Figure EV1. Comparison of Rtt103p CID structures bound to differently phosphorylated CTD.

A, B Comparison of Rtt103p CID (grey helices) bound to the (A) pThr⁴-CTD (magenta sticks; PDB ID: 5LVF) or (B) to the pSer²-CTD peptide (yellow sticks; PDB ID: 2L0I).

Rtt103p CID residues involved in the interaction with CTD are shown in grey sticks and labelled with blue font. Sequences of peptides used in structure determination are indicated below the structures. Peptide residues shown on the image are highlighted in red and black font; the residues highlighted in red have intermolecular contacts used for the structure calculation, residues in grey are not displayed for clarity reasons or are missing coordinates.

Figure EV2. Rtt103p CID interacts with pSer²-CTD and pThr⁴-CTD using the same canonical interface.

In order to identify the surface of the CID involved in interaction with pThr⁴-CTD, we performed a ¹H¹⁵N-TROSY NMR titration experiment, where ¹⁵N-labelled Rtt103 CID was titrated with pThr⁴ peptide. Titration confirmed that Rtt103p CID is interacting with pThr⁴-CTD peptide in an almost identical fashion as with pSer²-CTD, using the canonical surface of helices α₂, α₄ and α⁷ (Fig 1B and C). However, important rearrangements were observed for Val¹⁰⁹ and Ile¹¹², residues that lay in close proximity of P₃b and T₄b.

A Chemical shift perturbations (CSP) of the Rtt103p CID upon interaction with FAM-pSer² CTD (red) or FAM-pThr⁴ CTD (grey) peptides plotted against residue number of Rtt103p CID. Secondary structure elements are shown below the x-axis. Helices involved in the interaction with phospho-peptides are coloured in black. FAM, 5,6-carboxyfluorescein.

B Overlay of ¹H-¹⁵N TROSY spectra of complex of Rtt103p CID with FAM-pSer² CTD (red) and FAM-pThr⁴ CTD (blue).
Figure EV2.
Figure EV3. Structural integrity of Rtt103p CID mutants.
Comparison of $^1$H NMR spectra of the wild type (green), Y62A (blue) and H66A (red) mutants of Rtt103p CID; the region with NH backbone and side-chain resonances is shown. Data were collected on 850 MHz Bruker AVANCE III spectrometer at 293K.
Figure EV4. Structural sequence alignment of CIDs.
Sequence alignment of CIDs based on superposition of the CID structures (PDB IDs: 2KM4, 4NAC, 4FLB, 3CLJ, 2BFO, 3D9I) using Align tool of UCSF Chimera [40]. In this type of alignment, residue types are not used, only their spatial proximities. Yellow boxes highlight structured elements; red boxes show key residues responsible for the CTD recognition according to numbering of Rtt103p.

Figure EV5. Intermolecular contacts between the Y1c and Rtt103p CID.
Strip plots from 3D F1-13C/15N-filtered NOESY-[13C-1H]-HSQC showing intermolecular contacts between Y1c and Arg116 (left) and Ile118 (middle, right).
Figure EV5.

Arg116 Cα-Hα  Ile118 Cβ1-H51  Ile118 Cγ2-Qγ2
Figure EV6. Double phosphorylation of CTD does not influence serine–proline peptide bond isomerization state population.

A Scheme of cis- and trans-isomers of X-proline peptide bond (X stands for any amino acid). Peptide bond is highlighted in blue.

B To establish conformational populations of mono- and di-phosphorylated peptides, [\( ^{13}\text{C}, ^1\text{H} \)] HSQC spectra of CTD peptides were measured. Comparison of [\( ^{13}\text{C}, ^1\text{H} \)] HSQC spectra of PSYS\(^{13}\text{CpT}^{13}\text{PSYS} \) (left) and PSYpS\(^{13}\text{CpT}^{13}\text{PSYS} \) (right). \(^1\text{H}-^{13}\text{C} \) correlations of \( \beta \) and \( \gamma \) C-H pairs are shown. Trans-chemical shift region of \( \gamma/\beta \) highlighted in yellow [41].

C Overlay of [\( ^{13}\text{C}, ^1\text{H} \)] HSQC spectra from the NMR titration of the PSYS\(^{13}\text{CpT}^{13}\text{PSYS} \) peptide with non-labelled Rtt103p CID. Protein–peptide molar ratios for each titration step and corresponding colour of the spectrum are indicated on the right.
Figure EV6.