The antiobesity factor WDTC1 suppresses adipogenesis via the CRL4-WDTC1 E3 ligase

Beezly Groh, Mr. Matthew Smith, Feng Yan and Yue Xiong

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 30 April 2015

Thank you for your submission to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although all the referees find the topic of interest, they also find the study preliminary for publication here at this stage.

All of them have a number of largely overlapping concerns regarding the conclusiveness of the study -regarding the role of CRL4A-WDTC1 E3 ligase in adipogenic suppression, the conclusiveness of the comparison with CUL4A knockout mice and insufficient analysis of said mice. Given that all referees provide constructive suggestions on how to make the work more conclusive, I would like to give you the opportunity to revise your manuscript. As all concerns seem reasonable and are largely overlapping, they would all have to be addressed. Please note that a full elucidation of the mechanism whereby WDTC1 suppresses adipogenesis would not be required, but without the identification of relevant substrate(s), the referees clearly consider that the study does not go far enough. Especially given the caveats with comparing the phenotypes with those of the more pleitropic CUL4A knockout.

If the referee concerns can be adequately addressed, we would be happy to consider your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.
**REFEREE REPORTS**

Referee #1:

Groh et al. perform a well-executed series of studies to show that the interaction with CUL4A and DDB1 likely plays a role in WDTC1-mediated suppression of adipocyte differentiation. There are a few issues the authors need to address:

1. The heterozygous WDTC1+/− mice displayed dramatic obese phenotype, including those of appearance, body weight, and higher IWAT and GWAT deposits. What about the weight and appearance of Cul4a−/− mice? The fat to lean mass ratio should be compared between Cul4a−/− mice and the wild type control littermates.

2. The authors claim that WDTC1 "likely" functions as a substrate receptor and even state as much in the manuscript title but do not provide any direct evidence suggesting E3 ligase activity. The data indicates that the DDB1 interaction is important but there are currently no substrates identified and, even though many CRL4 substrate receptors (such as WDR5) are autoubiquitinated, WDTC1 is ubiquitinated at a fairly constant rate - regardless of its ability to bind DDB1. Without a relevant substrate for WDTC1, the mechanism by which WDTC1 works through CUL4A to antagonize obesity remains elusive.

3. Assuming that WDTC1 acts as a ubiquitin ligase as part of the CRL4 complex, a Cul4a−/− mouse model may not accurately recapitulate the loss of WDTC1 expression for two reasons: first, the CRL4A complex regulates numerous substrates and cellular processes through the use of multiple substrate receptors and second, due to their mutual use of DDB1 as a substrate adaptor, CUL4B may compensate for the loss of CUL4A expression. While CUL4A may be more relevant than CUL4B for WDTC1-associated effects in adipocytes, more in vitro data is needed to support this assertion.

4. Although there is an undesired increase in cell proliferation in CUL4Ai cells, Fig. S3D does show increased ORO staining in those cells. To ensure that CUL4A loss accurately recapitulates (or approximates) WDTC1 loss, CUL4Ai cells should also be examined for triglyceride production and changes in adipocyte-associated gene expression and these experiments should be included in the main manuscript (not in supplementary section).

5. WDTC1 binds to HDAC3 and inhibits PPARγ activity. Does CUL4A deletion or knockdown affect HDAC3 and PPARγ activities?

6. Why does DDB1 siRNA diminish WDTC1 expression in Fig 1F but not 1E? Please provide an explanation.

7 Some of the adipocyte markers that were quantified via RT-PCR. Western blot analysis should be provided for at least one of the key experiments detailed in Figures 2 and 3.

Referee #2:

The manuscript "The antiobesity factor WDTC1 mediates adipogenic suppression via the CRL4WDTC1 E3 ligase" by Groh et al. describes a new function for the evolutionarily conserved protein WDTC1. The authors present data suggesting that WDTC1 is a component of a CRL4 E3 ligase. Aboliing the interaction between WDTC1 and CRL4 ablates its effects on adipogenic suppression. The authors present data from biochemical studies using 3T3-L1 adipocytes and in vivo data from Cul4a knockout mice. This work provides a molecular basis for a previously unexplained phenomenon and addresses an area of broad importance to the readership of this journal. However, a number of unresolved questions need to be addressed in order to warrant publication.

1. The authors largely base their claim of adipogenic suppression on oil red O (ORO) staining,
triglyceride content, and the expression of a handful of general markers of adipogenesis. The authors need to do more to better explain their observations. They should assess the levels of genes involved in lipolysis, lipogenesis, and lipid oxidation to see if there are alterations that explain their observations.

2. This paper would be greatly strengthened by some mechanistic explanation for the findings presented. Specifically, how does a proposed component of an E3 ligase regulate adipocyte biology? Do the authors have any idea of possible substrates of CRL4WDTC1? How would these substrates in turn regulate gene expression and lipid accumulation?

3. On page 7, the authors explain that they are unable to make clear phenotypic assessments of Cul4a and Cul4b knockdown cells given the role of these proteins in cell cycle and other processes. If this is the case, then how do the authors justify using the Cul4a knockout mouse as a suitable model? How can they be sure the phenotype of this mouse, which appears interesting, is due to effects related to Wdtc1 as opposed to an independent function of Cul4a?

4. Several minor points also should be addressed. The authors should include size markers on their blots. They should also specify the genetic background of the Cul4a mutant mice.

Referee #3:
Wdtc1/Adp has been shown to suppress lipid accumulation is associated with obesity in mice and humans. Here, the authors show that disruption of WTC1-CRL4 interaction leads to loss of adipogenic suppression and TG accumulation by Wdtc1 to conclude that Wdtc1 is a component of CRL4 E3 ligase. By observing similar phenotype of Cul4a ablated mice to Wdtc1 heterozygote mice, the authors further attempt to demonstrate Wdtc1 as a component of CRL4 complex. The function of Wdtc1 as a component of CRL4 E3 ligase is new and worth reporting. It is unclear the component of CRL4 which Wdtc1 interacts and whether it is a direct interaction. More importantly, there is no indication as to the potential substrate(s) of the Wdtc1/CRL4 E3 ligase in affecting lipid accumulation and adipogenesis, which will enhance this work greatly. Furthermore, there are some conflicting or problematic results in the Cul4a ablated mice.

The authors indicate there was no changes in body weight in Fig. 4B while there was fat mass changes as shown in Fig.4C. It is unclear the age of mice when the body weights and fat pad pictures were taken. The authors need to document body weights and fat depots during the early to late adult stages of mice, especially since they do not observe altered body weights. High fat feeding may produce more clear differences. Furthermore, adipose tissue mass is a function of energy expenditure and intake. Although the supplementary data indicate there were no statistically significant differences in these parameters, use of global Cul4a knockout mice can be misleading. Adipose specific conditional knockout or overexpression would eliminate these concerns.

1st Revision - authors' response 28 August 2015

Point-by-point response

Referee #1:
Groh et al. perform a well-executed series of studies to show that the interaction with CUL4A and DDB1 likely plays a role in WDTC1-mediated suppression of adipocyte differentiation. There are a few issues the authors need to address:

We appreciate reviewer’s praise to our study and constructive comments. Below we address point-by-point reviewer’s concerns.

1. The heterozygous WDTC1+/- mice displayed dramatic obese phenotype, including those of appearance, body weight, and higher IWAT and GWAT deposits. What about the weight and
appearance of Cul4a/−/− mice? The fat to lean mass ratio should be compared between Cul4a/−/− mice and the wild type control littermates.

Response: We have examined the fat to lean mass ratio in female mice and found it to be insignificant. We recently noted that male, but not female Cul4a null mice, also developed heart hypertrophy, suggesting a possible gender difference of Cul4a function that we did not appreciate previously. To thoroughly examine this, it is necessary to generate age-matched male and female mice. We calculated the time frame for this work and determined that it would take conservatively 9 to 12 months to complete this study. Considering that we now have added significant new results on the identification of H2A as a putative substrate of WDTC1 (see below), that the Cul4a mice data provide supportive but not essential evidence to the main conclusion, and that this paper is a short Report, we have removed the mouse study from the revision.

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Response: To identify the relevant substrate for WDTC1, we first carried out two independent mass spectrometry screens of both wild-type and DDB1-binding deficient mutant WDTC1 complexes. A number of potential interacting proteins were identified, including Fatty Acid Synthase (FAS) and histone H2A. As a critical and positive regulator of lipid accumulation, we first focused on and extensively characterized the regulation of FAS by the CRL4<sub>WDTC1</sub> E3 ligase does not regulate FAS protein levels or target it for ubiquitylation.

We then investigated the possibility that histone H2A is as a substrate of WDTC1. 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 adipogenic suppressive function of WDTC1 is lost upon nuclear exclusion and WDTC1 physically interacts with ectopically expressed histones (Suh et al. 2007 Cell Metabolism, 6:195, PMID: 17767906).

We first confirmed that WDTC1, as well as CUL4A, CUL4B, DDB1 and ROC1, are present in the nucleus and further demonstrated that they bind to chromatin (New Fig 4A). We then performed a nuclear IP and demonstrated that WDTC1 associated with H2A, as well as H2B and H3 (New Fig 4B). In this experiment, we noted that WDTC1 interacted with histone H2A, but not the presumably monoubiquitylated form of H2A (indicated by an asterisk), an observation that is consistent with the possibility that CRL4<sub>WDTC1</sub> catalyzes H2AK119ub1. Chromatin co-immunoprecipitation assay confirmed the binding between WDTC1 and nucleosomal histone in vivo at physiologic levels (New Supplementary Fig S4E). Notably, the ΔH mutant, which disrupts the binding of WDTC1 with DDB1, retained histone binding activity, suggesting that WDTC1 has two separate binding activities, one for DDB1 and one for nucleosomes. We examined the global level of H2AK119ub1 in preadipocyte and induced 3T3-L1 cells stably expressing either a vector control, wild-type WDTC1 or WDTC1<sub>ΔH</sub> mutant (New Fig 4C). No significant changes in H2AK119ub1 levels were detected among the preadipocyte cells. In contrast, expression of wild-type WDTC1 in induced 3T3-L1 cells resulted in a modest, but significant and reproducible, increase in H2AK119ub1 while WDTC1<sub>ΔH</sub> expression caused a slight decrease of H2AK119ub1. This result suggests that WDTC1-promoted H2AK119ub1 is linked to adipogenesis and requires its binding with the CRL4 E3 ligase complex.
We next affinity purified and eluted the Flag-WDTC1 and Flag-WDTC1ΔH complexes (New Fig 4D, left panel), and incubated with recombinant H2A. After the reaction, the mixture was resolved by SDS-PAGE, followed by immunoblotting with H2AK119ub1 antibodies. The results show that H2A is robustly ubiquitylated by wild-type, but not WDTC1ΔH complexes (New Fig 4D, right panel). Multiple H2AK119 ubiquitylated species were detected after longer exposure, which likely represent in vitro ubiquitylation at additional sites in addition to H2AK119ub1. To confirm that K119 residue is the primary target of CRL4WDTC1, wild-type H2A and its mutants, H2AK118R and H2AK119R, were tested in in vitro ubiquitylation assays (New Fig 4E). While disrupting the K118 residue had very little effect, K119R mutation resulted in a substantial loss of H2A ubiquitylation by the CRL4WDTC1 E3 ligase. These results collectively suggest CRL4WDTC1 promotes H2AK119ub1 in vitro, and in vivo in an adipocyte lineage specific manner.
3. Assuming that WDTC1 acts as a ubiquitin ligase as part of the CRL4 complex, a Cul4a<sup>−/−</sup> mouse model may not accurately recapitulate the loss of WDTC1 expression for two reasons: first, the CRL4A complex regulates numerous substrates and cellular processes through the use of multiple substrate receptors and second, due to their mutual use of DDB1 as a substrate adaptor, CUL4B may compensate for the loss of CUL4A expression. While CUL4A may be more relevant than CUL4B for WDTC1-associated effects in adipocytes, more in vitro data is needed to support this assertion.

Response: We agree with the reviewer that CUL4A could assemble multiple CRL4A complexes to regulate numerous substrates and that the Cul4a<sup>−/−</sup> mutant mice may not accurately recapitulate the loss of WDTC1. The development of adipocyte hypertrophy and metabolic defects in Cul4 mutant mice, nevertheless, is consistent with and thus provide an in vivo support to our model that WDTC1 regulates adipogenesis through forming a CRL4A E3 ligase complex. This model is now further supported by our new results that CRL4A<sup>WDTC1</sup> is a putative H2AK119 E3 ligase.

Regarding the possibility that CUL4B may compensate for the loss of CUL4A expression, genetic analyses have shown that while Cul4a<sup>−/−</sup> mice are viable but exhibit resistance to UV-induced skin carcinogenesis (Li et al. 2009, Mol Cell 14:34, PMID: 19481525) and decreased proliferation in MEFs and impaired liver regeneration (Kopanja et al. 2009, Oncogene 28:2456, PMID: 19430492), Cul4b<sup>−/−</sup> mice die embryonically (Liu et al. 2012, Cell Res. 22:1258, PMID: 22453236, Jiang et al. 2012, PLoS One, 7:e37070, PMID: 22060329). These findings indicate that CUL4A and CUL4B each has a distinct function that cannot be compensated by the other. Whether Cul4b mutant mice, like Cul4a mice, also develop adipocyte hypertrophy and metabolic defects is an interesting question, but requires the creation of conditional mutant to study.

4. Although there is an undesired increase in cell proliferation in CUL4Ai cells, Fig. S3D does show increased ORO staining in those cells. To ensure that CUL4A loss accurately recapitulates (or approximates) WDTC1 loss, CUL4Ai cells should also be examined for triglyceride production and changes in adipocyte-associated gene expression and these experiments should be included in the main manuscript (not in supplementary section).

Response: CUL4A is known to assemble multiple CRL4A E3 complexes and therefore CUL4Ai would have pleiotropic effect on cell growth, differentiation and proliferation, whereas WDTC1 constitutes only one CRL4 E3 complex and WDTC1 loss, is therefore not expected to recapitulates the phenotype of CUL4Ai.

5. WDTC1 binds to HDAC3 and inhibits PPARγ activity. Does CUL4A deletion or knockdown affect HDAC3 and PPARγ activities?

Response: Although we did not detect an interaction between WDTC1 and HDAC3 or PPARγ from mass spectrometry based screens, we were curious about a potential connection as first suggested by Graff and colleagues (Suh et al. 2007 Cell Metabolism, 6:195, PMID: 17767906). We therefore examined both physical interaction between WDTC1 and HDAC3 and PPARγ. In our study, we did not detect HDAC3 (New Supplementary Fig S4B) or PPARγ (New Supplementary Fig 4C) binding with WDTC1 by co-immunoprecipitation experiments. Additionally, we also examined whether PPARγ is a substrate of CRL4 E3 ligase and obtained a negative result (New Supplementary Fig 4D). We therefore did not further pursue this line of inquiry.
6. Why does DDB1 siRNA diminish WDTC1 expression in Fig 1F but not 1E? Please provide an explanation.

**Response:** We also noted the slight decrease of ectopically expressed Flag-WDTC1 protein level in cells depleted for DDB1 in Fig 1F. There are three experimental differences: (1) the relative amount of Flag-WDTC1 plasmid DNA transfected per plate; 1.0 ug (Fig 1E) vs. 0.3 ug (Fig 1F), (2) the interval between Flag-WDTC1 transfection and siRNA DDB1 transfection; 9 hours (Fig 1E) and 24 hours (Fig 1F), (3) the degree of DDB1 depletion; DDB1 seemed to be more completely depleted in Fig 1F than in Fig 1E. We have not determined whether any of these differences might contribute to the subtle change in WDTC1 in Fig 1F. But this difference does not affect the conclusion made from these two figures (that ‘CRL4 does not regulate WDTC1 protein stability’).

7. Some of the adipocyte markers that were quantified via RT-PCR. Western blot analysis should be provided for at least one of the key experiments detailed in Figures 2 and 3.

**Response:** We have included data on PPARγ protein levels in 3T3-L1 preadipocyte and adipocyte cells stably expressing wild-type and mutant WDTC1 in the revised manuscript (new Supplementary Fig S2F).
Referee #2:

The manuscript "The antiobesity factor WDTC1 mediates adipogenic suppression via the CRL4\textsuperscript{WDTC1} E3 ligase" by Groh et al. describes a new function for the evolutionarily conserved protein WDTC1. The authors present data suggesting that WDTC1 is a component of a CRL4 E3 ligase. Abolishing the interaction between WDTC1 and CRL4 ablates its effects on adipogenic suppression. The authors present data from biochemical studies using 3T3-L1 adipocytes and in vivo data from Cul4a knockout mice. This work provides a molecular basis for a previously unexplained phenomenon and addresses an area of broad importance to the readership of this journal. However, a number of unresolved questions need to be addressed in order to warrant publication.

We appreciate reviewer’s praise to our study and constructive comments. Below we address point-by-point reviewer’s concerns.

1. The authors largely base their claim of adipogenic suppression on oil red O (ORO) staining, triglyceride content, and the expression of a handful of general markers of adipogenesis. The authors need to do more to better explain their observations. They should assess the levels of genes involved in lipolysis, lipogenesis, and lipid oxidation to see if there are alterations that explain their observations.

Response: We assessed the expression of a number of key genes regulating lipogenesis, lipolysis and fatty acid oxidation and found that the ectopic expression of wild-type WDTC1 led to a modest reduction of the expression of genes linked to lipogenesis (\textit{Fasn}, \textit{Dgat1} and \textit{Dgat2}), lipolysis (\textit{Agtl}) and β-oxidation of long chain fatty acids (\textit{Cpt2}). In contrast, ectopic expression of DDB1-binding deficient mutant WDTC1\textsuperscript{ΔH} led to opposite changes in the expression of these genes.

Additionally, we also found that WDTC1 likely acts downstream of the insulin signaling pathway. When insulin was replaced with rosiglitazone (a specific PPAR\textgamma agonist) in the induction cocktail, it did not alter the observed effect of WDTC1 expression on 3T3-L1 adipogenesis, although rosiglitazone promoted adipogenesis to a lesser extent (New Supplementary Fig S2E).
2. This paper would be greatly strengthened by some mechanistic explanation for the findings presented. Specifically, how does a proposed component of an E3 ligase regulate adipocyte biology? Do the authors have any idea of possible substrates of CRL4WDTC1? How would these substrates in turn regulate gene expression and lipid accumulation?

Response: To identify the relevant substrate for WDTC1, we first carried out two independent mass spectrometry screens of both wild-type and DDB1-binding deficient mutant WDTC1 complexes. A number of potential interacting proteins were identified, including Fatty Acid Synthase (FAS) and histone H2A. As a critical and positive regulator of lipid accumulation, we first focused on and extensively characterized the regulation of FAS by the CRL4WDTC1. However, our data thus far showed that CRL4WDTC1 E3 ligase does not regulate FAS protein levels or target it for ubiquitylation.

We then investigated the possibility that histone H2A is as a substrate of WDTC1. 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 adipogenic suppressive function of WDTC1 is lost upon nuclear exclusion and WDTC1 physically interacts with ectopically expressed histones (Suh et al. 2007 Cell Metabolism, 6:195, PMID: 17767906).
We first confirmed that WDTC1, as well as CUL4A, CUL4B, DDB1 and ROC1, are present in the nucleus and further demonstrated that they bind to chromatin (New Fig 4A). We then performed a nuclear IP and demonstrated that WDTC1 associated with H2A, as well as H2B and H3 (New Fig 4B). In this experiment, we noted that WDTC1 interacted with histone H2A, but not the presumably monoubiquitylated form of H2A (indicated by an asterisk), an observation that is consistent with the possibility that CRL4<sup>WDTC1</sup> catalyzes H2AK119ub1. Chromatin co-immunoprecipitation assay confirmed the binding between WDTC1 and nucleosomal histone in vivo at physiologic levels (New Supplementary Fig S4E). Notably, the ΔH mutant, which disrupts the binding of WDTC1 with DDB1, retained histone binding activity, suggesting that WDTC1 has two separate binding activities, one for DDB1 and one for nucleosomes. We examined the global level of H2AK119ub1 in preadipocyte and induced 3T3-L1 cells stably expressing either a vector control, wild-type WDTC1 or WDTC1<sup>ΔH</sup> mutant (New Fig 4C). No significant changes in H2AK119ub1 levels were detected among the preadipocyte cells. In contrast, expression of wild-type WDTC1 in induced 3T3-L1 cells 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 requires its binding with the CRL4 E3 ligase complex.

We next affinity purified and eluted the Flag-WDTC1 and Flag-WDTC1<sup>ΔH</sup> complexes (New Fig 4D, left panel), and incubated with recombinant H2A. After the reaction, the mixture was resolved by SDS-PAGE, followed by immunoblotting with H2AK119ub1 antibodies. The results show that H2A is robustly ubiquitylated by wild-type, but not WDTC1<sup>ΔH</sup> complexes (New Fig 4D, right panel). Multiple H2AK119 ubiquitylated species were detected after longer exposure, which likely represent in vitro ubiquitylation at additional sites in addition to H2AK119ub1. 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 (New 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 CRL4<sup>WDTC1</sup> promotes H2AK119ub1 in vitro, and in vivo in an adipocyte lineage specific manner.
3. On page 7, the authors explain that they are unable to make clear phenotypic assessments of Cul4a and Cul4b knockdown cells given the role of these proteins in cell cycle and other processes. If this is the case, then how do the authors justify using the Cul4a knockout mouse as a suitable model? How can they be sure the phenotype of this mouse, which appears interesting, is due to effects related to Wdtc1 as opposed to an independent function of Cul4a?

**Response:** The development of adipocyte hypertrophy and metabolic defects in *Cul4a* mutant mice is consistent with the phenotypes observed in *Wdtc1* heterozygous mice. Although we cannot rule out the possibility that additional substrate of CUL4A may also contribute to this phenotype, the mice results do provide in vivo evidence that is consistent with our model that WDTC1 regulates adipogenesis through a CRL4A E3 ligase complex. In a separate study involving *Cul4a* mice, we noted that male, but not female *Cul4a* null mice also developed heart hypertrophy. This suggests a possible gender difference of *Cul4a* function. To thoroughly examine this, however, it is necessary to generate age-matched male and female mice. We calculated the time frame for this work and determined that it would take conservatively 9 to 12 months to complete this study. Considering that we have now added significant new results on the identification of H2A as a putative substrate of WDTIC1, that the *Cul4a* mice data provide supportive but not essential evidence to the main conclusion, and that this paper is a short Report, we have removed the mouse study from the revision.

4. Several minor points also should be addressed. The authors should include size markers on their blots. They should also specify the genetic background of the Cul4a mutant mice.

**Response:** Size markers are included in the revised manuscript. The genetic background of the *Cul4a* mutant mice is C57BL/6; please see response #3.
Referee #3:

Wdtc1/Adp has been shown to suppress lipid accumulation is associated with obesity in mice and humans. Here, the authors show that disruption of WTC1-CRL4 interaction leads to loss of adipogenic suppression and TG accumulation by Wdtc1 to conclude that Wdtc1 is a component of CRL4 E3 ligase. By observing similar phenotype of Cul4a ablated mice to Wdte1 heterozygote mice, the authors further attempt to demonstrate Wdtc1 as a component of CRL4 complex. The function of Wdtc1 as a component of CRL4 E3 ligase is new and worth reporting.

Response: We appreciate reviewer’s praise to our study and constructive comments. Below we address point-by-point reviewer’s concerns.

1. It is unclear the component of CRL4 which Wdtc1 interacts and whether it is a direct interaction.

Response: Like other DDB1 binding WD40 proteins that are putative substrate receptors of CRL4 complexes, WTC1 directly interacts with DDB1, which is a linker protein that bridges the interaction between the substrate receptor and the CUL4 protein. Structural analysis by Ning Zheng’s group has previously reported that the direct interaction between WTC1 and DDB1 is mediated through an N-terminal α-helical motif termed the H-box (Li et al. 2010 Nat Struct Mol Biol 17:105, PMID: 19966799).

2. More importantly, there is no indication as to the potential substrate(s) of the Wdtc1/CRL4 E3 ligase in affecting lipid accumulation and adipogenesis, which will enhance this work greatly.

Response: To identify the relevant substrate for WDTC1, we first carried out two independent mass spectrometry screens of both wild-type and DDB1-binding deficient mutant WDTC1 complexes. A number of potential interacting proteins were identified, including Fatty Acid Synthase (FAS) and histone H2A. As a critical and positive regulator of lipid accumulation, we first focused on and extensively characterized the regulation of FAS by the CRL4WDTC1. However, our data thus far showed that CRL4WDTC1 E3 ligase does not regulate FAS protein levels or target it for ubiquitylation.
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Furthermore, there are some conflicting or problematic results in the Cul4a ablated mice. The authors indicate there was no changes in body weight in Fig. 4B while there was fat mass changes as shown in Fig. 4C. It is unclear the age of mice when the body weights and fat pad pictures were taken. The authors need to document body weights and fat depots during the early to late adult stages of mice, especially since they do not observe altered body weights. High fat feeding may produce more clear differences. Furthermore, adipose tissue mass is a function of energy expenditure and intake. Although the supplementary data indicate there were no statistically significant differences in these parameters, use of global Cul4a knockout mice can be misleading. Adipose specific conditional knockout or overexpression would eliminate these concerns.

Response: Total body weight was measured for approximately 1 year old wild-type and Cul4a−/− adult male and female mice. Fat pad pictures were taken for male mice between 9-12 months.

To address the concerns the reviewer raised, however, it is necessary to generate conditional KO mice. We calculated the time frame for this work and determined that it would take conservatively 9 to 12 months to complete this study. Considering that we now have added significant new results on the identification of H2A as a putative substrate of WDTC1, that the Cul4a mice data provide supportive but not essential evidence to the main conclusion, and that this paper is a short Report, we have removed the mouse study from the revision.

2nd Editorial Decision
28 October 2015

Thank you for the submission of your revised manuscript to EMBO reports. I am truly sorry for the delay in getting back to you but we have only now received the full set of referee reports.

As you will see, referee 2 now supports publication of the manuscript. However, while both referees 1 and 2 clearly appreciate the identification of a H2A as a substrate for WDTC1, referee 1 raises several concerns regarding the new experiments. Point 2 questions the role of H2AK119 ubiquitination and point 3 and 5 remark on technical problems of the ubiquitination assays. We normally do not allow the referees to raise new concerns during a revision, as however the removal of the CUL4A knockout data and the addition of a new series of experiments to identify and characterize a substrate for WDTC1 have considerably changed the study, these concerns should be addressed and I would like to give you the opportunity to revise your manuscript.

It is however not necessary to address point 1 experimentally. The effect on triglyceride production can be discussed in the manuscript and overstatements should be avoided.
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REFEREE REPORTS

Referee #1:

In the revised manuscript, the authors made significant changes by adding H2A as a substrate for WDTC1, and by deleting the exciting results of the Cul4a knockout mice. What was not addressed is whether H2A K119 monoubiquitination is functionally significant for adipogenesis.

1. While CUL4A assembles multiple CRL4 complexes and targets different substrates, WDTC1 was shown by the authors to regulate triglyceride production and adipocytic gene expression. The authors have yet to address the question raised in the prior review: does CUL4A RNAi change triglyceride production and adipocyte-associated gene expression? While they showed increased proliferation upon knocking down of CUL4A, there was no change of cell cycle profile.

2. Histone H2A was shown as a potential substrate of WDTC1. It is unclear what roles H2AK119ub by CUL4A/4B-WDTC1 play during adipogenesis. What is the status of H2A K119ub in 3T3-L1 cells overexpressing WDTC1, ΔH or RARA (Fig. 2B), or shWDTC1 cells expressing control, or rescuing cDNA of WDTC1, ΔH or RARA (Fig. 3B)?

3. In Fig. 4E, Flag-WDTC1 immunoprecipitated complex contains a mixer of other contaminated proteins besides DDB1, CUL4A, CUL4B and ROC1. The authors should use purified DDB1, CUL4A, CUL4B, ROC1 and WDTC1 to repeat this in vitro ubiquitination assay. H2AK119R should be used as a control for this in vitro ubiquitination assay.

4. WDTC1 also interacted with H2B and H3 (Figure 4B). Are H2B and H3 also substrates of WDTC1?

5. The increase in H2AK119ub in induced adipocytes is quite modest in Flag-WDTC1 overexpressing cells. A prolonged exposure of the anti-H2A blot (bottom panel) is necessary to reveal the H2AK119ub species, as in Figure 4B, to assess the increase in this putative ubiquitinated modified H2A.

6. The authors showed that knocking down WDTC1 enhanced 3T3-L1 differentiation. Does WDTC1 knockdown or overexpression affect cell cycle and cell proliferation?

7. In Figure EV3, CUL4A depletion by RNAi led to increased cell proliferation. However, there is no change of cell cycle phase distribution. CUL4B knockdown appeared to cause G1 arrest or delay, but did not have any significant effect on cell proliferation.

Referee #2:

The revised manuscript has addressed most of the criticisms raised after the initial review though the removal of the animal studies does lessen the broader impact of the findings. However, the authors have gone to great lengths to identify a substrate for WDTC1, which is to be commended.

One small point which could be addressed by textual changes relates to the gene expression experiment performed in EV2G. Most of these genes change a small amount and the authors are overstating their claim a bit in the text at the bottom of page 6, particularly regarding the effects of the WDTC1-deltaH mutant on expression of lipogenic, lipolytic genes.

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is whether H2A K119 monoubiquitination is functionally significant for adipogenesis.

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**Response:** In Supplement Fig. EV3D, we showed that knocking down CUL4A increased adipogenesis by Oil Red O staining, which is a reliable qualitative measurement of intracellular triglyceride production. We showed in Fig. 2 that ectopic expression of wildtype and DDB1-binding deficient ΔH mutant WDTC1 resulted in decreased and increased triglyceride production and adipocyte-associated gene expression, respectively. Measuring the effects of WDTC1 expression on adipogenesis and gene expression is more specific for determining the effect of the CRL4A<sup>WDTC1</sup> E3 ligase than examining CUL4A RNAi cells since CUL4A, as the reviewer pointed out, can assemble multiple CRL4 complexes and targets different substrates.

While they showed increased proliferation upon knocking down of CUL4A, there was no change of cell cycle profile.

**Response:** Our results suggest that WDTC1 regulates adipogenesis at least in part by assembling into an active CRL4A E3 complex to promote H2AK119 monoubiquitylation and thereby regulating the expression of genes involved in adipogenesis, rather than regulating the cell cycle progression which is also important for adipogenesis. We have added new result that ectopic expression of wildtype WDTC1 and two DDB1-binding WDTC1 mutants, ΔH and RARA, did not cause significant change in the cell cycle phase distribution when compared with empty vector control cells (new Fig. EV2E, see below).

2. Histone H2A was shown as a potential substrate of WDTC1. It is unclear what roles H2AK119ub by CUL4A/4B-WDTC1 play during adipogenesis.

**Response:** Monoubiquitylation at H2AK119 (H2AK119ub1) has been extensively investigated, especially during animal development and cell differentiation. Although this modification has been consistently associated with gene silencing, the detailed mechanism of how H2AK119ub1 represses transcription is not clear. We found that the ectopic expression of wildtype WDTC1 in adipogenically-induced 3T3-L1 cells are associated with increased H2AK119ub1, decreased expression of adipogenic genes and reduced adipogenesis, while the ectopic expression of DDB1-binding deficient ΔH mutant are associated with decreased H2AK119ub1, increased expression of adipogenic genes and enhanced adipogenesis. These results are consistent with the established role for H2AK119ub1 and led us to propose that CUL4A<sup>WDTC1</sup> E3 ligase promotes monoubiquitylation of H2AK119 which plays an important role of silencing the expression of adipogenic genes and inhibiting adipogenesis.

What is the status of H2A K119ub in 3T3-L1 cells overexpressing WDTC1, ΔH or RARA (Fig. 2B), or shWDTC1 cells expressing control, or rescuing cDNA of WDTC1, ΔH or RARA (Fig. 3B)?

**Response:** The status of H2AK119ub1 in 3T3-L1 cells overexpressing wildtype and mutant WDTC1 was determined in Fig. 4C. We found that while the genome-wide level of H2AK119ub1 was not affected by the expression of wildtype or mutant WDTC1 in the preadipocyte state, H2AK119ub1 was increased by the ectopic expression of wildtype WDTC1 and decreased by the ectopic expression of DDB1-binding deficient ΔH mutant WDTC1, respectively, following adipogenic induction. These changes seen in induced 3T3-L1 adipocytes were significant (P < 0.05) and reproducible when compared with the control cells transfected with empty vector. These results suggested that WDTC1 promotes H2AK119ub1 in an adipocyte-lineage specific manner and it is dependent on the formation of a CUL4-DDB1-ROC1 (CRL4) complex.

3. In Fig. 4E, Flag-WDTC1 immunoprecipitated complex contains a mixer of other contaminated proteins besides DDB1, CUL4A, CUL4B and ROC1. The authors should use purified DDB1, CUL4A, CUL4B, ROC1 and WDTC1 to repeat this in vitro ubiquitination assay. H2AK119R should be used as a control for this in vitro ubiquitination assay.
**Response:** We agree with the reviewer that like any other IP-based enzyme assay, we cannot exclude the possibility that the in vitro ligase assay shown in Fig. 4D is not contaminated by another E3 ligase that is responsible for the observed H2A monoubiquitylation. Reconstitution using purified CRL4 subunits is the ultimate way to prove that CRL4(WDTC1) catalyzes H2AK119ub1, but requires the purification of at least a dozen different proteins and has rarely been done on the characterization of a CRL E3 ligase. We provided in vivo H2AK119 ubiquitylation (Fig. 4C), included a mutant E3 substrate receptor (WDCT1-ΔH), used purified recombinant substrate H2A, and included two specific substrate mutants (H2AK118R and H2AK119R, Fig. 4E). These assays have exceeded the high standard in the current field for the CRL ubiquitylation assay.

4. WDTC1 also interacted with H2B and H3 (Figure 4B). Are H2B and H3 also substrates of WDTC1?

**Response:** In the assay shown in Fig. 4B, we only detected a slow migrating band for H2A, but not H2B, H3 or H4. Considering the amount of H2B, H3 and H4 expressed was actually slightly higher than that of H2A, we feel comfortable to conclude that at least under this experimental condition, WDCT1 preferentially, if not only, promotes H2A monoubiquitylation.

5. The increase in H2AK119ub in induced adipocytes is quite modest in Flag-WDTC1 overexpressing cells. A prolonged exposure of the anti-H2A blot (bottom panel) is necessary to reveal the H2AK119ub species, as in Figure 4B, to assess the increase in this putative ubiquitin-modified H2A.

**Response:** H2AK119ub1 in induced adipocytes was increased by 70% on average by the expression of wildtype WDTC1 when compared with the control cells transfected with empty vector (EV). This increase is significant (P < 0.05) and reproducible. Supporting the specificity of this change of H2AK119ub1 by WDTC1 is the significant (P < 0.05) decrease of H2AK119ub1 by 30% in induced 3T3-L1 cells expressing ΔH mutant WDTC1 when compared to the same cells transfected with empty vector. Considering the fact that H2AK119ub1 is very abundant and accounts for an estimated 5–15% of total H2A in higher eukaryotic cells (e.g. Goldknopf et al. 1975, JBC, PMID: 1165239; Goldknopf et al. 1977, PNAS, PMID, 265581), a 70% increase or 30% decrease are actually surprising to us and should significantly affect the expression of many genes.

6. The authors showed that knocking down WDTC1 enhanced 3T3-L1 differentiation. Does WDTC1 knockdown or overexpression affect cell cycle and cell proliferation?

**Response:** We analyzed the cell cycle distribution following adipogenic induction of 3T3-L1 cells ectopically expressing WDTC1 proteins. We found that ectopic expression of wildtype and two DDB1-binding mutants, ΔH and RARA, WDTC1 did not cause significant changes in the cell cycle phase distribution when compared with empty vector control cells (new Fig. EV2E).

7. In Figure EV3, CUL4A depletion by RNAi led to increased cell proliferation. However, there is no change of cell cycle phase distribution. CUL4B knockdown appeared to cause G1 arrest or delay, but did not have any significant effect on cell proliferation.
Response: The effects of CUL4A and CUL4B depletion are likely to be complex as each can assemble into multiple CRL4 E3 complexes. The substrates of CUL4A and CUL4B that are linked to cell cycle regulation has not been clearly defined, but the trend of knocking down either gene on cell proliferation is consistent with established role of cell proliferation being required for adipogenesis: depletion of CUL4A increased cell proliferation and adipogenesis while depletion of CUL4B reduced cell proliferation and inhibited adipogenesis.

Referee #2:

1. The revised manuscript has addressed most of the criticisms raised after the initial review though the removal of the animal studies does lessen the broader impact of the findings. Nevertheless, the authors have gone to great lengths to identify a substrate for WDTC1, which is to be commended.

Response: We appreciate reviewer’s praise to our study.

2. One small point which could be addressed by textual changes relates to the gene expression experiment performed in EV2G. Most of these genes change a small amount and the authors are overstating their claim a bit in the text at the bottom of page 6, particularly regarding the effects of the WDTC1-deltaH mutant on expression of lipogenic, lipolytic genes.

Response: We have clarified this in the manuscript. We interpret the modest alterations in the expression of genes regulating lipogenesis, lipolysis and fatty acid oxidation are likely to be downstream of altered adipogenesis in cells ectopically expressing WDTC1 proteins.

3rd Editorial Decision

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the enclosed report on it. As you will see, the referee finds the manuscript suitable for publication in EMBO reports. Nevertheless, the referee has raised two points that should be addressed. Given this evaluation, I would like to give you the opportunity for a final revision of your manuscript, with the understanding that the concerns of referee 1 must be fully addressed.

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REFEREE REPORT

Referee #1:

The authors have responded to most of the questions raised in the prior review. There are two remaining questions:
1. In Fig. 4C, a long exposure of the anti-H2A blot (bottom panel) is necessary to reveal mono-ubiquitinated H2AK119ub together with unmodified H2A, similar to what was shown in Fig. 4B (3rd panel from the bottom).
2. In Fig. 4D, the in vitro ubiquitination assay should at least include controls in the absence of Ub, E1 or E2.

4th Revision - authors' response

Point-by-point response

Referee #1:

The authors have responded to most of the questions raised in the prior review. There are two remaining questions:
1. In Fig. 4C, a long exposure of the anti-H2A blot (bottom panel) is necessary to reveal mono-ubiquitinated H2AK119ub together with unmodified H2A, similar to what was shown in Fig. 4B (3rd panel from the bottom).

**Response:** A longer exposure of the anti-H2A blot in Fig. 4C is attached below. In Fig 4B, transiently overexpressed HA-tagged histones are being detected by an anti-HA antibody in 293T cells, which detected all HA-tagged histones in this experiment (H2A, H2B, H3, H4 and possibly, ubiquitylated H2A). In Figure 4C, endogenous histone H2A is being detected in 3T3-L1 cells by an anti-H2A antibody. In ours hands, we have been unable to detect an endogenous H2AK119ub1 band probing with anti-H2A antibody in 3T3-L1 cells. One possibility is that H2AK119ub1 levels are relatively low in 3T3-L1 cells (Inagaki et al. 2015 J Biol Chem 290:7, PMID: 25533466) and as such, may be below the detection limit of the anti-H2A antibody we used.

![Long exposure of H2A blot in 4C](image)

2. In Fig. 4D, the in vitro ubiquitination assay should at least include controls in the absence of Ub, E1 or E2.

**Response:** As reviewer suggested, we repeated the in vitro ubiquitylation to include E1, E2, E3, substrate H2A and ubiquitin dropout controls (new Fig 4D; old figure was moved to Expanded View Figure EV4F). The results of this experiment demonstrated the specificity of the in vitro ubiquitylation reactions and confirmed that H2A is robustly ubiquitylated by wildtype WDTC1, but not the DDB1-binding mutant WDTC1ΔH complexes (below).

![New Figure 4D](image)

![New Figure EV4F](image)
I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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REFEREE REPORT

Referee #1
The authors have addressed all concerns raised in the prior review. Publication in EMBO Report is recommended.
EMBO REPORTS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

| Corresponding Author Name: Yue Xiong |
| Manuscript Number: EMBOR-2015-40500V4 |

Reporting Checklist For Life Sciences Articles

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research Issued by the NIH in 2010 (see link at top right) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under ‘Reporting Guidelines’ (see link at top right) for further information. See also: NIH guidelines for animal welfare on the animal care page. See also: PMI guidelines for European guidelines

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars only for independent experiments and sample sizes where the application of statistical tests is warranted. Error bars should not be shown for technical replicates.
- when n is small (n ≤ 5), the individual data points from each experiment should be plotted alongside an error bar.

Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the authorship guidelines on Data Presentation (see link at top right).

2. Captions

Each figure caption should contain the following information, for each panel where they are present:

- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.)
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, e.g. t-tests, Wilcoxon and Mann Whitney tests, can be unsuitably identified by name only, but more complex techniques should be described in the methods section;
  - tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - must statistical test results, e.g. P values * or > not P values < *
  - definition of 'center values' as median or average;
  - definition of error bars as sd or s.e.m.

Any descriptives too long for the figure legend should be included in the figures section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non-applicable).

B- Statistics and general methods

1. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?

A standard sample size of 3 was used for real-time qPCR assays and triglyceride quantitation. Figure legends for figures 2, 3 and EV2.

2. For animal studies, include a statement about sample size estimation even if no statistical methods were used.

A

3. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?

A

4. Were any steps taken to minimize the effects of subjective bias when allocating animals/cultures to treatment (e.g. randomization procedures)? If yes, please describe.

A

5. For animal studies, include a statement about randomization even if no randomization was used.

A

6. Were any steps taken to minimize the effects of subjective bias during group allocation or blind when assessing results (e.g. blinding of the investigator)? If yes, please describe.

A

7. For animal studies, include a statement about blinding even if no blinding was done.

A

8. For every figure, are statistical tests justified appropriately?

A

9. Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to meet it.

Normal distribution was assumed but not assessed. Figure legends for figures 2, 3 and EV2.

10. Is there an estimate of variation within each group of data?

Yes, mean ± SEM or mean ± SD. Figure legends for figures 2, 3 and EV2.

11. Is the variance similar between the groups that are being statistically compared?

The variance was assumed to be similar but not assessed. Figure legends for figures 2, 3 and EV2.

C- Reagents

1. To show that antibodies were profiled for use in the system under study (assay and species), provide:

A

2. Antibody information provided in materials and methods (page 12), and Table EV2.

A

3. Identify the source of reagents and report if they were recently authenticated (e.g., by IFR testing) and tested for mycoplasma contamination.

A

D- Animal Models

1. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.

A

2. For experiments involving live v ixes, include a statement of compliance with ethical regulations and identify the committee approving the experiments.

A

3. To ensure that all relevant aspects of animal studies are adequately reported, see author guidelines, under ‘Reporting Guidelines’ (see link at top right) for further information. See also: NIH guidelines for animal welfare on the animal care page. See also: PMI guidelines for European guidelines

E- Human Subjects

1. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.

A

2. For experiments involving live v ixes, include a statement of compliance with ethical regulations and identify the committee approving the experiments.

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5. For all hypertension, please see the table at the top-right of the document.
## F. Data Accessibility

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<td>19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal’s data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under ‘Expanded View’ or in unstructured repositories such as Dryad, JWS Online or Figshare). See link list at top right.</td>
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<td>20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right), EGA or EGA (see link list at top right).</td>
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<td>22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as BioModels. See link list at top right or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included as supplementary information.</td>
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## G. Dual use research of concern

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