The antiobesity factor WDTC1 suppresses adipogenesis via the CRL4<sup>WDTC1</sup> E3 ligase

Beezly S Groh<sup>1</sup>, Feng Yan<sup>2</sup>, Matthew D Smith<sup>2</sup>, Yanbao Yu<sup>1</sup>, Xian Chen<sup>1</sup> & Yue Xiong<sup>1,2,*</sup>

Abstract

**WDTC1/Adp** encodes an evolutionarily conserved suppressor of lipid accumulation. While reduced WDTC1 expression is associated with obesity in mice and humans, its cellular function is unknown. Here, we demonstrate that WDTC1 is a component of a DDB1-CUL4-ROC1 (CRL4) E3 ligase. Using 3T3-L1 cell culture model of adipogenesis, we show that disrupting the interaction between WDTC1 and DDB1 leads to a loss of adipogenic suppression by WDTC1, increased triglyceride accumulation and adipogenic gene expression. We show that the CRL4<sup>WDTC1</sup> complex promotes histone H2AK119 monoubiquitylation, thus suggesting a role for this complex in transcriptional repression during adipogenesis. Our results identify a biochemical role for WDTC1 and extend the functional range of the CRL4 complex to the suppression of fat accumulation.

**Keywords** adipose; adp; cullins; obesity; WDTC1

**Subject Categories** Metabolism; Post-translational Modifications, Proteolysis & Proteomics

**DOI** 10.15252/embr.201540500 | Received 6 April 2015 | Revised 25 February 2016 | Accepted 4 March 2016

Introduction

Over 50 years ago, Dr. Winifred Doane isolated and extensively characterized a naturally derived *D. melanogaster* mutant, termed adipose (*adp*) [1,2]. The most obvious mutant phenotype observed was fat body hypertrophy due to excessive lipid storage. *Adp* is evolutionarily conserved from flies to humans as a single gene and encodes a protein containing WD40 repeat domains and TPR motifs [3]. The mammalian homolog of *Adp* is *WDTC1* (WD40 and tetra-tripeptide repeats [1]). Loss of a single *Wdmc1* allele results in obese mice with poor metabolic profiles, and conversely, transgenic expression of *Wdmc1* in fat cells yields lean mice [4]. Recently, population studies have linked reduced *Wdmc1* expression to human obesity [5,6]. Despite strong genetic evidence linking WDTC1 to antidiapigenic function, its molecular mechanism remains unknown.

Ubiquitin pathway plays a critical role in virtually all cellular processes. Ubiquitylation proceeds via an enzymatic cascade where E1 and E2 enzymes catalyze the activation and conjugation of ubiquitin, while E3s confer reaction specificity through substrate recruitment [7,8]. Substrates can be polyubiquitylated, which often leads to proteasome-dependent degradation, or monoubiquitylated, which regulates the property and thus the function of the substrate. Comprising the largest family of E3 ligases is the cullin RING E3 ligase (CRL) complexes in which a cullin serves as the scaffold to bind small RING finger protein ROC1 (RBX1/HRT1) through a C-terminal domain, and a linker-substrate receptor dimer or a substrate receptor directly via a N-terminal domain. Mammalian cells express two cullin 4 (CUL4) proteins, CUL4A and CUL4B, that bind damaged DNA binding (DDB1) protein. DDB1 acts as the linker to bridge the interaction between CUL4 and a subset of DDB1 binding WD40 repeat proteins (DWDs or DCAFs for DDB1 cullin-associated factors) which function as substrate receptors to target specific substrates to the CRL4 E3 complexes [9–12]. The human genome encodes an estimated ~90 DWD proteins [9], but the functional interaction between CRL4 and DWDs remains unexplored for the vast majority. One of predicted DWD protein is WDTC1, raising the possibility that WDTC1 may function as a substrate receptor of CRL4 E3 ligase to inhibit fat accumulation. This study is aimed at determining this issue.

**Results and Discussion**

**WDTC1 is a putative substrate receptor for CRL4 E3 ligase**

Two structural features suggested that WDTC1 binds DDB1 (Fig 1A). First, WDTC1 contains two tandem DWD boxes with conserved WDXR submotifs, the signature motif present in nearly all WD40 proteins that bind DDB1 [9]. Second, the N-terminal regions of vertebrate WDTC1 contain an α-helical motif termed the H-box which is shared by several DWD proteins and some viral proteins that bind DDB1 [13]. Although the H-box of the DWD protein DDB2 makes a large contribution to DDB1 binding [11,13], a mutation in the WDXXR of DDB2 (R273) is found in human xero-derma pigmentosum patients and disrupts DDB2-DDB1 binding [14]. These observations underscore the importance and specificity of both DWD motif and H-box in mediating DDB1 binding, thus offering a unique opportunity for investigating the WDTC1–CRL4 interaction.

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1. Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC, USA
2. Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA
*Corresponding author. Tel: +1 919 966 2142; Fax: +1 919 966 8799; E-mail: yxiong@email.unc.edu

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Figure 1.
To test whether WDTC1 and CRL4 interact in vivo, endogenous CUL4A or CUL4B complexes were immunoprecipitated from 293T cells, and the presence of WDTC1 in the immunoprecipitates was determined by immunoblotting. We found that WDTC1 interacts endogenously with both CUL4A and CUL4B (Fig 1B). We then generated two WDTC1 mutants, one deleted the N-terminal 25 amino acid residues containing the H-box (referred to as ΔH) and one substituted the arginine residues in the tandem WDXRs to alanines (referred to as RARA; Fig 1A). We found that wild-type Flag-WDTC1 coimmunoprecipitated all CRL4 subunits tested, including DDB1, CUL4A, CUL4B, and ROC1 (Fig 1C). In contrast, deletion of the H-box (ΔH) completely ablated complex formation, while the RARA mutant showed a modest decrease in DDB1 binding and a marked reduction in CUL4 and ROC1 binding. A superimposed structure of WDTC1 is shown in complex with CRL4 (Figs 1D and EV1). As previously reported [13], WDTC1 is anchored through its H-box into the BPC propeller domain of DDB1. Similar to DDB2-DDB1 binding [15], the tandem WDXRs of WDTC1 are not predicted to make direct contact with DDB1 but are solvent-exposed on the bottom surface of the β-propeller. How these two signature arginine residues contribute to the binding of DWDs to DDB1 is presently unclear.

We previously reported the expression of an equivalent to a hypomorphic allele. This is consistent with genetic evidence indicating that WDTC1 exhibits dosage sensitivity [1, 4]. Additionally, WDTC1 likely acts downstream of the insulin signaling pathway as replacing insulin with the PPARγ agonist rosiglitazone did not alter the observed ORO phenotypes (Fig EV2F). Validating the phenotypes evaluated by ORO, ectopic expression of WT WDTC1 suppressed triglyceride accumulation, while expression of the WDTC1ΔH (3.5-fold) and WDTC1RARA (2.3-fold) mutants resulted in significantly higher triglyceride levels (Fig 2C). Further, expression of WT WDTC1 decreased adipogenic marker expression but WDTC1ΔH and WDTC1RARA mutants increased the expression of these genes with WDTC1ΔH again being more effective than WDTC1RARA (Figs 2D and EV2G). We also assessed expression of genes regulating lipogenesis, lipolysis, and fatty acid oxidation, and
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found modest alterations that are likely due to altered adipogenesis in cells expressing WT or mutant WDTC1 proteins (Fig EV2H). The expression of WT WDTC1 resulted in a modest reduction in the expression of genes linked to lipogenesis (Fig EV3A). While the re-expression of WT WDTC1 resulted in a modest reduction in the expression of WT WDTC1 proteins (Fig EV2H). The expression of WT WDTC1 resulted in a modest reduction in the expression of genes linked to lipogenesis (Fig EV3A), while 3T3-L1 adipogenesis was enhanced by CUL4A depletion, it was substantially inhibited by CUL4B depletion (Fig EV3D). It is presently unclear to us why the knockdown of Cul4a and Cul4b has opposite effects on 3T3-L1 adipogenesis. However, the function of CULAA in promoting adipogenesis needs to be cautiously interpreted as it is likely that multiple distinct CRLAA complexes are assembled in 3T3-L1 cells.

To more specifically demonstrate the function of CUL4–WDTC1 interaction in the suppression of 3T3-L1 adipogenesis, we therefore carried out rescue experiments. Utilizing a lentiviral knock-down-rescue vector strategy [18], we engineered 3T3-L1 cells to simultaneously knockdown endogenous Wdtc1 by shRNA and ectopically re-express shRNA-resistant WDTC1 encoding either WT or DDB1-binding mutants ΔH or RARA to levels comparable to 3T3-L1 cells. We found that knocking down Wdtc1 dramatically enhanced 3T3-L1 differentiation as assayed by ORO (Fig 3B), triglyceride accumulation (Fig 3C), and adipogenic gene expression (Fig 3D). While the re-expression of WT WDTC1 almost completely rescued the loss of adipogenic suppression in all three assays, adding back either ΔH or RARA mutants failed to

**Figure 2.** WDTC1 suppresses adipogenesis in a CRL4-dependent manner.

A 3T3-L1 preadipocytes were adipogenically induced, and lysates were prepared from cells collected at indicated days. Endogenous WDTC1 interaction with CRL4 subunits was evaluated by immunoprecipitation with anti-WDTC1 followed by immunoblot analyses with the indicated antibodies. Fatty acid synthase (FAS) levels were monitored to evaluate induction.

B–D 3T3-L1 stable cells expressing vector control (EV), Flag-tagged WT or mutant WDTC1 proteins were adipogenically induced. Adipogenesis was assessed by Oil Red O staining (ORO) (B); plate (top) and microscopic (bottom) views, 0.1 mm scale bar. Triglyceride levels were quantified by an enzymatic assay (C). Adipogenic gene expression was evaluated by RT–qPCR analysis (D).

Data information: In (C, D), bars represent the mean ± SEM from 3 independent experiments. *P < 0.05, **P < 0.005, significant change compared to EV control. Statistical significance was analyzed by paired two-tailed Student’s t-tests.

Source data are available online for this figure.
rescue the adipogenic defects resulting from the depletion of endogenous Wdtc1. Consistent with its partial disruption of DDB1 binding, the RARA mutant exhibited a weaker phenotype than the ΔH mutant, supporting the interpretation that the RARA mutant represents a hypomorphic form of WDTC1, while the ΔH mutant acts as a dominant negative. Collectively, these results demonstrate a strong parallel between CRL4-WDTC1 complex formation and WDTC1 function, underscoring the critical importance of the CRL4–WDTC1 interaction to the observed adipogenic suppression by WDTC1.

CRL4<sub>WDTC1</sub> promotes histone H2AK119 ubiquitylation

To gain insight into the mechanism by which CRL4<sub>WDTC1</sub> suppresses adipogenesis, we carried out two independent coupled immunoprecipitation and mass spectrometry (IP-mass spec) analyses of WDTC1 complexes in 3T3-L1 cells. Validating the IP-mass spec analyses, several known CRL4<sub>WDTC1</sub> components were identified, including DDB1, CUL4B, CUL4A, ROC1, and all nine subunits of COP9 signalosome subunits (Fig EV4A). One potential novel WDTC1-interacting protein is fatty acid synthase (FAS), a critical and positive regulator of lipid accumulation. We hypothesized that regulation of FAS by the CRL4WDTC1 complex may contribute to the antiadipogenic action of WDTC1. We therefore characterized the interaction between WDTC1 and FAS and performed preliminary characterization of a few other candidates as well. However, our data showed that CRL4WDTC1 E3 ligase does not appear to regulate FAS steady-state protein levels and reached similar conclusions for other candidates we examined such as HDAC3 and PPARγ-2 (Fig EV4B–D).

We then investigated the possibility that histone H2A is a CRL4WDTC1 substrate. This was inspired in part by the established
role of H2AK119 monoubiquitylation (referred to as H2AK119ub1 hereafter) as a key transcription repressive mechanism in the control of development and differentiation, and also because it has been demonstrated that the antiadipogenic function of WDTC1 is lost upon nuclear exclusion and WDTC1 physically interacts with ectopically expressed histones [4]. Further, ectopic WDTC1 expression is associated with reduced adipogenic marker expression (Fig 2D; [4]).

We first examined the subcellular distribution of Flag-WDTC1 and found that while it is abundantly recovered in the cytosolic fraction, it is also clearly present in the nuclear fractions, and in the MNase-digested chromatin fraction (Fig 4A). We then performed immunoprecipitation experiments with nuclear extracts and demonstrated that WDTC1 associated with H2A, as well as H2B and H3 (Fig 4B). In this experiment, we noted that WDTC1 interacted with H2A, but not the presumably monoubiquitylated form of H2A, an observation that is consistent with the possibility that CRL4<sup>WDTC1</sup> catalyzes H2AK119ub1. Chromatin coimmunoprecipitation assay confirmed the binding between WDTC1 and nucleosomal histones in vivo at physiologic levels (Fig EV4E). Notably, the DDB1 binding mutant, WDTC1<sup>ΔH</sup>, retained histone binding activity, indicating that histone binding is independent of CRL4 binding. Because WDTC1<sup>ΔH</sup> mutant disrupts DDB1 binding and thus disrupts probable ubiquitylation activity, and appears to functionally antagonize WDTC1 during adipogenesis, we took advantage of this mutant to investigate the effect of WDTC1 expression on histone H2AK119ub1 levels. We examined the global level of H2AK119ub1 in preadipocyte and induced 3T3-L1 cells stably expressing either a vector control, WT WDTC1 or WDTC1<sup>ΔH</sup> mutant (Fig 4C). We did not detect a notable change in H2AK119ub1 levels among these cell lines in the preadipocyte state. In contrast, expression of WT WDTC1 resulted in a modest, but significant and reproducible, increase in H2AK119ub1 while WDTC1<sup>ΔH</sup> expression caused a slight decrease of H2AK119ub1. This result suggests that WDTC1-promoted H2AK119ub1 is linked to adipogenesis and is dependent on the association with the CRL4 E3 complex.

To test whether H2AK119 is a direct target of CRL4<sup>WDTC1</sup> E3 ligase, we performed in vitro ubiquitylation assays. We affinity-purified Flag-tagged WT WDTC1 or WDTC1<sup>ΔH</sup> complexes from 293T cells and confirmed the presence of CRL4 components in the WT, but not ΔH, WDTC1 eluted fraction (Fig 4D, left). Recombinant H2A was incubated with purified WDTC1 complexes and reaction products were resolved by SDS–PAGE, followed by immunoblotting with H2AK119ub1 antibodies. The results show that H2A is robustly ubiquitylated by WT WDTC1, but not WDTC1<sup>ΔH</sup> complexes (Figs 4D, right, and EV4F), demonstrating that H2A was a specific substrate of CRL4<sup>WDTC1</sup> in this in vitro system. We noted that multiple ubiquitylated H2A forms were detected, perhaps due to the high efficiency nature of in vitro ubiquitylation reactions. To confirm that K119 residue is the primary target of CRL4<sup>WDTC1</sup>, wild-type H2A and its mutants, H2AK118R and H2AK119R, were tested in in vitro ubiquitylation assays (Fig 4E). While disrupting the K118 residue had very little effect, K119R mutation resulted in a substantial loss of H2A ubiquitylation by the CRL4<sup>WDTC1</sup> E3 ligase. These results collectively suggest that CRL4<sup>WDTC1</sup> promotes H2AK119ub1 in vitro and in vivo in an adipocyte lineage-specific manner.

The most extensively characterized H2A E3 ligase is the RING1A/B of PRC1 complex which plays a critical and evolutionarily conserved role in developmental control in flies and mammals. It has been unclear whether and how a single E3 ligase regulates different biological processes that involve epigenetic silencing, especially when considering the abundance of H2AK119ub1 in cells (5–15% of total H2A). Two reports support the latter possibility, 2A-HUB (DZIP3/hrUL138) that mediates selective repression of a specific set of chemokine genes in macrophages [19] and CRLAB<sup>RBBP4/C7</sup> that collaborates with PRC2 to repress various genes involved in cell growth and migration [20]. Our study supports the possibility that in addition to the historic PRC1, there may exist additional H2AK119 E3 ligases that function in epigenetic repression.

Multiple lines of genetic evidence from <i>Drosophila, C. elegans,</i> and mouse, and human epidemiology, have linked the function of WDTC1/Adp to the negative regulation of adipogenesis and fat metabolism. In this study, we demonstrate that the regulation of adipogenesis by WDTC1 requires its assembly into a CRL4 E3 ligase complex. A recent RNAi screen in <i>Drosophila</i> identified 47 genes which, when silenced, produced flies with high body fat (so-called antiobesity genes) [21]. Among them are <i>Cul4</i> and <i>Csn4</i>, which encodes a subunit of the COP9 signalosome that functions to activate CRL E3 ligases. These new findings provide additional support for the function of CRL4<sup>WDTC1</sup> as a negative regulator of adipogenesis and fat formation.

**Materials and Methods**

**Plasmids**

The human <i>WDTC1</i> (NM_015023.4) coding sequence was PCR-amplified from a HepG2 cDNA library and cloned into pENTR™/D-TOPO (Invitrogen) to generate a WDTC1 entry vector. WDTC1<sup>ΔH</sup> was subcloned from pENTR-WDTC1 and WDTC1<sup>RARA</sup> was generated by sequential QuikChange site-directed mutagenesis (Strata- gene) to pENTR-WDTC1. WDTC1 entry vectors were recombined with Gateway-adapted destination vectors (kind gift of Dr. K. I. Nakayama) p3XFLAG for transient and pMX-FLAG-puro for stable expressions. To generate WDTC1 knockdown-rescue vectors, shRNAs targeting mouse <i>Wdtc1</i> and <i>shRNA</i> refractory human <i>WDT</i>C1 coding sequences were cloned into pL-5.5-ires-EGFP (kind gift of Dr. J. Bear), as described previously [18,22]. Mouse <i>Cul4a</i> and <i>Cul4b</i> shRNA sequences were designed using BLOCK-IT RNAi tool (Invitrogen) and cloned into pMKO.1-puro retroviral vector for stable knockdown of respective mRNAs in 3T3-L1 cells. All plasmids were sequence-verified, and shRNA oligos are listed in Table EV1.

**Cell transfections, retroviral and lentiviral infections**

293T cells were transfected at 1:3 plasmid-to-reagent ratio using FuGene 6 transfection reagent (Promega) for transient overexpression or transfected with 50 nM of siRNA for knockdown using Lipolectamine 2000 reagent (Invitrogen) according to manufacturers’ protocols. Cells were incubated for 72 h for knockdown by siRNA; siRNA sequences were described previously [23] and listed in Table EV1. For retroviral or lentiviral production, 293T cells were

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Figure 4. CRL4<sup>WDTC1</sup> E3 ligase promotes H2AK119 ubiquitylation.

A. Induced 3T3-L1 cells stably expressing Flag-WDTC1 were subjected to subcellular fractionation. Aliquots of total cell extract (TCE) and subcellular fractions were analyzed by immunoblot as indicated; fraction purity was assessed by α-tubulin and histone H3 antibodies.

B. Chromatin-enriched nuclear extracts from 293T cells cotransfected with Flag-WDTC1 and HA-tagged core histones were immunoprecipitated (IP) with anti-FLAG and immunoblotted (IB) as indicated. The probable H2AK119ub1 band detected in the nuclear extract is indicated by an asterisk.

C. Soluble chromatin extracts were prepared from preadipocyte or induced 3T3-L1 cells stably expressing control (EV) and Flag-WDTC1 proteins. H2A monoubiquitylation was detected by H2AK119ub1-specific antibody and total H2A served as a loading control. Relative intensity represents ratio of H2AK119ub1 over total H2A signal normalized to EV control.

D. Affinity-purified Flag-WDTC1 wild-type and mutant ΔH complexes from 293T cells were the source of E3 ligase in in vitro ubiquitylation assays. Copurification of CRL4 E3 ligase proteins was confirmed by immunoblotting and Coomassie Blue staining (left panels). In vitro ubiquitylation assays of recombinant H2A were performed in the presence of the indicated proteins. Reaction products were resolved by SD5-PAGE, and ubiquitylated H2A was detected by anti-H2AK119ub1 (right panel).

E. In vitro ubiquitylation assays were performed with purified wild-type and mutant histone proteins as substrates. H2A ubiquitylation was assessed by immunoblotting with anti-ubiquitin and various antibodies as indicated.

Data information: In (C), bars represent the mean ± SD from 2 (preadipocyte) or 3 (induced) independent experiments. *P < 0.05, significant change compared to EV control. Statistical significance (induced only) was analyzed by paired two-tailed Student’s t-tests. Source data are available online for this figure.
Cells were visually selected for adipogenic induction. Lentivirally transduced pLL-5.5-IRES-EGFP with 4 drops of viral media twice within a 24-h interval. Cells were split to maintain preconfluence. Retrovirally transduced cells were selected with 4 μg/ml puromycin and maintained in selection media until adipogenic induction. Lentivirally transduced pLL-5.5-IRES-EGFP cells were visually selected for > 90% of cells with EGFP expression.

Antibodies, immunoprecipitation, and immunoblotting

A rabbit polyclonal antibody (Abgent, AP4944b) and a rabbit monoclonal antibody (Abcam, ab174294, [25]) were used to detect WDT1. DDB1, CUL4A, and ROC1 antibodies were generated by our laboratory as described previously [23], and all other antibodies used in this study are commercially available and listed in Table EV1. Cells were lysed on ice with a NP-40 lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1 mM EDTA, and 0.1% NP-40] supplemented with Halt protease/phosphatase inhibitor cocktail (PPIC). For immunoprecipitation experiments, clarified lysates prepared from Flag-WDT1 expressing cells were immunoprecipitated with anti-FLAG M2 affinity gel (Sigma) overnight at 4°C. Immunoprecipitates were washed 3–5 times in lysis buffer with rotation, eluted by boiling in Laemmli buffer, resolved by SDS-PAGE, transferred to PVDF membrane (Millipore), and detected by immunoblotting with indicated antibodies. Immunoblotting was performed following standard protocols; blots were cut into strips to allow probing of the same gel with multiple antibodies when possible. Blots were developed using an ECL reagent (GE Amersham). Protein band densitometry analyses were performed using ImageJ software (U.S. National Institutes of Health).

3T3-L1 differentiation, Oil Red O staining, and triglyceride assay

3T3-L1 cells were cultured and differentiated as described previously [26]. 3T3-L1 preadipocytes were cultured in DMEM supplemented with 1% penicillin-streptomycin solution (Corning) and 10% (v/v) fetal calf serum (Colorado Serum Co.). To induce differentiation, 2 day post-confluent 3T3-L1 cells (day 0) were treated with an induction media containing 1 mM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX), and 1 μg/ml insulin (all from Sigma) in 10% fetal bovine serum supplemented DMEM. Two days later, induction media was replaced with 1 μg/ml insulin only for the duration of the experiment and media was changed every 2 days. A schematic summarizing 3T3-L1 differentiation with time points indicated for different experimental procedures is shown (Fig EV2B). To stain lipid droplets, cells were fixed in 3.7% buffered formaldehyde and stained with 0.3% Oil Red O. Triglyceride levels were measured with the Triglyceride Quantitation Kit and manufacturer’s protocol (BioVision), and levels were normalized to sample protein concentrations by BCA assay (Thermo Sci.).

Real-time quantitative PCR

Total RNA was isolated from cells using Trizol (Invitrogen) and purified to remove residual phenol/chloroform using RNeasy Mini cleanup (Qiagen). First-strand cDNA was synthesized with 800 ng of RNA using Superscript II reverse transcriptase kit (Invitrogen). Quantitative PCR (qPCR) was performed in duplicate using 1 μl of cDNA and SYBR Green PCR master mix (Applied Biosystems) in an Applied Biosystems 7900HT Fast Real-Time PCR system. Gene expression was normalized to TBP levels and analyzed by ΔΔCT method. qPCR primers for Wdct1 and SREBP1c were generated using Primer-BLAST (NCBI), and all other primers were reported previously [27,28] and listed in Table EV1.

Subcellular fractionation and chromatin extraction

Cell fractionation and soluble chromatin extraction by MNase digestion was performed as described previously [29]. Pelleted 3T3-L1 cells (8 × 10⁶) were resuspended and incubated on ice for 5 min in 200 μl buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM DTT and Halt™ PPIC] plus 0.1% Triton X-100. Nuclei were recovered in pellet 1 (P1) after centrifugation (4 min, 1,300 g, 4°C), and the supernatant (S1) was further clarified by centrifugation to obtain cytosolic fraction (S2). For nucleoplasmic fraction, P1 pellet was washed once in buffer A and lysed in 100 μl buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and Halt™ PPIC), and centrifuged and the supernatant was recovered (S3). For chromatin-enriched fraction, P1 pellet was resuspended in buffer A and treated with 2 U MNase (1 min, 37°C) and centrifuged; treated nuclei were then lysed in 100 μl buffer B and the chromatin fraction was recovered in the supernatant (S3) of MNase treated nuclei. Immunoprecipitation with soluble chromatin extracts prepared by DNase treatment was performed as described previously [30].

In vivo and in vitro ubiquitylation assays

In vivo ubiquitination assays were performed as described previously [31]. For the detection of ubiquitylated WDT1C in vivo, 293T cells were first transfected with HA-ubiquitin, split ~10 h later, transfected again with various combinations of plasmids and siRNAs at 24 h post-HA-ubiquitin transfection to minimize variations in HA-ubiquitin expression across plates. At 67 h post-siRNA transfection, cells were treated with MG132 (10 μM) and collected 5 h later. Cells were lysed under denaturing conditions in 1% SDS buffer [50 mM Tris (pH 7.5), 0.5 mM EDTA, 1% SDS, 1 mM DTT] by boiling for 10 min, and clarified lysate was immunoprecipitated in 0.1% SDS by 10-fold dilution with NP-40 buffer. Flag-tagged WDT1C was immunoprecipitated by anti-FLAG M2 agarose beads, resolved by SDS–PAGE, and ubiquitylated WDT1C was detected by immunoblotting with anti-HA antibodies. For in vitro ubiquitylation assays, WDT1C immunocomplexes were purified from transiently transfected 293T cells expressing either Flag-WDT1C-WT or Flag-WDT1CΔH. Flag-tagged WDT1C was immunoprecipitated with anti-FLAG M2 agarose beads overnight on a rotator at 4°C. Immunocomplexes were washed three times in lysis buffer and twice in TBS followed by elution with molar excess of Flag peptide.
(Sigma). Ubiquitylation reactions were performed according to manufacturer’s protocol using an ubiquitin conjugation kit (Enzo Life Sci.). In 50 μl reaction volume, unless noted otherwise in figure, 100 nM of either WT or ΔH immunocomplexes as the source of E3 and 200 nM of human recombinant histone H2A substrate (New England Biolabs) were combined with a ubiquitylation buffer containing 100 nM EI, 1 μM E2-UbcH5c, 1 μM human recombinant WT ubiquitin or ubiquitin Mutant No K (Boston Biochem), 1 U inorganic pyrophosphatase, 1 mM DTT, and 5 mM Mg-ATP. Reactions were incubated at 37°C for 30 min and terminated by addition of SDS sample buffer. Reaction products were resolved by SDS–PAGE and detected by immunoblotting with either anti-H2AK119ub1 or anti-ubiquitin antibodies.

Mass spectrometry

WDTC1-associated proteins were isolated from 3T3-L1 preadipocytes and induced adipocytes stably expressing Flag-WDTC1-WT and Flag-WDTC1-ΔH proteins by FLAG affinity purification, and cells transfected with an empty vector (EV) served as contamination controls for Flag immunoprecipitation. For analyses of interacting proteins in preadipocytes, WDTC1-WT and WDTC1-ΔH complexes were purified from ten 15-cm plates at day 0. For analyses of interacting proteins in induced adipocytes, day 0 preadipocytes were induced to differentiate and 2 days post-induction, WDTC1 complexes were purified from five (WT) or ten (ΔH) 15-cm plates; twice the number of cells were used in the purification of WDTC1-ΔH complexes due to its lower stable expression compared to WDTC1-WT. Cell pellets were lysed for 30 min on a rotating platform at 4°C in NP-40 lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1 mM EDTA, and 0.1% NP-40] supplemented with Halt™ protease inhibitor cocktail (1 U inorganic pyrophosphatase, 1 mM DTT, and 5 mM Mg-ATP). Reaction products were resolved by SDS–PAGE and detected by immunoblotting with either anti-H2AK119ub1 or anti-ubiquitin antibodies.

Sequence alignment

WDTC1 protein sequences from Homo sapiens (Hs), Mus musculus (Mm), Xenopus laevis (Xl), Danio rerio (Dr), Drosophila melanogaster (Dm), Caenorhabditis elegans (Ce), and Arabidopsis thaliana (At) were aligned to compare H-box sequence similarity in different species. Sequences were aligned using CLUSTAL 2.1 multiple sequence alignment tool.

Molecular modeling of WDTC1 and CRL4WDTC1

Modeling was performed at the University of North Carolina R. L. Juliano Structural Bioinformatics Core. For modeling WDTC1, suitable templates for the H-box, WD40, and TPR domains of WDTC1 were identified using HHpred [32]. Three template structures were utilized as follows: WDTC1 H-box in complex with DDB1 (PDB 3I7N; [13]), DDB2 in complex with CRL4 (PDB 4A0K; [15]), and TPR domains of Sgf2 (PDB 3S7Z; [33]). The Hbox and WD40 domains were modeled simultaneously using the Modeller software package [34] and two template structures: PDB ID 3I7N and PDB: 4A0K. The TPR domains were modeled separately based on the template structure of Sgf2, a TPR structure from Aspergillus fumigatus (PDB ID 3S7Z). The model of the WDTC1–CRL4 complex was based on the structure of the DDB2–CRL4 complex (PDB ID 4A0K) with WDTC1 superimposed on and then replacing DDB2. The TPR domains were manually moved to a location near their insertion within the beta-propeller. Models were rendered using Pymol.

Expanded View for this article is available online.

Acknowledgements

We thank Dr. James Bear for providing pLL-5.5-IRES-EGFP plasmid and Dr. Brenda Temple for modeling WDTC1. We thank members of the Xiong laboratory, and Drs. William Marzluff and Brenda Temple for helpful discussions. This study was supported by U.S. National Institutes of Health grants NCI CPTAC 1U24CA160035 to X.C. and R01-GM067113 to Y.X.

Author contributions

BSG and YX designed research; BSG and FY performed experiments; MDS provided experimental support; YY and XC performed mass spectrometry analyses; and BSG and YX analyzed data and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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*EMBO reports* 10

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