Insights into the Regulation of the CRL4<sup>DTL</sup>

Ubiquitin Ligase

by

Crystal Jayne Lee

B.S. Molecular Biology
University of California, San Diego, 2003

SUBMITTED TO THE DEPARTMENT OF BIOLOGY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTORATE OF PHILOSOPHY

AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

February 2012

© 2012 Massachusetts Institute of Technology
All rights reserved

Signature of Author.............................

Crystal Jayne Lee
Department of Biology
November 1, 2011

Certified by........................................

Jacqueline A. Lees
Professor of Biology
Thesis Supervisor

Accepted by........................................

Alan D. Grossman
Professor of Biology
Chair, Biology Graduate Committee
Insights into the Regulation of the CRL4\textsuperscript{DTL} Ubiquitin Ligase

by

Crystal J. Lee

Abstract

The eukaryotic mitotic cell cycle is a strictly ordered process by which cells accurately duplicate their genome and divide into two. Ubiquitin-mediated degradation of key cell cycle regulators ensures that the cell cycle phases progress in a unidirectional and orderly manner. Cullin E3 ubiquitin ligases (CRLS) comprise a large family of multi-subunit complexes that selectively recruit substrates via a substrate receptor and facilitate substrate ubiquitination and degradation. The CRL4\textsuperscript{DTL} (Cullin 4 RING ligase, in association with the substrate receptor DTL/Cdt2/RAMP) ubiquitin ligase has recently emerged as a key regulator of cell cycle progression and genome integrity. Identified substrates CRL4\textsuperscript{DTL} play critical roles in S phase progression, replication, DNA repair processes, transcription, and chromatin regulation. CRL4\textsuperscript{DTL}-mediated targeting is restricted to S phase and after DNA damage through a PCNA-dependent mechanism. Recent studies have focused on elucidating the requirements within the substrates that dictate CRL4\textsuperscript{DTL}-mediated degradation. The majority of identified substrates have a specialized PCNA interaction peptide motif (PIP box) that distinguishes the substrates from the stable PIP box-containing proteins and couples interaction with chromatin-bound PCNA with CRL4\textsuperscript{DTL} recruitment.

Very few studies have explored the regulation of the substrate receptor DTL in the context of CRL4\textsuperscript{DTL} ligase activity. DTL contains multiple WD40 repeats in the N-terminus that are very highly conserved and a less conserved C-terminus that may have important regulatory function. We characterize DTL regulation during the cell cycle: DTL itself is degraded in an ubiquitin-dependent manner and degradation is dictated by an unidentified C-terminal determinant. DTL is also phosphorylated in the C-terminus. Here, we present the first study to directly examine the contribution of the C-terminus to CRL4\textsuperscript{DTL} ligase activity in the context of live cycling mammalian cells. We find that the DTL N-terminus can interact with substrates whether or not the substrates have bound PCNA. Importantly, we find that elements within the C-terminus are not required for CRL4\textsuperscript{DTL} ligase assembly, substrate recognition, and substrate degradation during S phase and after DNA damage.

Thesis Supervisor: Jacqueline A. Lees
Title: Professor of Biology
Acknowledgements

So many people have contributed in so many ways to my thesis and my experience as a graduate student. I must first thank my advisor, Jackie Lees, for her guidance and constant support over all these years. Thanks for sparking excitement from the very first day we met, for always being encouraging and excited about discussing data, and for helping me perform $^{31}P$ experiments. I would like to thank the members of my thesis committee, Steve Bell and Angelika Amon, for making my committee meetings so productive, keeping me on track, and helping me grow as a scientist. I also must thank Nancy Hopkins, who has supported my research and is endlessly fascinated by science.

I am so grateful to Chris Sansam, for being an incredible mentor, an amazing scientist, and a great baymate. Thank you for all your scientific guidance and teaching me how to be a graduate student. I want to thank Nelly Cruz, my classmate, labmate, baymate, and friend. Thanks for being there for this whole process with me! To Kurt Krummel, who was the first person to make me realize I could go to graduate school and get my Ph.D. Thanks for being a great boss, a teacher, and now, an awesome buddy.

To the rest of the Lees lab ladics! I couldn’t have survived the long timecourses, weekend tissue culture, and moving into a new building without you all. I hope I still get invited to social events! Tiziana- thanks for being just the best everything, for your words of wisdom, and for fighting to get that Nespresso machine. Keren, thanks for teaching me about sarcasm. Simona, your organizational powers are ever-inspiring. Mindy- I admire your running and work ethic. Amy- bonnie lassie, hearing your accent just lifts my spirits. Melissa- the best UROP with the most inspiring lab attitude. Ale- thanks for your dancing and singing. And Ali- for good talks and good laughs. Thanks to the entire CCR 5th floor and the Koch 4th floor, for providing a great scientific environment.

I have to thank my classmates, because I would not have survived the first year without them, especially Gina, Dan, Jadyn, and Christian. My dearest friends Jamie Newman and Kate Moreau: I really wouldn’t have made it without wine and cheese, popcorn, laughter and/or tears over the last 6 years. I’m so fortunate to have found such fun beautiful people to help me through some of the hardest days, to listen to me vent, and to watch trashy reality TV with. Jamie- thanks for all the coffee breaks and making me a “morning” person. Kate- it all started from epitope. I know the three of us will be dear friends until we’re as old as Kate’s Nana.

My friends from home, Tracy, Kathy, Caroline, and Emily, were the best long-distance support system a California girl could ask for. Thanks for calling, texting, gchatting, and occasionally visiting. I’m glad that California doesn’t feel too far away.

I am forever indebted to my family for being my rocks in life and for endlessly inspiring me to work and play hard. Thanks Mom, for giving me such a great work ethic. I live to make you proud. Thanks Dad, for bringing me to lab when I was young, and for wanting to do organic chemistry problems with me in college. I aspire to one day to be as good as a person, employee, and parent as you are. To my brother, Nathan, I’m glad that we are still 9 and 12 when we are together. You always know how to make me laugh. Thanks to my Aunt Sheric who sent me lovely handwritten letters and cards throughout my graduate studies.

And finally. To my Andreas. Thanks for putting up with my lab-induced mood swings for the last 6 years and loving me through it all. You are a constant inspiration to me. I am a better person because of you: you make me want to learn more, ask more questions, demand more from life and from myself. Thank you for always believing in me and I cannot wait to spend the rest of my life with you.
Dedication

I dedicate this thesis to my family.
# Table of Contents

**ABSTRACT** ........................................................................................................................... 3  
**ACKNOWLEDGEMENTS** ........................................................................................................ 5  
**DEDICATION** ......................................................................................................................... 6  
**TABLE OF CONTENTS** ....................................................................................................... 7  
**TABLE OF FIGURES AND TABLES** .................................................................................... 10  
**BIOGRAPHICAL NOTE** ....................................................................................................... 13  

## CHAPTER I: INTRODUCTION ............................................................................................. 15  
**THE EUKARYOTIC CELL CYCLE** .................................................................................. 16  
**UBIQUITIN-PROTEASOME SYSTEM** .............................................................................. 17  
  - Ubiquitination cascade ........................................................................................................ 18  
  - Cullin-RING E3 Ubiquitin Ligases ..................................................................................... 20  
  - CRL Architecture .............................................................................................................. 20  
  - Regulation of CRL activity ................................................................................................. 22  
  - CRL substrate recognition ............................................................................................... 25  
**CRL4<sup>DTL</sup>: REGULATOR OF CELL CYCLE PROGRESSION AND GENOME STABILITY** .................................................................................................................. 26  
  - CRL4<sup>DTL</sup> architecture ............................................................................................. 26  
  - Identification of DTL as a CRL4 substrate receptor .......................................................... 29  
  - Physiological consequences of DTL loss ......................................................................... 30  
  - Cdt1 destruction by CRL4<sup>DTL</sup> prevents rereplication .................................................. 31  
  - Substrates of the CRL4<sup>DTL</sup> ligase .............................................................................. 34  
**REGULATORY MECHANISMS OF CRL4<sup>DTL</sup> LIGASE ACTIVITY** .................................. 37  
  - PCNA-dependent substrate targeting ............................................................................... 38  
  - PCNA couples CRL4<sup>DTL</sup> activity to S phase and after DNA damage ....................... 38  
  - Identification of the PIP degron ......................................................................................... 39  
  - Assembly of the PCNA-substrate-CRL4<sup>DTL</sup> complex .................................................. 42  
  - Current Model for PIP degron-dependent degradation by CRL4<sup>DTL</sup> ......................... 44  
  - Additional mechanisms that regulate CRL4<sup>DTL</sup> activity ........................................... 45  

## CHAPTER II: THE C-TERMINUS OF DTL IS NOT REQUIRED FOR CRL4<sup>DTL</sup> SUBSTRATE REGULATION ........................................................................................................... 49  
**SUMMARY** ......................................................................................................................... 50  
**INTRODUCTION** ................................................................................................................. 51  
**RESULTS** ........................................................................................................................... 54  
  - Identification of a putative PIP box in the C-terminus of DTL ........................................ 54  
  - Generation of a DTL mutant lacking the less conserved C-terminus ............................... 57  
  - DTL C-terminus is not required for CRL4<sup>DTL</sup> ligase assembly ................................... 59  
  - DTL N-terminus binds to substrates Cdt1 and Set8 ........................................................ 60  
  - Generation of tet-inducible stable cell lines expressing DTL C-terminus mutant ........... 61  
  - DTL C-terminus is not required for normal cell cycle progression .................................. 63  
  - DTL C-terminus is not required for Cdt1 and Set8 degradation during S phase or after DNA damage ......................................................................................................................... 66  
**DISCUSSION** ....................................................................................................................... 69  
**EXPERIMENTAL PROCEDURES** .................................................................................... 71  
  - Cell culture, Drugs, and Transfection ............................................................................. 71
Plasmids ........................................................................................................................ 71
Generation of Stable pCW Tet-inducible Cell Lines ....................................................... 73
Immunoprecipitation ........................................................................................................ 73
Western Blot Analysis .................................................................................................... 74
Antibodies ....................................................................................................................... 74
FACS ............................................................................................................................... 74
Immunofluorescence ....................................................................................................... 75
ACKNOWLEDGEMENTS ................................................................................................. 75

CHAPTER III: FINAL DISCUSSION .............................................................................. 77
KEY CONCLUSIONS ...................................................................................................... 78
ASSEMBLY OF AN ACTIVE CRL4DTL UBIQUITIN LIGASE ...................................... 79
REVISITING THE ROLE OF PCNA ............................................................................... 80
IDENTIFICATION OF ADDITIONAL SUBSTRATES .................................................... 81
TARGETING CRL4DTL FOR THERAPEUTIC POTENTIAL ....................................... 82

APPENDIX A: CHARACTERIZATION OF DTL, THE SPECIFICITY FACTOR OF THE CRL4 LIGASE, DURING THE NORMAL CELL CYCLE ............................................................................ 85
SUMMARY ...................................................................................................................... 86
INTRODUCTION ............................................................................................................. 87
RESULTS ........................................................................................................................ 88
DTL protein is cell cycle regulated and post-translationally modified during normal cell cycle progression ................................................................................................................. 88
DTL interacts with DDB1 throughout its expression ...................................................................... 91
DTL is ubiquitinated and degraded in a proteasomal manner .................................................... 92
CRL4 ubiquitin ligase does not regulate DTL during mitosis ................................................. 94
APC complex does not regulate DTL during mitosis ......................................................... 96
DTL is post-translationally modified by phosphorylation ...................................................... 98
Mass spectrometry identifies several phosphorylation sites within DTL ............................... 100
Truncation mutants reveal multiple phosphorylation sites in the C-terminus ......................... 103

DISCUSSION .................................................................................................................. 106
Implications of DTL degradation in mitosis ........................................................................ 107
Coordination between phosphorylation and degradation ..................................................... 108
DTL phosphorylation sites and localization ......................................................................... 111
Identifying kinases that phosphorylate DTL ....................................................................... 113

EXPERIMENTAL PROCEDURES ................................................................................. 114
Cell culture, cell cycle synchronization, and FACS analysis ............................................. 114
Plasmids, siRNA, and transfections .................................................................................. 114
Generation of DTL mutants ............................................................................................ 115
Chromatin Fractionation and Phosphatase Assay ............................................................ 116
Immunoprecipitation ....................................................................................................... 116
Western Blot Analysis and Antibodies ............................................................................... 117
Immunofluorescence ....................................................................................................... 118
Mass spectrometry ........................................................................................................ 118
2D Phosphopeptide Mapping ......................................................................................... 118
ACKNOWLEDGEMENTS ............................................................................................... 119
Table of Figures and Tables

CHAPTER I: INTRODUCTION ............................................................................. 15

FIGURE 1. OVERVIEW OF THE UBIQUITIN-PROTEASOME PATHWAY .............. 19
FIGURE 2. ARCHETYPAL CULLIN RING E3 UBIQUITIN LIGASE (CRL) STRUCTURE .... 21
FIGURE 3. CAND1/NEDDYLATION/DENEDDYLATION REGULATES CRL LIGASE ACTIVITY .............................................................. 24
FIGURE 4. ARCHITECTURE OF CRL4 LIGASES AND DTL ............................ 28
FIGURE 5. CD T1 IS REQUIRED FOR REPLICATION ORIGIN LICENSING .......... 32
TABLE 1. CRL4DTL SUBSTRATES IDENTIFIED SO FAR ............................... 35
FIGURE 6. IDENTIFICATION OF THE PIP DEGRON ...................................... 41
FIGURE 4. CURRENT MODEL OF PCNA-CROMATIN-DEPENDENT DEGRADATION OF CRL4DTL SUBSTRATES .................................... 44

CHAPTER II: THE C-TERMINUS OF DTL IS NOT REQUIRED FOR CRL4DTL SUBSTRATE REGULATION ......................................................... 49

FIGURE 1. C-TERMINAL PIP BOX DOES IS NOT REQUIRED FOR CRL4DTL ACTIVITY .......................................................... 55
FIGURE 2. DTLAC IS NOT PHOSPHORYLATED, LOCALIZES TO THE CYTOPLASM, AND INTERACTS WITH THE CRL4 COMPLEX ..................... 58
FIGURE 4. TET-INDUCIBLE GFP-AC EXPRESSING CELLS CAN RESCUE THE G2/M CHECKPOINT ........................................................ 62
FIGURE 5. AC-EXPRESSING CELLS PROLIFERATE WITH THE SAME KINETICS AS WT ......................................................... 65
FIGURE 6. DC CAN DEGRADE CD T1 AND SET8 DURING S PHASE AND AFTER UV DAMAGE ......................................................... 68

CHAPTER III: FINAL DISCUSSION .................................................................. 77

APPENDIX A: CHARACTERIZATION OF DTL, THE SPECIFICITY FACTOR OF THE CRL4 LIGASE, DURING THE NORMAL CELL CYCLE ................................................................. 85

FIGURE 1. DTL PROTEIN LEVELS ARE CELL CYCLE REGULATED ..................... 90
FIGURE 2. DTL IS UBIQUITINATED AND DEGRADED IN A PROTEASOMAL-DEPENDENT MANNER ................................................................. 93
FIGURE 3. DTL IS NOT DEGRADED BY THE CRL4 LIGASE ............................ 95
FIGURE 4. DTL IS NOT DEGRADED BY THE APC .......................................... 97
FIGURE 5. DTL IS PHOSPHORYLATED .......................................................... 99
FIGURE 6. IDENTIFICATION AND FUNCTIONAL ANALYSIS OF DTL PHOSPHORYLATION SITES ............................................................... 102
FIGURE 7. C-TERMINAL TRUNCATION DTL MUTANTS REVEAL PHOSPHORYLATED REGIONS ................................................................. 105
FIGURE 8. AC MUTANT IS NOT DEGRADED LIKE WT .................................. 110

APPENDIX B: DTL LOSS CAUSES A ZEBRAFISH-SPECIFIC DAMAGE-INDUCED G2/M CHECKPOINT ................................................................. 121
FIGURE 1. DTL siRNA knockdown causes G2/M arrest in nonirradiated cells, but does not cause the IR-induced G2/M checkpoint defect.................................124
FIGURE 2. Co-depletion of Cdt1 or p21 does not reveal an IR-induced G2/M checkpoint defect. ......................................................................................................126
FIGURE 3. Nocodazole trapping experiment reveals loss of DTL causes mitotic delay defect.................................................................................................................128
REFERENCES.................................................................131
Biographical Note

Education

Ph.D. Massachusetts Institute of Technology, Department of Biology
Expected 2011 Cambridge, MA

B.S. University of California, San Diego
June 2003 La Jolla, CA
Major: Molecular Biology, Revelle College Provost's Honors

Research and Professional Experience

2006-2011 Graduate Research Assistant, MIT Department of Biology
Laboratory of Jacqueline A. Lees, Koch Institute for Integrative Cancer Research, Cambridge, MA

2010-2011 Intern, MIT Technology Licensing Office
MIT, Cambridge, MA

Jan, 2010 MIT Externship, Fish & Richardson, LLP
Boston, MA

2003-2005 Research Assistant, The Salk Institute
Laboratory of Geoff Wahl
La Jolla, MA

2000-2002 Undergraduate Assistant, UC San Diego
Laboratory of Martin Haas
La Jolla, MA

Summer 2000 Summer Intern, Cohesion Technologies, Inc.
Mountain View, CA

Publications


Chapter I: Introduction
The Eukaryotic Cell Cycle

All life depends on the ability of a cell to accurately duplicate its genome and successfully divide into two. This cycle of replication and division, referred to as the mitotic cell cycle, is strictly ordered by complex signaling pathways to ensure each phase is completed before proceeding to the next. The cycle is divided into four distinct phases: G1, S, G2, and M phase. DNA replication occurs during S phase, and segregation of the replicated genome and cell division occurs during M phase, or mitosis. Two gap phases (G1 and G2) separate S and M phases to allow for cell growth and preparation for the following phase. If cells in G1 do not receive the appropriate signals to divide, they may exit the cell cycle and enter a distinct quiescent state, known as G0. The majority of non-growing and non-proliferative cells in the human body remain in this resting state. Quiescent cells that receive signals to divide pass through the restriction point in mammalian cells (also known as START in yeast) and enter the cell cycle in G1 or before S phase. Once the cells have passed this point, they are committed to DNA replication and cell division.

An early study in dividing sea urchin embryos revealed a striking observation that one protein was synthesized but then rapidly destroyed just prior to each cell division (Evans et al. 1983). This observation was a critical starting point to the understanding that protein expression and proteolysis are tightly coordinated to ensure the precise ordering of cell cycle events. The protein was named cyclin B in reference to its periodic expression. Cyclins are a family of structurally related proteins that serve as the positive regulatory subunits of the cyclin-dependent-kinases (CDKs). Kinase activity of specific cyclin-CDK combinations is restricted to certain cell cycle stages, largely through proteolysis of the cyclin subunit. Activating or inhibitory phosphorylations and interaction with CDK inhibitor proteins also contribute to cyclin-CDK regulation (Lew and Kornbluth 1996; Malumbres and Barbacid 2009; Satyanarayana and Kaldis 2009). Further investigation of cyclin B degradation led to the discovery of the Anaphase...
Complex/Cyclosome (APC/C), which tags cyclin B with ubiquitin for subsequent degradation by the proteasome (King et al. 1995; Sudakin et al. 1995). Failure to degrade cyclin B blocks the metaphase-anaphase transition and prevents cytokinesis (Wheatley et al. 1997; Chang et al. 2003).

The APC/C is one of many multi-subunit protein complexes, called ubiquitin ligases, that targets important cell cycle regulators for destruction to ensure that key cell cycle events in each phase occur prior to the next phase. The relative timing of cell cycle events is extremely important: a cell must not replicate its genome more than once before dividing, and a cell must not separate its chromosomes before they have been fully replicated. Many cell cycle regulators are regulated by ubiquitin-mediated proteolysis at specific cell cycle phases. Importantly, failure to degrade these cell cycle regulators can be extremely deleterious for the cell, leading to abnormal cell cycle progression or cell cycle arrest. The ubiquitin ligase CRL4 (Cullin 4 RING Ligase), in association with a substrate recognition factor called Denticleless (DTL, also known as Cdt2), has been the subject of much attention due to its emerging importance in regulation of the cell cycle and maintenance of genome stability. The work of this thesis focuses on elucidating the regulation of the DTL substrate receptor and the determinants within DTL that facilitate substrate recognition and degradation.

**Ubiquitin-Proteasome System**

The proteolysis of proteins is critical for many biological processes, such as cell cycle regulation, signal transduction, cellular homeostasis, and embryonic development. The ubiquitin-proteasome system tags proteins for degradation by attaching a highly conserved and abundant 76 amino acid protein called ubiquitin to the targeted protein. Ubiquitinated proteins are recognized by the 26S proteasome and subsequently degraded. Destruction of specific cell cycle regulators at precise points in the cell cycle ensures the orderly progression through the cell cycle.
Ubiquitination cascade

Covalent attachment of ubiquitin to target proteins, or substrates, occurs through a three-step cascade. First, ubiquitin is activated through the formation of a thiol-ester bond with the E1 ubiquitin-activating enzyme in an ATP-dependent reaction. The activated ubiquitin is then transferred to the E2 ubiquitin conjugating enzyme, again via formation of a thiol-ester bond. Finally, the E3 ubiquitin ligase recruits the E2 and specific substrates and catalyzes the conjugation of ubiquitin to the substrate (Nakayama and Nakayama 2005) (Figure 1). An isopeptide bond is formed between the C-terminal glycine of ubiquitin and the ε-amino group of a lysine residue within the substrate (Ciechanover et al. 1980).

The configuration of the attached ubiquitins results in different consequences for the ubiquitinated protein. Monoubiquitinated substrates have only a single ubiquitin attached and are not recognized by the proteasome for degradation. Instead, monoubiquitination alters the function, localization, or binding partners of the target protein (Hicke 2001). Substrates are polyubiquitinated when additional ubiquitins are added via conjugation to lysines of the previously attached ubiquitin to form polyubiquitin chains. Targets with polyubiquitin chains linked through conjugation to the lysine 48 (K48) of each ubiquitin are recognized by the 19S regulatory particle of the proteasome (Verma et al. 2004). The ubiquitin chains are removed and recycled, while the protein is subsequently unfolded and directed into the 20S particle for proteolytic digestion. All seven lysines in ubiquitin can be used as ubiquitin acceptors to create alternative polyubiquitin chain linkages that change protein activity or localization. For example, proteins modified by K63-linked ubiquitin chains participate in nonproteolytic signaling or intracellular trafficking (Li and Ye 2008). Current efforts are being directed at decoding how the different chain linkages control the fate of the protein.
Figure 1. Overview of the ubiquitin-proteasome pathway.

Ubiquitin is a small protein, 76 amino acids, or about 8 kDa. It is transferred to an E1 activating enzyme via an ATP-dependent reaction. The activated ubiquitin is then transferred to the E2 ubiquitin conjugating enzyme, which often interacts with the substrate-recruiting E3 ubiquitin ligase. Finally, the ubiquitin is covalently attached to the target substrate, via an isopeptide bond. Formation of a polyubiquitinated chain (via lys 48 linkage) signals recognition by the 26S proteasome for degradation. The ubiquitin molecules are then recycled back through the ubiquitin-proteasome pathway. (Reprinted by permission from Nagayama et al. 2006.)
Cullin-RING E3 Ubiquitin Ligases

CRL Architecture

Cullin-RING E3 ubiquitin ligases (CRLs) are large multi-subunit complexes and constitute the largest category of E3 ubiquitin ligases. The central component of CRL complexes is a large cullin scaffold protein that links an E2-recruiting RING finger protein to the substrate recruiting components. Cullins are a family of evolutionarily conserved proteins; there are seven cullins (Cul 1, 2, 3, 4A, 4B, 5, and 7) and two distantly related cullin-like proteins (Cul 9 or PARC and APC2). RING finger proteins, such as Roc1 and Roc2 (Ring of Cullins, also known as Rbx1/Hrt1 and Rbx2/Hrt2), recruit the E2 ubiquitin-conjugating enzymes through their RING finger domain and bind to a highly conserved region in the cullin C-terminus (Skaar et al. 2007; Jackson and Xiong 2009). The N-terminus of the cullin interacts with an adaptor subunit, which in turn binds a substrate receptor. The substrate receptor contacts the targeted protein, bringing the substrate in close proximity to the associated E2 to facilitate ubiquitination (Figure 2). Each adaptor subunit can associate with a family of structurally related substrate receptors, and each substrate receptor targets a distinct subset of substrates. In this manner, a unique ubiquitin ligase is assembled for every specificity factor, thereby enabling each cullin-RING-adaptor-core the ability to target a wide array of substrates.
Figure 2. Archetypal Cullin RING E3 Ubiquitin Ligase (CRL) structure.

Schematic of the archetypal CRL structure. The N-terminus of the cullin protein interacts with a RING finger protein, which is responsible for recruiting the E2 ubiquitin conjugating enzyme. The C-terminus of the cullin binds to substrate recognition proteins, such as an adaptor protein that binds the substrate receptor. A single cullin catalytic core can often bind a family of structurally related substrate receptors. Each substrate receptor can assemble with the cullin-RING core to generate many unique CRLs. Each CRL in turn recognizes a distinct subset of substrates, therefore conferring diverse substrate specificity to a single cullin-RING core.
Regulation of CRL activity

Cullin proteins are modified by the covalent attachment of a small ubiquitin-like protein, Nedd8 (Neural precursor Expressed Developmentally Down-regulated protein 8), to enhance ubiquitin ligase activity. Nedd8 is conjugated to a conserved lysine present in the C-terminus of all cullins in a three-step enzymatic cascade similar to ubiquitination, called neddylation (Wada et al. 1999). Neddylation increases CRL activity in two ways: by promoting recruitment of some E2s through direct binding between Nedd8 and the E2 (Sakata et al. 2007), and by inducing conformational changes in the cullin to position the E2 and substrate in closer proximity for more efficient ubiquitination (Ohh et al. 2002; Duda et al. 2008).

The COP9 signalosome (CSN) is an eight-subunit complex that binds to CRLs to remove Nedd8 in a process called deneddylation. Nedd8 is cleaved from the cullin specifically by the Csn5 subunit, which has metalloprotease activity. Counterintuitively, CSN inactivation causes a reduction in CRL activity despite an increase in neddylated CRLs. The decrease in CRL activity is attributed to autoubiquitination and subsequent degradation of substrate receptors (Cope and Deshaies 2003). The CSN has deubiquitinating activity, perhaps through direct interaction with deubiquitinating enzymes, which counteracts the substrate receptor autoubiquitination (Cope and Deshaies 2003; Zhou et al. 2003). Therefore, CSN binding is critical for sustaining CRL activity. Substrate receptors of CRLs that are not bound to the CSN can be autoubiquitinated and degraded, thus freeing the CRL core to reassemble with new substrate receptors to target different substrates. Mechanisms that control CSN binding to CRLs are not yet understood.

A small protein called CAND1 (Cullin Associated and Neddylation Dissociated 1, also known as TIP120A) binds cullins that are neither neddylated nor associated with substrate recognition components. Studies of the CAND1-Cul1-Rbx1 crystal structure demonstrate that CAND1 binding blocks both the adaptor binding site and Nedd8 conjugation site (Zheng et al. 2007).
Thus, CAND1 could potentially sequester cullins and prevent their assembly into functional CRLs, thereby reducing CRL activity. Signals that release CAND1 from cullins are not well understood, though neddylation and association with adaptor proteins have been shown to dissociate CAND1 in some cases. Contrary to in vitro data, inactivation of CAND1 actually leads to inactivation of SCF\textsuperscript{Skp2} in vivo (Zheng et al. 2002). Therefore, the importance of CAND1 association may not be in inhibiting CRL activity, but in sequestering cullins from arbitrary adaptor and substrate receptor proteins in order to promote the assembly of CRLs with specific substrate receptors under the appropriate conditions.

The coordination of neddylation, deneddylation, and association with CAND1 modulates CRL ligase activity by stabilizing CRLs and facilitating substrate receptor switching. CAND1 sequesters "naked" cullins until neddylation or substrate recognition component association displaces CAND1, allowing the assembly of CRLs that are ready to target substrates. Association with CSN prevents the autoubiquitination of substrate receptors, thereby stabilizing the CRLs for continued ligase activity. Association with the CSN also results in deneddylation of the cullin. Upon dissociation of the CSN, the substrate recognition components can be autoubiquitinated and degraded, resulting in a deneddylated cullin that can be bound again by CAND1 until the ligase must be reassembled (Figure 3).
Figure 3. CAND1/Neddylation/Deneddylation regulates CRL ligase activity.

Cullin proteins that are neither bound to substrate recognition proteins (adaptors or substrate receptors) or neddylated can be bound by a small protein CAND1. The sequestration of “naked” cullins allows reshuffling of substrate receptors. Neddylation or interaction with adaptors or substrate receptors can displace CAND1. Neddylation, or covalent attachment of a Nedd8 moiety, enhances ubiquitination and subsequent degradation capacity. Association with the COP9 Signalosome inhibits autoubiquitination activity of the ligase of the adaptor and substrate receptor, thereby sustaining CRL ligase activity. Association with the CSN also results in Nedd8 cleavage from the cullin. Dissociation of the CSN leads to adaptor and substrate receptor ubiquitination, and then a “naked” cullin is left to potentially be sequestered by CAND1 binding until ligase assembly is required.
CRL substrate recognition

Many targets of the UPS are not degraded constitutively. One example is cyclin B, whose degradation must occur at a specific point during mitosis for proper mitotic progression. For some CRLs, association with the adaptor and substrate receptor is sufficient to activate ligase activity. Temporal regulation of CRL activity can also be achieved through post-translational modification of the substrate receptor or association with small molecule cofactors. Substrate receptors recognize substrates via short motifs within the substrates, called degrons, that can also be modified post-translationally.

Post-translational modification of ubiquitin ligase components can modulate substrate recognition. For example, phosphorylation plays an essential role in activating the APC/C-mediated destruction of cyclin B and other key regulators for proper mitotic exit. Association with one of its substrate receptors Cdc20 depends on phosphorylation of APC/C core subunits (Kramer et al. 2000; Kraft et al. 2003). CDK-dependent phosphorylation of the other substrate receptor Cdh1 inhibits interaction with the APC/C core while APC/C<sup>Cdc20</sup> is active. Upon dephosphorylation, Cdh1 assembles with the APC/C to target its substrates, which include Cdc20 (Zachariae et al. 1998; Jaspersen et al. 1999). Therefore, phosphorylation coordinates the ordered assembly of the specific ubiquitin ligase complexes APC/C<sup>Cdc20</sup> and APC/C<sup>Cdh1</sup> for targeted degradation of mitotic substrates in strictly ordered fashion.

The degron sequences within substrates can also be modified post-translationally to control recognition by the ubiquitin ligase. Many SCF-based ubiquitin ligases target substrates with phosphorylated degrons, called phosphodegrons. For example, the SCF substrate receptor β-TRCP targets substrates with the phosphodegron D-pS-G-X-X-pS while substrate receptor Fbw7 recognizes L-X-pT-P-P-X-pS (Ang and Wade Harper 2005). In the case of substrates Cdc6 and Skp2, phosphorylation seems to function as protection from ubiquitin ligase recognition (Mailand
and Diffley 2005; Gao et al. 2009). Modifications other than phosphorylation can also influence ligase activity. Hypoxic conditions trigger hydroxylation of the substrate HIF1’s (Hypoxia-Inducible Factor 1) degron, leading to recognition and degradation by the CRL2\(^{VHL}\) ligase (Ivan et al. 2001; Jaakkola et al. 2001).

**CRL4\(^{DTL}\): regulator of cell cycle progression and genome stability**

In recent years, the CRL4\(^{DTL}\) ubiquitin ligase has emerged as an essential regulator of cell cycle progression and DNA metabolic processes, such as replication and repair. The complex assembles on a cullin 4 scaffold, with E2 interaction occurring at one end and substrate recognition components binding at the other. Strikingly, loss of any subunit of the CRL4\(^{DTL}\) complex causes cell proliferation defects, rereplication, accumulation of DNA damage, activation of cell cycle checkpoints, and embryonic lethality in different models. The component that contributes substrate specificity is a WD40 repeat containing protein called Denticleless (also known as L2DTL, DCAF2, Cdt2, and RAMP), which I will refer to as DTL from here on.

**CRL4 architecture**

Crystal structure analyses suggest that the CRL4 ubiquitin ligase assumes the archetypal CRL structure. The RING finger protein Rbx1/Roc1 binds to the C-terminus of the cullin 4 scaffold and serves as the E2 docking component. At the cullin 4 N-terminus, an adaptor protein called DDB1 (DNA Damage Binding protein 1) interacts with a family of substrate receptors, called DCAFs (DDB1 and Cul4 Associated Factor) to recruit substrates for ubiquitination (Angers et al. 2006; Li et al. 2006b)(Figure 4).
Like other cullins, cullin 4 is evolutionarily conserved from fission yeast to humans. Mammalian cells express two closely related paralogs, Cul4A and Cul4B, with Cul4B containing an extended N-terminus. Cul4A and 4B share 80% similarity and have highly conserved homology at the N and C terminus where interaction with DDB1 and Roc1 occurs (Lee and Zhou 2007). Attempts to address whether there is redundancy between Cul4A and Cul4B by Cul4A transgenic mice have generated conflicting results. Mice lacking exon 1 of Cul4A are embryonic lethal (Li et al. 2002). In contrast, ablation of Cul4A function through deletion of the DDB1 or Roc1 binding domains result in viable mice with no obvious phenotypes (Kopanja et al. 2009; Liu et al. 2009). Although it is still unclear whether Cul4A and 4B are redundant in mammalian development, co-immunoprecipitation experiments in human cell lines have demonstrated that both Cul4A and 4B can assemble with DDB1 and DTL to form the CRL4DTL complex (Higa et al. 2003; Senga et al. 2006). However, it is unknown whether Cul4B is required for ubiquitination of CRL4DTL substrates, and most studies only consider Cul4A when referring to the CRL4DTL ligase.

DDB1 serves as the adaptor protein that facilitates recruitment of substrates to the ligase by linking substrate receptors to Cul4A/4B. Its unique structure comprises 21 total WD40-like repeats that fold into a triple β-propeller structure, with seven WD40 repeats in each propeller (BP-A, BP-B, BP-C), and a C-terminal α-helical fold (Li et al. 2006b). Crystal structure analysis of the Cul4-DDB1 complex revealed that Cul4A binds to the “bottom face” of β-propeller BP-B. The two remaining propellers fold into a double propeller to form a binding pocket for substrate receptors (Angers et al. 2006) (Figure 4).
Figure 4. Architecture of CRL4 ligases and DTL.

A) Crystal structure analysis of a CRL4 ligase (Rocl/Rbx1, Cul4A, DDB1, and SV5) hijacked by virus machinery that mimics DCAF substrate receptors, SV5. The Roc1/Rbx RING protein binds the C-terminus of Cul4A. Cul4A is an elongated protein made up of mostly alpha helices. DDB1 is a large WD40 repeat protein that folds into a 3-propeller structure, with each propeller containing 7 WD40 repeats. One propeller (BP-B) mediates interaction with the Cul4A N-terminus. The other two propellers interact with the substrate receptors (Reprinted with permission by Angers, 2010). B) Schematic of CRL4 ligase assembly. DCASs are a family of proteins that can serve as substrate receptors for the CRL4 ligase, and are characterized by containing multiple WD40 repeats. DCAFs are thought to bind to DDB1 either through conserved WDXR motifs located within WD40 repeats, or through an alpha-helical structure located N-terminal to the first WD40 repeat. C) Schematic of DTL protein and known domains. DTL contains 7 WD40 repeats in the N-terminus. Two residues within the 3rd and 4th WD40 repeat have been shown to mediate DDB1 interaction; however, there is also a conserved alpha-helix N-terminal of the WD40 repeats that may be important for DDB1 binding. The conservation between DTL metazoan orthologs is shown, as determined from a multiple sequence alignment of DTL orthologs from human, mouse, *Xenopus laevis*, zebrafish, and *Drosophila* by ClustalX.
The DCAF substrate receptors for the CRL4 ligase are characterized by multiple WD40 repeats. Genetic, proteomic, bioinformatic, and structural analyses have collectively identified 50 putative DCAFs (He et al. 2006; Higa et al. 2006b; Jin et al. 2006). However, only a few have been experimentally verified as bona fide substrate receptors; among these are DDB2 (DNA Damage Binding protein 2), CSA (Cockayne Syndrome A), and DTL. Comparative sequence analyses of the DCAFs identified relatively conserved WDXR or DXR motifs (consisting of the sequence W/Y-D-X-R/K) within the WD40 repeats of several DCAFs. Mutation of these motifs, specifically the arginine, abrogates interaction between the DCAF and DDB1 (Angers et al. 2006; Jin et al. 2006). A more recent study utilizing crystal structure analysis has demonstrated that a short α-helical motif located N-terminal to the first WD40 repeat of DCAFs, referred to as a H-box motif, is more likely to mediate interaction of some DCAFs with DDB1 than the WDXR motifs (Li et al. 2010).

The receptor-substrate pairs identified to date operate in a wide array of cellular processes, from chromatin remodeling to DNA repair. The function of many DCAFs is still unknown and their substrates have yet to be identified. In contrast, some substrates, such as Chk1 and p27, have not yet had a DCAF receptor assigned (Higa et al. 2006c; Leung-Pineda et al. 2009).

**Identification of DTL as a CRL4 substrate receptor**

Of all the identified DCAFs, DTL has been the most extensively studied. DTL is also known as DCAF2, Cdt2 (Cdc10-Dependent Transcript 2), RAMP (Retinoic Acid regulated nuclear Matrix-associated Protein). DTL was first identified as a transcription product of Cdc10, whose mRNA expression peaks at G1/S (Hofmann and Beach 1994). In Drosophila, denticleless homozygous mutants were embryonic lethal and exhibited a lack of ventral denticle belts, thus inspiring the designation “Denticleless” (Kurzik-Dumke et al. 1996). These initial studies did not uncover the role of DTL in ubiquitin-mediated proteolysis.
DTL was first identified as a substrate receptor for the S. pombe Cul4A homolog Pcu4-DDB1 ubiquitin ligase. DTL was shown to associate with the Pcu4-DDB1 complex to trigger Spd1 degradation during S phase and after DNA damage (Liu et al. 2005). Spd1 negatively regulates ribonucleotide reductase (RNR), which catalyzes the rate-limiting step for dNTP synthesis. Spd1 sequesters the small RNR subunit, Suc22, in the nucleus away from the cytoplasmic large RNR subunit, Cdc22. Upon degradation of Spd1 by the Pcu4-DDB1-DTL ligase, Suc22 is free to translocate into the cytoplasm and heterodimerize with Cdc22 to form active RNR. Elimination of Spd1 is essential to provide dNTPs for DNA replication and repair (Liu et al. 2003). Consistent with this, loss of components of the Pcu4-DDB1-DTL ligase causes severe proliferation and replication defects (Holmberg et al. 2005; Liu et al. 2005). Soon thereafter, several independent groups verified that DTL functions as a substrate receptor for the CRL4 ligase through biochemical and genetic approaches in the context of another substrate, Cdt1 (Higa et al. 2006a; Jin et al. 2006; Sansam et al. 2006).

**Physiological consequences of DTL loss**

Loss of DTL causes severe cell cycle proliferation defects and genome instability. Depletion of DTL in mammalian cells, C. elegans (Kim et al. 2008), and zebrafish gives rise to phenotypes that are indicative of rereplication (Jin et al. 2006; Sansam et al. 2006; Kim et al. 2008; Abbas and Dutta 2011). Cells accumulate in G2, exhibit >4N DNA content, and display enlarged nuclei (Melixetian et al. 2004; Takeda et al. 2005). Inappropriate rereplication activates the ATR-mediated G2/M DNA damage checkpoint causing cells to accumulate in G2 (Vaziri et al. 2003; Melixetian et al. 2004; Takeda et al. 2005).

*Del* knockout mice are early embryonic lethal at the 4-8 stage cell development, underscoring the importance of DTL in normal cell cycle progression (Liu et al. 2007). Because maternal mRNA stores can persist in zebrafish embryos for 24-48 hours, *Del* mutant embryos
survive for at least 32 hours post-fertilization. Prolonged survival of *Dti* zebrafish mutants uncovered additional cell cycle phenotypes: multipolar spindles and loss of cells in anaphase (Sansam et al. 2006). Prolonged arrest in G2 can cause formation of multipolar spindles and subsequent mitotic catastrophe and loss of anaphase cells, which suggests that these phenotypes may be a result of the rereplication and checkpoint activation caused by deregulation of the CRL4<sup>DTL</sup> substrate Cdt1. However, it is also plausible that these phenotypes result from deregulation of unidentified substrates.

*Cdt1 destruction by CRL4<sup>DTL</sup> prevents rereplication*

The replication licensing factor Cdt1 is tightly regulated to restrict the initiation of DNA replication to once per cell cycle. In eukaryotes, DNA replication initiates at thousands of sites, called replication origins, throughout the genome to facilitate rapid and efficient replication. In G1, replication origins are primed for replication initiation through the sequential recruitment of the ORC complex, Cdc6, Cdt1, and the Mcm2-7 helicase to form the pre-replicative complex (preRC). Cdt1, in coordination with Cdc6, loads the Mcm2-7 helicase to “license” the origins for replication initiation (Figure 5) (Gillespie et al. 2001; Bell and Dutta 2002). In some human cell lines, overexpression of Cdt1 is sufficient to induce rereplication (Vaziri et al. 2003; Nishitani et al. 2004). To ensure that each origin of replication initiates only once per cell cycle, inhibitory mechanisms negatively regulate each individual preRC component after replication initiation (Arias and Walter 2007).
Figure 5. Cdt1 is required for replication origin licensing.

In G1, replication origins are licensed through the sequential recruitment of factors to replication origins. The ORC complex first binds to the replication origins. Then replication licensing factors Cdc6 and Cdt1 bind to ORC, and together, they recruit and load the Mcm2-7 helicases onto the origins. At this point, pre-Replicative Complex (pre-RC) has been formed and the origins are now considered “licensed” for replication. Upon DNA synthesis initiation, the pre-RC components are negatively regulated to ensure that the genome is only replicated once. Three mechanisms exist for negative regulation of Cdt1: Geminin binding, degradation by phosphorylation-dependent ubiquitination by SCF$^{Skp2}$, and degradation by the CRL4$^{Ddb}$ ligase.
In metazoans, Cdt1 is negatively regulated by geminin binding and ubiquitin-mediated degradation by two different ubiquitin ligases, SCF\textsuperscript{Skp2} and CRL4\textsuperscript{DTL}. Geminin is a cell cycle regulated protein that binds Cdt1 and prevents Mcm2-7 origin loading (Wohlschlegel et al. 2000). Geminin itself is degraded by the APC/C during mitosis (McGarry and Kirschner 1998), which frees Cdt1 for recruitment to replication origins in the following G1 phase. Ubiquitin-mediated destruction of Cdt1 irreversibly inhibits origin licensing until Cdt1 protein is re-synthesized in mitosis. SCF\textsuperscript{Skp2} recognition of Cdt1 is dependent upon phosphorylation of Cdt1 by cyclinA-CDK2. Phosphorylation of Cdt1 threonine 29 creates the phosphodegron recognized by the Skp2 substrate receptor (Li et al. 2003; Liu et al. 2004; Takeda et al. 2005). The CRL4\textsuperscript{DTL} ligase, in cooperation with chromatin-bound PCNA, targets chromatin-bound Cdt1 (Arias and Walter 2006; Jin et al. 2006; Ishii et al. 2010; Roukos et al. 2011). The dependence on chromatin-bound PCNA suggests that CRL4\textsuperscript{DTL} negatively regulates Cdt1 at replication origins that have fired. The involvement of PCNA also temporally restricts CRL4\textsuperscript{DTL}-mediated destruction to S phase. The mechanism by which PCNA facilitates CRL4\textsuperscript{DTL} ubiquitination of Cdt1 and other substrates will be discussed in further detail below.

After exposure to DNA damage, Cdt1 must also be negatively regulated to prevent licensing and replication of damaged DNA. Under these conditions, only one ubiquitin ligase targets Cdt1 for rapid destruction: CRL4\textsuperscript{DTL}. Depletion or loss of CRL4\textsuperscript{DTL} components causes stabilization of Cdt1 after DNA damage, while depletion or loss of Skp2 does not perturb Cdt1 degradation (Higa et al. 2003; Hu et al. 2004; Arias and Walter 2006; Higa et al. 2006a). Cdt1 is completely degraded in asynchronously dividing cells after damage, indicating that, unlike undamaged cells, CRL4\textsuperscript{DTL} targeting is not temporally restricted to cells in S phase. Cdt1 ubiquitination in response to DNA damage is still dependent on PCNA and chromatin, but not dependent on the canonical DNA damage response signaling pathways mediated by ATM/ATR or Chk1/Chk2 (Higa et al. 2003).
Substrates of the CRL4<sup>DTL</sup> ligase

Over the last few years, substrates in addition to Cdt1 and S. pombe Spd1 have been shown to be targeted by the CRL4<sup>DTL</sup> ligase during S phase and after DNA damage. CRL4<sup>DTL</sup> targets have diverse roles: many essential cellular processes: cell cycle control, DNA replication and repair, transcription, and chromatin regulation. Importantly, identification of these substrates has assisted in elucidating the mechanism of CRL4<sup>DTL</sup> substrate recognition. A current list of substrates is summarized in Table 1.

E2F1 is a member of the E2F family of transcription factors whose transcriptional activation of cell cycle regulators in G1 is critical for S phase entry. In Drosophila, E2F1 is specifically destroyed in S phase in a CRL4<sup>DTL</sup>-dependent manner (Shibutani et al. 2008). It is unknown whether Drosophila E2F1 is destroyed after damage and if this process is also dependent on the CRL4<sup>DTL</sup> complex. Mammalian E2Fs employ other strategies to regulate E2F activity in S phase, and it is unlikely that they utilize CRL4<sup>DTL</sup>-mediated proteolysis.

Regulation of CDK inhibitors (CKIs) by the CRL4<sup>DTL</sup> ubiquitin ligase is conserved in higher eukaryotes. Human p21, Xenopus Xic1, and C. elegans CKI-1 are specifically targeted for destruction during S phase (Kim et al. 2007; Abbas et al. 2008; Kim et al. 2008; Kim et al. 2010). This degradation is critical for preventing rereplication by promoting nuclear export of Cdc6, which prevents relicensing of replication origins. Upon CKI destruction, CDK2 is free to phosphorylate Cdc6 for nuclear export. The purpose of p21 degradation after damage by CRL4<sup>DTL</sup> is less established. P21 inhibits DNA replication and repair through direct binding with PCNA. Therefore, it has been proposed that destruction of p21 frees participation in repair processes, such as translesion synthesis.
Table 1. CRL4<sup>DTL</sup>-substrates identified thus far.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Organisms</th>
<th>Purpose of degradation</th>
<th>S phase</th>
<th>After damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdt1</td>
<td>S. pombe, Xenopus, C. elegans, Drosophila, Danio Rerio, Human</td>
<td>Prevent relicensing of origins</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Epe1</td>
<td>S. pombe</td>
<td>Maintain boundaries between heterochromatin and euchromatin</td>
<td>Y</td>
<td>unknown</td>
</tr>
<tr>
<td>E2F1</td>
<td>Drosophila</td>
<td>Inhibit E2F1 target gene expression (S phase genes)</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>CDK inhibitors</td>
<td>Xenopus Xic1 C. elegans CKI-1 Human p21</td>
<td>Release of CDKs and PCNA for replication and repair</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>PCNA</td>
<td>Human</td>
<td>Monoubiquitination increases affinity for TLS polymerases for repair during normal S phase</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>Pol η</td>
<td>C. elegans</td>
<td>Protected from degradation by sumoylation, performs translesion repair</td>
<td>unknown</td>
<td>Y</td>
</tr>
<tr>
<td>Set8</td>
<td>Human</td>
<td>Potential role in origin licensing</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Spd1</td>
<td>S. pombe</td>
<td>Release of RNR subunit for dNTP synthesis for replication and repair</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>
The translesion synthesis polymerase itself, DNA polymerase η, is targeted by CRL4<sup>DTL</sup> in response to DNA damage in <i>C. elegans</i> embryos. Covalent attachment of a small protein called SUMO protects DNA pol η from CRL4<sup>DTL</sup>-mediated proteolysis to allow the polymerase to repair DNA lesions and avoid ATR checkpoint activation. Once bypass synthesis is completed, sumoylation of DNA pol η is reversed, and the error-prone polymerase is rapidly degraded by CRL4<sup>DTL</sup> (Kim and Michael 2008). Conservation of this function in higher eukaryotes has not been tested.

Interestingly, PCNA itself is also ubiquitinated by the CRL4<sup>DTL</sup> complex, however this does not result in proteasomal destruction. After exposure to DNA damage, PCNA is monoubiquitinated by the Rad6/Rad18 ubiquitin ligase to increase its affinity for members of the Y-family of DNA bypass polymerases for translesion syntheze (TLS), where DNA is synthesized across DNA lesions (Andersen et al. 2008). During normal cell cycle, DNA lesions are caused by DNA replication stress or errors at some frequency. Therefore, undamaged cells also employ monoubiquitinated PCNA to recruit DNA bypass polymerases for continued S phase progression, and PCNA monoubiquitination is mediated via the CRL4<sup>DTL</sup> complex (Terai et al. 2010).

Set8 (PR-Set7 or KMT5A) monomethylates histone H4 on lysine 20 (H4K20me) during G2 and mitosis. Set8 is critical for proper G2 progression and chromosome condensation prior to mitosis. There is also an unexplained connection between Set8, H4 monomethylation and replication origin licensing: silencing of Set8 prevents Mcm2-7 loading, while tethering of a catalytically active Set8 to genomic loci promoted loading of pre-RC proteins (Tardat et al. 2010). Failure to properly degrade Set8 during S phase reportedly causes widespread defects including: rereplication, spontaneous DNA damage, checkpoint activation and subsequent G2 arrest, increased expression in p53-dependent pro-apoptotic genes (Abbas et al. 2010; Centore et al. 2010; Oda et al. 2010; Tardat et al. 2010; Jorgensen et al. 2011). Like several other CRL4<sup>DTL</sup> substrates, Set8 is also targeted for degradation by the ligase after DNA damage (Abbas et al. 2010; Centore et al. 2010; Oda et al. 2010; Jorgensen et al. 2011).
*S. pombe* Epel (Enhancer of Position Effect 1) is an anti-silencing factor concentrated at the boundaries between heterochromatin and euchromatin (Zofall and Grewal 2006). The mechanism by which Epel prevents the spread of heterochromatin is unknown. CRL4\(^\text{DTL}\)-mediated degradation prevents accumulation of Epel within heterochromatin, thereby restricting its distribution to the boundaries of heterochromatin and preserving the silencing of chromatin regions (Braun et al. 2011).

Zebrafish and human cells lacking DTL fail to activate the early G2/M checkpoint in response to DNA damage. Loss of Cdt1 in Dtl mutant zebrafish does not rescue this defect (Sansam et al. 2006), implying that another protein other than Cdt1 must be destroyed by the CRL4\(^\text{DTL}\) ligase for proper checkpoint activation. Roles for the other identified CRL4\(^\text{DTL}\) substrates in this checkpoint have not been reported, therefore, an unidentified CRL4\(^\text{DTL}\) target may participate in the regulation of this checkpoint.

**Regulatory mechanisms of CRL4\(^\text{DTL}\) ligase activity**

Of all the identified CRL4\(^\text{DTL}\) substrates, the mechanism for CRL4\(^\text{DTL}\)-mediated destruction of the replication licensing factor Cdt1 is most well understood. A series of experiments in *Xenopus* egg extracts launched the studies that defined the PCNA-dependent regulation of Cdt1 ubiquitination and proteolysis. A consensus degron that combines a canonical PIP box with a critical residue that contacts DTL was identified in most CRL4\(^\text{DTL}\) substrates, provides a discrete mechanism for substrate recognition and ubiquitination. This PCNA-dependent mechanism has been most intensely studied, however other mechanisms that modulate the components of the ligase have been more recently addressed.
**PCNA-dependent substrate targeting**

*PCNA couples CRL4<sup>DTL</sup> activity to S phase and after DNA damage*

Experiments in *Xenopus* egg extracts first demonstrated that Cdtl ubiquitination occurs on chromatin and is strictly coupled to DNA replication. In this cell-free system, sperm chromatin is added to *Xenopus* egg cytoplasm and triggers pre-RC formation and a single round of DNA replication (Arias and Walter 2004). Ubiquitinated Cdtl was found on chromatin in the *Xenopus* extracts. Importantly, inhibition of DNA replication via depletion of DNA replication factors Cdc45, RPA, and DNA Polymerase α, treatment with aphidicolin, or addition of CDK inhibitor p27<sup>Kip</sup> prevented Cdtl destruction (Arias and Walter 2005; Jin et al. 2006). Taken together, these results suggested that DNA replication factors downstream of replication initiation regulated Cdtl destruction.

Subsequent studies identified PCNA (Proliferating Cell Nuclear Antigen) as an essential cofactor for triggering CRL4<sup>DTL</sup>-dependent proteolysis, fulfilling the prerequisite for chromatin binding and replication initiation observed for Cdtl degradation. PCNA assembles as a homotrimeric clamp encircling DNA and physically tethers polymerases to the DNA to achieve the replication efficiency required for duplication of the genome (Kelman and O'Donnell 1995). PCNA interacts with at least 50 proteins involved in many different cellular processes in addition to DNA replication, such as various DNA repair pathways and histone modification. Most PCNA-binding proteins contain a common binding motif called the PIP (PCNA Interaction Peptide) box: Q-X-X-H-X-X-A-A where ‘H’ represents residues with moderately hydrophobic side chains (V, L, I, or M), and ‘A’ represents residues with highly hydrophobic aromatic side chains (F or Y), and X is any residue (Moldovan et al. 2007). Depletion of PCNA from *Xenopus* extracts inhibited replication-dependent Cdtl degradation (Arias and Walter 2006). RNAi knockdown of PCNA
prevents Cdt1 destruction during normal cell cycle and after DNA damage (Hu et al. 2004; Higa et al. 2006a; Senga et al. 2006).

Interaction between Cdt1 and PCNA is required for Cdt1 ubiquitination by the CRL4<sup>DTL</sup> ligase. Interestingly, all metazoan Cdt1 orthologs contain a canonical PIP box in the extreme N-terminus (amino acids 3-9). Recombinant Cdt1 and PCNA interact <i>in vitro</i>, and affinity purification of overexpressed myc-tagged Cdt1 detected interacting PCNA peptides by mass spectrometry (Arias and Walter 2006; Hu and Xiong 2006). Importantly, interaction between endogenous Cdt1 and PCNA has been detected in human HeLa cells (Hu and Xiong 2006). In fact, deletion or mutation of the PIP box causes stabilization of Cdt1 during normal cell cycle and after DNA damage (Arias and Walter 2006; Higa et al. 2006a; Hu and Xiong 2006; Nishitani et al. 2006; Senga et al. 2006).

**Identification of the PIP degron**

The discovery that CRL4<sup>DTL</sup>-mediated destruction is dependent on substrates binding to PCNA raised the question of how the CRL4<sup>DTL</sup> ligase distinguishes substrates from the PCNA-associated proteins that should not be degraded? One study searched for a degron by alignment and comparison of the PIP boxes in Cdt1 from multiple species, other identified CRL4<sup>DTL</sup> substrates, and stable PIP box-containing proteins. This study identified a specialized PIP box present only in CRL4<sub>DTL</sub> targets (Havens and Walter 2009). This motif (Q-X-X-L/M/I/V-T-D-F/Y-F/Y-X-X-X-K/R), now called the PIP degron, contains two functional elements that are required for CRL4<sup>DTL</sup>-mediated degradation, a TD motif and B+4 residue (Figure 6).

The TD motif is embedded in the PIP box (positions 5 and 6), and confers high affinity binding to chromatin-bound PCNA. Crystal structure of soluble PCNA bound with p21’s PIP box shows that while essential PIP box residues interact directly with the hydrophobic pocket of PCNA, T5 and D6 protrude from the surface of PCNA into solution (Gulbis et al. 1996) (Figure 6). Mutation of T5 to alanine in human or <i>Xenopus</i> Cdt1 reduces replication-dependent and DNA
damage-dependent degradation. Mutation of D6 to alanine appears to slow Cdt1 destruction due to a significant reduction in PCNA binding, but does not prevent Cdt1 destruction (Senga et al. 2006; Havens and Walter 2009). Addition of the TD motif to the PIP boxes of normally stable Fen1 or DNA ligase dramatically increases their affinity for PCNA (Havens and Walter 2009; Michishita et al. 2011). The high affinity binding may serve to ensure processive ubiquitination and rapid destruction of substrates. Notably, not all identified CRL4<sup>DTL</sup> substrates contain a TD motif, presenting the possibility that other residues may contribute to increased binding efficiency to PCNA.

The B+4 element is a basic residue located four amino acids downstream from the PIP box that specifically contacts DTL to recruit the CRL4<sup>DTL</sup> complex to the PCNA-bound substrate. PCNA-p21-peptide crystal structure analysis revealed that basic residues at the +3 and +5 position interact with acidic residues within PCNA while the lysine at the +4 position protrudes into solution (Gulbis et al. 1996) (Figure 6). Consistent with the structural data, mutation of the +3 and +5 residues to alanines in human Cdt1 and p21 impair PCNA binding (Nishitani et al. 2008; Michishita et al. 2011). Strikingly, mutation of B+4 in Cdt1 and p21 stabilized the CRL4<sup>DTL</sup> substrates during normal S phase and after UV irradiation in human cell lines (Nishitani et al. 2008; Michishita et al. 2011). Additional experiments in Xenopus extracts demonstrated that although the basic residue in the +4 position from the PIP box is not essential for efficient PCNA binding, it is required for specific recruitment of DTL to PCNA-bound-substrates (Havens and Walter 2009). Unlike the TD motif, almost all identified substrates contain a B+4 residue (with the exception of <i>S. pombe</i> Epe1), suggesting that this residue is an indispensable determinant of the degron.
Figure 6. Identification of the PIP degron.

A) Crystal structure analysis of homotrimeric PCNA bound to the p21-PIP-box-peptide. B) Close up of the region where the p21-PIP box binds to PCNA. The PIP box residues at positions 1, 4, 7, and 8 make contact with PCNA. The TD motif within the PIP degron contacts PCNA and therefore confers high affinity binding. The B+4 residue does not contact PCNA and instead, juts out into soluble space. This residue putatively mediates interaction with DTL, however, the region/sequence on DTL that interacts with B+4 is unknown. C) Sequence alignments of the canonical PIP box with the PIP degron, in addition to all the PIP degrons from identified or closely evolutionarily related PIP boxes. (A) and (B) were reprinted from Havens and Walter 2011 with permission.
The PIP degron is also sufficient for binding to PCNA and recruitment of the CRL4\textsuperscript{DTL} ligase through contact with the substrate receptor. Addition of a 25 amino acid peptide containing the human Cdt1 PIP box caused CRL4\textsuperscript{DTL}-dependent degradation of GST (Nishitani et al. 2006; Senga et al. 2006). Also, introduction of T5 and B+4 to the PIP boxes of DNA ligase or Fen1 is sufficient to increase PCNA binding and trigger destruction of the proteins (Havens and Walter 2009; Michishita et al. 2011). Using the PIP degron consensus motif to search for additional substrates led to the identification of the Set8 as a novel CRL4\textsuperscript{DTL} substrate (Abbas et al. 2010).

Assembly of the PCNA-substrate-CRL4\textsuperscript{DTL} complex

Determination of the PIP degron’s bipartite functionality where PCNA and DTL form discrete contacts with the substrate led to the question: what is the order of events for PCNA-substrate-ligase complex assembly? Early studies in Xenopus extracts had already shown that both Cdt1 and DTL are loaded onto chromatin in during replication and after exposure to DNA damage (Arias and Walter 2005; Arias and Walter 2006). After induction of damage, both Cdt1 and DTL were found to rapidly localize to damaged chromatin with similar kinetics by fluorescence imaging in mammalian cells (Ishii et al. 2010; Roukos et al. 2011). Importantly, DTL is not required for PCNA-Cdt1 interaction while mutation of the Cdt1 PIP box significantly impaired loading of DDB1 and DTL onto chromatin (Arias and Walter 2006; Jin et al. 2006; Havens and Walter 2009), suggesting that PCNA-Cdt1 interaction occurs prior to CRL4\textsuperscript{DTL} recruitment.

The structure of the PIP degron also seems to support the idea that PCNA-substrate interaction is required for DTL recruitment. The close proximity of the PIP box and the B+4 residue supports a model where the substrate binds PCNA first to create an interface that is recognized by DTL. DTL would bind the B+4 residue within the substrate as well as additional residues in PCNA. However, there has not yet been any published data identifying residues in PCNA that can directly contact DTL.
Although initial studies defined the PCNA-dependent mechanism in the context of Cdt1 regulation, subsequent studies have shown that other CRL4\textsuperscript{DTL} substrates also depend upon PCNA interaction for their degradation. Mutation of PIP boxes inhibits degradation of human Set8, \textit{C. elegans} DNA Pol \(\eta\), \textit{Drosophila} E2F1, and Cdk inhibitors \(\text{p21}\) and \textit{Xenopus} Xic1 (Abbas et al. 2008; Kim and Michael 2008; Shibutani et al. 2008; Abbas et al. 2010; Centore et al. 2010; Kim et al. 2010). Because of the dependence on PCNA, it is assumed that CRL4\textsuperscript{DTL} recruitment to other substrates conforms to the mechanism defined for Cdt1.

There is some data that challenges this model for PCNA-substrate interaction occurring prior to DTL recruitment. First, there is some evidence that DTL can be recruited to chromatin or PCNA in the absence of substrates. For example, imaging of GFP-tagged DTL in live mammalian cells revealed that siRNA depletion of Cdt1 does not inhibit DTL recruitment to chromatin after damage (Roukos et al. 2011). Second, the C-terminal half of \textit{in vitro} translated DTL has been shown to interact with PCNA. \textit{In vitro} competition assays between Xic1, DTL, and PCNA reveal that Xic1 and DTL can compete for PCNA binding, suggesting that DTL interaction with PCNA occurs via a PIP box (Kim et al. 2010). And lastly, there have been some immunoprecipitation experiments in mammalian cells that have detected binding between components of the CRL4\textsuperscript{DTL} complex (DTL or DDB1) and substrates with mutated PIP boxes (Abbas et al. 2008; Kim et al. 2008; Abbas et al. 2010; Kim et al. 2010). A caveat of these experiments is that binding was only detected when proteins were overexpressed. Many questions and inconsistencies still remain regarding the precise ordering of the PCNA-substrate-CRL4\textsuperscript{DTL} complex; hopefully, these questions will be addressed in the future.
Current Model for PIP degron-dependent degradation by CRL4<sup>DTL</sup>

Taking together all the data discussed previously, a current model of CRL4<sup>DTL</sup>-mediated substrate recognition and degradation has been proposed as follows (Figure 7):

1) Substrates first dock onto chromatin-bound PCNA via its PIP box during S phase or after DNA damage.
2) CRL4<sup>DTL</sup> is recruited to the PCNA-bound substrate via DTL forming contacts with a basic residue four residues downstream of the PIP box to form a PCNA-substrate-CRL4<sup>DTL</sup> complex.
3) CRL4<sup>DTL</sup> mediates the covalent attachment of ubiquitins to the substrate, and the substrate is subsequently degraded by the proteasome.

Figure 4. Current model of PCNA-chromatin-dependent degradation of CRL4<sup>DTL</sup> substrates.

Schematic of prevailing model of CRL4<sup>DTL</sup> substrate degradation during active replication or after DNA damage. PCNA is recruited onto chromatin, either for replication during S phase or repair processes after DNA damage exposure. CRL4<sup>DTL</sup> substrates preferentially bind chromatin-bound PCNA via a PIP box/PIP degron within the substrate. Substrate bound PCNA then recruits DTL and the associated CRL4 ligase for interaction and ubiquitination. DTL interacts with the substrate, and may also interact with PCNA or the DNA. Ubiquitination is initiated and the proteasome recognizes the polyubiquitinated substrates for degradation.
Additional mechanisms that regulate CRL4<sup>DTL</sup> activity

Cul4A is neddylated in vitro on a conserved lysine in the C-terminus (Ohh et al. 2002), though the contribution of neddylation or deneddylation specifically for CRL4<sup>DTL</sup> activity has not been directly addressed. Recently, a small molecule inhibitor of CRL neddylation has been developed for cancer treatment. MLN4924 inhibits NAE (Nedd8-Activating Enzyme), thereby preventing the covalent attachment of Nedd8 to cullin proteins and subsequent activation of CRL ligase activity. Promising preclinical findings demonstrate that repeated doses in several xenograft cancer models effectively inhibited tumor growth (Soucy et al. 2009; Swords et al. 2010; Milhollen et al. 2011). Although the drug inhibits neddylation of all cullins, the inhibition of tumor cell proliferation appears to be caused by apoptosis and senesence resulting from stabilization of Cdt1. Moreover, this stabilization is mediated primarily via inhibition of CRL4<sup>DTL</sup> activity (Lin et al. 2010; Milhollen et al. 2011), indicating that neddylation of Cul4A has profound effects on CRL4<sup>DTL</sup> regulation.

Binding between CAND1 and Cul4A may also modulate CRL4<sup>DTL</sup> activity. CAND1 binds to Cul4A in human cells (Min et al. 2003; He et al. 2006); however, CAND1 and DDB1 binding to Cul4A are mutually exclusive (Hu et al. 2004). This finding suggests that CAND1 association with Cul4A prevents recruitment of substrates to the catalytic core would impair CRL4<sup>DTL</sup> activity, however the effects of CAND1 on CRL4<sup>DTL</sup> activity remains to be addressed in vivo.

The COP9 Signalosome (CSN) promotes CRL4<sup>DTL</sup> activity primarily through maintaining steady-state levels of DTL. All eight CSN subunits have also been detected in complex with Cul4A and DDB1 in human cells (Groisman et al. 2003; Higa et al. 2003; Liu et al. 2003). Consistent with this data, Csn1 and Csn2 are required for CRL4<sup>DTL</sup>-mediated degradation of Spd1 in S. pombe (Holmberg et al. 2005). In addition, a recent study in human cell lines demonstrated that depletion of Csn5 and Csn6 causes hyperneddylation of Cul4A and robust stabilization of Cdt1. Surprisingly,
depletion of Csn5 and Csn6 results in loss of DTL (Raman et al. 2011), suggesting that the CSN promotes CRL4<sup>DTL</sup> activity through the inhibition of DTL autoubiquitination.

Recent work identifying the E2 ubiquitin conjugating enzymes that cooperate with the CRL4<sup>DTL</sup> ligase found that different E2s promote the ubiquitination and degradation of distinct CRL4<sup>DTL</sup> substrates. RNAi knockdown experiments demonstrated that UBCH8 selectively promotes CRL4<sup>DTL</sup>-dependent ubiquitination and degradation of p21 during normal cell cycle progression and after UV irradiation. UBCH8 also promotes monoubiquitination of PCNA in unstressed cells and UV-induced ubiquitination of Set8 in cooperation with CRL4<sup>DTL</sup>. However, UBCH8 does not regulate Cdt1 stability; instead, UBE2G1 and UBE2G2 promote Cdt1 ubiquitination and degradation during S phase and after UV-irradiation (Shibata et al. 2011). The extent to which different UBCs influence the substrate specificity of ubiquitin ligases is unknown. Importantly, despite the fact that Cdt1 is a substrate of both CRL4<sup>DTL</sup> and SCF<sup>Skp2</sup> ligases, CRL4<sup>DTL</sup>-dependent ubiquitination cannot be promoted via the E2 that promotes SCF<sup>Skp2</sup> ligase activity, Cdc34 (Shibata et al. 2011).

Post-translational modification of substrates has also been shown to contribute to CRL4<sup>DTL</sup> activity. C. elegans DNA Pol η is degraded after DNA damage, however, sumoylation protects Pol η from CRL4DTL-mediated degradation through unknown mechanisms (Kim and Michael 2008). Phosphorylation of p21 on serine 114 is mediated by ATR-dependent signaling after UV-irradiation. This phosphorylation is required for p21 ubiquitination and degradation by CRL4<sup>DTL</sup> (Bendjennat et al. 2003; Abbas et al. 2008). Conversely, p21 phosphorylation on threonine 145 or serine 146 within the PIP box stabilizes the position after UV damage, likely by disrupting PCNA interaction (Arias and Walter 2006; Abbas et al. 2010; Centore et al. 2010). Degradation of Cdt1 and Set8 does not depend on ATR or ATM signaling after damage, so it seems unlikely that ATR-dependent phosphorylations promote their degradation after UV-irradiation (Arias and Walter 2006; Abbas et al. 2010; Centore et al. 2010). It remains to be seen whether post-translational modification of additional CRL4<sup>DTL</sup> substrates controls ubiquitination and degradation.
And lastly, cursory studies have identified phosphorylations within DTL, but have made no direct connections to CRL4DTL ligase activity. Two genome-wide proteomic studies utilizing mass spectrometry have identified phosphorylated DTL peptides; one phosphorylation occurs in response to DNA damage via ATM/ATR signaling, while other phosphorylations occur during normal cell cycle progression (Matsuoka et al. 2007; Dephoure et al. 2008). A separate study created DTL truncation mutants, assigned phosphorylations to the C-terminus, and proposed that Aurora B is the kinase responsible from comparison of the C-terminus with consensus motifs (Ueki et al. 2008). Aurora B has yet to be validated as the kinase that phosphorylates DTL. Importantly, the role of DTL phosphorylation has yet to be elucidated. One study has correlated DTL phosphorylation with presence on chromatin during S phase and after DNA damage, however, the contribution of the phosphorylation to DTL activity has yet to be directly addressed (Ishii et al. 2010).

In summary, the CRL4DTL ubiquitin ligase has recently emerged as a new master regulator of cell cycle progression and genome maintenance. CRL4DTL targets substrates that are involved in S phase progression, DNA replication, and DNA repair pathways. Substrate recognition depends on a specialized PIP degron that coordinates high affinity binding to PCNA with recruitment of the CRL4DTL ligase through the substrate receptor DTL. Loss of any ligase components results in reduced viability, proliferation defects, and accumulation of DNA damage or massive rereplication. Anti-tumorigenic effects after treatment with a recent novel and promising cancer therapeutic, MLN4924, seems to be caused through modulation of the CRL4DTL ligase specifically, which underscores the importance of this ubiquitin ligase and reveals its potential targeting for future therapeutic development.

Although extensive advances have been made in understanding the mechanism of PCNA-dependent CRL4DTL substrate targeting, there are still questions that remain. Does DTL directly contact PCNA, and if so, how does this interaction fit in the context of the PIP degron and binding
between the substrate and PCNA? What region of DTL contributes to the binding the B+4 residue of the PIP degron? Does PCNA function solely as a docking site to bring together the substrate and ligase on chromatin, or does PCNA somehow also modulate the activity of the ligase?

The work of this thesis aims to elucidate how different regulatory mechanisms of the substrate receptor DTL modulate CRL4<sup>DTL</sup> ligase activity. Specifically, we address which parts of the DTL protein are essential for assembly of the CRL4<sup>DTL</sup> complex, substrate recognition, and substrate degradation during S phase and after exposure to DNA damage. Here we present the first study to directly assess the contribution of regions of DTL to CRL4<sup>DTL</sup> ligase activity in the context of live cycling mammalian cells. We also characterize DTL protein expression and its post-translational modifications throughout the cell cycle. And finally, we explore the role of DTL in the DNA damage-induced early G2/M checkpoint in mammalian cells.
Chapter II: The C-terminus of DTL is not required for CRL4$^{DTL}$ substrate regulation

Crystal J. Lee and Jacqueline A. Lees

Experimental contribution:
C.J.L. performed all experiments presented in this chapter.
Summary

CRL4<sup>DTL</sup> ubiquitin ligase plays a critical role in regulating genome stability through timely degradation of substrates S phase and after exposure to DNA. Although many studies have focused on elucidating the elements of substrates that are required for recruiting the CRL4<sup>DTL</sup> ligase, little attention has been directed at addressing the elements of the ligase substrate receptor Denticleless (also known as DTL/Cdt2/RAMP) that are required for substrate recruitment or ligase activity. In metazoans, the DTL N-terminus is highly conserved while the C-terminus has no recognizable domains and is significantly less conserved. We hypothesize that the C-terminus contains motifs or post-translational modifications that may regulate DTL function in the context of CRL4<sup>DTL</sup> ligase activity. In this study, we identify a C-terminal putative PCNA-interaction motif (PIP box), however, disruption of the motif does not abrogate PCNA interaction or perturb cell cycle progression when expressed. We create a deletion mutant lacking the C-terminus (ΔC) and find that the DTL C-terminus is not required for ligase assembly or substrate degradation during normal cell cycle or after DNA damage. Importantly, we find that DTL can interact with substrates through the N-terminus.
Introduction

Regulated destruction of proteins is essential to many cellular processes, including cell cycle progression, DNA replication, transcription, and signaling. Proteins are tagged by the covalent attachment of a highly conserved and abundant 76 amino acid protein called ubiquitin through sequential action of three enzymes, an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase. Successive attachment of additional ubiquitin molecules to lysine 48 of the previously attached ubiquitin generates a polyubiquitin chain recognized by the 26S proteasome for proteolysis. E3 ubiquitin ligases are responsible for uniting E2 conjugating enzymes with selectively recruited substrates to facilitate the ubiquitination reaction.

Cullin RING ligases (CRLs) are a large family of E3 ubiquitin ligases that utilize substrate receptors to recognize proteins for targeted destruction. Two of the most well understood CRLs are the SCF (Skp1-Cullin 1-F-box protein) and the APC/C (Anaphase Promoting Cyclosome) ligases, which play critical roles in cell cycle progression (Nakayama and Nakayama 2006). The Cullin 4 RING E3 ubiquitin ligase (CRL4), in association with the substrate receptor DTL/Cdt2 (CRL4DTL), has recently emerged as a key regulator of cell cycle progression and genome stability. Crystal structure studies indicate that the cullin 4A or 4B protein acts as a scaffold, binding to the E2-recruiting RING protein Rbx1 through its C-terminus and substrate recognition components through its N-terminus. DDB1 serves as an adaptor protein that bridges the substrate receptor DTL and substrates to the cullin core (Angers et al. 2006; Li et al. 2010).

Loss of Cul4A, DDB1, or DTL in C. elegans, S. pombe, zebrafish, and mammalian cells leads to cell proliferation defects, checkpoint activation and subsequent G2 arrest, genome instability, and, in some cases, massive rereplication (Li et al. 2002; Holmberg et al. 2005; Arias and Walter 2006; Cang et al. 2006; Jin et al. 2006; Lovejoy et al. 2006; Sansam et al. 2006; Kim et...
al. 2007; Kim and Kipreos 2007; Liu et al. 2007; Abbas et al. 2008; Kim et al. 2008; Shibutani et al. 2008; Kopanja et al. 2009; Abbas et al. 2010; Centore et al. 2010). These phenotypes are attributed to CRL4DTL-dependent proteolysis of important cell cycle regulators involved in DNA replication and repair processes, S phase progression, and chromatin regulation. Two CRL4DTL substrates largely account for the rereplication and checkpoint activation observed upon inhibition of CRL4DTL-mediated degradation: the replication licensing factor Cdt1 and the histone methyltransferase Set8/PR-Set7. Cdt1 is required for loading the Mcm2-7 helicases onto replication origins during G1. CRL4DTL-mediated destruction is one of three mechanisms that negatively regulates Cdt1 once origins have fired to ensure that the genome is replicated only once per cell cycle (Arias and Walter 2007). Similarly, Set8 is targeted by CRL4DTL during S phase to prevent rereplication, though the precise connection between Set8 and replication licensing is unknown (Abbas et al. 2010; Tardat et al. 2010). Importantly, although redundant mechanisms control Cdt1 and Set8 activity in unperturbed cells, CRL4DTL alone negatively regulates both substrates in DNA-damaged cells. Other CRL4DTL substrates that are critical for S phase progression include S. pombe Spd1 (an inhibitor of ribonucleotide reductase), Drosophila E2F1, and CDK inhibitors, such as mammalian p21, C. elegans CKI-1, and Xenopus Xic1 (Liu et al. 2005; Kim et al. 2007; Abbas et al. 2008; Kim et al. 2008; Nishitani et al. 2008; Shibutani et al. 2008; Kim et al. 2010). CRL4DTL plays a critical role in translesion synthesis (TLS) DNA repair processes by monoubiquitinating PCNA and targeting C. elegans DNA Polymerase η for destruction after damage (Kim and Michael 2008; Terai et al. 2010). More recently, chromatin regulators Epe1 in S. pombe and mammalian histone acetyltransferase Gcn5 are degraded in a CRL4DTL-dependent manner (Braun et al. 2011; Li et al. 2011).

Previous studies have focused on the determinants within the substrates that contribute to CRL4DTL-dependent degradation. Most CRL4DTL substrates contain a PCNA interaction peptide motif (PIP box), and deletion of this PIP box inhibits CRL4DTL-mediated ubiquitination and destruction. Sequence alignments between CRL4DTL substrates identified a TD motif embedded
within the PIP box and a basic residue located four amino acids downstream of the PIP box (B+4), collectively referred to as the PIP degron. Further experiments in Xenopus extracts revealed that the PIP degron coordinates recruitment of CRL4\textsuperscript{DTL} to the substrate with high affinity binding to chromatin-bound PCNA, which couples CRL4\textsuperscript{DTL}-dependent destruction to active replication and after DNA damage. Recruitment of the ligase is thought to occur via interaction between DTL and the B+4 residue in the PIP degron once the substrates have bound to PCNA (Havens and Walter 2009). However, it is unclear DTL recognizes the B+4 residue in the substrate only or some interface formed between the associated PCNA and substrate. Also, it is unknown whether ligase recruitment to the substrate is sufficient to induce ubiquitination or if additional signals or modifications are needed.

We wanted to explore whether elements within the DTL C-terminus are required for interaction with PCNA-bound substrates or activation or CRL4\textsuperscript{DTL} activity. DTL consists of seven highly conserved WD40 repeats in the N-terminus and a less evolutionarily conserved C-terminus that contains no recognizable motifs. Direct interaction with DDB1 has been assigned to conserved arginines (R171 and R246) within WDXR motifs in the N-terminus (He et al. 2006; Jin et al. 2006), however, contribution of the C-terminus to DTL function is unknown. We hypothesized that the C-terminus may serve a regulatory role in higher eukaryotes. We were able to identify a conserved PIP box within the extreme C-terminus of metazoan DTL orthologs. However, mutation of this PIP box did not abrogate PCNA interaction or disrupt cell cycle progression, indicating that this motif is not required for CRL4\textsuperscript{DTL}-mediated substrate regulation.

We next generated a DTL mutant (AC) lacking the less evolutionarily conserved C-terminus. We find that AC is competent for assembly into the CRL4\textsuperscript{DTL} ligase and can bind overexpressed substrates Cdtl and Set8. In fact, our data shows that DTL recognizes substrates through the DTL N-terminus, and this interaction is not dependent on substrate-PCNA interaction. Using stable cell lines expressing tet-inducible GFP-DTL, we reveal the biological consequences of expressing AC on cell cycle progression. Importantly, motifs and post-translational modifications
within the DTL C-terminus are not required for ubiquitin-mediated degradation of substrates during S phase or after DNA damage.

Results

Identification of a putative PIP box in the C-terminus of DTL

Since PCNA is required for CRL4DTL-mediated degradation, we asked whether DTL contains any motifs that could mediate direct interaction with PCNA. It has been speculated that DTL may directly bind PCNA. In support of this, one study has shown that the C-terminus of Xenopus DTL is capable of binding directly to PCNA in vitro. Moreover, GST pulldown assays revealed that Xenopus Xic1 and DTL compete with each other for binding to PCNA (Kim et al. 2010). Together, these results argue that the C-terminus of Xenopus DTL binds directly to PCNA via a PIP box. With this in mind, we searched human DTL for sequences resembling the canonical PIP box, Q-X-X-(V/I/L/M)-X-X-(F/Y)-(F/Y) (Moldovan et al. 2007), and located a putative PIP box between residues 706-713. Despite low conservation of the DTL C-terminus overall, sequence alignment revealed high conservation of the putative C-terminal PIP box (Figure 1A). If direct interaction between PCNA and DTL mediated by this PIP box were required for ligase activity, we would expect that its mutation would prevent substrate degradation and trigger checkpoint activation and G2/M arrest.
PCLNA Interaction Peptide (PIP box) motif: QXX(V/I/L/M)XX(FY)(FY)

<table>
<thead>
<tr>
<th>CRL4&lt;sup&gt;DTL&lt;/sup&gt; substrates</th>
<th>Hs Cdt1</th>
<th>Hs Set8</th>
<th>Hs p21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hs Dtl</td>
<td>PSSMRKICTYFHRKSE</td>
<td>719</td>
<td></td>
</tr>
<tr>
<td>Mn Dtl</td>
<td>PSSMRKICTYFRRKTQD</td>
<td>718</td>
<td></td>
</tr>
<tr>
<td>Dr Dtl</td>
<td>SKPMHKISSYFHRRTQD</td>
<td>647</td>
<td></td>
</tr>
<tr>
<td>Xl Dtl</td>
<td>FGSMHKINCTYFRKSE</td>
<td>711</td>
<td></td>
</tr>
<tr>
<td>DtlΔPIP</td>
<td>PSSARKACTAAAHRKSE</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(DTL 1-730)

**Figure 1. C-terminal PIP box does is not required for CRL4<sup>DTL</sup> activity.**

(A) Schematic of PCNA interaction motifs in CRL4<sup>DTL</sup> substrates, DTL homologs, and ΔPIP mutant. Residues corresponding to PIP box consensus residues are in red. (B) HeLa cells were transfected with GFP-empty, WT and ΔPIP. Whole cell lysates were prepared and immunoprecipitated with anti-GFP and immunoblotted with PCNA. PCNA co-immunoprecipitated with WT and ΔPIP equally well. (C) Representative cell cycle profiles from siRNA rescue assay. SiDTL and GFP-tagged H2B control, WT, R171A, or ΔPIP were co-transfected into HeLa cells. 72 hours later, cells were harvested and stained with PI for FACS analysis. Red indicates GFP negative cells, blue indicates GFP positive cells. (D) siRNA rescue assay; GFP positive cells in G2/M were quantified using FACS when co-transfected with siDTL (black) or without siDTL (white).
We created a ΔPIP mutant by mutating the consensus PIP box residues (M706, I709, Y712, F713) to alanines by site-directed mutagenesis and assessed its ability to bind PCNA. Mutation of the equivalent PIP box residues within CRL4<sup>DTL</sup> substrates Cdt1, p21, and Set8 is sufficient to disrupt PCNA interaction (Arias and Walter 2006; Higa et al. 2006a; Abbas et al. 2008; Abbas et al. 2010). We transiently transfected N-terminally GFP tagged WT and ΔPIP in HeLa cells and immunoprecipitated DTL using an anti-GFP antibody. We found that the ΔPIP interacts with PCNA as efficiently as WT (Figure 1B), which suggests that this motif does not mediate direct interaction with PCNA. Because the mild cell lysis and immunoprecipitation conditions used may not capture the interaction occurring on chromatin where the complex is actively targeting substrates, we wanted to test the functional capacity of ΔPIP in the absence of endogenous DTL.

To this end, we designed a siRNA rescue assay to assess whether the DTL PIP box was required for proper cell cycle progression. SiRNA knockdown of DTL causes activation of the G2/M checkpoint and subsequent accumulation of cells in G2, presumably due to the failure to properly degrade substrates during S phase (Jin et al. 2006; Sansam et al. 2006). If PCNA interaction through this C-terminal PIP box is required for recruitment to the substrate-PCNA complex and substrate targeting, then expression of siRNA-resistant ΔPIP would not rescue the loss of endogenous DTL, and cells would arrest in G2/M. HeLa cells were co-transfected with DTL siRNA and siRNA-resistant GFP-DTL (WT, R171A, and ΔC), and cells were collected 72 hours later. SiRNA knockdown consistently results in complete ablation of endogenous DTL protein by western blot; however, co-transfection of GFP-DTL constructs only yielded 20-40% GFP positive cells. Thus, we specifically analyzed the cell cycle profiles of GFP positive cells to determine the functional contribution of each GFP-tagged DTL construct, and used the GFP negative cells as an internal control for DTL knockdown efficiency (Figure 1C).

We used the percentage of cells in G2/M that were GFP positive to score whether or not the transfected DTL construct could functionally rescue the loss of endogenous DTL. Expression
of a DDB1-binding deficient mutant R171A resulted in accumulation of cells in G2, similar to cells lacking endogenous DTL (Figure 1D), demonstrating that substrate-recognizing ligases could not be assembled to properly target substrates. Conversely, expression of WT restores the ability of the CRL4^{DTL} complex to target and degrade substrates, thus the percentage of cells in G2/M was similar to that of siRNA control cells (Figure 1D). Importantly, expression of ΔPIP was able to completely rescue the G2/M phenotype (Figure 1D), indicating that the putative PIP box is not required for DTL activity in the CRL4 ligase.

**Generation of a DTL mutant lacking the less conserved C-terminus**

As mentioned previously, the C-terminus in metazoan DTL is not very well conserved (Figure 2A) and may have evolved other regulatory elements that modulate CRL4^{DTL} ligase activity. To examine the contribution of elements within the DTL C-terminus to substrate targeting, we constructed a DTL mutant comprising residues 1-410 (ΔC) that retains the N-terminal WD40 repeats. We also constructed the complementary mutant comprising residues 411-730 (ΔN) (Figure 2B). We wanted to use the ΔC mutant to answer the following questions regarding the potential regulatory elements located within the C-terminus. First, does the C-terminus promote DTL assembly with DDB1 and Cul4A into the ligase? Second, does the C-terminus play a role in substrate recognition? And third, is the C-terminus required for substrate degradation during normal cell cycle or after DNA damage?
Figure 2. DTLΔC is not phosphorylated, localizes to the cytoplasm, and interacts with the CRL4 complex.

(A) Schematic depicting conservation between metazoan DTL orthologs including human, mouse, Xenopus laevis, zebrafish, and Drosophila. Multiple sequence alignment was performed using ClustalW2. (B) Schematic demonstrating the DTL truncation mutants ΔC and ΔN relative to full-length wildtype (WT). (C) GFP-tagged DTL constructs, WT, ΔN, and ΔC were transfected. Whole cell lysates were immunoprecipitated with GFP antibody and immunoprecipitates were immunoblotted with antibodies recognizing GFP, DDB1, and Cul4A. ΔC still associates into a complex with DDB1 and Cul4A. Background bands (*) and GFP-WT, R171A top arrows, ΔC bottom arrow. (D) Immunofluorescence of cells transfected with GFP-tagged WT, ΔN, ΔC. WT and ΔN are primarily localized to the nucleus; ΔC localizes primarily to the cytoplasm. GFP (green) indicates DTL localization and DAPI stain (blue) indicates nuclei.
**DTL C-terminus is not required for CRL4<sup>DTL</sup> ligase assembly**

We first addressed whether elements within the C-terminus are required for assembly into the CRL4<sup>DTL</sup> ligase complex. It has been previously demonstrated that elements within the N-terminus of DTL are critical for association with DDB1, either through conserved arginines R171 or R246 within the WD40 repeats (Jin et al. 2006; Sansam et al. 2006). To test if DTL C-terminal elements modulated the association with DDB1 and Cul4A, we overexpressed GFP-tagged WT, R171A, and ΔC and immunoprecipitated with GFP antibody. As expected, R171A is unable to bind to endogenous DDB1 or associate with Cul4A (Figure 2C), demonstrating that DTL association with the scaffold protein Cul4A occurs through its interaction with DDB1. WT is able to bind DDB1 and, therefore, also associates with Cul4A (Figure 2C). ΔC is also able to interact with both DDB1 and Cul4A. We consistently observe reduced WT levels in comparison to ΔC by transfection, and as a consequence, less WT is pulled down in the immunoprecipitation. However, similar amounts of endogenous DDB1 and Cul4A co-immunoprecipitated with WT and ΔC (Figure 2C), thus, ΔC may not bind to DDB1 as efficiently as WT. Visualization of GFP-ΔC via immunofluorescence revealed increased localization to the cytoplasm compared to WT (Appendix A), which may account for the slight impairment in Cul4A and DDB1 association if the majority of the complex assembles in the nucleus. Another possibility is that ΔC is just as efficient as WT for DDB1 binding, but there is an excess of ΔC compared to endogenous DDB1 and Cul4A available for complex formation.
**DTL N-terminus binds to substrates Cdt1 and Set8**

Since the C-terminus of DTL was not required for ligase assembly, we next asked if the C-terminus was required for recognition of the substrate. For these experiments, 293T cells were co-transfected with GFP-tagged DTL constructs (WT, R171A, ΔC, and ΔN) and wildtype (WT) HA-tagged Cdt1 or myc-tagged Set8. Complexes were co-immunoprecipitated with antibodies against GFP or myc. In order to exclude the possibility that DNA bridged the interactions detected, all immunoprecipitations were performed in the presence of ethidium bromide. Under these conditions, we find that WT, R171A, and ΔC were all able to associate with HA-Cdt1-WT (Figure 3A, lanes 3-5). However, loss of the N terminus (ΔN) abrogated association with tagged substrates (Figure 3A, lane 6). Co-immunoprecipitation of DTL mutants with myc-Set8 revealed the same trend: WT, R171A, and ΔC can interact with Set8 while ΔN is unable to associate with the substrate (Figure 3B, lanes 3-5).

Characterization of the B+4 residue within the PIP degron had prompted speculation that DTL is recruited to an interface formed through PCNA-bound substrates, however, residues within PCNA or DTL that participate in this ternary complex have yet to be identified (Havens and Walter 2009). Thus, we wanted to test whether the N or C-terminus DTL mutants could also bind to substrates deficient for PCNA binding (ΔPIP). As before, we coexpressed GFP-DTL constructs with HA-Cdt1-ΔPIP or myc-Set8-ΔPIP. After coimmunoprecipitation with either GFP or myc antibody, we found that WT, R171A, and ΔC can also bind substrates that have a non-functional PIP box (Figure 3A, lanes 8-10, Figure 3B, lanes 7-9). Although this seems to conflict with the prevailing model of PCNA-dependent CRL4\textsuperscript{DTL} degradation, other groups have also shown interaction between substrates and DTL or DDB1 (Abbas et al. 2008; Kim et al. 2008; Abbas et al. 2010; Kim et al. 2010). An obvious caveat is that in all cases, one or both proteins were overexpressed. Thus, we attempted to titrate both our GFP-DTL constructs and tagged-substrates to

60
endogenous levels, however we were unable to detect any interaction even between wildtype DTL and wildtype substrates.

We have demonstrated that the C-terminus is not required for association with substrates Cdtl and Set8, and this interaction occurs regardless of whether or not the substrate can associate with PCNA. Although the N-terminus of DTL is competent for interaction with the substrate and the Cul4A-DDB1 complex, the C-terminus may be required for mediating proper ubiquitination and degradation of substrates, as suggested by a previous study (Kim et al. 2010). Because these co-immunoprecipitation experiments were performed in cell lysates with overexpressed proteins, we wanted to test if the C-terminus was required for regulated degradation of substrates during normal cell cycle.

**Generation of tet-inducible stable cell lines expressing DTL C-terminus mutant**

We wanted to assess the biological consequence of expressing the ΔC mutant in the absence of endogenous DTL, however, the siRNA rescue assay presented earlier had some limitations. Expression of GFP-DTL constructs by transfection generates a largely heterogeneous population where only 20-40% of the cells express GFP-DTL, and each of these cells may express a variable level of GFP-DTL. To create a system where we could robustly examine substrate regulation in homogeneous populations that express a uniform level of GFP-DTL, we generated clonal stable HeLa cell lines that express siRNA-resistant GFP-tagged WT, R171A, and ΔC in a tet-inducible manner using the lentiviral construct pCW-Tre-rTTA. Upon administration of doxycycline, cells rapidly upregulated GFP-DTL within 24 hours, and expression levels can be titrated by varying the concentration of doxycycline (Figure 4C).
Figure 4. Tet-inducible GFP-ΔC expressing cells can rescue the G2/M checkpoint phenotype.

(A) Clonal stable cell lines (pCW) expressing WT, R171A, or ΔC were transfected with siControl or DTL siRNA and expression was induced with 1 μg/ml doxycycline. Cells were harvested after 72 hrs, stained with PI, and analyzed by FACs. G2/M population was calculated from the total population of cells. (B) Western blot analysis from a representative experiment in (A) showing expression of GFP-DTL, R171A, and ΔC detected by GFP antibody. Endogenous DTL, WT and R171A were detected by antibody recognizing the DTL C-terminus, residual ΔC signal from GFP blot is present. (C) WT and ΔC clonal line was treated with 0.1-1.0 μg/ml doxycycline for 24 hrs. (D) siRNA knockdown and induction at low doses of dox (0.05-0.2 μg/ml). Cells were harvested at 72h, stained with PI, and analyzed for GFP positive by FACs. (E) As in (D), percentage of cells in G2/M were determined from total population of cells (F) Cells were plated on coverslips and grown either in the absence or presence of 0.2 μg/ml dox for 24 hrs. Cells were stained with GFP-antibody and Alexa-488 and visualized by immunofluorescence.
**DTL C-terminus is not required for normal cell cycle progression**

To determine whether the C-terminus is essential for regulation of ligase activity in cycling cells, we performed the siRNA rescue assay in our stable tet-inducible cell lines. First, we transfected DTL siRNA and simultaneously induced siRNA-resistant GFP-DTL expression with 1 μg/ml doxycycline in asynchronously dividing stable cell lines. After 72 hours, we analyzed cell cycle progression by FACs analysis (Figure 4A). All cell lines exhibited greater than 85% GFP positive cells. Expression of WT rescued the G2/M checkpoint phenotype, as demonstrated by the significant reduction of the number of cells accumulated in G2/M compared to non-induced cells (Figure 4A). As expected, R171A-expressing cells exhibited a pronounced accumulation of cells in G2/M, indicating activation of the G2/M checkpoint as a result of deregulation of CRL4^{DTL} substrates. Strikingly, when we expressed ΔC, we observed that the percentage of cells in G2/M were similar to WT (Figure 4A), suggesting that the CRL4^{DTL} ligase formed with the N-terminus of DTL is sufficient to restore proper regulation of substrates. Western blot analysis confirmed induction of GFP-DTL expression after doxycycline treatment and efficient knockdown of endogenous DTL (Figure 4B). GFP antibody was used to detect GFP-tagged WT, R171A, and ΔC. An antibody recognizing the C-terminus of DTL was used to detect endogenous levels of DTL (denoted by *) and verify efficient DTL siRNA knockdown. We observe that induction of GFP-WT and GFP-R171A using 1 μg/ml doxycycline causes expression much greater than endogenous. We also observe residual GFP signal recognizing ΔC in western blot using the C-terminal DTL antibody (Figure 4B).

When we titrated down the concentration of doxycycline such that GFP-DTL levels were similar to endogenous by western blot (0.1-0.2 μg/ml) (Figure 4C), the majority (60-80%) of the cells appeared to be GFP negative by FACs analysis (Figure 4D). Therefore, either a small subset of cells was expressing GFP-DTL at high levels, or all cells were expressing GFP-DTL at levels that approached the threshold of detection by FACs. However, we still observed significant rescue
of the G2/M phenotype by WT and ΔC at 0.2 μg/ml doxycycline (Figure 4E), though the rescue was a bit more variable between experiments and between different clonal lines expressing the same GFP-DTL construct. Because we observe a rescue that is not proportional to the percentage of cells that are supposedly expressing GFP-DTL as detected by FACs, we reasoned that the level of GFP-DTL expression must be reaching the limits of FACs detection. To verify this hypothesis, we induced cells with 0.2 μg/ml doxycycline, stained cells with GFP antibody, and visualized the cells by immunofluorescence (Figure 4E). Even at this low dosage, most cells exhibited GFP signal compared to uninduced cells at the same exposure, indicating that our FACs analyses was not accurately detecting GFP signal when expression was similar to endogenous levels. More importantly, we found that expression of ΔC at endogenous levels is still able to rescue loss of endogenous DTL to the same extent as WT, signifying that the rescue observed from significantly overexpressed ΔC is not an artifact due to overexpression.

In parallel with our cell cycle analysis, we performed a growth curve assay to examine whether lack of regulation by elements in the C-terminus of DTL altered the kinetics of cell proliferation. We induced expression of siRNA resistant GFP-DTL at the time of siRNA transfection, and after 48 hours, monitored cell growth for 4 additional days (Figure 5A). Western blot analysis verified expression of each siRNA resistant GFP-tagged DTL construct over the four days of the growth assay, as well as the sustained loss of endogenous DTL via siRNA knockdown (Figure 5B). Loss of endogenous DTL concomitant with expression of the DDB1-binding deficient mutant R171A causes a profound G2/M arrest and cells ceased to proliferate (Figure 5C). In addition, non-induced and R171A expressing cells exhibited an increase in cells with >4N DNA content over time (Figure 5D), which is indicative of rereplication. Conversely, WT-expressing cells exhibit continuous exponential growth (Figure 5C), and do not display accumulation of G2/M cells or >4N DNA content over time (Figure 5D). Importantly, ΔC-expressing cells divided at a similar rate as WT-expressing cells over time (Figure 5C), demonstrating that lack of regulation
Figure 5. ΔC-expressing cells proliferate with the same kinetics as WT

(A) Schematic of experimental set-up. Stable tet-inducible HeLa cell lines (pCW) were transfected with 100 μM siDTL and GFP-WT, R171A, and ΔC expression was induced with 1 μg/ml Dox simultaneously. After 48 hours, cells were collected at the indicated days. (B) Experiment performed as in (A). Whole cell lysates were harvested corresponding to each day and immunblotted to detect GFP-tagged DTL (anti-GFP), endogenous and actin (loading control). (C) Experiment performed as in (A) with one set uninduced (-Dox, open points) and the other induced with 1 μg/ml (+Dox, filled points). After 48 hours, cells were plated at 1x10^5 and counted at the indicated days. Cells were counted on each indicated day. (D) As in (A), (C). Cells were stained with PI and analyzed by FACS analysis.
that may be conferred by the C-terminus of DTL does not adversely affect the coordination and
timing of each cell cycle phase. Cells expressing ΔC also did not display an accumulation of cells
in G2/M or >4N DNA content over time (Figure 5D). Together, this data indicates that the ΔC can
rescue loss of endogenous DTL, and that the C-terminus is not required for normal cell cycle
progression.

**DTL C-terminus is not required for Cdt1 and Set8 degradation during S
phase or after DNA damage**

To directly monitor substrate degradation during S phase, we synchronized our stable cell
lines and examined protein levels as cells progressed through S phase. Similar to previous
experiments, we transfected DTL siRNA and induced GFP-DTL expression simultaneously in our
tet-inducible cell lines (Figure 6A). After 48 hours, cells were arrested by nocodazole treatment,
and the mitotic cells were harvested by manual shake-off and released either induction media.
Samples were taken at the indicated timepoints (8, 12, and 16 hours after nocodazole release) to
capture cells in mitosis, G1, and S phases. Cell cycle profiles of mock infected, WT, and ΔC cells
after release from nocodazole are virtually indistinguishable (Figure 6C). Furthermore, endogenous
levels of Cdt1 and Set8 at each time point by western blot revealed that ΔC is able to degrade
substrates during normal cell cycle just as WT (Figure 6B). These results support our findings from
the previous cell proliferation assays and demonstrate that the DTL N-terminus is sufficient for
proper ubiquitination and degradation of Cdt1 and Set8 during normal S phase progression.

The CRL4\(^{\text{DTL}}\) complex also targets Cdt1 and Set8 in response to DNA damage to prevent
replication of damaged DNA. This occurs irrespective of cell cycle phasing because substrates are
degraded within 30 minutes after damage in asynchronously dividing cells (Hu et al. 2004; Abbas
et al. 2010; Ishii et al. 2010). During normal cell cycle progression, CRL4\(^{\text{DTL}}\) activity is restricted
to S phase, and so we wanted to determine if the C-terminus of DTL contributes to the ability of the CRL4DTL ligase to target substrates in phases in addition to S in response to DNA damage.

We utilized our stable cell lines to address whether the C-terminus contained elements that contribute to CRL4<sup>DTL</sup> ligase regulation after exposure to UV-irradiation (Figure 6C). Control cells were depleted of endogenous DTL, and without doxycycline induced GFP-DTL expression. These cells exhibited significantly increased levels of Set8 compared to mock infected (Figure 6D, lanes 3-8 compared to lane 1), consistent with previous studies (Abbas et al. 2010). Cells expressing R171A lack a functional CRL4<sup>DTL</sup> complex, and undamaged cells also exhibit high levels of Set8 (Figure 6D, lane 11). In contrast, induction of WT or ΔC expression restored CRL4<sup>DTL</sup> ligase function and levels of Set8 are equivalent to levels of undamaged cells without siRNA knockdown (Figure 6D lane 9 and 13). Upon exposure to UV, cells unable to assemble a functional CRL4<sup>DTL</sup> ligase, either as a result of endogenous DTL knockdown or R171A expression, could not promote Cdt1 or Set8 degradation (Figure 6D, lane 6 and 12). Cells expressing WT showed restoration of CRL4<sup>DTL</sup> activity via complete degradation of Cdt1 and Set8 after UV irradiation (Figure 6D, lane 10). Importantly, Cdt1 and Set8 were also completely degraded after DNA damage in cells expressing ΔC (Figure 6D, lane 14), indicating that regulatory elements in the C-terminus of DTL are not required for DNA-damage-dependent assembly of a functional CRL4<sup>DTL</sup> complex or subsequent ubiquitination and degradation of substrates.
Figure 6. DC can degrade Cdt1 and Set8 during S phase and after UV damage.

(A) Schematic of experimental set-up. Stable tet-inducible HeLaS (pCW) were transfected with 100 μM siDTL and GFP-WT or ΔC expression was induced by treatment with 1 μg/ml Dox. 48 hours later, cells were synchronized with 100 ng/ml nocodazole, and released. Cells were collected at the indicated time points, stained with PI and cell cycle phasing was quantified by FACS analysis. Mock infected cells were not transfected with siDTL and were used as control for cell cycle phasing. (B) Cells prepared as in (A). Whole cell lysates were harvested at the indicated time points and immunoblotted for detection of GFP-tagged WT or ΔC (anti-GFP), endogenous DTL (anti-DTL), Cdt1, Set8, and actin (loading control). (C) Cells prepared as in (A). Cells were stained with PI and analyzed by FACS. (D) Schematic of experimental set-up. Stable tet-inducible HeLa cells were transfected with 100 μM siDTL or siControl and GFP-WT, R171A (RA), and ΔC expression was induced by treatment with 1 mg/ml Dox. After, cells were treated with 50 J/m², and one hour later, cells were harvested. (D) Cells prepared as in (C). Whole cell lysates were immunoblotted for GFP-WT, R171A, and ΔC (anti-GFP), endogenous DTL (anti-DTL), Cdt1, Set8, and actin.
Discussion

Previous efforts directed at understanding the mechanisms that control CRL4\textsuperscript{DTL} activity have focused on defining the regulatory elements within each substrate that directs degradation. Studies have comprehensively demonstrated that PIP degrons within the substrates are required for proper substrate degradation, but no group has addressed the requirements of DTL that are required. Thus, the current model of CRL4\textsuperscript{DTL} activity is rather substrate-centric, with limited understanding of what regulatory mechanisms control the substrate receptor DTL in complex recruitment and initiation of CRL4\textsuperscript{DTL} ubiquitin ligase activity. Here we present the first study to directly assess the contribution of regions of DTL to CRL4\textsuperscript{DTL} ligase activity in the context of live cycling mammalian cells.

We hypothesized that the DTL C-terminus, due to its reduced conservation through evolution, may play a critical role in regulating DTL function within the CRL4\textsuperscript{DTL} complex in higher eukaryotes. Identification of a PIP box within the C-terminus of DTL that had high sequence similarity to the canonical PIP box and evolutionary conservation was promising. However, mutation of the motif did not abrogate detection of PCNA by immunoprecipitation. Though we were unable to find any other PIP box within DTL that conformed so well to the canonical sequence, DTL may contain additional degenerate PIP boxes or non-PIP box motifs that are competent for PCNA-binding. Another possibility is that we detected PCNA that was bound to an associated substrate.

Here we have demonstrated that the C-terminus (residues 411-730) is not required for ligase assembly, recognition of substrates, or substrate degradation during normal cell cycle progression or after DNA damage. Many groups, including us, have observed that the DTL C-terminus is post-translationally modified via phosphorylation (Matsuoka et al. 2007; Ueki et al. 2008). DTL phosphorylation has been associated with DNA damage and localization to chromatin,
however the consequences of non-phosphorylatable mutants on CRL4\textsubscript{DTL} activity had not been investigated (Matsuoka et al. 2007; Ueki et al. 2008; Ishii et al. 2010). Since many DNA damage response pathways signal through phosphorylation events, we hypothesized that CRL4\textsubscript{DTL}-mediated degradation of substrates after damage may be dependent upon a C-terminal phosphorylation. The ΔC mutant we have analyzed in this study is not phosphorylated (Appendix A), and thus, we find that DTL phosphorylation is not required for association with the CRL4 core or ligase activity during normal cell cycle or after DNA damage.

We also detected interaction between the N-terminus of DTL and substrates Cdt1 and Set8. WD40 repeats are known to fold into a β-propeller structure to mediate protein interactions. Two tandem WD40 motifs may interface with DDB1, but there are four other WD40 repeats that could bind to other proteins, such as substrates. It would be interesting to create N-terminal mutants to identify which WD40 repeats direct substrate interaction and determine if binding is mutually exclusive between substrates in mammalian cells. Truncation mutants of \textit{S. pombe} Cdt2 reveal that the 3rd and 4th WD40 repeats promote Spd1 binding via GST pulldown assays (Liu et al. 2005).

Strikingly, we find that interaction of DTL with Cdt1 and Set8 is not dependent upon substrate interaction with PCNA. We did not observe elimination of or even reduced interaction between WT, DDB1-binding deficient mutant R171A, and ΔC with PIP box mutant substrates, demonstrating that DTL binding is not dependent upon a PCNA-substrate interaction. Consistent with our finding, other groups have also shown interaction between the ΔPIP mutant substrates and DTL or DDB1 (Liu et al. 2005; Higa et al. 2006a; Kim et al. 2008; Abbas et al. 2010; Kim et al. 2010). In the current model, CRL4\textsuperscript{DTL} is recruited to substrate-bound PCNA via an interaction between a basic residue within the PIP degron of the substrate and DTL. Our immunoprecipitation data indicates that the B+4 residue interacts with the N-terminus of DTL, and this interaction may occur independently of PCNA-substrate interaction. If this is the case, then DTL and the CRL4\textsuperscript{DTL} ligase may be able to bind the substrates that are not on chromatin or bound to PCNA. This model
would suggest that PCNA or DNA could serve as a signal to trigger substrate ubiquitination once bound to chromatin. It would be interesting to specifically identify the WD40 repeat or motif within DTL that mediates interaction with the B+4 residue in the substrate.

**Experimental Procedures**

**Cell culture, Drugs, and Transfection**

HeLa and 293T cell lines were obtained from the American Type Culture Collection (ATCC) and cultured in DMEM (Invitrogen) supplemented with 10% FBS and L-glutamine. Tet-free media contained Tet-System Approved FBS (Clontech). 293FS cells were a kind gift from Dr. Keara Lane. UV treatment was performed with UV Stratalinker. Plasmids were transfected into cells using TransIT-LT1 Transfection Reagent (Mirus Bio) according to the manufacturer’s instructions. SiRNA targeting DTL (sense: GUAUGGGAUUUACGUAGAUU, Thermo Scientific Dharmacon) was transfected into cells at 100 nM using RNAi-max (Invitrogen), according to the manufacturer’s instructions.

**Plasmids**

DTL was cloned into pDEST-GFP as described in Chapter 2. DTL mutants were generated using site-directed mutagenesis using the following primers (5’ to 3’):

- siDTLres1-F: GCAGGAGCTGTGGATGGGATAATCAAAGTCTGGGACCTCCGTAAGAATTATACTGC
- siDTLres1-R: GCAGTATAATTCTTACGGAGGTCCAGACTTTGATTATCCCATCCACAGCTCCTGC
- siDTLres2-F: GGAGATAATCAAGTCTGGGACCTCAGGAAAAATTATACTGCTTATCGAACAAAG
- siDTLres2-R: TTCTTGTACAGTACGTAATTTTCTTCCGTAGGTCGACGACTTTGATTATCCC
- R171A-F: CATTATGGTCTGGGATACCGCGTGCAACAAAAAAGTGG
- R171A-R: CCCATCTTTTTTGTGACGCCGTATCCAGACACCATAATG
- ΔC-F: TGGGTGATATATGAGAAGTAAGAAGAGG
- ΔC-R: TCTTGACTCTTTTTTCTACTGAGGCCCAACCCA
- ΔPIP1-F: AGCTCCATGAGGAAATACTGCACAGCCGCCCATAGAAAGTCCCGAGG
- ΔPIP1-R: CTCCTGGGACCTTTCTATGGGCGGCTGTGCAGATTTTCCTCATGGAGCT
- ΔPIP2-F: TCACGCCCAGCTCCGAGGAAAGACCTGCACAGCCGCCC

71
Site-directed mutations were introduced by PCR amplification using the designated primers. PCR reactions were digested with DpnI to digest template DNA for 3 hours at 37°C. 2 μl of the PCR reaction are transformed into α-select competent cells and grown on plates with antibiotic resistance. ΔN was generated by amplifying the N-terminal fragment by PCR (primers F: CACCAAGAAAAAGAGTCAAGAC, R: CTATAATTCTTTGTTGTTTCA) for cloning into pENTR-TOPO, then transferred to pDEST-EGFP expression vector using LR Clonase Reaction II (Invitrogen). Nuclear localization sequence (NLS) comprising amino acids APLLLKL was added by annealing the following oligos F: CCGGATGGCTCCAAAGAAGAAGCGTAAGGTA, R: TACCGAGGTTTCTTTCCATACCATGCC and ligated into AgeI site upstream of GFP-DTL-AC. pCW-Tre-rtTA tet-inducible lentiviral vector and lentiviral packaging constructs A8.2 and VSV-G were a kind gift from Dr. Keara Lane, MIT. GFP-tagged DTL WT, R171A, and ΔC were amplified using primers and ligated into PacI and HpaI digested pCW-vector. The following primers were used:

GFP-WT/R171A:  
F: TTGTTAACATGGTGAGCAAGGG  
R: TTCGATCGCTACGCGCTTCTCGTT
GFP-ΔC:  
F: TTGTTAACATGGTGAGCAAGGG  
R: CCAGGTACCTTACGCTTCTTTGGAGCCAT

Myc-Set8-WT and myc-Set8-ΔPIP were a kind gift from Dr. Tarek Abbas and Dr. Anindya Dutta, University of Virginia. CDT1 cDNA was cloned into pENTR-TOPO, then transferred to pDEST-HA expression vector using LR Clonase Reaction II (Invitrogen). HA-CDT1-ΔPIP was generated using site-directed mutagenesis with the following primers:

Q3A, V6A  
F: CGCCATGGAGGCGCGGCACGCCGCCAGGAGCC  
R: GGGAGTCGGTGAGGCGGCCGCGCGCGCCGCCCTGCATGGCC
F9A, F10A  
F: CCGCGTCAGGAGCACGCGCGCGCGCGCGGCAGCGCGC  
R: CGGGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGG

ΔPIP2-R  
GGGCGGCTGTGCGAGGCTTTCCTCGCGGAGCTGGGCGTGA
Generation of Stable pCW Tet-inducible Cell Lines

Lentivirus was produced in 293FS cells by co-transfecting packaging constructs Δ8.2 and VSV-G. Viral supernatant was collected 48 hours after transfection, and filtered through 0.45 μM before adding 70% confluent HeLa cells in tet-free media for infection. Polybrene was added to a final concentration of 8 μg/ml. After 24 hours, the media was changed to fresh tet-free media. After an additional 24 hours (48 hours after transfection), HeLas were split and induced with 1 μg/ml doxycycline. Twenty-four hours later (or 72 hours after infection), cells were approximately 15-30% GFP positive and were collected for FACS sorting. The highest 80% green cells were collected and replated at extremely low density to facilitate ring cloning (estimated 50 cells per 15 cm plate) in tet-free media. Ten-fourteen days after sorting and plating, colonies were visible. Rings were used to collect clonal populations and each clone was expanded to test GFP-DTL expression after induction and cell cycle profile.

Immunoprecipitation

For immunoprecipitations, cells were lysed in an NP-40 lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% NP-40, protease inhibitor cocktail (Roche), 0.1 mM PMSF, 1 mM NaF, 10 mM β-glycerophosphate, 200 μM Na3VO4) or 0.1% TX-100 buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, and protease/phosphatase inhibitors as before) for 30 minutes on ice. Immunoprecipitation of DTL with substrates were performed in the presence of 5 μg/mL EtBr. Lysates were clarified by spinning at 4°C for 10 minutes at 14,000 rpm and the soluble supernatant was incubated with antibodies overnight at 4°C while rocking. Protein A/G, Protein A, or Protein G (Santa Cruz Biotechnologies) agarose beads were added for 1 hr at 4°C with rocking. The immunoprecipitates were washed three times with lysis buffer, resuspended in 2X Laemmli buffer, boiled for 10 minutes, and fractionated by SDS-PAGE gel electrophoresis. For 32P-labeled immunoprecipitations, cells were labeled for 4 hours with 32P (0.5 mCi per 10 cm
plate), lysed in 0.5% NP-40 lysis buffer (supplemented with protease and phosphatase inhibitors as described above) and immunoprecipitated (as above). Immunoprecipitates were fractionated by SDS-PAGE gel electrophoresis, and the gel was dried on a gel dryer (Hoefer) at 80°C for 2 hours before being exposed to autoradiography film.

**Western Blot Analysis**

Cells were lysed in RIPA buffer or IP buffer and quantified by Bradford protein assay (Bio-Rad). 30-60 μg of protein were boiled with Laemmli sample buffer, loaded on 6-8% SDS-PAGE gels, and run in Bio-Rad Mini-Protean apparatus. Gels were transferred to PVDF membrane (Millipore) at 4°C, and blocked with 4% dry milk. All antibodies were diluted in 4% dry milk; primary antibodies were incubated on membranes for 1 hour at room temperature or overnight at 4°C, and HRP-conjugated secondary antibodies (GE Healthcare) were diluted 1:5000 in 4% milk and incubated for 1 hour at room temperature. Blots were washed 5X with TBS-T (0.1% Tween). Protein bands were visualized by ECL (Perkin Elmer, GE Healthcare) and exposure to autoradiography film (Denville).

**Antibodies**

The following antibodies were used: Cul4A (100-401-A04, Rockland Immunochemicals), DDB1 (ab9194, Abcam), GFP (11814460001, Roche), Set8 (Cell Signaling Technology), HA (HA.11, Covance), CDT1 (H-300), myc (9E10), GFP, and actin (Santa Cruz Biotechnologies), a-tubulin (Sigma), and DTL (A300-947A, A300-948A) (Bethyl Laboratories).

**FACS**

For analysis, cells were trypsinized and resuspended in 70% ethanol/PBS at -20°C overnight. Fixed cells were reconstituted in PBS containing 50 mg/ml propidium iodide and 50 mg/ml RNase.
Samples were analyzed by FACScan (Becton Dickinson) and Flow Jo (Tree Star, Inc.) or ModFit LT (Verity Software). For sorting, cells were trypsinized and resuspended in 0.5 ml of media. Cells were sorted by MoFlo Cell Sorter (Becton Dickinson) into 15 ml conicals containing FBS.

**Immunofluorescence**

HeLa cells were grown on coverslips and were transfected with GFP-tagged DTL constructs for 24 hours. Cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature and washed 3X with PBS. Cells were permeabilized and blocked in PBS + 0.2% TX-100 and 10% goat serum. Coverslips were stained with anti-GFP (Roche) for 1 hour in PBS + 2% goat serum and AlexaFluor 488 (Invitrogen). Coverslips were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Inc.). Images were taken with Zeiss Axiophot II upright microscope and OpenLab software (PerkinElmer).

**Acknowledgements**

I would like to thank Chris Sansam and members of the Lees lab, particularly Nelly Cruz and Keren Hilgendorf, for helpful comments and discussions.
Chapter III: Final Discussion
Key Conclusions

The work comprising this thesis began with the studies discussed in Appendix A: characterization of DTL, the substrate receptor of the CRL4\textsuperscript{DTL} ligase, during normal cell cycle progression in human cells. We found that human DTL protein levels are expressed in G1, persisting past DTL mRNA transcriptional downregulation, until early mitosis. This sparked our interest in pursuing a greater understanding of the regulatory mechanisms of DTL, with the intent of gaining insight into CRL4\textsuperscript{DTL} activity and substrate regulation.

DTL itself is regulated by the ubiquitin-proteasome system during mitosis. Although we show that DTL is not degraded by the CRL4 or the APC ubiquitin ligases, we have evidence that signals within the C-terminus of DTL mediate its degradation. Surprisingly, expression of a DTL mutant lacking the C-terminus (ΔC1) does not adversely affect cell cycle progression or CRL4\textsuperscript{DTL} substrate regulation. These results demonstrate that human DTL is regulated differentially from \textit{S. pombe} CRL4\textsuperscript{DTL} or the APC, where overexpression of substrate recognition subunits is sufficient to induce substrate degradation (Visintin et al. 1997; Liu et al. 2005).

Our observation that DTL is phosphorylated at multiple sites during the cell cycle offered an attractive regulatory mechanism to control CRL4\textsuperscript{DTL} activity. Analysis of C-terminal deletion mutants revealed that DTL is phosphorylated (likely at multiple sites) between residues 590-730, with a possible additional phosphorylation in a subset of asynchronous cells between residues 500-590. Inability to phosphorylate DTL or stable DTL expression was not detrimental to cell cycle progression, nor did it affect substrate interaction or degradation during normal S phase and after DNA damage. We also show, for the first time, that the N-terminus of human DTL is sufficient for substrate binding in a PCNA-independent manner, and amend the model to specify that CRL4 ligase activity is recruited through the N-terminus of the specificity factor, DTL.
Assembly of an active CRL4\textsuperscript{DTL} ubiquitin ligase

CRL4\textsuperscript{DTL} activity has been shown to be restricted to S phase and chromatin-dependent (Arias and Walter 2005; Nishitani et al. 2006). Our initial characterization of DTL, Cul4A, and DDB1 protein levels, localization, and association throughout the normal cell cycle suggested that the CRL4\textsuperscript{DTL} ubiquitin ligase could assemble in any cell cycle compartment at any stage of the cycle, except in mitosis when DTL is degraded. Now we have also shown that the N-terminus associates with substrates, and this occurs whether or not substrates can bind to PCNA. This begs the question: what prevents the assembled ligase from associating and degrading substrates off of chromatin and in other phases? Other modes of ligase activation, other than the requirement of PCNA, have not been rigorously examined, but may also play a significant role in triggering CRL4\textsuperscript{DTL} activity.

We know that DTL expression is not sufficient for an active ligase because ectopic expression of DTL in mitosis and early G1 does not cause aberrant substrate degradation. One possible means to regulate CRL4\textsuperscript{DTL}-mediated destruction to S phase is through temporally restricting DTL-substrate interaction. We intend to determine whether DTL and substrates can co-immunoprecipitate in late G1. We are also interested in determining whether the N-terminal WD40 repeats mediate this interaction, and if so, which repeats. It seems highly likely that these structures, which are also found in SCF F-box specificity factors and mediate substrate recruitment, would mediate DTL interaction with substrates. By using data from \textit{S. pombe} N-terminal mutants (Liu et al. 2005) and protein modeling techniques to get a better sense of potential secondary structure, we plan to generate and analyze a panel of WD40 mutants and their abilities to bind distinct substrates.

Some studies have shown that the neddylation is required for cullin ubiquitin ligase activity in mammalian cells (Ohh et al. 2002). Cul4A can be neddylated \textit{in vitro} (Osaka et al. 1998), and functional and biochemical interaction between the CSN and Cul4A has been detected in \textit{S. pombe}
(Liu et al. 2003), Arabidopsis (Chen et al. 2006), and human cells (Groisman et al. 2003; Higa et al. 2003). A recent study demonstrated that the CSN subunits 5 and 6 prevent autoubiquitination of substrate receptors, and are required for proper ligase activity (Raman et al. 2011). It remains to be seen whether autoubiquitination of DTL by the CSN occurs specifically in mitosis or constitutively throughout the cell cycle.

And lastly, Cul4B is the paralog of Cul4A, and has been shown to assemble into a CRL4 ligase to regulate Cdt1 (Higa et al. 2003; Senga et al. 2006). Some functional redundancy between Cul4A and B has been suggested, however more recent CRL4<sup>DTL</sup> studies have only evaluated Cul4A-based ligases. It is unknown whether all, or just a subset of the currently identified CRL4<sup>DTL</sup> substrates can be regulated by a Cul4B-based ligase as well.

**Revisiting the role of PCNA**

Careful studies have shown that Cdt1 must have an intact PCNA interaction motif for CRL4<sup>DTL</sup>-dependent destruction (Arias and Walter 2006; Higa et al. 2006a; Hu and Xiong 2006; Jin et al. 2006; Senga et al. 2006). This key observation led to the development of a model in which substrates binding to chromatin-bound PCNA signals for CRL4<sup>DTL</sup> recruitment, which all subsequent studies have adhered to. The identification and dissection of the PIP degron in CRL4<sup>DTL</sup> substrates revealed bipartite organization: the TD motif confers high affinity binding with PCNA while the B+4 is critical for DTL recruitment. The close proximity between the PCNA binding site and the single residue that recruits DTL led to the hypothesis that other determinants for DTL recruitment must be located in PCNA, and the interaction between PCNA and substrates drives DTL recruitment and subsequent CRL4<sup>DTL</sup> activity (Havens and Walter 2009). Our results, presented in Chapter 2, show that the DTL N-terminus can interact with substrates that have mutations in key PCNA interaction residues (∆PIP mutants), demonstrating that there are no PCNA requirements needed for DTL-substrate interaction. Other studies also have shown experimental
evidence of ΔPIP mutants interacting with DTL or DDB1 (Abbas et al. 2008; Abbas et al. 2010; Kim et al. 2010), although no conclusions regarding this discrepancy were made.

Many groups have shown that ΔPIP substrates cannot be ubiquitinated in various in vitro ubiquitination assays, and overexpression of stabilized ΔPIP substrates results in rereplication and checkpoint activation phenotypes, similar to those after DTL knockdown (Arias and Walter 2006; Abbas et al. 2008; Abbas et al. 2010). We have no data to suggest that PCNA is not required for the ubiquitination and degradation of substrates, though it would be informative to use our tet-inducible DTL cell lines to assess whether ΔPIP substrates can be regulated normally by DTL-ΔC (N-terminus). Now that we have shown that DTL recruitment to substrates is not dependent on PCNA, we believe that the relationship between PCNA and ubiquitination of substrates is much more complicated than previously thought. The PIP degron contains both PCNA and DTL interaction determinants within a span of 11 amino acids, which seems small for two proteins to contact simultaneously. Kim et al. constructed p21 mutants that separated the PCNA interaction motif from the putative DTL interaction lysine, and found that the sites did not have to be adjacent to bind to PCNA or DTL (Kim et al. 2010). However p21 and evolutionarily related homologs may be regulated differently than other substrates, as we have seen overexpressed p21 interact with both the N and C terminus of DTL independently. Importantly, it has not been definitively shown that the same molecule of PCNA binds to a substrate and DTL at the same time. Altogether, PCNA is somehow required for CRL4DTL-mediated degradation, however the precise mechanism still warrants further investigation.

Identification of additional substrates

Independent studies have tried various approaches to identify additional substrates: genetic analysis, screening protein sequences for PIP degrons, and immunoaffinity purification of overexpressed components of the complex. The latter strategy, though powerful, has failed to
identify any substrates of the CRL4<sup>DTL</sup> complex. Identification of ubiquitin ligase substrates by IP/mass spectrometry approaches are challenging because often the interactions between specificity factors and substrates are very transient. An additional complication unique to the CRL4<sup>DTL</sup> ligase is that substrate interaction appears to occur on chromatin; immunoprecipitation buffers used to successfully detect associated proteins may not be stringent enough to isolate these interactions from the cell.

Currently, all known CRL4<sup>DTL</sup> substrates are targeted during S phase. The fact that DTL expression persists until mitosis implies that DTL function is also required outside of S phase. DTL expression expression in G1 may be required for appropriate regulation of downstream targets after DNA damage, however in G2, S-phase targets have already been degraded. Perhaps there are additional G2 targets that are regulated by DTL that contribute to the additional mitotic phenotypes observed in zebrafish Dtl mutant embryos (Sansam et al. 2006).

**Targeting CRL4<sup>DTL</sup> for therapeutic potential**

MLN4924 is an exciting new drug with promising anti-tumorigenic properties that specifically inhibits neddylation of cullin RING ligases (Soucy et al. 2009; Lin et al. 2010; Milhollen et al. 2011). Because of its therapeutic potential, the mechanism of action has been recently under intense investigation. MLN4924 treatment induces massive rereplication and subsequent checkpoint activation, ultimately leading to apoptosis or senescence. Neddylation inhibition theoretically affects activation of all cullin-based ubiquitin ligases, and the stabilization of substrates from distinct CRLs supports this (Soucy et al. 2009). However, recent experimental evidence demonstrated that the rereplication observed is caused by Cdt1 stabilization (Milhollen et al. 2011), which is targeted by both cullin ligases SCF<sup>Skp2</sup> and CRL<sup>DTL</sup>. Lin et al. went on further to show that the Cdt1 accumulation and rereplication observed after MLN4924 treatment is mediated primarily through inhibition of CRL4<sup>DTL</sup> (Lin et al. 2010). Because Set8 has been recently
identified as a CRL4\textsuperscript{DTL}-specific substrate (thus far) that can also cause rereplication, it would be informative to determine whether MLN4924-induced rereplication is also a function of Set8 stabilization. This would also solidify CRL4\textsuperscript{DTL} as the main mediator of the therapeutic response.

These findings have interesting implications for the importance of CRL4\textsuperscript{DTL} regulation in normal cell cycle and in cancer cells. Since cullin ligases target so many major cell cycle regulators, other potentially anti-tumorigenic or oncogenic factors, and a potential wealth of unidentified substrates, it is fascinating that the net result of inhibiting neddylation of all cullins may be attributed to one specific cullin ligase, CRL4\textsuperscript{DTL}. It elegantly demonstrates that deregulation of replication has extremely detrimental consequences, surpassing the consequences of any other pathway. The significance of replication fidelity and the role of CRL4\textsuperscript{DTL} as a protector of genome integrity cannot be underestimated.
Appendix A: Characterization of DTL, the specificity factor of the CRL4 ligase, during the normal cell cycle

Crystal J. Lee and Jaqueline A. Lees

Experimental contributions:
C.J.L. conducted all the experiments presented in this chapter, with the exception of Figure 4D, which was kindly performed by S. Rankin at the Oklahoma Medical Research Facility, and mass spectrometry analysis, which was performed by Swanson Biotechnology Core at the Koch Institute for Integrative Cancer Research.
Summary

Ubiquitin-mediated proteolysis of key cell cycle regulators ensures proper progression between cell cycle phases. The CRL4<sup>DTL</sup> ubiquitin ligase targets a number of critical proteins involved in replication and DNA repair processes, and loss of the substrate recognition subunit DTL causes massive rereplication, activation of the damage-induced G2/M checkpoint, and subsequent cell cycle arrest. However, not much is known about the regulation of DTL itself. In this study, we elucidate potential regulatory mechanisms that may play a role in controlling DTL and overall CRL4<sup>DTL</sup> ligase activity. We find that DTL protein expression is cell cycle regulated: it is at its highest during late G1, S, and early G2. While expressed, DTL is present in the cytoplasm and nucleus, and can associate with CRL4 complex. We observe a decrease of DTL protein level in mitosis, suggestive of ubiquitin-mediated proteolysis. Upon further analysis, degradation does not appear to be mediated by CRL4 or APC ubiquitin ligases. DTL is also phosphorylated within the C-terminus (between residues 500-730), likely at multiple sites, and we use a combination of mass spectrometry and mutational analysis to identify phosphorylation sites within DTL that may play a role in its regulation.
Introduction

Cullin-RING E3 ubiquitin ligases (CRLs) represent the largest family of E3 ubiquitin ligases in eukaryotic cells and play significant roles in various cellular processes including transcription, cell cycle control, proliferation, and DNA damage response. All CRLs adopt similar architectural structure: a cullin protein acts as a scaffold to interact with a specific adaptor protein and many different substrate specificity factors in a combinatorial manner. Each substrate receptor targets specific substrates, thereby conferring control over hundreds of proteins to a single cullin-adaptor pair (Petroski and Deshaies 2005).

The CRL4\textsuperscript{DTL} ubiquitin ligase comprises either cullin 4A or 4B, the adaptor protein DDB1 (Damage-specific DNA Binding protein-1), and the specificity factor DTL (Denticleless, also known as Cdt2/DCAF2/RAMP/L2DTL). Recent work demonstrated that CRL4\textsuperscript{DTL}-mediated degradation of key cell cycle regulators occurs specifically during active replication in S phase and after DNA damage (Higa et al. 2006a; Jin et al. 2006; Abbas et al. 2008; Kim and Michael 2008; Shibutani et al. 2008; Abbas et al. 2010; Kim et al. 2010). Loss of any component of the ligase causes deleterious effects, prompting intense investigation aimed at identifying additional substrates and regulatory mechanisms of the complex.

Because substrate receptors dictate substrate specificity to each CRL, it can be inferred that substrate receptor regulation is critical for ligase activity. In \textit{S. pombe}, the level of DTL ortholog protein is sufficient to drive activation of the CRL4\textsuperscript{DTL} ligase against one of its substrates Spdl. Furthermore, protein expression of the \textit{Sp}Dtl is regulated at the transcriptional level, with mRNA and protein levels peaking at S phase when CRL4\textsuperscript{DTL} actively targets substrates (Liu et al. 2005). In contrast, little was known about regulation of human DTL when we began our studies. DTL mRNA transcripts were found to be periodically expressed during the cell cycle in HeLa cells by a genome-wide study that performed cDNA microarray analysis of transcripts from 5 independent
synchronization experiments (Whitfield et al. 2002). DTL mRNA expression is highest in G1 and early S phase and decreases during S, G2, and M phases, similar to SpDtl mRNA expression. However, it was unknown whether DTL protein levels also control CRL4DTL ligase activity in human cells in an analogous manner to S. pombe.

In this study, we demonstrate that human DTL protein level oscillates and is phosphorylated during normal cell cycle progression in HeLa cells. Unlike DTL mRNA transcripts, DTL protein persists through G1, S, and G2 phases, and although CRL4DTL ligase activity is restricted to S phase, DTL can associate with DDB1 throughout its expression. DTL is ubiquitinated and degraded in a proteasomal dependent manner in mitosis. We constructed DTL mutants to identify the ubiquitin ligase that targets DTL. We find that the CRL4 and APC ligases do not degrade DTL. We also show that DTL is phosphorylated in the C-terminus between residues 500-590, and observed a correlation between phosphorylation and nuclear localization.

Results

**DTL protein is cell cycle regulated and post-translationally modified during normal cell cycle progression**

We wanted to first determine whether DTL protein expression correlated with transcriptional expression during the cell cycle. SpDTL protein levels match mRNA levels through the cell cycle; SpDTL mRNA and protein expression peaks during CRL4DTL activity in S phase (Liu et al. 2005). Because previous mRNA analysis revealed that DTL mRNA expression correlates with CRL4DTL activity (in S phase) (Whitfield et al. 2002), we wanted to determine whether DTL protein levels also correspond to mRNA expression levels. We utilized a polyclonal antibody (Sansam et al. 2006) that specifically recognizes the C-terminus of endogenous human DTL to characterize DTL protein expression throughout the cell cycle.
HeLa cells were synchronized by double thymidine block or nocodazole to analyze DTL expression from the G1/S transition or early mitosis, respectively. Samples were taken at the indicated timepoints for FACS and western blot analysis to determine cell cycle phasing and the corresponding levels of DTL expression. We observed two striking properties of DTL expression during the cell cycle. First, DTL expression decreases during late G2 or early mitosis, increases during G1, and reaches peak expression during S and G2 (Figure 1A and 1B). This differs from DTL transcriptional regulation, which peaks in S and decreases upon onset of G2 phase. Second, DTL exists in multiple mobility forms (Figure 1B). Further investigation of these different forms of DTL will be discussed in upcoming sections. Interestingly, expression of the CRL4 ubiquitin ligase components Cul4A and DDB1 remains constant throughout the cell cycle (Figure 1A and 1B). Taken together, these data indicate that DTL regulation in humans differs from *S. pombe*, and regulation of the cell cycle-dependent expression of DTL protein may play a critical role in CRL4<sup>DTL</sup> ligase activity.

CRL4<sup>DTL</sup> activity is restricted to S phase and after DNA damage by a PCNA and chromatin-dependent mechanism (Arias and Walter 2005; Jin et al. 2006). However, our data shows that DTL expression persists into G2, beyond known CRL4<sup>DTL</sup> activity. This observation led us to ask where the CRL4<sup>DTL</sup> components Cul4A, DDB1, and DTL are localized within the cell throughout the cell cycle. Since CRL4<sup>DTL</sup> activity is PCNA and chromatin-dependent, we expect the ligase components to be localized in the nucleus, specifically on chromatin, during S phase. Upon onset of G2, we reasoned that the CRL4DTL ligase may be no longer chromatin-bound, since its known activity on chromatin is restricted to S phase.
Figure 1. DTL protein levels are cell cycle regulated.

(A) HeLa cells were synchronized by double thymidine block, 2mM thymidine, released, and collected at the indicate timepoints. Cell cycle phasing was determined by FACS analysis of DNA content by PI staining (indicated by G1, S, G2, and M). Whole cell lysates were analyzed by western blot and show DTL decreases in late G2, early M phase. (B) HeLa cells were synchronized in mitosis by nocodazole block by treatment 100 ng/ml nocodazole for 16 h. Cell cycle phasing was determined by FACS analysis of DNA content by PI staining (indicated by G1, S, G2, and M). Whole cell lysates were analyzed by western blot to show DTL decreases in mitosis, while levels increased in late G1 through G2 phase. (C) HeLa cells were synchronized by double thymidine block and released through the cell cycle at the timepoints indicated. Cells were fractionated into cytoplasmic, soluble nuclear, and chromatin-enriched fractions and analyzed by western blot. Again, cell cycle phases were verified by PI staining and FACS analysis. Orc2 was used as a control for chromatin-enriched proteins (D) HeLa cells were synchronized by double thymidine block and cells were lysed in IP buffer. Endogenous DTL was immunoprecipitated by mouse antiserum raised against C-terminal human DTL, and immunoblotted for DDB1 interaction. Cul4A was not detected in immunoprecipitates.
To analyze DTL subcellular localization, we fractionated cells into cytoplasmic, nuclear soluble, and chromatin enriched fractions at various timepoints in synchronized cells. We found both DDB1 (Figure 1C) and Cul4A (data not shown) levels were unvaried and appeared in all fractions throughout the cell cycle, including the chromatin-enriched fraction. Interestingly, DTL was present in all fractions as well: its localization to the chromatin-enriched fraction was not restricted to S phase and occurs during G2 as well (Figure 1C). DTL levels in each fraction oscillated in a manner identical to that in the total cell lysates (Figure 1A and 1B). DTL localization to chromatin in G2 led us to wonder whether the CRL4^DTL ligase was assembled and active in G2.

**DTL interacts with DDB1 throughout its expression**

We assessed DTL association with DDB1 and Cul4A throughout the cell cycle by immunoprecipitating endogenous DTL from synchronized cells. Whole cell lysates from cells synchronized by double thymidine block were incubated with our polyclonal DTL antibody. Endogenous DTL associates with DDB1 in late G1 and G2, in addition to S phase (Figure 1D), implying that assembly of the complex is not restricted to S phase when it is known to be active. Unfortunately, we were unable to successfully detect Cul4A, likely because DTL interacts with Cul4A indirectly via DDB1. We also attempted to perform the same experiment using a DDB1 antibody to immunoprecipitate the complex, however the commercially available antibodies we tried were unsuccessful. A previous study has shown that Cul4A and DDB1 interacts stoichiometrically (Hu et al. 2004), therefore, we assume that any DDB1 in association with DTL will also be assembled with Cul4A. A caveat to consider is that our co-immunoprecipitation results may only represent assembled complex in the soluble (cytoplasmic) fraction of the cell because the conditions to release chromatin-associated complex components may be too stringent for successful immunoprecipitation of assembled complexes.

Localization of DTL to chromatin and detection of DDB1 association in G2 suggest that additional regulatory mechanisms must exist to restrict CRL4^DTL-mediated degradation to S phase.
The current model of CRL4<sup>DTL</sup> activity specifies complex recruitment to chromatin-associated substrates via DTL association with PCNA-bound substrates (Arias and Walter 2006; Higa et al. 2006a; Jin et al. 2006). Our data supports this model, suggesting that although DTL and the complex exists in chromatin-enriched fractions in phases other than S phase, an additional PCNA-dependent signal is required to bring the complex in direct contact with substrates to induce ubiquitin ligase activity. Alternatively, these results may indicate that the complex has activity in regulating unidentified substrates off of chromatin and also in other phases of the cell cycle.

**DTL is ubiquitinated and degraded in a proteasomal manner**

As shown earlier, DTL expression decreases in mitosis, and we wanted to determine whether this decrease represents regulation by the ubiquitination proteasome system. We treated asynchronous HeLa cells with cyclohexamide to inhibit protein synthesis and collected cells over time to show that DTL levels decrease with similar kinetics as other proteins that are degraded during the cell cycle, such as Cdt1 (Figure 2A). As expected, Cul4A, whose expression is constant throughout the cell cycle, does not decrease after translational inhibition (Figure 2A). When we treated asynchronous cells with proteasomal inhibitor MG132, DTL accumulates over time (Figure 2B), as does Cdt1, indicating that DTL levels are regulated in a proteasomal-dependent manner. Again, Cul4A levels did not accumulate.

To show that DTL is in fact ubiquitinated during normal cell cycle, we transfected HeLas with HA-tagged ubiquitin and immunoprecipitated endogenous DTL using our polyclonal antibody. The immunoprecipitation was performed under denaturing conditions to prevent detection of DTL-associated proteins that may also be ubiquitinated. By western blotting for HA, we were able to detect ubiquitinated DTL (Figure 2C). Because DDB1 was not detected in the immunoprecipitation, we conclude that DTL-associated proteins were not pulled down in the immunoprecipitate, which confirms that the ubiquitination detected is specific to DTL.
Figure 2. DTL is ubiquitinated and degraded in a proteasomal-dependent manner.

(A) HeLa cells were treated with 100 μg/ul cyclohexamide and whole cell lysates were collected over 10 hours at the indicated times for western blot and detection of endogenous DTL, Cul4A, Cdt1, and actin. (B) HeLa cells were treated with 10 μM MG132 over 6 hours and total cell lysates from each timepoint indicated were analyzed by western blot for detection of DTL, Cul4A, Cdt1, and actin. (C) HeLa cells were transfected with HA-tagged ubiquitin for 48 hours. Whole cell lysates were harvested in IP buffer and boiled for 10 minutes to denature the proteins. Endogenous DTL was immunoprecipitated using mouse antiserum and ubiquitinated DTL was detected by western blotting for the HA tag. DDB1 was detected in the input, but not in the IP lanes, indicating DTL did not pull down associated proteins.
**CRL4 ubiquitin ligase does not regulate DTL during mitosis**

Ubiquitin ligase substrate receptors are often autoubiquitinated and degraded by the ubiquitin ligase they associate with. Substrate receptor association with the core scaffold and adaptor protein brings them in close proximity to ubiquitin conjugation machinery. One such example is the substrate receptor DDB2, which is degraded by the CRL4 ligase (El-Mahdy et al. 2006; Li et al. 2006a). To determine whether DTL could be degraded by the CRL4 ligase as well, we disrupted DDB1 association to prevent association with the ligase. Disruption of two conserved arginines (R171A and R246) within two WDXR motifs in DTL has been shown to disrupt DDB1 binding (Jin et al. 2006). We generated these DDB1-binding deficient mutants by mutating these arginines to alanines to create two single mutants (R171A and R246A) and a compound mutant (RRAA). To verify that these mutations abrogate DDB1 binding, we overexpressed these mutants with HA-tagged DDB1 in 293T cells, and pulled down DDB1 using the HA-antibody. All three DTL mutants were unable to associate with DDB1, while wildtype DTL (WT) still interacted with DDB1 (Figure 3A). Cul4A was still able to interact with DDB1, demonstrating that loss of DTL binding does not destabilize the ligase core. We also immunoprecipitated through the DTL mutants and were unable to detect both DDB1 and Cul4A, verifying that the mutants could not interact with the complex.

Next we wanted to test whether the DDB1-binding-deficient mutants were degraded in mitosis. HeLa cells were transfected with mutant constructs (WT, R171A, R246A, and RRAA), and after 24 hours, were synchronized in early mitosis by nocodazole block. Western blot analysis revealed that all three DDB1-binding-deficient mutants were degraded with similar kinetics as overexpressed WT as cells progressed through mitosis (Figure 3B). Furthermore, cyclohexamide treatment showed that overexpressed RRAA mutant levels decrease similar to WT after translation inhibition (Figure 3C). Taken together, these experimental results indicate that DTL is not regulated by its own CRL4 ligase.
Figure 3. DTL is not degraded by the CRL4 ligase.

(A) HA-tagged DDB1 and GFP-tagged DTL mutants were co-transfected into 293T cells. After 48 hours, cells were harvested and DDB1 was immunoprecipitated using HA antibody. DTL constructs (WT and DDB1 binding mutants R171A, R246A, and RRAA) were detected by western blotting for the GFP tag. Cul4A was also detected, revealing HA-tagged DDB1 can interact with the ubiquitin ligase core. (B) Untagged DTL mutants were transfected into HeLa cells, and after 24 hours, cells were synchronized by nocodazole treatment for 16 hours. Cells were released and transfected DTL was detected by western blotting with DTL antibody. (C) GFP-tagged DTL-WT and DDB1-binding deficient mutant (RRAA) were transfected into HeLa cells for 24 hours, then treated with 100 ng/ml cyclohexamide for 8 hours. Whole cell lysates were analyzed by western blotting.
**APC complex does not regulate DTL during mitosis**

DTL degradation after release from nocodazole block is highly reminiscent of the degradation kinetics of known APC substrates, such as geminin and cyclin A (Figure 1B). Similar to CRLs, APC-mediated degradation is executed through association with substrate receptors. The two APC substrate recognition subunits, Cdc20 and Cdh1, target proteins that contain canonical degradation motifs D boxes (RxxL) and KEN boxes (KEN). Examination of the DTL protein sequence revealed three APC recognition motifs in the C-terminus: two D boxes (residues 408-411, 527-530) and one KEN box (residues 652-654) (Figure 4A). Of these motifs, one of the D-boxes and the KEN box are evolutionarily conserved. Therefore, we hypothesized that DTL is a substrate of the APC, and analyzed its interaction with the APC specificity factors Cdc20 and Cdh1, and the contribution of the degradation motifs to DTL stability.

To assay for interaction between DTL and either APC substrate receptor, we co-transfected untagged DTL and myc-Cdc20 or myc-Cdh1 in 293T cells and immunoprecipitated with myc antibody. We were able to reproducibly detect interaction between overexpressed DTL and both Cdc20 and Cdh1 (Figure 4B). Interestingly, interactions between DTL and both APC subunits increased significantly upon treatment with MG132, implying that the interaction is functional.

Previous studies have created non-degradable mutants by mutating the APC recognition motifs within other APC substrates, such as Cyclin B and Geminin (Shreeram et al. 2002; Chang et al. 2003). In a similar manner, we mutated the critical residues of the D box (R408 and L411, R527 and L530) and the KEN box (K652, E656, N657) to alanines to generate two mutants: one with the conserved D and KEN boxes mutated (KD), and the other with all three motifs mutated (KDD). Untagged mutants were transfected into HeLa cells and the levels were examined after nocodazole synchronization or cyclohexamide treatment. Expression levels of transfected DTL were significantly higher than endogenous (data not shown); therefore we were confident that our
Figure 4. DTL is not degraded by the APC.

(A) Multiple sequence alignment of DTL proteins in human, mouse, and zebrafish reveal two D-boxes and one KEN box, though only one D-box and one KEN box are evolutionarily conserved. (B) Myc-tagged APC subunits Cdc20 and Cdh1 were co-transfected with untagged wildtype DTL in 293T cells. After 48 hours, cells were treated with 10 μM MG132, and then harvested. Lysates were incubated with antibody against myc and DTL association was detected by western blot. (C) Untagged DTL constructs (WT, KD, or KDD) were transfected into HeLa cells for 24 hours. Cells were synchronized by treating with 100 ng/ml nocodazole for 16 hours and then released. DTL levels were detected by western blotting using DTL antibody. (D) Cyclin B, sororin, and DTL were translated in vitro, 35S-labeled, and incubated in Xenopus interphase egg extracts. Mitotic extracts were induced by addition of Cdc20 or recombinant Cdh1. Cyclin B and sororin were degraded by Cdc20 and Cdh1, respectively. DTL was not degraded by either Cdc20 or Cdh1 supplemented extracts. (*) indicates a background band from labeling present in all samples. IVT cyclin B1, sororin, and DTL are denoted by solid triangles.
subsequent analyses represented the exogenous DTL constructs. After release from nocodazole block, cells expressing KD or KDD forms of DTL all exhibited a reduction in levels in mitosis similar to WT (Figure 4C). In addition, upon cyclohexamide treatment, the KDD mutant still degraded over time with very similar kinetics to WT (Figure 4D). However, KD and KDD mutants were still able to interact with myc-tagged Cdc20 and Cdh1 when overexpressed in 293T cells. This suggests that additional motifs may be present within DTL that mediate interaction with the APC complex, and mutation of the canonical D and KEN boxes are not sufficient to disrupt APC targeting. This hypothesis is supported by the fact that some APC substrates contain other non-canonical motifs that confer APC-mediated destruction, such as the A box in Aurora kinases A (Littlepage and Ruderman 2002).

To definitively determine whether DTL is an APC target, we utilized an in vitro ubiquitination assay in collaboration with Dr. Susannah Rankin at Oklahoma Medical Research Foundation. *Xenopus* egg extracts were supplemented with either activated APC subunits Cdc20 or Cdh1 and cyclin B and sororin were in vitro translated in the presence of radiolabeled methionines. Sororin is a recently identified APC\textsuperscript{Cdh1} target, while cyclin B can be degraded by both APC\textsuperscript{Cdc20} and APC\textsuperscript{Cdh2} (Figure 4E). However, in vitro translated DTL is not degraded when incubated in extracts activated by either subunit, definitively demonstrating that DTL is not degraded by the APC (Figure E).

**DTL is post-translationally modified by phosphorylation**

During our characterization studies of DTL protein through the cell cycle, we observed that DTL exists in multiple mobility forms by western blot, indicating post-translational modifications (Figure 1B). Treatment of cell lysates with lambda phosphatase results in mobility shift, revealing that the slower mobility forms in untreated lysates detected by western blot are phosphorylated forms of DTL (Figure 5A). By labeling asynchronous HeLa cells with \(^{32}\text{P}-\text{orthophosphate and immunoprecipitating}
Figure 5. DTL is phosphorylated.

(A) HeLa cells were arrested by treatment with 100 ng/ml nocodazole for 16 hours. Whole cell lysates were harvested and either incubated with lambda phosphatase for 30 min. Samples were analyzed by western blotting with DTL antibody. (B) HeLa cells were labeled with $^{32}$P for 4 hours and cells were fractionated into cytoplasmic (C), nuclear (N), and chromatin-enriched (Ch) fractions. Lysates were immunoprecipitated with antibody against DTL and run out by SDS-PAGE electrophoresis. $^{32}$P-labeled DTL was detected by autoradiography, while total DTL levels were detected by western blot using DTL antibody. Orc2 detected primarily in the chromatin-enriched fraction as a control.
endogenous DTL from subcellular fractions, we verify that DTL is indeed phosphorylated. We also show that phosphorylated DTL is present in the cytoplasm, nucleus, and chromatin-enriched fractions (Figure 5B).

**Mass spectrometry identifies several phosphorylation sites within DTL**

We next wanted to identify the individual phosphorylation sites within DTL. We performed mass spectrometric analysis of immunoprecipitated endogenous DTL from nocodazole-arrested HeLa S3 cells using our anti-DTL antibody. Because we had previously observed the slowest mobility forms of DTL when arrested with nocodazole (Figure 1B), we reasoned that DTL isolated from nocodazole-arrested cells would be maximally phosphorylated and would reveal the whole spectrum of phosphorylations accumulated throughout the cell cycle. Endogenous DTL was immunoprecipitated, separated by SDS-PAGE gel electrophoresis, and the gel was subsequently stained with a coomassie stain. The indicated band containing proteins between 50 and 75 kDa was excised, digested with trypsin, and analyzed by mass spectrometry (Figure 6A). Recovered peptides represented 35% of the DTL protein and revealed 4 phosphorylation sites: serine 557, 697, and 510, 511, 512 (it was unclear which of the three was actually phosphorylated) and threonine 702 (Figure 6B). Notably, all the identified phosphorylation sites are located in the C-terminus of DTL and are relatively evolutionarily conserved.

Having identified phosphorylation sites in DTL by mass spectrometry, we constructed phosphorylation-deficient and phosphorylation-mimetic mutants to directly examine the function of each site. To generate phosphorylation-deficient mutants, we mutated the serines (S558, S697) and threonine (T702) identified in our screen to alanines which cannot be phosphorylated. We also generated double mutants by mutating serines 557 and 558 (SSAA) and both serine 697 and threonine 702 (STAA) because of their close proximity to each other. Because it was unclear which serine at 510, 511, and 512 was phosphorylated in our analysis, we mutated all three serines to alanines (SSSAAA). We examined the phosphorylation status of the mutants by transfecting
untagged WT, SSAA, STAA, and SSSAAA in HeLa cells and metabolically labeled cells with $^{32}$P-orthophosphate. DTL was immunoprecipitated from the asynchronous cells with anti-DTL mouse antiserum, which recognizes both endogenous and overexpressed DTL. None of the phosphorylation-deficient mutants exhibited a decreased $^{32}$P signal, suggesting that none of the mutations inhibited phosphorylation (Figure 6C). It is therefore likely that DTL is phosphorylated at multiple sites, and mutation of a few sites was not sufficient to reduce $^{32}$P signal.

Thus, we performed 2D tryptic $^{32}$P phosphopeptide mapping to compare maps of $^{32}$P-labelled WT with phosphorylation-deficient mutants SSAA and STAA. However, we found that there was no discernable loss or change in intensity of phosphopeptide spots on mutant maps when compared to WT (Figure 6D), indicating that mutation of the phosphorylation sites did not prevent DTL from being phosphorylated at the same tryptic peptides as WT. A caveat of this experiment is that the phosphorylation sites identified by mass spectrometry were from a nocodazole-arrested population of cells, while our 2D analyses were from an asynchronous population. If one of the sites represented a mitotic-specific event, it is likely that this phosphorylated peptide would not be visible in our 2D phosphopeptide map of asynchronous WT-expressing cells, since only 2-3% of cycling HeLas are in mitosis. Thus, even if mutants SSAA or STAA were non-phosphorylatable mutants for the mitotic phosphorylation, we would not be able to detect the loss of signal from the corresponding peptide compared to WT. Moreover, we were unable to successfully map SSSAAA mutant.

Because our phosphopeptide mapping was inconclusive, we generated phosphorylation-mimetic mutants to ascertain whether constitutive phosphorylation at these specific sites would perturb DTL function and cell cycle progression. We mutated the putative phosphorylation sites to glutamic acid to create three mutants: SSSEEE (S510E, S511E, S512E), SSEE (S557E, S558E), and STEE (S697E, T702E). We developed a rescue assay in which GFP-tagged siRNA resistant WT and phospho-mimetic mutants were cotransfected with DTL siRNAs. GFP-tagged histone protein 2B (H2B) was transfected as a control. The GFP-positive cells were analyzed to examine
Figure 6. Identification and functional analysis of DTL phosphorylation sites.

(A) Fifteen 15cm dishes of HeLa S3 cells were arrested by 100 ng/ml nocodazole for 16 hours. Cells were harvested and endogenous DTL was immunoprecipitated with mouse antiserum against C-terminal DTL. Lysate was run out by SDS-PAGE gel electrophoresis and the gel was stained using coomassie-based stain. The band between 50-75 kD proteins (black box) was excised and analyzed by MALDI-TOF mass spectrometry. (B) Representation of DTL peptides (in red) recovered from mass spectrometry. Identified phosphorylation sites (S510, S558, S697, T702) are located in the C-terminus (black circles). (C) HeLa cells were transfected with untagged DTL constructs (WT, nonphosphorylatable DTL constructs: SSSAAA, SSAA, STAA) for 24 hours and then labeled with $^{32}$P for 4 hours. DTL was immunoprecipitated by DTL antibody and detected by autoradiography. (D) 2D phosphopeptide maps of $^{32}$P-labeled DTL immunoprecipitated from HeLas transfected for 24 hours. Spots are numbered. (E) SiRNA-resistant GFP-tagged DTL constructs were co-transfected into HeLas with siDTL. Cells in G2/M were detected by PI staining and FACS. GFP-negative cells (black) and GFP-positive cells (white).
the effect of each DTL mutant on cell cycle progression in the absence of endogenous DTL. The GFP-negative cells indicated the efficiency of endogenous DTL knockdown. DTL knockdown causes re-replication and subsequent activation of the G2/M checkpoint (Jin et al. 2006; Sansam et al. 2006), which is typified by accumulation of cells in G2/M. Control cells co-transfected with GFP-H2B and DTL siRNA exhibit a substantial increase in cells in G2/M when compared to untransfected (NT) cells without DTL knockdown. GFP-WT expression rescues this G2/M arrest phenotype, while in contrast, RRAA (a DDB1-binding deficient mutant) cannot rescue the arrest (Figure 6E) due to its inability to associate with Cul4A and DDB1 and appropriately regulate substrates. All three phospho-mimetic mutants exhibited cell cycle profiles similar to WT, suggesting that either none of the mimetic mutations function as constitutively phosphorylated DTL or that constitutive phosphorylation at these sites serve no function in DTL regulation during normal cell cycle.

**Truncation mutants reveal multiple phosphorylation sites in the C-terminus**

Our previous analyses of individual phosphorylation sites did not identify sites that affect CRL4^DTL activity and normal cell cycle progression. Therefore, we constructed a series of N-terminally GFP-tagged deletion mutants to identify a region of DTL that could not be phosphorylated (Figure 7A). C-terminal truncations commenced after the N-terminal WD40 repeats to retain the amino acids and secondary structure required for DDB1 binding. The C-terminal mutant comprising residues 411-730 (ΔN) lacked the N-terminal WD40 repeats and did not bind to DDB1 (data not shown). By transfecting these deletion mutants into asynchronous HeLas, labeling with ^32P, and immunoprecipitating with an anti-GFP antibody, we observed that the ΔN mutant, who lacks the N terminus, is clearly phosphorylated (Figure 7B, lane 4).

Analysis of the different C-terminal deletion mutants revealed more specific regions of DTL that are likely to be phosphorylated (Figure 7B, lanes 5-9). Specifically, loss of ^32P signal
from mutants ΔC1 and ΔC2 indicates that phosphorylation occurs between residues 500 and 730 (Figure 7B). Because of the quantitative nature of radiolabeling, we can directly compare the intensity of $^{32}$P signal to protein expression detected by anti-GFP antibody on western blot to extrapolate the extent of phosphorylation of the mutants. Mutants ΔC3, ΔC4, and ΔC5 exhibit equivalent intensities of $^{32}$P signal to each other and similar level of protein expression by western blot. The significant decrease in $^{32}$P signal of ΔC2 (1-500) suggests that phosphorylations between 500 and 590 account for all the $^{32}$P signal of the three mutants. Interestingly, ΔC2 exhibited a very faint $^{32}$P signal, indicating that perhaps there is an additional phosphorylation site between residues 410 and 500. Another possibility is that only a defined subset of the ΔC2-expressing population is phosphorylated (i.e. ΔC2 in the cytoplasm is phosphorylated while ΔC2 in the nucleus is unphosphorylated between residues 410-500).

We also visualized the localization of these mutants by immunofluorescence and found a striking correlation between phosphorylation and nuclear localization. Full-length GFP-tagged DTL, ΔC3, ΔC4, and ΔC5 were all localized primarily in the nucleus with faint signal in the cytoplasm (Figure 7C). In contrast, mutants ΔC1 and ΔC2, which are mostly unphosphorylated, are largely excluded from the nucleus and are predominantly located in the cytoplasm. Taken together, the data indicates that phosphorylation between residues 500-590 is linked with nuclear localization. We were concerned that construction of ΔC1 and ΔC2 may have deleted a nuclear localization signal (NLS). Multiple sequence alignment of DTL metazoan orthologs identified an NLS between residues 197-203, which is retained in our ΔC1 and ΔC2 mutants. However, it is still possible that our mutants lack a less evolutionarily conserved NLS that we were unable to identify.

We wanted to determine whether mislocalization to the cytoplasm was sufficient for DTL hypophosphorylation. To this end, we added an NLS sequence upstream of the GFP tagged ΔC1. If nuclear exclusion prevented DTL phosphorylation via inaccessibility to a nuclear kinase, we hypothesized that restoration of nuclear localization would restore DTL status, so long as
Figure 7. C-terminal truncation DTL mutants reveal phosphorylated regions.

(A) Shematic of DTL truncation mutants. N-terminal WD40 repeats denoted by black boxes. (B) GFP-tagged DTL was immunoprecipitated from ³²P-labeled cells. Phosphorylated DTL was detected by autoradiography, and total DTL was analyzed by western blot with antibody recognizing GFP. (C) Immunofluorescence of transfected GFP-tagged DTL truncation mutants (green). Nuclei stained with DAPI (blue). (D) Immunofluorescence of transfected NLS-GFP-ΔC1 (green), and nuclei (blue). (E) NLS-GFP-ΔC1 was immunoprecipitated from ³²P-labeled cells. Phosphorylated DTL was detected by autoradiography, and total DTL levels were detected by GFP by western blot.
phosphorylation occurred in the N-terminal residues present in ΔC1. Immunofluorescence showed that NLS-GFP-ΔC1 was now localized to the nucleus (Figure 7D), similar to WT, however relocation to the nucleus did not change its phosphorylation status, as indicated by $^{32}$P labeling and immunoprecipitation (Figure 7E). This demonstrates that phosphorylation does indeed occur on the C-terminus.

Because ΔC1 exhibited altered localization and completely lacked phosphorylation modification, we were particularly interested in examining the consequences on ability to associate the CRL4 complex and appropriately target substrates. The biological consequences of ΔC1 on ligase assembly, substrate recognition, and substrate degradation in normal cell cycle and after DNA damage are discussed in Chapter 2.

**Discussion**

Regulation of DTL differs between yeast and humans. In *S. pombe*, CRL4$^{DTL}$ activity is controlled by DTL protein expression, which is regulated at the transcriptional level. Similar to *S. pombe*, human Dtl mRNA expression is high in G1 and S and low in G2 and mitosis, however, our studies show that DTL protein levels persist in G2 and decrease in mitosis, which suggest that DTL levels are also regulated post-transcriptionally. Another difference between yeast and human DTL protein is the presence of about 200 residues C-terminal to the WD40 repeats. This C-terminus is loosely conserved among higher eukaryotes, suggesting that additional regulatory mechanisms of DTL may have evolved in metazoans. To gain a better understanding of how CRL4$^{DTL}$ activity is controlled, we aimed to elucidate the mechanisms of DTL protein degradation and the function of DTL phosphorylation.
**Implications of DTL degradation in mitosis**

We were intrigued to find that DTL appeared to be degraded in mitosis. Concurrent with our studies, two other groups showed that DTL protein is cell cycle-regulated in breast and liver cancer cell lines in a similar manner as we have shown, with protein expression persisting through S and G2 and decreasing in mitosis (Pan et al. 2006; Ueki et al. 2008). However, other cell cycle synchronization experiments have shown that DTL protein expression is stable in HeLa cells (Abbas et al. 2010). We attribute this to differences in epitopes recognized by the DTL antibodies used. It is interesting to speculate that there is a specific form of DTL that is preferentially degraded in an ubiquitin-proteasomal dependent manner during mitosis. We attempted to show that DTL is ubiquitinated primarily in G2 or early mitosis, concomitant with its degradation, by immunoprecipitating endogenous DTL from synchronized cells expressing tagged-ubiquitin, however our results were inconclusive due to technical challenges.

Interestingly, the decrease of DTL levels coincides with accumulation of the most well characterized CRL4-DTL substrate Cdt1. Cdt1 levels increase during mitosis and remain high during G1 for proper licensing of replication origins prior to initiation of S phase. We wondered if, like in yeast, expression of DTL was sufficient for CRL4-DTL activity and therefore, degradation of DTL in mitosis is required for proper accumulation of Cdt1. The fact that both DTL and Cdt1 levels are elevated during G1 when CRL4-DTL is not active (Figure 1B) indicates that this may not be the case. To address this directly, we sought to identify the ubiquitin ligase that mediates DTL degradation in mitosis and create non-degradable mutants with the intent of expressing these mutants to assess the effects of ectopic DTL expression during mitosis on cell cycle progression and Cdt1 accumulation.

To our surprise, DTL is not regulated by the CRL4 or APC ubiquitin ligases. Initially, our preliminary data suggested DTL could be regulated by the APC: the kinetics of DTL degradation was remarkably similar to other APC substrates, Geminin and cyclin A, and we were able to detect a proteasomal-dependent interaction between both Cdh1 and Cdc20 with DTL (Figure 4B). An
independent study by Pan et al. proposed that the Cdh1-activated APC complex specifically degrades DTL. They showed that overexpression of the Cdh1 subunit of the APC triggers increased DTL degradation while overexpression of Cdc20 and a dominant negative Cdh1 mutant did not (Pan et al. 2006). We performed the same overexpression assay, but were unable to detect DTL degradation as a consequence of overexpressing either subunit. In addition, neither Cdh1 nor Cdc20-active extracts degrade DTL in our in vitro APC degradation assay (Figure 4D). However, one caveat is that the Cdc20-activated mitotic extracts contained endogenous Xenopus Cdc20 which may be unable to degrade the in vitro translated human DTL due to species-specific differences, and perhaps additional experiments are needed to further investigate whether the APC can degrade DTL.

A recent study has implicated subunits of the COP9 Signalosome (CSN) in regulating DTL ubiquitination and degradation. Depletion of Csn5 and Csn6 destabilizes DTL, suggesting that the CSN prevents DTL autoubiquitination (Raman et al. 2011). It is currently unknown whether DTL autoubiquitination and subsequent degradation occurs constantly throughout the cell cycle or at a specific timepoint (i.e. mitosis).

**Coordination between phosphorylation and degradation**

Though we were unable to create a non-degradable form of DTL through mutation of the APC recognition motifs and DDB1-interacting residues, we inadvertently created a non-degradable mutant when we deleted the C-terminus (AC1) for experiments aimed at probing the regulation of DTL phosphorylation. Protein levels of this mutant did not decrease during mitosis compared to WT when expressed in synchronized cells (Figure 8A) (other truncation mutants were not tested). The inability of this mutant to be phosphorylated is potentially relevant to its competency for degradation. First, we have demonstrated that DTL is additionally phosphorylated during mitosis before its degradation (Figure 1B), as indicated by the presence of a higher mobility form of DTL that was verified as a phosphorylation modification by phosphatase treatment (Figure 3A).
Interestingly, phosphorylation of in vitro translated human DTL in mitotic extracts was also detected in our in vitro APC ubiquitination (Figure 4D).

Second, we observed that prolonged treatment of MG132 results in accumulation of the higher mobility form of DTL (data not shown). Therefore, the signals that mediate DTL degradation are located in the C-terminus and may be dependent upon phosphorylation. The data obtained from our set of C-terminal truncation mutants can be utilized to refine the location of the degradation motif to a smaller region in the C-terminus.

It is interesting to note that in characterizing our truncation mutants, we observed a slight $^{32}\text{P}$ signal for $\Delta\text{C}2$ in comparison to the other mutants (Figure 7B), which we attribute to either a single phosphorylation site or a subset of the population being phosphorylated between residues 410-590. The latter supports a hypothesis that DTL is phosphorylated specifically between residues 410-590 during late G2 or early mitosis to create a phosphorylation-dependent degron or for re-localization to the cellular compartment where the targeting ubiquitin ligase is present. Many proteins are targeted through phosphorylated degrons; in fact, the SCF ligase complex targets phosphorylated motifs (phospho-degrons) that are specific to each F-box specificity factor. For example, the SCF$^{\text{p-TrCP}}$ ubiquitin ligase targets proteins with D-pS-G-X-X-S (Wu et al. 2003), such as Weel, the Cdc2 inhibitory kinase (Watanabe et al. 2004) and Bora, the cofactor of Aurora A (Chan et al. 2008; Seki et al. 2008). In contrast, SCF$^{\text{FW7/Cdc4}}$ recruits substrates containing the phosphodegron I/L-I/L-pT-K/R, where the positively charged residues are unfavored at the +4 position (Nash et al. 2001). Although DTL does not contain these particular consensus motifs, DTL could still be targeted in a phosphorylation dependent manner.
Figure 8. ΔC mutant is not degraded like WT

(A) GFP-tagged WT and ΔC were transfected in HeLa cells. 24 hrs after transfection, cells were treated with 100 mg/ml cyclohexamide for the time indicated. Whole cell lysates were analyzed by western blot analysis. GFP-DTL levels were detected by GFP antibody. (B) GFP-tagged WT and ΔC were transfected in HeLa cells. 24 hrs after transfection, cells were synchronized by double thymidine block and harvested at the indicated timepoints after release. GFP-DTL protein levels were detected by GFP antibody. (C) 24 hr 1μg/ml doxycycline induction of GFP-DTL in pCW stable cell lines causes different localization patterns of ΔC.
The critical question we are interested in answering is whether ectopic expression of non-degradable DTL will disrupt cell cycle progression as a consequence of inappropriate degradation of substrates. Overexpression of DTL in HeLa cell lines did not affect cell cycle progression or destabilize substrates, such as Cdt1 or Set8. Importantly, we found that expression of non-degradable ΔC1 in synchronized cells did not perturb cell cycle progression, Cdt1 accumulation in mitosis, or Cdt1 degradation in S phase (data and further discussion in Chapter 2). Thus, degradation of DTL is not required for cell cycle progression or regulation of known substrates; the functional significance of DTL degradation specifically occurring during mitosis, if any, remains unclear.

**DTL phosphorylation sites and localization**

While we were performing our studies, several other groups also reported that DTL is phosphorylated during normal cell cycle. Large-scale proteomic analyses of phosphorylated proteins identified phosphorylated DTL peptides in G1 or M-arrested HeLa cells (Dephoure et al. 2008). However, functional analyses of these phosphorylation sites and their role in CRL4 complex formation or substrate degradation were not performed. Some of the phosphorylation sites we identified by mass spectrometry (S510, S511, S512, S557) were also present in this genome-wide screen, which validated our results. Other phosphorylation sites were identified that were not represented in our analysis (S485, S490, S495, S508, S516, S676, S679, S681, S682, S684), however, it is unclear whether sites that were in close proximity to each other actually only represent a single site that was indistinguishable by analysis (similar to our experience with sites S510, S511, and S512). These sites also fall within the same regions we have identified by analysis of C-terminal deletion mutants to be phosphorylated (residues 500-730, with possible phosphorylations between residues 410-500). Mutational analysis of these sites may provide additional insight into phosphorylation-dependent regulatory mechanisms of DTL function.
We were unable to successfully generate non-phosphorylatable DTL mutants by point mutations to directly test the importance of specific phosphorylation sites on controlling CRL4\textsuperscript{DTL} activity. However, our deletion mutant approach successfully identified non-phosphorylatable deletion mutants, ΔC1 and ΔC2. Another group has generated three overlapping DTL deletion mutants and identified phosphorylation between residues 590 and 730 in breast cancer cells by phosphatase treatment (Ueki et al. 2008). We also found phosphorylations within residues 590-730.

We also identified a putative additional phosphorylation site between 500-590 that was not reported by Ueki et al. This can potentially be explained either by the fact that mobility shifts observed by western blot may not represent phosphorylations as faithfully as \textsuperscript{32}P labeling, or by differential regulation as a consequence of the different cell type and genetic background of the lines used.

Our observation that phosphorylation correlated with nuclear localization implies that these phosphorylation sites may play a critical role in CRL4\textsuperscript{DTL} activity. Although we have shown that phosphorylated DTL is present in all compartments of the cell via subcellular fractionation (Figure 3A), immunofluorescence of our non-phosphorylated mutants ΔC1 and ΔC2 were predominantly excluded from the nucleus (Figure 6B and 6C). Work by Ishii et al. demonstrated that phosphorylated DTL preferentially binds to chromatin during S phase and especially after DNA damage (Ishii et al. 2010), which provides a mechanism for activation of the CRL4\textsuperscript{DTL} ligase on chromatin via DTL phosphorylation. Altogether, these data show that DTL is phosphorylated between residues 500-730, and these phosphorylation(s) may facilitate nuclear import or chromatin association where the CRL4\textsuperscript{DTL} targets substrates. To test this hypothesis, we utilized ΔC1, which was unable to be phosphorylated and excluded from the nucleus, to examine the contribution of phosphorylation and localization to CRL4\textsuperscript{DTL} complex assembly and activity. The results of this study are presented in Chapter 2. Strikingly, we found that expression of ΔC in a stable cell line resulted in uniform expression of GFP-ΔC localized in the nucleus, very similar to WT (Figure
Therefore, we show that there are apparent differences in localization from transient overexpression compared to stable cell line expression. We find that with transient expression, the proteins are vastly overexpressed in fewer cells (Figure 7C). In contrast, doxycycline induction of stable cell lines resulted in less overexpressed GFP-tagged protein and localization was similar to WT. Thus, we conclude that massive overexpression of ΔC results in excess GFP-ΔC in the cytoplasm compared to the nucleus, however, when more moderately expressed, there is no defect in ΔC localization.

**Identifying kinases that phosphorylate DTL**

After finding phosphorylation sites by mass spectrometry, we were interested in identifying the kinases that regulate DTL phosphorylation and, potentially, function. Using the Scansite database, we were able to assign putative kinases to our identified phosphorylation sites via comparison of known consensus motifs and kinase binding sites. Scansite matched the phosphorylation of serine at 512 to either GSK3α kinase, which plays a role in Wnt signaling and glycogen synthesis, or MAPK1 (ERK), which plays a role in proliferation and differentiation signaling pathways. Phosphorylation site S558 was assigned to kinase CLK2 (Cdc2-like kinase 2), a serine/threonine kinase that has recently been implicated in S phase control and checkpoint activation (Collis et al. 2007; Røndtlev Danielsen et al. 2009). Inhibition of these kinases using RNAi technology or molecular inhibitors may useful to test if these kinases indeed phosphorylate DTL.

In this study, we have identified several potential regulatory mechanisms for DTL function and CRL4DTL activity. Elucidation of their role in CRL4DTL ligase assembly and substrate ubiquitination and degradation will further understanding of the complex mechanisms that drive faithful genome replication and cell division.
Experimental Procedures

Cell culture, cell cycle synchronization, and FACS analysis

HeLa, HeLa-S3, and 293T cell lines were obtained from the American Type Culture Collection (ATCC) and cultured in DMEM (Invitrogen) supplemented with 10% FBS and L-glutamine. HeLa cells were synchronized by double thymidine block or nocodazole and then released. For double thymidine block, cells were plated at 50% density and treated with 2 mM thymidine (Sigma) for 16 hrs. The cells were released for 8 hours by washing twice with warm PBS (phosphate buffered saline) and then incubating with fresh growth medium. Cells were subsequently blocked by adding 2 mM thymidine for 12 hours, and released again by washing with PBS and incubating in fresh growth medium. For the nocodazole block, cells were cultured in the presence of 100 ng/ml nocodazole (Calbiochem) for 16 hours. Mitotic cells were harvested by shake-off, washed in PBS, and replated in growth medium. Cells were harvested at the indicated timepoints. For FACS analysis, cells were trypsinized with 0.25% trypsin/1 mM EDTA and resuspended in 70% ethanol/PBS at -20°C overnight. Fixed cells were reconstituted in PBS containing 50 mg/ml propidium iodide and 50 mg/ml RNase. Samples were analyzed by FACScan (Becton Dickinson) and Flow Jo (Tree Star, Inc.) or ModFit LT (Verity Software).

Plasmids, siRNA, and transfections

The human DTL cDNA was PCR-amplified and cloned into pENTR/D-TOPO (Invitrogen) as previously described (Sansam et al. 2006) The cDNA was transferred to the pDs-Tolkit2-CS2 and pDEST-EGFP expression vectors using LR Clonase Reaction II (Invitrogen). 6xMyc-tagged Cdh1 (28127) and Cdc20 (111593) were purchased from Addgene. Plasmids were transfected into cells using Mirus Bio TransIT-LT1 Transfection Reagent according to the manufacturer’s instructions. SiRNA targeting DTL (sense: GUAUGGGUUUACGUAGAUU, Thermo Scientific
Dharmacon) was transfected into cells at 100 nM using RNAi-max (Invitrogen), according to the manufacturer’s instructions.

**Generation of DTL mutants**

Mutants were generated using oligonucleotide site-directed mutagenesis (Quickchange, Stratagene) and mutations were verified by sequencing. C-terminal deletion mutants were generated by point mutation to STOP codon. N-terminal mutant generated by amplifying the N-terminal fragment by PCR (primers F: CACCAAGAAAAAAGAGTCAGAC, R: CTATAATTCTGTTGTTGTTCA) for cloning into pENTR-TOPO, then transferred to pDEST-EGFP expression vectors using LR Clonase Reaction II (Invitrogen).

Primers (5’ to 3’) introducing mutations are as follows:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>R258A-F</td>
<td>GACCAAGATCATGTCTCCGGCCTACAGGCCCTTATTCCTGCTG</td>
</tr>
<tr>
<td>R258A-R</td>
<td>CACAGGAAATAGGAGCTTTCGCCGGAGACATGTCCTGTC</td>
</tr>
<tr>
<td>L531A-F</td>
<td>GTCTCCGGCAAAAGCCGCTATTCTGTGAGCCAG</td>
</tr>
<tr>
<td>L531A-R</td>
<td>CTGGCTCAGAGGAATAAGCGGCCTTTTGCCGGAGAC</td>
</tr>
<tr>
<td>K652A,E653A-F</td>
<td>GAGGGTGCTGAAATGAGTCACGCGCAATGATCTCTCCAGGAAATAA</td>
</tr>
<tr>
<td>K652A,E653A-R</td>
<td>TTATTCTCTGTTGGAACATTCTCGTCGCTCCTACATTTCAGACCCTTC</td>
</tr>
<tr>
<td>N654A-F</td>
<td>TGAAATGAGCGCGAGCGGTAGTTCCCCAGAGAATAAAA</td>
</tr>
<tr>
<td>N654A-R</td>
<td>TTATTCTCTGTTGGAACATTCTCGTCGCTCCTACATTTCAGACCCTTC</td>
</tr>
<tr>
<td>R171A-F</td>
<td>CATATGGTCTGGGATACCCCGCTGCAACAAAAAGATGGG</td>
</tr>
<tr>
<td>R171A-R</td>
<td>CCCATTTTTTTTTGTTGCAGCGGCTATCCAGACCATAATG</td>
</tr>
<tr>
<td>R246A-F</td>
<td>GTGGATGAGTAATACTCAAGATTTGAGATTAGCTGCTTAT</td>
</tr>
<tr>
<td>R246A-R</td>
<td>ATAGCGATATAATTCTAGCTAATCCACATCTTGGATTATCCATCCAC</td>
</tr>
<tr>
<td>S557A-F</td>
<td>AAAGAGGAGGCTAGACGCAAGCTGTGAGAG</td>
</tr>
<tr>
<td>S557A-R</td>
<td>CTCTCCAGACAGCGCTTTGGTCTAGCCCTTCTTCTTT</td>
</tr>
<tr>
<td>S558A-F</td>
<td>GAGGGAGGCTAGACGCTTCCAGCCCGTGAGAGTGTG</td>
</tr>
<tr>
<td>S558A-R</td>
<td>CACACTCTCCAGACAGCGCTTGATCTAGCCTCTCTCTC</td>
</tr>
<tr>
<td>S557A,S558A-F</td>
<td>GAGTAAAGAGGAGGCTAGACGCAAGCTGTGAGAGTGTGAAAC</td>
</tr>
<tr>
<td>S557A,S558A-R</td>
<td>GATTGACAGGCTGCTAGACGCCAGAGCGCTGTGAGAGTGTGAAAC</td>
</tr>
<tr>
<td>S557E,S558E-F</td>
<td>GTAAAGAGGAGGCTAGACGCAAGCTGTGAGAGTGTGAAAC</td>
</tr>
<tr>
<td>S557E,S558E-R</td>
<td>TTAGGGCCACACTCCACAGCAGCGCTCTGTAGGAGCTCTCTTCTTAC</td>
</tr>
<tr>
<td>S697A-F</td>
<td>GAGCGGAAAGACATCGCCAGCCCGGCTACC</td>
</tr>
<tr>
<td>S697A-R</td>
<td>GTGGAGCGGCTGCTGGCACATGCTGTTCCCGCTC</td>
</tr>
<tr>
<td>T702A-F</td>
<td>CCGTCACCACTGCGCCAGCCAGCTCCA</td>
</tr>
<tr>
<td>T702A-R</td>
<td>TGGAGCTGGGCGGATGTTGAGCGG</td>
</tr>
</tbody>
</table>

115
Chromatin Fractionation and Phosphatase Assay

Cells were fractionated into a cytoplasmic soluble fraction (S1), soluble nuclear fraction (S2), and a chromatin-enriched fraction (S3) as previously described in Mendez and Stillman, 2000. For immunoprecipitation of cellular fractions, the P2 pellet containing the chromatin-enriched fraction was lysed using RIPA buffer. For the phosphatase assay, cells were lysed in RIPA lysis buffer with or without phosphatase inhibitors. 50 μg of protein was incubated with 2 U of λ phosphatase (New England Biolabs) at 30°C for 30 minutes.

Immunoprecipitation

For immunoprecipitations, cells were lysed in an NP-40 lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% NP-40, protease inhibitor cocktail (Roche), 0.1 mM PMSF, 1 mM NaF, 10 mM...
β-glycerophosphate, 200 μM Na3VO4) for 30 minutes on ice. Lysates were clarified by spinning at
4°C for 10 minutes at 14,000 rpm and the soluble supernatant was incubated with antibodies
overnight at 4°C while rocking. Protein A/G (Santa Cruz Biotechnologies) agarose beads were
added for 1 hr at 4°C with rocking. The immunoprecipitates were washed three times with lysis
buffer, resuspended in 2X Laemmli buffer, boiled for 10 minutes, and fractionated by SDS-PAGE
gel electrophoresis. For 32P-labeled immunoprecipitations, cells were labeled for 4 hours with 32P,
lysed in 0.5% NP-40 lysis buffer (supplemented with protease and phosphatase inhibitors as
described above) and immunoprecipitated (as above). Immunoprecipitates were fractionated by
SDS-PAGE gel electrophoresis, and the gel was dried on a gel dryer (Hoefer) at 80°C for 2 hours
before being exposed to autoradiography film.

**Western Blot Analysis and Antibodies**

Cells were lysed in RIPA buffer or IP buffer and quantified by Bradford protein assay (Bio-Rad).
30-60 μg of protein were boiled with Laemmli sample buffer, loaded on 6-8% SDS-PAGE gels,
and run in Bio-Rad Mini-Protean apparatus. Gels were transferred to PVDF membrane (Millipore)
at 4°C, and blocked with 4% dry milk. All antibodies were diluted in 4% dry milk; primary
antibodies were incubated on membranes for 1 hour at room temperature or overnight at 4°C, and
HRP-conjugated secondary antibodies (GE Healthcare) were diluted 1:5000 in 4% milk and
incubated for 1 hour at room temperature. Blots were washed 5X with TBS-T (0.1% Tween).
Protein bands were visualized by ECL (Perkin Elmer, GE Healthcare). The following antibodies
were used: Cul4A (100-401-A04, Rockland Immunochemicals), DDB1 (ab9194, Abcam), GFP
(11814460001, Roche), CDT1 (H-300), Geminin, HA (Y-11), myc (9E10), GFP, Orc2, cyclin A,
and actin (Santa Cruz Biotechnologies), α-tubulin (Sigma), and DTL (A300-947A, A300-948A)
(Bethyl Laboratories). Mouse polyclonal antiserum was generated against endogenous human DTL
as previously described (Sansam et al. 2006).
**Immunofluorescence**

HeLa cells were grown on coverslips and were transfected with GFP-tagged DTL constructs for 24 hours. Cells were fixed with cold methanol for 20 minutes at -20°C and washed 3X with PBS. Coverslips were stained with DAPI and mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Inc.). Images were taken with Zeiss Axiophot II upright microscope and OpenLab software (PerkinElmer).

**Mass spectrometry**

Fifteen 15 cm plates of HeLa S3 cells were grown in normal growth medium with 100 ng/ml nocodazole for 20 hours to synchronize cells at the metaphase-anaphase transition. Cells were collected and washed with PBS, lysed in 10 ml 0.5% NP-40 lysis buffer, and lysate was clarified by spinning for 1 hour at 10,000 rpm at 4°C. The soluble supernatant was transferred to a 50 ml conical tube and antibodies recognizing endogenous DTL were added for incubation overnight at 4°C with rocking. 2 ml of pre-washed Protein A-sepharose beads (GE Healthcare) were added for incubation at 4°C with rocking for 2 hours. Immunoprecipitate was washed five times with 10 ml of lysis buffer and eluted with SDS elution buffer. Eluate was concentrated by spinning through microcon for 20 minutes at 4°C. 5X Laemmli sample buffer was added and the sample was fractionated through SDS-PAGE gel electrophoresis and the gel was stained with GelCode Blue Stain Reagent (Pierce) overnight. A band between 50 and 75 kDa was excised using sterile technique and sent to The Swanson Biotechnology Center for tryptic digestion and MALDI-TOF mass spectrometric and data analysis.

**2D Phosphopeptide Mapping**

HeLa cells were transfected with indicated plasmids for 24 hours and then incubated with 4 mCi $^{32}$P-orthophosphate for 4 hours in sodium pyruvate-free medium. Cells were washed 2X PBS and
lysed in 0.5% NP-40 lysis buffer with protease and phosphatase inhibitors. Lysates were incubated with anti-DTL antibodies overnight at 4°C with rocking. Then protein A/G beads were added for 1 hour at 4°C with rocking. Immunoprecipitates were washed 3X 0.5%NP-40 lysis buffer and resuspended in 40 ml 2X Laemmli Sample Buffer. Samples were loaded onto 8% SDS-PAGE gel and separated by electrophoresis. The gel was dried at 80°C for 2 hours, then exposed to autoradiography film for 1-4 hours. Bands were cut out of the dried gel and peptides were prepared as described in Boyle et al., 1991. Peptides were separated on 20 x 20 cm cellulose (EM Science) using the Hunter Thin Layer Peptide Mapping Electrophoresis System (HTLE-7002). Second dimension separation was performed for 10 hours in a chromatography tank and plates were dried for 1 hour before exposure to autoradiography film.

Acknowledgements

I would like to thank Chris Sansam for generating the mouse antisera against C-terminal DTL, for general reagents, and helpful advice and discussion. I would also like to thank Susannah Rankin at OMRF for performing the in vitro APC ubiquitination assay.
Appendix B: DTL loss causes a zebrafish-specific damage-induced G2/M checkpoint

Crystal J. Lee and Jacqueline A. Lees
Background information

DTL was previously identified in a screen for novel DNA damage response regulators in zebrafish embryos (Sansam et al. 2006). This screen was performed in the Hopkins zebrafish collection, which contains approximately 500 heterozygote lines carrying retroviral insertional mutations in over 300 different genes required for embryonic development (Amsterdam and Hopkins 2004). Zebrafish have an intact DNA damage response; embryos can robustly activate an ATM/ATR-dependent G2/M arrest after exposure to DNA damage.

For the screen, heterzygotes were intercrossed and subjected to ionizing radiation (IR), and were stained with an antibody recognizing serine 10 of phosphorylated Histone H3 (anti-PH3), which is a marker for mitotic cells. One hour after exposure to IR, anti-PH3-positive cells are largely absent in wildtype embryos. Mutant embryos that did not exhibit a decrease in anti-PH3-positive cells were unable to initiate an IR-induced G2/M checkpoint. Two mutant lines containing unique insertions within the Dtl gene that resulted in Dtl loss displayed this mitosis after irradiation phenotype, suggesting that DTL is required for activation of the early IR-induced G2/M checkpoint.

Sansam et al. showed that cell cycle defects in non-irradiated Dtl mutant zebrafish are caused by deregulation of Cdt1, and DTL is the substrate receptor for the CRL4 ligase. Loss of DTL causes deregulation of CRL4_DTL substrates, most notably Cdt1, and subsequently, cells initiate rereplication and accumulate DNA damage. Co-depletion of Dtl and Cdt1 in zebrafish embryos by morpholino injections rescues the G2 arrest in non-irradiated cells, but did not rescue the damage-induced G2/M checkpoint phenotype. This suggests that Cdt1 deregulation does not cause this phenotype and that CRL4_DTL regulates another substrate critical for this checkpoint.

We were interested in elucidating the mechanism of how CRL4_DTL regulates activation of the DNA-damage induced G2/M checkpoint, and identifying this unknown substrate. We then
moved into human tissue culture, where many biochemical and molecular tools for dissection DNA damage response pathways are available and validated. Our first goal was to reproduce the G2/M checkpoint phenotype in human cells.

Results and Discussion

*DTL siRNA knockdown recapitulates G2 accumulation phenotype but not damage-induced G2/M phenotype*

Sansam et al. was able to recapitulate the cell cycle defects observed in non-irradiated *Dil* mutant zebrafish embryos and the damage-induced G2/M checkpoint by transient transfection of a siRNA that targeted DTL in HeLa cells (Figure 1A). However, when we utilized the same siRNA, we were able to recapitulate the G2 arrest in undamaged cells (Figure 1B), induced by Cdt1 deregulation, but were unable to reproduce the damage-induced G2/M checkpoint phenotype by FACS analysis (Figure 1C). Cells that were transfected with siRNA exhibited efficient knockdown of endogenous DTL protein by western blot (Figure 1D), however, two hours after IR treatment, DTL knockdown cells displayed a reduction in mitotic cells to a level very similar to WT (Figure 1C).

We devised various strategies to troubleshoot: varying the concentration of siRNA in transfection, performing a timecourse to identify the window of optimal knockdown and maximal phenotype, transfecting siRNA into a panel of human cell lines including U2OS, HCT116, 293T, and SaOS2, and designing retroviral short hairpins against DTL. However, we were still not able to recapitulate an irradiation-induced G2/M checkpoint defect compared to control.
Figure 1. DTL siRNA knockdown causes G2/M arrest in nonirradiated cells, but does not cause the IR-induced G2/M checkpoint defect.

(A) Sansam et al. 2006, reprinted with permission. siRNA knockdown in HeLa by transfection for 72h, then 10Gy IR for 2h. Cells were fixed and stained with anti-PH3 to detect mitotic cells. Anti-PH3 positive cells and PI staining was quantified by FACS analysis. siGFP was used as a control. (B) Helas were transfected with DTL siRNA and harvested after 72h. Cell cycle profiles were analyzed by PI staining and FACS. (C) siDTL knockdown in HeLa for 72h. Cells were irradiated with 10Gy for 2h before cells were fixed. Cells stained with anti-PH3 and PI, and analyzed by FACS. (D) 72h siDTL knockdown in HeLa. Whole cell lysates were immunoblotted for endogenous DTL (anti-DTL) and actin.
DTL loss results in reduced mitotic index in normally cycling cells

We noticed DTL-depleted HeLa cells exhibited a reduced number of anti-PH3 positive cells in non-irradiated samples compared to their control counterparts. In addition, this mitotic index was also less than the mitotic index of the DTL knockdown cells in experiments performed by Sansam et al. (Figure 1C, and compared to 1A). We hypothesized that depletion of DTL in non-irradiated cells activated such a robust cell cycle checkpoint (as a consequence of substrate stabilization) that DTL-depleted cells were no longer progressing through mitosis, and therefore, obscuring the mitotic phenotype after damage. Thus, we tried to alleviate the checkpoint activation by co-depleting CRL4DTL substrates. Sansam et al. showed that co-depletion of DTL and Cdt1 rescues the G2 arrest but not the damage-induced G2/M checkpoint phenotype (Sansam et al. 2006). However, we found that co-depletion of DTL was did not relieve the G2/M checkpoint in non-irradiated cells, though Cdt1 knockdown may not have been robust (Figure 2A).

Another way we tried to circumvent the G2 arrest was to knockdown DTL in a p21 deficient background. Cells that undergo rereplication accumulate DNA damage, which triggers the G2/M checkpoint and causes a G2 arrest. This arrest is mediated through the ATM/ATR-dependent damage response pathway (Vaziri et al. 2003). Kim et al. showed that co-depletion of DTL and p21 causes suppression of the G2 arrest (Kim et al. 2008). We utilized HCT116 cells that were null for p53 or p21 (Bunz et al. 1998), and knocked down DTL by siRNA transfection. Loss of DTL caused robust activation of the G2/M checkpoint in non-irradiated cells, regardless of p21 or p53 status, and we were unable to observe an abrogated radiation-induced G2/M checkpoint (Figure 2B).
Figure 2. Co-depletion of Cdt1 or p21 does not reveal an IR-induced G2/M checkpoint defect.

(A) and (B) HeLa cells were co-transfected with the combination of siRNAs indicated. After 72 hours, cells were irradiated with 10Gy for 2h, fixed, then stained with anti-PH3 and PI. Cells were quantified by FACS. (A) shows % cells in G2/M determined by PI staining, (B) shows % mitotic cells determined by anti-PH3 staining. (C) HCT116 WT, p53⁻/⁻, and p21⁻/⁻ cells were transfected with the indicated siRNAs for 72h, then subjected to 10GY for 2h, fixed, then stained with anti-PH3 and PI. Mitotic cells were determined by PH3 signal by FACS.
Identification of a HeLa cell line with an irradiation-induced G2/M checkpoint defect

We obtained a different HeLa cell line and found that DTL knockdown in these cells resulted in mild irradiation-induced checkpoint defect. The percentage of anti-PH3 positive cells in unirradiated cells between control and DTL knockdown cells were comparable. After irradiation, both DTL knockdown cells exhibit increased anti-PH3 positive cells compared to control (Figure 3A). Importantly, we have shown that multiple HeLa cell lines exhibit different IR-induced checkpoint phenotypes as a result of DTL knockdown by siRNA. This finding demonstrates how prolonged growth in the laboratory can create different “strains” of even a well-characterized cell lines such as HeLa cells. Altering signaling pathways in different “strains” may result in different phenotypical outcomes, as we have demonstrated here.

DTL does not regulate the damage-induced G2/M checkpoint in human cells

To determine whether the high mitotic index after IR resulted from a failure to prevent entry into mitosis following DNA damage or a failure to efficiently exit mitosis, we utilized a nocodazole trapping experiment described previously (Sansam et al. 2006). In this assay, cells are treated with the nocodazole to “trap” cells in mid-mitosis. An accumulation of anti-PH3 positive cells during treatment demonstrates cells are entering mitosis. After IR, zebrafish Dtl mutants exhibit an accumulation of anti-PH3 positive cells in the presence of nocodazole, verifying that these cells cannot activate a G2/M checkpoint in response to damage (Sansam et al. 2006). As expected, unirradiated cells exhibited an increase in mitotic cells after nocodazole trapping. However, after IR, control cells trigger a G2/M checkpoint to arrest cells at the G2/M transition, and there is no accumulation of anti-PH3 positive cells. Strikingly, we found that human DTL-
Figure 3. Nocodazole trapping experiment reveals loss of DTL causes mitotic delay defect.

(A) New HeLa cells were transfected with the indicated siRNAs. After 72h, cells were irradiated with 10Gy for 2h, fixed and stained with anti-PH3 and PI. Cells were analyzed by FACS to determine PH3-positive cells. (B) New HeLas were transfected with the indicated siRNAs. After 72h, cells were pre-treated with 100 ng/ml nocodazole for 1h prior to irradiation of 10Gy for 2h. Cells were fixed, stained with anti-PH3 and PI, and analyzed by FACS to determine mitotic cells.
depleted cells did not accumulate anti-PH3 positive cells after damage in the presence of nocodazole compared to in the absence of nocodazole (Figure 3B). This indicates that loss of DTL in human cells does not abrogate the activation of the damage-induced G2/M checkpoint. We did find that DTL loss did lead to some sort of mitotic defect, as cells were not able to efficiently exit mitosis. Whether or not this is damage-dependent in these cells has not been addressed.

Zebrafish lack components of some DNA damage response pathways that are critical in mammals, such as p19/ARF and BRCA1 (personal communication, C. Sansam). Thus, zebrafish may utilize the CRL4<sup>DTL</sup> ubiquitin ligase to regulate an unknown substrate in a zebrafish-specific DNA damage response pathway. The nocodazole trapping experiment also revealed DTL knockdown leads to a mitotic delay or arrest after IR, which we have not addressed further.

**Experimental Procedures**

**Cell culture, Drugs, and Transfection**

HeLa and 293T cell lines were obtained from the American Type Culture Collection (ATCC). New HeLas were a gift from S. Vyas at MIT. HCT116 WT, p53<sup>-/-</sup>, and p21<sup>-/-</sup> cells were from Dr. B. Vogelstein at Johns Hopkins University. SaOS2 cells were a gift from Dr. A. Ianari. All cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS and L-glutamine. SiRNA targeting DTL (sense: GUAUGGGAUUUACGUAAGAUU, Thermo Scientific Dharmacon) was transfected into cells at 100 nM using Oligofectamine (Invitrogen), according to the manufacturer’s instructions.

**G2 checkpoint assay (PH3) and FACS**

For FACS analysis, cells were trypsinized and resuspended in 70% ethanol/PBS at -20°C overnight. Fixed cells were reconstituted in PBS containing 50 μg/ml propidium iodide and 50
\( \mu g/ml \) RNase. Samples were analyzed by FACScan (Becton Dickinson) and Flow Jo (Tree Star, Inc.) or ModFit LT (Verity Software).

For the G2 checkpoint assay, cells were exposed to 10Gy of IR from a \( ^{60} \)Co source. Two hours after irradiation, cells were trypsinized and fixed in 70\% ethanol/PBS at -20\(^{\circ}\)C overnight. Fixed cells were washed in PBS, then spun at 4000 rpm for 5 min then aspirated. Cells were permeabilized in PBS + 0.25\% Triton X-100 for 10 minutes on ice. Cells were washed with PBS + 1\% BSA (PBSA), then resuspended in dilution of anti-PH3 antibody (Santa Cruz) in PBSA for 2h at room temperature. Cells were washed with PBSA and resuspended in 1:200 dilution of secondary (Alexa-Fluor488 FITC goat anti-rabbit) for 30 min. Cells were washed one last time before resuspension in 50 \( \mu g/ml \) propidium iodide and 50 \( \mu g/ml \) Rnase.

**Acknowledgements**

I would like to thank Chris Sansam for sharing his data, designing the DTL siRNAs, teaching me the G2 (PH3) checkpoint assay and how to do FACS, and invaluable discussions on troubleshooting the data presented.
References


Lee, J. and Zhou, P. 2007. DCAFs, the missing link of the CUL4-DDB1 ubiquitin ligase. Mol Cell 26(6): 775-780.


