

When two is not enough: a CtIP tetramer is required for DNA repair by Homologous Recombination.

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Homologous recombination (HR) is central to the repair of double-strand DNA breaks that occur in S/G2 phases of the cell cycle. HR relies on the CtIP protein (Ctp1 in fission yeast, Sae2 in budding yeast) for resection of DNA ends, a key step in generating the 3'-DNA overhangs that are required for the HR strand-exchange reaction. Although much has been learned about the biological importance of CtIP in DNA repair, our mechanistic insight into its molecular functions remains incomplete. It has been recently discovered that CtIP and Ctp1 share a conserved tetrameric architecture that is mediated by their N-terminal domains and is critical for their function in HR. The specific arrangement of protein chains in the CtIP/Ctp1 tetramer indicates that an ability to bridge DNA ends might be an important feature of CtIP/Ctp1 function, establishing an intriguing similarity with the known ability of the MRE11-RAD50-NBS1 complex to link DNA ends. Although the exact mechanism of action remains to be elucidated, the remarkable evolutionary conservation of CtIP/Ctp1 tetramerisation clearly points to its crucial role in HR.

Introduction

The appropriate choice of DNA double-strand break (DSB) repair mechanism is critical for maintenance of genomic stability¹. During the S and G2 phases of the cell cycle, when sister chromatids become available due to ongoing or completed DNA replication, cells can repair DSBs by HR. Extensive resection of DNA ends commits cells to HR, by generating 3'-DNA overhangs that are the required substrate for the strand-exchange reaction promoted by the RAD51 recombinase².

CtIP (CtBP-interacting protein), also known as RBBP8 (Retinoblastoma-binding protein 8), is an evolutionarily conserved DNA-repair factor with a critical role in HR³. It is now well established that a shared function of CtIP and its orthologues in HR-mediated DSB repair is to promote DNA-end resection^{4,5} in a cell-cycle dependent fashion^{6,7}. Accumulating evidence from studies in budding yeast supports a model where the CtIP orthologue, Sae2, is required for effective resection of DNA ends that are blocked by a lesion or the presence of bound proteins^{8,9}. The critical function of CtIP/Ctp1/Sae2 at DNA ends is carried out in close functional and physical cooperation with the DNA damage sensor and repair complex MRE11-RAD50-NBS1 (MRN, where NBS1 is Xrs2 in budding yeast)¹⁰⁻¹⁵. The central role of CtIP/Ctp1/Sae2 in DSB repair is further highlighted by its complex regulation, mediated by an array of post-translational modifications that include phosphorylation by cyclin-dependent and DNA-damage activated kinases, acetylation, ubiquitylation, NEDDylation and proline isomerisation³.

Despite its importance to DSB repair, our mechanistic understanding of how CtIP promotes resection remains incomplete. Inspection of its amino acid sequence offers little insight into the molecular details of its possible function, as regions of high sequence conservation are limited to its N- and C-terminal ends and display no similarity to known protein domains. The CtIP sequence has diverged radically in evolution relative to its orthologues in lower eukaryotes; this is highlighted by its increased size in metazoans, a phenomenon shared with other mediators of DNA repair such as BRCA2. These observations suggest that CtIP might function to promote repair via multiple, regulated protein-protein interactions. However, nuclease activity has also been reported for Sae2 and CtIP¹⁶⁻¹⁸, indicating that CtIP might have both catalytic and non-catalytic roles in processing of DNA ends. The complexity of the available evidence highlights the need for additional work to further define the biochemical roles of CtIP in DNA repair.

Here, we comment on two recent structure-function studies that have provided fresh insight into CtIP function^{19,20}. The new evidence uncovers a remarkable oligomerisation mechanism that is shared by human CtIP and its fission-yeast orthologue Ctp1: juxtaposition of their conserved N-terminal regions results in a specific tetrameric structure that is necessary for effective DSB repair by HR.

Structural basis of CtIP/Ctp1 tetramerisation

Although published reports had already provided evidence that CtIP could self-associate²¹⁻²³, the precise oligomeric state of CtIP and orthologues had remained undefined. Our own new data¹⁹, together with the work by Andres

and colleagues²⁰, has now solved this issue for human CtIP and fission yeast Ctp1, respectively. Both reports provide biophysical and structural evidence that CtIP and Ctp1 exist as constitutive tetramers and share a mode of self-association that has remained remarkably conserved over a billion years of evolutionary history. Furthermore, the crystallographic analyses reveal that tetramerisation is mediated by short sequence motifs present at the start of parallel coiled-coil segments located in the amino-terminal regions of both CtIP and Ctp1. The splayed ends of two coiled-coil dimers come together in an interlocking interaction, generating a tetrameric arrangement of protein chains that is best described as a dimer-of-dimers architecture (Fig. 1A).

Superposition of the crystal structures of tetrameric CtIP and Ctp1 reveals the common molecular determinants for self-association. Tetramerisation is mediated by an amphipatic two-turn helix (tetramerisation helix) spanning the CtIP sequence 20-FKDLWTKL-27 (12-WSIVYRQL-19 in Ctp1) (Fig. 1B). Aromatic and hydrophobic residues F20, L23, W24 and L27 (W12, V15, Y16 and L19 in Ctp1) generate a stable hydrophobic core by intermeshing interactions that result from the antiparallel packing of the tetramerisation helices in the four CtIP chains (Fig. 1C). In Ctp1, the tetramerisation domain is extended by one additional helical turn spanning leucine residues 22 and 23. The transition from coiled-coil structure to the splayed helices of the tetramerisation domain is promoted by a bulky aromatic residue in position 'd' of the heptad repeat, H31 for CtIP and Y26 for Ctp1.

The biophysical evidence coming from size-exclusion chromatography - multi-angle laser scattering (SEC-MALS) measurements shows that, rather than

forming heterogeneous oligomeric mixtures, both CtIP₁₈₋₁₄₅ and full-length Ctp1 exist predominantly as tetrameric species in solution, in agreement with their extensive tetramerisation interfaces revealed by the crystallographic analysis. This tetrameric architecture must therefore be considered as the constitutive oligomeric state for both CtIP and Ctp1. However, it remains possible that their tetrameric arrangement may be altered by the regulated intervention of other proteins, and/or by post-translational modifications of the CtIP/Ctp1 N-terminal region. The striking similarity between the tetrameric architectures of CtIP and Ctp1 strongly implies that their presence must be widespread among their eukaryotic counterparts. Indeed, the functional orthologue of CtIP in budding yeast, Sae2, has been reported to exist in multimeric form^{16,24} and a single-point mutant in its N-terminal region, L25P, abolishes its ability to self-associate²⁵.

Although the crystallographic analysis of the CtIP N-terminus was limited to the first 52 amino acid residues, secondary structure prediction of the CtIP sequence shows that its parallel coiled-coil structure extends to include the first ~150 residues. Intriguingly, the coiled-coil region is interrupted in its middle by a zinc-binding motif comprising conserved cysteines 89 and 92¹⁹. The tetrahedral coordination of zinc is presumably satisfied by shared coordination of one metal atom between two CtIP polypeptides. The likely structural alterations induced by zinc binding on the regular coiled-coil structure, as well as the functional role of the zinc-binding motif, are currently not understood. Zinc binding by the N-terminal domain appears to be an exclusive feature of vertebrate CtIP sequences, as the cysteines that serve as ligands for the metal are absent in CtIP orthologues from simpler eukaryotes.

Functional implications of CtIP tetramerisation

The best-characterized cellular phenotype of human CtIP deficiency is a profound defect in DNA-end resection, resulting in impaired DNA repair for all pathways that require generation of single-stranded DNA overhangs at a DSB site. These include HR by gene conversion⁵ or single-strand annealing²⁶, as well as microhomology-mediated end-joining (MMEJ; also known as alternative end-joining²⁶). In yeast, Ctp1 or Sae2 mutants are also hypersensitive to DNA-damaging agents^{27,28} and show defective resolution of meiotic recombination intermediates^{29,30}.

We exploited our structural insight into CtIP oligomerisation to design a single-residue substitution, replacing leucine at position 27 with glutamate, which prevented tetramer formation whilst preserving CtIP's ability to dimerise. Remarkably, we found that the L27E CtIP mutant phenocopies CtIP deficiency in terms of defective resection and gene conversion¹⁹, highlighting the critical role of the tetrameric CtIP architecture for recombinational DNA repair. Interestingly, the L27E CtIP mutant also shows a strong defect in accumulating at DNA-damage sites¹⁹, providing a molecular explanation to the functionally-null phenotype observed in the complementation assays.

Why would CtIP's accrual at DNA-damage sites depend on its tetrameric state? CtIP localization to DSBs is thought to occur in two different ways: through recruitment by the NBS1 component of the MRN complex^{31,32} and by direct interaction with the Fanconi Anemia related protein FANCD2^{33,34}. However, L27E CtIP shows no defect in its interaction with either NBS1¹⁹ or FANCD2 (our unpublished data). We note that the conserved C-terminal

domain (CTD) of CtIP and Ctp1 can bind DNA^{19,20} and that mutation of Ctp1 residues critical for DNA binding causes hypersensitivity to DNA-damaging agents²⁰. The tetrameric architecture adopted by CtIP/Ctp1 thus appears well suited to simultaneously position multiple CTDs on distinct DNA molecules. Although a CtIP tetramer could in principle locate its CTDs on four different DNA molecules, we favour the possibility that a 'dimer of dimers' CtIP could locate pairs of CTDs at the two ends of a DNA DSB²⁰, in preparation for simultaneous processing of DNA ends (Fig. 2). Thus, tetrameric CtIP would help link DNA ends while at the same time providing two copies of its CTD to promote end-resection, a task shared with the MRN complex, which also contains dimeric versions of its core components MRE11 and RAD50³⁵. Failure to connect CtIP dimers, as in the case of the L27E CtIP mutant, could result in weaker DNA binding and improper retention of CtIP at DNA-damage sites. Interestingly, a requirement for CtIP tetramerisation does not seem to be equally important to all forms of CtIP-dependent DNA repair, as CtIP dimers appear to retain some functionality in MMEJ¹⁹. This opens the possibility of functional regulation of CtIP oligomeric states, a feature already described for budding yeast Sae2²⁴, even though tetrameric oligomerisation seems to be CtIP's natural state in unperturbed cells¹⁹.

The newly uncovered architectural parallelism between CtIP and the MRN complex is functionally intriguing, although the similarity breaks down in one important respect: the size of CtIP, unlike that of RAD50 (the architectural component of the MRN complex), is not evolutionary conserved. Thus, human CtIP is about three times larger than Ctp1 (Fig. 1A), and in general a direct correlation is observed between organismal complexity and size of the CtIP

orthologue. In addition, the coiled-coil region of CtIP comprises only one sixth of its length, whereas most of its remaining sequence shows no clear signs of structural conservation. Consequently, although the ability of CtIP to operate concomitantly at the two ends of a DNA break is clearly important as suggested by its 'dimer-of-dimers' structure, it is unlikely that CtIP will have the principal mechanical role in bridging DNA ends. Important aspects of the biochemical function of the tetramerisation region might also differ between human CtIP and its orthologues, as highlighted by the observation that the N-terminal domain of fission yeast Ctp1 possesses DNA-binding abilities²⁰ that have not so far been observed for the NTD of human CtIP¹⁹.

Figure Legends

Figure 1. Tetrameric architecture of human CtIP and fission yeast Ctp1. (A) Comparison of the crystal structures of the tetramerisation domains of CtIP and Ctp1. The structures are shown as ribbons, with the tetramerisation region of the structure coloured in purple and the coiled-coil region in yellow. The N- and C-termini of one chain are marked in each tetrameric structure. The position and extent of the structure is mapped onto a drawing of the CtIP and Ctp1 sequences, colour-coded as the structural ribbons. An asterisk marks the position of the zinc-binding motif in the coiled-coil region of CtIP. The position of the conserved C-terminal CtIP/Ctp1/Sae2 homology region is indicated in green. (B) Structure-based sequence alignment of CtIP and Ctp1 N-terminal regions. Sequences are colour-coded as in A. Residues forming the tetramerisation motif shared between CtIP and Ctp1 are highlighted in white. (C) Details of tetramerisation interactions in human CtIP and fission

yeast Ctp1. The left-side panel shows a superposition of the crystal structures, highlighting side chains of their shared tetramerisation motifs. The right-side panel shows a conserved mode of interaction of the aromatic side chains of W24 in CtIP and Y16 in Ctp1, involved in the antiparallel association between tetramerisation motifs.

Figure 2. A model for bridging DNA ends by CtIP and the MRN complex at a DSB site. The dimer-of-dimers architecture of a CtIP tetramer might serve to position a pair of its conserved CTDs at each end of a DNA DSB, where it would cooperate with the MRN complex to promote the initial DNA-end resection event. The structural elements responsible for oligomerisation of the MRN complex (RAD50's zinc hook³⁶) and CtIP (N-terminal tetramerisation domain¹⁹) are shown as helical ribbons. The zinc-binding motifs in the N-terminus of each CtIP dimer are also shown.

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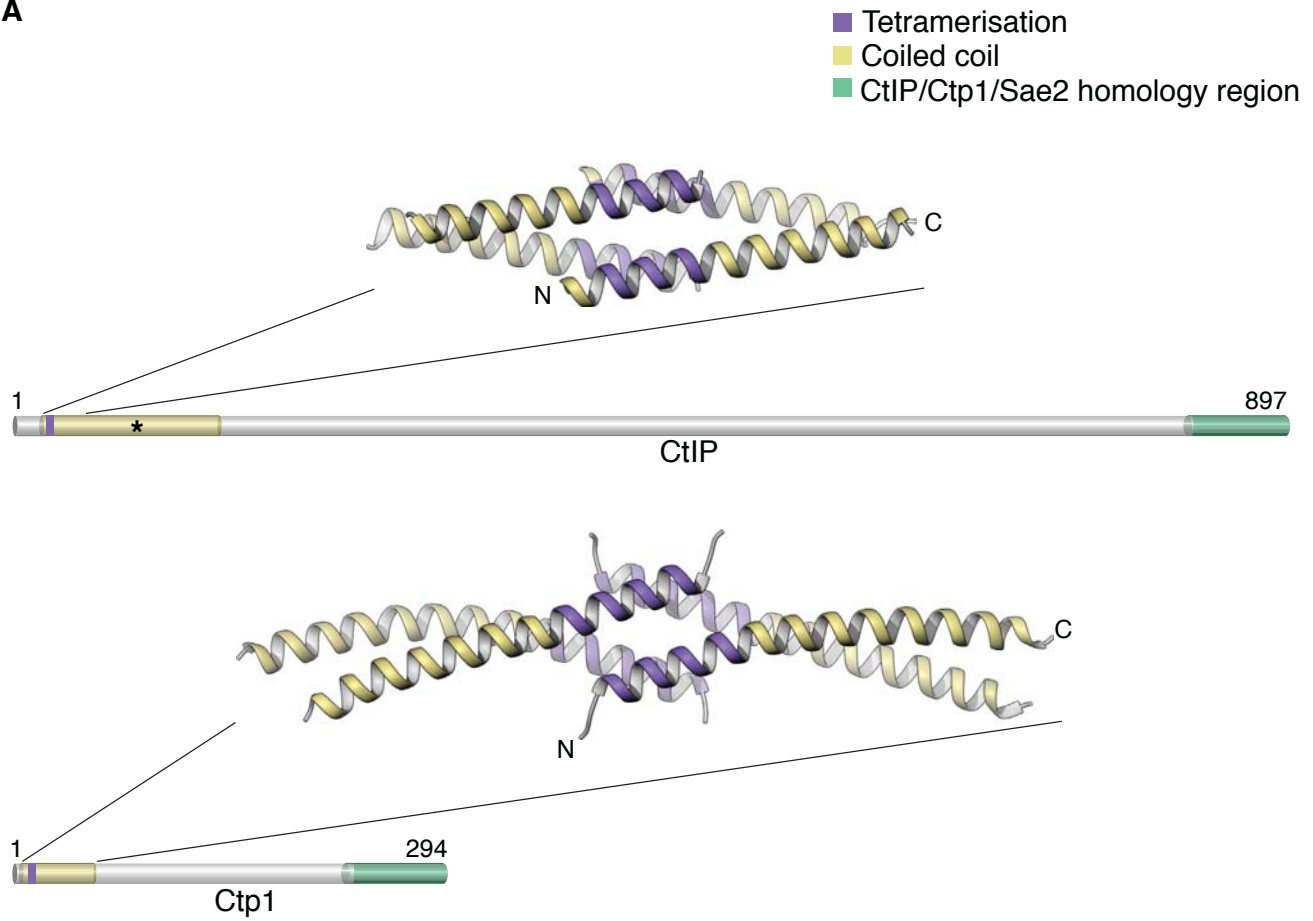
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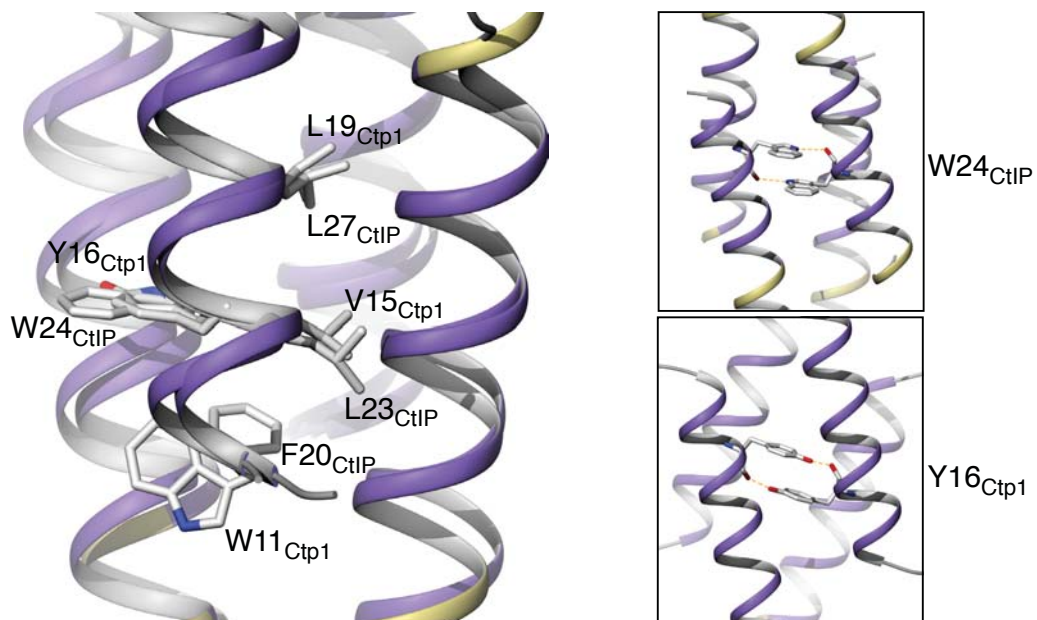
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A**B**

19	D	FKDLWTKLKECH	DRE VQGLQVKVTKLKQERILD	52	CtIP
11	H	WSIVYRQLGNLLEQY	EVEIARLKSQLVLEKKLR	45	Ctp1

C**Figure 1**

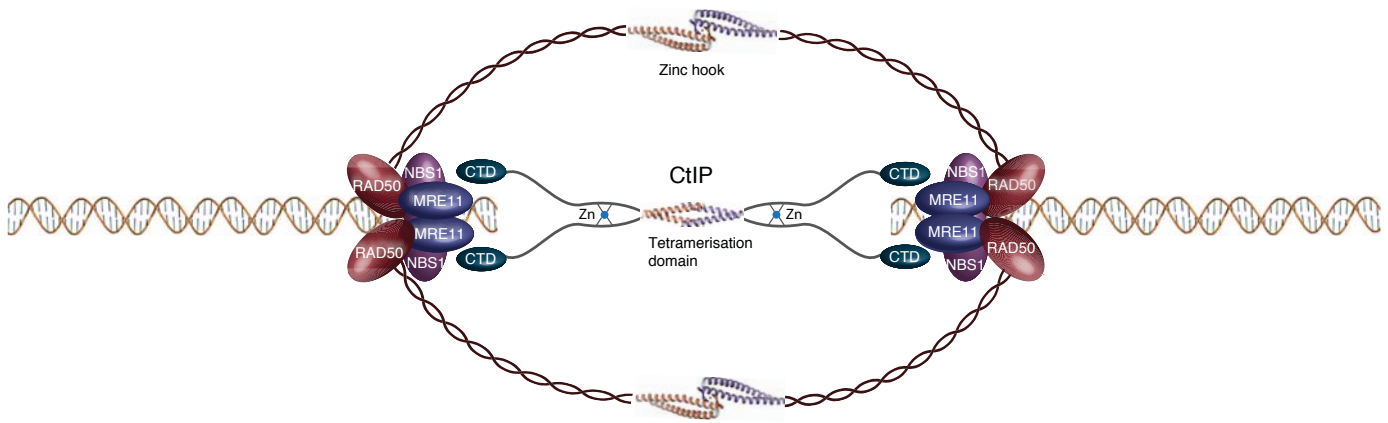


Figure 2