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002).
Figure 1.7 TSC-mTOR pathway in the regulation of translation.

mTOR plays a central role in the regulation of protein synthesis. TSC1/2 are the major inhibitors of mTOR function. TSC-mTOR pathway is regulated by different signals, including growth factors, nutrients and ATP depletion. X represents unknown factors. IRS: insulin receptor substrate. Adapted from Inoki, K., Corradetti, M.N., and Guan, K.L. Nat Genet 37, 19-24, 2005.
PTEN, a potent tumor-suppressor, acts as a 3’ phosphotase to decrease the PtdInsP3 level, thereby relieving the inhibition on TSC2 (Kaper et al., 2006). The essential role of PTEN in regulation of TSC2-mTOR pathway has been highlighted by the discoveries that PTEN mutation causes several hamartoma syndromes, including Cowden disease, Bannayan-Riley-Ruvalcaba syndrome (BRRS), Lhermitte-Duclos disease and Proteus syndrome (Eng, 2003; Inoki et al., 2005). In response to low intracellular ATP/AMP ratio, the 5’ AMP-activating protein kinase (AMPK) phosphorylates and activates TSC2, thereby inhibiting mTOR function (Hardie et al., 1998; Hawley et al., 2003; Inoki et al., 2003b). Notably, LKB1 (also known as STK11), an upstream of AMPK, has been shown inactivated in an hamartoma syndrome: Peutz-Jegher syndromes (Hawley et al., 2003; Hong et al., 2003).
Summary

This dissertation describes the work I have taken to explore the mechanism by which CUL4 targets substrates and the regulation of DNA replication, cell cycle and cell growth by CUL4-DDB1-ROC1 ubiquitin ligase.

Chapter II presents the paper “Targeted ubiquitination of CDT1 by the DDB1-CUL4A-ROC1 ligase in response to DNA damage” (Hu et al., 2004), in which I am the first author.

Chapter III presents the paper “An evolutionarily conserved function of proliferating cell nuclear antigen for CDT1 degradation by the Cul4-Ddb1 ubiquitin ligase in response to DNA damage” (Hu and Xiong, 2006), in which I am the first author.

Chapter IV describes my investigation on the role of CUL4-DDB1-FBW5-ROC1 ubiquitin ligase in degrading TSC2.
Chapter II

CUL4-DDB1-CDT2-ROC1 TARGET CDT1 FOR DEGRADATION IN RESPONSE TO DNA DAMAGE

(Including modified portions of “CUL4-DDB1-ROC1 TARGET CDT1 FOR DEGRADATION IN RESPONSE TO DNA DAMAGE,” Jian Hu, Chad McCall, Tomohiko Ohta, and Yue Xiong, Nat Cell Biol, 2004 Oct;6(10):1003-9)
Summary

Cullins assemble a potentially large number of ubiquitin ligases by binding to the RING protein ROC1 to catalyze polyubiquitination, as well as binding to various specificity factors to recruit substrates. The *CUL4A* gene is amplified in human breast and liver cancers, and loss-of-function of *Cul4* results in the accumulation of the replication licensing factor CDT1 in *Caenorhabditis elegans* embryos and ultraviolet (UV)-irradiated human cells. Here, I present that human UV-damaged DNA-binding protein DDB1 associates stoichiometrically with CUL4A *in vivo*, and binds to an amino-terminal region in CUL4A in a manner analogous to SKP1, VHL, SOCS and BTB binding to CUL1, CUL2, CUL5 and CUL3, respectively. As with SKP1–CUL1, the DDB1–CUL4A association is negatively regulated by the cullin-associated and neddylation-dissociated protein, CAND1. DDB1 and CDT1 associate with each other, and ectopically expressed DDB1 bridges CDT1 to CUL4A. I also describe that a DWD (DDB1-binding WD40) box protein CDT2 recruits CDT1 to CUL4-DDB1. Silencing *DDB1* or *CDT2* prevented UV-induced rapid CDT1 degradation *in vivo* and CUL4A-mediated CDT1 ubiquitination *in vitro*. I suggest that DDB1 and CDT2 targets CDT1 for ubiquitination by a CUL4A-dependent ubiquitin ligase, CDL4A^{DDB1-CDT2}, in response to UV irradiation.
Background

E3 ubiquitin ligases contain two distinct activities: catalysis of isopeptide bond formation and recruitment of substrates to this catalytic activity (Hershko and Ciechanover, 1998). The cullins are a family of evolutionarily conserved proteins that assemble a large family of cullin-dependent E3 ligases (CDL). The human cullin family includes six closely related proteins (CUL1, CUL2, CUL3, CUL4A, CUL4B and CUL5) and three distantly related proteins (CUL7, PARC and APC2). All cullins contain a conserved carboxy-terminal domain of approximately 100 amino acids, which binds to a small RING finger protein: ROC1 (RING of Cullins, also known as Hrt1 and Rbx1), ROC2 or APC11 (Deshaies, 1999; Jackson et al., 2000). ROC proteins activate an E2 ubiquitin-conjugating enzyme to catalyze polyubiquitination through their RING finger, and bind with cullins through an N-terminal sequence flanking the RING domain to recruit the catalytic function of the E2 to cullins (Furukawa et al., 2002). A unique feature of CDLs is that the cullins, through a conserved N-terminal domain, interact with a specificity factor — either directly or through an adaptor protein or adaptor complex — to recruit specific substrates, rather than binding to substrates directly as most other ligases do. The SKP1 adaptor bridges an F-box protein to CUL1-dependent ligases (Bai et al., 1996; Feldman et al., 1997; Skowyra et al., 1997; Zheng et al., 2002b), a heterodimeric elongins B and C complex brings VHL (von Hippel-Lindau) box proteins and SOCS (suppressor of cytokine signalling) box proteins to CUL2-dependent and CUL5-dependent ligases, respectively (Kamura et al., 2001; Kamura et al., 2004; Kamura et al., 1998; Stebbins et al., 1999; Zhang et al., 1999), whereas CUL3
binds directly to the BTB domain (Broad-Complex C (BR-C), Tramtrack (Ttk) and Bric-a-brac) (Furukawa et al., 2003; Geyer et al., 2003; Pintard et al., 2003; Xu et al., 2003). The presence of numerous substrate specificity factors — mammals express more than 60 F-box, 40 SOCS and 200 BTB proteins — suggests that individual cullins may assemble into multiple E3 ligase complexes.

CUL4 has been highly conserved during evolution, with closely related orthologues present in fission yeast, plants, worms, flies and mammals. Loss-of-function of the Cul4 gene results in elongated cells with decondensed chromosomes in fission yeast (Osaka et al., 2000) and massive DNA re-replication in C. elegans embryos (Zhong et al., 2003), and deletion of CUL4A results in early embryonic lethality in mice (Li et al., 2002). Human CUL4A is genomically amplified or overexpressed in a portion of breast and liver tumours (Chen et al., 1998; Yasui et al., 2002), suggesting an oncogenic function of CUL4A. Loss-of-function mutation of CUL4B gene has been reported to cause a X-linked mental retardation syndrome (Tarpey et al., 2007; Zou et al., 2007). Together, these results indicate an essential function for CUL4 (or CUL4A) in cell-cycle control, genomic stability and development. Degradation of several substrates has been linked to the function of CUL4, including CDT1 (Higa et al., 2003; Zhong et al., 2003), HOXA9 (Zhang et al., 2003b), STAT1 and STAT3 (Andrejeva et al., 2002; Ulane and Horvath, 2002; Ulane et al., 2003), and c-Jun (Wertz et al., 2004). The substrate-targeting mechanism of CUL4-dependent ligases is not well understood. It was recently proposed that DDB1
participates in targeting the substrate c-Jun to the CUL4–ROC1 ligase by interacting with an undefined motif present in the human De-etiolated-1 (DET1) protein (Wertz et al., 2004).

In this chapter, I report the CUL4 utilizes DDB1 and CDT2 as adaptor complex to target CDT1 for ubiquitination and degradation in response to DNA damage.
Experimental procedures

Plasmids, cell culture and cell transfection

Plasmids expressing, and antibodies recognizing, human CUL4A, ROC1 and CAND1, and procedures for yeast two-hybrid assay, immunoprecipitation and immunoblotting were as described (Furukawa et al., 2003; Liu et al., 2002; Ohta et al., 1999). Plasmids expressing DDB1 and CDT1 were obtained from collaborators P. Raychaudhuri (University of Illinois at Chicago, IL) and X. Wu (Scripps Research Institute, CA). Mutations were introduced by site-directed mutagenesis using the Quick-Change Kit (Stratagene, La Jolla, CA) and verified by DNA sequencing. All human cells were cultured in DMEM containing 10% FBS in a 37 °C incubator with 5% CO2. Cell transfections were performed using a calcium-phosphate buffer.

Antibodies, proteins, immunological procedures and mass spectrometric analysis

Antibodies to haemagglutinin (HA) (12CA5; Boehringer-Mannheim, Mannheim, Germany), to Myc (9E10; NeoMarker, Fremont, CA), to T7 (Novagen, Madison, WI), to FLAG (M2; Sigma, St Louis, MO) and to CSN5 (JAB1-2A10.8; GeneTex, San Antonio, TX) were purchased. Rabbit polyclonal antibody to ROC1 has been described (Ohta et al., 1999) and rabbit polyclonal antibodies to CUL4A (N-MADEAPRKGSFSALVGRTNG-C), CDT1 (N-ADLAHITARLAHQTRAEEGL-C) and DDB1 (N-REKEFNKGPWKQENVE-H) were raised against a synthetic peptide derived from the respective human proteins. The rabbit polyclonal antibody to GST–CDT1 (amino acids 267–546) was a gift from X. Wu
The rabbit polyclonal antibody to CDT2 was a gift from H. Zhang (Yale University, CT). DDB1 protein purified from insect cells was a gift from N. Zheng (University of Washington, WA). Procedures for protein purification, immunoprecipitation and immunoblotting have been described (Furukawa et al., 2003; Ohta et al., 1999). To purify the endogenous ROC1 complex, HeLa cells were lysed with a 0.5% NP-40 lysis buffer and lysates were pooled. To purify the CUL4A complex, 24 150-mm plates of BT474 breast cancer cells were lysed with a 0.5% NP-40 lysis buffer and lysates were pooled (150 mg total). Lysates were incubated with affinity purified anti-ROC1 (18 μg) or anti-CUL4A (3 μg) antibodies. Immunocomplexes were precipitated by Protein-A or Protein-G agarose beads and then eluted by incubating with a molar excess of antigen peptide. Eluted immunocomplexes were resolved on an SDS–PAGE gel, stained with Coomassie blue and protein bands digested with trypsin before mass spectrometric analysis at the University of North Carolina Proteomics Core Facility.

**RNA interference**

All siRNA oligonucleotides were synthesised with 3' dTdT overhangs by Dharmacon (Lafayette, CO) in a purified and annealed duplex form. The sequences targeting each human gene were as follows: *Ddb1*, 5'-CCUGUUGAUUGCCAAAAAC-3'; *Ddb2*, 5'-GAGCGAGAUCCGAGUUUAC-3'; *CUL4A*, 5'-GAACUCCAGACAGAGACC-3'; *Cul4B*, 5'-AAGCCUAAAUUACCAGAAA-3'; *Cand1*, 5'-AATGATTGATGACGGAACCTG-3'; *Cdt2*,
5’-CAAUGGACACCAGAACUCUACCUUU -3’.

OPTI-MEM medium (500 μl) was mixed with Lipofectamine 2000 reagent (10 μl) for 5 min and then incubated with 10 μl (20 mM) of siRNA for 20 min at room temperature. DMEM–10%-FBS medium (1.5 ml) was added to the mixture, and the entire 2.5 ml was added to HeLa cells cultured on a 60-mm plate at 30–40% confluence. The cells were transfected once with CUL4A, Ddb1, Cdt2 and Ddb2 siRNA and analysed 48–72 h after transfection, and transfected three times with Cand1 siRNA every 24 h and analysed 24 h after the last transfection.

In vitro ubiquitin ligation assays.

The procedures for ubiquitin labeling were as described (Furukawa et al., 2003; Liu et al., 2002). Briefly, to purify substrate, Myc-tagged CDT1 was ectopically expressed in 293T cells, extracted in RIPA lysis buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% DOC and 0.1% SDS), and immunoprecipitated using an anti-Myc antibody.

To purify CUL4A ligases from HeLa cells, CUL4A immunocomplexes were immunoprecipitated using a CUL4A antibody from untreated cells or HeLa cells transfected with Ddb1 or Ddb2 siRNA oligonucleotides, immobilised on protein A–agarose beads and washed three times with an NP-40 buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.5% NaCl and 50 mM NaF) and twice with a ligase assay buffer (25 mM Tris-HCl at pH 7.5, 50 mM sodium chloride, 1 mM EDTA, 0.01% NP-40 and 10% glycerol). For the in vitro CDT1 ubiquitination, the CUL4A immunocomplex was mixed with Myc–CDT1 substrate and the mixtures were added to a ubiquitin ligation reaction (final volume 30 μl) containing
the following: 50 mM Tris-Cl at pH 7.4, 5 mM MgCl₂, 2 mM sodium fluoride, 10 nM okadaic acid, 2 mM ATP, 0.6 mM dithiothreitol, 12 μg bovine ubiquitin, 1 μg HA–ubiquitin (Sigma), 60 ng E1, 300 ng E2 (hUbc5c), 5 μg Myc peptide and 5 μg CUL4A peptide. Reactions were incubated at 37 °C for 60 min, terminated by boiling for 5 min in an SDS sample buffer containing 0.1 M dithiothreitol and resolved on an SDS–PAGE gel before immunoblotting with the anti-CDT1 antibody to examine ubiquitin ladder formation.
Results

*DDB1 associates with ROC1 and CUL4A in vivo*

Our initial investigation into the function of DDB1 in CUL4–ROC1 ligases was inspired by the co-purification of DDB1 with ROC1 from HeLa cells (Figure 2.1A). As previously reported, transfection-immunoprecipitation assays demonstrated that when overexpressed, DDB1 readily associates with both CUL4A and CUL4B (Shiyanov et al., 1999) and data not shown). To view quantitatively the DDB1–CUL4A association in vivo, I immunopurified an endogenous CUL4A complex from HeLa cells, which express a high level of CUL4A, and visualized the CUL4A complex by silver staining. CUL4A associated nearly stoichiometrically with DDB1, and associated abundantly with subunits of the COP9 signalosome (Figure 2.1B), indicating that DDB1 is a major partner of CUL4A. In the same purification, as in ROC1 immunopurification from HeLa cells, we did not detect any DDB2. This indicates that DDB2, if associated with DDB1–CUL4A in HeLa cells, is present as a minor component.

*DDB1 binds to an N-terminal region in CUL4A*

To gain mechanistic insight into the DDB1–CUL4A interaction, I took advantage of the defined functions of different domains in cullins, and determined what sequence in CUL4A is involved in binding with DDB1. Deletion of the N-terminal 97 residues in CUL4A (▲N97) completely abolished DDB1 binding. Conversely, a fragment of CUL4A retaining the N-terminal 406 residues (N406) exhibited a DDB1-binding activity that is similar to that
Figure 2.1 DDB1 associates with ROC1 and CUL4A *in vivo*.

(A) Clarified lysates derived from HeLa cells were immunoprecipitated with an anti-ROC1 antibody and resolved on an SDS–PAGE gel before silver staining. Specific ROC1-interacting proteins were identified by being competed off after addition of a molar excess of antigen peptide. The identities of proteins determined by mass spectrometric analysis are indicated.

(B) An anti-CUL4A immunoprecipitate derived from 150 mg of clarified lysate from logarithmically growing HeLa cells was resolved by SDS–PAGE before Sypro Ruby staining. Specific bands were excised and subjected to mass spectrometric analysis; the identified proteins are indicated.
Various deletion mutants and site-specific mutants in the H2, H5 or both H2 and H5 helices of CUL4A were ectopically expressed in 293T cells by transient transfection, and assayed for binding with DDB1 by immunoprecipitation and western blot analysis. Asterisks indicate individual deletion mutants of myc-tagged CUL4A (A, middle). The concentration of endogenous DDB1 in each transfected cell population was determined by direct immunoblotting (B, bottom).
of wild-type CUL4A (Figure 2.2A). Hence, an N-terminal sequence in CUL4A is both necessary and sufficient for binding to DDB1. Two hydrophobic helical surfaces in the N-terminal tip of CUL1, H2 and H5 pack with hydrophobic and polar residues from SKP1 to form a large interface. The N-terminal regions of other cullins form similar H2 and H5 helices, which contain residues that are invariably conserved in orthologues, but are different in paralogues (Zheng et al., 2002b). The substitution of residues in the H2 (from Leu 86-Tyr 87-Gln 88-Ala 89-Val 90 to Ala 86-Ala 87-Ala 88-Ala 89-Ala 90) or H5 (from Trp 139-Gln 140-Asp 141-His 142 to Ala 139-Ala 140-Asp 141-Ala 142) helices of CUL4A substantially reduced DDB1–CUL4A binding (Figure 2.2B). Together, these results demonstrate that DDB1 binds to CUL4A in a manner similar to SKP1–CUL1 binding.

**DDB1 and CAND1 bind to CUL4A in a mutually exclusive manner**

Mutations in either the H2 or H5 helices also abolished the CAND1–CUL4A association (Figure 2.2B), suggesting that CAND1 and DDB1 may competitively bind to an overlapping sequence in CUL4A. Supporting this idea, no CAND1 was detected in the DDB1 immunocomplex; and conversely no DDB1 was detected in the CAND1 immunocomplex, under conditions where CUL4A was readily detected in both DDB1 and CAND1 complexes (Figure 2.3A). Notably, DDB1 associated with both unneddylated and neddylated forms of CUL4A, whereas CAND1 selectively associated with unneddylated CUL4A. Suppression of DDB1 expression by RNA interference (RNAi) considerably increased the CAND1–CUL4A association, whereas it had little effect on the steady state
level of either protein (Figure 2.3B). Conversely, knocking down CAND1 by RNAi did not affect the steady state levels of CUL4A and DDB1, but did increase the CUL4A–DDB1 association (Figure 2.3C). The competitive interaction between CAND1 and DDB1 for binding with CUL4A is very similar to the competitive interaction between CAND1 and SKP1 for binding with CUL1 (Liu et al., 2002), providing additional evidence that DDB1 binds to CUL4A in a similar manner to SKP1–CUL1 binding.

**DDB1 binds to CDT1 and bridges CDT1 to CUL4A**

To test whether DDB1 might function as a linker for CUL4A–ROC1 ligases to recruit substrates, I examined the possibility that DDB1 may target CDT1 for CUL4-mediated ubiquitination. The DDB1–CDT1 association can be detected readily and reciprocally by coupled transfection and co-immunoprecipitation assays (Figure 2.4A). Although DDB1 binds to both fast- and slow-migrating forms of CDT1, it preferentially associated with a slow-migrating form of CDT1 (Figure 2.4A), which may correspond to phosphorylated CDT1 (Li et al., 2003). When overexpressed in cultured human cells, CDT1 and CUL4A did not appreciably associate with each other, but a CDT1–CUL4A complex became readily detectable when these two proteins were co-expressed with DDB1 (Figure 2.4B). I postulate that the CDT1–CUL4A association bridged by endogenous DDB1 may be rapidly dissociated after ubiquitination and thus escape detection. However, the high concentrations of the CDT1–DDB1–CUL4A ternary complex assembled in cells overexpressing all three proteins might saturate endogenous components (for example, ROC1) required for rapid ubiquitination and degradation of CDT1.
Figure 2.3 DDB1 and CAND1 bind to CUL4A in a mutually exclusive manner.

(A) Total cell lysates were prepared from 293T cells transiently transfected with a plasmid expressing either T7–DDB1 or Myc–CAND1 and subjected to either direct immunoblotting or immunoprecipitation and western blot analysis with the indicated antibodies. (B, C) Total cell lysates were prepared from cells transiently transfected with siRNA silencing the expression of either DDB1 or CAND1. The level of individual proteins was determined by direct immunoblotting and CUL4A–DDB1 and CUL4A–CAND1 complexes were examined by immunoprecipitation and western blot analysis.
DWD protein CDT2 functions as a receptor to recruit substrates to CUL4-DDB1 ligase

In a recently published paper "DDB1 functions as a linker to recruit receptor WD40 proteins to CUL4–ROC1 ubiquitin ligases" (He et al., 2006), my colleagues and I described that proteins containing a DWD (DDB1 binding WD40) motif function as receptors to recruit various substrates to CUL4-DDB1 ligase (Figure 2.5A). CDT2 is a DWD protein, and it is under the regulation of the same transcription factor of CDT1, Cdc10 (Hofmann and Beach, 1994), and it has been reported to associate with CDT1 in fission yeast, *Xenopus* and human (Higa et al., 2006a; Higa et al., 2006b; Jin et al., 2006; Ralph et al., 2006; Sansam et al., 2006). IP-western assay in mammalian cells with overexpressed CUL4A, CUL4B, DDB1 or/add CDT2 showed that CDT2 associates stably with CUL4A, CUL4B and DDB1, suggesting that CDT2 is a receptor protein for CUL4-DDB1 ligase (Figure 2.5B). Consistent with the notion that CDT2 might target CDT1 to CUL4-DDB1 ligase, overexpressed CDT2 destabilized steady state level CDT1 (Figure 2.5B).

CUL4, DDB1 and CDT2 are required for degradation of CDT1 in response to DNA damage

Consistent with a recent report that CDT1 is degraded by the CUL4–ROC1 ligases after DNA damage (Higa et al., 2003), UV irradiation caused rapid degradation of CDT1 in a proteasome-dependent manner (Figure 2.6A). Knocking down expression of both CUL4A and CUL4B, prevented UV-induced CDT1 degradation, but had no detectable effect on CDT1 degradation in the absence of DNA damage (Figure 2.6C). Remarkably, knocking down CDT2 and DDB1 effectively prevented UV-induced CDT1 degradation, but had no
discernible effect on the concentration of CDT1 in non-irradiated cells (Figure 2.6B, C). In addition, knocking down DDB2 expression had no appreciable effect on the concentration of CDT1 in the presence or absence of DNA damage (Figure 2.6C). We noted that DDB2 itself was decreased to a nearly undetectable level after UV irradiation (Figure 2.6C); however, the significance and mechanism underlying the UV-induced DDB2 decrease remains to be determined.

Figure 2.4 DDB1 binds to CDT1 and bridges CDT1 to CUL4A.

(A) Total 293T-cell lysates prepared from cells co-transfected with plasmids expressing both T7–DDB1 and Myc–CDT1 were analysed for DDB1–CDT1 association by immunoprecipitation and western blot analysis. Note that DDB1 preferentially associates with the slower migrating (most probably phosphorylated) form of CDT1. (B) The association of CUL4A and CDT1 was examined by immunoprecipitation and western blot analysis in 293T cells ectopically transfected with plasmids expressing both proteins with or without additional co-expression of DDB1. The levels of ectopically expressed CDT1 in each transfected cell population were determined by direct immunoblotting of the same cell lysates (bottom).
Figure 2.5 CDT2 functions as a receptor for CUL4-DDB1 ligase to target CDT1.

(A) CUL4-DDB1 ligase utilizes DWD (DDB1 binding WD40) proteins as receptors to target substrates. (B) 293T cells were transfected with plasmids expressing T7-DDB1, Flag-CDT2, Myc3-CUL4A or and Myc3-CUL4B. Cell lysates were immunoprecipitated by anti-Flag antibody. The levels of proteins were detected by anti-Myc, anti-Flag, anti-DDB1, anti-tubulin and anti-CDT1 antibodies.
**CUL4, DDB1 and CDT2 are required for ubiquitination of CDT1 in vitro**

The CUL4A as well as CUL4B and CDT2 immunocomplexes efficiently catalyzed polyubiquitination of CDT1 *in vitro* in an E1- and E2-dependent manner (Figure 2.7A and C). Deletion of either the N-terminal 203 or 97 residues from CUL4A, which disrupted DDB1–CUL4A association, abolished the ability of the CUL4A immunocomplex to ubiquitinate CDT1. Knocking down *DDB1*, but not *DDB2*, by RNAi substantially reduced the efficiency of CUL4A-catalyzed CDT1 polyubiquitination (Figure 2.7B). Together, these results indicate that DDB1 and CDT2 are required for UV-induced CDT1 degradation *in vivo* and CUL4A-mediated CDT1 ubiquitination *in vitro*. DDB2 has long been linked with DDB1 functionally, and forms a complex with DDB1 (Keeney et al., 1993). The exact role of DDB2 in connection with CUL4 remains unclear, but it has been suggested to be a substrate of, or to recruit substrates to, the DDB1–CUL4 ligase (Groisman et al., 2003; Nag et al., 2001). My results also suggest that DDB2 has only a minor, if any, role in mediating CDT1 ubiquitination by the DDB1–CUL4A–ROC1 ligase in response to UV irradiation. This notion is supported by the fact that DDB2 is not present in non-mammals, whereas the other five components (DDB1, CUL4, CDT2, ROC1 and CDT1) are evolutionarily conserved.
Figure 2.6 CUL4, DDB1 and CDT2 are required for the degradation of CDT1 in response to DNA damage

(A) UV irradiation induced a rapid and proteasome-dependent degradation of CDT1. HeLa cells were irradiated by UV (50 J/m²) with or without MG132 treatment. The level of CDT1 protein was determined by direct immunoblotting of total-cell lysate prepared 30 min after UV treatment. (B, C) CUL4, DDB1 and DCT2 are required for the UV-induced rapid degradation of CDT1. HeLa cells were transfected with siRNA oligonucleotides silencing CDT2, DDB1, DDB2 or a combination of both CUL4A and CUL4B. Cells were UV-irradiated (50 J/m²) 48 h after transfection and then lysed for 30 min. The steady state levels of CDT1, as well as CDT2, DDB1, DDB2 and CUL4A, were determined by direct immunoblotting.
Figure 2.7 CUL4, DDB1 and CDT2 are required for the ubiquitination of CDT1 *in vitro*

(A) The N-terminal sequence of CUL4A is required for *in vitro* CDT1 ubiquitination. Myc-tagged wild-type and mutant CUL4A were ectopically expressed in 293T cells, immunoprecipitated using an anti-Myc antibody and incubated with separately precipitated Myc–CDT1 from transfected 293T cells in the presence or absence of various components as indicated. The Myc antigen peptide was added to the mixtures to elute the ligases and the substrates. The ubiquitination reaction mixtures, as well as the eluate (middle) and proteins remaining on the protein G agarose beads (bottom), were resolved on an SDS-PAGE gel, before immunoblotting with an anti-CDT1 or anti-Myc antibody. (B) DDB1 is required for *in vitro* CUL4A-mediated ubiquitination of CDT1. The CUL4A immunocomplex was precipitated from untransfected HeLa cells or HeLa cells transfected with siRNA oligonucleotides silencing *DDB1* or *DDB2*. The targeted reduction of *DDB1* or *DDB2* expression was verified by direct immunoblotting (bottom). The substrate, Myc–CDT1, was immunoprecipitated from separately transfected 293T cells and incubated with the CUL4A immunocomplex in the presence or absence of various components as indicated. A CUL4A peptide and the Myc peptide were added to the mixtures to elute the ligases and the substrates, respectively. After an *in vitro* ubiquitination reaction, the mixtures were resolved on an SDS–PAGE gel and CDT1 ubiquitination was examined by immunoblotting with an anti-CDT1 antibody (top). (C) CDT2 is required for *in vitro* CDT1. Myc-tagged CUL4A and CUL4B and Flag-tagged CDT2 were ectopically expressed in 293T cells, immunoprecipitated using an anti-Myc or anti-Flag antibodies and incubated with separately precipitated HA-CDT1 from transfected 293T cells in the presence or absence of various components as indicated. The Myc, Flag or HA antigen peptide was added to the mixtures to elute the ligases and the substrates. The ubiquitination reaction mixtures were resolved on an SDS-PAGE gel, before immunoblotting with an anti-HA antibody.
Discussion

DDB1 was first discovered as a component of the damaged DNA binding protein complex (DDB), which contains the other component DDB2, using a two-step chromatographic purification composed of a hydroxylapatite column and an UV-irradiated DNA cellulose column (Takao et al., 1993). DDB2 mutations have been linked to xeroderma pigmentosum Group E, a human hereditary disease characterized by defective nucleotide excision repair and increase of skin cancer (Dualan et al., 1995; Rapic-Otrin et al., 2003). DDB1 mutation, on the other hand, has not been identified in any xeroderma pigmentosum patients. The role of DDB1 in DNA repair has been elusive for quite a long time after multiple contradictory studies (Li et al., 2006a; Reardon and Sancar, 2003; Wakasugi et al., 2002; Wakasugi et al., 2001; Wittschieben et al., 2005). My work shows that the major function of DDB1 is an adaptor for CUL4 to target substrates for ubiquitination. There are four lines of evidence supporting the conclusion that DDB1 plays a role as a major adaptor in CUL4 complex to target substrates, CDT1 specifically, for ubiquitination: first, DDB1 binds stochiometrically with CUL4A in vivo; second, CUL4A-DDB1 association is negatively regulated by CAND1 in a manner similar with CUL1-SKP1 association; third, depletion of DDB1 abolishes the degradation of CDT1 in response DNA damage; finally, depletion of DDB1 decreases the ubiquitination level of CDT1 catalyzed by CUL4A immunocomplex in vitro.

One remarkable feature of CUL1-, CUL2- and CUL3-dependent ligases is the assembly of various distinct complexes through interaction with a common motif present in
multiple adaptor proteins. To look for the specificity factors of CUL4-DDB1 ligase, Yizhou He, a former technician in our lab, aligned four reported CUL4-DDB1 associating WD40 proteins, including DDB2, RbBP7, CSA and COP1. He found that there is a common motif in these four WD40 proteins, and this motif is not present in another WD40 protein CDC20 which does not bind to CUL4-DDB1. We named this motif DWD (DDB1 binding WD40) (He et al., 2006). He searched other WD40 proteins and found over 100 containing DWD motif and he picked 12 of them to test the bindings with CUL4-DDB1. IP-western assays showed that all these 90 DWD proteins bind to CUL4-DDB1, suggesting that CUL4-DDB1 utilize DWD proteins as receptors to target various substrates. My work demonstrated that CDT2 is a DWD protein which is required for CDT1 ubiquintation and degradation in response to DNA damage, which supports Yizhou's conclusion.

DDB1 is conserved from fission yeast to human. Deletion of DDB1 gene in fission yeast causes genomic instability, increase of spontaneous mutation rate and failure to pass the meiosis (Bondar et al., 2003; Bondar et al., 2004; Holmberg et al., 2005). Deletion of DDB1 in Drosophila leads to early developmental lethality (Takata et al., 2002). Inactivation CUL4 or DDB1 in C. elegans causes CDT1 accumulation and DNA re-replication (Kim and Kipreos, 2006; Zhong et al., 2003). Deletion of CUL4A (Li et al., 2002) or DDB1 (Cang et al., 2006) in mice leads to early embryonic lethality. Deletion of DDB1 in mouse brain, lens and epidermis causes genomic instability and apoptosis in proliferating progenitor cells. Removal of p53 rescues the lethality of many mitotic cells from death and allows them proliferate aberrantly (Cang et al., 2006).
Since the first substrate CDT1 was identified, CUL4-DDB1 has been shown to ubiquitinate multiple substrates. Paramyxovirus V proteins have been reported to hijack CUL4-DDB1 to ubiquitinate and degrade STAT1/3 (Ulane and Horvath, 2002; Ulane et al., 2003). C-Jun, an oncogene is recruited to CUL4-DDB1 by DET1-COP1 complex for ubiquitination (Wertz et al., 2004). CDT2 targets not only CDT1 but also Spd1, an inhibitor of ribonucleotide reductase in \textit{S. pombe} (Bondar et al., 2004; Liu et al., 2005). The ubiquitination catalyzed by CUL4-DDB1 is involved in not only proteasome-dependent degradation, but also in non-proteolytic regulation. CUL4-DDB1 has been reported to target XPC (El-Mahdy et al., 2006) and histones (Kapetanaki et al., 2006; Wang et al., 2006) for non-proteolytic ubiquitination through receptor DDB2. The polyubiquitination chain through the linkage on K48 of ubiquitin is believed to be transported to proteasome, while K63 polyubiquitin chain is involved in proteasome independent regulation. How CUL4-DDB1 ligase catalyzes both types of polyubiquitin chain remains intriguing. HOXA9 (Zhang et al., 2003a) and p27 (Bondar et al., 2006) were also reported to be substrates of CUL4-DDB1.

CDT1 is a DNA replicative licensing factor. The degradation of CDT1 in response to DNA damage prevents the aberrant DNA replication and serves a novel checkpoint. CDT1 is also degraded during the S and G2 phases of the cell cycle. However, my study showed that CUL4 and DDB1 RNAi did not accumulate CDT1 in the absence of DNA damage reagent. Recently, Nishitani reported that during the S and G2 phases of the cell cycle, CDT1 is ubiquitinated by two redundant ubiquitin ligases CUL4-DDB1 and SCF-SKP2 in
mammalian cells (Nishitani et al., 2006). Later in the third chapter, I will show that in fission yeast, the degradation of CDT1 in the normal cell cycle is only dependent on CUL4-DDB1, but not SCF-SKP2, suggesting that SCF-SKP2 are evolved later to ensure the precise regulation of CDT1.
PCNA IS REQUIRED FOR CDT1 DEGRADATION BY CUL4-DDB1-ROC1 IN RESPONSE TO DNA DAMAGE

(Including modified portions of “An evolutionarily conserved function of proliferating cell nuclear antigen for CDT1 degradation by the Cul4-Ddb1 ubiquitin ligase in response to DNA damage,” Jian Hu and Yue Xiong, J Biol Chem. 2006 Feb; 281(7):3753-6.)
Summary

The DNA replication licensing factor CDT1 is degraded by the ubiquitin-proteasome pathway during S phase of the cell cycle, to ensure one round of DNA replication during each cell division and to halt DNA replication in response to DNA damage. Constitutive expression of CDT1 causes DNA re-replication and is associated with the development of a subset of human non-small cell-lung carcinomas. In mammalian cells, DNA damage-induced CDT1 degradation is catalyzed by the CUL4-DDB1-ROC1 E3 ubiquitin ligase. We report here that overexpression of the proliferating cell nuclear antigen (PCNA) inhibitory domain from the CDK inhibitors p21 and p57, but not the CDK-cyclin inhibitory domain, blocked CDT1 degradation in cultured mammalian cells after UV irradiation. In vivo soluble CDT1 and PCNA co-elute by gel filtration and associate with each other physically. Silencing PCNA in cultured mammalian cells or repression of pen1 expression in fission yeast blocked CDT1 degradation in response to DNA damage. Unexpectedly, deletion of Ddb1 in fission yeast also accumulated CDT1 in the absence of DNA damage. We suggest that the CUL4-DDB1 ligase evolved to ubiquitinate CDT1 during normal cell growth as well as in response to DNA damage and a separate E3 ligase, possibly SCF(Skp2), evolved to share the function of CDT1 ubiquitination during normal cell growth and that PCNA is involved in mediating CDT1 degradation by the CUL4-DDB1 ligase in response to DNA damage.
Background

CDT1, first identified in the fission yeast *Schizosaccharomyces pombe* as a G1 START component Cdc10-dependent transcript whose loss-of-function prevents DNA replication (Hofmann and Beach, 1994), binds to the origin recognition complex (ORC) with Cdc6 at the origins of replication and together with Cdc6 and ORC recruits the minichromosome maintenance 2–7 (MCM2–7) to assemble the prereplication complexes during G1, thereby controlling the initiation of DNA replication (Bell and Dutta, 2002). Constitutive expression of CDT1 alone in *Caenorhabditis elegans*, with Cdc6 in *S. pombe* or with Cdc6 and cyclin A-cdk2 in p53-deficient mammalian cells causes DNA re-replication (Vaziri et al., 2003; Yanow et al., 2001; Zhong et al., 2003). Constitutive expression of CDT1 is also associated with the development of a subset of human non-small lung carcinomas (Karakaidos et al., 2004), indicating the critical importance of regulating CDT1 level for both initiating DNA replication and maintaining genome integrity.

In addition to Cdc10-dependent transcriptional regulation, at least four mechanisms have been proposed for controlling CDT1 function at the protein level: CDT1 is exported from the nucleus in S phase in the budding yeast *Saccharomyces cerevisiae* (Tanaka and Diffley, 2002), is inhibited by the binding of geminin from S to M phase in metazoans (Wohlschlegel et al., 2000), is degraded in human cell lines, possibly by the SCFSkp2 E3 ubiquitin ligase (Li et al., 2003; Nishitani et al., 2000), and is degraded in response to DNA damage and during normal *C. elegans* embryogenesis by the CUL4-DDB1-ROC1 ubiquitin
E3 ligase (Higa et al., 2003; Hu et al., 2004; Zhong et al., 2003). Evolving multiple distinct mechanisms to negatively regulate the level of CDT1 protein presumably functions to meet the needs of stopping DNA replication irreversibly at multiple stages of the cell cycle and development and rapidly in response to genotoxic insults.

The mechanisms underlying the ubiquitin-mediated degradation of CDT1 remain incompletely understood. In particular, the identity and components of the CDT1 E3 ubiquitin ligase are currently confusing. Two different E3 ligases, a CUL1-dependent SCF^{Skp2} E3 (Li et al., 2003; Sugimoto et al., 2004) and a CUL4-DDB1-dependent E3 (Higa et al., 2003; Hu et al., 2004; Zhong et al., 2003), have been linked to CDT1 degradation. Critically missing from the current understanding of these two ligases in promoting CDT1 ubiquitination is the signal(s) that recruits CDT1 to either ligase. I described in the Chapter II that DDB1 preferentially associated with a slow migrating form of CDT1, which likely corresponds to phosphorylated CDT1. The nature of this DNA damage-induced CDT1 kinase and whether CDT1 phosphorylation is required for binding with DDB1 are yet to be determined. Separately, a cyclin A-dependent CDK has been reported to promote CDT1 phosphorylation and subsequent binding with the F-box protein Skp2 (Liu et al., 2004; Sugimoto et al., 2004), resulting in CDT1 degradation during the normal cell cycle.

Co-immunoprecipitation analysis showed that cells treated with the proteasome inhibitor MG132 prior to UV irradiation had increased Skp2-CDT1 association (Kondo et al., 2004), raising the possibility that both SCF^{Skp2} and CUL4-DDB1 could mediate CDT1
degradation following UV-induced DNA damage. In an effort to clarify the role of CDKs in regulating CDT1 ubiquitination, I examined how CDK inhibitors may affect CDT1 degradation during the DNA damage response. This investigation did not identify any evidence for the requirement of a CDK in regulating CDT1 degradation in response to DNA damage but instead led to the unexpected finding that the proliferating cell nuclear antigen (PCNA) is required for CDT1 degradation.
Experimental procedures

Plasmids, Cell Culture, and Cell Transfection

Plasmids expressing human p21, p21<sup>ΔPCNA</sup> (M148A/F151A), p27, p57QT (residues 142–198), and p57QT<sup>ΔPCNA</sup> (L271A/F275A) were described previously (Watanabe et al., 1998), and the procedures for immunoprecipitation and immunoblotting were as described in the Chapter II. All human cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in a 37 °C incubator with 5% CO<sub>2</sub>. Cell transfections were performed using a calcium phosphate buffer.

Antibodies, Proteins, Immunological Procedures, Mass Spectrometric Analysis, and Size Exclusion Chromatography

Antibodies to hemagglutinin (HA) (12CA5; Roche Applied Science, Mannheim, Germany), to protein A (Sigma), to PCNA (PC10, Santa Cruz Biotechnology, Santa Cruz, CA), to actin (NeoMarkers, Fremont, CA), and to p21, p27, and p57 (Foster and Galloway, 1996; Watanabe et al., 1998) were either purchased commercially or described previously. Rabbit polyclonal antibodies to CUL4A, Ddb1, and CDT1 have been described in the Chapter II. To purify the CDT1 complex, 10 150-mm plates of HEK 293T cells were transfected with pcDNA3-myc3-CDT1 and lysed with a 0.5% Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF). Pooled lysates were incubated with affinity purified anti-CDT1 (10 µg) antibody. Immunocomplexes
were precipitated by protein A-agarose beads and then eluted by incubating with a molar excess of antigen peptide. Eluted immunocomplexes were resolved on an SDS-PAGE gel, stained with Coomassie Blue, and protein bands digested with trypsin before mass spectrometric analysis at the University of North Carolina-Duke Proteomics Core Facility.

For size exclusion chromatography, HeLa cells were lysed in 0.5% Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF) and fractionated on a Sepharose 200 gel-filtration column. The fractions were resolved by SDS-PAGE before the levels of CDT1 and PCNA were determined by immunoblotting.

RNA Interference

All siRNA oligonucleotides were synthesized with 3'-NN overhangs by Dharmacon (Lafayette, CO) in a purified and annealed duplex form. The sequences targeting each human gene were as follows: Ddb1, 5'-CCUGUUGAUUGAAAAAC-3’ and PCNA, 5’-UCAAGGACCUCAUACGA-3’. Opti-MEM medium (500 µl) was mixed with Lipofectamine 2000 reagent (10 µl) for 5 min and then incubated with 10 µl (20 mM) of siRNA for 20 min at room temperature. Dulbecco's modified Eagle's, 10% fetal bovine serum medium (1.5 ml) was added to the mixture, and the entire 2.5 ml was added to HeLa cells cultured on a 60-mm plate at 30–40% confluence.

S. pombe Genetics and Strain Construction
Strain construction, gene deletion, and epitope tagging were performed by standard genetic techniques (Bahler et al., 1998). $Nmt81\text{-}Pcn1$ was a gift from Dr. Teresa Wang of Stanford University (Arroyo and Wang, 1999).
Results

Inhibition of PCNA by p21 or p57 prevents the degradation of CDT1 in response to UV

To determine how CDK activity may affect CDT1 degradation in response to DNA damage, I overexpressed the CDK inhibitors p21 and p27 in HEK 293T cells and determined the steady-state levels of CDT1 protein following UV irradiation. Overexpression of p21 considerably inhibited CDT1 degradation following UV irradiation (Figure 3.1A). Although this finding is consistent with the notion that CDK may cause CDT1 phosphorylation and thereby promote its degradation in response to DNA damage, surprisingly, overexpression of p27 had no detectable effect on CDT1 degradation in the same experiment. Both p21 and p27 have indistinguishable CDK substrate specificity regions in their N termini. The major difference between these two CDK inhibitors lies in their C-terminal regions; while p21 contains a PCNA binding and inhibitory domain in its C-terminal region, the C-terminal sequence of p27 plays roles in regulating the phosphorylation of p27 and stability but has no PCNA binding activity. This prompted us to determine which region in p21, the N-terminal CDK-cyclin binding or C-terminal PCNA binding sequence, is required for inhibiting CDT1 degradation following UV irradiation.

The C-terminal PCNA binding domain in p21 has been extensively characterized and residues required for the binding of p21 to PCNA have been identified (Chen et al., 1995; Luo et al., 1995; Nakanishi et al., 1995; Warbrick et al., 1995). Combined mutations of Met_{148} and Phe_{151} to Ala in p21 (referred to as p21_{ΔPCNA}) completely disrupted its binding
with PCNA (Nakanishi et al., 1995; Warbrick et al., 1995) and its ability to block CDT1 degradation following UV irradiation (Figure 3.1B). The p57KIP2 CDK inhibitor also contains a C-terminal domain, often referred to as the QT domain, that shares sequence homology with the C-terminal PCNA binding domain of p21 and binds with and inhibits the function of PCNA (Watanabe et al., 1998). To further confirm the role of PCNA in regulating CDT1 degradation, I ectopically expressed the p57QT domain and examined CDT1 degradation following UV irradiation. Overexpression of the p57QT peptide, but not a L271A/F275A mutant peptide (referred to as p57QTΔPCNA) that cannot bind with PCNA (Watanabe et al., 1998), blocked CDT1 degradation after UV irradiation (Figure 3.1C), demonstrating that inhibition of PCNA blocked CDT1 degradation in response to DNA damage. These results also indicate that CDKs that are inhibited by p21 and p27 (CDK1–CDK6) are unlikely to be required for DNA damage-induced CDT1 degradation. In the absence of DNA damage, overexpression of both p21 and p27 increased the steady-state level of CDT1 protein, particularly the slower migrating form (Figure 3.1A). This observation suggests that either a p21/p27-sensitive CDK(s) is involved in regulating CDT1 stability during normal cell growth, or the CDT1 increase resulted indirectly from the G1 cell cycle arrest caused by p21 or p27 overexpression. My subsequent study was focused on the control of CDT1 stability during the DNA damage response.

**PCNA and CDT1 co-exist in size exclusive fractionation**

To obtain further evidence corroborating the function of PCNA in controlling CDT1
Figure 3.1 Inhibition of PCNA prevents the degradation of CDT1 in response to UV.

HEK 293T cells were transfected with plasmids expressing p21 and p27 (A), a p21 mutant deficient in binding with PCNA (p21ΔPCNA) (B), and the PCNA binding domain of p57 (HA-p57QT) or a mutant of p57QT deficient in PCNA binding (HA-p57QTΔPCNA) (C). Cells were UV-irradiated (50 J/m²) 24 h after transfection and then lysed 30 min later. The steady-state levels of CDT1 as well as p21, p27, p57, Ddb1, and actin proteins were determined by direct immunoblotting.
degradation, I examined the distribution of these two proteins by gel filtration. Size exclusion chromatography analyses indicated that soluble (non-chromatin-associated) PCNA and CDT1 co-eluted in a fraction at molecular mass of less than 158 kDa that is consistent with the formation in vivo of a complex containing PCNA and CDT1 (Figure 3.2). CUL4A, on the other hand, is present in high molecular mass fractions and is not detected in the low molecular mass fractions that contain both CDT1 and PCNA. The patterns of co-elution of CDT1 and PCNA and mutually exclusive distribution of PCNA-CDT1 from CUL4A are consistent with the possibility that PCNA is involved in promoting CDT1 ubiquitination and subsequent degradation of CDT1 by the CUL4A ligase.

**CDT1 and PCNA associate with each other in vivo**

Using the CDT1 antibody described in Chapter II, I immunopurified CDT1 complexes from an Nonidet P-40-soluble extract and examined them by Coomassie blue staining (Figure 3.3A). CDT1-interacting proteins were identified as the polypeptides that were competed off by a molar excess of antigen peptide. In addition to a 60-kDa band that was identified as CDT1 (see below), the only prominent polypeptides visualized were several bands clustered at a molecular mass of around 35 kDa. Mass spectrometry analyses identified, in addition to the 60-kDa CDT1, two proteins: geminin and PCNA (Figure 3.3B). The close migration of geminin and PCNA prevented us from determining the relative stoichiometric ratio of CDT1 and these two proteins. Overexpression of p21 reduced the PCNA-CDT1 association (Figure 3.3C), suggesting that one possible mechanism by which
Figure 3.2 PCNA and CDT1 co-exist in size exclusion chromatography

HeLa cells were lysed in 0.5% Non-ident P-40 lysis buffer and fractionated on a Sepharose 200 gel-filtration column. The fractions were resolved by SDS-PAGE before the levels of CUL4A, CDT1, and PCNA were determined by immunoblotting.
p21 stabilizes CDT1 is to hinder PCNA-CDT1 association. This result, however, needs to be interpreted cautiously as the possibility remains that p21 could also impede CDT1-PCNA association by inhibiting CDK-mediated CDT1 modification.

**PCNA is required for CDT1 degradation in response to DNA damage in mammalian cells**

To seek *in vivo* evidence supporting a role of PCNA in CDT1 degradation, I first performed RNAi-based experiments in cultured mammalian cells to determine how silencing the expression of PCNA affects the steady-state level of CDT1 protein. Consistent with the data presented in Chapter II, knocking down DDB1 by siRNA accumulated CDT1 in UV-treated HeLa cells (Figure 3.4). Notably, reduction of the steady-state level of PCNA by siRNA accumulated CDT1 following UV irradiation as well (Figure 3.4, comparing *lanes 1* and *3*) but did not increase CDT1 protein level in non-irradiated cells (comparing *lanes 2* and *4*). The partial effect of PCNA RNAi on degradation of CDT1 is likely due to incomplete depletion of PCNA protein.
<table>
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<th>Peptides identified</th>
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<tr>
<td>CDT1</td>
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<td>58^DQARPPAR^67</td>
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<tr>
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</table>

A Proteins Peptides identified

B Proteins Peptides identified

C

HA-CDT1 + + +

IP: anti-CDT1

HA-CDT1 + + +

PCNA

p21

p21

Direct IB
Figure 3.3 CDT1 and PCNA associate with each other in vivo and in vitro

(A and B) an anti-CDT1 immunocomplex was purified from HEK 293T cells ectopically expressing myc3-CDT1 and was resolved by SDS-PAGE. After Coomassie Blue staining, specific bands were identified by being competed off by a molar excess of antigen peptide, excised, and subjected to mass spectrometric analysis. The identified peptide sequences are indicated, and the numbers refer to the position of the first and last amino acid residue of the identified peptide in the full-length protein. (C) HEK 293T cells were transfected with plasmids expressing HA-CDT1 with or without p21. Cells were lysed in 0.5% Nonidet P-40 lysis buffer, and cleared lysates were immunoprecipitated with an antibody to CDT1 (left panel). The steady-state levels of CDT1 and PCNA protein were determined by direct immunoblotting.
Figure 3.4 PCNA is required for CDT1 degradation in response to DNA damage in mammalian cells

Logarithmically growing HeLa cells were transfected with siRNA oligonucleotides silencing $DDB1$ and $PCNA$. Cells were UV-irradiated (50 J/m$^2$) 48 h after transfection and then lysed after 30 min. The steady-state levels of CDT1, as well as DDB1, PCNA, and actin, were determined by direct immunoblotting.

**PCNA is required for CDT1 degradation in response to DNA damage in fission yeast**

For further *in vivo* experiments, I utilized fission yeast, which has conserved PCNA, CDT1, and the Cul4-Ddb1 E3 ligase. I constructed fission yeast strains expressing TAP-tagged CDT1 under the control of its native promoters in both $Ddb1^+$ and $Ddb1^\Delta$ backgrounds. The steady-state level of CDT1-TAP is much higher in $Ddb1^\Delta$ than in $Ddb1^+$ cells (Figure 3.5A comparing lanes 1 and 3). This result provides the first evidence that in fission yeast Ddb1 is also involved in controlling CDT1 degradation as in mammalian cells and that a Ddb1-mediated E3 ligase in fission yeast cells also controls CDT1 degradation during normal cell growth in the absence of DNA damage. I then examined the level of
CDT1 in CDT1-TAP, Ddb1Δ and CDT1-TAP, Ddb1+ cells sustaining either inhibition of DNA replication or DNA damage. Unexpectedly, unlike DNA damage (see below), inhibition of DNA replication by treatment of cells with hydroxyurea (HU) increased, rather than decreased, the steady-state level of CDT1-TAP (Figure 3.5B, comparing lanes 1 and 2). To confirm this result, we constructed yeast strains expressing TAP-tagged Spd1 (Spd1-TAP) under the control of its native promoters in both Ddb1+ and Ddb1Δ backgrounds. Spd1 is an inhibitor of ribonucleotide reductase and is also targeted for degradation by the Cul4-Ddb1 ligase both during S phase and in response to DNA damage in S. pombe (Bondar et al., 2004; Holmberg et al., 2005; Liu et al., 2003). Consistent with previous reports, inhibition of DNA replication caused complete degradation of Spd1 and deletion of Ddb1 blocked HU-induced Spd1 degradation (Figure 3.5B, comparing lanes 1 and 3). We also noted that during the normal cell cycle, the steady-state level of Spd1 is higher in Ddb1+ cells than in Ddb1Δ cells (Figure 3.5B, comparing lanes 2 and 4), confirming that Ddb1 in fission yeast cells is involved in degradation of Spd1 both during normal cell cycle and when DNA replication is blocked. HU inhibits ribonucleotide reductase, and thus dNTP production, therefore arresting the DNA replication fork. Our results reveal a major difference between CDT1 and Spd1 degradation during the normal cell cycle. We speculate that during S phase, Spd1 and CDT1 are degraded before and after replication fork formation, respectively, and an additional factor(s) may contribute differently to the ubiquitination of these two proteins by the same Ddb1-mediated E3 ligase.
In \textit{ddb1}Δ cells, CDT1 accumulated to a much higher level (comparing \textit{lanes 1 and 3 of Figure 3.5A} or \textit{lanes 6 and 8 in Figure 3.5C}) than Spd1 (comparing \textit{lanes 2 and 4 of Figure 3.5B}), indicating that although Ddb1 is required for the degradation of both proteins, the level of these two proteins is regulated by an additional mechanism(s). We postulate that there may exist a feedback regulatory mechanism to repress the expression of Spd1, but not CDT1, in \textit{ddb1}Δ cells during each cycle. As a result, CDT1 continuously accumulates to a very high level that, although not inhibiting cell growth, would contribute to deregulated DNA replication and thus genomic instability.

Treatment of cells with methylmethane sulfonate (MMS), a DNA damage reagent, caused CDT1 degradation in \textit{Ddb1}+ cells (Figure 3.5C, comparing \textit{lanes 3 and 4} or \textit{lanes 5 and 6}) but had no effect on the steady-state level of CDT1 in \textit{ddb1}Δ cells (\textit{lanes 7 and 8}), indicating that DNA damage-induced, Ddb1-mediated CDT1 degradation had evolved as early as in yeast cells. To investigate whether PCNA is required for CDT1 degradation in response to DNA damage in \textit{S. pombe}, I generated a strain in which PCNA encoding gene \textit{Pcn1} expression is under the control of the thiamine-repressible \textit{nmt81} promoter and TAP is tagged after the \textit{CDT1} gene (\textit{nmt81-pcn1, CDT1-TAP}). Repression of \textit{Pcn1} expression by thiamine addition was confirmed by direct blotting with an antibody specific to PCNA (Figure 3.5C, comparing \textit{lanes 1 and 2} and \textit{lanes 3 and 4}). Loss of PCNA had no significant effect on the steady-state level of CDT1-TAP in the absence of DNA damage (comparing \textit{lanes 2 and 4}) but blocked the degradation of CDT1 in response to DNA damage.
(comparing lanes 1 and 3). Hence PCNA is required for CDT1 degradation in response to DNA damage in *S. pombe*. Less CDT1 was accumulated in PCNA-depleted cells than in Ddb1-null cells. This is likely because depletion of PCNA arrests the cell cycle and thus prevents further CDT1 accumulation, while Ddb1 null cells continue to proliferate and accumulate CDT1.
Figure 3.5 PCNA is required for CDT1 degradation in response to DNA damage

(A and B) S. pombe strains CDT1-TAP and CDT1-TAP, ddb1Δ or Spd1-TAP and Spd1-TAP, ddb1Δ were treated with 20 mM HU for 4 h or untreated. Yeast cells were collected and lysed with acid-washed glass beads. Steady-state levels of CDT1-TAP and Spd1-TAP proteins were determined by direct immunoblotting. (C) S. pombe strain nmt81-Pcn1, CDT1-TAP was treated with 150 mM thiamine for 12 h and then treated with 0.1% MMS for 3 h. S. pombe strains CDT1-TAP and CDT1-TAP, ddb1Δ were treated with 0.1% MMS for 3 h or untreated. Yeast were collected and lysed with acid-washed glass beads. Steady-state levels of CDT1-TAP and PCNA were determined by direct immunoblotting.
Discussion

In this study, I present three separate lines of evidence that collectively support a role of PCNA in mediating CDT1 degradation by CUL4-DDB1 in response to DNA damage. First, overexpression of the PCNA inhibitory domain of either p21 or p57, but not the CDK-cyclin inhibitory domain, blocked CDT1 degradation in UV-irradiated cells. Second, soluble PCNA and CDT1 co-exist in the same fractions and PCNA and CDT1 physically associate with each other in vivo. Third, knocking down PCNA in cultured mammalian cells and repression of PCNA expression in fission yeast both block CDT1 degradation following DNA damage. Together with the findings that DDB1 is required for DNA damage-induced CDT1 degradation in mammalian cells described in the Chapter II and in fission yeast (Figure 3.5C), I suggest that PCNA-dependent CDT1 degradation by CUL4-DDB1 ligase in response to DNA damage evolved quite early and has been conserved during evolution.

My study also raises three new questions. First, what is the exact biochemical role of PCNA in promoting CDT1 degradation? I previously described that DDB1 can target CDT1 through CDT2, suggesting that PCNA is not required for recruiting CDT1 to the CUL4-DDB1 ligase. Thus far, my in vitro immunoprecipitation-based CDT1 ubiquitination assay has neither identified a significant amount of PCNA nor revealed a role of PCNA in CDT1 ubiquitination by the CUL4-DDB1 ligase. One factor obviously missing from these assays is subcellular localization. In particular, given that all four proteins, CDT1, PCNA, DDB1, and CUL4, are associated with chromatin, it is likely that CDT1 ubiquitination may occur in situ at the site of damaged DNA on chromatin and that
PCNA plays a critical role in bringing about in situ CDT1 ubiquitination, a function that is not possible to be detected in the current in vitro assay or by reconstitution. In fact, in *Xenopus*, CDT1 is degraded during the course of DNA replication on chromatin (Arias and Walter, 2005). Whether this replication-dependent CDT1 degradation on chromatin is catalyzed by the CUL4-DDB1 ligase or regulated by CDK remains to be determined.

The second issue concerns the role of DDB1, a factor that was initially identified as a component of a damaged DNA binding activity and has long been associated with DNA repair processes, during normal cell growth in the absence of DNA damage. Surprisingly, deletion of *Ddb1* in fission yeast cells accumulated CDT1 as well as Spd1 in non-damaged cells (Figure 3.5), revealing a function of Ddb1 in controlling normal cell growth.

Related to this issue is the role of Skp2 in CDT1 degradation. Accumulating evidence has suggested that another E3 ligase, possibly SCF^{Skp2}, is involved in causing CDT1 ubiquitination and degradation in mammalian cells (Li et al., 2003; Liu et al., 2004; Sugimoto et al., 2004). Consistent with the presence of multiple E3 for CDT1 degradation, knocking down Ddb1, while accumulating abundant CDT1 following DNA damage, was not sufficient to accumulate CDT1 during normal cell growth (Higa et al., 2003; Hu et al., 2004). I have thus far obtained no evidence in fission yeast that would suggest the involvement of another E3 ligase in promoting CDT1 ubiquitination. Deletion of *Ddb1* accumulated CDT1 to a very high level (Figure 3.5C), arguing that if there exists another E3 ligase in fission yeast that controls CDT1 ubiquitination, its activity must be very minor in comparison with
that of Ddb1. Recently, Kim Y. et al reported that SCF^{\text{Skp2}} is not required for CDT1 degradation in the normal cell cycle in *C. elegans* (Kim and Kipreos, 2006). I suggest that the CUL4-DDB1 ligase evolved early to ubiquitinate CDT1 both during normal cell growth as well as in response to DNA damage. Later in evolution, a separate E3 ligase evolved to share the function of CDT1 ubiquitination during normal cell growth, but DNA damage-induced CDT1 degradation continues to be carried out by the CUL4-DDB1 ligase.
Chapter IV

CUL4-DDB1-FBW5-ROC1 TARGET TSC2 FOR DEGRADATION
Summary

Tuberous sclerosis (TSC) is an autosomal dominant disease characterized by hamartoma formation in various organs and is caused by mutations targeting either the TSC1 or TSC2 genes. TSC1 and TSC2 proteins form a functionally interdependent dimeric complex. Phosphorylation of either TSC subunit by different kinases regulates the function of TSC and represents a major mechanism to integrate various signals into a centralized cell growth pathway. The majority of disease-associated mutations targeting either TSC1 or TSC2 results in a substantial decrease in protein level, suggesting that protein turnover also plays a critical role in TSC regulation. Here I report that TSC2 protein binds to FBW5, a DDB1-binding WD40 (DWD) protein, and is recruited by FBW5 to the DDB1-CUL4-ROC1 E3 ubiquitin ligase. Overexpression of FBW5 or CUL4A promotes TSC2 protein degradation, and silencing FBW5, DDB1 or CUL45A/B conversely stabilizes TSC2. Mutations targeting either Ddb1 or Cul4 in Drosophila result in TSC2 protein accumulation and cause growth defects that can be partially rescued by TSC2 reduction. These results indicate that FBW5-DDB1-CUL4-ROC1 is an E3 ubiquitin ligase regulating TSC2 protein stability.
Background

mTOR plays a central role in controlling cell growth, proliferation and metabolism, and regulates numerous cellular processes, including translation, transcription, mRNA turnover, protein stability, actin cytoskeletal organization and autophagy (Inoki et al., 2005; Jacinto and Hall, 2003). mTOR belongs to the phosphoinositide kinase-related kinase family, but it phosphorylate proteins instead of phosphoinositides (Harris and Lawrence, 2003). Ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4E binding protein 1 (4EBP1), two essential regulators of protein translation, are the best-characterized substrates of mTOR (Harris and Lawrence, 2003). S6K is the major kinase for the ribosomal protein S6, phosphorylation of which selectively increases the translation of mRNAs containing a tract of pyrimidines motif. These mRNAs usually translate ribosomal proteins and other translation regulators, through which S6K enhances overall protein synthesis of the cell (Avruch et al., 2001; Meyuhas, 2000). 4EBP1, another target of mTOR, plays a role as a translation inhibitor by binding eukaryotic initiation factor 4E (eIF4E), which recognizes 5’ end cap of eukaryotic mRNAs (Gingras et al., 1999). Phosphorylation of 4EBP1 by mTOR dissociates the interaction between eIF4E and 4EBP1, thereby relieving the inhibition of 4EBP1 on eIF4E (Gingras et al., 1999). eIF4E increases the translation of a subset of growth stimulating factors, including Cylin D1, c-Myc and vascular endothelial growth factor (VEGF) (Mamane et al., 2004). mTOR links the cell growth to multiple extracellular and intracellular signals, including growth factors, nutrients and energy (Harris and Lawrence, 2003; Inoki et al., 2003b; Jacinto and Hall, 2003). All these signals act on
mTOR through the tuberous sclerosis complex (TSC) gene product TSC1 and TSC2.

Tuberous sclerosis is an autosomal dominant disease that causes benign tumors (hamartomas) in a variety of organs with an incidence estimated to be 1 in 6000 live births. TSC1 (also known as hamartin) and TSC2 (also known as tuberin) are two genes whose mutations are believed to cause this disease (Young and Povey, 1998). TSC2 gene encodes a 200 kDa protein containing a coiled-coil domain and a GAP (GTPase activating protein) domain, which inhibits the small G protein Rheb, an activator of mTOR (Inoki et al., 2003a).

Ubiquitin-dependent proteolysis pathway has been reported to regulate a massive array of cellular functions. Recently, two groups reported that PTEN, a regulator of the cell growth pathway, is ubiquitinated by a HECT domain ubiquitin ligase NEDD4-1 (Trotman et al., 2007; Wang et al., 2007). TSC2 is a short-lived protein and it is readily ubiquitinated (Chong-Kopera et al., 2006). Many disease-associated mutations in TSC1 and TSC2 result in a substantial decrease in the level of hamartin and tuberin, respectively (Inoki et al., 2002; Nellist et al., 2005), suggesting that protein turnover plays a critical role in TSC regulation. Identification of the ubiquitin ligase for TSC2 would shed light on the understanding of the regulation of TSC and cell growth pathways. In Chapter II, I described that CUL4-DDB1 utilize DWD proteins as receptors to target various substrates. In the effort of identifying ubiquitin ligase of TSC2, my colleague Stuart Shumway and I discovered that Fbw5, a DWD protein, binds to TSC2 very tightly. Following this discovery, I discovered that CUL4-DDB1-Fbw5-ROC1 complex targets TSC2 for ubiquitination.
Experimental procedures

Plasmids, cell culture and cell transfection

Plasmids expressing, and antibodies recognizing, human CUL4A, CUL4B, DDB1, ROC1 and CAND1, and procedures for immunoprecipitation and immunoblotting were as described in Chapter II. Plasmids expressing GFP, SKP1, SKP2, CUL1, CUL2, CUL3 and CUL5 were described previously (Michel and Xiong, 1998). Plasmids expressing CDT2 and myc3-ubiquitin were obtained from collaborators Hui Zhang (Yale University, CT) and Anton Jetten (NIEHS, NC), respectively. Plasmids expressing Flag-Ubiquitin, TSC1, TSC2 and TSC2 mutants were obtained from Kunliang Guan (University of Michigan at Ann Arbor, MI). Plasmids expressing Fbw5 and Fbl6 were cloned from a HeLa cDNA library. Mutations were introduced by site-directed mutagenesis using the Quick-Change Kit (Stratagene, La Jolla, CA) and verified by DNA sequencing. All human cells were cultured in DMEM containing 10% FBS in a 37 °C incubator with 5% CO₂. Cell transfections were performed using a calcium-phosphate buffer.

Antibodies, proteins and immunological procedures

Antibodies to haemagglutinin (HA) (12CA5; Boehringer-Mannheim, Mannheim, Germany), to Myc (9E10; NeoMarker, Fremont, CA), to T7 (Novagen, Madison, WI) and to FLAG (M2; Sigma, St Louis, MO) were purchased. Rabbit polyclonal antibody to ROC1 has been described (Ohta et al., 1999) and rabbit polyclonal antibodies to CUL4A (N-MADEAPRKGSFSALVGRTNG-C), CDT1 (N-ADLAHITARLHQTRAEEGL-C) and
DDB1 (N-REKEFKGPWKQVENKE-H) were raised against a synthetic peptide derived from the respective human proteins. Antibodies to TSC1 and TSC2 were described previously (Shumway et al., 2003; Zacharek et al., 2005). Procedures for protein purification, immunoprecipitation and immunoblotting have been described (Furukawa et al., 2003; Ohta et al., 1999).

**RNA interference**

All siRNA oligonucleotides were synthesised with 3’ dTdT overhangs by Dharmacon (Lafayette, CO) in a purified and annealed duplex form. The sequences targeting each human gene were as follows: *Ddb1*, 5’-CCUGUUGAUUGCCAAAAAC-3’; *Ddb2*, 5’-GAGCGGAGAUCCGAGUUUAC-3’; *CUL4A*, 5’-GAACUUCCGAGACAGACCU-3’; *Cul4B*, 5’-AAGCCUAAAUUACCAGAAA-3’; *Cand1*, 5’-AATGATTTGATGACGGAACTG-3’; *Fbw5*, 5’-CCAAGGAGGGCUUGCGACUUUCU -3’. OPTI-MEM medium (500 μl) was mixed with Lipofectamine 2000 reagent (10 μl) for 5 min and then incubated with 10 μl (20 mM) of siRNA for 20 min at room temperature. DMEM–10%-FBS medium (1.5 ml) was added to the mixture, and the entire 2.5 ml was added to HeLa cells cultured on a 60-mm plate at 30–40% confluency. The cells were transfected once with *CUL4A+CUL4B*, *Ddb1* and *Fbw5* siRNA and analysed 48–72 h after transfection, and transfected three times with *Cand1* siRNA every 24 h and analysed 24 h after the last transfection.
In vivo ubiquitin ligation assays.

The U2OS cells transfected with plasmids expressing HA3-TSC2 and Flag-Ubiquitin were further transfected with siRNAs targeting Fbw5, DDB1 or CUL4A+CUL4B, or scrambled siRNA. The cells were lysed with SDS lysis buffer (50 mM Tris-HCl at pH 8.0, 100 mM NaCl, 2% Triton X-100 and 1% SDS) and then the lysates were diluted with 10 times of NP-40 buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.5% NaCl and 50 mM NaF). The diluted lysates were applied to immunoprecipitation with anti-HA antibody and immunoblotting with anti-Flag antibody.

In vitro ubiquitin ligation assays.

The procedures for ubiquitin labeling were as described (Furukawa et al., 2003; Hu et al., 2004; Liu et al., 2002). Briefly, to purify substrate, GST-TSC2 was ectopically expressed in 293T cells, extracted in RIPA lysis buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% DOC and 0.1% SDS), and pulled down with Glutathione Sepharose™ 4B beads (Amersham Biosciences, Uppsala, Sweden) followed by 10 mM Glutathione elution. To purify CUL4A, CUL1, FBL6 and FBW5 ligases, Myc3-CUL4A, Myc3-CUL1, Myc3-FBL6 and Myc3-FBW5 immunocomplexes were immunoprecipitated using anti-Myc antibody from transfected 293T cells, immobilized on protein A–agarose beads and washed three times with an NP-40 buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.5% NaCl and 50 mM NaF) and twice with a ligase assay buffer (25 mM Tris-HCl at pH 7.5, 50 mM sodium chloride, 1 mM EDTA, 0.01% NP-40 and 10% glycerol).
Immobilized immunocomplexes were eluted by incubating with a molar excess of Myc peptide. For the \textit{in vitro} TSC2 ubiquitination, the different combination of CUL4A, CUL1, Fbw5 and Fbl6 immunocomplexes were mixed with GST-TSC2 substrate and the mixtures were added to an ubiquitin ligation reaction (final volume 30 μl) containing the following: 50 mM Tris-HCl at pH 7.4, 5 mM MgCl₂, 2 mM sodium fluoride, 10 nM okadaic acid, 2 mM ATP, 0.6 mM dithiothreitol, 12 μg bovine ubiquitin, 1 μg ubiquitin (Sigma), 60 ng E1 and 300 ng E2 (hUbc5c). Reactions were incubated at 30°C for 60 min, terminated by boiling for 5 min in an SDS sample buffer containing 0.1 M dithiothreitol and resolved on an SDS–PAGE gel before immunoblotting with the anti-GST antibody to examine ubiquitin ladder formation.

\textit{Drosophila} mutants

\textit{Cul4}\textsuperscript{11L}, \textit{Cul4}\textsuperscript{6P}, and \textit{Ddb1}\textsuperscript{PL12c} alleles were generated by imprecise excision of the \textit{P} element in \textit{Cul4}\textsuperscript{EP2518} and \textit{Ddb1}\textsuperscript{Y01408} chromosomes, respectively, and will be described in detail elsewhere. The \textit{Cul4}\textsuperscript{EP2518}, \textit{Ddb1}\textsuperscript{pic2}, \textit{Ddb1}\textsuperscript{S026316}, and \textit{Ddb1}\textsuperscript{Y01408} alleles were obtained from the Bloomington Stock Center. \textit{gig}\textsuperscript{192} was a kind gift of DJ Pan.

Quantification of mean eclosion day and percentage of eclosed flies.

\textit{DDB1}\textsuperscript{PL12c}/ TM3 Sb, \textit{Ser P[act-GFP]} females were crossed to either \textit{DDB1}\textsuperscript{pic2}/TM3 Sb, \textit{Ser P[act-GFP]} or \textit{DDB1}\textsuperscript{pic2} \textit{gigas/TM3} x \textit{DDB1}\textsuperscript{PL12c}/TM3 males, and \textit{Ddb1} mutant and heterozygous sibling progeny were counted. For mean eclosion day calculations, the first
day any fly from the culture eclosed established day 1, and the genotype of all flies were scored each day for 9 and 12 successive days at 25°C and 18°C, respectively. The experiment was performed in triplicate. A 95% Confidence Interval test was used to obtain the range of the true mean of the combined data at each temperature. Two tailed t-test assuming unequal variances was used to compare eclosion day data between different genotypes. The percentage of eclosed flies was calculated using the combined data at each temperature and different genotypes were compared using Fisher’s exact test.
Results

FBW5 binds to TSC1/2 and CUL4-DDB1

To identify the TSC2 ubiquitin ligase, a yeast two-hybrid screen was performed using TSC2 as bait by Stuart Shumway, a former Post-doc in the lab, and several potential TSC2 binding proteins were identified. Coupled immunoprecipitation and immunoblotting (IP-Western) analyses were then carried out to confirm these bindings in cells ectopically expressing TSC2 and individual prey proteins. Two F-box proteins, FBL6 and FBW5, were found to bind with TSC2 (Figure 4.1A and data not shown). Three additional control F-box proteins, SKP2, β-TrCP and FBX5, did not bind with TSC2 in the same assay (data not shown), indicating specificity of the FBL6-TSC2 and FBW5-TSC2 interaction and also suggesting that F-box motif is not sufficient for binding to TSC2. Consistent with TSC1-TSC2 dimerization, FBW5 also associates with TSC1 (Figure 4.1A). The genetic study in Drosophila (see below) conducted by our collaborator Robert Duronio led me to focus on determining the function and mechanism of the FBW5-TSC interaction. The functional significance of FBL6-TSC interaction remains to be determined.

Human FBW5 is a 60 kDa (566 residues) protein containing two recognizable domains; an N-terminal located F box motif and a WD40 repeat occupying most of the rest of the sequence. The F box motif functions to bridge a substrate protein to the CUL1-ROC1 E3 ligase via the SKP1 adaptor (Bai et al., 1996; Feldman et al., 1997; Skowyra et al., 1997). Recently, we and others found that DDB1 binds to a subset of
Figure 4.1 CUL4/DDB1, TSC1/2 and Fbw5 associate with each other

(A and B) 293T cells were transfected with plasmids expressing indicated proteins and protein-protein association was examined by coupled IP-Western analysis. (C) Endogenous TSC2-CUL4A association was determined by IP-Western analysis.
WD40 proteins, referred to as DWD proteins (also known as DCAF for DDB1- and CUL4 and DDB1-associated WD40 repeat proteins), and bridges them to CUL4-ROC1 to CUL4-associated factors or CDW to constitute a potentially large and distinct family of DWD-DDB1-CUL4-ROC1 E3 ubiquitin ligases (Angers et al., 2006; He et al., 2006; Higa et al., 2006b; Jin et al., 2006). FBW5 contains two recognizable DWD box between residues 436 to 451 and 484 to 499 (He et al., 2006), which led me to determine whether it can bind to and target TSC2 for ubiquitination by the DDB1-CUL4-ROC1 ligase.

I first examined the association between ectopically expressed TSC2 and DDB1-CUL4A complex in 293T cells. TSC2, as well as its partner TSC1, was readily detected in both DDB1 and CUL4A immunocomplexes (Figure 4.1B). Both TSC1 and TSC2 proteins were also readily detected in CUL4A immunocomplexes from un-transfected 293T or U2OS cells (Figure 4.1C), demonstrating association between endogenous TSC1-TSC2 and DDB1-CUL4A. The amount of TSC2 protein detected in CUL4A complexes is similar to or slightly greater than that detected by direct Western blotting using one tenth of the cell extract, suggesting that at least 10% of the total TSC2 is associated with DDB1-CUL4A in both 293T cells and U2OS cells. I therefore conclude that the DDB1-CUL4 ligase plays a direct and important role in regulating the TSC complex in vivo.

To determine which domain, the F-box or the WD40 repeats, is required for FBW5 to bridge TSC1-TSC2 to DDB1-CUL4A, Stuart generated a mutant FBW5, FBW5ΔF, with a
**Figure 4.2** F box is not required for bridging FBW5 to associate with DDB1-CUL4A.

293T cells transfected with plasmid expressing myc-tagged wild-type or mutant FBW5 deleting the F-box (FBW5ΔF), followed by P-Western analysis to examine protein complex formation.
deletion of the F box motif. FBW5ΔF mutant cannot bind to SKP1, but retains the ability to bridge TSC1 and TSC2 with DDB1-CUL4A (Figure 4.2 A and B), suggesting that the WD40 repeats in FBW5 mediate TSC2 binding to DDB1.

**FBW5-DDB1-CUL4 ubiquitinate and degrade TSC2 in mammalian cells**

To determine whether FBW5-DDB1-CUL4 is an E3 ubiquitin ligase for TSC2 and promotes TSC2 degradation, I co-expressed TSC2 with a series of individual components of different E3 ubiquitin ligases. Overexpression of FBW5 substantially reduced the steady state level of ectopically expressed TSC2 protein, while overexpression of another DWD protein, CDT2, or two F-box proteins, SKP2 and FBL6, did not exhibit any detectable effect on the level of TSC2 (Figure 4.3A). In the same assay, overexpression of CUL4A also reduced the level of TSC2 protein. Overexpression of ROC1 or DDB1 did not show any effect on TSC2 levels, suggesting that a sufficient amount of both proteins is present in the cells to support the ligase activity toward TSC2. Overexpression of CUL1 also noticeably reduced the level of TSC2, albeit to a lesser extent than CUL4A overexpression, raising the possibility that TSC2 may be regulated by an additional, CUL1-mediated E3. Consistent with the notion that FBW5ΔF is sufficient to bridge TSC2 to DDB1-CUL4A, overexpression of FBW5ΔF reduced TSC2 levels with a potency similarity to that of wild-type (Figure 4.3B). Degradation of TSC2 by FBW5 or FBW5ΔF was inhibited by the proteasome inhibitor MG132 (Figure 4.3B), indicating that FBW5-promoted TSC2 degradation is proteasome-dependent.
Figure 4.3 Overexpression of FBW5 decreases TSC2.

(A) HA-tagged TSC2 was co-transfected with plasmids expressing various indicated proteins into 293T cells. The steady state level of TSC2 protein was determined by direct immunoblotting. (B) FBW5 promotes TSC2 degradation in a proteasome-dependent mechanism. 293T cells were co-transfected with plasmids expressing indicated proteins and were either untreated or treated with proteasome inhibitor MG132. The steady state level of TSC2 protein was determined by direct immunoblotting.
Figure 4.4 A C-terminal domain of TSC2 is both required and sufficient for FBW5-promoted degradation.

Various TSC2 deletion mutants were co-transfected with FBW5 and the steady state level of TSC2 protein was determined by direct immunoblotting.
A series of deletion mutants of TSC2 were generated to map the region in TSC2 required for its degradation by FBW5-DDB1-CUL4A. All mutants containing amino acids from 1321 to 1765 were effectively degraded by the co-expression of FBW5, while all mutants missing this fragment were resistant to FBW5-promoted degradation (Figure 4.4C). These results suggest that fragment 1321-1765 of TSC2, which contains the Rap-GAP domain and is highly conserved during evolution, is necessary and sufficient to mediate TSC2 degradation by the FBW5-DDB1-CUL4 ligase.

To further demonstrate the function of FBW5, DDB1 and CUL4A/B in targeting TSC2 protein degradation, I blocked protein newly synthesis with cycloheximide treatment and determined the half-life of TSC2 protein. TSC2 is a short-lived protein with a half-life around 3 hours in control U2OS cells transfected with scrambled siRNA oligo (Figure 4.5A). Silencing either DDB1 or the combination of CUL4A and CUL4B increased the half-life of TSC2 to over 8 hours (the duration of the experiment), and silencing FBW5 increased the half-life of TSC2 protein to around 6 hours (Figure 4.5A). These results suggest that FBW5-DDB1-CUL4 is an active E3 ubiquitin ligase which continuously ubiquitinates TSC2 and promotes its degradation.

I next determined whether the FBW5-DDB1-CUL4-ROC1 ligase ubiquitinates TSC2. U2OS cells were co-transfected with plasmids expressing HA-tagged TSC2 and FLAG-tagged ubiquitin, and twelve hours later the cells were transfected with siRNA oligo
silencing *DDB1*, *FBW5* or a combination of *CUL4A/B*. TSC2 *in vivo* ubiquitination in these cells was analyzed by measuring the amount of HA-TSC2-FLAG-ubiquitin conjugates by IP/western. TSC2 protein is actively ubiquitinated and the level of TSC2 ubiquitination was substantially reduced by knocking down individually FBW5, DDB1 or CUL4A, with FBW5 knock down exhibiting the most pronounced effect (Figure 4.6A).

To demonstrate that the FBW5-DDB1-CUL4-ROC1 ligase directly promotes TSC2 ubiquitination *in vitro*, I immunoprecipitated CUL4A complexes from transfected 293T cells and used it as a source of E3 ligase. Incubation of purified GST-TSC2 substrate with CUL4A and FBW5 immunocomplexes resulted in robust TSC2 polyubiquitination in the presence of E1, E2-UBC5, ubiquitin and ATP (lane 1, Figure 4.7A). Omitting either CUL4A (lane 3) or FBW5 (lane 5) or substitution of FBW5 with FBL6 (lane 2) abolished GST-TSC2 polyubiquitination. To further confirm GST-TSC2 ubiquitination by the FBW5-DDB1-CUL4A-ROC1 ligase, we performed dropped out experiments (Figure 4.7B). Omitting individually E1 (lane 3), E2-UBC5 (lane 4), ATP (lane 5) and ubiquitin (lane 6) each eliminated GST-TSC2 polyubiquitination. Consistent with the finding that the F-box motif is not required for mediating TSC2 ubiquitination by DDB1-CUL4A, FBW5

Together, these results demonstrate that FBW5 recruits TSC2 to the DDB1-CUL4A-ROC1 ligase for polyubiquitination in vitro.
Figure 4.5 Ablation of CUL4, DDB1 or Fbw5 stabilizes TSC2.

(A) Silencing either DDB1, FBW5 or combination of CUL4A and CUL4B in mammalian cells increases the half-life of TSC2 protein. U2OS cells transfected with siRNA targeting indicated gene were treated with cycloheximide for different times. The steady state level of endogenous TSC2 as well as TSC1 was determined by direct immunoblotting. (B) Accumulation of dTsc2/Gigas protein in dCul4 or dDdb1 mutant. Total protein extracts were prepared from larvae of wild type, two dCul4 mutants and one dDdb1 mutant, and the steady state levels of dTsc2/Gigas protein were determined by direct immunoblotting.
Figure 4.6 *In vivo* ubiquitination of TSC2 by FBW5-DDB1-CUL4 ligase.

CUL4A, DDB1 and FBW5 are required for TSC2 ubiquitination *in vivo*. U2OS cells were co-transfected with plasmids expressing HA-TSC2 and FLAG-Ubiquitin and siRNA targeting CUL4A+CUL4B, DDB1 or FBW5. Cells were lysed 24 hours after transfection and TSC2 ubiquitination was analyzed by immunoprecipitating HA-TSC2 and blotted with an antibody recognizing FLAG.
Figure 4.7 *In vitro* ubiquitination of TSC2 by FBW5-DDB1-CUL4 ligase.

(A) In *vitro* ubiquitination of TSC2 by FBW5 and CUL4A. 293T cells were co-transfected with plasmids expressing indicated myc-tagged proteins. Individual protein complex was eluted by incubating with molar excess myc antigen peptide and mixed as indicated with substrate GST-TSC2 in the presence of E1, E2, ubiquitin and ATP. Reaction mixtures were incubated for 30 minutes, terminated by boiling directly in SDS-sample buffer, resolved by SDS-PAGE and immunoblotted with indicated antibodies. (B) Drop-out assay was carried out as described in (A) to determine the specificity of *in vitro* TSC2 ubiquitination.
**CUL4-DDB1 ubiquitinate and degrade TSC2 in Drosophila**

To seek further *in vivo* evidence for FBW5-DDB1-CUL4-mediated TSC2 degradation, my colleagues Bob Duronio, Sima Zacharek and Kate Lee turned to *Drosophila melanogaster* in which both the TSC-mediated cell growth control pathway and the DDB1-CUL4 E3 ligase are conserved. *Drosophila* also contains a gene, CG9144, encoding an F-box and WD40 protein that exhibits 40% identity and 58% similarity to human FBW5, and thus represents an FBW5 homologue. Sima generated and characterized a series of lethal mutations in the single *Cul4* and *Ddb1* genes (Zacharek et al. in preparation). *Cul4*\textsuperscript{6AP} and *Cul4*\textsuperscript{11L} were generated by P-element-mediated excision mutagenesis, and encode predicted proteins containing C-terminal deletions of 82 and 65 amino acids, respectively. *Cul4*\textsuperscript{11L} produces no protein and is therefore null while *Cul4*\textsuperscript{6AP} produces a stable truncated protein (Figure 4.5B). *Ddb1*\textsuperscript{SO26316} and *Ddb1*\textsuperscript{EY01408} are P-element insertion null alleles that produce no detectable protein (Figure 4.5B). Both *Cul4* and *Ddb1* null mutations cause growth arrest in the first (*Cul4*) or second (*Ddb1*) instar larvae, with the larvae capable of surviving for over 10 days on rich medium without growing (Figure 4.8A). Immunoblot analysis of whole larval extracts revealed that TSC2 protein dramatically accumulates in both *Cul4* and *Ddb1* mutants (Figure 4.5B). The *Cul4*\textsuperscript{11L/6AP} genotype results in the accumulation of more TSC2 protein than the *Cul4*\textsuperscript{6AP/6AP} genotype, suggesting that the truncated *Cul4*\textsuperscript{6AP} protein retains some function, although the growth arrest phenotype between the two allele combinations is the same. These results correlate TSC2 accumulation with the severity of Ddb1-Cul4 ligase impairment and provide
direct \textit{in vivo} evidence that DDB1-CUL4 ligase controls TSC2 protein turnover.

Sima also isolated and characterized viable Ddb1 hypomorphic alleles that cause growth defects. It was determined by complementation analysis that a previously defined locus termed \textit{piccolo} (\textit{pic}) was allelic to \textit{Ddb1}. Semi-lethal \textit{piccolo} mutants were originally characterized based on shared irregularities in bristle, wing, and tergite growth (Clark and Chovnick, 1985, 1986; Rushlow and Chovnick, 1984). This type of growth defect is reminiscent of phenotypes observed in hypomorphic \textit{myc} mutants and \textit{Minute} mutants, which are heterozygous for null alleles of ribosomal protein genes (Figure 4.8B). \textit{pic}^2 is an X-ray induced hypomorphic allele of \textit{Ddb1} encoding a G21$\rightarrow$D substitution that results in severely reduced Ddb1 protein levels (Zacharek et al. in prep.). Sima generated another hypomorphic allele of \textit{Ddbl} (\textit{Ddb1}^{pl12c}) by excision of the \textit{Ddb1}^{EY01408} P element located in the 5’UTR. \textit{Ddb1}^{pic2pl12c} mutants develop into viable adults, but these flies show a significant developmental delay and eclose on average 2 and 4 days later than control siblings at 25°C and 18°C, respectively (Figure 4.8C). In addition, these flies have cuticle defects including missing bristles and small bristles that resemble Min mutants (the piccolo phenotype; Figure 4.8B). Reduction of TSC2 gene dose by half substantially suppressed the late eclosion phenotype and the semi-lethality of \textit{Ddb1}^{pic2pl12c} mutants (Figure 4.8C), but not the bristle defects. These data suggest that TSC accumulation contributes to some of the growth defects caused by \textit{Ddb1} mutations, and also that Ddb1-Cul4 E3 ligases may have other targets.
A

B

C

<table>
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<th>Genotype</th>
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<tr>
<td></td>
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<td>% expected</td>
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<td>67</td>
</tr>
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<td>33</td>
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<td>67</td>
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<tr>
<td>Ddb1^{iso} gig1^{102} /Ddb1^{11L}</td>
<td>661</td>
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#P = 0.0001  *95% confidence interval  ^P < 0.05  $P > 0.05
Figure 4.8 Growth defects in *Drosophila Cul4* and *Ddb1* mutants.

(A) *Drosophila Cul4* mutant shows developmental arrest. The comparison of the first instar larvae of Cul4 heterozygosity Cul4^{11L/+} and homozygosity Cul4^{11L/11L}.

(B) *Drosophila Ddb1* mutant shows small or missing bristles. The comparison of the second instar larvae of wild type, Min and *Ddb1* mutant *Ddb1^{pic}.*

(C) Growth defects of *Cul4* and *Ddb1* mutants are rescued by heterozygosity of *gig* (*TSC2*). Total eclosed flies and mean eclosion time of *Ddb1^{pic} sib*, *Ddb1^{pic}/Ddb1^{pl12c}*, *Ddb1^{pic2}^{192} sib* and *Ddb1^{pic2}^{192}/Ddb1^{pl12c}* were measured at 18 °C and 25 °C.

These figures were made by Sima Zacharek and Kate Lee.
Discussion

In this study, I provided five lines of evidence supporting that CUL4-DDB1-FBW5-ROC1 ubiquitin ligase target tumor suppressor TSC2 for ubiquitination and degradation. First of all, CUL4, DDB1 and FBW5 associate with TSC and TSC2. Second, overexpression of FBW5 and CUL4A decrease the ectopically expressed TSC2 in cultured mammalian cells. Third, depletion of CUL4, DDB1 or FBW5 in cultured mammalian cells by RNAi increases the half-life of TSC2, and fly TSC2 is stabilized in Cul4 and Ddb1 mutants. Fourth, CUL4, DDB1 and FBW5 are required for the ubiquitination of TSC2 in vivo and in vitro. Finally, fly DDB1 hypomorphical alleles cause growth defects which can be rescued by reduction of TSC2 expression by half.

TSC2 is a central regulator of growth pathway and it is under precise regulation, mostly by phosphorylation. Previous studies have shown that TSC2 activity can be inhibited by the kinase AKT and enhanced by as many as five other kinases: GSK3, AMPK, RSK, ERK and MAPKAPK2 upon various signals. In this study, I showed that TSC2 can also be regulated by ubiquitin-proteasome pathway. However, the signal(s) beyond this regulation remains mysterious. TSC2 is a short-lived protein and it is turned over quickly, and the half-life of TSC2 can be increased by CUL4, DDB1 or Fbw5 RNAi (Figure 4.5A), so CUL4-DDB1-FBW5-ROC1 ubiquitin ligase might contribute to the constant degradation of TSC2 which does not need to be triggered by any signals. However, the steady state level of TSC2 does not change in CUL4, DDB1 or FBW5 RNAi treated cultured cells (data not shown). This could be due to a feed back loop involved by transcriptional and
post-translational regulation in cultured mammalian cells, which needs to be determined by further investigation. This negative feedback loop may not exist or be partially disrupted in the early developmental stage in fly, because the steady-state TSC2 accumulates in Cul4 and Ddb1 mutants.

The initial TSC2 yeast two-hybrid screen identified an F-box protein FBL6, whose binding with TSC2 was confirmed by IP-western (data not shown). The solid binding of FBL6 and FBW5 with TSC2 suggests that potentially there are three ubiquitin ligases targeting TSC2 for ubiquitination: CUL4-DDB1-FBW5, CUL1-SKP1-FBL6 and CUL1-SKP1-FBW5. Why TSC2 requires so many different ubiquitin ligases and under what circumstances these ligases play their roles needs to be further studied.

Both Cul4 and Ddb1 fly mutants show typical growth defects, such as developmental delay, late eclosion and small or missing bristles, which supports the notion that TSC2 is a substrate of CUL4-DDB1. Consistently, the developmental delay and late eclosion phenotypes of Ddb1 hypomorphical alleles can be rescued by reduction of TSC2 level by half. However, the small and missing bristle phenotype can not be rescued by TSC2 heterozygosity, which suggests the tissue specificity of TSC2 function.

Human FBW5 gene localizes at chromosome 9q34.3 subtelomeric gene-rich region. Submicroscopic deletion del(9)(q34.3) is a rare constitutional microdeletion syndrome with around 40 cases reported (Faraone et al., 2006; Gao et al., 2007; Hampshire et al., 2006; Harada et al., 2004; Iwakoshi et al., 2004; Kleefstra et al., 2006a; Kleefstra et al., 2006b; Kleefstra et al., 2005; Medlej-Hashim et al., 2002; Saar et al., 1999; Sanger et al., 2005;
Some clinical features of the del(9)(q34.3) syndrome have been related to the gene Euchromatin Histone Methyl Transferase1 (Eu-HMTase1) (Kleefstra et al., 2006a; Kleefstra et al., 2005), but other features need to be linked to other specific genes. Yatsenko et al. summarize the clinical features and corresponding genotypes of 15 del(9)(q34.3) patients (Figure 4.9). \textit{FBW5} gene localizes between two genes \textit{TRAF2} and \textit{COBRA1}, and according to Yatsenko et al., there are four clinical features could be linked to \textit{FBW5} gene: infections, brain anomaly, epilepsy or seizures and vascular and cardio anomalies. Whether these features are caused by \textit{FBW5} mutation or deletion needs to be further determined.
**Figure 4.9 Clinical features linked to 9q34.3 region.** (A) Physical map of the 9q34.3 region (top) including known markers and their megabase position. The microsatellite markers are ordered approximately to scale according to their physical location in the contig. Horizontal lines show respective locations of the FISH probes (below). Filled black dots above clone 48C7 represent fosmid clones. (B) Schematic representation of the 9q34.3 deletions in 15 patients determined by FISH and genotype analyses. Five individuals (KCL1, KCL2, KCL3, KCL4, KCL5) (Yatsenko et al., 2005) were characterised by FISH in the present study. Clinical and molecular data on 10 informative patients reported elsewhere include patients P2, P5–9, P12, (Stewart et al., 2004) and cases B, C, and E (Harada et al., 2004). The left part of the figure shows the extent of deletion in each patient. Non-deleted chromosome regions are represented by filled horizontal bars. The hatched bars identify either uninformative or inconclusive areas. The deleted regions in each patient are indicated by a double dashed line. On the right side of the figure the clinical features in the patients are given according to the initial letters of anomaly (Trigonocephaly, Genitourinary anomaly, Brain anomaly, Epilepsy or seizures, congenital Vascular and cardiac anomalies, Laxity of joints, Infection susceptibility, Craniofacial dysmorphism, Hypotonia, Obesity, Microcephaly, Speech impairment). +: feature present; –: feature absent; blank: feature not reported or not verified. (C) Phenotypic differences correlated with 9q34.3 deletion size. Open rectangles represent the proposed position of critical regions for individual clinical findings. The critical intervals defined are based on the subject with the particular phenotypic feature and the smallest deletion. Transcribed genes in the critical interval are represented by arrows; *EMHT1* is represented by a broken arrow to indicate alternative splicing. From Yatsenko, S.A., Cheung, S.W., Scott, D.A., et al. *Journal of medical genetics* 42, 328-335, 2005.
Chapter V

CONCLUSION AND PERSPECTIVES
When I began to study ubiquitin-proteasome pathway in Dr. Xiong lab around four years ago, there was little known about CUL4's function: neither the substrates nor the substrate-targeting mechanism. We knew, at that point, CUL4 is a member of cullin family proteins, there are two CUL4s in human: CUL4A and CUL4B, and CUL4A gene is amplified in human liver and breast cancers. Through the hard work of our lab and many other labs, the function of CUL4 is becoming clearer. The data presented here and the studies published by other labs enriched our knowledge of the substrates and the substrate-targeting mechanism of CUL4, in the meantime, they have also spurred a myriad of questions perplexing the field. The ideas described below are the results of the ongoing discussions in our lab and worth to be pursued in the future.

**How many substrates can CUL4 target?**

As already described, DDB1 is the most abundant binding protein in CUL4A complex (Figure 2.1), which associates with CUL4A nearly stoichiometrically. Up to now, there are nine substrates of CUL4 have been reported: CDT1, H2A, H3, H4, CSB, XPC, HOXA9, c-Jun and Spd1, among which CDT1, H2A, H3, H4, CSB, XPC, c-Jun and Spd1 are recruited to CUL4 through the adaptor protein DDB1. The targeting mechanism through which HOXA9 is recruited to CUL4 is not clear. My colleagues and I have described that CUL4-DDB1 could target multiple substrates through one of DWD (DDB1 binding WD40) proteins. Consistent with this notion, CDT1 and Spd1 have been reported to be recruited to CUL4-DDB1 through a DWD protein CDT2. Similarly, recruitment of
substrates H2A, H3, H4 and XPC have been linked to DWD protein DDB2, CSB has also been linked to CSA, and H3 has been linked to WDR5. It has been established that the SKP1 adaptor bridges an F-box protein to CUL1-dependent ligases (Bai et al., 1996; Feldman et al., 1997; Skowyra et al., 1997; Zheng et al., 2002a), a heterodimeric elongins B and C complex brings VHL (von Hippel-Lindau) box proteins and SOCS (suppressor of cytokine signaling) box proteins to CUL2-dependent and CUL5-dependent ligases, respectively (Kamura et al., 2001; Kamura et al., 2004; Kamura et al., 1998; Stebbins et al., 1999; Zhang et al., 1999), whereas CUL3 binds directly to the BTB domain (Broad-Complex C (BR-C), Tramtrack (Ttk) and Bric-a-brac) (Furukawa et al., 2003; Geyer et al., 2003; Pintard et al., 2003; Xu et al., 2003). The presence of numerous substrate specificity factors — mammals express more than 60 F-box, 40 SOCS and 200 BTB proteins — suggests that individual cullins may assemble into multiple E3 ligase complexes. We and other labs have shown that there are more than 100 DWD proteins in human, which suggests that CUL4-DDB1 could target at least 100 different substrates. An ongoing project in our lab is to test the interaction of CUL4-DDB1 and DWD proteins one by one and identify DWD protein associated proteins, through which the substrate pool of CUL4-DDB1 could be expanded.

WD40 proteins form a propeller-like structure, typically with seven blades, each composed of four anti-parallel β-sheets. There are >700 human WD40 repeat protein entries in the current PFAM database (http://www.sanger.ac.uk/Software/Pfam/index.shtml), corresponding to 320 unique genes after eliminating duplicates and truncated forms. A
16-residue stretch that we refer to as the DWD box (DDB1-binding and WD40 repeat) (Figure 5.1) is defined by three features. (1) The first 14 residues in the DWD box correspond to the second half of a WD40 repeat that is more conserved than other WD40 repeats. These 14 residues include three highly conserved residues, Asp7, Trp13, and Asp14; five hydrophobic residues (Ile, Leu, or Val) at positions 1, 2, 10, 12, and 15; and three small residues (Ala, Gly, Ser, or Thr) at position 3, 4, and 5. (2) Arg16 following the WD dipeptide is a signature residue of the DWD box. The crystal structure of the heterotrimeric G protein β1 subunit, a DWD protein, has been solved and shows that several conserved residues, including the Arg16, within the DWD box are located on the protein surface (Sondek et al., 1996), suggesting that these residues might participate in binding with other proteins. (3) A DWD protein usually contains one and sometimes two DWD boxes, but rarely three. According to the definition of the DWD box, Arg16 is the critical residue for the binding between DWD protein and DDB1, and we presented some data in the paper that mutation of Arg16 in CSA, PWP1 and APG16L disrupts or reduces the binding with DDB1. However, further study in our lab showed that mutation of Arg16 in some other DWD proteins, such as FBW5, WSB1, RBBP4 and GRWD1, did not change the binding with DDB1, suggesting that Arg16 is not the residue required for the binding. This discrepancy needs to be addressed by further study, especially crystal structural analysis.
Figure 5.1 Identification of a putative DDB1-binding motif in WD40 proteins, the DWD box. The DWD box is predicted to be present in >100 human WD40 repeat proteins.
Are CPSF160, SAP130 and Rik1 adaptors for CUL4?

If the primary sequence of DDB1 protein was aligned in NCBI database, no homologous sequence would be fished out. Neuwald A. and Poleksic A. combined a multiple alignment procedure based on hidden Markov models (HMMs) that incorporates rather specific structural features with a PSI-BLAST search (Altschul et al., 1997) initialized with a profile corresponding to the HMM alignment and identified three structural homologies of DDB1: CPSF160 (Cleavage and polyadenylation specificity factor 160 kDa subunit) and SAP130 (Spliceosome-associated protein 130) in human and Rik1 in fission yeast, which were all predicted to share β-propeller domains with DDB1 (Neuwald and Poleksic, 2000) (Figure 5.2).

Could CPSF160, SAP130 and Rik1, like DDB1, be adaptors for CUL4? To test this hypothesis, I determined the binding between ectopically expressed CPSF160/SAP130 and CUL4A/B using IP-Western assay which showed positive (data not shown). If CPSF160 and SAP160 are adaptors for CUL4, they might also use DWD proteins to target substrates. To test this possibility, Paula de Marval in our lab determined the binding between CPSF160 and a few DWD proteins, and she found that CPSF160 binds to DDB2, CSA, FBW5 and FBW8 (data not shown), suggesting that like DDB1, CPSF160 and SAP130 could target various substrates through different DWD proteins. As previously described in the chapter II, DDB1 is the major adaptor for CUL4, then if CPSF160 and SAP130 play a role as adaptors for CUL4, they must play a minor role or function under certain circumstances.
Figure 5.2 The propeller cluster structure of DDB1 family proteins.

(A) Structural domain arrangement of DDB1 as determined from the DDB1 crystal. The four domains of DDB1 are colored differently with the conventionally named top faces of each propeller indicated. The a-d strands of BPA’s blade 2 are individually labeled. (B) Schematic diagram of the secondary structure and domain arrangement of DDB1. The top surface loops of each propeller, which connect interblade d-a and b-c strands, are drawn in solid lines, whereas the bottom surface loops, which link intrablade a-b and c-d strands, are plotted in dash lines. (C) Domain architectures for UV-DDB-127 repeat proteins. Repeats are colored red proportional to their likelihood scores using lighter shades for less conserved repeats. Protein names are color coded according to families. The numbers of repeats are indicated in parentheses. (A) and (B) are from Li T. et al. Cell. **124**:105-17, 2006; (C) is from Neuwald A. and Poleksic A. Nucleic Acids Research. **28**: 3570-3580, 2000.
Although DDB1, CPSF160 and SAP130 have been shown to bind DWD proteins ectopically, the endogenous bindings and the physiological relevance of these bindings need to be further determined. The subsequent question is whether DDB1, CPSF160 and SAP130 bind the whole pool of the DWD proteins or only subset of it. Addressing these questions could greatly enrich our knowledge on the function of CUL4.

**Important potential substrates of CUL4**

Null mutation of *DDB1* gene in mouse causes early embryonic lethality (Cang et al., 2006; Cang et al., 2007). Conditional inactivation of *DDB1* in brain, lens and epidermis leads to genomic instability, aberrant cell cycle and elimination of proliferating progenitor cells. Considering the myriad substrates of the CUL4-DDB1 ubiquitin ligases, the phenotypes of *DDB1* null mice could be the result of malfunction of multiple cellular processes. Consistent with previously published papers, several reported substrates of CUL4-DDB1, Cdt1, c-Jun and p27 are accumulated in *DDB1* null mice. p53 and p21, two proteins which are not supposed to be the substrates of CUL4-DDB1, are also up-regulated in *DDB1* null epidermis. *DDB1*/- phenotype can be partially rescued by crossing with p53-/-. p53 accumulation, however, might be an indirect effect of *DDB1* inactivation, which could be caused by genomic instability and aberrant cell cycle. Nevertheless, the study in our lab showed that p53 is readily detectable in CUL4A immunocomplex (data not shown). p53 has been reported to be targeted for ubiquitination by COP1, a WD40 protein which associates with CUL4-DDB1-DET1 complex (Dornan et al., 2004). A reasonable
hypothesis is that one of the CUL4-DDB1 ubiquitin ligases targets p53 for ubiquitination, probably through COP1 or one of DWD proteins. COP1 is composed of a RING finger domain and a WD40 repeat, and its catalytic activity could be boosted by recruiting another RING finger protein, ROC1, through CUL4-DDB1-DET1 complex. These hypotheses are worth to be tested in the future and would strengthen our understanding on CUL4 function, cell cycle regulation and apoptosis. p21, on the other hand, is more likely to be an authentic substrate of CUL4-DDB1, because p21 is accumulated in both DDB1-/- and DDB1-/- p53-/- double null mice, suggesting that p21 up-regulation is not due to transcriptional enhancement. One way to find out which DWD protein recruits p21 to CUL4-DDB1 is to test the bindings between p21 and DWD proteins one by one. This method may look tedious, but could be very effective, and is definitely worth to try.

Hijacking CUL4-DDB1 by viral proteins

So far, there are three viral proteins have been reported to hijack CUL4-DDB1: paramyxovirus V protein (Andrejeva et al., 2002; Li et al., 2006b; Lin et al., 1998; Precious et al., 2005; Ulane et al., 2005; Ulane et al., 2003), hepatitis B virus X protein (Bergametti et al., 2002; Bontron et al., 2002; Leupin et al., 2005; Leupin et al., 2003; Sitterlin et al., 2000) and HIV1 Vpr (Le Rouzic et al., 2007; Schrofelbauer et al., 2007). The mechanism through which paramyxovirus V protein hijacks CUL4-DDB1 is rather clear: V protein plays a role as a substrate-targeting molecule to recruit STAT1/2/3 to CUL4-DDB1 and promote their ubiquitination, thereby suppresses the immune response of the host (Precious et al.,
However, the functions of hepatitis B virus X protein and Vpr still remain elusive. Chad McCall, a graduate student in our lab, identified a Vpr binding protein (VprBP) in CUL4A immunocomplex. VprBP is a DWD protein and it has been shown to bind Vpr directly through direct yeast-two hybrid assay (Zhang et al., 2001). In a recently published paper, Schrofelbauer B. et al. identified a large amount of DDB1 from Vpr immunoprecipitation (Schrofelbauer et al., 2007). The exact function of Vpr hijacking CUL4-DDB1 is still not clear. Vpr has been shown to cause UNG2 (uracil-DNA glycosylase) degradation (Schrofelbauer et al., 2005), G2-arrest and apoptosis (Jowett et al., 1995; Nishizawa et al., 2000; Shostak et al., 1999), all of which have been shown to be dependent on CUL4-DDB1 (Le Rouzic et al., 2007; Schrofelbauer et al., 2007). The role of VprBP in CUL4-DDB1-Vpr complex and the molecular basis for G2-arrest and apoptosis are being pursued in our lab currently.
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