Research Article

An interaction network of the mammalian COP9 signalosome identifies Dda1 as a core subunit of multiple Cul4-based E3 ligases

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Summary

The COP9 signalosome (CSN) is an evolutionarily conserved macromolecular complex that interacts with cullin-RING E3 ligases (CRLs) and regulates their activity by hydrolyzing cullin-Nedd8 conjugates. The CSN sequesters inactive CRLs, which rapidly dissociates from the CSN upon DNA damage. Here we systematically define the protein interaction network of the mammalian CSN through mass spectrometric interrogation of the CSN subunits Csn1, Csn3, Csn4, Csn5, Csn6 and Csn7a. Notably, we identified a subset of CRL complexes that stably interact with the CSN and thus might similarly be activated by dissociation from the CSN in response to specific cues. In addition, we detected several new proteins in the CRL-CSN interactome, including Dda1, which we characterized as a chromatin-associated core subunit of multiple CRL4 proteins. Cells depleted of Dda1 spontaneously accumulated double-stranded DNA breaks in a similar way to Cul4A-, Cul4B- or Wdr23-depleted cells, indicating that Dda1 interacts physically and functionally with CRL4 complexes. This analysis identifies new components of the CRL family of E3 ligases and elaborates new connections between the CRL and CSN complexes.

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Key words: Ubiquitin-dependent proteolysis, Cullin-RING E3 ligases, Neddylolation, Deneddylolation, Dda1

Introduction

Multisubunit cullin-RING type ubiquitin ligases constitute the most prominent family of ubiquitin ligases, in which the cullin protein functions as an assembly platform (Petroski and Deshaies, 2005). Cullins recruit the RING finger protein Rbx1 (also known as Roc1 and Hrt1) through their conserved C-terminus and the substrate recognition module through their N-terminal domains. The human genome encodes at least six cullins including Cul1, Cul2, Cul3, Cul4A, Cul4B and Cul5 (Kipreos et al., 1996), which bind distinct substrate recognition modules. The SCF complexes use the Bric-a-brac, Transtrack, Broad-complex (BTB)-fold adaptor Skp1 to connect Cul1 to a myriad of F-box proteins, which recognize their substrates mostly through WD40- or leucine-rich repeats (LRR). Likewise, the cullin-RING E3 ligases (CRLs) CRL2 and CRL5 use the BTB-fold adaptor ElonginC to connect Cul2 and Cul5 to BC-VHL box and BC-SOCS box proteins, respectively. In CRL3, a single polypeptide containing a BTB domain and a substrate-binding interface merges the function of Skp1-F-box or ElonginC-B box heterodimers (Pintard et al., 2004). Finally, heterodimers composed of the large Ddb1 protein, which does not harbor a BTB-fold but three seven-bladed β-propellers, and a member of the DCAF family (Ddb1-Cul4-associated factor), function as the substrate recognition modules of CRL4s (Angers et al., 2006; Jin et al., 2006). The small subunit Dda1 may also be part of some CRL4s (Jin et al., 2006), but its molecular function and regulation is still poorly understood.

Despite the fact that they display distinct molecular compositions, all CRLs are regulated by the covalent linkage of the ubiquitin-like protein Nedd8 to a conserved C-terminal lysine residue of the cullins (Pan et al., 2004). Neddylolation is essential for viability in all tested species, except budding yeast. Nedd8 activates CRLs by promoting a drastic conformational change of the C-terminal part of the cullin that frees Rbx1 and allows it to adopt multiple orientations that stimulate substrate ubiquitination in vitro (Duda et al., 2008).

The COP9 signalosome (CSN) complex physically interacts with CRLs and counteracts neddylation by hydrolyzing cullin-Nedd8 conjugates (Lyapina et al., 2001; Schwechheimer et al., 2001). Several lines of evidence indicate that the main function of the CSN is to inactivate CRLs by deneddylating cullins, at least in part by preventing the autocatalytic instability of their substrate-specific adaptors and subunits (Wee et al., 2005; Wu et al., 2005).

Although significant progress has been made in understanding how specific cues regulate substrate modification and in turn CRL binding, much less is known about how substrate binding is coordinated with cullin neddylation and deneddylation. Interestingly, several recent reports indicate that the availability of the adaptor bound to its substrate may promote CRL assembly and activation through cullin neddylation. For example, the availability of the F-box protein Skp2 along with its substrate p27, triggers assembly of the SCFTskp2 ubiquitin ligase and its activation through neddylation of the Cul1 subunit in vitro (Bornstein et al., 2006). Likewise, cullin
mutants unable to bind substrate recognition modules exhibit reduced neddylation (Chew and Hagen, 2007). However, not all CRLs appear to be regulated by this mechanism, as substrate recruitment does not stimulate neddylation of Keap1-associated Cul3 (Chew and Hagen, 2007). Interestingly, specific cues may trigger dissociation of fully assembled but inactive CRLs from the CSN, and in turn their activation through cullin neddylation. For example, inactive CRL4Ddb2 ubiquitin ligase is tightly bound to the CSN but is rapidly activated upon ultraviolet (UV) irradiation by dissociation from the CSN and subsequent neddylation (Groisman et al., 2003). These observations raise the possibility that, in a similar way to the CRL4Ddb2 complex, some fully assembled CRLs might be sequestered and kept inactive by the CSN in the absence of specific cues.

In this study, we sought to identify all pre-assembled CRLs that are tightly bound to the CSN in mammalian cells. We stably expressed and purified each FLAG-tagged CSN subunit from HEK 293T cells, and systematically identified CSN-associated polypeptides by sensitive tandem mass spectrometry (LC-MS-MS). Interestingly, we not only identified a total of fifteen associated CRLs but also found new components such as Dda1 as core subunits of multiple CRL4 complexes. Our subsequent functional analysis indicates that Dda1 acts as a positive regulator of several CRL4s.

**Results**

The proteomic interaction network of the mammalian COP9 signalosome

To systematically map the protein interaction network of the mammalian COP9 signalosome, we generated cell lines (HEK 293T) that stably expressed CSN subunits (Csn1, 3, 4, 5, 6 and 7a) fused to three repeats of the FLAG epitope (Fig. 1A), and analyzed anti-FLAG immunoprecipitates by sensitive LC-MS-MS. Although fibroblasts expressing FLAG-Csn2 exist (Huang et al., 2005), we were unable to obtain a cell line stably expressing FLAG-Csn2, thus Csn2 and 8 are the only subunits missing in our analysis.

Importantly, immunopurification of FLAG-Csn1, 3, 4 and 6 led to the identification of a common set of associated proteins, thus defining the CSN interaction network (Fig. 1B; supplementary material Table S3). By contrast, immunopurification of FLAG-Csn5 or FLAG-Csn7a was rather poorly efficient in pulling down this set of associated proteins. In both cases these results are expected because Csn5 also exists as a monomeric form (Tomoda et al., 2002) and thus a large fraction of FLAG-Csn5 was not incorporated into the CSN complex as revealed by gel filtration experiments (Fig. 1A), and recombinant FLAG-Csn7a subunit compete for incorporation into the CSN complex not only with endogenous Csn7a but also with the Csn7b subunit. Indeed, Csn7b was not recovered in the FLAG-Csn7a immunoprecipitate (supplementary material Table S3), indicating that the incorporation of Csn7a and Csn7b into the CSN complex is mutually exclusive.

![Image 315x453 to 565x719](https://example.com/image.png)

**Fig. 1.** The protein interaction network of the mammalian CSN. (A) Protein extracts prepared from HEK 293T cells stably expressing the indicated FLAG-tagged CSN subunits were fractionated on a Superose 6 column (bed volume: 24 ml) and 1-ml fractions were collected and analyzed by SDS-PAGE with specific FLAG, Csn1 and Cul3 antibodies. (B) CSN-interacting CRL subunits are highlighted in purple (SCF), blue (CRL2 and CRL5), green (CRL3) and red (CRL4). Subunits highlighted in orange have been previously found associated with CRL4s. (C) CSN-cullin interactions observed by LC-MS-MS were confirmed by co-immunoprecipitation experiments combined with western blot identification.

Although the COP9 signalosome has been shown previously to interact with a myriad of factors (Wei and Deng, 2003), our systematic proteomic analysis of the indicated COP9 signalosome exclusively identified subunits of CRLs [SCF (purple), CRL2 and CRL5 (blue), CRL3 (green), and CRL4 (red and orange)]. The presence of Cul1, Cul2, Cul3, Cul4A and Cul4B in FLAG-Csn4 immunoprecipitates was confirmed by immunoblotting with specific antibodies (Fig. 1C). Conversely, immunoprecipitations with Cul3 antibodies specifically recovered CSN subunits (Fig. 2A and supplementary material Table S5) demonstrating that cullins physically interact with the CSN. In addition to cullin proteins, we readily identified Rbx1, and the linkers Skp1, ElonginC, ElonginB and Ddb1, which bridge the interaction of cullins with their specific substrate adaptors (Fig. 1B; supplementary material Table S3). Besides these core CRL subunits, we systematically found the same set of substrate recruitment factors including one F-box (Fbg4-Fbx17), three BC-box (Lrrl, Lrcc14 and Fem1), four BTB (Btbd1, Btbd2, Klhdc5 and Klhl18), and several DCAF (Ddb1- and Cul4-associated factor) proteins including Csa-Errc8 and Ddb2, which have been shown previously to specifically rearrange from the CSN in response to DNA damage (Groisman et al., 2003). Conversely mass spectrometry analysis of FLAG-Lrcc14 and FLAG-Lrcc1 immunoprecipitates identified Cul2, ElonginC, ElonginB, Rbx1 and every CSN subunit (Fig. 2G; supplementary material Table S4). Therefore, among the 16 BC-box proteins that were recently identified in FLAG-ElonginB pulldowns (Mahrour et al., 2008), only Lrcc14, Lrr1 and Fem1 stably associated with the CSN. Similarly, only four BTB proteins (Btbd1, Btbd2, Klhdc5 and Klhl18) were systematically identified in the CSN immunoprecipitates (Fig. 1B), whereas at least five additional BTB-substrate adaptors (Klh9, Klhl13, Kctd10, Klhl22 and Kbtbd6) were recovered in endogenous Cul3 immunoprecipitates (Fig. 2A and supplementary material Table S5). Indeed, the FLAG-Csn4 readily co-immunoprecipitated with the endogenous Btbd1-Btbd2.
accompanied by accumulation of endogenous Dda1 (Fig. 3C). Moreover, siRNA-mediated depletion of Cul4A and Cul4B was strongly accumulated in cells treated with MG132, and FLAG-Dda1 was degraded with a half-life of approximately 1 hour (Fig. 3A). Interestingly, single-cell analysis using indirect immunofluorescence revealed that HeLa cells depleted for Cul4A by RNAi exhibited low abundance, or were primarily detected when cells were treated with the proteasome inhibitor MG132, among them Dda1. Dda1 is a small evolutionarily conserved protein that has been shown to interact with Ddb1; however, its role and regulation remain elusive (Jin et al., 2006).

In addition to substrate-recruitment factors, we identified several subunit of multiple Cul4 ubiquitin ligases but not with Klb9-Klb13, which preferentially immunoprecipitated neddylated Cul3 (Fig. 2B). Likewise, several DCAF proteins were also found to be stably associated with the CSN by MS-MS analysis (Fig. 1B; Fig. 2D), supporting the notion that the CSN may sequester many pre-assembled CRL4 complexes. Accordingly, co-immunoprecipitation experiments with HA-tagged versions of Ddb2, Wdr23 and H326 confirmed the presence of Csn2 and Csn3 (Fig. 2E) and Csn subunits were recovered in endogenous Vprbp immunoprecipitates (data not shown) (McCall et al., 2008). Taken together, these results suggest that the CSN stably associates with a specific subset of CRLs in vivo (Fig. 2F).

**The CSN-interacting protein Dda1 is an unstable nuclear core subunit of multiple Cul4 ubiquitin ligases**

In addition to substrate-recruitment factors, we identified several proteins that did not contain any recognizable domain (Fig. 1B, highlighted in orange). Some of these factors were recovered at low abundance, or were primarily detected when cells were treated with the proteasome inhibitor MG132, among them Dda1. Dda1 is a small evolutionarily conserved protein that has been shown previously to interact with Ddb1; however, its role and regulation remain elusive (Jin et al., 2006).

Consistent with our mass spectrometry data, FLAG-Dda1 strongly accumulated in cells treated with MG132, and FLAG-Dda1 was degraded with a half-life of approximately 1 hour (Fig. 3A). Interestingly, single-cell analysis using indirect immunofluorescence revealed that HeLa cells depleted for Cul4A by RNAi exhibited high levels of FLAG-Dda1 compared with control cells (Fig. 3B). Moreover, siRNA-mediated depletion of Cul4A and Cul4B was accompanied by accumulation of endogenous Dda1 (Fig. 3C), suggesting that Dda1 is degraded by Cul4 complexes, most likely through an autocatalytic mechanism.

To investigate whether Dda1 associates with Ddb1 in assembled Cul4 ubiquitin ligases, we analyzed FLAG-Dda1 immunoprecipitates by LC-MS-MS (left panel). (B) Klb9 and 13, and Btb1 and 2 were immunoprecipitated from 293T cells expressing FLAG-Csn4, separated by SDS-PAGE and immunoblotted with specific antibodies against the indicated proteins. The arrows mark neddylated (Cul3Nedd8) and unneddylated Cul3. (C) Endogenous Cul4A immunoprecipitates were separated by SDS-PAGE and analyzed by tandem mass spectrometry. (D) Cul4A immunoprecipitates were separated by SDS-PAGE and blotted with specific antibodies directed against Cul4A, Ddb1, Ddb2, Cand1, Csn2, Csn3, Cul3, Vprbp, Wdr23 and Dda1. (E) HA-tagged DCAF proteins H326, Wdr23 and Ddb2 were expressed in HeLa cells and immunoprecipitated. The immunoprecipitates were separated by SDS-PAGE and blotted with specific antibodies directed against the HA peptide, Cul4A, Cul4B, Ddb1, Cul3, Csn2 and Csn3. (F) Summary of the LC-MS-MS analysis of FLAG-Csn, Cul3 and Cul4A immunoprecipitates. Green squares indicate presence in the immunoprecipitate and red indicate absence. Cul3 and CRL4 core subunits (Cul4A, Cul4B and Ddb1) as well as BTB and DCAF adaptors are presented. (G) Summary of the LC-MS-MS analysis of FLAG-Lrr1, -Lrrc14 immunoprecipitates and co-immunoprecipitation analysis of Btb1 and 2, Klb9 and 13 and HA-H326, -Ddb2 and -Wdr23 immunoprecipitates. Green squares indicate presence in the immunoprecipitate and red indicate absence.

The localization of Dda1 is cell cycle regulated

To investigate the molecular function of Dda1, we determined its expression levels during the cell division cycle using a double thymidine block-release synchronization protocol (Sumara et al., 2007). Dda1 was detected throughout the cell cycle with lower levels of expression during mitosis (Fig. 4A). Indirect immunofluorescence
experiments confirmed that Dda1 levels were reduced in mitotic cells, whereas Cul4A levels remained unchanged (data not shown). Dda1 was predominantly nuclear during interphase and resisted extraction procedures that solubilize nucleoplasmic proteins (Fig. 4B), suggesting that, like Cul4A, Dda1 may be associated with chromatin. Indeed, biochemical purification of chromatin from S-phase-arrested cells confirmed that significant fractions of Dda1, Ddb1 and Cul4A were found in chromatin preparations and could be solubilized by nuclease treatment (Fig. 4C). Interestingly, immunofluorescence analysis revealed that in contrast to Cul4A, Dda1 staining was strongly reduced on chromosomes aligned on the metaphase plate or on segregating chromosomes during anaphase, and Dda1 was only recruited to chromatin during telophase when the chromosomes start to decondense (Fig. 4D). However, the total amount of Dda1 co-precipitating with Cul4A was comparable in cells arrested in S-phase or mitosis (Fig. 4F), suggesting that Dda1 only dissociates from a small fraction of Cul4 complexes during mitosis.

Dda1 is a positive regulator of CRL4s

Cul4-type E3 ligases are involved in controlling DNA metabolism such as DNA replication and repair (O’Connell and Harper, 2007). Indeed, cells depleted of Cul4A and Cul4B spontaneously accumulate double-stranded DNA breaks that can be visualized by staining phosphorylated H2A.X foci using appropriate antibodies (Fig. 5A). Importantly, Dda1-depleted cells similarly accumulate spontaneous DNA breaks, though to a lesser extent compared with Cul4A- and Cul4B-depleted controls. Quantification by FACS analysis showed that almost 20% and 40% of Dda1 and Cul4A-B-depleted cells accumulated phosphorylated H2A.X foci, respectively (Fig. 5B,C). Likewise, we found that inactivation of the uncharacterized Dda1-interacting DCAF protein, Wdr23, specifically resulted in an accumulation of DNA breaks (Fig. 5C). Analysis of cell cycle repartition revealed that these cells were primarily in S-phase (data not shown), suggesting that the observed double-stranded breaks may occur during DNA replication leading in turn to the activation of the S-phase checkpoint. As double-stranded breaks have also been observed in cells depleted for Vprbp (Hrecka et al., 2007), these observations indicate that Dda1 may act as a positive regulator of multiple Cul4 ubiquitin ligases during DNA replication in vivo. The effect of Dda1 in suppressing the formation of double-stranded DNA breaks likely depends on Ddb1, as Dda1 partially accumulated in the cytoplasm in Ddb1-depleted cells (Fig. 5E). Likewise, a truncated version of Dda1(ΔN26), which failed to bind Ddb1 in co-immunoprecipitation assays (Fig. 5F), also localized to the cytoplasm (data not shown), indicating that
nuclear accumulation of Dda1 requires its interaction with Ddb1. However, consistent with previous findings (Pick et al., 2007), we did not detect any effect resulting from Dda1 inactivation on UV-induced degradation of Cdt1 by the CRL4Cdt2 ligase (data not shown), indicating that Dda1 is not an essential regulator of all Cul4-based E3 ligases.

Discussion

We report a comprehensive interaction network of the mammalian CSN, an evolutionarily conserved macromolecular complex that regulates CRLs by promoting deneddylation of cullin subunits. Interestingly, we found fifteen CRLs that are stably associated and sequestered by the CSN but may dissociate in response to specific cues, in a similar way to the CRL4Ddb2 complex upon DNA damage (Grosman et al., 2003). In addition, we identified several poorly characterized proteins, including the evolutionarily conserved protein Dda1. We show that Dda1 is an unstable subunit of multiple CRL4 complexes, which associates with chromatin in a cell cycle-dependent manner. Our results suggest that Dda1 is a chromatin-associated CRL4 subunit that may specifically regulate a subset of Cul4 complexes, which associates with chromatin in a cell cycle-dependent manner. Several CRL4 complexes control chromatin-associated processes such as silencing of gene expression, DNA replication and repair (O’Connell and Harper, 2007). For example, Cul4 co-purifies with chromatin-bound complexes involved in RNAi-mediated mechanisms (Hong et al., 2005). Moreover, the CRL4Ddb2 complex is required to degrade the replication licensing factor Cdt1 on chromatin after S-phase entry or upon DNA damage, thereby restricting DNA replication to once per cell cycle or preventing DNA replication in conditions of damaged DNA (Jin et al., 2006). Although the precise molecular mechanisms by which Dda1 regulates CRL4 function remain unclear, several lines of evidence indicate that Dda1 may contribute to the spatiotemporal regulation of chromatin-bound CRL4. Indeed, Dda1 is recruited to chromatin via Ddb1, and is required to prevent the accumulation of DNA damage during S-phase. Several CRL4 complexes control chromatin-associated processes such as silencing of gene expression, DNA replication and repair (O’Connell and Harper, 2007). For example, Cul4 co-purifies with chromatin-bound complexes involved in RNAi-mediated mechanisms (Hong et al., 2005). Moreover, the CRL4Ddb2 complex is required to degrade the replication licensing factor Cdt1 on chromatin after S-phase entry or upon DNA damage, thereby restricting DNA replication to once per cell cycle or preventing DNA replication in conditions of damaged DNA (Jin et al., 2006). Ddb1 and Cul4A were also implicated in S-phase-dependent genomic instability and nucleotide excision repair (Higa and Zhang, 2007), and indeed, the CRL4Ddb2 ligase is also required to prevent the formation of double-stranded breaks during S-phase. These functions are reminiscent of the yeast cullin Rtt101p, which regulates progression of replication forks through nucleosome-
dense chromosomal regions or sites of DNA damage (Luke et al., 2006). In the absence of Rtt101p function, replication forks often collapse, resulting in the formation of double-stranded breaks. Based on these data, we speculate that Dda1 may be necessary to activate a subset of Cul4 ligases during DNA replication, perhaps by stabilizing the association of Ddb1 with chromatin-associated DCAFs. Interestingly, in contrast to Cul4A, Dda1 is cell cycle regulated and specifically excluded from chromatin during mitosis. It is thus conceivable that this chromatin exclusion prevents the activity of Dda1-dependent Cul4 ligases during chromosome segregation.

The CSN stably binds a subset of CRLs predominantly involved in DNA-metabolism

Bioinformatic sequence analysis indicates that the human genome codes for over 600 potential substrate-specific adaptors of cullin-based E3 ligases that are characterized by specific motifs such as the F-box, BC box, BTB domain or WDXR motifs (Willems et al., 2004). However, despite this complexity, we systematically recovered only 15 substrate adaptors in CSN purifications, which belong to all four major cullin subfamilies and include Fbg4, Lrr1, Fem1, Lrrc14, Btbd1, Btbd2, Klhdc5, Klhl18, Ddb2, Vprbp, Csa, Wdr23, Wdr32, H326 and Cdt2. Likewise, a SILAC-based mass spectrometry approach similarly identified cullins 1, 2, 3, 4A and 4B, Bxb1, Ddb1 as well as the adaptors Vprbp, Klhdc5 and Ddb2 associated with Csn5 complexes (Fang et al., 2008). It is unlikely that this limited set can simply be explained by differences in expression levels, as several other substrate-specific adaptors are readily detected in cell lysates and/or in immunoprecipitation experiments with specific cullin antibodies. For example, Klhl9 and Klhl13 readily immunoprecipitate Cul3 but failed to associate with the CSN. Likewise, several DCAFs were present in Cul4A or Dda1-immunoprecipitates, but were not found in CSN immunopurifications. Interestingly, Klhl9 and Klhl13 precipitated a significant fraction of neddylated Cul3, whereas predominantly unneddylated Cul3 was bound to the Btbd1 and Btbd2 adaptors. Finally, we have performed reciprocal immunopurifications and confirmed that the CRL2 adaptors Lrr1 and Lrrc14, the CRL3 adaptors Btbd1 and Btbd2, and the CRL4 adaptors Ddb2, Wdr23, and H326 are indeed part of the CSN interaction network. For example, LC-MS-MS analysis of FLAG-Lrr1 immunoprecipitates identified CRL2 components and every CSN subunit (Csn1, 2, 3, 4, 5, 6, 7a, 7b, 8). Likewise, Cdt2 has been shown to co-precipitate each CSN subunits in Schizosaccharomyces pombe (Liu et al., 2006).
2005), and more recently, CSN subunits were readily identified in Vprbp immunoprecipitates from human cells (McCall et al., 2008). Taken together, these data suggest that the CSN stably binds a small but specific subset of substrate adaptors.

What could be the molecular function of this association with the CSN complex? Interestingly, Ddb2 stably interacts with the CSN but rapidly dissociates in response to DNA damage, in particular during global genome repair (Grosman et al., 2003). Released CRL4Ddb2 complexes bind damaged chromatin, and process DNA lesions in part through ubiquitylation of the repair protein XPC (Xeroderma pigmentosum group C protein). Post-recovery repair involves a-association of the CSN with the CRL4Ddb2 complex and its inactivation through deubiquitylation. Thus, in this case, the CSN sequesters and thereby inactivates preassembled CRL4Ddb2 ubiquitin ligase in response to UV irradiation and ATR-dependent phosphorylation of CSN subunits. In particular, ATM-related), including Csn1, Csn3 and Csn7a (Matsuoka et al., 2007). It is thus conceivable that phosphorylation of CSN subunits may regulate their interaction with specific CRLs. In particular, ATM-ATR-dependent phosphorylation of CSN subunits may release the CSN complex on the one hand to prevent replication of damaged DNA and on the other hand to initiate DNA repair. Further experiments are now required to test this attractive model. In particular, it will be crucial to identify the specific signals and pathways that trigger the regulated release of the stably CSN-associated CRLs, thus determining their cellular function and targets.

Materials and Methods

DNA recombinant work

Standard procedures were used for Gateway cloning (Invitrogen) and DNA manipulations (Sambrook et al., 1989). To construct the pMT3989 and pMT4149 mammalian gateway vectors, the cassette EcoRV-FLAG3(X)Aattb1-Ccdb-Cm rr Aattb2-EcoRV was PCR amplified and cloned into pBluescript (pBKS) vector. After verification by direct sequencing, the cassette was subcloned into the pMX-pie vector (gift from T. Pawson) (EcoRV site) or into the pcMV5 (Small site) to generate pMT3989 (Luke-Glaser et al., 2007) and pMT4149, respectively. Csn1, Csn5, Csn7a cDNA and the various mutant versions of Dda1 were PCR amplified and cloned into the entry vector pDONR201 (Invitrogen). Csn3, Csn4, Cdt2, Lrc14 and Dda1 cDNA directly cloned into pDONR223 were obtained from Open Biosystems. Full-length Lr1 entry clone was purchased from GeneCopeia cat. No. T2299. The cDNAs of Ddb2, H326 and Wdr23 (ImaGenes, Berlin, Germany) were amplified and extended with the restriction site pairs BamHI-XhoI by PCR, cloned into pcDNA3.1(+)-HA(term) and verified by direct sequencing. The plasmids used in this study are listed in the supplementary Table S1.

Cell cycle and FACS analysis, and drug treatment

Cells were synchronized in G1 phase of the cell cycle using a double-thymidine block release as previously described (Sumara et al., 2007). Drug treatments were applied for 12 hours using the following concentrations: 2 µM nocodazole and 2 µg/ml aphidicolin prepared in 0.1% DMSO.

For flow cytometry analysis to assay the cell cycle position of γH2A.X-positive cells, cells were stained with a cocktail of monoclonal antibodies against histone H2AX and β-tubulin. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software.

RNA interference knockdown

RNA interference experiments were performed either using Dharmacon’s siGENOME SMARTpool siRNAs or single siRNA duplexes (Microscyn, Balgach, Switzerland) to deplete endogenous levels of Ddb1, Dda1, Cul4A, Cul4B and Cul3 in HeLa cells. The siRNAs used are listed in supplementary information (supplementary material Table S2).

HeLa cells were transfected with 50 nM siRNAs using Oligofectamine (Invitrogen) for 48 hours or 72 hours. For simultaneous knockdown and overexpression of genes, 0.4 µg DNA, 200 nM siRNAs and 2 µl Lipofectamine 2000 were used in 12-well dishes for 48 hours. Equal amounts of cells were harvested and directly resuspended in Laemmli buffer. The lysates were separated by SDS-PAGE, transferred onto a PVDF membrane and immunoblotted with the indicated antibodies. Stable HeK 293T cell lines were generated as described (Luke-Glaser et al., 2007).

Interaction map of the COP9 signalosome

HeLa and HEK 293T cell culture and stable cell line selection

HeLa and Human Embryonic Kidney (HEK) 293T cells were grown in Dulbecco’s modified Eagle’s high-glucose medium (DMEM, high glucose) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and antibiotic-antimycotic (Gibco) or with 10% FBS (PAV) and 1 unit/ml penicillin, 1 µg/ml streptomycin, 0.25 µg/ml amphotericin B (Gibco). Transient transfections were carried out for 24 hours in 10-cm dishes with 12 µg of DNA or in 6-cm dishes using 3 µg of DNA using FuGENE (Roche) (HA-V4D010PS,FLAG-Dda1 overexpression in Cul4A-RNAi cells). For western blotting, equal amount of cells were harvested and directly resuspended in Laemmli buffer. The lysates were separated by SDS-PAGE, transferred onto a PVDF membrane and immunoblotted with the indicated antibodies. Stable HEK 293T cell lines were generated as described (Luke-Glaser et al., 2007).

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Antibodies

Antibodies directed against the following polypeptides were used in this study: anti-FLAG (Sigma), anti-Cul2 [Rockland Immunocinics Inc. (Chemicon)], anti-Cul3 (Sumara et al., 2007), anti-Cul4A (this study), anti-Vprbp (this study), anti-Csn1 (Bethyl), anti-Csn2 (Biotron), anti-Csn3 (Abcam), anti-Ddb1 and anti-Ddb2 (this study), anti-Ddb1 (Bethyl), anti-Dda1 (Heck et al., 2007), anti-Tubulin (Sigma), anti-HA (Covance), anti-Klb9/Btbd13 (Sumara et al., 2007), anti-Wdr23 (this study), anti-Ddb2 (Santa Cruz), anti-Cand1 (Santa Cruz), anti-Cyclin E (Santa Cruz), anti-PH3 (Upstate), anti-γH2A.X (Upstate). Secondary antibodies coupled to peroxidase were purchased from Amersham or from Sigma. Gel filtration experiments were performed as described (Luke-Glaser et al., 2007).
Immunoprecipitations, protein extracts, immunoblotting, and chromatin purification

Standard procedures were used (Sambrook et al., 1989). For immunoprecipitation experiments, cellular extracts were prepared in extraction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM CHAPS, 20 mM beta-glycerophosphate, 10% glycerol, 0.5 mM DTT, 1 tablet complete protease inhibitor cocktail 50 ml). Affinity-purified antibodies were coupled to Affigel protein A beads (BioRad) in a ratio of 1 mg antibody to 1 ml buffer. The antibody beads were rotated end-over-end in HeLa cell extracts for 2 hours at 4°C. A ratio of 10 μl beads to 1–2 mg of protein in the extract was used. The beads were washed several times with extraction buffer and eluted using 100 mM glycine, pH 2.3. The eluates were neutralized using 1.5 M Tris pH 9.2, and 4× Laemmli buffer. For diphosphorylation assays, 250 μg of total protein were treated with 400 Units of active or heat-inactivated (1 hour at 65°C) lambda-phosphatase (NEB) for 1 hour at 30°C. The reaction was stopped by addition of 4× Laemmli buffer.

For chromatin purification, cell pellets were resuspended in lysis buffer (100 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 10 mM HEPES, pH 7.5, 0.5% Triton X-100) and lysed using a syringe. Lysates were layered over lysis buffer containing 1 M sucrose and centrifuged at 8000 g for 30 minutes at 4°C. The supernatant was collected (cytosol-nucleoplasm) and the pellet was digested with micrococcal nuclease at 37°C for 1 hour in lysis buffer containing 5 mM CaCl₂ and 350 mM KCl. After centrifugation for 10 minutes at 16,100 g, the supernatant was collected as solubilized chromatin.

Immunostaining

Cells were washed three times with warm PBS and fixed with paraformaldehyde (PFA) 4% for 10 minutes at room temperature. Where indicated, cells were pre-extracted by incubation with ice-cold pre-extraction buffer (300 mM sucrose, 3 mM MgCl₂, 1 mM EDTA, 50 mM NaCl, 25 mM HEPES, pH 7.5, 0.5% Triton X-100) for 5 minutes and washed with PBS before fixation. In some cases cells were spun down onto a glass slide using a cytospin centrifuge as previously described (Sumara et al., 2007). PFA was washed away with PBS, cells were permeabilized in PBS-0.1% Triton X-100 (PBST) and incubated for 1 hour at room temperature in the blocking solution (5% BSA) for 5 minutes before fixation. In some cases cells were fixed overnight with 4% PFA for 10 minutes at room temperature. Where indicated, cells were pre-treated with 100 μg/ml of the proteasome inhibitor MG132 (10 M) or its vehicle DMSO.

Immunopurification

LC-MS-MS analysis of endogenous Cul3 and Cul4 protein complexes

Immunopurification

The immunoprecipitated hCul3 and hCul4A complexes were washed several times with extraction buffer containing 0.5% CHAPS, 50 mM NaCl, 0.1% Tween 20, 1 μg/ml leupeptin-pepsatin A, 1 μg/ml aprotinin, 100 μg/ml PMSF and 100 μg/ml NaVO₄ and discarded. MS-MS data were searched using MASCOT Version 2.1 (Matrix Science Ltd, UK) against a human subset of IPI Uni-Prot database (EBD) (May 2005). All basic Mascot searches were performed using a maximum missed-cleavage value of 1, variable methionine oxidation modification (+16 Da), and protein N-term acetylation modification (+42 Da), fixed cysteine carbamidomethylation modification (+57 Da) and phospho modification (+79 Da). The MS and MS/MS ion data was selected for 80% sequence coverage with at least two unique peptides assigned to each identified protein. The immunopurified hCul3 and hCul4A complexes were washed several times with extraction buffer containing 500 mM and 150 mM NaCl.

Limited electrophoresis

After removal of the supernatant, beads were eluted by boiling in 50 μl electrophoresis buffer. The supernatant was loaded on an 8% polyacrylamide gel and proteins were stained with Coomassie Blue. Distances of 18 cm and 38 cm stained molecular weight markers were run in parallel to ensure that proteins up to 250 kDa had entered the separating gel at time of stop. After a quick fixing and Coomassie-staining steps (15 minutes), every gel lane was cut into slices corresponding to molecular weight regions.
Digestion, HPLC and mass spectrometry

Bands or gel regions were excised from gels as cubes of 2.0-mm length. Proteins were manually in-gel digested with trypsin according to a described protocol (Shevchenko et al., 1996). Tryptic peptides were recovered in the supernatant of the digestion, concentrated by evaporation to 15 μl and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS-MS) on a SCIEX QSTAR Pulsar 1 (Concord, Ontario, Canada) hybrid quadrupole-time of flight instrument equipped with a nanoelectrospray source and interfaced to an LC-Packsings Ultimate (Amsterdam, Holland) HPLC system. Separation was performed on a PepMap (LC-Packsings, Amsterdam) reversed-phase capillary C18 (75 μm ID × 15 cm) column at a flow rate of 200 nl/minute along a 52-minute gradient of acetonitrile (0-40%). The Analyst QS 1.1 instrument controlling software was used to perform peak detection and automatically selecting sequentially eluting peptides for collision-induced fragmentation (CID). The two most intense ions in the mass range 400-1200 with charge state 2+ to 4+ were selected for analysis after a 1-second survey scan. CID spectra were accumulated for 3 seconds for every precursor. Analyzed ions were excluded for 180 seconds from further analysis (the tolerance window for exclusion was 0.075 amu).

CID data generation

Collections of tandem mass spectra for database searching were generated from the Analyst files with the script Mascot.dll version 1.6b4 (Matrix Science, London). The tool was set to try determine precursor charge state from the survey scan, after centroiding peaks at 50% height and merging data points within a distance of 0.1 amu. Charge state information thus determined was used whenever available, while all the default charge states 2+, 3+ and 4+ were written when charge state could not be auto determined. CID spectra from the same precursor were added and averaged to yield one spectrum if they fell within a mass window of 1.0 amu and a time window of 10 measurement cycles (maximum of 2.5 minutes). Spectra containing fewer than ten peaks before treatment were discarded. Accepted CID spectra were processed as follows: peaks below 0.5% of the base peak were removed; remaining peaks were not smoothed but were centroided at 50% height with a merge time window of 10 measurement cycles (maximum of 2.5 minutes). Spectra were auto determined. CID data generation was 0.075 amu).

CID spectra were accumulated for 3 seconds for every precursor. Analyzed ions were excluded for 180 seconds from further analysis (the tolerance window for exclusion was 0.075 amu). A cullin E3 ligase complex includes Rbx1 and the C4 histone H3-K9 methyltransferase and is required for RNAi-mediated heterochromatin formation. RNA B 2, 106-111.

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