LETTERS

The conserved protein DCN-1/Dcn1p is required for cullin neddylation in *C. elegans* and *S. cerevisiae*

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SCF-type E3 ubiquitin ligases are multi-protein complexes required for polyubiquitination and subsequent degradation of target proteins by the 26S proteasome[1]. Cullins, together with the RING-finger protein Rbx1, form the catalytic core of the ligase, and recruit the substrate-recognition module[1–4]. Cycles of covalent modification of cullins by the ubiquitin-like molecule Nedd8 (neddylation)[5] and removal of Nedd8 by the COP9 signalosome (deneddylation) positively regulate E3 ligase activity[6,7]. Here we report the identification and analysis of a widely conserved protein that is required for cullin neddylation in the nematode *Caenorhabditis elegans* and the yeast *Saccharomyces cerevisiae*. *C. elegans* DCN-1 and *S. cerevisiae* Dcn1p (defective in cullin neddylation) are characterized by a novel UBA-like ubiquitin-binding domain and a DUF298 domain of unknown function. Consistent with their requirements for neddylation, DCN-1 and Dcn1p directly bind Nedd8 and physically associate with cullins in both species. Moreover, overexpression of Dcn1p in yeast results in the accumulation of Nedd8-modified cullin Cdc53p. Both *in vivo* and *in vitro* experiments indicate that Dcn1p does not inhibit deneddylation of Cdc53p by the COP9 signalosome, but greatly increases the kinetics of the neddylation reaction.

In *C. elegans* the cullin CUL-3 forms a ligase that is required to degrade MEI-1 (ref. 8), a subunit of the microtubule-severing complex katanin[9]. *C. elegans* katanin is active during meiosis[10] but is inactivated by the CUL-3 ligase before mitosis, to allow for the stable assembly and positioning of the first mitotic spindle in the one-cell zygote[7,11,12]. Loss of the CUL-3 ligase leads to abnormally high levels of katanin during mitosis and associated defects in microtubule-dependent processes.

To identify new proteins involved in CUL-3-dependent degradation of MEI-1/katanin, we screened time-lapse movies of embryonic cell divisions deposited in databases of large-scale *C. elegans* RNA interference (RNAi) screens[12,13] for defects resembling those caused by loss of CUL-3 function. In the publicly accessible database of the chromosome III RNAi screen[12], we identified a new gene that we named *dcn-1* (open reading frame H38K22.2), which is required for the post-meiotic degradation of MEI-1. In embryos depleted of DCN-1 by RNA interference (hereafter referred to as *dcn-1(RNAi)* embryos), MEI-1 tagged with green fluorescent protein (GFP) accumulated on the mitotic spindle, and endogenous MEI-1 was present at higher levels (Fig. 1a, d; see also Supplementary Videos 1 and 2). Consequently, astral microtubules appeared shorter, and mitotic spindles were mis-positioned (Fig. 1b, c). These mitotic spindle defects in *dcn-1(RNAi)* embryos were rescued by simultaneous reduction of MEI-1 function (Fig. 1c). We conclude that DCN-1 acts with, or in parallel, to the CUL-3/MEL-26 (where MEL-26 is the substrate-specific adapter of the CUL-3 ligase) E3 ligase to downregulate MEI-1/katanin.

C. elegans DCN-1 is a novel 295-amino-acid protein with a carboxy-terminal DUF298 domain of unknown function (PF03556) (Fig. 2a). This domain is evolutionarily conserved, with a single gene encoding family members in *C. elegans*, *S. cerevisiae* and *Schizosaccharomyces pombe*, and a limited number of homologues in other eukaryotes (Fig. 2b; see also Supplementary Fig. S1). Database searches with generalized profiles constructed from the amino termini of representative DUF298 proteins also revealed a significant similarity ($P < 0.001$) to a number of non-DUF298 proteins. These matches include the human Fas-associated factor FAF1 and the TNF-receptor-associated TTRAP protein, as well as the p97/Cdc48 cofactor p47/Shp1 from several organisms. Inclusion of these proteins in a subsequent iteration of profile searches[14] yielded highly significant matches ($P < 0.001$) to a number of established UBA domains (Supplementary Fig. S2). UBA domains have been found in a variety of proteins linked to the ubiquitin pathway[14], where they have been shown to interact directly with ubiquitin[15]. Indeed, purified full-length DCN-1, as well as the UBA-like domain by itself, strongly interacted with immobilized ubiquitin (Fig. 2c), with an affinity significantly stronger than that of the human p47 UBA domain. Interestingly, full-length DCN-1 also directly interacted with the ubiquitin-like protein Nedd8 (Fig. 2c). In contrast to its interaction with ubiquitin, the DCN-1 UBA-like domain by itself only weakly interacted with Nedd8, suggesting that the UBA-like domain is specific for ubiquitin, and that Nedd8 binding is probably mediated by a different part of DCN-1. We obtained similar results with the *S. cerevisiae* DCN-1 homologue Dcn1p (YLR128W), suggesting that both ubiquitin- and Nedd8-binding of these proteins is evolutionarily conserved. Although many of the DUF298-domain-containing proteins contain an N-terminal UBA-like domain, it is absent in some of the DCN-1 homologues in other organisms (Fig. 2b). Notably, *C. elegans* DCN-1 is expressed in two different splice forms, with the UBA-like domain absent in the shorter one (Supplementary Fig. S3), suggesting that DCN-1 and DCN-1-like proteins may perform functions independent of their ubiquitin-binding properties and more directly related to Nedd8 binding.

Because DCN-1 and Dcn1p both bind Nedd8, and *dcn-1(RNAi)* results in defects similar to those observed in mutant *C. elegans* embryos lacking the neddylation pathway, we investigated whether DCN-1 and Dcn1p are involved in cullin neddylation. In *C. elegans* embryos expressing a GFP-tagged version of DCN-1, the GFP signal was highest in the nuclei of early embryonic cells but was also present at lower levels in the cytoplasm (Supplementary Fig. S4). We observed an identical expression pattern using anti-DCN-1 antibodies and indirect immunofluorescence to stain fixed embryos

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(Supplementary Fig. S4). This distribution of DCN-1 in *C. elegans* embryonic cells is identical to that previously reported for *C. elegans* Nedd8 and for the COP9 signalosome subunit CSN-5 (refs 7, 8), consistent with a role for DCN-1 in cullin neddylation. We next used western blots to compare the status of CUL-3 neddylation in extracts prepared from wild-type and *dcn-1(RNAi)* embryos. Interestingly, endogenous CUL-3 was mainly present in its un-neddylated form in *dcn-1(RNAi)* extracts, comparable to embryos lacking the Nedd8 E1 activating enzyme RFL-1 (Fig. 3a). In budding yeast, deletion mutations of the genes affecting the neddylation status of Cdc53p (Cul1) are not lethal, but they lower the restrictive temperature for conditional alleles of Cdc53p and other components of this E3 ligase[16,17]. Accordingly, deletion of *S. cerevisiae DCN1* in yeast carrying the temperature-sensitive *cdc53-1* allele resulted in lethality at lower temperatures compared with *cdc53-1* cells with wild-type *DCN1* (Fig. 3c); neddylated Cdc53p was barely detectable in *dcn1Δ*

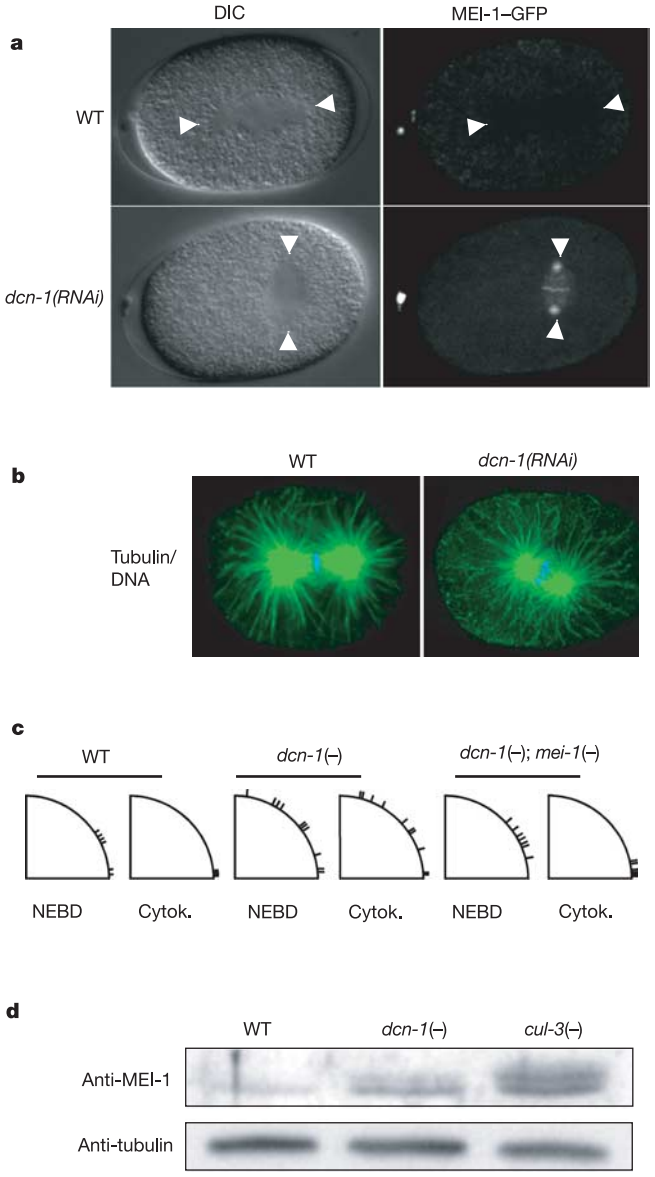

Figure 1 | *dcn-1(RNAi)* embryos stabilize MEI-1 protein. **a**, Nomarski and MEI-1–GFP micrographs of wild-type (top row) and *dcn-1(RNAi)* (bottom row) embryos during the first cell division. Loss of DCN-1 results in spindle orientation defects (arrowheads) and ectopic MEI-1 localization to the centrosomes and the mitotic apparatus (bottom-right panel). **b**, Confocal micrographs of α-tubulin and DNA in one-cell embryos fixed for indirect immunofluorescence. Compared with wild-type spindles, *dcn-1(RNAi)* spindles are shorter, mis-oriented and many astral microtubules terminate before reaching the cortex. **c**, Spindle orientation in multiple wild-type, *dcn-1(RNAi)* and *dcn-1(RNAi); mei-1(RNAi)* embryos, at nuclear envelope breakdown (NEBD) and during cytokinesis (Cytok.). Alignment along the anterior–posterior axis corresponds to 0°. Each black bar represents one embryo. Simultaneous reduction of MEI-1 function restores a wild-type spindle orientation in *dcn-1(RNAi)* embryos. **d**, MEI-1 levels in extracts prepared from wild-type, *dcn-1(RNAi)* and *cul-3(RNAi)* embryos.

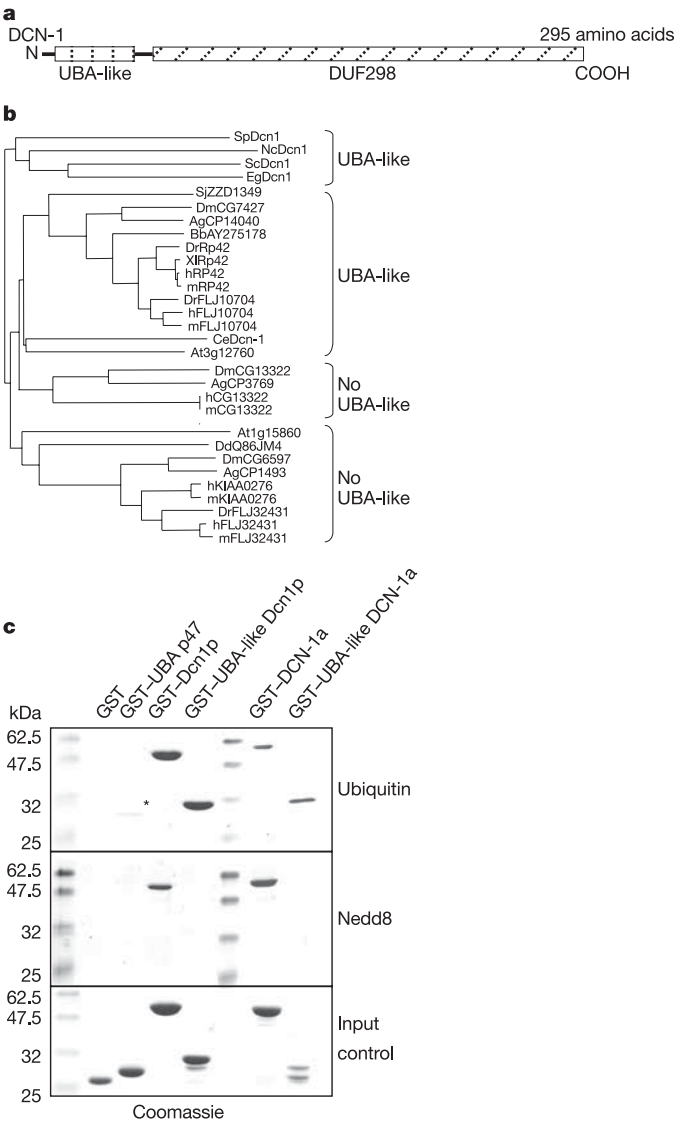

Figure 2 | DCN-1 and Dcn1p are evolutionarily and functionally conserved and bind ubiquitin and Nedd8. **a**, *C. elegans* DCN-1 is characterized by a C-terminal DUF298 domain of unknown function (PF03556) and a novel N-terminal UBA-like domain. **b**, Neighbour-joining tree of representative DUF298 domains. Only two of the major subgroups are found associated with UBA-like domains. Ag, *Anopheles gambiae*; At, *Arabidopsis thaliana*; Bb, *Branchiostoma belcheri*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Eg, *Eremothecium gossypyii*; h, *Homo sapiens*; m, *Mus musculus*; Nc, *Neurospora crassa*; Sc, *S. cerevisiae*; Sj, *Schistosoma japonicum*; Sp, *S. pombe*; Xl, *Xenopus laevis*. **c**, DCN-1a and Dcn1p directly bind to ubiquitin and Nedd8. A tenfold molar excess of full-length and the indicated domains of DCN-1 and Dcn1p were expressed as GST fusion proteins, and assayed for direct binding to immobilized ubiquitin or Nedd8. The known ubiquitin-binding UBA domain of human p47 (asterisk) served as a positive control. The bottom panel shows the protein input (10% for ubiquitin-binding assay; 2.5% for Nedd8-binding assay).

cells (Fig. 3b). Thus, DCN-1 and Dcn1p function is required for cullin neddylation in two highly diverged species.

Because Dcn1p was found to physically interact with Cdc53p in a genome-wide two-hybrid screen[18], we reasoned that Dcn1p might promote Nedd8 conjugation in part by binding to the cullin. We first verified the interaction of Dcn1p with the CUL1 homologue Cdc53p using a LexA-based yeast two-hybrid system (Fig. 3d). Notably, this interaction is conserved, because endogenous *C. elegans* CUL-3 in nematode extracts associated with glutathione *S*-transferase (GST)–DCN-1, but not with control GST, in pull-down experiments (Fig. 3e). Moreover, the interaction of DCN-1 and Dcn1p with two different types of cullins indicates that they may act on other cullin subclasses. Indeed, the neddylation state of the yeast cullin Rtt101p is also affected by the deletion of *DNC1* (Supplementary Fig. S4), and a large-scale yeast two-hybrid analysis in *Drosophila melanogaster* revealed that the *Drosophila* DUF298 domain proteins CG7427 and CG13322 interact with *Drosophila* Cul5 and Cul4, respectively[19].

Loss of DCN-1/Dcn1p function could result in the accumulation of un-neddylated cullin in either of two ways: a defect in neddylation, or a failure to inhibit deneddylation by the COP9 signalosome. To distinguish between these two possibilities, we deleted both *DCN1* and the core subunit of the signalosome *RRI1* in budding yeast. If loss of Dcn1p promotes deneddylation, the double mutant should resemble an *rri1Δ* single mutant, and exclusively accumulate neddylated Cdc53p. In contrast, if Dcn1p is required for neddylation, the double mutant should resemble a *dcn1* single mutant, with reduced levels of neddylated Cdc53p. Notably, we detected both neddylated and un-neddylated Cdc53p in the *rri1Δ dcn1Δ* double mutant (Fig. 4a), implying that Dcn1p is not essential for the neddylation reaction in the absence of deneddylation activity, but increases its efficiency *in vivo*. Importantly, overexpression of Dcn1p promoted a shift to the neddylated form of Cdc53p (Fig. 4a) and also lowered the permissive temperature of *cdc53-1* temperature-sensitive mutants (Fig. 4b), comparable to the deletion of the deneddylase *RRI1* (ref. 20). Together, these observations suggest that Dcn1p may be a limiting factor that promotes cullin neddylation *in vivo*.

To confirm that the Nedd8 modification machinery indeed works less efficiently in cells for which *DCN1* has been deleted, we expressed haemagglutinin (HA)-tagged Cdc53p from the inducible *GAL* promoter in both *rri1Δ* and *rri1Δ dcn1Δ* mutants. After induction of HA-Cdc53p in galactose-containing medium for 1 h, its expression was shut off transcriptionally by the addition of glucose. We then examined the neddylation state of HA-Cdc53p over a period of 3 h after shut off. Unlike endogenous Cdc53p, overexpressed HA-tagged Cdc53p is not completely neddylated in *RRI1*-deleted cells (Fig. 4c). However, most of the protein is modified and the levels of neddylated Cdc53p did not change over time (Fig. 4c). In contrast, only a small amount of HA-Cdc53p was modified in *rri1Δ dcn1Δ* double mutants 1 h after induction of the protein. The amount of modified HA-Cdc53p slowly increased over time, but even at 3 h after the shut off, neddylated HA-Cdc53p was less abundant than in *rri1Δ* single mutants (Fig. 4c). Thus, we conclude that Dcn1p greatly promotes cullin neddylation *in vivo*.

We next tested whether Dcn1p can also promote cullin neddylation *in vitro*. Previous work had shown that neddylation can be achieved *in vitro* when the cullin is in a complex with Rbx1 (ref. 21), suggesting that Rbx1 might function as the ligase for Nedd8. As expected, a small fraction of Cdc53p was neddylated *in vitro* in the purified Cdc53p–Rbx1p complex in the presence of Nedd8, the E1

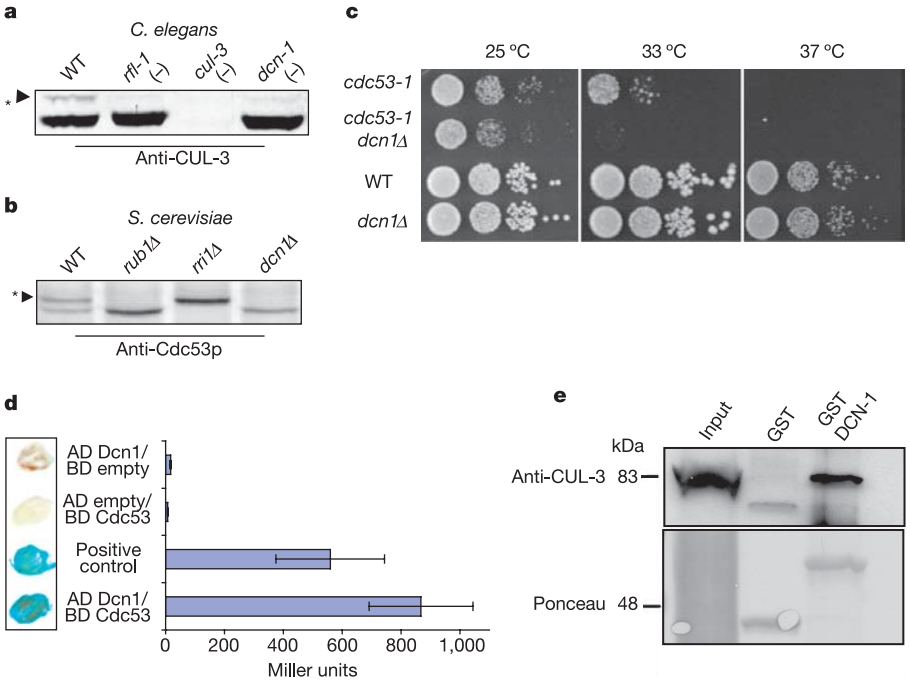

Figure 3 | DCN-1 and Dcn1p proteins are required for Nedd8 modification of cullins in yeast and nematodes, and physically associate with cullins. **a**, *C. elegans* extracts prepared from wild-type, Nedd8 E1 activating enzyme mutants *(rfl-1)*, *cul-3(RNAi)* and *dcn-1(RNAi)* mutant embryos were subjected to immunoblotting with anti-CUL-3 antibody. Nedd8-modified Cul-3 is marked with an asterisk. **b**, Extracts from wild-type, *rub1Δ*, *rri1Δ* and *dcn1Δ* yeast cells were subjected to immunoblotting with anti-Cdc53p antibody. Deletion of the deneddylase *RRI1* resulted in accumulation of Nedd8/Rub1p-modified Cdc53p (asterisk), whereas in *rub1Δ* and *dcn1Δ* cells only unmodified Cdc53p was detected. **c**, Deletion of *S. cerevisiae DCN1* lowers the restrictive temperature of *cdc53-1* conditional mutants. **d**, The interaction of Dcn1p (AD; activation domain) and Cdc53p (BD; binding domain) was determined by a two-hybrid assay by LacZ-reporter activity on filters (left) and quantitatively in liquid assays (right). Error bars indicate s.d. of at least three independent experiments. **e**, GST–DCN-1 and GST were expressed in *E. coli* and incubated with nematode extracts. Immunoblotting was performed using anti-CUL-3 antibody (top panel). The bottom panel shows Ponceau S staining of the same blot.

activating complex APP–BP1-UBA3 and the E2 UbcH12 (Fig. 4d). Importantly, the fraction of neddylated Cdc53p was significantly increased by the addition of yeast extract expressing GFP–Dcn1p, but to a much lower extent by addition of an extract prepared from *dcn1Δ* cells. Quantification of time course experiments revealed that the presence of GFP–Dcn1p reduced the $t_{1/2}$ of the reaction from 42 min to 2.4 min (Fig. 4e, f), showing that Dcn1p increases the kinetics of the neddylation reaction approximately 20-fold. Interestingly, purified Dcn1p expressed in *Escherichia coli* or SF9 cells was not sufficient to promote neddylation of Cdc53p (data not shown), implying that either a post-translational modification of Dcn1p or additional components in the extract may be involved. It is interesting to note in this regard that even addition of *dcn1Δ* extracts slightly increased the basal neddylation activity of the Cdc53p–Rbx1p complex (Fig. 4d).

Cullin neddylation and deneddylation are highly conserved processes that influence the activity of most SCF-type E3 ligases. The function of cullin neddylation remains elusive, but it has been proposed that the Nedd8 moiety initiates E3 ligase assembly[22,23] and increases the affinity of the E2 to the ligase[24]. As with ubiquitination, neddylation of cullins requires an E1 activating and E2 conjugating enzyme; an E3 ligase for Nedd8 conjugation has not been identified, although *in vitro* reconstitution assays suggested that Rbx1 may perform this function[21]. Our results suggest that DCN-1 and Dcn1p may function either as a regulator or part of a Nedd8 E3 ligase. This conclusion is consistent with our finding that the *in vivo* and *in vitro* kinetics of the neddylation reaction are significantly increased by the presence of Dcn1p. Furthermore, overexpression of GFP–Dcn1p in *S. cerevisiae* converts the cullin Cdc53p into its Nedd8-modified form. Finally, DCN-1 and Dcn1p physically associate with cullins in both *C. elegans* and *S. cerevisiae*, and directly bind to Nedd8. However, we do not believe that Dcn1p acts as a Nedd8 E3 ligase by itself, because bacterially or baculovirus-expressed purified Dcn1p was not able to enhance the kinetics of the neddylation reaction *in vitro*. Moreover, *dcn1Δ* extracts mildly stimulated Cdc53p neddylation *in vitro*, further suggesting the presence of additional positive regulators of neddylation.

Although the exact molecular mechanism of DCN-1/Dcn1p function remains unclear, it is likely that direct Nedd8 binding to DCN-1 and Dcn1p has an important role. The Nedd8 E2 Ubc12 has been shown to associate with Rbx1 (ref. 25), and the addition of Rbx1 is necessary to reconstitute neddylation activity *in vitro*[21]. It is possible that DCN-1/Dcn1p and Rbx1 function together to recruit the Nedd8-charged E2 Ubc12 to the cullin and thus facilitate neddylation. Additionally, it is important to emphasize that DCN-1 and Dcn1p contain a strong UBA-like ubiquitin-binding domain at their N termini. How ubiquitin binding may contribute to efficient neddylation is not immediately apparent, and it is tempting to speculate that the ubiquitin- and Nedd8-binding properties of DCN-1 and Dcn1p are required to execute two independent functions.

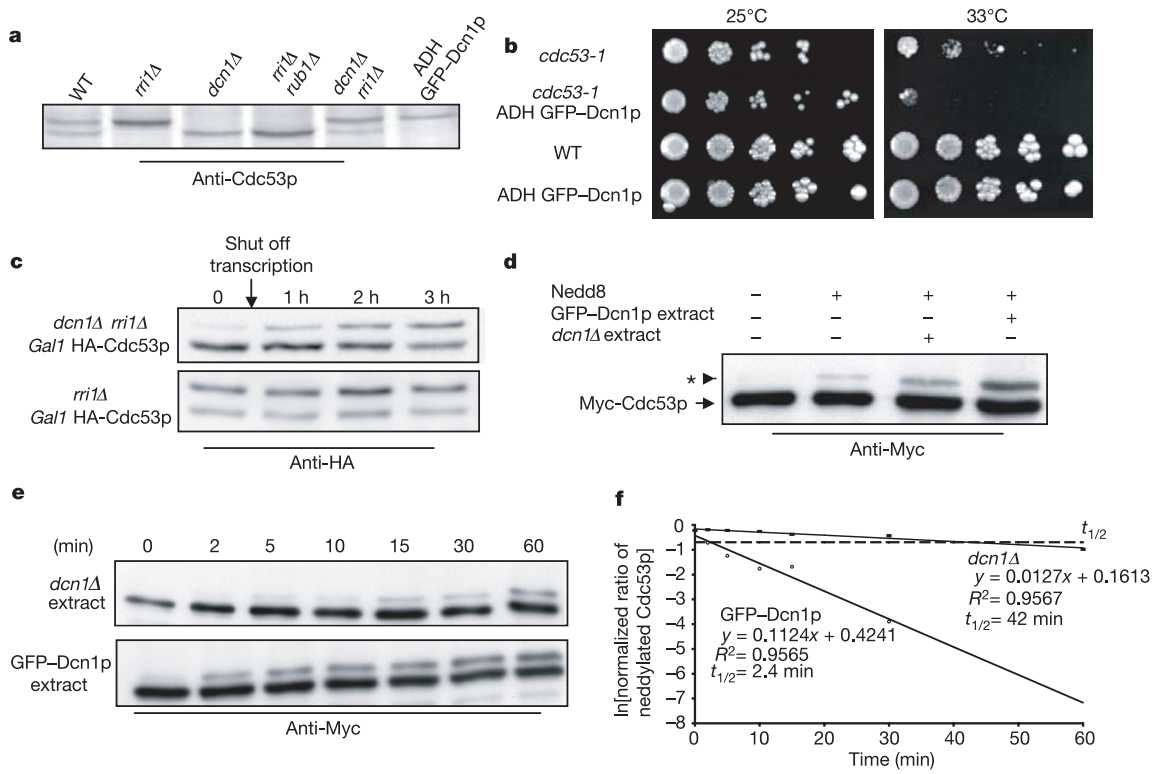

Figure 4 | DCN-1 and Dcn1p catalyse cullin neddylation *in vivo* and *in vitro*. **a**, **b**, The neddylation state of Cdc53p was determined by a western blot of yeast cell extracts prepared from *rri1Δ dcn1Δ* and *rub1Δ rri1Δ* cells. Constitutive *ADH* overexpression of GFP–Dcn1p results in accumulation of Nedd8-modified Cdc53p (**a**), and lowers the permissive temperature of *cdc53-1* temperature-sensitive mutants (**b**). **c**, The kinetics of Cdc53p neddylation *in vivo* was compared in *dcn1Δ rri1Δ* double (top panel) and *rri1Δ* single mutants (bottom panel) by inducing expression of HA-tagged Cdc53p from the *GAL* promoter. The transcription of HA-Cdc53p was shut off 1 h after induction, and the neddylation state of Cdc53p was followed for 3 h after shut off. **d**, Dcn1p promotes the neddylation of Cdc53p *in vitro*. Purified Myc-Cdc53p–Rbx1p complex was used as substrate for a 15 min *in vitro* neddylation reaction containing Nedd8, yeast extract, Nedd8 E1, Nedd8 E2 and ATP. The asterisk marks the neddylated form of Cdc53p. **e**, **f**, *In vitro* neddylation in the presence of *dcn1Δ* or GFP–Dcn1p extract over time. Reactions were started by the simultaneous addition of Nedd8 and yeast extract, and samples were taken at the time points indicated. The fraction of neddylated Myc-Cdc53p at each time point was quantified and plotted as a function of the neddylation state against time. The fraction of neddylated cullin was determined as $-\ln([\text{Nedd–Myc-Cdc53p}]_\infty - [\text{Nedd–Myc-Cdc53p}]_t)$, whereas the 60-min time point for GFP–Dcn1p was chosen as $[\text{Nedd–Myc-Cdc53p}]_\infty$. The different concentrations for $[\text{Nedd–Myc-Cdc53p}]_t$ were normalized to total [Myc-Cdc53p] in each reaction.

METHODS

Strains and manipulations. *C. elegans* Bristol strain N2 was used as wild type. Injection RNAi was performed as described previously[8] or by feeding nematodes *E. coli* expressing RNA from L4440-derived feeding vectors. To identify new genes involved in MEI-1 degradation, movies deposited in databases from large-scale RNAi screens (http://www.wormbase.org) were visually screened for defects resembling *cul-3* loss of function and inactivated by RNAi in GFP–MEI-1-expressing nematodes.

Yeast strains are listed in Supplementary Table 1. Standard yeast growth conditions and genetic manipulations were used[26]. To test for an enhancement of *cdc53-1* temperature sensitivity, the strains indicated in Figs 2c and 4b were grown to mid-log phase at permissive temperature, and subsequently fivefold serial dilutions of an equal number of cells were spotted onto YPD medium and grown at 25 °C, 33 °C and 37 °C for 2–3 days.

Alignments and sequence analysis. Database and Blast searches were carried out at NCBI (http://www.ncbi.nlm.nih.gov), SGD (http://www.yeastgenome.org) and Wormbase (http://www.wormbase.org). Multiple alignments and general sequence analysis was performed using T-coffee, Boxshade 3.2 and MacVector software. Generalized profile construction and searches were run locally using the *pftools* package, version 2.1. (program available from ftp://ftp.isrec.isb-sib.ch/sib-isrec/pftools/). Profiles were constructed using the BLOSUM45 substitution matrix and default penalties of 2.1 for gap opening and 0.2 for gap extension[20]. A significance threshold of $P < 0.01$, derived from database shuffling analysis, was used as an acceptance criterion for iterative profile refinement[27].

Protein extracts, antibodies and immunoblotting. *C. elegans* embryonic extracts and yeast protein extracts were prepared as described previously[7,28]. The following antibodies were used in this study: anti-MEI-1, anti-CUL-3, anti-DCN-1, anti-α-tubulin (Sigma clone DM1α), anti-HA (Babco) and anti-Cdc53p (Santa Cruz; yN-18). Secondary antibodies conjugated to horseradish peroxidase were purchased from BioRad and used according to the manufacturers specifications.

Polyclonal antibodies to *C. elegans* DCN-1 were generated by injecting purified GST-fused full-length protein into rabbits. For affinity purifications, GST fusions were cleaved using Precision protease (Amersham Pharmacia Biotech), DCN-1 was coupled to HiTrap-NHS columns (Amersham Pharmacia Biotech) and rabbit sera were affinity purified against coupled columns.

***In vivo* and *in vitro* Cdc53p neddylation.** Full-length HA-Cdc53p was cloned under the inducible *GAL1,10* promoter and transformed into *rri1Δ* (YTK46) and *rri1Δ dcn1Δ* (YTK43) yeast. The cells were grown in 2% raffinose to log phase, when HA-Cdc53p expression was induced by the addition of 2% galactose. After 1 h, HA-Cdc53p expression was shut off by addition of 2% glucose, and samples were taken until 3 h after shut off.

For *in vitro* neddylation, the substrate (Myc3-Cdc53–6 × His-Rbx1 complex) immobilized on Ni-NTA beads was added to the reaction buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 2 mM ATP, 50 μM dithiothreitol (DTT)) containing human Nedd8 E1 (Boston Biochem) and human Nedd8 E2 (Boston Biochem). The reaction was started by the addition of human 6 × His-Nedd8 (Boston Biochem) or a mixture of 6 × His-Nedd8 and yeast extract and incubated at 30 °C. The reaction was stopped at the appropriate time by addition of SDS sample buffer. Neddylated Cdc53p was detected by western blotting using anti-Myc antibodies (Gramsch Laboratories). High concentration yeast spheroblast extract was essentially prepared as described previously[29].

Ubiquitin- and Nedd8-binding assays. The respective complementary DNAs were cloned into pGEX4T (Amersham) and expressed in BL21 D3 Star cells (Invitrogen). All proteins were purified to homogeneity and concentrated to 1 mM in storage buffer (50 mM KCl, 25 mM Tris pH 7.4 and 5% glycerol). Binding experiments were carried out at 4 °C in 1 ml of binding buffer. Binding buffer contained 150 mM KCl, 50 mM Tris pH 8.0, 2 mM DTT, 5% glycerol and 0.1% Triton X-100 for the *S. cerevisiae* proteins and 150 mM KCl, 50 mM Tris pH 7.4, 2 mM DTT, 5% glycerol and 0.1% Triton X-100 for the *C. elegans* proteins. For one reaction, 5 μl (50 μg) of agarose-immobilized ubiquitin or Nedd8 (Boston Biochem) were extensively washed with binding buffer, incubated with 50 μl (50 nmol) of each protein for 3 h at 4 °C on a wheel, subsequently washed four times for 15 min with 1 ml binding buffer, and eluted with SDS buffer. Bound proteins were detected on a 10% SDS gel by Coomassie staining.

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