Functional analyses of the CENP-O complex in mice

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ABSTRACT

During cell division, it is important to ensure that the genomic information is faithfully transmitted to daughter cells. To achieve correct segregation of chromosomes, specialized apparatus are built and complexly regulated during eukaryotic cell division. The kinetochore is one of these apparatus. The kinetochore forms a large structure on centromere DNA and plays important roles in mitotic progression by microtubule attachment. The kinetochore of higher eukaryotes has a trilaminar structure that consists of the inner plate, outer plate and midzone. The inner plate functions as the foundation of kinetochore formation. While a large number of proteins that localize to the inner kinetochore have been identified recently, precise functions of each kinetochore protein are poorly understood and little is known about their functional role especially in organismal context.

The CENP-O complex that consists of CENP-50/U, O, P, Q, and R constitutively localizes into the kinetochore and is required for recovery from spindle damage in chicken DT40 cells. While DT40 cells with KO of most proteins localized in inner kinetochore die, DT40 cells lacking CENP-O complex proteins are still viable. Therefore, we focus on functional role of the CENP-O complex proteins in organismal context.

Previous study showed that mouse ES cells and mice lacking CENP-50/U die within several days after depletion of the protein and during early development, respectively, while chicken DT40 cells with KO of CENP-50/U are viable. This observation suggests that the CENP-O complex plays an essential role in the early development of mice. Notably, knockout phenotype of CENP-R in DT40 cells is different from that of other CENP-O complex proteins in some aspects and kinetochore localization of CENP-R occurs downstream of other complex proteins. CENP-R may play a different role from that of other CENP-O complex proteins. It is important to examine function of CENP-O complex including CENP-R in organismal context.

In this study, ES cell lines and mice lacking CENP-R were created to elucidate the function of CENP-O complex proteins in mice, and their phenotypes were compared with that of CENP-50/U.

Although mice with knock out of CENP-50/U died during early embryogenesis, CENP-R null mice were viable and normally produced offspring, suggesting that

CENP-R is not essential in mice and has different role from that of other CENP-O complex proteins.

To elucidate that the lethality of CENP-50/U deficient mice is caused at a cellular level, ES cells lacking CENP-50/U or R were generated. Although ES cells with KO of CENP-50 /U were not viable and died within several days, ES cells lacking CENP-R were normally propagated. In addition, mitotic index and the number of anaphase with lagging chromosomes were increased in ES cells lacking CENP-50/U. These results suggest that the lethality of mice lacking CENP-50/U is caused at a cellular level, and each cell in mice lacking CENP-50/U dies due to some mitotic defects. I also confirmed that the kinetochore structure and the dependencies of kinetochore localization of CENP-O complex proteins are conserved between chicken DT40 cells and mice ES cells.

Chicken DT40 cells are derived from chicken B cells, and DT40 cells lacking CENP-50/U are viable. Therefore, I expected that the viabilities of mice cells lacking CENP-50/U are different among cell types. To examine this, I generated inducible knockout mice, in which CENP-50/U is removed by ERT2-conjugated Cre recombinase. I isolated mouse embryonic fibroblast cells and B-cells from adult inducible knockout mice of CENP-50/U. As a result, both mouse B cells and mouse embryonic fibroblast cells were viable and normally propagated even after CENP-50/U was removed from kinetochore by OHT addition although ES cells lacking CENP-50/U are lethal. I concluded that the importance of CENP-50/U in mitosis is different among cell types in mice.

In living organism, there are various cell divisions such as meiosis, segmentation of fertilized egg or asymmetric cell division in which machinery of chromosome segregation, growth rate, mitotic regulation are different in detail. In addition, the frequency of chromosome missegregation is different among cell types.

It is possible that the kinetochore has a function in strict regulation of these particular cell divisions, however, little is known about kinetochore functions and variety in organismal context. In this study, I showed that the importance of CENP-50/U differs among cell types. From this result, I suggested that CENP-50/U may be required for these strict regulations of mitosis in mice.

INTRODUCTION

During cell division, it is important to ensure that genomic information is transmitted faithfully to daughter cells. In mitosis, specialized apparatus is built and is regulated complexly during eukaryotic cell division to achieve correct segregation of chromosomes.

A kinetochore is one such apparatus that functions in mitosis progression. In mitosis, all duplicated chromosomes are aligned on a metaphase plate. They are then captured by spindle microtubules extending from opposite spindle poles, and separated equally into daughter cells. In this process, a multi-protein structure called a kinetochore forms on the centromeric region on chromosomal DNA and serves as an attachment site of spindle microtubules, as a cohesion site of chromatid during mitosis and as a center of the spindle checkpoint (1, 2, 3). The kinetochore plays important role in mitosis. When the function of kinetochore and the number of kinetochore are disturbed, mitotic defect and aneuploidy are caused. To elucidate kinetochore functions is medically- and biologically-important matter.

The kinetochore was traditionally visualized using electron micrographs. The kinetochore of higher eukaryotes has a trilaminar disk-like structure comprising the inner plate, outer plate, and midzone (4 and Fig. 1A). The inner and outer plates are determined as electron dense regions. It was believed that the inner plate, which contains inner kinetochore proteins and centromeric DNA, functions as the foundation of recruitment of other outer proteins, and that the outer plate consists of outer plate proteins that are necessary for attachment to microtubule or spindle checkpoints. However, molecules which are involved in inner and outer plate have not been unclear.

Recently, various studies have revealed that more than 100 proteins localize into the kinetochore plate, where they work cooperatively to form the functional kinetochore (3). To elucidate the chromosome segregation mechanism, it is important to study the detailed functions of these proteins.

Among various centromere proteins, we specifically examine constitutive centromere associate proteins (CENP). These proteins, which are highly conserved in yeast to human, are constitutively localized into the inner plate of a trilaminar structure throughout the cell cycle. They are important for establishment of the kinetochore structure and functions (3, 5). Traditionally, CENP-A protein (a variant

of histone H3), CENP-B (a binding protein for repetitive sequence in pericentromeric heterochromatin), and CENP-C (DNA binding protein) are well known. Recently, newly CENP-H, I, K, L, M, N, O, P, Q, R, S, T, 50/U, W, and X are identified as CENPs by proteomics analysis (3, 6–15).

Regarding chicken DT40 cells, studies have revealed that they are divided into several complexes by the phenotype of knockout cells or their biochemical interactions (7, 9, 10): the CENP-C, the CENP-B, the CENP-A, the CENP-T/W/S/X complex, and the CENP-H complex which consists of the CENP-H/I/K, the CENP-L/M/N, and the CENP-O/P/Q/50(-U)/R complex. The CENP-A is a variant of histone H3, and the CENP-A - containing nucleosomes identify functional centromere. The CENP-C is a DNA-binding protein and located into inner kinetochore. The CENP-C is a mediator protein between the centromeric chromatin and other outer kinetochore proteins. The CENP-C is required for chromosome alignment and kinetochore assembly. The CENP-T, W, S and X have a histone-fold domain, and they have a DNA binding activities. The CENP-T and the CENP-W are tightly binding. Recently, it is shown that N-terminal region of CENP-T binds directly to the Ndc80 complex (microtubule-binding surface). The CENP-H/I/K complex acts as a mediator between CENP-A nucleosomes and outer proteins and essential for kinetochore functions. This complex is required for localization of newly synthesized CENP-A to centromere. The CENP-L, M, and N are associated with CENP-H/I/K. However, their detailed functions are not well understood. The CENP-O/P/Q/50(-U)/R forms a heterogeneous complex and have an important roles in recovery from spindle damage. Severe damage that is observed after release from the nocodazole block is partially rescued by treatment of proteasome inhibitor MG132 (20). This recovery after spindle damage is related to phosphorylation of CENP-50/U by Plk1.

Thus, many CENP-proteins have been identified and analyzed. Although, various findings related to the kinetochore have come from research of cultured cells, little is known about kinetochore in an organismal context. Various cell divisions are known to occur in living organisms. During cleavage of fertilized egg or division of stem cells, where the spindle and plane of division are placed asymmetrically, cells and their components are divided asymmetrically. During cleavage of a fertilized egg, the mitotic rate and cell size change according to the stage. With respect to meiosis, the chromosome number is reduced by half, and the cell cycle is blocked until appropriate stimulations (hormone or insemination) induce further cell

cycling. Consequently, considering that cell cycle regulators differ subtly depending on the cell type, various cell divisions exist in organisms. Despite the existence of various cell divisions in living organisms, it has not been studied sufficiently whether the mitotic apparatus including kinetochore is common among various cell types. Analysis of each kinetochore protein in living animals is important to understand the functions or differences of kinetochore proteins among various cell divisions. Therefore, we strove to analyze the function of CENP proteins in mice.

The number of report about mice lacking centromere protein is very small. To date, studies of knockout mice lacking CENP-A, CENP-B, or CENP-C have been reported, and the phenotypes of knockout mice are consistent with those of cultured cells with respective knockouts. Actually, CENP-A is known to be necessary to establish kinetochore, and cells lacking CENP-A die. Mice lacking CENP-A show strong embryonic lethality at E5.5 with severe mitotic problems including chromosome missegregation, fragmentation, micronulei or macronuclei formation, and metaphase arrest (16). Similarly, cells with knockout of CENP-C are not viable, and mice lacking CENP-C also die at E3.5 (17). It is known that cells with knockout of CENP-B are healthy. Then, despite a decrease in body weight, testis size, and number of offspring, mice lacking CENP-B are viable(18, 19).

In this study, I focus on these CENP-O complex proteins to elucidate the kinetochore specificity in multicellular organisms. Chicken CENP-O complex proteins were first reported in our laboratory, and its homologues were conserved among a vertebrate genome.

Previously, we analyzed CENP-O complex proteins and determined several of their features. The CENP-O complex is one of the subcomplex of CENP-H complex that constitutively localizes to the inner kinetochore plate from interphase to metaphase. The CENP-O complex consists of five proteins: O, P, Q, 50/U, and R (7 and Fig. 1A). These proteins form a stable complex and have dependency on kinetochore localization (20). Localizations of CENP-O complex proteins occur downstream of CENP-H or CENP-M subcomplex, another subgroup of the CENP-H complex. In CENP-O complex proteins, localization of CENP-O, P, Q, and 50/U is interdependent and localization of CENP-R is downstream of other CENP-O complex members.

The CENP-50/U is unique in terms of cell viability. As described previously, the many proteins existing around the CENP-O complex are important for kinetochore

establishment, and chicken DT40 cells lacking most inner kinetochore proteins die with severe mitotic problems. However, DT40 cells lacking CENP-O complex proteins are viable, although cells lacking other subcomplex members of the CENP-H complex (CENP-H class or CENP-M) are lethal (7, 20 and Fig. 1A). In cultured cells, CENP-O complex proteins are not necessary for normal cell division. Therefore, we expected that these proteins work in a specific situation in animal mitosis. To ascertain the functions of CENP-O complex proteins in animal mitosis, we chose mice as the experimental material. As a first step for understanding the functions of CENP-O complex proteins, it is important to determine whether these proteins are essential or not in mice.

Herein, CENP-50/U and R are specifically examined. As mentioned previously, chicken CENP-O complex proteins have a hierarchy of kinetochore localization. Because the localizations of CENP-O, P, Q, and 50/U are interdependent, CENP-50/U was selected as representative of CENP-O, P, Q, and 50/U. When CENP-50/U is removed from chicken DT40 cells, other CENP-O, P and Q are unable to localize into kinetochore. So, it is expected that phenotypes of mice lacking CENP-O, P and Q are similar to that of CENP-50/U.

However, the localization of CENP-R is downstream of other CENP-O, P, Q, and 50/U. Furthermore, the knockout phenotype of CENP-R in DT40 cells differs from that of other CENP-O complex proteins in some aspects. First, knockout cell lines of all CENP-O complex proteins are viable respectively, but the respective proliferation rates of CENP-O, P, Q and 50/U deficient cells are lower than that of WT cells. The proliferation of cells lacking CENP-R is similar to that of wild type cells. Next, CENP-O, P, Q, and 50/U, except CENP-R, are necessary for recovery from spindle damage. These slight differences observed between CENP-R and other members (O, P, Q and 50/U) suggest that CENP-R plays a different role in the CENP-O complex.

To elucidate the roles of CENP-O complex proteins in mice, it is important to compare knockout phenotype of CENP-R deficient mice with that of CENP-50/U. First, mice lacking CENP-50/U were generated in our laboratory (Hori, T. unpublished data). Mice lacking CENP-50/U shrank and died during early embryo genesis (E8.5), although chicken DT40 cells with KO of CENP-50/U are viable. This observation suggests that CENP-50/U plays a necessary role in early embryonic mitosis.

In this study, mice and ES cells lacking CENP-R were generated, after which their phenotypes were compared with that of CENP-50/U. Then it is described

that mice or ES cells lacking CENP-R are viable, although mice or ES cells lacking CENP-50/U die. These findings suggest that the function of CENP-R is different from that of CENP-50/U in the mouse CENP-O complex. The author also generated mice with an OHT-inducible CENP-50 knockout allele. In this line, mouse embryonic fibroblast cells and lymphocytes lacking CENP-50/U are healthy, indicating that the requirement of CENP-50/U for mitosis differs among cell types.

RESULTS

Mice lacking CENP-R were generated to determine whether CENP-R is essential in mice and whether lethality shown in mice lacking CENP-50/U is conserved in mice.

1.1 Isolation of mouse CENP-R

Previously, CENP-R was reported in human and chicken, and a homologue of CENP-R was predicted computationally among vertebrates, including mice. First, the putative mouse homologue of CENP-R was isolated using RT-PCR. As presented in Fig. 2-A, CRNP-R protein is highly conserved among vertebrates. Furthermore, mouse CENP-R has 64% amino acid identity with human CENP-R, and 35% amino acid identity with chicken CENP-R. Amino acids conserved in all three species are boxed. Then, several domains are predicted on the CENP-R gene. As the coiled–coil region, NLS, DD1 domain, and LXXIL motif are predicted on exons 3 and 4 of the mouse CENP-R gene, targeting vectors are designed in this work to disrupt exons 3 or 4. When exon 3 or 4 is disrupted, downstream amino acids are fully disrupted by the frame shift. Next, to confirm the kinetochore localization of putative mouse CENP-R, a polyclonal antibody against mouse CENP-R was raised. Mouse L-cell lines stably expressing CENP-A-GFP protein were established and were immunostained with the anti-CENP-R antibody (Fig. 2B). Colocalization of CENP-R with CENP-A was observed as punctate signals, indicating that mouse CENP-R localizes into the kinetochore. We also examined CENP-R localization in several embryonic tissues (Fig. 2C). The CENP-R signals showed a typical punctate pattern in the brain or skin of E14.5 embryo.

1.2 Generation of exon 4-targeted ES cells

To disrupt the mouse CENP-R gene in ES cells, a promoterless targeting vector was constructed. The targeting strategy for the mouse CENP-R gene is depicted in Fig. 3. First, using a mouse CENP-R cDNA fragment as a probe, a genomic 129/Sv phage library was screened and three clones harboring exons 3–4 of CENP-R were isolated (λ#9, 6, 10). Next, conditional targeting constructs to disrupt exon 4 (Fig. 3A) was created from these genomic clones inserted into pBluescrypt KS+ vector. In this construct, exon 4 is flanked by the loxP sequences, and the SA-Ires-neomycin marker was inserted upstream of exon 4. The targeting vector was linearized and

transfected into ES cells to generate homologous recombinant.

After G418 selection, we isolated two clones in which the expected homologous recombination occurred (Fig. 3B). The DNAs of G418 resistant clones were isolated and were double digested with Nco I and ApaL I. The 3' probe detected a 9.6 kb band in wild type allele and a novel 7.7 kb in recombinant allele. Fifteen ES clones in which expected homologous recombination occurred were isolated from 253 G418-resistant clones.

1.3 Production of CENP-R null mice

To generate chimeric animals, targeted ES clones were injected into C57BL/6 blastocysts. Then blastocysts were transferred to recipient pseudopregnant MCM females (by Dr Sado, T.).

Male chimeric mice were mated with C57BL/6 females to generate mice having Ires-geo allele heterozygously. Germline transmission was identified by agouti coat color and PCR genotyping. Then the two ES lines were transmitted to the germ line.

To delete exon 4, male mice with genotype of CENP-R +/Ires-geo were crossed to female deleter mice expressing CAG-Cre recombinase in its germline. Then mice with CENP-R +/-, CAG-Cre -/- genotype were selected by PCR genotyping of tail tip or Southern hybridization (Figs. 3B and 3C). In oocytes containing maternal Cre recombinase, the paternal exon 4 flanked by loxP sequence was deleted effectively (- allele) and intact Ires-geo allele did not transmit to progeny.

Next, CENP-R null mice were generated by crossing heterozygous males with females. The CENP-R null mice were born normally. This was confirmed by southern hybridization using the same probe described previously. In this line, CENP-R protein depletion was confirmed using Western blot analysis. Subsequent immunocytochemical analysis using anti-CENP-R antibody revealed that CENP-R proteins were depleted completely in MEF cells isolated from CENP-R null embryo (Figs. 3D and 3E).

1.4 CENP-R null mice are healthy and have normal meiosis

Although knockout mice or DT40 cells lacking cell cycle-related genes usually show severe mitotic defect such as chromosome missegregation, apoptosis, aneuploid, mitotic arrest, or multiple nuclei, and eventually die, CENP-R null mice do not seem to have mitotic defect (Fig. 3F). For confirmation, we analyzed the

segregation ratio of offspring from crosses between heterozygous animals. As presented in Table 4, the -/- offspring were born in accordance with the Mendelian rule. Mice lacking CENP-R were regarded as showing normal development.

Next, we checked whether CENP-R null mice have meiotic defects. To analyze the fertility of null animals, offspring were created by crosses between CENP-R null males and females. As Table 5 shows, null animals can produce equal ratios of males and females. These results show that mice lacking CENP-R have normal meiosis.

Consequently, mice lacking CENP-R are viable and show no apparent differences between WT and KO, although mice lacking CENP-50/U die during early development. Actually, CENP-R is not required in mitosis under normal development. These results demonstrate that lethality of knockout mice is not conserved among CENP-O complex members, which suggests that CENP-R has different functions from those of CENP-50/U in CENP-O complex.

1.5 Generation of ES cells lacking CENP-R

Although CENP-50/U is not required for normal cell proliferation in cultured chicken DT40 cells, mice lacking CENP-50/U die during early embryogenesis. To ascertain whether this lethality occurs at a cellular level or whether the kinetochore structure is conserved between species, ES cells with knockout of CENP-R were generated. Then its phenotypes were compared with those of ES cells lacking CENP-50/U (generated by Hori, T.).

To disrupt mouse CENP-R alleles in ES cells, targeting vectors of two types were constructed. The targeting strategy for mouse CENP-R gene is portrayed in Fig. 4. First, conditional targeting constructs used to disrupt exon 3 (Fig. 4A) of the first CENP-R allele were created from genomic clones on pBluescrypt II KS+ vector. In this construct, exon 3 is flanked by a lox sequence. Then the promoterless SA-Ires-neomycin marker was inserted upstream of exon 3.

The targeting vector was transfected into ES cells. Then several G418 resistant clones were isolated, in which expected homologous recombination occurred (Fig. 4C). The DNAs of G418 resistant clones were digested with Acc I, and the 5' probe detected a 9.4 kb band in wild type allele and a novel 11.3 kb in recombinant allele. Ten ES clones in which expected homologous recombination occurred were isolated from 123 G418-resistant clones.

Second, the inserted drug resistance gene (ß-geo) was deleted by transient expression of Flp recombinase. Genomic DNAs isolated from colonies were digested with Acc I, and deletion of the drug resistance gene was identified by Southern blot using the probe described above. As portrayed in Fig. 4C, marker-deleted allele (flox allele) was detected as a novel 9.8 kb band instead of an Ires-geo band (11.3 kb).

Next, the second allele was disrupted using another targeting vector (Fig. 4B) to substitute exon 4 with the SA-ires geo drug resistance gene. After transfection and G418 selection, G418 resistant clones were isolated. Southern blot analysis showed that the novel 14.4 kb band instead of wild type 9.4 kb band, and that 5 clones out of 35 G418-resistant ones had correctly targeted the second allele (Fig. 4C).

LoxP-flanked exon 3 of the first allele was removed by transient expression of Cre recombinase. As portrayed in Fig. 4C, deletion of exon 3 was detected as a band shift from 9.8 kb (loxP) to 7.9 kb (exon4) on the Southern blot. Finally, CENP-R null ES cell lines were generated (Fig. 4C).

In these cell lines, we confirmed by Western blot and immunocytochemical analysis using anti-CENP-R antibody that CENP-R protein was deleted completely (Figs. 4E and 4F).

1.6 Kinetochore architecture of CENP-O complex proteins is conserved between mouse ES cells and chicken DT40 cells

In chicken DT40 cells, CENP-O complex proteins have hierarchies of kinetochore localization. First, CENP-H localizes into kinetochores. Subsequently, CNEP-O, P, Q, and 50/U are loaded interdependently. Then the localization of CENP-R occurs downstream of other subunits (Fig. 1B). To ascertain whether the fundamental architecture of CENP-O complex proteins is conserved between chicken DT40 cells and mouse ES cells, the dependency of kinetochore localization of CENP-50/U or CENP-R in ES cells was examined.

In ES cells lacking CENP-R, immunofluorescence analysis showed that CENP-O components except for CENP-R still localize into the kinetochore, indicating that CENP-R localizes into kinetochore downstream of these proteins in mice ES cells. In ES cells with KO of CENP-50/U, its downstream protein CENP-R and interdependent protein CENP-O do not localize into kinetochore, and the upstream protein CENP-H remains. This result indicates that CENP-50/U is a protein intermediate between CENP-R and CENP-H (Fig. 5A).

The protein amounts in the chromatin fraction reflect this dependency.

Suppression of the CENP-50/U amount diminished the CENP-O and CENP-R amount, and depletion of CENP-R does not affect the amount of other CENP-O members (Fig. 5B).

Thus, I confirmed that the dependency of kinetochore localization of CENP-O complex proteins is conserved between mouse and chicken cells. Apparently, the difference of lethality between mice and chicken DT40 cells is not caused by the difference of kinetochore architecture.

1.7 ES cells lacking CENP-R normally propagated although ES cells lacking CENP-50/U died

To determine whether the lethality observed in mice lacking CENP-50/U occurs at a cellular level, ES cells lacking CENP-50/U (by Hori, T.) or CENP-R (this work) were created. Then the growth rates of ES cells lacking CENP-R or CENP-50/U were examined.

In the targeting step of CENP-R, it was possible to isolate ES cells having the knockout allele homozygously, which indicated that ES cells lacking CENP-R are viable. To verify this viability, colony formation assay was performed. The colony number of ES cells with CENP-50/U flox/- genotype resembles that of ES cells lacking CENP-R or wild type. However, the colony number is strongly reduced significantly when ES cells with CENP-50/U flox/- are treated with OHT (Fig. 6A).

Next, the growth rates of wild type and CENP-R deficient cells (Fig. 6E) were compared. Results show that the growth rates of CENP-R deficient cells closely approximated that of wild type (Fig. 6C). The ES cells lacking CENP-R propagated normally.

We expected that ES cells lacking CENP-50/U are lethal because mice lacking CENP-50/U are lethal. Therefore, we generated ES cells harboring loxP-flanked CENP-50/U allele and mutated estrogen receptor conjugated Cre recombinase (Mer-Cre-Mer). In this line, CENP-50/U proteins are depleted by addition of OHT (Fig. 6D). After addition of OHT, ES cells lacking CENP50/U stopped proliferation after 3 days. Most cells died within 7 days, indicating that depletion of CENP-50/U caused growth arrest and subsequent cell death (Fig. 6B).

Immunofluorescence analysis revealed that cells lacking CENP-50/U signals on kinetochore were counted, and the percentage of CENP-50/U negative cells was calculated at each time point after OHT induction (Figs. 6F, 6G). The percentage of CENP-50/U deficient ES cells decreased rapidly and finally reached around 0% in 7 days, suggesting that CENP-50/U deficient cells gradually die and that only ES

cells in which CENP-50/U remains are propagated.

Lethality of CENP-50/U deficient mice is probably determined at a cellular level.

1.8 ES cells lacking CENP-50/U showed an increased mitotic index and chromosome missegregation

When inner kinetochore proteins are disrupted, cells usually show severe mitotic defect and die. In previous studies, chicken DT40 cells lacking CENP-O complex proteins showed no severe mitotic defect. Therefore, to examine whether ES cells lacking CENP-O complex proteins have mitotic defect, the mitotic index and chromosome segregation during anaphase were investigated. The mitotic index of ES cells lacking CENP-R or 50/U was determined by counting H3P10 (histone H3 phosphorylated at serine 10) positive cells. The H3P10 is commonly used as a mitotic marker.

Results show that the mitotic index of ES cells lacking CENP-50/U is slightly increased, and the mitotic index of ES cells lacking CENP-R closely resembles that of wild type (Fig. 7A), which suggests that some CENP-50/U-deficient cells are not able to finish metaphase.

To detect evidence of chromosome missegregation, the percentage of ES cells with a lagging chromosome in anaphase cells was examined. The percentage of lagging anaphase in CENP-50/U deficient cells (Fig. 7B) is five-fold higher than that in either wild type or CENP-R-deficient ES cells (Fig. 7C).

These results suggest that the lethality of ES cells lacking CENP-50/U might ascribe to defects in chromosome segregation during anaphase.

1.9 Generation of inducible CENP-50/U deficient mice

Although ES cells and mice lacking CENP-50/U are lethal, chicken DT40 cells lacking CENP-50/U are viable. This study demonstrated that the dependency of the kinetochore localization of CENP-O complex proteins was conserved between chicken and mouse, meaning that the kinetochore architecture of CENP-O complex protein is conserved between them. Then, the difference of lethality might result from a difference between species or from a difference of cell types.

To determine whether the lethality of CENP-50/U deficient cells is conserved among cell types, mice in which CENP-50/U is removed inductively by OHT were generated. Mice harboring knockout allele of CENP-50/U heterozygously (+/-) and mice having an inducible knockout allele of CENP-50/U homozygously (lox/lox) were generated previously (Dr. Hori, T.), and mice with ERT2-Cre (estrogen

receptor conjugated Cre enzyme were received from Dr. Saga Y.). By crossing these mice, mice harboring both CENP-50/U (lox/-) and ERT2-Cre (+/-) were generated.

1.10 Mouse embryonic fibroblast lacking CENP-50/U normally proliferated, although ES cell lacking CENP-50/U died

To determine whether the lethality of CENP-50/U deficient cells is conserved in mouse embryonic fibroblast cells, MEF cells were isolated in which CENP-50/U protein had been removed by OHT induction. Mouse embryos were harvested from crosses between mice with CENP-50/U-lox/-, ERT2 Cre +/- allele, and mice with CENP-50/U lox/lox allele. The MEF cells were isolated from them. MEF cells having both CENP-50/U lox/ - and ERT2-Cre +/- allele were identified by PCR genotyping (Fig. 8A). In these MEFs, CENP-50/U was removed from kinetochores using OHT induction (Fig. 8B). After OHT induction, the MEF cells lacking CENP-50/U apparently propagated normally, and morphological abnormity was not observed.

Further examination of the viability of MEF cells lacking CENP-50/U was necessary, so the respective growth rates of CENP-50/U- inducible knockout MEF cells and pre-treatment cells were compared. Results show that the growth rate of CENP-50/U-deficient cells closely resembled those of pre-treatment cells (Fig. 8C).

Using immunofluorescence analysis, cells lacking CENP-50/U signals on kinetochore were counted. Then the percentage of CENP-50/U negative cells was calculated for each time point after OHT induction (Fig. 8D). Two days after OHT treatment, the percentage of CENP-50/U deficient cells was increased rapidly to 39% in the OHT-treated group, and sustained for 6 days. This result contrasts to that of ES cells lacking CENP-50/U (Fig. 6F, G) and suggests that MEF cells lacking CENP-50/U propagate normally although ES cells lacking CENP-50/U die.

Based on this result, lethality of CENP-50/U deficient ES cells is not conserved among cell types.

1.11 Mouse lymphocytes lacking CENP-50/U are normally propagated

DT40 cells were derived from chicken B cell and are viable when CENP-50/U is disrupted. To ascertain whether mouse B cells propagate normally in the absence of CENP-50/U, mouse lymphocytes and propagated mice B cells were isolated under mitogen stimulation with LPS. Isolated mouse lymphocytes were cultured for two days in LPS and OHT-containing medium. After washing the OHT, lymphocytes were cultured in mitogen-supplemented medium for another 4 days.

As shown in Fig. 9B, the cell number of lymphocytes had increased five-fold by 4 days after OHT-treatment. The growth ratio of OHT treated cells approximates that of non-treated cells. Using immunofluorescence analysis, the percentage of cells lacking CENP-50/U negative cells was calculated at each time point after OHT induction (Figs. 9A, 9C). Two days after OHT treatment, the percentage of CENP-50/U deficient cells had increased rapidly to 60% in the OHT-treated group, and was sustained for 4 days after OHT treatment. This result apparently indicates that B cells lacking CENP-50/U are normally propagated similarly to MEF cells. The lethality observed in ES cells lacking CENP-50/U is not conserved in B cells.

DISCUSSION

The CENP-O complex proteins—O, P, Q, 50/U, and R—were initially identified as members of the CENP-H/I complex in chicken DT40 cells. Although DT40 cells lacking other CENP-H/I members die with severe mitotic defect, DT40 cells lacking CENP-O complex proteins normally propagate (7). Earlier, we reported that DT40 cells lacking CENP-O complex proteins show a slight delay in growth rate and have a defect in recovery from spindle damage. After release from nocodazole treatment, DT40 cells lacking CENP-O complex proteins, except CENP-R, are not able to propagate (20). Based on these observations, it was inferred that the CENP-O complex proteins are necessary for strict mitotic regulation, which is not essential or which is rarely needed in normal cultured cells. Because various cell divisions and regulations of cell cycle are present in mice, it was expected that CENP-O complex proteins are necessary for one of many mitoses in mice. The present study was undertaken to ascertain the role of CENP-O complex proteins through analysis of mice deficient for CENP-O complex proteins.

First, mice lacking CENP-50/U (by Hori, T.) or CENP-R (this work) were generated to examine whether CENP-O complex proteins are essential in mice or which cell type they are necessary for. Results show that mice lacking CENP-50/U died by E8.5 embryo (Hori, T., unpublished data), indicating that CENP-50/U is necessary for mitosis regulation during early embryogenesis. In contrast, mice lacking CENP-R were born normally, subsequently propagating normally and producing offspring (Tables 4 and 5). Consequently, severe mitotic defect and lethality are not observed and CENP-R is unnecessary for mice mitosis under normal breeding.

CENP-50/U and CENP-R are highly conserved in vertebrates and form a tightly connected complex in chicken DT40 cells (20). However knockout of CENP-50/U or CENP-R caused different phenotypes in mice, although their knockout caused similar viability in chicken DT40 cells. Probably, the difference between CENP-50 and CENP-R deficient mice arises from the functional difference between the two proteins. This idea corresponds to the observation that DT40 cells lacking CENP-50/U are necessary for recovery after spindle damage although DT40 cell lacking CENP-R is not required (20). Localization pattern of CENP-R is also different from that of CENP-O, P, Q, and 50/U: CENP-R localization to kinetochore occurs after localization of CENP-50/U, O, P, and Q. CENP-R may play a role

accessorily at the downstream of CENP-O, P, Q, and 50/U.

Our previous observations revealed that DT40 cells lacking CENP-50/U propagate more slowly than wild type cells do. It is important to ascertain whether the lethality of CENP-50/U deficient mice results from cell death at a single cell level or by developmental delay attributable to the slow growth rate. We produced ES cells in which CENP-50/U is removed inductively by OHT. After OHT addition, ES cells lacking CENP-50/U showed severe cell death. Their growth rate was greatly reduced (Fig. 6B). As depicted in Fig. 6G, the percentage of CENP-50/U-deficient ES cells decreased rapidly and finally reached around 0% in 7 days, suggesting that CENP-50 deficient cells died. These observations suggest that not delayed growth rate but cell death in several or most cell types required for embryogenesis causes embryonic lethality in CENP-50/U deficient embryo. The mitotic index and chromosome missegregation are increased in ES cells lacking CENP-50/U (Fig. 7). Furthermore, chicken DT40 cells lacking CENP-50/U have defects in recovery after spindle damage, and polo-like kinase plays a role in this defect. These results suggest that ES cells lacking CENP-50/U die because of some mitotic defect(s).

Although DT40 cells lacking CENP-50/U are healthy, mouse ES cells lacking CENP-50/U are lethal. Several possibilities can be regarded as causing the difference of lethality between DT40 cells and ES cells. The difference might arise from structural differences of the CENP-O complex proteins between chicken and mice, functional differences between species, or differences of cell types. This difference might result from differences between cultured cells and organisms.

First, the difference of kinetochore structure was examined. The kinetochore structure around CENP-O complex proteins might be different between mice ES cells and chicken DT40 cells. To ascertain whether the mouse kinetochore structure is identical to that of chicken or not, dependence of the kinetochore localization of CENP-O complex proteins in ES cells was analyzed. Results show that the CENP-O complex proteins of mouse ES cells localized into kinetochore in the same manner as those of chicken DT40 cells (Fig. 5), indicating that the kinetochore structure around CENP-50/U is conserved between chicken DT40 cells and mice ES cells. In addition, this dependency of kinetochore localization was conserved in mouse embryonic fibroblast or lymphocyte (data not shown). These results indicate that differences of lethality between chicken DT40 cells and mouse ES cells does

not arise from differences of the kinetochore structure in two species.

Next, the importance of CENP-50/U might differ among cell types or species. To ascertain whether the importance of CENP-50/U differs among cell types in mice, mice in which CENP-50/U is inductively removed by OHT were generated. Then, mouse embryonic fibroblast (MEF) or lymphocyte cells were isolated and examined to determine their viability after depletion of CENP-50/U by OHT. In contrast to ES cells, MEF lacking CENP-50/U were viable. They propagated normally, and the percentage of CENP-50/U deficient cells was sustained for 6 days after OHT treatment (Fig. 8). Furthermore, lymphocytes lacking CENP-50/U showed similar viability to that of MEF cells (Fig. 9). Based on these results, I concluded that importance of CENP-50/U in mitosis progression differs among cell type.

In chicken, it is difficult to discuss the presence of a cell type in which CENP-50/U is essential, as they are in mouse ES cells, because ES cells or knockout animals are not easily available in chicken. Probably, chicken lymphocytes lacking CENP-50/U are viable like mouse lymphocytes (mainly B cell stimulated by LPS), because DT40 cells in which CENP-50/U is not required were isolated from chicken B cells. The importance of CENP-50/U in mitosis might differ among cell types and might be conserved among species.

Various cell types are assumed in which mitotic regulation differs in detail. Some cell types might use strict regulation, which is not required in cultured cells such as DT40 cells. Based on those assumptions, CENP-50/U might be necessary for the strict regulation of mitosis, and the importance of CENP-50/U differs among cell types depending on the stringency of mitotic regulation. According to this idea, depletion of CENP-50/U causes mitotic defects and cell death in mouse ES cells. However, in another cell type such a MEF or B cell, CENP-50/U is not necessary for mitosis, and cells lacking CENP-50/U are healthy. Mitotic regulation of ES cells may be stricter than that of MEF cells or B-cells. It seems that CENP-50/U-requirement cell and non-requirement cell are present in mice embryo. Then, CENP-50/U-deficient embryos might die at E8.5 because cells requiring CENP-50/U cause mitotic defects and die after exhaustion of maternal CENP-50/U. It is important to examine which kinds of cells die and how long maternal CENP-50/U remains at kinetochore in CENP-50/U null mice.

The next question is why requirements of CENP-50/U differ among cell types. This variety of CENP-50/U requirement might arise from the difference between

undifferentiated cells and differentiated cells, and between growth rates or frequencies of chromosome mis-segregation. Alternatively, cells requiring CENP-50/U might propagate under some stressful circumstances that inhibit correct chromosome segregation. To resolve this question, it is important to study kinetochore structure or associated proteins further in cells requiring CENP-50/U.

Our preliminary analysis indicated that the size of inner-kinetochore plate and CENP-T amount in the kinetochore tend to increase in chicken DT40 cells lacking CENP-50/U (Suzuki A.), which suggests that CENP-50/U has a function in determination of kinetochore range on chromosome. It is important to determine whether the size of inner-kinetochore plate is altered in mouse ES cells and MEF cells lacking CENP-50/U. the importance of kinetochore range or amount of kinetochore proteins may be different depending on the cell types.

Although CENP-50/U is necessary for mitosis in some cell groups in mice, mice lacking CENP-R are healthy. This study did not identify cells in which CENP-R is necessary for propagation. Because gene redundancy of CENP-R has not been found by searching databases or through biochemical analyses, CENP-R and CENP-50/U apparently play different roles in the same protein complex. To elucidate the function of CENP-R in CENP-O complex proteins, further analysis of mice lacking CENP-R is important.

Under usual breeding conditions, CENP-R is inferred not to be involved in normal mitosis. However, when mice are exposed to particular conditions such as carcinogenesis, recovery from injury or disease, or other stressful circumstances, CENP-R might be necessary to complete mitosis under difficult circumstances.

Moreover, transcriptome analysis using microarrays suggests that depletion of CENP-R affects the expression levels of some neuron-specific genes. These data might be clues to the elucidation of CENP-R functions.

MATERIALS AND METHODS

3.1 Isolation of mouse CENP-R

The mouse CENP-R cDNA was isolated by RT-PCR of mRNA extracted from mouse brain. The sequences of primers were 5'-cgGGATCCatgccagttaaaagatcactgaaactg and 5'-cccaagctttcagttcaaaatggctttaaggaattcaaagc that were designed for amplification of ORF of CENP-R gene. PCR reaction was performed with Phusion High-Fidelity DNA polymerase (FINNZYMES) under the condition of 35 cycles with 10 sec at 98°C for denaturing, 2 0 sec at 72°C for annealing and extension. Amplified sequence that was inserted in pET 28a expression vector were sequenced and identified as ORF sequence of the mouse CENP-R.

I used the mouse CENP-R cDNA as a probe to isolate genomic clones specific for the mouse CENP-R from ES cells ↖ 129/terSV/J1↗ genomic library constructed in λFIX vector (Stratagene). By plaque hybridization, several independent clones were isolated and inserted into pBluescript vector. The genomic structures of isolated clones were determined by partial sequenceing.

3.2 Antibodies

To raise Anti-mCENP-R antibody, expression construct was created in pET 28a vector (Novagen). The histidine-tagged recombinant protein was expressed in E. coli BL21 (DE3) after 3h induction with 0.5mM isopropyl-β-D-thiogalactopyranoside and purified recombinant mCENP-R was used for antigen. Immunization and affinity purification of antibody were performed at MBL. Anti-mCENP-50/U, H, O antibody was raised by Hori, T. Phospho-Histone H3-S10 Antibody (monoclonal) is gifted from Kimura, H. Anti-αtubulin (Monoclonal) was purchased from SIGMA (T9029).

3.3 Western blotting

For the detection of mCENP-R protein, whole cell extracts or chromatin fraction were prepared from MEF or ES cells, respectively, and were separated by SDS PAGE. After separation, proteins were transferred onto Hybond-P nitrocellulose membrane (GE Healthcare) using Trans-blot SD Semi-Dry Transfer Cell (Bio-Rad). Membranes were blocked for 1h with 5% skim milk/TBS-T, and incubated for 1h at room temperature with primary antibody diluted to an appropriate concentration (Table 1). After washing with TBS-T, blots were incubated for 1h at RT with

HRP-conjugated secondary antibody (1:15000), and developed by ECL plus kit (GE healthcare). The blots were detected by Storm imaging system (Molecular Dynamics).

3.4 Southern hybridization

Genomic DNA isolated from ES cells or mouse tail tips were digested with appropriate restriction enzyme and separated by agarose gel electrophoresis. After 0.25M HCl treatment, the DNA was neutralized in gel, and transferred to Hybond-N+ nylon membrane. Probe DNA was radiolabeled by Megaprime DNA Labelling System (GE Healthcare), and used for hybridization in Curch Mix at 60-65℃ for overnight. Membranes were washed three times with 2xSSC containing 0.5% SDS for 20 min, and image was detected by X-ray film or Storm phosphor imager (Molecular Dynamics).

3.5 Immunofluorescence

Mouse ES cells, MEF cells and mouse lymphocyte suspensions were collected onto slides with cytocentrifuge, and fixed in 3% paraformaldehyde in 250mM Hepes at room temperature for 15min and permeabilized in 0.5% NP-40 in PBS for 15min at room temperature or cooled 100% methanol for 20min. The fixed cells were incubated with appropriate primary antibody diluted with 0.5% BSA for 1h at 37℃ or for overnight at 4℃. After washing, primary antibodies binding to antigen were detected with FITC or Cy3 conjugated secondary antibodies diluted with 0.5% BSA/PBS. DNA was counterstained with 4, 6-diamidino-2-phenylindole (DAPI) at 0.2μg/ml in Vectorshield Antifade (Vector Laboratories, Burlingame, CA). Immunofluorescence images were collected with a cooled EM CCD camera (QuantEM, Roper Scientific Japan) mounted on an Olympus IX71 inverted microscope with a 100×objective lens together with a filter wheel and DSU confocal system. Z-section images were collected at 0.2μm interval and analyzed using Metamorphe software (Molecular Device Japan). Antibody and dilution ratio use in immunocytochemistry was listed in Table 2.

3.6 Cell culture condition

Mouse embryonic fibroblast cells and mouse L-cells were cultured in Dulbecco's modified medium supplemented with 10% fetal calf serum, 100μM of beta-mercaptoethanol and antibiotic Penicillin-Streptomycin (GIBCO 15140). Cell cuture was performed at the density of 25-80% confluent.

Mouse ES cells were cultured in Dulbecco's modified medium supplemented with 15% fetal calf serum, 0.1mM non essential amino acid (GIBCO11140), 500U/ml or 1000U/mil LIF(ESGRO, Millipore), 100μM of beta-mercaptoethanol and antibiotic Penicillin-Streptomycin (GIBCO 15140). Cells were cultured on mitotically inactivated embryonic feeder cells on gelatin-treated dish. The cell cuture were performed at low density. When dish was about 80% confluent or when the size of colonies is big, cells were trypsinized with 0.25% trypsin (GIBCO) and split.

Mouse lymphocyte was cultured in RPMI-1640 medium containing 10% fetal calf serum,50μM of beta-mercaptoethanol and Penicillin-Streptomycin at the density of 0.5×10^6cells/2cm^2(24well plate).

All incubations were performed in a humidified 37℃, 5% CO_2 conditions.

3.7 Isoration of primary cells

Mouse embryogenic fibroblast (MEF) were prepared from day E14.5 embryo with wild type or CENP-50 lox/-, ERT2-Cre +/- genotypes according to manipulating mouse embryo third edition (21). After removing fetal liver, heart, or head, the rest of embryo was washed and cut into small pieces in a PBS. The embryo was trypsinized with 0.05% trypsin ↖GIBCO↗ at 37℃ for 1h. After that, the cell suspension was transferred to 10cm dish and cultured in MEF medium for 2day (passage 0). A third passage cells were used for growth rate analysis or immunocytochemistry.

Mouse lymphocyte was prepared by standard method (22, 23). Briefly, spleens of mice (8-12 week old) were crushed through the 18-gauge syringe needle in 5ml of medium. The Cell suspensions from spleen was layered onto 5ml of Lympholyte-M (CEDARLANE) and spun at room temperature for 20min at 500g. A middle layer containing white blood cell was collected, and cells were washed twice with 10ml of medium. Red blood cells were lysed with 0.84% ammonium chloride and removed. For cell proliferation assay, B-cells were cultured in medium containing 50μg/ml of LPS (from E.coli 055:B5, SIGMA).

3.8 Cell line

ES cells (129/terSV/J1ES) and G418 resistant feeder cells for generation of knockout mice were gifted by Dr. Sado, T. (Division of Epigenomics, Med. Inst. Bioreg., Kyushu. Univ).

ES cells with OHT-inducible CENP-50/U knockout allele (CENP-U lox/-, Mer-Cre-Mer +/-) were previously generated by Hori, T. (2008). ES cells lacking

CENP-R were created in this work.

3.9 Mouse line and breeding

! All animals were maintained in accordance with the Animal experiment Handbook at NIG. The animals were fed standard chow and water in 12-h light/dark cycle under SPF condition. C57BL/6 strain was used for recipient of targeted ES cells, and background strain of all mutants in this study.

Mice carrying CENP-50/U knockout allele heterozygouly (CENP-50/U +/-) and mice carrying CENP-50/U inducible knockout allele homozygouly (CENP-50/U lox/lox) were created by Hori, T. (2008 unpublish). Mice carrying CENP-R null allele (CENP-R -/-) were generated in this work. Mice having ERT2-Cre homozygouly (ERT2-Cre +/+) and CAG-Cre deleter mice were gifted from Mammalian development lab (NIG).

3.10 Genotyping of mice

For PCR genotyping, mouse tail DNA was extracted using hot alkaline method. The tail tip was incubated in 300µl of 50mM NaOH at 95℃ 30min and neutralized by adding 75µl of 1M Tris-HCl (pH 8.0). One µl of crude extract was used as a PCR template. PCR reaction was performed with Ex-taq DNA polymerase (Takara) under the condition of 35 cycles with 30 sec at 95°C for denaturing, 30 sec at 60°C for annealing and 30sec at 72℃ for extension. Primer sequences used in genotyping of mice were listed in Table 3.

3.11 Cell proliferation assay

To examine growth curve of MEF cells, a third passage of MEF was treated with 100○M of OHT for 24h and plated on 24well plate at 60000 cells/well and cultured. When cells reached subconfluent, they were split at 1:4 ratios. Every two days, cultures were harvested by trypsinization with 0.25% trypsin/EDTA and counted to determine cell number.

ES cells that were treated with OHT in the same way mentioned previously were plated on feeder-free 6well plate at 1×10^6 cells/well and cultured. cells were harvested and counted everyday, and replated on new 6well plate at 1×10^6 cells/well.

The mouse lymphocyte isolated from mouse spleen was cultured in 24well plate after adding 50µg/ml of LPS and OHT for two days. After that, OHT was removed by medium exchange, and cells were cultured in LPS-containing medium. Cell

number was counted every two days.

3.12 Mitotic index determination

ES cells with inducible CENP-50/U knockout allele and ERT2-Cre gene were treated with 100ＯM OHT for 24h. After that, the cells were washed out and cultured for 6 days. The cells attaching to the surface of dish and floating in culture supernatant were collected on the slide with cytocentrifuge. After fixation, cells were co-stained with anti H3P10 antibody (mouse monoclonal) and anti-CENP-50/U antibody (rabbit polyclonal).

I categorized stained cells as CENP-50/U-lacking or CENP-50/U-remaining groups, and the percentage of mitotic cells was calculated, respectively.

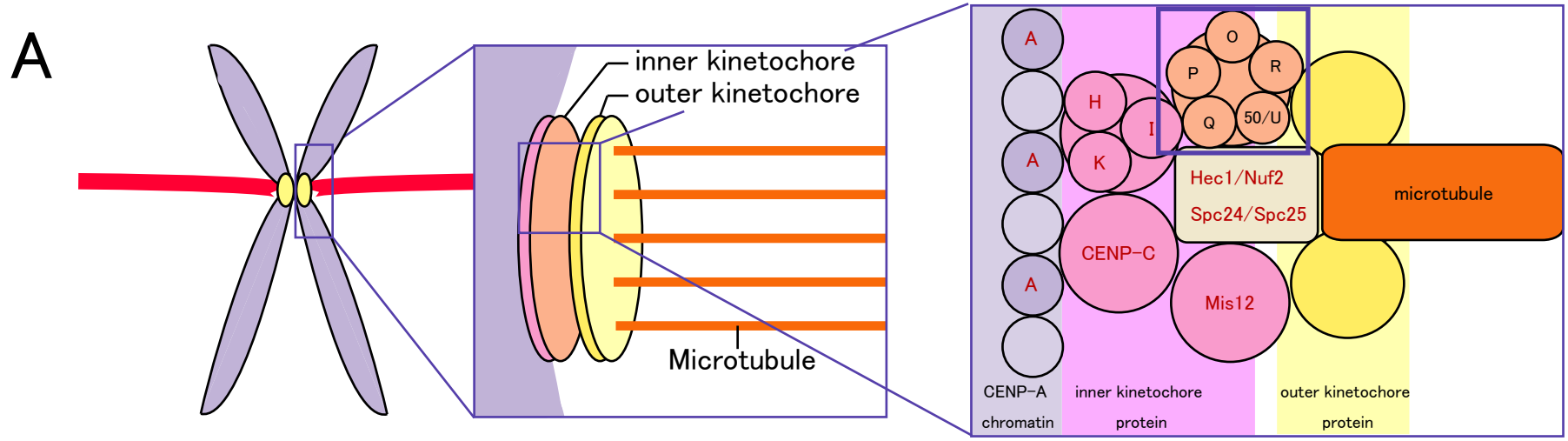
3. 13 Creation of CENP-R knockout mice

Mouse ES cells with CENP-R +/Ires-geo allele were used for transfection experiment to generate homologous recombinant. The cells ($2x10^7$cell) were electroporated with 30μg of linealized construct DNA at 500uF, 240V and ∞Ω (Bio Rad Gene pulser) and grown on G418 resistant feeder cells. After 24h, G418 selection was started at a concentration of 250μg/ml. Resistant colonies were picked 7 day later and cell lines were established. The cells were grown up to confluent, and genomic DNA was extracted for southern hybridization.

Targeted ES cell lines were propagated, injected into C57BL/6 blastocysts, which were transferred into recipient pseudopregnant mice by Dr. Sado, T. Chimeric mice were selected by coat color and mated with C57BL/6 mice to generate heterozygotes. CENP-R heterozygous mice (+/-) were established by Cre-mediated excision of lox-flanked CENP-R exon 4 in the female germline by mating of CAG-Cre transgenic mice. CENP-R null mice (-/-) were generated by crossing between CENP-R heterozygous (+/-) mice.

3.14 Generation of ES cells lacking CENP-R

To generate ES cells with CENP-R null (-/-) genotype, ES cells carrying inducible knockout allele heterozygously (+/Ires-geo) were created using #3 vector in the same method mentioned above, and the ßgeo marker cassette was depleted by transient expression of a Flp recombinase (+/loxP) using Nucleofection kit (Amaxa). The remaining wild-type allele was disrupted using the targeting strategy shown in Figure 4 (-/loxP). Then, the loxP-flanked exon 3 of the first allele was depleted by transient expression of a Cre recombinase(-/-).



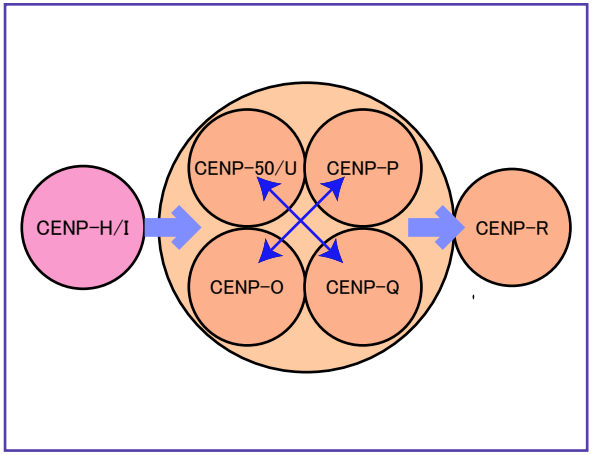


Fig. 1: The kinetochore structure in chicken DT40 cells and CENP hierarchy around CENP-O class proteins. (A). The Kinetochore forms a large structure on centromere DNA and plays important roles in M phase progression (left). Knetochore has a trilaminar disk-like structure that consists of the inner plate, outer plate and midzone (middle). The CENP-O complex that consists of CENP -50/U, O, P, Q, and R constitutively localizes into the innerkinetochore (right). chicken DT40 cells lacking proteins shown by red are lethal. (B). CENP-O class proteins have hierarchy in Kinetochore localization (arrow indicates dependency of localization). Localizations of CENP-O class proteins occur downstream of CENP-H. CENP-O, P, Q and 50/U is interdependent and localization of CENP-R is downstream of other CENP-O class members.

A

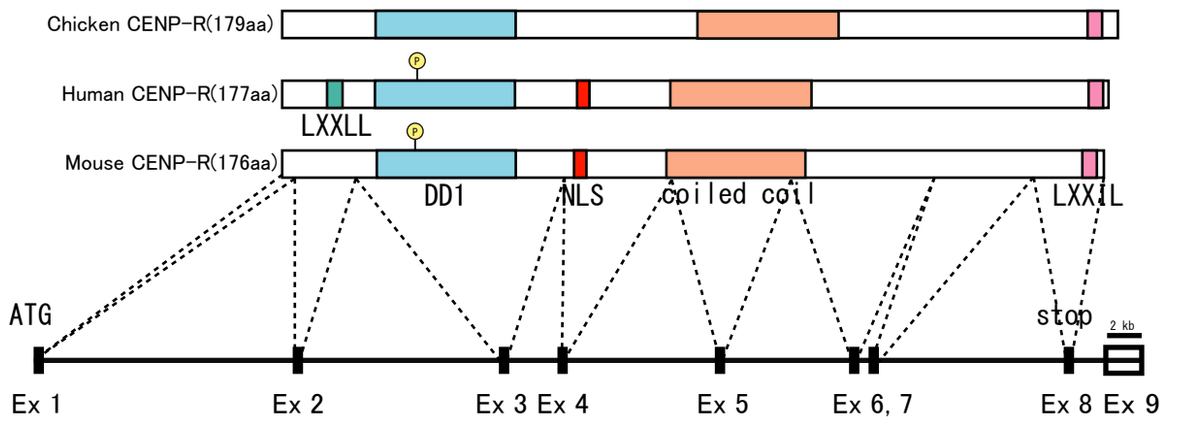
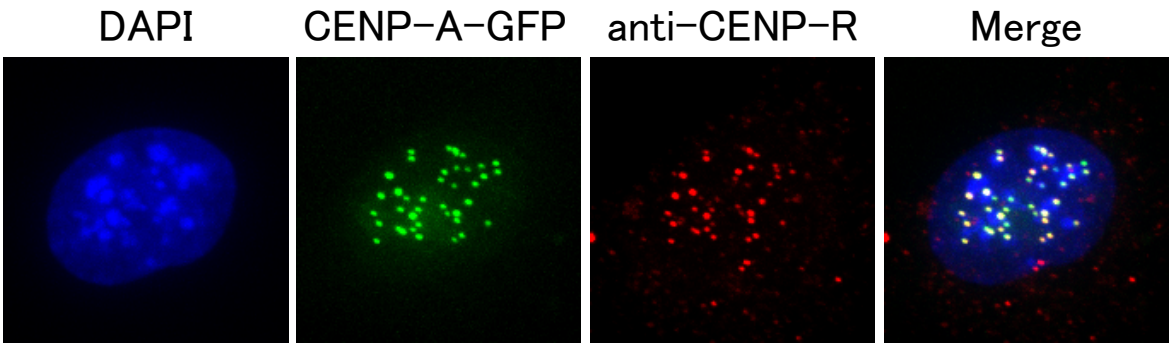


Fig. 2: Isolation of mouse CENP-R. (A) Comparison of amino acid sequences of human,mouse, and chicken CENP-R proteins. CENP-R is highly conserved among vertebrate. Amino acids conserved in all three species are boxed. Predicted coiled-coil region,NLS, DD1 domain and LXXIL motif are present in exon 3 and 4 of mouse CENP-R gene.

B



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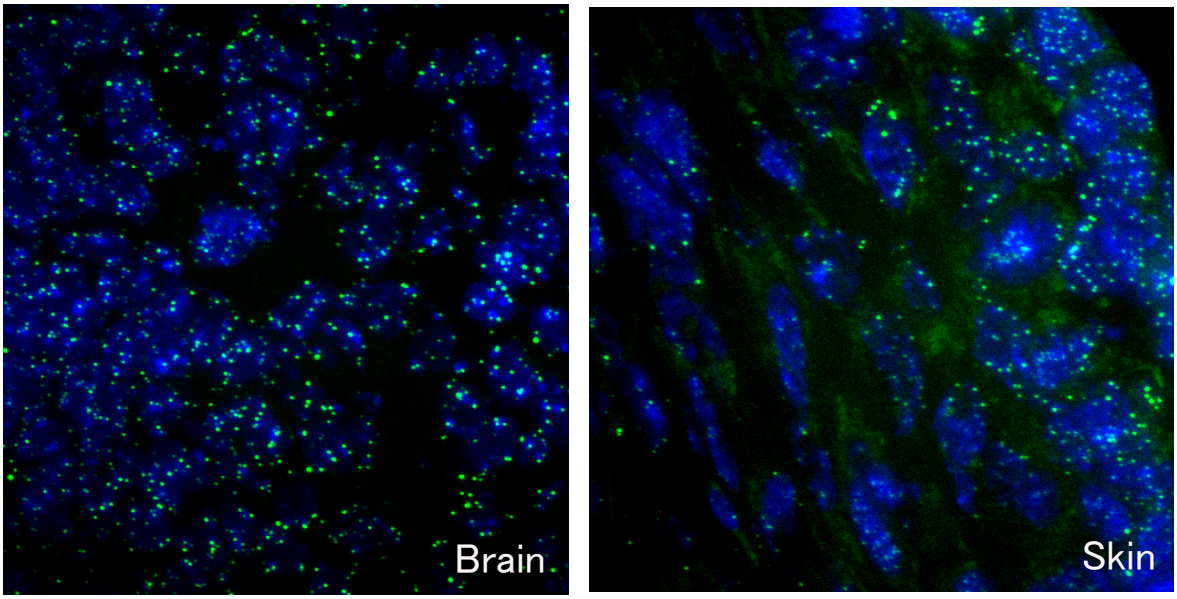


Fig.2: (B) Co-localization of mouse CENP-R and CENP-A-GFP.
Mouse L cell expressing CENP-A-GFP protein was stained with anti CENP-R antibody. (C) CENP-R signals showed a typical dotted pattern in various tissues of E14.5 embryo.

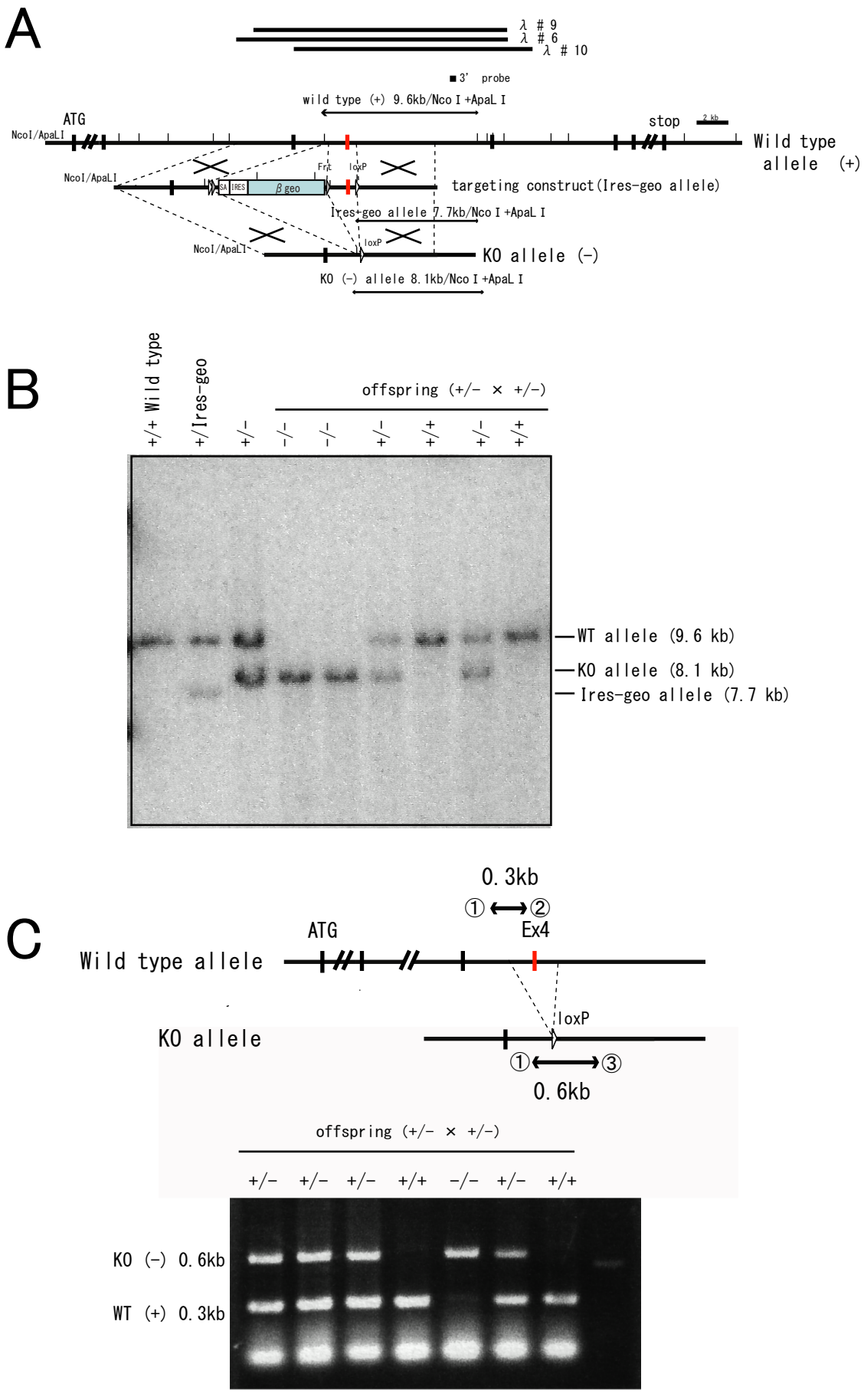


Fig. 3: Generation of mice lacking CENP-R.

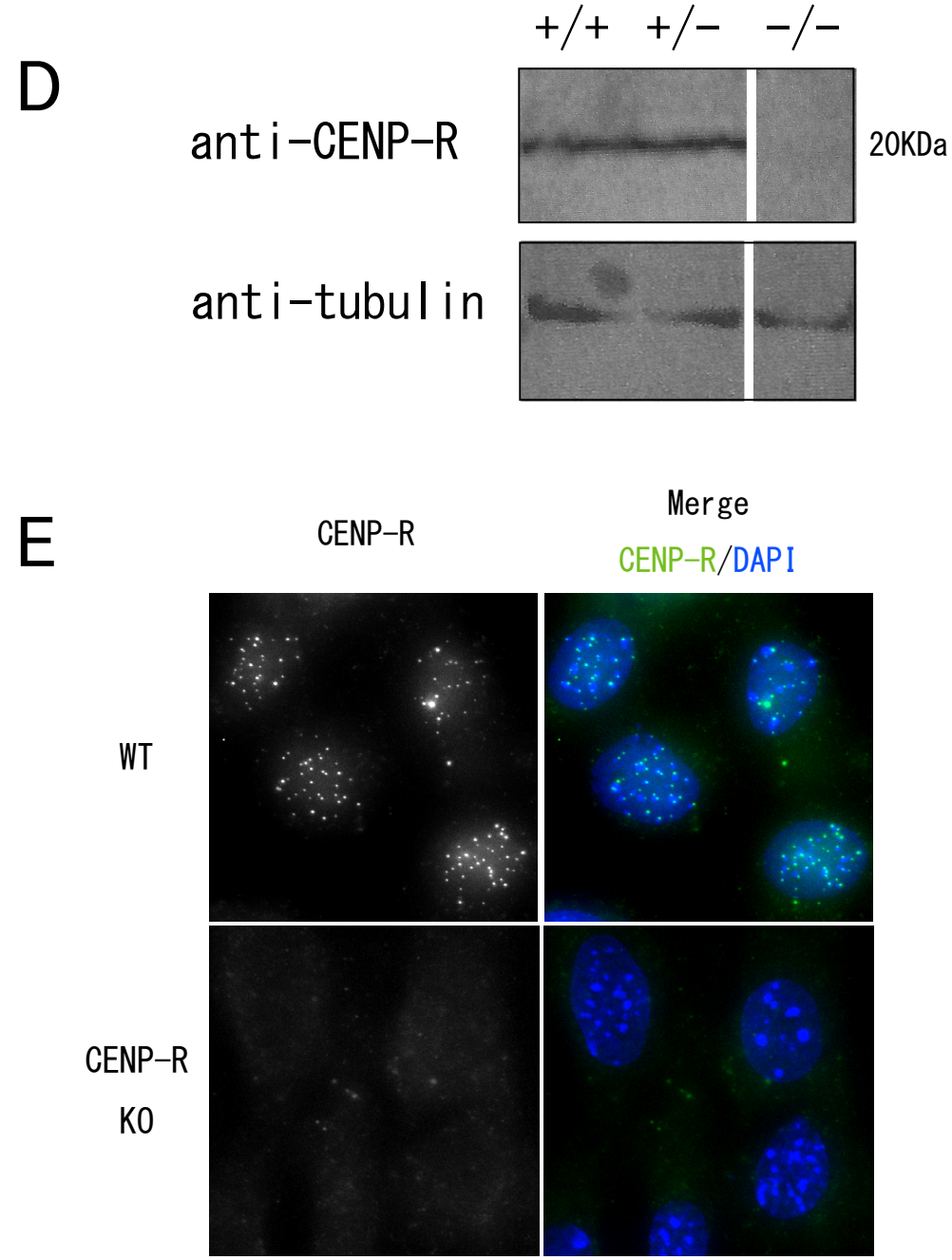


Fig. 3: Generation of mice lacking CENP-R.

Fig. 3: Generation of mice lacking CENP-R. (A) Targeting strategy to generate mice lacking CENP-R. CENP-R allele was targeted using the vector to disrupt Exon 4 (red). The targeted ES cells were used to produce mice having this allele heterozygously. loxP-flanked exon 4 was deleted by crossing with Cre deleter-mice. After that, null mice were created by intercrossing of heterozygous mice. (B) Genotyping results at each step by southern analysis. Genomic DNA was digested by Nco Ⅰ and ApaL Ⅰ, and detected with 3' external probe.(C) Primer positions and Example of PCR genotyping. Primer sequences are shown in table 3. primer names are ①: Nhe1SeqFw, ②: 3SeqRv and ③: Bgl1-Rv. (D) Western blot analysis of whole cell extracts with anti CENP-R antibodies. (E) Immunocytochemistry analysis of MEF cells using anti CENP-R antibody. (F) Appearances of E14.5 embryo and 17 weeks mice lacking CENP-R.

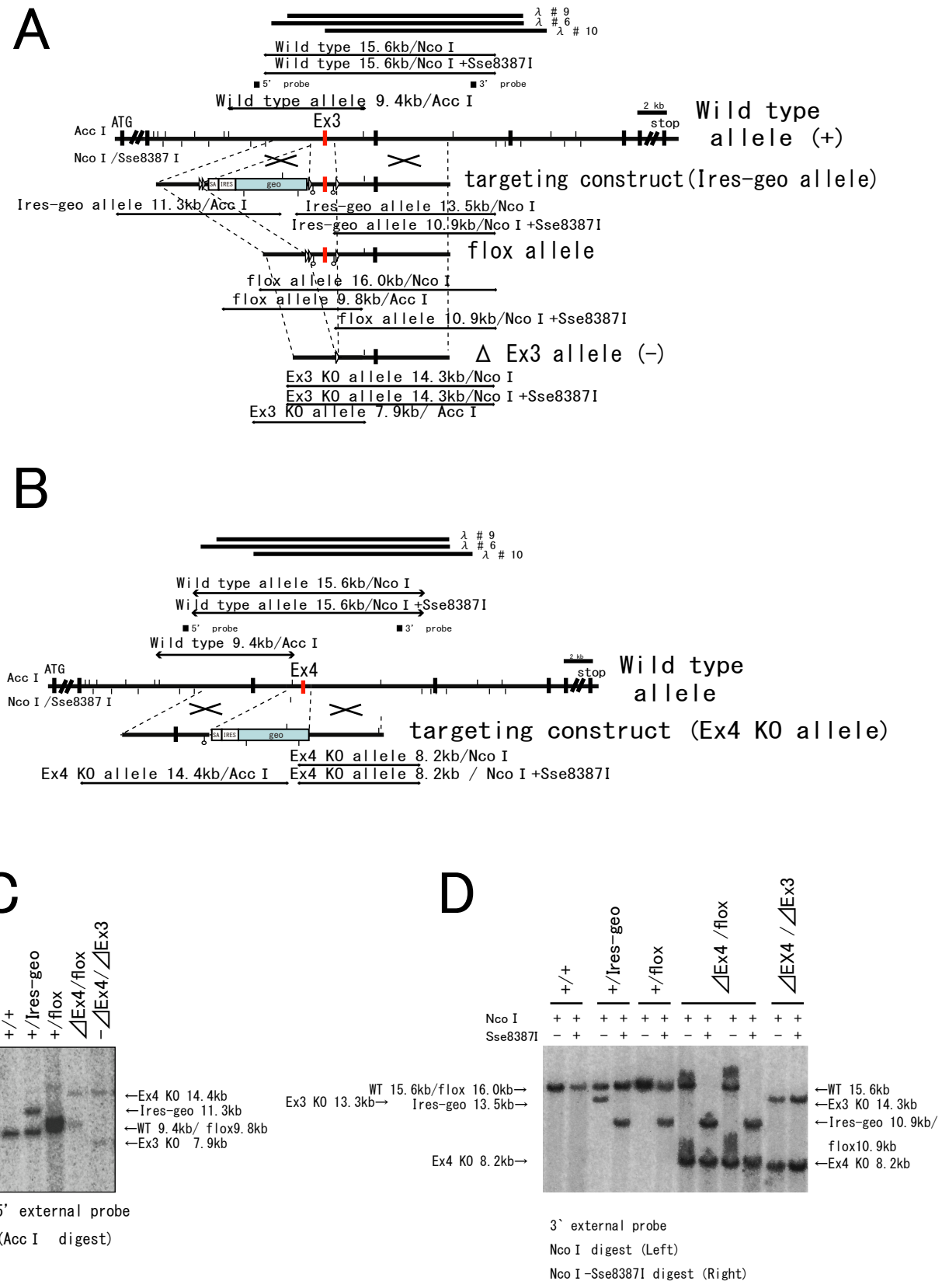
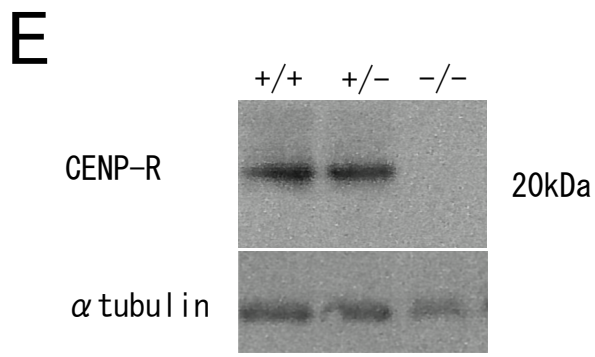


Fig. 4 :Generation of ES cells lacking CENP-R.



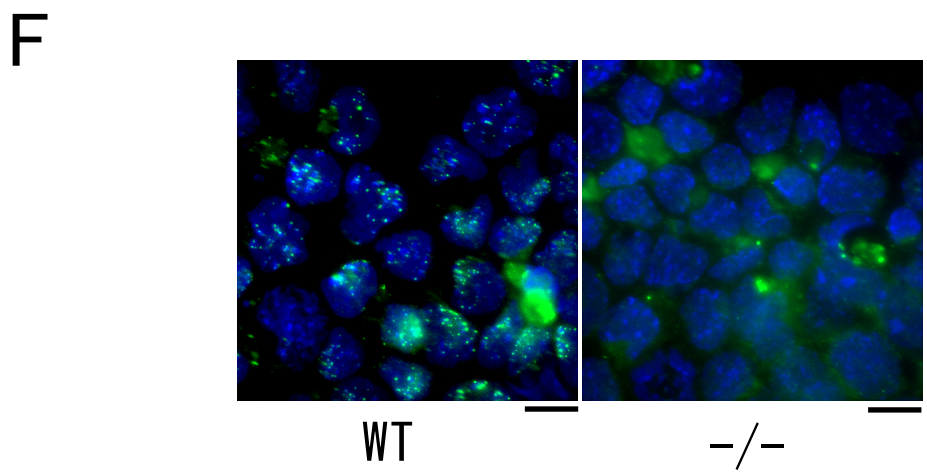


Fig. 4 : Generation of ES cells lacking CENP-R. (A)(B) Targeting strategy to generate ES cells lacking CENP-R allele. The exon 3 of the CENP-R allele (red)was targeted as shown in (A)(+/Ires-geo), and the βgeo marker cassette was depleted by transient expression of a Flp recombinase (+/flox). Next, the exon 4 of the second allele was disrupted using the targeting strategy shown in (B) (Ex4 KO/flox). Finally, the loxP-flanked exon 3 of the first allele was depleted by transient expression of a Cre recombinase(Ex4 KO/Ex3 KO). (C, D): Genotyping results at each step by southern analysis. (E) Western blot analysis of whole cell extracts with anti CENP-R antibodies. CENP-R protein was completely deleted. (F) Immunofluorescence analysis of WT or CENP-R KO ES cells with anti CENP-R.

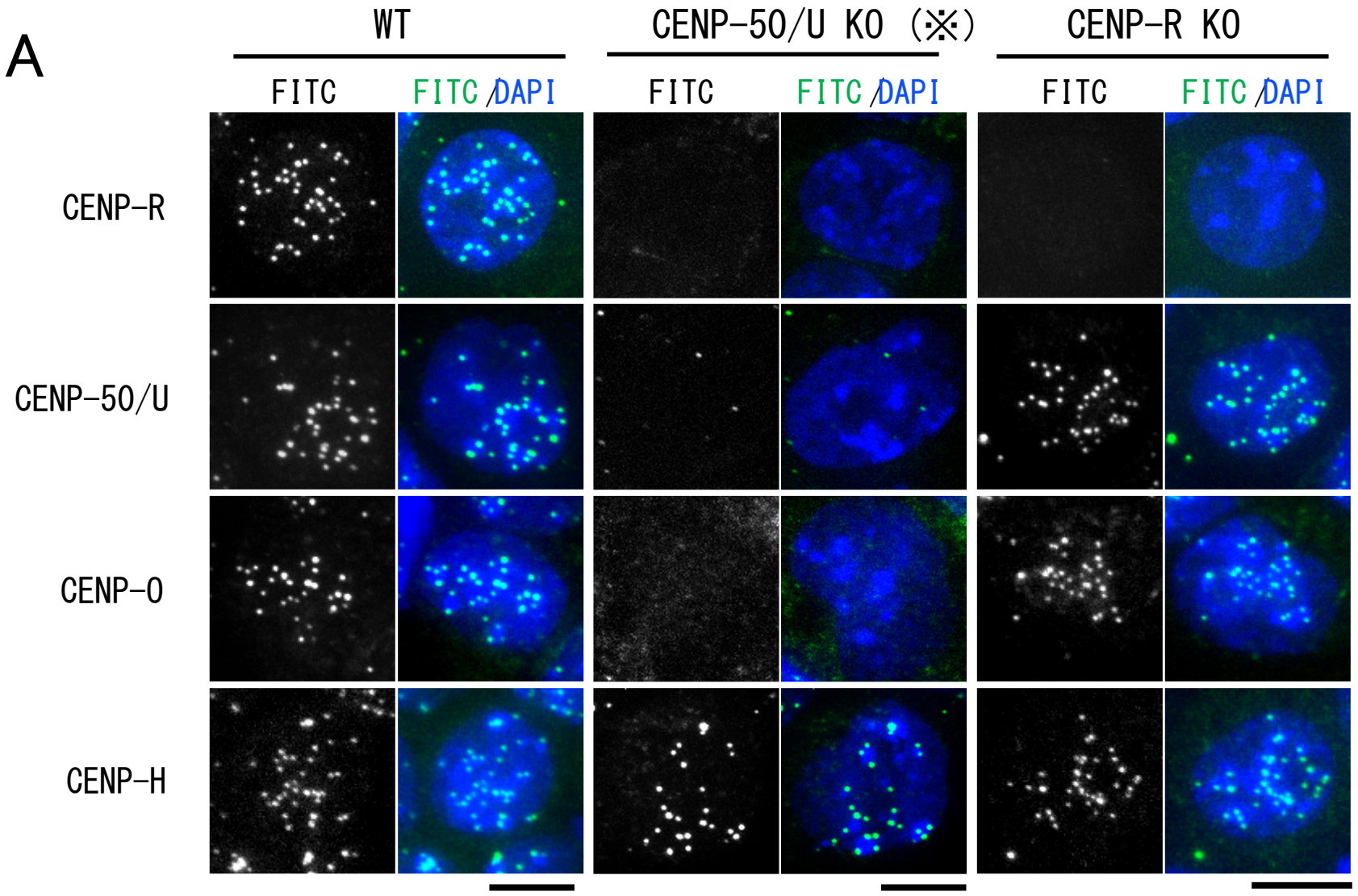


Fig. 5 : The kinetochore structure is conserved between chicken DT40 cells and mouse ES cells.

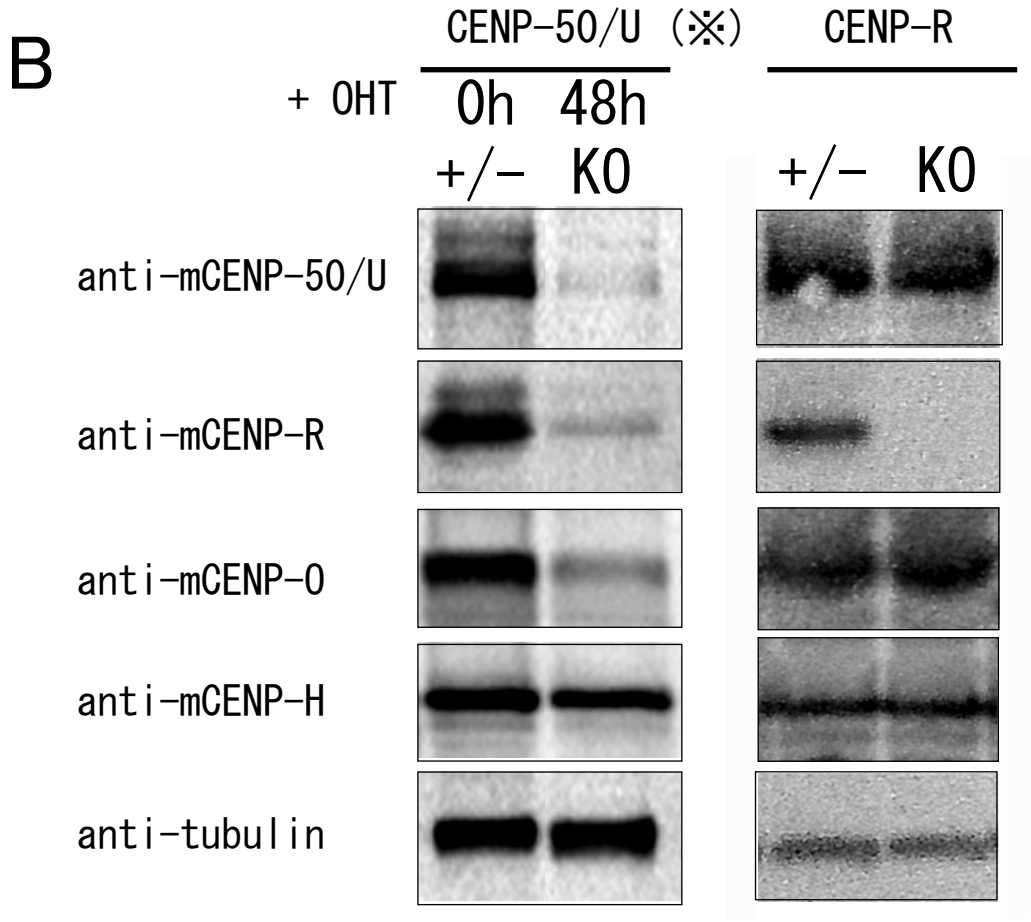


Fig. 5 : The kinetochore structure is conserved between chicken DT40 cells and mouse ES cells. (A) Immunofluorescence analysis of WT, OHT-indusable CENP-50/U KO and CENP-R KO ES cells with anti CENP-R, 50/U, O, and H antibodies. In ES cell with KO of CENP-50, its downstream protein CENP-R and interdependent protein CENP-O do not localize into kinetochore, and upstream protein CENP-H remains. This result indicates that CENP-50/U is protein intermediate between CENP-R and H. In ES cell with KO of CENP-R, other subunits remain at the kinetochore, which indicates CENP-R localizes into kinetochore downstream of these proteins in mice ES cells. (B) Western blot analysis of chromain fractions with anti CENP-O complex proteins antibodies. Suppression of CENP-50/U amount causes diminish of CENP-O and R amount, and depletion of CENP-R does not affect the amount of other CENP-O members. It was confirmed that the dependency of kinetochore localization of CENP-O complex is conserved between chicken DT40 cells and mouse ES cells. ES cells lacking CENP-R was generated and analyzed in this work,and ES cells lacking CENP-50/U was generated and analysis by Hori, T.(※)

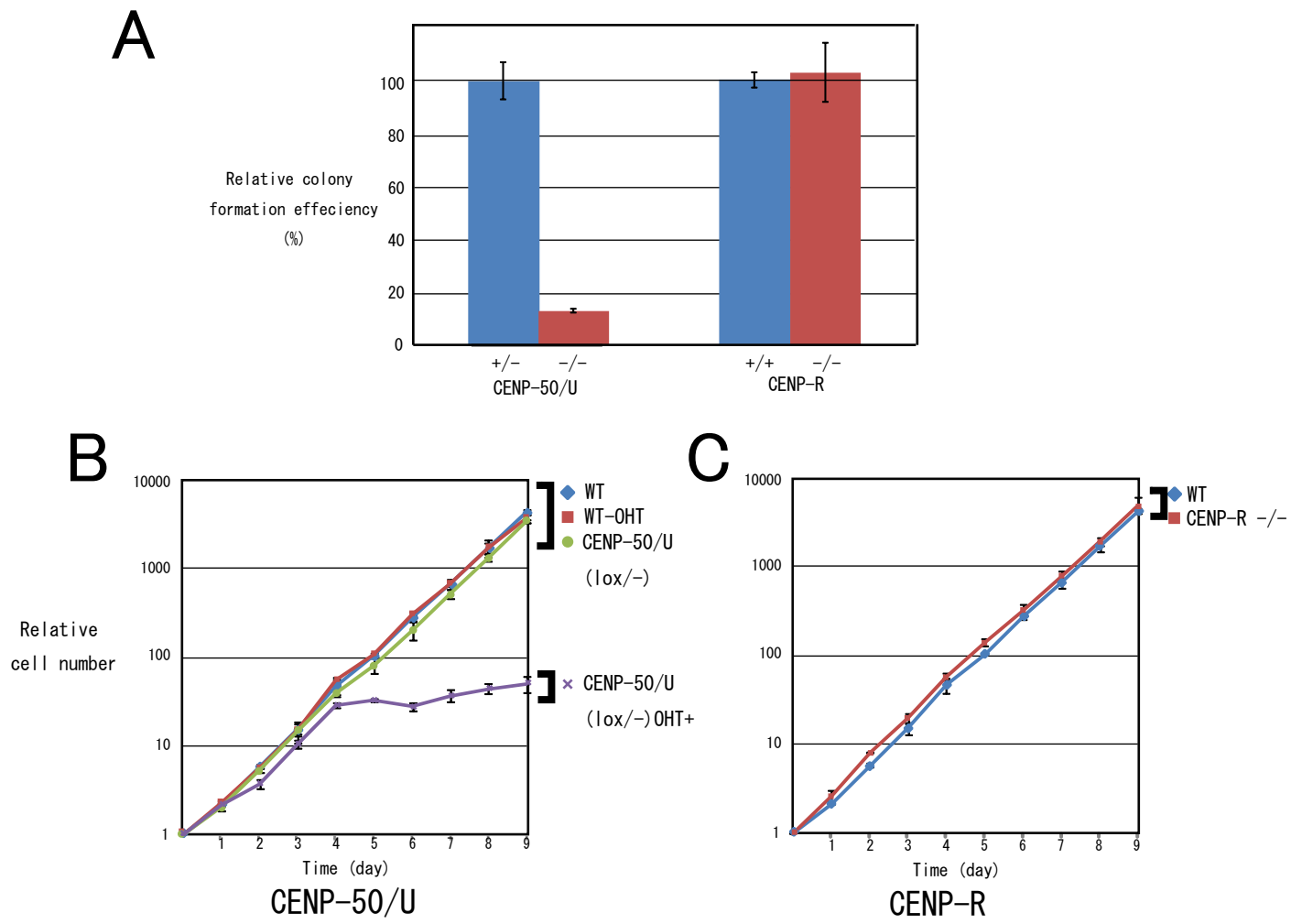


Fig. 6: Viability of ES cells lacking CENP-50/U or CENP-R.
(A) The colony formation ability of ES cells lacking CENP-50/U and R. Each cell lines was treated with OHT for 24h and plated at a density of 3000 cells/plate. The colony numbers were counted 7 days after. (B)(C) Growth curve of ES cells lacking CENP-50/U or CENP-R deficient cells. ES cells lacking CENP-R are very healthy, however ES cells lacking CENP-50/U show severe lethality.

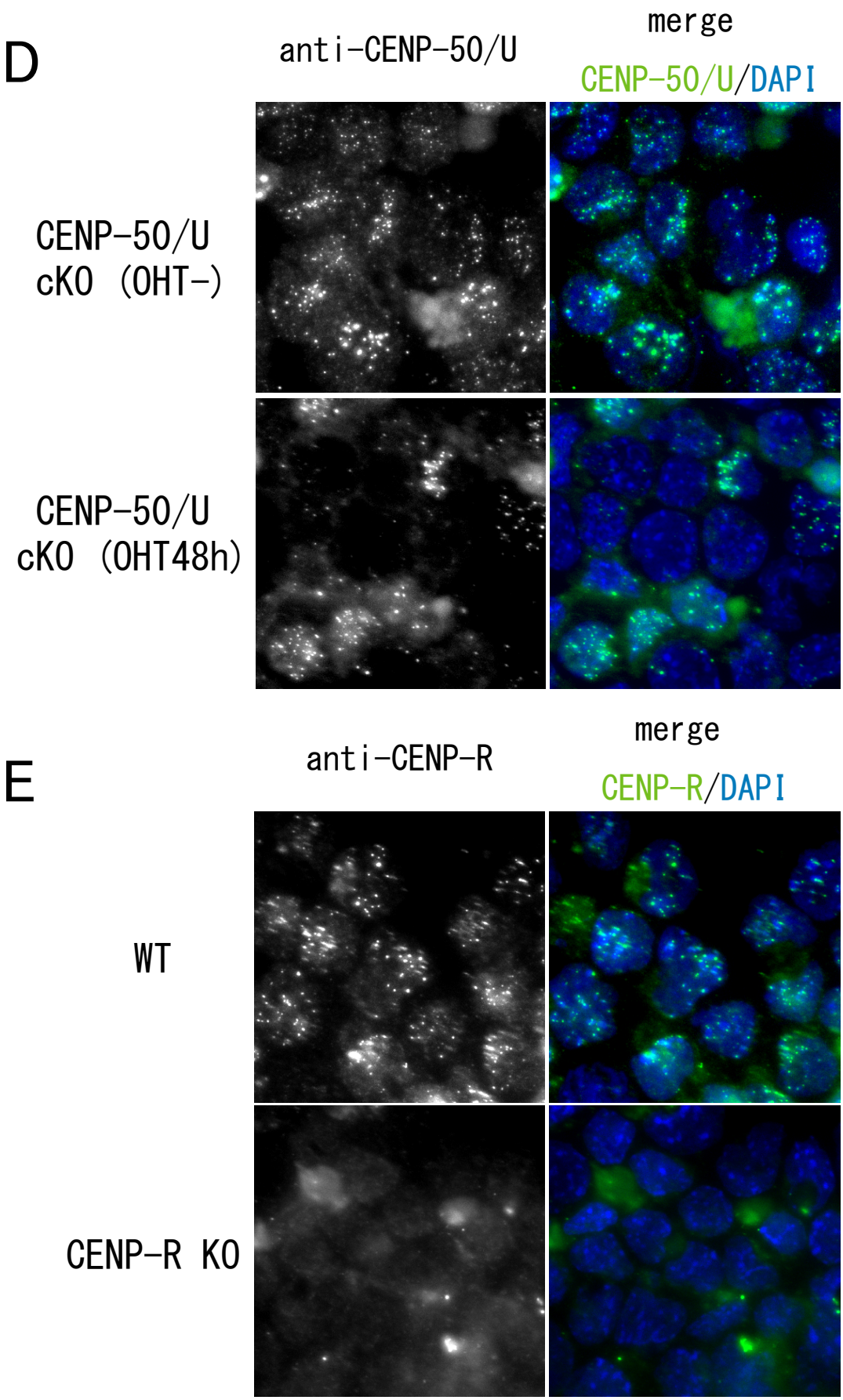


Fig. 6: Viability of ES cells lacking CENP-50/U or CENP-R.

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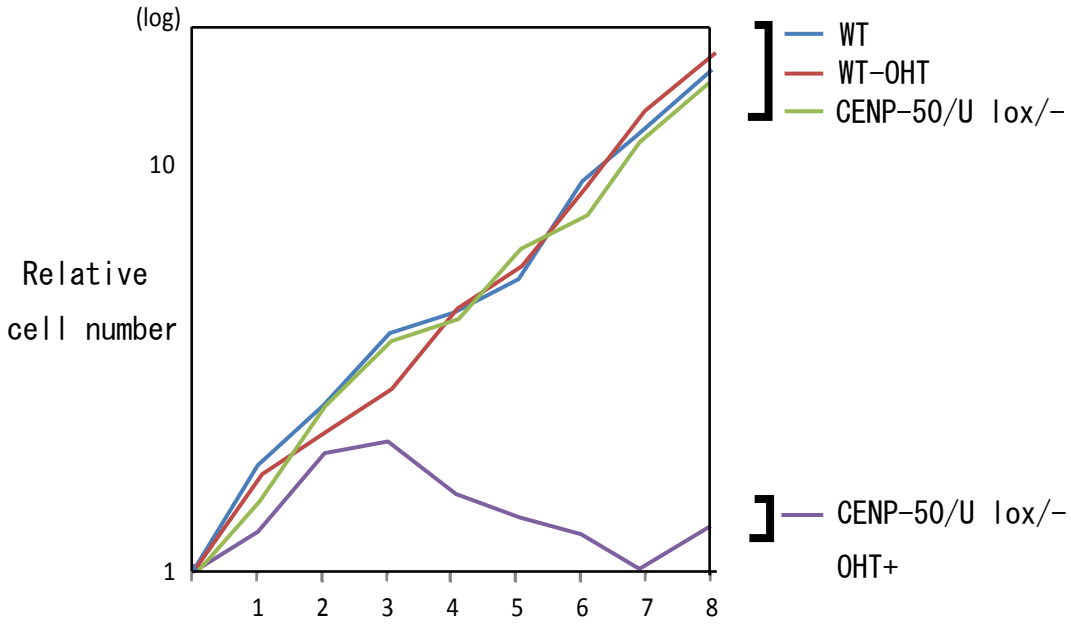
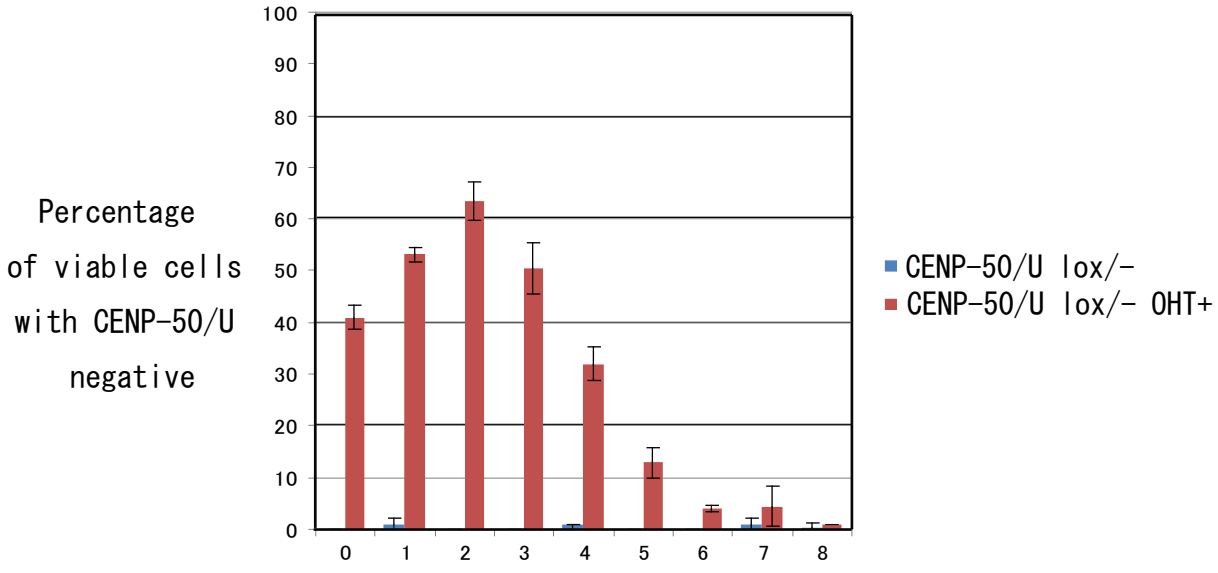


CENP-50/U

G



CENP-50/U

Fig. 6: (D) Immunofluolescence analysis of ES cells with anti CENP-50/U antibody. ES cells with CENP-50/U flox/- and CAG-Cre +/- alleles were treated with OHT. At 48h after OHT treatment, most CENP-50/U were removed from kinetochore. (E) Immunofluolescence analysis of ES cells lacking CENP-R with anti CENP-R antibody. (F) The growth rate of CENP-50/U deficient cells after OHT treatment. (G)The percentage of CENP-50/U deficient ES cells in OHT-treated population. After staining with anti-CENP-50/U antibody, cells lacking CENP-50/U were counted at each time point shown in (F).

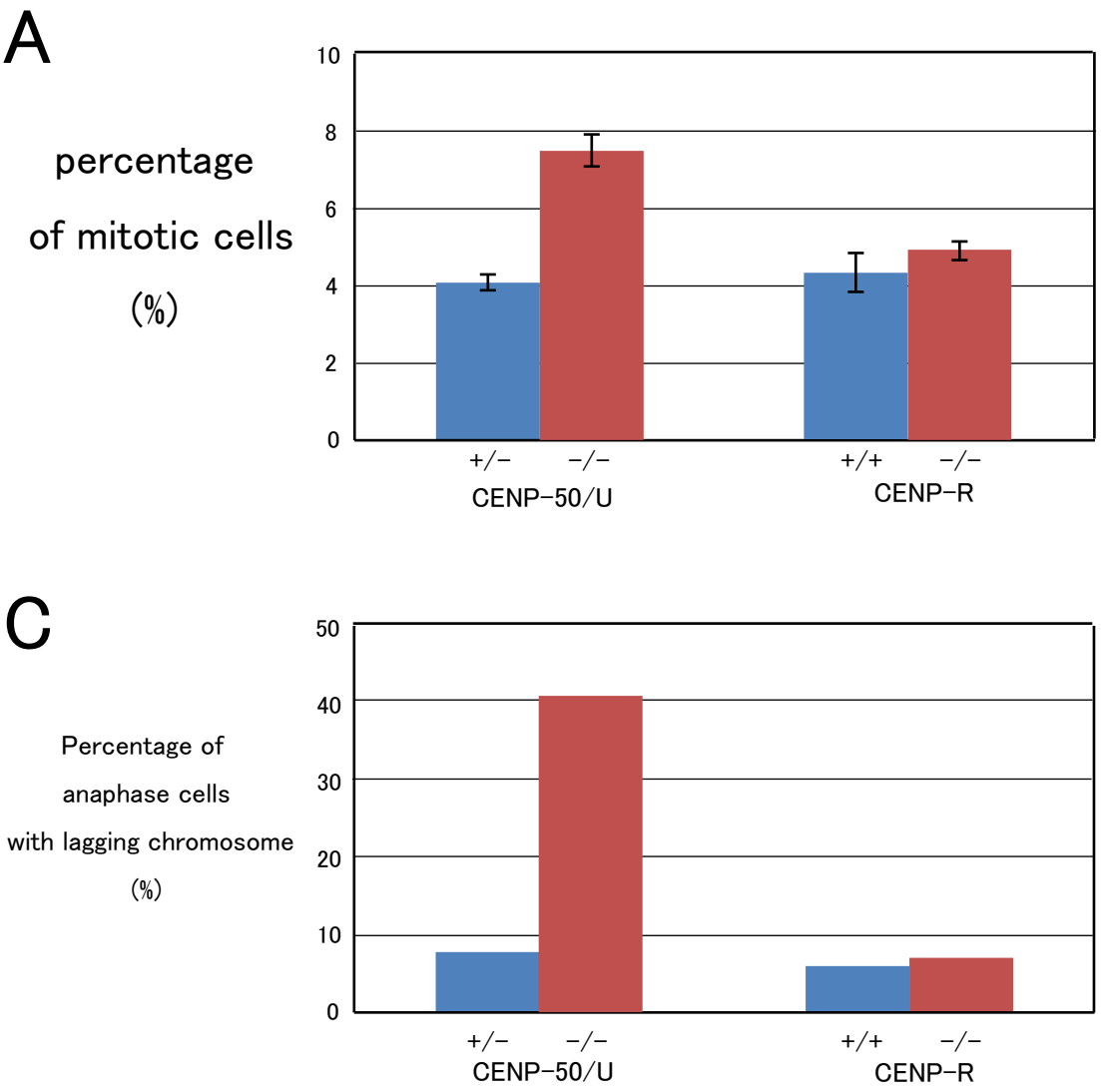


Fig. 7: ES cells lacking CENP−50/U show slight mitotic defects.
(A) Mitotic defect in ES cells lacking CENP−50/U.(A)Mitotic index of ES cells lacking CENP−50/U (4day after OHT treatment) or CENP−R. Mitotic index is slightly increased in ES cells lacking CENP−50.(B)Image of anaphase cells lacking lacking CENP−R or CENP−50/U. Arrow indicates lagging chromosome. (C) The percentage of ES cells with lagging chromosome in anaphase cellls. The percentage of anaphase cells with lagging chromosome is fivefold higher in CENP50/U null ES cells than in wild type or CENP−R null ES cells.

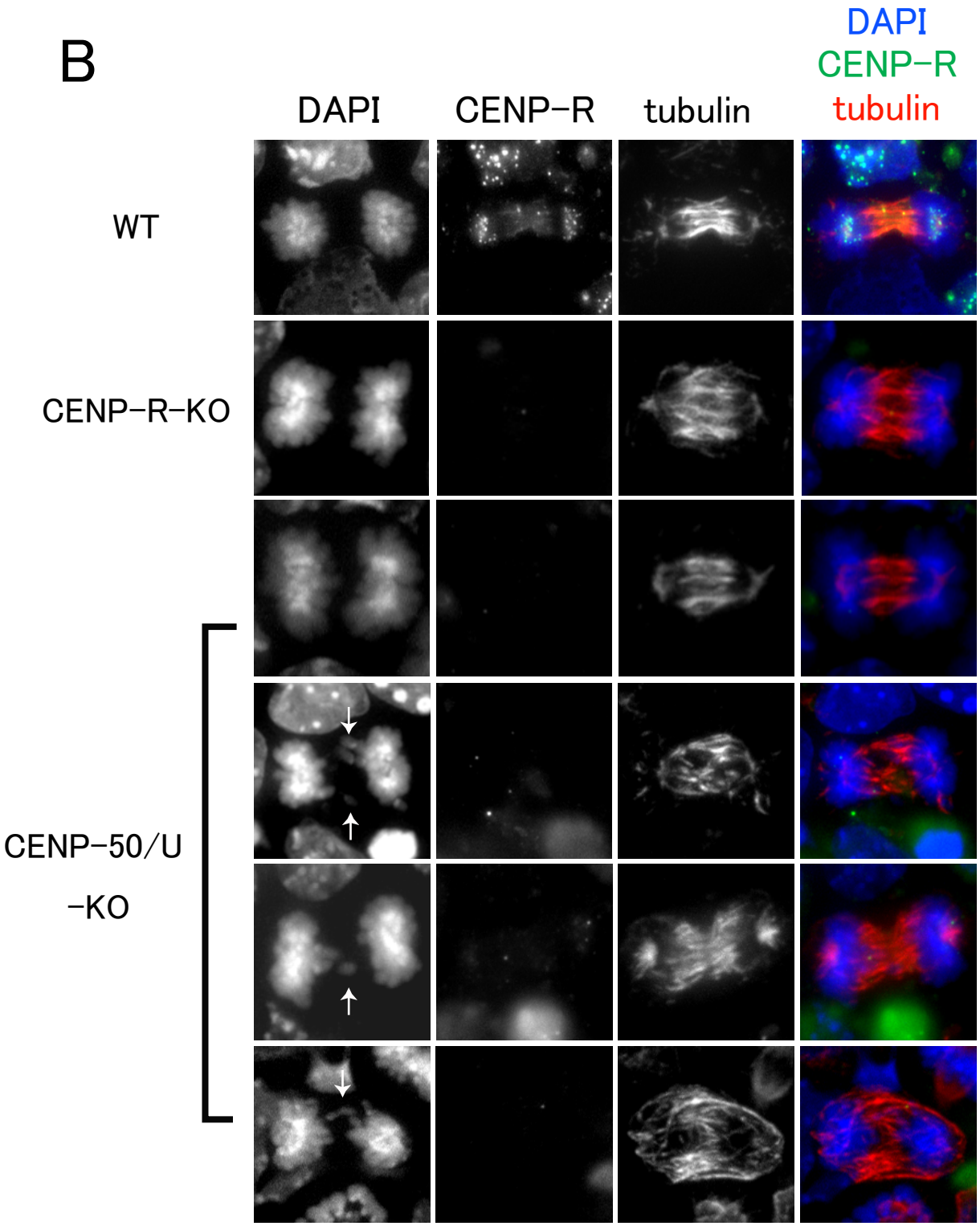


Fig. 7: ES cells lacking CENP-50/U showe slight mitotic defects.

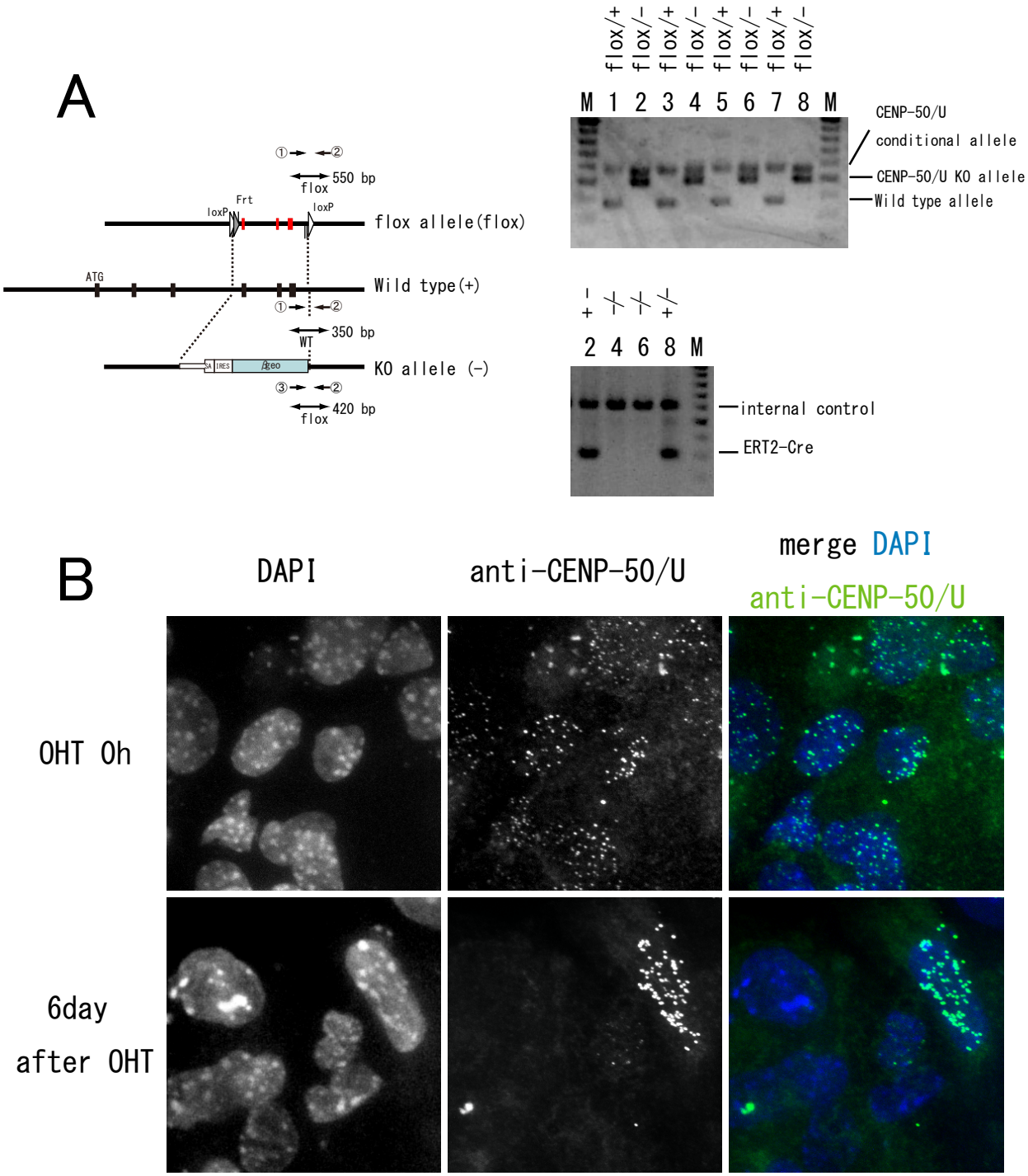
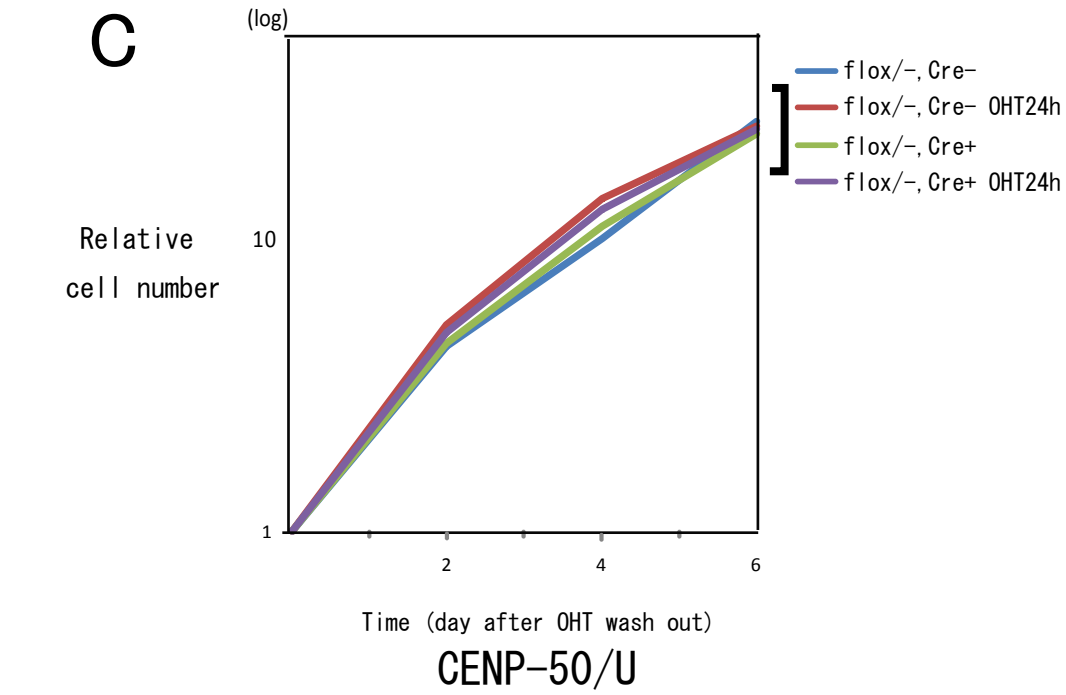


Fig. 8: Viability of MEF cells lacking CENP-50/U. (A) Primer positions and Example of PCR genotyping. Primer sequences are shown in table 3. (Primer names are ①:3F, ②: 3R and ③: Neo3). Embryo with CENP-50/U flox/− and ERT2-Cre +/− was selected.The rane numbers indicate each embryo. (B) Immunofluolescence analysis of MEF cells with anti CENP-50/U antibody. MEF cells with CENP-50/U flox/− and CAG-Cre +/− alleles were treated with OHT. At 6 days after OHT treatment, most CENP-50/U was removed from kinetochore.



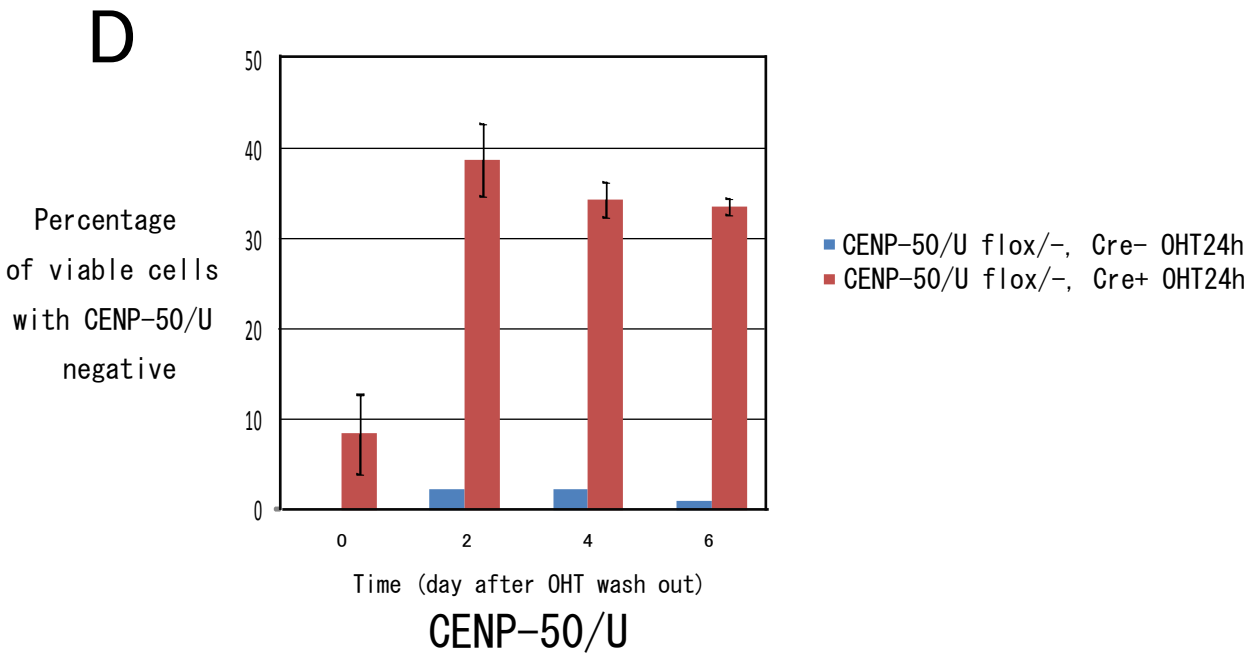


Fig. 8: (C) Growth rate of MEF cells with OHT inducible CENP-50/U KO allele after OHT treatment. (D)The percentage of CENP-50/U deficient MEF cells in OHT-treated population. After staining with anti-CENP-50/U antibody, cells lacking CENP-50/U were counted at each time point shown in (C).

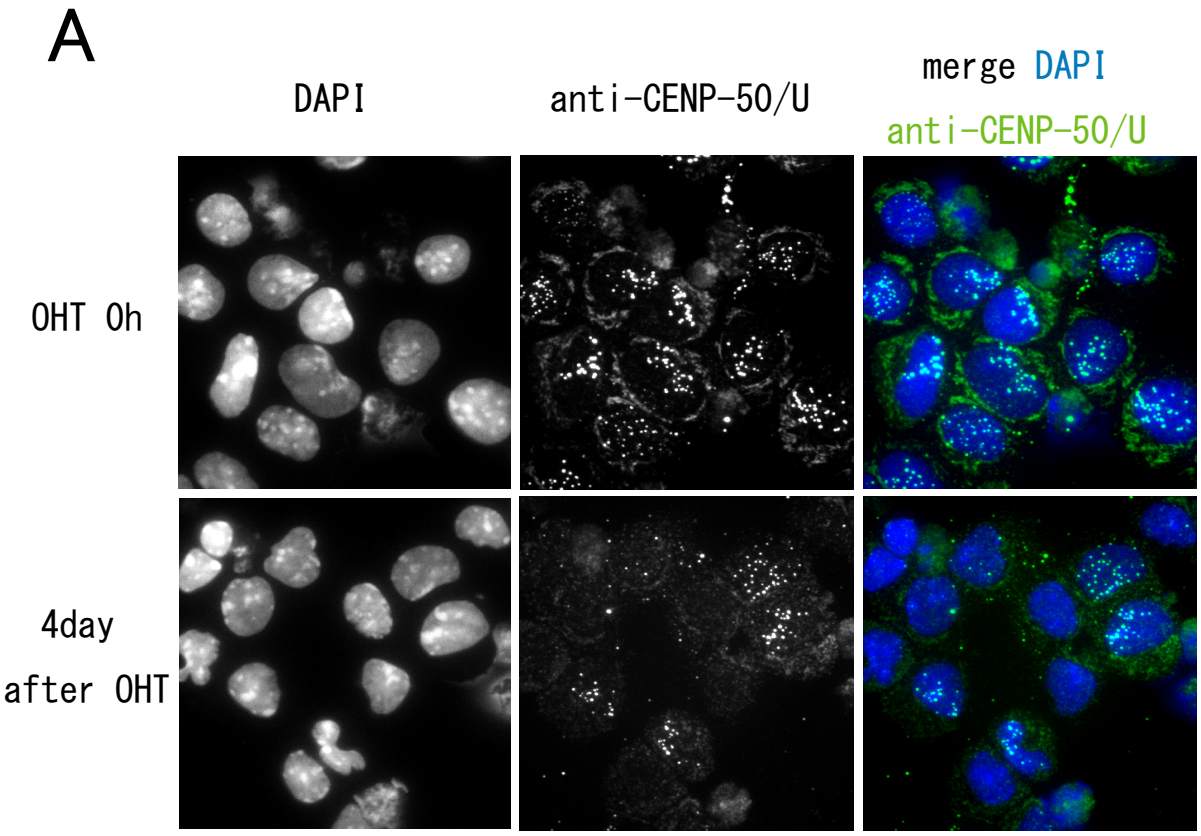
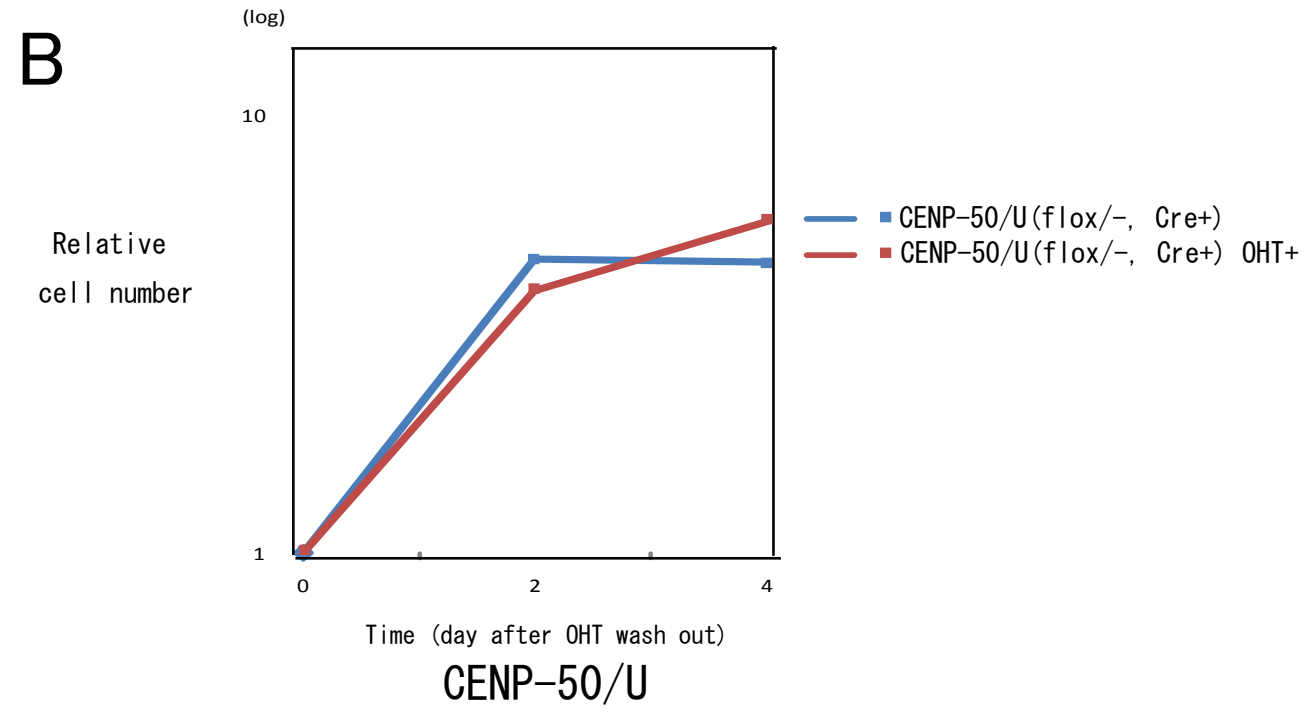


Fig. 9 : Viability of lymphocytes lacking CENP-50/U.
(A) Immunofluolescence analysis of lymphocyte with anti CENP-50/U antibody. Lymphocytes with CENP-50/U flox/- and CAG-Cre +/- alleles were treated with OHT. At 4 days after OHT treatment,CENP-50/U was removed from kinetochore.



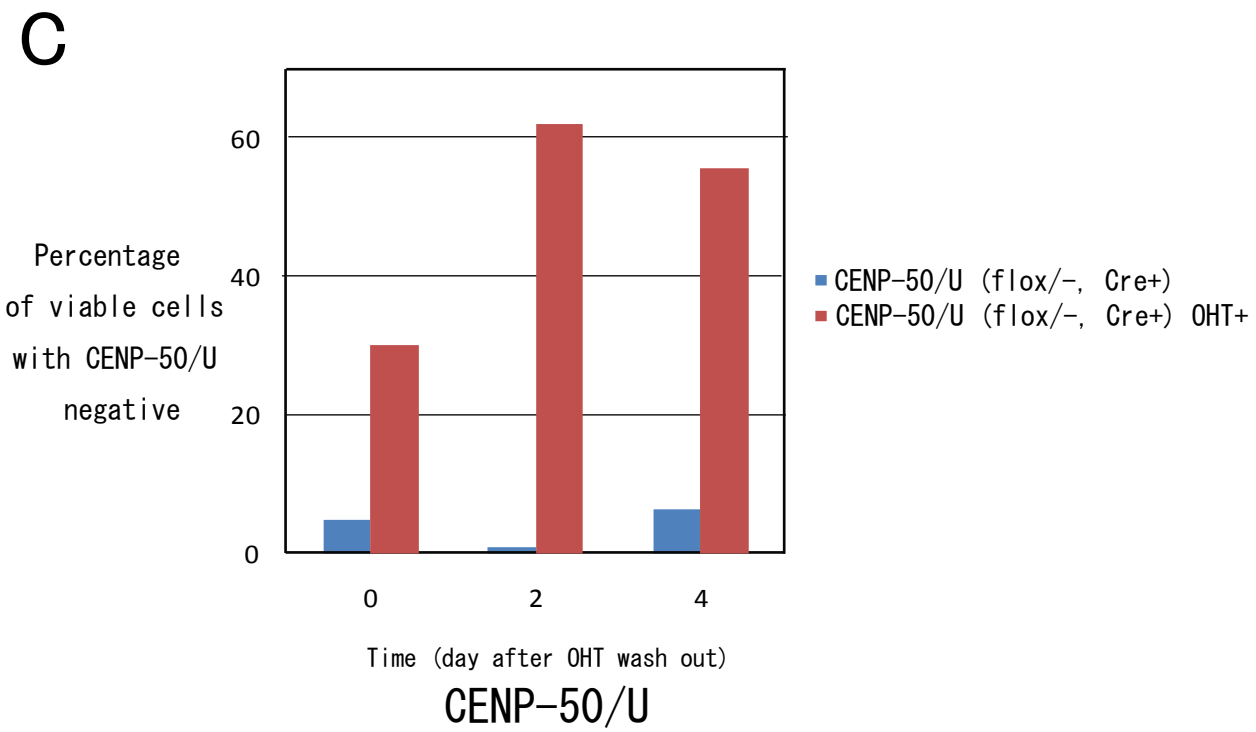


Fig. 9: (B) Growth rate of lymphocyte cells with OHT inducible CENP-50/U KO allele after OHT treatment. (C) The percentage of CENP-50/U deficient B-cells in OHT-treated population. After staining with anti-CENP-50/U antibody, cells lacking CENP-50/U were counted at each time point shown in (B).

Table 1 Primary or secondary antibodies for immunobloting

Antibody	dilution rate	Reference or supplier
rabbit-anti-mouse CENP-O (polyclonal)	1:1000	
rabbit-anti-mouse CENP-50/U (polyclonal)	1:1000	Hori, T.
rabbit-anti-mouse CENP-H (polyclonal)	1:1000	
rabbit-anti-mouse CENP-R (polyclonal)	1:1000	this work
Monoclonal anti-α-tubulin	1:5000	Sigma (T-5168)
HRP-conjugated Goat anti-mouse IgG	1:15000	Jackson Immuno Research Lab (111-035-003)
HRP-conjugated rabbit anti-mouse IgG	1:10000	Jackson Immuno Research Lab (315-035-003)

Table 2 Primary or secondary antibodies for immunocytochemistry

Antibody	dilution rate	Reference or supplier
rabbit-anti-mouse CENP-O (polyclonal)	1:1000	
rabbit-anti-mouse CENP-50/U (polyclonal)	1:1000	Hori, T.
rabbit-anti-mouse CENP-H (polyclonal)	1:1000	
rabbit-anti-mouse CENP-R (polyclonal)	1:1000	this work
FITC conjugate anti-α-tubulin (Monoclonal)	1:100	Sigma (F-2168)
Phospho-Histone H3-S10 Antibody (monoclonal)	1:1000	Kimura, H.
Cy3-conjugated goat anti-rabbit IgG	1:2500	Amersham (PA43004)
Cy3-conjugated goat anti-mouse IgG	1:1000	Jackson Immuno Research Lab
FITC-conjugated Fab goat anti-rabbit IgG	1:1000	Jackson Immuno Research Lab
FITC-conjugated rabbit anti-mouse IgG	1:200	Jackson Immuno Research Lab (315-095-003)

Table 3 Primer list used in mouse genotype

allele	primer name	sequence (5'-3')
CENP-50/U	3F	tataccctgttgctctctt
	3R	gcagaaagtcttccacctgt
	Neo3	ggatctcatgctggagttct
CENP-R	Nhe1SeqFw	GACTTCTTTCCATACTTAGTCTTGC
	3SeqRv	GTCATCACTGAATCTGGACTAC
	Bgl1-Rv	CTTACAACAGAAGACCATGAGC
GFP	GFP-F	TACGGCAAGCTGACCCTGAA
	GFP-R	tgtgatcgcgcttctcgttg

Table 4 Frequency of possible genotypes from crosses between CENP-R heterozygous animals

		WT	+/-	-/-	total
offspring	♂	13	23	15	51
	♀	16	26	13	55
total(%)		29(27%)	49(46%)	28(26%)	106(100%)

Table 5 Sex ratio of offspring from crosse between CENP-R null males and females

	♂	♀	total
offspring(%)	15 (46%)	18 (54%)	33 (100%)

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