Recruitment of the inhibitor Cand1 to the cullin substrate adaptor site mediates interaction to the neddylation site

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ABSTRACT Cand1 inhibits cullin RING ubiquitin ligases by binding unneddylated cullins. The Cand1 N-terminus blocks the cullin neddylation site, whereas the C-terminus inhibits cullin adaptor interaction. These Cand1 binding sites can be separated into two functional polyptides which bind sequentially. C-terminal Cand1 can directly bind to unneddylated cullins in the nucleus without blocking the neddylation site. The smaller N-terminal Cand1 cannot bind to the cullin neddylation region without C-terminal Cand1. The separation of a single cand1 into two independent genes represents the in vivo situation of the fungus Aspergillus nidulans, where C-terminal Cand1 recruits smaller N-terminal Cand1 in the cytoplasm. Either deletion results in an identical developmental and secondary metabolism phenotype in fungi, which resembles csn mutants deficient in the COP9 signalosome (CSN) deneddylase. We propose a two-step Cand1 binding to unneddylated cullins which initiates at the adaptor binding site and subsequently blocks the neddylation site after CSN has left.

INTRODUCTION Cullins represent stalk-like eukaryotic scaffold proteins with three repeats of a five-helix bundle. They serve as a platform for the formation of various multisubunit complexes (Zheng et al., 2002b; Wu et al., 2003). The minimal set of a eukaryotic cell comprises the three cullins Cul1, Cul3, and Cul4; humans express seven cullins and additional proteins with a cullin homology domain (Pintard et al., 2004; Petroski and Deshaies, 2005). The cullin C-terminus forms a globular domain and stably interacts with the RING H2 finger protein Roc1/Rbx1/Hrt1 (Kamura et al., 1999; Seol et al., 1999; Tan et al., 1999), which attracts E2 ubiquitin-conjugating enzymes. The cullin RING ligases (CRLs) make up the largest group of E3 ubiquitin ligases. They contain a variable substrate recognition subunit (SRS) and in most cases an adaptor that links the SRS to the complex. The CRLs are the specificity factors for the covalent binding of ubiquitin to substrates. They regulate a wide range of dynamic cellular and developmental responses by triggering 26S proteasome-mediated protein degradation. The largest group of CRLs are Cul1-based Skp1-Cul1-F-box (SCF) protein complexes. The F-box proteins represent the large class of substrate recognition proteins, which are linked to Cul1 by the adaptor protein Skp1 (Feldman et al., 1997; Skowyra et al., 1997).

Activation of CRLs requires neddylation, which is the posttranslational covalent linkage of the ubiquitin-like protein Nedd8 to a conserved C-terminal lysine residue of the cullin (Pan et al., 2004). Neddylution opens the closed conformation of the compact globular domain formed by cullin’s C-terminus and the RING protein. This frees the RING domain from interactions with cullin and enables flexible positioning of the attached E2 for substrate ubiquitination (Duda et al., 2008). Neddylation-induced activation therefore increases the interaction surface between CRLs and charged E2 ubiquitin-conjugating enzyme (Kawakami et al., 2001; Sakata et al., 2007). CRL activity is inhibited in vitro by the COP9 signalosome (CSN), which is a deneddylase removing Nedd8 from CRLs (Cope et al., 2002; Cope and Deshaies, 2003).
Cullin-associated Ned8-dissociated protein 1 (Cand1) is another in vitro inhibitor of CRLs, which stably binds to unneddylated cullin-RING complexes and colocalizes with cullin mainly in the nucleus (Yogosawa et al., 1996; Zheng et al., 2002a; Oshikawa et al., 2003). Cand1 inhibits the cullin interaction of proteins which, as the DDB1 homologue SAP130, interact to neddylated cullins in vivo (Menon et al., 2008). Cand1 binds to the closed conformation of the cullin-RING complex and the numerous contacts between Cand1’s C-terminus and cullin induce a slightly less curved conformation of cullin’s N-terminal domain. In the crystal structure, Cand1 blocks the Lys720 neddylation site in the catalytic C-terminal part of Cul1 of the Cand1-cullin-RING complex. In addition, the Skp1 adaptor binding site in the Cul1 N-terminal region is blocked by the β-hairpin protrusion of the C-terminal Cand1 (Goldenberg et al., 2004).

Impairment of Cand1 or CSN results in decreased CRL activities in vivo, which is called a CSN paradox because it is in contrast to the observed biochemical CRL inhibition in vitro (Liu et al., 2002; Busch et al., 2003; Feng et al., 2004; Bosu and Kipreos, 2008). This is presumably due to an increased autoubiquitination activity of CRLs that lack substrates. This results in the destabilization of F-box substrate binding proteins. Deneddylation and inhibition by CSN seem necessary for stabilization of CRL subunits, thus promoting CRL activity in vivo (Zheng et al., 2002a; Wei et al., 2005; Cope and Deshaies, 2006; Chew et al., 2007; Dubiel, 2009; Schmidt et al., 2009). The composition of CRLs has been proposed to be regulated by cycles of assembly and disassembly resulting in active neddylated CRLs and inactive unneddylated cullin-RING subcomplexes, respectively. A small fraction of unneddylated subcomplexes is sequestered by Cand1 for stability-independent recycling of CRL substrate recognition proteins (Lo and Hannink, 2006). When a new substrate becomes available, Cand1 can be replaced by Skp1 and another F-box protein for a new round of CRL assembly (Borningstein et al., 2006; Sierjeijk et al., 2009). Replacement of Cand1 by the substrate adaptors might be facilitated by additional factors or the neddylation of Cand1 itself. This was recently observed for the Cand1 homologue Lag2 in baker’s yeast (Sierjeijk et al., 2009). Thus, availability of substrates transfers CRLs from a Cand1 cycle to a CSN cycle, which starts with substrate binding followed by cullin neddylation. The Cand1 cycle thus allows the incorporation of rare adapters into a subset of CRL complexes (Bosu and Kipreos, 2008; Schmidt et al., 2009).

The exchange of different substrate binding proteins owing to changes in substrate levels for CRLs is important during the development of multicellular organisms. CSN dysfunction results in early lethality or developmental defects in animals, plants, and filamentous fungi (Castle and Meinke, 1994; Freilich et al., 1999; Lykke-Andersen et al., 2003; Busch et al., 2007). Cand1 is present during all analyzed stages of development of plants or mice, and plant cand1 mutants show severe defects in fertility, photomorphogenesis, and flowering (Aoki et al., 1999; Yogosawa et al., 1999; Cheng et al., 2004; Chuang et al., 2004).

We show that Cand1 can be split into two functional proteins binding to each other and to cullins. Binding of the C-terminal Cand1 peptide to cullin’s N-terminal adaptor interaction site mediates the binding of the N-terminal Cand1 entity to the neddylation site on cullin’s C-terminus. C-terminal Cand1 can only associate to unneddylatable cullin in vivo. N-terminal Cand1 is unable to interact with any form of cullin without prior binding of the C-terminal part. The separation of a single Cand1 encoding gene corresponds to the in vivo situation of the filamentous fungus Aspergillus nidulans. Deletion of either of the two Cand1 encoding genes in the fungus results in identical phenotypes and an even stronger impact on development than deletion of genes for CSN subunits.

RESULTS
Aspergillus nidulans Cand1 is encoded by two separated genes, candA-N and candA-C
Whereas the crystal structure of the Cand1-cullin complex has been resolved, the molecular process of how the Cand1 protein binds to unneddylated cullins is unknown. The genome of the mold A. nidulans (Galagan et al., 2005) revealed a cand1 homologue divided into candA-N (AN10306.3) encoding the smaller Cand1 N-terminus and candA-C (AN24583.3) for the larger C-terminal part (Figure 1A). Both genes are located on chromosome VII in relative proximity to each other, separated by four open reading frames of conserved hypothetical proteins. The distribution of a split Cand1 protein system in the fungal kingdom is restricted to the Eurotiomata including various Aspergilli. Other fungi possess a single cand1 encoding an approximately 1300-amino-acid protein as in plants or animals (Figure 1B). The percentage of identities of A. nidulans CandA to other Cand1 sequences ranges from 77% for Cand1 of other Aspergilli to 26% for human Cand1. The identity of A. nidulans CandA is only 10% to the recently discovered Cand1/Lag2 of Saccharomyces cerevisiae, which differs from the actual Cand1 in sequence and in a much smaller size of only 660 amino acids (Sierjeijk et al., 2009). There is high conservation of both HEAT repeats A1/B2 and the β-hairpin protrusion of the mammalian Cand1 (Goldenberg et al., 2004). A1/B2 is located in the smaller CandA-N of A. nidulans, representing the N-terminal part of the Cand1 protein, and blocks the binding site for the ubiquitin-like modifier Ned8 on Cul1 in mammalian cells. In CandA-N, residues Asp22 and Asp24 are conserved, corresponding to the two important residues of Cand1, which block the cullin Lys720 neddylation site. The β-hairpin protrusion domain, which partially occupies the adaptor binding site on Cul1, is located in the larger CandA-C corresponding to the C-terminal part of Cand1.

Sequencing of candA-C genomic and cDNA confirmed two introns at the 3’-end of candA-C. The S’-untranslated region of the transcript was determined and is located 99 base pairs upstream of the start codon corresponding to the first AUG of the mRNA. The deduced sequence of 1041 amino acids results in a 113.5-kDa protein. Genomic and cDNA of candA-N also revealed two introns and a deduced 313-amino-acid protein of 33.6 kDa making up one quarter in length of the combined CandA protein. Therefore, both candA genes of A. nidulans are transcribed and can result in two separate proteins with a combined size of the Cand1 of higher eukaryotes (Figure 1A). The restriction of the split cand1 genes to a small group of organisms suggests that the common ancestor of these filamentous fungi had a single cand1 that has been separated by a recombination event.

Both candA genes are required for fungal development
Single-deletion strains of candA-N and candA-C as well as a double-deletion strain candA-N/candA-C were constructed. All phenotypes could be complemented by ectopic integration of the corresponding wild-type genes. The three ΔcandA strains were similar and appeared dark red when grown on an air-medium interface that induces development (Figure 2). The red color was not present in wild type but was reminiscent to various A. nidulans mutants impaired in the CSN (Busch et al., 2003, 2007). A. nidulans induces the sexual cycle in the dark and under oxygen limitation. First, filaments aggregate and form so-called nests. Then primordia are formed, which finally differentiate to closed fruit bodies termed cleistothecia. All ΔcandA strains were able to initiate the cycle but were blocked in the initial stage of early nest formation. Hypheae aggregated to the small white or yellow nest structure including the
We investigated the time points when CandA-N and CandA-C were present. Replacement by gfp::candA-N and candA-C::gfp including the native promoter complemented the phenotypes of the corresponding deletion strains. A single band representing the CandA-C-GFP protein was present during vegetative growth and during early asexual or sexual development (Figure 1C). During later development, CandA-C was unstable, resulting only in the smaller band representing the stable GFP-tag. The GFP-CandA-N fusion only resulted in a stable GFP signal, suggesting that the functional fusion is not very stable. This suggests that at least CandA-C fulfills its function primarily during vegetative growth and at the beginning of asexual or sexual development.

All three cand deletions strains exhibited identical reduction in asexual and block in sexual development. A genetic crossing experiment was performed to examine whether the ΔcandA-N and ΔcandA-C deletions present in different nuclei can complement each other. Hyphae of the ΔcandA-N strain were able to fuse to hyphae of ΔcandA-C strains and to develop primordia within the nests and ultimately mature cleistothecia (Figure 2C). This supports a common function of CandA-N and CandA-C within the cell and suggests a cooperation of both proteins on the molecular level.

candA mutants produce the same pattern of secondary metabolites and separation of CandA-N and CandA-C can be reversed by gene fusion

The red color of the ΔcandA-N, ΔcandA-C, and ΔcandA-NΔcandA-C mutant strains suggested a similar defect in secondary metabolism. The culture filtrate contained several substances especially with ammonium as a nitrogen source that are only produced in deletion strains but not in wild type (Figures 3A and 3B). The culture filtrate was extracted with ethyl acetate, and the organic phase was concentrated in vacuum and analyzed by thin-layer chromatography and high-performance liquid chromatography/mass spectrometry (HPLC/MS/UV) detection (Figures S1 and S2). Metabolites were isolated by column chromatography, gel chromatography, and HPLC to yield the pure compounds in microscale. The structural elucidation was performed using spectroscopic methods and databases and revealed orcinol, violaceol II, violaceol I, cordyol C, and diocinol that were isolated from all ΔcandA strains, but not from wild type (Figures 3C and 3D). These data indicate that the lack of CandA-N, CandA-C, or both proteins resulted in an identical change in secondary metabolite production and further supports a common function of both fungal CandA proteins.

We examined whether CandA of A. nidulans has to be split into CandA-N and CandA-C to fulfill its function. This could reflect a change in molecular mechanism for the split fungal CandA during evolution in comparison to the single Cand1 proteins present in
most eukaryotic organisms. A candA-N::C fusion under the control of the native candA-N promoter and candA-C terminator (Figure 2D) was integrated ectopically into the ΔcandA-N/candA-C strain. As a control, both candA-N and candA-C genes were reintegrated separately into the double-deletion strain. The candA-N::C fusion strain lost the mutant colony color and grew like the wild type as the strain where the double mutation was complemented by the two separate candA genes. The development of asexual spores and the formation of sexual fruit bodies including mature sexual spores could also be restored (Figure 2E). This shows that both the candA-N::C fusion protein and the combination of the two CandA proteins fulfill their molecular function in the fungus and support a molecular mechanism for the split fungal CandA similar to that for the single peptide Cand1 of higher eukaryotes.

CandA-N requires CandA-C for nuclear localization and only CandA-C but not CandA-N interacts with cullins in the yeast two-hybrid system

Functional mRFP-CandA-N and CandA-C::GFP fusions under the control of the inducible alcA promoter were expressed in the ΔcandA-N/candA-C strain (Figure S3A) to examine where both CandA proteins are localized. The strain with both fusions was phenotypically indistinguishable from wild type and both fusion proteins were exclusively detected in nuclei (63% of n = 30; Figure 4A). This suggests functions for both CandA proteins in the nucleus. We further analyzed whether CandA-N depends on CandA-C to be transported into the nucleus, because only CandA-C harbors a predicted nuclear localization sequence (NLS) (RKRRR). Therefore, we expressed mRfp::candA-N/candA-C::gfp2-5 separately in the ΔcandA double-deletion strain. CandA-C::GFP accumulated in the nuclei (64% of n = 62), whereas we never detected RFP-CandA-N in the nucleus (n = 60) (Figure 4A). This suggested that CandA-C is essential for the transport of CandA-N into the nucleus. We fused the RKRRR peptide N-terminally to CandA-N to investigate the importance of the putative NLS of CandA-C for CandA-N. The functional mRFP-RKRRR-CandA-N fusion localized in the nuclei (84% of n = 64; Figure S3B) in the candA-N single-deletion strain. The fusion also localized in the nuclei of the double-deletion strain (83% of n = 66; Figure 4B) but was unable to complement the candA deletion phenotype. This demonstrated that CandA-N cannot fulfill its function in the nucleus without CandA-C.

The ability of CandA-N and CandA-C to interact with A. nidulans cullins Cul1/CulA and Cul4/CulD (Busch et al., 2007) was analyzed in the yeast two-hybrid system (Figure 5A). An interaction of CandA-C which contains the protrusion domain that partially occupies the adaptor-binding site on Cul1 was observed with both fungal cullins. In analogy to mammals, CandA-C interacted with neither CsnB/CSN2 nor any other CSN subunit (data not shown). In contrast, CandA-N containing the HEAT repeats that blocks the binding site for Nedd8 did not interact with any cullin. We replaced both putative neddylation sites, Lys710 of CulA and Lys826 of CulD, respectively, by arginine to exclude the possibility that neddylation-rubbylation of A. nidulans cullins impedes the binding of CandA-N to the cullins. These proteins, CulA* and CulD*, did not bind to CandA-N in the yeast two-hybrid assay either (Figure 5B). Thus, our results suggest that the 1041 amino acid–long CandA-C has to mediate the binding of the 313 amino acid–long protein CandA-N into a putative complex consisting of CandA-N, CandA-C, and a cullin.

**FIGURE 2:** αcandA-N and ΔcandA-C mutants impaired in development and secondary metabolism. (A) Sexual development of same strains grown for 6 d on a sealed agar plate in the dark. Close-ups for stop of development of ΔcandA strains at early nests (en) when wild type produces mature fruit bodies (c: cleistothecia). Bar, 200 μm. (B) Asexual development of A. nidulans ΔcandA-N (AGB264) and ΔcandA-C (AGB262), double mutant (AGB268) and wild-type AGB160 (wt) at 37°C for 6 d in light. Conidia shown in the close-up (bar, 600 μm) (C) Cross of ΔcandA-N to ΔcandA-C. Bar, 200 μm. (D) candA-N::C fusion construct (plasmid pME3310) with primer and restriction sites. The candA-N stop codon was replaced by Spel restriction site. (E) Development of integrated candA-N::C fusion (AGB332) or integrated single genes candA-N and candA-C (AGB331) into the ΔcandA-N/candA-C deletion strain (AGB268) complementing the deletion phenotype. Arrows indicate mature cleistothecia (left and middle) and early nest structures (right). Bar, 200 μm.
Cand\(\Delta\)N interacts only in combination with Cand\(\Delta\)C with deneddylated cullin in vivo, whereas Cand\(\Delta\)C can bind on its own.

We investigated Cand\(\Delta\)N–Cand\(\Delta\)C–cullin complexes in vivo. The C-terminal half of eyfp (ceyfp) was fused to cand\(\Delta\)A-C and the N-terminal half of eyfp (neyfp) to CUL1/cuLA. These fusions were integrated at the wA locus and controlled by the bidirectional nitrate promoter in a \(\delta\)cand\(\Delta\)A–C as well as a \(\delta\)cand\(\Delta\)A–C/\(\delta\)cand\(\Delta\)N background still carrying the wild-type cuLA because deletion is essential. The cand\(\Delta\)A:C:ceyfp fusion is functional. A bimolecular fluorescence complementation resulted in nuclear signals (79% in \(n = 63\)) in the \(\delta\)cand\(\Delta\)A–C strain (Figure 6A). When Cand\(\Delta\)A-N was missing, however, fluorescent nuclei significantly decreased (9% of \(n = 63\)), indicating that Cand\(\Delta\)A is dependent on Cand\(\Delta\)N to bind wild-type cuLA in vivo (Figure 6B). The interaction of Cand\(\Delta\)A–C with the CuLA* variant, which cannot be neddylation due to the K710R substitution, revealed a different pattern. Again, when Cand\(\Delta\)A was present, Cand\(\Delta\)A–C was bound to CuLA* (88% of \(n = 51\); Figure 6A), but, even in the absence of Cand\(\Delta\)A–N, Cand\(\Delta\)A–C interacted with CuLA* (81% of \(n = 57\); Figure 6B). This result supports that Cand\(\Delta\)A–C can interact with deneddylated cullin but is unable to sequester the deneddylated cullin subpopulation from the cullin pool without Cand\(\Delta\)A–N. As expected, the regenerated Cand\(\Delta\)A–N–C fusion, which simulates the situation in higher eukaryotes, interacts as well to cuLA in nuclei (90% of \(n = 52\)) as to the CuLA* variant (81% of \(n = 52\); Figure 6C). These results further confirm that the molecular mechanism of Cand1 function can tolerate splitting Cand1 into two polypeptide entities, as happened in an ancestor of the Aspergilli. Both proteins act in concert to bind cullins similarly as the single polypeptide chain Cand1.

We then examined Cand\(\Delta\)A–N's ability to bind the two variants of cuLA in the presence or absence of Cand\(\Delta\)A–C. The yeast two-hybrid data did not support a Cand\(\Delta\)A–N interaction to cullins, but Cand\(\Delta\)A–N harbors the conserved binding site covering cullin's neddylation site, which might be important for Cand1 in vivo to discriminate between the unneddylation and neddylation domain. We N-terminally fused cand\(\Delta\)A–N to neyfp and furthermore included the sequence encoding the NLS of Cand\(\Delta\)A–C (RKRKR) to overcome the need for Cand\(\Delta\)A–C to enter the nucleus. The functional neyfp::RKRKR::cand\(\Delta\)A–N fusion resulted in signals like the controls (Figure 5A) and could not interact with either cuLA or CuLA* when Cand\(\Delta\)A–C was missing (\(n = 60\) and \(n = 60\); Figure 6D). Cand\(\Delta\)A–N's affinity to unneddylation cullin was obviously too low to cause substantial, visible interaction. Complex formation of Cand\(\Delta\)A–N with both variants of CuLA was observed in vivo, however, in a combined action of the two Cand\(\Delta\)A proteins when Cand\(\Delta\)A–C was present in the hyphae (Figure 6E; 83% of \(n = 70\) for cuLA, 87% of \(n = 94\) for CuLA*).

These experiments demonstrate that Cand\(\Delta\)A–N needs Cand\(\Delta\)A–C in vivo not only for transport into the nucleus but also to support its binding to unneddylation cullins. Thus, in vivo binding of the larger Cand\(\Delta\)A–C is a prerequisite for subsequent binding of the smaller Cand\(\Delta\)A–N, which blocks the neddylation site and completes the interaction of Cand\(\Delta\)A to deneddylated cullin.

DISCUSSION

A single Cand1 protein is typical for most eukaryotes, whereas the filamentous fungus A. nidulans and its relatives are an exception. A. nidulans possesses two genes for Cand1, but only the combination of the two genes cand\(\Delta\)A–N and cand\(\Delta\)A–C corresponds to mammalian Cand1. The two open reading frames oppose each other and are separated by four open reading frames. This suggests that an originally fused Cand1 gene was torn apart by DNA rearrangement. Both gene parts had to end up with all signal sequences, and the molecular mechanism of Cand1 function had to operate as a single peptide or as two peptides. The appearance of a split gene in only one taxonomic order suggests a single occasion for the rearrangement during evolution. The ancient recombination event is reversible, because a fused gene derived from the split candA genes is functional. This makes the split fungal CandA an interesting model to explore the molecular Cand1 function.

Deletions of both fungal candA genes resulted in identical developmental phenotypes and include an intense red color. The candA mutants synthesize orsellinic acid derivatives, which are produced when A. nidulans is under stress conditions (Schroeckh et al., 2009). Similar phenotypes including the red color are the result of deletions for genes encoding COP9 subunits (Busch et al., 2003, 2007). This corroborates the cross-talk between CandA and CSN at a developmental level in fungi. Both types of mutants are unable to form sexual fruit bodies, although sexual development stops even earlier in \(\delta\)cand\(\Delta\)A than in \(\delta\)cns strains. This is different in Cand1 defective
plants, which flower later than wild type, whereas csn mutants stop in early development (Castle and Meinke, 1994; Freilich et al., 1999; Lykke-Andersen et al., 2003; Cheng et al., 2004; Chuang et al., 2004; Feng et al., 2004; Busch et al., 2007). However, none of the described plant mutants corresponded to full deletions (Cheng et al., 2004; Chuang et al., 2004; Feng et al., 2004; Alonso-Peral et al., 2006). A plant deletion strain should elucidate the significance of the csn and cand1 mutant phenotypes between plants and fungi. The stronger developmental impact of fungal candA in comparison to csn deletions might reflect yet unexplored additional de neddy lase activities in A. nidulans.

Fungal CandA-C is only present during vegetative growth and at the beginning of development. This might reflect a function for CandA at a specific developmental transition point. Similarly, plant mutants defective in Cand1 are impaired in the vegetative to reproductive growth transition of the primary shoot apical meristem (Cheng et al., 2004; Chuang et al., 2004; Feng et al., 2004; Alonso-Peral et al., 2006). However, Cand1 is expressed in all developmental stages in plants and in mouse embryos (Aoki et al., 1999; Yogosawa et al., 1999). A developmental transition point might have a special need for reorganization of CRLs by Cand1 and CSN assisted cycles of disassembly and assembly (Lyapina et al., 2001; Cope and Deshaies, 2003; Wu et al., 2006; Schmidt et al., 2009; Figure 7). Special developmental stages might require specific regulatory proteins, which have to be substituted by others during subsequent phases of development. Accordingly, in mammalian cells, silencing of Cand1 leads to a stabilization of the cell-cycle-dependent kinase inhibitor p27, whose ubiquitination is SCF dependent (Zheng et al., 2002a). In plant cand1 mutants, the developmental regulator protein Hy5 and the gibberellin hormone pathway repressor RGA are stabilized (Feng et al., 2004). In A. nidulans, the SCF F-box protein GnA is expressed in late sexual development and is required to form fertile sexual spores (Krappmann et al., 2006). This suggests that a SCF<sup>Cand1</sup> substrate has to be degraded for complete development and CSN and Cand1 might be necessary for accurate CRL formation.

Wrapping of Cand1 around the cullin-RING complex covers the adaptor binding and the neddylation site and sequesters them from interacting with other proteins (Goldenberg et al., 2004). The two fungal CandA proteins bind to different sites on cullin and therefore allow a comparison of their cellular function. Alignment with the determined crystal structures suggests that fungal CandA-C can contact the stalk-like N-terminal domain (NTD) of CulA via three major anchoring sites (Goldenberg et al., 2004; Duda et al., 2008). The junction between both Candida proteins colocalizes with the region around Asp267 of Cand1. This is located on the opposite side of cullin’s C-terminal domain (CTD) in comparison to its winged helix binding (WHB) domain, Roc1 and the neddylation site. Thus, CandA-C’s N-terminus presumably forms only a small interaction surface for cullin’s four-helix...
binds to cullin in a yeast two-hybrid assay, in contrast to the smaller CandA-N, and has the potential to interact even in the absence of CandA-N. CandA-N can form a heterodimer with CandA-C to be transported into the nucleus. However, the number of binding sites of the six HEAT repeats of CandA-N is not sufficient to mediate a stable interaction to cullins in the absence of the CandA-C partner protein. CandA-N is not even able to interact alone with unneddylatable cullin variants where the described Cand1 binding site of cullin WHB and RING domains is permanent.

The CandA-C/CandA-N heterodimer binds to cullin in the nucleus as stably as a single polypeptide chain in vivo. This requires a sequence of molecular interactions (Figure 7): 1) The two CandA proteins form a heterodimer in the cytoplasm. CandA-N needs CandA-C to enter the nucleus, because only CandA-C harbors a functional NLS. The A. nidulans CandA-C NLS is ancient, as it is conserved not only in split but also in fused Cand1/CandA homologues of distant fungal relatives. 2) CandA-C NLS interaction with importins results in nuclear transport. 3) CandA-C sites initiate cullin interaction, which 4) mediates binding between CandA-N and cullin’s CTD leading to 5) full inhibition of CRL activity.

CandA-C alone can only stably interact with unneddylatable cullin in vivo. Cullin interacts with numerous proteins, and in vivo binding of CandA-C might require that no other proteins with higher affinity compete for cullin. The affinity of CandA-N to CullA is too low to bind CullA alone in either form. The affinity of Cand1 to CUL1 is presumably higher than that of the deneddylating CSN. Therefore, Cand1 enhances the deneddylation reaction by sequestering unneddylated cullin (Min et al., 2005). The CSN binds to cullin’s 4HB and α/β domain via subunits 1, 2, 4, and 5, blocking access of Cand1. The region of amino acids 414 to 600 of CUL1 is important for CSN binding and is exclusively covered by CandA-N. Therefore, CandA-C binding might represent a still unstable intermediate after deneddylation when CSN still occupies CullA. Additional proteins might be involved in the cross-talk between Cand1 and CSN (Bosu and Kipreos, 2008).

The CandA-C/CandA-N heterodimer binds to cullin in the presence of intact untagged CandA-N. (B) Same as (A), but in strains that lack CandA-N (CulA: AGB559 and CulA*: AGB560). (C) BIFC with CandA-N-C fusion and both CulA variants in combination with nuclear CandA-N (AGB570) and in the absence CandA-C (AGB568). (E) Same as (D), but in strains expressing candA-C (CulA: AGB569 and CulA*: AGB567). Bar, 10 μm. Right-hand schemes summarize interactions.
adaptor binding site is supported by the finding that adapters competing with Cand1’s C-terminal part are required for dissociation of Cand1’s/Lag2 from cullin (Bornstein et al., 2006; Siergiejuk et al., 2009). Alternatively, displacement could start at the neddylation site, resulting in a transient intermediate of Cullin/Rbx1 and CandA-C. A CandA-C-cullin could even be a substrate to neddylation, because cullin can be neddylated while bound to Cand1 (Liu et al., 2002). Removal of the N-terminal part and exposure of the neddylation site could facilitate neddylation but would circumvent the process of adapter acquisition as a prerequisite for CRL function.

Cand1 release could also start with the modification of CandA-N mediating binding between CandA-N and cullin’s C-terminal domain. This leads to full inhibition of cullin-E3-ligase activity. Removal of Nedd8 through CSN activity destabilizes the cullin complexes through recruitment of an adapter protein (A) and subsequent neddylation (N). Removal of Nedd8 through CSN activity destabilizes the cullin complexes through recruitment of an adapter protein (A) and subsequent neddylation (N).

**FIGURE 7:** Molecular function of split fungal CandA. 1) CandA-C/N forms heterodimer in the cytoplasm. 2) CandA-C nuclear localization signal interacts with importins (I) for transport through the nuclear pore (NP). 3) CandA-C sites initiate cullin (Cull) interaction, which 4) mediates binding between CandA-N and cullin’s C-terminal domain. 5) This leads to full inhibition of cullin-E3-ligase activity. 6) Release of CandA from cullin allows the formation of new cullin-E3-ligase complexes through recruitment of an adapter protein (A) and subsequent neddylation (N). 7) Removal of Nedd8 through CSN activity destabilizes the cullin complexes and starts the CandA cycle.

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**MATERIALS AND METHODS**

**Cultivation of organisms**

E. coli strains were propagated in Luria Bertani medium (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) at 37°C. For selection, 100 μg/ml ampicillin, 25 μg/ml chloramphenicol, 20 μg/ml kanamycin, or 25 μg/ml Zeocin (Cayla, Toulouse, France) was used.

_S. cerevisiae_ strains were grown at 30°C under nonselective conditions in yeast extract peptone dextrose (2% peptone, 1% yeast extract, 2% glucose) or under selective conditions in SC medium (0.15% yeast nitrogen base without amino acids, 0.5% (NH₄)₂SO₄, 0.2 mM myo-Inositol, 0.2% amino acid mix containing either 2% glucose or 2% galactose/1% raffinose) supplemented as described (Guthrie and Fink, 1991). A. nidulans strains were grown at 37°C in or on minimal medium (7 mM KCl, 11.2 mM KH₂PO₄ [pH 5.5], 2 mM MgSO₄, trace elements; Käfer, 1965). As a carbon source, 1% glucose was used. As nitrogen sources, 70 mM NaNO₃, 10 mM NaNO₂, or 10 mM NH₄Cl were added. The medium was supplemented with 4.8 μM pyridoxine HCl and/or 5 mM uridine when required. For plates, 2% agar was added. Selection for the ble marker of _Streptothalloteichus hindustanus_ was performed using 10 μg/ml phleomycine (Cayla). For _ptr_- (TAKARA BIOMARK, Junki, Poland), 100 ng/ml pyrithiamine (Sigma-Aldrich Chemie GmbH, Munich, Germany) and for transformants containing the nourseothricin (nat) resistance (Goldstein and McCusker, 1999), 100 μg/ml nourseothricin dihydrogen sulfate (clonNAT, Werner BioAgents, Jena, Germany) was added to the medium.

Vegetative mycelia were obtained from submerged liquid cultures, inoculated with 10⁶ spores/ml and grown on a rotary shaker for 14–30 h. For induction of development, 10³ spores were spread on agar plates.

Transformation procedures

For general cloning procedures, Escherichia coli DH5α [F−, _Φ_80dλlacZEM15−, _Δ_(lacZYA-argF)-U169, _relA1_, _endA1_, _hsdR17_ (rK, mK), supE44, _λ−_, thi1, _gyrA96_, relA1] (Woodcock et al., 1989) was used. Homologous and ectopic integration of constructs, respectively, was confirmed using PCR and/or Southern hybridization analyses (Southern, 1975). Recombinant DNA technologies were performed according to standard methods (Sambrook et al., 1989). For PCR reactions, _Taq_ (Fermentas GmbH, St. Leon-Rot, Germany), _Pfu_ (Fermentas), _Kod_ (Novagen, Nottingham, UK), or _Phusion_ (Finzymes OY, Espoo, Finland) polymerases were used. Custom oligonucleotides were ordered from Operon Europe (Cologne, Germany) or Invitrogen GmbH (Karlsruhe, Germany). Restriction enzymes were ordered from Fermentas. 5′RACE was performed using the GeneRacer Kit (Invitrogen) according to the manual.

Transformations of _E. coli_, _S. cerevisiae_, and _A. nidulans_ were performed as described (Inoue et al., 1990; Elble, 1992; Eckert et al., 2000). Strains are listed in Table 1; primer sequences and plasmids in Supplemental Tables S1 and S2.

The _A. nidulans_ BAC library (obtained from Clemson University, Clemson, SC) was screened for _candA-C_ using a gene-specific probe, amplified from genomic wild-type DNA. Probes used for _Bacillus_ filter hybridization were [α-32P]-dATP labeled with the HexaLabel DNA
| Strain | Genotype | Reference/Construction |
|--------|-----------|------------------------|
| A4     | A. nidulans Glasgow wild type | FGSC* (Nayak et al., 2006) |
| TNO2A3 | pyrG89; pyroA4; ΔnkuA::argB | (Busch et al., 2003) |
| AGB152 | pyrG89; pyroA4 | (Busch et al., 2003) |
| AGB160 | pyrG89/acy-4; pyroA4 | pME3115 in AGB152 |
| AGB262 | ΔcandA-C::pyr-4; pyrG89; pyroA4 | pME3116 in AGB262 |
| AGB263 | ΔcandA-C::pyr-4; candA-C::ble; pyrG89; pyroA4 | pME3306 in AGB262 |
| AGB264 | ΔcandA-C::pyr-4; candA-C::ble; pyrG89; pyroA4 | pME3308 in AGB264 |
| AGB265 | candA-C::gfp2-5; pyrG89; pyroA4 | pME3120 in AGB262 |
| AGB266 | ΔcandA-C::pyr-4; candA-C::ble; pyrG89; pyroA4 | pME3127 in AGB264 |
| AGB331 | ΔcandA-C::pyr-4; candA-C::ble; pyrG89; pyroA4 | pME3311 and pME3314 in AGB268 |
| AGB332 | candA-C::ble; pyrG89; pyroA4 | pME3310 in AGB268 |
| AGB333 | gfp2-5::candA-C::nkuA::argB; candA-C::ble; pyrG89; pyroA4 | pME3490 in AGB264 |
| AGB334 | candA-N::pyr-4; ΔnkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3394 and pME3395 in AGB268 |
| AGB354 | candA-C::ble; pyrG89; pyroA4; nkuA::argB | pME3727 in TNO2A3 |
| AGB544 | ΔcandA-N::nat6; ΔcandA-N::ble; pyrG89; pyroA4; nkuA::argB | pME3601 in AGB553 |
| AGB545 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB | pME3740 in AGB553 |
| AGB546 | ΔcandA-C::ble; pyrG89/acy-4; pyroA4; ΔnkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3741 in AGB553 |
| AGB547 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3742 in AGB553 |
| AGB548 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3744 in AGB553 |
| AGB549 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3745 in AGB554 |
| AGB550 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3746 in AGB555 |
| AGB551 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3747 in AGB555 |
| AGB552 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3748 in AGB554 |
| AGB553 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3749 in AGB554 |
| AGB554 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3750 in AGB554 |
| AGB555 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3751 in AGB554 |
| AGB556 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3752 in AGB554 |
| AGB557 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3753 in AGB554 |
| AGB558 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3754 in AGB554 |
| AGB559 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3755 in AGB554 |
| AGB560 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3756 in AGB554 |
| AGB561 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3757 in AGB554 |
| AGB562 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3758 in AGB554 |
| AGB563 | ΔcandA-C::ble; pyrG89; pyroA4; nkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3759 in AGB554 |
| AGB564 | ΔcandA-C::ble; pyrG89; pyroA4; ΔnkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3760 in TNO2A3 |
| AGB565 | ΔcandA-C::ble; pyrG89; pyroA4; ΔnkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3749 in aGB564 |
| AGB566 | ΔcandA-C::ble; pyrG89; pyroA4; ΔnkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3749 in AGB554 |
| AGB567 | ΔcandA-C::ble; pyrG89; pyroA4; ΔnkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3748 in AGB564 |
| AGB568 | ΔcandA-C::ble; pyrG89; pyroA4; ΔnkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3748 in AGB554 |
| AGB569 | ΔcandA-C::ble; pyrG89; pyroA4; ΔnkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3747 in AGB564 |
| AGB570 | ΔcandA-C::ble; pyrG89; pyroA4; ΔnkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3747 in AGB554 |
| AGB571 | ΔcandA-C::ble; pyrG89; pyroA4; ΔnkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3750 in AGB268 |
| AGB572 | ΔcandA-C::ble; pyrG89; pyroA4; ΔnkuA::argB; ΔnkuA::ble; ΔnkuA::argB; ΔnkuA::ble | pME3750 in AGB264 |

* Fungal Genetics Stock Center (University of Missouri, Kansas City, MO).

**TABLE 1:** Aspergillus nidulans strains used and constructed in this study.
Labeling Kit (MBI Fermentas GmbH, St. Leon-Rot, Germany) and detection was performed using the BioMaxMS film (Kodak Molecular Imaging, New Haven, CT). A 9.3-kb Hpal fragment from BAC library clone E19, plate 9, containing the complete candA-C coding region was cloned into pBluescript II SK+ (MBI Fermentas GmbH) cut with EcoRV yielding plasmid pME3609.

**Plasmid construction**
Details are given in the Supplemental Material.

**Yeast two-hybrid analyses**
The yeast two-hybrid test was described (Golemis and Brent, 1996). Details are given in the Supplemental Material.

**Protein isolation and analysis**
Protein isolation has been described (Busch et al., 2007). Mouse anti-GFP (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France), rabbit anti-RFP (Abcam, Cambridge, UK), and mouse anti-β-tubulin (Sigma-Aldrich, St. Louis, MO) antibodies were used as primary antibodies. Horseradish peroxidase-coupled goat anti-mouse IgG (Jackson Immuno Research Laboratories, Westgrove, PA) or goat anti-rabbit IgG (Invitrogen, Eugene, OR), respectively, were used as secondary antibodies.

**In silico analyses**
Basic Local Alignment Search Tool searches were performed using data from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Clustal W (http://www.ddbj.nig.ac.jp/) and MultAlin (Corpet, 1988) (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html) were used for alignments.

**DNA isolation and hybridization**
Isolation of genomic DNA of A. nidulans was described (Busch et al., 2007). Southern analysis was performed with the Gene Images Random-Prime DNA labeling kit and the Gene Images CDP-Star Detection Kit (GE Healthcare Life Sciences, Munich, Germany). For signal detection, Amersham Hyperfilm ECL was used (GE Healthcare Life Sciences, Munich, Germany).

**Microscopy**
A Kappa PS30 digital camera and ImageBase software (KAPPA opto-electronics GmbH, Gleichen, Germany) was used in combination with an Olympus SXZ12 binocular (Olympus, Hamburg, Germany) or a ZEISS Axioslab (ZEISS AG, Oberkochen, Germany) light microscope. Fluorescence microscopy was conducted as described (Helmstaedt et al., 2008).

**Chemical analysis**

$^1$H NMR spectra were recorded on Varian Inova 600 and Unity 300 spectrometers, respectively, at 600 and 300 MHz at 298 K (VARIAN Inova, Palo Alto, CA). Infrared spectra were recorded on a Perkin-Elmer Fourier transform IR 1600 spectrometer as KBr pellets (Perkin-Elmer, Waltham, MA). Solvents for extraction and chromatography were of technical grade and distilled before use. Thin-layer chromatography (TLC) was carried out: type A, on silica gel 60 F$_{254}$ plates/0.2 mm (Merck, Darmstadt, Germany) using CHCl$_3$:MeOH (9:1) as solvent; or type B, on RP-18 F$_{3445}$:plates/0.2 mm (Merck) using MeOH:H$_2$O (7:3) as solvent. Silica gel 60/0.040–0.063 mm (Machery & Nagel, Düren, Germany) and Sephadex LH-20 (Pharmacia, Germany) were used for column chromatography. Flash column chromatography was performed using silica gel 60/0.025–0.040 mm (Machery & Nagel). Medium-pressure liquid chromatography was performed with Knauer Wellchrom Maxi-Star K 1000 pumps (Knauer, Berlin, Germany) using a Merck LiChroprep RP-18/0.040–0.063 mm column (B, 310–25). HPLC was carried out on JASCO HPLC systems with PU-2080 Plus or PU-1587 pumps (JASCO, Gross-Umstadt, Germany), respectively, using column A (Macherey & Nagel, Superspher-100 RP-18 endc., 4 µm, 100 x 2 mm, flow rate 0.3 ml min$^{-1}$), column B (Macherey & Nagel, Superspher-100 RP-18 endc., 4 µm, 100 x 20 mm, flow rate 18.0 ml min$^{-1}$), or column C (JASCO Kromasil, 100 C-18, 5 µm, 250 x 8 mm, flow rate 2.5 ml min$^{-1}$). HPLC/MS/diode array detection (DAD) analysis was carried out using Flux Instruments Rheos 4000, PDA detector (Finnigan Surveyor), and MS-LC-Q detector (Finnigan) with Xcalibur 1.3 software (Finnigan).

**Metabolite analysis and isolation**

In general, 2-l minimal medium cultures containing 10 mM NH$_4$Cl as a nitrogen source grown in the light or in the dark were used for metabolite analysis and isolation. The culture broth was separated from the mycelium by filtration using a Miracloth filter (Calbiochem, Merck Biosciences, Nottingham, UK). The mycelium was extracted with MeOH:acetone (3:2, 3 x 1 l). The culture filtrate was adjusted to pH = 5.0 and extracted with ethyl acetate (3 x 2 l), and the solvent was removed by evaporation to yield the crude residue. The crude extracts were dissolved each in 1 ml of MeOH and analyzed by TLC. For HPLC/DAD/MS analysis, the crude extracts (5 mg/ml) and pure compounds (1 mg/ml) were dissolved in MeOH. Analytical HPLC was performed to investigate crude extracts, fractions, and pure compounds. Preparative purification of compounds proceeded from subjection to silica gel chromatography (50 x 2 cm, cyclohexane:ethyl acetate:methanol (5:10:1), which yielded diorcinol). Subsequent size exclusion chromatography (100 x 2.5 cm, Sephadex LH-20, acetone) and reverse phase HPLC gave the other compounds. The metabolites orcinol (up to 1.3 mg), diorcinol (up to 16 mg/l), cordyol C (up to 2.4 mg/l), violaceol I (up to 0.8 mg/l), and violaceol II (up to 2.0 mg/l) were obtained as pure compounds. Ad-
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