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CAND1 exchange factor promotes Keap1 integration into cullin 3-RING ubiquitin ligase during adipogenesis

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ABSTRACT

Adipogenesis is governed by a plethora of regulatory proteins which are most commonly controlled by the ubiquitin proteasome system. Here, we show that the differentiation of LiSa-2 preadipocytes is associated with an increase of cullin-associated and neddylation-dissociated 1 (CAND1), COP9 signalosome (CSN), neddylated cullin 3 (Cul3) and the BTB protein Keap1. Silencing of CAND1 leads to a decrease and reduced integration of Keap1 into Cul3-RING ubiquitin ligases (CRL3) and to a retardation of adipogenesis. Transient transfection of LiSa-2 cells with CAND1 targeting miRNA148a also reduces Keap1 and slowed down adipogenesis of LiSa-2 cells. These results demonstrate for the first time that CAND1 acts as a BTB-protein exchange factor for CRL3 complexes. The specific increase of neddylated Cul3 might be explained by the recruitment of Cul3 or CRL3 in a membrane-bound location during adipogenesis. Together, the results show that during adipogenesis in LiSa-2 cells a CAND1-dependent remodeling and activation/neddylation of CRL3 complexes take place.

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1. Introduction

Obesity is a recognized worldwide epidemic with more than 1 billion adults classified as overweight. It is associated with co-morbidities such as heart diseases, diabetes mellitus type II, hypertension and cancer. Dysfunction of adipogenesis, the differentiation of preadipocytes to adipocytes, is a hallmark of obesity. Therefore, elucidation of the molecular mechanisms that control adipogenesis would help to identify therapeutics for treatment of obesity and other metabolic diseases.

Adipocyte differentiation comprises a plethora of transcription factors including proliferator-activated receptor gamma (PPARγ) and members of the CCAAT/enhancer binding protein (C/EBP) family (Cristancho and Lazar, 2011; Farmer, 2006; Morrison and Farmer, 1999; White and Stephens, 2010). Most of these transcription factors are regulated by the ubiquitin proteasome system (UPS), a major proteolytic system in eukaryotic cells controlling the stability of numerous essential cellular regulators. Recently it has been shown that PPARγ itself is a ubiquitin ligase (Hou et al., 2012) and targets MUC1-C oncoprotein for proteolysis (Hou et al., 2014). On the other hand, PPARγ is ubiquitinated by MKRN1 resulting in its degradation and prevention of adipocyte differentiation (Kim et al., 2014). Moreover, C/EBPα (Yoshida et al., 2013) as well as C/EBP homologous protein (CHOP) (Li et al., 2006), a dominant negative form of C/EBP family members are degraded by the UPS. CHOP is a negative regulator of adipogenesis and has to be degraded for process progression (Batchvarova et al., 1995). The stability of CHOP is controlled by a cullin-RING ubiquitin ligase (CRL) containing cullin3 (Cul3) and the BTB and Kelch-like ECH-associated protein 1 (Keap1) called $CRL3^{Keap1}$ (Huang et al., 2012). CRLs constitute the largest family of E3s with approximately 250 members (Deshaies and Joazeiro, 2009). They consist of a cullin (Cul1–7) as scaffold, a RING protein Rbx1 or Rbx2 and a substrate recognition subunit. CRL1 complexes possess F-box proteins as substrate recognition subunits linked to Cul1 via the adaptor protein Skp1. In CRL3 complexes BTB proteins function as substrate recognition subunits and are directly linked to Cul3. Interestingly, CRL3s form dimers mediated by their BTB proteins as it has been shown for Keap1 and SPOP (Ogura et al., 2010; Zhuang et al., 2009), which might optimize ubiquitination function.

CRLs are regulated by Cullin-Associated and Neddylation-Dissociated 1 (CAND1) and by the COP9 signalosome (CSN). CAND1

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acts as a protein exchange factor for CRL1 complexes. It augments the dissociation of F-box protein-Skp1 modules by million-fold (Pierce et al., 2013). This allows the recruitment of new F-box proteins to Cul1 complexes. Depletion of CAND1 in *Schizosaccharomyces pombe* (Wu et al., 2013), in *Saccharomyces cerevisiae* (Zemla et al., 2013) or in human cells (Dubiel et al., 2013; Pierce et al., 2013) profoundly alters the cellular repertoire of CRL1 complexes. In addition, it is required for Keap1 recycling in CRL3 complexes (Lo and Hannink, 2006). However, its role as a protein exchange factor for CRL3 complexes is not clear.

During adipogenesis CAND1 increases and releases the F-box protein Skp2 from CRL1s resulting in the required accumulation of p27 (Dubiel et al., 2013). This causes cell cycle exit necessary for cell differentiation (Hsieh et al., 2000; Morrison and Farmer, 1999).

Cullins are activated by covalent modification with the ubiquitin-like protein Nedd8 (or Rub1 in *S. cerevisiae*) (Liakopoulos et al., 1998). Cycles of neddylation and deneddylation are crucial for CRL activity as well as reassembly (Pierce et al., 2013; Wu et al., 2013; Zemla et al., 2013). Nedd8 conjugation on a C-terminal highly conserved lysine changes the conformation of cullins. As a consequence Ub molecules are efficiently transferred from E2 enzymes to the targeted substrate (Boh et al., 2011; Duda et al., 2008; Saha and Deshaies, 2008). CRLs are inactivated by CSN-mediated deneddylation (Cope et al., 2002). The removal of Nedd8 by the CSN is also a prerequisite for CAND1 action (Pierce et al., 2013; Wu et al., 2013; Zemla et al., 2013). Obviously CAND1 and the CSN collaborate in the control of CRL1 remodeling. The CSN deneddylates all cullins which raises the question whether CAND1 can act as a protein exchange factor for all CRLs as well.

Here we show that CAND1 is necessary for the BTB protein Keap1 exchange in CRL3 complexes. Permanent silencing of CAND1 in LiSa-2 cells reduces the integration of Keap1 into CRL3 complexes associated with the retardation of adipogenesis. In addition, we show a specific increase of neddylated Cul3 during the differentiation of LiSa-2 cells.

2. Materials and methods

2.1. Cell culture, cell differentiation and lysis

Human liposarcoma LiSa-2 cells were used as an adipocyte differentiation model growing in ISCOVE/RPMI (4:1) medium containing 10% fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin as described (Wabitsch et al., 2000). The differentiation is induced by culturing cells in serum-free medium with supplements including 1 nM insulin, 20 pM triiodothyronine and 1 mM cortisol according to a protocol by Wabitsch et al. (2000). During differentiation the medium was changed every 2 or 3 days. Differentiation is monitored by formation of lipid droplets assessed by Oil Red O (ORO) staining, followed by visualizing of nuclei with hemotoxylin (Huang et al., 2012).

Differentiated cells were harvested on different days of differentiation and lysed with ice-cold triple-detergent lysis buffer (50 mM Tris–HCl, pH 8.5; 150 mM NaCl; 0.02% (w/v) sodium azide; 0.1% (w/v) SDS; 1% (v/v) NP-40; 0.5% (w/v) sodium deoxycholate) or single-detergent lysis buffer (50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA, pH 8.0; 1% Triton X-100) with freshly added PMSF (1 mg/ml) and aprotinin (10 μg/ml).

2.2. RNA knockdowns and transient transfections

LiSa-2 cells permanently downregulating CAND1 (shCAND1-LiSa-2 cells) were generated as described before (Peth et al., 2007a, 2007b). Cells with permanently downregulating GFP (shGFP-LiSa-2 cells) were used as control cells. For transient transfection cells were transfected with 100 nM miRNA148a (LifeTechnologies) or control miRNA using Lipofectamine 2000 (Invitrogen). Similarly, 100 nM siRNA against Keap1 (cell signalling) or against control (siGFP) were transfected into cells. At different time points after transfection cells were lysed and analyzed by SDS-PAGE and western blotting. Transient Keap1 overexpression was performed with Flag-tagged Keap1 (Addgene) using outlined DNA amounts in presence of Lipofectamine 2000.

2.3. Immunoprecipitation, glycerol gradient, western blotting and densitometry

Immunoprecipitations with the antibody against Cul3 (Santa Cruz) were carried out as before (Peth et al., 2007b). Glycerol gradient centrifugation analysis was performed as described (Huang et al., 2012). Fractions with different density (5% to 22% glycerol) were separated on SDS-PAGE and analyzed by western blotting. Density gradient was standardized with purified human CSN (about 400 kDa, fractions 8–10) and 20S proteasome (about 700 kDa, fractions 11–12) from red blood cells. Western blots were carried out using antibodies against Cul3 (BD Transduction Laboratories), Cul1, Cul4, CAND1 and Keap1 (Santa Cruz), Cul2 (Invitrogen), CSN5 (GeneTex) and CSN8 (Enzo Life Sciences).

Densitometry was carried out using ImageJ software. Statistics were calculated with GraphPad InStat3. Error bars are standard deviations (SD) and unpaired Student's *t*-test was applied for statistical analysis.

3. Results

3.1. Impact of CAND1 on abundance of CRLs and CSN components

For better understanding the role of CAND1 in adipogenesis we established LiSa-2 cells permanently expressing CAND1 shRNA. As shown in Fig. 1A and C, an increase of CAND1 was confirmed in control cells (shGFP-LiSa-2 cells), which is significantly retarded in shCAND1-LiSa-2 cells during differentiation (see Fig. 1B and D). Initially CAND1 expression in shCAND1-LiSa-2 cells is reduced by more than 60% (see Fig. 1A–D, 1 day of differentiation). However, after 8 days of differentiation despite shCAND1 an increase of CAND1 protein was observed. In parallel with CAND1, the subunits of the CSN, CSN5 and CSN8, increased during adipogenesis (Fig. 1A). Because the expression of other CSN subunits was elevated as well (data not shown), an increase of the holo CSN complex during adipogenesis was assumed. CAND1 silencing reduced CSN5 and CSN8 during the first 8 days of adipogenesis (Fig. 1B). Cul1 did not change during adipogenesis in control cells and silencing of CAND1 had no effect on its neddylation/deneddylation ratio. In contrast in shGFP-LiSa-2 cells a clear adipogenesis-dependent increase of neddylated Cul3 was observed (Fig. 1A and C), which was slightly suppressed in shCAND1-LiSa-2 cells (Fig. 1B and D). In parallel with Cul3 neddylation, there was an upregulation of Keap1 during the first 8 days of adipogenesis which was significantly retarded by downregulation of CAND1 (Fig. 1A–D).

Permanent downregulation of CAND1 had consequences for lipid droplet formation (Fig. 1E). After 8 and 15 days of differentiation a retardation of lipid droplet formation was observed in shCAND1-LiSa-2 cells compared to shGFP-LiSa-2 cells as visualized by ORO staining.

Similar effects as with CAND1 shRNA were obtained with miRNA148a that targets CAND1 (Murata et al., 2010) (see Fig. 2). Transient transfection of miRNA148a into LiSa-2 cells reduced the expression of CAND1 by approximately 60%. As expected, this was accompanied by a significant decrease of p27 in undifferentiated as well as in 6-days differentiated LiSa-2 cells (Fig. 2A). CSN8 and

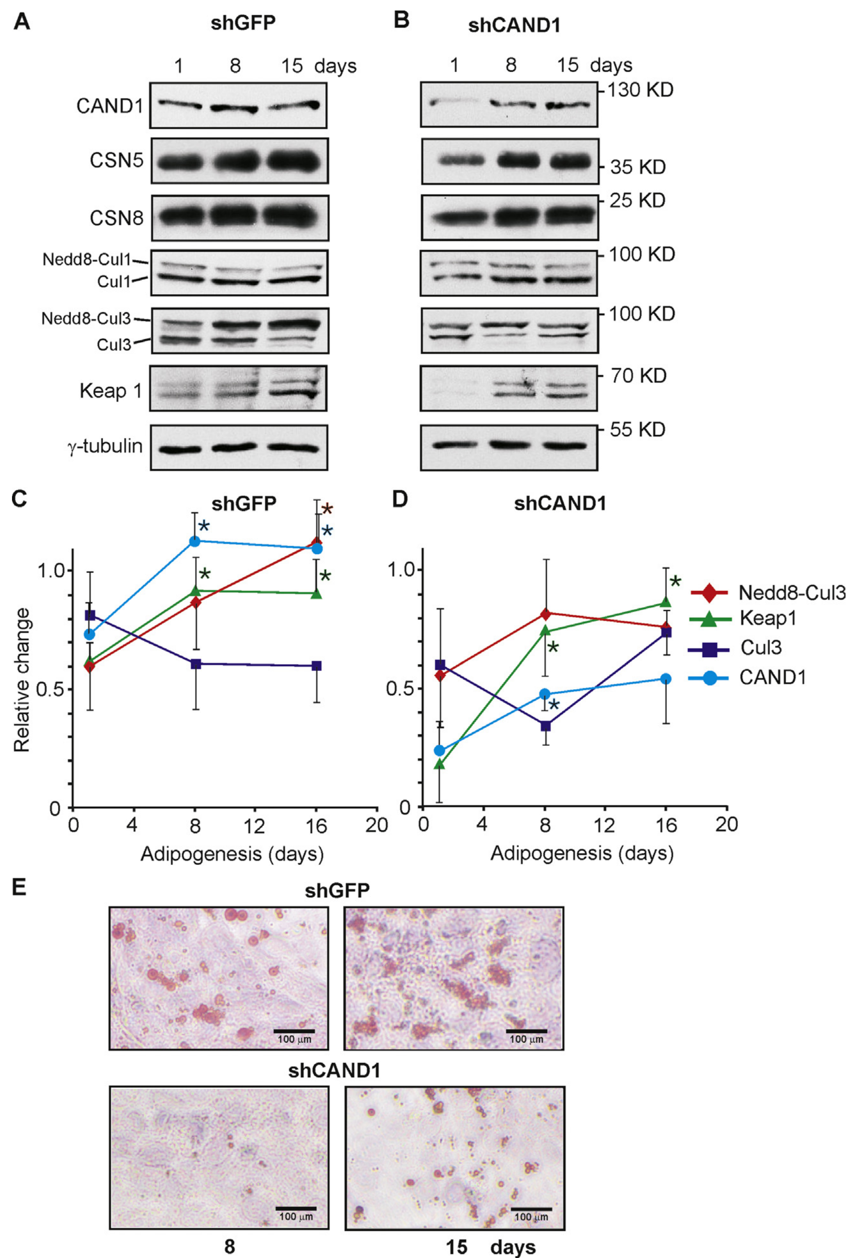

Fig. 1. Impact of CAND1 silencing on abundance of CSN and CRLs components during differentiation of LiSa-2 cells. (A) shGFP-LiSa-2 cells and (B) shCAND1-LiSa-2 cells were induced to differentiation by a hormone cocktail as described in Section 2. At different time points of differentiation the cells were lysed with triple-detergent buffer. Lysates were separated by SDS-PAGE, followed by western blot analysis using indicated antibodies. (C and D) Nedd8-Cul3, Keap1, Cul3 and CAND1 data from shGFP-LiSa-2 cells (C) or shCAND1-LiSa-2 cells (D) as shown in Fig. 1A and B were quantified by densitometry, normalized to γ-tubulin and plotted as relative change against days of adipogenesis. In case of Keap1 the two distinct bands were added. Data are expressed in means ±SD of 3–4 independent experiments. Statistical significance in comparison to day 1 of differentiation is indicated by stars (*$P < 0.05$). (E) Formation of lipid droplets was visualized by ORO staining as outlined in Section 2 after 8 and 15 days of differentiation in shGFP- and shCAND1-LiSa-2 cells. Microscopy was performed with 200-fold magnification.

Cul3 were not much affected by miRNA148a. In contrast, there was a clear reduction of Keap1 steady state levels by transient transfection with miRNA148a in undifferentiated and differentiated cells (Fig. 2A). The differentiation of LiSa-2 cells was clearly retarded in the presence of miRNA148a as shown for day 8 of differentiation by ORO staining (Fig. 2B).

3.2. Keap1 is integrated into CRL3 complexes in a CAND1-dependent manner

CAND1 is an exchange factor for F-box proteins of CRL1 complexes (Pierce et al., 2013). Because CAND1 binds Cul3, we asked whether CAND1 is an exchange factor for the integration of Keap1 into Cul3 complexes as well. To study this we performed density gradient centrifugation with lysates from shGFP-LiSa-2 control cells and shCAND1-LiSa-2 cells without differentiation and after 8 days of differentiation (Fig. 3A and B). Data confirmed downregulation of Keap1 as a result of CAND1 silencing (see also Fig. 1B and D) in undifferentiated LiSa-2 cells. Moreover, after 8 days of differentiation in control cells Keap1 expression increased and the BTB protein migrated into fractions with higher density (fractions 8–10). Increase of Keap1 as well as spreading into fractions 8–10 was retarded in shCAND1-LiSa-2 cells after 8 days of differentiation.

To verify the observation that CAND1 silencing reduces Keap1 integration into Cul3 complexes we carried out specific immunoprecipitations. In shGFP-LiSa-2 and in shCAND1-LiSa-2 cells

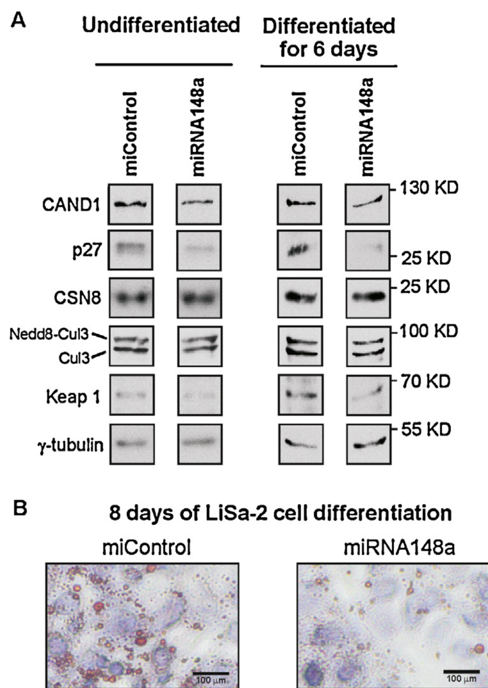

Fig. 2. The impact of CAND1 targeting miRNA148a on Keap1 protein level and on adipogenesis. (A) LiSa-2 cells were transfected with 100 nM miRNA148a and control miRNA using Lipofectamine 2000. After 48 h of transfection the cells were lysed (undifferentiated). In addition, 48 h after transfection another portion of cells was induced to differentiation and 6 days later these cells were lysed (differentiated for 6 days). Lysates were analyzed using western blotting with indicated antibodies. (B) After 48 h of miRNA transfection LiSa-2 cells expressing miRNAs were induced to differentiation. On day 8 of differentiation lipid droplets were stained with ORO as described above. Cell nuclei are visualized by hemotoxylin.

Flag-Keap1 was transiently transfected and 48 h later adipogenesis was induced. After 6 days of adipogenesis there was approximately the same Flag-Keap1 expression in both cell lines (Fig. 4A, input). At this time point immunoprecipitations with a specific anti-Cul3 antibody were performed and Cul3-associated Flag-Keap1

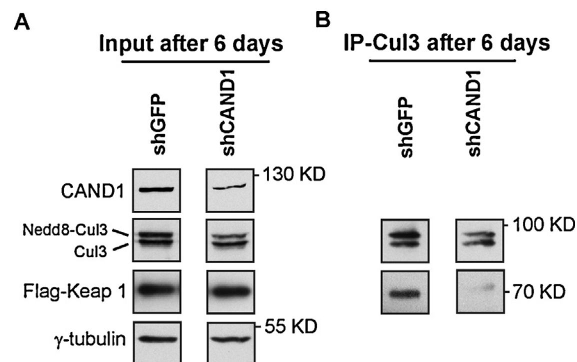

Fig. 4. Immunoprecipitation of Cul3 and co-immunoprecipitation of Keap1 in shGFP- and shCAND1-LiSa-2 cells. (A) shGFP- and shCAND1-LiSa-2 cells were transfected with 10 µg Flag-tagged Keap1 DNA. After 48 h cells were stimulated for differentiation. On day 6 of differentiation the cells were lysed in triple detergent buffer, followed by western blotting with indicated antibodies. (B) Same lysates as in (A) were subjected to immunoprecipitation with the anti-Cul3 antibody (Santa Cruz), followed by western blotting using anti-Flag antibody to detect co-immunoprecipitation of Flag tagged Keap1.

was detected by western blotting with the anti-Flag antibody. Data demonstrated in Fig. 4B clearly show that in shCAND1-LiSa-2 cells significantly less Flag-Keap1 co-immunoprecipitated with Cul3 after 6 days of adipogenesis compared to control cells.

3.3. Specific adipogenesis-dependent neddylation of Cul3

In parallel with elevated Keap1 integration into Cul3 complexes a clear increase of neddylated Cul3 was monitored during adipogenesis (Fig. 1). This raised the question whether neddylation of Cul3 is specific for adipogenesis or whether other cullins are neddylated as well. Therefore cullins 1–4 were analyzed by western blotting after day 1 and 8 of LiSa-2 cell differentiation using specific antibodies. Data shown in Fig. 5A confirm previous results (Fig. 1A) that neither neddylated nor deneddylated Cul1 changed during adipogenesis. Moreover, there was no change of neddylated/deneddylated Cul2. A marginal increase of neddylated Cul4

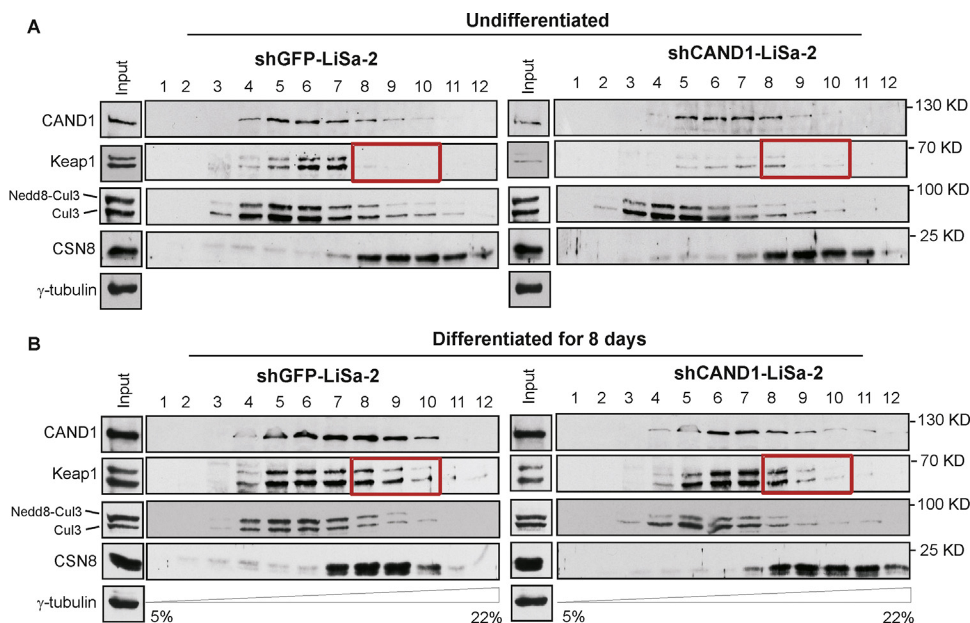

Fig. 3. CAND1 silencing reduces Keap1 expression and migration to fractions with high density in glycerol gradients. Undifferentiated (A) or 8-day differentiated (B) shGFP- and shCAND1-LiSa-2 cells were lysed using triple detergent buffer and loaded onto 5–22% glycerol density gradients. Sedimentation of protein complexes in fractions with different density was detected using western blotting with appropriate antibodies as shown. Input – western blots of lysates loaded on density gradients.

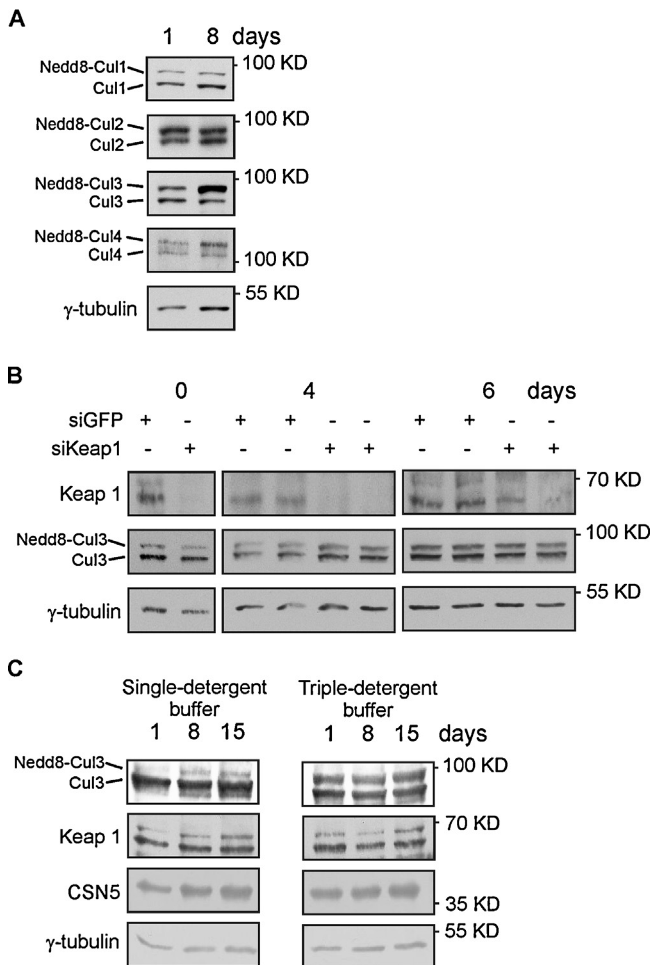

Fig. 5. Accumulation of neddylated Cul3 during adipogenesis. (A) In LiSa-2 cells at indicated days of differentiation ratios of neddylated/deneddylated cullins were determined with western blotting using appropriate antibodies. (B) Silencing of Keap1 has no impact on the neddylation of Cul3. LiSa-2 cells were transfected with 100 nM siRNA against Keap1 and control siRNA (siGFP). After 48 h LiSa-2 cells were induced to differentiation. At different time points during differentiation the ratio of deneddylated/neddylated Cul3 in lysates was determined with antibody against Cul3. (C) Neddylated Cul3 was detected in the membrane/nuclei fraction. At indicated days of differentiation LiSa-2 cells were lysed with different lysis buffers and analyzed by western blotting with antibodies against Cul3, Keap1 and CSN5.

after 8 days of differentiation was observed. Whether it has any functional consequence is not known at the moment. In contrast, data confirmed a significant elevation of neddylated Cul3 after 8 days of adipogenesis.

The increase of neddylated Cul3 during adipogenesis cannot be explained by the expression of the CSN deneddylase, since it also increased during the process (see Fig. 1). Elevation of neddylated Cul3 might be the result of CRL3^{Keap1} dimerization (Canning and Bullock, 2013; Ogura et al., 2010) assuming that CRL3$^{Keap1\text{-}Keap1}$CRL3 dimers possess reduced accessibility for the CSN. To test this possibility Keap1 was transiently silenced. Data shown in Fig. 5B demonstrate that Keap1 downregulation had no appreciable impact on Cul3 neddylation during 6 days of adipogenesis.

Compartmentalization of CRL3 complexes associated with limited accessibility for the CSN deneddylase might be another reason for specific neddylation of Cul3 during adipogenesis. To prove this hypothesis LiSa-2 cells were lysed with different detergent buffers. We used a single-detergent buffer which releases exclusively cytosolic proteins from LiSa-2 cells. These results were compared with proteins obtained with a triple-detergent buffer releasing also membrane-bound proteins. Data in Fig. 5C show that only the triple-detergent lysis buffer revealed neddylated Cul3 in the western blot pointing to a membrane-bound localization.

4. Discussion

Here we confirm that CAND1 is an essential regulator of adipogenesis. As demonstrated before it acts as a protein exchange factor releasing Skp2 from CRL1 complexes resulting in the stabilization of p27 (Dubiel et al., 2013). For the first time we show that CAND1 also acts as a protein exchange factor for CRL3 complexes allowing the integration of Keap1 during the differentiation of LiSa-2 preadipocytes. CAND1 function as an exchange factor for Keap1/CRL3 complexes is evidenced by the following facts. First, Keap1 stabilization during adipogenesis is diminished by CAND1 silencing and by miRNA148a. As it has been shown for Skp2 (Dubiel et al., 2013), Keap1 integration into CRL3 complexes most likely leads to stabilization of the BTB protein. Since downregulation of CAND1 retards integration of Keap1, it also destabilizes the protein. Second, CAND1 allows co-sedimentation of Keap1 with larger Cul3 complexes. Density gradient centrifugation shows that only after 8 days of differentiation Keap1 sediments into fractions with high density (fraction 8–10) containing also Cul3. This process is retarded by CAND1 silencing. Third, there is a significant reduction of Keap1 co-immunoprecipitation with Cul3 in cells permanently expressing shRNA against CAND1 after 6 days of LiSa-2 cell differentiation. Using HeLa cells and ectopically expressed HA-Cul3 and Keap1-CBD, Lo and Hannink obtained different results (Lo and Hannink, 2006). Although transient CAND1 silencing confirmed reduction of steady state Keap1 expression, in contrast to our data there was an increase in HA-Cul3 and Keap1-CBD binding shown by chitin pulldowns. The deviating results might be due to different conditions used, e.g. ectopic expression of all components including Cul3.

Taken together our data clearly show that Keap1 is integrated into CRL3 complexes in a CAND1-dependent manner making CAND1 besides its function as an F-box protein exchange factor to a BTB protein exchange factor. Because CAND1 interacts with all tested cullins, it might function as a universal CRL exchange factor. Both the CAND1-dependent remodeling of CRL1 (Dubiel et al., 2013) and CRL3 complexes (this paper) are essential for LiSa-2 cell differentiation most likely as an adaptation of CRLs to new substrate specificity requirements during adipogenesis. The CRL3^{Keap1}-dependent ubiquitination and subsequent degradation of CHOP is one example for adipogenesis-specific proteolysis.

The fact that neddylated Cul3 specifically increases during the differentiation of LiSa-2 cells points to a unique role of CRL3 complexes in the process of adipogenesis which goes beyond the degradation of CHOP. None of the tested cullins (Cul1–4) changes as much as Cul3 manifested in an increase of Cul3 expression as well as neddylation during differentiation of LiSa-2 cells. Presumably Cul3 neddylation is accompanied with activation of CRL3 complexes. One possible reason for Cul3 neddylation/activation might be a blockade of specific deneddylation by the CSN during adipogenesis. Since CSN expression increases during LiSa-2 cell differentiation, reduced accessibility of the CSN to neddylated Cul3 could be responsible for elevated Cul3 neddylation. There are at least two reasons for reduced accessibility of activated CRL3s: dimerization and compartmentalization.

Dimerization has been demonstrated for CRL3s forming two substrate binding sites and two catalytic cores enhancing the ubiquitination activity of the E3 (Canning and Bullock, 2013; Ogura et al., 2010; Zhuang et al., 2009). CRL3 dimers are neddylated but whether they are efficiently deneddylated by the CSN has not been reported. Therefore, we downregulated Keap1 to block

Keap1-mediated dimerization of CRL3s. Since Keap1 silencing had no impact on Cul3 neddylation, it is unlikely that Keap1-mediated CRL3 dimerization causes Cul3 neddylation during adipogenesis. However, at the moment we cannot rule out that other BTB proteins form CRL3 dimers, which might be responsible for reduced Cul3 deneddylation.

Another possible scenario is the recruitment and neddylation of Cul3 at the plasma membrane via DCNL3 as it has been described before (Meyer-Schaller et al., 2009). This might point to a role of Cul3 in protein trafficking (Huotari et al., 2012). Recently Cul3 emerged as an important regulator of intracellular trafficking pathway (Hubner and Peter, 2012), which plays a pivotal role in the formation of lipid droplets during adipogenesis (Fridolfsson et al., 2014; Le Lay et al., 2009). Using triple-detergent lysis buffer we were able to detect neddylated Cul3 in LiSa-2 cell western blots, which was impossible using single-detergent buffer. This argues for a membrane association of neddylated Cul3 and points to a protection against CSN-mediated deneddylation via compartmentalization. The exact localization and role of membrane-bound neddylated Cul3 has to be elucidated in the future.

Summing up, adipogenesis in LiSa-2 cells is associated with three important events: First, the coordinated increase of CSN subunits indicating a pivotal role of the CSN presumably in CRL remodeling, second, the elevation of CAND1 as the protein exchange factor that in cooperation with the CSN organizes CRL remodeling and, third, the increase of neddylated Cul3/CRL3 which is most likely responsible for the ubiquitination of regulatory proteins of adipogenesis.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

Batchvarova, N., Wang, X.Z., Ron, D., 1995. Inhibition of adipogenesis by the stress-induced protein CHOP (Gadd153). EMBO J. 14, 4654–4661.

Boh, B.K., Smith, P.G., Hagen, T., 2011. Neddylation-induced conformational control regulates cullin RING ligase activity in vivo. J. Mol. Biol. 409, 136–145.

Canning, P., Bullock, A.N., 2013. New strategies to inhibit KEAP1 and the Cul3-based E3 ubiquitin ligases. Biochem. Soc. Trans. 42, 103–107.

Cope, G.A., Suh, G.S., Aravind, L., Schwarz, S.E., Zipursky, S.L., Koonin, E.V., et al., 2002. Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. Science 298, 608–611.

Cristancho, A.G., Lazar, M.A., 2011. Forming functional fat: a growing understanding of adipocyte differentiation. Nat. Rev. Mol. Cell Biol. 12, 722–734.

Deshaies, R.J., Joazeiro, C.A., 2009. RING domain E3 ubiquitin ligases. Annu. Rev. Biochem. 78, 399–434.

Dubiel, D., Gierisch, M.E., Huang, X., Dubiel, W., Naumann, M., 2013. CAND1-dependent control of cullin 1-RING Ub ligases is essential for adipogenesis. Biochim. Biophys. Acta 1833, 1078–1084.

Duda, D.M., Borg, L.A., Scott, D.C., Hunt, H.W., Hammel, M., Schulman, B.A., 2008. Structural insights into NEDD8 activation of cullin-RING ligases: conformational control of conjugation. Cell 134, 995–1006.

Farmer, S.R., 2006. Transcriptional control of adipocyte formation. Cell Metab. 4, 263–273.

Fridolfsson, H.N., Roth, D.M., Insel, P.A., Patel, H.H., 2014. Regulation of intracellular signaling and function by caveolin. FASEB J. 28, 3823–3831.

Hou, Y., Gao, J., Xu, H., Xu, Y., Zhang, Z., Xu, Q., et al., 2014. PPARgamma E3 ubiquitin ligase regulates MUC1-C oncoprotein stability. Oncogene 33, 5619–5625.

Hou, Y., Moreau, F., Chadee, K., 2012. PPARgamma is an E3 ligase that induces the degradation of NFkappaB/p65. Nat. Commun. 3, 1300.

Hsieh, F.F., Barnett, L.A., Green, W.F., Freedman, K., Matushansky, I., Skoultchi, A.I., et al., 2000. Cell cycle exit during terminal erythroid differentiation is associated with accumulation of p27(Kip1) and inactivation of cdk2 kinase. Blood 96, 2746–2754.

Huang, X., Ordemann, J., Muller, J.M., Dubiel, W., 2012. The COP9 signalosome, cullin 3 and Keap1 supercomplex regulates CHOP stability and adipogenesis. Biol. Open 1, 705–710.

Hubner, M., Peter, M., 2012. Cullin-3 and the endocytic system: New functions of ubiquitination for endosome maturation. Cell Logist. 2, 166–168.

Huotari, J., Meyer-Schaller, N., Hubner, M., Stauffer, S., Katheder, N., Horvath, P., et al., 2012. Cullin-3 regulates late endosome maturation. Proc. Natl. Acad. Sci. U. S. A. 109, 823–828.

Kim, J.H., Park, K.W., Lee, E.W., Jang, W.S., Seo, J., Shin, S., et al., 2014. Suppression of PPARgamma through MKRN1-mediated ubiquitination and degradation prevents adipocyte differentiation. Cell Death Differ. 21, 594–603.

Le Lay, S., Blouin, C.M., Hajduch, E., Dugail, I., 2009. Filling up adipocytes with lipids. Lessons from caveolin-1 deficiency. Biochim. Biophys. Acta 1791, 514–518.

Li, X., Huang, H.Y., Chen, J.G., Jiang, L., Liu, H.L., Liu, D.G., et al., 2006. Lactacystin inhibits 3T3-L1 adipocyte differentiation through induction of CHOP-10 expression. Biochem. Biophys. Res. Commun. 350, 1–6.

Liakopoulos, D., Doenges, G., Matuschewski, K., Jentsch, S., 1998. A novel protein modification pathway related to the ubiquitin system. EMBO J. 17, 2208–2214.

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

Meyer-Schaller, N., Chou, Y.C., Sumara, I., Martin, D.D., Kurz, T., Katheder, N., et al., 2009. The human Dcn1-like protein DCNL3 promotes Cul3 neddylation at membranes. Proc. Natl. Acad. Sci. U. S. A. 106, 12365–12370.

Morrison, R.F., Farmer, S.R., 1999. Role of PPARgamma in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18(INK4c) and p21(Waf1/Cip1), during adipogenesis. J. Biol. Chem. 274, 17088–17097.

Murata, T., Takayama, K., Katayama, S., Urano, T., Horie-Inoue, K., Ikeda, K., et al., 2010. miR-148a is an androgen-responsive microRNA that promotes LNCaP prostate cell growth by repressing its target CAND1 expression. Prostate Cancer Prostatic Dis. 13, 356–361.

Ogura, T., Tong, K.I., Mio, K., Maruyama, Y., Kurokawa, H., Sato, C., et al., 2010. Keap1 is a forked-stem dimer structure with two large spheres enclosing the intervening, double glycine repeat, and C-terminal domains. Proc. Natl. Acad. Sci. U. S. A. 107, 2842–2847.

Peth, A., Berndt, C., Henke, W., Dubiel, W., 2007a. Downregulation of COP9 signalosome subunits differentially affects the CSN complex and target protein stability. BMC Biochem. 8, 27.

Peth, A., Boettcher, J.P., Dubiel, W., 2007b. Ubiquitin-dependent proteolysis of the microtubule end-binding protein 1, EB1, is controlled by the COP9 signalosome: possible consequences for microtubule filament stability. J. Mol. Biol. 368, 550–563.

Pierce, N.W., Lee, J.E., Liu, X., Sweredoski, M.J., Graham, R.L., Larimore, E.A., et al., 2013. Cand1 promotes assembly of new SCF complexes through dynamic exchange of F box proteins. Cell 153, 206–215.

Saha, A., Deshaies, R.J., 2008. Multimodal activation of the ubiquitin ligase SCF by Nedd8 conjugation. Mol. Cell 32, 21–31.

Wabitsch, M., Bruderlein, S., Melzner, I., Braun, M., Mechtersheimer, G., Moller, P., 2000. LiSa-2, a novel human liposarcoma cell line with a high capacity for terminal adipose differentiation. Int. J. Cancer 88, 889–894.

White, U.A., Stephens, J.M., 2010. Transcriptional factors that promote formation of white adipose tissue. Mol. Cell Endocrinol. 318, 10–14.

Wu, S., Zhu, W., Nhan, T., Toth, J.I., Petroski, M.D., Wolf, D.A., 2013. CAND1 controls in vivo dynamics of the cullin 1-RING ubiquitin ligase repertoire. Nat. Commun. 4, 1642.

Yoshida, A., Kato, J.Y., Nakamae, I., Yoneda-Kato, N., 2013. COP1 targets C/EBPalpha for degradation and induces acute myeloid leukemia via Trib1. Blood 122, 1750–1760.

Zemla, A., Thomas, Y., Kedziora, S., Knebel, A., Wood, N.T., Rabut, G., et al., 2013. CSN- and CAND1-dependent remodelling of the budding yeast SCF complex. Nat. Commun. 4, 1641.

Zhuang, M., Calabrese, M.F., Liu, J., Waddell, M.B., Nourse, A., Hammel, M., et al., 2009. Structures of SPOP-substrate complexes: insights into molecular architectures of BTB-Cul3 ubiquitin ligases. Mol. Cell 36, 39–50.