DCAF26, an Adaptor Protein of Cul4-Based E3, Is Essential for DNA Methylation in Neurospora crassa

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Abstract

DNA methylation is involved in gene silencing and genome stability in organisms from fungi to mammals. Genetic studies in Neurospora crassa previously showed that the CUL4-DDB1 E3 ubiquitin ligase regulates DNA methylation via histone H3K9 trimethylation. However, the substrate-specific adaptors of this ligase that are involved in the process were not known. Here, we show that, among the 16 DDB1- and Cul4-associated factors (DCAFs) encoded in the N. crassa genome, three interacted strongly with CUL4-DDB1 complexes. DNA methylation analyses of dcaf knockout mutants revealed that dcaf26 was required for all of the DNA methylation that we observed. In addition, histone H3K9 trimethylation was also eliminated in dcaf26 mutants. Based on the finding that DCAF26 associates with DDB1 and the histone methyltransferase DIM-5, we propose that DCAF26 protein is the major adaptor subunit of the CUL4-DDB1-DCAF26 complex, which recruits DIM-5 to DNA regions to initiate H3K9 trimethylation and DNA methylation in N. crassa.

Introduction

The CUL4-DDB1 complex, a major class of cullin-RING ubiquitin ligases (CRLs), is evolutionarily conserved from yeasts to humans. Previous studies have indicated that Cul4-DDB1-regulated ubiquitination is linked to multiple processes, such as cell cycle regulation, DNA replication licensing, DNA repair, and gene expression processes [1–3]. In the CRLs, cullin associates with substrates via adaptor molecules in the N terminus, and interacts with the E2 enzyme via the RING finger protein Hrt1/ROC1/ Rbx1 in the C terminus [4]. Substrate-specific adaptors, such as the F-box-containing proteins in SCF complexes and the BTB domain-containing proteins in Cul3-based ubiquitin ligases, determine substrate-specific ubiquitination in many biological processes [5]. Although the different structural states of DDB1 may allow it to directly recruit substrates to the Cul4-based E3 platform, studies have demonstrated that ubiquitination of several characterized CUL4-DDB1 substrates requires additional substrate-specific adaptors [6,7].

Recent studies showed that a class of adaptors called DCAFs (DDB1- and Cul4-associated factors) [4] are employed by Cul4-based E3 ligases to identify specific proteins for ubiquitination. Most DCAFs are WD40-containing proteins with relatively conserved “WDXR” motifs that interact with DDB1 protein. However, several DCAF proteins lacking this conserved motif are still able to bind DDB1 in vivo [3,4,8,9].

Among the well-characterized DCAF proteins, mammalian WDR5 and RBBP5 are essential components of the histone methyltransferase complex that methylates histone H3 on lysine 4 (H3K4) [10–12]. Further studies showed that Cul4-DDB1 can interact with WDR5 and RBBP5 and regulate histone H3K4 methylation. Down regulation of each of these genes by siRNA severely reduces the tri- and monomethylation of histone H3K4, but not H3K4 dimethylation [13]. Interestingly, inactivation of Cul4 or DDB1 also causes a significant inhibition of histone H3K9 and H3K27 trimethylation [13]. However, none of these DCAFs were shown to be involved directly in DNA methylation in eukaryotes.

In fission yeast, the Cul4-Rik1 E3 ubiquitin ligase associates with the histone methyltransferase Clr4 on heterochromatin regions to methylate histone H3K9, contributing to heterochromatin assembly and maintenance [14]. The catalytic activity of Cul4 is required for its proper function in heterochromatin formation. This study suggests that the activity of Cul4-based E3 ligase is required for histone H3K9 methylation. In addition, a WD-40-containing protein, Raf1/Dos1/Clr8/Cmc1, is required for histone H3K9 methylation and heterochromatin formation in S. pombe [15–18]. Thus, these studies imply that Raf1/Dos1/Clr8/Cmc1 functions as an adaptor protein associated with Cul4-Rik1 complex in S. pombe.

We previously demonstrated that Cul4-DDB1 E3 ligase is essential for DNA methylation in N. crassa by regulating histone H3K9 trimethylation [19]. These results suggest that Cul4-DDB1 ubiquitin ligase is required for epigenetic control in higher eukaryotes. However, the substrate-specific adaptors of Cul4-DDB1 E3 ligase and the requirement of DCAFs as the substrate adaptors in DNA methylation are unknown in N. crassa.
Author Summary

DNA associates with histones to form chromatin in eukaryotes. Epigenetics refers to DNA and histone modifications in chromatin that persist from one cell generation to the next, controlling gene expression and genome stability. These epigenetic changes are crucial for the development and differentiation of the various cell types in eukaryotes. In this study, we identified DCAF26 as a crucial regulator of DNA methylation. Inactivation of this gene in N. crassa resulted in loss of both DNA methylation and histone H3K9 trimethylation. We found that the resulting severe defects in the development and growth of dcaf26 mutants were similar to those found in cul4, ddb1, and dim-5 mutants, suggesting that these four genes function in the same pathway. Furthermore, we showed that DCAF26 functioned as an adapter protein for the Cullin4-DDB1 complex to recruit the histone methyltransferase DIM-5 and regulate trimethylation of histone H3K9, which marks DNA for methylation. Our results reveal important roles for DCAF26 in H3K9 trimethylation and DNA methylation in N. crassa and suggest a conserved mechanism for DNA methylation in eukaryotic organisms.

Here, we identified three DCAFs that could strongly interact with DDB1 protein in vivo, out of 16 candidates in the N. crassa genome. DNA methylation analysis in knockout strains of the 12 dcaf genes showed that dcaf26 was essential for DNA methylation in N. crassa. The dcaf26 deletion mutant also lost histone H3K9 trimethylation. Our protein interaction results suggest that DCAF26 functions as an adaptor subunit of the Cul4-DDB1-DCAF complex by recruiting histone methyltransferase DIM-5 to DNA methylation regions in N. crassa. In addition, we show that the interaction of DCAF26 with DDB1 was required to enable the Cul4-DDB1-DCAF26-DIM-5 complex to regulate H3K9 trimethylation and DNA methylation in this organism.

Results

Comparative analysis of N. crassa DCAF proteins

We searched the N. crassa genome for WD-40-containing proteins with WDXR and DxR motifs (also known as a DWD motif). The conserved 16 amino acid motif (IFV[2]-[IFV][2]-[AGST][AGST][x][DE][x][2]-[IFV][2]-[x][IFV][2]-[AT][DE][AT][DE][RK][20] was used as the seed to search for WD-40-containing proteins. Five putative DCAFs met the criteria (Table 1). Based on the sequences of hundreds of DCAF proteins from yeast to human that have been identified by protein interaction experiments and bioinformatics analysis, we identified another 11 putative DCAF proteins (Table 1). In total, we identified 16 putative DCAFs in the N. crassa genome (Figure 1A).

To identify the true DCAFs in N. crassa, we examined the interactions between DDB1 and the predicted DCAF proteins. We first made constructs in which the DCAF ORF was under the control of a quinic acid (QA)-inducible promoter [21]. To facilitate the detection of DCAF expression, five copies of the c-Myc epitope and six histidine residues [22] were inserted into the N terminus of the DCAF ORF. The constructs were then transformed into a wild-type strain and Myc-DCAF expression in the resulting transformants in the presence of QA was confirmed by western blot analysis using the c-Myc antibody. Afterwards, immunoprecipitation assays were used to examine the interactions between Myc-DCAF proteins and DDB1 in the transformants. As shown in Figure 1B, three Myc-tagged DCAFs, DCAF11, DCAF2, and DCAF26, interacted strongly with the DDB1 protein in vivo. Our DDB1 antibody depleted with tissue of the ddb1KO strain specifically recognized a 129-kDa band in the wild-type strain, but not in the ddb1 mutant (Figure 1B). DCAF11 and DCAF2 had two conserved WDXR motifs, while DCAF26 had only one conserved WDXR motif (WNVR). In contrast, only weak interactions were detected between DDB1 and the other Myc-DCAFs (Figure S2). These results suggest that DCAF11, DCAF2, and/or DCAF26 could be the adaptor(s) in the Cul4-DDB1 E3 ligase complex in N. crassa.

Knockout of N. crassa dcaf genes

Our previous study showed that the Cul4-DDB1 E3 complex is required for DNA methylation in N. crassa [19]. To investigate the function of the putative DCAFs in DNA methylation, we tried to generate deletion strains of the 16 candidate dcaf genes by gene replacement in the ku70RIP strain. However, we could not obtain the homokaryotic deletion strains of four genes (NCU01595, NCU02759, NCU05426, and NCU09521), suggesting that they are important for cell viability in N. crassa. Several independent homokaryotic knockout strains of the other 12 dcaf genes were obtained by microconidia purification. PCR analysis confirmed the integration of the knockout cassette at the endogenous dcaf locus, and no dcaf ORF signals were detected in these knockout mutants.

DCAF26 is essential for DNA methylation

To identify the DCAF protein(s) required for DNA methylation, we measured the cytosine methylation states of all homokaryotic dcaf mutants. Genomic DNA of the wild-type strain and ku70RIP, dim-2KO, and dcaf mutants was digested with DpnII or BstUI (Sau3AI). Undigested and digested DNA was then used as templates for PCR. Three representative methylated regions, the ku70RIP locus, ζ-η, and ψ63, were examined in the wild-type strain, ku70RIP, dim-2KO, and dcaf mutants. As shown in Figure 2, no PCR products were detected with DpnII- or BstUI-digested genomic DNA as templates in the dcaf26KO mutant, the same as for the dim-2KO mutant. To further confirm these results, we tested the known methylation region on centromere VII of N. crassa. As expected, methylation on this DNA region was also lost in the dcaf26KO mutant (Figure 2), indicating that DCAF26 plays an essential role in DNA methylation. In contrast, the other 11 homokaryotic dcaf mutants exhibited normal cytosine methylation patterns at ζ-η and ψ63 regions, the same as those in the wild-type strain (Figure S3). This demonstrated that they were not essential for DIM-2-dependent DNA methylation (Table 1). Taken together, these results suggest that DCAF26 was a key regulator of DNA methylation in N. crassa.

DCAF26 is required for H3K9 trimethylation

In N. crassa, proper DNA methylation depends on histone H3K9 trimethylation, which is controlled by the histone methyltransferase DIM-5 [23,24]. When we compared the growth and developmental phenotypes of the dcaf knockout strains to those of the wild-type strain and cul4KO, ddb1KO, and dim-5KO mutants, we found that the dcaf26 mutant exhibited dense, cauliflower-like growth patterns with abnormal hyphae and asexual spores on plates (Figure 3A). This was similar to the phenotypes of the cul4, ddb1, and dim-5 deletion strains [19] (Figure 3A). The dcaf26KO mutant also exhibited slow growth rates on racetubef compared to that of the wild-type strain (Figure 3B); these rates were similar to those of the cul4KO, ddb1KO, and dim-3KO strains. These results suggest that DCAF26 affected DNA methylation by regulating
H3K9 trimethylation in the same pathway as the Cul4-DDB1 complex. Therefore, we next examined H3K9 trimethylation levels by chromatin immunoprecipitation (ChIP) in the wild-type strain, the *ka7*KO strain, and the *dcaf26* mutant. Chromatin samples were immunoprecipitated with antibody against trimethylated Lys9 of histone H3 and analyzed by PCR with primers targeted to methylated DNA regions. As shown in Figure 3C, trimethylated H3K9 was associated with the methylated *ka7*KO region, whereas trimethylated H3K9 in the *ka7*KO region was abolished in the *dcaf26* mutant. Similarly, trimethylated H3K9 at the ζ-1 and ζ-3 regions in the *ka7*KO strain was observed at levels comparable to the wild-type strain (Figure 3C). In contrast, H3K9 trimethylation was lost in the *dcaf26* mutant (Figure 3C). Takeda et al. also showed that the upper band in the input and the Myc-DCAF26-interacting Cul4 band were neddylated Cul4 (Figure 4A), similar to the association between DIM-5 and neddylated Cul4 species in *N. crassa* [19]. We then examined the interactions between DCAF26 and DIM-5. As expected, the Flag antibody pulled down the Myc-tagged DCAF26 in the strain that coexpresses Flag-DIM-5 and Myc-DCAF26 (Figure 4B), indicating that DCAF26 was a component of the Cul4-DDB1-DIM-5 complex. These data explain the similar phenotypes in *N. crassa* [19].

### Table 1. Putative DCAFs in *Neurospora crassa.*

| DCAFs | NCU Number | Gene name in *Neurospora* database | Homolog | WDXR motif | Required for DIM-2-dependent DNA methylation |
|-------|------------|----------------------------------|---------|------------|--------------------------------------------|
| DWD motif | DCAF20 | 00202.3 | coronin-6 | Coronin | 1 | × |
| DCAF21 | 01389.3 | mitogen-activated protein kinase organizer 1 | MAPK01 | 1 | × |
| DCAF22 | 06483.3 | F-box and WD repeat-containing protein | FBW7 | 0 | × |
| DCAF23 | 06679.3 | histone acetyltransferase type B subunit 2 | CAF1C | 1 | × |
| DCAF24 | 07724.3 | mitochondrial division protein 1 | MDV1 | 2 | × |
| homologue search | DCAF25 | 00794.3 | ribosome biogenesis protein Rsa4 | NLE1/Notchless | 0 | × |
| DCAF13 | 01595.3 | SOF1 | DCAF13/WDOSO1 | 0 | Not tested |
| DCAF26 | 01656.3 | conserved hypothetical protein | Rsf1/Crb/Dos1/Cmc1 | 1 | √ |
| DCAF11 | 02151.3 | WD repeat containing protein 23 | DCAF11/WDR23 | 2 | × |
| DCAF27 | 02729.3 | transducin family protein | PWP1 | 0 | Not tested |
| DCAF28 | 03244.3 | WD repeat protein | WDR5 | 1 | × |
| DCAF2 | 03668.3 | WD domain-containing protein | DCAF2/CDT2 | 2 | × |
| DCAF29 | 04534.3 | nuclear migration protein nudF | Nudf/LIS1 | 1 | × |
| DCAF30 | 05426.3 | WD repeat protein | WDR39/CIAO1 | 0 | Not tested |
| DCAF31 | 05797.3 | US snRNP complex subunit | WDR57 | 3 | × |
| DCAF32 | 09521.3 | ribosome biogenesis protein | GRWD1/RRB1 | 2 | Not tested |

DCAF26 is the key component for recruiting DIM-5 to the Cul4-DDB1 complex. Having identified DCAF26 as a DCAF protein that interacted strongly with DDB1 protein, we next tested whether DCAF26 was a key component in the Cul4-DDB1-DIM-5 complex for DNA and histone H3K9 methylation. We did so by performing an immunoprecipitation assay to detect interactions between Myc-DCAF26 and Flag-Cul4 and between Myc-DCAF26 and Flag-DIM-5. As shown in Figure 4A, Myc-tagged DCAF26 specifically interacted with one of two Flag-Cul4 species (neddylated/unneddylated Cul4). To determine whether DCAF26 preferentially interacts with neddylation or unneddylated Cul4, we loaded protein extract from a *com 2*KO strain, in which Cul4 remained in a hyperneddylated state, side by side with Myc-DCAF26/Flag-Cul4 to show that the upper band in the input and the Myc-DCAF26-interacting Cul4 band were neddylation Cul4 (Figure 4A), similar to the association between DIM-5 and neddylation Cul4 species in *N. crassa* [19].

Next, we investigated the function of DCAF26 in this complex. We checked the interactions of DDB1-DIM-5 in the *dcaf26*KO strain and in a *dim-5*KO, qa-Myc-Cul4 transformant. As shown in Figure 4C, the interactions between DDB1 and DIM-5 were severely impaired in the *dcaf26*KO strain, while DIM-5 strongly interacted with DDB1 in the presence of DCAF26. This indicates that DCAF26 was required for recruiting DIM-5 to the Cul4-DDB1 complex. Furthermore, the interaction between DDB1 and Cul4 was not affected in the *dcaf26*KO mutant (Figure 4D), confirming that DCAF26 was an adapter protein in the Cul4-DDB1-DIM-5 complex.

Interactions between DCAF26 and DDB1 are essential for DNA and H3K9 methylation. The finding that *N. crassa* DCAF26 interacts with DDB1 and neddylation Cul4 to form a complex prompted us to investigate the functional importance of the interaction between DCAF26 and

![Image](https://example.com/image.jpg)
DDB1. As shown in Figure 5A, DCAF26 and Cul4 protein interactions were totally abolished in the ddb1KO mutant, suggesting that DDB1 served as a bridge between Cul4 and DCAF26 to form the complex. This result suggests that DDB1, DCAF26, and their interactions contributed the H3K9 and DNA methylation functions of the Cul4-DDB1-DCAF26-DIM-5 complex.

Figure 1. Putative DDB1- and Cul4-associated factors (DCAFs) in N. crassa. (A) Schematic representation of 16 putative DCAFs with predicted domains. (B) Interactions between Myc-tagged DCAFs and DDB1 protein. c-Myc antibody was used for immunoprecipitation, followed by western blot analysis using DDB1 or c-Myc antibodies. The wild-type strain and wild-type expressing Myc-tagged MCB (the regulatory subunit of cAMP-dependent protein kinase) strain were used as negative controls.

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DCAF26 (NCU01656.3) contains some interesting sequence features, including one WDXR motif (WNVR) and WD-40 repeat regions. When the DCAF26 protein sequence was queried in a blast search against protein databases, DCAF26 was found to be similar to various fungal homologs. Many DCAF26 homologs contain one WDXR motif and one WDTA, or another WDXR motif located separately between consecutive "propeller blade" folds of the protein (Figure 5B).

To determine which domains of DCAF26 are involved in the DCAF26-DDB1 interaction, we mutated arginine 976 to alanine in the WNVR motif (Figure 5C) in qa-Myc-His-DCAF26 construct. As shown in Figure 5D, this mutation of DCAF26 reduced binding with DDB1 compared to the binding between wild-type DCAF26 and DDB1 in dcaf26KO transformants. However, this DCAF26 mutation did not affect the interactions between DCAF26R(976)A and DIM-5 (Figure 5E). We then deleted 24 amino acids (960–983) from DCAF26; this amino acid stretch included the WNVR motif and the third WD-40 domain (Figure 5C). As shown in Figure 5D, the interaction of DCAF26dWD3 with DDB1 was totally abolished in dcaf26KO qa-Myc-DCAF26dWD3 transformants. However, this DCAF26 deletion did not affect interactions between DCAF26dWD3 and DIM-5 (Figure 5E). These results indicate that the WNVR motif and adjacent amino acids were important for interactions with DDB1, not interactions with DIM-5.

To further examine the function of the interaction between DCAF26 and DDB1, we investigated the function of these mutated DCAF26 in dcaf26KO transformants. As shown in Figure 6A, the expression of Myc-tagged wild-type DCAF26 fully rescued the growth and developmental defects of the dcaf26KO mutant, resulting in a similar growth rate as the wild-type strain on plates. Importantly, the DNA methylation (Figure 6B) and the H3K9 trimethylation (Figure 6C and 6D) in the dcaf26KO, qa-Myc-His-DCAF26 transformant were also restored. The expression of Myc-DCAF26R(976)A mutant protein can restore the growth and developmental phenotypes (Figure 6A) as well as DNA methylation (Figure 6B) and H3K9 trimethylation (Figure 6C and 6D) of dcaf26KO mutant. These results indicated that the weak interaction of DCAF26-DDB1 is sufficient for the formation of Cul4-DDB1-DCAF26-DIM-5 complex. In contrast, the expression of Myc-DCAF26dWD3 mutant protein failed to rescue the growth and developmental phenotypes (Figure 6A) and the defects of DNA methylation (Figure 6B) and H3K9 trimethylation (Figure 6C and 6D) of dcaf26KO mutant, indicating that the interaction of DCAF26-DDB1 was required for the proper function of Cul4-DDB1-DCAF26-DIM-5 complex. Taken together, these results further demonstrate that DCAF26 is a critical component in the Cul4-DDB1 ubiquitin ligase in N. crassa.

Purification of DCAF26 and identification of DCAF26-associated proteins

Recent studies demonstrate the biochemical function of the DCAF protein DDB2 as a substrate receptor for XPC.
ubiquitination in DDB1-DDB2-CUL4A-Rbx1 complex in human cell lines [25]. Since we have demonstrated that DCAF26 is required for recruiting DIM-5 to the Cul4-DDB1 complex, we wondered whether there are other substrates associated with the complex.

To better understand the substrate adaptor role of DCAF26 for the Cul4-DDB1 E3 ligase, we attempted to purify this protein in N. crassa. To do so, we expressed Myc-His-DCAF26 protein by inoculating the dcaf26KO qa-Myc-His-DCAF26 strain in liquid media containing quinic acid. Then Myc-His-DCAF26 protein was purified by nickel-column followed by immunoprecipitation using the c-Myc monoclonal antibody. As shown in Figure 7A, several major protein bands were specifically observed in the Myc-His-DCAF26 sample but not in the negative control (WT lane). The LC-MS/MS analysis of excised gel bands led to the identification of five well-known proteins, DCAF26, Cul4, DDB1, Nedd8, DIM-5 (the histone H3K9 methyltransferase in N. crassa) (Figure 7A and 7B). All proteins were represented with three or more peptides. The coexistence of Nedd8 peptides with Cul4 was consistent with previous results showing that DCAF26 preferentially interacted with neddylated Cul4 proteins in vivo, further confirming that DCAF26 is a key component of Cul4-DDB1 E3 ligase. In addition, we also identified other four DCAF26 co-purified proteins (Figure 7A and 7B) encoded by NCU07855, NCU04152, NCU06123, and NCU11350, respectively. Among these proteins, NCU04152 (DIM-7) is also required for DNA methylation and H3K9 trimethylation (Figure S4). Since the submission of this paper, similar results were recently shown by Lewis et al. [26]. Furthermore, the absence of ubiquitin peptides in the purification products suggests that either the ubiquitinated substrate is released immediately from Cul4-DDB1 complex or its ubiquitination level is too low to be detected.
Cul4-based E3 ubiquitin ligases are evolutionarily conserved multifunctional complexes in eukaryotes. In this study, using a genetic screen to find genes that encode proteins interacting with DDB1 and Cul4 in *N. crassa*, we identified DCAF26 as a new component required for DNA methylation. *dcaf26KO* mutants exhibited the loss of all known DNA methylation and H3K9 trimethylation. Our data demonstrate that DCAF26 interacts with DDB1, which in turn recruits DIM-5 via the Cul4-DDB1-DCAF26 complex to regulate H3K9 trimethylation and DNA methylation. DCAF26 is an essential component in the Cul4-DDB1 complex for recruiting DIM-5 to regulate H3K9 trimethylation.

Recent studies demonstrated that three proteins, DIM-5 [23], HP1 [27], and DIM-2 [28], are essential for DNA methylation in *N. crassa*. In this study, we identified DCAF26 as a new component required for DNA methylation.
N. crassa, which function in histone H3K9 trimethylation and DNA methylation. To understand the regulation of DNA methylation in N. crassa, we sought to identify the proteins operating upstream of DIM-5. Previously, we showed that DDB1 and Cul4 function in an early step of DNA methylation by forming a complex with DIM-5 to regulate H3K9 trimethylation in N. crassa [19]. To determine the precise function of this complex, we sought to identify the DDB1- and Cul4-associated factors (DCAFs) involved in this pathway. In mammalian cells, the DCAF protein WDR5 is a core component of the histone methylation complex essential for H3K4 methylation [12], and loss of WDR5 specifically affects tri- and monomethylated H3K4 [13]. However, there is limited evidence to suggest that histone H3 methylation is directed by DCAF protein, which recruits a specific histone methyltransferase. In fission yeast, a putative DCAF protein, Dos1/Raf1/Clr8/Cmc1 [15–18], interacts with Cul4, Rik1, and Clr4 (histone H3K9 methyltransferase) to regulate the heterochromatin formation. It was reported that Dos1, which is associated with Rik1 and important for the function of the Clr4-Rik1 complex, is essential for the recruitment of Clr4 in the RNAi-dependent heterochromatin pathway [17].

To find out how N. crassa DCAF proteins participate in histone H3K9 methylation and DNA methylation, we explored potential DCAF proteins that might interact with Cul4-DDB1 E3 ubiquitin ligase. We found that DCAF26 was associated with DDB1 and was essential for H3K9 trimethylation and DNA methylation. Protein interaction experiments revealed that, as with DIM-5 [19], DCAF26 preferentially interacted with neddylated Cul4 species. Interactions between DDB1 and DIM-5 were dependent on DCAF26, this indicates that DCAF26 served as a link between Cul4-DDB1 and DIM-5 in this pathway, thus regulating H3K9 trimethylation. Interestingly, in fission yeast, the loss of H3K9 methylation in dos1 deletion mutants is similar to that in rik1 and clr4 mutants, and more severe than in RNAi mutants [17], suggesting that Dos1 is required for histone H3 modification in the same pathway as Rik1 and Clr4.

Interaction between DDB1 and DCAF26 is essential for H3K9 trimethylation and DNA methylation

Immunoprecipitation experiments revealed that DCAF26 bridges the Cul4-DDB1 complex to DIM-5 and functions in the same DNA methylation pathway in N. crassa. If DCAF26 protein interacts with DDB1, Cul4, and DIM-5 to form a complex, we would expect the association of DCAF26-DDB1 to play an essential role in the function of the Cul4-DDB1-DCAF26-DIM-5 complex. Recent studies showed that many DCAF proteins contain two conserved WDXR motifs that are the key interacting modules with DDB1 in Cul4-DDB1 E3 ubiquitin ligases [3,4,15,29,30]. In this study, we showed that deletion of a region with a WDXR motif in DCAF26, but not the single-amino acid substitution of WNVR976A, eliminated DCAF26-DDB1 interactions and H3K9 trimethylation and DNA methylation in vivo, indicating that the interaction between DCAF26 and DDB1 is required for H3K9 trimethylation and DNA methylation. Although several studies showed that the arginines in conserved WDXR motifs in DCAF proteins are required for the association of DCAF proteins with ubiquitin ligases, these studies did not examine the functional importance of these interactions in vivo.

Figure 7. Identification of DCAF26-associated proteins. (A) Silver-stained SDS-PAGE showing the two-step purification of N. crassa DCAF26. Wild-type strain was used as the negative control. Asterisks indicate the two IgG bands. (B) Eight DCAF26-associated proteins were identified by LC-MS/MS.
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between DCAFs and DDB1, some DCAFs that lack the conserved motifs can still interact with DDB1 and Cul4 proteins [6,9]. Interestingly, S. pombe Dos1/Raf1/Cht8/Cmc1 contains two conserved WDXR motifs [4]; however, mutagenesis studies of these motifs to examine the interactions with Rik1 protein have not yet been performed. Alignment of these two motifs and adjacent regions in S. pombe Dos1 with the corresponding region in N. crassa showed that they share a high similarity, suggesting that this region is important for interactions with DDB1. Indeed, the results of our deletion and mutagenesis studies revealed that the WDXR-containing WD3 region, but not the arginine residue, was necessary for productive assembly with DDB1 into a functional H3K9 methyltransferase complex under physiological conditions. In addition, we found that the WDXR-containing WD3 region was not required for interactions between DCAFs and DIM-5, and confirmed that the association of DCAF26-DDB1 was necessary for productive assembly with DDB1 into a functional WDXR-containing WD3 region, but not the arginine residue, which was necessary for productive assembly with DDB1 into a functional H3K9 methyltransferase complex under physiological conditions. In addition, we found that the WDXR-containing WD3 region was not required for interactions between DCAFs and DIM-5, and confirmed that the association of DCAF26-DDB1 was essential for recruiting DIM-5 to regulate H3K9me3. Thus, the neddylation and DCAF26, and then recruits DIM-5 to specific DNA regions neddylated Cul4 promotes the complex formation with DDB1 and DCAF26, in which assembly of the Cul4-DDB1-DCAF26 complex, in which recruiting DIM-5 or to ubiquitinate a specific substrate(s) to the complex during H3K9 methylation and DNA methylation analysis. No enzyme was added to the control samples. The PCR primers

The substrate(s) of the Cul4-DDB1-DCAF26 ubiquitin ligase

Our results indicate that DCAF26 protein was necessary for recruiting DIM-5 into the Cul4-DDB1 complex. In addition, both DCAFs and DIM-5 preferentially interacted with neddylated Cul4 species. These data suggest that the Cul4-DDB1-DCAF26 complex is required to recruit DIM-5 or to ubiquitinate a specific substrate(s) to the complex during H3K9 methylation and DNA methylation analysis. In contrast, histones H2B and H4 were copurified with ubiquitin, Cmc1, and Cht4 in Rik1 purification products and were suggested as the likely targets of Cul4-mediated ubiquitination in fission yeast [15]. In mammalian cells, XPC was identified as a substrate directly targeted by the DCAF protein DDB2 to the complex containing Cul4-Rik1-Raf1-Raf2-Clr4 [16]. In mammalian cells, the putative targeting substrate of Cul4-DDB1-WDR5 is histone H3 [13]. In human cells, Cul4-DDB1-DCAF26 complex mainly recruits DCAF26 to regulate H3K9 trimethylation and DNA methylation. In contrast, histones H2B and H4 were copurified with ubiquitin, Cmc1, and Cht4 in Rik1 purification products and were suggested as the likely targets of Cul4-mediated ubiquitination in fission yeast [15]. In mammalian cells, XPC was identified as a substrate directly targeted by the DCAF protein DDB2 to the complex containing Cul4-Rik1-Raf1-Raf2-Clr4 [16]. In mammalian cells, the putative targeting substrate of Cul4-DDB1-WDR5 is histone H3 [13]. In human cells, Cul4-DDB1-DCAF26 complex mainly recruits DCAF26 to regulate H3K9 trimethylation and DNA methylation. In contrast, histones H2B and H4 were copurified with ubiquitin, Cmc1, and Cht4 in Rik1 purification products and were suggested as the likely targets of Cul4-mediated ubiquitination in fission yeast [15]. In mammalian cells, XPC was identified as a substrate directly targeted by the DCAF protein DDB2 to the complex containing Cul4-Rik1-Raf1-Raf2-Clr4 [16]. In mammalian cells, the putative targeting substrate of Cul4-DDB1-WDR5 is histone H3 [13]. In human cells, Cul4-DDB1-DCAF26 complex mainly recruits DCAF26 to regulate H3K9 trimethylation and DNA methylation. In contrast, histones H2B and H4 were copurified with ubiquitin, Cmc1, and Cht4 in Rik1 purification products and were suggested as the likely targets of Cul4-mediated ubiquitination in fission yeast [15]. In mammalian cells, XPC was identified as a substrate directly targeted by the DCAF protein DDB2 to the complex containing Cul4-Rik1-Raf1-Raf2-Clr4 [16]. In mammalian cells, the putative targeting substrate of Cul4-DDB1-WDR5 is histone H3 [13]. In human cells, Cul4-DDB1-DCAF26 complex mainly recruits DCAF26 to regulate H3K9 trimethylation and DNA methylation. In contrast, histones H2B and H4 were copurified with ubiquitin, Cmc1, and Cht4 in Rik1 purification products and were suggested as the likely targets of Cul4-mediated ubiquitination in fission yeast [15]. In mammalian cells, XPC was identified as a substrate directly targeted by the DCAF protein DDB2 to the complex containing Cul4-Rik1-Raf1-Raf2-Clr4 [16]. In mammalian cells, the putative targeting substrate of Cul4-DDB1-WDR5 is histone H3 [13]. In human cells, Cul4-DDB1-DCAF26 complex mainly recruits DCAF26 to regulate H3K9 trimethylation and DNA methylation. In contrast, histones H2B and H4 were copurified with ubiquitin, Cmc1, and Cht4 in Rik1 purification products and were suggested as the likely targets of Cul4-mediated ubiquitination in fission yeast [15]. In mammalian cells, XPC was identified as a substrate directly targeted by the DCAF protein DDB2 to the complex containing Cul4-Rik1-Raf1-Raf2-Cl...
for ku70, ζ-η, Ψ63, and cen VII regions are listed in Table 2. PCR was performed using 1 μL of the digested DNA as a template in a 50 μL reaction system, with a program of 5 min at 94°C, followed by 31 cycles at 94°C (30 sec), 53°C (30 sec), and 72°C (1 min). The PCR products were resolved by electrophoresis on 2% agarose gels. Each experiment was performed independently at least three times.

ChIP analysis

For the ChIP assay, tissues were fixed in minimal media containing 1% formaldehyde for 10 min at 25°C. ChIP was performed using 8 μL of antibody to H3K9me3 (07-442, Upstate Biotechnology) or 10 μL of antibody to H3 (06-755, Upstate Biotechnology) for 6 mg/ml protein. After washing with 70% ethanol, extracted DNA pellets were resuspended in 30 μL of double-distilled water, and 0.5 μL of the DNA solution was used for PCR. The primers for ku70, ζ-η, Ψ63, and hH4 regions are listed in Table 2. PCR conditions were as follows: 5 min at 94°C, 26–30 cycles at 94°C (30 sec), 53°C (30 sec), and 72°C (1 min). Different PCR cycles were tested to ensure that DNA amplification was within the exponential amplification range. PCR products were resolved by electrophoresis on 2% agarose gels. ChIP assays with anti-Myc antibody or no antibody were used as negative controls. Each experiment was independently performed at least three times.

Western blot analysis of histone H3 and trimethylated H3K9

N. crassa histone proteins were extracted from the wild-type strain, dcaf26KO, ddb1KO, cul4KO, and dim-5KO strains, as described previously [34]. Equal amounts of histone protein extracts were loaded onto 15% SDS-PAGE gels. Western blot analysis was performed using antibodies against trimethylated H3 Lys9 (07-442 Upstate Biotechnology) or H3 (06-755 Upstate Biotechnology). The PCR products were resolved by electrophoresis on 2% agarose gels. Western blot analysis was performed using antibodies against trimethylated H3 Lys9 (07-442 Upstate Biotechnology) or H3 (06-755 Upstate Biotechnology). The PCR products were resolved by electrophoresis on 2% agarose gels. Western blot analysis was performed using antibodies against trimethylated H3 Lys9 (07-442 Upstate Biotechnology) or H3 (06-755 Upstate Biotechnology).

Table 2. List of primers used in this study.

| Target | Location | Range on Contig | Length | Primers | Sequence (5' to 3') | Purpose |
|--------|----------|-----------------|--------|---------|--------------------|---------|
| ku70   | Chromosome IV Contig 53 | 147012–147629 | 618 bp | ku70.1F | GAAGAATTGAGAGAAGACAGGG | DNA methylation H3K9me3 ChIP |
|        |          |                 |        | ku70.2R | TGGGAGATAATTCCGTCGTC |         |
|        |          |                 |        | ku70.3F | ATGATTCCGGCATTGGCA | DNA methylation H3K9me3 ChIP |
|        |          |                 |        | ku70.4R | GACATATTGCTCTGCTAGG |         |
|        |          |                 |        | ku70.5F | GCGAAGCTCTCAAAGATGC | DNA methylation H3K9me3 ChIP |
|        |          |                 |        | ku70.6R | AAGTCTCTAACTAGCAGG |         |
| ζ-η    | Chromosome I Contig 9 | 945683–945860 | 178 bp | ζ-η.1F | ACACCTAGATTGCTGCTGTC | DNA methylation H3K9me3 ChIP |
|        |          |                 |        | ζ-η.2R | GTACGGATCTATCGGCTAC |         |
| Ψ63    | Chromosome IV Contig 49 | 26128–26691 | 564 bp | Ψ63.1F | ACATACGACCATACACCTACG | DNA methylation H3K9me3 ChIP |
|        |          |                 |        | Ψ63.2R | GGTCAGGGATATTTGGAGG |         |
| centromere | Chromosome VII Contig 55 | 144426–145009 | 584 bp | cenVELFF | CTAACCCTATGCGTCTACTTTC | DNA methylation H3K9me3 ChIP |
|        |          |                 |        | cenVELFR | CTACAGAAGCTCCTCAAAGATG |         |
| hH4    | Chromosome II Contig 5 | 428931–429351 | 421 bp | hH4.5 | AACCAGGAAACCGTAGAGGTA | DNA methylation H3K9me3 ChIP |

Supporting Information

Figure S1 Specificity of the DDB1 antibody. Western blot analysis of DDB1 protein in the wild-type and ddb1KO strains using anti-DDB1 serum (A) or DDB1 antibody after depletion using tissue of the ddb1KO strain (B) as the primary antibody. Asterisks indicate nonspecific bands detected by our DDB1 antibody. The wild-type strain was used as a negative control, followed by western blot analysis using the DDB1 and c-Myc antibodies. The wild-type strain was used as a negative control, followed by western blot analysis using the DDB1 and c-Myc antibodies. The wild-type strain was used as a negative control, followed by western blot analysis using the DDB1 and c-Myc antibodies. The wild-type strain was used as a negative control, followed by western blot analysis using the DDB1 and c-Myc antibodies.

Figure S2 Interactions between the other 11 DCAF11 and DDB1. c-Myc antibody was used for immunoprecipitation, followed by western blot analysis using the DDB1 and c-Myc antibodies. The wild-type strain was used as a negative control, followed by western blot analysis using the DDB1 and c-Myc antibodies. The wild-type strain was used as a negative control, followed by western blot analysis using the DDB1 and c-Myc antibodies. The wild-type strain was used as a negative control, followed by western blot analysis using the DDB1 and c-Myc antibodies.

Figure S3 DNA methylation analysis in other dcafKO strains. DNA methylation in the wild-type strain (WT) and ku70KO, dim-2KO, and dcafKO strains on (A) the ζ-η region and (B) the Ψ63 region.
detected by methylation-sensitive restriction digestion. The knockout mutants were in the \(^{60}kA76^{60}\) background. Genomic DNA digested by 5cm-sensitive BstCI (B) or its 5cm-insensitive isochizomer, DpnII (D), was amplified by PCR with the labeled primers. Untreated genomic DNA as template for PCR was used as the control (C) or CB.

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**Figure S4** DIM-7 required for H3K9 trimethylation and DNA methylation in *N. crassa*. (A) Dense, cauliflower-like growth pattern of *dim-7KO* strain (\(^{60}kA76^{60}\) background) on plate with minimal media (30°C, 32 hr). (B) DNA methylation in the wild-type strain (WT) and demethylation in the *dim-7* strain at the \(\phi 63\) region. (C) Loss of histone H3K9 trimethylation at \(\phi 63\) region in the *dim-7* strain. Levels of H3K9 trimethylation at the \(\phi 63\) region were determined by ChIP assay. Myc antibody was used as a negative control. H3 antibody was used as the positive control and as the control for integrity of the nucleosome structure. (D) Western blot analysis of global H3 and H3K9 trimethylation in the wild-type and *dim-7KO* strains.

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**Author Contributions**

Conceived and designed the experiments: H Xu, Q Hu, Q He. Performed the experiments: H Xu, J Wang, Y Quan, H Chen, Y Cao. Analyzed the data: H Xu, Q Hu, H Chen, C Li, Y Wang, Q He. Contributed reagents/materials/analysis tools: C Li. Wrote the paper: H Xu, Q He.

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