Human Ccr4–Not complexes contain variable deadenylase subunits

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The Ccr4–Not complex is evolutionarily conserved and important for regulation of mRNA synthesis and decay. The composition of the yeast complex has been well described. Orthologues of the yeast Ccr4–Not components have been identified in human cells including multiple subunits with mRNA deadenylation activity. In the present study, we examine the composition of the human Ccr4–Not complex in an in-depth proteomic approach using stable cell lines expressing tagged CNOT proteins. We find at least four different variants of the human complex, consisting of seven stable core proteins and mutually exclusive associated mRNA deadenylase subunits. Interestingly, human CNOT4 is in a separate ~200 kDa complex. Furthermore, analyses of associated proteins indicate involvement of Ccr4–Not complexes in splicing, transport and localization of RNA molecules. Taken together, human Ccr4–Not complexes are heterogeneous in composition owing to differences in their deadenylase subunits, which may reflect the multi-functionality of these complexes in cellular processes.

Key words: Ccr4–Not complex, gene expression, mRNA deadenylation, proteomics, transcription regulation.

INTRODUCTION

The evolutionarily conserved Ccr4–Not complex is important for multiple cellular functions. It is involved in several aspects of the mRNA regulatory pathways (for reviews, see [1,2]). Besides that, the E3 ligase activity of yeast Not4p and human CNOT4 subunits [3,4] implicates the Ccr4–Not complex in the protein ubiquitination/degradation pathways [3,5]. Moreover, members of the Ccr4–Not complex are associated with various functions in several species, both in the nucleus and in the cytoplasm. These include involvement in DNA repair and histone methylation in yeast [6,7], spindle positioning and regulation of microtubule length in Caenorhabditis elegans [8] and spermatogenesis in mice [9].

The Ccr4–Not complex may influence the synthesis of mRNA molecules in several ways. It has been reported that some yeast Ccr4–Not subunits interact both physically and genetically with TBP (TATA-binding protein), TAFs (TBP-associated factors), and other regulators of TBP binding, indicating direct regulation of gene expression at the core promoter through binding of these basal transcription factors (reviewed in [10]). The Ccr4–Not complex may also influence gene transcription by affecting chromatin structure [7,11,12] or by repressing the nuclear receptor-mediated transcription in a ligand-dependent manner via CNOT1 [13]. Two other human CNOT proteins, CNOT2 and CNOT9/hRed1/hCAF40, are also implicated in repression of promoter activity [11]. The major repression function of CNOT2 was found in a conserved protein motif called the Not-Box, which is also present at the C-terminus of CNOT3 [11]. Furthermore, the Ccr4–Not complex also plays a direct role in mRNA degradation and aberrant mRNA surveillance (reviewed in [14]). The deadenylase subunits in the complex are responsible for these events. Ccr4p is the major mRNA deadenylase in yeast [15,16], whereas mammalian cells require both CNOT6/hCcr4 and CNOT7/hCAF1 activities for constitutive deadenylation [17].

The composition of the conserved Ccr4–Not complex has been well studied in yeast. Initially, it was identified as physically and functionally separate subcomplexes of Ccr4-associated proteins and of Not proteins [18]. Not1p/Cdc39p, Not2p/Cdc36p, Not3p, Not4p/Mot2p and Not5p form the Not module, which regulates transcription initiation and elongation (reviewed in [1,2]). They were first isolated as repressors of HIS3 gene transcription from a non-canonical TATA element in yeast (reviewed in [10]). The Ccr4 module encompasses the two deadenylases Ccr4p and Caf1p in association with Caf40p and Caf30p [19,20], and is involved in mRNA degradation. Surprisingly, biochemical analyses revealed that the Ccr4 module also binds Not proteins [19,20], resulting in the Ccr4–Not protein complex. Interestingly, the Ccr4p subunit affects transcription both negatively and positively [20]. Orthologues for most of the yeast Ccr4–Not components have been identified in human cells [21,22]. Not3p and Not5p are similar yeast proteins, and the human genome contains only one orthologous protein, CNOT3 [21]. In contrast, two human orthologues have been found for yeast Ccr4p (CNOT6/hCcr4a and CNOT6L/hCcr4b) [23] and for yeast Caf1p (CNOT7/hCAF1 and CNOT8/hPOPOP2/CAF1) [21]. These four deadenylases have been identified as components of human Ccr4–Not complexes [23,24], but the exact composition of the human complex is less well studied.

In the present study, we determine the composition and the interactors of human Ccr4–Not complexes from stable HeLa cell derivatives, which express epitope-tagged Ccr4–Not subunits.
at near endogenous levels. We identified the interactors of the purified complexes from these cell lines by MS. Strikingly, the CNOT7 and CNOT8 proteins specify the distinct human Ccr4–Not complexes, whereby CNOT6 and CNOT6L associate more tightly with CNOT7 than with CNOT8. Besides the known Ccr4–Not proteins, we invariably and abundantly co-purified TAB182 and C2ORF29 proteins in human Ccr4–Not complexes, indicating that these subunits are novel core complex components. Moreover, our results imply that association of CNOT4 with the Ccr4–Not complex may be a regulated event. Furthermore, additional GO (Gene Ontology) analyses of interactors of the complex suggest direct involvement of CNOT7-containing complexes in mRNA splicing, transport and localization, whereas CNOT8-containing complexes seem to play a role in splicing only. Taken together, the results of the present study support the model wherein Ccr4–Not complexes are involved from RNA synthesis in the nucleus to RNA decay in the cytoplasm.

MATERIALS AND METHODS

Antibodies and immunoblotting

CNOT1, CNOT2 and CNOT3 antibodies have been previously described [13]. Crude serum raised against CNOT8 (described in [21]) was affinity-purified via immunoblotting against a GST (glutathione transferase)–CNOT8 fusion protein and elution from the membrane using 100 mM glycine/HCl (pH 2.5). Antibodies directed against CNOT7 (clone 2F6), HA (haemagglutinin; clone 3F10), α-tubulin (DM1A) and TAB182 were obtained from Abnova, Roche Applied Science, Calbiochem and Novus Biologicals respectively. The HA (12CA5) antibody was purified from the hybridoma cell line 12CA5 (A.T.T.C.).

Protein complexes or extracts were separated using SDS/PAGE and were then transferred on to PVDF membranes. The membranes were blocked using 5 % (w/v) non-fat dried skimmed milk, probed with primary antibodies overnight at 4 °C and then with HRP (horseradish peroxidase)-conjugated secondary antibodies for 45 min at room temperature (21 °C). Detection was performed by ECL (enhanced chemiluminescence) according to the manufacturer’s protocol (PerkinElmer Life and Analytical Sciences).

cDNA sequences of human CNOT genes

Human cDNAs of CNOT1 (partial), CNOT2, CNOT3S, CNOT3L, CNOT4N, CNOT4L, CNOT8 and CNOT9 have been previously described [11,21]. cDNA sequences of the human CNOT6 ORF (open reading frame) was obtained by PCR using pGal4-hCCR4 [25] as a template. Full-length cDNAs of human CNOT7 and CNOT10 were purchased from RZPD (German Science Centre for Genome Research, Germany). All cDNA constructs were verified by DNA sequencing.

Generation of stable cell lines

Human CNOT cDNAs were introduced by GATEWAY cloning [26] into a retroviral destination plasmid derived from pBABE-puro carrying an N-terminal FlagPreScissionHA-tag (for CNOT2, CNOT3L, CNOT4L, CNOT6, CNOT8, CNOT9 and CNOT10) or a C-terminal HAPreScissionFlag-tag (for CNOT3S and CNOT7). Details of primer sequences and the cloning strategy are available upon request from the corresponding author. HeLa S3 cell clones expressing a tagged CNOT protein were obtained by retroviral transduction and puromycin selection. Positive clonal cell lines were identified by immunoblotting using antibodies directed against HA, CNOT2, CNOT3, CNOT7 or CNOT8. HeLa S3 cells with integrated empty pBABE-puro plasmid served as a control.

Overexpression in HEK (human embryonic kidney)-293T cells

Human CNOT7 and CNOT8 cDNAs were introduced by GATEWAY cloning into pMT2SM carrying an N-terminal HA tag. HEK-293T cells were transfected with 3 μg of pMT2SM–HA–CNOT7 or pMT2SM–HA–CNOT8 using FuGene™6 according to the manufacturer’s instructions (Roche Applied Science). Cells were extracted 40 h post-transfection in lysis buffer [20 mM Hapes/NAOH (pH 8.0), 5 % (v/v) glycerol, 150 mM NaCl, 0.5 mM EDTA, 5 mM MgCl2, 0.5 % (v/v) Nonidet P40, 0.5 mM PMSF, 0.5 mM DTT (dithiothreitol) and Complete™ protease inhibitor cocktail (Roche Applied Science)] and analysed by immunoblotting.

Cell culture conditions

HeLa S3 stable cell lines and HEK-293T cells were grown in DMEM (Dulbecco’s modified Eagle’s medium; 1 g/l glucose) with 10 % (v/v) FBS (fetal bovine serum), 2 mM L-glutamine and 100 units/ml penicillin/streptomycin (1 μg/ml puromycin for HeLa S3 cells). HeLa S3 cells were grown in bioreactors in MEM (modified Eagle’s medium with Hank’s balanced salt solution), 10 % (v/v) FBS, 2 mM L-glutamine, 1 % 100 x non-essential amino acids, 100 units/ml penicillin/streptomycin and 1 μg/ml puromycin. All reagents were obtained from Cambrex, except for puromycin (Sigma).

Protein extraction and affinity purification

HeLa cells were lysed by douncing in hypotonic lysis buffer [10 mM Hapes/NAOH (pH 8.0), 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT and 0.5 mM PMSF]. Proteins were extracted in 1/3 volume of 4 x extraction buffer [80 mM Hapes/NAOH (pH 8.0), 50 % (v/v) glycerol, 1.68 M NaCl, 0.8 mM EDTA, 3 mM MgCl2, 1 mM DTT and 4 % (v/v) protease inhibitor cocktail (Sigma)], bringing the ionic strength to 420 mM NaCl. Lysates were centrifuged at 168 000 g for 45 min at 4 °C. Supernatants were dialysed overnight against dialysis buffer [20 mM Hapes/NAOH (pH 8.0), 20 % (v/v) glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT].

Dialysed extracts (10–20 mg/ml) were incubated for 5 h with 1/20 volume of M2 affinity resin (Sigma) pre-equilibrated in BC150 buffer [40 mM Tris/HCl (pH 7.9), 20 % (v/v) glycerol, 150 mM KCl, 0.2 mM EDTA, and 0.1 % (v/v) Nonidet P40]. The resin was subsequently washed with 60 column volumes of BC300 buffer [20 mM Tris/HCl (pH 7.9), 20 % (v/v) glycerol, 300 mM KCl, 0.2 mM EDTA and 0.1 % (v/v) Nonidet P40]. Proteins were eluted twice with two column volumes of BC300 buffer containing 200 μg/ml FLAG peptide (Sigma). FLAG elution fractions were pooled and subsequently incubated overnight with HA affinity resin pre-equilibrated in BC300 buffer. HA affinity resin was prepared by covalently binding HA (12CA5) antibody to Dynabeads protein A (Dynal Biotech) using dimethyl pimelimidate dihydrochloride (Pierce) according to the manufacturer’s instructions. After overnight incubation, resin was washed with 15 resin volumes of BC300 buffer. Bait and interacting proteins were eluted for 20 min at 30 °C with 2 mg/ml HA peptide (Sigma–Genosys), followed by precipitation [27] and identification by MS, or proteins were separated on 4–12 % Bis-Tris gels (NuPAGE; Invitrogen) for silver-stain detection.

For affinity purification at lower salt conditions, cells were lysed in hypotonic lysis buffer, dounced and proteins were extracted in 1/3 volume of 4 x extraction buffer containing 600 mM NaCl, bringing the ionic strength to 150 mM NaCl, followed by
overnight dialysis. Extracts were purified via the FLAG tag as described above, using buffers containing 150 mM NaCl. Purified complexes were subjected to immunoblotting.

MS/MS

Precipitated Ccr4–Not complexes were dissolved in 8 M urea in 50 mM ammonium bicarbonate (pH 8.0), and were incubated with 1 μg of LysC (Roche Diagnostics) for 4 h at 37°C. Following reduction and alkylation using 2 mM DTT and 4 mM iodoacetamide respectively, the sample was diluted to 2 M urea with 50 mM ammonium bicarbonate (pH 8.0), and incubated overnight with 2 μg of trypsin at 37°C. Protein digests were desalted using a small plug of C18 material (3 M Empore C18 extraction disk) packed into a GELoader Tip in a similar manner as described in [28]. The eluate was dried completely by vacuum centrifugation and subsequently reconstituted in 20% (v/v) acetonitrile and 0.05% (v/v) formic acid.

SCX (strong cation exchange) was performed using two Zorbax BioSCX-Series II columns (internal diameter, 0.8 mm; length, 50 mm; particle size, 3.5 μm), a Famos autosampler (LCpackings), a Shimadzu LC-9A binary pump and a SPD-6A UV-detector (Shimadzu). After injection of the peptides, the first 10 min were run isocratically at 100% solvent A [0.05% (v/v) formic acid in 8:2 (v/v) water/acetonitrile (pH 3.0)], followed by a linear gradient of 1.3% min⁻¹ solvent B [500 mM NaCl in 0.05% (v/v) formic acid in 8:2 (v/v) water/acetonitrile (pH 3.0)]. A total number of 24 SCX fractions (1 min each, i.e. a 50 μl elution volume) were manually collected and dried in a vacuum centrifuge.

These residues were reconstituted in 20% (v/v) acetonitrile and 0.05% (v/v) formic acid.

Argentina was visualized on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)-containing plates at 30°C.

Gel filtration

Cell extracts of the CNOT4L-tagged HeLa S3 stable cell line were prepared as described above with the following adaptations: hypotonic lysis buffer also contained 2% (v/v) protease inhibitor cocktail (Sigma), cells were lysed by syringe douncing and proteins were extracted by addition of 1 vol. of extraction buffer [20 mM Hepes/NaOH (pH 8.0), 20% (v/v) glycerol, 300 mM NaCl, 0.4 mM EDTA, 1.5 mM MgCl₂, 0.2% (v/v) Nonidet P40 and 0.5 mM DTT]. Extracts were analysed on a Superose 6 HR 10/30 column (GE Healthcare) in 20 mM Hepes/NaOH (pH 7.9 at 4°C), 150 mM NaCl, 10 μM ZnCl₂, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin A, followed by immunoblot analyses of the 0.4 ml fractions.

RESULTS

Stable HeLa S3 cell lines with FLAG-/HA-tagged CNOT proteins under endogenous conditions

The human Ccr4–Not complex is biochemically less well defined than its yeast counterpart [18,23,24]. To obtain more insight into the composition of the human complex, we generated HeLa cell lines that stably expressed FLAG-/HA-tagged versions of human CNOT (Ccr4–Not subunits). The expression levels of these tagged proteins within the distinct cell lines were similar with the exception of the more highly expressed CNOT9 (Figure 1A). Immunoblot analyses using specific antibodies against CNOT2, CNOT7 or CNOT8 showed near endogenous expression levels of the tagged protein (Figure 1B). Immunoprecipitation of tagged CNOT3 or CNOT9 resulted in co-purification of the known...
Figure 1 Integration of FLAG-/HA-tagged CNOT proteins in endogenous Ccr4–Not complexes

(A) Cell extracts from stable human cell lines expressing the indicated tagged CNOT protein were analysed by immunoblotting using anti-HA antibodies. Asterisks indicate epitope-tagged proteins. Detection of α-tubulin serves as a loading control. Control cells are HeLa S3 stably transfected with pBABE-puro. Molecular mass markers are indicated in kDa on the left-hand side of the gel. (B) Some extracts from (A) were analysed by specific antibodies. Both endogenous and exogenous tagged proteins can be detected. (C) Immunoblot analyses of FLAG/HA purifications of CNOT3L, CNOT9 or control cells using specific antibodies. (D) Silver stain of purified Ccr4–Not complexes. Asterisks indicate epitope-tagged CNOT3L, CNOT7 and CNOT9 protein bands respectively from left to right. Molecular mass markers (kDa) are given on the left-hand side of the gel. (E) Cell extracts of CNOT3L, CNOT9 and control cell lines were FLAG-/HA-purified and analysed by immunoblotting using specific antibodies against TAB182. Flow-through (FT) is also shown.

subunits of the Ccr4–Not complex, such as CNOT2, CNOT3 and CNOT7 (Figure 1C), indicating that the tagged proteins were integrated into larger protein complexes. Notably, similar amounts of CNOT2 and CNOT7 could be co-immunoprecipitated from cell extracts containing tagged CNOT3L or the higher expressed tagged CNOT9 (Figures 1A and 1C). This indicated that the higher expression levels of CNOT9 did not disrupt normal Ccr4–Not complex formation. In addition, silver-stain analysis of large-scale affinity purifications of CNOT3L, CNOT7 and CNOT9 cell lines revealed co-purification of other proteins, which were identified as Ccr4–Not complex subunits by MS (Figure 1D and Table 1). Thus the generated HeLa S3 cell lines expressed their epitope-tagged proteins at near endogenous levels and were integrated into endogenous Ccr4–Not complexes.

Human Ccr4–Not complexes differ in deadenylase subunits

To analyse the composition of human Ccr4–Not complexes from different entry points, tagged HeLa S3 cells were grown in bioreactors for large-scale affinity purifications of Ccr4–Not complexes, which were subjected to in-solution tryptic digestion, SCX fractionation and nano-reversed phase liquid chromatography coupled to high-resolution MS [LTQ (linear ion trap)-FTICR] analyses. Table 1 summarizes the results in NSAFs to indicate the relative abundances of co-purified proteins among the individual CNOT purifications [30] (Supplementary Figure S1, at http://www.BiochemJ.org/bj/422/bj4220443add.htm, is a coloured representation of Table 1). In the present study, we define a core subunit of the Ccr4–Not complex as being identified by MS with at least an NSAF value of 0.4 in all Ccr4–Not purifications and with no peptides in a control purification. We define a subunit as variable when the NSAF value is at least 0.4, but not in all purifications. According to these criteria, the deadenylases CNOT6, CNOT6L, CNOT7 and CNOT8 are variable subunits within the human complex and CNOT4 E3 ligase is not an integral subunit of human Ccr4–Not (Table 1). CNOT1, CNOT2, CNOT3, CNOT9, CNOT10 and the new interactors TAB182 and C2ORF29 were identified as core subunits of the human complexes (Table 1). The C2ORF29 protein is predicted...
FLAG/HA affinity-purified complexes of a tagged Ccr4–Not subunit were analysed by MS. The control is a HeLa S3 cell line stably transfected with pBABE-puro. Numbers represent 10-fold NSAFs (as described in [30]) for identified proteins. The molecular masses (kDa) of these interactors are given in the right-hand column. The sum of unique peptides and sum of all identified peptides of identified proteins per purification are shown. Bold numbers indicate the NSAF values of deadenylase subunits in the purified Ccr4–Not complexes using a tagged deadenylase. The numbers in italics indicate the NSAF values of the tagged CNOT protein.

Table 1 NSAFs of identified Ccr4–Not components

<table>
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<tr>
<th>Identified protein</th>
<th>CNOT2</th>
<th>CNOT3L</th>
<th>CNOT6</th>
<th>CNOT7</th>
<th>CNOT8</th>
<th>CNOT9</th>
<th>CNOT10</th>
<th>Control</th>
<th>CNOT3S</th>
<th>Molecular mass (kDa)</th>
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<td>0.6</td>
<td>1.3</td>
<td>1.0</td>
<td>1.3</td>
<td>0.5</td>
<td>0.8</td>
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<td>2.1</td>
<td>1.0</td>
<td>1.5</td>
<td>1.6</td>
<td>2.1</td>
<td>1.3</td>
<td>0</td>
<td>0</td>
<td>60</td>
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<td>CNOT3</td>
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<td>0.8</td>
<td>1.2</td>
<td>1.4</td>
<td>1.0</td>
<td>1.1</td>
<td>0</td>
<td>0.9</td>
<td>82</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>71</td>
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<td>0.2</td>
<td>0</td>
<td>0</td>
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<td>63</td>
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<td>0.3</td>
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<td>0.7</td>
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<td>0.5</td>
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<td>1.9</td>
<td>1.3</td>
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<td>1.3</td>
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<td>0</td>
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</tr>
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<td>TAB182</td>
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<tr>
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<td>1039</td>
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<td>638</td>
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</table>

from the human genome sequence with no known function and TAB182 has been described as a binding protein for tankyrase 1, which plays a role in telomere length and mitotic spindle composition [34,35]. Immunoblot analysis showed that a small, but significant, proportion of cellular TAB182 co-purified with CNOT3L and CNOT9 complexes (Figure 1E), confirming that TAB182 is a stable interactor of the human Ccr4–Not complex. Unfortunately, no antibodies against C2ORF29 are available, precluding confirmation by Western blot analysis.

The detailed MS results revealed several interesting properties of human Ccr4–Not complexes (Table 1). In particular, the variable deadenylase subunits showed interesting differences among the tagged CNOT6, CNOT7 and CNOT8 purifications (shown in bold in Table 1). No significant CNOT7 was detected in CNOT8 purifications and vice versa, suggesting that CNOT7 and CNOT8 are present in distinct Ccr4–Not complexes. CNOT6 and CNOT6L were identified in CNOT7 purifications, whereas they were absent from CNOT8 purifications, suggesting that CNOT6/6L proteins stably interact with CNOT7, but not with CNOT8. No CNOT6L could be identified in two CNOT6 purifications (Table 1 and the Tranche database), indicating mutual exclusiveness of these subunits as expected from their distinct functions; CNOT6L, but not CNOT6, impairs cell growth [23]. It is interesting to note that the deadenylases CNOT6, CNOT6L, CNOT7 and CNOT8 are relatively lower represented in the purifications than the other CNOT proteins, except when they are the bait of the purification. Moreover, the deadenylase subunits could not be detected in CNOT10-purified complexes. This could, however, be due to the lower amounts of recovered proteins in this purification. Importantly, Ccr4–Not complexes obtained via other tagged subunits inversely identified CNOT10. Furthermore, comparison of full-length CNOT3L (residues 1–753) to CNOT3S lacking the Not-Box (residues 1–609) showed that the smaller isoform did not interact with any Ccr4–Not subunit. This indicates that the Not-Box is required for the stable interaction of CNOT3 with the rest of the complex [11,21,36]. Finally, human CNOT4 could hardly be detected in purified Ccr4–Not complexes in contrast with yeast [18,20], indicating that CNOT4 is not stably integrated within human complexes. In conclusion, purified Ccr4–Not complexes from human cells using seven different entry points indicate the existence of several Ccr4–Not complexes differing only in the deadenylase subunits CNOT6, CNOT6L, CNOT7 and CNOT8.

Analyses of substoichiometric interactors suggest Ccr4–Not functioning in RNA splicing and nuclear export

Besides subunits of Ccr4–Not complexes, the MS results identified 498 proteins with at least two unique peptides and no peptides in a control purification. These proteins have low NSAF values and are not present in all purifications. We propose that these 498 proteins represent substoichiometric interactors of tagged Ccr4–Not proteins (Supplementary Table S2 at http://www.BiochemJ.org/bj/422/bj4220443add.htm). Approx. 47% of these associated proteins were shared among the deadenylase subunits and the other CNOT proteins, indicating that almost half of them bind both the deadenylases as well as transcriptional Ccr4–Not subunits. In addition, 70% of the interactors were co-purified by at least two CNOT proteins, 44% by at least three Ccr4–Not components, and 26% by at least four subunits. Importantly, these proteins were not identified in a control purification, suggesting that these substoichiometric interactors are relevant for the function of the Ccr4–Not complex. We searched the HomoMINT database [37] for interologues to find seven interactors shared between our MS/MS-based human dataset and the yeast dataset. These shared interologues are rather diverse in function and we found only a few peptides of each interactor (Supplementary Table S3 at http://www.BiochemJ.org/bj/422/bj4220443add.htm).

To gain insight into the biological functions of human Ccr4–Not complexes, GO term over-representation analyses were performed with the CNOT subunits and the substoichiometric Ccr4–Not interactors. The results are given in Supplementary Figure S2 (at http://www.BiochemJ.org/bj/422/bj4220443add.htm) of which the interesting clusters are represented in Figure 2. Clusters containing gene transcription and RNA stability-related terms were identified (Figures 2A and 2B), indicating that expected functions of Ccr4–Not can be retrieved from these data analyses. Interestingly, many subunits gave association with mRNA splicing (Figure 2C), and mRNA transport and...
Interactors identified by at least two unique peptides and without peptides in a control purification (keratins excluded) were analysed for GO term over-representation (see also Supplementary Figure S2 at http://www.BiochemJ.org/bj/422/bj4220443add.htm). Colours represent the log2 P values of the Pearson correlation clustering multiplied by −1 on a scale of 0–35. (A) Transcription cluster. (B) RNA stability cluster. (C) Splicing cluster. (D) RNA transport and localization cluster. (E) Nuclear envelope cluster. (F) Nuclear localization cluster.

localization (Figure 2D), except for CNOT6 and CNOT8 respectively. Moreover, CNOT6, CNOT8 and CNOT10 have low probability to interact with the nuclear envelope (Figures 2E and 2F), suggesting that these proteins may not be involved directly in nuclear mRNA export. Most Ccr4–Not subunits co-purified NUPs (nuclear pore complex proteins) located at the nuclear basket, the cytosolic filaments and inside the nuclear membrane (Supplementary Figure S3 at http://www.BiochemJ.org/bj/422/bj4220443add.htm). The majority of the NUPs inside the nuclear membrane were co-purified. However, more peptides were retrieved from the identification of NUPs on the cytosolic or nuclear membrane side (Supplementary Figure S3). This suggests that the Ccr4–Not complex may interact with the intact nuclear pore complex. Thus GO term over-representation of substoichiometric interactors indicates physical interactions of human Ccr4–Not complexes with proteins involved in mRNA processing and nuclear export, suggesting involvement of Ccr4–Not in these cellular processes.
CNOT4 binds CNOT4, and CNOT6 binds CNOT8 in yeast two-hybrid assays

To further explore the composition of human Ccr4–Not complexes, we investigated binary interactions between known components of the complex by yeast two-hybrid interaction experiments [38]. Instead of using full-length CNOT1 protein, this large protein (2376 residues) was split up into smaller fragments (Figure 3A). Positive binary interactions between a B42-fusion and a LexA-fusion CNOT protein in yeast are shown in Figure 3(B). The majority of the subunits, except for CNOT3L and CNOT6, interacted with the C-terminal part of CNOT1 (CNOT1L: residues 648–2376). This is in concordance with earlier findings that a shorter C-terminal CNOT1 (CNOT1C: residues 1106–2376) interacts with CNOT2, CNOT4N and CNOT8 [21]. In addition, CNOT2 and CNOT9 interacted with the N-terminus of CNOT1 (CNOT1N: residues 1–716). These observations indicate a scaffold function for CNOT1 as suggested previously for its yeast orthologue Not1p [18]. Moreover, none of the examined subunits interacted with themselves, suggesting that they occur as monomers. Interestingly, CNOT3L only interacted with CNOT2 (Figure 3B), suggesting that CNOT3 integrates within the Ccr4–Not complex by CNOT2 interaction. Furthermore, CNOT6 interacted with CNOT7 and vice versa. Also, CNOT6 showed a binary interaction with CNOT8 in this assay, whereby the reciprocal interaction could not be performed owing to self-activation properties of LexA–CNOT8. It is interesting to note that CNOT4N and CNOT1L also interacted in this assay.

CNOT4 resides in a smaller complex outside the human Ccr4–Not complex

To further investigate the association of CNOT4 with the Ccr4–Not complex, we have generated a cell line that stably expresses the largest isoform of CNOT4, CNOT4L [21]. Extracts of these cells were separated by gel filtration under physiological salt conditions and were subjected to immunoblot analyses (Figure 4). Tagged CNOT4L protein resided in a ~200 kDa complex, whereas the subunits CNOT1, CNOT3, CNOT7 and CNOT8 co-eluted in fractions corresponding to ~1.2 MDa. In addition, a small fraction of CNOT8 and of CNOT2 was seen at a single protein size (results not shown). It would be interesting to determine the composition of the ~200 kDa complex. However, the low abundance of this complex precluded our first attempts in this direction (results not shown).

Taken together, gel-filtration analyses confirm the MS results that CNOT4 is not stably interacting with human Ccr4–Not components, but rather forms a distinct protein complex in human cells. The identity of CNOT4-associated subunits could reveal how interaction of CNOT4 with the Ccr4–Not complex would be regulated.

CNOT6 interacts more strongly with CNOT7 than with CNOT8

To further analyse the interactions among the four human deadenylases, we first determined the specificity of antibodies directed against CNOT7 and/or CNOT8 as these deadenylases are 75% identical in their primary structure. Upon transient overexpression of CNOT7 and/or CNOT8, the CNOT7 antibodies specifically recognized CNOT7 (Figure 5A, upper left-hand panel), whereas antibodies directed at CNOT8 (middle left-hand panel) detected both CNOT7 (upper band) and CNOT8 (lower band). The CNOT7 antibodies also specifically recognized endogenous CNOT7 (Figure 5A, upper right-hand panel), whereas the CNOT8 antibodies detected endogenous CNOT8, but also displayed considerable background bands (Figure 5A, middle right-hand panel). Thus the specificity of CNOT7 antibodies is exclusive for CNOT7 proteins, and antibodies directed against CNOT8 detect both CNOT7 and CNOT8 subunits, which can be distinguished by different mobilities in protein gels.

Immunoblot analyses using these antibodies showed the absence of CNOT7 proteins in CNOT8-purified complexes, and no CNOT8 proteins could be detected in CNOT6- and CNOT7-purified complexes (Figure 5B). As expected, both CNOT7 and CNOT8 were present in CNOT2-purified complexes (Figure 5B). These observations confirm the MS results (Table 1 and Supplementary Table S1) of the mutual exclusiveness of CNOT7 and CNOT8 within Ccr4–Not complexes. In addition, the interaction between CNOT6 and CNOT8 was further studied by immunoblot analyses using the described antibodies (Figure 5C). Yeast two-hybrid analysis showed CNOT6 binding to CNOT8,
whereas MS results did not reveal CNOT6 in CNOT8 complexes and vice versa (Table 1 and Supplementary Table S1). One possibility is the loss of the CNOT6–CNOT8 interaction during the two-step purification prior to MS identification. To address this hypothesis, one-step FLAG purifications of Ccr4–Not complexes were performed under low- or high-salt concentrations (Figure 5C). The association of CNOT7 within CNOT6- or CNOT2-purified complexes was not sensitive to salt, indicating strong binding of CNOT7 to these complexes. Notably, CNOT8 could be detected just above background in CNOT6-purified complexes under low-salt conditions only, whereas CNOT8 was clearly detectable in CNOT2-purified complexes independent of the salt concentration. Taken together, this indicates that the interaction between CNOT6 and CNOT8 is sensitive to the salt concentration. Unfortunately, the lack of CNOT6 antibodies does not allow us to perform the converse experiment using CNOT7- and CNOT8-purified complexes. In conclusion, these experiments confirm that CNOT7 and CNOT8 deadenylases are mutually exclusive subunits of human Ccr4–Not complexes, whereby CNOT6 seems to interact less stably with CNOT8 than with CNOT7.

**DISCUSSION**

In the present study, we have investigated the composition and the protein interactors of human Ccr4–Not complexes by an in-depth proteomic effort. We have defined the core proteins of human Ccr4–Not complexes including two new subunits, TAB182 and C2ORF29. These complexes are heterogeneous in composition by their associated mRNA deadenylase subunits, which may reflect functional differences in coupling mRNA synthesis and processing to nuclear export. Size-fractionation experiments showed that the human CNOT4 E3 ligase resides in a separate ∼200 kDa protein complex. Interestingly, a yeast two-hybrid assay displayed a CNOT4 interaction with the scaffold protein CNOT1. Moreover, CNOT3 requires its Not-Box to integrate into the human Ccr4–Not complex via CNOT2. Taken together, the results of the present study define the composition of human Ccr4–Not complexes and have revealed the co-existence of multiple Ccr4–Not complexes differing in their deadenylase subunits.

**Defining the human Ccr4–Not complexes**

In the past, the components and the composition of the yeast Ccr4–Not complex have been revealed by a variety of biochemical purifications and genetic experiments [18–20]. Gel filtration of the yeast complex revealed two peaks of 1.9 and 1.0 MDa [18,19]. In contrast, we observed a rather broad peak for the human complex at ∼1.2 MDa (Figure 4). Gavin et al. [39] have purified the human Ccr4–Not complex via a TAP-tagged version of CNOT2, but they failed to identify CNOT3, CNOT4 and CNOT6 as Ccr4–Not components. Our MS results indicate a core of seven subunits for human Ccr4–Not complexes consisting of CNOT1, CNOT2, CNOT3, CNOT9, CNOT10, TAB182 and C2ORF29 with the associated deadenylases CNOT6, CNOT6L, CNOT7 and CNOT8 (Figure 6). The combined molecular mass of these components in the human complex is ∼0.9 MDa, which corresponds with the gel-filtration results (Figure 4).

The overall composition of human Ccr4–Not complexes is similar to the yeast complex [19]. We found that several CNOT proteins (CNOT2, CNOT7, CNOT8 and CNOT9) interact with the largest subunit of the complex, CNOT1 (Figure 3B), indicating that this protein acts as a scaffold similar to yeast Not1p. However, we noted several differences between the human and yeast Ccr4–Not complexes. First, human cells contain multiple deadenylases, which have different binding properties. Our salt titration experiments indicate that CNOT6 interacted less stably with CNOT8 compared with CNOT7 (Figure 5C). Notably, CNOT6 only showed binary interactions with CNOT7 and CNOT8 in a yeast two-hybrid assay (Figure 3B), indicating that these two deadenylases link CNOT6 to the rest of the Ccr4–Not complex as proposed in [21,40]. Secondly, CNOT3 did not bind directly to CNOT1 as reported for the yeast Ccr4–Not complex [18,19], but it did bind CNOT2 (Figure 3B). Moreover, MS analyses showed that CNOT3 lacking the Not-Box (CNOT3S) could not interact with any Ccr4–Not subunits (Table 1). This suggests that CNOT3 integrates in the human complex via a
CNOT7 and CNOT8 form different human Ccr4–Not complexes

Not-Box-mediated interaction with CNOT2. Finally, in contrast with yeast Not4p [18,19], CNOT4 was not stably integrated in human Ccr4–Not complexes (Table 1 and Figure 4), but it could interact with the scaffold protein CNOT1 in a yeast two-hybrid assay (Figure 3B). In addition, human CNOT4 is a true orthologue of yeast NOT4 as it can complement not4Δ not5Δ lethality in yeast [21]. This suggests that the CNOT4 interaction with the Ccr4–Not complex may be a regulated event in human cells. Possibly, association of a core subunit precludes interaction of CNOT4 with CNOT1. It is also possible that interactors of CNOT4 or post-translational modifications are involved in restricting CNOT4 integration into the human complex. A model of the distinct Ccr4–Not complexes in human cells is depicted in Figure 6.

Figure 5 CNOT6 interacts more strongly with CNOT7 than with CNOT8

(A) Cell extracts of HEK-293T cells with transiently overexpressed deadenylases (left-hand panel) or stable HeLa cell line extracts (right-hand panel) were immunoblotted using antibodies directed against CNOT7 or CNOT8. Tagged (tag) or endogenous (endo) CNOT7 and CNOT8 proteins are indicated by arrows. (B) Extracts of cells containing epitope-tagged CNOT proteins or control cell extracts were affinity-purified and analysed by specific antibodies. Asterisks indicate the cross-reactivity of the CNOT8 antibody with the CNOT7 protein. (C) Cell extracts of CNOT6, CNOT2 or control cells were FLAG-epitope affinity-purified under conditions of 150 mM NaCl (left of triangle) or 300 mM NaCl (right of triangle). Purified complexes were analysed by immunoblotting using specific antibodies. Asterisks indicate the cross-reactivity of the CNOT8 antibody with CNOT7 protein.
Multiple human Ccr4–Not complexes differing in mRNA deadenylase subunits

The yeast Ccr4–Not complex harbours two deadenylases, Ccr4p and Caf1p, of which Caf1p is dispensable for Ccr4p deadenylase activity [15,16]. Human cells contain two orthologues of yeast Ccr4p, CNOT6/hCcr4a and the recently identified CNOT6L/hCcr4b, and two homologous Caf1p orthologues, CNOT7 and CNOT8. All of these Ccr4p/Caf1p orthologues display in vitro 3′→5′ poly(A) exoribonuclease activity [23,41]. The results of the present study revealed that the highly homologous CNOT7 and CNOT8 proteins are in separate complexes (Table 1 and Figure 5B). Only a small portion of CNOT8-containing complexes harbour CNOT6, which seems to be less stable than the CNOT6 interaction in CNOT7-containing complexes (Figure 5C). It is plausible that CNOT7 and CNOT8 are competing for the same binding site on CNOT1 allowing association of either CNOT7 or CNOT8. In addition, CNOT6 and CNOT6L may also be mutually exclusive as no unique peptides of CNOT6L were found in two purifications of CNOT6 and CNOT6L may also be mutually exclusive as no unique peptides of CNOT6L were found in two purifications of CNOT6L-tagged complexes (Table 1 and the Tranche database). This is also supported by earlier results showing that CNOT6L is functionally distinct from CNOT6 [23]. Furthermore, our results showed co-purification of CNOT6–CNOT7 and CNOT6L–CNOT7 (Table 1), which was previously reported in a transient overexpression purification of CNOT6–CNOT7 and CNOT6L and CNOT8. All of these Ccr4p/Caf1p orthologues are competing for the same binding site on CNOT1 allowing association of either CNOT7 or CNOT8. In addition, CNOT6 and CNOT6L may also be mutually exclusive as no unique peptides of CNOT6L were found in two purifications of CNOT6L-tagged complexes (Table 1 and the Tranche database). This is also supported by earlier results showing that CNOT6L is functionally distinct from CNOT6 [23]. Furthermore, our results showed co-purification of CNOT6–CNOT7 and CNOT6L–CNOT7 (Table 1), which was previously reported in a transient overexpression approach [40]. Thus all of these findings support a model of distinct Ccr4–Not complexes allowing only a single Ccr4p and a single Caf1p orthologue (Figure 6). It is interesting to note that the substrate specificity for the enzymatic activity of CNOT7 and CNOT8 is different [41], which may allow differential regulation of mRNA degradation.

Human Ccr4–Not complexes play various roles in mRNA synthesis to degradation

GO term over-representation analyses of associated Ccr4–Not proteins provided insights into the functional roles of human Ccr4–Not complexes such as in gene transcription and RNA stability (Figures 2A and 2B). A role in mRNA transport and localization has also been indicated for CNOT7-, but not for CNOT8-, containing complexes (Figures 2D–2F). Indeed, multiple NUPs interacted with all CNOT proteins tested, except for CNOT8 (Supplementary Figure S3). Interestingly, CNOT6 and CNOT7 proteins were reported to shuttle between the nucleus and cytoplasm [40]. In general, more peptides were retrieved from nuclear and cytosolic NUPs than from NUPs within the nuclear envelope (Supplementary Figure S3). This may indicate a shuttling function of the Ccr4–Not complex, which may link its nuclear and cytosolic functions. In addition, involvement in mRNA splicing is also suggested by GO term over-representation analyses, which included the CNOT8-containing complexes (Figure 2C). Taken together, these findings support the view that human Ccr4–Not complexes can influence the mRNA metabolism at multiple levels.

In conclusion, the present study provides comprehensive composition analyses of Ccr4–Not complexes in human cells. We identified multiple Ccr4–Not complexes differing in their deadenylase subunits and in complex stability. Our proteomic analyses provide a platform for further functional studies of human Ccr4–Not complexes defining the regulation of mRNA metabolism by this evolutionarily conserved protein complex.

AUTHOR CONTRIBUTION

Nga-Chi Lau generated the stable cell lines, performed large-scale affinity purifications, gel filtration and Western blot analyses, analysed the MS data, prepared figures and wrote the manuscript. Annemieke Kolkman analysed the purified Ccr4–Not complexes by high-resolution MS. Richard van Schaik performed the yeast two-hybrid study as described in Figure 3. Klaas Mulder performed the bioinformatics analyses resulting in Figure 2. Pim Flyvbjerg developed the techniques to perform large-scale affinity purifications of protein complexes. Albert Heck conceived the experiments. Marc Timmers conceived the experiments and wrote the manuscript.

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We thank Dr M.A. Collart (University of Geneva, Geneva, Switzerland) for the gift of the CNOT1 antibody, Dr V.J. Bardwell (University of Minnesota, MN, USA) for the partial CNOT1 cDNA constructs and Dr T. Aoki (Chiba University, Chiba, Japan) for the gift of the CNOT3 antibody. We also thank Fred Koch for technical assistance in generating and purifying the HeLa S3 stable cell line expressing CNOT7, and Danny Navarro for the submission of the Scaffold data to the ProteomeCommons/Tranche database. We are grateful to Dr P. Kemmeren (University Medical Center Utrecht, Utrecht, The Netherlands) for bioinformatic assistance. We thank members of the Timmers and Heck laboratories for discussions, and Dr Nikolay Dutchievouk and Loes van de Pasch for critical reading of this manuscript prior to submission.

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REFERENCES

SUPPLEMENTARY ONLINE DATA

Human Ccr4–Not complexes contain variable deadenylase subunits

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Table S1 Unique peptide counts of identified Ccr4–Not components after MS analyses of purified Ccr4–Not complexes using distinct entry points

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Figure S1 Heatmap of NSAFs of identified Ccr4–Not components

NSAF values of Table 1 are represented by colours on a scale of 0–3.1.

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Figure S2  For legend see facing page
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Table S2  Identified proteins in Ccr4–Not complex purifications

Interactors of tagged CNOT proteins, identified by at least two unique peptides and no peptides in a control purification, are shown in the Table by their gene names. The core and variable Ccr4–Not subunits are given in bold. The entry points for the purifications are indicated on the top. The peptide counts (unique and total), identified peptide sequences and protein identifiers can be downloaded from ProteomeCommons.org Tranche (https://proteomecommons.org/tranche/), using the following Tranche hash: EKYhwbHIfbis7ocZqc18hDwmT7WZvSLAWp1dVKjhBF9W9i2ubb6E5vjuh2WjuQMvyBpaPAT/HbYy6edmHPMeNo)10AAAABAAADPw==. The data can also be downloaded from https://proteomecommons.org/dataset.jsp?i=72595.

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Figure S2  GO term over-representation of Ccr4–Not interactors

All interactors identified by at least two unique peptides and no peptides in a control purification (keratins excluded) were analysed for GO term over-representation. Heatmap is presented in two parts in colours that represent log2 P values of Pearson correlation clustering multiplied by $-1$ on a scale of 0–35. Selected clusters are highlighted in Figure 2.
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Table S3  Overlap of substoichiometric Ccr4–Not interactors with yeast interologues from the HomoMINT database

Unique peptide counts of the overlapping Ccr4–Not interactors (identified protein given as gene name) after MS analyses of purified Ccr4–Not complexes using the indicated tagged protein are shown.

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Figure S3  Ccr4–Not subunits interact with the nuclear pore complex

Unique peptide counts of NUPs and XPO1 in all purifications are represented by colours on a scale of 0–16. Cellular localization of nuclear pore complex proteins are indicated: nu, nuclear basket; env, nuclear envelope; and cyt, cytoplasmic side.

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