EVALUATING DOWNSTREAM TARGETS OF CULLIN4-DEPENDENT E3 LIGASES AND OF D-TYPE CYCLINS: IMPLICATIONS FOR THE DYSREGULATION OF UBIQUITINATION AND CELL GROWTH IN CANCER

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ABSTRACT

SIMA ZACHAREK: Evaluating downstream targets of cullin4-dependent E3 ligases and of D-type cyclins: implications for the dysregulation of ubiquitination and cell growth in cancer
(Under the direction of Yue Xiong)

Cyclin D1 and cullin4 (CUL4) are two proteins known to be upregulated in cancer. Cyclin D1 functions to regulate the cell division cycle and cell growth, while CUL4 assembles E3 ubiquitin ligase complexes that function to ubiquitinate target proteins, often marking them for degradation. However, the downstream effectors of their oncogenic activities are not fully characterized. Therefore, the aim of this study was to discover novel targets of the D-type cyclins and of CUL4, and to better describe the E3 ubiquitin ligase complexes assembled by CUL4. We identified the TSC1-TSC2 tumor suppressor complex, a key negative regulator of cell growth, as a cyclin D-interacting complex, and demonstrated that D-type cyclins could down-regulate the activity of TSC1-TSC2 by both CDK (Cyclin Dependent Kinase) -dependent and –independent mechanisms. In a separate line of studies, I conducted a genetic analysis of mutants of *Cul4* and one of its putative substrate receptor molecules, *Ddb1* (Damaged DNA Binding protein 1) in *Drosophila*, and established that $CUL4^{DDB1}$ plays an essential role in cell growth, proliferation, and development. These studies suggested a number of novel substrates of the $CUL4^{DDB1}$ ligase, and also served to clarify the role of $CUL4^{DDB1}$ in controlling the degradation of the replication licensing factor CDT1/DUP during the cell cycle. Collectively, these analyses of the D-type cyclins and CUL4 broaden our understanding of the consequence of their disruption in cancer development.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

CHAPTER I

Introduction

Of the numerous of cell divisions that occur over a lifetime, most transpire under normal constraints and remarkably without incident. The occurrence of cancer, which results from abnormal and uncontrolled cell division, in fact arises less than once per human lifetime. Multiple checks and balances are in place to prevent the development of cancer, but if adequate restraints on proliferation are breached, a somatic cell can acquire the ability to propagate limitlessly under conditions in which their normal cellular counterparts arrest, become senescent, or undergo programmed cell death. The development of cancer is an evolutionary process in which the acquisition of genetic and epigenetic alterations confers a selective advantage to the cell at specific stages of tumorigenesis, leading to unimpeded clonal expansion of the neoplastic cell (Foulds, 1954; Lowe et al., 2004).

Genetic alterations in cancer

Cancer is a genetic disease, arising from direct and indirect alterations in gene expression. Factors such as environmental influences, infectious agents, and aging also contribute to cancer development. Some cancers are initiated by cancer predisposition syndromes such as retinoblastoma, Cowdens disease, Li-Fraumeni syndrome, or von Hippel-Lindau syndrome, and stem from inherited germline mutations affecting the pRb, PTEN, p53/ Chk2, or VHL genes, respectively (Turnbull and Hodgson, 2005). Mutations in these

and many other genes can also arise sporadically in somatic cells, initiating the neoplastic process. With the subsequent stepwise accumulation of further somatic mutations and alterations, gross chromosomal abnormalities and extensive changes in gene expression patterns often develop in the neoplastic cell.

Genes frequently altered in cancer can be classified as oncogenes, tumor suppressor or 'gatekeeper' genes, and stability or 'caretaker' genes (Vogelstein and Kinzler, 2004). While oncogenes and tumor suppressor genes are generalized as promoting or antagonizing, respectively, cellular proliferation and survival, stability genes act to maintain genomic integrity through prevention of chromosomal instability and repair of damaged DNA. The process of tumor formation is induced by reactivation, constitutive activation, or amplification of oncogenes, coupled with inactivation of tumor suppressor and stability genes. Such alterations in gene expression can result from direct genomic mutation, from epigenetic events, which alter gene expression without affecting the primary DNA sequence, i.e. by promoter methylation, or from defects in the control of mRNA or protein stability, i.e. by microRNAs or ubiquitination (Alvarez-Garcia and Miska, 2005; Baylin and Ohm, 2006; Devoy et al., 2005; Hall and Russell, 2005).

Tumor suppressor loci, such as those of the transcription factor p53, the CDK inhibitor $p27^{Kip1/Cip1}$, or the lipid phosphatase PTEN, can be haploinsufficient for tumor suppression (Cook and McCaw, 2000; Fero et al., 1998; Sulis and Parsons, 2003; Venkatachalam et al., 1998), requiring the inactivation of only one allele in order to promote tumorigenesis. In different settings, these and other tumor suppressor genes such as pRb,

BRCA1 and BRCA2 have often been characterized as undergoing a "two-hit" mode of inactivation, in which a primary inactivating event is followed by loss of heterozygosity (LOH), or inactivation of the second allele (Knudson, 1971; Payne and Kemp, 2005; Vogelstein and Kinzler, 2004). Ultimately, the outcome of such genetic alterations is dependent on the sequence of their appearance and on the context, at both the micro- and macroenvironmental levels, in which they occur.

Context of cancer development

The contexts in which cancers arise are diverse. There are more than 100 different tumor types, though more than half arise in the lung, prostate, breast, colon, and rectum. Each tumor type is distinguishable by a unique microenvironment defined by the specific milieu of cell types characteristic of the tissue in which the tumor arises. Each microenvironment is individually governed by interactions between the tumor cells and the surrounding stromal cells and extracellular matrix (Bissell and Radisky, 2001).

As the genetic alterations leading to cancer within the different cell types are beginning to be elucidated, it is becoming evident that the prevalence of some mutations are tumor-type specific. For example, even though the BRCA1 (Breast Cancer 1) gene product appears to be widely required across all cell types to play key roles in homologous recombination, inherited BRCA1 mutations have been associated with familial breast and ovarian cancers, but not other cancers (Turnbull and Hodgson, 2005). In addition, somatic BRCA1 mutations have not been reported in sporadic breast or ovarian cancers. This paradox has been proposed to be attributable to the weak selective advantage caused by loss

of BRCA1, coupled with temporal differences in the sensitivity to such alterations during development in different tissues. Additionally, functional redundancy of signaling pathways that compensate for the loss of BRCA1, for example, may protect some cell populations, but not those lacking similar safeguards (Sherr, 2004).

The cancers arising from cancer predisposition syndromes are in fact relatively rare. Most cancers appear to arise sporadically, and have not revealed overt inheritance patterns. It is thought, however, that many low penetrance genes that confer increased cancer susceptibility in combination with environmental factors may be contributing to the variability of tumor development observed in different individuals. Such modifier loci have yet to be defined in humans, but appear to play an important role in tumorigenesis in mice of different genetic backgrounds (Loeb et al., 2003).

Global principles of tumorigenesis

As increasing numbers of molecular alterations associated with tumorigenesis become evident, several global principles guiding the neoplastic process have emerged. Given the common molecular machinery governing basic cellular processes of proliferation, differentiation, and death across all cell types, such rules are can be broadly applicable. Some of the salient traits shared by many human tumors include: self-sufficiency in growth signals, insensitivity to anti-growth signals, defective DNA damage/repair pathways and genetic instability, evasion of apoptosis, limitless replicative potential (immortalization), sustained angiogenesis, and in more advanced malignancies, tissue invasion/metastasis (Hahn and Weinberg, 2002; Hanahan and Weinberg, 2000). Roughly four to seven such

rate-limiting events appear to be necessary for cancer development in human cells, and even fewer are required in rodent cells (Hanahan and Weinberg, 2000). Fewer alterations seem to be required in the formation of 'liquid' (leukemias or lymphomas), versus those required in 'solid' (epithelial or mesenchymal) tumors, partly because of the migratory behavior already innate to precursors of liquid tumors (Vogelstein and Kinzler, 2004).

In becoming self-sufficient in growth, the cancer cell loses its reliance on extracellular growth factor, mitogenic, and nutrient signals in the microenvironment to grow and proliferate. Their relationship with the surrounding extracellular matrix and communications transmitted by cell-cell interaction molecules that normally contribute to homeostatic growth often becomes altered (Bianco et al., 2006). Dysregulation of growth factor receptor signaling pathways, which are normally under stringent control, is one of the most common alterations in the cancer cell. Members of the Receptor Protein Tyrosine Kinase (RPTK) family, including the EGFR (epidermal growth factor receptor) and InsR (insulin receptor) subfamilies, for example, are often dysregulated in cancer, which can promote ligand-independent signaling and pathway hyperactivation (Bennasroune et al., 2004). Alterations of RPTKs commonly observed in cancer include gain-of function mutations, genomic rearrangements creating RPTK-containing fusion proteins, or over-expression resulting from genomic amplification. Mutations in the downstream effectors of growth factor receptor signaling are also prevalent. For example, deregulated Ras–Raf–ERK or PI3K–AKT–mTOR signaling at multiple points along either pathway commonly eliminates the requirement for extrinsic growth factor stimulation (Blume-Jensen and Hunter, 2001).

Cancer cells must also be able to evade anti-growth signals to maintain continued growth. Anti-growth signaling normally disrupts active proliferation by driving cells into a quiescent (G0) state, or into a post-mitotic, terminally differentiated state. These states are largely governed by signaling that regulates the cell division cycle or cell growth, i.e. the pRb pathway or the Myc pathway, and are consequently frequent targets in cancer (Sherr, 2004).

Since the measured rate of mutation in human cells is lower than that deduced from the natural occurrence of neoplastic growth observed in the general population, the development of cancer cells is presumably hastened by the acquisition of a 'mutator' phenotype (Loeb, 1991). A mutator phenotype most often stems from mutations in 'caretaker' genes responsible for monitoring genomic integrity, repairing damaged DNA, and ensuring proper chromosomal segregation during mitosis (Raptis and Bapat, 2006). The inability to repair double stranded breaks in DNA, for example, and failure to trigger an appropriate mitotic checkpoint to arrest growth under such conditions, can lead to gross chromosomal changes such as translocations or amplifications, leading to chromosomal instability, and more rapid accumulation of further genetic alterations.

With normal cellular checkpoints in place, the occurrence of such oncogenic events as malfunctioning cellular networks and genomic instability would normally trigger an innate tumor suppressor response involving proliferative arrest and often programmed cell death or apoptosis. Cancer cells therefore must acquire the ability to circumvent such checkpoints. Once apoptosis is activated by extrinsic (i.e., TNF and FAS) and intrinsic signals (a balance

of pro- and anti- apoptotic BH3-containing family of proteins, i.e., Bax and Bcl-2), the cleavage of a cascade of caspases is activated, which drive the destruction of the cell (Fadeel and Orrenius, 2005). Cancer cells become resistant to apoptosis through a variety of mechanisms, the most common of which is the inactivation of the p53 tumor suppressor pathway. p53 is a master regulator of the cellular response to stresses such as hypoxia, nutrient deprivation, DNA damage, and oncogene hyper-activation. By disrupting the p53 pathway, a cell presented with such stresses fails to initiate pro-apoptotic signals such as activation of Bax, or mitochondrial cytochrome c release, resulting in predomination of survival signaling (Klein and Vassilev, 2004).

When confronted with dyregulated signaling and growth, most mammalian cells have another mechanism, termed senescence, to autonomously block further growth. The normal cellular senescence program causes irreversible cell-cycle arrest accompanied by epigenetic changes in chromatin and altered cell morphology. As cells normally age, chromosome ends—telomeres, progressively shorten in length, eventually leading to (replicative) senescence. Senescence can also be triggered in response to oncogenic or genotoxic stress, as has been observed in response to Ras activation (Serrano et al., 1997). Senescence, like apoptosis, therefore represents an internal tumor suppressor mechanism in place that limits replicative potential and oncogenic transformation (Lowe et al., 2004). If the pRb or p53 pathways are disrupted, however, the senescence program can be circumvented. The resulting progressive shortening of telomeres can lead to fusion of unprotected chromosome ends, chromosomal instability, and entry into 'crisis'. Variants that can emerge from the crisis state exhibit activation of telomerase (hTERT) or have utilized recombination-based

interchromosomal exchange pathways to restore telomere length, so that an immortalized cell with even greater oncogenic potential persists (Blasco, 2005; Dimri, 2005).

Given the ability to proliferate limitlessly, tumors will be incapable of growing to large sizes (greater than 2 mm in diameter) unless provided with a sustained blood supply for exchange of nutrients, oxygen, and metabolic waste. Oxygen deprivation in the tumor microenvironment activates the expression of HIF1 (Hypoxia Inducible Factor 1), which can contribute to the expression and secretion of stimulatory growth factors and cytokines that recruit endothelial and stromal cells, and allow angiogenesis—the development of new blood vessels, for the growing neoplasm (Kaelin, 2005). VEGF (vascular endothelial growth factor) and FGF (basic fibroblast growth factor) serve as such angiogenic factors, and are frequently over-expressed in cancer (Carmeliet, 2005).

Tumors often remain benign if their growth is constrained within their tissue of origin, but can become malignant once they have acquired the capacity to invade other tissues and metastasize to and proliferate in distant sites. Metastastic tumors in fact account for the vast majority of cancer patient deaths (Sporn, 1996). The ability of metastatic tumor cells to mobilize through and invade a variety of tissues is not well understood molecularly, but appears to be driven by altered interactions with extracellular matrix components and the microenvironment, activation of extracellular proteases, and epithelial-mesenchymal transition (EMT), a process whereby epithelial cells acquire mesenchymal, fibroblast-like properties and show reduced intercellular adhesion and increased motility. EMT and metastasis have been demonstrated to be linked to several oncogenic pathways involving Ras,

integrins, Wnt/beta-catenin, and PI3K/ AKT, coupled with down-regulation of the cell adhesion molecule E-cadherin (Larue and Bellacosa, 2005; Wittekind and Neid, 2005).

Signaling pathways commonly altered in cancer

As the signaling pathways driving these global principles of cancer progression are becoming better defined, their recurrent involvement across many different tumor types is becoming more evident. Several pathways, some of which were highlighted above, have been defined as critical targets in tumorigenesis, and include the p53, pRb, PI3K, RTKs, TGFß / SMAD, Bcl/apoptotis, APC (Adenomatous polyposis coli)/ ß-catenin, Hedgehog/ Gli (glioma-associated oncogene), and HIF1/ hypoxia pathways (Vogelstein and Kinzler, 2004). Many of these pathways are commonly found to be co-opted by viruses to drive malignancies; for example, the large T antigen expressed by the SV40 DNA tumor virus promotes tumorigenesis by disrupting both the pRb and p53 pathways.

The pathways listed above govern a wide array of biological processes, including the cell division cycle, cell growth, metabolism, DNA repair, survival, apoptosis, development, and differentiation (Bianco et al., 2006). Several of these pathways applicable to the studies described herein are illustrated in Figure 1.1. The identification and circuitry of these pathways have been pieced together in the study of normal cellular processes, with important contributions from biochemical studies in mammalian cells, coupled with seminal genetic studies in model organisms such as the fruit fly *Drosophila melanogaster* and the budding yeast *Saccharomyces cerevisiae* (Hiesinger and Hassan, 2005; Oliver, 2006; Sprinzak and Elowitz, 2005; Vidal and Cagan, 2006).

As the gene products that comprise these signaling pathways are better characterized, an 'exclusivity principle' of pathway disruption in cancer has become clearer, and dictates that a single alteration within a given signaling pathway is sufficient to promote tumorigenesis. The p53 pathway, for example, has been demonstrated to be inactivated in most, if not all, human tumors (Levine, 1997). In different settings, the p53 pathway has been found to be inactivated by direct mutation of the p53 gene, over-expression of the p53 antogonist HDM2 (an E3 ubiquitin ligase), or inactivation of the HDM2 antagonist, $p19^{ARF}$, but not by more than one of these events in a single tumor. Such an exclusivity principle has been found to be broadly applicable across known cellular pathways (Sherr, 2004).

The cell division cycle and cell growth

The balanced growth of a cell, and its equal division into two daughter cells, are highly regulated processes governed by conserved signaling pathways that have been well characterized (Mitchison, 2003; Sherr and McCormick, 2002).

Cyclins, CDKs, retinoblastoma (pRb), and the cell cycle

The pRb pathway is a central point of convergence of signaling that governs the cell division cycle: the ordered set of events that results in DNA replication, cell growth, and division into two daughter cells (Sherr and McCormick, 2002). The pRb pathway regulates entry into and transition through the first gap phase (G1) of the cell cycle (Figure 1.1). The G1 phase serves as a critical period in which many signals coalesce to control proliferation and differentiation. It is not surprising then that disruption of the pRb pathway, which would

permit unrestrained passage through this most important transition of the cell cycle, is one of the most frequently encountered events in cancer. The pRb protein is part of a family that includes p107 and p130. During G1, the pRb family proteins act to inhibit entry into the DNA synthesis (S) phase of the cell cycle, primarily by binding to and inhibiting the E2F family of transcription factors. As the cell cycle progresses, the pRb proteins lose their ability to repress E2Fs due to inactivating phosphorylation events driven by cyclin dependent kinases (CDKs), allowing expression of E2F responsive genes required for DNA synthesis. CDKs are positively regulated by their cyclin binding partners, whose levels fluctuate throughout the cell cycle—increasing upon induction by mitogenic signaling, and decreasing again rapidly by targeted degradation. CDK4 and CDK6 bind D-type cyclins, and CDK2 binds E-type cyclins; once activated, CDK4/6 and CDK2 act sequentially to phosphorylate and disable the pRb family proteins. CDK4/6 are inactivated by the INK4 (inhibitors of CDK4: $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, $p19^{INK4d}$) and Cip/Kip ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$) families of CDK inhibitors. The pRb pathway has been found to be altered in cancer by disruption of $p16^{INK4a}$ or of pRb itself, by over-expression of cyclin D or cyclin E, or by constitutive activation of CDK4, but never by more than one of these alterations, since the functional consequence of any one of these events is similar (Sherr, 2004).

Genetic analyses in mice provide further evidence for the importance of the pRb pathway in tumor suppression. Mutations disrupting D-type cyclins or CDK4 inhibit oncogenic signaling and tumor development (Malumbres and Barbacid, 2006). Similarly, inactivating mutations of *Rb*, *INK4a*, *INK4c*, or constitutive activation of CDK4 (Mittnacht, 2005) resulted in the development of many tumor types in mice. Since disruption of the pRb pathway is often necessary for neoplastic progression, insight into the function and regulation

of the components of this pathway will advance our understanding of cancer development, and therefore guided the focus of the studies described in chapters IV and V.

Given the vulnerability of cells to tumorigenesis upon disruption of the pRb pathway, strict mechanisms have evolved to control entry and progression through the cell cycle. Strict controls are also in place to regulate cell growth (increase in cell mass), which must be coordinately regulated with the cell cycle to maintain cell size within physiological limits. Although the term 'growth' is often used interchangeably with 'proliferation' or in describing cell cycle progression, growth and the cell cycle are in fact distinct, separable processes. Stable proliferation results from a coordinate balance between growth and cell cycle progression. If uncoupled, i.e. if the cell cycle and cell division proceeded in the absence of cell growth, progressively smaller daughter cells would result, while conversely, cell growth in the absence of cell division can lead to cellular hyperplasia (Mitchison, 2003).

Gene expression microarray analysis of neoplastic versus normal cell counterparts almost invariably exhibit up-regulation of genes not only controlling the cell cycle, but also those that regulate cell growth. Such an expression profile, referred to as a proliferation signature, is a strong indicator of poor prognostic outcome in cancer patients (Whitfield et al., 2006). Although the controls guiding cell growth and cell cycle progression can overlap, they can be distinguished as arising from distinct signaling events.

Cell growth, mTOR, and the Tuberous Sclerosis Complex (TSC)

Cell growth in metazoan cells is regulated in large part by the well-conserved PI3K/ AKT/ TOR signaling pathway (Figure 1.1). The lipid kinase PI3K (phosphoinostide 3-kinase) is activated by growth factor signaling, such as through insulin growth factor receptor (IGFR, a RTK) activation by extracellular insulin-like growth factors. Activated PI3K generates phosphatidylinositol-3,4,5-trisphosphate (PI(3, 4, 5)P3), which serves as a second messenger essential for activation of AKT (PKB, protein kinase B). PI3K activity is antagonized by the lipid phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) (Parsons, 2004; Sansal and Sellers, 2004).

Central to the growth signaling pathway is the mammalian Target of Rapamycin (mTOR or TOR) serine/threonine kinase that, as its name indicates, is inhibited by rapamycin, a clinically important drug used as an immunosuppressant, an anti-fungal, and as a potential anti-cancer drug (Georgakis and Younes, 2006). mTOR assembles with the scaffolding molecule raptor and GßL to form a functionally distinct complex, mTORC1, which is rapamycin-sensitive, while an alternative complex, mTORC2, composed of mTOR, rictor and GßL, is rapamycin-insensitive. mTORC1 regulates ribosomal biogenesis and mRNA translational control primarily through phosphorylation of its downstream substrates/ effectors, S6K (ribosomal S6 kinase 1) and 4EBP1 (eukaryotic initiation factor 4E binding protein 1). TOR is positively regulated by Rheb, that itself is negatively regulated by the tuberous sclerosis complex gene products, TSC1 (hamartin) and TSC2 (tuberin). TSC2 is a GAP (GTPase activating protein) that directly inactivates the small GTPase Rheb (Kwiatkowski and Manning, 2005; Nobukini and Thomas, 2004).

TSC2 and TSC1 physically bind and stabilize one another as a heterodimer. *TSC1* and *TSC2* were originally identified as tumor suppressor genes disrupted in the autosomal-dominant disorder, tuberous sclerosis complex (TSC), which is diagnosed in roughly 1 in 6,000 newborns. Mutations in either *TSC1* or *TSC2* can account for TSC, though *TSC2* mutations are more prevalent. TSC is characterized by the development of hamartomas, which are tumorlike malformations that are typically benign, and frequently result from loss of heterozygosity of the remaining wild type *TSC1* or *TSC2* allele, and subsequent dysregulation of growth and development (Astrinidis and Henske, 2005). Hamartomas of TSC patients have been found to develop in a number of different tissues, including the brain, kidneys, skin, heart, and lungs. The severity of the TSC disease is typically dependent on the location at which the hamartomas arise. TSC patients can experience relatively mild symptoms, such as facial angiofibromas, to more severe manifestations, such as development of renal cysts and renal cell carcinoma, or development of cerebral cortical tubers, which can cause of seizures, mental retardation, and autism (Consortium, 1993; Roach et al., 1998).

The *in vivo* function of TSC1-TSC2 have recently been elucidated by studies in *D. melanogaster* that identified the TSC genes (*dTSC1* and *dTSC2*) as important regulators of cell growth (Pan et al., 2004), which was reinforced by multiple studies in mammalian cells (Inoki et al., 2005b). As a complex, TSC1-TSC2 has been found to serve as a central node within the growth signaling pathway, integrating signals from growth factors, nutrients, stress, and cellular energy levels (Inoki et al., 2005b). TSC1-TSC2 receives inputs from at least three major signaling pathways, including the PI3K-AKT pathway, the ERK1/2-RSK1

pathway and the LKB1-AMPK pathway (Kwiatkowski and Manning, 2005). Growth factor signaling via the PI3K-AKT pathway or the MAPK-ERK-RSK1 pathway can act to disable the TSC complex, through direct phosphorylation of TSC2 by AKT, or by ERK1/2 or RSK1. The LKB1-AMPK pathway senses fluctuations in cellular energy levels (i.e., ATP and AMP levels); under energy-starved conditions, the TSC complex is activated by direct phosphorylation of TSC2 by AMPK (Hardie, 2005). Therefore, mutations that alter TSC1 or TSC2 result in the disruption of a central node of regulation of the cell growth pathway.

Many of the proteins that promote cell growth and are known to be activated in cancer (PI3K and AKT) are classified as oncoproteins, and conversely, the proteins that restrict cell growth and are inactivated in neoplastic events (i.e., PTEN, TSC1 and TSC2) are commonly labeled as tumor suppressors (Inoki et al., 2005a), emphasizing the importance of cell growth regulators in tumorigenesis.

The ubiquitin—proteasome system

Many biological processes, such as cell cycle progression and proper developmental control, are dependent on the regulated rise and fall in gene expression. This control can be mediated by a number of mechanisms, one of which involves regulated proteolysis. The ubiquitin-proteasome pathway is the predominant cellular pathway controlling regulated protein destruction across nearly every aspect of eukaryotic cell biology, and not surprisingly, is commonly dysregulated in cancer (Yamasaki and Pagano, 2004). The von Hippel-Lindau (VHL) syndrome, for example, is a cancer predisposition system resulting from mutations in the *VHL* gene, whose normal gene product serves to ubiquitinate the pro-angiogenic

transcription factor, HIF1α, marking it for degradation. Loss of VHL function in VHL patients or by sporadic inactivation in somatic cells results in stabilization of HIF1α, and predisposition to a variety of highly vascularized tumors (Kaelin, 2002).

The regulatory modification of cellular proteins by ubiquitination has been found to be dysregulated in a number of different oncogenic settings, affecting a wide array of substrate proteins (Fig. 1.2). For example, in colorectal cancer, the APC gene is frequently mutated, which prevents the oncoprotein transcription factor beta-catenin from being phosphorylated and targeted for ubiquitination, leading to its accumulation (Xiong and Kotake, 2006). Cervical cancer most often results from infection by the human papilloma virus. One of its proteins, E6, hijacks the cellular ubiquitination machinery to target the tumor suppressor protein p53 for degradation (Mantovani and Banks, 1999). In familial cases of breast cancers, the *BRCA1* gene is often mutated; BRCA1 is in fact thought to be involved in ubiquitinating cellular proteins involved in chromosomal stability (Starita and Parvin, 2006). In a number of other cancers, the oncoproteins HDM2 or Skp2 are constitutively activated or over-expressed, leading to destruction of the tumor suppressor p53, or the CDK inhibitor p27, respectively (Burger and Seth, 2004; Mani and Gelmann, 2005; Pagano and Benmaamar, 2003).

Ubiquitin-mediated proteolysis is a multi-step process, and requires three sequential enzymatic activities supplied by E1 (ubiquitin activating), E2 (ubiquitin conjugating) and E3 (ubiquitin ligating) enzymes (Figure 1.2; Hershko and Ciechanover, 1998). The process of ubiquitination initiates with formation of an ATP-dependent thio-ester bond between

ubiquitin (an 8.6 kDa protein) and the E1 enzyme. Activated ubiquitin is then transferred to an E2. An E3 ubiquitin ligase largely determines the specificity of the reaction, in that it is responsible for recruiting the substrate and mediating the transfer of the ubiquitin from the E2 to the substrate. The substrate becomes bound by ubiquitin through an isopeptide linkage on one or multiple lysine residues, and can subsequently undergo multiple rounds of ubiquitination, leading to the formation of polyubiquitin chain(s). Polyubiquitinated proteins are typically rapidly delivered to and degraded by the 26S proteasome, a 2.5 MDa multi-subunit complex (Pickart, 2001). Polyubiquitin chains linked through lysine residue 48 of ubiquitin (K48) to substrates commonly mark these proteins for destruction by the proteasome. Ubiquitin linked through K63 to a substrate, often resulting in a monoubiquitinated protein, does not result in proteasome-dependent destruction, but rather can play important signaling roles affecting protein localization and DNA repair (Haglund and Dikic, 2005; Sun and Chen, 2004).

E3 ubiquitin ligase families

The large array of proteins targeted by ubiquitin ligases is matched by a diverse array of E3s, which can be categorized into two large families. The HECT family of E3s is characterized by the HECT domain, homologous to the E6AP carboxyl terminus, and is functionally distinct in its ability to directly form thioester linkages with ubiquitin (Ardley and Robinson, 2005; Huibregtse et al., 1995). The RING family of E3s contains either an intrinsic RING finger domain or an associated RING subunit essential for their ubiquitin ligase activity that does not form a direct thioester linkage with ubiquitin, but mediates the transfer of ubiquitin from the E2 to substrate (Deshaies, 1999).

Cullin-dependent ubiquitin ligases belong to the RING family of E3s. The cullins are a well-conserved family of genes that in mammals include *CUL1, CUL2, CUL3, CUL4A, CUL4B* and CUL5, and three related genes (*CUL7*, *Parc* and *APC2*). Cullins serve as scaffolding molecules that assemble multi-subunit E3 ligases (Petroski and Deshaies, 2005). CUL1 is the best-characterized of the Cullins, and the SCF (Skp-CUL1-F-Box) complex, whose crystal structure has been solved (Figure 1.2; Zheng et al., 2002b), is seen as the prototype of Cullin complexes. A highly conserved domain within the C-terminus of CUL1 is required for its association with the RING finger protein ROC1 (RING of cullins) (also known as Rbx1 and Hrt1). CUL2, CUL3, and CUL4 also associate with ROC1, while CUL5 preferentially associate with ROC2, a ROC1 paralogue (Donaldson et al., 2004; Kamura et al., 2004). ROCs recruits and allosterically activates an E2 ubiquitin conjugating enzyme, forming the catalytic core of the complex (Joazeiro and Weissman, 2000; Ohta et al., 1999; Seol et al., 1999).

The N-terminal region of Cullins is the most variable, and therefore not surprisingly serves as the region that determines substrate specificity. The N-terminus of CUL1 is necessary for binding to SKP1, an "adaptor protein". SKP1 in turn serves to bridge CUL1 to a "substrate-targeting molecule," an F-box protein, which directly binds phosphorylated substrate (Jackson et al., 2000; Joazeiro and Weissman, 2000). Once fully assembled, the SCF complex brings substrates into close range of the ROC-E2 catalytic core, promoting regulated substrate polyubiquitination. The *Drosophila* genome contains three *ROC* genes, *ROC1A*, *ROC1B*, and *ROC2*. Recent genetic studies in *Drosophila* indicate that the identity

of the ROC protein associated with the SCF may functionally distinguish its activity in vivo, thereby adding another level of specificity to the complex (Donaldson et al., 2004; Noureddine et al., 2002).

Other Cullin family members have been shown to form analogous complexes (Figure 1.3), though the specific array of subunits of each complex are likely unique to each Cullin. CUL2, for example, binds directly to a heterodimeric complex containing Elongin C, a SKP1-related protein, which binds a SOCS-box protein (for example, VHL, von Hippel-Lindau), which in turn associates with substrates such as HIF1α (Lisztwan et al., 1999; Maxwell et al., 1999; Ohh et al., 2000). CUL3 associates with BTB proteins, which then directly bind substrate molecules (Furukawa et al., 2003; Geyer et al., 2003; Pintard et al., 2003; Xu et al., 2003). Cullins 4, 5, 7 and Parc are much less understood. Evidence that CUL5, like CUL2, associates with Elongins B and C, is becoming clearer, as is the distinction between ligase specificity deriving from preferential association with ROC2 versus ROC1, which dictate different subsets of BC-box interacting proteins as substrate specificity factors (Donaldson et al., 2004; Kamura et al., 2004). CUL4A does not bind to either SKP1 or Elongin C (Kamura et al., 1999; Michel and Xiong, 1998), but does associate with DDB1 (Damaged DNA Binding Protein 1) (Shiyanov, 1999; (Groisman et al., 2003; Liu et al., 2003; Shiyanov et al., 1999; Ulane and Horvath, 2002; Ulane et al., 2003; Wertz et al., 2004), a strong candidate adaptor protein.

Regulation of cullin complexes by NEDD8, the COP9 signalosome, and CAND1

All cullins are covalently modified by a small ubiquitin-like protein, NEDD8 (neuronally expressed developmentally downregulated) through a process termed

neddylation (Hori et al., 1999; Lammer et al., 1998; Osaka et al., 1998). NEDD8 forms an isopeptide bond between its C-terminal glycine, and a conserved Cullin lysine residue (Osaka et al., 1998). Cullins are the only identified substrates of NEDD8, and require the association of ROC1 or ROC2 to be modified by NEDD8 (Furukawa et al., 2000). Modification by NEDD8 appears to be necessary for normal Cullin function. Neddylation has been shown to be necessary for activation of CUL1, CUL2, CUL3, and CUL4 E3 ligases (Furukawa et al., 2000; Morimoto et al., 2000; Morimoto et al., 2003; Osaka et al., 1998; Osaka et al., 2000; Podust et al., 2000; Read et al., 2000) for facilitating the recruitment of E2 enzymes (Kawakami et al., 2001), and thereby promoting polyubiquitination (Wu et al., 2000; Wu et al., 2002).

The removal of NEDD8 from Cullins can be accomplished by the COP9 signalosome (CSN) (Lyapina et al., 2001; Mundt et al., 2002; Schwechheimer and Calderon Villalobos, 2004; Zhou et al., 2001; Zhou et al., 2003). The CSN is a large complex consisting of 8 core subunits that was first identified in Arabidopsis as being required for photomorphogenesis (Wei et al., 1994). It is evolutionarily conserved in other plants, fungi, worms, flies, and humans, and has since been implicated in a wide variety of biological processes such as development, DNA repair, and cell-cell communication (Chamovitz et al., 1996; Freilich et al., 1999; Wei and Deng, 2003; Wei et al., 1994). The CSN is not only associated with deneddylase activity, but also with kinase and with deubiquitinating activities. The CSN-associated kinase activity phosphorylates a number of transcription factors including c-jun and p53 (Bech-Otschir et al., 2001; Bech-Otschir et al., 2002; Seeger et al., 2001; Seeger et al., 1998). Additionally, the CSN suppresses the autoubiquitination activity of bound Cullin

complexes; this activity has been attributed to the CSN5 subunit (Groisman et al., 2003), and to Ubp12, a deubiquitinating enzyme that associates with the CSN (Zhou et al., 2003). The CSN is therefore important in both the assembly and stability of cullin complexes (Lyapina et al., 2001). The CSN has been reported to co-purify with the *S. pombe* CUL4 orthologue, Pcu4, and Csn1 and Csn2 were both found to be required for CUL4 function (Liu et al., 2003). Human CUL4 complexes have also been shown to co-purify with the CSN (Groisman et al., 2003; Liu et al., 2003).

Cullins are additionally regulated by binding with CAND1 (cullin associated, NEDD8 dissociated protein). CAND1 forms a tight association with both the N- and C-terminal domains of cullins, as determined biochemically, and recently confirmed by the solved crystal structure (Goldenberg et al., 2004; Hwang et al., 2003; Liu et al., 2002; Lo and Hannink, 2006; Min et al., 2003; Min et al., 2005; Oshikawa et al., 2003; Zheng et al., 2002a). The ternary complex of CUL1, ROC1, and CAND1 prevents the binding of SKP1, and thereby inhibits SCF ligase activity. Data from our lab supports a model in which CAND1 negatively regulates the assembly of active ligase complexes by preventing association between a Cullin its adaptor molecule. Concomitantly, CAND1 regulation of Cullins may prevent complex instability by inhibiting inappropriate autoubiquitination of the complex (Galan and Peter, 1999; Zhou and Howley, 1998). Binding of CAND1 is alleviated by neddylation of CUL1, allowing the rapid assembly of the SCF ligase into an active complex. Through coordinated activities of NEDD8, CAND1 and the CSN, Cullin E3 ligases may cycle through multiple rounds of assembly and disassembly (Cope and Deshaies, 2003; Liu et al., 2002).

Composition of a CUL4-DDB complex and its link to cancer

CUL4 is of particular interest amongst the cullin family because the CUL4A gene has been found to be amplified in primary breast tumors, primary hepatocellular carcinomas, and primary esophageal squamous carcinoma (Chen et al., 1998; Yasui et al., 2002), and therefore may impose oncogenic activity that contributes to cancer development. However, unlike its well-characterized homologues CUL1 and CUL2, little is known about the *in vivo* functions of CUL4 or the multisubunit complexes formed by CUL4, and were therefore a major focus of the studies described in chapters II and III.

It is established that CUL4 interacts with ROC1 in mammalian cells, and ROC1a in Drosophila cells (Donaldson et al., 2004; Ohta et al., 1999). There is also accumulating evidence that CUL4 interacts with DDB1, the Damaged DNA binding protein 1. DDB1 forms a heterodimer with another subunit, DDB2, in mammalian cells. The DDB complex binds tightly to UV-irradiated DNA (Tang and Chu, 2002), and mutations in DDB2 give rise to the cancer predisposition syndrome, Xeroderma Pigmentosum- group E (XP-E) (Cleaver, 2005). DDB1 has co-purified with CUL4 in *S. pombe* (Liu et al., 2003), in HeLa cells (Groisman et al., 2003), and in 293T cells, in association with the paramyxovirus V protein (Li et al., 2006b; Lin et al., 1998; Precious et al., 2005; Ulane and Horvath, 2002; Ulane et al., 2003). A CUL4-DDB1 complex has also been described which additionally associates with COP1 (constitutively photomorphogenic-1), and DET1 (de-etiolated 1) as substrate specificity factors (Wertz et al., 2004).

It has been unclear, though, whether DDB1 and DDB2 act as components of the CUL4 E3 ligase, or whether they simply represent direct substrates of CUL4, since ubiquitination of both DDB1 and DDB2 is induced by CUL4 (Chen et al., 2001; Galan and Peter, 1999; Nag et al., 2001; Zhou and Howley, 1998). An identifiable *DDB2* orthologue has not been found in lower organisms, but *DDB1* is well-conserved, with close orthologues identified in plants, worms, flies, and fission yeast. Interestingly, in *S. pombe*, accumulation of a replication inhibitor, Spd1, can result from disrupting either *DDB1*, or the *CUL4* orthologue, *Pcu4*, or components of the CSN (Bondar et al., 2004; Liu et al., 2003). In addition, human CUL4A interacts stoichiometrically with DDB1, and DDB1 is required for UV-dependent degradation of the known CUL4A/B substrate CDT1 (Hu et al., 2004). Therefore, we expected that CUL4 targets a number of proteins for ubiquitination through a multi-subunit complex containing DDB1 as an adaptor molecule.

A number of proteins containing WD-40 protein: protein interaction domains, including DDB2, CSA, COP1, KIAA0800, PWP1, and WDR23 interact with both DDB1 and CUL4A (Groisman et al., 2003; Wertz et al., 2004). One prediction of the model in Figure 1.3C is that other WD-40-containing proteins may also interact with DDB1/CUL4 through their WD-40 domains, providing specificity in a manner analogous to the F-box proteins in the SCF complex.

CUL4 functions in vivo

The *CUL4* gene is evolutionarily conserved, with a single homologue in *S. pombe*, *C. elegans*, *D. melanogaster*, and 2 highly related genes, *CUL4A* and *CUL4B* in mammals.

CUL4A and CUL4B are 82% identical, making it likely that they rely on similar adaptor complexes, and are partially redundant. Both CUL4A and CUL4B have in fact been shown to target the same substrate, CDT1, for degradation (Higa et al., 2003). Analysis of mRNA expression levels indicated that CUL4A and CUL4B transcripts are broadly expressed, and can be found in the same tissue (Chen et al., 1998). However, CUL4A null mice are lethal, suggesting that CUL4B cannot compensate for all of the functions carried out by CUL4A (Li et al., 2002).

The *Drosophila* genome contains a *CUL4* orthologue (referred to as *CUL4*) encoding a protein that is 66% identical to CUL4A and 63% identical to CUL4B, but that is more distantly related to human CUL1 (29%), CUL2 (26%), CUL3 (34%) and CUL5 (26%). There is also a DDB1 orthologue in *Drosophila*, DDB1 which shares 60 % identity (74% similarity) to the human DDB1 protein, and has been proposed to have a role in cell cycle and development (Takata et al., 2002; Takata et al., 2004b). Genetic studies of *ROC1a, ROC1b, ROC2*, *NEDD8, CSN4, CSN5, CUL1*, and *CUL3* mutant alleles in *Drosophila* (Doronkin et al., 2002; Doronkin et al., 2003; Freilich et al., 1999; Noureddine et al., 2002; Oron et al., 2002; Ou et al., 2002; Suh et al., 2002) have already contributed significantly to our understanding of the complexity of cullin regulation. These factors offer a unique opportunity to examine the *in vivo* function of *CUL4* in cell cycle control, in response to DNA damage, and during development.

Genetic studies of cullins in many different model organisms have indicated essential roles in cell cycle control and development (Dealy et al., 1999; Feng et al., 1999; Kipreos et

al., 1996; Singer et al., 1999; Wang et al., 1999; Willems et al., 1996). Deletion of the *S. pombe* homologue of *CUL4*, *Pcu4*, resulted in cells that were slower growing and elongated with decondensed chromosomes, but was not lethal (Osaka et al., 2000). A null allele of *CUL4A* in mouse caused early embryonic death (before day 7.5) (Li et al., 2002). *CUL4A* heterozygous mice are haploinsufficient, being observed nearly half as often as expected (Li et al., 2002), implying that proper CUL4A expression is important for embryonic development.

In vitro studies of mammalian CUL4A have also suggested a role in cell cycle progression. A stable cell line that over-expresses CUL4A failed to arrest at G2-M following ionizing radiation (Gupta et al., 2002). In addition, overexpression of CUL4A in cultured myeloid cells promoted proliferation and attenuated differentiation (Li et al., 2003a). Both reports indicated that normally cycling cells were unaffected by overexpression of CUL4A, but that overexpression continued to drive the cell cycle in cells that would otherwise be arrested.

CUL4, DDB1, and Nucleotide Excision Repair

CUL4 and DDB1 have recently been implicated in nucleotide excision repair (NER) (Groisman et al., 2003). Two proteins that play critical roles in NER, DDB2 and CSA, were found in identical complexes containing CUL4A, DDB1, ROC1, and the CSN. NER is a central cellular defense against DNA damage caused by UV or environmental carcinogens. It consists of two pathways: global genomic repair (GGR) and transcription-coupled repair (TCR); GGR removes lesions nonspecifically from the entire genome, while TCR

preferentially removes lesions from the transcribed strand of expressed genes (Mellon, 2005; Reardon and Sancar, 2005; Sancar et al., 2004; Scicchitano and Mellon, 1997). DDB2, in association with DDB1, is recruited to chromatin and binds to UV-damaged DNA, and through an unknown mechanism stimulates GGR (Hwang and Chu, 1993; Hwang et al., 1998; Tang and Chu, 2002; Tang et al., 2000).

CSA, on the other hand, is involved in TCR. *CSA* is one of the two genes associated with Cockayne syndrome (CS), the other being the *CSB* gene, whose protein has been shown to interact directly with the CSA (Henning et al., 1995). Mutation of either CSA or CSB results in Cockayne syndrome, which is characterized by sun hypersensitivity, developmental and neurological defects, and premature aging. Cells lacking either CSA or CSB, if subjected to UV damage, accumulate polyubiquitinated RNA polymerase II (Bregman et al., 1996). Given that CUL4A assembles with CSA as an TCR-induced E3 ligase, and because RNA Pol II has also been found to co-purify with the complex (Groisman et al., 2003), it is plausible that CUL4A/DDB1 may be responsible for the ubiquitination of RNA polymerase II.

Potential CUL4 substrates involved in development, proliferation, and DNA repair

Recent years have seen an emergence of the identity of a number of CUL4 substrates. In *S. pombe*, the replication inhibitor, Spd1, was found to accumulate in *Pcu4* or *DDB1* deficient cells (Bondar et al., 2003; Liu et al., 2003). Though metazoans do not share an Spd1 orthologue, other CUL4 substrates have been suggested to be involved in proliferation. The replication licensing factor, CDT1, was stabilized upon disruption of *CUL4* expression

in *C. elegans* (Zhong et al., 2003), or in *Drosophila* or mammalian cells (Higa et al., 2003), and was shown to be specifically polyubiquitinated by CUL4 *in vitro* in response to ultraviolet or ionizing radiation (Higa et al., 2003; Hu et al., 2004). CUL4 also seems to play a role controlling the activities of a DNA damage recognition protein, XPC (Xeroderma Pigmentosum –group C), the DNA damage checkpoint kinase Chk1, and histone H2A following DNA damage via monoubiquitination, reinforcing the importance of a CUL4-DDB1 complex in regulating the cellular response to DNA damage (Kapetanaki et al., 2006; Sugasawa et al., 2005; Zhang et al., 2005). The HOXA9 homeodomain protein has also been demonstrated to be polyubiquitinated by CUL4A, resulting in disruption in the ability of HOXA9 to promote granulocyte differentiation (Zhang et al., 2003b). Finally, the ubiquitination of the transcription factor, c-jun, was shown to be catalyzed by a novel CUL4 complex containing DDB1, ROC1, COP1, and DET1 (Wertz et al., 2004).

These newly described CUL4 substrates have aided in our understanding of the pleiotropy of functions controlled by CUL4 and DDB1, guiding our genetic study of the $CUL4^{DDB1}$ E3 ligase in *Drosophila*, described in chapters II and III. Chapters IV and V are dedicated to the study of another oncogene of interest, cyclin D, and its interaction with the tumor suppressor proteins, TSC1 and TSC2.

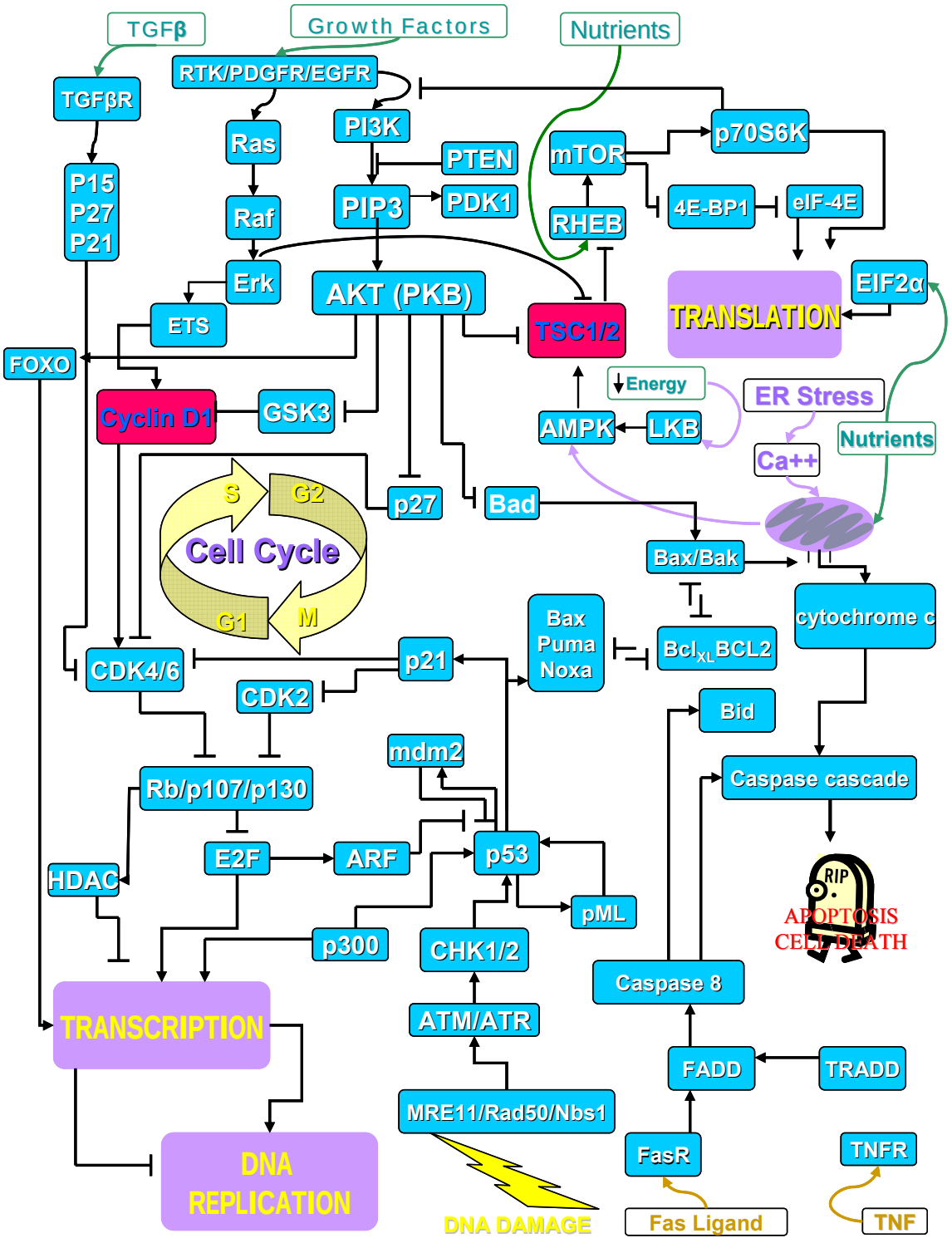

Figure 1.1. Major cellular signaling networks affected in tumorigenesis Adapted from O'Shea, 2005

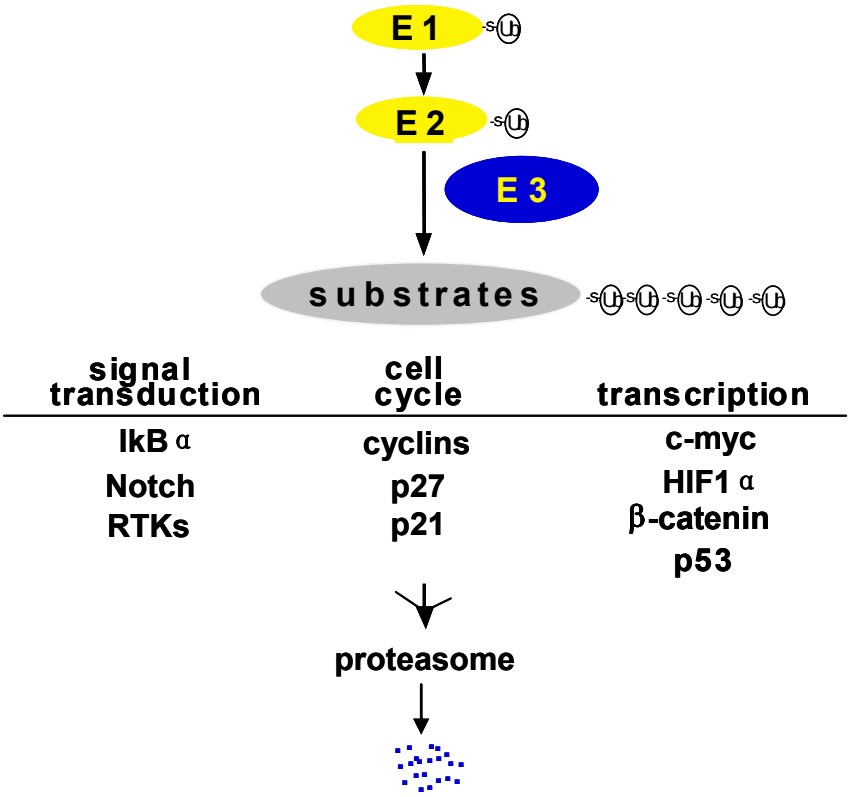

Figure 1.2. The ubiquitin-proteasome system and substrates affected in cancer. Ubiquitination occurs through a cascade of enzymatic activities: E1, ubiquitin activating; E2, ubiquitin conjugating; E3, ubiquitin ligating. E3 ligases mediate much of the specificity of the reaction via recruitment of cellular proteins; recruited substrates are then covalently modified by ubiquitin. The proteins listed here are representative examples of proteins whose dysregulated ubiquitination has been described in cancer.

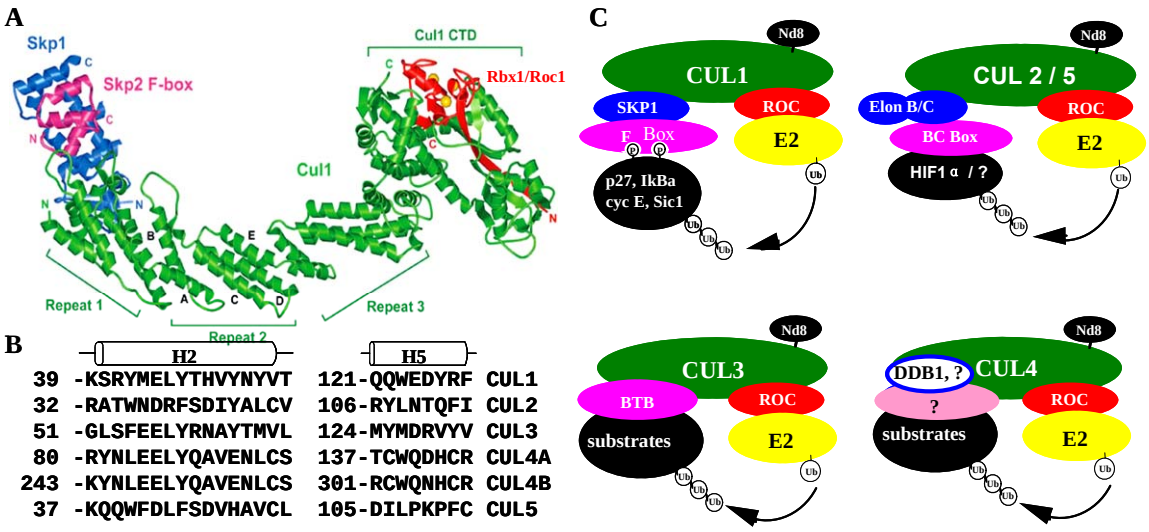

Figure 1.3. Structure, domains, and complexes of cullin-dependent E3 ligase.
(A) Solved structure of SCF-ROC1 (Zheng, 2002). CUL1 serves as a molecular scaffold, binding SKP1–SKP2 complex at the helical N-terminal domain, and ROC1 at the globular C-terminal domain.
(B) H2 and H5 are hydrophobic helical surfaces in the N-terminal region of CUL1 interact with SKP1 and SKP2. Surface residues in the SKP1-SKP2 binding site of CUL1 are conserved only in CUL1 orthologues, but not in paralogues; the same is true of the other cullins, suggesting that all cullins have a protein-binding site in their NH2-terminal regions conferring different specificity for substrate receptor modules.
(C) CUL1 assembles into multiple SCF ligases and ubiquitinates various substrates. CUL2 interacts directly with a SKP1-like protein, Elongin C, and indirectly with a BC-Box proteins that in turn binds with substrates such as HIF1alpha.. CUL5 assembles an analogous complex. CUL3 associates with substrates through just one class of intermediary proteins, BTB proteins. The substrate targeting mechanism of CUL4 is thought to function through DDB1 as a substrate receptor molecule, though it is not clear if other substrate receptor molecules or adaptor molecules also associate with the CUL4 complex.

CHAPTER II

The Role of $CUL4^{DDB1/piccolo}$ in the control of growth and CDT1/double parked levels during Drosophila development

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Summary

CDT1/DUP is an essential replication licensing factor that is degraded at the onset of S phase via ubiquitin-mediated proteolysis to ensure that the genome is replicated only once per cell cycle. The $CUL4^{DDB1}$ E3 ubiquitin ligase is necessary for the regulated proteolysis of CDT1/DUP after DNA damage, but whether it plays an essential role in the destruction of CDT1/DUP at the beginning of S phase is unclear. In order to examine this issue and to determine the *in vivo* function of $CUL4^{DDB1}$ we isolated and characterized mutations in the essential *Drosophila Cul4* and *Ddb1* genes. *Cul4* and *Ddb1* null mutants develop until the 1st or 2nd larval instar stage, and display phenotypes consistent with a growth defect: the mutant animals can survive for up to 10 days without developing further and fail to incorporate BrdU in most cells. We discovered that the previously described piccolo (pic) mutations, which cause growth defects affecting adult bristle, tergite, leg, and wing development, represent viable, hypomorphic alleles of *Ddb1*. Clones of *Ddb1* null mutant cells generated by mitotic recombination in larval imaginal discs are reduced in size relative to control clones. Similarly, *Cul4* mutant cells grow slowly and are eventually eliminated from the imaginal epithelia most likely via competition with phenotypically normal neighboring cells. Depletion of either CUL4 or DDB1 in homozygous mutant larvae or by RNAi in cultured S2 or HeLa cells results in hyper-accumulation of CDT1/DUP. DDB1 and CDT1/DUP were detected in CUL4 immunocomplexes. However, clones of either *Ddb1* or *Cul4* mutant imaginal cells demonstrated normal CDT1/DUP degradation at the G1-S transition, suggesting that $CUL4^{DDB1}$ is not necessary for cell cycle regulated CDT1/DUP degradation and that the observed hyper-accumulation may be due to growth or cell cycle arrest. *Cul1* mutant clones were also found to degrade CDT1/DUP normally. However,

Roc1a mutant cell clones, in which both CUL4- and CUL1-dependent E3 ubiquitin ligases are inactivated, were compromised in degrading CDT1/DUP during S phase. These data suggest redundancy between CUL4 and CUL1 E3 ligases in the control of CDT1/DUP degradation during the cell cycle.

Introduction

To maintain genomic integrity, replication of the genome must occur only once during the cell cycle. Replication is therefore a highly regulated process controlled at multiple levels (Blow and Dutta, 2005). During late mitosis/ early G1 phase of the cell cycle, a pre-replication complex (pre-RC) assembles on chromatin at origins of DNA replication. The core of the pre-RC is the origin recognition complex (ORC), which associates with the origin and recruits binding of Cdc6 and CDT1, which in turn facilitate the loading of MCM2-7 thereby licensing the origin for replication. Subsequent activation of the MCM2-7 helicase allows unwinding of chromosomal DNA, recruitment of DNA polymerases, and progression through S phase (Diffley, 2004). Once replication has initiated, re-replication is prevented through various mechanisms that prevent re-assembly of the pre-RC. Critical among these is the inhibition of CDT1 via binding to its negative regulator, geminin, and via ubiquitin-mediated proteolysis during S phase (Li and Blow, 2005; Nishitani et al., 2001; Saxena and Dutta, 2005; Takeda et al., 2005; Thomer et al., 2004).

Ubiquitination, or the process by which substrate proteins become covalently modified by ubiquitin, is carried out by a series of three different enzymes: the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin ligase, which

mediates the covalent conjugation of ubiquitin to the substrate. If polyubiquitinated, substrate proteins are often targeted for degradation by the 26S proteasome (Pickart, 2004). Since E3 ligases are principally responsible for substrate recognition, they provide much of the specificity to the ubiquitination reaction.

Several E3 ligases have been proposed to be involved in the ubiquitination of CDT1 upon replication initiation. The SCF^{Skp2} (Skp1-Cul1-F box) and a CUL4-containing ubiquitin ligase have each been linked to the cell cycle-dependent and/or DNA damage-induced degradation of CDT1 (Higa et al., 2003; Hu et al., 2004; Kondo et al., 2004; Li et al., 2003b; Liu et al., 2004; Nishitani et al., 2004; Sugimoto et al., 2004; Zhong et al., 2003). Although silencing of CUL4 in *C. elegans* results in massive re-replication of DNA in seam cells that can be rescued by reduction in CDT1 levels (Li et al., 2003b), evidence for CUL4-dependent degradation of CDT1 in higher organisms has only been demonstrated in response to genotoxic stress (Higa et al., 2003; Hu et al., 2004). CUL1 has been proposed to be involved in a CDK-dependent mechanism of CDT1 degradation during the cell cycle, and Skp2 binds to CDT1 that has been phosphorylated by cyclin-dependent kinases (Li et al., 2003b; Liu et al., 2004; Sugimoto et al., 2004). However, CDK-specific phospho-mutants of CDT1 are still efficiently degraded during S phase (Takeda et al., 2005; Thomer et al., 2004). Therefore, the mechanism by which CDT1 is targeted for timely degradation during each cell cycle is still unclear.

CUL1 and CUL4 belong to an evolutionarily conserved family of proteins known as cullins, which act as molecular scaffolds to assemble multi-subunit E3 ubiquitin ligase

complexes *in vivo*. Cullins share the greatest homology at their C-terminus, where the RING-finger proteins ROC1 or ROC2 bind and recruit E2 conjugating enzymes (Donaldson et al., 2004; Kamura et al., 1999; Kamura et al., 2004; Ohta et al., 1999; Seol et al., 1999; Skowyra et al., 1999). The N-terminus of cullins is required for substrate recruitment through binding of substrate receptor modules. Different cullins bind different substrate receptor modules, and consequently the cullin NH2-terminus is divergent. CUL4 is of particular interest amongst the cullin family in that the CUL4A gene is amplified in primary breast tumors, in hepatocellular carcinomas, and in esophageal squamous carcinoma (Chen et al., 1998; Yasui et al., 2002), suggesting an oncogenic activity that contributes to cancer development. However, unlike its better-characterized family member CUL1, the multi-subunit complexes formed by CUL4 are not as completely defined.

There is accumulating evidence from *S. pombe* and mammalian cells that the Damaged DNA Binding protein 1 (DDB1) serves as an adaptor for substrate recruitment by the Cul4 E3 ligase (Groisman et al., 2003; Hu et al., 2004; Kulaksiz et al., 2005; Liu et al., 2003; Shiyanov et al., 1999; Ulane and Horvath, 2002; Wertz et al., 2004). In mammalian cells, a large subset of cellular DDB1 forms a heterodimer with another subunit, DDB2, which binds tightly to damaged DNA (Kulaksiz et al., 2005; Sancar et al., 2004; Wittschieben and Wood, 2003; Wittschieben et al., 2005). DDB2 is mutated in individuals with Xeroderma Pigmentosum- group E (XP-E), a cancer predisposition disorder caused by a defect in the cellular response to DNA damage (Cleaver, 2005). Complexes containing Cul4, DDB1, and DDB2 or CSA (Cockayne Syndrome-A) have been implicated in the regulation of nucleotide excision repair (NER) (Groisman et al., 2003).

It has been unclear, though, whether DDB1 serves as the only adaptor molecule for the Cul4 E3 ligase, and what *in vivo* functions CUL4-DDB1 may have in metazoans in the absence of genotoxic stress. In order to better characterize the *in vivo* functions of CUL4 and DDB1 and their role in CDT1 degradation, we generated and analyzed *Cul4* and *Ddb1* mutants in *Drosophila*. Both *Cul4* and *Ddb1* were found to be essential for development. *Cul4* and *Ddb1* mutants share overlapping phenotypes, including proliferation and growth defects and melanotic tumor formation. *Cul4* mutants exhibited more severe proliferation defects relative to that of *Ddb1* mutants, suggesting that CUL4 utilizes additional adaptor molecules besides DDB1 for substrate targeting. Mutation of $CUL4^{DDB1}$ does not affect CDT1/DUP degradation during the cell cycle. Interestingly, CDT1/dup was also efficiently degraded in the absence of CUL1, but accumulated in Roc1a mutants or when both CUL1 and CUL4 were disrupted in either fly or human cells, indicating a redundant, well-conserved mechanism of CDT1/DUP regulation.

Results

Isolation and characterization of Drosophila Cul4 mutants

The *Drosophila* genome contains a single *Cul4* orthologue encoding a protein that is 66% identical to human CUL4A and 63% identical to human CUL4B. There are two P-element insertion alleles of *Drosophila Cul4* publicly available, one (*KG02900*) located in the 5'UTR and one (*EP2518*) located in the 3'UTR (Fig. 2.1A). Homozygous $Cul4^{EP2518}$ flies are viable, and $Cul4^{KG02900}$ causes recessive lethality that is reverted after precise excision of the *KG02900* P-element. Because $Cul4^{KG02900}$ is a hypomorph (see below), we isolated additional *Cul4* alleles by mobilizing the *EP2518* P-element and screening for

excision mutations that failed to complement the lethality of *Cul4*^{KG02900} and therefore likely represent new alleles of *Cul4*. Three different mutant alleles were identified from 400 independent excision events: *Cul4*^{6AP}, *Cul4*^{11L}, and *Cul4*^{11R}. All three *Cul4* excision mutants arrested during development as first instar larvae, either as homozygotes or in trans to each other or over the deficiency (*Df(2R)CA53*) that uncovers *Cul4*. The *Cul4*^{KG02900} allele is less severe, and *Cul4*^{KG02900}/ *Df(2R)CA53* mutants arrest as second instar larvae. Strikingly, although *Cul4* mutants display early developmental arrest, they do not die and can survive for over 10 days without growing (Fig. 2.3).

We generated an antibody specifically recognizing the N-terminus of fly CUL4 and detected full length CUL4 (Fig. 2.1C) in wild type larvae, but not in homozygous mutants *Cul4*^{6AP}, *Cul4*^{11L}, and *Cul4*^{11R} (Fig. 2.1C, lanes 1-3). Homozygous *Cul4*^{KG02900} mutants expressed reduced levels of CUL4, although the ratio of neddylated to unneddylated CUL4 was increased, relative to wild type larvae (Fig. 2.1C). The specific breakpoints of each excision mutant were determined by sequencing and revealed predicted protein C-terminal deletions of 18 residues in *Cul4*^{11L}, 65 residues in *Cul4*^{6AP} and 82 residues in *Cul4*^{11R}. Truncated proteins corresponding to the predicted molecular weights were detected by Western blot in both *Cul4*^{6AP} and *Cul4*^{11R} mutants as single, un-neddylated bands (Fig. 2.1C), whose stability could be partially attributable to their inability to be neddylated (Wu et al., 2005). The *Cul4*^{11L} allele, which is likely destabilized by the fused P-element sequence, resulted in undetectable protein levels by Western blot and appears to be a null allele (Fig. 2.1C). All three truncation mutants retain the ROC binding site, but lack the NEDD8 conjugation site (K767) and a highly conserved C-terminal domain (Fig.. 2.1B), providing *in*

vivo evidence supporting the essential function of NEDD8 conjugation and/ or the C-terminal domain of CUL4 (Feldman et al., 1997; Furukawa et al., 2002; Furukawa et al., 2000; Kipreos et al., 1996).

Isolation and characterization of Drosophila Ddb1/ piccolo mutants

Biochemical analyses using cultured S2 cells demonstrated that as in mammalian cells, CUL4 and DDB1 physically interact as assayed by co-immunoprecipitation, either when ectopically expressed (Fig. 2.2A), or as endogenous proteins (Fig. 2.2B). Like *Cul4*, *Drosophila Ddb1* is an essential, well-conserved gene, encoding a protein that is 61% identical to human DDB1. $Ddb1^{EY01408}$, a lethal P-element allele (Fig. 2.2C), causes growth arrest developmentally early during second larval instar. Precise excision of the *EY01408* P-element rescued the lethality of $Ddb1^{EY01408}$ flies, and imprecise repair of *EY01408* excision events yielded multiple additional *Ddb1* alleles with a range of severity. The most severe *Ddb1* alleles caused second instar lethality, while less severe alleles allowed survival until third instar or pupal stages. The least severe *Ddb1* alleles resulted in adult flies with reduced viability and fertility, and notable growth defects including missing/ thin bristles (Figs. 2.3B, C) when compared with wild type flies (Fig. 2.3A).

Upon scanning the cytological map surrounding the *Ddb1* locus on 3R, we recognized that a previously defined locus termed *piccolo* (*pic*), mapped roughly to a location on 3R adjacent to the *rosy* locus by complementation analyses of 40 previously-generated X-ray and EMS mutants (Flybase), caused growth abnormalities similar those caused by hypomorphic *Ddb1* mutants. Viable *piccolo* mutants were originally characterized based on

shared irregularities in bristle, wing, and tergite growth (Clark and Chovnick, 1986; Hilliker et al., 1980; Rushlow and Chovnick, 1984; Schalet et al., 1964). This type of growth defect, manifest in short, thin bristles and delayed development, is reminiscent of phenotypes observed in hypomorphic *myc* (*diminuitive*) and haploinsufficient ribosomal subunit *Minute* mutants (Lambertsson, 1998; Schreiber-Agus et al., 1997).

We obtained flies carrying $pic^{S026316}$, pic^{drv3}, and pic^{2} alleles, and found that they caused 2nd ($pic^{S026316}$ and pic^{drv3}) to 3rd (pic^{2}) instar lethality, and failed to complement the lethality caused by $Ddb1^{EY01408}$. By Western blot, we confirmed that $Ddb1^{EY01408}$, $pic^{S026316}$, and pic^{drv3} are null *Ddb1* alleles, while pic^{2} is a hypomorphic allele that expresses DDB1 at reduced levels (Fig. 2C). The pic^{2} X-ray allele was sequenced and found to contain a Gly21-to-Asp substitution in *Ddb1*, thereby altering a single residue that is positioned at a turn in propeller A of the tertiary structure of DDB1 (Li et al., 2006b), and is well conserved across species. Pic^{drv3} resulted from an uninverted transposition event following UV irradiation (Clark and Chovnick, 1986), leaving a large segment of genomic DNA inserted within the *Ddb1* locus. Sequence surrounding the *SO26316* P-element was available, and corresponded to the 5' UTR of *Ddb1* (Flybase; Szeged Stock Center). The pic^{2} allele combined with other weak $Ddb1^{EY01408}$ excision alleles resulted in viable flies that were piccolo in phenotype, with a subset also afflicted with abnormal wing (Fig. 2.3D) and leg development (data not shown). Taken together, these data confirmed that *piccolo* is in fact *Ddb1*. Importantly, $Ddb1^{EY01408}$, $pic^{SO26316}$, pic^{drv3}, and pic^{2} mutants all phenocopied the early growth arrest with continued survival observed in CUL4 mutants (Fig. 3G), suggesting that a CUL4-DDB1-dependent E3 ubiquitin ligase may control a substrate(s) important in cell growth control.

Another phenotype shared by both *Cul4* and *Ddb1* mutant larvae is the development of melanotic masses/ tumors with varying severity (Figs. 2.3H-J) but high penetrance. These melanotic masses were also detected in hypomorphic *Ddb1* mutant adult flies (Figs. 2.3B, D), and therefore did not prevent development to adulthood. Melanotic tumors have previously been described as arising in *Drosophila* larvae in which *Ddb1* had been silenced by RNAi (Takata et al., 2004a), and are thought to result from abnormal hemocyte development that elicits an auto-immune response (Dearolf, 1998; Rizki and Rizki, 1983).

Growth and proliferative defects of Cul4 and Ddb1 mutants

To further analyze the consequence of *Cul4* or *Ddb1* disruption *in vivo*, we generated mutant clones of each via FLP-FRT-mediated mitotic recombination (Xu and Rubin, 1993) during larval development. Larvae were heat-shocked during first instar to induce expression of FLP and subsequent mitotic recombination, and wing and eye-antennal discs were dissected and analyzed at third instar. The eye imaginal disc is especially useful in the study of cell cycle regulators, since cells within the eye imaginal disc enter a synchronized wave of division, in which they arrest in G1 anterior and within the morphogenetic furrow (MF), and then undergo a final division, or second mitotic wave (SMW), before differentiating into photereceptor cells in the posterior margin. Cells undergoing S phase within the SMW, or within the asynchronously dividing populations anterior to the MF in the eye imaginal disc or within wing imaginal discs, were marked by the incorporation of BrdU (Fig. 2.4A).

Under wild type conditions, a twin spot resulting from a single recombination event is visible as a patch of GFP positive cells and of GFP negative cells that are roughly equal in size, and contain levels of BrdU incorporation that are indistinguishable from other surrounding wild type cells (Figs. 2.4A1,2). In contrast, when *Ddb1* mutant clones (GFP-/-) were similarly generated, they incorporated BrdU at reduced levels and demonstrated a 1:4 growth disadvantage relative to wild type clones, in either wing or eye imaginal discs (Figs. 2.4B, 2.4J). *Cul4* mutant clones, however, were undetectable under the same heat-shocking regimen of generating clones (Fig. 2.4D), presumably due to competitive elimination of the slower growing mutant cells during larval development. When recombination was induced at late second instar, however, *Cul4* mutant clones were visible, but showed reduced BrdU incorporation (Fig. 2.4C).

Proliferative defects resulting from disruption of either *Cul4* or *Ddb1* were also apparent in tissues dissected from mutant larvae. Hypomorphic *Ddb1* mutants (pic^{2}/ $Ddb1^{\Delta EY01408}$ transheterozygotes) surviving until third larval instar contained imaginal discs that were smaller in size relative to wild type (Figs. 2.4E, F; data not shown), and showed irregular/ reduced BrdU incorporation in eye imaginal disc cells within the SMW (Fig. 2.4F). We also assessed BrdU incorporation within first instar larval tissues, and observed that while cells within wild type brains undergoing S phase were abundant (Fig. 2.4G), reduced numbers of BrdU positive cells were found in *Ddb1* mutant brains (Fig. 2.4H), and even far fewer in *Cul4* mutants (Fig. 2.4I). Indeed, RNAi of CUL4 or DDB1 in S2 cells causes a marked G1 arrest (Bjorklund et al., 2006; Higa et al., 2006; Li et al., 2006a).

Collectively, these data indicate that ablation of either CUL4 or DDB1 inhibits proliferation and growth. *Cul4* mutant cells, however, appear to be phenotypically more severely affected than *Ddb1* mutants. Since CUL4 is more stable than DDB1, having a longer half-life than DDB1 (Fig. 2.4K), the milder phenotype observed in DDB1 mutants is probably not due to greater perdurance of DDB1 versus CUL4 levels in each respective mutant. Therefore, although CUL4 and DDB1 appear to function in common pathways to affect growth, proliferation, and development, CUL4 also potentially has DDB1-independent functions.

Involvement of $CUL4^{DDB1}$ *in the regulation of DUP/CDT1 levels*

A key cell cycle regulator, the replication licensing factor, CDT1/ Double Parked/ DUP, had previously been described as being regulated by CUL4 in a subset of cells in *C. elegans* (Zhong et al., 2003), and by $CUL4^{DDB1}$ following DNA damage (Higa et al., 2003; Hu et al., 2004). We detected DUP in a CUL4 immunocomplex (Fig. 2.2B), and unneddylated CUL4 in a DUP immunocomplex (Fig. 2.5D), suggesting that the $CUL4^{DDB1}$ E3 ligase may act to regulate DUP protein levels, even in the absence of genotoxic stress.

To test whether DUP is regulated by $CUL4^{DDB1}$ *in vivo*, we carried out immunostaining of DUP in imaginal discs in which $Cul4^{11L}$ or $Ddb1^{EY01408}$ mutant clones had been generated (Fig. 2.4). Other substrates have previously been shown to accumulate in mitotic clones mutant for components of E3 ubiquitin ligases, indicating the efficacy of such an assay in characterizing potential substrates (Jiang and Struhl, 1998; Noureddine et al., 2002; Ou et al., 2002). Under wild type conditions, DUP is primarily nuclear, and is most

abundant in cells in late mitosis and early G1. In eye imaginal disc cells, DUP staining is pronounced in cells anterior to the SMW, but deficient within the SMW due to degradation before the onset of S phase (Fig. 2.3A); (Thomer et al., 2004). We were surprised to find that DUP did not hyper-accumulate in *Cul4* or *Ddb1* mutant clones (Fig. 2.4). In fact, DUP appeared to be efficiently degraded in these mutants during S phase, as BrdU positive cells within *Cul4* or *Ddb1* mutant clones did not stain positively for DUP, as under wild type conditions (Fig. 2.4). This was especially unexpected, since DUP levels were found to be elevated in homozygous CUL4 or DDB1 mutants, relative to wild type larvae (Figs. 2.1C, 2.2D). Similarly, when CUL4 or DDB1 were silenced by RNAi in S2 or Hela cells, respectively, CDT1/DUP levels accumulated modestly (Fig. 2.5D, E). The observed discrepancy in the control of DUP levels in imaginal disc epithilia versus whole mutant larvae or S2 cells could be due to differences in the sufficiency of the $CUL4^{DDB1}$ ligase to regulate DUP in different cell types (May et al., 2005; Zhong et al., 2003).

Redundancy in the regulation of DUP by Cullin-dependent E3 ligases

Since $CUL4^{DDB1}$ appeared insufficient in controlling DUP levels, we questioned whether other E3 ubiquitin ligases may be required to degrade DUP at the G1-S transition during larval development. CUL1 was also detected in a DUP immunocomplex (Fig. 2.5D), indicating that it may be involved in DUP regulation. To test the potential involvement of CUL1 in controlling DUP levels, we generated homozygous null $Cul1^{EX}$ clones, and analyzed DUP levels and BrdU incorporation. In wing or eye imaginal discs containing Cul1 mutant clones, DUP was detected at normal levels and with normal distribution, showing no overlap with cells incorporating BrdU (Fig. 2.5A).

CUL1 and CUL4 independently did not appear to be sufficient to control DUP at the G1-S transition during larval development, but might be required to act cooperatively to degrade DUP. To test this idea, we generated *roc1a*^{G1} clones, in which CUL1-, CUL3-, and CUL4-dependent E3 ligases are inactivated (Donaldson et al., 2004). We positively marked *roc1a* clones with GFP using the MARCM method (Lee and Luo, 2001). *Roc1a* mutant clones contain few cells, largely due to elimination of *roc1a* null cells by apoptosis (Noureddine et al., 2002). Although many *roc1a* clones appeared to degrade DUP normally (data not shown), some appeared to accumulate DUP cytoplasmically at greater levels than surrounding phenotypically wild type cells (Fig. 2.5B). Additionally, some *roc1a* mutant cells containing BrdU positive cells also showed elevated DUP levels, compared to neighboring GFP negative, BrdU positive cells (Fig. 2.5C). Similarly, when CUL1 (or SKP2) and CUL4 (or DDB1) were co-silenced, versus any protein alone, in HeLa cells, CDT1 levels were found to be most hyper-accumulated (Fig. 2.5F). Interestingly, CUL1 and CUL4 not only appear to collaborate functionally, but were also found to interact physically, as detected by co-immunoprecipition from S2 cells (Fig. 2.5D) and HeLa cells (unpublished observation, C. McCall).

Since depletion of CUL4 or DDB1 results in arrest in G1 (Bjorklund et al., 2006; Higa et al., 2006; Li et al., 2006a), the cell cycle stage at which DUP levels are naturally elevated (Nishitani et al., 2001; Thomer et al., 2004), we cannot rule out the possibility that the observed hyper-accumulation of DUP was an indirect consequence of the G1 arrest caused by disrupted expression of these proteins, rather than a direct effect of inhibited ubiquitination and degradation of DUP. However, given the biochemical evidence of a direct

interaction between CDT1/DUP and CUL1 and CUL4 (Figs. 2.1, 2.5) and ubiquitination of CDT1/DUP promoted by CUL1- and CUL4- dependent ligases (Hu et al., 2004; Li et al., 2003b; Liu et al., 2004), the observed hyper-accumulation of DUP in these studies most likely represent a direct effect of altered ubiquitination by cullin-dependent E3 ligases.

Discussion

Cullins have been identified to control critical functions in diverse physiological processes, including cell cycle control, gene transcription, the DNA damage response, apoptosis, and development (Nakayama and Nakayama, 2005; Petroski and Deshaies, 2005; Schwechheimer and Calderon Villalobos, 2004). Our study demonstrates an essential role for CUL4 during the cell cycle, growth, and development in metazoans. This is in agreement with studies from other organisms; a null allele of *Cul4A* in mice caused early embryonic death and was haploinsufficient (Li et al., 2002), and silencing of *cul-4* by RNAi in *C. elegans* results in developmental arrest at the L2 larval stage (Zhong et al., 2003). *In vitro* studies of mammalian CUL4A have also suggested a role in cell cycle progression, as overexpression of CUL4A in cultured myeloid cells promoted proliferation and attenuated differentiation (Li et al., 2003a). In *S. pombe*, although *Cul4* (*Pcu4*) does not appear to be essential, its deletion results in elongated, very slow-growing cells with decondensed chromosomes (Osaka et al., 2000).

We also provide evidence in support of a role for DDB1 during the cell cycle, growth, and development, in the absence of DNA damage. Previous studies had also indicated that silencing of *Ddb1* in *Drosophila* results in early developmental arrest, and that transcription

of *Ddb1* is controlled by the DRE/DREF system (Takata et al., 2002; Takata et al., 2004b), indicating that *Ddb1* is a cell cycle-regulated gene. In *S. pombe*, like *Pcu4* mutants, *Ddb1* is not essential, and its depletion results in slow-growing, elongated cells with abnormal nuclei (Bondar et al., 2003; Bondar et al., 2004; Zolezzi et al., 2002). *Pcu4* and *Ddb1* mutants exhibit slow S-phase progression, and are incapable of entering premeiotic S phase, largely due to accumulation of Spd1, an inhibitor of RNR (ribonucleotide reductase) unique to *S. pombe* (Bondar et al., 2004; Holmberg et al., 2005; Liu et al., 2005; Liu et al., 2003). It has been unknown, though, whether the control of entry into S phase by $CUL4^{DDB1}$ is functionally conserved in higher organisms.

This study demonstrates that CUL4 and DDB1 participate in S phase entry in *Drosophila*, since their disruption inhibited BrdU incorporation in developing larval tissues (Figs. 2.4, 2.5). A key regulator of replication licensing and S phase entry, CDT1/ DUP, is under tight control by geminin and ubiquitin-dependent proteolysis, the latter of which in part appears to be regulated by CUL4 and DDB1 during normal development (Figs. 2.1, 2.2, 2.5). Although $CUL4^{DDB1}$ appeared insufficient to control DUP at the G1-S phase transition (Fig. 2.4), these studies revealed redundancy in the replication-dependent proteolysis of CDT1/DUP, since the inactivation of both CUL1- and CUL4-dependent E3 ligases by RNAi or in *Roc1a* mutant clones resulted in stabilization of CDT1/ DUP (Fig. 2.5). This coordinate control of DUP appears to be conserved in mammalian cells (Fig. 2.5E). While this manuscript was in preparation, several other studies on the regulated ubiquitination of CDT1 in *Xenopus* (Arias and Walter, 2006) and HeLa cells (Nishitani et al., 2006; Senga et al., 2006) were reported. The degradation of CDT1 had previously been established to be

coupled to the onset of DNA replication (Arias and Walter, 2005; May et al., 2005), and an interaction between $CUL4^{DDB1}$ and PCNA on chromatin appears to trigger CDT1 degradation at the G1-S transition, while CUL1-Skp2 appear to principally control CDT1 levels during S-G2 phases (Arias and Walter, 2006; Nishitani et al., 2006; Senga et al., 2006).

The importance in controlling CDT1/DUP function during the G1/S transition is underscored by the multiple mechanisms in place to limit its activity. Other examples of single substrates controlled by multiple E3 ubiquitin ligases are beginning to emerge, including the control of c-jun by $CUL4^{DDB1}$-based and Itch E3 ligases (Gao et al., 2004; Wertz et al., 2004), cubitus interruptus by CUL1- and CUL3-dependent E3 ligases (Ou et al., 2002), p53 by MDM2, COP1, Pirh2, TOPORS, ARF-BP1 E3 ligases (Brooks and Gu, 2006). Interestingly, CUL1 and CUL4 seem to cooperate in targeting a number of substrates, including p27, cyclin E (Higa et al., 2006), and Chk1 (Zhang et al., 2005). The finding that CUL1 and CUL4 co-immunoprecipitate (Fig. 2.5D) suggests that their colocalization in a multi-subunit complex may contribute to their cooperative activity in controlling DUP levels.

Our data also supports the emerging view that DDB1 serves as one but not the only adaptor molecule for the CUL4 E3 ubiquitin ligase. Several recent studies identified Rik1, a DDB1-like protein unique to *S. pombe*, as an adaptor for a CUL4-based E3 ligase that regulates Clr4 (Su(var)39) methyltransferase activity to affect methylation of histone H3-K9 and heterochromatin formation, though the relevant substrate is unknown (Horn et al., 2005; Jia et al., 2005; Li et al., 2005; Thon et al., 2005). DDB1 and Rik1 also share homology with other beta-propeller domain-containing proteins, including SAP130 (Splicing and

Polyadenylation factor) and CPSF160 (Cleavage and Polyadenylation Specificity Factor) (Li et al., 2006b; Neuwald and Poleksic, 2000) which could potentially serve as alternate adaptors for the CUL4 complex to affect an even broader array of potential substrates. Although CUL4-DDB1 may not require an additional specificity factor to target CDT1 for degradation following DNA damage (Hu et al., 2004), the *S. pombe* Pcu4-Ddb1 complex requires the WD-40-containing protein Cdt2 (denticleless) to target Spd1 for degradation (Liu et al., 2005), and the Pcu4-Rik1 complex associates with another WD-40 protein, Raf1/ Dos1/ Clr8, along with Raf2/ Dos2/ Clr7 to control heterochromatin formation (Horn et al., 2005; Li et al., 2005; Thon et al., 2005). In mammalian cells, a complex containing CUL4, DDB1, DET1, and the WD-40 protein COP1 assembles to target c-jun for ubiquitination (Wertz et al., 2004), and CUL4 and DDB1 have also been described as forming distinct complexes with the WD-40 proteins CSA or DDB2 to affect global genomic-NER (and ubiquitination of histone H2B) or transcription coupled-NER, respectively (Groisman et al., 2003; Kapetanaki et al., 2006). Additionally, the V protein of paramyxoviruses associates with the CUL4-DDB1-Roc1 core ligase as an adaptor to target STAT1/2 for degradation (Andrejeva et al., 2002; Li et al., 2006b; Lin et al., 1998; Precious et al., 2005; Ulane and Horvath, 2002; Ulane et al., 2003). CUL4 therefore appears unique amongst the cullins in the diversity of complexes it assembles for substrate targeting.

The lethality caused by the CUL4 C-terminal truncation mutants isolated in our mutagenesis screen (Fig. 2.1) confirm the essential nature of neddylation and/ or the conserved C-terminal domain to CUL4 function. *Drosophila Cul3* mutants lacking the NEDD8 conjugation site (Zhu et al., 2005) or the conserved C-terminal domain (Mistry et al.,

2004) were also found to be inactive *Cul3* alleles, as were similar C*ul4* mutants in *S. pombe* (Jia et al., 2005). We did not recover any excision mutants that ablated the ROC binding site within CUL4, which perhaps would have resulted in dominant-negative mutants. Indeed, a *Xenopus* C-terminal CUL1 truncation mutant lacking its ROC binding site behaves as a dominant-negative mutant whose overexpression caused accumulation of a known Cul1 substrate, Beta-catenin (Voigt and Papalopulu, 2006).

The observation that both C*ul4* and *Ddb1* mutants develop melanotic tumors (Fig. 2.3) suggests that a $CUL4^{DDB1}$ ligase may regulate a pathway(s) controlling hemocyte development. A CUL4-dependent ligase has in fact been shown to regulate mammalian hematopoeisis; silencing of CUL4A was found to inhibit granulocyte differentiation, primarily due to stabilization of the HOXA9 homeodomain protein (Zhang et al., 2003b). The dysregulation of the hop-scotch (JAK/STAT) pathway (Harrison et al., 1995; Muller et al., 2005), the Toll/ Rel/ Cactus (IL-1R/ NF-kappaB/ IkappaB) pathway, the ribosomal S6 protein (Dearolf, 1998) have also been implicated in inducing over-proliferation of hemocytes and resulting melanotic tumor formation, and therefore may represent other possible $CUL4^{DDB1}$ targets. Similarly, given that both *cul4* and *ddb1/ piccolo* mutants were also found to affect growth control during development (Figs. 2.3, 2.4), other possible classes of $CUL4^{DDB1}$ substrates may include negative regulators of the PI3K-dTOR growth signaling pathway.

Materials and Methods

Fly stocks and P-element excision-mediated mutagenesis. Stocks carrying *Cul4* mutant alleles *EP2518* and *KG02900* (stocks 17253 and 13335), and *Ddb1/ piccolo* mutant alleles *EY01408*, pic^{2}, and pic^{drv3} (stocks 15350, 4278, 1979) were obtained from the Bloomington Stock Center (Bloomington, Indiana). The $pic^{SO26316}$ line was from the Szeged Stock Center (stock 444, Szeged, Hungary). The *EP2518* P-element in the 3' UTR of *Cul4* was mobilized by crossing to *w-; Sp/CyO; Dr, Δ 2-3/TM6* flies. Resulting mosaic males were crossed to *Pin/Cyo* flies, and white-eyed flies representing EP2518 excision events were screened for novel *Cul4* mutant alleles by crossing with the $Cul4^{KG02900}$ line. The breakpoints of $Cul4^{6AP}$, $Cul4^{11L}$, and $Cul4^{11R}$ were confirmed by sequencing. The *EY01408* P-element in the 5' UTR of *Ddb1* was similarly mobilized, and resulting white-eyed flies were tested for complementation with the $Ddb1^{S026316}$ allele.

Mitotic recombination and clonal analysis. Mitotic recombination was carried out using the FLP/FRT technique(Xu and Rubin, 1993). To generate clones, *hs-FLP; FRT42B Ubi-GFP/ FRT42B* $Cul4^{11L}$, or *hs-FLP; FRT82B Ubi-GFP/ FRT82B* $Ddb1^{EY01408}$, or *hs-FLP; FRT42D Ubi-GFP/ FRT42D* $Cul1^{EX}$, or FRT19A *GAL80/ FRT19A* $Roc1a^{G1}$*; hsFLP, UAS-GFP*; *Act-GAL4* (MARCM method) larvae were heat-shocked for one hour at 37 °C, 48-80 hours after egg deposition, and dissected as third instar larvae. For BrdU incorporation, dissected larvae were incubated with 10 uM BrdU in Schneider's media for 1 hour prior to fixation.

Immunochemistry procedures. For Western blot analyses, larvae and cells were lysed in RIPA (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 0.1% SDS, 0.1% Triton X-

100, 0.5% sodium deoxycholate), supplemented with 1 mM DTT, 1 mM PMSF, 1 m M sodium vanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 150 μg/ml benzamidine, and cleared by high speed centrifugation. Larval lysate were further clarified through 0.65 micrometer centrifugal low binding durapore membrane filters (Ultrafree-MC, Millipore). Lysates were resolved by SDS-PAGE and analyzed by Western blot. For immunofluorescence analyses, dissected larval tissues were fixed in 4% formaldehyde/ PBS-T, blocked in 5% NGS, and immunostained using standard procedures. Stained tissues were analyzed using a Zeiss confocal microscope.

Antibody specific for the N-terminus of *Drosophila* CUL4 was generated in rabbits, using a synthetically generated peptide (MSAAKKYKPMDTTELHEN) coupled to KLH (Pocono Farms), and affinity purified. A C-terminal anti-CUL4 antibody was a gift from Dr. Hui Zhang (Yale, CT). Anti-DDB1 was generated in mice using a GST fusion protein containing 2/3 of the N-terminal portion of human DDB1 (Zymed). Rabbit anti-CUL4B was generated in rabbits using the synthetic N-terminal peptide (MMSQSSGSGDGNDDEATTSK), coupled to KLH (Pocono Farms). Guinea pig anti-DUP was kindly provided by Dr. Terry Orr-Weaver (MIT, MA), and mouse anti-dCyclin A was from the Developmental Studies Hybridoma Bank (University of Iowa). Rabbit anti-Cul1 (ZL18, Zymed) was used to detect *Drosophila* CUL1. Antibodies recognizing human CUL1, CUL4, and CDT1 were generated in rabbits (Pocono Farms) and previously described (Hu et al., 2004; Michel and Xiong, 1998). Mouse anti-HA (12CA5, NeoMarkers), mouse anti-tubulin (NeoMarkers, and goat anti-Skp2 (N-19, Santa Cruz) were obtained commercially.

Cell culture, transfection, and RNAi. HeLa cells were cultured in 5%CO_2 in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% fetal bovine serum (Sigma), penicillin, and streptomycin (Invitrogen), and transfected with siRNAs using Oligofectamine (Invitrogen). S2 cells were cultured in Schneider's/ 10% FBS at 25 °C, and were transfected using Lipofectamine (Invitrogen). RNAi in S2 cells was performed using dsRNA, as previously described (Clemens et al., 2000).

Figure Legends

Figure 2.1. Drosophila CUL4 mutants are early larval lethal and partially accumulate the replication licensing factor DUP. (A) The *Drosophila CUL4* locus is located on chromosome 2R at 44B1 and contains 12 exons (filled boxes). Coding and untranslated region (UTR) are represented by black and grey boxes, respectively. The P-elements *KG02900* and *EP2518* are located in the 5' UTR and 3' UTR, respectively, of the *CUL4* locus. Three additional *CUL4* mutant alleles, *11R*, *6AP* and *11L*, were isolated by imprecise repair after mobilizing the *EP2518* P-element, and are marked at their respective breakpoints within *CUL4*. (B) Wild type CUL4 protein domains, and truncations resulting from the *6AP*, *11R,* and *11L* excision mutants. Substrate receptor modules (i.e. DDB1) associate with a well-conserved domain within the amino-terminus of CUL4, marked in blue. The C-terminus contains the ROC1a binding domain (red), another highly conserved region at the extreme C-terminus (orange) of unknown function, and the lysine residue (K767) utilized for conjugation with the ubiquitin-like modifier, NEDD8. Mutants 6AP and 11R are stably expressed C-terminal truncation mutants, lacking 65 and 82 amino acids, respectively, and therefore lack the neddylation site and the C-terminal domain, but retain the ROC binding domain. Additional P element sequence located at the mutants' C-termini is colored green; mutants 11R and 6AP contain 3 and 4 additional nonsense C-terminal residues, respectively, while 11L contains 54 additional residues. The 11L mutant lacks 18 amino acids at the CUL4 C-terminus, thus retaining the NEDD8 conjugation site. (C) CUL4 mutants partially accumulate DUP in the absence of DNA damage. Homozygous mutant *6AP*, *11L*, *11R*, or transheterozygous *KG/Df(2R)* (*KG02900 / Df(2R)CA53) or 11L/ Df2R* 1st instar larvae were collected, homogenized, and analyzed by Western blot. Mutants 6AP and 11R express truncation mutants of the predicted molecular weight (red arrows), while the 11L mutant does not express detectable levels of CUL4, and appears null. KG02900 mutants express hypomorphic levels of CUL4. The green arrow indicates a non-specific band. DUP levels correlate with functional CUL4 levels: The CUL4 hypomorphic mutant KG02900 has elevated DUP levels, while mutants 11R, 6AP, and 11L contain higher DUP levels, relative to that observed wild type 1st instar larvae.

Figure 2.2. DDB1/ piccolo is required for viability, and participates in controlling DUP levels. (A) CUL4 and HA-DDB1 co-immunoprecipitate. HA-DDB1 was ectopically expressed in S2 cells, and HA-DDB1 was immunoprecipitated, analyzed by SDS-PAGE and Western using anti-CUL4 and anti-HA antibodies. (B) Endogenous CUL4, DDB1, and DUP co-immunoprecipitate. Anti-CUL4 antibodies specific for the amino-terminus (C4-N) or carboxy-terminus (C4-C) were used to immunoprecipitate endogenous CUL4 complexes. Western blotting using anti-DUP and anti-DDB1 antibodies revealed the binding of DUP and DDB1 with CUL4. (C) The *Drosophila DDB1/ piccolo* locus is located on 3R at 87D10 and contains 7 exons (labeled as in Figure 1). Two *DDB1* P-element stocks were obtained, containing *EY01408* or *SO26316*, both of which are located in the 5' UTR and are lethal when homozygous. The transversion event within the pic^{2} allele (G→A), which causes a G21D point mutation at the amino acid level, is located at the 5'end of exon 2, as indicated. (D) *DDB1* mutants partially accumulate DUP in the absence of DNA damage. Homozygous *SO26316*, *EY01408*, or transheterozygous pic^{2}*/ Df[3R]* or pic^{Drv3}*/ Df[3R]* mutants were

collected as second instar larvae, homogenized, analyzed by SDS-PAGE and Western, probing for DUP, DDB1, and tubulin.

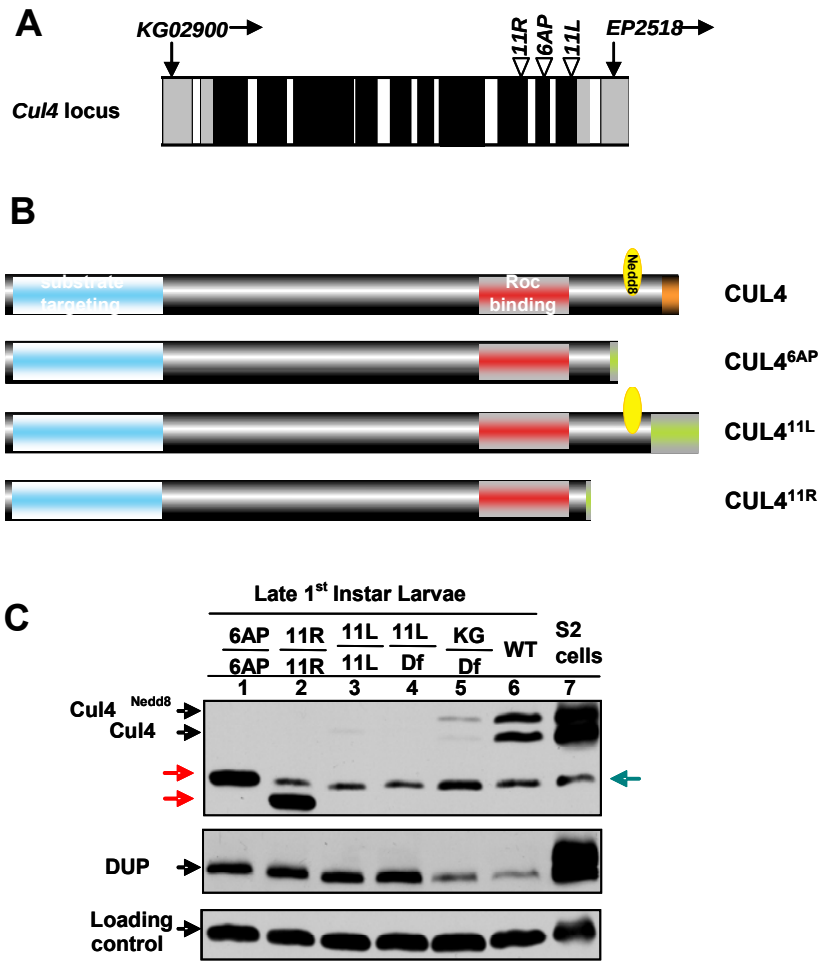
Figure 2.3. CUL4 and DDB1 mutants share overlapping phenotypes, including growth defects and melanotic tumor formation. (A) Wild type fly. (B-D) The piccolo phenotype affects bristle and wing development. *DDB1*$^{\Delta EY01408}$*/pic*^{2} mutants survive until adulthood, and most exhibit thin/ missing bristles ((C) is an enlarged image from (B)) and abnormal wings (D). Melanotic tumor formation is prevalent in *piccolo/DDB1* mutants (B and D). (E, F) CUL4 -/- (*KG02900* mutants shown here), growth arrest early, but survive at a retarded size for > 1 week; shown here is the size difference between homozygous and heterozygous mutants observed at day 5. GFP balancer chromosomes (F) were used to distinguish heterozygous (GFP +/-) from homozygous (GFP-/-) mutants. (G) DDB1 -/- larvae (*EY01408* mutants shown here) also growth arrest early (2nd instar), but continue to survive for > 1 week. (H-J) Both *DDB1* (*I, J*) and *CUL4* (*H*) mutants develop melanotic tumors during larval development. H. *CUL4*$^{11L/11L}$ mutants, shown here at early to late 1st instar. (I, J) Roughly 20% of DDB1 mutants develop extensive melanotic tumors, while the majority typically develop smaller masses (J).

Figure 2.4. DDB1 mutants partially recapitulate CUL4 mutant phenotypes. (A, B) Wild type mitotic clones within eye (A) or wing (B) imaginal discs from third instar larvae. The GFP+/+ clones and GFP-/- clones within a single twin spot are roughly equal in size. BrdU immunolabeling marks S phase cells (blue) and exhibits a distinct, non-overlapping pattern from DUP immunostaining (red), which is most abundant in early G1 phase cells. (C, D) *DDB1*^{EY01408} mutant clones (GFP-/-) in either eye (C) or wing (D) imaginal discs are small in size and proliferate poorly, incorporating reduced levels of BrdU relative to phenotypically WT surrounding cells. (E, F) *CUL4*^{11L} mutant clones fail to proliferate when mitotic recombination is induced by heat shock at 1st larval instar (F), but are detectable when heat-shocked at 2nd larval instar. (E) Like *DDB1* mutants, *CUL4* mutant clones incorporate BrdU poorly, and contain normal levels of DUP. (F, G) Eye discs dissected from *pic*^{2}*/ Df[3R]* (G) versus wild type (F) 3rd instar larvae are reduced in size, and have reduced/ irregular BrdU incorporation. (I, J, K) CUL4 and DDB1 homozygous mutants exhibit reduced proliferation. First instar brain lobes dissected from *DDB1*^{EY01408} (K) or *CUL4*^{11L} (J) mutants, compared to wild type (I), show reduced BrdU incorporation. (L) *DDB1*^{EY01408} clones are 75% smaller in size than wild type clones. The size of GFP+/+ versus GFP-/- clones from wild type or DDB1 twin spots were measured in area, in arbitrary units (pixels). (M) CUL4 has a longer half-life than DDB1. S2 cells were treated with cycloheximide (CHX, 40 ug/mL), and collected over an 8 hour timecourse. Lysates were resolved by SDS-PAGE, and analyzed by Western to detect the half-lives of CUL4, DDB1, and cyclin A.

Figure 2.5. The degradation of CDT1/ DUP may be redundantly controlled by CUL4-DDB1 and CUL1-dependent E3 ubiquitin ligases during S phase. (A) *CUL1*^{EX} clones degrade DUP normally. Immunolabeling of *CUL1*^{EX} clones (GFP-/-) for BrdU incorporation and DUP levels revealed distinct, non-overlapping distributions of S phase and DUP positive cells. (B, C) Dysregulated DUP levels are observed in few *ROC1a* mutant clones. *ROC1a*^{G1} clones were positively marked (GFP +/+), and immunostained as in (A). Some ROC1a mutant clones have elevated cytoplasmic DUP levels (B), and others that are BrdU positive also

inappropriately contain DUP (C). (D) DUP, CUL1, and CUL4 co-immunoprecipitate. Immunoprecipitations using anti -DUP, -CUL1, -CUL4, and –myc (control) and S2 cell lysates were resolved by SDS-PAGE, and probed by Western blot as indicated. (E) $CUL4^{DDB1}$ control DUP levels in S2 cells, in the presence or absence of DNA damage. $CUL4^{DDB1}$ was silenced in S2 cells by treatment with CUL4 or DDB1 dsRNA for 4 days, and subjected to ionizing radiation (110 Gy), or left untreated. (F) Disabling both CUL1-SKP2 and CUL4-DDB1 E3 ligases by RNAi in HeLa cells more completely protects CDT1 from degradation during S phase than disrupting either E3 ligase alone. HeLa cells were transfected with synthetic siRNAs corresponding to the indicated genes, lysed 48 hours later, and analyzed by Western blot.

Figure 2.1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Roc binding
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11R 11R
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Figure 2.2

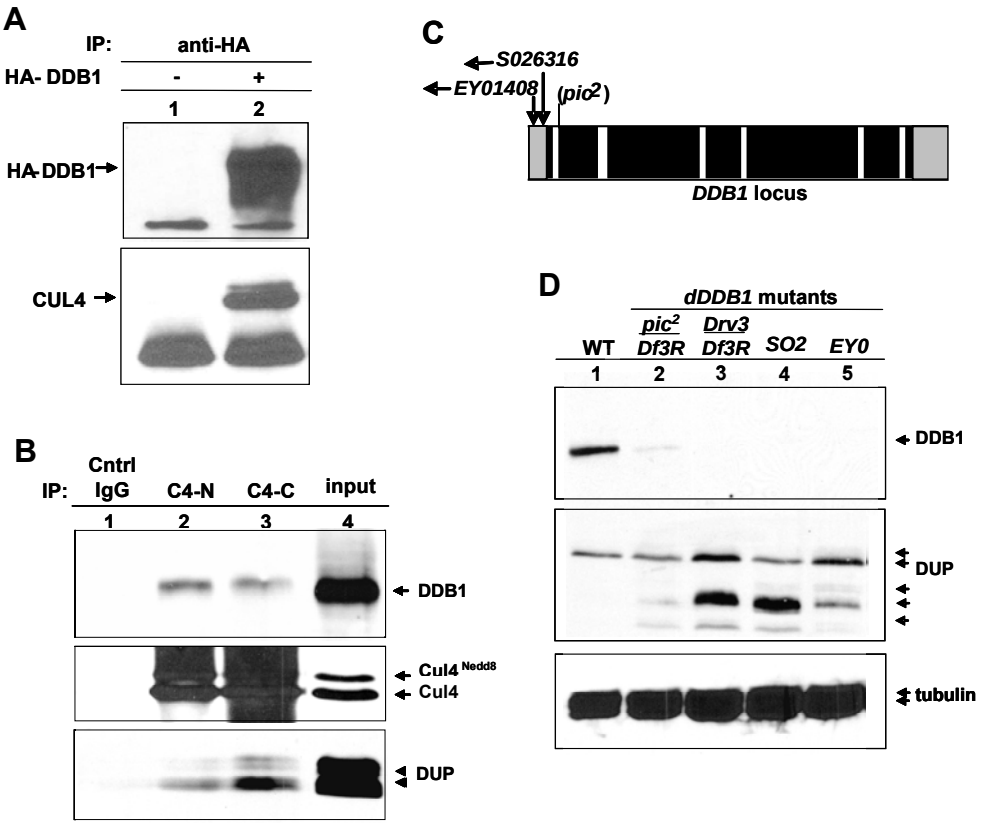
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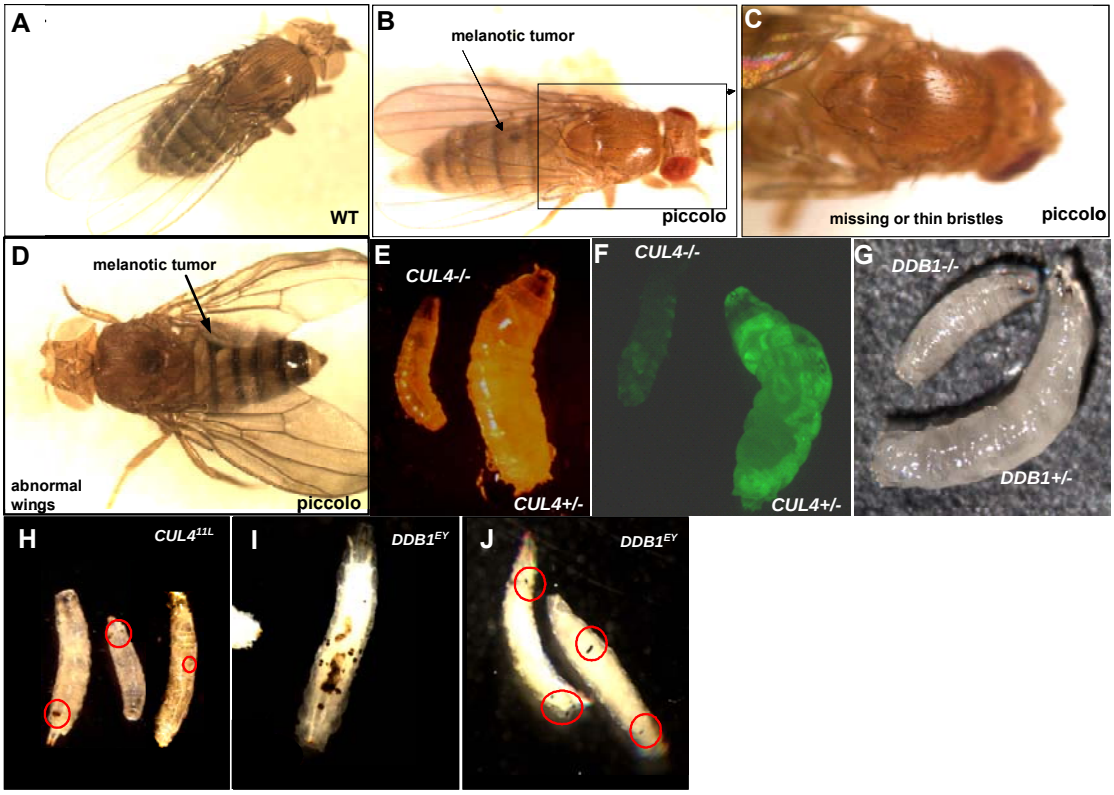
Figure 2.3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piccolo
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D
melanotic tumor
abnormal wings
piccolo
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CUL4+/-
F
CUL4-/-
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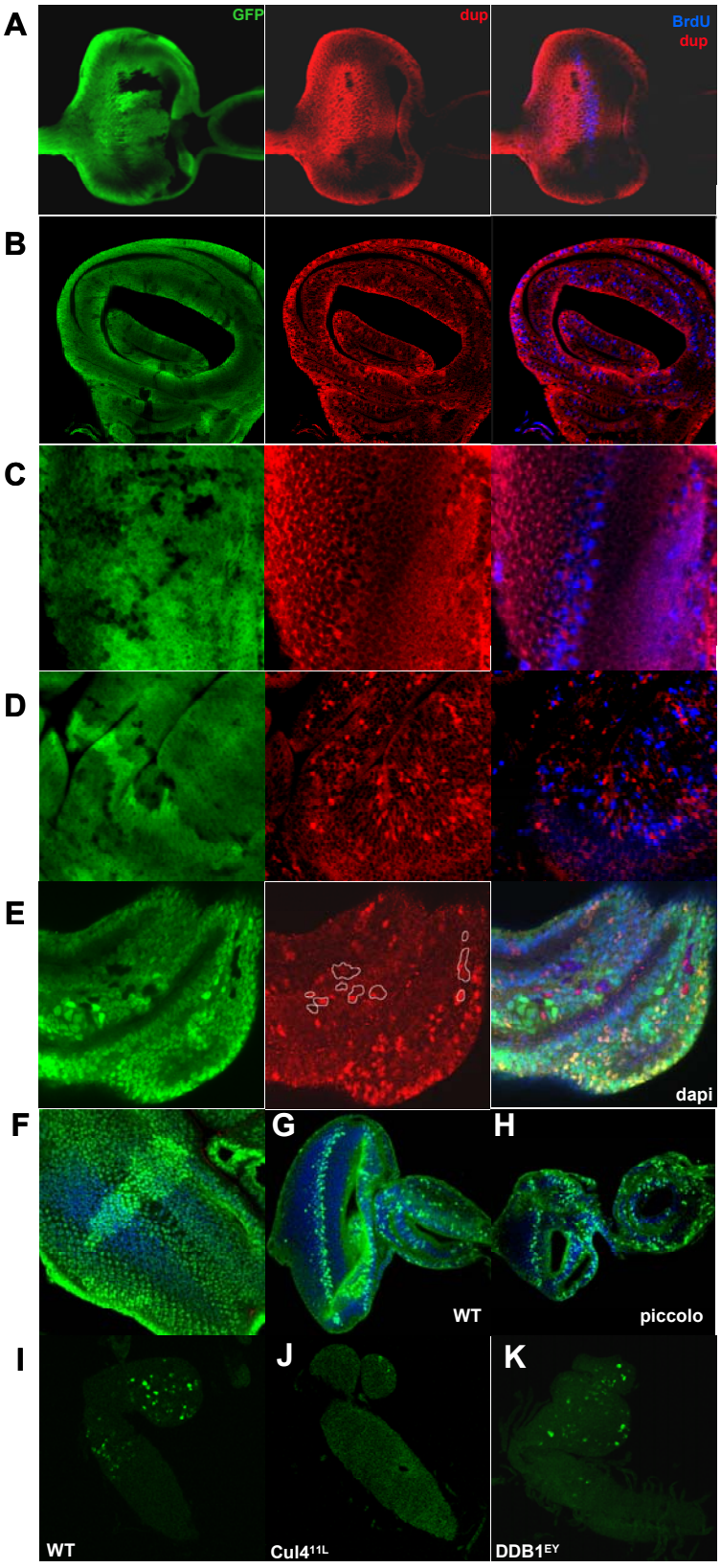
DDB1^EY
J
DDB1^EY

Figure 2.4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BrdU
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dapi
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piccolo
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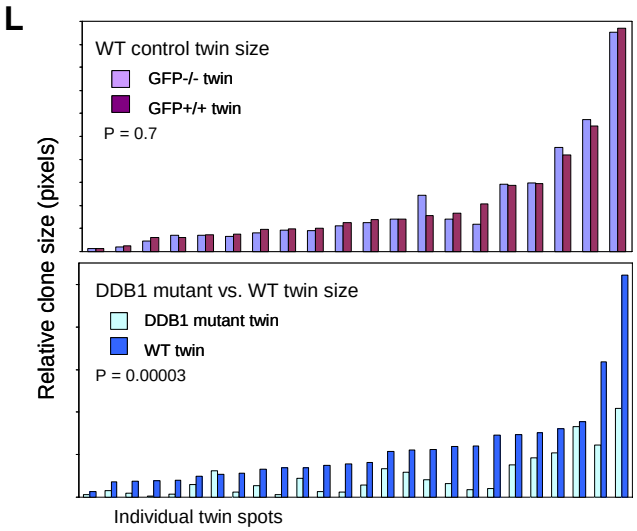
Cul4^11L
DDB1^EY

Figure 2.4, continued



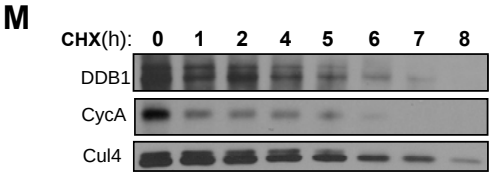
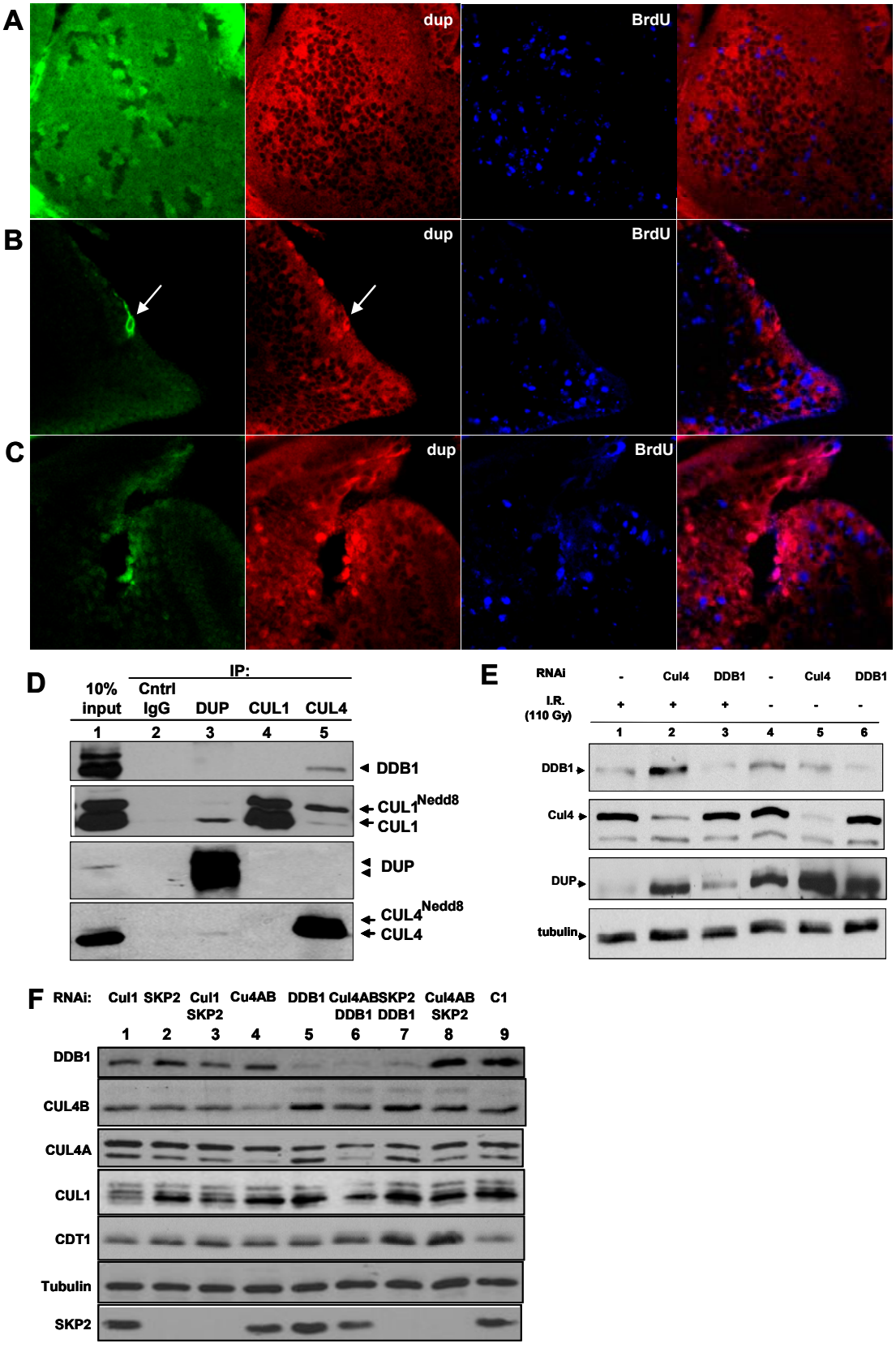


Figure 2.5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B
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BrdU
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D
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Cntrl IgG
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CUL4
1 2 3 4 5
DDB1
CUL1Nedd8
CUL1
DUP
CUL4Nedd8
CUL4
E
RNAi - Cul4 DDB1 - Cul4 DDB1
I.R. (110 Gy) + + + - - -
1 2 3 4 5 6
DDB1
Cul4
DUP
tubulin
F
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1 2 3 4 5 6 7 8 9
DDB1
CUL4B
CUL4A
CUL1
CDT1
Tubulin
SKP2

CHAPTER III

Alternate substrate adaptors and potential substrates of the CUL4-dependent E3 ubiquitin ligase

DDB1-dependent and –independent functions of CUL4

Our studies of CUL4 and DDB1 in *Drosophila* (Chapter II) coupled with genetic studies in other model organisms, including *S. pombe*, *C. elegans*, and mice have established that CUL4 and DDB1 are essential for development, and have pleiotropic functions that extend beyond the DNA damage response. These studies have also indicated that DDB1 serves as a substrate receptor molecule for CUL4, but that it does not account for the full scope of CUL4 activity, suggesting an even broader array of complexes assembled and potential substrates targeted by the CUL4 E3 ubiquitin ligase (Figure 3.3). Studies in *S. pombe* have very recently demonstrated a role for CUL4 in heterochromatin formation, by assembling with a DDB1-like molecule, Rik1 (unique to *S. pombe*), and two additional adaptor molecules (Raf1/ Dos1/ Clr8 and Raf2/ Dos2/ Clr7) (Horn et al., 2005; Li et al., 2005; Thon et al., 2005).

Structural analyses of DDB1 support the genetic evidence for the existence of additional DDB1-like molecules that may associate with CUL4. Other well-conserved beta-propeller domain-containing proteins that structurally resemble DDB1, including SAP130 (Splicing and Polyadenylation factor) and CPSF160 (Cleavage and Polyadenylation

Specificity Factor) (Li et al., 2006b; Neuwald and Poleksic, 2000) are likely candidate substrate receptor molecules for the CUL4 ligase. Preliminary data in our lab suggests that CUL4A can in fact co-immunoprecipitate with SAP130 and CPSF160 when ectopically expressed in mammalian cells (personal communication, Y. Xiong, unpublished results). Such potential associations are suggestive of possible roles for the CUL4 E3 ligase in regulating factors involved in mRNA processing events (Figure 3.3). Further studies will be required to evaluate the functional consequence of such interactions and possible substrates that may be targeted.

Roles for CUL4 and DDB1 in proliferation and growth

A deeper understanding of the full range of functions of the $CUL4^{DDB1}$ E3 ligase awaits further analysis as well. Our studies in *Drosophila* support existing data indicating a role for the $CUL4^{DDB1}$ ligase in regulating CDT1/DUP levels during S phase, and following DNA damage (chapter II). The expression of CUL4 and DDB1 fluctuates slightly during the course of development, but with similar patterns that are coincident throughout (Fig 3.2), indicating that association between DDB1 and CUL4 would be viable at all developmental stages in which CUL4 is expressed. As disruption of *DDB1* appears less severe (and presumably would affect fewer cellular processes) than *CUL4* disruption in *Drosophila*, and since DDB1 does not appear to have CUL4-independent functions, a more detailed analysis of the growth and proliferation defects of *DDB1* mutants could point to other potential substrates of the $CUL4^{DDB1}$ ligase.

The growth defect observed in CUL4 and DDB1 mutants (Chapter II) indicate that $CUL4^{DDB1}$ could be involved in controlling a negative regulator of the growth signaling pathway. For example, the potential involvement of $CUL4^{DDB1}$ in regulating PTEN or TSC1/ TSC2, could be suggestive of the oncogenic function of CUL4 observed in breast and other cancers. The growth defect in *DDB1* mutants is evident as a *piccolo* phenotype with clear disruption of bristle growth (Chapter II). Given that the piccolo phenotype is highly visible and traceable, rescue experiments using *Drosophila* DDB1 mutants could be used to test candidate growth effectors as $CUL4^{DDB1}$ substrates. For example, testing whether reducing levels of PTEN, TSC1, or TSC2 in DDB1 mutants can rescue the piccolo phonotype could quickly establish whether these growth regulators are controlled by $CUL4^{DDB1}$. In addition, a scaled-up screen could be conducted to identify other genes that may be able to rescue the piccolo phenotype, which could potentially lead to the identification not only of possible $CUL4^{DDB1}$ substrates, but could perhaps also identify novel growth regulators.

CUL4-interacting proteins

In attempting to identify other potential CUL4-interacting proteins, we have also employed biochemical assays, such as immunoprecipitation and mass spectrometric analyses. Using our anti-CUL4-N antibody and S2 cell lysates, CUL4 immunocomplexes were purified and analyzed by mass spectrometry (Figure 3.1). Immunoprecipitations (IPs) of CUL4 were incubated in the absence or presence of CUL4-N competing peptide (a 15 amino acid peptide corresponding to the epitope used for anti-CUL4-N production), to ascertain specific interactions with the anti-CUL4-N antibody. Bands that were present in the minus-peptide IP, but which were competed away in the plus-peptide IP, were submitted for mass spectrometric

analysis. Two prominent bands were positively identified as CUL4, likely representing neddylated and unneddylated forms. Although previously identified CUL4-interacting proteins, such as CAND1, signalosome subunits, DDB1, or ROC proteins, were not identified in this experiment, and some identified bands such as the heat shock protein (AE003708 NID) are notoriously "sticky" proteins that probably represented non-specific interactions, other bands identified could be relevant to CUL4 function. Interestingly, the ribosomal protein (L6) was found to co-immunoprecipitated with CUL4, which could be indicative of a growth-related regulatory function of CUL4. Other proteins, including CG30069 (uncharacterized protein with putative cell cycle function) and CG10102 (RNA-directed DNA polymerase) are newly identified interactions and could be involved in CUL4-mediated regulation of proliferation. Another interesting interaction with the large subunit of RNA polymerase II was also identified (Fig. 3.1), as was an interaction with the small subunit of RNA polymerase II, in a separate IP-Mass spec experiment using wild type *Drosophila* embryos (data not shown). RNA polymerase II has previously been identified in association with CUL4, DDB1, and CSA, as part of a complex involved in transcription-coupled repair of damaged DNA (Groisman et al., 2003). Further exploration of the functional consequence of the interaction between CUL4 and RNA Polymerase II in the presence or absence of DNA damage will lead to a greater understanding of $CUL4^{DDB1}$ function.

CUL4 promotes both mono- and poly-ubiquitination of substrates

Recent studies in mammalian cells have indicated another unique property of CUL4 amongst the cullin family; CUL4 is not only able to promote the polyubiquitination and

subsequent degradation of target substrates, but may also serve to monoubiquitinate select substrates to regulate their function. For example, monoubiquitination of histone H2A and XPC (the xeroderma pigmentosum group-C protein involved in damaged DNA recognition) by a $CUL4^{DDB1\text{-}DDB2}$ complex in response to DNA damage appears to promote the ability of these molecules to contribute to repair of damaged DNA, and does not lead to their destruction by the proteasome (Kapetanaki et al., 2006; Sugasawa et al., 2005). Therefore, newly identified CUL4-interacting proteins/ substrates may not necessarily be subjected to targeted polyubiquitination and degradation, but may be activated, inactivated, re-localized, or otherwise functionally altered by CUL4-mediated monoubiquitination.

CUL4 utilizes additional substrate receptors through association with DDB1 family proteins

CUL4 often appears to require more than DDB1 or other DDB1-like proteins in recruiting substrates for ubiquitination (Figure 3.3). Several WD-40 domain-containing proteins have been identified in CUL4 complexes in association with DDB1, including DDB2, CSA, DET1, Cdt1, and Clr8 (through Rik1 in *S. pombe*), and appear to be required for ubiquitination of target substrates. It is conceivable and likely that these WD-40-containing proteins belong to a larger, yet undefined family of proteins, analogous to the F-box family of proteins interacting with SKP1 of the CUL1 complex, that preferentially associate with DDB1 or DDB1-like proteins to specify substrate recruitment. Several of these identified mammalian WD-40 proteins share close homologues in Drosophila, and can potentially be tested genetically for interactions with CUL4 and DDB1, SAP130, or CPSF160. One of these proteins, Cdt2 (in *S. pombe*), has a close homologue, *denticleless*, in *Drosophila*. *denticleless* is required for development and proper denticle formation during

embryogenesis, and is an E2F-responsive gene (Cathy Silver Key, personal communication), and would therefore be interesting to evaluate whether it could be partly involved in CUL4-mediated control of proliferation. Since CUL4 functions are highly pleiotropic, the full scope of such functions may be masked in CUL4 mutants *in vivo*. Therefore, an *in vivo* study of other proteins that mediate substrate specificity of the CUL4 complex, and therefore regulate a smaller subset of CUL4 function, will undoubtedly lead to a greater understanding of CUL4 function.

Targeting of CUL4 in viral infection and tumorigenesis

The cellular proteins targeted by viruses during viral infection often also represent proteins targeted in tumorigenesis (O'Shea, 2005). CUL4 is known to be hijacked by two different mechanisms during viral infection by hepadnaviruses or paramyxovirus. Hepadnaviruses encode the C protein, which associates with DDB1 to control viral genome replication. The $CUL4^{DDB1}$ complex is co-opted by the V proteins of paramyxoviruses (mumps virus), thereby polyubiquitinating STAT1 and STAT3 and marking them for degradation (Ulane and Horvath, 2002; Ulane et al., 2003). Interestingly, the development of melanotic tumors in *CUL4* and *DDB1* mutants (chapter II), which can result from dysregulated JAK/STAT signaling, suggests that $CUL4^{DDB1}$ may utilize other cellular adaptors to ubiquitinate STATs *in vivo*. Further studies aimed at determining the physiological substrate(s) responsible for melanotic tumor formation in *CUL4* or *DDB1* *Drosophila* mutants, could be another means of fully appreciating $CUL4^{DDB1}$ function *in vivo*, and in the context of its over-expression in breast and other cancers.

Genomic amplification and overexpression of human CUL4A in breast cancer suggests a potential function of CUL4 in promoting cell survival. Generation of transgenic flies over-expressing CUL4 could provide an opportunity to test this possibility at the organismal or cellular level, as flies have been utilized as a model system of tumorigenesis (Higa et al., 2003; Hu et al., 2004). Using the UAS/ GAL4 system of expression, CUL4 can be preferentially over-expressed in the whole organism, in a tissue-specific manner, or in clones of cells via the FLP/FRT system (Xu and Rubin, 1993), and examined to determine whether normal proliferation and/or differentiation of affected tissue is perturbed.

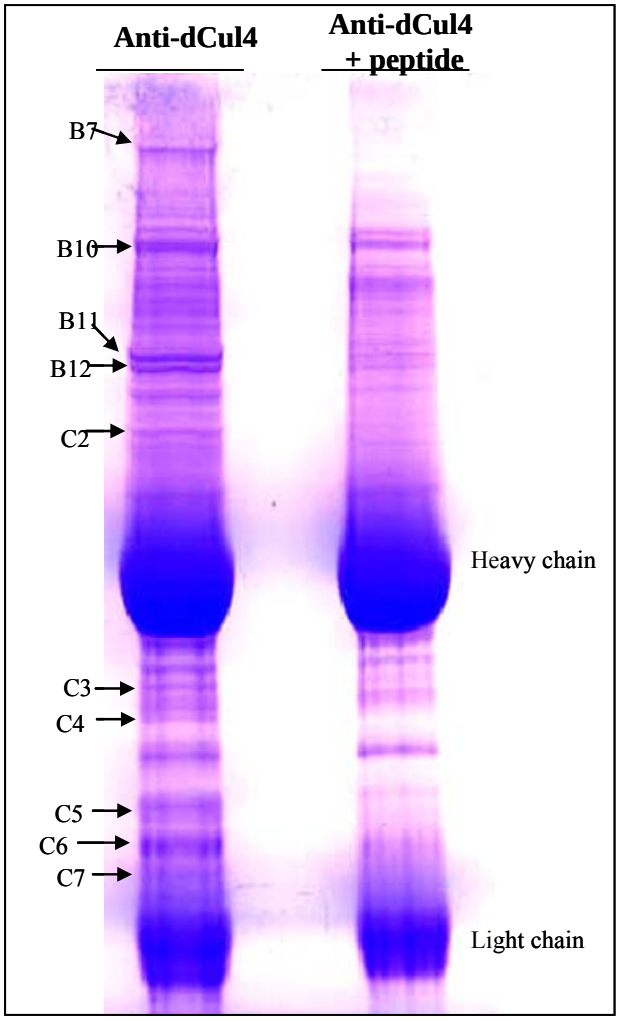

Figure 3.1. Mass spectrometric analysis of CUL4 complexes, purified from S2 cells. CUL4 immunocomplexes were purified from *Drosophila* S2 cell lysates in the presence or absence of CUL4 competing peptide, and were resolved by SDS-PAGE. Bands were visualized using Coomassie blue. Marked bands indicate those that bound to CUL4 specifically, as indicated by their reduced association with CUL4 in the presence of competing peptide. Noted bands were excised and identified by mass spectrometric analysis: B7: CG30069, cell cycle/ unknown function; B10: DNA-directed RNA Pol II; B11: neddylated dCul4; B12: dCul4; C2: heat shock protein; C3: Fructose 1,6 bisphosphate; C4: Fibrillarin; C5: Ribosomal Protein L6; C6: ADP/ATP translocase; C7: RNA-directed DNA polymerase.

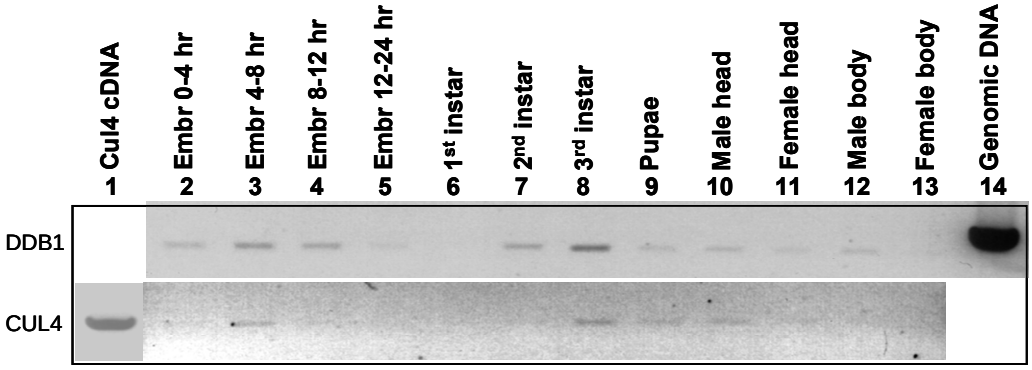

Figure 3.2. CUL4 and DDB1 transcripts exhibit similar expression patterns during *Drosophila* development. RT-PCR of CUL4 and DDB1 first strand cDNAs generated from *Drosophila* mRNA isolated throughout development (Rapid-Scan Gene Expression Panel; OriGene Technologies) .

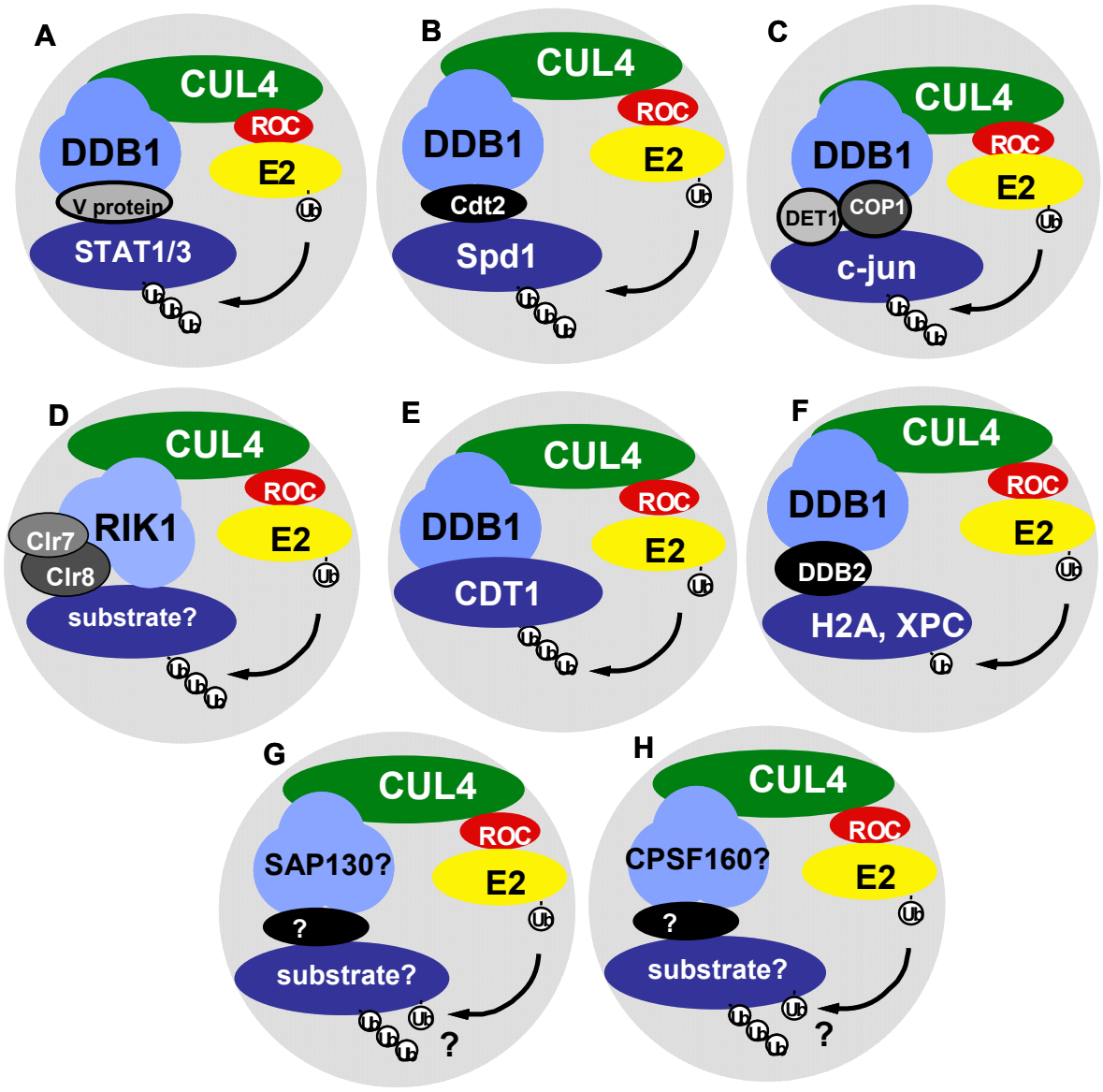

Figure 3.3. Complexes assembled by CUL4 E3 ligases. (A) Hijack of the $CUL4^{DDB1}$ ligase by the mumps V protein targets STAT1 or STAT3 for ubiquitination. (B) In *S. pombe*, CUL4 associates with DDB1 and CDT2 to target the ribonucleotide reductase inhibitor, Spd1, for ubiquitination. (C) A complex containing CUL4, DDB1, DET1, and COP1 has been described to ubiquitinate c-jun. (D) In *S. pombe*, CUL4 also assembles with RIK1, a DDB1-like protein, along with additional adaptor proteins Clr7 and Clr8, to promote heterochromatin formation, though the relevant substrate(s) is unknown. (E, F) In mammalian cells subjected to UV, $CUL4^{DDB1}$ interacts directly with the substrate CDT1, leading to its degradation, and preventing S phase entry (E). (F) Upon DNA damage, DDB2 appears to be important for localizing the $CUL4^{DDB1}$ complex to sites of DNA damage, allowing the monoubiquitination of histone H2A and XPC (a damaged DNA binding protein), and repair of DNA. (G, H) CUL4 may also associate with two other proteins that share homology with DDB1: SAP130 (G), and CPSF160 (H), implicating CUL4 in processes affecting splicing and mRNA processing.

CHAPTER IV

Negative Regulation of TSC1-TSC2 by mammalian D-type cyclins*

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* Published in *Cancer Research* (Zacharek et al., 2005).

Summary

The metazoan cell cycle is driven by the timely and composite activities of cyclin-dependent kinases (CDKs). Among these, cyclin D-dependent kinases phosphorylate the pRb family proteins early in the G1 phase of the cell cycle and thereby advance cells beyond the restriction point. Increasing evidence suggests that cyclin D-dependent kinases might affect events other than Rb pathway-mediated entry into S phase, such as accumulation of cell mass. However, little is known about cyclin D activity toward Rb-independent pathway(s) or non-pRb substrates. We designed a yeast three-hybrid screen to identify potential regulators and substrates of cyclin D1-CDK6 and isolated TSC2 as a cyclin D1 binding protein. In cultured cells, co-expression of cyclin D1-CDK6 leads to increased phosphorylation and decreased detectable levels of both TSC1 and TSC2 proteins, and promotes the phosphorylation of the mTOR substrates 4E-BP1 and S6K1, two key activators of cell growth that are negatively regulated by the TSC1-TSC2 complex. At the cellular level, ectopic expression of cyclin D1 restored the cell size decrease caused by TSC1-TSC2 expression. Intriguingly, down regulation of TSC proteins was also observed by the expression of a mutant cyclin D1 that is unable to bind to CDK4/6, or by the co-expression of cyclin D1 with either an INK4 inhibitor or with catalytically inactive CDK6, indicating that cyclin D may regulate TSC1-TSC2 independent of CDK4/6. Together, these observations suggest that mammalian D-type cyclins participate in cell growth control through negative regulation of TSC1-TSC2 function.

Introduction

Studies in *S. cerevisiae* that laid the groundwork for our understanding of the cell division cycle also established its critical link to cell growth control—regulating the accumulation of cellular mass (Hartwell and Unger, 1977; Nurse, 1975). Cell cycle control and cell growth control must be coordinately regulated to maintain homeostatic cell size, yet the two processes are separable (Jorgensen and Tyers, 2004). The use of temperature sensitive cell division cycle mutants demonstrated that yeast cells blocked from progressing through the cell cycle could still continue to increase in size. However, when cell growth was blocked by nutrient deprivation or by inactivating key biosynthetic genes, the cell cycle could no longer proceed (Hartwell and Unger, 1977). Although disruptions in the control of the cell cycle, and more recently of cell growth, have been widely recognized as major contributors to tumorigenesis (Deshpande et al., 2005; Shamji et al., 2003), the molecular mechanisms linking the two processes are not well understood.

Entry into the proliferative cell cycle and progression through G1 is initiated by extracellular mitogenic signaling, which in mammalian cells leads to the synthesis of D-type cyclins. Two catalytic subunits, CDK4 and CDK 6 (CDK4/6), can interact with any of three D-type cyclins (D1, D2, and D3) to form up to six distinct kinase holoenzymes (collectively referred to as cyclin Ds-CDK4/6). Although individual D-type cyclins, and to lesser extent CDK4 and CDK6, are expressed differentially in a tissue-specific manner, different cyclin Ds-CDK4/6 complexes are biochemically similar if not indistinguishable. CDK4/6 are negatively regulated by two families of CDK inhibitors, the INK4 (inhibitors of CDK4) family, which can bind CDK4/6 and prevent their association with D-type cyclins, and the

CIP/KIP family, which can bind to and inhibit cyclin Ds-CDK4/6 in a ternary complex (Pei and Xiong, 2005). Active cyclin Ds-CDK4/6 phosphorylate pRb and two other pocket proteins p107 and p130, thereby lifting repression of the E2F transcription factors and permitting expression of genes necessary for DNA replication during S phase.

However, some aspects of cyclin D and CDK4/6 biology cannot be reconciled by their kinase activity toward pRb alone. For example, as some cells undergo senescence and permanently withdraw from the cell cycle, such as during myotube differentiation, the level of cyclin D in the cell actually accumulates rather than decreases (Franklin and Xiong, 1996). Furthermore, a cyclin D1 mutant ineffective at targeting pRb retains transforming ability in cooperation with Ras (Zwicker et al., 1999). In a similar vein, disruption of cyclin D-CDK4/6 function by $p16^{INK4a}$ over-expression in melanocytes exhibited phenotypically distinct consequences from pRb inactivation (Yu et al., 2003). Cyclin D1 and CDK4 are dispensable for proliferation in flies and mice, but do appear to play a role in growth control, as disruption of either cyclin D or CDK4 results in reduced cell and overall organism size (Datar et al., 2000; Kozar et al., 2004; Meyer et al., 2000)). In fact, over-expression of cyclin D and CDK4 in flies leads to an increase in cell and organ size in an pRb-independent manner (Datar et al., 2000; Xin et al., 2002). Genetic studies have suggested a role for cyclin D-CDK in cell growth control by acting upstream of Hif-1 prolyl hydroxylase (Hph) and the mitochondrial ribosomal protein mRpL12 (Frei and Edgar, 2004; Frei et al., 2005).

Despite the broad effects of cyclin D-CDK on cell cycle and cell growth control, little is known about its substrates other than the pRb family. Recently, Smad3 was identified as a

direct cyclin Ds-CDK4/6 substrate, linking cyclin D-CDK activity with another effector of proliferative control (Matsuura et al., 2004). In addition, CDK-independent activation of several transcription factors by cyclin D have been described (Fu et al., 2004). The identification of other cyclin D-CDK4/6 targets could lead to a greater understanding of its role in cell cycle progression, cell growth control, and tumorigenesis. The limited number of identified cyclin Ds-CDK4/6 substrates could be partly due to the technical difficulties associated with identifying the transient association of a kinase and substrate. In this study, we describe a protein yeast three-hybrid system capable of assembling a cyclin-CDK complex designed to identify potential binding proteins. We isolated the cell growth regulator TSC2 using this system, and further characterization of this interaction suggests that the TSC1-TSC2 complex may be a novel target of cyclin D-CDK4/6 activity involved in cell growth control.

Results

Design of a protein yeast three-hybrid system

The conventional two-hybrid system is capable of detecting only binary protein-protein interactions. We reasoned that because some protein interactions with CDKs may be cyclin-dependent, the use of a cyclin-CDK complex as "bait" may allow the identification of novel cyclin-CDK-interacting proteins. In order to investigate this possibility, we designed a modified version of the yeast two-hybrid system that allows the formation of a ternary protein complex in yeast. Similar systems have been described previously (Gordon and Buchwald, 2003; Licitra and Liu, 1996; Pause et al., 1999; Sandrock and Egly, 2001; Tirode et al., 1997). To facilitate interactions of potential CDK substrates, we used a CDK6 point

mutant ($CDK6^{K43M}$) that is incapable of binding to ATP and therefore could form a more stable complex with the substrate. A similar mutant has previously been shown to stabilize the interaction of cyclin D-CDK4 with pRb (Kato et al., 1993). We fused $CDK6^{K43M}$ to the GAL4 DNA binding domain (GAL4BD) and cyclin D1 to the GAL4 nuclear localization signal (NLS). Expression of GAL4BD-$CDK6^{K43M}$ and NLS-cyclin D1 proteins was driven by distinct promoters within a single plasmid carrying the *TRP* marker. The prey cDNA was fused to the GAL4 activation domain (GAL4AD) and expressed from a conventional two-hybrid plasmid expressing the *LEU* marker. Interaction of bait and prey results in the reconstitution of the GAL4 transcriptional activator and drives expression of the *HIS3* gene, allowing growth on media lacking histidine. This system is capable of detecting binary interactions between $CDK6^{K43M}$ and the prey as well as ternary interactions involving $CDK6^{K43M}$, cyclin D1, and the prey.

In order to confirm the efficacy of the three-hybrid system, we tested for binding of proteins known to interact with CDK6 in a cyclin D-dependent manner. We assessed the ability of the CDK6 substrates pRb or p130 to interact with $CDK6^{K43M}$ either alone or in the presence of cyclin D1 or cyclin D3. As expected, pRb and p130 were able to interact with $CDK6^{K43M}$ in the presence of cyclin D1 or D3, but failed to interact with $CDK6^{K43M}$ alone (Figs. 4.1a, b). The CDK4/6 inhibitors, $p15^{INK4b}$ and $p18^{INK4c}$, are known to interact with CDK4/6 independently of cyclin D (Guan et al., 1994). Both $p15^{INK4b}$ and $p18^{INK4c}$ showed positive interaction with $CDK6^{K43M}$, either in the presence or absence of cyclin D1 or D3 (Figs. 4.1a, b). These interactions were confirmed by β-galactosidase activity in yeast co-transformed with $CDK6^{K43M}$-cyclin D1 and either p21, pRb, or $p15^{INK4b}$ (data not shown).

Detection of CDK6/cyclin D interacting proteins using a yeast three-hybrid library screen

After confirming the feasibility of the three-hybrid system, we sought to identify proteins that could interact with the $CDK6^{K43M}$-cyclin D1 complex by screening a human cDNA library generated from the HaCaT keratinocyte cells. Of an estimated 10^6 colonies screened, 512 were isolated on histidine-minus growth medium. Of 135 colonies further analyzed, 96 were positive for β-galactosidase staining. A more stringent assay revealed that 71 of the β-galactosidase positive colonies were able to sustain growth in the presence of 30 mM 3-amino-triazole (3-AT), a HIS3 inhibitor. Sequence analysis of these clones indicated that the majority represented known CDK6-cyclinD1 interacting proteins, including 31 isolates encoding D-type cyclins, 14 isolates representing members of the INK4 family of CDK inhibitors, 9 isolates representing the CDK inhibitor p21, and 3 isolates representing p130, a pRb family member. Of the genes encoding proteins previously unrecognized as interacting with CDK6-cyclin D1, a truncated form of TSC2 was isolated 20 times and formed a stable interaction with the $CDK6^{K43M}$-cyclin D1 complex, as judged by strong β-galactosidase activity and growth in the presence of 30 mM 3-AT. The prey plasmid encoding the carboxyl-terminus of human TSC2 (TSC2-C) was found to initiate at amino acid 1297, yielding a protein of 511 amino acids with a calculated molecular weight of 56 kDa. When re-transformed into yeast, the interaction of TSC2-C with $CDK6^{K43M}$ was dependent on the presence of either cyclin D1 or D3 and was also capable of interacting with both cyclins in a binary fashion (Fig. 4.1b). An interaction between $p18^{INK4c}$ and $CDK6^{K43M}$ confirms expression of the kinase (Fig. 4.1b). A similar two-hybrid screen of the HaCaT cDNA library using GAL4BD-$CDK6^{K43M}$ as bait alone revealed that of 39 isolated colonies,

neither p21, p130 nor TSC2 were represented (data not shown), consistent with the notion that these proteins interact preferentially, if not only, with cyclin D-CDK complexes.

TSC2 interacts with the D-type cyclins

To confirm the interaction of TSC2 with cyclin D proteins in mammalian cells, 293T cells were transiently transfected with Flag-tagged full length TSC2 in combination with one of the three D-type cyclins. Anti-Flag immunoprecipitates were resolved by SDS-PAGE and each of the three D-type cyclins were present in the Flag-TSC2 immunocomplexes (Fig. 4.2a). The cyclin box of cyclin D1, the region that is sufficient for association with CDK4/6, does not bind to TSC2 (Fig. 4.2b). Rather, the amino and carboxyl termini of cyclin D1 retain ability to associate with TSC2, consistent with the formation of a ternary complex between TSC2-cyclin D1-CDK6 observed in the yeast three-hybrid assay.

The TSC1-TSC2 complex acts to negatively regulate cell growth by inhibiting the protein kinase mTOR (Gao et al., 2002; Inoki et al., 2002; Tee et al., 2002). The TSC complex is sensitive to growth conditions—in the presence of growth factors and mitogens TSC2 is multiphosphorylated, rendering TSC1-TSC2 inactive, and in their absence TSC2 is hypophosphorylated, allowing TSC1-TSC2 to actively represses mTOR (Han et al., 2004; Inoki et al., 2002; Li et al., 2003c; Ma et al., 2005; Manning et al., 2002; Potter et al., 2002; Roux et al., 2004; Tee et al., 2002). To determine whether growth conditions might impact the association of cyclin D1 with TSC2, we performed co-immunoprecipitation experiments from serum starved cells with or without serum stimulation (Fig. 4.2c). Consistent with previous observations, we observed more stable detection of TSC2 when its plasmid was co-

expressed with one encoding TSC1 (Benvenuto et al., 2000). Though TSC2 was detectable in the anti-cyclin D1 immunocomplex from serum starved cells, its association increased as the cells were stimulated with 10% serum (Fig. 4.2c, lanes 13-18). Together these data demonstrate an association between cyclin D1 and TSC2 that allows for CDK4/6 binding and is subject to regulation by serum-derived growth signals.

Cyclin D1 overexpression abrogates the growth inhibitory effects of TSC1-TSC2

In the absence of functional TSC1-TSC2, metazoan cells are quantitatively larger and conversely, excess TSC1-TSC2 causes a measurable decrease in overall cell size (Gao and Pan, 2001; Potter et al., 2002; Rosner et al., 2003; Tapon et al., 2001). A physical interaction of cyclin D1-CDK with the TSC1-TSC2 complex implies a role for cyclin D1-CDK in cell growth control, consistent with previous conclusions based on *Drosophila* genetics (Tapon et al., 2001). We therefore evaluated the effect of cyclin D1 expression on TSC1-TSC2 function by using flow cytometry to measure changes in cell size—determined by the forward scatter (FSC) of light of transfected cells. As expected, when TSC1 and TSC2 were ectopically expressed in U2OS cells, a decrease in mean FSC of G1 phase cells was observed (Fig. 4.3a). A similar but less pronounced effect on cell size was evident in G2/M phase cells (data not shown). The addition of cyclin D1 to cells over-expressing TSC1-TSC2 nearly restored the average cell size to control levels (Fig. 4.3a). TSC1 and TSC2 have also been proposed to affect cell cycle progression (Miloloza et al., 2000; Potter et al., 2001; Soucek et al., 1997; Tapon et al., 2001). TSC1-TSC2 over-expression caused only subtle alterations in cell cycle distribution, leading to a slight increase in the G1 population, which again was abrogated when cyclin D1 was co-expressed (Fig. 4.3a).

Mammalian cell growth is regulated by the kinase activity of mTOR directed at either inhibiting or activating, respectively, two primary targets—4E-BP1 and S6K1 (Fingar et al., 2002). The activity of mTOR, in turn, is negatively regulated by the TSC1-TSC2 complex which itself may be controlled in part by cyclin D-CDK4/6 (Fig. 4.3a). Therefore, to determine whether the antagonizing effect of cyclin D1 toward TSC complex is mediated through the mTOR activity, we assayed for mTOR-dependent phosphorylation of 4E-BP1 and S6K1 under conditions of cellular proliferation or quiescence. 293T cells were transfected with plasmids encoding either HA-4E-BP1 alone or with cyclin D and CDK4, and cultured either with 10% serum or in the complete absence of serum for one hour before harvesting. Under normal growth conditions, HA-4E-BP1 can be seen both as a faster migrating non-phosphorylated band and as a more slowly migrating phosphorylated band (Fig. 4.3b lane 2). Upon serum withdrawal, HA-4E-BP1 collapses to the non-phosphorylated form (Fig. 4.3b). However, under the same conditions co-expression of cyclin D1-CDK4 substantially prevented the loss of phosphorylated HA-4E-BP1. Serum induced phosphorylation of 4E-BP1 is dependent on mTOR (Burnett et al., 1998; Gingras et al., 1998), and the repression of mTOR following serum depletion requires the activity of the TSC1-TSC2 complex (Jaeschke et al., 2002; Kwiatkowski et al., 2002). Therefore, these data are consistent with the suggestion that cyclin D1-CDK4 acts to protect the mTOR signaling pathway from TSC1-TSC2 function.

Similar results were obtained when the activity of S6K1 was examined under conditions of serum withdrawal. Plasmid encoding HA-S6K1 was transfected into 293T cells, either independently or in conjunction with cyclin D1-CDK4. Twenty-four hours following

transfection, serum was removed and the cells were collected 2, 4, or 8 hours later. HA-S6K was immunoprecipitated from cell lysates and assayed for its ability to phosphorylate GST-S6 (Fig. 4.3c). Under standard growth conditions, HA-S6K efficiently phosphorylated GST-S6, but as the duration of serum starvation increased, HA-S6K activity diminished (Fig. 4.3c lanes 3, 5, 7). However, when plasmids encoding cyclin D1-CDK4 were co-expressed with HA-S6K, a partial attenuation of the rapid decline in S6K activity was observed at each time point (Fig. 4.3c lanes 4, 6, 8). Furthermore, while overexpression of TSC1 and TSC2 can reduce the activating phosphorylation of S6K1 at threonine 389 by mTOR, the co-expression of cyclin D1 either alone or in combination with CDK6 restores mTOR-dependent phosphorylation of S6K1 (Fig. 4.3d). Thus, high cyclin D-CDK4/6 levels repress the ability of the TSC1-TSC2 complex, when boosted by either serum depletion or its overexpression, to inhibit mTOR signaling.

TSC1 and TSC2 are phosphorylated in a CDK-dependent manner

Two lines of evidence suggest that cyclin D-CDK4/6 may promote the phosphorylation of TSC1 and/or TSC2: (i) physical interaction of the TSC1-TSC2 complex with cyclinD-CDK4/6 (Figs. 4.1 and 4.2), and (ii) a retarded mobility of both TSC1 and TSC2 on SDS-PAGE when cyclin D and CDK4/6 were co-expressed (Fig. 4.3d). To examine the phosphorylation state of TSC2 in the presence of CDKs *in vivo*, Saos-2 cells were transfected with plasmids encoding HA-tagged TSC2-C and cyclin D1, CDK6, or cyclin D1-CDK6 and labeled with ^{32}P-orthophosphate. We observed a marked increase in the phosphorylation state of TSC2-C in the presence of both cyclin D1 and CDK6 (Fig. 4.4a) relative to either subunit alone. This observation was also made *in vitro* using bacterially

expressed TSC2-C and extracts from insect Sf9 cells expressing cyclin D1-CDK6 (data not shown). In order to evaluate the potential kinase activity of other CDKs toward TSC2, cDNAs for TSC2 and myc-TSC1 were co-transfected into U2OS cells with different cyclin and CDK combinations, and total cell lysates were resolved by SDS-PAGE (Fig. 4.4b). As expected, co-expression of cyclin D1 and CDK6 caused a mobility shift of TSC2, while the catalytically inactive $CDK6^{K43M}$ mutant failed to alter the mobility of TSC2 (Fig. 4.4b). Calf intestinal phosphatase (CIP) treatment of extracts confirmed that the mobility shift was due to phosphorylation (Fig. 4.4c). Interestingly, the mobility of TSC1 was also retarded by co-expression with cyclin D1-CDK6, but not by cyclinD1- $CDK6^{K43M}$. The phosphorylation of TSC1 on at least three sites, T417, S584, and T1047, by cyclin B-CDK1 has been described (Astrinidis et al., 2003; Ballif et al., 2005). We found that, in fact, several different cyclin-CDK pairs, including cyclin D-CDK4 (data not shown), cyclinE-CDK2, cyclinA-CDK1, and cyclinA-CDK2 promoted the apparent phosphorylation of both TSC1 and TSC2 upon co-expression (Fig. 4.4b). These results suggest that both TSC1 and TSC2 can be phosphorylated in a CDK-dependent manner, either directly or indirectly, and offer one potential mechanism for the regulation of the TSC1-TSC2 complex by cyclin-CDK partners.

Down-regulation of TSC1-TSC2 by cyclin D

Another notable consequence of the concomitant expression of plasmids encoding cyclin D-CDK6 (WT or K43M) and the TSC1-TSC2 complex is a decrease of both TSC1 and TSC2 protein levels (Figs. 4.3d and 4.4b). Overexpression of cyclin D1 alone is able to cause the down-regulation of co-expressed myc-TSC1 and HA-TSC2, although this effect is dependent on growth conditions (Fig. 4.2b). HA-TSC2 co-expressed with myc-TSC1 and

cyclin D1 is most stable under serum-starved conditions, and becomes increasingly sensitive to cyclin D1-mediated down-regulation upon serum re-stimulation (Fig. 4.2b, lanes 4, 8, 12). Cyclins D2 and D3 elicit a similar effect on decreasing TSC1-TSC2 under the same assay conditions (data not shown). To determine whether expression of cyclin D1 alone might down-regulated TSC protein levels through the activation of endogenous CDK4/6, we utilized cyclin $D1^{K112E}$, which is a cyclin box mutant that fails to bind or activate either CDK4 or CDK6 (Inoue and Sherr, 1998; Zwijsen et al., 1997). As shown in Figure 4.5a, cyclin $D1^{K112E}$ is also capable of down-regulating myc-TSC1 and HA-TSC2, demonstrating the kinase-independence of this activity.

For a further confirmation of a CDK-independent down regulation of ectopic TSC1 and TSC2 by co-expressed cyclin D1, we took advantage of the differential inhibitory modes of the two families of CDK inhibitors, the INK4 vs. the CIP/KIP family. $p16^{INK4a}$, which forms a binary complex with CDK4/6, is unable to protect myc-TSC1 or HA-TSC2 from down-regulation by cyclin D1 expression (Fig. 4.5b). In contrast, $p21^{CIP}$, which forms a ternary complex with cyclin D-CDK4/6, is able to attenuate the down-regulation of myc-TSC1 and HA-TSC2 caused by either cyclin D1 alone or cyclin D1-CDK6 (Fig. 4.5b). A three-fold increase in transfected cyclin D1 expression plasmid causes a correspondingly larger decrease in both myc-TSC1 and TSC2 levels (Fig. 4.5c), indicating that the effect of cyclin D1 on TSC1 and TSC2 protein levels is equivalent. We failed to inhibit cyclin D1-mediated TSC1-TSC2 down-regulation with MG132, chloroquine, and leupeptin (data not shown), ruling out the potential involvement of the proteasome, lysosome, or calpain. Detection of either TSC1 or TSC2 is increased when co-expressed with the second TSC

complex member (Fig. 4.2b), suggesting that proper folding or protein stability requires TSC1-TSC2 complex formation. TSC1 and, to a lesser extent, TSC2, fractionate in an NP-40-insoluble fraction when expressed alone, and are found predominantly in an NP-40-soluble fraction when co-expressed (Fig. 4.5c; (Nellist et al., 2001). Cyclin D1 expression causes a dose-dependent shift of both TSC1 and TSC2 from the NP-40 soluble fraction to the insoluble fraction (Fig. 4.5c). In addition, binding of ectopically expressed TSC2 and cyclin D1 is disrupted by co-expression of TSC1 (Fig. 4.2c, lanes 14, 16, 18), indicating that stable TSC1-TSC2 complex formation is incompatible with cyclin D1 association. Together, these data suggest that cyclin D1 overexpression is able to negatively regulate co-expressed TSC1 and TSC2 via a CDK-independent mechanism, possibly through complex disruption.

Discussion

The "pRb pathway" controls the transition from the G1 to the S phase of the cell cycle and is often denoted as a linear sequence of interactions: INK4 proteins—|cyclin D-dependent kinases—|pRb family proteins (Sherr and McCormick, 2002). The importance of this pathway is underscored by the likelihood that it is deregulated in most, if not all, human tumors. Interestingly, the aberrant activation of CDK4/6, by either loss of INK4 inhibitor genes or amplification and overexpression of cyclin D, CDK4, or CDK6 genes, is observed disproportionately more often than is inactivation of pRb proteins in diverse cancers. This observation suggests that inappropriate cyclin D-CDK4/6 activity confers more growth advantages to cells than mere loss of pRb function, and implies that inactivation of cyclin D-CDK4/6 targets in addition to pRb may contribute to its oncogenic potency (Deshpande et al., 2005). We have shown that cyclin D, either alone or in conjunction with CDK6 can physically interact with the tumor suppressor protein, TSC2, and down-regulate the growth suppressive function of the TSC1-TSC2 heterodimer.

TSC1 and TSC2 were originally identified as two separate loci linked to the congenital disorder, tuberous sclerosis complex (TSC, (Consortium, 1993; van Slegtenhorst et al., 1997)). The *in vivo* function of TSC1-TSC2 had been difficult to pinpoint until studies in *D. melanogaster* identified the TSC genes (*dTSC1* and *dTSC2*) as important regulators of cell growth (Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001). Numerous studies, both in *Drosophila* and mammalian systems, have linked TSC1-TSC2 with several other gene products involved in the insulin→PI3K→AKT→TOR cell growth signaling network (Gao and Pan, 2001; Gao et al., 2002; Inoki et al., 2002; Manning et al., 2002; Potter et al.,

2001; Potter et al., 2002; Rosner et al., 2003; Tee et al., 2002). The TSC1-TSC2 complex is a key negative regulator of the TOR kinase activity, integrating diverse inputs from growth factors, oxygen and nutrient availability, and energy status. The TOR inhibitory activity of TSC1-TSC2 stems from the GAP (GTPase-activating protein) domain of TSC2, which inactivates the GTPase Rheb and prevents it from stimulating the kinase activity of mTOR (Garami et al., 2003; Inoki et al., 2003; Li et al., 2004; Saucedo et al., 2003; Stocker et al., 2003; Tee et al., 2003; Zhang et al., 2003a).

The coupling of cell division with cell growth in multicellular organisms, as opposed to unicellular organisms, intuitively requires more complex regulatory mechanisms (Conlon and Raff, 1999). Whereas unicellular organisms must constantly adapt to changing nutrient and energy availability, cells of a muticellular organism are maintained within an environment of relatively constant and abundant nutrient and energy supply, and rely on extracellular cues, in the form of mitogenic or growth factor signaling, to drive cell division or cell growth, respectively. Cyclins are regarded as potential "cell growth sensors" or "translational sizers" that may transmit growth stimuli to proliferative pathways since they are rate-limiting for cell cycle progression and their expression, or accumulation, is sensitive to the rate of protein synthesis (Fingar and Blenis, 2004; Jorgensen and Tyers, 2004). Cyclin D might be considered a bilateral relay for proliferation signals since its activation of CDK is derived from mitogens and/or growth factors and because it can simultaneously promote cell cycle progression as well as cell growth (Fig. 4.6).

We have demonstrated that the TSC1-TSC2 heterodimer may be a molecular target of cyclin D-CDK's growth control activity. An analogous association has also been observed in *Drosophila* where *dTSC1* and *dTSC2* have been found to interact genetically with *cycD* and *CDK4* (Tapon et al., 2001). While dTSC1-dTSC2 over-expressed in the eye resulted in a pronounced reduction in overall eye size, co-overexpression of cyclin D-CDK4 rescued this defect. Interestingly, cyclin E co-overexpression was also capable of rescuing the small eye phenotype, and reduction in cyclin E or cyclin A levels resulted in even smaller eyes. These studies provide a premise to our findings that in mammalian cells cyclin D-CDK could act upstream of and inhibitory to TSC1-TSC2. Additionally, the studies in *Drosophila* also suggest the potential involvement of other cyclins in negatively regulating TSC1-TSC2. Consistent with this, we have detected interactions between TSC2 and cyclins A, B, and E (data not shown), and interactions between TSC2 and cyclin A, cyclin B, and CDK1 have been reported previously (Astrinidis et al., 2003; Catania et al., 2001).

The co-expression of TSC1-TSC2 with multiple cyclin-CDK wild-type pairs, but not kinase dead ones, results in the phosphorylation of both TSC1 and TSC2 (Fig. 4.4). Though in additional experiments we have failed to unambiguously demonstrate direct phosphorylation of TSC1 or TSC2 by cyclin D1-CDK4/6 (data not shown), we cannot exclude this possibility. Astrinidis et al. described the direct phosphorylation of TSC1 by cyclin B-CDK1 in nocodazole treated cells (Astrinidis et al., 2003). The shared ability of multiple cyclin-CDK pairs to bind to, and possibly phosphorylate, TSC1-TSC2 suggests that throughout all stages of the cell cycle the TSC complex is under the negative regulation of cyclin-CDK complexes, ensuring continuous protein synthesis and growth to meet the needs

of a dividing cell. In fact, early studies have shown that growth of metazoan cells is not confined to a single phase of the cell cycle but rather is continuous throughout (Mitchison, 2003). Consistent with this, cells require a sufficient nutrient supply to pass the restriction point in G1. However, once past the restriction point cells continue to divide even after nutrients have been withdrawn (Zetterberg et al., 1995).

The down-regulation, or decrease in detection, of TSC1 and TSC2 does not require their phosphorylation promoted by the co-expression of cyclin Ds-CDK4/6, nor does it require binding of CDK4/6 by cyclin D1 (Figs. 4.4 and 4.5). Cyclin D, therefore, appears to affect TSC1 and TSC2 through both kinase-dependent and kinase-independent mechanisms. The cyclin D-dependent down-regulation of TSC1-TSC2 coincides with their shift from a soluble to an insoluble fraction of cell lysate, consistent with a reduced ability of the TSC1 and TSC2 monomers to form a dimeric complex (Nellist et al., 2001). Since the effect of cyclin D overexpression on TSC1 and TSC2 steady state levels was most pronounced on ectopically expressed proteins (data not shown), these data suggest that cyclin D might interfere with the folding of nascent TSC1 or TSC2, thereby preventing stable complex formation. The effect, if any, of cyclin D-CDK driven phosphorylation of TSC1 and TSC2 might be masked by the CDK independent ability of cyclin D to down-regulate the TSC1-TSC2 complex.

In summary, we have described the inhibition of the tumor suppressor complex TSC1-TSC2, a key negative regulator of mTOR activity, by cyclin D1, itself a well known proto-oncogene frequently amplified in various types of human cancers. mTOR stimulates

cell growth through ribosome biogenesis and activity, and the importance of its regulation in suppressing unrestrained cell growth and proliferation is manifest in many cancers (Ruggero and Pandolfi, 2003). Further study of the cellular setting(s) and mechanism(s) whereby cyclin D1, with or without its CDK partners, is able to antagonize TSC1-TSC2 activity will be crucial to our understanding of the interplay between cell cycle and cell growth regulation, and may validate the targeting of mTOR in tumors where cyclin D1 is over-expressed.

Materials and Methods

Construction of yeast three-hybrid system. To conduct a yeast three-hybrid screen, two vectors, pGBT6 and pGBT7, were constructed. Both plasmids were derived from pGBT8, a modified form of pGBT9 that has been widely used for two-hybrid screening. pGBT8 was cut with the restriction enzymes XhoI and PstI and the vector backbone was gel purified. This fragment was then ligated to two annealed oligonucleotides, pGBT-3 and pGBT-4 (pGBT-3 sequence is: 5' TCG AGG CCT GAT CAT GGC CAC TAG TGG TAC CGC GGA TCG ATG CA 3'; PGBT-4 sequence is: 5' TCG ATC CGC GGT ACC ACT AGT GGC CAT GAT CAG GCC 3'). A 915 bp AatII restriction fragment was generated from pGBT8 by PCR that contains an ADH promoter followed by a sequence encoding a nuclear localization signal (NLS, the first 73 amino acids of the GAL4-BD), a unique MCS and the ADH termination sequence. These primers were GBT-5 and GBT-6 (GBT-5 sequence is 5' TAG ACG TCG CTT GCA TGC AAC TTC 3'; GBT-6 is 5' ATG ACG TCC GGC ATG CCG GTA GAG GTG 3'). This cassette was inserted into the AatII site of pGBT8, resulting in pGBT7. A similar experimental methodology was used to generate pGBT6 that does not retain the NLS. Both pGBT7 and pGBT6 were confirmed by restriction mapping and partial sequencing.

Yeast three-hybrid assays. The HF7c strain of *S. cerevisiae* was co-transformed with bait and prey plasmids and grown on selective media. Additional growth assays were performed as above in the presence of either 10mM or 30mM 3-amino-triazole. beta-galactosidase assays were performed according to the manufacturer's instructions (Clontech).

Cell Culture, transfections, plasmids, and reagents. Saos-2, U2OS, or HEK293T cells were grown at 37º C with 5%CO_2 in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% fetal bovine serum (Sigma), penicillin, and streptomycin (Invitrogen). Cells were transiently transfected using the calcium phosphate precipitation method, or Lipofectamine (Invitrogen) or FuGene (Roche) according to the manufacturers' instructions. After transfection cells were typically cultured for 24-48 hours prior to treatment or harvest for flow cytometry, immunoprecipitation (IP), or Western analysis. To serum-starve cells, transiently transfected cells were cultured in DMEM without serum overnight (18 hours), followed by re-stimulation with 10% serum over a 4 hour timecourse. The cDNA encoding rat TSC2 was kindly provided by Dr. Ray Yeung, and was subcloned into pcDNA3 in-frame with an HA-epitope tag. Flag-tagged human TSC2 was kindly provided by Dr. Lewis Cantley. Full-length cDNA encoding human TSC1 was PCR amplified from a HeLa cDNA library and subcloned in-frame with a Myc-epitope tag. Constructs encoding HA-p70S6K and HA-4E-BP1 were kindly provided by Dr. Kun-Liang Guan and Dr. John Blenis, respectively. Those encoding cyclins D1, D2, D3, E1,and A2, CDKs 1, 2, 2^{K33M}, 4, 6, 6^{K43M}, and GFP-spectrin are from lab stocks. Site-directed mutagenesis of cyclin D1 was performed by standard PCR techniques using the QuikChange kit (Stratagene). All constructs were verified by partial DNA sequencing.

Flow cytometry. Cells were transiently transfected with cDNAs of interest along with GFP-spectrin, cultured for 48 hours, collected by trypsinizing, washed and resuspended in cold PBS, and fixed by adding EtOH to a final concentration of 75%. Fixed cells were resuspended in PBS/ 0.1 % Triton/ 0.1 mg/mL RNase, and DNA was labeled with propidium

iodide (1 mg/mL) overnight at 4º C. Prepared cells were analyzed using a flow cytometer (FACScan, Becton-Dickinson), gating specifically on GFP-positive, transfected cells, using Summit software (version 3.0, BD Biosciences) for data processing.

Immunochemistry procedures and antibodies. Cells analyzed by Western blot or IP were lysed in NP-40 lysis buffer (50 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.5% Nonidet P-40, 1 mM PMSF, 1 mM dithiothreitol, 50 m M sodium fluoride, 1 m M sodium vanadate, and protease inhibitors: 2 µg/ml aprotinin, 2 µg/ml leupeptin, 10 µg/ml trypsin inhibitor, and 150 µg/ml benzamidine) and cleared by centrifugation. NP-40 insoluble fractions were solubilized using an SDS lysis buffer (50 m M Tris-HCl pH 7.5, 0.5 m M EDTA, 1% SDS, 1 mM PMSF, 1 mM dithiothreitol, 50 m M sodium fluoride, 1 m M sodium vanadate, and protease inhibitors). Extracts treated with CIP (Calf Intestinal alkaline Phosphatase, 25 units; NEB) were prepared by lysing cells in NP-40 lysis buffer lacking sodium vanadate and sodium fluoride. Clarified total cell lysates were quantified using BioRad protein assay kits. Immunoprecipitations were incubated overnight at 4º C, using 0.5-1 mg of total protein lysate, 1-2 ug affinity purified antibody, and Protein A or G agarose beads (Invitrogen). Western blotting was performed with 50-100 µg of protein extract separated by SDS-PAGE and transferred to nitrocellulose membrane (Osmonics, Inc.). Polyclonal antibody to TSC2 was raised in rabbits (Pocono Rabbit Farms, PA) using a C-terminal TSC2 peptide (corresponding to residues 1788-1807) coupled to KLH as an immunogen, and later affinity purified (Pierce Biotechnology). Rabbit polyclonal antibodies to cyclin D1/2, cyclin D3, CDK6, and p16 were similarly generated by our lab. Affinity-purified antibodies to Myc (clone 9E10, NeoMarkers), HA (clone 12CA5, NeoMarkers), Flag (M2, Sigma), cyclins D1, D2, D3

(G124-259, PharMingen), tubulin (NeoMarkers) and phosphorylated Thr^{389} of S6K (#9205, Cell Signaling Technologies) were purchased commercially.

In vivo phosphorylation and kinase assays. Transiently transfected cells were grown for 48 hours, and then depleted of phosphate by culturing in phosphate-free DMEM (Gibco) for 2 hours. 100 μCi ^{32}P-orthophosphate was added to media and cells were incubated for 30 minutes. Cells were rinsed twice with phosphate buffered saline and lysed using NP-40 lysis buffer. Anti-HA immunoprecipitates were purified and separated by SDS-PAGE. The gel was stained with coomassie blue to determine equal loading of protein, then dried and analyzed by autoradiography. For kinase assays 293T cells in 6-well plate were transfected as indicated and serum starved for 2 to 8 hours. One half of anti-HA- immunoprecipitate was used for Western blot analysis and the other half was washed twice in kinase assay buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) and incubated with 0.5 μg GST-S6 and 50 μM ATP, and 2 μCi $^{[32]}$P-γ-dATP and incubated for 15 minutes at 30˚C. Reactions were separated by SDS-PAGE, dried and visualized by autoradiography.

Figure Legends

Figure 4.1. D-type cyclins interact with the C-terminus of TSC2. (A) Yeast three-hybrid assay was performed by cotransforming yeast with *TRP*+ plasmids encoding "bait," listed first and fused to GAL4DB along with *LEU*+ plasmids encoding "prey," listed second and fused to GAL4AD. Transformed yeast were plated on selective media to confirm expression (middle panel) and positive interaction (*HIS*+, right panel). Where two "bait" proteins are indicated, both were expressed from a single *TRP*+ plasmid. (B) Directed yeast two- or three-hybrid assay was performed as in (A).

Figure 4.2. TSC2 co-immunoprecipitates with cyclin D. (A) 293T cells were transfected with plasmids encoding Flag-TSC2 and/or the indicated cyclin D. Whole cell lysate (lanes 1-3) or anti-Flag immunoprecipitates (lanes 4-7) were separated by SDS-PAGE and blotted with antibody against TSC2 (upper panel) or cyclins D1-3 (lower panel). (B) Myc-cyclin D1 was divided into three domains (amino acids 1-42, pRb binding; 42-153, cyclin box; 153-295, C-terminus), expressed in 293T, and immunoprecipitated with anti-Myc antibody to test for association with co-transfected HA-TSC2. Equal expression of HA-TSC2 was confirmed in whole cell lysates (data not shown). (C) 293T cells were transfected with equal amounts of *HA-TSC2* cDNA with or without *Myc-TSC1* and/or *cyclin D1* cDNAs. Following treatment, whole cell lysate (lanes 1-12) or anti-cyclin D1 immunoprecipitates (lanes 13-18) were run on SDS-PAGE and analyzed by Western blot as indicated.

Figure 4.3. Cyclin D-CDK4/6 abrogates the growth inhibitory effects of TSC1-TSC2. (A) U2OS were transfected with *GFP-spectrin* alone (control), or with *GFP-spectrin*, *TSC1* and *TSC2*, with or without *cyclin D1*, and analyzed 48 hours later by flow cytometry to assess cell cycle and cell size (FSC) profiles. Shown here is the cell size distribution of G1 phase cells. Results are representative of three independent experiments. (B) HA-4E-BP1 was transiently expressed in 293T alone, or with cyclin D1 and CDK4. After 24 hours, cells were either left untreated, or were starved of serum for 1 hour prior to harvest. Whole cell lysates were run on SDS-PAGE and blotted with anti-HA antibody. (C) 293T cells were transfected with empty vector or *HA-S6K*, with or without *cyclin D1* and *CDK4*, and cultured for 24 hours under normal growth conditions. Before harvest, cells were either left untreated (0 hour), or were serum-starved for 2, 4, or 8 hours. HA-S6K was immunoprecipitated from each total cell lysate; one quarter was separated by SDS-PAGE and probed with anti-HA antibody (lower panel), and the remainder of each IP was used in a kinase assay with purified GST-S6 and ^{32}P-ATP, separated by SDS-PAGE, and analyzed by autoradiography (upper panel). The band intensities of ^{32}P -GST-S6 were quantified by densitometric analysis, and represented as relative units (lowest panel). (D) U20S were transfected with the indicated plasmids, harvested 48 hours later, and analyzed by SDS-PAGE and Western, blotting with anti-HA, anti-Myc, and anti-phosph-T389-S6K antibodies.

Figure 4.4. CDKs promote the phosphorylation of TSC1 and TSC2. (A) Saos-2 cells were transiently transfected with plasmids encoding HA-TSC2-C, cyclin D1, and/ or CDK4 and metabolically labeled with $^{[32]}$P-orthophosphate. HA-TSC2-C was immunoprecipitated, separated by SDS-PAGE, and analyzed by autoradiography. Equal loading of protein per lane was confirmed by Coomassie blue staining (data not shown). (B) U2OS were

transfected with plasmids encoding myc-TSC1 and TSC2, alone or in conjunction with various cyclin-CDK combinations, and whole cell lysates were separated by SDS-PAGE and analyzed by Western blot. (C) U2OS were transfected with the indicated plasmids and analyzed as in (B), except that each whole cell lysate was either treated with 25 units CIP, or left untreated.

Figure 4.5. Down-regulation of TSC1-TSC2 by cyclin D. (A) Myc-TSC1 and HA-TSC2 were ectopically expressed in U2OS alone, or in conjunction with WT or mutant (K112E) cyclin D1. Total cell lysates were analyzed by SDS-PAGE and Western blot, as indicated. (B) U2OS were transiently transfected with plasmids encoding HA-TSC2 and Myc-TSC1, along with cyclin D1 and/or CDK6, and p16 or HA-p21, as indicated, and analyzed by SDS-PAGE and Western blot, probing with anti-HA, -Myc, -CDK6, -cyclin D1, -p16, and –tubulin antibodies. (C) U2OS were transiently transfected with 100 ng each of *pcDNA3-Myc-TSC1* and *pcDNA3-HA-TSC2* alone, or with 100 ng or 300 ng (3x) *pcDNA3-cyclin D1*. Transfected cells were collected and lysed in NP-40 lysis buffer (soluble fraction), and the remaining pellet (insoluble fraction) was solubilized in an SDS lysis buffer. The resulting samples were run on SDS-PAGE and analyzed by Western blot.

Figure 4.6. Model of cyclin D-CDK4/6 activity in cell division and growth. Early in G1, mitogenic and growth factor signaling induce the synthesis of D-type cyclins, leading to activation of cyclin D-CDK4/6. The INK4 family of CDK inhibitor proteins is induced by as-yet-undefined pathways and specifically inhibits CDK4/6. When activated, cyclin Ds-CDK4/6 and cyclin Es-CDK2 (not shown here) cooperatively phosphorylate pRb family proteins, derepressing E2F to allow transcription of E2F target genes, thereby permitting G1 to S transition. In concert, cyclin D-CDK4/6 may also inactivate the TSC1-TSC2 heterodimer, thereby activating the TOR growth signaling pathway. Thus cyclin D-CDK4/6 may be seen as a bilateral relay for proliferation signals, integrating mitogenic and growth factor signaling to stimulate DNA synthesis as well as protein synthesis, thereby driving both the cell cycle and cell growth. Proto-oncogenic proteins are shaded in gray and tumor suppressor proteins in black.

Figure 4.1. D-type cyclins interact with the C-terminus of TSC2

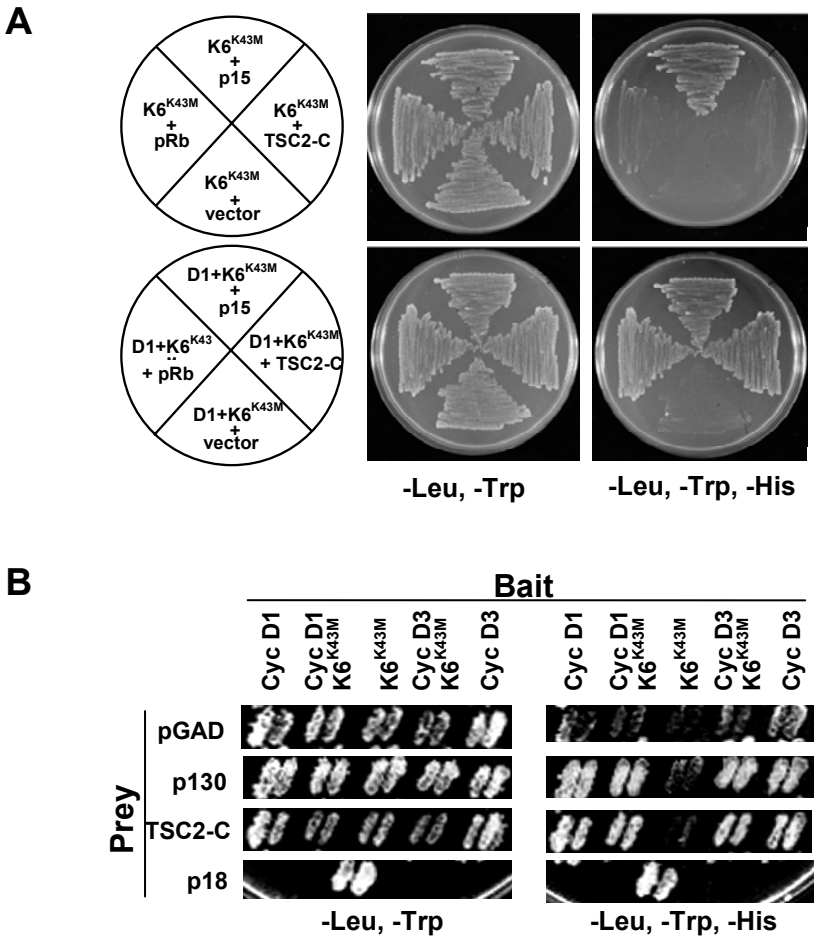


Figure 4.2. Co-immunoprecipitation of TSC2 and cyclin D

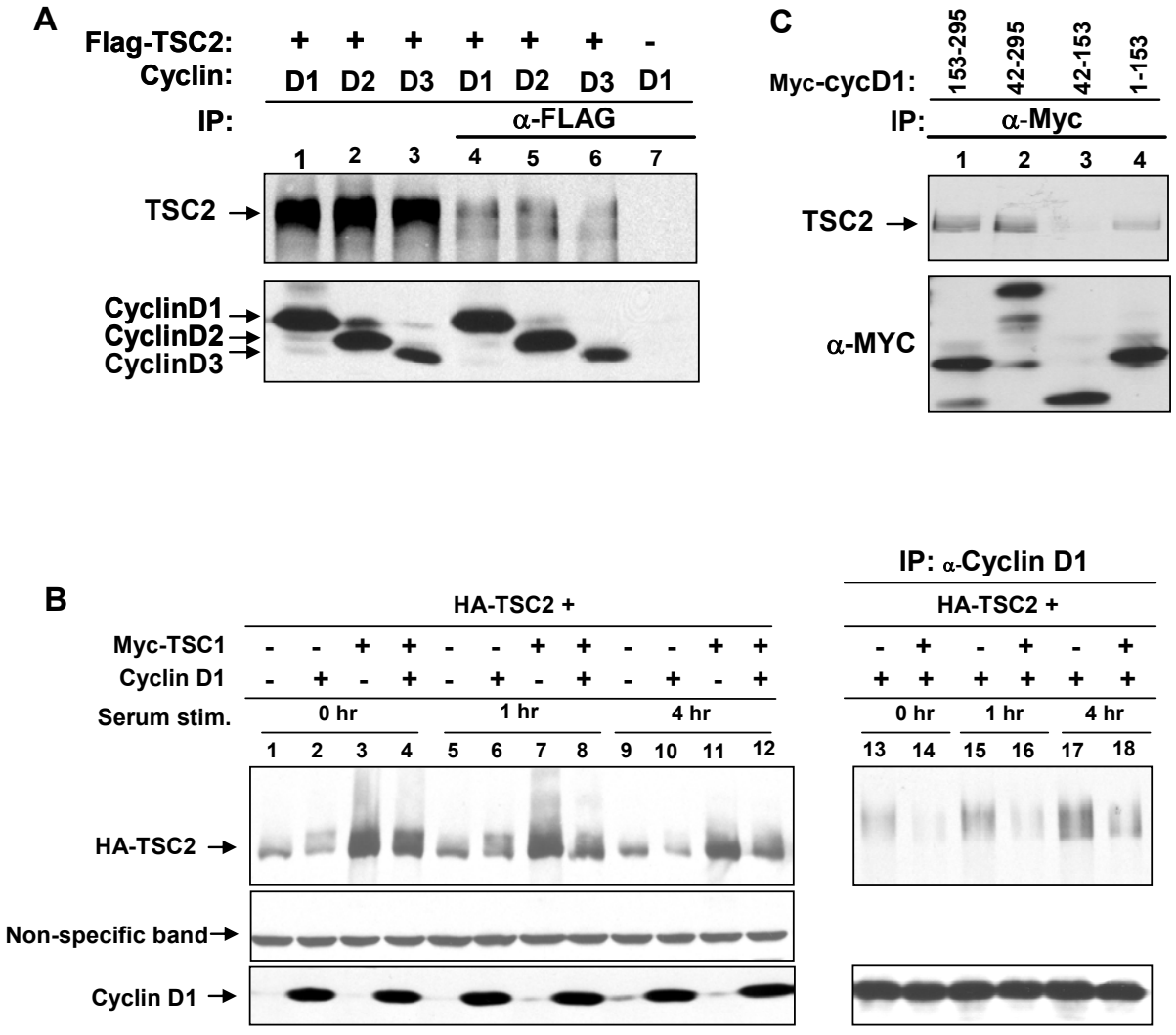
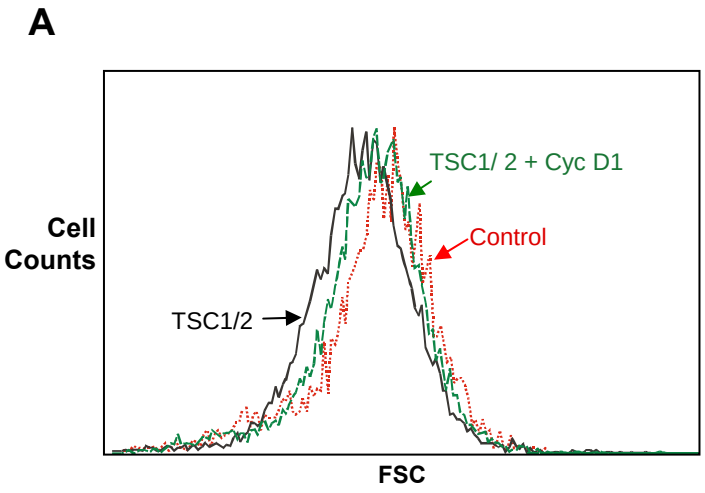
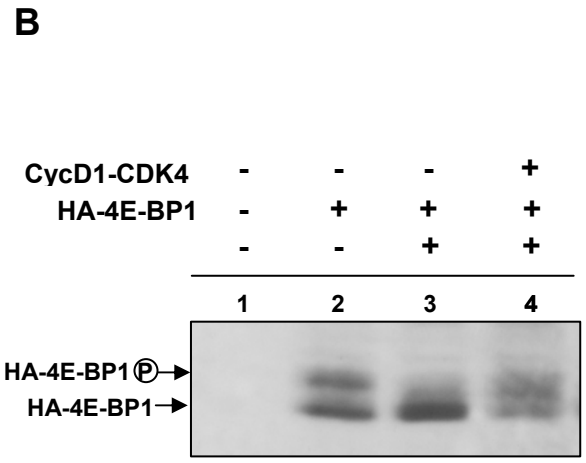


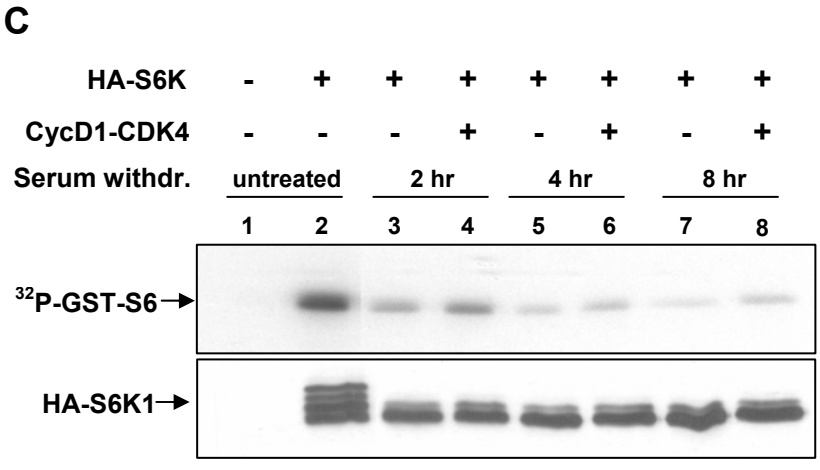
Figure 4.3

Cyclin D-CDK6 abrogates the growth inhibitory effects of TSC1/ TSC2



Transfection	G1	S	G2/M	Mean FSC
Control	39.2	34.1	22.6	95.0
TSC1+TSC2	42.6	30.6	23.8	90.0
TSC1+TSC2+CycD1	37.8	34.3	24.9	93.6





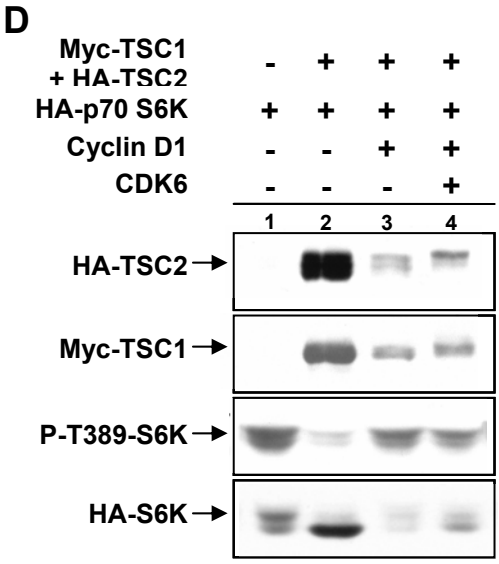


Figure 4.4. CDKs promote the phosphorylation of TSC1 and TSC2

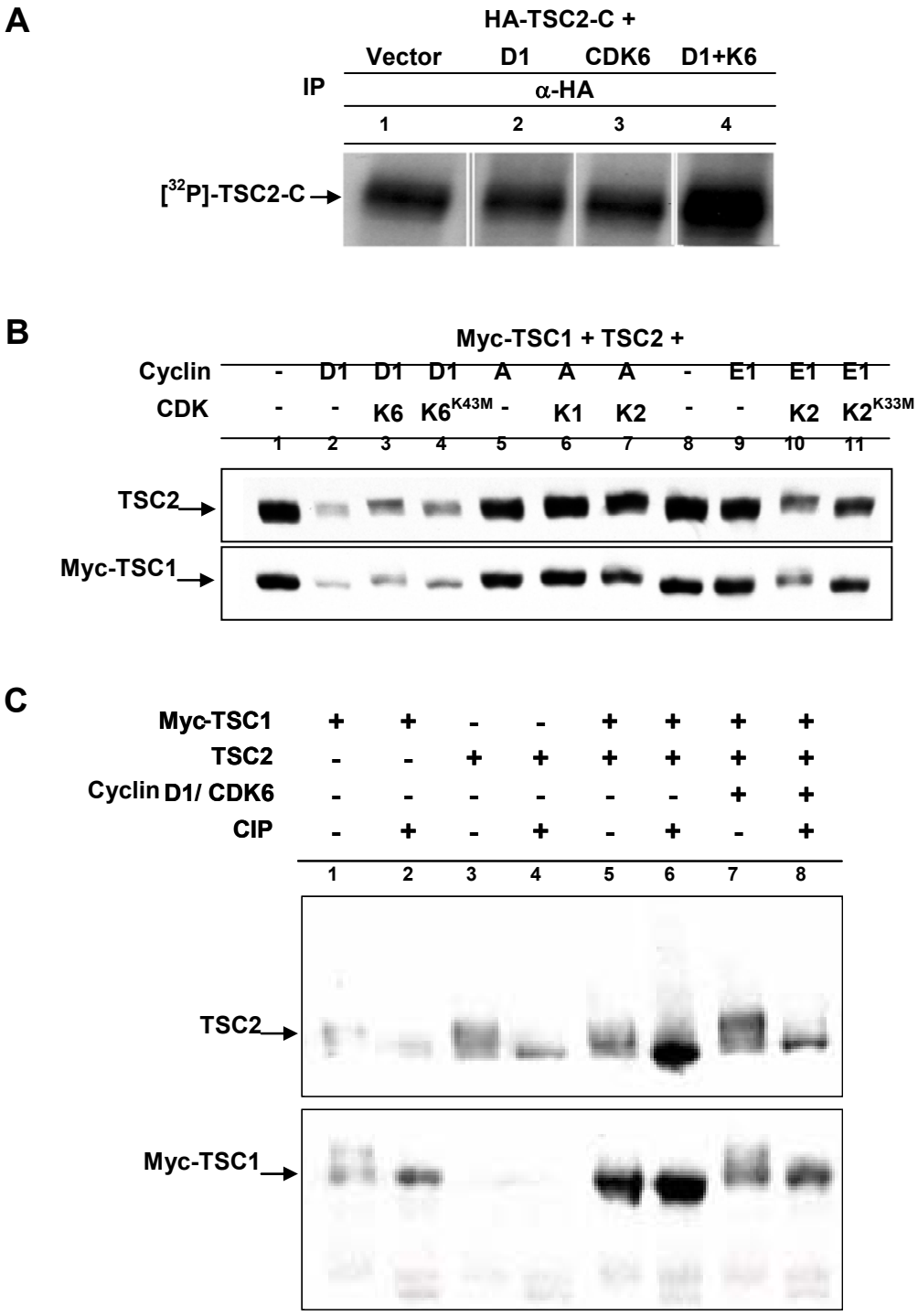


Figure 4.5. Down-regulation of TSC1-TSC2 by cyclin D

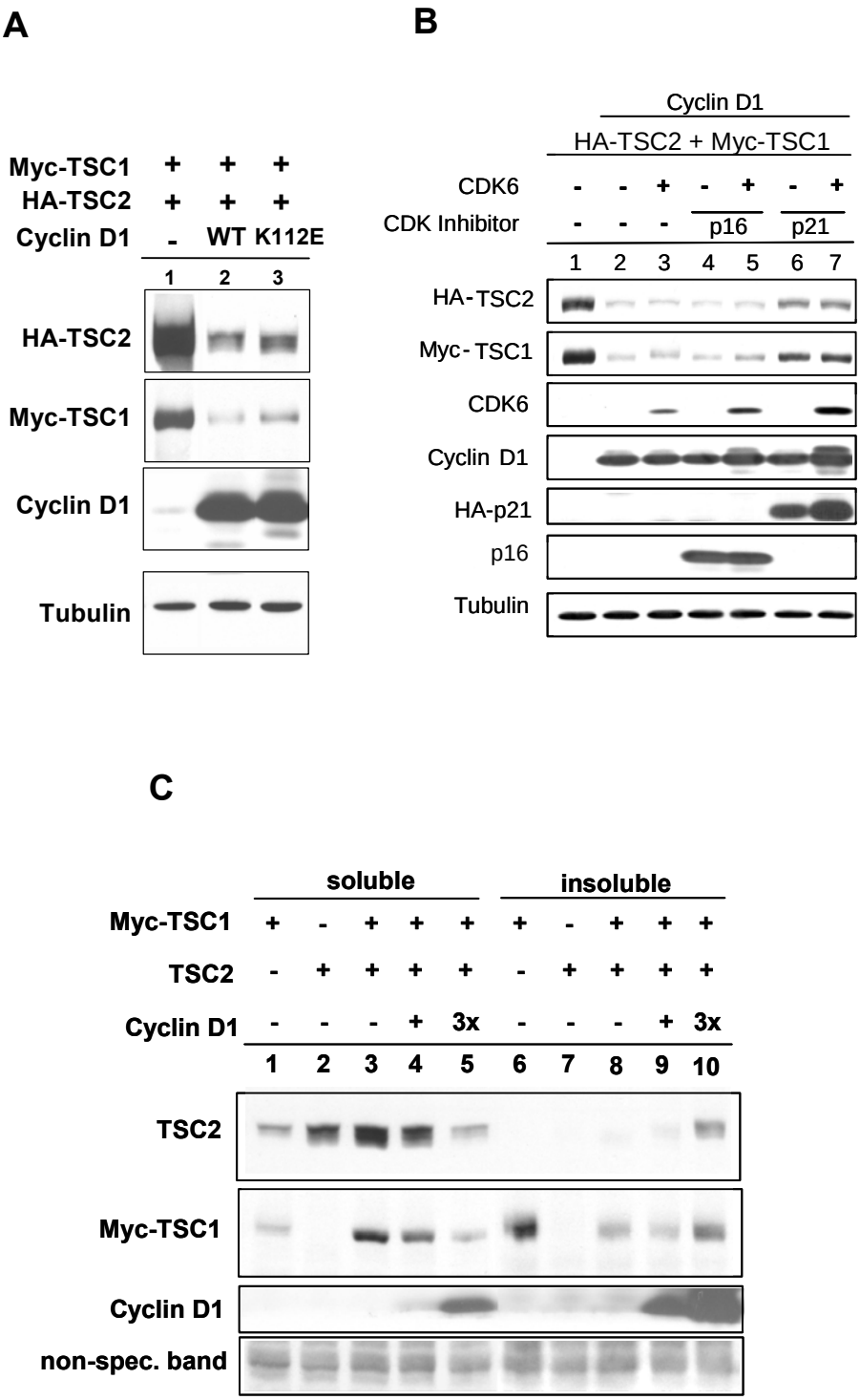
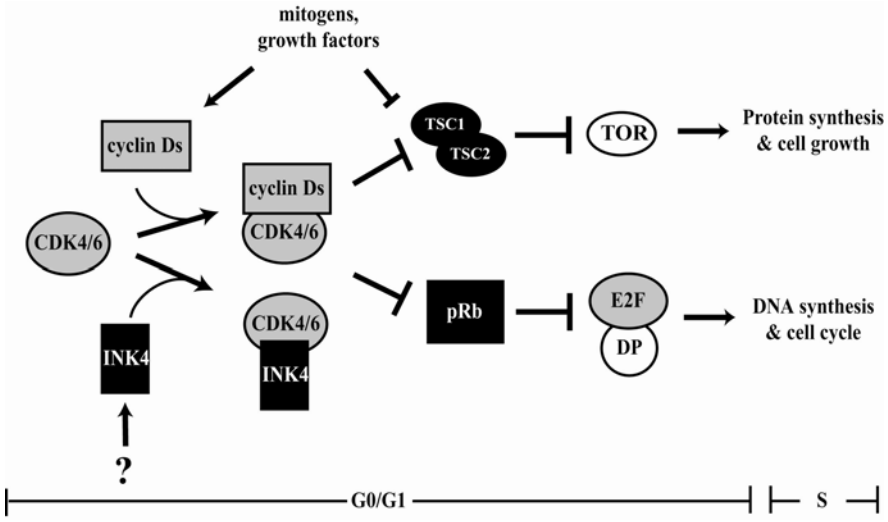


Figure 4.6. Model of cyclin D-CDK4/6 activity in cell division and growth.



CHAPTER V

Evaluating the potential control of TSC1-TSC2 by the cell cycle machinery *in vivo*

TSC1 and TSC2 are important regulators of cell growth, integrating signaling resulting from nutrient and oxygen availability, cellular stress, energy status, and growth factor signaling. Our studies in chapter IV suggest another possible input into the TSC1-TSC2 growth regulatory node that stems from the cell cycle machinery. Evidence from *Drosophila* had also previously suggested that cyclin D and CDK4 could interact genetically with TSC1-TSC2, and that cyclin D-CDK4 have critical functions in cellular growth control, in addition to roles in cell cycle regulation (Datar et al., 2000; Kozar et al., 2004; Meyer et al., 2000; Tapon et al., 2001). We have shown that over-expression of D-type cyclins and CDK4/6 can regulate the ability of ectopically expressed TSC1-TSC2 to control cellular growth of tissue culture cells (chapter IV). Whether this regulatory interaction occurs *in vivo*, however, is still unclear.

CDK-independent down-regulation of TSC1-TSC2 by D-type cyclins

The ability of over-expressed D-type cyclins to down-regulate ectopically expressed TSC1 and TSC2 appears specific, since the effect is dose-dependent, and the stability of several other co-expressed proteins, including HA-ARF and HA-S6K, was unaltered by cyclin D1 over-expression (Fig. 5.1). Conversely, expression of a number of different

proteins other than cyclins, such as GFP, CDK inhibitors, or $p70^{S6K}$, to name a few, had no effect on protein levels of co-expressed TSC1-TSC2 (data not shown).

In order to test whether TSC1-TSC2 protein levels are regulated by cyclin D1 *in vivo*, we evaluated endogenous levels of TSC1 and TSC2 in cell types which endogenous cyclin D1 levels were altered (Figs. 5.2, 5.3, 5.4, 5.5). Early passage cyclin D1 null MEFs (mouse embryo fibroblasts) were lysed, and TSC2 protein levels were assessed (Fig. 5.2). Full-length TSC2 levels in cyclin D1 null MEFs were found to be similar to those observed in wild type MEFs. Lower molecular weight bands of TSC2 of ~85 kDa and ~100 kDa were also detected by C-terminal- or N-terminal-specific anti-TSC2 antibodies, respectively, in WT MEFs but not in cyclin D1 null MEFs. Since the combined sizes of these bands approximately add up to the molecular weight of full-length TSC2, they could potentially represent cleavage products produced in WT cells, but not in cyclin D1 null MEFs. However, when cyclin D1 expression was restored in the cyclin D1 null MEFs by retroviral expression, the lower molecular weight bands were not observed (data not shown); therefore, the identity and function of the 85 kDa and 100 kDa bands are unclear, and the disruption of cyclin D1 in MEFs does not appear to alter TSC2 protein levels.

In the opposite case, in which various cancer cell lines expressing elevated cyclin D1 levels (MCF7, breast; A431, cervix; FaDu, pharynx; and U2OS, bone) were compared to other cell lines expressing cyclin D1 within a normal range (293T, kidney; HeLa, cervix; C33A, cervix, SAOS2, bone), TSC1 and TSC2 levels did not appear to correspond with cyclin D1 levels (Fig. 5.3). Phospho-T389-$p70^{S6K}$ levels, which are dependent on active

mTOR (and therefore expected to be elevated under conditions in which TSC1-TSC2 activity is reduced), were also assessed in this panel of cell lines, but also failed to show any correlation with cyclin D1 levels. The stability of TSC1 and TSC2 is dependent on their heterodimerization, and disruption of their association is destabilizing (Inoki et al., 2003; Inoki et al., 2002); (chapter IV). Therefore, we assessed the binding between TSC1 and TSC2 in several of these cell lines, but did not observe any alterations in their association in the presence of amplified cyclin D1 (Fig. 5.4). However, it is difficult to interpret comparisons between protein levels and interactions derived from different cell types. Comparisons between two cell lines of common origin, such as the osteosarcoma cell lines U2OS and SAOS2, may be more valid; TSC1 (and to a much lesser degree, TSC2) levels do in fact appear to be reduced in U2OS cells, in which cyclin D1 levels are elevated, relative to SAOS2 cells, which express normal levels of cyclin D1. Perhaps a more comprehensive analysis of TSC1-TSC2 levels and activity in tissue type-matched cells with or without cyclin amplification would more clearly establish whether cyclins participate in regulating TSC function *in vivo*.

When the same panel of cell lines described above was subjected to cyclin D1 RNAi, endogenous TSC1 and TSC2 levels were not stabilized (Fig. 5.5). Collectively (Figs. 5.2, 5.3, 5.4, 5.5), these data suggest that cyclin D1 is insufficient to control endogenous TSC1 and TSC2 protein levels. These findings corroborated with additional observations of the cell cycle protein expression profiles of TSC1 and TSC2. It could be expected that if D-type cyclins alone were able to regulate the stability of TSC1 and TSC2, TSC levels would be decreased during the stage in which cyclin D1 levels are up-regulated (G1 phase). However,

TSC1 and TSC2 levels did not fluctuate significantly during the course of the cell cycle in the primary cell line NHF2 (Fig. 5.6).

Potential involvement of other cyclins in the regulation of TSC1-TSC2

Recent reports have suggested that TSC1 and TSC2 may interact with cyclins other than cyclin D (Astrinidis et al., 2003; Catania et al., 2001). Therefore, I tested whether TSC2 could associate with other cyclins. TSC2 and cyclins A1, B1, D1, E1, and N1, with or without CDK1, CDK2, CDK6, or CDK10, were ectopically expressed in 293T cells. IP-Western analysis revealed that each cyclin co-immunoprecipitated with TSC2 (Fig. 5.7). Other cyclins were also found to down-regulate TSC1-TSC2 levels (data not shown). Therefore, the lack of variance in TSC1 and TSC2 protein levels during the cell cycle (Fig. 5.6) could be explained by a potential continuum of cyclins that associate with and modulate the activities of TSC1 and TSC2 during the course of the cell cycle (see chapter IV).

Potential phosphorylation of TSC1 and TSC2 by multiple cyclin-CDKs

Significant shifts in mobility have also been observed when several different CDKs were co-expressed with their cyclin partners and TSC1-TSC2; cyclin E1-CDK2, for example, clearly promoted the phosphorylation of TSC2 (lane 9, Fig. 5.7). Phosphorylation of both TSC1 and TSC2 has in fact been observed to be induced upon co-expression with CDK1, CDK2, CDK4, and CDK6 (chapter IV; data not shown). These experiments have typically been performed using asynchronous populations of cells, which appears to affect the occurrence of CDK-induced phosphorylation of TSC1 and TSC2 (Fig. 5.7; data not shown). Most frequently, however, TSC1 seems to be more highly phosphorylated than TSC2 when

co-expressed with cyclins-CDKs, as in Figure 5.8 (lane 5), in which TSC1 is prominently phosphorylated when co-expressed with cyclin D and CDK6, but not with the catalytically inactive mutant CDK6^{K43M}, or in the presence of p16 (lanes 6,7).

Although kinase assays designed to test the direct phosphorylation of TSC2 by CDK4/6 have been inconclusive (data not shown), we have not yet analyzed the direct phosphorylation of TSC2 or TSC1 by the other CDKs. TSC1 in fact is a heavily phosphorylated protein (data not shown); (Sarbassov dos et al., 2005), which contains 3 putative CDK consensus sites, while TSC2 contains only one (Ballif et al., 2005); (Scansite). While TSC2 has been shown to be phosphorylated by numerous kinases, including AKT, AMPK, ERK, p90^{RSK}, and MK2, the study of the kinases involved in phosphorylating TSC1 has been lagging and are largely unknown. Therefore, a more comprehensive analysis and mapping of direct phosphorylation of TSC2 and especially TSC1 by the CDKs is warranted. Given the redundancy of function of the CDKs (Pagano and Jackson, 2004), it will be challenging to decipher which CDKs act to phosphorylate TSC1 or TSC2 under normal physiological conditions, and in which contexts, but such insight will undoubtedly aid in our understanding of crosstalk between the cell cycle and cell growth machineries.

Analyzing the consequence of the cyclin/CDK—TSC1/TSC2 interaction in vivo

Aside from assessing the phosphorylation status of TSC1-TSC2 as a readout of regulation by cyclins-CDKs, the recent finding that TSC2 acts as a GAP (GTPase activating protein) specifically toward the small GTPase Rheb (Kwiatkowski and Manning, 2005) has provided a direct functional assay by which to assess TSC1-TSC2 function. Preliminary

studies designed to test the impact of cyclin D-CDK4 on endogenous Rheb activity (Fig. 5.9) support our conjecture that cyclin D-CDK4 can influence the function of TSC1-TSC2 in regulating cell growth effectors. Rheb has been detected *in vivo* to have a highly active basal state that is predominantly GTP-bound (Li et al., 2004). HA-Rheb ectopically expressed in 293T cells, purified, and analyzed by thin layer chromatography was primarily GTP-bound, as expected (Fig. 5.9, lane 1). Upon serum starvation, TSC1-TSC2 is activated, thereby increasing levels of GDP-bound Rheb (Fig. 5.9, lane 2). Under serum-starved conditions, the co-expression of cyclin D-CDK4 (but not the kinase dead complex of cyclin D-CDK4^{R31C}, lane 4), with HA-Rheb shifted its activity back to a more highly GTP-bound state (Fig. 5.9, lane 3). By assaying Rheb function, it is now possible to directly analyze the function of TSC1-TSC2. Extending these studies to assess endogenous Rheb activity *in vivo*, in different contexts in which cyclin D or CDK4/6 (or other cyclins-CDKs) are aberrantly over-expressed or activated, or in which CDK inhibitors are inactivated, will broaden our understanding of the impact of dysregulated cell cycle machinery on cell growth control during tumorigenesis.

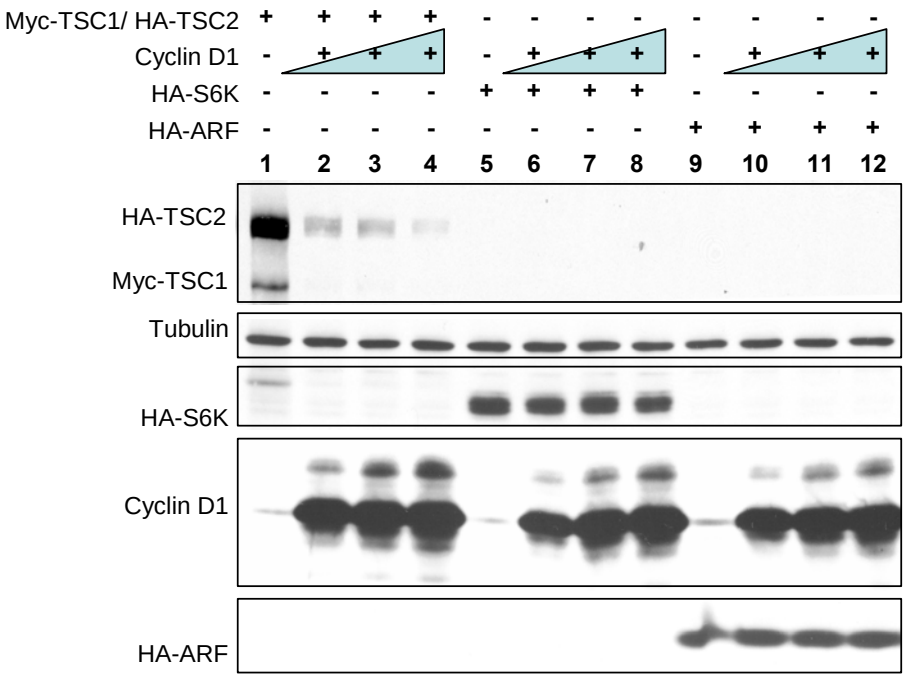


Figure 5.1 Down-regulation of TSC1/TSC2 by cyclin D is dose-dependent and appears specific. U2OS cells were transfected with plasmids myc-TSC1/ HA-TSC2, HA-S6K, or HA-ARF, plus increasing amounts of cyclin D, to assess the specificity of regulation of TSC1/TSC2 by cyclin D1. Western blotting revealed a graded reduction in TSC1/TSC2 levels by increasing levels of cyclin D1 expression, while levels of HA-S6K and HA-ARF were unaffected by cyclin D1 overexpression.

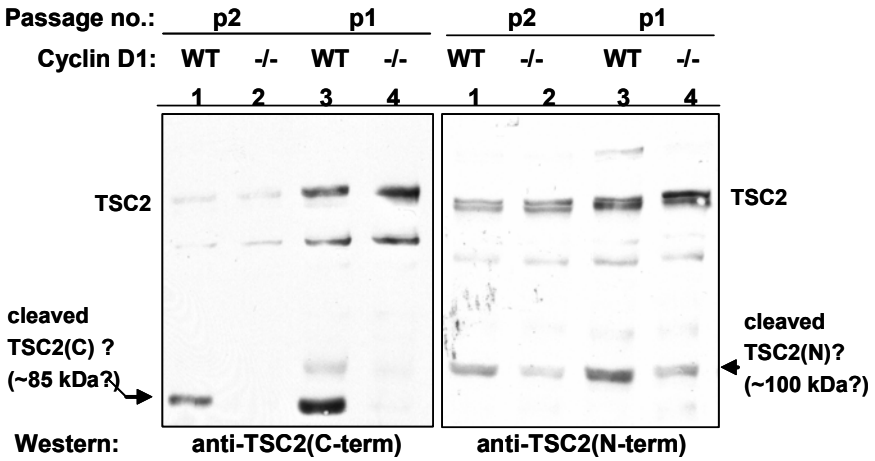


Figure 5.2. TSC2 protein levels are not elevated in cyclin D1 null MEFs. Lysates from cyclin D1 null (-/-) and wild type MEFs (mouse embryo fibroblasts) were analyzed by Western, probing for TSC2 using anti-TSC2 antibodies specific for the C-terminus or N-terminus of TSC2. Levels of full length TSC2 were unaltered in cyclin D1 null cells. Lower molecular weight bands of ~85 kDa and 100 kDa were detected by the anti-TSC2 (C-term) and anti TSC2 (N-term) antibodies, as indicated, in wild type but not in cyclin D1 null MEFs.

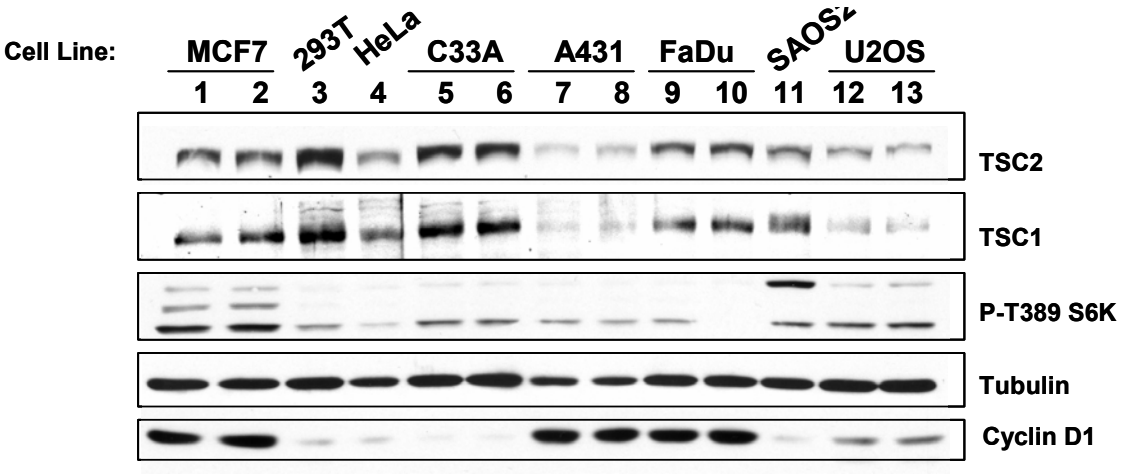


Figure 5.3. Cell lines over-expressing Cyclin D do not show decreased endogenous protein levels of TSC1/ TSC2. Cell lines expressing wild type levels (293T, HeLa, C33A, SAOS2) and amplified levels (MCF7, A431, FaDu, and U2OS) of cyclin D1 were analyzed by Western blot, probing for the indicated proteins; P-T389 S6K is a readout of mTOR function. Several cell lysates were loaded in duplicate.

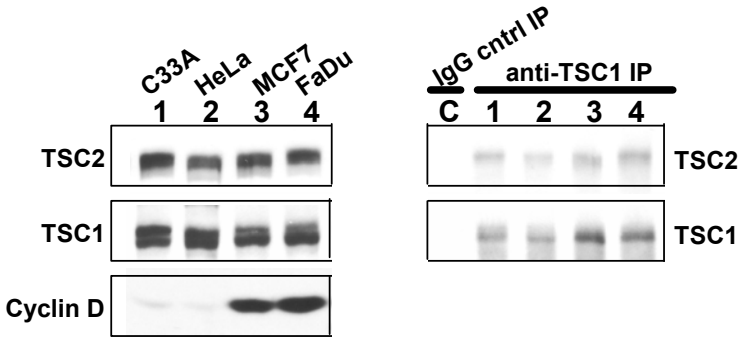


Figure 5.4. Elevated levels of Cyclin D1 do not affect the interaction between TSC1 and TSC2. Binding between TSC1 and TSC2 in cell lines with wild type (C33A, HeLa) versus amplifed (MCF7, FaDu) levels of cyclin D1 was assayed by immunoprecipitation using anti-TSC1 antibody, resolved by SDS-PAGE, and analyzed by Western blot.

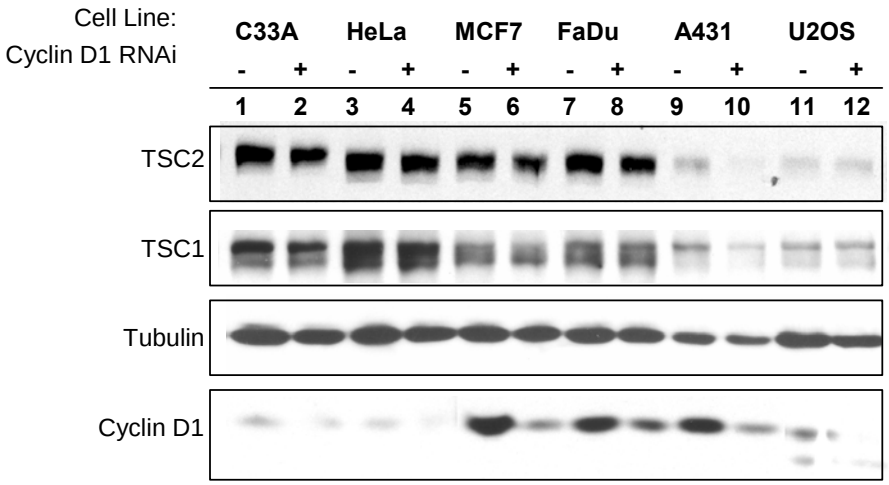


Figure 5.5. RNAi of Cyclin D1 in various cell lines does not increase steady state levels of TSC1/ TSC2. Cyclin D1 was silenced using synthetic siRNAs, and TSC1/TSC2 levels were assessed by Western blot.

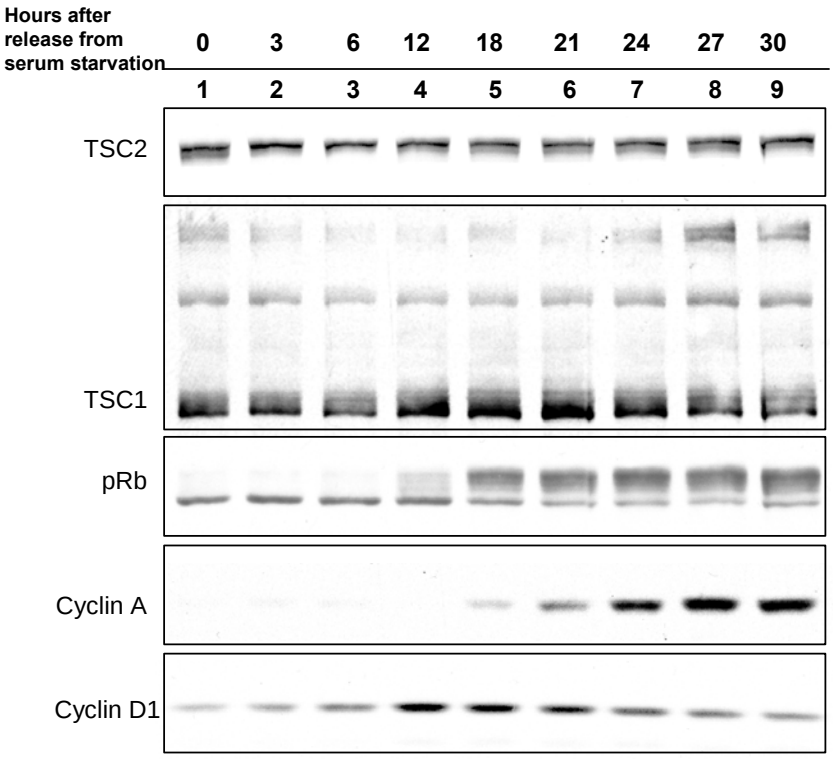


Figure 5.6. TSC1 and TSC2 protein levels do not significantly fluctuate over the course of the cell cycle in NHF2 cells. Following serum starvation of NHF2 cells to allow synchronization in G0, the cells were restimulation with serum, and collected over a timecourse of 30 hours. pRb and cyclins A and D1 were immunostained to confirm effective synchronization.

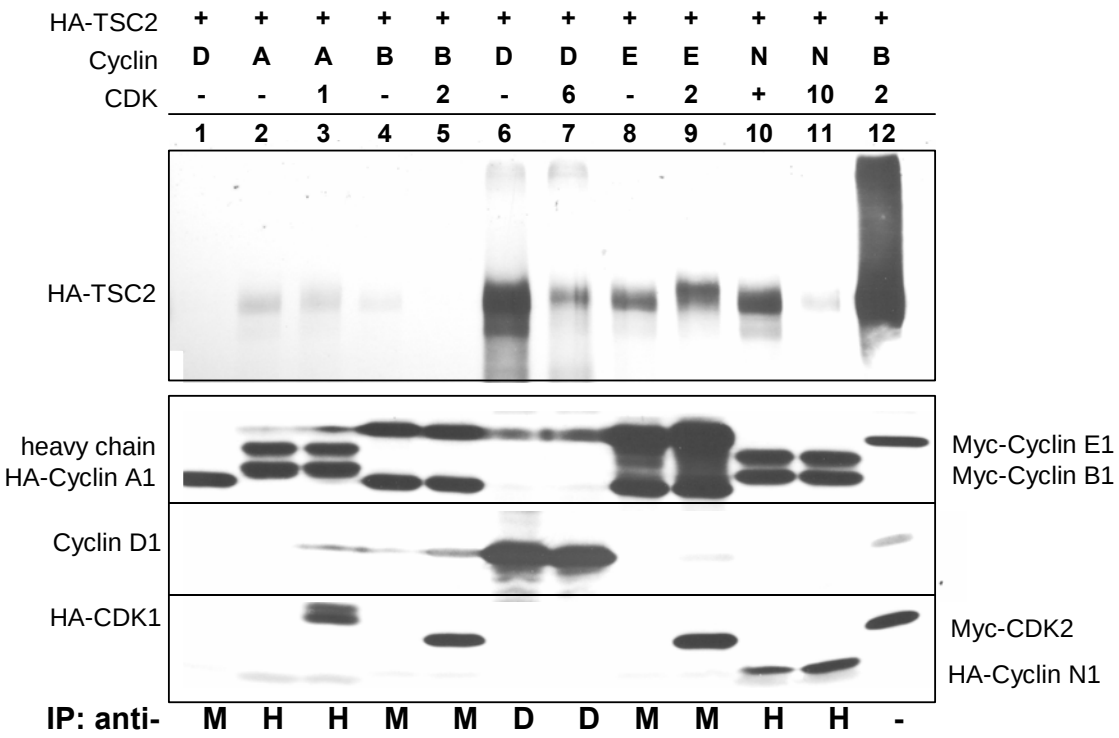


Figure 5.7. TSC2 not only co-immunoprecipitates with cyclin D, but also with cyclin A, cyclin B, cyclin E, and cyclin N. 293T cells were transfected with plasmids encoding HA-TSC2, along with HA-cyclin A1, Myc-cyclin B1, cyclin D1, Myc cyclin E1, or HA-cyclin N, with or without its CDK binding partner (HA-CDK1, Myc-CDK2, CDK6, or CDK10). These ectopically expressed cyclins were immunoprecipitated using anti-myc (M), anti-HA (H), or anti-cyclin D1 antibodies, and analyzed by Western blot.

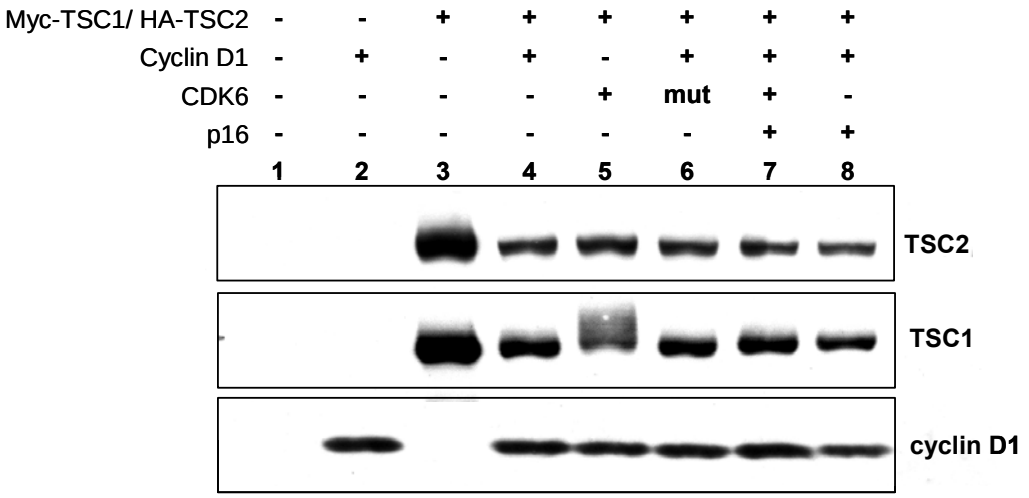


Figure 5.8. TSC1 is highly phosphorylated in response to cyclin D-CDK6 co-expression. U2OS cells were transfected with the indicated cDNAs (mut CDK6 is a catalytically inactive mutant), treated with the proteasome inhibitor MG132 for 4 hours, lysed, and evaluated by Western.

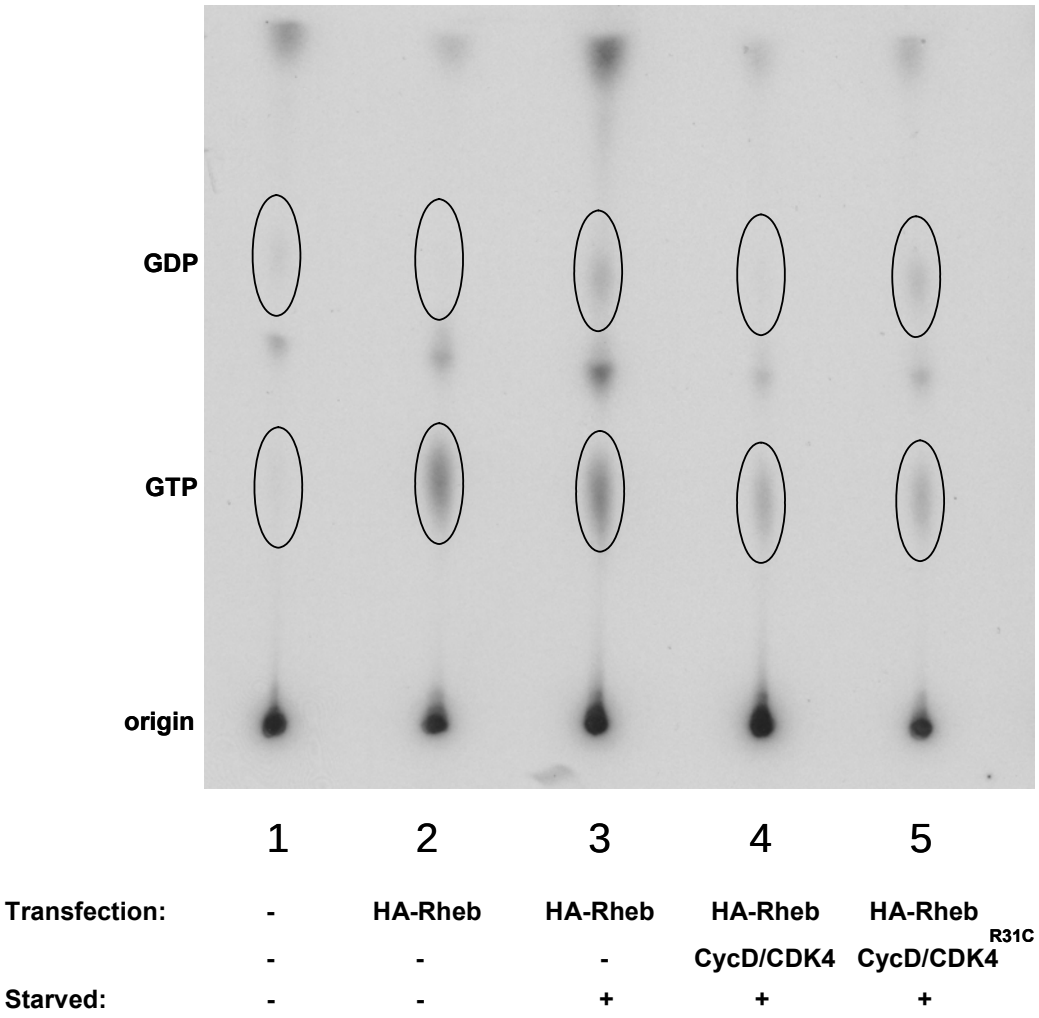


Figure 5.9. Cyclin D-CDK4 positively regulates Rheb GTPase activity. 293T cells were transfected with plasmids encoding HA-Rheb alone, or with cyclin D1 and CDK4 (wild type or catalytically inactive mutant, $CDK4^{R31C}$). 48 hours post-transfection, the culture media was replaced with phosphate-free media, with or without serum, for 1 hour; metabolically labeled with 32P-orthophosphate for 4 hours, and lysed. HA-Rheb immunoprecipitates were purified, bound GDP/ GTP was eluted, and resolved by thin layer chromatography.

CHAPTER VI

Conclusions

Implications of the dysregulation of cell growth and ubiquitination in cancer

The dysregulation of cell growth is an often over-looked but important contributing factor in tumorigenesis, given the frequent targeting of the PI3K-AKT-TOR pathway in cancer. Though cell growth is separable from the cell cycle, these processes are closely tied. Our studies of the interaction between cyclin-CDKs and TSC1/TSC2 demonstrate a potential link between the cell cycle and cell growth signaling networks (chapters IV, V). Therefore, disruption of the CDK inhibitor—cyclin—CDK cell cycle machinery in neoplastic progression can have implications that extend beyond pRb to include the cell growth signaling pathway, and points to the targeting of cell growth pathways, i.e. by the mTOR inhihitor rapamycin, in such cancer settings.

Our studies of the $CUL4^{DDB1}$ E3 ligase in *Drosophila* have also unexpectedly indicated its potential involvement in regulating cell growth (chapters II, III). Though the relevant substrates targeted by the $CUL4^{DDB1}$ E3 ligase are as yet unknown, they likely represent negative regulators of growth, and can be easily tested using our existing *piccolo DDB1* fly model system. *CUL4* is a gene of pleiotropic function whose gene product unquestionably assembles a wide array of E3 ligase complexes, to target an even larger array of substrates for ubiquitination. The analysis of *CUL4* and *DDB1* mutants in *Drosophila*

have reinforced this idea, and established the $CUL4^{DDB1}$ ligase as an important regulator of proliferation and development. The $CUL4^{DDB1}$ ligase plays a role in replication licensing by regulating CDT1 levels during the cell cycle, and appears to be required for proper hemocyte differentiation and development (chapter II). Our genetic analysis has also indicated that CUL4 carries additional functions that are independent of DDB1, which has also been suggested by studies in *S. pombe* (Horn et al., 2005; Jia et al., 2005; Li et al., 2005; Thon et al., 2005). Thus CUL4 has been linked to other functions such as regulating heterochromatin formation, and given its likely interaction with SAP130 and CPSF160, may also be involved in regulating mRNA processing events. Finally, CUL4 plays an important role in response to genotoxic stress by regulating XPC, histone H2A, Chk1, and CDT1, and allowing repair of damaged DNA to proceed. Therefore, the dysregulated amplification or over-expression of CUL4 in cancer would have broad implications, affecting the ability of the cell to repair damaged DNA, control cellular growth and proliferation, or properly differentiate. The identification of additional substrates that are targeted by CUL4-dependent E3 ligases will contribute to our understanding of the oncogenic function of CUL4A in settings such as breast cancer.

A better understanding of cyclin D-CDK4/6 and CUL4-dependent E3 ligases and their downstream effectors will add to the growing knowledge of the pathways altered in tumorigenesis. It is becoming clearer that there is a recurring collection of cellular hubs that are disrupted in the process of driving aberrant proliferation. The challenge then will be to understand how these hubs are connected within a larger signaling network, and to envision how together they create the tumor cell phenotype, so that we can develop therapeutic

strategies to eliminate it (O'Shea, 2005). Efforts are also currently underway to quantitatively model normal versus neoplastic cell signaling networks, taking into account *in vivo* data from the past several decades and the from the advent of high throughput expression and interactome data. These novel systems-level approaches seem to be propelling us into a new computational era, leading to better-defined signaling networks and improving our predictive capabilities on rational drug targeting in cancer.

Looking ahead

Such efforts will hopefully aid in our gaining a better appreciation of the unique vulnerabilities of cancer cells. Cancer cells have in fact been characterized as being highly dependent, or 'addicted' to the oncogenes or alterations in tumor suppressor genes that acted to drive their growth. Addiction to oncogenes and hypersensitivity to tumor suppressor genes indicates that tumor progression is not simply an additive process of the responsible genetic alterations, but rather a complex process dependent on interactions due to the cellular and microenvironmental context (Weinstein, 2002). Such addictions reveal points of susceptibility in cancer treatment. For example, cancers in which the p53 pathway has been inactivated are particularly sensitive to the reintroduction of wild type p53, or inhibition of Bcl2 (Bykov et al., 2003). Similarly, cancer cells in which the apoptotic program had been inactivated due to the over-activation of the PI3K/AKT pathway (but not by other parallel or downstream pathways) respond favorably to the mTOR inhibitor rapamycin, by re-activating the apoptotic program (Wendel et al., 2004; Wendel and Lowe, 2004). Such observations reinforce the rationale for recent initiatives to map out the aberrations common to the different tumor types, and the eventual goal of mapping of an individual cancer patient's

neoplastic alterations, so as to allow tailoring of cancer therapy to individual neoplastic disease (Lowe et al., 2004).

A large scale endeavor to map the Cancer Genome Atlas is currently underway, whose organizers aspire to scan the genomes of tumors and catalog the genetic alterations associated with cancer. The p53 mutation databases have already helped enumerate key alterations in the oncogenic process, and serve as a paradigm for future analyses of other important tumor suppressor genes, caretaker genes, and oncogenes (Soussi, 2005; Soussi et al., 2006).

Currently, we are now aware of some of the genes dysregulated in tumorigenesis, but we are still unaware of the identity of the cell population(s) susceptible to tumorigenesis for the majority of human cancers (Polyak and Hahn, 2006). The cell population(s) affected in tumorigenesis generally have attributes typically associated with stem cells: they are multipotent cells that self-renew limitlessly, and they also have the capacity to differentiate. Many signaling pathways implicated in the maintenance of normal stem cells are in fact found to be mutated in human cancers. However, the true nature of the cancer stem cell is still unclear; whether the cancer stem cell only phenotypically resemble a stem cell or whether the cancer stem cell represents an altered early progenitor cell, is currently being investigated.

With a better understanding of the cellular origins and signaling events driving tumor progression in patients, along with the development of methods to streamline the detection an

individual's unique polymorphisms (Gresham et al., 2006) so that the contribution of our genetic backgrounds can be taken into account in managing disease, we seem to be entering a new era of highly individualized, targeted treatments. The National Cancer Institute recently revealed an initiative to reduce the death and suffering due to cancer within the next ten years; it seems that their goal could just be attainable.

REFERENCES

Alvarez-Garcia, I., and Miska, E. A. (2005). MicroRNA functions in animal development and human disease. Development *132*, 4653-4662.

Andrejeva, J., Young, D. F., Goodbourn, S., and Randall, R. E. (2002). Degradation of STAT1 and STAT2 by the V proteins of simian virus 5 and human parainfluenza virus type 2, respectively: consequences for virus replication in the presence of alpha/beta and gamma interferons. J Virol *76*, 2159-2167.

Ardley, H. C., and Robinson, P. A. (2005). E3 ubiquitin ligases. Essays Biochem *41*, 15-30.

Arias, E. E., and Walter, J. C. (2005). Replication-dependent destruction of Cdt1 limits DNA replication to a single round per cell cycle in Xenopus egg extracts. Genes Dev *19*, 114-126.

Arias, E. E., and Walter, J. C. (2006). PCNA functions as a molecular platform to trigger Cdt1 destruction and prevent re-replication. Nat Cell Biol *8*, 84-90.

Astrinidis, A., and Henske, E. P. (2005). Tuberous sclerosis complex: linking growth and energy signaling pathways with human disease. Oncogene *24*, 7475-7481.

Astrinidis, A., Senapedis, W., Coleman, T. R., and Henske, E. P. (2003). Cell cycle-regulated phosphorylation of hamartin, the product of the tuberous sclerosis complex 1 gene, by cyclin-dependent kinase 1/cyclin B. J Biol Chem *278*, 51372-51379.

Ballif, B. A., Roux, P. P., Gerber, S. A., MacKeigan, J. P., Blenis, J., and Gygi, S. P. (2005). Quantitative phosphorylation profiling of the ERK/p90 ribosomal S6 kinase-signaling cassette and its targets, the tuberous sclerosis tumor suppressors. Proc Natl Acad Sci U S A *102*, 667-672.

Baylin, S. B., and Ohm, J. E. (2006). Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? Nat Rev Cancer *6*, 107-116.

Bech-Otschir, D., Kraft, R., Huang, X., Henklein, P., Kapelari, B., Pollmann, C., and Dubiel, W. (2001). COP9 signalosome-specific phosphorylation targets p53 to degradation by the ubiquitin system. Embo J *20*, 1630-1639.

Bech-Otschir, D., Seeger, M., and Dubiel, W. (2002). The COP9 signalosome: at the interface between signal transduction and ubiquitin-dependent proteolysis. J Cell Sci *115*, 467-473.

Bennasroune, A., Gardin, A., Aunis, D., Cremel, G., and Hubert, P. (2004). Tyrosine kinase receptors as attractive targets of cancer therapy. Crit Rev Oncol Hematol *50*, 23-38.

Benvenuto, G., Li, S., Brown, S. J., Braverman, R., Vass, W. C., Cheadle, J. P., Halley, D. J., Sampson, J. R., Wienecke, R., and DeClue, J. E. (2000). The tuberous sclerosis-1 (TSC1)

gene product hamartin suppresses cell growth and augments the expression of the TSC2 product tuberin by inhibiting its ubiquitination. Oncogene *19*, 6306-6316.

Bianco, R., Melisi, D., Ciardiello, F., and Tortora, G. (2006). Key cancer cell signal transduction pathways as therapeutic targets. Eur J Cancer *42*, 290-294.

Bissell, M. J., and Radisky, D. (2001). Putting tumours in context. Nat Rev Cancer *1*, 46-54.

Bjorklund, M., Taipale, M., Varjosalo, M., Saharinen, J., Lahdenpera, J., and Taipale, J. (2006). Identification of pathways regulating cell size and cell-cycle progression by RNAi. Nature *439*, 1009-1013.

Blasco, M. A. (2005). Telomeres and human disease: ageing, cancer and beyond. Nat Rev Genet *6*, 611-622.

Blow, J. J., and Dutta, A. (2005). Preventing re-replication of chromosomal DNA. Nat Rev Mol Cell Biol *6*, 476-486.

Blume-Jensen, P., and Hunter, T. (2001). Oncogenic kinase signalling. Nature *411*, 355-365.

Bondar, T., Mirkin, E. V., Ucker, D. S., Walden, W. E., Mirkin, S. M., and Raychaudhuri, P. (2003). Schizosaccharomyces pombe Ddb1 is functionally linked to the replication checkpoint pathway. J Biol Chem *278*, 37006-37014.

Bondar, T., Ponomarev, A., and Raychaudhuri, P. (2004). Ddb1 is required for the proteolysis of the Schizosaccharomyces pombe replication inhibitor Spd1 during S phase and after DNA damage. J Biol Chem *279*, 9937-9943.

Bregman, D. B., Halaban, R., van Gool, A. J., Henning, K. A., Friedberg, E. C., and Warren, S. L. (1996). UV-induced ubiquitination of RNA polymerase II: a novel modification deficient in Cockayne syndrome cells. Proc Natl Acad Sci U S A *93*, 11586-11590.

Brooks, C. L., and Gu, W. (2006). p53 ubiquitination: Mdm2 and beyond. Mol Cell *21*, 307-315.

Burger, A. M., and Seth, A. K. (2004). The ubiquitin-mediated protein degradation pathway in cancer: therapeutic implications. Eur J Cancer *40*, 2217-2229.

Burnett, P. E., Barrow, R. K., Cohen, N. A., Snyder, S. H., and Sabatini, D. M. (1998). RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. Proc Natl Acad Sci U S A *95*, 1432-1437.

Bykov, V. J., Selivanova, G., and Wiman, K. G. (2003). Small molecules that reactivate mutant p53. Eur J Cancer *39*, 1828-1834.

Carmeliet, P. (2005). VEGF as a key mediator of angiogenesis in cancer. Oncology *69 Suppl 3*, 4-10.

Catania, M. G., Mischel, P. S., and Vinters, H. V. (2001). Hamartin and tuberin interaction with the G2/M cyclin-dependent kinase CDK1 and its regulatory cyclins A and B. J Neuropathol Exp Neurol *60*, 711-723.

Chamovitz, D. A., Wei, N., Osterlund, M. T., von Arnim, A. G., Staub, J. M., Matsui, M., and Deng, X. W. (1996). The COP9 complex, a novel multisubunit nuclear regulator involved in light control of a plant developmental switch. Cell *86*, 115-121.

Chen, L. C., Manjeshwar, S., Lu, Y., Moore, D., Ljung, B. M., Kuo, W. L., Dairkee, S. H., Wernick, M., Collins, C., and Smith, H. S. (1998). The human homologue for the Caenorhabditis elegans cul-4 gene is amplified and overexpressed in primary breast cancers. Cancer Res *58*, 3677-3683.

Chen, X., Zhang, Y., Douglas, L., and Zhou, P. (2001). UV-damaged DNA-binding proteins are targets of CUL-4A-mediated ubiquitination and degradation. J Biol Chem *276*, 48175-48182.

Clark, S. H., and Chovnick, A. (1986). Studies of normal and position-affected expression of rosy region genes in Drosophila melanogaster. Genetics *114*, 819-840.

Cleaver, J. E. (2005). Cancer in xeroderma pigmentosum and related disorders of DNA repair. Nat Rev Cancer *5*, 564-573.

Clemens, J. C., Worby, C. A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B. A., and Dixon, J. E. (2000). Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. Proc Natl Acad Sci U S A *97*, 6499-6503.

Conlon, I., and Raff, M. (1999). Size control in animal development. Cell *96*, 235-244.

Consortium, T. E. C. T. S. (1993). Identification and characterization of the tuberous sclerosis gene on chromosome 16. The European Chromosome 16 Tuberous Sclerosis Consortium. Cell *75*, 1305-1315.

Cook, W. D., and McCaw, B. J. (2000). Accommodating haploinsufficient tumor suppressor genes in Knudson's model. Oncogene *19*, 3434-3438.

Cope, G. A., and Deshaies, R. J. (2003). COP9 signalosome: a multifunctional regulator of SCF and other cullin-based ubiquitin ligases. Cell *114*, 663-671.

Datar, S. A., Jacobs, H. W., de la Cruz, A. F., Lehner, C. F., and Edgar, B. A. (2000). The Drosophila cyclin D-Cdk4 complex promotes cellular growth. Embo J *19*, 4543-4554.

Dealy, M. J., Nguyen, K. V., Lo, J., Gstaiger, M., Krek, W., Elson, D., Arbeit, J., Kipreos, E. T., and Johnson, R. S. (1999). Loss of Cul1 results in early embryonic lethality and dysregulation of cyclin E. Nat Genet *23*, 245-248.

Dearolf, C. R. (1998). Fruit fly "leukemia". Biochim Biophys Acta *1377*, M13-23.

Deshaies, R. J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. Annu Rev Cell Dev Biol *15*, 435-467.

Deshpande, A., Sicinski, P., and Hinds, P. W. (2005). Cyclins and cdks in development and cancer: a perspective. Oncogene *24*, 2909-2915.

Devoy, A., Soane, T., Welchman, R., and Mayer, R. J. (2005). The ubiquitin-proteasome system and cancer. Essays Biochem *41*, 187-203.

Diffley, J. F. X. (2004). Regulation of Early Events in Chromosome Replication. Current Biology *14*, R778-R786.

Dimri, G. P. (2005). What has senescence got to do with cancer? Cancer Cell *7*, 505-512.

Donaldson, T. D., Noureddine, M. A., Reynolds, P. J., Bradford, W., and Duronio, R. J. (2004). Targeted disruption of Drosophila Roc1b reveals functional differences in the Roc subunit of Cullin-dependent E3 ubiquitin ligases. Mol Biol Cell *15*, 4892-4903.

Doronkin, S., Djagaeva, I., and Beckendorf, S. K. (2002). CSN5/Jab1 mutations affect axis formation in the Drosophila oocyte by activating a meiotic checkpoint. Development *129*, 5053-5064.

Doronkin, S., Djagaeva, I., and Beckendorf, S. K. (2003). The COP9 signalosome promotes degradation of Cyclin E during early Drosophila oogenesis. Dev Cell *4*, 699-710.

Fadeel, B., and Orrenius, S. (2005). Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. J Intern Med *258*, 479-517.

Feldman, R. M., Correll, C. C., Kaplan, K. B., and Deshaies, R. J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. Cell *91*, 221-230.

Feng, H., Zhong, W., Punkosdy, G., Gu, S., Zhou, L., Seabolt, E. K., and Kipreos, E. T. (1999). CUL-2 is required for the G1-to-S-phase transition and mitotic chromosome condensation in Caenorhabditis elegans. Nat Cell Biol *1*, 486-492.

Fero, M. L., Randel, E., Gurley, K. E., Roberts, J. M., and Kemp, C. J. (1998). The murine gene p27Kip1 is haplo-insufficient for tumour suppression. Nature *396*, 177-180.

Fingar, D. C., and Blenis, J. (2004). Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. Oncogene *23*, 3151-3171.

Fingar, D. C., Salama, S., Tsou, C., Harlow, E., and Blenis, J. (2002). Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. Genes Dev *16*, 1472-1487.

Foulds, L. (1954). The experimental study of tumor progression: a review. Cancer Res *14*, 327-339.

Franklin, D. S., and Xiong, Y. (1996). Induction of p18INK4c and its predominant association with CDK4 and CDK6 during myogenic differentiation. Mol Biol Cell *7*, 1587-1599.

Frei, C., and Edgar, B. A. (2004). Drosophila cyclin D/Cdk4 requires Hif-1 prolyl hydroxylase to drive cell growth. Dev Cell *6*, 241-251.

Frei, C., Galloni, M., Hafen, E., and Edgar, B. A. (2005). The Drosophila mitochondrial ribosomal protein mRpL12 is required for Cyclin D/Cdk4-driven growth. Embo J *24*, 623-634.

Freilich, S., Oron, E., Kapp, Y., Nevo-Caspi, Y., Orgad, S., Segal, D., and Chamovitz, D. A. (1999). The COP9 signalosome is essential for development of Drosophila melanogaster. Curr Biol *9*, 1187-1190.

Fu, M., Wang, C., Li, Z., Sakamaki, T., and Pestell, R. G. (2004). Minireview: Cyclin D1: normal and abnormal functions. Endocrinology *145*, 5439-5447.

Furukawa, M., He, Y. J., Borchers, C., and Xiong, Y. (2003). Targeting of protein ubiquitination by BTB-Cullin 3-Roc1 ubiquitin ligases. Nat Cell Biol *5*, 1001-1007.

Furukawa, M., Ohta, T., and Xiong, Y. (2002). Activation of UBC5 ubiquitin-conjugating enzyme by the RING finger of ROC1 and assembly of active ubiquitin ligases by all cullins. J Biol Chem *277*, 15758-15765.

Furukawa, M., Zhang, Y., McCarville, J., Ohta, T., and Xiong, Y. (2000). The CUL1 C-terminal sequence and ROC1 are required for efficient nuclear accumulation, NEDD8 modification, and ubiquitin ligase activity of CUL1. Mol Cell Biol *20*, 8185-8197.

Galan, J. M., and Peter, M. (1999). Ubiquitin-dependent degradation of multiple F-box proteins by an autocatalytic mechanism. Proc Natl Acad Sci U S A *96*, 9124-9129.

Gao, M., Labuda, T., Xia, Y., Gallagher, E., Fang, D., Liu, Y. C., and Karin, M. (2004). Jun turnover is controlled through JNK-dependent phosphorylation of the E3 ligase Itch. Science *306*, 271-275.

Gao, X., and Pan, D. (2001). TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. Genes Dev *15*, 1383-1392.

Gao, X., Zhang, Y., Arrazola, P., Hino, O., Kobayashi, T., Yeung, R. S., Ru, B., and Pan, D. (2002). Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. Nat Cell Biol *4*, 699-704.

Garami, A., Zwartkruis, F. J., Nobukuni, T., Joaquin, M., Roccio, M., Stocker, H., Kozma, S. C., Hafen, E., Bos, J. L., and Thomas, G. (2003). Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Mol Cell *11*, 1457-1466.

Georgakis, G. V., and Younes, A. (2006). From Rapa Nui to rapamycin: targeting PI3K/Akt/mTOR for cancer therapy. Expert Rev Anticancer Ther *6*, 131-140.

Geyer, R., Wee, S., Anderson, S., Yates, J., and Wolf, D. A. (2003). BTB/POZ domain proteins are putative substrate adaptors for cullin 3 ubiquitin ligases. Mol Cell *12*, 783-790.

Gingras, A. C., Kennedy, S. G., O'Leary, M. A., Sonenberg, N., and Hay, N. (1998). 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. Genes Dev *12*, 502-513.

Goldenberg, S. J., Cascio, T. C., Shumway, S. D., Garbutt, K. C., Liu, J., Xiong, Y., and Zheng, N. (2004). Structure of the Cand1-Cul1-Roc1 complex reveals regulatory mechanisms for the assembly of the multisubunit cullin-dependent ubiquitin ligases. Cell *119*, 517-528.

Gordon, S. M., and Buchwald, M. (2003). Fanconi anemia protein complex: mapping protein interactions in the yeast 2- and 3-hybrid systems. Blood *102*, 136-141.

Gresham, D., Ruderfer, D. M., Pratt, S. C., Schacherer, J., Dunham, M. J., Botstein, D., and Kruglyak, L. (2006). Genome-wide detection of polymorphisms at nucleotide resolution with a single DNA microarray. Science *311*, 1932-1936.

Groisman, R., Polanowska, J., Kuraoka, I., Sawada, J., Saijo, M., Drapkin, R., Kisselev, A. F., Tanaka, K., and Nakatani, Y. (2003). The Ubiquitin Ligase Activity in the DDB2 and CSA Complexes Is Differentially Regulated by the COP9 Signalosome in Response to DNA Damage. Cell *113*, 357-367.

Guan, K. L., Jenkins, C. W., Li, Y., Nichols, M. A., Wu, X., O'Keefe, C. L., Matera, A. G., and Xiong, Y. (1994). Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. Genes Dev *8*, 2939-2952.

Gupta, A., Yang, L. X., and Chen, L. (2002). Study of the G2/M cell cycle checkpoint in irradiated mammary epithelial cells overexpressing Cul-4A gene. Int J Radiat Oncol Biol Phys *52*, 822-830.

Haglund, K., and Dikic, I. (2005). Ubiquitylation and cell signaling. Embo J *24*, 3353-3359.

Hahn, W. C., and Weinberg, R. A. (2002). Rules for making human tumor cells. N Engl J Med *347*, 1593-1603.

Hall, P. A., and Russell, S. H. (2005). New perspectives on neoplasia and the RNA world. Hematol Oncol *23*, 49-53.

Han, S., Santos, T. M., Puga, A., Roy, J., Thiele, E. A., McCollin, M., Stemmer-Rachamimov, A., and Ramesh, V. (2004). Phosphorylation of tuberin as a novel mechanism for somatic inactivation of the tuberous sclerosis complex proteins in brain lesions. Cancer Res *64*, 812-816.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hardie, D. G. (2005). New roles for the LKB1-->AMPK pathway. Curr Opin Cell Biol *17*, 167-173.

Harrison, D. A., Binari, R., Nahreini, T. S., Gilman, M., and Perrimon, N. (1995). Activation of a Drosophila Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects. Embo J *14*, 2857-2865.

Hartwell, L. H., and Unger, M. W. (1977). Unequal division in Saccharomyces cerevisiae and its implications for the control of cell division. J Cell Biol *75*, 422-435.

Henning, K. A., Li, L., Iyer, N., McDaniel, L. D., Reagan, M. S., Legerski, R., Schultz, R. A., Stefanini, M., Lehmann, A. R., Mayne, L. V., and et al. (1995). The Cockayne syndrome group A gene encodes a WD repeat protein that interacts with CSB protein and a subunit of RNA polymerase II TFIIH. Cell *82*, 555-564.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu Rev Biochem *67*, 425-479.

Hiesinger, P. R., and Hassan, B. A. (2005). Genetics in the age of systems biology. Cell *123*, 1173-1174.

Higa, L. A., Mihaylov, I. S., Banks, D. P., Zheng, J., and Zhang, H. (2003). Radiation-mediated proteolysis of CDT1 by CUL4-ROC1 and CSN complexes constitutes a new checkpoint. Nat Cell Biol *5*, 1008-1015.

Higa, L. A., Yang, X., Zheng, J., Banks, D., Wu, M., Ghosh, P., Sun, H., and Zhang, H. (2006). Involvement of CUL4 ubiquitin E3 ligases in regulating CDK inhibitors Dacapo/p27Kip1 and cyclin E degradation. Cell Cycle *5*, 71-77.

Hilliker, A. J., Clark, S. H., Chovnick, A., and Gelbart, W. M. (1980). Cytogenetic analysis of the chromosomal region immediately adjacent to the rosy locus in Drosophila melanogaster. Genetics *95*, 95-110.

Holmberg, C., Fleck, O., Hansen, H. A., Liu, C., Slaaby, R., Carr, A. M., and Nielsen, O. (2005). Ddb1 controls genome stability and meiosis in fission yeast. Genes Dev *19*, 853-862.

Hori, T., Osaka, F., Chiba, T., Miyamoto, C., Okabayashi, K., Shimbara, N., Kato, S., and Tanaka, K. (1999). Covalent modification of all members of human cullin family proteins by NEDD8. Oncogene *18*, 6829-6834.

Horn, P. J., Bastie, J. N., and Peterson, C. L. (2005). A Rik1-associated, cullin-dependent E3 ubiquitin ligase is essential for heterochromatin formation. Genes Dev *19*, 1705-1714.

Hu, J., McCall, C. M., Ohta, T., and Xiong, Y. (2004). Targeted ubiquitination of CDT1 by the DDB1-CUL4A-ROC1 ligase in response to DNA damage. Nat Cell Biol *6*, 1003-1009.

Huibregtse, J. M., Scheffner, M., Beaudenon, S., and Howley, P. M. (1995). A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc Natl Acad Sci U S A *92*, 2563-2567.

Hwang, B. J., and Chu, G. (1993). Purification and characterization of a human protein that binds to damaged DNA. Biochemistry *32*, 1657-1666.

Hwang, B. J., Toering, S., Francke, U., and Chu, G. (1998). p48 Activates a UV-damaged-DNA binding factor and is defective in xeroderma pigmentosum group E cells that lack binding activity. Mol Cell Biol *18*, 4391-4399.

Hwang, J. W., Min, K. W., Tamura, T. A., and Yoon, J. B. (2003). TIP120A associates with unneddylated cullin 1 and regulates its neddylation. FEBS Lett *541*, 102-108.

Inoki, K., Corradetti, M. N., and Guan, K. L. (2005a). Dysregulation of the TSC-mTOR pathway in human disease. Nat Genet *37*, 19-24.

Inoki, K., Li, Y., Xu, T., and Guan, K. L. (2003). Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev *17*, 1829-1834.

Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K. L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol *4*, 648-657.

Inoki, K., Ouyang, H., Li, Y., and Guan, K. L. (2005b). Signaling by target of rapamycin proteins in cell growth control. Microbiol Mol Biol Rev *69*, 79-100.

Inoue, K., and Sherr, C. J. (1998). Gene expression and cell cycle arrest mediated by transcription factor DMP1 is antagonized by D-type cyclins through a cyclin-dependent-kinase-independent mechanism. Mol Cell Biol *18*, 1590-1600.

Jackson, P. K., Eldridge, A. G., Freed, E., Furstenthal, L., Hsu, J. Y., Kaiser, B. K., and Reimann, J. D. (2000). The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. Trends Cell Biol *10*, 429-439.

Jaeschke, A., Hartkamp, J., Saitoh, M., Roworth, W., Nobukuni, T., Hodges, A., Sampson, J., Thomas, G., and Lamb, R. (2002). Tuberous sclerosis complex tumor suppressor-mediated S6 kinase inhibition by phosphatidylinositide-3-OH kinase is mTOR independent. J Cell Biol *159*, 217-224.

Jia, S., Kobayashi, R., and Grewal, S. I. (2005). Ubiquitin ligase component Cul4 associates with Clr4 histone methyltransferase to assemble heterochromatin. Nat Cell Biol *7*, 1007-1013.

Jiang, J., and Struhl, G. (1998). Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb. Nature *391*, 493-496.

Joazeiro, C. A., and Weissman, A. M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. Cell *102*, 549-552.

Jorgensen, P., and Tyers, M. (2004). How cells coordinate growth and division. Curr Biol *14*, R1014-1027.

Kaelin, W. G., Jr. (2002). Molecular basis of the VHL hereditary cancer syndrome. Nat Rev Cancer *2*, 673-682.

Kaelin, W. G., Jr. (2005). The von Hippel-Lindau protein, HIF hydroxylation, and oxygen sensing. Biochem Biophys Res Commun *338*, 627-638.

Kamura, T., Koepp, D. M., Conrad, M. N., Skowyra, D., Moreland, R. J., Iliopoulos, O., Lane, W. S., Kaelin, W. G., Jr., Elledge, S. J., Conaway, R. C.*, et al.* (1999). Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. Science *284*, 657-661.

Kamura, T., Maenaka, K., Kotoshiba, S., Matsumoto, M., Kohda, D., Conaway, R. C., Conaway, J. W., and Nakayama, K. I. (2004). VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases. Genes Dev *18*, 3055-3065.

Kapetanaki, M. G., Guerrero-Santoro, J., Bisi, D. C., Hsieh, C. L., Rapic-Otrin, V., and Levine, A. S. (2006). The DDB1-CUL4ADDB2 ubiquitin ligase is deficient in xeroderma pigmentosum group E and targets histone H2A at UV-damaged DNA sites. Proc Natl Acad Sci U S A.

Kato, J., Matsushime, H., Hiebert, S. W., Ewen, M. E., and Sherr, C. J. (1993). Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev *7*, 331-342.

Kawakami, T., Chiba, T., Suzuki, T., Iwai, K., Yamanaka, K., Minato, N., Suzuki, H., Shimbara, N., Hidaka, Y., Osaka, F.*, et al.* (2001). NEDD8 recruits E2-ubiquitin to SCF E3 ligase. Embo J *20*, 4003-4012.

Kipreos, E. T., Lander, L. E., Wing, J. P., He, W. W., and Hedgecock, E. M. (1996). cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family. Cell *85*, 829-839.

Klein, C., and Vassilev, L. T. (2004). Targeting the p53-MDM2 interaction to treat cancer. Br J Cancer *91*, 1415-1419.

Knudson, A. G., Jr. (1971). Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A *68*, 820-823.

Kondo, T., Kobayashi, M., Tanaka, J., Yokoyama, A., Suzuki, S., Kato, N., Onozawa, M., Chiba, K., Hashino, S., Imamura, M.*, et al.* (2004). Rapid degradation of Cdt1 upon UV-induced DNA damage is mediated by SCFSkp2 complex. J Biol Chem *279*, 27315-27319.

Kozar, K., Ciemerych, M. A., Rebel, V. I., Shigematsu, H., Zagozdzon, A., Sicinska, E., Geng, Y., Yu, Q., Bhattacharya, S., Bronson, R. T.*, et al.* (2004). Mouse development and cell proliferation in the absence of D-cyclins. Cell *118*, 477-491.

Kulaksiz, G., Reardon, J. T., and Sancar, A. (2005). Xeroderma pigmentosum complementation group E protein (XPE/DDB2): purification of various complexes of XPE and analyses of their damaged DNA binding and putative DNA repair properties. Mol Cell Biol *25*, 9784-9792.

Kwiatkowski, D. J., and Manning, B. D. (2005). Tuberous sclerosis: a GAP at the crossroads of multiple signaling pathways. Hum Mol Genet *14 Spec No. 2*, R251-258.

Kwiatkowski, D. J., Zhang, H., Bandura, J. L., Heiberger, K. M., Glogauer, M., el-Hashemite, N., and Onda, H. (2002). A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells. Hum Mol Genet *11*, 525-534.

Lambertsson, A. (1998). The minute genes in Drosophila and their molecular functions. Adv Genet *38*, 69-134.

Lammer, D., Mathias, N., Laplaza, J. M., Jiang, W., Liu, Y., Callis, J., Goebl, M., and Estelle, M. (1998). Modification of yeast Cdc53p by the ubiquitin-related protein rub1p affects function of the SCFCdc4 complex. Genes Dev *12*, 914-926.

Larue, L., and Bellacosa, A. (2005). Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. Oncogene *24*, 7443-7454.

Lee, T., and Luo, L. (2001). Mosaic analysis with a repressible cell marker (MARCM) for Drosophila neural development. Trends Neurosci *24*, 251-254.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. Cell *88*, 323-331.

Li, A., and Blow, J. J. (2005). Cdt1 downregulation by proteolysis and geminin inhibition prevents DNA re-replication in Xenopus. Embo J *24*, 395-404.

Li, B., Jia, N., Kapur, R., and Chun, K. T. (2006a). Cul-4A targets p27 for degradation and regulates proliferation, cell cycle exit, and differentiation during erythropoiesis. Blood.

Li, B., Ruiz, J. C., and Chun, K. T. (2002). CUL-4A is critical for early embryonic development. Mol Cell Biol *22*, 4997-5005.

Li, B., Yang, F. C., Clapp, D. W., and Chun, K. T. (2003a). Enforced expression of CUL-4A interferes with granulocytic differentiation and exit from the cell cycle. Blood *101*, 1769-1776.

Li, F., Goto, D. B., Zaratiegui, M., Tang, X., Martienssen, R., and Cande, W. Z. (2005). Two novel proteins, dos1 and dos2, interact with rik1 to regulate heterochromatic RNA interference and histone modification. Curr Biol *15*, 1448-1457.

Li, T., Chen, X., Garbutt, K. C., Zhou, P., and Zheng, N. (2006b). Structure of DDB1 in complex with a paramyxovirus V protein: viral hijack of a propeller cluster in ubiquitin ligase. Cell *124*, 105-117.

Li, X., Zhao, Q., Liao, R., Sun, P., and Wu, X. (2003b). The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation. J Biol Chem.

Li, Y., Inoki, K., and Guan, K. L. (2004). Biochemical and functional characterizations of small GTPase Rheb and TSC2 GAP activity. Mol Cell Biol *24*, 7965-7975.

Li, Y., Inoki, K., Vacratsis, P., and Guan, K. L. (2003c). The p38 and MK2 kinase cascade phosphorylates tuberin, the tuberous sclerosis 2 gene product, and enhances its interaction with 14-3-3. J Biol Chem *278*, 13663-13671.

Licitra, E. J., and Liu, J. O. (1996). A three-hybrid system for detecting small ligand-protein receptor interactions. Proc Natl Acad Sci U S A *93*, 12817-12821.

Lin, G. Y., Paterson, R. G., Richardson, C. D., and Lamb, R. A. (1998). The V protein of the paramyxovirus SV5 interacts with damage-specific DNA binding protein. Virology *249*, 189-200.

Lisztwan, J., Imbert, G., Wirbelauer, C., Gstaiger, M., and Krek, W. (1999). The von Hippel-Lindau tumor suppressor protein is a component of an E3 ubiquitin-protein ligase activity. Genes Dev *13*, 1822-1833.

Liu, C., Poitelea, M., Watson, A., Yoshida, S. H., Shimoda, C., Holmberg, C., Nielsen, O., and Carr, A. M. (2005). Transactivation of Schizosaccharomyces pombe cdt2+ stimulates a Pcu4-Ddb1-CSN ubiquitin ligase. Embo J *24*, 3940-3951.

Liu, C., Powell, K. A., Mundt, K., Wu, L., Carr, A. M., and Caspari, T. (2003). Cop9/signalosome subunits and Pcu4 regulate ribonucleotide reductase by both checkpoint-dependent and -independent mechanisms. Genes Dev *17*, 1130-1140.

Liu, E., Li, X., Yan, F., Zhao, Q., and Wu, X. (2004). Cyclin-dependent kinases phosphorylate human Cdt1 and induce its degradation. J Biol Chem *279*, 17283-17288.

Liu, J., Furukawa, M., Matsumoto, T., and Xiong, Y. (2002). NEDD8 Modification of CUL1 Dissociates p120(CAND1), an Inhibitor of CUL1-SKP1 Binding and SCF Ligases. Mol Cell *10*, 1511-1518.

Lo, S. C., and Hannink, M. (2006). CAND1-mediated substrate adaptor recycling is required for efficient repression of Nrf2 by Keap1. Mol Cell Biol *26*, 1235-1244.

Loeb, L. A. (1991). Mutator phenotype may be required for multistage carcinogenesis. Cancer Res *51*, 3075-3079.

Loeb, L. A., Loeb, K. R., and Anderson, J. P. (2003). Multiple mutations and cancer. Proc Natl Acad Sci U S A *100*, 776-781.

Lowe, S. W., Cepero, E., and Evan, G. (2004). Intrinsic tumour suppression. Nature *432*, 307-315.

Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D. A., Wei, N., Shevchenko, A., and Deshaies, R. J. (2001). Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. Science *292*, 1382-1385.

Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P., and Pandolfi, P. P. (2005). Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. Cell *121*, 179-193.

Malumbres, M., and Barbacid, M. (2006). Is Cyclin D1-CDK4 kinase a bona fide cancer target? Cancer Cell *9*, 2-4.

Mani, A., and Gelmann, E. P. (2005). The ubiquitin-proteasome pathway and its role in cancer. J Clin Oncol *23*, 4776-4789.

Manning, B. D., Tee, A. R., Logsdon, M. N., Blenis, J., and Cantley, L. C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol Cell *10*, 151-162.

Mantovani, F., and Banks, L. (1999). The interaction between p53 and papillomaviruses. Semin Cancer Biol *9*, 387-395.

Matsuura, I., Denissova, N. G., Wang, G., He, D., Long, J., and Liu, F. (2004). Cyclin-dependent kinases regulate the antiproliferative function of Smads. Nature *430*, 226-231.

Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R., and Ratcliffe, P. J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature *399*, 271-275.

May, N. R., Thomer, M., Murnen, K. F., and Calvi, B. R. (2005). Levels of the origin-binding protein Double parked and its inhibitor Geminin increase in response to replication stress. J Cell Sci *118*, 4207-4217.

Mellon, I. (2005). Transcription-coupled repair: a complex affair. Mutat Res *577*, 155-161.

Meyer, C. A., Jacobs, H. W., Datar, S. A., Du, W., Edgar, B. A., and Lehner, C. F. (2000). Drosophila Cdk4 is required for normal growth and is dispensable for cell cycle progression. Embo J *19*, 4533-4542.

Michel, J. J., and Xiong, Y. (1998). Human CUL-1, but not other cullin family members, selectively interacts with SKP1 to form a complex with SKP2 and cyclin A. Cell Growth Differ *9*, 435-449.

Miloloza, A., Rosner, M., Nellist, M., Halley, D., Bernaschek, G., and Hengstschlager, M. (2000). The TSC1 gene product, hamartin, negatively regulates cell proliferation. Hum Mol Genet *9*, 1721-1727.

Min, K. W., Hwang, J. W., Lee, J. S., Park, Y., Tamura, T. A., and Yoon, J. B. (2003). TIP120A associates with cullins and modulates ubiquitin ligase activity. J Biol Chem *278*, 15905-15910.

Min, K. W., Kwon, M. J., Park, H. S., Park, Y., Yoon, S. K., and Yoon, J. B. (2005). CAND1 enhances deneddylation of CUL1 by COP9 signalosome. Biochem Biophys Res Commun *334*, 867-874.

Mistry, H., Wilson, B. A., Roberts, I. J., O'Kane, C. J., and Skeath, J. B. (2004). Cullin-3 regulates pattern formation, external sensory organ development and cell survival during Drosophila development. Mech Dev *121*, 1495-1507.

Mitchison, J. M. (2003). Growth during the cell cycle. Int Rev Cytol *226*, 165-258.

Mittnacht, S. (2005). The retinoblastoma protein--from bench to bedside. Eur J Cell Biol *84*, 97-107.

Morimoto, M., Nishida, T., Honda, R., and Yasuda, H. (2000). Modification of cullin-1 by ubiquitin-like protein Nedd8 enhances the activity of SCF(skp2) toward p27(kip1). Biochem Biophys Res Commun *270*, 1093-1096.

Morimoto, M., Nishida, T., Nagayama, Y., and Yasuda, H. (2003). Nedd8-modification of Cul1 is promoted by Roc1 as a Nedd8-E3 ligase and regulates its stability. Biochem Biophys Res Commun *301*, 392-398.

Muller, P., Kuttenkeuler, D., Gesellchen, V., Zeidler, M. P., and Boutros, M. (2005). Identification of JAK/STAT signalling components by genome-wide RNA interference. Nature *436*, 871-875.

Mundt, K. E., Liu, C., and Carr, A. M. (2002). Deletion mutants in COP9/signalosome subunits in fission yeast Schizosaccharomyces pombe display distinct phenotypes. Mol Biol Cell *13*, 493-502.

Nag, A., Bondar, T., Shiv, S., and Raychaudhuri, P. (2001). The xeroderma pigmentosum group E gene product DDB2 is a specific target of cullin 4A in mammalian cells. Mol Cell Biol *21*, 6738-6747.

Nakayama, K. I., and Nakayama, K. (2005). Regulation of the cell cycle by SCF-type ubiquitin ligases. Semin Cell Dev Biol *16*, 323-333.

Nellist, M., Verhaaf, B., Goedbloed, M. A., Reuser, A. J., van den Ouweland, A. M., and Halley, D. J. (2001). TSC2 missense mutations inhibit tuberin phosphorylation and prevent formation of the tuberin-hamartin complex. Hum Mol Genet *10*, 2889-2898.

Neuwald, A. F., and Poleksic, A. (2000). PSI-BLAST searches using hidden markov models of structural repeats: prediction of an unusual sliding DNA clamp and of beta-propellers in UV-damaged DNA-binding protein. Nucleic Acids Res *28*, 3570-3580.

Nishitani, H., Lygerou, Z., and Nishimoto, T. (2004). Proteolysis of DNA replication licensing factor Cdt1 in S-phase is performed independently of geminin through its N-terminal region. J Biol Chem *279*, 30807-30816.

Nishitani, H., Sugimoto, N., Roukos, V., Nakanishi, Y., Saijo, M., Obuse, C., Tsurimoto, T., Nakayama, K. I., Nakayama, K., Fujita, M.*, et al.* (2006). Two E3 ubiquitin ligases, SCF-Skp2 and DDB1-Cul4, target human Cdt1 for proteolysis. Embo J *25*, 1126-1136.

Nishitani, H., Taraviras, S., Lygerou, Z., and Nishimoto, T. (2001). The human licensing factor for DNA replication Cdt1 accumulates in G1 and is destabilized after initiation of S-phase. J Biol Chem *276*, 44905-44911.

Nobukini, T., and Thomas, G. (2004). The mTOR/S6K signalling pathway: the role of the TSC1/2 tumour suppressor complex and the proto-oncogene Rheb. Novartis Found Symp *262*, 148-154; discussion 154-149, 265-148.

Noureddine, M. A., Donaldson, T. D., Thacker, S. A., and Duronio, R. J. (2002). Drosophila Roc1a encodes a RING-H2 protein with a unique function in processing the Hh signal transducer Ci by the SCF E3 ubiquitin ligase. Dev Cell *2*, 757-770.

Nurse, P. (1975). Genetic control of cell size at cell division in yeast. Nature *256*, 547-551.

O'Shea, C. C. (2005). Viruses - seeking and destroying the tumor program. Oncogene *24*, 7640-7655.

Ohh, M., Park, C. W., Ivan, M., Hoffman, M. A., Kim, T. Y., Huang, L. E., Pavletich, N., Chau, V., and Kaelin, W. G. (2000). Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. Nat Cell Biol *2*, 423-427.

Ohta, T., Michel, J. J., Schottelius, A. J., and Xiong, Y. (1999). ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. Mol Cell *3*, 535-541.

Oliver, S. G. (2006). From genomes to systems: the path with yeast. Philos Trans R Soc Lond B Biol Sci *361*, 477-482.

Oron, E., Mannervik, M., Rencus, S., Harari-Steinberg, O., Neuman-Silberberg, S., Segal, D., and Chamovitz, D. A. (2002). COP9 signalosome subunits 4 and 5 regulate multiple pleiotropic pathways in Drosophila melanogaster. Development *129*, 4399-4409.

Osaka, F., Kawasaki, H., Aida, N., Saeki, M., Chiba, T., Kawashima, S., Tanaka, K., and Kato, S. (1998). A new NEDD8-ligating system for cullin-4A. Genes Dev *12*, 2263-2268.

Osaka, F., Saeki, M., Katayama, S., Aida, N., Toh, E. A., Kominami, K., Toda, T., Suzuki, T., Chiba, T., Tanaka, K., and Kato, S. (2000). Covalent modifier NEDD8 is essential for SCF ubiquitin-ligase in fission yeast. Embo J *19*, 3475-3484.

Oshikawa, K., Matsumoto, M., Yada, M., Kamura, T., Hatakeyama, S., and Nakayama, K. I. (2003). Preferential interaction of TIP120A with Cul1 that is not modified by NEDD8 and not associated with Skp1. Biochem Biophys Res Commun *303*, 1209-1216.

Ou, C. Y., Lin, Y. F., Chen, Y. J., and Chien, C. T. (2002). Distinct protein degradation mechanisms mediated by Cul1 and Cul3 controlling Ci stability in Drosophila eye development. Genes Dev *16*, 2403-2414.

Pagano, M., and Benmaamar, R. (2003). When protein destruction runs amok, malignancy is on the loose. Cancer Cell *4*, 251-256.

Pagano, M., and Jackson, P. K. (2004). Wagging the dogma; tissue-specific cell cycle control in the mouse embryo. Cell *118*, 535-538.

Pan, D., Dong, J., Zhang, Y., and Gao, X. (2004). Tuberous sclerosis complex: from Drosophila to human disease. Trends Cell Biol *14*, 78-85.

Parsons, R. (2004). Human cancer, PTEN and the PI-3 kinase pathway. Semin Cell Dev Biol *15*, 171-176.

Pause, A., Peterson, B., Schaffar, G., Stearman, R., and Klausner, R. D. (1999). Studying interactions of four proteins in the yeast two-hybrid system: structural resemblance of the pVHL/elongin BC/hCUL-2 complex with the ubiquitin ligase complex SKP1/cullin/F-box protein. Proc Natl Acad Sci U S A *96*, 9533-9538.

Payne, S. R., and Kemp, C. J. (2005). Tumor suppressor genetics. Carcinogenesis *26*, 2031-2045.

Pearson, R. B., Dennis, P. B., Han, J. W., Williamson, N. A., Kozma, S. C., Wettenhall, R. E., and Thomas, G. (1995). The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain. Embo J *14*, 5279-5287.

Pei, X. H., and Xiong, Y. (2005). Biochemical and cellular mechanisms of mammalian CDK inhibitors: a few unresolved issues. Oncogene *24*, 2787-2795.

Petroski, M. D., and Deshaies, R. J. (2005). Function and regulation of cullin-RING ubiquitin ligases. Nat Rev Mol Cell Biol *6*, 9-20.

Pickart, C. M. (2001). Mechanisms underlying ubiquitination. Annu Rev Biochem *70*, 503-533.

Pickart, C. M. (2004). Back to the future with ubiquitin. Cell *116*, 181-190.

Pintard, L., Willis, J. H., Willems, A., Johnson, J. L., Srayko, M., Kurz, T., Glaser, S., Mains, P. E., Tyers, M., Bowerman, B., and Peter, M. (2003). The BTB protein MEL-26 is a substrate-specific adaptor of the CUL-3 ubiquitin-ligase. Nature *425*, 311-316.

Podust, V. N., Brownell, J. E., Gladysheva, T. B., Luo, R. S., Wang, C., Coggins, M. B., Pierce, J. W., Lightcap, E. S., and Chau, V. (2000). A Nedd8 conjugation pathway is essential for proteolytic targeting of p27Kip1 by ubiquitination. Proc Natl Acad Sci U S A *97*, 4579-4584.

Polyak, K., and Hahn, W. C. (2006). Roots and stems: stem cells in cancer. Nat Med *12*, 296-300.

Potter, C. J., Huang, H., and Xu, T. (2001). Drosophila Tsc1 functions with Tsc2 to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size. Cell *105*, 357-368.

Potter, C. J., Pedraza, L. G., and Xu, T. (2002). Akt regulates growth by directly phosphorylating Tsc2. Nat Cell Biol *4*, 658-665.

Precious, B., Childs, K., Fitzpatrick-Swallow, V., Goodbourn, S., and Randall, R. E. (2005). Simian virus 5 V protein acts as an adaptor, linking DDB1 to STAT2, to facilitate the ubiquitination of STAT1. J Virol *79*, 13434-13441.

Raptis, S., and Bapat, B. (2006). Genetic instability in human tumors. Exs, 303-320.

Read, M. A., Brownell, J. E., Gladysheva, T. B., Hottelet, M., Parent, L. A., Coggins, M. B., Pierce, J. W., Podust, V. N., Luo, R. S., Chau, V., and Palombella, V. J. (2000). Nedd8 modification of cul-1 activates SCF(beta(TrCP))-dependent ubiquitination of IkappaBalpha. Mol Cell Biol *20*, 2326-2333.

Reardon, J. T., and Sancar, A. (2005). Nucleotide excision repair. Prog Nucleic Acid Res Mol Biol *79*, 183-235.

Rizki, T. M., and Rizki, R. M. (1983). Blood cell surface changes in Drosophila mutants with melanotic tumors. Science *220*, 73-75.

Roach, E. S., Gomez, M. R., and Northrup, H. (1998). Tuberous sclerosis complex consensus conference: revised clinical diagnostic criteria. J Child Neurol *13*, 624-628.

Rosner, M., Hofer, K., Kubista, M., and Hengstschlager, M. (2003). Cell size regulation by the human TSC tumor suppressor proteins depends on PI3K and FKBP38. Oncogene *22*, 4786-4798.

Roux, P. P., Ballif, B. A., Anjum, R., Gygi, S. P., and Blenis, J. (2004). Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. Proc Natl Acad Sci U S A *101*, 13489-13494.

Ruggero, D., and Pandolfi, P. P. (2003). Does the ribosome translate cancer? Nat Rev Cancer *3*, 179-192.

Rushlow, C. A., and Chovnick, A. (1984). Heterochromatic position effect at the rosy locus of Drosophila melanogaster: cytological, genetic and biochemical characterization. Genetics *108*, 589-602.

Sancar, A., Lindsey-Boltz, L. A., Unsal-Kacmaz, K., and Linn, S. (2004). Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem *73*, 39-85.

Sandrock, B., and Egly, J. M. (2001). A yeast four-hybrid system identifies Cdk-activating kinase as a regulator of the XPD helicase, a subunit of transcription factor IIH. J Biol Chem *276*, 35328-35333.

Sansal, I., and Sellers, W. R. (2004). The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol *22*, 2954-2963.

Sarbassov dos, D., Ali, S. M., and Sabatini, D. M. (2005). Growing roles for the mTOR pathway. Curr Opin Cell Biol *17*, 596-603.

Saucedo, L. J., Gao, X., Chiarelli, D. A., Li, L., Pan, D., and Edgar, B. A. (2003). Rheb promotes cell growth as a component of the insulin/TOR signalling network. Nat Cell Biol *5*, 566-571.

Saxena, S., and Dutta, A. (2005). Geminin-Cdt1 balance is critical for genetic stability. Mutat Res *569*, 111-121.

Schalet, A., Kernaghan, R. P., and Chovnick, A. (1964). Structural and Phenotypic Definition of the Rosy Cistron in Drosophila Melanogaster. Genetics *50*, 1261-1268.

Schreiber-Agus, N., Stein, D., Chen, K., Goltz, J. S., Stevens, L., and DePinho, R. A. (1997). Drosophila Myc is oncogenic in mammalian cells and plays a role in the diminutive phenotype. Proc Natl Acad Sci U S A *94*, 1235-1240.

Schwechheimer, C., and Calderon Villalobos, L. I. (2004). Cullin-containing E3 ubiquitin ligases in plant development. Curr Opin Plant Biol *7*, 677-686.

Scicchitano, D. A., and Mellon, I. (1997). Transcription and DNA damage: a link to a kink. Environ Health Perspect *105 Suppl 1*, 145-153.

Seeger, M., Gordon, C., and Dubiel, W. (2001). Protein stability: the COP9 signalosome gets in on the act. Curr Biol *11*, R643-646.

Seeger, M., Kraft, R., Ferrell, K., Bech-Otschir, D., Dumdey, R., Schade, R., Gordon, C., Naumann, M., and Dubiel, W. (1998). A novel protein complex involved in signal transduction possessing similarities to 26S proteasome subunits. Faseb J *12*, 469-478.

Senga, T., Sivaprasad, U., Zhu, W., Park, J. H., Arias, E. E., Walter, J. C., and Dutta, A. (2006). PCNA Is a Cofactor for Cdt1 Degradation by CUL4/DDB1-mediated N-terminal Ubiquitination. J Biol Chem *281*, 6246-6252.

Seol, J. H., Feldman, R. M., Zachariae, W., Shevchenko, A., Correll, C. C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S.*, et al.* (1999). Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. Genes Dev *13*, 1614-1626.

Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593-602.

Shamji, A. F., Nghiem, P., and Schreiber, S. L. (2003). Integration of growth factor and nutrient signaling: implications for cancer biology. Mol Cell *12*, 271-280.

Sherr, C. J. (2004). Principles of tumor suppression. Cell *116*, 235-246.

Sherr, C. J., and McCormick, F. (2002). The RB and p53 pathways in cancer. Cancer Cell *2*, 103-112.

Shiyanov, P., Nag, A., and Raychaudhuri, P. (1999). Cullin 4A associates with the UV-damaged DNA-binding protein DDB. J Biol Chem *274*, 35309-35312.

Singer, J. D., Gurian-West, M., Clurman, B., and Roberts, J. M. (1999). Cullin-3 targets cyclin E for ubiquitination and controls S phase in mammalian cells. Genes Dev *13*, 2375-2387.

Skowyra, D., Koepp, D. M., Kamura, T., Conrad, M. N., Conaway, R. C., Conaway, J. W., Elledge, S. J., and Harper, J. W. (1999). Reconstitution of G1 cyclin ubiquitination with complexes containing SCFGrr1 and Rbx1. Science *284*, 662-665.

Soucek, T., Pusch, O., Wienecke, R., DeClue, J. E., and Hengstschlager, M. (1997). Role of the tuberous sclerosis gene-2 product in cell cycle control. Loss of the tuberous sclerosis gene-2 induces quiescent cells to enter S phase. J Biol Chem *272*, 29301-29308.

Soussi, T. (2005). The p53 pathway and human cancer. Br J Surg *92*, 1331-1332.

Soussi, T., Ishioka, C., Claustres, M., and Beroud, C. (2006). Locus-specific mutation databases: pitfalls and good practice based on the p53 experience. Nat Rev Cancer *6*, 83-90.

Sporn, M. B. (1996). The war on cancer. Lancet *347*, 1377-1381.

Sprinzak, D., and Elowitz, M. B. (2005). Reconstruction of genetic circuits. Nature *438*, 443-448.

Starita, L. M., and Parvin, J. D. (2006). Substrates of the BRCA1-Dependent Ubiquitin Ligase. Cancer Biol Ther *5*, 137-141.

Stocker, H., Radimerski, T., Schindelholz, B., Wittwer, F., Belawat, P., Daram, P., Breuer, S., Thomas, G., and Hafen, E. (2003). Rheb is an essential regulator of S6K in controlling cell growth in Drosophila. Nat Cell Biol *5*, 559-565.

Sugasawa, K., Okuda, Y., Saijo, M., Nishi, R., Matsuda, N., Chu, G., Mori, T., Iwai, S., Tanaka, K., Tanaka, K., and Hanaoka, F. (2005). UV-induced ubiquitylation of XPC protein mediated by UV-DDB-ubiquitin ligase complex. Cell *121*, 387-400.

Sugimoto, N., Tatsumi, Y., Tsurumi, T., Matsukage, A., Kiyono, T., Nishitani, H., and Fujita, M. (2004). Cdt1 phosphorylation by cyclin A-dependent kinases negatively regulates its function without affecting geminin binding. J Biol Chem *279*, 19691-19697.

Suh, G. S., Poeck, B., Chouard, T., Oron, E., Segal, D., Chamovitz, D. A., and Zipursky, S. L. (2002). Drosophila JAB1/CSN5 acts in photoreceptor cells to induce glial cells. Neuron *33*, 35-46.

Sulis, M. L., and Parsons, R. (2003). PTEN: from pathology to biology. Trends Cell Biol *13*, 478-483.

Sun, L., and Chen, Z. J. (2004). The novel functions of ubiquitination in signaling. Curr Opin Cell Biol *16*, 119-126.

Takata, K., Ishikawa, G., Hirose, F., and Sakaguchi, K. (2002). Drosophila damage-specific DNA-binding protein 1 (D-DDB1) is controlled by the DRE/DREF system. Nucleic Acids Res *30*, 3795-3808.

Takata, K., Shimanouchi, K., Yamaguchi, M., Murakami, S., Ishikawa, G., Takeuchi, R., Kanai, Y., Ruike, T., Nakamura, R., Abe, Y., and Sakaguchi, K. (2004a). Damaged DNA binding protein 1 in Drosophila defense reactions. Biochem Biophys Res Commun *323*, 1024-1031.

Takata, K., Yoshida, H., Yamaguchi, M., and Sakaguchi, K. (2004b). Drosophila damaged DNA-binding protein 1 is an essential factor for development. Genetics *168*, 855-865.

Takeda, D. Y., Parvin, J. D., and Dutta, A. (2005). Degradation of Cdt1 during S Phase Is Skp2-independent and Is Required for Efficient Progression of Mammalian Cells through S Phase. J Biol Chem *280*, 23416-23423.

Tang, J., and Chu, G. (2002). Xeroderma pigmentosum complementation group E and UV-damaged DNA-binding protein. DNA Repair (Amst) *1*, 601-616.

Tang, J. Y., Hwang, B. J., Ford, J. M., Hanawalt, P. C., and Chu, G. (2000). Xeroderma pigmentosum p48 gene enhances global genomic repair and suppresses UV-induced mutagenesis. Mol Cell *5*, 737-744.

Tapon, N., Ito, N., Dickson, B. J., Treisman, J. E., and Hariharan, I. K. (2001). The Drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. Cell *105*, 345-355.

Tee, A. R., Fingar, D. C., Manning, B. D., Kwiatkowski, D. J., Cantley, L. C., and Blenis, J. (2002). Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. Proc Natl Acad Sci U S A *99*, 13571-13576.

Tee, A. R., Manning, B. D., Roux, P. P., Cantley, L. C., and Blenis, J. (2003). Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr Biol *13*, 1259-1268.

Thomer, M., May, N. R., Aggarwal, B. D., Kwok, G., and Calvi, B. R. (2004). Drosophila double-parked is sufficient to induce re-replication during development and is regulated by cyclin E/CDK2. Development *131*, 4807-4818.

Thon, G., Hansen, K. R., Altes, S. P., Sidhu, D., Singh, G., Verhein-Hansen, J., Bonaduce, M. J., and Klar, A. J. (2005). The Clr7 and Clr8 directionality factors and the Pcu4 cullin mediate heterochromatin formation in the fission yeast Schizosaccharomyces pombe. Genetics *171*, 1583-1595.

Tirode, F., Malaguti, C., Romero, F., Attar, R., Camonis, J., and Egly, J. M. (1997). A conditionally expressed third partner stabilizes or prevents the formation of a transcriptional activator in a three-hybrid system. J Biol Chem *272*, 22995-22999.

Turnbull, C., and Hodgson, S. (2005). Genetic predisposition to cancer. Clin Med *5*, 491-498.

Ulane, C. M., and Horvath, C. M. (2002). Paramyxoviruses SV5 and HPIV2 Assemble STAT Protein Ubiquitin Ligase Complexes from Cellular Components. Virology *304*, 160-166.

Ulane, C. M., Rodriguez, J. J., Parisien, J. P., and Horvath, C. M. (2003). STAT3 ubiquitylation and degradation by mumps virus suppress cytokine and oncogene signaling. J Virol *77*, 6385-6393.

van Slegtenhorst, M., de Hoogt, R., Hermans, C., Nellist, M., Janssen, B., Verhoef, S., Lindhout, D., van den Ouweland, A., Halley, D., Young, J.*, et al.* (1997). Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. Science *277*, 805-808.

Venkatachalam, S., Shi, Y. P., Jones, S. N., Vogel, H., Bradley, A., Pinkel, D., and Donehower, L. A. (1998). Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. Embo J *17*, 4657-4667.

Vidal, M., and Cagan, R. L. (2006). Drosophila models for cancer research. Curr Opin Genet Dev *16*, 10-16.

Vogelstein, B., and Kinzler, K. W. (2004). Cancer genes and the pathways they control. Nat Med *10*, 789-799.

Voigt, J., and Papalopulu, N. (2006). A dominant-negative form of the E3 ubiquitin ligase Cullin-1 disrupts the correct allocation of cell fate in the neural crest lineage. Development *133*, 559-568.

Wang, Y., Penfold, S., Tang, X., Hattori, N., Riley, P., Harper, J. W., Cross, J. C., and Tyers, M. (1999). Deletion of the Cul1 gene in mice causes arrest in early embryogenesis and accumulation of cyclin E. Curr Biol *9*, 1191-1194.

Wei, N., and Deng, X. W. (2003). The COP9 signalosome. Annu Rev Cell Dev Biol *19*, 261-286.

Wei, N., Kwok, S. F., von Arnim, A. G., Lee, A., McNellis, T. W., Piekos, B., and Deng, X. W. (1994). Arabidopsis COP8, COP10, and COP11 genes are involved in repression of photomorphogenic development in darkness. Plant Cell *6*, 629-643.

Weinstein, I. B. (2002). Cancer. Addiction to oncogenes--the Achilles heal of cancer. Science *297*, 63-64.

Wendel, H. G., De Stanchina, E., Fridman, J. S., Malina, A., Ray, S., Kogan, S., Cordon-Cardo, C., Pelletier, J., and Lowe, S. W. (2004). Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. Nature *428*, 332-337.

Wendel, H. G., and Lowe, S. W. (2004). Reversing drug resistance in vivo. Cell Cycle *3*, 847-849.

Wertz, I. E., O'Rourke, K. M., Zhang, Z., Dornan, D., Arnott, D., Deshaies, R. J., and Dixit, V. M. (2004). Human De-etiolated-1 regulates c-Jun by assembling a CUL4A ubiquitin ligase. Science *303*, 1371-1374.

Whitfield, M. L., George, L. K., Grant, G. D., and Perou, C. M. (2006). Common markers of proliferation. Nat Rev Cancer *6*, 99-106.

Willems, A. R., Lanker, S., Patton, E. E., Craig, K. L., Nason, T. F., Mathias, N., Kobayashi, R., Wittenberg, C., and Tyers, M. (1996). Cdc53 targets phosphorylated G1 cyclins for degradation by the ubiquitin proteolytic pathway. Cell *86*, 453-463.

Wittekind, C., and Neid, M. (2005). Cancer invasion and metastasis. Oncology *69 Suppl 1*, 14-16.

Wittschieben, B. B., and Wood, R. D. (2003). DDB complexities. DNA Repair (Amst) *2*, 1065-1069.

Wittschieben, B. O., Iwai, S., and Wood, R. D. (2005). DDB1-DDB2 (xeroderma pigmentosum group E) protein complex recognizes a cyclobutane pyrimidine dimer, mismatches, apurinic/apyrimidinic sites, and compound lesions in DNA. J Biol Chem *280*, 39982-39989.

Wu, J. T., Lin, H. C., Hu, Y. C., and Chien, C. T. (2005). Neddylation and deneddylation regulate Cul1 and Cul3 protein accumulation. Nat Cell Biol *7*, 1014-1020.

Wu, K., Chen, A., and Pan, Z. Q. (2000). Conjugation of Nedd8 to CUL1 enhances the ability of the ROC1-CUL1 complex to promote ubiquitin polymerization. J Biol Chem *275*, 32317-32324.

Wu, K., Chen, A., Tan, P., and Pan, Z. Q. (2002). The Nedd8-conjugated ROC1-CUL1 core ubiquitin ligase utilizes Nedd8 charged surface residues for efficient polyubiquitin chain assembly catalyzed by Cdc34. J Biol Chem *277*, 516-527.

Xin, S., Weng, L., Xu, J., and Du, W. (2002). The role of RBF in developmentally regulated cell proliferation in the eye disc and in Cyclin D/Cdk4 induced cellular growth. Development *129*, 1345-1356.

Xiong, Y., and Kotake, Y. (2006). No exit strategy? No problem: APC inhibits beta-catenin inside the nucleus. Genes Dev *20*, 637-642.

Xu, L., Wei, Y., Reboul, J., Vaglio, P., Shin, T. H., Vidal, M., Elledge, S. J., and Harper, J. W. (2003). BTB proteins are substrate-specific adaptors in an SCF-like modular ubiquitin ligase containing CUL-3. Nature *425*, 316-321.

Xu, T., and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. Development *117*, 1223-1237.

Yamasaki, L., and Pagano, M. (2004). Cell cycle, proteolysis and cancer. Curr Opin Cell Biol *16*, 623-628.

Yasui, K., Arii, S., Zhao, C., Imoto, I., Ueda, M., Nagai, H., Emi, M., and Inazawa, J. (2002). TFDP1, CUL4A, and CDC16 identified as targets for amplification at 13q34 in hepatocellular carcinomas. Hepatology *35*, 1476-1484.

Yu, B. D., Becker-Hapak, M., Snyder, E. L., Vooijs, M., Denicourt, C., and Dowdy, S. F. (2003). Distinct and nonoverlapping roles for pRB and cyclin D:cyclin-dependent kinases 4/6 activity in melanocyte survival. Proc Natl Acad Sci U S A *100*, 14881-14886.

Zacharek, S. J., Xiong, Y., and Shumway, S. D. (2005). Negative regulation of TSC1-TSC2 by mammalian D-type cyclins. Cancer Res *65*, 11354-11360.

Zetterberg, A., Larsson, O., and Wiman, K. G. (1995). What is the restriction point? Curr Opin Cell Biol *7*, 835-842.

Zhang, Y., Gao, X., Saucedo, L. J., Ru, B., Edgar, B. A., and Pan, D. (2003a). Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nat Cell Biol *5*, 578-581.

Zhang, Y., Morrone, G., Zhang, J., Chen, X., Lu, X., Ma, L., Moore, M., and Zhou, P. (2003b). CUL-4A stimulates ubiquitylation and degradation of the HOXA9 homeodomain protein. EMBO J *22*, 6057-6067.

Zhang, Y. W., Otterness, D. M., Chiang, G. G., Xie, W., Liu, Y. C., Mercurio, F., and Abraham, R. T. (2005). Genotoxic stress targets human Chk1 for degradation by the ubiquitin-proteasome pathway. Mol Cell *19*, 607-618.

Zheng, J., Yang, X., Harrell, J. M., Ryzhikov, S., Shim, E. H., Lykke-Andersen, K., Wei, N., Sun, H., Kobayashi, R., and Zhang, H. (2002a). CAND1 Binds to Unneddylated CUL1 and Regulates the Formation of SCF Ubiquitin E3 Ligase Complex. Mol Cell *10*, 1519-1526.

Zheng, N., Schulman, B. A., Song, L., Miller, J. J., Jeffrey, P. D., Wang, P., Chu, C., Koepp, D. M., Elledge, S. J., Pagano, M*., et al.* (2002b). Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. Nature *416*, 703-709.

Zhong, W., Feng, H., Santiago, F. E., and Kipreos, E. T. (2003). CUL-4 ubiquitin ligase maintains genome stability by restraining DNA-replication licensing. Nature *423*, 885-889.

Zhou, C., Seibert, V., Geyer, R., Rhee, E., Lyapina, S., Cope, G., Deshaies, R. J., and Wolf, D. A. (2001). The fission yeast COP9/signalosome is involved in cullin modification by ubiquitin-related Ned8p. BMC Biochem *2*, 7.

Zhou, C., Wee, S., Rhee, E., Naumann, M., Dubiel, W., and Wolf, D. A. (2003). Fission yeast COP9/signalosome suppresses cullin activity through recruitment of the deubiquitylating enzyme Ubp12p. Mol Cell *11*, 927-938.

Zhou, P., and Howley, P. M. (1998). Ubiquitination and degradation of the substrate recognition subunits of SCF ubiquitin-protein ligases. Mol Cell *2*, 571-580.

Zhu, S., Perez, R., Pan, M., and Lee, T. (2005). Requirement of Cul3 for axonal arborization and dendritic elaboration in Drosophila mushroom body neurons. J Neurosci *25*, 4189-4197.

Zolezzi, F., Fuss, J., Uzawa, S., and Linn, S. (2002). Characterization of a Schizosaccharomyces pombe strain deleted for a sequence homologue of the human damaged DNA binding 1 (DDB1) gene. J Biol Chem *277*, 41183-41191.

Zwicker, J., Brusselbach, S., Jooss, K. U., Sewing, A., Behn, M., Lucibello, F. C., and Muller, R. (1999). Functional domains in cyclin D1: pRb-kinase activity is not essential for transformation. Oncogene *18*, 19-25.

Zwijsen, R. M., Wientjens, E., Klompmaker, R., van der Sman, J., Bernards, R., and Michalides, R. J. (1997). CDK-independent activation of estrogen receptor by cyclin D1. Cell *88*, 405-415.