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学位論文題目	Biochemical and cell biological studies on the constitutive kinetochore proteins: the CENP-T-W-S-X complex functions as a critical scaffold in kinetochore assembly
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Faithful chromosome segregation during mitosis is essential for the accurate transmission of genetic material. To facilitate this process, replicated each sister chromatid assembles the multi-protein kinetochore complex on centromeric DNA which forms a dynamic interface with spindle microtubules. In most eukaryotes, the kinetochore is formed at a single genome locus on each chromosome and the kinetochore specification is occurred by a sequence-independent epigenetic mechanism. To establish a functional kinetochore structure, a subset of kinetochore proteins must make strong and specific contacts with centromeric DNA. Nucleosomes containing the centromere-specific histone H3 variant CENP-A might provide an important epigenetic mark to establish a kinetochore-specific chromatin structure. However, although CENP-A deposition is necessary for kinetochore specification, it is not strictly sufficient for the formation of functional kinetochores in vertebrate cells. This suggests there are additional proteins that are required to direct sequence-independent kinetochore assembly. In addition to CENP-A, a group of 16 chromatin-proximal proteins termed as the Constitutive Centromere-Associated Network (CCAN) is present at centromeres throughout the cell cycle in vertebrate cells. Some CCAN proteins such as CENP-T or CENP-C possess DNA-binding activity and these proteins might be candidates to function as a platform for kinetochore assembly. Our laboratory has previously shown that CENP-T forms a complex with CENP-W (CENP-T-W complex) and the complex does not directly associates with CENP-A-containing nucleosomes. As these data suggest that this complex has a distinct function from CENP-A, I focused on the CENP-T-W complex to define the mechanisms that direct kinetochore assembly and examined this complex with chicken DT40 cells in my thesis.

Chicken CENP-T is a 639 amino acids (aa) protein and contains histone-fold domain in its C-terminal 109 aa region, which is responsible to bind to DNA and to form a complex with another histone-fold containing protein CENP-W. In contrast, function of the 530 aa N-terminal region is unclear. To clarify the functional role of the CENP-T N-terminus, I analyzed DT40 cells in which endogenous CENP-T is replaced with several CENP-T mutants with deletion in the N-terminus. I found that cells expressing a CENP-T mutant with 100 aa deletion in N-terminus showed mitotic abnormalities and growth defects. I found that Ndc80, which directly binds to spindle microtubules, does not localize to kinetochores in these cells, suggesting that the N-terminal 100 aa region of CENP-T is required for the Ndc80 complex to localize into kinetochores. Next, to directly address whether the CENP-T N-terminus is sufficient for localization of the Ndc80 complex into kinetochores, I created a GFP-fusion construct in which the C-terminal DNA-binding domain of CENP-T was replaced with LacI (GFP-CENP-TΔC-LacI) and ectopically localized the GFP-CENP-TΔC-LacI protein into

non-centromeric LacO region. Interestingly, I found accumulation of many outer kinetochore proteins including Ndc80, Nuf2, Mad2 and ZW10 on the LacO region in cells expressing GFP-CENP-TΔC-LacI, suggesting that the CENP-T N-terminus is sufficient for outer kinetochore assembly. Finally, I tested whether the outer kinetochore proteins recruited by GFP-CENP-TΔC-LacI on the non-centromeric LacO region induces a functional kinetochore. For this assay, I deleted the endogenous centromere by a genetic engineering method and examined behavior of chromosome with outer kinetochore assembly on the LacO region. I found that these chromosomes with the ectopic outer kinetochore assembly divided equally at least several cell-cycles even in the absence of the endogenous centromere. As CENP-A was not detected on the LacO region, I conclude that ectopic localization of the CENP-T N-terminus results in functional kinetochore assembly independent of CENP-A. These results indicate that the CENP-T-W complex functions as a structural platform for kinetochore assembly in vertebrate cells.

I showed that the N-terminus of CENP-T provides a structural platform for kinetochore assembly independent of CENP-A in Chapter I. However, it remains unclear how CENP-T makes a strong contact with endogenous centromeric chromatin. To address this question, I examined the C-terminal DNA binding region of CENP-T in Chapter II. Firstly, I found that the histone-fold domain of CENP-T forms a complex with CENP-W and the complex binds to DNA in sequence independent manner. To analyze detail feature of the histone-fold region of the CENP-T-W complex, I also focus on a second group of histone-fold containing CCAN proteins, the CENP-S-X complex and found that the CENP-S-X complex forms a tetramer and has the DNA binding activity. Next, I showed that the CENP-T-W complex associated with the CENP-S-X complex by both in vivo and in vitro analyses to generate a stable CENP-T-W-S-X heterotetramer, which showed structural similarity to canonical histone complex in the nucleosome. Finally, I identified critical amino acids in CENP-T for its kinetochore localization, which are independent of DNA binding activity. Based on my biochemical and cell biological data combined with other results in our laboratory, I propose that tetramer formation of CENP-T-W-S-X is essential for kinetochore assembly.

Structural and biochemical analyses of the CENP-T-W-S-X heterotetramer suggest that the CENP-T-W-S-X complex form a distinct structure with DNA, like canonical histones with DNA (nucleosome structure). Then, in Chapter III, I analyzed DNA binding mode of the CENP-T-W-S-X complex. The canonical histone octamer wraps DNA along the surface of the nucleosome. As basic amino acid residues for the DNA binding surface of the CENP-T-W-S-X heterotetramer are similar to those of canonical histones, it is possible that DNA is wrapped along the DNA binding surface of the CENP-T-W-S-X heterotetramer. To test this hypothesis, I used a DNA supercoiling assay and found that the CENP-T-W-S-X complex induces supercoils into plasmid DNA as canonical histones do, suggesting that the CENP-T-W-S-X complex forms a

nucleosome-like structure. Mutants designed to compromise the supercoiling activity result in abnormal kinetochore assembly in vivo, suggesting that the formation of nucleosome-like structure of DNA-CENP-T-W-S-X complex is essential for proper kinetochore assembly. I further examined topological state of the DNA-CENP-T-W-S-X complex. So far, topological state of the centromeric chromatin is unclear. Some data using minichromosomes combined with mutants for kinetochore components in *S. cerevisiae* suggested that a centromeric nucleosome in *S. cerevisiae* might induce positive supercoiling. On the other hand, reconstituted human CENP-A nucleosomes induce negative supercoils into plasmid DNA in vitro as canonical histone H3 containing nucleosomes. Under this situation, it is important to determine the topological status of the nucleosome-like DNA-CENP-T-W-S-X complex. My data indicated that the reconstituted CENP-T-W-S-X complex induces positive supercoils into DNA, suggesting that the CENP-T-W-S-X complex provides special topological feature on centromeric chromatin. In total, I propose that the unique nucleosome-like structure of the CENP-T-W-S-X complex functions as a critical scaffold in kinetochore assembly.

細胞分裂時には、紡錘糸が染色体上の動原体に結合し、染色体は娘細胞へと分配されていく。動原体は染色体側のインナーキネトコアと紡錘糸が結合するアウターキネトコアからなり、多くの構成タンパク質が種々のサブ複合体を形成してこの構造を造っている。しかし、個々の構成因子の機能については未だ良く分かっていない。竹内康造君は、インナーキネトコアのタンパク質でヒストン様の構造(ヒストン・フォールド)を持つ、CENP-T, CENP-W, CENP-S, CENP-X に注目し、生化学的手法とニワトリ DT40 細胞を用いた遺伝子改変により機能解析を行った。

本論文は3章からなり、第1章では CENP-T タンパク質の N 末側の機能について述べられている。CENP-T は、C 末側にヒストン・フォールドがあり N 末側は単独では構造をとらない。この N 末側を欠くとインナーキネトコアにある CENP-H は動原体に局在するが、アウターキネトコアにある Ndc80 は局在しなくなる。そこで、CENP-T の N 末側だけを Z 染色体の動原体以外の場所に人工的に結合させると、アウターキネトコアにある Ndc80 やスピンドルチェックポイントタンパク質が同じ場所に局在することが分かった。さらに Z 染色体の動原体を除き、同じ染色体に CENP-T の N 末側を結合させると、染色体は細胞分裂に伴って効率よく分配されたことから、この系で機能的な動原体が出来たと結論した。このことは、CENP-T が、動原体集合の構造的なプラットフォームになることを意味している。

第2章では、CENP-T がどのように動原体クロマチンと結合するのかを調べている。この結合には CENP-T の C 末側のヒストン・フォールドが必要である。CENP-T は C 末のヒストン・フォールドを介して CENP-W とダイマーを、CENP-S と CENP-X はそれぞれ2分子からなるヘテロテトラマーを造るが、これらを混ぜ合わせるとそれぞれ1分子からなるヘテロテトラマーを形成し、非特異的に2本鎖 DNA に結合することを示した。さらに、CENP-T, -W, -S, -X からなるヘテロテトラマーが形成でき、DNA へも結合するが、動原体クロマチンへの局在が起こらない CENP-T のヒストン・フォールド内の変異を見いだし、この CENP-T 変異領域がクロマチン結合に必要であることを示唆している。

第3章では、CENP-T, -W, -S, -X ヘテロテトラマーの DNA の構造への影響について調べている。そして、この複合体が 100 bp 程度をカバーするように DNA に結合すること、そして 2 重鎖 DNA に対して正のねじれを導入することを明らかにした。通常のヒストンオクタマーは反対に負のねじれを導入することから、この複合体はユニークな性質を持っているらしい。

本研究は動原体構成因子 CENP-T, -W, -S, -X の機能を明らかにし、動原体研究に新たな知見を付け加えるものである。特に、これらの因子が動原体形成のプラットフォームになることを初めて示したもので、関連研究領域への寄与は大きい。さらに、これら4つのタンパク質が複合体を形成して DNA に結合すると共に、通常のヒストンとは反対方向に2重鎖 DNA にねじれを加えている可能性を示しており、これら因子の分子機作解明への糸口となるものである。以上の理由から、竹内康造君の学位提出論文は博士号授与の要件を満たすと審査員全員一致で判断した。