

Chapter 13

The Ccr4-Not Complex: Architecture and Structural Insights

Martine A. Collart and Olesya O. Panasenko

Abstract The Ccr4-Not complex is an essential multi-subunit protein complex that plays a fundamental role in eukaryotic mRNA metabolism and has a multitude of different roles that impact eukaryotic gene expression. It has a conserved core of three Not proteins, the Ccr4 protein, and two Ccr4 associated factors, Caf1 and Caf40. A fourth Not protein, Not4, is conserved, but is only a stable subunit of the complex in yeast. Certain subunits have been duplicated during evolution, with functional divergence, such as Not3 in yeast, and Ccr4 or Caf1 in human. However the complex includes only one homolog for each protein. In addition, species-specific subunits are part of the complex, such as Caf130 in yeast or Not10 and Not11 in human. Two conserved catalytic functions are associated with the complex, deadenylation and ubiquitination. The complex adopts an L-shaped structure, in which different modules are bound to a large Not1 scaffold protein. In this chapter we will summarize our current knowledge of the architecture of the complex and of the structure of its constituents.

Keywords Ccr4-Not complex • Eukaryotic gene expression • Deadenylation • Ubiquitination • RING E3 ligase • mRNA decay • Transcription

13.1 Introduction

The Ccr4-Not complex is a functionally diverse machine essential and conserved in all eukaryotes (for review *see* Collart 2016). It performs two different and conserved catalytic functions, as well as essential scaffolding functions that are key for the regulation of eukaryotic gene expression. The former are well understood, enzymatically and structurally, while we are only gradually grasping at the complexity and importance of the Ccr4-Not scaffolding platform. Ccr4 and Caf1 are the subunits responsible for the first catalytic activity, deadenylation, which involves the shortening of the cytoplasmic mRNA poly(A) tail. This represses mRNA translation

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and is the rate-limiting step that initiates mRNA degradation. Appropriate control of this step in the gene expression pathway is essential in many biological processes. Not4 is the subunit responsible for the second catalytic activity, ubiquitination, which involves covalent attachment of one or multiple 8 kDa ubiquitin moieties at lysine residues of the target protein. Poly-ubiquitination generally targets proteins for degradation by the proteasome. In contrast, mono-ubiquitination serves multiple functions, ranging from modification of protein function to interaction with partners for targeting of proteins to degradation by the lysosome. Appropriate control of ubiquitination is essential for many biological processes and for cellular proteostasis. Finally, Not1 is the major protein relevant for the scaffolding function of the Ccr4-Not complex. The other Ccr4-Not complex subunits dock onto this very large scaffold protein, which is tethered to different mRNA substrates. The tethering to mRNAs is mediated by Not1 itself, by other Ccr4-Not subunits, or by other factors such as RNA binding proteins or the microRNA machinery that interact with different complex subunits (for review see Collart 2016). Hence, Not1 brings together components interacting with different Ccr4-Not complex interfaces and mRNA targets. It seems likely that the Ccr4-Not subunits that have no identified catalytic function play important roles as interaction surfaces on the Not1 scaffold.

Genes encoding subunits of the Ccr4-Not complex were first identified by genetic selections in yeast designed to identify transcription factors (for review see Collart 2003). Since these early studies implicating the complex in transcriptional regulation, many studies have demonstrated that the complex is important for transcription, mRNA quality control and export, mRNA translatability, co-translational quality control, translation regulation and mRNA decay. It also regulates post-translational protein modifications and protein decay (reviewed in Collart 2016). The implication of the complex at all stages of gene expression makes it a key player in gene expression homeostasis and in response to extracellular signaling. Not surprisingly, the complex has a role in many diverse physiological processes including cell proliferation, apoptosis, gametogenesis and embryogenesis, heart function, bone formation, energy metabolism and many more (for review see Shirai et al. 2014).

Our current knowledge of the Ccr4-Not complex has been summarized recently in a large number of different reviews (Collart et al. 2012; Collart and Panasenko 2012; Doidge et al. 2012; Miller and Reese 2012; Panepinto et al. 2013; Reese 2013; Wahle and Winkler 2013; Chapat and Corbo 2014; Inada and Makino 2014; Panasenko 2014; Shirai et al. 2014; Temme et al. 2014; Xu et al. 2014; Villanyi and Collart 2015). A major recent achievement has been the resolution of many structures of Ccr4-Not constituents and the definition of the global architecture of the complex by cryo-EM as will be outlined below.

13.2 Composition of the Ccr4-Not Complex

The composition of the complex in different organisms where it has mostly been studied is summarized in Table 13.1. There are subunits that are common to the different eukaryotes and can be thought of as the core subunits. These are Not1, Not2, Not4, Not5, Caf1, Ccr4 and Caf40. Some confusion exists concerning Not5 in yeast, named CNOT3 in human and/or Not3 in other eukaryotes. In budding yeast there are 2 genes, *NOT3* and *NOT5*, which encode similar proteins. When the human gene was first isolated it was considered to be more similar to yeast Not3 than yeast Not5. However Not5 is the most relevant functional subunit in *S. cerevisiae*. Not4 is a core subunit since the gene is present in all eukaryotes and the human protein can complement the absence of the protein in *S. cerevisiae*. But it is a stable subunit of the complex only in budding and fission yeast. There are additional species-specific subunits of the complex. The main ones are Caf130 in budding yeast, the RNA binding protein Mmi1 in fission yeast, and Not10 and Not11 in human and flies.

The Ccr4-Not complex is built around Not1, a large scaffold protein of more than 200 kDa. The other subunits are organized in at least 4 different functional and physical separable modules that dock onto the Not1 scaffold (Fig. 13.1). Ccr4 and Caf1 constitute a deadenylase module, Not4 a ubiquitination module, a third NOT module is defined by the Not2-Not3-Not5 proteins, and finally Caf40 is module 4. It is likely that Caf130 in yeast, and Not10-Not11 in human or flies, define a fifth functional module. Consistent with different functions mediated the different modules, these dock onto different areas of the Not1 scaffold (Fig. 13.1). This docking is essential for the recruitment of the different modules to the target mRNAs.

Table 13.1 Name of *CCR4-NOT* genes in yeast, flies and human

Core name	<i>Drosophila</i> gene name	<i>S. cerevisiae</i> gene name	<i>S. pombe</i> gene name	<i>H. sapiens</i> gene name
Not1	Not1	NOT1/CDC39	NOT1	CNOT1
Not2	Regena (Rga)	NOT2/CDC36	NOT2	CNOT2
Not5	Not3	NOT5	NOT3	CNOT3
Caf1	Pop2	CAF1/POP2	CAF1	Caf1a/CNOT7/CAF1 Caf1b/CNOT8/POP2/CALIF
Ccr4	twin	CCR4	CCR4	CCR4a/CNOT6C CCR4b/CNOT6L
Caf40	Rcd1	CAF40	CAF40	CNOT9/Rcd1/CAF40/RQCD1
Not4		NOT4/MOT2/SIG1	NOT4	CNOT4
Not10	Not10			CNOT10
Not11	Not11			CNOT11/C2orf29
Caf130		CAF130		
Not3		NOT3		
Mmi1			MMI1	

Collart (2016)

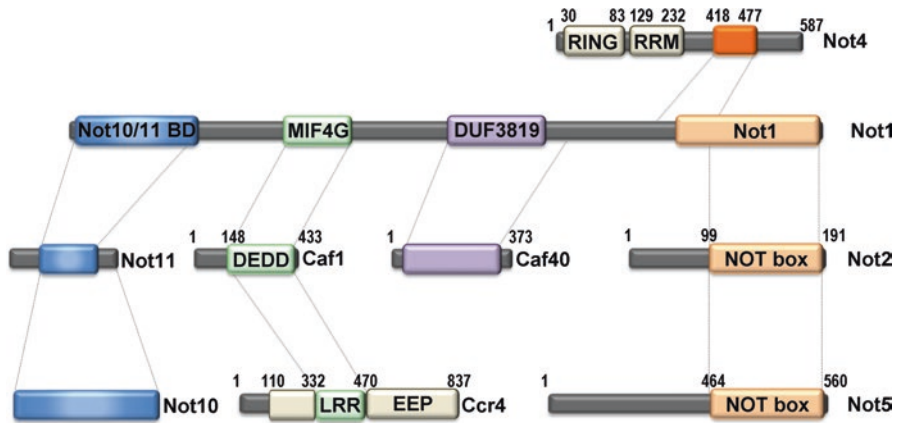


Fig. 13.1 Schematic representation of the Not1 scaffold of the Ccr4-Not complex with docking sites for the other Ccr4-Not subunits (Adapted from Bawankar et al. (2013), Collart (2016)). The numbering refers to amino acids of the yeast subunits that are discussed in the chapter. The domains of interaction between the subunits are indicated, as are additional domains of the proteins that have been identified. The human-specific Not10, Not11 subunits and their site of docking on the human Not1 scaffold are included. For each subunit, the known signature motifs are indicated by their given names (RING, RRM, MIF4G, DUF3819, DEDD, EEP, LRR and NOT box)

13.3 Architecture of the Ccr4-Not Complex

The global architecture of the complex was determined first in budding yeast in 2011 (Nasertorabi et al. 2011) and more recently in fission yeast (Ukleja et al. 2016). In the first study (Nasertorabi et al. 2011), the scaffold of the complex, Not1, was tagged at its C-terminus with a Tap-tag (consisting of a calmodulin binding peptide, a cleavage site for the Tobacco Etch Virus nuclear-inclusion-a endopeptidase (TEV) and finally a protein A moiety). Not1 was purified from total extracts on IgG sepharose beads and eluted with TEV cleavage. The Not1 eluate was then cross-linked by glutaraldehyde and fractionated on a glycerol gradient. This yielded a complex of all nine Ccr4-Not subunits with a size somewhat greater than 1 MDa. The mild cross-linking was necessary because the complex shows an inherent instability. Electron microscopy of negatively stained particles were obtained at a resolution of 33 Å. Projections of the three-dimensional map obtained revealed an L-shaped particle with 2 arms of similar lengths (180 and 190 Å) (Fig. 13.2a). The shorter arm was evaluated to account for approximately 600 kDa, the longer thinner arm for 300 kDa and the hinge for 100 kDa. The relative arrangement of the arms was observed to be variable, indicating flexibility of the whole assembly. Moreover the arms defined also a cavity in the center that could provide a platform for controlled interaction with RNA and accessory factors.

In the more recent study, the Not2 subunit was tagged at its C-terminus with the same tag used previously for Not1. The tagged Not2 was purified first by IgG sepharose and eluted by TEV cleavage. Second it was separated on a glycerol gradient.

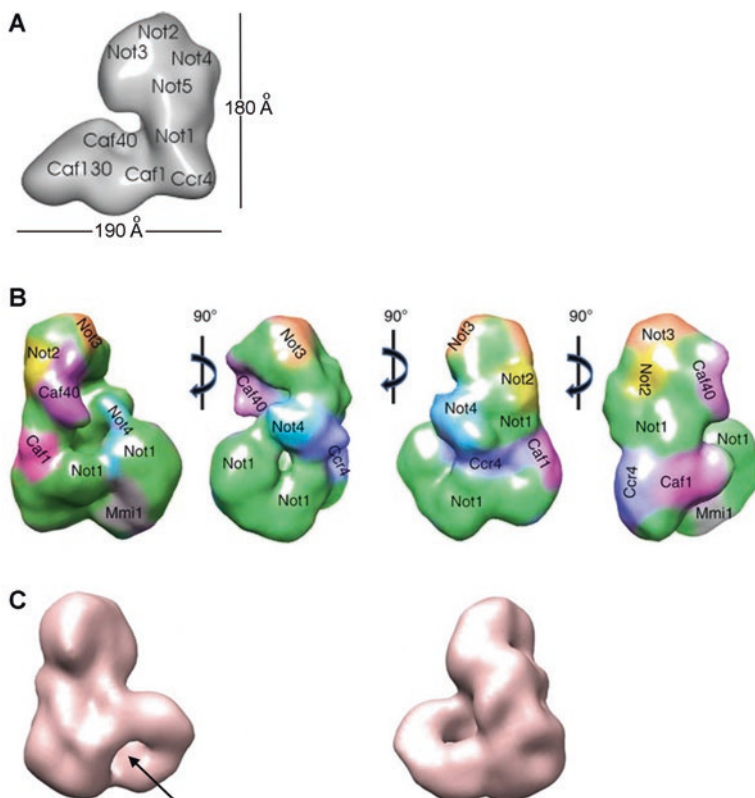


Fig. 13.2 EM structures of the Ccr4-Not complex. (a) EM structure of the *S. cerevisiae* Ccr4-Not complex with a proposed location of the nine subunits (Nasertorabi et al. 2011). The structure in the EMBD data base is ID 1901. (b) EM structure of the *S. pombe* Ccr4-Not complex with the mapped positions of the Ccr4-Not subunits and the RNA binding protein Mmi1 (Ukleja et al. 2016). The 3D reconstruction code in EMBD is EMD3232. (c) Structure of the *S. pombe* Ccr4-Not complex without Mmi1. The “hole” from lack of Mmi1 is indicated by an *arrow* in the left structure. 2 orientations are shown

High salt conditions were used to avoid co-purification of non-specific components. This procedure yielded the core Ccr4-Not components and an additional fission yeast-specific subunit Mmi1. Negative staining and 3D-reconstruction defined the same L-shaped structure described previously, with slightly shorter arms (140 and 150 Å). For cryo-EM the authors repeated the procedure with low glutaraldehyde concentrations in the glycerol gradient as used in the first study. They obtained a volume at 20 Å resolution very similar to the negative staining EM but with additional details (Fig. 13.2b).

Mmi1 is an RNA binding protein that is important for removal of meiosis-specific transcripts during vegetative growth. Its presence in the purified fission yeast Ccr4-Not complex seems to indicate a role of Ccr4-Not in regulation of meiosis in *S. pombe*. A complex without the essential Mmi1 protein was obtained using a bio-

chemical trick. The 3D reconstruction rendered a volume that was very similar, but with a small channel in the shorter mass (Fig. 13.2c).

The EM structures of the Ccr4-Not complex from fission and budding yeast are very similar, indicating that the subunit organization within the complex is conserved. For the *S. pombe* complex, immuno-EM was used to localize each subunit tagged with the green fluorescent protein (GFP) within the 3D reconstruction. The protrusions in the different images enabled the localization of the different subunits on the L-shaped front view (Fig. 13.2b).

13.4 The Ccr4-Not Complex Consists of Different Functional Modules

13.4.1 The Not2-Not5 Heterodimer

13.4.1.1 Physiological Relevance of the NOT Module

The Not2 and Not5 proteins form a heterodimer that associates with a C-terminal region of Not1 (Fig. 13.1). This forms the NOT module very important for the integrity of the entire Ccr4-Not complex. Budding yeast lacking either one of these 2 subunits have a doubling time more than 3 times longer than wild-type cells (Maillet et al. 2000) and deletions of either one of the genes encoding these 2 subunits is embryonic lethal in all animals (Maeda et al. 2001; Kamath and Ahringer 2003). In addition specific functions have been attributed to Not2 and Not5. In mouse and in human this module is important for self-renewal of ES cells, and not surprisingly mutations in *CNOT3*, the human ortholog of yeast Not5 (Table 13.1) have been identified in several cancers. For instance *CNOT3* was identified as a tumor suppressor in T-cell acute lymphoblastic leukemia and its knockdown in a sensitized *Drosophila melanogaster* model caused cancer (De Keersmaecker et al. 2013). Mutations in *CNOT3* have also been defined as a modifier of mutations in *PRPF31* responsible for retinitis pigmentosa, preventing retinal degeneration (Venturini et al. 2012). Along the same lines, a recent study modeled changes in expression of *CNOT2* and showed that this was predictive for tumor cell metastatic potential *in vivo* (Faraji et al. 2014).

An important role of the NOT module for the ability of animals to stock and mobilize nutrients has been suggested by the observation that heterozygous *CNOT3*^{+/-} mice are born smaller than wild type mice with a particular reduction of liver and adipose tissues and a higher metabolic rate (Morita et al. 2011). Such heterozygous mice show osteopenia and exacerbated ageing-induced osteoporosis (Watanabe et al. 2014). They have also revealed that *CNOT3* is a critical regulator of heart function (Neely et al. 2010). Conditional knock out of *CNOT3* has provided knowledge about additional specific functions. In B lineage cells, it revealed that *CNOT3* is important for B cell differentiation because it controls *Igh* rearrangements and it is important for p53 mRNA destabilization (Inoue et al. 2015). In

mouse embryonic fibroblasts instead, it promotes necroptosis, and this correlates with induction of mRNAs encoding cell-death inducing proteins (Suzuki et al. 2015). Clearly the Not2-Not5 module is essential for a wide-range of physiological phenomena.

13.4.1.2 Molecular Mechanism of NOT Module Action

At the cellular level, the function of the Not2-Not5 module is still unclear. Neither protein has a catalytic domain. It seems likely that this module serves as a protein interaction surface that can regulate the enzymatic activities of the Ccr4-Not complex. Indeed in several studies down-regulation of either protein was shown to have consequences on specific mRNA levels, or less frequently on length of poly(A) tails, probably because it is important for the recruitment of the Ccr4-Not complex to specific mRNAs (Morita et al. 2011). It has also been reported to contribute to decapping (Muhlrad and Parker 2005; Alhusaini and Coller 2016). One has to keep in mind that any function identified by phenotypes of cells lacking Not2 or Not5 could be indirectly due to decreased Ccr4-Not complex integrity. There is nevertheless at least one example in *Drosophila* where Not3 binds an RNA-binding protein Bicaudal-C and thereby contributes to the recruitment of the entire Ccr4-Not complex to mRNAs for their deadenylation (Chicoine et al. 2007). Moreover the structural characterization of the NOT module has shown that Not2 and Not5 NOT boxes create a V-shaped surface that displays patches of positively charged residues and have the ability within the NOT module to bind poly(U) RNA (Bhaskar et al. 2013).

Mostly this module has been connected to transcription. It interacts with a diversity of general and specific transcription factors, is present at sites of transcription and has an impact on the presence of transcription factors at promoters and on global histone acetylation levels (for review see Collart 2016). The Not2-Not5 heterodimer is also important during translation, since it is needed during synthesis of the largest subunit of RNA polymerase II to prevent its aggregation (Villanyi et al. 2014).

13.4.1.3 Structure of the Not Module

A 2.8 Å resolution crystal structure of a core of Not proteins from budding yeast (Bhaskar et al. 2013) and a corresponding structure from a core of human Not proteins at 3.2 Å resolution (Boland et al. 2013) are available (Fig. 13.3a, b). In both cases the stoichiometry is 1:1:1. The C-terminal region of Not1 that interacts with the Not2-Not5 heterodimer adopts an L-shape with two perpendicularly arranged stacks of alpha helices (shown for human CNOT1 on Fig. 13.3c with a cartoon representation on Fig. 13.3d). The topology is typical of that observed in HEAT repeat proteins where the repeats are characterized by a helix A-turn-helix B motif and are arranged in tandem in an almost parallel way. The Not1 C-terminal domain organization does not change in the trimeric NOT module (cartoon on Fig. 13.3e).

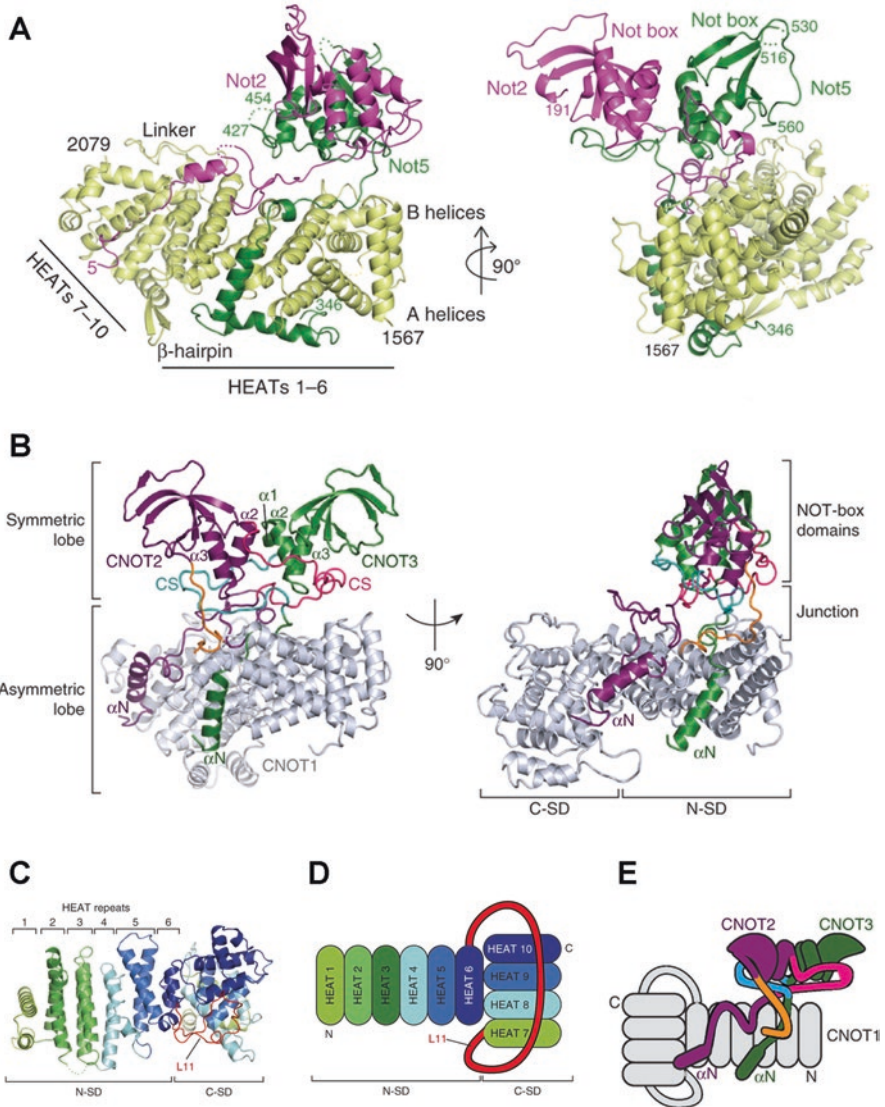


Fig. 13.3 Structure of the NOT module of the Ccr4-Not complex. **(a)** Structure of the *S. cerevisiae* NOT module (accession code PDB 4B46) (Boland et al. 2013). Not1 is depicted in yellow (residues 1567–2079), Not2 in pink (residues 5–191) and Not5 in green (residues 346 to 560). Disordered regions of Not2 (14–29 and 44–48) and two loops of Not5 (428–453) are excluded. Different structural elements are indicated. **(b)** Structure of the human NOT module (PDB 4C0D) (Bhaskar et al. 2013). The human hetero-trimeric complex contains CNOT1 indicated in silver (1842–2353), CNOT2 in pink (350–540) and CNOT3 in green (607–748). The symmetric lobe defined by the NOT box domains and the asymmetric lobe comprising the N-terminal extensions of CNOT2 and CNOT3 interacting with CNOT1 are indicated. **(c)** Structure of the C-terminal HEAT domain of CNOT1 that associates with the Not heterodimer (PDB 4C0E). **(d)** Cartoon scheme of the structure of the C-terminal Not1 HEAT domain presented in (c) (Bhaskar et al. 2013). **(e)** Cartoon scheme of the human NOT module showing that the structure of the CNOT1 domain does not change upon CNOT2-CNOT3 binding (Bhaskar et al. 2013)

Not5 and Not2 share a conserved C-terminal domain called the Not box (Fig. 13.1). This domain is a homo- or hetero-dimerization domain, and it is very similar for both proteins, human or yeast. The Not5 and Not2 homo-dimers differ in the conformation adopted by their N-terminal helices (compare Fig. 13.4a for CNOT3 homo-dimers and Fig. 13.4b for CNOT2 homo-dimers). The hetero-dimer adopts the same arrangement as the Not5 homo-dimer (Fig. 13.3a, b). The Not box is located between residues 99 and 191 for yeast Not2 and residues 464–560 for yeast Not5. Extensive interactions between the 2 proteins are centered on Phe114 and Leu115 of Not2, including Glu177, Trp176, Thr111, Phe107 and Leu106 and centered on the corresponding Phe479 and Ile480 of Not5, including Lys471, Phe472, Thr476 and Trp541 (Fig. 13.4c).

A 3D model obtained by negative EM staining of the Not2-Not5 dimer from *S. cerevisiae* shows 2 connected masses, one of about 110 Å long and one of about 70 Å long (Ukleja et al. 2016). The central part of the volume encompasses a spacious cavity most likely occupied by the C-terminal part of Not1 (Fig. 13.4d, upper row). This volume was located within the cryo-EM reconstruction of the entire Ccr4-Not complex with some manual fitting (Fig. 13.4d, lower row).

Besides their C-terminal Not box, Not2 and Not5 have extended N-terminal domains (residues 5–75 for yeast Not2 and 346–404 for yeast Not5) that span more than 100 Å and wrap around an important surface on the Not1 interaction domain (cartoon on Fig. 13.3e). The N-terminal extension of Not2 is organized in 3 segments. The first segment of Not2 (residues 5–13) binds the alpha helices of HEAT repeats 9 and 10 of the Not1 C-terminal domain (Fig. 13.5a). Leu9 of Not2 inserts into a hydrophobic pocket of Not1 defined by a number of residues including F2064, I2071, L2027, L2013 and Y1982. Residues Leu6 and Pro11 of Not2 also make important contacts. The second segment of Not2 (residues 31–64) formed by a short helix and a hairpin docks back and forth between the HEAT repeats 4–6 of the Not1 C-terminal domain (Fig. 13.5b). The helix positions itself on a hydrophobic surface centered around Arg1811 and Leu1814 of HEAT 5 with residues Leu34, Met37 and Leu41 contributing to dock the helix. The hairpin fits into a groove of HEAT repeats 4 and 5 defined by residues Phe1751 to Ile1812, such that residues Trp60, Pro59 and Phe56 can make relevant contacts (Fig. 13.5b). Finally, the third segment of Not2 between residues 65–75 interacts over the beta helices of HEAT 3 of the Not1 C-terminal domain (Fig. 13.5c). The extended N-terminal domain of Not5 is also divided in 3 relevant segments that cover HEAT repeats 1–5 of the Not1 C-terminal domain. First apolar interactions occur between residues 346–373 within an alpha helix of Not5, in particular residues Trp362, Phe350, Pro353 and Phe354, and an alpha helix of Not1 above Val 1673 and Val 1671 (Fig. 13.5d). The second segment (residues 374–391), in particular residues Met380, Leu384 and Leu388 of an alpha helix of Not5, makes hydrophobic contacts with an intra-repeat region of HEATs 3–5 of the Not1 C-terminal domain, around residues Phe1788, Pro1743 and Phe1751 (Fig. 13.5e). Finally the third segment of Not5 (residues 392–404) makes both polar and apolar contacts, involving in particular Asp393, Asp396, Pro400 and Leu402, in the vicinity of Arg65 of Not2, and on top of beta helices between HEAT repeats 1–3 of the Not1 C-terminal domain (Fig. 13.5c).

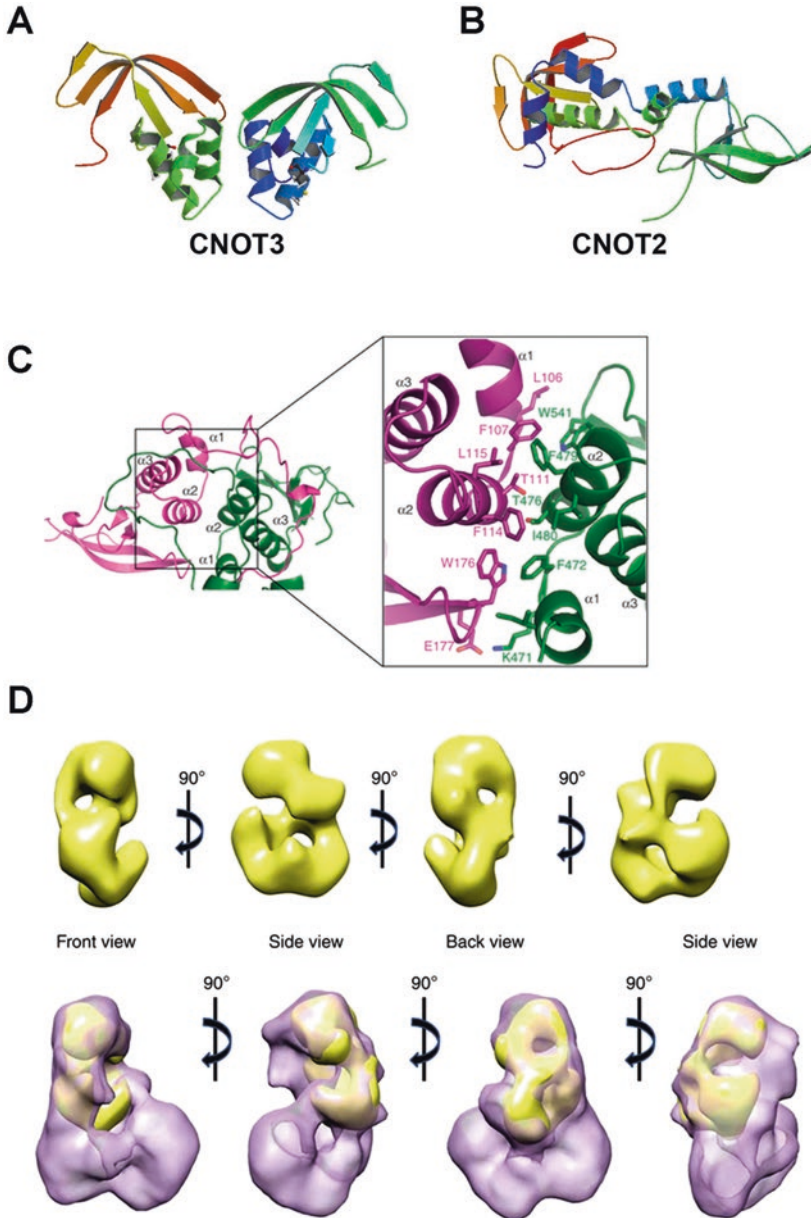


Fig. 13.4 Structures of the NOT box dimers. (a) The human CNOT3 homo-dimer is depicted (PDB 4C0G) (Boland et al. 2013). (b) The human CNOT2 homo-dimer is depicted (PDB 4C0F). (c) The Not box interaction surface between yeast Not2 and yeast Not5 is depicted (Bhaskar et al. 2013) (PDB 4BY6). Not2 is indicated in *pink* and Not5 in *green*. (e) 3D reconstruction of the *S. cerevisiae* Not2-Not5 heterodimer at 16 Å resolution (*upper row*) and manual docking of this reconstruction onto the *S. pombe* cryo-EM 3D reconstruction shown above in Fig. 13.3 (Ukleja et al. 2016)

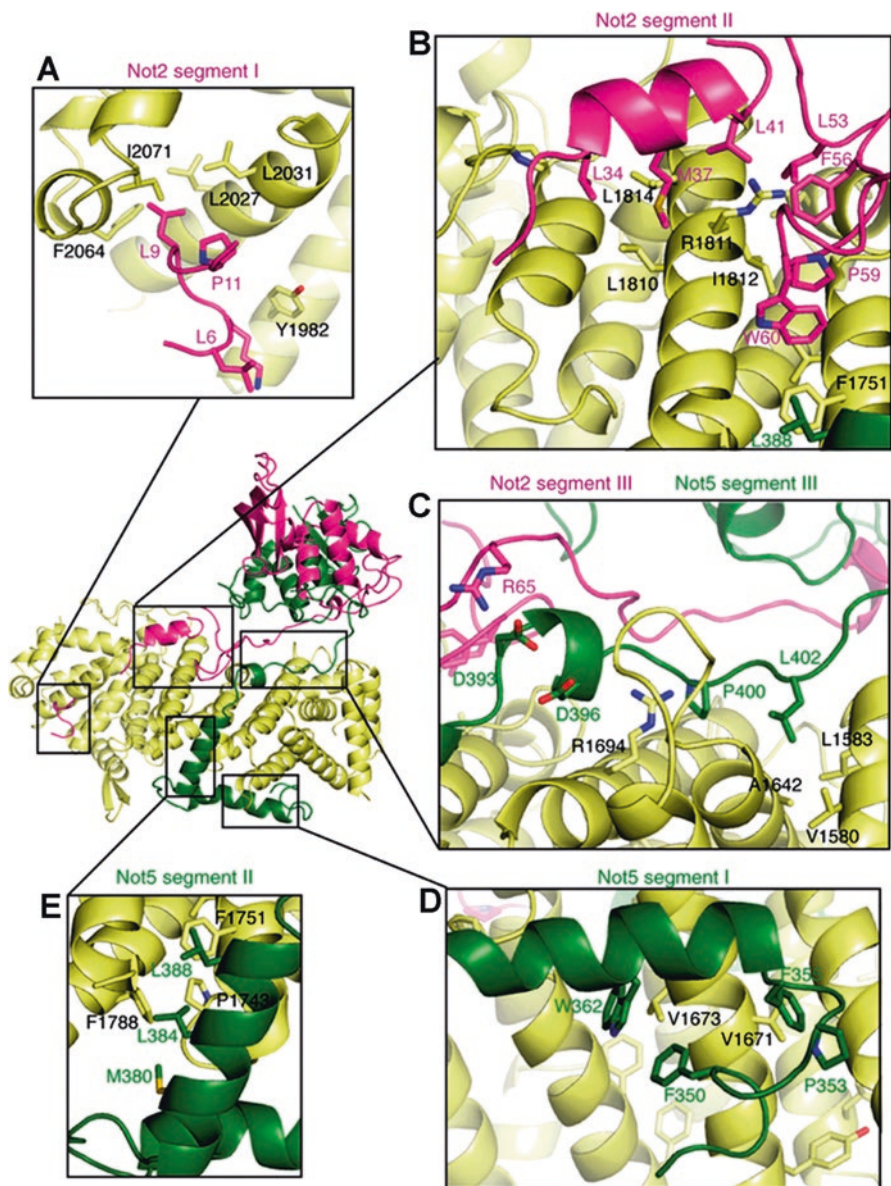


Fig. 13.5 Expanded view of the interaction surfaces between yeast Not1, Not2 and Not5 (Bhaskar et al. 2013). Conserved residues that participate in interactions are indicated. Not1 is in yellow, Not2 in pink and Not5 in green. (a) The Not2 segment 1 that interacts with Not1 is expanded. (b) The Not2 segment 2 that interacts with Not1 is expanded. (c) The Not2 and Not5 segments 3 that interact with each other and with Not1 are expanded. (d) The Not5 segment 1 that interacts with Not1 is expanded. (e) The Not5 segment 2 that interacts with Not1 is expanded

These structures reveal that the formation of the NOT module requires the cooperative binding of the N-terminal domains of Not2 and Not5 that extend from the Not box to the C-terminal domain of Not1. The Not2-Not5 Not box heterodimer forms a symmetric lobe above the conserved helical surface of the more N-terminal portion of the Not1 interacting domain (Fig. 13.3a, b).

13.4.2 *The Ubiquitylation Module*

13.4.2.1 *Physiological Role of Not4*

Our knowledge of Not4's physiological role is less extensive than for the other core Ccr4-Not subunits, and it concerns mostly yeasts. In *Candida albicans* Not4 is necessary for hyphal development and pathogenicity (Krueger et al. 2004). In *K. lactis* it is important for carbon metabolism (Mazzoni et al. 2005). In *S. cerevisiae* Not4 is necessary for resistance to DNA replication stress induced by hydroxyurea (Mulder et al. 2005) probably because it is needed for destabilization of Cdc17 itself needed for resistance to hydroxyurea (Haworth et al. 2010). Not4 is also needed during oxidative stress in budding yeast for degradation of cyclin C, that with the Cdk8 kinase represses a subset of stress genes needed for resistance to oxidative stress (Cooper et al. 2012). It also contributes to degrade the oxidative stress-induced transcriptional activator Yap1, and thereby limits the duration of the stress response induction (Gulshan et al. 2012). In human and in flies, Not4 is a positive regulator of the JAK/STAT pathway essential for innate immunity, organogenesis and stress responses (Gronholm et al. 2012).

13.4.2.2 *Molecular Mechanism of Not4 Action*

Not4 contains an N-terminal RING domain (Fig. 13.1) and harbors E3 ligase activity in yeast and in human. It depends upon its interaction with the Ubc4/5 E2 enzymes in yeast and the ortholog UbcH5 in humans (Albert et al. 2002; Mulder et al. 2007). It ubiquitylates a wide range of substrates (Panasenکو et al. 2006; Mersman et al. 2009; Cooper et al. 2012; Gulshan et al. 2012; Panasenکو and Collart 2012; Sun et al. 2015; Zhang et al. 2015), in the nucleus and in the cytoplasm, and as indicated above, this activity is certainly responsible for many of the physiological roles assigned to Not4. Curiously however, mutations that abolish Not4's interaction with its partner E2 enzymes in budding yeast has only very mild phenotypes compared to deletion of the entire RING domain, or C-terminal truncation of the protein that abolishes Not4's binding to Not1 (reviewed in Collart 2013). Thus the importance of Not4's docking onto Not1 and function as E3 ligase still needs to be clarified in the context of global Not4 function.

Recent data has linked Not4 to translation and in particular to co-translational quality control (reviewed in Collart 2013; Panasenکو 2014). However the function

of Not4 during translation is rather controversial. It is reported to poly-ubiquitinate proteins at stalled ribosomes (Dimitrova et al. 2009), repress translation initiation from mRNAs carrying stalled ribosomes (Preissler et al. 2015) or finally preserve translation from mRNAs carrying transiently stalled ribosome complexes (Halter et al. 2014). A model to reconcile these different observations suggests that Not4's action may occur in 2 steps, first prevent repression of translation initiation in case the ribosome can resume elongation, and second let repression occur and allow mRNA decay to proceed if the ribosome is definitively stalled (Collart 2016).

A ribosome-associated chaperone and a ribosomal protein are Not4 substrates, and interestingly these substrates are not de-stabilized by ubiquitination but accumulate instead as stable mono-ubiquitinated proteins. However whether these ubiquitination events mediate the roles of Not4 in translation is not known. Not4 also has a putative RNA-Recognition-Motif (RRM) and it docks onto the Not1 scaffold via a C-terminal domain (Fig. 13.1).

13.4.2.3 Structure of the Ubiquitination Module

The structure of the RING domain of CNOT4 was solved first (Hanzawa et al. 2001) and is unusual in the sense that it folds via a C4C4 motif in which zinc is coordinated by 8 cysteine residues. A structure of the RING domain of yeast Not4 with the yeast Ubc4 E2 enzyme (Fig. 13.6a) became available recently (Bhaskar et al. 2015). It is very similar to the human Not4 RING domain in isolation and also to the previously determined structure of Ubc4 in isolation (Cook et al. 1993). This structure allowed for the suggestion that the specificity of Not4 for the Ubc4 E2 enzyme is due to subtle differences in the more extensive interaction network possible between Ubc4 and Not4 compared to what could occur between Not4 and other E2 enzymes.

A complex between the C-terminal region of yeast Not4 (residues 418–477) and residues 1541–2093 of yeast Not1 led to crystals that diffracted to a resolution of 3.6 Å (Bhaskar et al. 2015). The HEAT repeat structure of Not1 in this structure is similar to that in the NOT module (see above). The C-terminal domain of Not4 that binds Not1 folds into an alpha helix that is flanked by regions lacking defined secondary structural elements (Fig. 13.6b). Several amino acids are critical for three different points of contact between Not1 and Not4 (Fig. 13.6c). The mutations of 2 residues in the first interacting segment of Not4, L463E, F464E or F464E, W466E, is sufficient to disrupt the association of the 2 proteins. The resolution of this structure has provided some explanation for the curious observation that Not4 is a stable subunit of the complex in budding and fission yeast but not in metazoa (Lau et al. 2009; Temme et al. 2010). Indeed, the third segment of Not4, essential for stable binding to Not1 in yeast, is not present in CNOT4.

The NOT module and the ubiquitination module bind to a similar C-terminal domain of Not1. However, a superposition of the yeast NOT structure and the structure of the ubiquitination module suggests that the binding of the Not2-Not5 heterodimer and Not4 enzyme onto Not1 are independent (Fig. 13.6d).

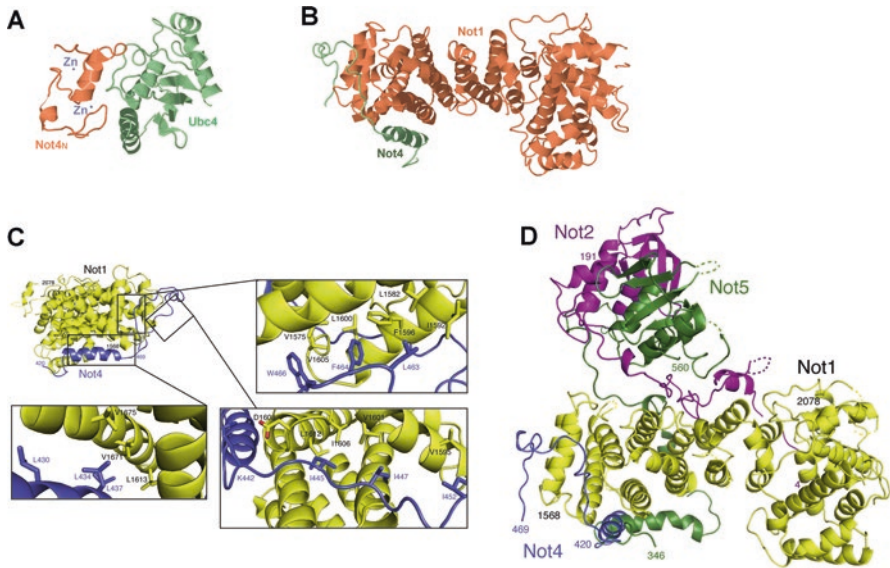


Fig. 13.6 Structure of the ubiquitination module of the Ccr4-Not complex (Bhaskar et al. 2015). (a) Structure of the complex between the *S. cerevisiae* N-terminal Not4 RING E3 domain (residues 30–83) depicted in *orange* and the *S. cerevisiae* Ubc4 E2 (residues 1–148) depicted in *green* (PDB 5AIE). 2 ions of Zn^{2+} are indicated. (b) Structure of the C-terminal domain of *S. cerevisiae* Not1 (residues 1541–2093) depicted in *orange* and the *S. cerevisiae* Not4 C-terminal domain (residues 418–477) depicted in *green* (PDB 5AJD). (c) Expanded view of the interaction interface between Not1 and Not4. Not1 is in *yellow* and Not4 is in *purple*. The residues involved in interactions are shown as *sticks* and their positions are labeled. (d) Superposition of the NOT and ubiquitination modules determines that Not4 C-terminal domain and the Not2-Not5 heterodimer bind independently. Not1 is indicated in *yellow*, Not2 in *pink*, Not5 in *green* and Not4 in *green* (Not1 interaction domain) and *blue* (Bhaskar et al. 2015)

13.4.3 Caf40

13.4.3.1 Physiological Relevance of Caf40

Caf40 was first known under the name Rcd1 (Required for Cell Differentiation) when it was identified as a factor essential for nitrogen-starvation-induced sexual differentiation in fission yeast (Okazaki et al. 1998). In mammalian cells it was described as a critical transcriptional co-factor for retinoic acid-induced differentiation of teratocarcinoma cells and for lung development (Hiroi et al. 2002). It is frequently up-regulated in breast cancer biopsies and cell lines, and its knock down suppresses their proliferation (Ajiro et al. 2009). Very recently a recurrent mutation in Caf40, P131L, was identified in cutaneous melanoma (Wong et al. 2015). In budding yeast, Caf40 has not been specifically characterized by mutant phenotypes, and it was identified biochemically as a component of the Ccr4-Not complex (Chen et al. 2001).

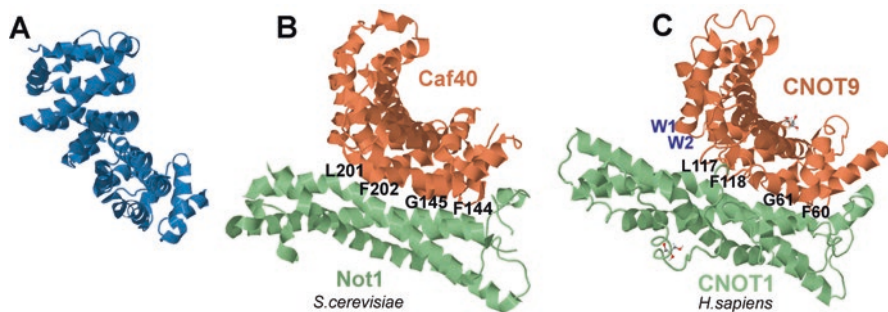


Fig. 13.7 Structure of the Caf40 module of the Ccr4-Not complex. (a) Cartoon representation of human CNOT9 (PDB 2FV2). (b) Structure of the *S. cerevisiae* Caf40 in complex with the DUF Not1 binding domain (PDB 4CV5). Not1 is in *green* and Caf40 is in *orange* (Mathys et al. 2014). Caf40 residues that contribute to interaction are indicated on the interface in a cartoon way. (c) Structure of human CNOT9 in complex with the CNOT1 DUF domain (PDB 4CT7) in the same color code as for panel B (Mathys et al. 2014). CNOT9 residues that contribute to the interaction are indicated above the interface. W1 corresponds to CNOT1 Trp1603 that is included in the structure despite being within an unstructured region and W2 is an additional Trp that gets included in the structure upon addition of an excess of free L-Trp (Chen et al. 2014). These Trp residues reveal that Caf40 has Trp-binding pockets

13.4.3.2 Molecular Mechanism of Caf40 Action

Until recently very little was understood about the mechanism of Caf40 action. It interacts with transcription factors and alters their activation potential (Hiroi et al. 2002; Haas et al. 2004; Garapaty et al. 2008). It can bind single stranded DNA polymers, but surprisingly not poly(A) sequences (Garces et al. 2007). Finally, it is important for the interaction of an RNA helicase Mtr4 and an exonuclease Rrp6, which are co-factors of the nuclear exosome (Azzouz et al. 2009). A breakthrough has been the recent finding that the interaction of Caf40 with Not1 provides binding sites for GW182/TNRC6 proteins of the microRNA machinery and thereby contributes to gene silencing by microRNAs (Chen et al. 2014; Mathys et al. 2014). A common theme between these different observations is that Caf40, through its association with Not1 and ability to bind single stranded nucleic acids, might provide surfaces that tether together proteins and nucleic acids. The recent structural resolution of Caf40-Not1 complexes makes it now possible to test Caf40 derivatives that do not associate with Not1 and progress in our understanding of this functional module of Ccr4-Not.

13.4.3.3 Structure of the Caf40 Module

A first crystal structure of human Caf40 revealed that the protein has an Armadillo repeat formed by a bundle of alpha helices arranged in a clockwork spiral (Garces et al. 2007) (Fig. 13.7a). A positively charged cleft was suggested to be important

for nucleotide binding while the super-helix produced by the folded Armadillo repeats thought to provide a versatile platform for protein-protein interactions.

Caf40 and its orthologs dock onto a DUF3819 domain in the middle of the Not1 protein (Fig. 13.1). A complex of yeast Caf40 (residues 102–370) and yeast Not1 (residues 1071–1282) was solved at 3.8 Å resolution (Fig. 13.7b) (Mathys et al. 2014). A similar complex of the human orthologs, CNOT9 (residues 16–284) and CNOT1 (residues 1351–1588), was obtained and refined at 2 Å resolution (Fig. 13.7c) (Mathys et al. 2014). Another structure at 1.65 Å resolution of a complex between CNOT9 and a fragment of CNOT1 composed of residues 1356–1607 was reported independently (Chen et al. 2014). The new structures of Caf40 and CNOT9 in complex with Not1 and CNOT1 are virtually identical to the previous one of CNOT9 in isolation, suggesting that CNOT9 does not undergo major rearrangements upon CNOT1 binding. It was observed that CNOT9 in isolation forms homo-dimers, but the interaction surface between CNOT9 and CNOT1 partially overlaps with the homo-dimerization domain of CNOT9 and it is more extensive, indicating that CNOT9 and CNOT1 complexes are probably favored over the homo-dimer in vivo. Multiple mutations are needed to disrupt this interface.

The CNOT9-binding domain of CNOT1 is composed of 7 alpha helices. It differs from the other docking domains of Not1 organized into HEAT repeats and it is quite a unique folding domain. The specific region of CNOT1 that contacts CNOT9 is very much disordered. It participates in an important way in the complex however, because it inserts Trp1603 into a Trp-binding pocket of CNOT9 and it is therefore required for crystallization. This Trp residue is clearly defined in the structure (indicated W1 on Fig. 13.7c). A second Trp-binding pocket in CNOT9 is visible when excess of L-Trp is added in the crystallization solution (W2 on Fig. 13.7c). The distance between the two Trp pockets is 20–25 Å. They are not relevant for CNOT9 homo-dimerization. However, if the 2 pockets are mutated (A248F, Y203A, R244A for pocket 1 and P165G, R205A, H208A for pocket 2), CNOT9 in complex with its CNOT1 docking domain fails to bind TNRC6 in vitro. This would suggest that CNOT9 has the primary Trp-binding sites yet in vivo CNOT9 does not bind TNRC6 if it is defective for interaction with CNOT1. Moreover full-length CNOT1 mutants that no longer interact with CNOT9 can bind TNRC6. These observations indicate that CNOT1 must carry additional sites for TNRC6 besides those available on CNOT9. Similar observations were made with the *Drosophila* proteins indicating that these interactions are conserved. They are consistent with many studies indicating that GW182/TNRC6 proteins bind CNOT1 through multiple W-containing motifs. Moreover binding redundancy correlates also with functional redundancy for mRNA repression.

13.4.4 The Ccr4-Caf1 Nuclease Module

13.4.4.1 Physiological Roles of Ccr4 and Caf1

Most eukaryotic mRNAs are heterogeneously polyadenylated. This ensures their stability in the cytoplasm and their translatability. Ccr4 and Caf1 are 2 different deadenylating enzymes. They come into action after an initial shortening of poly(A) tails to a rather homogenous length of approximately 110 nucleotides by the Pan2-Pan3 deadenylases. They function at the rate-limiting step, to deadenylate mRNAs further to a rather heterogenous length between 20 and 110 nucleotides. This initiates mRNA degradation and translational repression. This process concerns all polyadenylated mRNAs and is the major route for mRNA degradation in eukaryotes.

Since Ccr4 and Caf1 contribute to decay of all mRNAs, one might expect that these proteins are essential. However, this is not the case either in higher eukaryotes or in yeast, where they are encoded by single copy genes. Hence one has to conclude that deadenylation by Ccr4 and Caf1, even though it is the major route for mRNA decay of all polyadenylated mRNAs in eukaryotes, is only essential under certain circumstances. In most cases, either cells use alternative routes to degrade mRNAs in the absence of Ccr4 and Caf1, such as the 3' to 5' exonuclease called the exosome, or cells adapt to increased mRNA stability by reducing transcription, or more likely both mechanisms co-exist.

Ccr4

Genetic and RNA interference experiments have defined the role of Ccr4 in many different species ((Goldstrohm and Wickens 2008) and for review see (Shirai et al. 2014)). In yeast only Ccr4 but not Caf1 is active as an enzyme, whereas in contrast in *Trypanosoma brucei* no ortholog of Ccr4 has been identified. In *Drosophila* cell lines, depletion of Ccr4 has no major effect on bulk poly(A). However adult flies bearing a null allelic combination of its encoding gene *twin* have an increased steady-state bulk poly(A) tail length, and decay of specific mRNAs is slower than in wild type. The females are affected in their reproductive capacity. Moreover, embryos derived from *twin* mutant mothers have a reduced viability (for review see Temme et al. 2014). Self-renewal of germ line stem cells needs recruitment of Ccr4 by the specific RNA binding proteins Nanos and Pumilio. In *C. elegans* germ cells, depletion of *CCR4* leads to a global increased poly(A) tail length (Nousch et al. 2013). In mammals, the 2 Ccr4 orthologs have partially overlapping functions, but distinctive tissue-specific distribution, with CNOT6L being ubiquitous and CNOT6 restricted to testis ovary, thymus and spleen (Chen et al. 2011). Consistently, CNOT6L seems to play a more major role. Nevertheless, mice lacking either ortholog are viable and fertile. Both human orthologs contribute to growth of the MCF-7 breast cancer cell line, but only CNOT6L contributes to growth of murine NIH3T3

fibroblasts (Morita et al. 2007). Moreover depletion of CNOT6L has more important effects on mRNA levels than CNOT6 (Morita et al. 2007; Mittal et al. 2011). Recent data has linked changes in expression of the Ccr4 orthologs to several cancers. Loss of CNOT6L copy number in human colon adenocarcinoma samples (Tay et al. 2011) or reduced expression of CNOT6L in ALL or AML leukemia cells with slight up-regulation of CNOT6 (Maragozidis et al. 2012) have been reported, as well as a significant association of SNPs in CNOT6 with B-cell ALL susceptibility (Gutierrez-Camino et al. 2014).

Caf1

The biological significance of Caf1 varies among eukaryotes. In *S. cerevisiae* Caf1 is essential for deadenylation because it tethers Ccr4 to Not1, but it does not play a catalytic role (Tucker et al. 2002). In *Drosophila* the catalytic activity of Caf1 is thought to be more potent than that of Ccr4 (Temme et al. 2004, 2010) and in *Trypanosoma brucei* it is the only deadenylase (Schwede et al. 2008). In mammals the two orthologs are ubiquitously expressed. They have overlapping roles in repressing expression of anti-proliferative genes and allowing cell proliferation (Aslam et al. 2009). CNOT7 is a downstream effector in interferon pathways and degrades STAT1-regulated mRNAs (Chapat et al. 2013). Its knockout in mice results in severe infertility associated with a defect in spermatogenesis (Berthet et al. 2004; Nakamura et al. 2004). The mice also show increased bone mass because CNOT7 functions as a suppressor of bone morphogenetic protein signaling (Washio-Oikawa et al. 2007). Like CNOT6L, CNOT7 is down-regulated in ALL and AML leukemia cells. CNOT8 on the other hand is elevated in colorectal carcinoma and metastatic lesions compared to the normal mucosa (Seiden-Long et al. 2006).

13.4.4.2 Molecular Mechanism of Ccr4 and Caf1 Action

Ccr4 and Caf1 are both deadenylases, but they belong to distinct families based upon their nuclease domain signature. Both hydrolyze the RNA in the 3' to 5' direction with the concomitant release of 5'AMP. Caf1 in yeast, or the CNOT7 and CNOT8 human orthologs, belong to the DEDD-type nucleases that have conserved catalytic Asp and Glu residues. Ccr4 in yeast, or CNOT6L and CNOT6 in human, are members of the exonuclease-endonuclease-phosphatase (EEP) family. The catalytic roles of Ccr4 and Caf1 differ in different organisms. In addition, Caf1 serves in all eukaryotes an additional scaffolding or tethering function.

Deadenylation is catalyzed by two metal ions coordinated by acidic residues. Deadenylating enzymes require an active catalytic site and must be tethered to their substrates, because they do not degrade cytoplasmic mRNA poly(A) tails indiscriminately. They get tethered to their target mRNAs in several different ways. As mentioned above, Ccr4 is tethered to the Ccr4-Not complex via Caf1. In turn the complex will be recruited to mRNAs. Components of the antiproliferative BTG-

TOB family of proteins serve as one set of adapter proteins that interact with CNOT7 or CNOT8 to tether the Ccr4-Not complex to mRNAs. Tob1 for instance, interacts with the Poly(A) binding protein PABP for general recruitment of the deadenylase to mRNAs. The Caf1 proteins can also interact with CEBP3 bound to a cis element upstream of the poly(A) site for specific recruitment (for review see Doidge et al. 2012). RNA binding proteins, independently of BTG-TOB proteins, also recruit the deadenylase to mRNAs. One particularly well-studied example is tristetraprolin (TTP) that recognizes AU-rich elements and mediates rapid mRNA decay (Sandler et al. 2011). Another example is Nanos2, an RNA binding protein required for development of germ cells and male fertility (Suzuki et al. 2010) (see further) or the pumilio protein Mpt5 in yeast (Goldstrohm et al. 2006). In several cases, different subunits of the Ccr4-Not complex have been shown to associate with RNA binding proteins to tether Not1 to mRNAs. One example is CNOT3 that interacts with Bialdal-C (Chicoine et al. 2007). Finally the miRNA machinery recruits the Ccr4-Not deadenylase to mRNAs, by interacting with Not1 and Caf40 that both have Trp-binding pockets (see above, and reviewed in Collart 2016).

Deadenylation activity *in vivo* requires the Ccr4-Not complex even though *in vitro* the enzymes are active without being integrated into the complex (Basquin et al. 2012). This seems curious, since in many cases CNOT7 and CNOT8 are at the same time the catalytic subunit and the Ccr4-Not subunit that is tethered to the mRNA. Maybe the Ccr4-Not complex is important *in vivo* for stability of the deadenylases, but this is probably not the only explanation. Indeed, in *Drosophila*, a Caf1 mutant that does not interact with Not1 is dominant-negative (Petit et al. 2012).

13.4.4.3 Structures of the Ccr4 and Caf1 Deadenylases

The crystal structure of Caf1 was first defined for the *S. cerevisiae* protein in 2003 (Thore et al. 2003). The overall structure displays 13 alpha helices and 6 beta strands (Fig. 13.8a). It has the common fold of DEDD-type nucleases, but has instead a SEDQ motif and lacks essential residues to be catalytically active. Caf1 from the fission yeast *S. pombe* is very similar (Fig. 13.8b), but reveals an intact DEDD motif that can chelate the metal ions (Fig. 13.8c). The metal ions have a great importance on the rate of deadenylation. The presence of Zn²⁺ in the catalytic site for instance slows the reaction whereas high activity is observed with Mn²⁺ (Andersen et al. 2009). A structure of the mammalian ortholog, CNOT7, in complex with a BTG-TOB protein was solved (Horiuchi et al. 2009) (Fig. 13.8d), and the CNOT7 within this structure has high structural similarity to the yeast Caf1 proteins (reviewed in Bartlam and Yamamoto 2010). Like for the yeast protein the highest activity for CNOT7 was observed with Mn²⁺.

Ccr4 is the major cytoplasmic deadenylase in yeast. It has a non-conserved N-terminal region that is rich in glutamines and asparagines and was originally described to interact with the transcription machinery. The central region of Ccr4 contains tandem copies of a leucine-rich-repeat domain (LRR) that interacts with Caf1 and docks Ccr4 onto the Not1 scaffold (Fig. 13.1) (Malvar et al. 1992; Draper

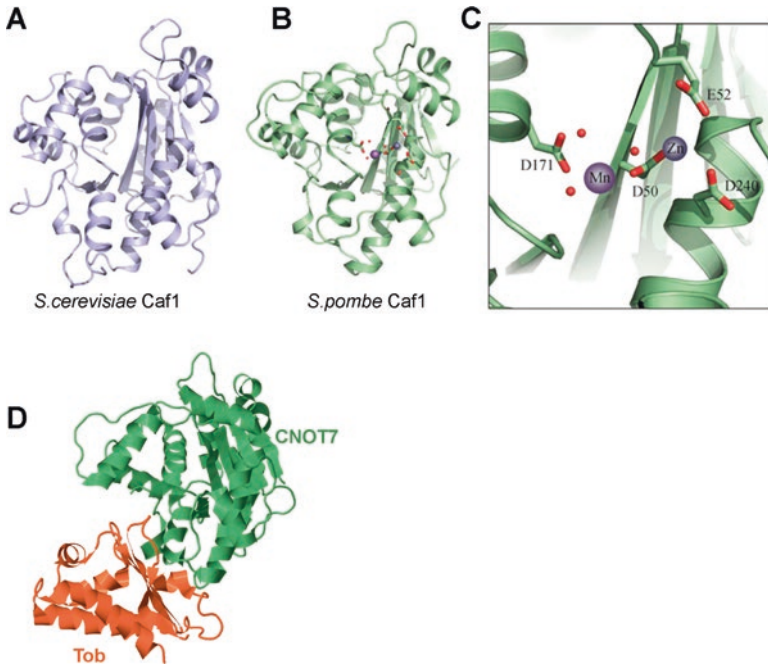


Fig. 13.8 Cartoon representations of Caf1 structures (Bartlam and Yamamoto 2010). (a) The structure of *S. cerevisiae* is represented (PDB 1U0C). (b) The structure of *S. pombe* is represented (PDB 2P51). (c) Expanded view of the catalytic site with Mn²⁺ is represented. (d) Structure of human CNOT7 (green) in complex with Tob1 (orange) is depicted (PDB 3DJU)

et al. 1995; Liu et al. 2001). The C-terminal region contains the deadenylase domain typical of the EEP superfamily, with conserved catalytic Asp and His residues in the active site. An intact Ccr4 nuclease domain structure for human CNOT6L is available (Wang et al. 2010). It has a heart-like shape in which outer layers of alpha helices sandwich 2 interior beta sheets (Fig. 13.9a). It reveals a prominent active-site pocket at the top of the molecule with 2 bound Mg²⁺ ions. Glu240 coordinates the first ion and is essential, whereas Asp410, Asn412, and His529 coordinate more weakly the second one (Fig. 13.9b). Activity is Mg²⁺-dependent and targets strictly poly(A) RNA substrates. Structural studies have indicated that residues Pro365, Asn412, and Phe484 are important for substrate recognition and have explained substrate specificity. Indeed, even the purine base G, which shares the most similarity with A, has a carbonyl oxygen in position 6' of the base that would clash with Asn412, while the pyrimidine bases could not be accommodated within the recognition pocket.

The deadenylase module docks onto the Not1 MIF4G domain via the Caf1 subunit. A structure of the human CNOT1 MIF4G domain (residues 1093–1317) alone (Fig. 13.10a) and in complex with CNOT7 (Fig. 13.10b) was solved. The very N- and C-terminal residues of CNOT7 are not visible in the structure and are probably flexible or disordered. The Not1 MIF4G domain comprises five helical hairpins

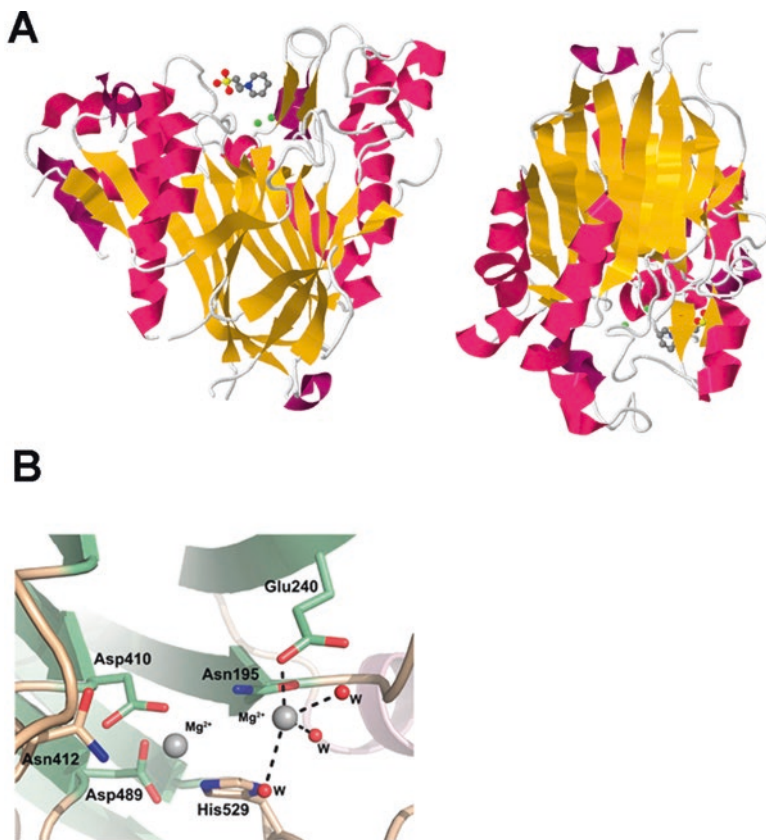


Fig. 13.9 Structure of the Ccr4 nuclease domain. (a) Cartoon representation of CNOT6L (PDB 3NGQ) in 2 different orientations. 3-pyridinium-1 YLPropane-1-sulfonate (PPS) used for the crystallization is visible in the structure close to 2 Mg^{2+} ions shown in green. (b) Expanded view of the catalytic site with 2 Mg^{2+} ions (Wang et al. 2010)

organized in HEAT-like repeats that are followed by a C-terminal extension that wraps around alpha helices 9 and 7 in a structurally conserved manner (Fig. 13.10a). An alignment of Not1 MIF4G domains shows a conservation of 41 residues throughout the domain. Many of these conserved residues lie within the hydrophobic core, or within internal interaction regions between the helices, indicating that these conserved residues are needed for the global integrity of the domain. Some conserved residues are also in loops between helices, solvent exposed, and thus are likely to engage in protein-protein interactions. The loops between helices 6 and 7 and 8 and 9 are the ones that are involved in Caf1 binding. Many important proline residues are also conserved (see further).

A structural view of a complete deadenylase module has been reported for the yeast complex (Fig. 13.10c). This structure contains only a partial Ccr4 nuclease domain, but the structure of the nuclease domain of CNOT6L can be superimposed

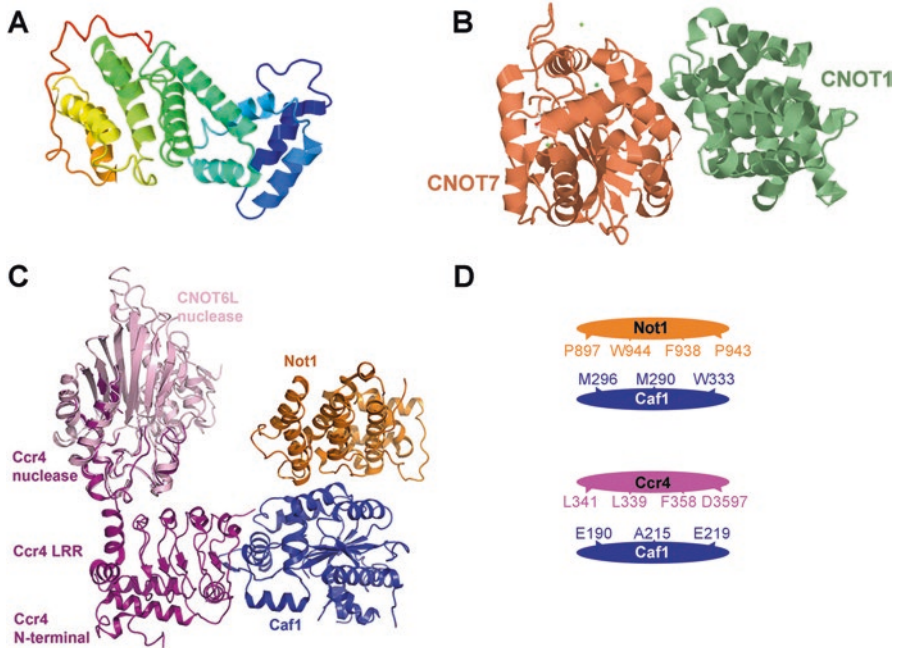


Fig. 13.10 Structure of the Ccr4-Not nuclease module. (a) Structure of the MIF4G domain of CNOT1 (PDB 4GML). (b) Structure of the complex of human CNOT7 (orange) with the CNOT1 MIF4G binding site (green) (PDB 4GMJ). (c) Structure of the entire nuclease module of *S. cerevisiae* (PDB 4B8C) (Basquin et al. 2012) with the additional superposition of the complete CNOT6L nuclease domain to complete the structure (Xu et al. 2014). The Not1 MIF4G domain is orange, Caf1 is in blue, yeast Ccr4 is in dark pink and the CNOT6L nuclease domain is in light pink. The different domains of Ccr4 are indicated. (d) Cartoon indication of the important residues within the interaction interfaces between Not1 and Caf1, as well as between Caf1 and Ccr4 (Basquin et al. 2012)

to give a complete view (Xu et al. 2014). The N-terminal arm of Not1 (residues 754–1000) shows the same HEAT motif as the human protein, with its two antiparallel helices and successive HEAT motifs packed together nearly parallel. The domain of Caf1 that associates with Not1 is opposite to its active site pocket, such that, like for CNOT7 in complex with the MIF4G domain of CNOT1, very little of the total accessible solvent area is masked within the Not1-Caf1 interface. Ccr4 interacts with Caf1 via its LRR domain. It makes no direct interaction with Not1 and its nuclease domain remains accessible. There is a disordered 70 amino acid linker between the yeast-specific N-terminal domain of Ccr4 and the LRR domain, and inter-molecular interactions build a single structural unit. The part of the Ccr4 nuclease domain that is resolved in this nuclease module is the helices that interact with the LRR domain. Hydrophobic and polar contacts are defined in the surface between the LRR and nuclease domains. A cartoon of the important residues defining interactions between the 3 subunits of the nuclease module is shown (Fig. 13.10d)

(Basquin et al. 2012). The L339E, L341E double mutation in Ccr4 disrupts the interaction between Ccr4 and Caf1 but does not touch the interaction between Caf1 and Not1, while a M290K, M296K double mutation in Caf1 disrupts the interaction with Not1 but does not affect the interaction with Ccr4 (Basquin et al. 2012).

13.4.5 *Non-conserved Ccr4-Not Subunits*

There is no structural information concerning the non-conserved subunits, except for the RNA binding protein Mmi1, which is present in the cryo EM structure of *S. pombe* Ccr4-Not. In the case of Not10 and Not11, only the subunits' docking site onto the very first amino acids of Not1 have been defined (Fig. 13.1) (Mauxion et al. 2013).

13.5 Additional Structural Information

Several additional structures between Not1 and other proteins are available. For instance there are structures for CNOT1 and the RNA binding protein TTP (Fabian et al. 2013) or Nanos (Bhandari et al. 2014), or for the DEAD-box protein DDX6 and CNOT1, with or without additionally the 4E-T protein (Chen et al. 2014; Mathys et al. 2014; Ozgur et al. 2015).

The Nanos proteins interact with the same C-terminal domain of CNOT1 that interacts with the CNOT2-CNOT3 heterodimer, within the so-called superfamily homology domain (SHD). They insert conserved aromatic residues into a hydrophobic pocket on the CNOT1 surface. A crystal structure between the CNOT1 SHD (residues 1833–2361) and the interaction peptide of Nanos 1 (amino acids 40–56) was resolved at 2.8 Å (Fig. 13.11a, b). The overall arrangement of the CNOT1 domain in the CNOT1-Nanos 1 structure does not change compared to its arrangement in the NOT module, except in an alpha helix that displays 2 additional turns in the CNOT1-Nanos 1 complex. This observation suggests that the Nanos binding pocket is not preformed, but instead is induced by binding.

The binding site for TTP on the Not1 scaffold is located between amino acids 800–1015 of CNOT1. A structure at 1.5 Å resolution of this domain with a peptide of human TTP covering residues Ala312 to Glu326 was obtained (Fig. 13.11c). The N-terminal 20 residues of CNOT1 of this domain are disordered, while the region between residues 820 and 999 consists of 8 alpha helices stacked as a series of helix-turn-helix motifs, similar to the typical HEAT repeats found throughout Not1. The TTP binding site is formed by a highly conserved hydrophobic groove between 2 alpha helices flanked by negatively charged patches (Fig. 13.11c).

Dhh1 in yeast, or DDX6 in human and flies, interacts with Not1 through the MIF4G domain. This protein is thought to contribute to gene repression induced by tethering of the Ccr4-Not complex to mRNAs. A co-crystal of the CNOT1 MIF4G

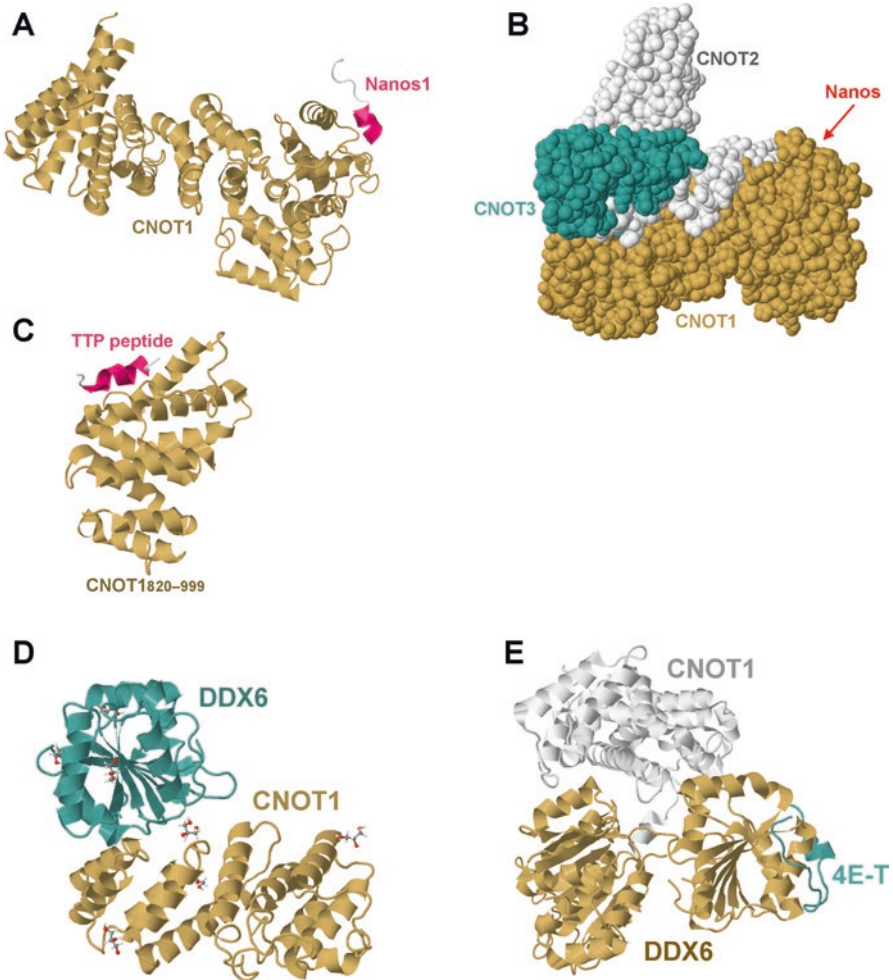


Fig. 13.11 (a) Structure of the Nanos peptide with the C-terminal domain of CNOT1 (PDB 4CQ0). (b) Representation of the NOT module with an *arrow* to indicate where the Nanos peptide binds (Bhandari et al. 2014). (c) Structure of the complex between TTP and CNOT1. CNOT1 (residues 820–999) indicated in *yellow* in complex with the TTP peptide (depicted in *pink*) is shown (PDB 4J8S) (Fabian et al. 2013). (d) Structure of the complex between the DDX6 RecA domain and the CNOT1 MIF4G domain. Glycerol used for the crystallization is visible in *red*. (e) Cartoon representation of the structure between the human CNOT1 MIF4G domain, DDX6 RecA1 and A2 domains, and the 4E-T CHD domain (PDB 5ANR)

domain with the C-terminal RecA domain of DDX6 was solved at 1.75 Å (Fig. 13.11d). The interaction of the RecA domain of DDX6 and the concave surface of the MIF4G domain of CNOT1 is similar to the eIF4G and eIF4A interaction, with helix 1 and loops L3, 5 and 7 of the RecA DDX6 domain interacting with the first two N-terminal HEAT repeats of the MIF4G domain. This structure reveals that

the binding of DDX6 is compatible with the simultaneous binding of Caf1 occurring on an opposite surface of the MIF4G domain.

Several studies have pointed to 4E-T (eIF4E transporter) as a key translational repressor connected to DDX6. A structure between the MIF4G domain of CNOT1, DDX6 and 4E-T was solved at 2.1 Å. It includes residues 216–238 of 4E-T, residues 95–462 of DDX6 and residues 1065–1309 of CNOT1. The DDX6-CNOT1 portion of the ternary complex is similar to the binary complex described above. The concave surface of CNOT1 MIF4G faces DDX6 and interacts via its first and last HEAT repeats to contact the RecA2 and RecA1 domains of DDX6 (Fig. 13.11e).

13.6 Perspectives

The eukaryotic Ccr4-Not complex is an extremely sophisticated regulator of gene expression. The multitude of site and type of actions of the complex has been truly tantalizing. With the available structures we can start to visualize how the complex is tethered to its mRNA substrates and can act to modify these substrates at different functional sites in different ways. We are still far from a complete integrated view, but we can address specific questions by mutating selected interfaces. The need to use cross-linking for the cryo EM structures has confirmed that the complex is very dynamic, and hence we will need to consider that there may be alternative conformations from the ones for which we have structures. Moreover, the assembly of the complex in time and space within the cell is a question that has to be tackled to truly understand Ccr4-Not function in the nucleus and cytoplasm and how these are connected. This is certainly the biggest and exciting challenge ahead.

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