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14. ABSTRACT: Low or absent expression of CDK inhibitor p27Kip1 is associated with malignant prostatecarcinomas. Loss of tumor suppressors p53 and Pten is also associated with prostate cancers.We found that p27 is regulated by both SCF-SKP2 and a novel ubiquitin E3 ligase containingCUL4-DDB1-WD40-repeat proteins. In addition, we have identified that a specific CUL4A-DDB1-L2DTL/CDT2-PCNA ubiquitin E3 ligase binds and targets p53 polyubiquitination-dependentproteolysis in cooperation with MDM2. By interacting with a WD40-repeat proteins as asubstrate targeting subunit, the CUL4-DDB1 ubiquitin E3 ligase regulates a wide array ofbiological events including cell cycle regulation, genome stability, and histone methylation;alteration of these events have been shown to associate with the development of prostatecarcinogenesis. We in addition found that expression of SKP2 and Pten heterozygosity do notcooperate in the mouse prostate carcinoma models, suggesting that SKP2 may act downstream ofPten pathway. Our work provides novel insights into the mechanism of prostate tumorigenesis.

15. SUBJECT TERMS
p53, p27, SKP2, SCF, CUL4, DDB1, WD40, histone methylation, prostate, tumor, ubiquitin E3ligase, protein degradation

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Table of Contents

Introdution;
Low or absent expression of CDK inhibitor $p27^{Kip1}$ is associated with malignant prostate carcinomas (*1-3*). P27 is primarily regulated by the ubiquitin E3 ligase SCF^{SKP2}, which binds and targets p27 for ubiquitin-dependent proteolysis (*4*). SKP2 is the critical component of SCF^{SKP2} complex that is over-expressed in many prostate carcinomas (*3, 5, 6*). The inverse correlation between the low level of p27 and elevation of SKP2 in these cancers indicates that SKP2 is rate limiting for p27 degradation (*1, 3*). We have previously shown that expression of SKP2 under the androgen-responsive probasin promoter control is sufficient to downregulate p27 and induce hyperplasia, dysplasia and low grade carcinomas in the mouse prostate gland (*7*). The original proposal of the grant is to develop high titer SKP2 adenovirus to acutely deliver and express SKP2 in the prostate epithelium to determine the effect on cell proliferation and p27 regulation. The SKP2 transgenic mice will also be crossed into Pten and Nkx3.1 mice which have been shown to cause prostatic neoplasm in the mouse (*8-10*). These compound mice will be examined for the synergism between oncoproteins and tumor suppressors for prostate tumor development. Finally, the sensitivity of SKP2 transgenic mice to certain nutrient factors such as vitamin D3 will be examined. In addition to the inactivation of Pten and Nkx3.1, loss of tumor suppressor p53 is also associated with prostate carcinomas and recent studies indicate that combined loss of p53 and Pten in the mouse prostate gland causes the formation of large, aggressive, invasive, and metastatic prostate carcinomas (*11*). Malignant prostate carcinmas often display chromosome polypoloidy and aneuploidy (*12*). Understanding these regulations would provide novel insights into the mechanism of prostatic carinogenesis and new strategy for diagnosis, prognosis, and treatment of prostate cancer.

Body:
The first aim is to acutely express SKP2 in the form of a recombinant SKP2 adevnovirus in the prostatic epithelium to determine whether SKP2 can downregulation p27 in diferentiated epithelial cells and induce the entry into the cell cycle and possibly tumor formation. Krasimir Spasov, a technician in the lab, has been producing the adevovirus-SKP2. However, Mr. Spasov had substantial problem in making the SKP2 adenovirus in sufficient quantity and high titer to acutely express SKP2 in the mouse prostate gland. Mr. Spasov left the lab early last year so this aim was not fully developed.

The second aim is to determine the cooperativity between SKP2 expression and loss of Pten. We have obtained Pten-knockout and Pten-conditional knockout mice, as well as the prostate-specific Cre-recombinase transgenic mice for the conditional knockout of Pten in the mouse prostate gland. We have made the compound Pten-/+ and SKP2 transgenic mice and determined the effect of losing one copy of Pten genes in the SKP2 transgenic background. While SKP2 transgenic mice produced hyperplasia, dysplasia, and low grade carcinomas in the mouse prostate gland in 4-7 months, as we have previously reported (*7*), loss of one copy of Pten genes produced mostly prostatic hyperplasia and dysplasia in about 8-10 months. The SKP2-transgene and Pten+/- compound mice displayed the same phenotypes as that of SKP2-transgenic mice in the frequency of incidences (about 70%), time (usually within 4-7 months), and tumor grade.

We concluded that heterozygous Pten deficiency does not have detectable synergistic effect on the prostate tumorigenesis induced by SKP2 expression.

To determine whether loss of both copies of Pten genes can cooperate with SKP2 expression, we used the Pten conditional knockout mice which were bred with SKP2 transgenic mice and then with the prostate-specific Cre-combinase mice to excise the Pten genes. It has been very difficult to get the homozygous Pten deletion in the Pten conditional mice. The expression of Cre-recombinase was very low in the prostate gland and this might be one reason that we could not obtain the efficient excision of Pten genes in the mice. An additional problem arised when Dr. Jianyu Zheng, the postdoctoral associate for these experiments, left my lab for an independent position at University of Connecticut. The remaining work was picked up by the technicain, Krasimir Spasov, who had been doing the adevovirus-SKP2 and the vitamin D3 work. However, Mr. Spasov had substantial problem in the genotyping of the mice to produce sufficient mice for the required work.

The third aim is to determine whether SKP2 can cooperate with the loss of Nkx3.1, a putative tumor suppressor for prostate cancer (*13, 14*). We never got the Nkx3.1 mice from MMHCC since they have to be generated from frozen embryos. Because of these problems, we ordered the Myc-transgene mice under the probasin promoter control, as well as the p53 conditional knockout mice, from MMHCC to see whether they could have effects on SKP2-induced prostatic tumorigenesis. Both Myc-transgenic and loss of p53 genes in mice have been shown to promote prostate carcinomas, respectively (*15-17*). The effect of p53 loss has been shown to cooperate with the loss of Pten (*11*). We wish to see whether these genetic alterations can promote SKP2-mediated prostatic tumorigenesis in mice. Krasimir Spasov started these experiments. However, he left my lab last year and these experiments were not finished.

In parallel to these studies, two graduate students in my lab, Leigh Ann Higa and Damon Banks, found that the protein stability of p27 is also regulated by a novel ubiquitin E3 ligase containing CUL4-DDB1 (*18*). However, the relationship between SCF^{SKP2} and CUL4-DDB1 ubiquitin E3 ligases in p27 degradation was not clear. In light of these findings, part of the funds was used to support the work of Leigh Ann Higa and Damon Banks. Damon found that not only the protein stability of p27, but also that of tumor suppressor p53, is regulated by the CUL4-DDB1 E3 ligase (*19*). Leigh Ann's studies indicated that loss of CUL4 or DDB1 in cells by siRNA-mediated gene silencing did not affect SCF^{SKP2}-mediated p27 degradation in a cell free extract system for p27 degradation (*18*). Thus, these studies provide evidence that CUL4-DDB1 may regulate p27 stability independent of SKP2. As CUL4A gene is amplified in many breast carcinomas and hepatocellular carcinomas (*20, 21*), it is likely that CUL4-mediated proteolysis may be altered in other cancers including prostate carcinomas.

Since CUL4-DDB1 ubiquitin E3 ligase is a novel protein complex that regulates cell cycle progression and genome stability by targeting p27, p53, and CDT1, a critical replication licensing protein, for ubiquitin-dependent degradation in response to cell signaling and DNA damage (*18, 19, 22, 23*), we have conducted substantial work on

identification of the composition, function, and regulation of CUL4-DD1 complexes. We found that CUL4-DDB1 interacts with a novel WD40-repeat protein, L2DTL/CDT2. L2DTL/CDT2 in turn interacts with PCNA. CDT1, p53, and MDM2 can also interact with PCNA and each of these proteins contains a PCNA-interacting protein motif (PIP) that mediates their interaction with PCNA and the CUL4-DDB1-L2DTL complex (*19, 22*). We found L2DTL and PCNA are part of the CUL4-DDB1 ubiquitin E3 ligase that regulates the protein stability of CDT1 and p53, as well as that of MDM2 after UV-irradiation. Current work has been focused on the identification of the specific WD40-repeat protein for the regulation of p27 turnover. In addition, we found that CUL4-DDB1 interacts with a large group of WD40 repeat proteins, including L2DTL, WDR5, and EED, and use them as substrate-specific subunits, similar to the role of F-box proteins in the SCF ubiquitin E3 ligases, to regulate protein degradation (*24*). WDR5 and EED are essential components of histone methyl-transferases, such as MLL1 and EZH2, for histone H3 methylation at lysine 4 (K4), lysine 9 (K9) and lysine 27 (K27), respectively (*24*). We found CUL4-DDB1 regulates these histone methylation events through interacting with WD40-repeat proteins WDR5 and EED. Please see the attached publications for details. We are currently knocking down CUL4A, CUL4B, DDB1 and L2DTL/CDT2 in mice. We have germline transmission with the CUL4A, DDB1, and L2DTL mice. The genotype of CUL4B is still under investigation. These studies will collectively provide novel mechanism for the low or absent expression of CDK inhibitor p27 in malignant prostate carcinomas.

Key Research Accomplishments:

We have found that a novel ubiquitin E3 ligase containing CUL4-DDB1 ubiquitin E3 ligase regulates the protein stability of p27 independent of SCF^{SKP2} ubiquitin E3 ligase. CUL4-DDB1 ubiquitin E3 ligase also regulates the levels of p53, CDT1, and histone methylation by employing a family of WD40-repeat proteins as the substrate targeting subunits. These studies provide significant progress towards our understanding of the cullin ubiquitin E3 ligases in regulating the cell cycle, genome stability, histone methylation, and tumorigenesis. Our work provides a novel insight into the mechanism by which the protein stability of p27, as well as that of p53, is regulated by ubiquitin-dependent proteolysis. We also found that Pten heterozygous background does not affect the frequency, time of incidences, and tumor grade of prostate tumors/carcinomas the SKP2-transgenic mice.

Reportable outcomes:

Our work has resulted in four reportable publications:

1) 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/$p27^{Kip1}$ and Cyclin E Degradation. *Cell Cycle* 5:1, 71-77.

2) Higa, L. A., Banks, D, Wu, M., Kobayashi, R., Sun, H., Zhang, H. (2006). L2DTL/CDT2 Interacts with the CUL4/DDB1 Complex and PCNA and Regulates CDT1 Proteolysis in Response to DNA Damage. *Cell Cycle* 5:15, 1675-1680.

3) Banks, D., Wu, M., Higa, L. A., Gavrilova, N., Guan, J., Ye, T., Kobayashi, R., Sun, H., Zhang, H. (2006). L2DTL/CDT2 and PCNA interact with p53 and regulate p53 Polyubiquitination and Protein Stability through MDM2 and CUL4A/DDB1Complexes. *Cell Cycle* 5:15, 1719-1729.

4) Higa, L. A., Wu, M., Ye, T., Kobayashi, R., Sun, H., Zhang, H. (2006). CUL4/DDB1 Ubiquitin Ligase Interacts with Multiple WD40-repeat Proteins and Regulates Histone Methylation. *Nature Cell Biology*, 8:1277-1283.

Additional puplications regarding the effect of CUL4A, CUL4B, and DDB1 knockout in mice and their effect on SCF^{SKP2}-mediated p27 degradation and prostate carcinogenesis will be reported later.

List of Personnel receiving pay from the research efforts:
Hui Zhang, Ph. D., Principal Investigator
Jianyu Zheng, Ph. D., Postdoctoral Associate
Krasimir Spasov, M.S., Technician
Lei Ann Higa, B. S., Ph. D. Graduate student
Damon Banks, B. S., Ph. D. Graduate student

Conclusion:
Low or absent expression of CDK inhibitor $p27^{Kip1}$ is associated with malignant prostate carcinomas. Loss of tumor suppressors p53 and Pten is also associated with prostate cancers. We found that p27 is regulated by both SCF-SKP2 and a novel ubiquitin E3 ligase containing CUL4-DDB1-WD40 repeat proteins. In addition, we have identified that a specific CUL4A-DDB1-L2DTL/CDT2-PCNA ubiquitin E3 ligase binds and targets p53 polyubiquitination-dependent proteolysis, in cooperation with MDM2. By interacting with a WD40-repeat proteins as a substrate targeting subunit, the CUL4-DDB1 ubiquitin E3 ligase regulates a wide array of biological events including cell cycle regulation, genome stability, and histone methylation; alteration of these events have been shown to associate with the development of prostate carcinogenesis. We also found that expression of SKP2 and Pten heterozygosity do not cooperate in the mouse prostate carcinoma models, suggesting that SKP2 may act downstream of Pten pathway.

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Appendices

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Report

Involvement of CUL4 Ubiquitin E3 Ligases in Regulating CDK Inhibitors Dacapo/p27^{Kip1} and Cyclin E Degradation

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KEY WORDS

CUL4, Dacapo, p27^{Kip1}, cyclin E, SCF, Proteolysis

ABBREVIATIONS

SCF	the SKP1, CUL1, F-box protein, ROC1/RBX1 complex
DDB1	UV-damaged DNA-binding protein 1
CDK	cyclin-dependent kinase
CSN	the COP9-signalosome complex

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ABSTRACT

The CUL4 (cullin 4) proteins are the core components of a new class of ubiquitin E3 ligases that regulate replication and transcription. To examine the roles of CUL4 in cell cycle regulation, we analyzed the effect of inactivation of CUL4 in both *Drosophila* and human cells. We found that loss of CUL4 in *Drosophila* cells causes G_1 cell cycle arrest and an increased protein level of the CDK inhibitor Dacapo. Coelimination of Dacapo with CUL4 abolishes the G_1 cell cycle arrest. In human cells, inactivation of CUL4A induces CDK inhibitor p27^{Kip1} stabilization and G_1 cell cycle arrest which is dependent on the presence of p27, suggesting that this regulatory pathway is evolutionarily conserved. In addition, we found that the *Drosophila* CUL4 also regulates the protein level of cyclin E independent of Dacapo. We provide evidence that human CUL4B, a paralogue of human CUL4A, is involved in cyclin E regulation. Loss of CUL4B causes the accumulation of cyclin E without a concomitant increase of p27. The human CUL4B and cyclin E proteins also interact with each other and the CUL4B complexes can polyubiquitinate the CUL4B-associated cyclin E. Our studies suggest that the CUL4-containing ubiquitin E3 ligases play a critical role in regulating G_1 cell cycle progression in both Drosophila and human cells.

INTRODUCTION

The CUL1 (cullin 1)-containing SCF (SKP1, CUL1/CDC53, F-box proteins) ubiquitin E3 ligases are key regulators of cell cycle progression from yeast to human.[1,2] The SCF E3 ligases use different F-box proteins to bind and target various cell cycle regulators for ubiquitin-dependent proteolysis. In mammalian cells, it has been shown that SKP2, an F-box protein, primarily binds and targets phosphorylated CDK inhibitors p27^{Kip1} and p21^{Cip1} for ubiquitin-dependent proteolysis, while another F-box protein, human CDC4/AGO/FBXW7 regulates the proteolysis of phosphorylated cyclin E protein.[3-10] In mammalian cells, the G_1 cell cycle is regulated by the relative abundance of G_1 cyclin/CDKs and CDK inhibitors such as p27 and p21.[1,2,11,12] Similarly, the Drosophila G_1 cell cycle is regulated by the balance between the CDK inhibitor Dacapo, which shares substantial homology to p27, and cyclin E.[13,14] While cyclin E is regulated by the conserved Drosophila SCF^{Ago} E3 ligase,[10] it is not clear how the level of Dacapo is regulated in the cell cycle.

Like other cullin family members, CUL1 is regulated by the covalent linkage of an ubiquitin like protein, NEDD8, through the neddylation activating enzyme E1 (APPBP1 and UBA3) and the E2 enzyme, UBC12.[15,16] Neddylation of CUL1 dissociates CAND1, an inhibitor of SCF, from CUL1, and consequently promotes the binding of SKP1 and F-box proteins such as SKP2 to CUL1 and the assembly of the SCF E3 ligase complex.[17,18] The neddylation of CUL1 is removed (deneddylated) by the peptidase activity of the COP9-signalosome complex (CSN).[16,19] Many lines of evidence suggest that the activity of cullins is regulated by the elegant balance between the neddylation and deneddylation activities.[16]

Cullin 4 (CUL4) is a conserved core component of a new class of ubiquitin E3 ligase that also contains the UV-damaged DNA-binding protein 1 (DDB1) and Ring finger protein ROC1 (also called RBX1 or HRT1).[1] Unlike Drosophila or other metazoans, mammals encode two paralogues of CUL4, CUL4A and CUL4B.[20,21] CUL4A and CUL4B are coexpressed in many cells but the functional differences between them remain unclear.[21] Like other cullin E3 ligases, the CUL4 proteins also bind to CAND1 and CSN, and are regulated by neddylation and deneddylation processes.[17,22] Our previous studies

suggest that CUL4-containing E3 ligase complexes and CSN regulate the proteolysis of replication licensing protein CDT1 in response to UV or gamma-irradiation.[21] Additional studies suggest that DDB1, a potential SKP1-like adaptor for CUL4 E3 ligase,[23] is also involved in UV-induced CDT1 proteolysis.[24] The CUL4A-DDB1 complex also regulates the proteolysis of c-Jun and DDB2.[25,26] However, the roles of CUL4-containing ubiquitin E3 ligases in cell cycle regulation remain uncharacterized. We have been investigating the regulation of cell cycle regulators by neddylation and CAND1. Here we report the unexpected finding that CUL4 E3 ligase plays a critical role in regulating G_1 cell cycle progression.

MATERIALS AND METHODS

The human lung carcinoma H1299 cells were obtained from the American Type Culture Collection and cultured in RPMI-1620 medium supplemented with 10% fetal bovine serum and antibiotics. The *Drosophila* Schneider cells (SD2) were cultured in Schneider's *Drosophila* medium while human HEK 293 and HeLa cells were cultured in Dulbecoo's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics as described before.[17,27] The double stranded RNA (dsRNA)- or siRNA-mediated RNA interferences in *Drosophila* SD2 cells or human cells were conducted as described previously.[17,21] The cell cycle was analyzed by flow-cytometry using propidium iodide staining as described before.[21] The anti-*Drosophila* CUL4 antibodies against the carboxy terminal (CUL4CT) and amino terminal (dmCUL4NT) regions, dmGeminin, human CUL4A, CUL4B, the carboxy terminal region (CUL4CT), CUL1, CUL2, SKP2, SKP1, p27 and cyclin E antibodies were described previously.[3,17,21,27] The anti-human DDB1 antibody was kindly provided by Dr. Yue Xiong (University of North Carolina). The monoclonal antibody (N2 7A1) against *Drosophila* Armadillo was obtained from Developmental Studies Hybridoma Bank. The anti-dmCUL1 and dmCAND1 rabbit polyclonal antibodies were raised against the peptides of the last 25 amino acids at the carboxy termini of the proteins after coupling to keyhole limpet hemocyanin (KLH) while GST-dmcyclin E and GST-Dacapo fusion proteins were used to raise polyclonal anti-dmcyclin E and Dacapo antibodies, respectively.

RESULTS

We have previously shown that the CUL1 binding protein CAND1 only interacts with unneddylated CUL1 and inhibits the association between CUL1 and SKP1/SKP2.[17] Neddylation of CUL1 dissociates CAND1 and promotes the binding of SKP1 and SKP2 to CUL1, thus facilitating the assembly of the SCF^{SKP2} E3 ligase complex. To further investigate the biological function of CAND1, we examined the effect of CAND1 inactivation in *Drosophila* SD2 cells on the protein levels of two known SCF substrates, Armadillo/β-catenin (Arm) and cyclin E, which are regulated by $SCF^{\beta\text{-TRCP/Slimb}}$ and $SCF^{Ago/CDC4/FBXW7}$ E3 ligase complexes, respectively.[1] Double-stranded RNA-mediated silencing of *Drosophila* CAND1 homologue, dmCAND1 (CG5366), which shares extensive homology to human CAND1, led to a substantial increase in the protein level of Armadillo (Fig. 1A). This is consistent with our observation that loss of human CAND1 induces a modest increase of CDK inhibitor $p27^{Kip1}$ in human cells.[17] Similar effects of CAND1 deficiency on other SCF substrates have been reported.[28,29] However, when we examined the silencing effects of CAND1 deficiency on *Drosophila* cyclin E, we surprisingly did not see a detectable change in the protein level of dmcyclin E (Fig. 1A). Since the binding of CAND1 to CUL1 is regulated by CUL1 neddylation and deneddylation,[1] we further tested the RNAi effect of the neddylation activating E1 enzyme, APPBP1, and CSN5, a critical component of deneddylation enzyme CSN complex in the presence or absence of CAND1 RNAi. While loss of APPBP1 induces an increase of Armadillo, further accumulation of Armadillo protein is induced if CAND1 and APPBP1 are cosilenced (Fig. 1A). However, there is again no detectable change on the dmcyclin E protein level either in the APPBP1 single knockout or cosilencing of APPBP1 and CAND1 (Fig. 1A). In striking contrast, the protein level of dmcyclin E is greatly increased after silencing of CSN5 or CSN1 (Fig. 1A and Fig. 3A), components of the CSN complex, while there is no significant alteration in the protein level of Armadillo in CSN5 silenced SD2 cells (Fig. 1A). Since Armadillo/β-catenin and cyclin E have been reported to be regulated by $SCF^{\beta\text{-TRCP/Slimb}}$ and $SCF^{Ago/CDC4/FBXW7}$,[1] it is quite unexpected that these two SCF substrates are regulated differentially by CAND1/ APPBP1 and CSN.

To investigate the mechanism of the differential regulation of Armadillo and dmcyclin E by CAND1/APPBP1 and CSN, we explored the possibility that these proteins may be regulated by cullin E3 ligases other than SCF. *Drosophila* has at least six conserved cullin family members (CUL1-CUL6)[1,21] and we have silenced each of them and determined their effects on the protein levels of Armadillo and dmcyclin E (Fig. 1B). While silencing of CUL1 caused the accumulation of both Armadillo and dmcyclin E proteins, a dramatic increase in the protein level of dmcyclin E, but not that of Armadillo, was observed when CUL4 is silenced (Fig. 1B). To determine whether CAND1 can cooperate with CUL1 or CUL4, we silenced all six cullins in the presence or absence of CAND1 RNAi (Fig. 1C). Compared with CUL1 single knockout, cosilencing of CUL1 and CAND1 caused further accumulation of cyclin E or Armadillo proteins (Fig. 1C). However, there is no significant change in dmcyclin E levels between CUL4 single and CUL4/CAND1 double knockout cells (Fig. 1C). These results suggest that while Armadillo is regulated by CUL1, dmcyclin E is regulated by both CUL1 and CUL4. In addition, CAND1 silencing appears to have greater effects on the substrates of SCF E3 ligases than that of CUL4 ligases (Fig. 1C), an effect that may reflect a structural or compositional difference in these two cullin E3 ligase complexes.

In *Drosophila* cells, it has been shown that cyclin E/CDK complexes are regulated by the inhibitory binding of the CDK inhibitor Dacapo, a *Drosophila* protein that shares significant homology to mammalian CDK inhibitors p27 and p21[13,14] Since cyclin E degradation by $SCF^{Ago/CDC4/FBXW7}$ requires cyclin E phosphorylation and since the binding of a CDK inhibitor can inhibit such a phosphorylation,[1] we examined whether CUL4 deficiency-induced dmcyclin E accumulation is dependent on the presence of Dacapo in SD2 cells. We found that cosilencing of Dacapo with either CUL1 or CUL4 did not abolish the increase of dmcyclin E produced by the silencing of either CUL1 or CUL4 (Fig. 1D), suggesting CUL4 regulates dmcyclin E independent of Dacapo. Further characterization indicates that CUL4 regulates cyclin E protein via a post-transcription mechanism since no corresponding increase of dmcyclin E mRNA is observed after CUL4 silencing (Fig. 1D). We also tested whether there is an interaction between CUL4 and cyclin E. We found that cyclin E can bind to CUL4 when it is ectopically expressed in SD2 cells. (Fig. 1E)[21] These findings raise the possibility that CUL4 directly regulates dmcyclin E protein stability via ubiquitin-dependent proteolysis.

To measure the biological significance of CUL4 silencing, we examined the cell cycle progression in control and CUL4 silenced SD2 cells by flow-cytometry. Surprisingly, we found that loss of CUL4 caused a marked G_1 cell cycle arrest (Fig. 2A), an unexpected consequence if dmcyclin E is induced by CUL4 silencing. This observation suggests that there are additional targets of CUL4 in G_1 cell cycle regulation. We tested whether Dacapo is responsible for the G_1 cell cycle arrest induced by CUL4 silencing. Cosilencing of Dacapo and CUL4 counteracted CUL4 RNAi-induced G_1 cell cycle arrest (Fig. 2C). Analysis of the Dacapo protein level in CUL1, CUL4, and CSN silenced cells revealed that Dacapo accumulates in both CUL4 and CSN1 silenced cells but not in those with CUL1 silencing (Fig. 3A). The effect of CUL4 occurs at the protein level of Dacapo, since there is no corresponding change in the Dacapo mRNA in CUL4 silenced cells (Fig. 3B). These studies indicate that CUL4 regulates G_1 cell cycle progression and the primary target of CUL4 in G_1 phase is the CDK inhibitor Dacapo.

To determine whether the CUL4-regulated G_1 events are conserved from *Drosophila* to human cells, we examined the cell cycle effects of silencing human CUL4A and its paralogue CUL4B in cell cycle progression and the levels of CDK inhibitors p27 and p21 by flow-cytometry and Western blotting (Figs. 4 and 5). Initial experiments were conducted in cells containing a

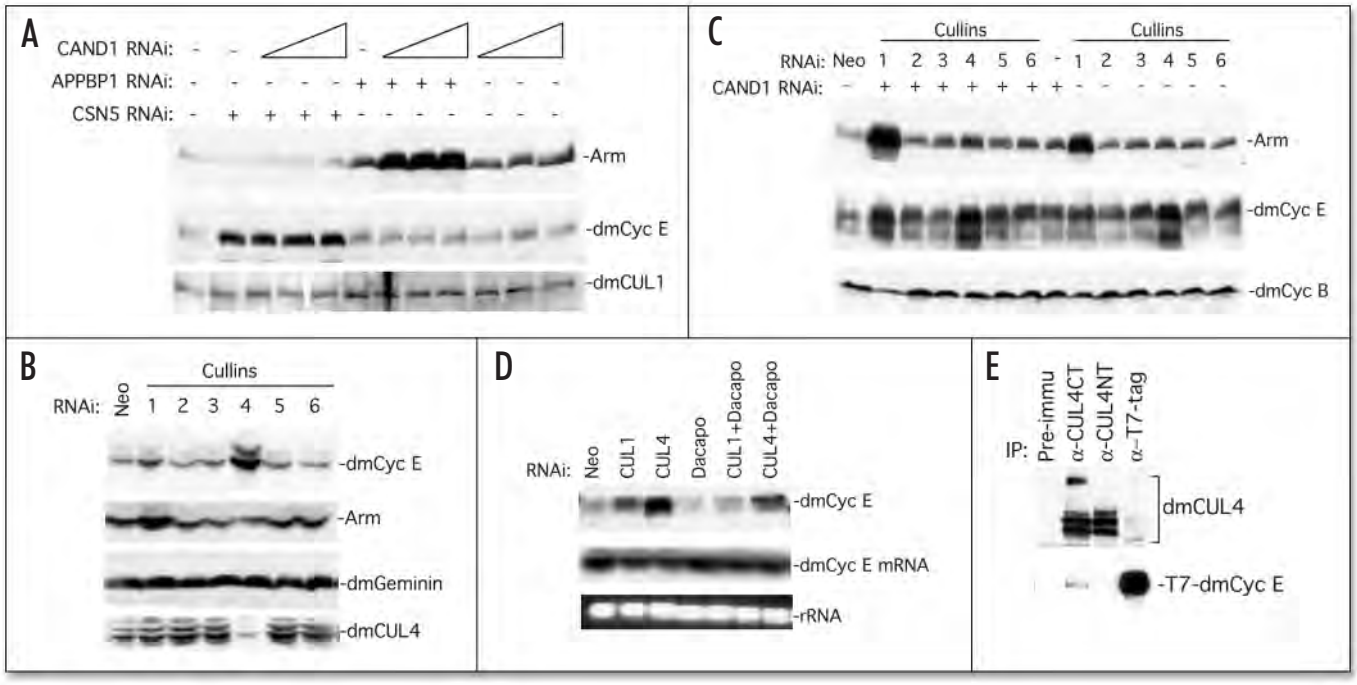

Figure 1. Cyclin E is regulated by both CUL1 and CUL4 independent of Dacapo in *Drosophila* cells. (A) Inactivation of *Drosophila* CAND1, APPBP1, or CSN has differential effects on Armadillo/β-catenin (Arm) and cyclin E proteins. *Drosophila* SD2 cells were transfected with increasing amounts (0, 5, 10, 15 μg) of double-stranded RNA (dsRNA) of CAND1 in the absence or in presence of either APPBP1 or CSN5 dsRNA (each 15 μg) as indicated. The total amount of dsRNAs was compensated with control dsRNA from the bacterial Neo gene. Sixty hours post-RNAi treatment, the protein levels of Armadillo and cyclin E were analyzed by western blotting using their specific antibodies. CUL1 was used as a control. (B) Cyclin E is regulated by both CUL1 and CUL4. Six *Drosophila* cullins (CUL1-CUL6) were silenced with their respective dsRNAs (15 μg each) for 60 hours in SD2 cells. The protein levels of *Drosophila* Armadillo, cyclin E, Geminin, and CUL4 were analyzed by western blotting. (C) Silencing of CAND1 has differential effects in CUL1 and CUL4 substrates. Six *Drosophila* cullins (CUL1-CUL6) were silenced in the presence or absence of 15 μg CAND1 dsRNA as indicated. The levels of Armadillo, cyclin E, and cyclin B were analyzed. (D) *Drosophila* CUL4 regulates cyclin E at the protein level in the absence of Dacapo. The SD2 cells were silenced with dsRNAs (each 15 μg) against control (Neo), CUL1, CUL4, Dacapo, CUL1+Dacapo, and CUL4+Dacapo as indicated. The total RNA and proteins were isolated and the levels of cyclin E protein and mRNA were analyzed by western and northern blots. (E) *Drosophila* cyclin E interacts with CUL4. A T7-tagged cyclin E expression construct was transfected into SD2 cells for 72 hours. The CUL4 complexes and T7-cyclin E were immunoprecipitated with anti-CUL4CT (CUL4 carboxy terminal peptide), CUL4NT (CUL4 amino terminal peptide), and T7 antibodies and western blotted with either CUL4CT or T7 antibodies. Preimmu: preimmune serum control for CUL4CT antibody.

wild-type p53 such as HeLa or U2OS. We found that inactivation of CUL4A by siRNA is sufficient to cause the accumulation of p27 and p21 (Fig. 5B). Further analysis indicates p21 accumulation is a secondary effect caused by p53 accumulation after CUL4A silencing (Fig. 5B) (will be published elsewhere). To avoid the p53/p21 effect, we used human lung carcinoma H1299 cells in which p53 is inactivated by mutation.[30] As indicated in Figure 4, we found that silencing of CUL4A, but not CUL4B, by siRNA is sufficient to induce a marked G_1 cell cycle arrest in H1299 cells. This G_1 cell cycle arrest is dependent on the presence of p27, since p27 markedly accumulated in CUL4A silenced cells and cosilencing of p27 and CUL4A by siRNA eliminated the G_1 cell cycle arrest induced by CUL4A silencing alone (Figs. 4 and 5A). These studies suggest that like Dacapo in *Drosophila* cells, p27 is a primary target for CUL4A mediated G_1 cell cycle arrest in human cells.

Since DDB1 interacts with both CUL4A and CUL4B and serves as an essential component of the CUL4 E3 ligase complexes, we asked if inactivation of DDB1 affects p27 protein level. Loss of DDB1 by siRNA also caused a marked accumulation of p27 protein in both asynchronous growing and hydroxyurea synchronized S phase H1299 cells (Fig. 5D), suggesting that the DDB1-CUL4 E3 ligase complexes are involved in p27 regulation. Further examination indicates that loss of CUL4A or DDB1 stabilized p27 protein, as there is no corresponding increase of p27 mRNA in CUL4A deficient cells, while 27 protein decay rate is reduced in the presence of cycloheximide after silencing of CUL4A or DDB1 (Figs. 6A and B),

Previous studies indicate that p27 stability is regulated by SCF^{SKP2} E3 ligase,[3,31] leading us to ask whether silencing of CUL4A or CUL4B affects the levels of the components of SCF^{SKP2}. We found that CUL1, SKP1, and SKP2 proteins are not detectably affected in CUL4A or CUL4B silenced H1299 cells (Fig. 5A). To determine whether the decreased levels of CUL4A or DDB1 affect the ability of SCF^{SKP2} to target the phospho-threonine 187 dependent degradation of p27 in p27 degradation extracts,[3] we prepared cell extracts from control, DDB1, and CUL4A silenced H1299 cells. The SCF-dependent degradation activity of these extracts was assayed with ^{35}S-methionine labeled p27 in the presence or absence of recombinant cyclin E/CDK2. Our studies suggest that even though DDB1 and CUL4A proteins are markedly reduced by their specific siRNAs, the control and DDB1 or CUL4A deficient extracts retained their ability to proteolyze p27 in the presence of cyclin E/CDK2 (Fig. 6C). These data provide evidence that CUL4A-DDB1 mediated p27 proteolysis may be independent of SCF^{SKP2}, although more rigorous experiments using CUL4A or DDB1 null cells will be needed to address these questions.

In addition to the induction of p27, we have observed that cyclin E also accumulated after inactivation of CUL4A by siRNA (Fig. 5A). Interestingly, while silencing of CUL4A causes an increase in both p27 and cyclin E levels, silencing of CUL4B often caused the accumulation of cyclin E without a significant and concomitant increase of p27 (Fig. 5A). The effect of CUL4B inactivation on cyclin E accumulation occurs at the protein level, since there is no corresponding increase of cyclin E mRNA in CUL4B silenced cells (Fig. 6A). Since *Drosophila* cyclin E accumulates in CUL4 silenced cells independent of Dacapo (Figs. 1 and 3), we further tested the dependence of cyclin E accumulation on p27 in H1299 cells. Cosilencing of p27 with CUL4A could still cause a slight but detectable increase of cyclin E protein

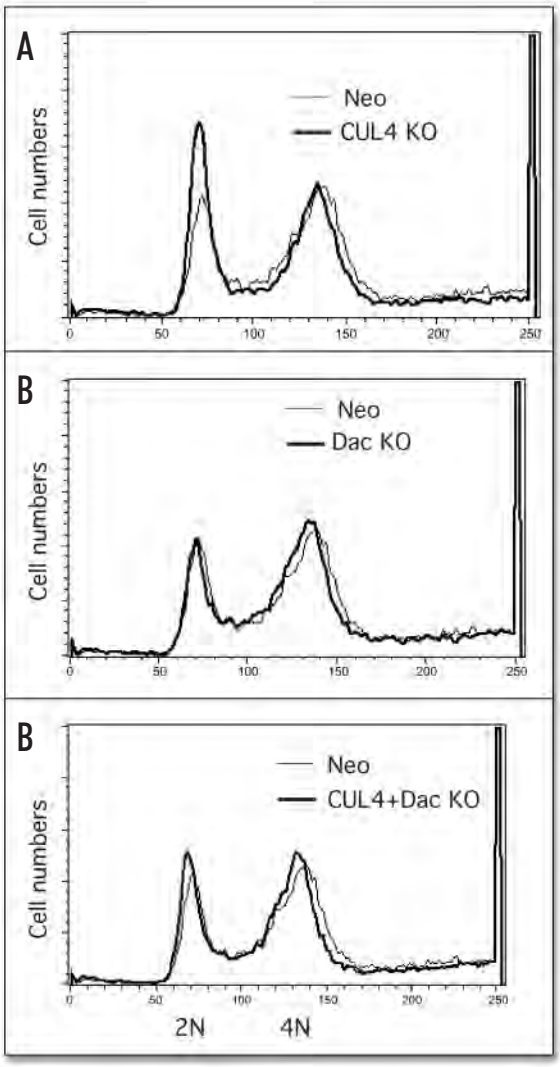

Figure 2. Inactivation of CUL4 causes a Dacapo-dependent G_1 cell cycle arrest in *Drosophila* cells. (A–C) The SD2 cells were transfected with dsRNAs (each 15 μg) of control Neo, CUL4, Dacapo, CUL4+Dacapo for 60 hours as indicated. The cells were harvested, fixed, and stained with propidium iodide (PI) and analyzed by flow-cytometry. Dac: Dacapo. The cell cycle distributions of the RNAi treated cells: Neo RNAi control: 20% G_1, 38% S, 42% G_2/M; CUL4A RNAi: 31% G_1, 32% S, 37% G_2/M; Dacapo RNAi: 18% G_1, 47% S, 35% G_2/M; Dacapo and CUL4 RNAi: 21% G_1, 41% S, and 38% G_2/M.

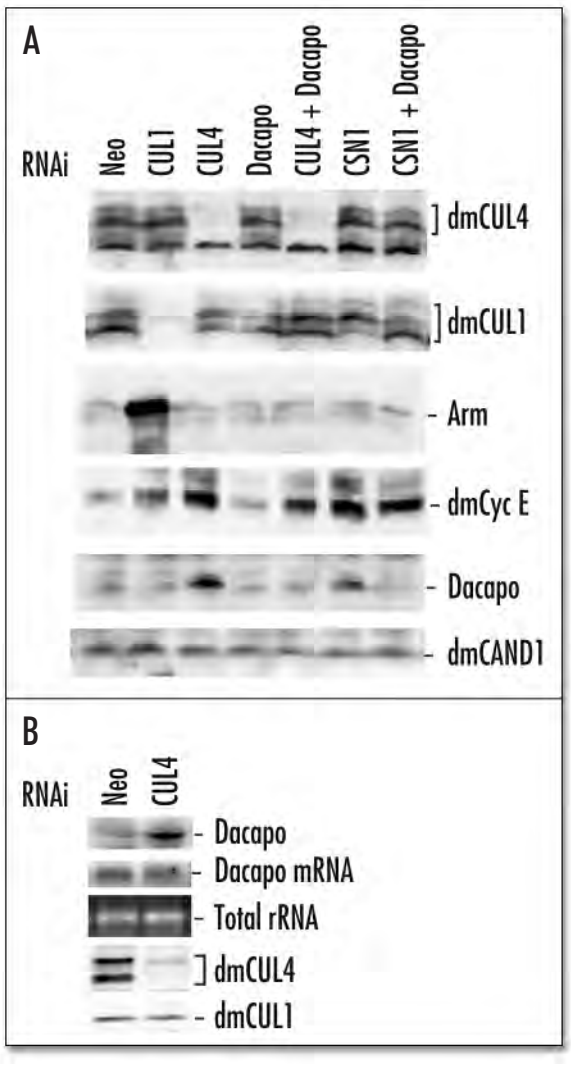

Figure 3. CUL4 and CSN regulate Dacapo protein in *Drosophila* cells. (A) The SD2 cells were transfected with dsRNAs for Neo, CUL1, CUL4, Dacapo, CUL4+Dacapo, CSN1, and CSN1+Dacapo for 60 hours as in Figure 1A. The levels of *Drosophila* CUL4, CUL1, Armadillo, cyclin E, Dacapo, and CAND1 were analyzed by western blotting using their respective antibodies as indicated. (B) CUL4 regulates Dacapo post-transcriptionally. The SD2 cells were silenced with Neo or CUL4 dsRNA as in Figure 1A. The levels of Dacapo mRNA and protein were analyzed by northern and western blotting.

level (Fig. 5C). The slight effect of CUL4A on cyclin E in p27 silenced cells raises the possibility that the effect of CUL4A on cyclin E may be dependent on p27 and that CULA and CUL4B may differentially regulate p27 and cyclin E in human cells. To determine the regulation of cyclin E, we further examined whether CUL4 interacts with cyclin E. We found that the endogenous cyclin E is present in anti-CUL4B immunoprecipitates from H1299 cells (Fig. 7A). Conversely, anti-cyclin E antibodies also coimmunoprecipitated small amounts of CUL4B (Fig. 7A). We also tested whether CUL4 complexes can polyubiquitinate cyclin E. Since cyclin E interacts with CUL4, we transfected a T7-tagged cyclin E expression construct into 293 cells, immunoprecipitated CUL1, CUL2, CUL4A and CUL4B ligase complexes, and assayed the ability of these cullin complexes to polyubiquitinate the associated T7-cyclin E in the presence of E2, E1, ubiquitin and ATP.[21] Our data showed that T7-cyclin E associated with CUL1, CUL4A, and CUL4B, and the cullin-associated T7-cyclin E can be polyubiquitinated in vitro (Fig. 7B). These data provide evidence suggesting that CUL4 complexes may regulate cyclin E stability through interaction and polyubiquitination in vivo.

DISCUSSION

In this study, we report our finding that loss of CUL4 E3 ligases causes a G_1 cell cycle arrest that is dependent on CDK inhibitors Dacapo in *Drosophila* and p27 in human cells. The regulation of Dacapo and p27 by CUL4 E3 ligases occurs at the post-transcriptional levels of protein stability. Although so far we could not demonstrate that p27 can be directly polyubiquitinated by the CUL4 E3 ligase complexes in vitro due to technical difficulties, our study raises the possibility that CUL4 E3 ligases may regulate Dacapo or p27 by directly targeting them for ubiquitin-dependent proteolysis. Several lines of evidence support this hypothesis. We showed that Dacapo protein is regulated by CUL4 but not by CUL1 in *Drosophila* cells (Fig. 3A). Although in human cells, SCF^{SKP2} regulates p27, there is no structural and functional evidence that SKP2 is conserved in *Drosophila* cells.[2,32,33] In addition, although

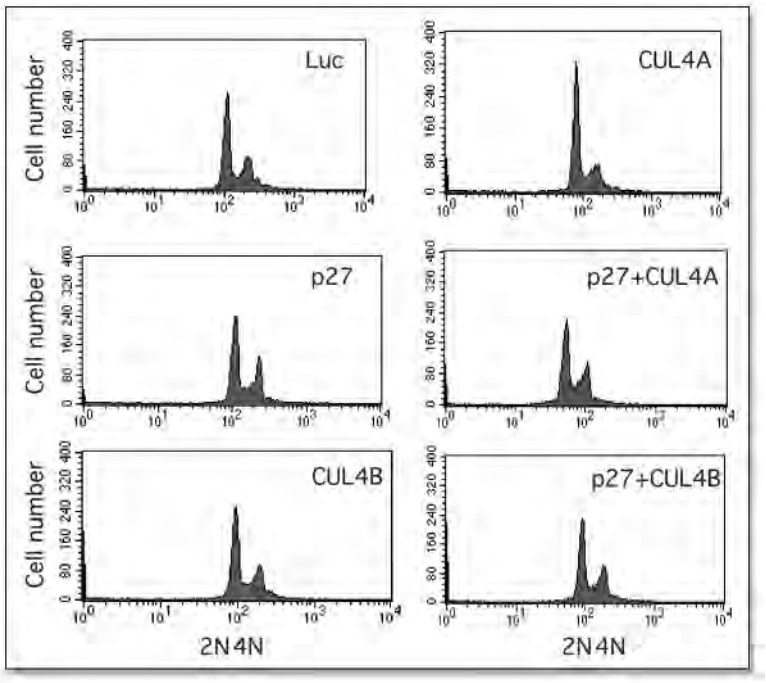

Figure 4. Inactivation of human CUL4A causes $p27^{Kip}$-dependent G_1 cell arrest. Human lung carcinoma H1299 cells were transfected with siRNAs (50 nM each) for luciferase (Luc, a control), CUL4A, p27, p27+CUL4A, CUL4B, and p27+CUL4B for 72 hours. The cells were collected, fixed, and stained with PI. The cell cycle profiles were analyzed by flow-cytometry as indicated. The cell cycle distributions of the cells are: Luciferase siRNA: 56% G_1, 9% S, 35% G_2/M; CUL4A siRNA: 65% G_1, 7% S, 28% G_2/M; p27 siRNA: 55% G_1, 12% S, 33% G_2/M; p27+CUL4A siRNAs: 55% G_1, 9% S, 36% G_2/M; CUL4B siRNA: 57% G_1, 9% S, 34% G_2/M; p27+CUL4B siRNAs: 55% G_1, 12% S, and 33% G_2/M.

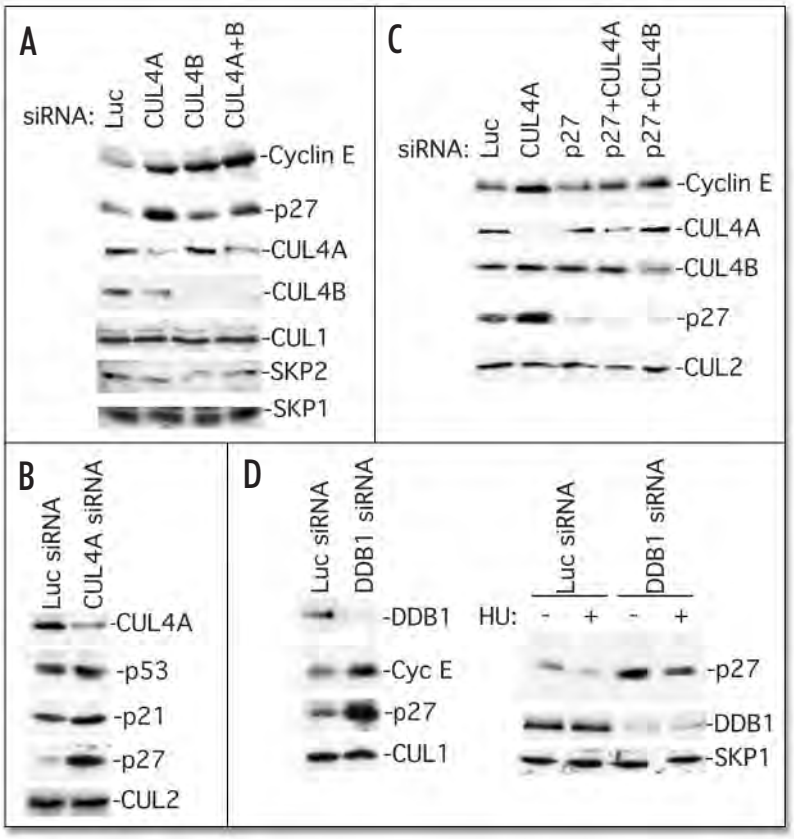

Figure 5. Loss of CUL4A induces p27 accumulation while silencing of CUL4A and CUL4B causes an increase in cyclin E protein. (A) p53 deficient human H1299 cells were transfected with siRNAs (50 nm) for luciferase, CUL4A, CUL4B, CUL4A and CUL4B for 72 hours. The levels of cyclin E, p27, CUL4A, CUL4B, CUL1, SKP2 and SKP1 were analyzed. (B) The same as in (A) except human cervical carcinoma HeLa cells were used and p53 and p21 levels were also analyzed. (C) H1299 cells were transfected with siRNAs for luciferase, CUL4A, p27, p27+CUL4A, and p27+CUL4B as in (A). The protein levels of cyclin E, CUL4A, CUL4B, p27, and CUL2 were analyzed by western blotting. (D) Left panel: H1299 cells were transfected with control luciferase siRNA or DDB1 siRNA for 72 hours. The protein levels of DDB1, cyclin E, p27, and CUL1 were analyzed. Right panel: the same as the left panel except at 60 hours, one set of cells were treated with 5 mM hydroxyurea (HU, +) for 12 hours while the other set (-) remained untreated before harvesting the cells.

Dacapo shares substantial homology to p27 or p21 in the core region that mediates cyclin or CDK binding,[13,14] it diverges greatly at the carboxy terminal end with p27 in which the critical threonine 187 is located for the SCF^{SKP2}-dependent proteolysis of p27 (this threonine is absent in Dacapo).[3,4,34] Furthermore, we found that there are no significant differences in the SCF-dependent p27 degradation between extracts derived from the control and DDB1 or CUL4A siRNA treated cells (Fig. 6C), suggesting that reduced levels of DDB1 and CUL4A proteins does not significantly affect SCF^{SKP2} activity. However, these experiments do not completely rule out the possibility that CUL4A/DDB1 are catalytically involved in SCF^{SKP2}-mediated p27 degradation since small amounts of DDB1 and CUL4A proteins remain in the siRNA treated cells. Moreover, although SKP2 represents a major proteolysis pathway for regulating p27 degradation in S phase of human cells,[1] substantial evidence suggests there are additional pathways that regulate the stability of CDK inhibitors.[35,36] For example, it was found that the *Xenopus* p27 homologue $p27^{Xic1}$ is polyubiquitinated on chromatin only when DNA replication starts in the *Xenopus* egg extracts.[36] We found that replication licensing protein CDT1 is proteolyzed by CUL4/ROC1 E3 ligase in response to UV or gamma-irradiation.[21] CDT1 is also degraded in S phase in mammalian cells and such an event can be reproduced in *Xenopus* egg extracts in which CDT1 was found to undergo ubiquitin-dependent proteolysis once DNA replication starts.[37,38] In *C. elegans*, loss of CUL4 stabilizes CDT1 in S phase and causes the accumulation of polyploid nuclei containing 100C DNA content.[39] It is possible that CUL4 may also regulate the proteolysis of Dacapo or p27 in similar processes in *Drosophila* or human cells.

We also found that cyclin E protein accumulates in CUL4 silenced *Drosophila* and human cells (Figs. 1, 3, 5 and 6) often in the absence of CDK inhibitors Dacapo or p27. Although this effect is more pronounced in *Drosophila* cells, the CUL4 E3 ligase may represent one of several pathways that regulate cyclin E in response to certain signals in mammalian cells.[8-10,40,41] It has been shown that CUL1- and CUL3-containing E3 ligases regulate cyclin E stability in mammalian cells.[42,43] Cyclin E expression and its protein stability are also regulated by an E2F/DP-1 dependent process.[44,45] We found that cyclin E

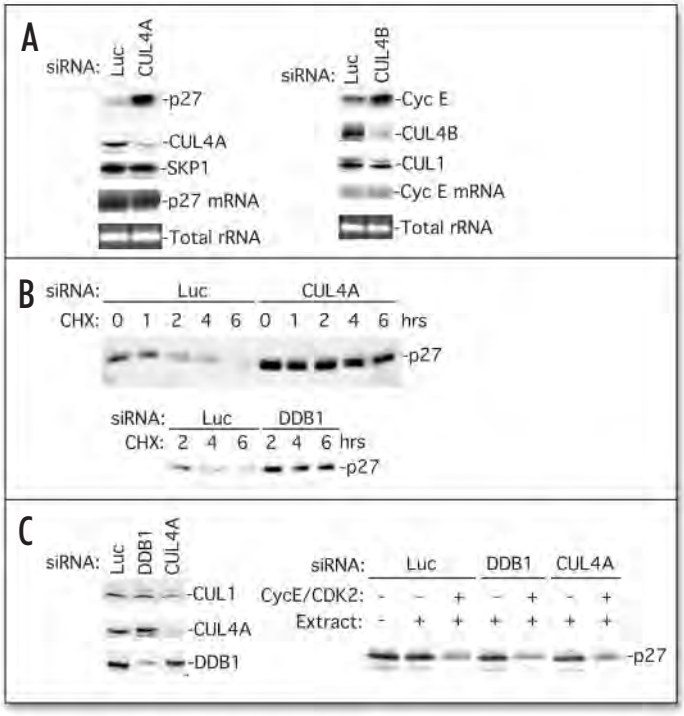

Figure 6. CUL4A regulates p27 stability. (A) H1299 cells were transfected with luciferase, CUL4A, or CUL4B siRNA. The mRNA and protein levels of p27 and cyclin E were analyzed by Western and northern blotting. (B) Inactivation of CUL4A and DDB1 stabilizes p27 protein. H1299 cells were transfected with luciferase, CUL4A or DDB1 siRNAs for 60 hours. The control and CUL4A or DDB1 siRNA treated cells were treated with 100 μg/ml protein synthesis inhibitor cycloheximide (CHX) for 0, 1, 2, 4 and 6 hours and the cells were harvested and analyzed for p27 protein by Western blotting as indicated. (C) Silencing of DDB1 or CUL4A by siRNA does not significantly affect the cyclin E/CDK2-dependent p27 degradation in extracts. H1299 cells were transfected with siRNAs for luciferase, DDB1, or CUL4A for 72 hours. The cytosolic extracts were prepared. The ^{35}S-methionine labeled p27 was in vitro translated in reticulocyte lysates. It was then incubated with extracts for one hour at 30°C in the absence or presence of recombinant GST-cyclin E/CDK2 as indicated. After incubation, the remaining amount of p27 was determined by immunoprecipitation and phospho-image analysis.

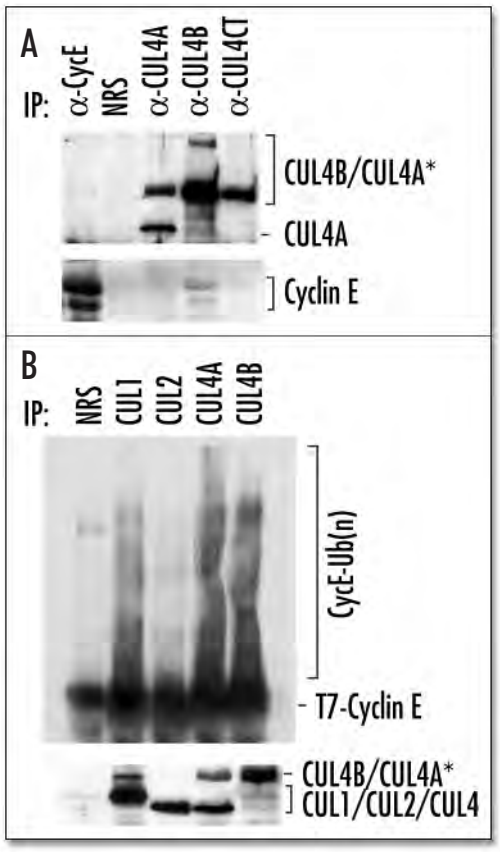

Figure 7. CUL4B interacts with cyclin E and both CUL4A and CUL4B immunocomplexes can polyubiquitinate cyclin E in vitro. (A) Lysates were prepared from actively growing H1299 cells and immunoprecipitated with anti-cyclin E, control normal rabbit serum (NRS), CUL4A, CUL4B and CUL4CT (CUL4 carboxy terminal peptide) antibodies. The immunocomplexes were blotted with a mixture of anti-CUL4A and CUL4B antibodies (top) and anti-cyclin E antibodies (bottom). (B) The T7-tagged cyclin E expression construct was transfected into 293 cells for 36 hours. The cells were then treated with 50 μg/ml MG132, an inhibitor of the 26S proteosome, for 4 hours. The CUL1, CUL2, CUL4A and CUL4B complexes were isolated by immunoprecipitation and the associated T7-cyclin E was analyzed for polyubiquitination by incubating with ubiquitin E2 (CDC34, 100 ng), ubiquitin activating enzyme E1 (50 ng), ubiquitin (2 μg), and 2 mM ATP at 30°C for 1 hour. The ubiquitinated cyclin E was detected by western blotting using anti-T7 antibody.

directly interacts with *Drosophila* CUL4 and human CUL4B and the isolated CUL4A or CUL4B immunocomplexes can polyubiquitinate the associated cyclin E in vitro (Fig. 1E and Fig. 7). These observations raise the possibility that cyclin E may also be a direct ubiquitination target of CUL4 E3 ligases in vivo.

Our studies indicated that loss of CAND1, APPBP1, or CSN has differential effects on Armadillo/β-catenin and cyclin E (Fig. 1). This effect could be partly explained by our observation that while Armadillo is regulated by CUL1-containing SCF ligase, cyclin E is controlled by both CUL1 and CUL4 E3 ligases (Fig. 1B and C). We also provide evidence that the effects of CAND1, APPBP1 or CSN deficiency on the substrates of various cullin E3 ligases may be different (Fig. 1C). Further analysis is required to investigate the mechanisms for these observations.

Our data reveal that CUL4 E3 ligase represents a novel and conserved pathway from *Drosophila* to human cells in regulating CDK inhibitors and cyclin E. In the G_1 cell cycle, the CDK inhibitors Dacapo and p27 appear to be the primary targets of CUL4 E3 ligases, as loss of CUL4 in *Drosophila* or CUL4A in human leads to the G_1 cell cycle arrest rather than enhanced S phase entry. Since the gene encoding CUL4A is amplified in many breast cancers and hepatocellular carcinomas[46,47] and since low or absent expression of p27 is often associated with malignant cancers,[48] our studies also highlight how altered regulation of CUL4 E3 ligase may contribute to the genesis and progression of human cancers.

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Report

L2DTL/CDT2 Interacts with the CUL4/DDB1 Complex and PCNA and Regulates CDT1 Proteolysis in Response to DNA Damage

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KEY WORDS

CUL4, DDB1, CDT1, PCNA, L2DTL/CDT2, proteolysis

ABBREVIATIONS

CDT1	CDC10-dependent transcript 1
DDB1	UV-damaged DNA-binding protein 1
L2DTL/CDT2	lethal(2) denticleless/ CDC10 transcript 2
CSN	The COP9-signalosome complex
PCNA	proliferation cell nuclear antigen

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ABSTRACT

The CUL4 (cullin 4) proteins are the core components of a new class of ubiquitin E3 ligases that regulate cell cycle, DNA replication and DNA damage response. To determine the composition of CUL4 ubiquitin E3 ligase complex, we used anti-CUL4 antibody affinity chromatography to isolate the proteins that associated with human CUL4 complexes and identified them by mass-spectrometry. A novel and conserved WD40 domain-containing protein, the human homologue of Drosophila lethal(2) denticleless protein (L2DTL) or fission yeast CDT2, was found to associate with CUL4 and DDB1. L2DTL also interacts with replication licensing protein CDT1 in vivo. Loss of L2DTL in Drosophila S2 and human cells suppressed proteolysis of CDT1 in response to DNA damage. We further isolated the human L2DTL complexes by anti-L2DTL immuno-affinity chromatography from HeLa cells and found it associates with DDB1, components of the COP9-signalosome complex (CSN) and PCNA. We found that PCNA interacts with CDT1 and loss of PCNA suppressed CDT1 proteolysis after DNA damage. Our data also revealed that in vivo, inactivation of L2DTL causes the dissociation of DDB1 from the CUL4 complex. Our studies suggest that L2DTL and PCNA interact with CUL4/DDB1 complexes and are involved in CDT1 degradation after DNA damage.

INTRODUCTION

CUL4 (cullin 4) is a conserved core component of a new class of ubiquitin E3 ligases that regulate genome stability, chromatin structure, replication and transcription.[1] CUL4 binds to the UV-damaged DNA-binding protein 1 (DDB1), a putative adaptor protein for CUL4 E3 ligase and Ring finger protein ROC1 (also called RBX1 or HRT1) to form an active ubiquitin E3 ligase complex.[1] Previous studies show that DDB1 also interacts with DDB2 and CSA which are implicated in nucleotide excision repair (NER).[2,3] One of the best-characterized substrates of CUL4 E3 ligase is the replication licensing protein CDT1. In metazoans, CDT1 is targeted for proteolysis by the CUL4/ROC1 E3 ligase complexes in response to UV or gamma-irradiation.[4] DDB1 is also involved in the UV-induced CDT1 degradation.[5] Aberrant activation of CDT1, either by the loss of its inhibitor Geminin or its unscheduled expression,[6,7] causes rereplication of the genome and the formation of polyploid nuclei. In Caenorhabditis elegans, loss of CUL4 stabilizes CDT1 in S phase and induces the accumulation of polyploid nuclei containing 100C DNA content.[8]

Our recent studies indicate that CUL4/DDB1 E3 ligase complexes also control the G_1/S transition by regulating the protein stability of CDK inhibitors Dacapo/$p27^{Kip1}$ in both Drosophila and human cells.[9] Loss of Drosophila CUL4 or human CUL4A causes G_1 cell cycle arrest and such an arrest can be prevented by coinactivation of Dacapo or p27 with CUL4. The CUL4/DDB1 complex also regulates cyclin E protein turnover.[9] The CUL4A encoding gene is amplified in many breast cancers and hepatocellular carcinomas,[10,11] indicating altered expression of CUL4A may contribute to tumorigenesis.

Although the CUL4/DDB1 E3 ligase complex plays a critical role in regulating the cell cycle progression, replication and DNA damage, the composition of the complex and its regulation remain largely unknown. In this report, we have identified L2DTL as a novel protein that interacts with the CUL4/DDB1 complex. Further analysis of the L2DTL complex revealed its association with PCNA. We also show that both L2DTL and PCNA are involved in CDT1 proteolysis in response to DNA damage.

MATERIALS AND METHODS

Isolation of the CUL4 and L2DTL complexes. Affinity purified rabbit CUL4B or L2DTL polyclonal antibodies were covalently linked to Protein A Sepharose beads using a method described previously.[12] Lysates from twenty liters of suspension HeLa cells were passed through the immobilized CUL4B or L2DTL antibody/protein A chromatography and the proteins in the complexes were eluted, separated in protein gel and each protein band was excised and identified by mass-spectrometry as described previously.[12] Various peptides corresponding to DDB1 and the components of CSN were identified. Three peptides of L2DTL (LYNTESQSFRK, LVTAAGDQTAK and GSVSSVSPKPPSSFK) were obtained from the CUL4B complex while one peptide of PCNA (FSASGELGNGNIK) was obtained from the L2DTL complex. These peptides matched exactly to the human L2DTL and PCNA protein sequences, respectively. The cDNAs of the genes encoding identified proteins were obtained from American Type Culture Collection (ATCC) and used for binding assays. Drosophila cDNAs were obtained from the Drosophila Genomics Resource Center (DGRC).

Cells, RNAi and siRNA and transfection. Human HEK293, HeLa, U2OS, RKO and U87MG cells and Drosophila Schneider D2 cells were cultured as described previously.[4,12] The sequence of human L2DTL siRNA is AAUCCCUUCUGACUGGUUAUC. The siRNAs for CUL4, DDB1 and PCNA were synthesized as previously described.[4,9,13,14] Silencing of Drosophila CUL4 and L2DTL were conducted using dsRNAs in vitro transcribed from CUL4 and L2DTL cDNAs as described previously.[4]

Antibodies. The CUL2, CDT1, CUL4A, CUL4B, CUL4CT and DDB1 antibodies were used as described.[4,6,9] Drosophila L2DTL peptide LASPRLQSLRQSECSPRIHASPRRRISHT and human L2DTL peptide CRKICTYFHRKSQEDFCGPEHSTEL were synthesized and used for raising rabbit polyclonal antibodies using the method described previously.[4] We also raised rabbit polyclonal antibodies against the glutathione-S-transferase (GST) fused with the full-length human L2DTL protein, GST-L2DTL. Anti-PCNA antibody (PC10) was purchased from Santa Cruz Biotechnology, Inc., California, and anti-CSN5 and CSN2 antibodies were from Boston Biochem.

RESULTS AND DISCUSSION

Isolation of L2DTL as a CUL4 interacting protein. We have previously shown that CUL4/ROC1 E3 ligase targets CDT1 for ubiquitin-dependent proteolysis in response to UV or gamma-irradiation.[4] Since the CUL4 complex represents a new type of cullin-containing E3 ligases that is very different from the SCF, CUL2 and CUL3 ubiquitin E3 ligase complexes, we attempted to determine the protein composition of the CUL4 complex to understand the mechanism of substrate recognition and its regulation. To identify these proteins, we utilized the anti-CUL4 antibody affinity chromatography to isolate CUL4 complexes from human cells. As shown in Fig. 1A, mass-spectrometry analysis revealed that the isolated human CUL4B complex from HeLa cells contained DDB1, components of the CSN complex and the human homologue of Drosophila lethal(2) denticleless protein (Fig. 1A),[15] L2DTL, a conserved WD40 repeat-containing protein which is essential for Drosophila embryogenesis but without any known molecular function (Fig. 1D). L2DTL also shares homology with the *S. pombe* CDT2.[16,17]To further determine the interaction, we raised rabbit polyclonal antibodies against human L2DTL protein.

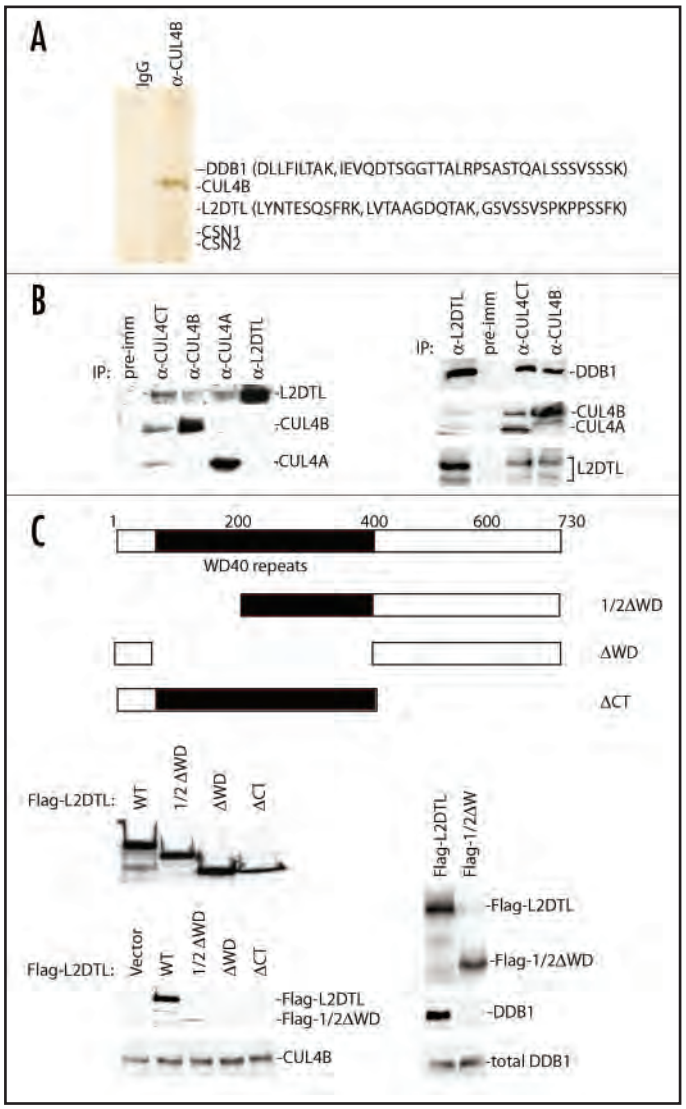

Figure 1. Isolation of human homologue of L2DTL that interacts with CUL4 and DDB1. (A) Human CUL4B complexes were immuno-affinity purified using the affinity purified anti-CUL4B antibody and the peptides derived from the associated DDB1, human L2DTL, and CSN proteins were identified by mass-spectrometry as indicated. IgG: control antibodies. (B) Human L2DTL interacts with CUL4A, CUL4B and DDB1. The CUL4 and L2DTL complexes were immunoprecipitated from HeLa cells and western blotted with anti-L2DTL, CUL4A, CUL4B, and CUL4CT (recognizes both CUL4A and 4B), and DDB1 antibodies as indicated. Pre-immun: pre-immune serum for L2DTL antibody control. (C) Human L2DTL contains a conserved WD40 repeat domain and both the WD40 repeats and carboxy terminal region are required for binding to CUL4 and DDB1. The human L2DTL and deletion mutants 1/2 WD (a.a. 206-730), ΔWD (a.a. 1-65, 390-730), and ΔCT (a.a. 1-429), were epitope-tagged at the amino terminus with the Flag-tag (pCMV-Tag 2B). The expression constructs of wild-type and the mutants were transfected into 293 cells and assayed for their binding to CUL4B and DDB1 by immunoprecipitation and western blotting as indicated.

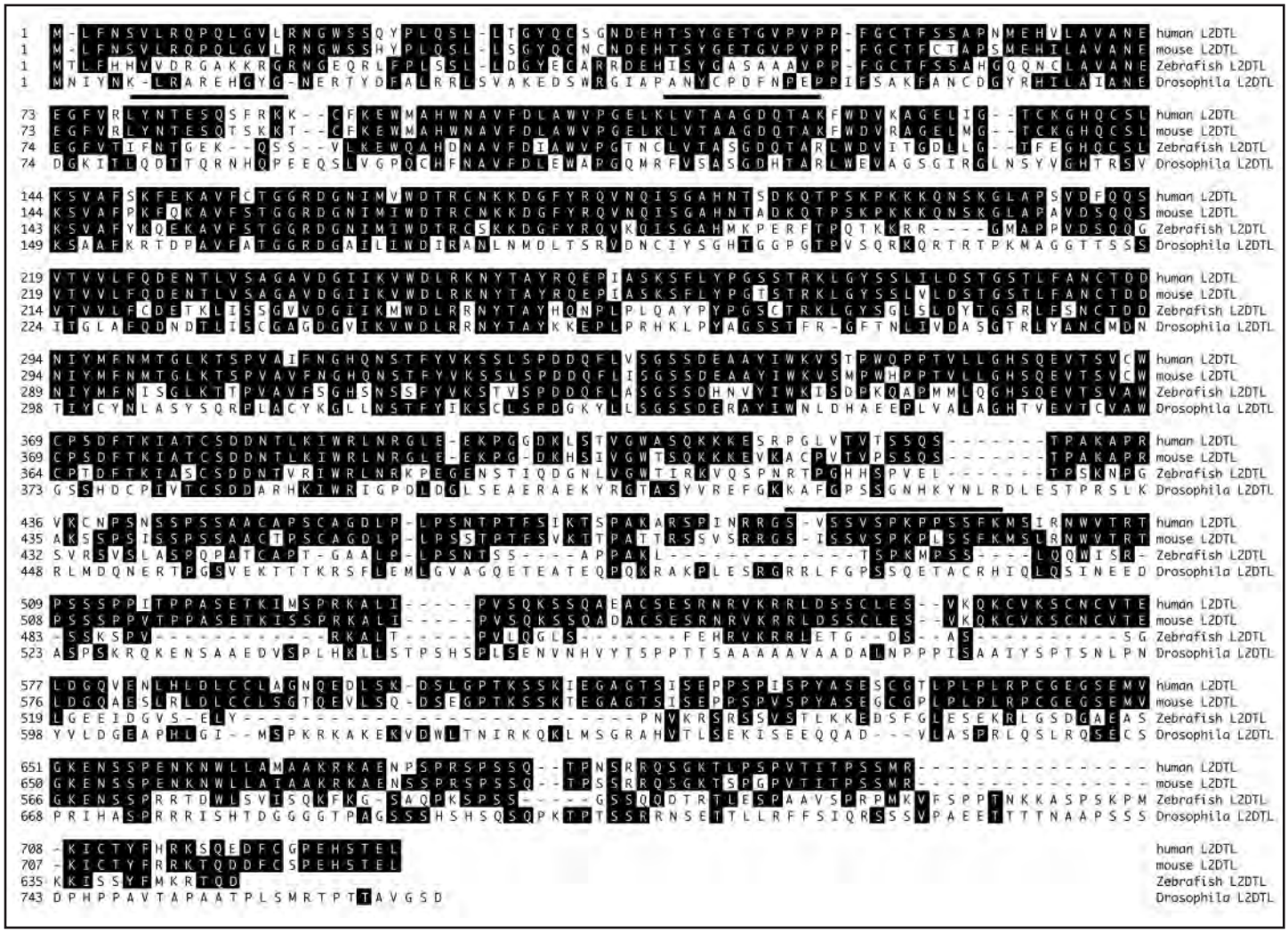

Figure 1D. Isolation of human homologue of L2DTL that interacts with CUL4 and DDB1. D) Protein sequence alignment of L2DTL from different species. The peptide sequences of human L2DTL identified by mass-spectrometry are indicated by the heavy lines above the human L2DTL protein.

Coimmunoprecipitation performed with L2DTL antibodies confirmed that human L2DTL indeed interacts with endogenous CUL4A, CUL4B and DDB1 in human cells (Fig. 1B). While the anti-L2DTL antibodies consistently coimmunoprecipitated DDB1 (Fig. 1B), the interaction between L2DTL and CUL4A or CUL4B varies between experiments (Fig. 1B), suggesting L2DTL may preferentially interact with DDB1. However, the lack of a DDB1 antibody that can immunoprecipitate the CUL4/DDB1/L2DTL complexes prevented us from further exploring this possibility. We also raised an antibody against the Drosophila L2DTL protein. Our data indicate that the Drosophila L2DTL protein can be detected in the anti-CUL4 immuno-complex in Drosophila Schneider D2 (S2) cells (Fig. 2A).

Structurally, the L2DTL protein contains a conserved WD40 repeat region at its amino terminus and a less conserved carboxy half (Fig. 1C).[15] To determine the region in L2DTL that mediates its interaction with CUL4 and DDB1, we made deletion mutants that lack either the WD40 repeats or the carboxy terminal region and analyzed their binding to CUL4 or DDB1. While wild-type L2DTL binds both CUL4 and DDB1, loss of either the WD40 repeats or the carboxy terminal region severely impaired the association between L2DTL and CUL4 or DDB1 (Fig. 1C, data not shown), suggesting that both domains are required for L2DTL interaction with DDB1-CUL4 complexes.

L2DTL is required for CDT1 degradation in response to DNA damage. One of the best characterized CUL4 substrates is replication licensing protein CDT1.[4] To determine L2DTL function, we initially silenced the expression of Drosophila L2DTL in S2 cells by RNA interference (RNAi) because of highly efficient gene silencing in these cells.[6] We then examined the effect of L2DTL inactivation on CDT1 proteolysis induced by gamma-irradiation. While CDT1 is rapidly degraded in response to gamma-irradiation in S2 cells treated with control dsRNA (Neo), inactivation of L2DTL by RNAi completely blocked CDT1 proteolysis after radiation (Fig. 2B). This effect is similar to inactivation of CUL4 on CDT1 in parallel experiments (Fig. 2B),[4] indicating that Drosophila L2DTL is required for CDT1 proteolysis in response to DNA damage.

L2DTL interacts with DDB1, CSN and PCNA. We have previously shown that replication licensing protein CDT1 serves as a substrate of CUL4 ubiquitin E3 ligase complex.[4] The inactivation of Drosophila L2DTL suppresses CDT1 proteolysis in response to DNA damage (Fig. 2). We therefore tested whether L2DTL can interact with CDT1. Since Drosophila anti-L2DTL antibodies do not immunoprecipitate L2DTL complex (Fig. 2A and data not

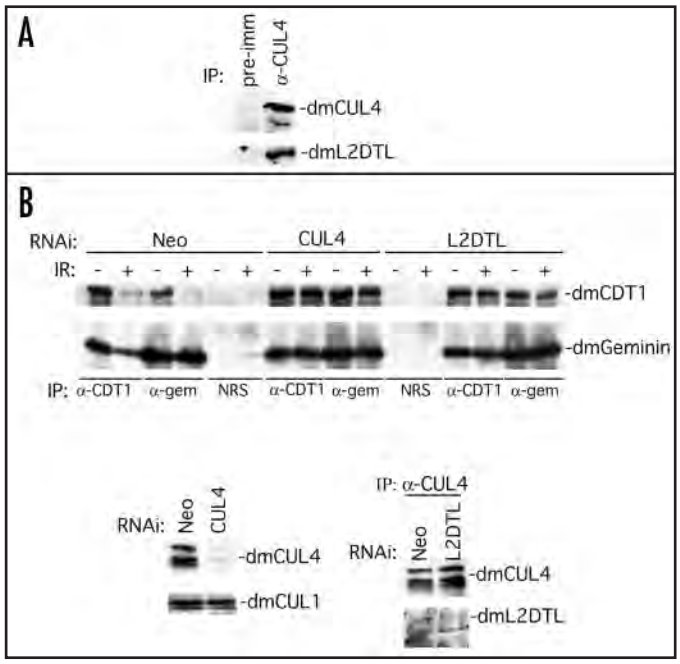

Figure 2. L2DTL interacts with CDT1 and regulates CDT1 protein stability in response to gamma-irradiation in Drosophila. (A) Drosophila L2DTL interacts with CUL4. Lysates from Drosophila S2 cells were immunoprecipitated with pre-immune or anti-CUL4 antibodies followed by western bloting with anti-Drosophila CUL4 and L2DTL antibodies as indicated. (B) Silencing of Drosophila L2DTL by RNAi suppresses CDT1 degradation in response to γ-irradiation. The Drosophila S2 cells were treated with 15 μg dsRNAs of neomycin (Neo, control), CUL4, and L2DTL for 60 hours. Half of the cells were gamma-irradiated (IR) with 100 Gy as indicated and all cells were harvested 30 minutes later. The protein levels of CDT1, geminin, CUL4, CUL1, and CUL4-associated L2DTL were analyzed by specific antibodies to assess the effects of radiation and RNAi.

shown), we characterized the interaction between L2DTL and CDT1 in human cells. L2DTL can be detected in the anti-CDT1 immunocomplexes while CDT1 is also present in anti-L2DTL complexes, albeit the signal is relatively weak under our immunoprecipitation conditions. Our data indicate that there is an interaction between the endogenous L2DTL and CDT1 proteins (Fig. 3A). These studies are consistent with our previous findings and observations from other groups that CUL4 and its associated DDB1 interact with CDT1 in vivo.[4,5]

Because of the relative weak interaction between L2DTL and CDT1 under various conditions, we considered the possibility that there may be additional subunits in the CUL4 complexes that regulates CDT1 stability. To further identify the proteins that associate with L2DTL and CUL4 complexes, we affinity purified L2DTL complexes using anti-L2DTL antibodies as the affinity resins for chromatography from HeLa cell lysates. Mass-spectrometry was used to analyze proteins that are specifically associated with L2DTL complexes (Fig. 3B). Peptides corresponding to DDB1 and subunits of the CSN complex were obtained from the isolated protein bands in L2DTL complexes (Fig. 3B). One of the peptides in L2DTL complexes corresponds to PCNA (Fig. 3B). To confirm these interactions, we performed immunoprecipitation of cell lysates prepared from various human cells, followed by western blotting using anti-L2DTL, CSN5 and PCNA antibodies. Our data confirmed that endogenous CSN5 and PCNA proteins are indeed present in L2DTL complexes isolated from various mammalian cells (Fig. 3C).

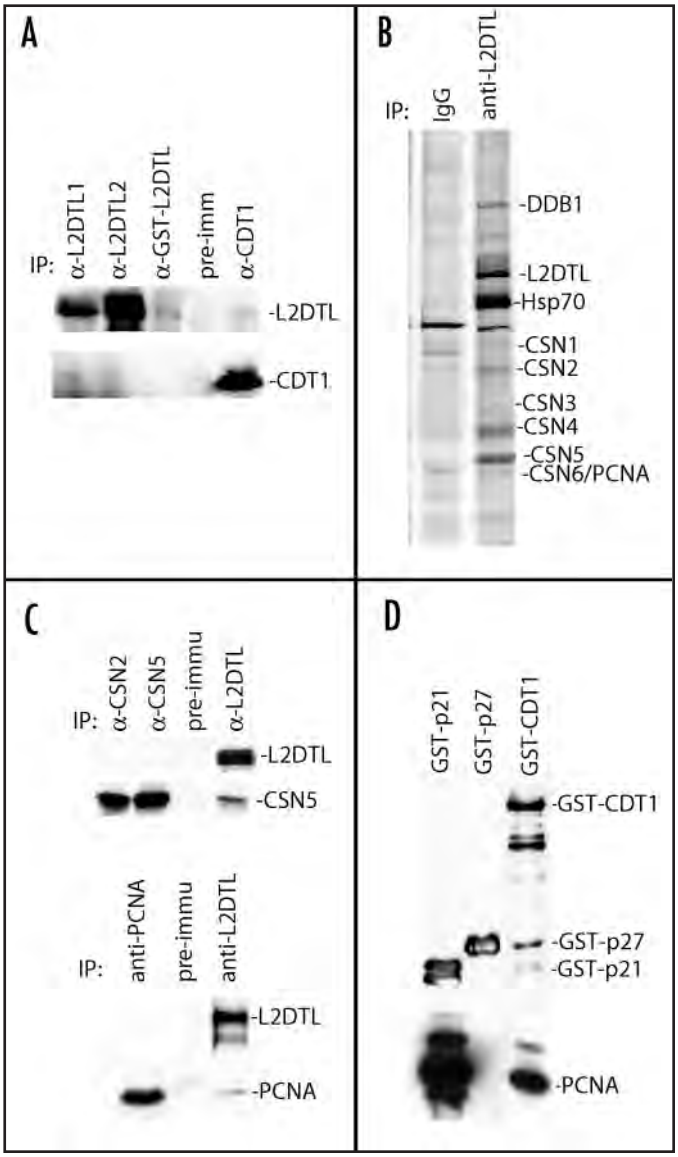

Figure 3. The isolated L2DTL complexes contain DDB1, components of CSN, and PCNA and both L2DTL and PCNA interact with CDT1. (A) Human L2DTL interacts with CDT1. Human L2DTL and CDT1 complexes were immunoprecipitated with three different L2DTL antibodies and a CDT antibody as indicated. They were western blotted with L2DTL and CDT1 antibodies. (B) The L2DTL complex associates with DDB1, CSN, and PCNA. The L2DTL complex was isolated from HeLa cells by anti-L2DTL antibody affinity chromatography. The L2DTL-associated proteins were identified by mass-spectrometry. DDB1, CSN, and PCNA peptides were found in the L2DTL complex as described in Materials and Methods.(C) L2DTL interacts with endogenous CSN and PCNA in vivo. The L2DTL, CSN2, CSN5, and PCNA complexes were immunoprecipitated from RKO cells after treating cells with MG132 for three hours. The immunoprecipitated proteins were blotted with anti-CSN5, PCNA (PC10), and anti-L2DTL antibodies. (D) PCNA directly interacts with CDT1. SF9 insect cells were co-infected with baculovirus encoding PCNA and either GST-p21, GST-p27 or GST-CDT1. The complexes were isolated by glutathionine beads, washed and blotted with anti-PCNA antibody. The GST-proteins were detected by an anti-GST antibody.

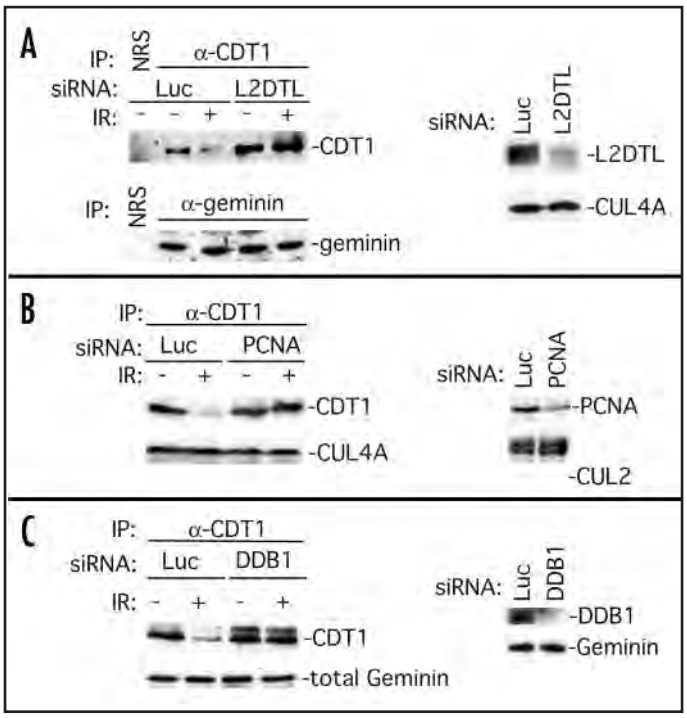

Figure 4. L2DTL and PCNA regulate CDT1 proteolysis in response to DNA damage. (A) Loss of human L2DTL suppresses CDT1 degradation in response to gamma-irradiation. HeLa cells were treated with 50 nM siRNAs for Luciferase (Luc, control) or L2DTL for 72 hours. The cells were irradiated by gamma-irradiation (10 Gy) and the levels of L2DTL, CUL4A, CDT1, and geminin were analyzed after one hour. (B, C): Loss of PCNA (B) or DDB1 (C) stabilizes CDT1 in response to gamma-irradiation. The human H1299 cells were treated with 50 nM siRNAs against luciferase, PCNA, or DDB1 for 72 hours as indicated. Half of the cells were irradiated with gamma-irradiation as in Fig. 4A and after one hour, the levels of CDT1, PCNA, CUL4A, and Geminin were examined by western blotting as indicated.

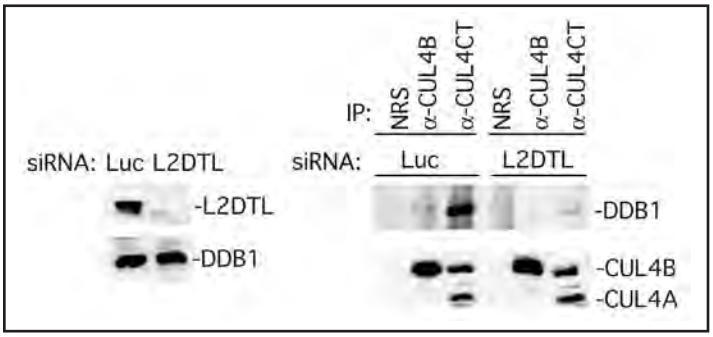

Figure 5. Loss of L2DTL reduces DDB1 binding to CUL4 complex. Expression of L2DTL was silenced by L2DTL siRNA in HeLa cells as in Fig. 4. The CUL4 complexes were isolated by anti-CUL4B and CUL4CT (CUL4A and CUL4B) immunoprecipitation and assayed for DDB1 binding by western blotting. The total protein levels of DDB1 and silencing effect of L2DTL by siRNA were also examined in parallel.

L2DTL and PCNA are required for CDT1 degradation in mammalian cells. To determine whether L2DTL functions to regulate CDT1 stability in human cells, we silenced the expression of human L2DTL in HeLa, U2OS, or other human cell lines by small interfering RNA (siRNA). Loss of human L2DTL in these cells also suppresses CDT1 degradation in response to gamma-irradiation (Fig. 4A). These studies demonstrate that L2DTL is a novel protein that associates with CUL4 and DDB1 and is required for CDT1 degradation in response to DNA damage in both Drosophila and human cells. Sometimes, we have observed that CDT1 is stabilized in L2DTL silenced cells in the abscence of irradiation (Fig. 4A). This effect was also sometimes observed in DDB1 or CUL4 silenced cells (data not shown). Since the protein stability of CDT1 is regulated in S phase,[8,16] it is possible that L2DTL and DDB1/CUL4 complexes regulate CDT1 proteolysis in S phase cells.

Since inactivation of L2DTL prevented CDT1 degradation in response to DNA damage, we also silenced the expression of PCNA in human cells. Similar to L2DTL or DDB1 silenced cells (Figs. 4A and C), inactivation of PCNA by siRNA prevented CDT1 proteolysis in response to gamma irradiation (Fig. 4B). We also examined whether CDT1 interacts with PCNA. We found that the recombinant CDT1 and PCNA directly interact in insect SF9 cells infected with baculoviruses encoding CDT1 and PCNA cDNAs (Fig. 3D). These studies suggest that PCNA is involved in regulating DNA-damage induced proteolysis of CDT1. These data, together with our previous observation, suggest that CDT1 proteolysis after DNA damage requires the presence of L2DTL, PCNA and the DDB1/CUL4 E3 ligase complexes.

Loss of L2DTL causes the dissociation of DDB1 from CUL4 complex. Since L2DTL binds to DDB1 and CUL4, we explored the mechanism for the requirement of L2DTL by the CUL4 complex. We found that loss of L2DTL by siRNA sometimes reduced the binding of DDB1 to CUL4 complexes (Fig. 5). These observations suggest that one function of L2DTL may be to facilitate the interaction between DDB1 and CUL4A complexes in vivo.

In this report, we found that a novel WD40 repeat-containing protein, L2DTL, binds to DDB1, CUL4, PCNA and CSN (Figs. 1 and 3). Our studies further indicate that PCNA associates with L2DTL and CDT1 (Fig. 3). Similar to the effect of loss of CUL4 and DDB1, inactivation of either L2DTL or PCNA prevented CDT1 proteolysis in response to DNA damage. In contrast, inactivation of CSN5 or CSN2, components of CSN complex, by siRNAs did not alter CDT1 proteolysis after DNA damage, even though the protein levels of CSN2 or CSN5 were substantially reduced. This differs from our previous data, which demonstrated loss of CSN complex abolished CDT1 degradation in Drosophila S2 cells after gamma-irradiation.[4] It is possible that the siRNAs we have used against human CSN2 or CSN5 were still not sufficient to silence the expression and the activity of CSN to the level that can impact CDT1 proteolysis. Alternatively, because cullin deneddylation can be mediated by CSN and DEN1,[1,17,18] it is possible that the function of CSN and DEN1 may overlap. In the accompanying paper, our studies also showed that CUL4A, DDB1, L2DTL and PCNA also interact with p53 and MDM2 in human cells and are required for the CUL4-mediated p53 polyubiquitination activity. These studies suggest that L2DTL and PCNA may be part of the CUL4 complexes that regulate the protein stability of CUL4 substrates such as CDT1 and p53. In this regard, we found that loss of L2DTL often leads to the dissociation of DDB1 from CUL4 complex. It is possible that L2DTL may play a role in promoting and/or stabilizing the interaction between DDB1 and CUL4 complex. Consistently, the fission yeast CDT2 was recently isolated as a CUL4 binding protein. Since L2DTL and PCNA interact with CDT1 and p53/MDM2, our studies suggest that L2DTL and PCNA also contribute to substrate recognition of the DDB1/CUL4 E3 ligase complex.

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Report

L2DTL/CDT2 and PCNA Interact with p53 and Regulate p53 Polyubiquitination and Protein Stability through MDM2 and CUL4A/DDB1 Complexes

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ABSTRACT

The CUL4-ROC1 E3 ligase complex regulates genome stability, replication and cell cycle progression. A novel WD40 domain-containing protein, L2DTL/CDT2 and PCNA were identified as proteins associated with CUL4/DDB1 complexes. Inactivation of CUL4A, L2DTL, PCNA, DDB1 or ROC1 induced p53 stabilization and growth arrest. L2DTL, PCNA and DDB1/CUL4A complexes were found to physically interact with p53 tumor suppressor and its regulator MDM2/HDM2. The isolated CUL4A complexes display potent and robust polyubiquitination activity towards p53 and this activity is dependent on L2DTL, PCNA, DDB1, ROC1 and MDM2/HDM2. We also found that the interaction between p53 and CUL4 complex is regulated by DNA damage. Our data further showed that MDM2/HDM2 is rapidly proteolyzed in response to UV irradiation and this process is regulated by CUL4/DDB1 and PCNA. Our studies demonstrate that PCNA, L2DTL and the DDB1-CUL4A complex play critical and differential roles in regulating the protein stability of p53 and MDM2/HDM2 in unstressed and stressed cells.

INTRODUCTION

The tumor suppressor protein p53 is encoded by the most frequently mutated gene in human cancers.[1-4] p53 regulates cell cycle progression and cell survival in response to various cellular stresses including DNA damage and replication perturbation.[2,4,5] The level of p53 is primarily controlled by ubiquitin-dependent proteolysis.[3,4] In vivo, MDM2 (also called HDM2 in human) binds to p53 and is implicated in promoting p53 polyubiquitination and subsequent proteolysis.[4,5] However, the exact role of MDM2 in regulating p53 polyubiquitination remains unclear and controversial. Although p53 can be polyubiquitinated in vitro in the presence of high molar excess of MDM2,[6] several studies suggest that at the physiological levels, MDM2 preferentially monoubiquitinates p53 in vitro.[3,4,7,8] Many lines of evidence suggest that MDM2 requires additional cellular proteins to polyubiquitinate p53.[3,7,8] The recombinant amino-terminal region of p300 has been shown to act as an ubiquitin E4 enzyme in vitro to polyubiquitinate p53 only when p53 is monoubiquitinated by MDM2.[7] In addition, the YY1 protein has been reported to promote p53 polyubiquitination in vitro in the presence of MDM2 by binding to both p53 and MDM2 to increase the local concentrations of MDM2 on p53.[8] The p53 protein is usually stabilized by various cellular stresses including UV- or γ-irradiation, genotoxic drugs and depletion of nucleotide pools.[4,5] The activation of DNA damage or replication checkpoints causes the phosphorylation of p53, primarily on several serines and threonines at the amino-terminus of p53 and consequently disrupts the interaction between p53 and MDM2.[3-5] However, it remains unclear whether p300 and/or YY1 are the major in vivo ubiquitin-dependent proteolysis pathways that regulate p53 stability and whether there are additional mechanisms or cellular proteins that are involved in p53 polyubiquitination-dependent proteolysis in vivo.

CUL4 (cullin 4) E3 ligase represents a new class of ubiquitin E3 ligases that regulates cell cycle progression, replication, DNA damage and genome stability.[9] CUL4 forms a complex with the putative adaptor protein DDB1 and Ring finger protein ROC1 (also called RBX1 or HRT1) to form an active ubiquitin E3 ligase complex.[9] The replication licensing protein CDT1 is one of the best characterized CUL4 E3 ligase substrates which is targeted for proteolysis by the DDB1/CUL4/ROC1 E3 ligase complex and the COP9-Signalosome complex (CSN) in response to UV or gamma-irradiation.[10] In *Caenorhabditis elegans*, loss of CUL4 induces the accumulation of polyploid nuclei containing

100C DNA content that correlates with the stabilization of CDT1.[11] Recently, we found that in *Drosophila* cells, the CUL4/DDB1 complex regulates the G_1/S transition by controlling the protein stability of Dacapo,[12] a *Drosophila* CDK inhibitor that shares substantial homology to the mammalian CDK inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. This pathway appears to be conserved in human cells.[12] Inactivation of either human CUL4A or DDB1 led to the stabilization of CDK inhibitor p27 and G_1 cell cycle arrest.[12] In addition, we also found that inactivation of human CUL4A led to the accumulation of p53 and its downstream CDK inhibitor p21 in human cells.[12] Similarly, mouse embryonic cells deficient in CSN2, a component of CSN that removes the covalent attachment of an ubiquitin-like protein NEDD8 (neddylation) in CUL4 and other cullin family members,[9] causes p53 accumulation and embryonic lethality.[13] These studies suggest that CUL4 complex may play a role in p53 regulation. The CUL4A encoding gene is amplified in many breast cancers and hepatocellular carcinomas,[14,15] indicating altered expression of CUL4A may contribute to tumorigenesis. Here we report that L2DTL (also called human CDT2), a novel WD40 repeat domain-containing protein and PCNA, physically interact with the DDB1/CUL4 complex, MDM2 and p53 and regulate p53 polyubiquitination through MDM2 and the DDB1/ CUL4A complex in unstressed human cells. We also provide evidence that PCNA and DDB1/CUL4 complex also regulate the proteolysis of MDM2 in response to UV-irradiation.

MATERIALS AND METHODS

Cells, RNAi and siRNA and transfection. Human HEK293, HeLa, U2OS, RKO and U87MG cells and mouse embryonic fibroblasts were cultured as described previously,[10] The p53 and MDM2 double null mouse embryonic fibroblasts were kindly provided by Dr. Guillermina Lozano. The sequence of human L2DTL siRNA is AAUCCCUUCUGACUGGUUAUC. The DDB1, ROC1, CUL4A, CUL4B, PCNA and p53 siRNAs were synthesized and transfection of the siRNAs was conducted as previously described.[10,12,16-18] For stable Flag-tagged p53 expression, Flag-p53 was cloned under the CMV promoter control (pCMV-2B) and after transfection, the stable expressing lines are selected by G418 and the Flag-p53 protein was isolated as described.[10]

Antibodies, plasmids and p53 polyubiquitination reaction. Anti-p53 (DO1) and MDM2 (SMP14 and D12) antibodies were purchased from Santa Cruz, California. Mouse monoclonal antibody OP115 against MDM2 was obtained from Calbiochem. The CUL2, CUL4A, CUL4B, CUL4CT, L2DTL and DDB1 antibodies were used as described.[10,12,19] Immunoprecipitation, western blotting, the isolation of Flag-p53 protein and polyubiquitination of p53 by MDM2 and CUL4 ligase complexes were conducted as described before.[10,12] Unless specified in the figure legends or text, polyubiquitination of p53 by CUL4 and MDM2 was conducted using a combination of CDC34 and UbcH5C as described.[10] The baculoviruses containing PCNA, GST-p27, GST-p21, GST-CDT1, GST-p53, GST-MDM2 were constructed to produce the recombinant fusion proteins in insect SF9 cells and used for binding assays.[10,20] For PCNA binding, we also used GST-p53, GST-MDM2 and their various deletion mutant proteins isolated from bacteria as described previous.[21,22]

p53 expression and retrovirus infection. The p53 retrovirus was expressed in Phenix cells, harvested and used as described previously.[23] The RVY- and CTF-U2OS cells were constructed by infecting an empty retrovirus vector (RVY) or the retrovirus containing a dominant negative p53 (CTF, kindly provided by Dr. Daniel DiMaio, Yale University, Connecticut) and selected by hygromycin resistance.[24] Cell cycle analysis after bromodeoxyuridine incorporation by flow-cytometry was conducted as described.[10] Apoptosis was analyzed using APO-BRDU kit from BD Biosciences PharMingen, California.

RESULTS

Loss of CUL4A, DDB1, L2DTL and PCNA causes p53 stabilization. Our previous studies have shown that CUL4 regulates a conserved proteolysis pathway that controls the protein stability of *Drosophila* and human CDK inhibitors Dacapo and $p27^{Kip1}$ in *Drosophila* S2 and p53 deficient human cells, respectively.[12] We also found that inactivation of human CUL4A causes the accumulation of tumor suppressor p53 and the CDK inhibitor $p21^{Waf1/Cip1}$, as well as G_1 cell cycle arrest in cells that contain an intact p53 (Figs. 1A and 2A).[12] Since p21 is transcriptionally regulated by p53,[3] we investigated whether p53 acts upstream of p21 in CUL4A deficient cells. Cosilencing of p53 and CUL4 greatly reduced p21 accumulation and G_1 cell cycle arrest (Figs. 1B and 2A), suggesting p53 may contribute to p21 accumulation in CUL4A deficient cells. However, the remaining p21 still accumulated in the p53 silenced cells after inactivation of CUL4 (Fig. 1B), suggesting that a p53-independent regulation of p21 by the CUL4A complex may exist.

We have recently identified a novel WD40 repeat-containing protein, L2DTL, that interacts with the DDB1/CUL4/ROC1 and CSN complexes (see the accompanying paper). To determine whether loss of L2DTL and the CUL4A/DDB1/ROC1 complex is required for p53 induction, we inactivated L2DTL, DDB1 and ROC1 by siRNA[25] and examined their effects on p53. In each case, we found that loss of L2DTL and the components of the CUL4 complex profoundly induced p53, suggesting that p53 is regulated by the components or activity of the CUL4A complex (Fig. 1C–E). Like inactivation of CUL4A in these cells, loss of L2DTL or DDB1 also caused induction of p21, growth arrest and apoptosis (Fig. 1C and D and data not shown). Measurement of the p53 decay rate by treating CUL4A and L2DTL silenced cells with cycloheximide demonstrated that loss of either CUL4A or L2DTL reduced p53 decay rate (Fig. 1F and G), indicating that L2DTL and the CUL4A complexes regulate p53 stability in vivo. p53 regulates both cell cycle progression and cell survival.[3] Our data indicate that induction of p53 after CUL4A inactivation caused an enhanced apoptosis in response to UV-irradiation in U2OS cells (Fig. 2B). Such an effect is abolished if a dominant negative p53 is expressed in these cells (Fig. 2B and C).

To characterize the mechanism of p53 induction, we examined whether L2DTL and the CUL4A/DDB1 complex interact with p53. Both endogenous p53 and MDM2 can often be detected in the immunoprecipitated human CUL4A complex, but not that of its paralogue CUL4B (Fig. 3A and B).[10] In some cells, the interactions between p53/MDM2 and CUL4A complex are enhanced by the presence of MG132, an inhibitor of the 26S proteosome that proteolyzes the polyubiquitinated proteins, suggesting that CUL4A complex may be involved in p53 polyubiquitination. We also examined whether L2DTL and DDB1 interact with p53 and MDM2. We found that both ectopically expressed Flag-L2DTL and endogenous L2DTL bind p53 in many human cell lines, including 293, RKO and HCT116 (Fig. 3C and unpublished data). A stable interaction

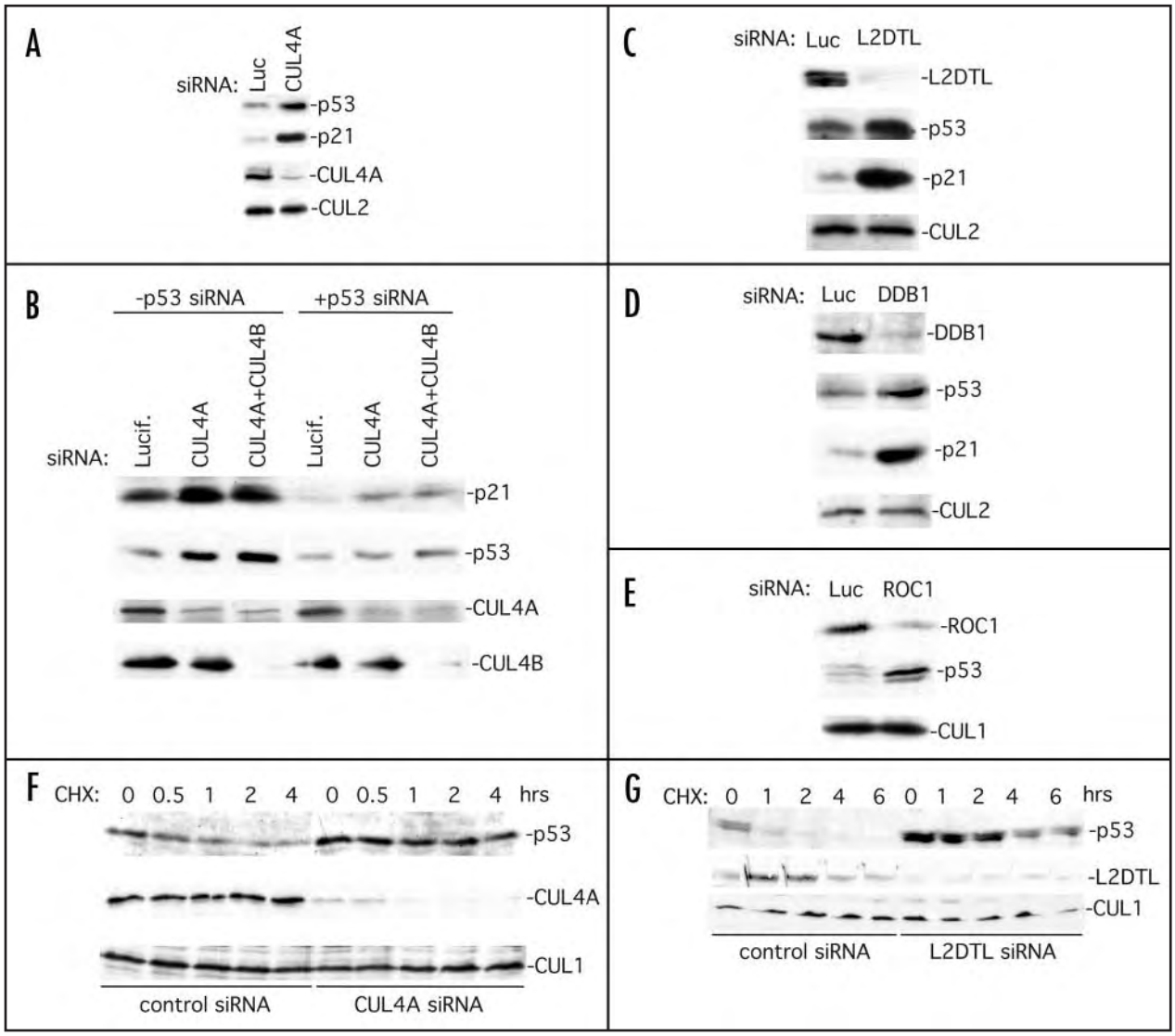

Figure 1. Inactivation of CUL4A, L2DTL, DDB1 or ROC1 stabilizes p53. (A) Silencing of CUL4A is sufficient to induce p53 and p21 accumulation. Human RKO cells containing an intact p53 were treated with 50 nM siRNAs for luciferase (control) or CUL4A for 72 hours. The total protein levels of p53, p21, CUL4A and CUL2 (loading control) were examined by Western blotting using respective antibodies as indicated. (B) p53 is responsible for p21 accumulation after inactivation of CUL4. Human glioblastoma U87MG cells were treated with 50 nM siRNAs for luciferase, CUL4A and CUL4A + CUL4B in the presence or absence of 50 nM p53 siRNA as indicated. The protein levels of p53, p21, CUL4A and CUL4B were analyzed 60 hours post-treatment by Western blotting using specific antibodies. (C–E) Loss of L2DTL, DDB1 or ROC1 causes the accumulation of p53 and p21. Human U2OS cells were treated with 50 nM siRNAs for luciferase (Luc), L2DTL, DDB1 or ROC1. The protein levels of p53, p21, L2DTL, DDB1 or ROC1 were analyzed by specific antibodies after 72 hours as indicated. (F) Inactivation of CUL4A stabilized p53. U2OS cells were treated with CUL4A siRNA and a control siRNA (luciferase) for 60 hours as indicated. Cycloheximide (100 μg/ml) was then added and the protein levels of p53, CUL4A and CUL1 were analyzed at various times as indicated on top of the lanes. (G) Loss of L2DTL stabilized p53 protein. HeLa cells were transfected with 50 nM L2DTL siRNA or luciferase siRNA for 60 hours. Protein synthesis inhibitor cycloheximide (100 μg/ml) was then added and the protein levels of p53, L2DTL and CUL1 (as a control) were analyzed at various times as indicated on top of the lanes.

between the endogenous L2DTL and MDM2 can often be detected in these cell lines (Fig. 3D). Examination of various human cell lines indicated that DDB1 interacts with endogenous p53 and MDM2 (Fig. 3E). These studies, together with the observation that loss of each of these proteins causes p53 protein stabilization (Fig. 1), suggest that L2DTL and CUL4A complexes may be involved in regulating p53 stability.

PCNA associates with L2DTL, CUL4, p53 and MDM2. To further characterize the relationship between L2DTL and the CUL4/DDB1 complex, we affinity purified L2DTL complexes using anti-L2DTL antibodies from HeLa cells. Mass-spectrometry analysis of proteins associated with L2DTL complexes revealed the presence of DDB1, components of CSN and PCNA. These interactions were further confirmed by immunoprecipitation of L2DTL complexes from various human cell lines followed by western blotting with DDB1, CSN5 and PCNA antibodies (see the accompanying paper).

Since inactivation of L2DTL and DDB1 causes p53 accumulation, we also silenced the expression of PCNA in human cells and examined its effect on p53 protein. Loss of PCNA potently induced the accumulation of p53 under normal culture conditions (Fig. 4A), suggesting that PCNA may be involved in regulating p53. Because L2DTL interacts with CUL4, p53, PCNA and MDM2 (Fig. 3), we

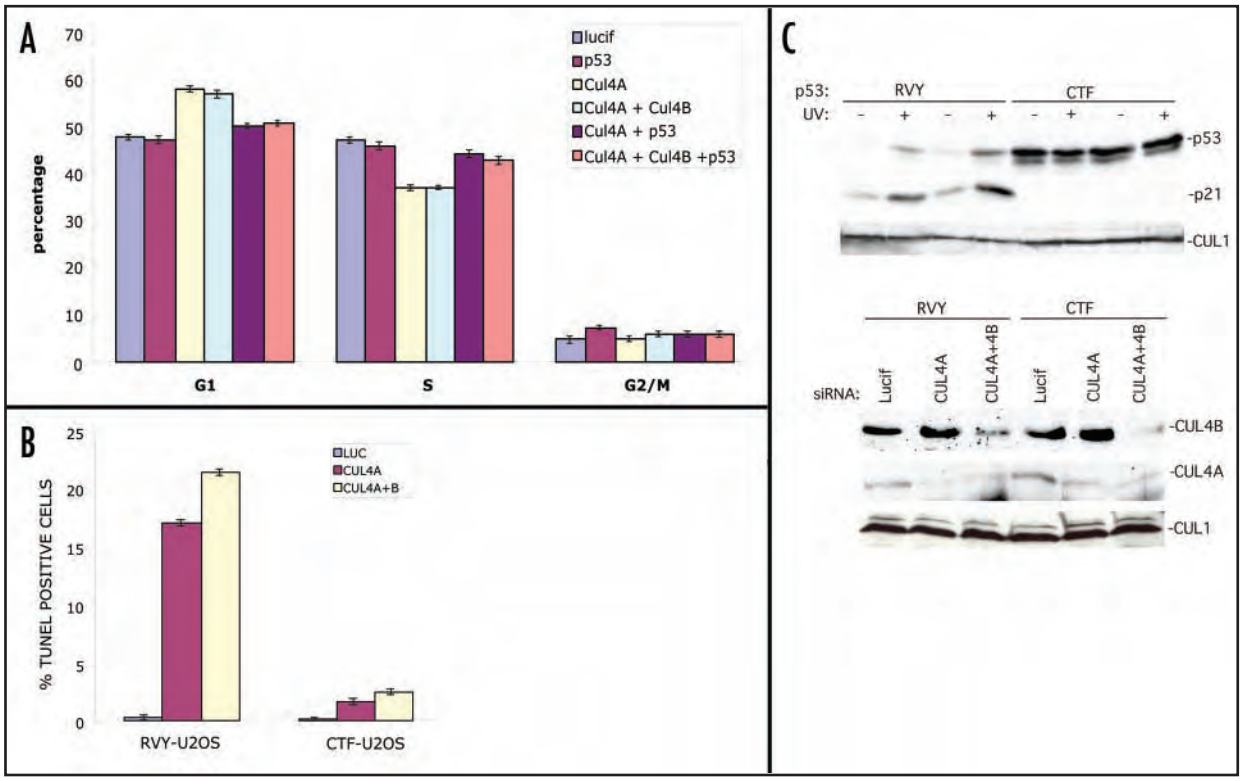

Figure 2. Inactivation of CUL4 causes G_1 cell cycle arrest. (A) Human U87MG cells were treated with 50 nM siRNAs for luciferase (control), CUL4A and CUL4A and CUL4B in the presence or absence of 50 nM p53 siRNA as indicated. Seventy-two hours-post transfection, the cells were analyzed for cell cycle effect by flow-cytometry. Loss of CUL4A or CUL4A and CUL4B induces G_1 arrest (48% G_1 in control versus 57% G_1 cells in CUL4 deficient cells) and a corresponding decrease of S phase cells (47% to 38%). Silencing of p53 significantly suppresses the effect of CUL4 deficiency on G_1 cell arrest (from 57% of CUL4A to 48% of CUL4A plus p53 siRNA). Duplicate experiments were conducted in parallel and essentially the same conclusion was obtained with three experimental repeats. The silencing efficiency was analyzed by western blotting in Figure 1B. (B) CUL4A deficiency enhances p53 dependent and UV-induced apoptosis. CUL4A deficiency enhances UV-induced apoptosis in p53 positive cells (U2OS) but not in cognate p53 negative cells expressing a dominant p53. U2OS cells were infected with an empty retrovirus (RVY) or a retrovirus containing a dominant negative p53 (CTF) and stable cells were selected by hygromycin. The cells were treated with 50 nM siRNAs for luciferase, CUL4A and CUL4A and CUL4B for 48 hours. One set of cells were irradiated with 20 J/M^2 and processed for apoptosis assay by the TUNEL assay after 24 hours. The percentage of apoptotic cells was plotted. (C) Another set of cells of (B) were analyzed by western blotting to examine the effect of CUL4 knockdown and the levels of p53 and p21 after UV and CUL4 elimination. The U2OS-RVY cells responded to UV irradiation and treatment of CUL4 siRNAs by stabilizing p53 and inducing p21 while no changes in p53 and p21 were observed in U2OS-CTF cell in which p53 is inactivated (Top panels).

examined whether PCNA also binds to these protein complexes. Immunoprecipitation of p53, MDM2 and CUL4 complexes from mouse embryonic fibroblasts revealed that PCNA indeed interacts with these proteins or protein complexes in vivo (Fig. 4B). Reciprocal immunoprecipitation of PCNA complexes revealed the presence of p53 and MDM2 in the complex, suggesting a stable association between these proteins (Fig. 4B). To further characterize these interactions, we used recombinant p53, MDM2 or CDK inhibitor p21, which is known to bind to PCNA,[26,27] and CDT1, a substrate of CUL4 complex, to examine PCNA interaction. Interestingly, we found that recombinant p53, MDM2, p21 and CDT1 proteins all interact with the recombinant PCNA protein in vitro. These interactions are specific, since GST-p27, a protein that does not interact with PCNA, did not bind to PCNA under these conditions (Fig. 4C).

PCNA interacts with CDK inhibitor p21 through a PCNA interacting protein motif (PIP), a conserved QXXHXXAA (H, hydrophobic amino acid residues; A, aromatic amino acid residues)[26,27] that is present in many PCNA binding proteins including CDK inhibitors Dacapo and p21.[28] Examination of the protein sequences revealed that p53, MDM2 and CDT1, all contain at least one potential PCNA interacting protein motif (Fig. 4D–H). We have subcloned these PIP regions in p53 and MDM2 and found that these fragments can interact with PCNA in vitro (Fig. 4D–G). These studies indicate that both p53 and MDM2 are PCNA interacting proteins, although the exact role of these PCNA binding regions in p53 or MDM2 regulation remains to be investigated. However, since loss of PCNA induces p53 accumulation, it suggests that one of these PCNA binding sites in MDM2 or p53 may play a role in regulating p53 stability.

The interaction between p53 and CUL4A complex is regulated by γ- and UV-irradiation. The p53 protein is rapidly stabilized in response to DNA damage. This regulation is in part mediated through the phosphorylation of the multiple serines/threonines at the amino terminus of p53 by the ATM/ATR and CHK1/CHK2 checkpoint kinases, particularly the serine 15 (S15) and serine 20 (S20).[3-5] Phosphorylation of these serine residues in p53 causes its dissociation from MDM2 and promotes p53 protein stability.[4] We found that the interaction between the CUL4A complex and p53 is rapidly reduced in response to gamma-irradiation in both 293 and U2OS cells (Figs. 5A and B). The association between p53 and MDM2 was also abolished by gamma-irradiation (Fig. 5C). To further determine

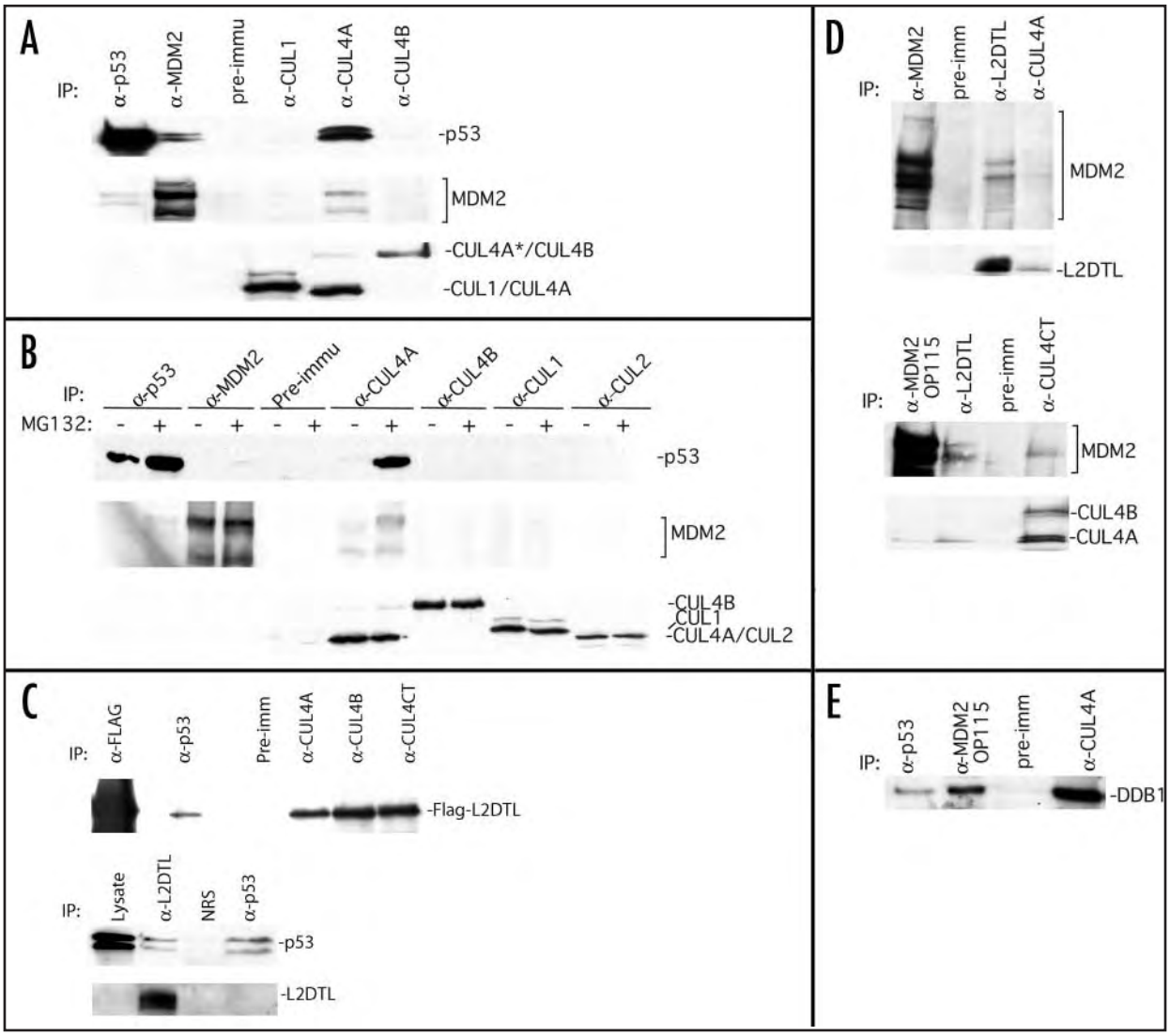

Figure 3. The CUL4A, DDB1 and L2DTL complexes interact with p53 and MDM2. (A and B) Interaction between CUL4, MDM2 and p53 in 293 (A) or U2OS (B) cells. A. Human HEK 293 cell lysates were immunoprecipitated with preimmune (preimmu) serum, monoclonal anti-p53 (DO1) and MDM2 (SMP14) antibodies and anti-CUL1, CUL4A and CUL4B antibodies followed by western blotting analysis for p53, MDM2 and cullins as indicated. B. Human U2OS were pretreated with either dimethyl sulfoxide (DMSO) or 50 μg/ml MG132 for 2 hours as indicated. The interaction between CUL1, CUL2, CUL4A and CUL4B with p53 and MDM2 were analyzed by immunoprecipitation and Western blotting with respective antibodies. (C) L2DTL interacts with p53. Top panel: Flag-tagged L2DTL was transfected into 293 cells and assayed for its binding with p53, CUL4A, CUL4B and CUL4CT by immunoprecipitation and western blotting as indicated. Lower panel: endogenous L2DTL and p53 were immunoprecipitated from 293 cells with specific antibodies and their interactions were analyzed by western blotting as indicated. Preimm: preimmune serum for L2DTL. NRS: normal rabbit serum. Similar results were obtained from RKO and HCT116 cells (data not shown). (D) L2DTL interacts with MDM2. The cell lysates from 293 cells were immunoprecipitated with anti-MDM2, preimmune, L2DTL and CUL4A antibodies. They were blotted with anti-MDM2, L2DTL, CUL4A (against amino terminus of CUL4A) and CUL4CT (against the conserved carboxy terminus of CUL4A and CUL4B) antibodies as indicated. Similar results were obtained from RKO or HCT116 cells (data not shown). (E) Interaction between p53 and MDM2 with DDB1. The p53 and MDM2 complexes were isolated as in (A and B), the presence of DDB1 was analyzed by an anti-DDB1 antibody. The same results were obtained from RKO cells.

the regulation of p53 dissociation, we treated cells with Wortmannin, an inhibitor of the ATM/ATR kinase family.[4] Prior treatment of cells with Wortmannin suppressed the dissociation of p53 from the CUL4A complex in response to irradiation (Fig. 5B), suggesting the involvement of ATM/ATR kinase family members in regulating the p53-CUL4A complex interaction. The dissociation of p53 from both CUL4A and MDM2 in response to γ-irradiation also suggests CUL4A may depend on MDM2 in regulating p53 (see below).

We also examined the interaction between CUL4A and p53 in UV-irradiated cells. While the interaction between p53 and CUL4A is again prevented by UV-irradiation (Fig. 5D), we found that MDM2 is rapidly proteolyzed in response to UV irradiation (Fig. 5D). The proteolysis of MDM2 by UV-, but not γ-irradiation, has been observed[29] and our studies are consistent with these observations (Fig. 5D). The proteolysis of MDM2 after UV irradiation may in part explain the dissociation of p53 and CUL4A complexes in response to UV-irradiation if CUL4A binding is affected by dissociation or loss of MDM2 (Fig. 4D). Our studies thus suggest that the interaction between p53 and CUL4A complexes is regulated by checkpoint activation and the protein stability of MDM2.

A dual function of PCNA and DDB1/CUL4 complexes in

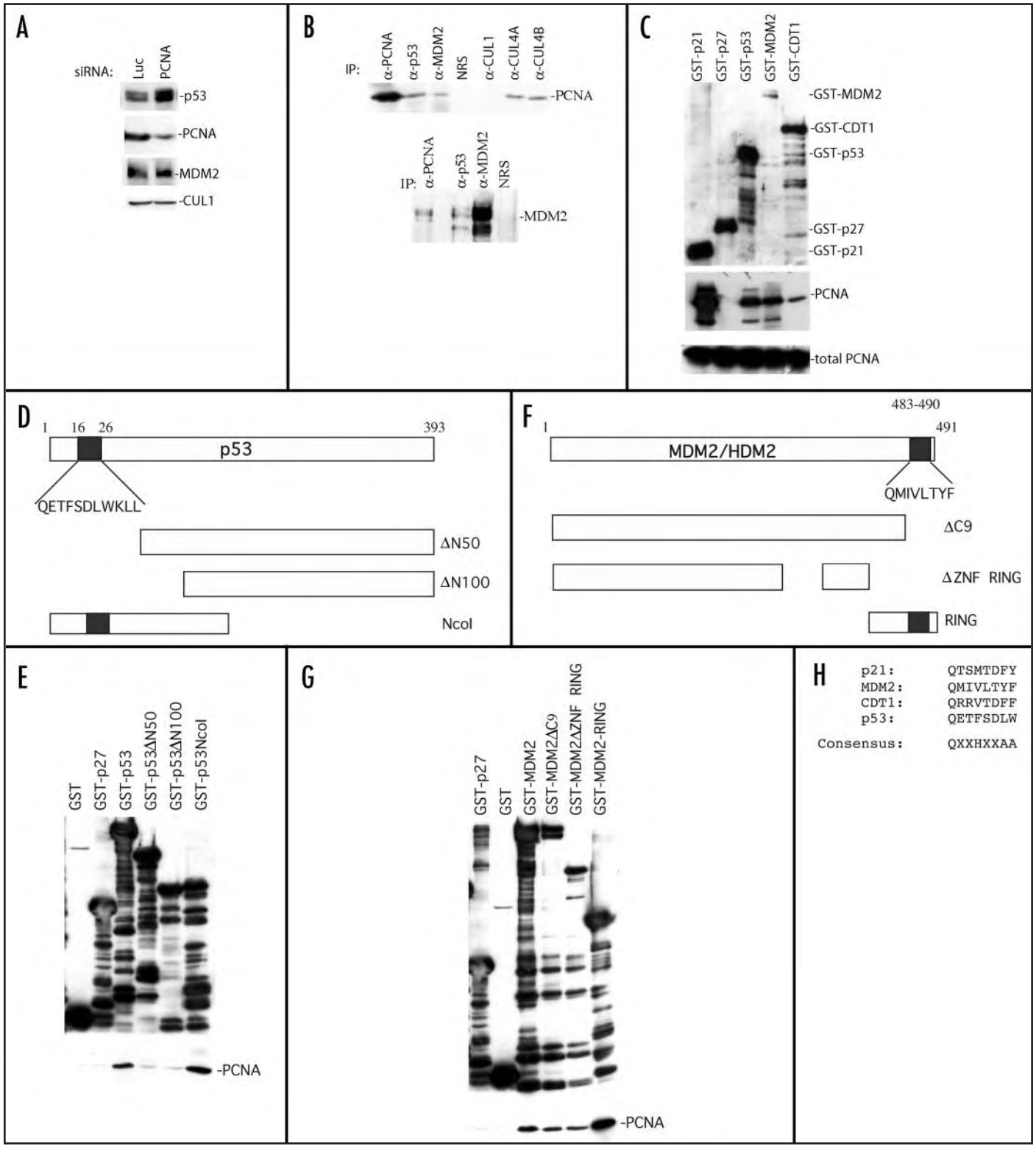

Figure 4. See Figure Legend, page 1725.

regulating p53 stability in unstressed cells and MDM2 proteolysis in UV-irradiated cells. The UV-sensitivity of p53 and CUL4 interaction and MDM2 proteolysis after UV-irradiation prompted us to examine the effect of inactivation of PCNA and DDB1/CUL4 complex on the protein stabilities of p53 and MDM2 in the presence or absence of UV-irradiation. We found that loss of PCNA or DDB1 greatly elevated p53 protein levels regardless of the presence or absence of UV-irradiation (Fig. 5E and F, also see Fig. 4A). In addition, although inactivation of PCNA and DDB1 did not have significant effects on MDM2 protein levels in the absence of DNA damage (Fig. 4A and 5E and F), silencing of PCNA and DDB1 by specific siRNAs prevented MDM2 degradation in response to UV-irradiation

Figure 4. Interaction between PCNA and p53, MDM2 and CUL4 complexes. (A) Loss of PCNA causes the accumulation of p53. RKO cells were treated with 50 nM control siRNA (luciferase) or PCNA siRNA for 72 hours and the levels of p53, MDM2, PCNA and CUL1 were examined by Western blotting. (B) PCNA interacts with p53, MDM2 and CUL4 complexes in mouse embryonic fibroblasts. The PCNA, p53, MDM2, CUL1, CUL4A and 4B immunocomplexes were isolated from actively growing primary mouse embryonic fibroblasts by respective antibodies as indicated on top of each lane. They were blotted with anti-PCNA (top) or MDM2 (bottom) antibodies, respectively. (C) p53 and MDM2 interact with PCNA directly. SF9 insect cells were coinfected with baculovirus encoding PCNA and one of the following baculoviruses encoding either GST-p21, GST-p27, GST-p53, GST-MDM2 or GST-CDT1. The protein complexes were isolated by glutathione beads, washed and blotted with anti-PCNA antibody. The GST-proteins were detected by an anti-GST antibody. (D) Mapping the PCNA binding site(s) in p53. The GST-p53 and deletion mutants of GST-p53 were used to map PCNA interaction site(s). ΔN50 and ΔN100 mutants represent p53 mutants that were deleted the amino terminal 50 or 100 amino acid residues (amino acid residues 1–50 or 1–100) while the NcoI mutant contains only the amino terminal fragment (amino acid residues 1–160) in p53. The potential PCNA binding site in p53 (amino acid residues 16–23) is shown. This site overlaps with the known MDM2 binding site (amino acid residues 19–26) in p53. (E) The GST, GST-p27, GST-p53 and GST-p53 mutants were expressed in *E coli*. the proteins were isolated. Their binding to a baculovirus expressed PCNA were analyzed after GST-pulling down and western blotted with the anti-PCNA antibody. The deletion of amino terminus 50 residues greatly abolished p53 binding to PCNA while the amino terminal fragment (NcoI) in p53 is sufficient to mediated PCNA binding. (F) Wild type MDM2/HDM2 and its deletion mutants. One of the conserved PCNA binding sites is located at the carboxy terminus of MDM2 (amino acid residues 483-490). The MDM2 mutant ΔC9 contains a 9 amino acid residues deletion at the carboxy terminus while the RING domain mutant contains only the RING domain located at the carboxy terminal part (amino acid residues 438–491) of MDM2. The ΔZNFΔRING contain double deletions in the zinc finger domain (amino acid residues 299–328) and in the RING finger domain (amino acid residues 438-491) in MDM2. (G) The binding of GST-MDM2 and its deletion mutants to PCNA. The bindings were conducted as in (E). While the presence of the carboxy terminal region (RING) is sufficient to mediate PCNA binding to MDM2, the deletion of 9 amino acid residues at the carboxy terminus of MDM2 significantly reduced but not completely abolished PCNA binding, suggesting the presence of PCNA binding sites in MDM2 in addition to the PCNA interacting motif at the carboxy terminus of MDM2. (H) p53 and MDM2 contain conserved PCNA interacting protein motifs. The PCNA interaction domains in p21 (a.a. 144–151), MDM2 (a.a. 483–490), CDT1 (a.a. 3–10) and p53 (a.a. 16–23) are aligned. H, hydrophobic amino acid sidechains; A, aromatic amino acid sidechains.

(Fig. 5E and F). Since UV-irradiation induces a DDB1/CUL4-dependent proteolysis of CDT1,[10,30] we also found that loss of PCNA and DDB1 prevents the UV-induced CDT1 degradation (Fig. 5E and F). These studies suggest that PCNA is involved in regulating the UV-induced proteolysis of MDM2 and CDT1 and this process is mediated through the DDB1/CUL4 E3 ligase activity.

Isolated CUL4A complexes contain a potent polyubiquitination activity towards p53. To test the involvement of CUL4A complex in p53 polyubiquitination, we developed in vitro p53 polyubiquitination assays to monitor the roles of L2DTL, DDB1, ROC1 and CUL4A in p53 polyubiquitination. Since the exact composition of CUL4 complexes remains to be further elucidated, we first examined whether CUL4-associated p53 can be directly polyubiquitinated by the immunoprecipitated CUL4 complexes.[10] A Flag-epitope tagged p53 was transfected into 293 cells and this transfected p53 can be immunoprecipitated by antibodies against MDM2 and CUL4A and weakly by CUL4B (Fig. 6A). The Flag-p53 associated with the MDM2 and CUL4 immunocomplexes was further incubated in a polyubiquitination reaction containing the ubiquitin conjugating enzyme E2, the ubiquitin activating enzyme E1, ubiquitin and ATP.[10] We found that the CUL4A-associated p53 could be robustly polyubiquitinated by the CUL4A complexes (Fig. 6A) while immunoprecipitated MDM2 exhibited weak p53 ubiquitination activity (Fig. 6A). These observations are consistent with previous reports that MDM2 may require additional cellular proteins for p53 polyubiquitination.[7,8]

To conduct a more detailed analysis of p53 polyubiquitination by the CUL4 complex, we developed further p53 polyubiquitination assay using isolated p53 protein and a protocol adapted from CDT1 polyubiquitination reaction by CUL4 complex (Fig. 6B).[10] The Flag-p53 protein was isolated from 293 cells by anti-Flag antibody/protein A Sepharose beads and eluted from the beads by Flag-peptide.[10] The purified Flag-p53 was added directly to the isolated CUL4 or MDM2 immunocomplexes for polyubiquitination.[10] Using this assay, we found that p53 again can be robustly polyubiquitinated by the CUL4A complexes isolated with two independent CUL4 antibodies, respectively (Fig. 6B).[10] This reaction is dependent on exogenously added ubiquitin, E2 and E1 (Fig. 7C, data not shown) and is catalyzed specifically by CUL4 complexes since other cullin ligases such as CUL2 E3 ligase cannot polyubiquitinate p53 in these assays (Fig. 6B).

Requirement of MDM2 in CUL4A-mediated p53 polyubiquitination. Since MDM2 interacts with L2DTL, PCNA and CUL4A ligase complexes (Figs. 3 and 4), we asked whether MDM2 status affects CUL4A ligase activity. We compared the p53 polyubiquitination activity of CUL4 complexes isolated from mouse embryonic fibroblasts (MEFs) containing either the wild-type MDM2$^{+/+}$ or the MDM2 null mutation (MDM2 $^{-/-}$).[31] Surprisingly, the CUL4A ligase complex isolated from the MDM2 null MEFs contained a substantially diminished ubiquitination activity for p53 (Fig. 6C), as compared with the CUL4A E3 ligase complexes isolated from the wild-type MEFs. Since the MDM2 null MEFs we used are also deficient in p53 (p53$^{-/-}$) to circumvent growth arrest due to p53 accumulation,[31] it is possible that the effect on CUL4-dependent p53 polyubiquitination is not due solely to MDM2 status. To address this possibility, we tested whether reintroduction of MDM2 into the MDM2 and p53 null cells can restore the ability of CUL4A complex to polyubiquitinate p53. Human MDM2 was expressed under the CMV promoter control in MDM2 and p53 double null MEFs and the CUL4A complexes were isolated and assayed for p53 polyubiquitination. We found that expressed MDM2 can bind to CUL4A complexes and MDM2 expression alone is sufficient to restore the ability of CUL4A complexes to polyubiquitinate p53 in the MDM2 and p53 double null cells (Fig. 6D). These studies indicate that the CUL4A complex requires MDM2 to polyubiquitinate p53.

CULA complex requires DDB1, L2DTL, PCNA and ROC1 for p53 polyubiquitination. Since loss of CUL4A, L2DTL, PCNA and DDB1 caused p53 stabilization (Figs. 1 and 4), we investigated the contribution of L2DTL, DDB1 or PCNA to the polyubiquitination activity of CUL4A complex towards p53. The CUL4A complexes were isolated from control cells or cells in which the expression of L2DTL, DDB1 or PCNA was silenced by siRNA and their polyubiquitination activity towards p53 assayed. While the CUL4A complexes isolated from control cells displayed robust p53 polyubiquitination activity, this activity is lost if the CUL4A complexes were isolated from L2DTL, DDB1 or PCNA deficient cells (Figs. 7A and B). These observations indicate that L2DTL, DDB1 and PCNA are required for p53 polyubiquitination by the CUL4A complex.

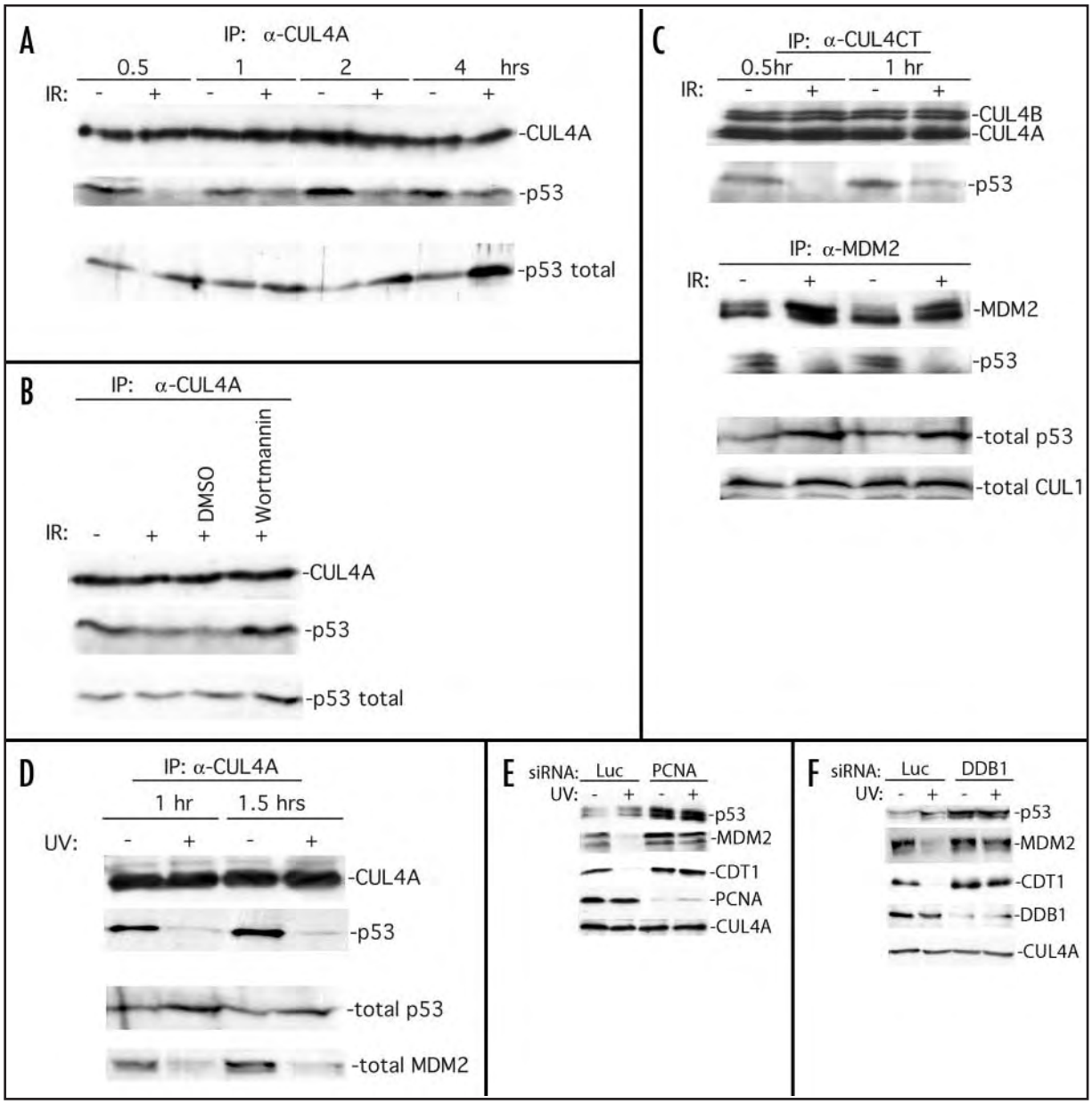

Figure 5. The interaction between p53 and CUL4 complex is regulated by DNA damage checkpoints. (A) p53 dissociates from CUL4A complex after DNA damage. Human U2OS cells were irradiated with or without gamma-irradiation (10 Gy). Cells were harvested at the indicated time points and the CUL4 complexes were isolated and its association with p53 were examined by western blotting. (B) The same as in (A) except some U2OS cells were treated with 10 μM Wortmannin or DMSO for 2 hours before irradiation and continue the treatment until harvesting all the cells one hour after irradiation as indicated. The dissociation of p53 from CUL4A in response to DNA damage is abolished by Wortmannin. All the cells were also treated with MG132 for 1.5 hours to stabilize p53 on CUL4A complexes. (C) A stable 293 cell line expressing p53 Flag-tagged at the amino terminus was treated with or without γ-irradiation (10 Gy). The complexes were immunoprecipitated by anti-CUL4CT or anti-MDM2 antibodies, respectively. The presence of Flag-tagged p53 in the CUL4 or MDM2 immunocomplexes was detected with anti-Flag immunoblot. The total protein levels of Flag-p53 and CUL1 were included as controls. (D) The mouse embryonic fibroblasts were treated with or without UV irradiation (50 J/M2). The CUL4A complex were isolated and its association with p53 was examined by Western blotting. Total p53 and MDM2 proteins were also examined before and after UV irradiation. MDM2 was found to disappear after UV irradiation. (E) Loss of PCNA and DDB1 causes the accumulation of p53 and prevents the proteolysis of MDM2 and CDT1 after UV-irradiation. The RKO cells were treated with 50 nM siRNAs against luciferase and PCNA for 72 hours. Half of the cells were irradiated with UV at 100 J/M2 and after 1 hour, the levels of p53, MDM2, CDT1, PCNA and CUL4A were examined by Western blotting. (F) The same as (E), except DDB1 siRNA was used to determine the effect of DDB1 inactivation on p53, MDM2 and CDT1 proteins in the presence or absence of UV-irradiation.

The CUL4-containing E3 ligase complexes, like other cullin-containing E3 ligases, use their bound Ring finger protein ROC1 as a catalytic subunit for E2 binding and ubiquitin transfer reactions.[9] To determine whether p53 polyubiquitination by CUL4A complexes requires ROC1, we silenced the expression of ROC1 in HeLa cells by siRNA and isolated CUL4A complexes from these cells. As an additional control, we also isolated the CUL4A immunocomplex from cells in which CUL4A expression was silenced by siRNA. While the CUL4A complexes isolated from control treated cells displayed robust polyubiquitination activities towards p53, such activities are lost in CUL4A complexes isolated from ROC1 or CUL4A silenced cells (Fig. 7C). Conversely, we also examined the

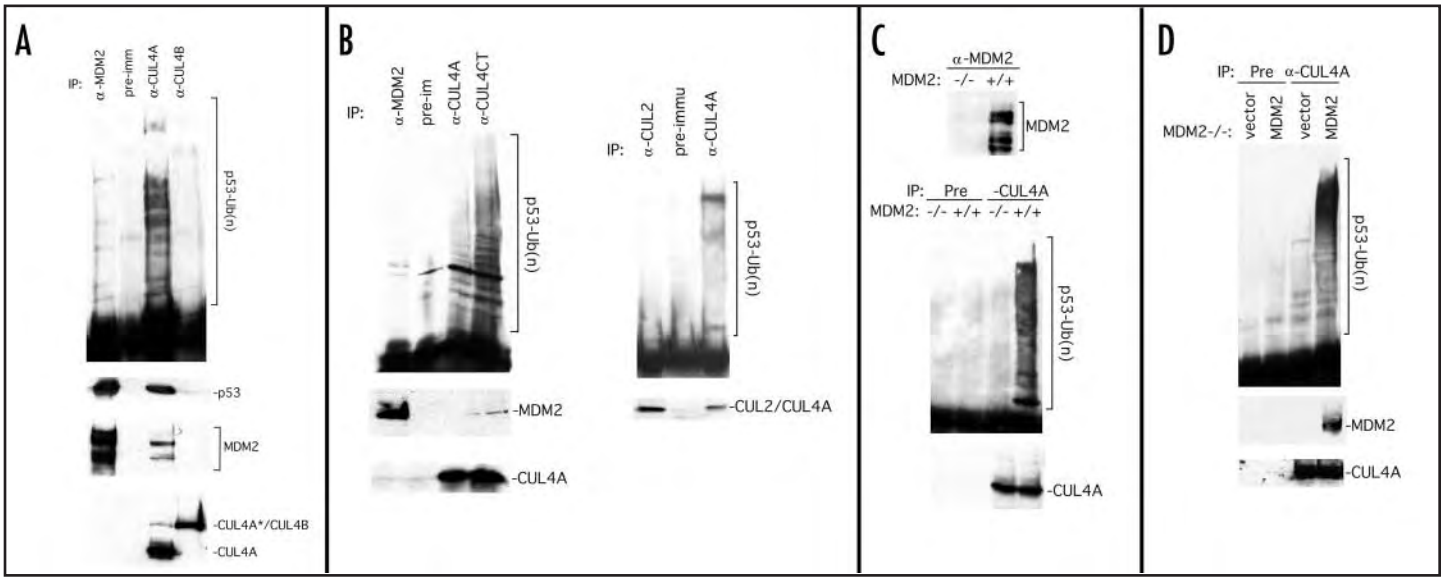

Figure 6. The CUL4A E3 ligase complexes exhibit an intrinsic and robust polyubiquitination activity towards p53 and require MDM2 for p53 polyubiquitination. (A) The CUL4-associated p53 can be ubiquitinated by CUL4 complex. The Flag-tagged p53 expression DNA was transfected into 293 cells. The MDM2- and CUL4-associated Flag-p53 was immunoprecipitated by anti-MDM2 (SMP-14), CUL4A or CUL4B antibodies. The immunoprecipitates were further incubated with 500 ng E2, 50 ng of E1, 10 μM ubiquitin and 2 mM ATP for 30 minutes. The polyubiquitinated p53 was visualized by anti-Flag immunoblot. The blot was stripped and reblotted with anti-p53, MDM2, CUL4A and CUL4B antibodies as indicated. (B) Characterization of CUL4A-mediated p53 polyubiquitination. Left: MDM2 and CUL4 immunocomplexes were isolated by anti-MDM2 (SMP-14), preimmune serum or anti-amino and anti-carboxy termini of CUL4A antibodies (CUL4A and CUL4CT), respectively, from U2OS cells. The immunoprecipitated complexes were incubated with isolated Flag-p53 and polyubiquitination reactions were conducted as in A. The polyubiquitinated p53 was visualized by anti-Flag immunoblot. The blot was stripped and reblotted with anti-MDM2 and CUL4A antibodies. Right: CUL4A E3 ligase complex specifically polyubiquitinates p53. CUL2 and CUL4A complexes were immunoprecipitated from U2OS cells. The Flag-p53 was incubated with CUL2 and CUL4A complexes for polyubiquitination. (C) CUL4A complex requires MDM2 to polyubiquitinate p53. Left: CUL4A complexes isolated from MDM2 and p53 double null mouse fibroblasts (MDM2 and p53 -/- MEFs) display significant reduction in polyubiquitination activity towards p53 as compared to CUL4A complex isolated from the wild-type cells (MDM2 and p53 +/+). The CUL4A complexes were isolated by immunoprecipitation from wild-type MEFs (MDM2+/+) and MDM2/p53 null MEFs (MDM2-/- and p53-/-) and assayed for their polyubiquitination activity against Flag-tagged p53 as in B. (D) Expression of MDM2 in MDM2 and p53 double null MEFs (MDM2-/-) is sufficient to restore the polyubiquitination activity of CUL4A complex toward p53. The MDM2 cDNA under the CMV promoter control and the control empty vector were transfected into the MDM2 and p53 double null MEFs and expressed for 48 hours. The CUL4A complexes were isolated by anti-CUL4A antibodies from these cells and assayed for p53 polyubiquitination using Flag-p53 as a substrate as in 6B.

effect of over-expression of ROC1 on p53 polyubiquitination by CUL4A complex. Human ROC1 was over-expressed under the CMV promoter control in HeLa cells. The ectopic expression of ROC1 significantly increased its binding to CUL4A (Fig. 7D) and this increased interaction is associated with a concomitant stimulation of p53 polyubiquitination by CUL4A complexes (Fig. 7D). These studies demonstrate that polyubiquitination of p53 by the CUL4A complex is dependent on the presence of ROC1 in the cell.

DISCUSSION

In this report, we found that the CUL4A-DDB1 complex regulates p53 polyubiquitination in cooperation with MDM2. A novel WD40 repeat-containing protein, L2DTL, binds to both DDB1-CUL4 and MDM2-p53 complexes and regulates p53 stability (Fig. 1). Our studies further indicate that PCNA associates with p53, MDM2, L2DTL and CUL4 complexes and is required for CUL4A-mediated p53 polyubiquitination (Figs. 4–7). In the accompanying paper, we also showed that both L2DTL and PCNA are required for the proteolysis of replication licensing protein CDT1 in response to DNA damage. Although it remains unclear how CUL4/DDB1 complexes, L2DTL and PCNA regulate p53 in unstressed cells while they control the proteolysis of CDT1 only in the presence of DNA damage, our studies suggest that L2DTL and PCNA are required for the proteolysis of both types of CUL4 substrates.

In addition to p53 and CDT1, our studies also revealed that MDM2 is rapidly proteolyzed in response to UV irradiation. Since both CDT1 and MDM2 are proteolyzed by UV irradiation and the proteolysis of both proteins are regulated by PCNA and DDB1/CUL4 complexes, our studies suggest that PCNA and DDB1/CUL4 complexes may provide a general mechanism for the proteolysis of many proteins in response to UV irradiation and other types of DNA damage.

Our studies indicate that both MDM2 and p53 are PCNA interacting proteins.[27,32] However, since p53 is stabilized in response to DNA damage such as UV irradiation while MDM2 is destabilized after UV-irradiation, it is not clear how PCNA and CUL4 complexes mediate these two different proteolysis events. Our finding that the interaction between p53 and CUL4 complex is reduced after γ- and UV-irradiation may in part provide an explanation for these opposite effects. Our preliminary data suggest that MDM2 and p53 may have more than one PCNA binding site in each protein, and it would be interesting to further determine which PCNA binding region(s) in p53 or MDM2 is required for p53 or MDM2 degradation in stressed or unstressed cells through the CUL4 complexes.

Our study reveals that CUL4A complex is a potent and critical regulator of p53 polyubiquitination. PCNA, L2DTL and the DDB1-CUL4A complex interact with p53 and MDM2 and the isolated CUL4A complex targets p53 for polyubiquitination in vitro in an MDM2-dependent process. This CUL4 A-mediated

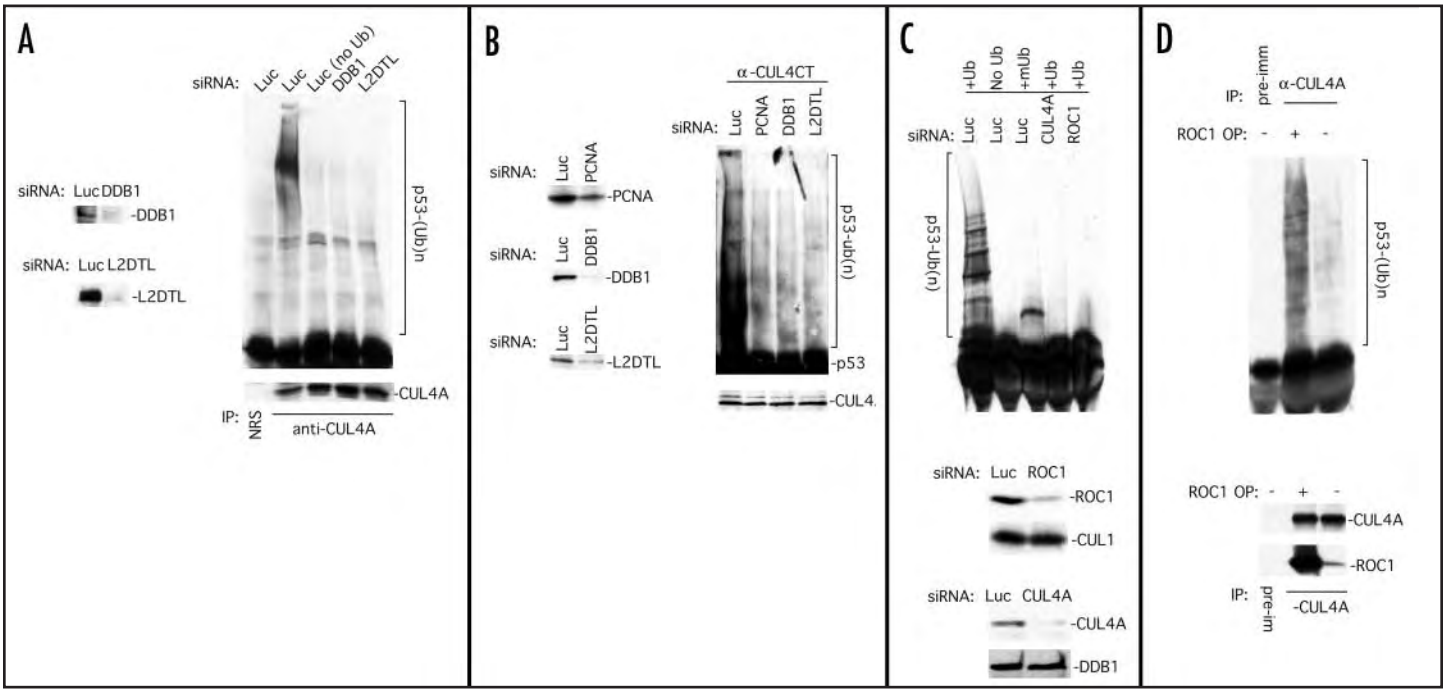

Figure 7. The p53 polyubiquitination activity of CUL4A complex requires DDB1, L2DTL, PCNA and ROC1. A. p53 polyubiquitination by CUL4A complexes is abolished in L2DTL or DDB1 deficient cells. HeLa cells were treated 50 nM siRNAs for luciferase, DDB1 or L2DTL, respectively, for 72 hours. The CUL4A complexes were isolated and assayed for p53 polyubiquitination as described in 6B. The silencing effects of DDB1 and L2DTL by their specific siRNAs were also examined by western blotting. The same results were obtained from RKO and HCT116 (see Fig. 6F and data not shown). B. Loss of PCNA inactivates p53 polyubiquitination by CUL4A complex. RKO cells were silenced with 50 nM siRNA against PCNA, DDB1 and L2DTL for 72 hours. The CUL4A complexes were immunoprecipitated with anti-CUL4CT or CUL4A amino terminal antibodies and normal rabbit serum (NRS). The p53 polyubiquitination was examined as in 6B. C. p53 polyubiquitination by CUL4A is dependent on the catalytic Ring finger protein ROC1. HeLa cells were treated with 50 nM siRNAs for luciferase, CUL4A and ROC1 for 60 hours. The CUL4A complex were isolated by immunoprecipitation and assayed for Flag-p53 polyubiquitination activity in the presence or absence of ubiquitin (Ub) or methylated ubiquitin (mUb) as indicated. Flag-p53 polyubiquitination was detected by anti-Flag antibodies as in B. D. Human ROC1 cDNA is over-expressed (OP) in HeLa cells under the CMV promoter control and CUL4A complexes were isolated from cells with or without ROC1 expression after 48 hours. Polyubiquitination of Flag-p53 by the isolated CUL4A complexes were assayed as described in 6A.

polyubiquitination activity is dependent on L2DTL, PCNA, DDB1 and ROC1, suggesting that the catalytic activity of CUL4 complex is required for p53 polyubiquitination. These findings help explain the observations that loss of CSN2, a component of COP9-signalosome (CSN) that regulates the neddylation of CUL4 and other cullins,[9] causes p53 accumulation while expression of CUL4A can downregulate p53.[13,33] Our studies have provided strong evidence for a unique role of PCNA, L2DTL and the DDB1-CUL4A-ROC1 complex in polyubiquitinating p53 in unstressed cells. In this reaction, the CUL4A complex appears to cooperate with MDM2 to facilitate the polyubiquitination of p53 for subsequent degradation of p53 by the 26S proteosome. Since MDM2 can monoubiquitinate p53, our studies suggest that the CUL4 complex may act as an ubiquitin E4 enzyme for p53 polyubiquitination.[34] However, further experiments are required to demonstrate this possibility.

Our previous studies indicate that CUL4 and CSN complexes regulate the protein stability of *Drosophila* CDK inhibitor Dacapo.[12] In mammalian cells, CUL4A complex regulates the protein stability of CDK inhibitor $p27^{Kip1}$ and G_1 cell cycle arrest in p53 deficient cells.[12] Our current study indicates that CUL4A complex also controls the expression of CDK inhibitor p21 through regulating the polyubiquitination of p53 tumor suppressor and its protein stability, although it remains unclear whether p21 is also regulated by the CUL4 complex in the absence of p53 (Fig. 1B). Nevertheless, our data indicate that DDB1/CUL4 complex is a critical and conserved regulator of the transition from G_1 to S phases by modulating the levels of CDK inhibitors and p53 tumor suppressor. In addition, our data indicate that the DDB1/CUL4 complex also regulates the DNA damage response by targeting CDT1 and MDM2 for proteolysis. Since the gene encoding CUL4A is amplified in many breast cancers and hepatocellular carcinomas,[14,15] our studies provide a novel insight into the mechanisms by which altered regulation of CUL4 E3 ligase may contribute to tumorigenesis.

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CUL4–DDB1 ubiquitin ligase interacts with multiple WD40-repeat proteins and regulates histone methylation

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The CUL4–DDB1–ROC1 ubiquitin E3 ligase regulates cell-cycle progression, replication and DNA damage response[1–4]. However, the substrate-specific adaptors of this ligase remain uncharacterized. Here, we show that CUL4–DDB1 complexes interact with multiple WD40-repeat proteins (WDRs) including TLE1-3, WDR5, L2DTL (also known as CDT2) and the Polycomb-group protein EED (also known as ESC). WDR5 and EED are core components of histone methylation complexes that are essential for histone H3 methylation and epigenetic control at K4 or K9 and K27, respectively[5–7], whereas L2DTL regulates CDT1 proteolysis after DNA damage through CUL4–DDB1 (ref. 8). We found that CUL4A–DDB1 interacts with H3 methylated mononucleosomes and peptides. Inactivation of either CUL4 or DDB1 impairs these histone modifications. However, loss of WDR5 specifically affects histone H3 methylation at K4 but not CDT1 degradation, whereas inactivation of L2DTL prevents CDT1 degradation but not histone methylation. Our studies suggest that CUL4–DDB1 ligases use WDR proteins as molecular adaptors for substrate recognition, and modulate multiple biological processes through ubiquitin-dependent proteolysis.

The CUL4–DDB1–ROC1 complex is a new class of cullin-containing ubiquitin E3 ligases[1]. Previous studies have indicated that the CUL4–DDB1 E3 ligase regulates the proteolysis of the replication licensing protein CDT1 or fission yeast ribonucleotide reductase inhibitor, Spd1, in response to DNA damage or replication[2,3,9]. The CUL4 protein shares significant homology with other cullin family members including CUL1–3 and CUL-5 (ref. 1). Cullin E3 ligases use specific substrate-targeting adaptors, such as F-box proteins in SCF (SKP1–CUL1–F-box) complexes or BTB–POZ proteins in CUL3–ubiquitin E3 complexes, for substrate recognition[1]. CUL4 may use a similar substrate-specific adaptor mechanism to target various proteins for ubiquitin-dependent proteolysis. The CUL4–ROC1 complex binds to DDB1 (UV-damaged DNA-binding protein 1), which has been proposed to be adaptor protein for substrate-targeting[1,10]. However, it remains unclear whether the CUL4–DDB1 E3 ligase requires additional proteins to define substrate specificity.

We recently isolated a novel human WDR protein, L2DTL, which binds to the CUL4–DDB1 complex and CDT1 (ref. 8). We showed that L2DTL is required for CDT1 proteolysis in response to DNA damage through the CUL4–DDB1 ubiquitin E3 ligase[8]. L2DTL requires the WD40-repeat domain for DDB1 binding[8] and DDB1 also directly interacts with WDR proteins DDB2 and the Cockayne syndrome protein, CSA[11]. The CSA-containing CUL4A–DDB1 E3 ligase has recently been shown to target CSB for proteolysis, whereas DDB2 regulates the UV radiation-induced XPC translocation within chromatin[12,13]. Importantly, the binding of DDB2 and CSA to DDB1 seems to be mutually exclusive[11]. These observations raise the possibility that WDR proteins constitute the substrate-specific adaptors that recruit substrates to the CUL4–DDB1–ROC1 core complexes. Here, we show that the isolated CUL4–DDB1 complex contains several additional WDR proteins. Our studies suggest that WDR proteins act as substrate-specific adaptors for the CUL4–DDB1 ubiquitin E3 ligases.

We previously isolated the CUL4B complex from human HeLa cells by affinity chromatography using the anti-CUL4B antibodies as the affinity resin[8]. The proteins associated with the CUL4B complexes were identified by mass spectrometry. This led to the identification of a novel WD40-repeat protein, human L2DTL, in the CUL4B–DDB1 complex[8], which regulates CDT1 proteolysis in response to DNA damage[8]. Using similar purifications from293 cells, several other WD40-repeat proteins were identified, including WDR26, WD40 repeat–transducin-like enhancer proteins 1-3 (TLE1-3), WDR82, glutamate-rich WD40-repeat protein 1 (GRWD1) and Suppressor of mec-8 and unc-52 (SMU1), in addition to L2DTL, DDB1 and components of the COP9–signalosome complex (CSN; Fig. 1)[8]. To confirm these interactions, these WDR proteins were tagged with a Flag-epitope tag and expressed in human cells by transfection (Fig. 2). Our studies confirmed that these WDR proteins interact with the CUL4–DDB1 complexes *in vivo* (Fig. 2 and data not shown). Endogenous WDR proteins such as TLE2 and L2DTL were observed in complexes with the CUL4–DDB1 E3 ligases (Fig. 3a–d).

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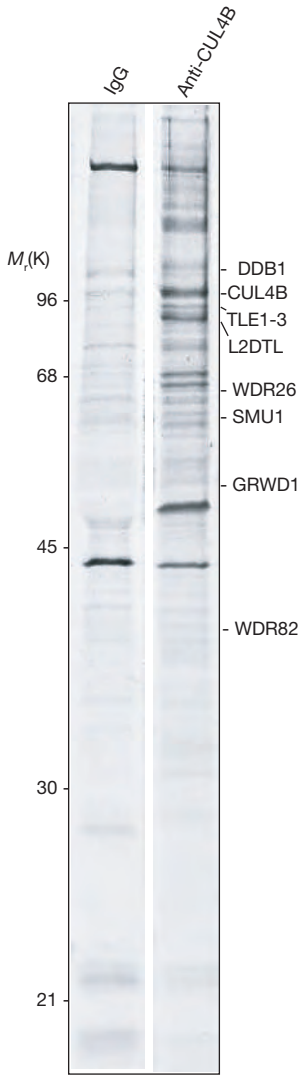

Figure 1 Isolation of various WDR proteins that interact with CUL4B. Human CUL4B complexes were immuno-affinity purified from 293 cells using the affinity purified anti-CUL4B antibody and the associated WDR proteins were identified by mass spectrometry as indicated. IgG, control antibodies.

As WDR proteins share substantial sequence homology within the WD40 repeat domain[14,15], we tested the association of additional WDR proteins with the CUL4–DDB1 complexes. More than 20 WDR proteins were analysed, including WDR5, RBBP5, EED–ESC1–HEED, COP1 and Notchless (NLE1; see Supplementary Information, Fig. S1), and found that they can interact with the CUL4–DDB1 complexes when they are expressed *in vivo* (Figs 2, 3). The interaction of the endogenous CUL4–DDB1 with COP1, WDR5, RBBP5 and EED proteins was verified using their specific antibodies (Fig. 3d–h). These studies indicate that CUL4–DDB1 complexes can interact with a large number of WDR proteins and raise the possibility that these WDR proteins may serve as substrate-recognition subunits or adaptors of the CUL4–DDB1 ubiquitin E3 ligases. As the function of many WDR proteins was not previously defined, we named these proteins CDW proteins for CUL4- and DDB1-associated WDR proteins (see Supplementary Information, Fig. S1).

To determine the structural basis of the interaction between WDR proteins and CUL4–DDB1, the protein sequences of L2DTL proteins from fission yeast to human, as well as the WDR proteins originally isolated from CUL4B association, was compared (Fig. 1). This alignment revealed the presence of a centrally positioned and conserved tandem repeat of a DXXXR/KXWDXR/K motif (R/K; either Arg or Lys), between L2DTL, WDR82, GRWD1 and TLE1-3 proteins (see Supplementary Information, Fig. S2). This subdomain of WD40 repeats is also conserved in CSA and DDB2. The importance of the arginine (Arg 273) in DDB2 within the WDXR submotif is underscored by its mutation to histidine (R273H) in a xeroderma pigmentosum group E patient and the subsequent inability to bind DDB1 (ref. 16). This motif is present in most of the WDR proteins we found (see Supplementary Information, Fig. S3), suggesting that it is a key determinant for the interaction with DDB1. The crystal structures of WDR5 and TLE1 WD40-repeat domains reveal that the Asp and Arg/Lys in the WDXR/K submotif are exposed on the bottom surface of the barrel-shaped WD40 propeller folds[17,18], and are thus available for interaction with CUL4–DDB1. The Arg/Lys in the DXXXR/K submotif is on the side to the bottom of the barrel, whereas the first Asp is on the opposite side of the barrel that binds to histone in WDR5. Thus the R/KXWDXR/K motif is more likely to be involved in DDB1 binding. However, we have noticed that although the last Arg/Lys in WDXR/K is required for DDB2 binding to DDB1, it is not strictly observed among the WDR proteins we identified. A few WDR proteins lack the first or second Arg/Lys in the WDXR/K submotif but still bind to CUL4–DDB1 (see Supplementary Information, Fig. S3). Interestingly, although EED strongly interacts with CUL4A–DDB1 (Fig. 3e), it does not have a recognizable WDXR/K submotif (it has WNIQ in the N repeat of the WD40-repeat domain and WRIN in the N+1 repeat instead; see Supplementary Information, Fig. S3 and data not shown)[19]. Thus, although WDR proteins containing the R/KXWDXR/K motif are enriched in CUL4–DDB1 complexes, other structural motifs may also mediate the interaction between WDR proteins and CUL4–DDB1.

If CUL4–DDB1 can associate with different WDR proteins to target distinct substrates, loss of L2DTL, but not other WDR proteins, should prevent CDT1 degradation. To test this hypothesis, we silenced the expression of various WDR proteins, such as *Drosophila* WDS (the orthologue of WDR5), RBBP5, ESC, GRWD1, and WDR26 in *Drosophila* S2 cells and analysed CDT1 degradation in response to DNA damage. As a positive control, we used *Drosophila L2DTL*, *CUL4* and *CSN5* RNA interference (RNAi) to suppress CDT1 degradation after DNA damage[3,8].

As shown in Fig. 4a and b, although the treatment of *Drosophila* S2 cells with either *CUL4*, *CSN5* or *L2DTL* double-stranded RNAs prevented the degradation of CDT1 in response to γ-irradiation, the inactivation of other WDR proteins by RNAi did not alter CDT1 proteolysis after DNA damage.

We also examined the requirement for WDR5, RBBP5 and WDR26 for proteolysis of CDT1 in human cells. Again, only the inactivation of human L2DTL prevented CDT1 proteolysis in response to γ-irradiation, whereas loss of other WDR proteins did not block CDT1 degradation after irradiation (Fig. 4c, d). These studies suggest that L2DTL is the only WDR protein tested so far that is required for CDT1 proteolysis.

If distinct WDR proteins define specific biological events for CUL4–DDB1 regulation, the function of CUL4–DDB1 complex should be required for biological processes specifically regulated by a particular WDR protein. We focused on testing whether CUL4–DDB1 regulates histone H3 methylation at K4, K9 and K27, as WDR proteins WDR5, RBBP5 and EED interact with the CUL4–DDB1 complexes

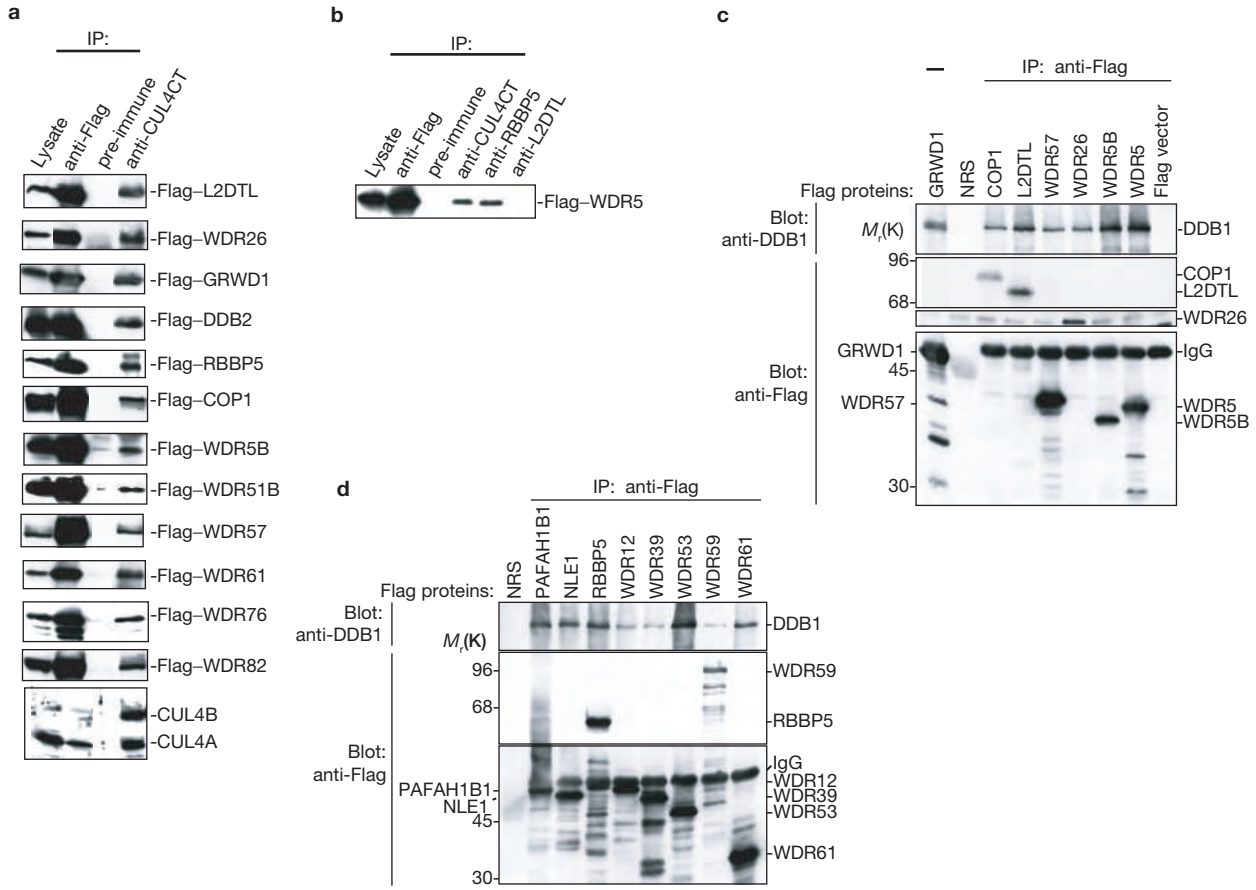

Figure 2 WDR proteins interact with the CUL4–DDB1 complexes. (**a**) Human L2DTL and other WDR proteins were Flag-epitope tagged and expressed in 293 or H1299 cells. The protein complexes were isolated from transfected cell lysates by immunoprecipitation with the anti-Flag antibody, pre-immune serum, and anti-CUL4CT antibodies that recognize both CUL4A and CUL4B and blotted for Flag-tagged and CUL4 proteins as indicated. (**b**) Flag-tagged WDR5 was transfected as in **a** and the complexes were immunoprecipitated by anti-Flag, CUL4, RBBP5 and L2DTL antibodies as indicated, then blotted with anti-Flag antibodies. (**c**, **d**) Flag-tagged WDR proteins were transfected as in **a**, their complexes immunoprecipitated by anti-Flag and the proteins separated by SDS–PAGE. The top half of the gel was blotted with anti-DDB1 antibodies and the bottom half with anti-Flag antibodies. Because the top halves also contain Flag–COP1, L2DTL and WDR26 in **c**, and RBBP5 and WDR59 in **d**, the anti-DDB1 blots were stripped and reblotted with anti-Flag antibodies for these proteins. The Flag–WDR26 blot in **c** was from a different exposure.

(Figs 2, 3). WDR5 is an essential component of the MLL (mixed-lineage leukaemia) histone methylation complexes[7], which are homologous to the *Saccharomyces cerevisiae* Set1 and the *Drosophila* Trithorax complexes that catalyze the critical trimethylation at K4 on histone H3 (ref. 5). RBBP5 is another WDR protein invariably associated with the MLL–WDR5 complexes[20,21]. The trimethylation at K4 marks transcription start sites of almost all active promoters, including the homeobox genes (Hox)[7,22]. It has been shown that WDR5 directly interacts with the methylated K4 of histone H3 in cell lysates and this interaction is required for the K4 trimethylation by the Set-domain containing MLL methylation complexes[7]. Loss of WDR5 by siRNA-mediated gene silencing has been shown to severely and specifically block the tri- and mono-methylation of histone H3 at K4, whereas dimethylation at this lysine is only modestly affected[7].

Inactivation of WDR5 by small interfering RNA (siRNA) severely inhibited the tri- and mono-methylation of histone H3 at K4 (Fig. 5d), whereas K4 dimethylation was only slightly affected (Fig. 5d), consistent with previous reports[7]. Treatment with *WDR5* siRNA did not significantly affect total histone H3 levels or trimethylation at K9 (Fig. 5d)[7]. To determine whether the regulation of histone H3 methylation at K4 is unique to WDR5–RBBP5, we examined whether loss of L2DTL or other WDR proteins affects this methylation. We found that the loss of L2DTL or DDB2 did not significantly affect K4 methylation status (Fig. 5c). These observations indicate that WDR5 and RBBP5 are specific WDR proteins for these K4 modifications in histone H3, as other WDR proteins, including L2DTL, cannot replace their function.

Our previous studies showed that CUL4–DDB1 and L2DTL can interact with CDT1 to regulate its stability after DNA damage[3,8]. If WDR5 or RBBP5 are adaptors for CUL4–DDB1, CUL4–DDB1 should interact with the methylated histone H3 at K4. Furthermore, loss of either CUL4 or DDB1 should mimic the effects of WDR5 or RBBP1 inactivation on histone H3 methylation at K4 if the CUL4–DDB1 complexes are functionally involved. We tested this possibility by examining whether CUL4–DDB1 can interact with methylated mononucleosomes. Previous studies indicate that WDR5 directly binds to histone H3 containing di- and tri-methylated H3 at K4 (ref. 7) and this binding is considered as an essential and critical step that allows the MLL methylation complexes to methylate histone H3 at K4 (ref. 7). To monitor CUL4–DDB1 binding, the soluble mononucleosomes were released from the nuclear fraction after digestion with micrococcal nuclease[23]. Mononucleosomes containing tri- and

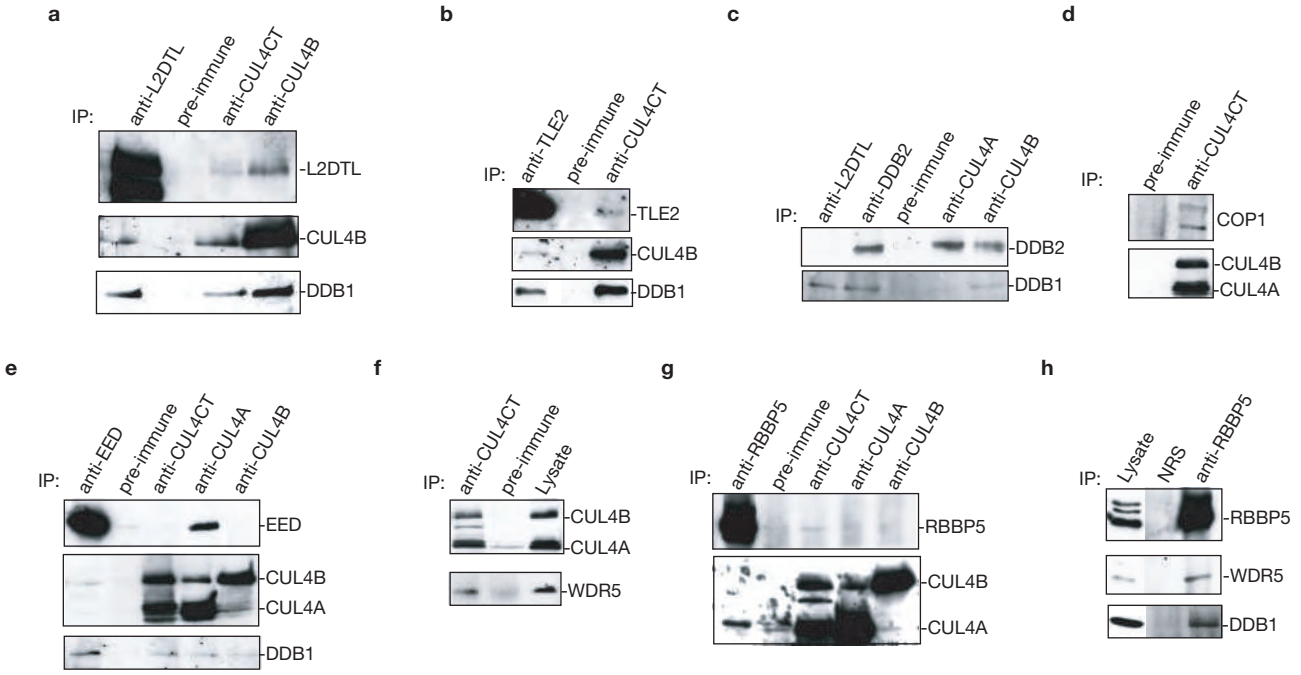

Figure 3 Endogenous WDR proteins interact with the CUL4–DDB1 complexes. (**a**–**h**) Protein complexes containing human WDR proteins L2DTL, TLE2, DDB2, COP1, EED and RBBP5, as well as CUL4A and CUL4B were isolated by specific antibodies as indicated from human H1299 or HeLa cells. Their association with CUL4A, CUL4B, DDB1 and WDR5 was analysed by western blotting as indicated. NRS, normal rabbit serum. In **h,** the proteins in the lysates are from a different exposure.

di-methylated histone H3 at K4 were then immunoprecipitated with ChIP grade anti-tri- and di-methylated K4 antibodies and their association with WDR5 or DDB1–CUL4 was examined[7]. Our studies revealed that WDR5 and DDB1 are indeed present in the nucleosomes immunoprecipitated by the di- and trimethylated K4 antibodies (Fig. 5a). In addition, DDB1 is associated with nucleosomes containing monomethylated histone H3 at K4, and trimethylated histone H3 at K9 and K27 (Fig. 5b). As the WDR protein EED regulates the methylation of histone H3 at K9 and K27 (refs 5, 6), CUL4–DDB1 in complex with EED may participate in regulating the methylation of H3 at K9 and K27 (Fig. 3e).

We found that CUL4 was also present in the H3 methylated nucleosome fractions. Interestingly, CUL4A, but not its paralogue CUL4B, is enriched in methylated histone H3 nucleosomes (Fig. 5b), although only trace amounts of CUL4 can be detected with H3 methylated at K9 and K27. It has been shown that WDR5 can bind to the amino terminal peptide of histone H3 only when K4 is methylated[7]. We found that WDR5, DDB1 and CUL4A can interact with the H3 N-terminal peptide containing methylated K4, but not its unmodified form in cell lysates, suggesting that these interactions are specific for methylated histone H3 (Fig. 5c). In these experiments, although we could relatively easily find CUL4–DDB1 in the methylated nucleosomes, we could not detect L2DTL in these fractions, consistent with its inability to alter histone H3 methylation at K4 (Fig. 5a–c, and data not shown).

As CUL4–DDB1 interact with WDR5, RBBP5 and EED, and both DDB1 and CUL4 associated with methylated histone H3, we examined whether the CUL4–DDB1 complex is involved in histone H3 methylation. As predicted, inactivation of either CUL4 (CUL4A and CUL4B) or DDB1 by siRNA severely reduced the tri- and monomethylations of histone H3 at K4, with little or no significant effect on the H3K4 dimethylation (Fig. 5e). This selective inhibition of tri- and monomethylations of histone H3 at K4 after CUL4 or DDB1 inactivation mirrors the effect of WDR5 and RBBP5 inactivation, and supports the notion that WDR5 and RBBP5 function as adaptor proteins for CUL4–DDB1 to regulate H3 K4 methylation (Fig. 5d)[7]. In these experiments, inactivation of CUL4 or DDB1 caused a significant inhibition of histone H3 trimethylation at K9 and K27 (Fig. 5e). This effect is relatively modest compared to the impact of CUL4–DDB1 inactivation on histone H3 tri- and monomethylation at K4 (Fig. 5e). As this effect is not observed after WDR5 and RBBP5 inactivation (Fig. 5d), CUL4–DDB1 may use other WDR proteins for the regulation of histone H3 methylation at K9 and K27. The CUL4–DDB1 complex also associates with EED (Fig. 3e), suggesting that EED is a strong candidate for the CUL4–DDB1 effect on trimethylation of histone H3 at K9 and K27. The human Polycomb-group protein EED is the homologue of *Drosophila* PcG protein Esc, which forms a core complex with the SET-domain containing protein enhancer of zeste (E(Z), or its human homologue EZh2) and suppressor of zeste-12 (Su(z)12) to methylate K9 and K27 in histone H3 (refs 24, 25) and repress transcription[5,6,26]. The trimethylation at K27 in histone H3 is also associated with mammalian X chromosome inactivation and genomic imprinting, whereas K9 trimethylation is responsible for the transcriptionally repressive state in heterochromatin[5,6,27]. However, because our *EED* siRNAs did not efficiently silence the expression of EED, we were not able to determine whether *EED* siRNA severely affects K9 and K27 methylation, as reported in mouse or *Drosophila*[5,6,27]. However, the loss of L2DTL, WDR5, RBBP5 or DDB2, do not affect the trimethylation of histone H3 at K9 and K27. These studies indicate that the CUL4–DDB1 E3 ligase complexes interact with distinct WDR proteins (such as WDR5 and EED proteins) to differentially regulate histone methylation at K4, K9 and K27, and that they interact with L2DTL to target CDT1 for proteolysis in response to DNA damage.

We have shown that the CUL4–DDB1 ubiquitin E3 ligase interacts with multiple WDR proteins. WDR proteins constitute one of the largest gene families in human genome with more than 150

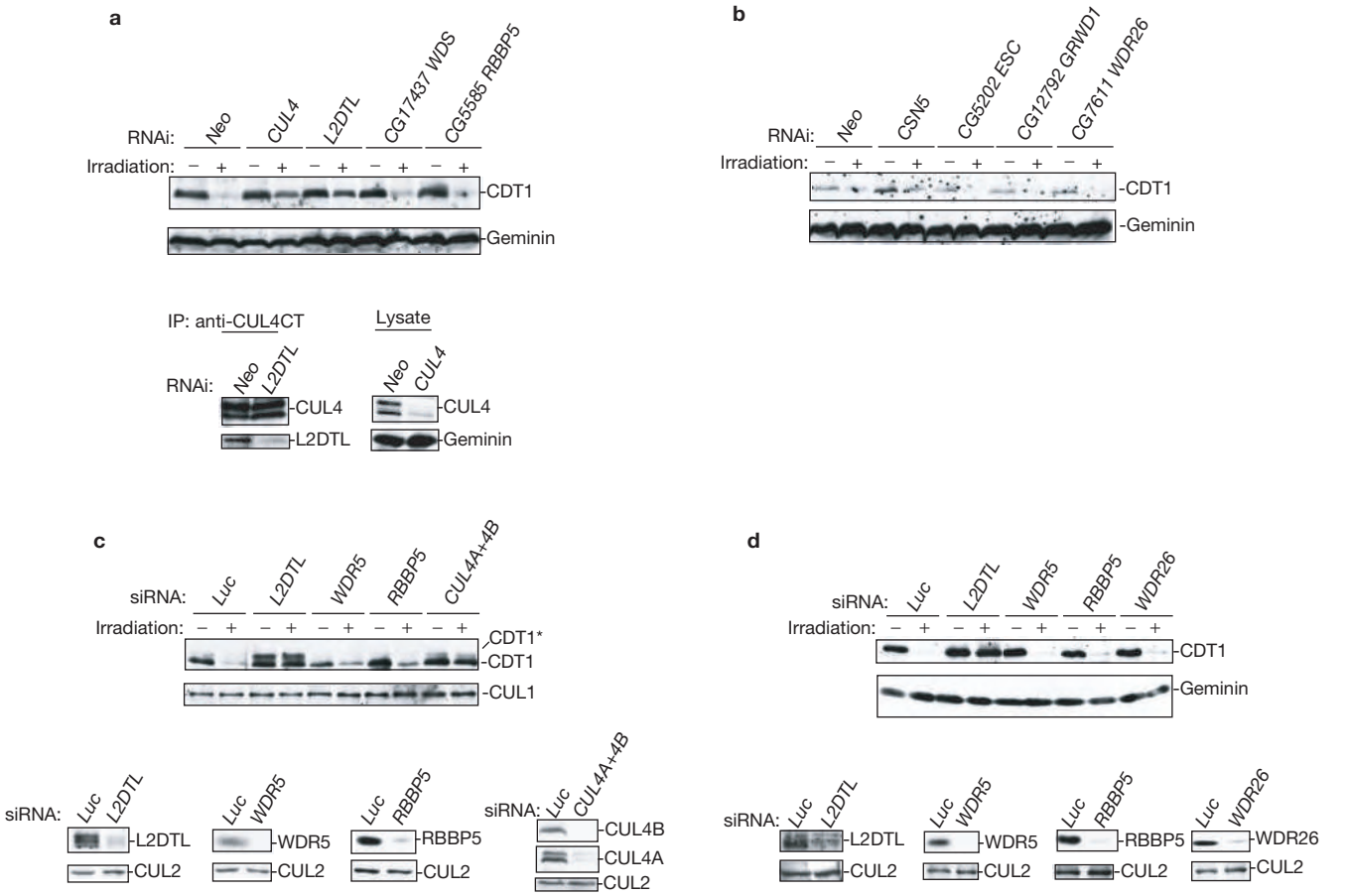

Figure 4 L2DTL, but not other WDR proteins, specifically targets CDT1 for proteolysis in response to DNA damage. (**a**, **b**) *Drosophila* S2 cells were treated with 15 μg each of specific double-stranded RNAs for *Neo* (control) and Drosophila orthologues of *CUL4*, *L2DTL*, *CSN5*, *WDR5* (*WDS*), *EED*, *RBBP5*, *GRWD1* and *WDR26* for 60 h. The cells were γ-irradiated at 100 Gy and cells were harvested in SDS containing buffer 1 h later. The total CDT1 and geminin proteins were analysed by specific *Drosophila* anti-CDT1 and geminin antibodies. (**c**, **d**) Human L2DTL is specific for CDT1 proteolysis after DNA damage. Human H1299 (**c**) and HeLa (**d**) cells were transfected with 50 nM siRNAs for *Luciferase* (*Luc*; control), *L2DTL*, *WDR5*, *RBBP5*, *CUL4A* and *CUL4B*, and *WDR26* for 72 h as indicated. The cells were γ-irradiated (10 Gy) and harvested after 1 h. The protein levels of CDT1, L2DTL, WDR5, RBBP5, WDR26, geminin, CUL2, and CUL4A and B were analysed by specific antibodies as indicated. CTD1*, a possible modified CDT1 protein.

members[14,28]. As WDR proteins have been shown to be involved in regulating an array of important biological processes (such as transcription, cell-cycle regulation, signalling and chromatin modification[14,29]), it is possible that these processes are also controlled by the CUL4–DDB1 ubiquitin E3 ligase complex. Our data suggest that WDR proteins function as the substrate-specific adaptors for the core CUL4–DDB1 ubiquitin E3 ligase complex in specific biological events, such as histone methylation or CDT1 degradation after DNA damage. These data are consistent with the observation that CUL4, DDB1 or its paralogues are involved in regulating histone methylation in fission yeast[30]. As L2DTL, DDB2 and CSA directly interact with DDB1, it is likely that DDB1 provides an interaction interface between WDR proteins and the CUL4–ROC E3 ligase complex to promote substrate recognition and polyubiquitination. Further work is required to determine how CUL4–DDB1 ubiquitin E3 ligases regulate a wide range of biological processes by targeting specific proteins for ubiquitin-dependent proteolysis. □

Note added in proof: while this manuscript was in press, two papers were published that demonstrated that WD40-repeat domain proteins can act as substrate-specific adaptors for CUL4–DDB1 ubiquitin E3 ligases[31,32].

METHODS

Isolation of the CUL4B complexes. Affinity purified CUL4B antibodies were covalently linked to protein A–Sepharose beads by a previously described method using 50 litres of suspension 293 cells[8]. The lysate was passed through the immobilized CUL4B antibody–protein A chromatography and the proteins in the complexes were eluted, separated and identified by mass spectrometry[8]. Peptides of DDB1, L2DTL, CSN and other WDR proteins were obtained. The peptides from WDR–TLE1-3 (TLIVVGGEASTLTLTIWDLASPTPR; DAPTSPASVASSSTPSSK; SPMVSFGAVGFDPHPPMR; NDAPTPGTSTTPGLR; DNLLNAWR; ATVYEVIY; IWDISQPGSK; TPYGASIFQSK; LWTGGLDNTVR; VPTWGPLR); WDR protein SMU1 (DSSQILSASFDQTIR and MSIEIESSDVIRLIMQYLK); WDR82 (VVALSMSPVDDIFISGSLDK); glutamate rich WD-repeat protein GRWD1 (VSWLGEEPVAGVWSEK); and WDR26 (SELPIAELTGHTR; GYNFEDLTDR; LQTYLPPSVMLPPR; NIVQEDHPIMSFTISK and MSQSHEDSLTSVAWNPDGK) were obtained. These peptides matched exactly to the corresponding human protein sequences, respectively. The cDNAs of the genes encoding identified proteins and other WDR proteins were obtained from American Type Culture Collection (ATCC, Manassas, VA) unless specified.

Cells, RNAi and siRNA, and transfection. Human HEK293, HeLa, RKO and H1299 cells and *Drosophila* Schneider S2 cells were cultured as previously described[3]. The *DDB1*, *CUL4A*, *CUL4B* and *L2DTL* siRNAs were synthesized and transfection of the siRNAs was conducted as previously described[4]. The siRNAs for human *RBBP5* (GAGCCGAGATGGTCATAAA), *WDR26* (CTACCAAATTCCGAAATCA) and *DDB2* (AGAGCGAGATCCGAGTTTA),

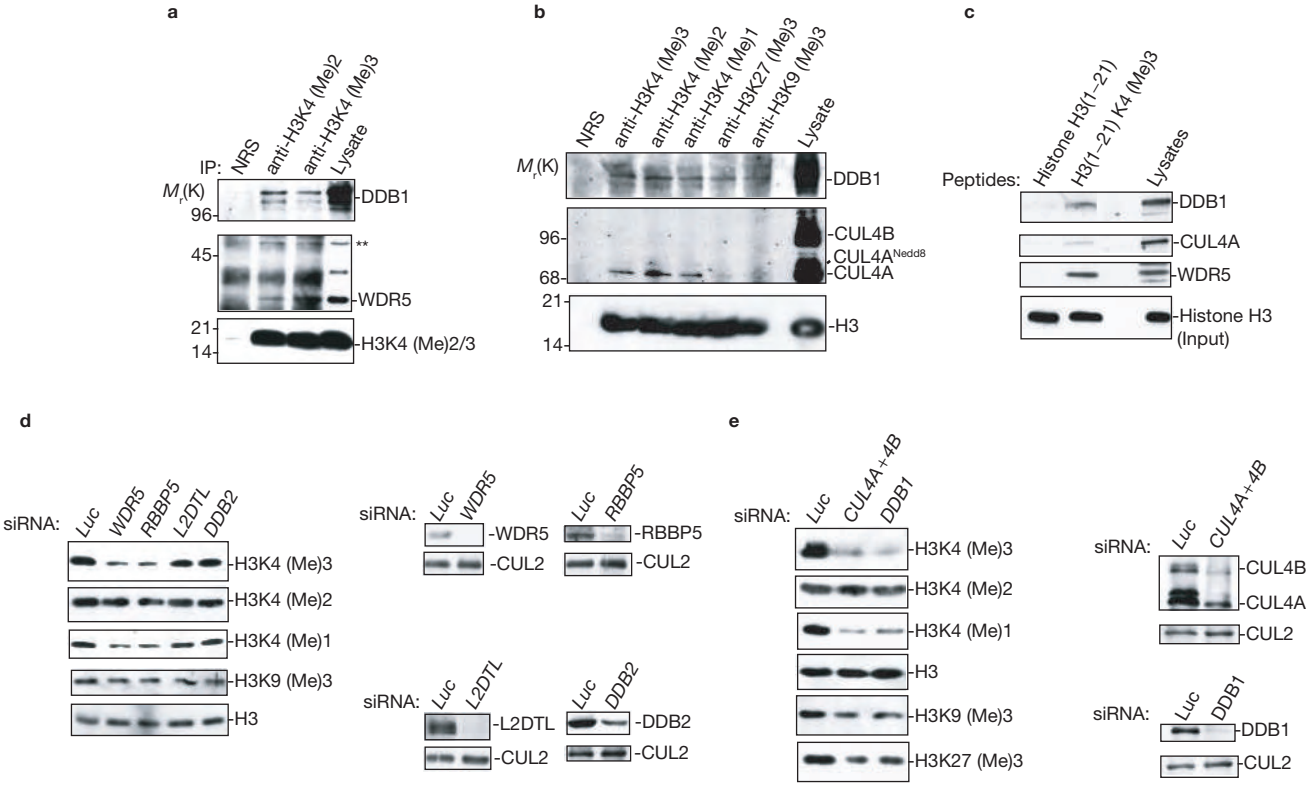

Figure 5 CUL4–DDB1 associates with histone H3 methylated at K4, K9 and K27 and regulates H3 methylation *in vivo*. (**a**) Association of DDB1 and WDR5 with histone H3 methylated at K4. HeLa cells were harvested and lysed as described in the Methods. The mononucleosomes containing the methylated histone H3 were released from the insoluble chromatin preparation by micrococcal nuclease. The soluble nucleosomes were clarified by centrifugation and used as the source for immunoprecipitation with control normal rabbit serum (NRS), and antibodies against histone H3 that is tri- and dimethylated at K4 as indicated H3K4 (Me)2/3. The association of DDB1 and WDR5 with nucleosomes was analysed by western blotting using the indicated antibodies. The asterisk indicates another protein detected by the WDR5 antibody. (**b**) DDB1 is associated with histone H3 methylated at K4, K9 and K27 whereas CUL4A is enriched in histone H3 methylated at K4. The experiment was performed as in **a,** except mononucleosome fractions were immunoprecipitated with antibodies against tri-, di- and monomethylated histone H3K4; and trimethylated H3K9 and H3K27. These complexes were blotted with anti-CUL4A and CUL4B antibodies. CUL4A^{Nedd8} is a ubiquitin-like modification of CUL4A by Nedd8. (**c**) The biotinylated N-terminal peptides of histone H3, with or without K4 methylation, was linked to streptavidin–Sepharose beads and incubated with the nuclear extract from HeLa cells. The binding of CUL4A, DDB1 and WDR5 to the peptides were analysed. (**d**) WDR5, but not other WDR proteins, regulates histone H3K4 methylatiom. Human HeLa cells were treated with 50 nM siRNAs for *luciferase, WDR5, RBBP5, L2DTL* and *DDB2* for 72 h. Total cell lysates were prepared by lysing cells in SDS sample buffer and the levels of tri-, di- and monomethylated histone H3K4 and trimethylated H3K9 were analysed. Total histone H3 was used as a control. (**e**) CUL4 and DDB1 regulate histone methylation at H3K4, H3K9 and H3K27. HeLa cells were treated with 50 nM siRNAs for *luciferase, CUL4A* and *CUL4B,* and *DDB1* for 72 h. The total cell lysates were prepared and the levels of tri-, di- and monomethylated histone H3K4 and trimethylated H3K9 and H3K27 were analysed. Total histone H3 was used as a control.

as well as siRNA smart pools for human *WDR5* and *EED–HEED* were synthesized and obtained from Dharmacon, Lafayette, CO. The double-stranded RNAs for *Drosophila* homologues of *WDR5 (*WDS, CG17437), *RBBP5* (CG5585), *EED–HEED* (ESC1, CG5202), *GRWD1* (CG12792), *WDR26* (CG7611), and *CUL4, CSN5*, and *L2DTL* were obtained, prepared and used for RNAi in *Drosophila* S2 cells as previously described[3]. The WDR proteins were fused in frame and tagged with Flag-epitope tag in p3XFlag–CMV10 (Sigma, St Louis, MO) and expressed by transfection in 293 or H1299 cells as previously described[3]. CDT1 degradation in *Drosophila* and human cells was conducted as previously described[3].

Antibodies and chromatin methylation. Anti-Flag, L2DTL, DDB1, CUL4A, CUL4B, CUL4 carboxy terminal antibodies that recognize both CUL4A and CUL4B were previously described[8]. The COP1 peptide (CVCWRALPDGESNV LIAANSQGTIKVLELV) was used for raising rabbit polyclonal antibodies. The antibodies for TLE1-3 and DDB2 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, and the anti-histone 3, H3 mono-, di-, and tri-methylation at K4 and tri-methylation at K9 and K27, EED, and WDR5 were from Abcam, (Cambridge, MA) or Upstate (Lake Placid, NY) . The histone H3 amino terminal peptide (amino acids 1–21) with or without K4 methylation was from Upstate and the binding assays were conducted as previously described[7]. The RBBP5 antibody was from Bethyl Laboratory (Montgomery, TX). Nuclei containing the intact chromatin were isolated according to published protocols and mononucleosomes were prepared from the isolated chromatin preparation after digestion with micrococcal nuclease in the presence of calcium[23].

GenBank accession numbers. The GenBank accession numbers for CDW2 to CDW14 are EF011612 to EF011624, respectively.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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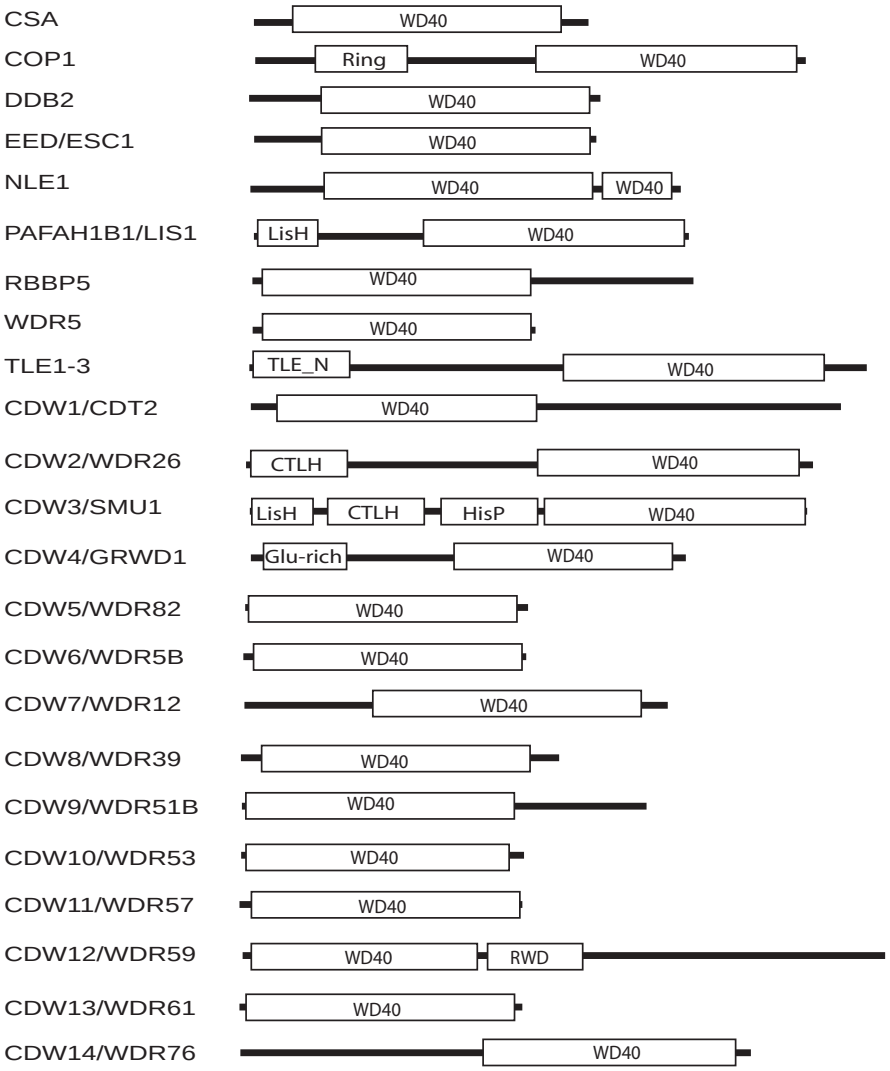

Figure S1 The WDR proteins used in our experiments.

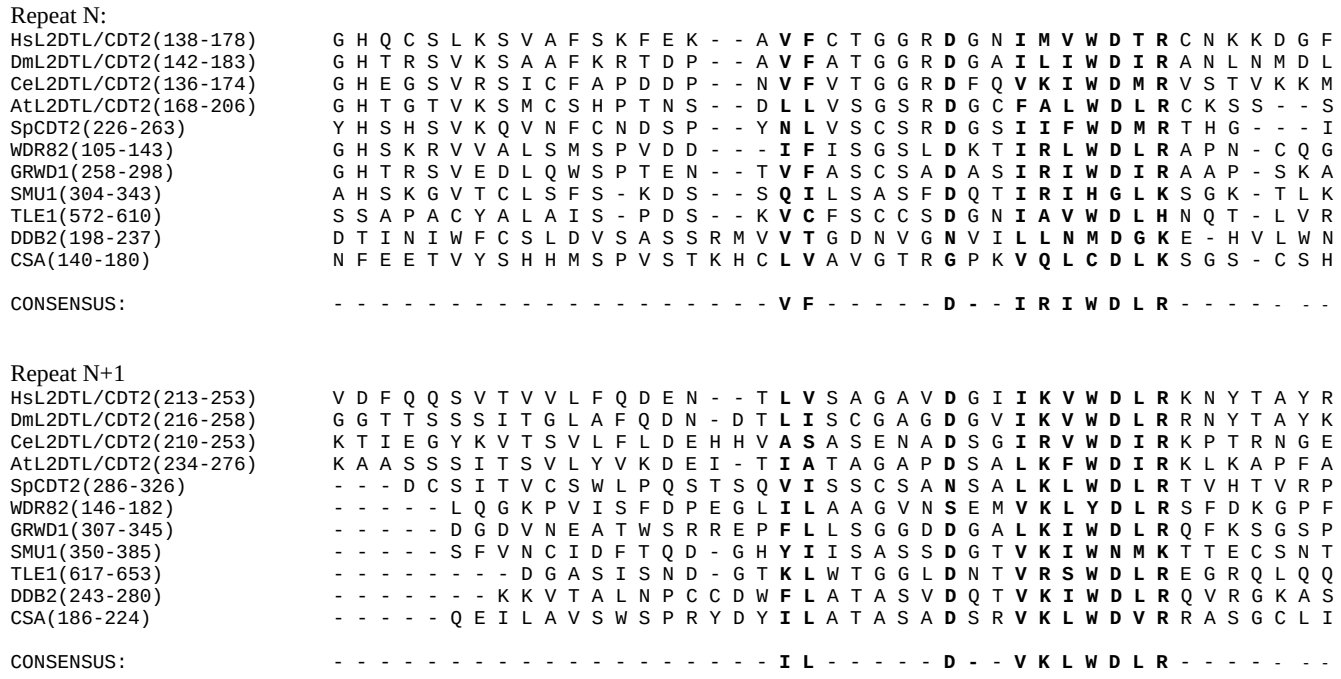

Figure S2 The conserved W/Y-D-H-R/K motif between L2DTL/CDT2 between different species and WDR proteins isolated by their association with CUL4B complex in Figure 1. CSA and DDB1 are also included as a comparison.

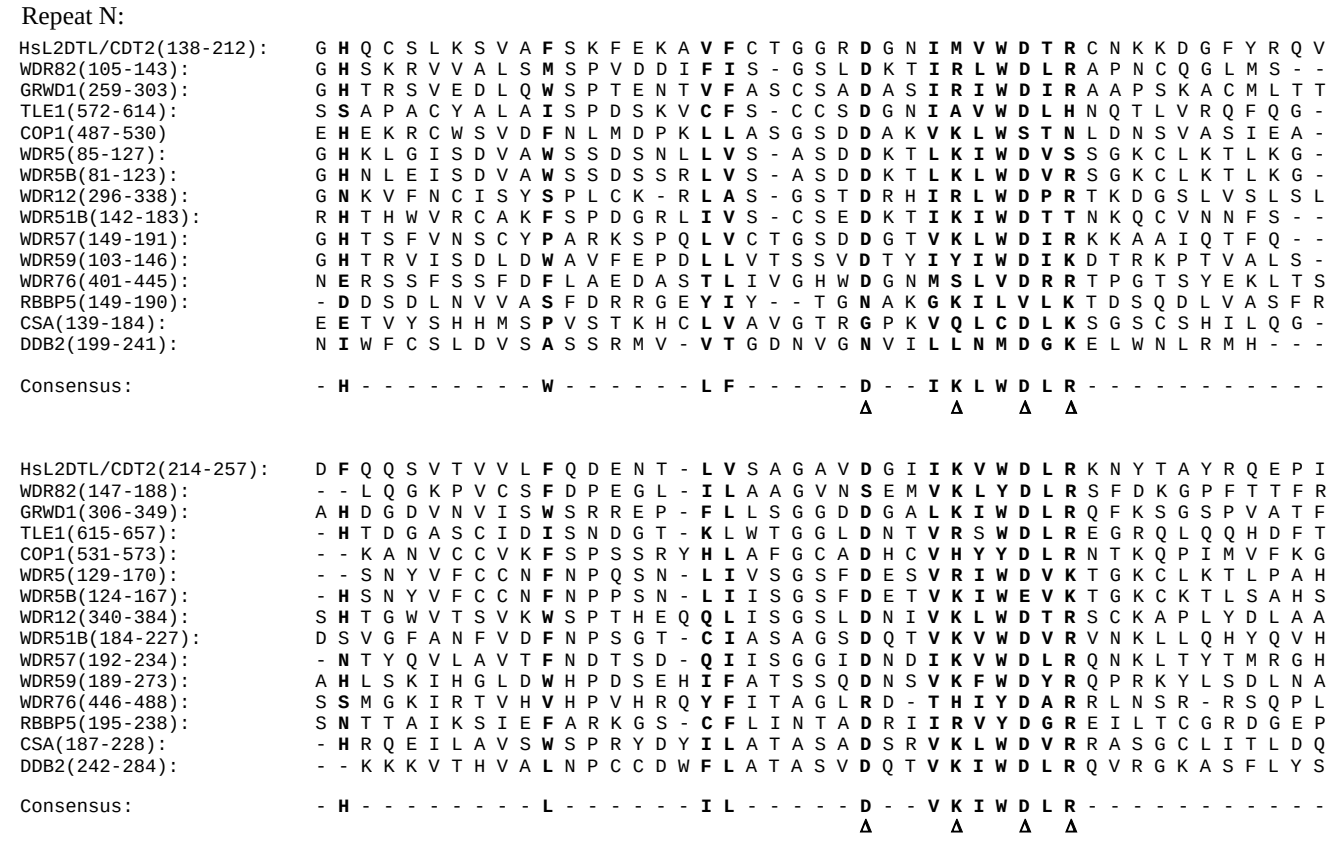

Figure S3 The alignment of various WDR proteins in two consecutive and central repeats of WD40 domains. The conserved amino acid residues are in bold face. D denotes the exposed amino acid residues which also mark the DXXXR/KXWDXR/K motif.

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