氏　　名	三上　剛和
学位（専攻分野）	博士（理学）
学位記番号	総研大乙第149号
学位授与の日付	平成１７年９月３０日
学位授与の要件	学位規則第６条第２項該当
学位論文題目	The functional region of CENP-H interacts with the Nuf2 complex that localizes to centromere during mitosis
論文審査委員	主　査　教授　佐々木　裕之
	教授　荒木　弘之
	教授　吉森　保
	教授　仁木　宏典
	主任研究員　原口　徳子（情報通信研究機構）

論文内容の要旨

The centromere plays a fundamental role in accurate chromosome segregation during mitosis and meiosis in eukaryotes. Its functions include sister chromatid adhesion and separation, microtubule attachment, chromosome movement, formation of the heterochromatin structure, and mitotic checkpoint control. One essential function of the centromere is the formation of the kinetochore, which is the structure responsible for microtubule binding and chromosome movement. Although chromosome segregation errors cause genetic diseases including some cancers, the mechanism by which the kinetochore interacts with the microtubules of the spindle apparatus during cell division is not fully understood.

Traditional electron microscopy of chromosomes revealed that the kinetochore of vertebrate cells is a trilaminar button-like structure on the surface of the centromeric heterochromatin. The inner kinetochore plate plays an essential role in kinetochore assembly. The outer kinetochore plate serves both as a microtubule binding structure and as a mitotic checkpoint structure that includes the Bub and Mad complexes. The inner kinetochore contains the centromeric DNA as well as centromere proteins (CENPs) A and C. CENP-H is also one of the inner kinetochore proteins. CENP-H localizes to the centromere throughout the cell cycle, and is found only in active centromeres, including neocentromeres. In this thesis, I investigated the function of CENP-H.

Firstly, I attempted to generate a conditional knockout of CENP-H in chicken DT40 cells. Analysis of CENP-H-deficient cells revealed that CENP-H is essential for cell growth and mitotic progression.

Secondly, the functional region of CENP-H was identified by using a CENP-H conditional knockout cell line. The minimal region of CENP-H required for centromere localization and cell viability was determined by a series of GFP-tagged deletion derivatives of CENP-H. On the basis of the complementation assay and cellular localization data from the CENP-H deletion derivatives, the minimal region necessary for centromere targeting (aa 72 to 225) was identified, which was also essential for cell viability.

Thirdly, interaction of CENP-H with other proteins was investigated. A yeast two-hybrid screening was performed using CENP-H as a bait, and I picked up 24 proteins (104 positive clones) including Hec1. In this thesis, I focused on the relationship between Hec1 and CENP-H. Hec1 was originally identified as a retinoblastoma protein-associated protein, and it was shown that microinjection of Hec1 antibodies into cultured cells disrupts mitotic progression. The two-hybrid system was used to map the region in chicken CENP-H that is responsible for the interaction with Hec1. This analysis showed that the Hec1 interaction region contains the minimal region for CENP-H function identified by the complementation assay. Because CENP-H interacts with Hec1 in the yeast two-hybrid system, cellular localization of both proteins was investigated. A DT40 cell line that co-expressed CENP-H-Flag and Hec1-GFP was created, and immunostaining with anti-Flag antibodies was performed at various cell cycle stages. Hec1 localized to the centrosome but not to the centromere during G1 and S phases. Hec1 moved into the nucleus and localized to centromeres in G2, and it remained associated with the centromere during mitosis. During G2 and mitosis,

CENP-H-Flag and Hec1-GFP signals were colocalized at centromeres. To investigate interactions between CENP-H and Hec1 in DT40 cells further, a coimmunoprecipitation assay was performed. A cell line that expressed CENP-H-Flag was prepared to perform this assay. Hec1 was detected in the immunoprecipitates when the exstract was treated with anti-Flag antibodies. Hec1 is a member of the Nuf2 complex, which contain Nuf2, Spc24, and Spc25. Nuf2 was also coimmunoprecipitated with CENP-H. A cell line that co-expressed CENP-H-Flag and Spc24-GFP and another cell line that coexpressed CENP-H-Flag and Spc25-GFP were also created. Coimmunoprecipitation experiments with anti-Flag antibodies using these cells were performed and the immunoprecipitates were analyzed by Western blotting with anti-GFP antibodies. Both Spc24-GFP and Spc25-GFP signals were detected. These results indicate that CENP-H interacts with the Nuf2 complex at centromeres in DT40 cells.

Lastly, photobleaching experiments were carried out on Hec1 and CENP-H. An iFRAP (inverse fluorescence recovery after photobleaching) analysis was performed to examine the stability of the Hec1-GFP association with centromeres. The fluorescence intensities of the unbleached area and of the whole cell were measured across time. During G2, the fluorescence intensity of the unbleached region decreased gradually, while that of the bleached region was recovered. After approximately 150s, the signal intensities of the whole cell and of the unbleached region became equivalent. In contrast, little loss of fluorescence intensity of Hec1-GFP in the unbleached region was observed during mitosis, and the fluorescence intensity of the bleached region remained constant. These findings indicate that Hec1-GFP associates stably with the centromere during mitosis. The stability and mobility of CENP-H-GFP during both mitosis and interphase was then investigated. The fluorescence intensity remained unchanged in both bleached and unbleached regions for at least 30 min, suggesting that CENP-H is stable at centromeres throughout the cell cycle. These findings indicate that CENP-H and Hec1 form a stable association with the centromere during mitosis.

Considering the results of these analyses, it is proposed that the Nuf2 complex, including Hec1, is stably associated with the centromere through the interaction with CENP-H during mitosis and provides a site for assembly of the checkpoint proteins (outer kinetochore proteins) that regulate cell cycle progression. The Nuf2 complex may serve as a connector between the inner and outer kinetochores.

論文の審査結果の要旨

有糸分裂時に染色体のセントロメア領域に形成されるキネトコアは染色体分配に重要な役割を果たす。キネトコアは3層構造からなるが、その内板と呼ばれる部分には有糸分裂の進行に必須な CENP-H 蛋白質が存在する。申請者の三上剛和くんはこの CENP-H について、ニワトリ DT40 細胞を用いた一連の機能的解析を行い、キネトコアの構成と機能について新たな知見を得た。

まず、蛍光性の GFP タグを付けた一連の CENP-H 部分欠失体を CENP-H の条件的ノックアウト DT40 細胞に導入し（相補試験）、72 番目から 225 番目のアミノ酸までの領域が細胞の生存とセントロメアへの局在に必須であることを示した。次に、CENP-H と相互作用する蛋白質を同定するため、酵母を用いた two-hybrid スクリーニングを行い、幾つかの蛋白質を同定した。そのうち Hec1 は有糸分裂の進行に必須だということが分かっている。そこで、三上くんは CENP-H と Hec1 との関係を中心に詳細な研究を行った。

まず、酵母の two-hybrid システムを用いて Hec1 と相互作用する CENP-H の部分を解析したところ、上述の CENP-H の機能に必須な部分を含む領域であることが分かった。また、DT40 細胞にタグ付きの蛋白質を発現させて免疫染色法で観察したところ、CENP-H が細胞周期を通してセントロメアに局在するのに対し、Hec1 は G2 期と M 期にセントロメアに局在した。一方、Hec1 は Nuf2 などの蛋白質と複合体を作ることが知られている。そこで、DT40 細胞の抽出物中でタグ付きの CENP-H と共沈する蛋白質を調べ、Hec1、Nuf2、Spc24、Spc25 など、この複合体の構成成分を検出した。つまり、CENP-H は Nuf2 複合体と相互作用することが分かった。最後に、CENP-H と Hec1 のセントロメア局在の安定性を調べるため、GFP タグを付けた蛋白質を細胞に導入し iFRAP（inverse fluorescence recovery after photobleaching）法で解析した。その結果、CENP-H は細胞周期を通して非常に安定にセントロメアに存在すること、一方 Hec1 は M 期においてのみ安定にセントロメアに局在することが分かった。

以上の結果は、Hec1 を含む Nuf2 複合体が M 期のキネトコア構造の構成成分の1つであることを強く示唆する。三上くんは、キネトコア内板の CENP-H と Hec1 が相互作用するという自分の発見と、Nuf2 複合体がキネトコア外板の Mad2 複合体と相互作用するという報告とを考慮し、Nuf2 複合体がキネトコアの内板と外板を架橋する役割を担うという新しいモデルを提唱した。

以上のように、本申請論文の内容は脊椎動物のセントロメアの構造と機能の解明を大きく進めるものであり、科学的に高く評価できる。これらの研究は国立遺伝学研究所の深川竜郎助教授のもとで行われたもので、三上くんはすでに2報の論文を国際誌に発表しており、うち1報では筆頭著者として本申請論文の主な研究成果を報告している。したがって、遺伝学専攻において論文博士を授与する基準を十分に満たしている。以上を総合的に評価して、審査委員会は、提出された申請論文が博士の学位を授与するに足ると結論した。