Crystal structure of CENP-TWSX: A novel histone-fold complex at eukaryotic centromere

During eukaryotic cell division, each and every one of replicated chromosome is faithfully segregated into two daughter cells to keep their genome integrity. Failure in this process could result in chromosome instabilities, which may cause cell death, genomic disorder or even tumor formation. To prevent such catastrophic events, cells tightly regulate the chromosome segregation.

Two mechanisms coordinate to ensure faithful chromosome segregation. One is sister chromatid cohesion which hold the two sister chromatids upon their generation during replication until the onset of anaphase. Another is microtubule attachment to chromosomes. In mitosis, spindle microtubules emanate from two daughter centrosomes and capture chromosomes (Fig. 1(a)). Only the chromosome that is correctly captured by microtubules from two different centrosomes generates a tension that is balanced between the pulling force from microtubules and the resisting force from sister chromatid cohesion. These bi-oriented chromosomes are aligned to metaphase plate during mitosis. Other attachments are futile and are corrected by spindle checkpoint pathway. When all the chromosomes are correctly captured and aligned to metaphase plate, spindle checkpoint is released. This enables sister chromatid cohesion to be dissolved and segregates chromosomes into two daughter cells to complete mitosis.

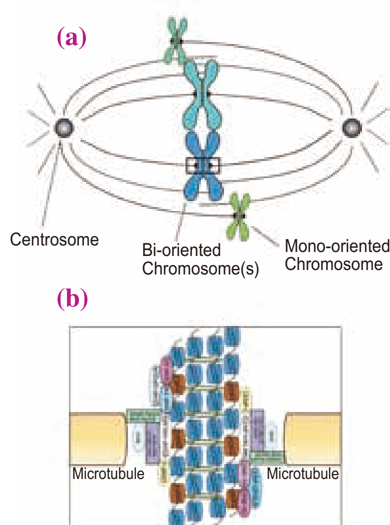

Fig. 1. **(a)** Schematic diagram of eukaryotic chromosome segregation. Replicated chromatids are shown as X-shape and kinetochore is formed at each chromosome denoted by black circles. Spindle microtubules emanate from centrosomes and capture chromosomes through kinetochore. Bioriented chromsomes that are captured by two spindle microtubules align to the metaphase plate. **(b)** Close-up view of the centromere-microtubule attachment site boxed in (a). Only major kinetochore components that are involved in chromosome-microtubule attachments are depicted. Two sister chromatids are held together by sister chromatid cohesion rings.

Robust binding between chromosome and microtubule is mediated by proteinacious structure called a kinetochore that is built at the centromeric region on chromosome [1]. Proteomic analysis of eukaryotic kinetochore revealed that it is comprised of more than 100 components. The function of these components varies from protein-protein interaction to protein-nucleic acid interaction (Fig. 1(b)). These components form an intricate network to connect chromosome and microtubules. One of the mysteries that remain in the field is how the centromere DNA is connected to the microtubule.

To address this question we identified the putative DNA binding kinetochore complexes from both human and chicken cells and analyzed them. CENP-S, -T, -W, and -X are kinetochore components containing histone fold within its sequence. CENP-T and CENP-W forms a heterodimer and CENP-S and CENP-X forms a heterodimer [2,3]. CENP-T and -W are essential for cell viability and deletion/knockdown of CENP-S or CENP-X also causes chromosome segregation defect, although cells with their knockout are still viable. To understand the structural basis of these complexes, we crystallized chicken CENP-TW histone fold and chicken CENP-SX. Diffraction data were collected at **BL38B1** and **BL44XU**. Both CENP-TW and CENP-SX structures were determined by selenium single anomalous dispersion using selenomethionine derivative crystals. Mercury derivative was also used for determination of CENP-SX crystal structure. CENP-TW was a heterodimer and CENP-SX was a tetramer (Figs. 2(a) and 2(b)) [4]. Overall structure of both CENP-TW and CENP-SX was similar to canonical histones and other histone fold complexes. In addition to three helices which are conserved among histone fold proteins, CENP-T contained two extra helices at its carboxyl-terminus (C-terminus) which formed a v-shape. CENP-S similarly contained an extra helix at its C-terminus. Apart from these regions, CENP-T contains ~500 residues of flexible amino-terminus (N-terminus) and CENP-S contains ~40 residues of C-terminal basic tail. Both regions were omitted from crystallization. Surface charge plot of CENP-TW and CENP-SX revealed that both proteins were highly basic and in particular, CENP-TW was globally positively charged with pI of 10.1 whereas pI of CENP-SX was 8.7 (Fig. 2(a) and 2(b)). Based on *in vitro* DNA binding experiment using naked double stranded DNA, we demonstrated that these complexes indeed associate with DNA [4].

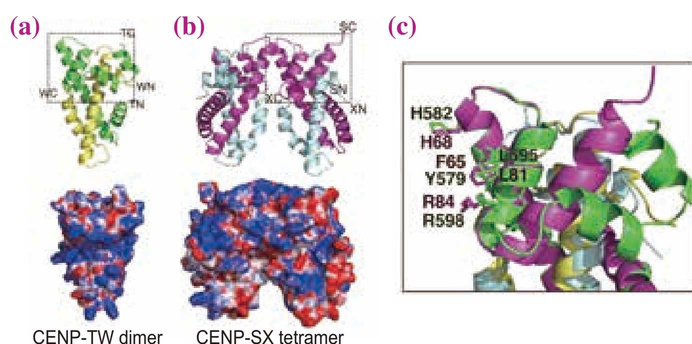

Fig. 2. **(a)** Crystal structure and electrostatic potential of CENP-TW dimer. CENP-T and CENP-W are shown in ribbon diagram and are shown in green and in yellow, respectively. N and C terminus of T or W are labeled. **(b)** Crystal structure and electrostatic potential of CENP-SX tetramer. CENP-S and CENP-X are shown in ribbon diagram and are shown in magenta and cyan, respectively. N and C terminus of S or X are labeled as in (a). **(c)** superposition of CENP-TW and CENP-SX dimer. Boxed regions in (a) and (b) are superimposed. Residues shown in stick model are conserved between CENP-T and CENP-S.

More intriguing fact upon structure determination was that CENP-SX and CENP-TW are more similar to each other than to other histones (Fig. 2(c)). The similarity between the two complexes was also observed in the regions within CENP-SX involved in tetramer formation. Although CENP-TW only forms a dimer in solution, the residues were almost identical. This suggested that CENP-SX and CENP-TW might form a higher order complex. Upon mixture, CENP-SX and CENP-TW formed a stoichiometric complex. We further purified the complex and crystallized them. Two different crystal forms were obtained and the diffraction data were collected at **BL38B1** and **BL44XU**. The structure was determined by molecular replacement using CENP-TW and CENP-SX heterodimer as a template (Fig. 3(a)). Crystal structure visualized the details of CENP-TWSX complex and they formed a heterotetramer using the conserved tetramer interface (Fig. 3(b)) [4].

Biochemical analysis of CENP-TW, CENP-SX, and CENP-TWSX showed that they all possess supercoiling activity and wrapped DNA using basic regions [4]. However, CENP-TW, CENP-SX and CENP-TWSX bound to DNA with different mode. CENP-TW and DNA formed a larger complex upon mixture and there was no distinct complex. On the other hand, CENP-SX had a preference toward ~50 bp dsDNA while CENP-TWSX favored ~80 bp dsDNA. Micrococcal nuclease digestion showed that CENP-TW binds tightly and CENP-SX binds less tightly. CENP-TWSX heterotetramer binding was intermediate showing that this new complex possesses characteristics of both complexes. We created a chicken DT40 cell line in which wild-type CENP-T was replaced with tetramer defective CENP-T mutant and found that the tetramer defective CENP-T mutant did not recruit CENP-S or CENP-X to kinetochore and these cells fail to build functional kinetochore [4]. Similarly, we demonstrated that DNA binding mutant of CENP-TW or CENP-SX loses kinetochore integrity *in vivo*. Thus, we conclude that CENP-TWSX complex forms a structural core for kinetochore formation on centromere using its DNA binding activity.

In addition to the histone fold complex, both chicken and human CENP-T contain long flexible N-terminus where most of the region is predicted to be unstructured. Within the extreme N-terminal 100 residues, there are several cyclin-dependent kinase (CDK) phosphorylation sites and these sites are phosphorylated during mitosis [5]. Phosphorylation of CENP-T enables Ndc80 complex to associate to centromere and this complex binds microtubules. It has recently been shown that homologous complex is conserved not only in higher eukaryotes but also in fission yeast, filamentous fungi and budding yeast. Thus, CENP-TWSX complex seems to be present in most eukaryotes to form a new centromeric chromatin structure and to build kinetochore structure.

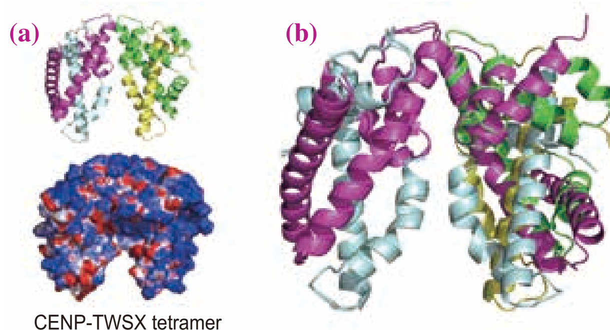

Fig. 3. **(a)** Crystal structure and electrostatic potential of CENP-TWSX tetramer. CENP-T, -W, -S and -X are shown in ribbon diagram. Coloring scheme is same as in Fig. 2. **(b)** Superposition of CENP-SX tetramer and CENP-TWSX tetramer. Two tetramers were aligned through CENP-SX.

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