RWD domain: a recurring module in kinetochore architecture shown by a Ctf19–Mcm21 complex structure

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The proteins Ctf19, Okp1, Mcm21 and Ame1 are the components of COMA, a subassembly of budding-yeast kinetochores. We have determined the crystal structure of a conserved COMA subcomplex—the Ctf19–Mcm21 heterodimer—from *Kluyveromyces lactis*. Both proteins contain ‘double-RWD’ domains, which together form a Y-shaped framework with flexible N-terminal extensions. The kinetochore proteins Csm1, Spc24 and Spc25 have related single RWD domains, and Ctf19 and Mcm21 associate with pseudo-twofold symmetry analogous to that in the Csm1 homodimer and the Spc24–Spc25 heterodimer. The double-RWD domain core of the Ctf19–Mcm21 heterodimer is sufficient for association with Okp1–Ame1; the less conserved N-terminal regions may interact with components of a more extensive ‘CTF19 complex’. Our structure shows the RWD domain to be a recurring module of kinetochore architecture that may be present in other kinetochore substructures. Like many eukaryotic molecular machines, kinetochores may have evolved from simpler assemblies by multiplication of a few ancestral modules.

Keywords: COMA; mitosis; X-ray crystallography; yeast

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INTRODUCTION

Kinetochores are the multiprotein assemblies that attach eukaryotic chromosomes to spindle microtubules during meiosis and mitosis. They transmit forces that move chromosomes and originate the spindle assembly checkpoint signals. Kinetochores of budding yeast, such as *Saccharomyces cerevisiae*, which connect an approximately 150–230 base-pair ‘point centromere’ to a single microtubule, seem to represent a phylogenetically conserved unit, repeated in multimodular kinetochores of most

other eukaryotes [1]. The proteins associated with centromeric DNA compose the ‘inner kinetochore’, and those in extensive contact with microtubules compose the ‘outer kinetochore’. A framework of ‘linker’ proteins connects the two.

Of the more than 60 proteins now assigned to the *S. cerevisiae* kinetochore, many form discrete protein complexes [2]. COMA [2,3], which includes the proteins Ctf19 (chromosome transmission fidelity 19) [4], Okp1, Mcm21 (minichromosome maintenance 21) [5] and Ame1, as well as the complexes MIND, NDC80 and SPC105, seem to be the most important constituents of the linker framework [1,2]. Each *S. cerevisiae* kinetochore probably contains two or three copies of COMA [6]. These form part of the larger, isolable CTF19 complex, which includes eight more kinetochore proteins [7]. Recombinant *S. cerevisiae* COMA associates *in vitro* with MIND [8]. The *S. pombe* orthologues for Ctf19 and Mcm21, Fta2 and Mal2, respectively [9], are also part of a multiprotein complex, Sim4 [10], with analogies to the CTF19 complex. In vertebrates, CENP-P and CENP-O (also Mcm21R) designate the Ctf19 and Mcm21 orthologues, respectively, which are components of the multiprotein constitutive centromere-associated network complex [11].

Ctf19 and Mcm21 are required for the fidelity of chromosome separation, maintenance of genomic stability and bipolar sister chromatid orientation. *S. cerevisiae* with certain mutations in Ctf19 or Mcm21 shows marked chromosome missegregation [3–5,12] and defects in cohesion of pericentromeric DNA [13]. Mcm21 becomes essential for chromosome biorientation in cells with reduced function of Ipl1, the Aurora B kinase ortholog [14]. In *S. pombe*, Fta2 and Mal2 are essential for viability. Cells with certain Fta2 mutants show severe chromosome missegregation with a substantial amount of unseparated chromatin [9]. Similarly, inactivation of Mal2 causes an elevated rate of chromosome missegregation [15]. Depletion of CENP-O in human cells by RNA silencing results in pronounced defects in bipolar spindle assembly and chromosome congression, aberrant kinetochore–microtubule attachment and delayed centrosome separation [16–18]. Cells with deletions of CENP-O and CENP-P show defects in mitosis, including a delay in cell cycle progression [19], chromosome misalignment and abnormally condensed chromatin [11].

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We have reconstituted the Ctf19–Mcm21 complex (Ctf19–Mcm21) from the point-centromere yeast *Kluyveromyces lactis* and determined its crystal structure. The two proteins have closely related structures, each with a partly disordered N-terminal segment followed by tandem ‘RWD’ domains [20]. Similar domains are interaction modules in three other kinetochore proteins—Spc24, Spc25 and Csm1 [21,22]. The similarity among the RWD domains of all five kinetochore proteins is substantial enough that we imagine them to be paralogues and we suggest that other kinetochore components also have related interaction modules.

RESULTS AND DISCUSSION

Structure determination

Sedimentation equilibrium analytical ultracentrifugation shows that purified Ctf19–Mcm21 (Fig 1) is a 1:1 heterodimer. It crystallizes in space group $P6_222$, with two heterodimers in the asymmetric unit (supplementary Figs S1 and S2 online), and we determined the crystal structure to $\sim 3.9\text{Å}$ resolution (supplementary Table S1 online). Both proteins fold into a globular part, with interpretable electron density, and disordered, flexible N-terminal regions with no significant level of continuous electron density (Fig 2). We modelled continuous polypeptide chain for Ctf19 from positions 96/97 to 269 (of 270). A less well-ordered, mainly α -helical segment, spanning residues 69–92, protrudes from the complex. For Mcm21, we traced residues 99 to the C terminus (293) and a U-shaped segment that includes residues 44–59, located between Ctf19 and Mcm21 (Fig 2). We have assigned the disconnected segments (Ctf19, residues 66–92, and Mcm21, residues 44–59) to the heterodimer with which they are in most extensive molecular contact. Connections to a crystallographically or non-crystallographically related molecule would be unusually long for the number of residues in the gap and would disagree with the stable, non-associating heterodimeric state in solution (Fig 1). Coordinates and structure factors have been deposited in the Protein Data Bank with accession number 3ZXU.

RWD domains in Ctf19 and Mcm21

The polypeptide chains of Ctf19 and Mcm21 from residue 107 to the C terminus are structurally homologous (Table 1) and form a rigid scaffold. Each protein has two similarly folded $\alpha+\beta$ sandwiches (Figs 2 and 3A,B), arranged in tandem, and connected by a central α -helix $\sim 40\text{Å}$ in length. The two subunits are related by pseudo-twofold rotational symmetry, with the axis located between a short coiled coil of $\alpha 2$ in Ctf19 and $\alpha 1$ in Mcm21 (Fig 3C). The N-terminal $\alpha+\beta$ sandwiches form most of the intersubunit contacts; the C-terminal $\alpha+\beta$ sandwiches protrude away from each other, conferring a Y-shaped outline on the complex (Fig 2). The extensive molecular interface includes $\sim 2,710$ and $\sim 2,670\text{Å}^2$ of buried area from Ctf19 and Mcm21, respectively ($\sim 21\%$ of the calculated total solvent accessible area). The tight association explains the observed cellular interdependence of the Ctf19 and Mcm21 orthologues in *S. pombe*: localization of either to the kinetochore depends on the presence of the other [9].

The $\alpha+\beta$ sandwiches of Ctf19 and Mcm21 are structurally similar to the globular domains of the kinetochore proteins Csm1 [22], Spc24 and Spc25 [21] (Fig 3; Table 1). Csm1 is a subunit of the monopolin complex; Spc24–Spc25 form the

Fig 1 | Characterization of purified Ctf19–Mcm21, COMA and COMA with N-terminally truncated Ctf19–Mcm21. Size-exclusion chromatograms (absorbances at 260 and 280 nm) and Coomassie blue-stained SDS-PAGE of fractions across the principal peak from (A) full-length Ctf19–Mcm21, (B) full-length COMA with histidine-tagged Mcm21 (His-Mcm21), (C) COMA with N-terminally truncated Ctf19 (residues 107–270) and His-Mcm21 (108–293). The overloaded initial lane shows the column input (A and B: 10% Bis-tris gel, NuPAGE, Life Technologies; C: 4–20%, Tris–HCl TGX gel, Bio-Rad).

Fig 2 | Molecular architecture of Ctf19-Mcm21. (A,B) Secondary structure representation of Ctf19-Mcm21. Flexible regions without interpretable electron density are indicated by dotted lines. Apart from minor variations in the C-terminal helix of Mcm21 and the penultimate helix in Ctf19, the globular parts of the two heterodimers in the asymmetric unit superimpose without substantial differences in secondary structure, indicating that they form a rigid scaffold. A linear guide to the polypeptide chains is shown below the ribbon representations. These and the following molecular illustrations were prepared with PyMol (<http://www.pymol.org/>). N-term, N-terminus.

centromere-proximal part of the NDC80 complex. The pseudo-twofold relationship between Ctf19 and Mcm21 is similar to that between the globular domains of Spc24 and Spc25 in the heterodimer and to the twofold relationship in the Csm1 homodimer (Fig 3G-I). The homology in structure and assembly implies a common evolutionary origin of these protein complexes. The $\alpha + \beta$ sandwiches in Csm1, Ctf19, Mcm21, Spc24 and Spc25 are RWD domains (Fig 3; Table 1), a module first identified by sequence comparisons [23] and exemplified by the structure of murine GCN2, the α -subunit of the eIF2 α kinase (supplementary Fig S3 online) [20]. The RWD domain is especially common among ubiquitin (E2)-conjugating enzymes [24]. Its presence in three different kinetochore subcomplexes (among the few for which structures are known) shows that this fold is an important recurring feature of kinetochore molecular architecture.

Ctf19 and Mcm21 also have pronounced structural similarity to the central domain of the *Drosophila melanogaster* Fanconi anaemia core complex (FANCL; Fig 3C; Table 1), an E3 ubiquitin ligase [25]. Like Ctf19 and Mcm21, FANCL has two RWD domains (RWD-N and RWD-C) arranged in tandem, the first-described example of a double-RWD domain (D-RWD). In FANCL, the D-RWD is required for binding to FANCI-FANCD2 [25]. Single RWD domains are also protein interaction modules in functionally different proteins. They show a substantial variety of interaction modes, but intermolecular contacts often include amino-acid residues from the solvent-exposed, structurally

variable part corresponding to $\alpha 2$ and $\alpha 3$ [20] (supplementary Fig S3 online). In Csm1, a conserved cluster of hydrophobic amino-acid side chains, several of which are located on the surface of a Csm1-specific element between $\alpha 2$ and $\alpha 3$ of the RWD domain, interacts with the protein Dsn1, a subunit of the MIND complex, and potentially with Mif2, a component of the inner kinetochore [22]. The RWD domains of Spc24-Spc25 also interact with MIND [26,27], and several of the most conserved amino acids are in the C-terminal helices of Spc24 and Spc25 [21].

The loop between $\beta 3$ and $\beta 4$ in most currently characterized RWD proteins contains residues, often in an YPXXXP motif (supplementary Fig S3 online), that are important for folding and stability [20]. Two temperature-sensitive, single-amino-acid variants in the corresponding turn ($\beta 7$ - $\beta 8$) of the RWD-C domain of Fta2 (supplementary Fig S6B online), the *S. pombe* Ctf19 orthologue, corresponding to Pro232 and Ser233 in *K. lactis* Ctf19 (Fig 3A), show severe chromosome missegregation and defects in bipolar attachment [9]. Overexpression of the Mcm21 orthologue, Mal2, alleviates this phenotype, suggesting that, as in other RWD domains, the contacts from this turn contribute to stability and that high concentrations of the partner protein can compensate when forming the heterodimer [9]. In our structure, residues in a P₂₃₂SPYP₂₃₆ sequence in the Ctf19 $\beta 7$ - $\beta 8$ turn make intermolecular contacts with $\alpha 3$ of Mcm21, rather than intramolecular contacts with residues from the C-terminal helices as in most RWD domains. The same applies to the $\beta 3$ - $\beta 4$ turn of Ctf19,

Table 1 | Comparison of the D-RWD and RWD domains of Ctf19 and Mcm21 with a selection of structurally similar RWD domains identified by C α superposition with DALI

Domain comparison	DALI Z-score	r.m.s.d.	N	% Ident	Ref
<i>Ctf19 D-RWD (107–269)</i>					
Mcm21 D-RWD (3ZXU)	6.8	4.5	142	8	This report
FANCL D-RWD (3K1L)	5.9	4.5	127	10	[25]
<i>Mcm21 D-RWD (107–293)</i>					
FANCL D-RWD (3K1L)	6.0	4.4	131	17	[25]
<i>Ctf19 RWD-N (107–190)</i>					
Mcm21 RWD-N (107–206)	6.3	4.0	79	13	This report
Mcm21 RWD-C (207–293)	2.1	3.2	53	8	This report
Ctf19 RWD-C (191–269)	3.9	4.7	59	8	This report
Spc24 (2FTX)	4.1	3.0	55	9	[21]
Spc25 (2FTX)	6.0	3.5	77	6	[21]
Csm1 (3N4R)	4.9	3.8	74	14	[22]
GCN2 (1UKX)	4.3	3.3	68	15	[20]
Ubc13 ubiquitin ligase (1JBB)	3.4	3.1	66	8	[37]
<i>Ctf19 RWD-C (191–269)</i>					
Mcm21 RWD-N (107–206)	2.9	2.7	54	9	This report
Mcm21 RWD-C (207–293)	3.8	2.9	66	14	This report
Spc25 (2FTX)	3.0	2.8	53	0	[21]
Csm1 (3N4R)	2.7	2.9	51	6	[22]
GCN2 (1UKX)	4.0	2.9	70	4	[20]
Ubc13 ubiquitin ligase (1JBB)	3.5	2.6	66	8	[37]
<i>Mcm21 RWD-N (107–206)</i>					
Mcm21 RWD-C (207–293)	2.6	3.4	61	11	This report
Spc24 (2FTX)	3.3	3.8	55	15	[21]
Spc25 (2FTX)	5.6	3.7	76	4	[21]
Csm1 (3N4R)	4.5	3.2	73	8	[22]
GCN2 (1UKX)	3.2	3.9	77	13	[20]
Ubc13 ubiquitin ligase (1JBB)	3.3	3.3	74	9	[37]
<i>Mcm21 RWD-C (207–293)</i>					
Spc25 (2FTX)	2.0	3.0	54	15	[21]
Csm1 (3N4R)	3.4	2.7	57	12	[22]
GCN2 (1UKX)	3.3	3.1	72	8	[20]
Ubc13 ubiquitin ligase (1JBB)	4.0	2.8	74	5	[37]

RWD protein domains were aligned with DALI [38]. All proteins are from *S. cerevisiae*, except for Ctf19 and Mcm21 (*K. lactis*), GCN2 (*M. musculus*), and FANCL (*D. melanogaster*). A DALI Z-score of 2.0 was chosen as a threshold for the inclusion of structural homologues in this list; r.m.s.d. is the root-mean-squared distance between the N α -carbon positions aligned; the percent sequence identity (ident) for aligned residues is shown in the next to last column. Protein Data Bank accession numbers are in parentheses after each comparison.

suggesting that Ctf19 is stabilized by Mcm21. Thus, the structure is consistent with available mutational data from related proteins and our observation that recombinant Ctf19 requires the presence

of Mcm21 for overexpression and production of soluble protein in *Escherichia coli* (data not shown).

N-terminal segments

Preceding the RWD-N domains in the polypeptide chains of Ctf19 and Mcm21 are segments spanning positions 69–92 in Ctf19 and 44–59 in Mcm21, both of which have overall higher thermal displacement (*B*) factors than most other parts of the structure. Their N termini extend in parallel away from the body of the complex towards a large solvent channel in the crystal, with ample space to accommodate the polypeptide chain without traceable electron density (Fig 2; supplementary Fig S4 online). In the absence of interacting proteins, these parts are evidently disordered, consistent with predictions based on their amino-acid sequences (supplementary Fig S5 online).

Conserved and divergent regions in Ctf19 and Mcm21

We compared the amino-acid sequences from *K. lactis* Ctf19 and Mcm21 with their orthologues in fungi, metazoa and plants (supplementary Fig S6 online). Mcm21 sequences vary less than those of Ctf19, but neither sequences nor lengths are particularly conserved. Mcm21 from *S. cerevisiae* is 75 residues longer than its *K. lactis* orthologue, and both have ~27% sequence identity (~57% similarity); Ctf19 from *S. cerevisiae* is 99 amino acids longer than its *K. lactis* orthologue, with ~18% identity (~50% similarity). The sequences of *S. pombe*, Fta2 and Mal2, and the metazoan orthologues, CENP-P and CENP-O, have diverged even further (supplementary Fig S6B,D online). Much of the variation lies in the N-terminal regions that are disordered in our crystals. Sequence conservation at key positions in the D-RWD domains implies structural conservation of the core framework of the heterodimer in all eukaryotes.

Sequence conservation between Ctf19 and Mcm21 is very low, despite their close structural relationship. The two subunits have the same or closely related residues at only a few equivalent positions. These include the following pairs in Ctf19 and Mcm21, respectively: Thr 123–Ser 123; Arg 134–Arg 138; Phe 138–Phe 153; Arg 143–Lys 158; Leu 158–Leu 174; Gln 197–Gln 217; Lys 201–Lys 221; Glu 204–Glu 223; and Arg 210–Arg 220. An asparagine caps the C terminus of the fourth β -strand of each RWD domain, and its side chain forms hydrogen bonds with main-chain atoms of the preceding β -strand.

Ctf19–Mcm21 interactions in kinetochore assembly

Localization of COMA to kinetochores depends on Ame1 [2,28], whereas *Okp1* mutants impair the localization only of Ctf19, Mcm21 and *Okp1*, and not that of Ame1. Thus, Ame1 seems to function as a kinetochore ‘adaptor’ for COMA. We have purified recombinant *K. lactis* COMA (Fig 1, Materials and methods, in supplementary information online) and carried out limited proteolysis of both the full COMA complex and the Ctf19–Mcm21 subcomplex (supplementary Table S2 online). For the disordered parts in our structure, the patterns are essentially identical. It is therefore likely that these regions are also disordered in the full COMA heterotetramer and do not interact substantially with *Okp1* or Ame1. We have confirmed this suggestion by purifying N-terminally truncated Ctf19 (residues 107–270) and Mcm21 (residues 108–293) together with *Okp1* and Ame1 (Fig 1C). The D-RWD regions of Ctf19 and Mcm21 are

Fig 3 | Comparison of the molecular structures of D-RWD domains, RWD domains in kinetochore proteins and the quaternary structure of the Ctf19–Mcm21, the Csm1–Csm1 and the Spc24–Spc25 kinetochore complexes. Comparison of the D-RWD domains of (A) Ctf19 (residues 107–269), (B) Mcm21 (residues 107–293), (C) FANCL (residues 111–286; Protein Data Bank accession number 3K1L) and the single RWD domains of (D) Csm1 (70–180; 3N4R), (E) Spc24 (132–221; 2FTX) and (F) Spc25 (155–213; 2FTX), and schematic diagrams of their secondary structures are shown below. Spc24 lacks β 4 and has only one C-terminal α -helix. Ribbon representations in orientations obtained by superposing C α positions on those of Ctf19 (107–269). The β -turn between β 7 and β 8 of Ctf19 is displayed as ball and stick. (G–I) Dimeric complexes are shown in orientations obtained by superposing C α positions. The segments with residues 69–92 in Ctf19 and 44–59 in Mcm21 are semitransparent. Lens symbols show locations of pseudo- and twofold rotational axes.

Fig 4 | Amino-acid sequence conservation mapped onto the Ctf19–Mcm21 structure. (A,B) Semitransparent molecular surface representation of Ctf19–Mcm21, coloured by sequence conservation score, with secondary structure below. A key to the colour coding for the normalized sequence conservation score is shown. The complex is oriented as shown in Figure 2A,B. Three surface areas, conserved among point-centromere yeast (supplementary Fig S6 online), are highlighted: a hydrophobic groove in Mcm21; hydrophobic residues in $\alpha 5$ and $\alpha 6$ of Ctf19; and Lys162 in Mcm21. Sequence conservation scores, based on TCooffee [35] alignments, were calculated with ConSurf [36] and mapped onto the coordinates.

alone sufficient to associate with Okp1–Ame1. Further truncation of Ctf19 and Mcm21, by deleting the C-terminal RWD domains from either partner, did not yield stable protein complexes from recombinant overexpression in *E. coli*, consistent with our observations above with regard to the role of the $\beta 7$ – $\beta 8$ loop in RWD domain stability.

Localization of a number of proteins to the kinetochore depends on COMA components. Binding partners probably include Sli15, the adaptor for Ipl1 [29], and other components of the CTF19 complex [13,30,31]. Ctf19–Mcm21 has four spatially separated RWD domains (Fig 2), each presenting potentially independent protein interaction surfaces. Most of the surface residues conserved among point-centromere yeasts are on one face of the heterodimer (Fig 4). A conserved binding partner, such as Okp1, is likely to interact with this surface, consistent with our data showing that Okp1–Ame1 associates with the core D-RWD of Ctf19–Mcm21. The less conserved N-terminal regions of Ctf19 and Mcm21, disordered in our crystals, could be potential interaction sites for other partners, such as subunits of the CTF19 complex.

The several apparent contact surfaces presented by the four RWD domains in Ctf19–Mcm21 probably reflect its functional role as a linker. Although conserved in all eukaryotes and essential in *S. pombe*, Ctf19 and Mcm21 are not essential for viability in *S. cerevisiae*. The known structures of kinetochore subcomplexes indicate that a kinetochore resembles more closely a cross-connected framework than a solid entity. Its molecular architecture probably incorporates important but redundant components and cooperative interactions among them. The links established by partners of Ctf19–Mcm21 might be dispensable in some contexts but not in others, because of the variable strengths of compensating interactions.

The observation that RWD domains are principal interaction modules in at least five different kinetochore proteins suggests that

we will find this structural element in several more kinetochore components. The kinetochore is not the only elaborate, subcellular assembly that contains distinct, but related building blocks. The subunits of the eukaryotic proteasome [32] and the ‘ancestral coatamer element’, common to several of the ~ 30 nucleoporins and to COPII vesicle proteins [33], are further examples. Like these other molecular machines, kinetochores may have evolved from a small number of ancestral protein modules.

METHODS

Molecular cloning and protein preparation. Coding regions of the four COMA components were amplified by polymerase chain reaction from *K. lactis* genomic DNA. The construction of di- and tetracistronic vectors, using backbone of pET3aTR [34], expression and purification of the Ctf19–Mcm21, as well as full and truncated COMA complexes, are described in supplementary information online.

Protein characterization. We used sedimentation equilibrium analytical ultracentrifugation to determine the oligomeric state of Ctf19–Mcm21. The cleavage sites of the fragments of Ctf19–Mcm21 and COMA from limited proteolysis were determined by mass spectrometry (details in supplementary information online).

Crystallization and structure determination. The Ctf19–Mcm21 complex was crystallized using 7–10% (wt/vol) polyethylene glycol 8,000 14–20% (vol/vol) ethylene glycol, 30 mM CaCl_2 , 30 mM MgCl_2 and 100 mM bicine/Trizma, pH 8.1–8.9, at ~ 293 K. The crystals were in space group $P6_222$, $a = b = 239.0$, $c = 179.5$ Å, with two heterodimeric complexes in the asymmetric unit. X-ray diffraction data were recorded at NE-CAT beamline 24-ID-C at the Advanced Photon Source, Argonne National Laboratory. Data processing, phase determination by single-wavelength anomalous diffraction, model building and refinement are described in supplementary information online.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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Author contributions: F.S. planned and carried out the experiments; both authors analysed data and wrote the paper.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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