CAND1-dependent control of cullin 1-RING Ub ligases is essential for adipogenesis

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A R T I C L E   I N F O

Article history:
Received 14 September 2012
Received in revised form 20 December 2012
Accepted 7 January 2013
Available online 14 January 2013

Keywords:
CPD9 signalsome
F-box proteins
Preadipocytes
p27
Skp2

A B S T R A C T

Cullin-RING ubiquitin (Ub) ligases (CRLs) are responsible for ubiquitylation of approximately 20% of all proteins degraded by the Ub proteasome system (UPS). CRLs are regulated by the COP9 signalsome (CSN) and by Cullin-associated Nedd8-disassociated protein 1 (CAND1). The CSN is responsible for removal of Nedd8 from cullins inactivating CRLs. CAND1 modulates the assembly of F-box proteins into cullin 1–RING Ub ligases (CRL1s). We show that CAND1 preferentially blocks the integration of Skp2 into CRL1s. Suppression of CAND1 expression in HeLa cells leads to an increase of the Skp2 assembly into CRL1s and to significant reduction of the cyclin-dependent kinase (CDK) inhibitor p27. In contrary, CAND1 overexpression causes elevation of p27. The observed CAND1-dependent effects and CAND1 expression are independent of the CSN as demonstrated in CSN1 knockdown cells. Increase of p27 is a hallmark of preadipocyte differentiation to adipocytes. We demonstrate that the accumulation of p27 is associated with an increase of CAND1 and a decrease of Skp2 during adipogenesis of human LiSa-2 preadipocytes. CAND1 knockdown reduces p27 and blocks adipogenesis. Due to the impact of CAND1 on Skp2 control, CAND1 could represent an important effector molecule in adipogenesis, but also in cancer development.

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1. Introduction

The Ub ligases, E3s, play a pivotal role in the UPS, because they confer substrate specificity to intracellular proteolysis [1]. Members of the cullin family (Cul1, Cul2, Cul3, Cul4A, Cul4B, Cul5, Cul7, PARC and APC2) provide the scaffold of CRL complexes responsible for a large portion of UPS-mediated proteolysis. The C-terminus of the cullin binds a RING H2 finger protein, Rbx1 or Rbx2, which recruits the E2 with the activated Ub. The N-termini of cullins bind substrate–adaptor proteins (Skp1, elongin C or DDB1) that recruit substrate-recognition subunits (SRSs) to the complex [2].

F-box proteins (FBPs) function as SRS in case of Cul1 complexes (CRL1) [2,3]. They can be subdivided into 3 groups: FBPs with WD40 domain (FBXWs) including β-TrCP and Fbw7, FBPs with leucine-rich repeats (FBXLs) such as Skp2 and FBPs with other diverse domains (FBXOs) [4]. β-TrCP targets regulators of cell cycle or apoptosis for ubiquitylation. It binds specifically phosphorylated β-catenin, β-catenin, PARC and CDC25A [5] and is an oncoprotein because it targets tumor suppressors such as PDCD4. Fbw7 recognizes its substrates also via a phospho-degron that is present in proto-oncogenes such as c-Myc, N-Myc, c-Jun, but also in Notch, cyclin E1 and SREBP1. These substrates are implicated in proliferation and oncogenesis but also in brain development and neurogenesis [6,7]. Skp2 is a high abundant FBP [8] and involved in the regulation of cell cycle by targeting CDK inhibitors p21 and p27 [5]. Skp2 recognizes p27 upon CDK-mediated phosphorylation on Thr187. It is an oncogene and increase of Skp2 expression was observed in a wide range of human tumors [5]. Skp2 regulates entry into S phase by mediating the degradation of p27. It becomes unstable in G1 after ubiquitylation by the anaphase-promoting complex/cyclosome (APC/C) [9]. Typically the abundance of integrated FBPs is regulated by autoubiquitylation within the CRL complex. Knockdown of the CSN subunit 5 (CSN5) and suppression of CSN-mediated removal of Nedd8 (deneddylation) reduce FBPs levels in human cells [10,11] indicating that a major function of the CSN is the protection of FBPs against degradation [10–12].

CSN and CAND1 are essential regulators of CRLs in eukaryotic cells. Interestingly, both regulators inhibit CRL activities in vitro but promote CRL functions in vivo [11,13]. In mammals the CSN consists of 8 subunits (CSN1–8) [14] and exhibits an intrinsic metalloprotease activity mapped to the MPN+/JAMM motif of CSN5 [15]. This activity is responsible for the removal of the Ub-like protein Nedd8 from cullins [16,17]. Recent studies demonstrated a significant conformational change of CRL1 Skp2 upon Nedd8 conjugation [18]. The neddylated form of the CRL1.Skp2 allows the initiator Ub to bridge a 50 Å gap between E2 and the substrate [2] and therefore neddylation accelerates the process of ubiquitylation [19] and, accordingly, deneddylation inhibits CRLs. Hence CSN-mediated deneddylation prevents autoubiquitylation and degradation of FBPs [11]. Interestingly, CSN containing mutant CSN5 still efficiently protects FBPs.
indicating that the binding of the CSN is important for this protective function [12]. In the presence of a specific substrate the CSN is perhaps released from the CRL1 until the substrate is completely ubiquitylated and degraded [20,21]. In addition, the CSN is associated with a variety of proteins including protein kinases [22–24] and the Ub-specific protease, USP15, a deubiquitylating enzyme, which protects non–FBP52s proteins against autoubiquitylation and degradation [11,25–30].

CAND1 specifically binds to free, unmodified cullin-Rbx complexes. The C-terminus of CAND1 blocks the substrate adaptor/SRS site of Cul1, whereas the N-terminus binds directly the Nedd8 acceptor lysine of Cul1 preventing neddylation [31]. It has been shown that the Skp1–Skp2 complex abrogates the inhibitory effect of CAND1 and in the presence of the substrate p27 an active, neddylated CRL1Skp2 complex can be formed [32]. It seems that CAND1 does not function by sequestering all cullins and protecting CRL components, since only a small portion of unmodified Cul1 is associated with CAND1 in cells [33]. On the other hand, CAND1 deletion leads to developmental defects in Aspergillus nidulans [34] and Caenorhabditis elegans [35], where it seems to be required for the activity of distinct CRLs. Moreover, in Schizosaccharomyces pombe CAND1 helps rare FBPs to be integrated into CRLs [11].

Here we demonstrate that CAND1 differentially influences the integration of the FBPs into CRL1 complexes. Suppression of CAND1 expression specifically promotes the incorporation of Skp2 into CRL1 complexes accompanied with a degradation of p27. The function of CAND1 is essential for the differentiation of human LiSa-2 preadipocytes, and suppression of CAND1 expression abrogates adipogenesis.

2. Materials and methods

2.1. Cell culture, cell differentiation

HeLa cells and Flag-CSN2 B8 mouse fibroblasts were cultured under standard conditions [36] 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) with freshly added PMSF (1 mg/ml) and aprotinin (10 μg/ml). We used human liposarcoma cells (LiSa-2) to study adipocyte differentiation in vitro. LiSa-2 cells were grown in Iscove/RPMI 4:1 with 10% fetal calf serum (FCS; Biochrom, Berlin, Germany). 100 U/ml penicillin and 0.1 mg/ml streptomycin or serum-free, basal medium (DMEM/F12 (1:1)) supplemented with 10 mg/ml transferin, 15 mM NaHCO3, 15 mM HEPES, 33 mM biotin, 17 mM pantothenate, 100 U/ml penicillin and 0.1 mg/ml streptomycin. To induce differentiation of confluent LiSa-2 cells, cells were cultured in serum-free medium supplemented with 1 mM insulin, 20 mM triiodothyronine and 1 mM cortisol (adipogenic medium). LiSa-2 cells were plated on a 6-well-plate and cultured for 24 h with Iscove/RPMI medium. During differentiation the medium was changed every other day. Differentiation was monitored by a protocol described before [37,38] and assessed by Oil Red O (ORO) staining using the Thermo Scientific HyClone complete AdvanceSTEM™ Adipogenic differentiation kit (Thermo Fisher Scientific, Schwerte, Germany). In this protocol provided by the manufacturer lipid droplets are stained with ORO and nuclei with hematoxylin. Differentiated cells were harvested after indicated days of differentiation and lysed using triple-detergent lysis buffer. ORO was quantified according to [39]. In brief, after washing cells four times in PBS, the ORO stained lipids were extracted with 4% NP-40 and 96% isopropanol. The supernatants were measured spectrophotometrically at 520 nm.

2.2. siRNA knockdowns and transient transfections

HeLa cells permanently downregulating CSN1 (siCSN1 cells) or CSN5 (siCSN5 cells) were produced as described [40,41]. For transient transfection cells were transfected with 50, 100 or 150 nM of siRNA against CAND1 (Eurogentec) or against control siGFP using Lipofectamine 2000 (Invitrogen). 24 h after transfection cells were lysed and supernatants were analyzed by SDS-PAGE and Western blotting. CAND1 overexpression was performed with 3xmyc-CAND1 (Addgene) and indicated DNA amounts using Lipofectamine 2000.

2.3. Immunoprecipitation, Flag-pulldowns, glycerol gradients, Western blotting and statistics

Immunoprecipitations with the anti-CSN7 and the anti-CAND1 antibodies were carried out as described [41]. Flag-pulldowns using Flag-CSN2 B8 cells were outlined before [36]. Just in brief, lysates were loaded onto the prepared ANTI-FLAG M2 affinity column (Sigma). After washing with 20 column volumes of 1 x TBS (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA), proteins were eluted by competition with the Flag peptide (100 μg/ml) as recommended by the manufacturer (Sigma). Eluted proteins were used for Western blots. Glycerol gradient centrifugation analysis was performed as described [38]. Fractions with different density (5% to 22% glycerol) were analyzed by Western blots. Density gradient was calibrated with purified CSN (about 400 kDa, fractions 5–7) and with purified 20S proteasome (about 700 kDa, fractions 8–9). Proteins were separated and analyzed by SDS-PAGE and Western blotting using antibodies against Cul1, γ-tubulin, CAND1, Fbxw7, c-Jun, c-Myc, Skp2, p27, β-TrCP, β-catenin and PPAR-γ (all antibodies from Santa Cruz).

Densitometry was carried out applying the ImageJ software. Statistics were calculated with Microsoft Excel and with GraphPad InStat3 software. Error bars are standard deviations (SD) or standard error of mean (SEM) and unpaired Student’s t-test was used for statistical analysis.

3. Results

3.1. CAND1 regulates Skp2 integration into CRL1s and p27 degradation

Based on the hypothesis that in the presence of the CSN and the specific substrate FBPs are stabilized by integration into CRL1 complexes and that CAND1 modulates their integration [11], we studied CAND1-dependent FBP steady state levels. Moreover, the function of the FBPs Fbxw7, Skp2 and β-TrCP was investigated by estimating steady state levels of their typical substrates c-Myc, p27 as well as β-catenin, respectively. To study a possible functional cooperation between CAND1 and the CSN, and the impact on the assembly of CRL1 complexes we used HeLa cells permanently expressing siRNA against CSN1 (siCSN1 cells), siCSN5 cells [42] were characterized by reduced CSN1 protein steady state level, which is connected with approximately 60% reduction of the whole CSN complex [40,42] and a slight reduction of total FBPs (Fig. 1A) confirming earlier observations [10,11].

First, CAND1 expression was transiently suppressed by transfecting 50, 100 or 150 nM siCAND1 into siCSN1 cells, or appropriate control cells permanently expressing siRNA against GFP (siGFP cells). Treatment of the cells with 150 nM siCAND1 led to suppression of CAND1 protein levels by more than 70% (Fig. 1A and B). As a consequence of CAND1 knockdown a significant increase of Skp2 expression in siGFP cells as well as in siCSN1 cells was observed (Fig. 1A and B). Quantification of Skp2 showed that the protein amount increased more than 2-fold in siGFP and in siCSN1 cells suggesting an enhanced incorporation of Skp2 substrate c-Myc, p27 as well as β-catenin, respectively. To study a possible functional cooperation between CAND1 and the CSN, and the impact on the assembly of CRL1 complexes we used HeLa cells permanently expressing siRNA against CSN1 (siCSN1 cells), siCSN5 cells [42] characterized by reduced CSN1 protein steady state level, which is connected with approximately 60% reduction of the whole CSN complex [40,42] and a slight reduction of total FBPs (Fig. 1A) confirming earlier observations [10,11].
downregulation in siCSN1 cells were obtained in siCSN5 cells [40] (data not shown).

To verify the impact of CAND1 on the integration of Skp2 into CRL1 complexes we used for additional studies density gradient centrifugation after treatment of HeLa cells with 100 nM of CAND1 siRNA. After 24 h cells were lysed and cellular proteins were separated by density gradients and analyzed by Western blotting. As shown in Fig. 2 knockdown of CAND1 led to a shift of Skp2 from fractions with low density (Fig. 2A, fractions 2–4) into fractions with higher density (Fig. 2B, fractions 5–7). Obviously Skp2 was integrated into larger complexes, most likely CRL1s responsible for the promotion of p27 degradation. The presence of both Cul1 and Skp2 in the same fractions reflects the assembled CRL1 complexes which peak in CAND1 knockdown cells. The anti-CSN8 antibody marks the position of the CSN complex. Density gradients were calibrated with purified CSN and 20S proteasome.

To visualize increased incorporation of Skp2 into CRL1 complexes after CAND1 silencing more specifically, we performed Flag-pulldowns. Flag-CSN2-B8 fibroblasts [36] were transfected with siCAND1 or siGFP and after 24 h cells were lysed and Flag-pulldowns and subsequent Western blots were carried out. As seen in Fig. 2C, CAND1 downregulation led to a reduction of CAND1 protein to less than 50%. The Flag-pulldown precipitated the CSN complex as demonstrated by the anti-CSN6 antibody together with CRL1 complex indicated by Cul1. There was an increased Skp2 incorporation into the CRL1 complex upon CAND1 silencing as demonstrated by the anti-Skp2 antibody (Fig. 2C).

Although ectopic expression of CAND1 was not very efficient, there was an approximately 2.5-fold increase of p27 caused by CAND1 overexpression in siGFP as well as siCSN1 cells (Fig. 3Aa and B). This was not reflected by a significant decrease of Skp2 steady state levels. Fbxw7 and β-TrCP also did not change upon CAND1 overexpression.

3.2. CAND1 does not interact with the CSN

For better understanding the interplay between CAND1, CSN and CRLs we carried out protein-protein interaction studies. To investigate physical interaction of CAND1 with the CSN we performed Flag-pulldowns as in Fig. 2C using Flag-CSN2 stable transfected B8 fibroblasts [36] upon CAND1 overexpression. Herein, we found no detectable CAND1/CSN interaction in the Flag-pulldowns. Further, overexpression of CAND1 had no impact on CSN binding to Cul1 (Fig. 4A). The CSN becomes assembled in supercomplexes with CRLs.

Fig. 1. CAND1 knockdown stabilizes Skp2 and reduces p27. (A) siGFP cells or siCSN1 cells were transfected with 50, 100 and 150 nM siGFP (control) or siCAND1. After 24 h cells were lysed and proteins analyzed by Western blotting using indicated antibodies. (B) Blots as shown in (A) of CAND1 (n = 4), Skp2 (n = 3) and p27 (n = 3) were quantified by densitometry, normalized against γ-tubulin and expressed as means of relative amounts. Error bars are standard deviations (SD). Unpaired Student’s t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.005.

Fig. 2. Incorporation of Skp2 into large complexes upon CAND1 downregulation. (A) HeLa cells were transfected with siGFP. 24 h after transfection cells were lysed and lysates loaded on glycerol density gradients. Fractions with different density were analyzed by Western blots using antibodies against CAND1, Cul1, Skp2, p27 and CSN8. The density gradient was calibrated with purified CSN (approximately 400 kDa) and purified 20S proteasome (approximately 700 kDa). (B) HeLa cells were transfected with siCAND1 and 24 h later glycerol density gradient centrifugation and Western blots were performed as in (A). (C) Flag-CSN2 B8 cells were transfected with 100 nM siGFP (control) or siCAND1. After 24 h cells were lysed and proteins of lysate and Flag-pulldown analyzed by Western blotting using indicated antibodies.
in which CSN2 interacts with cullins and CSN6 with Rbx1 [16,17]. Our previous data [27] and data from other groups [43,44] indicate that the CSN interacts with all cullins of all studied CRLs, and also CAND1 interacts with all cullins in human cells [33]. Therefore we asked whether the CSN and CAND1 co-immunoprecipitate. Using the anti-CSN7 antibody which precipitates the entire CSN complex and associated proteins [22] we could not recognize CAND1 in the precipitate (Fig. 4B), which confirms earlier studies [45,46]. Moreover, immunoprecipitation with an anti-CAND1 antibody did not precipitate the CSN (Fig. 4B).

3.3. CAND1 is essential for adipogenesis

CAND1 deletions in C. elegans and in A. nidulans led to severe developmental phenotypes [34,35]. Recently we found that the CSN, another regulator of CRLs, promotes the differentiation of LiSa-2 preadipocytes to adipocytes (adipogenesis) [38]. Therefore, we were interested to address the impact of LiSa-2 peptidocies on adipogenesis. For this purpose LiSa-2 peptidocies were stimulated by insulin, cortisol and triiodothyronine and incubated for 22 days. At days 1, 8, 15 and 22 cells were lysed and investigated by Western blotting. As shown in Fig. 5A and B an increase of CAND1 during adipogenesis was observed. The amount of CAND1 was elevated more than 2-fold at day 22. Concomitant with the increase of CAND1, there was a slight decrease of Skp2 and a significant increase of p27 starting from day eight of adipogenesis observed (Fig. 5A and B). Cul1 and the ratio between neddylied and unneddylated Cul1 did not change. The nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ) acts as a master switch in adipocyte differentiation. It is degraded by the UPS [47,48]. In our experiments (Fig. 5A) PPARγ increased about 4-fold on day 22. Changes of Fbxw7 and β-TrCP were not detected and c-Jun and β-catenin decreased only marginally.

CAND1 was essential for the differentiation of LiSa-2 peptidocies. This was demonstrated by transient transfection of 100 nM siCAND1 48 h prior to induction of differentiation (Fig. 6). As shown before in HeLa cells CAND1 knockdown led to a significant reduction of CAND1 steady state levels after 1 day of differentiation (corresponding to 3 days after transfection) and after 3 days of differentiation (corresponding to 5 days after transfection). After 8 days of differentiation and 10 days of transfection, CAND1 levels in siCAND1 treated cells recovered to almost control values. Nevertheless, suppression of CAND1 expression for 3 and 8 days led to a significant effect on cell differentiation. As shown by quantification of ORO staining lipids accumulated in control cells transfected with control siGFP as a hallmark of normal adipocyte differentiation. In contrast, CAND1 silencing led to a significant reduction of ORO staining by approximately 30% after
Our data demonstrate that CAND1 knockdown in studied human cells significantly increases the integration of the FBP Skp2 into CRL complexes. This is shown by stabilization of Skp2 steady state levels as well as by enhanced degradation of p27, a substrate of CRL1 complexes. Therefore, CAND1 knockdown reduces p27 and leads to inhibition of adipogenesis as measured by reduced lipid droplet formation. These experiments demonstrate that CAND1 is essential for LiSa-2 preadipocyte differentiation. Because of its high preference for Skp2 inactivation, CAND1 could represent an important factor in adipogenesis and in cancer development. Therefore, CAND1 is a distinguished target for the treatment of obesity and of different types of cancer.

Acknowledgments

This work was supported by grants from the Deutsche Forschungsgemeinschaft SPP 1365 to WD (DU 229/12-2) and MN (NA 292/9-1).

Fig. 5. CAND1 becomes upregulated during differentiation of LiSa-2 preadipocytes and stabilizes p27. (A) Western blot analysis of cells prepared on day 1, 8, 15 and 22 after induction of adipogenesis were carried out using indicated antibodies. Unneddylated (Cul1) and neddylated (Nedd8-Cul1) are specifically indicated. There is an increase of CAND1 and of p27, whereas Fbxw7 and β-TrCP did not change, while Skp2 declines. (B) CAND1, Skp2 and p27 data were quantified by densitometry, normalized against γ-tubulin and plotted as relative change against days of adipogenesis. Error bars are standard deviations of 3 independent experiments. Unpaired Student’s t-test revealed statistical significance between 1 day and 22 days of CAND1 steady state levels (*P<0.05) and between 1 day and 22 days of p27 levels (*P<0.05).

3 days and 40% after 8 days of differentiation (Fig. 6B). In addition, microscopy images nicely demonstrate reduced lipid droplet formation in particular after 8 days as a consequence of CAND1 knockdown (Fig. 6C).

4. Discussion

Our data demonstrate that CAND1 knockdown in studied human cells significantly increases the integration of the FBP Skp2 into CRL complexes. This is shown by stabilization of Skp2 steady state levels as well as by enhanced degradation of p27, a substrate of CRL1 complexes. We hypothesize that the integration of Skp2 into CRL1 complex upon CAND1 silencing occurs under the protection of the CSN and results in a stabilization of Skp2. Even in our siCSN1 cells in which the total FBPs are reduced confirming earlier observations [10–12], we did detect a protection/stabilization of Skp2. Nevertheless, we cannot exclude an induction of Skp2 expression upon CAND1 downregulation. Our experiments using density gradients showed the shift of Skp2 to large complexes, most likely CRL1Skp2 complexes, upon suppression of CAND1 expression. Moreover, pulldowns of Flag-CSN-CRL1 complexes revealed an increase of Skp2 incorporation into CRL1 complexes upon silencing of CAND1.

There was no effect of CAND1 knockdown neither on the stability of Fbxw7 or β-TrCP, nor on the degradation of c-Myc or β-catenin. These data demonstrate a preferential effect of CAND1 on Skp2 integration. In contrast to the knockdown, overexpression of CAND1 significantly stimulated steady state levels of p27, although Skp2 did not change significantly. This might be due to a quick recovery of control Skp2 steady state levels after CAND1 overexpression. Again, no influence was observed on Fbxw7 and β-TrCP upon CAND1 overexpression.

Interestingly, there was no dependency between CAND1 and the CSN observed, thus, CAND1 acts independently of the CSN on CRLs. This was also shown by CAND1 overexpression and subsequent CSM pulldowns in Flag-CSN2 B8 mouse fibroblasts (Fig. 4A). Further, there was no competition between CAND1 and the CSN for binding to Cul1 recognized. The absence of CAND1 in immunoprecipitates of the CSN (Fig. 4) confirms most of the earlier results [45,46] with the exception of pulldowns from human T cells showing CAND1 in CSN precipitates [49], which might be mediated by additional proteins.

Our data support the notion of CAND1 as a regulator of CRL assembly [50] that differentially affects the composition of CRL1 complexes [11]. We conclude that it acts as an inhibitor of the integration of the highly abundant Skp2 into CRLs. The exact mechanism how CAND1 specifically blocks the incorporation of Skp2 into CRL complexes is not known at the moment. Herein, the fact that the Skp2–Skp1 dimer is unable to inhibit CSN-mediated deneddylation in contrast to the β-TrCP–Skp1 and Fbxw7–Skp1 dimers might be important [21].

Because CAND1 stabilizes p27, it could act as a tumor suppressor. Interestingly, its suppression has been reported in lung tumors [51] and accelerated degradation of p27 is a hallmark of many tumor cells [5].

p27 is an important regulator of cell differentiation. Accumulation of p27 causes cell cycle exit necessary for the erythroid differentiation [52]. During 3T3-L1 preadipocyte differentiation the exit from the cell cycle into a pre-differentiation state of post-mitotic growth arrest was characterized by significant increase in p27. Interestingly this increase of p27 was predominantly independent of gene expression occurring via unknown post-transcriptionally controlled pathways [53]. Here we show for the first time that CAND1 is an essential regulator of adipogenesis, since it protects p27 against Ub-dependent degradation. However, we cannot exclude that CAND1 regulates other factors in addition to p27. CAND1 increases during differentiation of LiSa-2 preadipocytes and in parallel the p27 steady state amounts. This effect is presumably due to the CAND1 blockade of the integration of Skp2 into CRL complexes. Therefore, CAND1 knockdown reduces p27 and leads to inhibition of adipogenesis as measured by reduced lipid droplet formation. The experiments demonstrate that CAND1 is essential for LiSa-2 preadipocyte differentiation. Because of its high preference for Skp2 inactivation, CAND1 could represent an important factor in adipogenesis and in cancer development. Therefore, CAND1 is a distinguished target for the treatment of obesity and of different types of cancer.
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