Analysis for the CCAN dependent CENP-A incorporation

CCAN に依存する CENP-A の導入に関する解析

Jinghui Cao

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Abstract

The functional centromere plays a critical role in ensuring chromosome stability during cell division, primarily governed by epigenetic mechanisms. A key epigenetic marker, CENP-A (Histone H3 variant), is exclusively present in nucleosomes at the centromere region. These CENP-A nucleosomes serve as a crucial platform for recruiting inner kinetochore proteins (CCAN) throughout the cell cycle, while the CCAN recruit microtubule binding outer kinetochore proteins (KMN network) during the M phase. This orchestrated process ensures the proper attachment of microtubules and the equal distribution of sister chromatids into daughter cells.

Remarkably, we made a discovery where new centromeres could be induced ectopically, and CENP-A could be recruited by CCAN, in addition to known CENP-A incorporation machinery components such as HJURP and Knl2. To investigate this intriguing phenomenon, I employed the ectopic tethering assay in combination with the auxin-inducible degron (AID)-based protein knockout method. Our findings revealed that tethering CENP-C or CENP-I resulted in the incorporation of CENP-A at a non-centromeric locus, even in the absence of Knl2. Additionally, I observed that CENP-C co-immunoprecipitated with HJURP independently of Knl2.

Based on these results, I propose a mechanism wherein CENP-C can recruit CENP-A through direct binding to HJURP, leading to the formation of artificial new centromere. While Knl2-HJURP predominantly contributes to the deposition of new CENP-A into centromeres, our findings suggest that CENP-C or CENP-I possess CENP-A recruitment activity independent of Knl2 for artificial new centromere formation in chicken DT40 cells.

Catalog

Chapter 1: General introduction

1. Cell division

1.1 Overview

Cell division is a fundamental process in eukaryotic organisms, playing a crucial role in development, growth, and repair. There are two main types of cell division: mitosis and meiosis. Mitosis is a form of cell division that takes place in somatic cells, which are non-reproductive cells. It results in the formation of two genetically identical daughter cells, each with the same number of chromosomes as the parent cell. On the other hand, meiosis is a type of cell division that occurs in reproductive cells (sperm and egg cells). This process produces four genetically diverse daughter cells, each containing half the number of chromosomes as the parent cell.

To ensure proper cell division, eukaryotic cells rely on a series of coordinated events called the cell cycle. This cycle encompasses a variety of processes, including DNA replication, mitosis or meiosis, and cytokinesis. The cell cycle is a tightly regulated process that ensures the faithful transmission of genetic material from parent to daughter cells. Any errors in this process can result in chromosomal abnormalities, which may lead to diseases such as cancer.

1.2 Mitotic cell division

The mitotic cell division cycle (**Figure 1-1**) is a highly intricate process that can be conceptually divided into two major stages: interphase and M phase. Interphase is the longer stage during which the cell duplicates its entire cellular content, while M phase is the stage when the cell physically divides to generate two genetically identical daughter cells. Specifically, DNA replication occurs during a distinct phase of interphase known as the ‘S phase,’ while the actual separation of DNA takes place during mitosis (M phase). Within M phase, the chromatin undergoes condensation in prophase, forming its condensed structure - the chromosome. Subsequently, chromosomes align at the cell's center during metaphase, followed by the separation of sister chromatids in to opposite poles of cell during anaphase. Ultimately, cytokinesis, occurring at the conclusion of M phase, completes the cell cycle by segregating the

cellular content, leading to the formation of two separate daughter cells. At this point, the cell has the option to either exit the cell cycle or initiate a new round of cell division. Interphase is further subdivided into three distinct periods: G1, S, and G2. Historically, G1 and G2 were termed the 'gap phases' as they represent intervals between the two primary events of the cell cycle - DNA duplication and segregation. During G1, the cell undergoes a critical decision-making process to determine whether it should proceed with the cell cycle or not. Similarly, during G2, the cell initiates the processes that lead to chromosome segregation. Cells crucially employ cell cycle checkpoints to safeguard against the accumulation and dissemination of genetic anomalies throughout the process of cell division. These checkpoints hinge upon highly conserved evolutionary signaling pathways that diligently scrutinize various facets of cell cycle progression. Specifically, they monitor interphase for DNA damage, denoted as the DNA damage checkpoint, assess the integrity of DNA replication forks during the S phase, referred to as the DNA replication stress checkpoint, and evaluate the completeness of spindle assembly during the M phase, identified as the Spindle assembly checkpoint. These surveillance mechanisms collectively play an essential role in preserving genomic integrity. (Matthews et al., 2021).

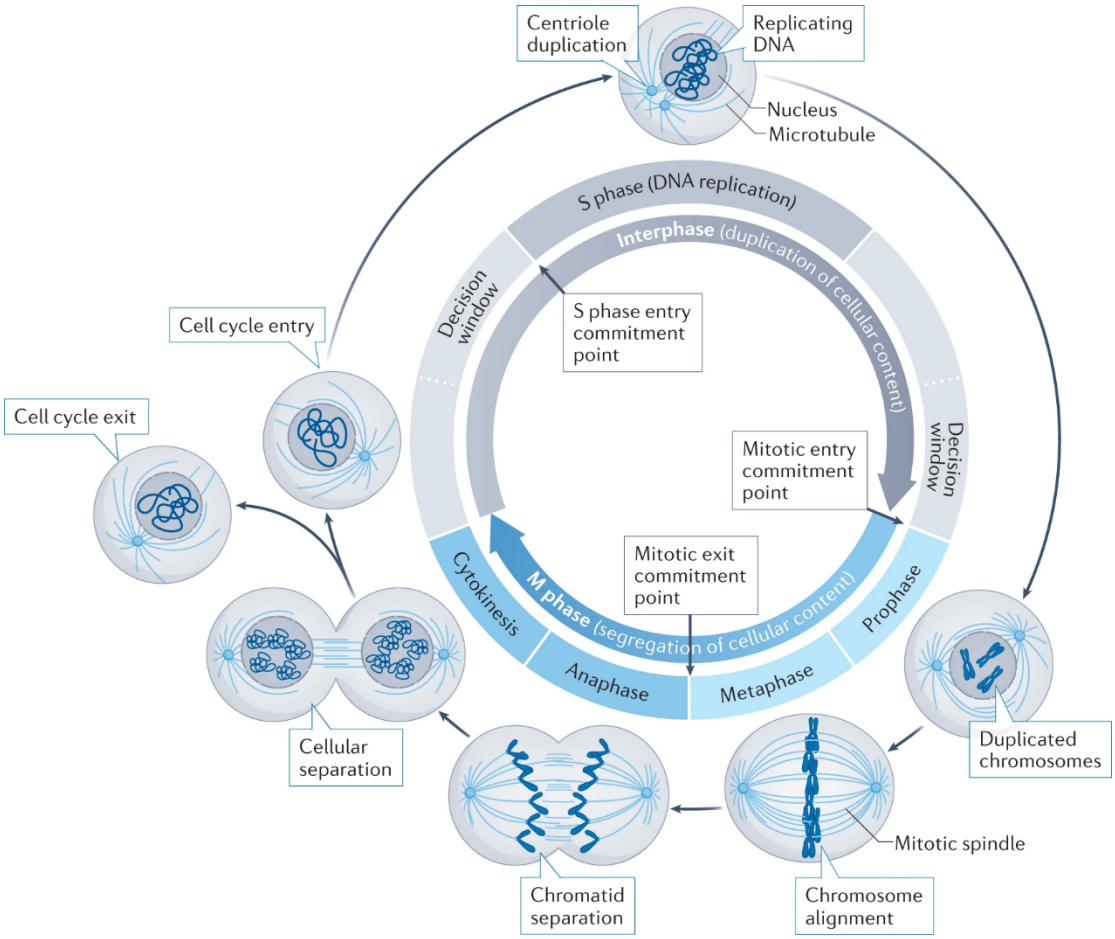


Figure 1-1. Mitotic cell division cycle
When cell entry into the mitotic cell division cycle, it goes through gap1 (G1), then

comes into S phase for DNA replication, subsequently undergoes gap2 (G2), finally entries M phase for segregation of cellular content to finish this cell division cycle. Figure adapted from Helen K. Matthews, Cosetta Bertoli & Robertus A. M. de Bruin, 2021

1.3 Spindle assembly checkpoint

The spindle assembly checkpoint (SAC) system is a critical regulatory mechanism that governs the progression from metaphase to anaphase during mitosis or meiosis. It serves as a safeguard, ensuring that the separation of duplicated chromosomes (anaphase) does not occur until each chromosome is properly attached to the spindle. This process is crucial in maintaining genomic stability, as improper attachment of chromosomes to the spindle can result in aneuploidy, characterized by an abnormal number of chromosomes in a cell, which finally causes the cancer or cell death (Holland & Cleveland, 2009).

The critical events in the process of mitosis involve the orchestrated separation of chromosomes during anaphase, followed by cytokinesis, which culminates in the formation of two daughter cells from a single mother cell, achieved through the orchestrated involvement of actin and microtubules in cytoplasmic division (**Figure 1-1**). These intricately regulated events are meticulously governed by the anaphase-promoting complex/cyclosome (APC/C), aptly named for its role in promoting anaphase progression. APC/C serves as an E3 ubiquitin ligase with the task of targeting inhibitors of anaphase and cytokinesis, such as cyclin B1 and securin, for degradation. It is of utmost significance to prevent the activation of APC/C until all chromosomes are firmly attached to spindle microtubules, ensuring accurate segregation. To maintain this control, the Spindle Assembly Checkpoint (SAC) is activated by default at the commencement of each mitotic phase within the cell division cycle. Its primary function is to restrain the activity of APC/C targeting its anaphase substrates. Shortly after the key mitotic cell cycle regulatory complex, CDK1–cyclin B1, triggers the breakdown of the nuclear envelope and activates SAC, the checkpoint begins its vigilant surveillance of kinetochore–microtubule interactions. The outermost region of the kinetochore, facing the cytoplasm, is composed of protein complexes KNL1, MIS12, and NDC80, collectively known as the KMN network, which plays a pivotal role in binding to microtubules. This microtubule-binding capacity is essential for kinetochores as it enables them to withstand the forces involved in chromosome separation during anaphase. SAC signaling proteins associate with KMN network complexes in the absence of microtubule interactions, thereby regulating the production of anaphase inhibitor molecules. Each kinetochore can be viewed as an autonomous SAC signaling platform that harmoniously integrates the mechanisms of microtubule attachment with those governing the production of anaphase inhibitors. On a cellular level, the SAC only reaches satisfaction when both kinetochores of sister chromatid

pairs have firmly established stable, end-on interactions with microtubules connected to opposite spindle poles. This configuration, termed amphitelic attachments, results in chromosome bi-orientation. Before achieving bi-orientation, chromosomes may undergo various intermediate and occasionally erroneous microtubule attachments, including interactions with the microtubule lattice (referred to as lateral attachments) during initial capture attempts or interactions where both kinetochores engage with microtubules emanating from the same spindle pole (known as syntelic attachments). Error correction mechanisms are in place to rectify these erroneous attachment states, returning them to an unattached kinetochore status. This restorative process involves a combination of Aurora A and Aurora B-dependent phosphorylation of Hec1 within the Ndc80 complex, effectively reducing its affinity for interactions with microtubules (Iemura et al., 2021). Subsequently, SAC proteins assemble at these unattached kinetochores, ensuring that the onset of anaphase is postponed until all attachment corrections are completed (McAinsh & Kops, 2023).

At the heart of the spindle checkpoint lies a pivotal factor known as Mps1, a kinase enzyme. Mps1 assumes a pivotal role in the intricate checkpoint process by virtue of its recruitment to unattached kinetochores, notably the Hec1 and Nuf2 in Ndc80 complex, during the course of mitosis. Recruited Mps1 leads to the phosphorylation of the Met–Glu–Leu–Thr (MELT) motif in the N-terminal region of KNL1, facilitating the assembly of the Mitotic Checkpoint Complex (MMC). The MMC, in turn, functions to suppress the activity of the Anaphase-Promoting Complex/Cyclosome (APC/C). And the Reversine (Mps1 selective ATP-competitive inhibitor) caused compromised error correction and the spindle checkpoint (Santaguida et al., 2010). Consequently, Mps1 is believed to activate the spindle checkpoint, initiating the signaling cascade that ultimately ensures proper chromosome alignment and attachment before anaphase can proceed. In vitro, MPS1 exhibits a preference for binding when the NDC80 complex is unassociated with microtubules. This observation has presented a compelling model for understanding the mechanisms involved in silencing the Spindle Assembly Checkpoint (SAC) at kinetochores following microtubule attachment (Ji et al., 2015).

2. Chromosome

2.1 Overview

Chromosomes (mitotic form of chromatin) carries most of the genetic information of a cell. In Hierarchical chromatin-folding model (**Figure 1-2**), the basic repeating structural and functional unit of chromatin is the nucleosome (diameter: 11nm). A nucleosome consists of eight histone octamer (two H2A, two H2B, two H3, and two H4 proteins) which together bind and wrap around 146 base pairs of DNA. The addition

of an H1 protein further wraps an additional 20 base pairs, forming a chromatosome with two complete turns around the octamer (Luger et al., 1997; van Holde, 1989). The appearance of chromatin as beads on a string under electron microscopy provided early evidence for the existence of nucleosomes (Olins & Olins, 1974; Woodcock et al., 1976). The chromatin is further coiled into an even shorter, thicker fiber, approximately 30 nanometers in diameter, known as the "30-nanometer fiber." During eukaryotic cell division, genomic DNA must be equally partitioned into both daughter cells. To achieve this, the chromatin becomes most compacted form in metaphase (chromosome), which are visible under a light microscope. Once cell division is complete, the chromosomes uncoil again, returning to less compacted interphase form (chromatin).

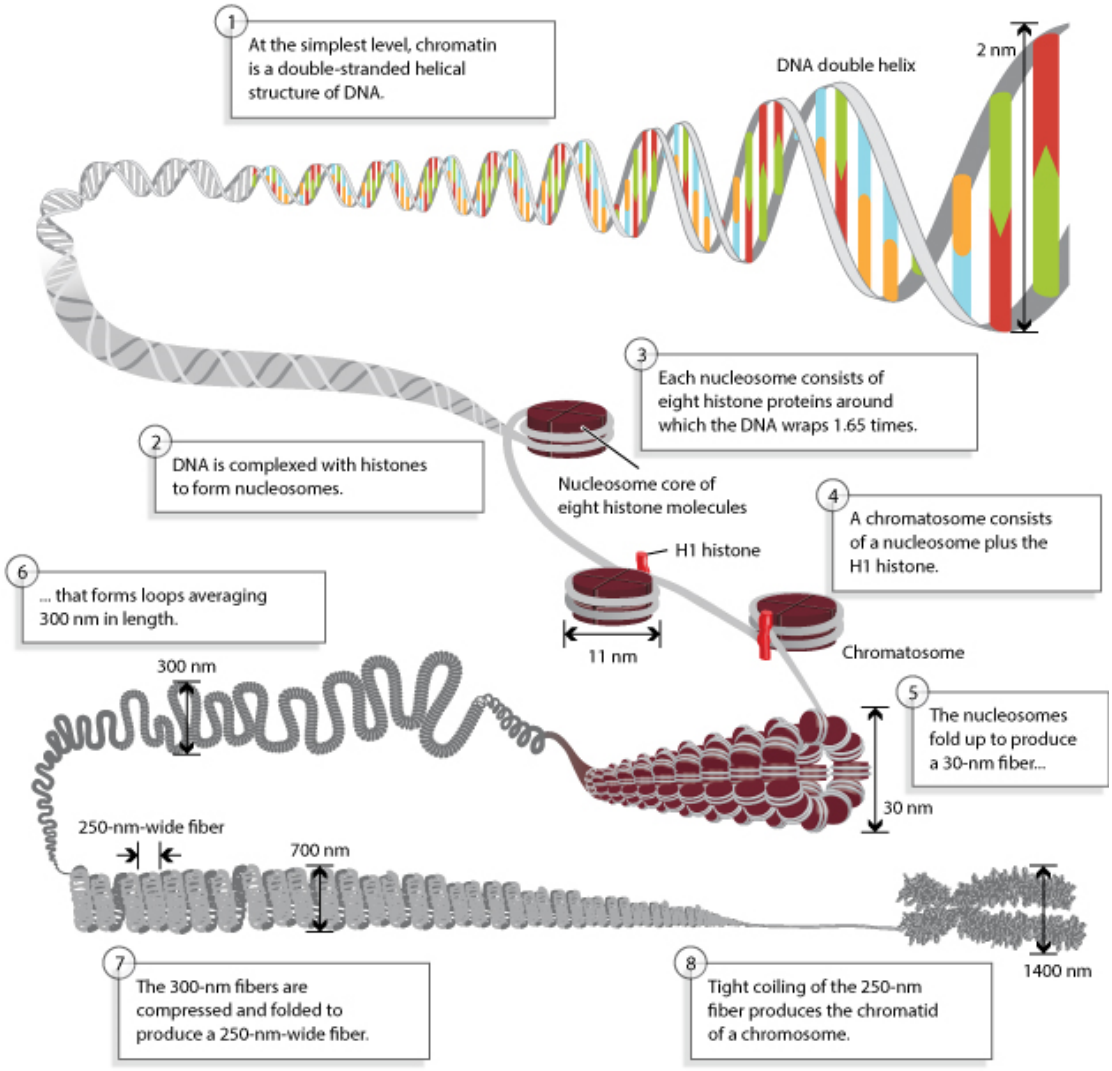


Figure 1-2. The classical hierarchical chromatin-folding model
At simplest level, chromatin is a double-stranded helical structure of DNA. Then the DNA is complexed with histones to form nucleosomes which consists of eight histone octamers around with 1.65 times DNA wrapping. Next, with the addition of H1, the nucleosomes fold up to produce a 30 nm fiber following an averaging 300 nm loops

forms. The 300-nm fibers are then compressed and folded to produce a 250-nm-wide fiber. The tight coiling of 250-nm fiber produces the most compacted form of chromatin in mitosis-chromosome.
Figure adapted from Pierce, Benjamin, 2013

The hierarchical chromatin-folding model mentioned above, originally formulated based on observations in vitro reconstituted samples, has recently undergone significant refinement. This refinement has been made possible through the development of a labeling method designed to enhance DNA contrast in electron tomography (Ou et al., 2017). Consequently, these advancements have led to the discovery that human chromatin in its natural, in situ state exists as a disordered fiber with a diameter ranging from 5 to 24 nm (**Figure 1-3**). This fiber exhibits varying concentration densities within both interphase nuclei and mitotic chromosomes. In the context of interphase nuclei, it is noteworthy that each decondensed chromosome occupies a distinct nuclear space, which is referred to as chromosomal territories (Cremer & Cremer, 2010). Furthermore, the interphase chromatin can be subdivided into two distinct compartments: the A compartment, primarily characterized by gene-rich domains concentrated at the central region of the nucleus, and the B compartment, which encompasses gene-poor domains occupying the periphery of the nuclear region (Boyle et al., 2001). Significant progress has been made in elucidating the chromatin's structural organization at higher resolutions, thanks to the advent of higher resolution Hi-C mapping techniques (Dixon et al., 2012). These advanced methods have revealed the presence of topologically associated domains (TADs). TADs are discrete spatial domains characterized by high-frequency self-interactions occurring within each unit at a resolution of 40 kbp. Importantly, TADs are separated by distinct boundary regions where chromatin contacts abruptly terminate. In mammalian cells, these boundary regions are typically marked by the presence of the chromatin insulator CTCF and cohesion proteins. Despite these recent advancements, the precise relationship between chromatin compartments and TADs remains a subject of ongoing investigation, warranting further exploration to comprehensively understand the intricacies of chromatin organization within the cell nucleus (**Figure 1-4**) (Misteli, 2020).

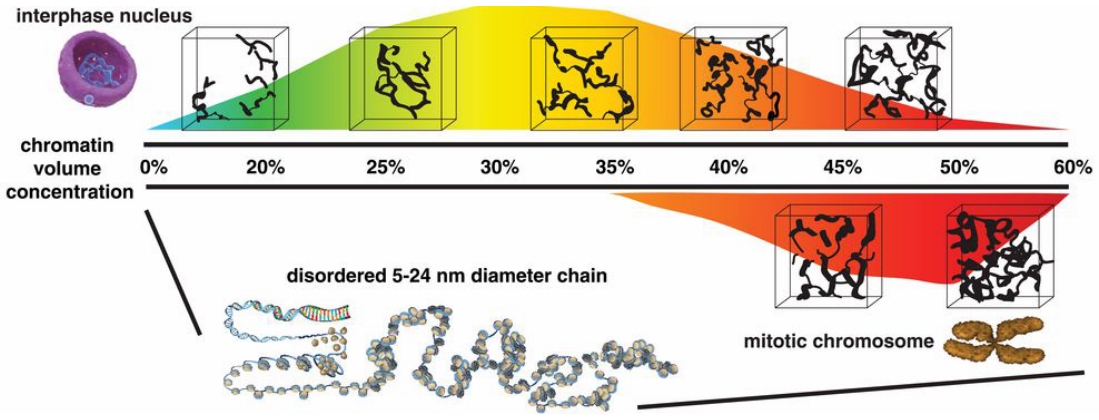


Figure 1-3 Higher-disorder 3D chromatin packing

Chromatin can be characterized as a flexible, disordered chain with a diameter ranging from 5 to 24 nm. It is intricately arranged within the three-dimensional space of interphase nuclei and mitotic chromosomes, exhibiting varying concentration density distributions.
Figure adapted from Ou et al., 2017

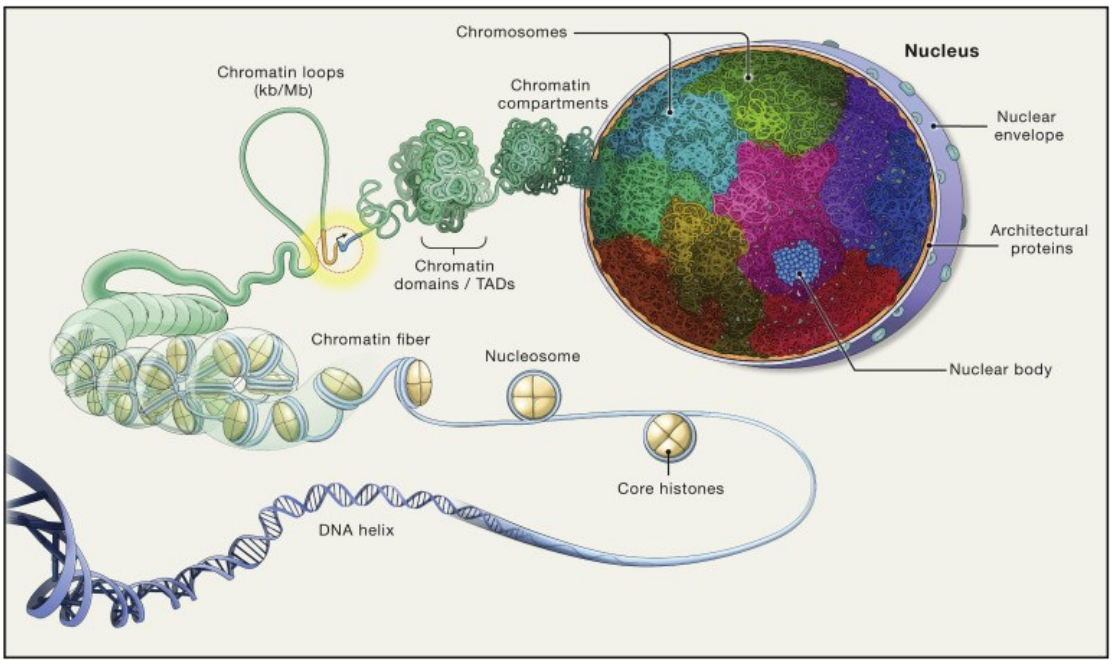


Figure 1-4 The recent model of organization of the eukaryotic genome
Genomes exhibit a hierarchical organization spanning multiple levels. At the fundamental level, DNA becomes intricately entwined around nucleosomes, each comprising an octamer of core histones. These nucleosomes collectively give rise to the chromatin fiber, which, in turn, undergoes folding into compact loops. Beyond this, the chromatin fiber further coalesces into larger chromatin domains known as Topologically Associating Domains (TADs), establishing a sophisticated structural hierarchy. These TADs interact with one another to form chromatin compartments, contributing to the three-dimensional configuration of the genome. Consequently, the DNA of each chromosome occupies a discrete spatial volume referred to as a "chromosome territory" within the confines of the cell nucleus.
Figure adapted from Misteli, 2020

2.2 Chromosome segregation

Chromosome segregation is a vital process in eukaryotes, wherein two sister chromatids, formed through DNA replication, or paired homologous chromosomes, separate and migrate towards opposite poles of the nucleus. This crucial mechanism

takes place during both mitosis and meiosis. The centromere, a specialized chromatin region located within each condensed chromosome, plays a pivotal role in this process. It serves as the foundation for kinetochore assembly and functions as a site for spindle attachment. During mitotic chromosome segregation, the spindle microtubules attach to the chromosome at the centromere region via the kinetochore complex (**Figure 1-5**). Subsequently, the microtubules exert force to pull apart the sister chromatids, ensuring their equal distribution into the resulting daughter cells.

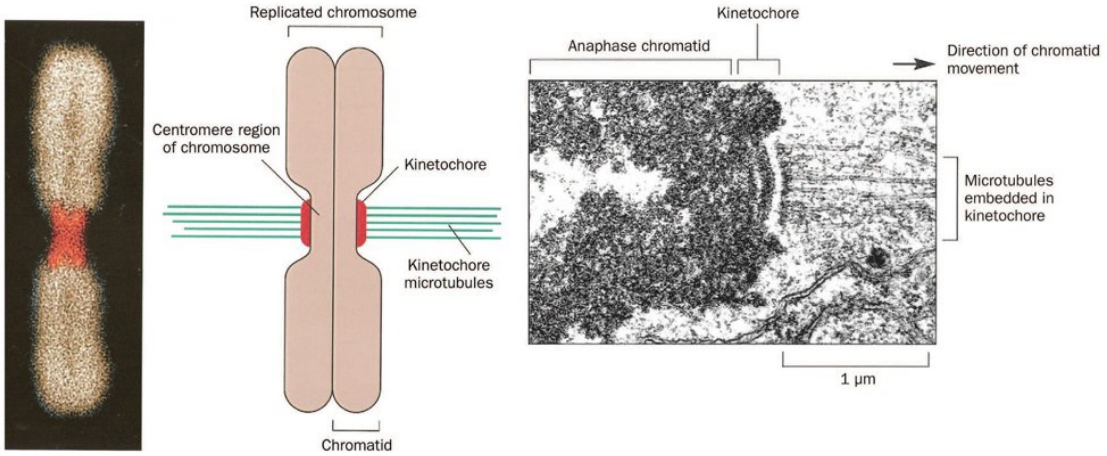


Figure 1-5. Machinery for mitotic chromosome segregation
Schematic representation of the machinery for mitotic chromosome segregation (middle) and their light (left) and electron (right) microscope levels. The kinetochore has a trilaminar structure with outer and inner electron-opaque layers and a middle electron-translucent layer. The inner lamina attaches the centromere region of sister chromatids and the outer lamina binds spindle microtubules during prometaphase.
Figure adapted from Johnson, M. K., and Wise, D. A. 2009.

3. Centromere

3.1 Overview

The centromere is a crucial region within a chromosome that plays a fundamental role in the segregation of chromosomes during mitosis and meiosis in eukaryotic cells. It serves as the site for the assembly of the kinetochore, which is essential for the attachment of microtubules to the chromosomes during mitosis. Additionally, the centromere is involved in the division of the chromosome into a short arm (p) and a long arm (q).

There are two main types of centromeric organization found in chromosomes:

mono-centricity and holo-centricity. Mono-centric chromosomes possess a single centromere, which forms a narrow constriction. This type of centromere is the most common structure and is composed of highly repetitive DNA sequences in plants and animals. Monocentric chromosomes are prevalent across various organisms. In contrast, holo-centric chromosomes possess multiple kinetochores along their length instead of a single centromere. The existence of holo-centric chromosomes was first described in cytogenetic experiments in 1935 (Schrader, n.d.). One distinctive characteristic of holo-centric chromosomes is their ability to attach to spindle microtubules along their entire length (Guerra et al., 2010; Steiner & Henikoff, 2014). They can be found in a wide range of animal and plant species.

Within the category of monocentric chromosomes, two subtypes are recognized: point centromeres and regional centromeres. Point centromeres are characterized by specific DNA sequences that attract mitotic spindle fibers. In these cases, specific proteins bind to these DNA sequences, providing the foundation for the attachment of mitotic spindle fibers. Point centromeres display high efficiency in binding to proteins that recognize particular DNA sequences. Consequently, any DNA fragment containing the point centromere DNA sequence has the potential to form a centromere if present in the appropriate species. On the other hand, regional centromeres represent the majority of centromeres and are typically formed on regions with preferred DNA sequences. However, they can also form on other DNA sequences. Unlike point centromeres, regional centromeres are not defined by a specific DNA sequence.

The faithful inheritance of centromeres is crucial for the proper functioning of monocentric chromosomes during cell division. Failure to maintain the centromere at a single site on each chromosome can have severe consequences. One such scenario involves the loss of the centromere, rendering the chromosome unable to attach to microtubules during mitosis. This leads to the chromosome being excluded from the daughter cells, disrupting the overall genomic stability (McClintock, 1939). Additionally, the presence of more than one distinct locus for microtubule anchoring within a single chromosome can result in chromosome breakage during the segregation process. This aberrant separation may lead to structural abnormalities in the daughter cells, affecting their genetic integrity and causing potential genetic disorders (Koshland et al., 1987). Ensuring the precise inheritance of centromeres is thus essential for the accurate segregation of chromosomes during cell division, preventing detrimental outcomes and maintaining genomic stability throughout successive generations.

3.2 Genetics of centromere

The DNA sequences that form centromeres are vary between species. The centromere of S. cerevisiae is a region of DNA, approximately 150 bp in length (point centromere), containing three important sequence elements. Each of the functional centromere sequences contains a high (91% to 95%) AT region (centromere DNA element II), 78 to 86 bp in length, flanked by two conserved DNA sequences

(centromere DNA elements I and III). Centromere DNA elements I and III are responsible for interacting with CBF1 and CBF3 complex for centromere specification and function. These three Centromere DNA elements (I II III) are sufficient to define the functional centromere of S. cerevisiae.

In the other hand, vertebrate centromeres are usually highly repetitive. Human centromeres are composed of alpha satellite DNA. Alpha satellite is based on a 171 bp monomer arranged in a tandem, head-to-tail fashion. Individual monomers share 50%–70% sequence identity. An integral number of monomers give rise to a higher order repeat (HOR) unit that is itself repeated in a largely uninterrupted fashion so that within a given centromeric locus, the alpha satellite array can span from 250 to 5,000 kb. Humans carry large numbers of CENP-B boxes (a 17 bp motif) in alpha satellite DNA which binds Centromere protein B (CENP-B) specifically. However, this highly repetitive form does not define the vertebrate centromere. One counter example is that in some human cells, people found some chromosome containing a new centromere that form at a place on the chromosome that is usually not centromeric which people called neocentromere. And this neocentromere don't have highly repetitive DNA sequence (Marshall, Chueh, et al., 2008). Another counter example is that for some vertebrates like chicken, its cells contain two kinds of centromere, repetitive form and non-repetitive form (Piras et al., 2010; Shang et al., 2010). The important thing is that specific DNA sequences are neither strictly necessary nor sufficient for centromere specification.

3.3 Epigenetics of centromere

In vertebrates, centromeres are thought epigenetically defined by the histone H3 variant, centromere protein A (CENP-A), which replaces the canonical histone H3 in centromeric nucleosomes (Fukagawa & Earnshaw, 2014). CENP-A was originally identified as a histone like protein in sperm cells, which shares homology with histone H3 (Palmer et al., 1991; Sullivan et al., 1994). Much evidences suggests that centromeres are epigenetically defined by the location of nucleosomes containing CENP-A, independently of the DNA sequences where they are located. Active centromeres are often marked by the presence of CENP-A. The CENP-A nucleosome is interspersed with canonical histone-H3 nucleosome in core centromere region which is surrounded by pericentromeric heterochromatin usually marked by H3K9 or H3K27 methylations. It was reported that at core centromere H3K4 di-methylation is accumulated which related to prevent surrounding heterochromatin invading. Distinct from both euchromatin and heterochromatin, centromeric chromatin showcases a unique pattern of histone modifications (Van Hooser et al., 2001). By using chicken non-repetitive centromere, H4K20 mono-methylation was found at core centromere which involved in kinetochore assembly (Hori et al., 2014). H4K5 and H4K12 acetylation was also found at core centromere especially at G1 phase which involved in new CENP-A incorporation (Shang et al., 2016).

Furthermore, the phosphorylation of CENP-A by Aurora B kinase assumes an unexpected role in cytokinesis completion (Zeitlin, Shelby, et al., 2001). Interestingly, the phosphorylation of CENP-A by Aurora A during prophase is imperative for the enrichment of Aurora B at inner centromeres and for the effective functioning of kinetochores (Kunitoku et al., 2003). Additionally, a study has highlighted the requirement of CENP-A K124 ubiquitylation for the deposition of CENP-A at the centromere (Niikura et al., 2015).

3.3.1 CENP-A

The histone H3 variant known as CENP-A replaces canonical H3 histones in a specific subset of nucleosomes within centromeric chromatin, forming what is referred to as CENP-A nucleosomes. These CENP-A nucleosomes play a critical role in kinetochore assembly. Once established at the centromere, the CENP-A nucleosomes are recognized by additional proteins to facilitate the formation of a functional kinetochore. The CENP-A targeting domain (CATD) and the carboxy-terminal tail of CENP-A directly interact with core kinetochore components, CENP-N, and CENP-C, respectively (Ariyoshi et al., 2021; Carroll et al., 2009, 2010; Guse et al., 2011; Kato et al., 2013; Logsdon et al., 2015). The CENP-A protein epigenetically defines the centromere's position on each chromosome, determining the site for kinetochore assembly and sister chromatid cohesion during mitosis. Domain analysis of CENP-A has revealed that CATD is necessary and sufficient for centromeric targeting (Black, Brock, et al., 2007; Black, Jansen, et al., 2007). Studies have shown that centromeric localization is lost when CENP-A lacks CATD, but interestingly, a mutant form of H3, in which the loop-1 and α2-helix regions are replaced with CENP-A CATD, can confer centromeric localization in human cells. CENP-A nucleosomes also possess unique structural properties, particularly due to the characteristics of CATD. Comparisons of hydrogen-deuterium exchange have demonstrated that the free (CENP-A-H4) tetramer is more conformationally rigid than the (H3-H4) tetramer, resulting in a more compact structure.

3.3.1.1 CENP-A study history

The journey of understanding the role and characteristics of CENP-A began with its initial identification, alongside CENP-B and CENP-C, through sera from patients exhibiting scleroderma CREST symptoms (W. Earnshaw et al., 1986; W. C. Earnshaw & Rothfield, 1985). Subsequently, CENP-A was found to be copurified with nucleosome core particles, suggesting its association with histones (Palmer et al., 1987). This histone-like nature was further supported by the similarity of certain CENP-A

sequences to histone H3 regions, while other segments were distinct, possibly contributing to centromeric localization or specific functions (Palmer et al., 1991). Investigations into CENP-A revealed that its histone fold domain, akin to histone H3, was vital for centromeric targeting (Shelby et al., 1997; Sullivan et al., 1994). The chromatin containing CENP-A and α-satellite DNA was identified as a major component of the inner kinetochore plate (Vafa & Sullivan, 1997). Notably, at the same time, the formation of functional neo-centromeres composed of non-alpha-satellite DNA was observed, which indicated repeat form of centromeric DNA is not required for functional centromere (Sart et al., 1997). Further, in vitro studies indicated that human CENP-A could substitute histone H3 in nucleosome reconstitution, supporting its distinct nucleosome formation ability, which strongly support CENP-A with a nucleosome form to execute centromeric function (Yoda et al., 2000). Subsequent investigations highlighted the uncoupling of centromeric chromatin replication from other genome regions (Shelby et al., 2000) and revealed the requirement for specific steps in kinetochore assembly, including precise CENP-A targeting and microtubule-associated protein targeting (Van Hooser et al., 2001). At the same time, differential phosphorylation patterns of CENP-A and histone H3 in G2/M supported the CENP-A's unique role in centromeres (Zeitlin, Barber, et al., 2001). And neocentromeres with altered replication timing were also identified (Lo, Craig, et al., 2001; Lo, Magliano, et al., 2001). Later, histone acetylation and replication timing were shown to be insufficient for specifying CENP-A deposition sites (Ouspenski et al., 2003), and functional complementation studies between yeast and human CENP-A were conducted, which showed the functional similarity of CENP-A within evolution. (Wieland et al., 2004). Then, proteolysis was found to contribute to restricted CENP-A localization in yeast and Drosophila (Collins et al., 2004; Moreno-Moreno et al., 2006). In the same time, it was shown that CENP-A incorporation levels correlated with chromosome size and origin (Irvine et al., 2004). Further investigations revealed the role of CENP-A in forming functional ectopic kinetochores in Drosophila (Heun et al., 2006) and highlighted its incorporation during early anaphase in Drosophila embryo (Schuh et al., 2007). And, for CENP-A itself, the CENP-A Targeting Domain was recognized as a determinant of centromere identity (Black, Jansen, et al., 2007), while the necessity of heterochromatin and RNAi for CENP-A chromatin establishment was also found (Folco et al., 2008). A three-dimensional localization studies revealed the arrangement of centromeric CENP-A containing chromatin in a coiled 30-nm fiber, contributing to higher-order structures (Marshall, Marshall, et al., 2008). CENP-A's stable incorporation through a "loading-only" mechanism in G1 phase have been found in the same year (Hemmerich et al., 2008), and the direct recognition of CENP-A nucleosomes by CENP-N was identified as a contributor to centromere assembly (Carroll et al., 2009). In the same period, the CENP-A specific chaperone HJURP was discovered to mediate CENP-A incorporation (Dunleavy et al., 2009; Foltz et al., 2009), and its counterpart Scm3 (HJURP) in fission yeast was shown to be crucial for stable CENP-A assembly (Pidoux et al., 2009; Williams et al., 2009). Structural studies unraveled the features marking centromeres through the elucidation of $(CENP\text{-}A–H4)_2$ structure (Sekulic et al., 2010), and the importance of CENP-A nucleosomes recognized

by CENP-N and CENP-C for centromere assembly was underscored (Carroll et al., 2010). The interaction between HJURP and CENP-A was established, emphasizing the former's role in deposition (Shuaib et al., 2010), while Cdc42 and Rac GTPases were shown to stabilize newly incorporated CENP-A for epigenetic centromere maintenance (Lagana et al., 2010). An E3 ubiquitin ligase was identified to prevent ectopic CENP-A localization through its Centromere Targeting Domain (Ranjitkar et al., 2010). Later, the regulatory role of Diaphanous formin mDia2 in CENP-A levels and centromere movement was uncovered (C. Liu et al., 2018; C. Liu & Mao, 2016). Additionally, SENP6-mediated M18BP1 deSUMOylation was shown to regulate CENP-A centromeric localization (Fu et al., 2019).

3.3.1.2 CENP-A inheritance

As a critical epigenetic marker of the centromere, CENP-A plays a key role in centromere inheritance which is also commonly referred to as CENP-A inheritance. Once incorporated into the centromeres, CENP-A's position remains stable. During the S phase, when DNA replication occurs, CENP-A is evenly distributed to sister chromatids, forming nucleosomes similar to other canonical histones (Bodor et al., 2013; Falk et al., 2015; Jansen et al., 2007).

To maintain the precise centromere position for the next cell division, new CENP-A must be incorporated into the existing centromere region to form novel CENP-A nucleosomes after each cell division. However, unlike canonical histones, the deposition of new CENP-A is not coupled with DNA replication. In human cells, the exact timing for new CENP-A incorporation into the centromere is early G1 phase (Bodor et al., 2013; Jansen et al., 2007), meaning that the levels of CENP-A in chromatin are half-maximal from the S phase until the next G1 phase.

Domain analysis of CENP-A has revealed that the CENP-A Targeting Domain (CATD) is necessary and sufficient for centromeric targeting (Black, Brock, et al., 2007; Black, Jansen, et al., 2007). Mutants of H3, in which loop-1 and α2-helix regions were replaced with CENP-A CATD, could confer centromeric localization in human cells, further supporting the role of CATD in centromere targeting (Black, Brock, et al., 2007; Black, Jansen, et al., 2007).

Additionally, a CENP-A specific chaperone, known as Holiday Junction Recognition Protein (HJURP), has been identified (Dunleavy et al., 2009; Foltz et al., 2009). HJURP forms a complex with CENP-A/H4, but not with H3/H4, through its N-terminal CENP-A Binding Domain (CBD) (Barnhart et al., 2011; Hu et al., 2011; Shuaib et al., 2010; Zhou et al., 2011). HJURP is essential for the CENP-A incorporation process and can direct CENP-A incorporation at ectopic loci (Barnhart et al., 2011; Hori et al., 2013; Perpelescu et al., 2015; Shono et al., 2015).

Subsequently, the Mis18 complex, known as the centromere licensing factor, recognizes the CENP-A/H4/HJURP complex(Hori et al., 2020; Hu et al., 2011) (**Figure**

1-6). The Mis18 complex comprises Mis18α, Mis18β, and Mis18BP1 (Knl2) (Fujita et al., 2007; Maddox et al., 2007). Mis18α and Mis18β directly interact with HJURP in the CENP-A/H4/HJURP complex (Nardi et al., 2016; Pan et al., 2017, 2019), while Mis18BP1 (Knl2) localizes to the centromere by recognizing existing CENP-A nucleosomes. In chicken cells, Mis18BP1 (Knl2) localizes to the centromere through direct interaction with existing CENP-A nucleosomes (Hori et al., 2017; Jiang et al., 2023) (**Figure 1-7**). In human cells, Mis18BP1 (Knl2) requires CENP-C as a bridge to connect with existing CENP-A nucleosomes (Dambacher et al., 2012; Nardi et al., 2016).

These findings indicate that new CENP-A incorporation occurs at loci where old CENP-A nucleosomes exist, with the assistance of the CENP-A specific chaperone (HJURP) and the CENP-A centromere licensing factor (Mis18 complex) (**Figure 1-8**).

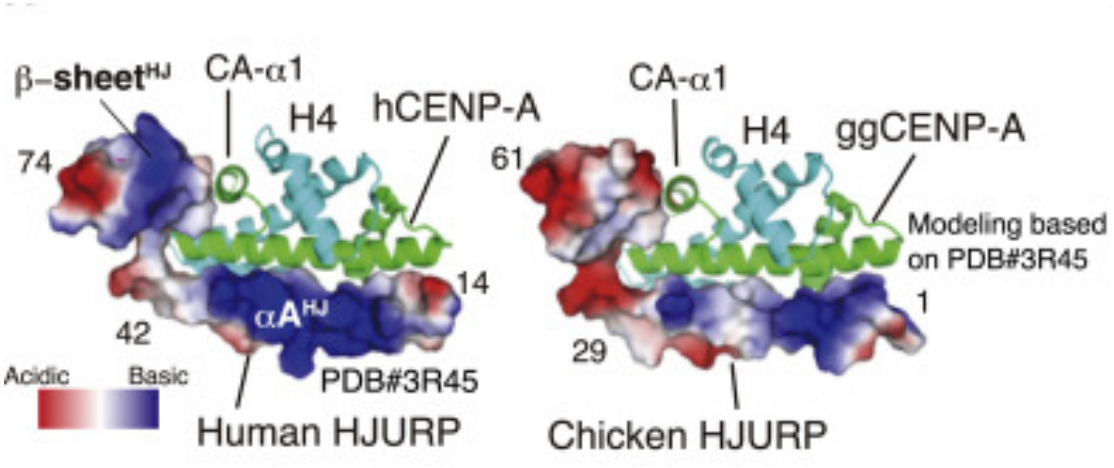


Figure 1-6 Structure of HJURP in the complex with CENP-A/H4
Structure of HJURP-CENP-A-H4 complexes in human (PBD: 3R45) and chicken (Modelling based on PBD: 3R45). HJURP is shown in charged mode. CENP-A is depicted with ribbon presentations colored in green and cyan respectively.
Figure adapted from Hori et al., 2020

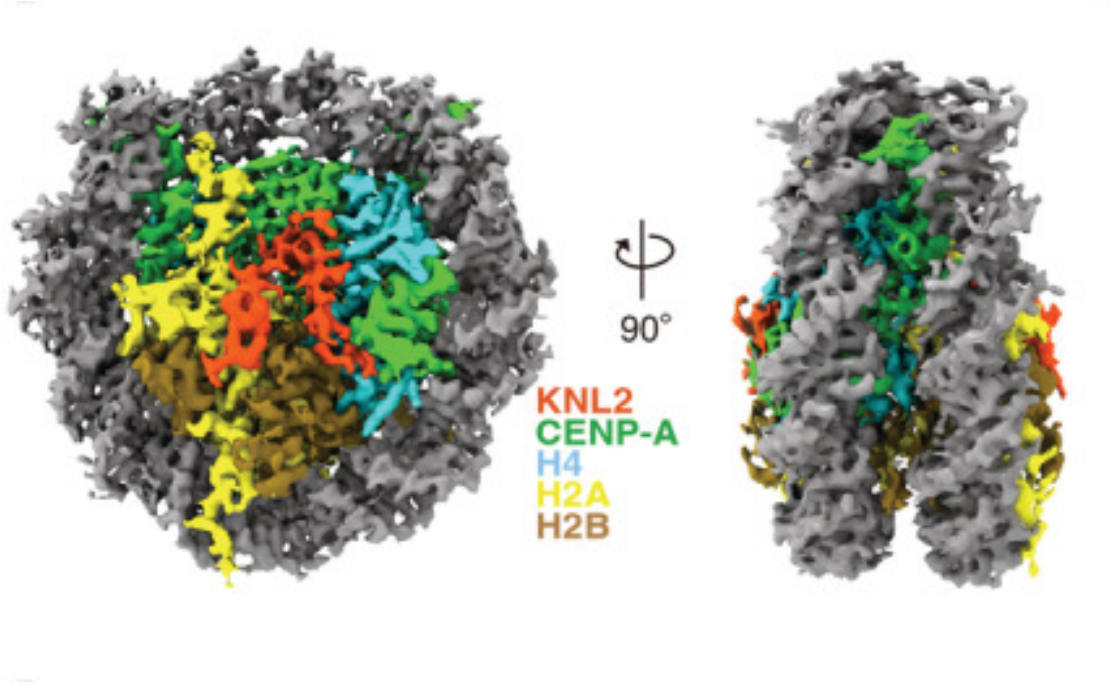


Figure 1-7 Structure of Knl2 in complex with CENP-A nucleosome
Cryo-EM density map of the chicken CENP-A nucleosome in complex with the Knl2(517-560) peptide at 3.42 Å resolution. The side views of the complex along the two-fold axis are shown. The density corresponding to each molecule in the complex is color coded as indicated in the figure.
Figure adapted from Jiang et al., 2023

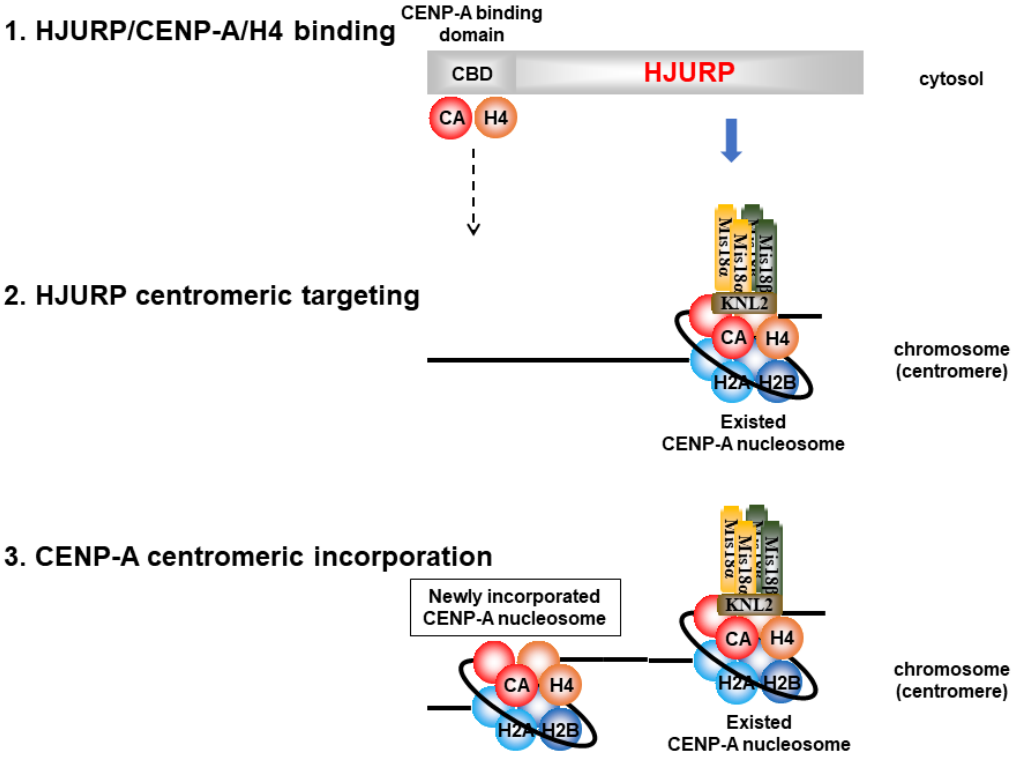


Figure 1-8. CENP-A incorporation in Chicken
Schematic representation of CENP-A incorporation in chicken DT40 cells. The process begins with the CENP-A specific chaperone utilizing its CENP-A binding domain to capture the CENP-A/H4 tetramer. Subsequently, HJURP recognizes the Mis18α/β subunit in the Mis18 complex through its C-terminal region. The Mis18 complex recognize centromere by directly interacts with existing CENP-A nucleosomes through its main component, Knl2. Thus, with the assistance of HJURP, new CENP-A incorporation occurs in close proximity to the existing CENP-A nucleosomes.

3.4 Neocentromere

Neocentromeres, a fascinating phenomenon in chromosomal biology, represent new centromeres that form at non-centromeric regions of the chromosome. These structures typically arise as a consequence of disruption or loss of the native centromere. Unlike typical centromeres, neocentromeres often lack the characteristic highly repeated DNA sequences. Despite their unconventional nature, cells with neocentromeres are capable of dividing normally during both mitosis and meiosis (Marshall, Chueh, et al., 2008). In the context of chicken cells, a noteworthy observation has been made where artificially removing the native centromere of

chromosome Z triggers the formation of neocentromeres on the same chromosome. Notably, this process occurs with a certain proportion (approximately 1 in 50,000 cells). Furthermore, a significant number of these newly formed centromeres tend to localize near the region where the native centromere was removed (Shang et al., 2013).

4. Kinetochore

4.1 Overview

The kinetochore is a protein structure that forms in the centromere, of a duplicated chromosome (Jokelainen, 1967; Luykx, 1965). It consists of an inner region and an outer region. The inner region is bound to chromosomal DNA, while the outer region connects to spindle fibers. The kinetochore mediates chromosome segregation at cell division by linking chromosomes to spindle microtubules. It is made of more than 100 protein species in mammalian cells. Molecular tools are presently revealing the biochemical interactions and regulatory mechanisms that ensure proper kinetochore function. The inner kinetochore, the constitutive centromere associated network (CCAN), comprises a group of 16 proteins that play a crucial role in the centromere-kinetochore interface. Throughout the cell cycle, these proteins localize to the centromere. In vertebrates, these proteins are designated with alphabetical CENP names, including CENP-C, CENP-H, CENP-I, CENP-K, CENP-L, CENP-M, CENP-N, CENP-O, CENP-P, CENP-Q, CENP-U, CENP-R, CENP-T, CENP-W, CENP-S, and CENP-X. Within the CCAN, these proteins can be categorized into five groups: CENP-C, the CENP-L-N complex, the CENP-H-I-K-M complex, the CENP-O-P-Q-U-R complex, and the CENP-T-W-S-X complex (Amano et al., 2009; W. C. Earnshaw & Rothfield, 1985; Foltz et al., 2006; Izuta et al., 2006; Minoshima et al., 2005; Nishihashi et al., 2002; Okada et al., 2006; Saitoh et al., 1992; Sugata et al., 1999). Each of these subcomplexes forms numerous direct physical interactions, creating an extensive meshwork. This network is dynamic, as different subcomplexes rely on specific interactions during different stages of the cell cycle. Among the CCAN proteins, only CENP-C and CENP-N have been reported to directly bind to nucleosomes. They achieve this by recognizing the structural distinctions between CENP-A and H3. Additionally, several CCAN proteins, including CENP-C, CENP-Q, and the CENP-T-W-S-X complex, bind directly to DNA. Once assembled at the centromere, the CCAN serves as a platform for the assembly of the outer kinetochore. Notably, CENP-C and CENP-T contribute parallel but non-redundant pathways for recruiting the essential microtubule binding proteins of the kinetochore, forming the KNL1-MIS12-NDC80 (KMN) network. Remarkably, targeting fragments of CENP-C or CENP-T to an ectopic chromosomal locus is sufficient to recruit the KMN network and generate a kinetochore-like structure capable

of directing chromosome segregation.

4.2 Constitutive centromere associated network (CCAN)

The constitutive centromere associated network (CCAN) is an integral subcomplex within the kinetochore, specifically referred to as the inner kinetochore complex. It remains localized at the centromere throughout the entire cell cycle. The CCAN is comprised of multiple proteins, namely CENP-C, CENP-H, CENP-I, CENP-K, CENP-M, CENP-L, CENP-N, CENP-T, CENP-W, CENP-S, CENP-X, CENP-O, CENP-P, CENP-Q, CENP-U, and CENP-R (**Figure 1-9**). The primary function of the CCAN is to establish and maintain an association with centromere chromatin, playing a critical role in centromere function and chromosome segregation processes.

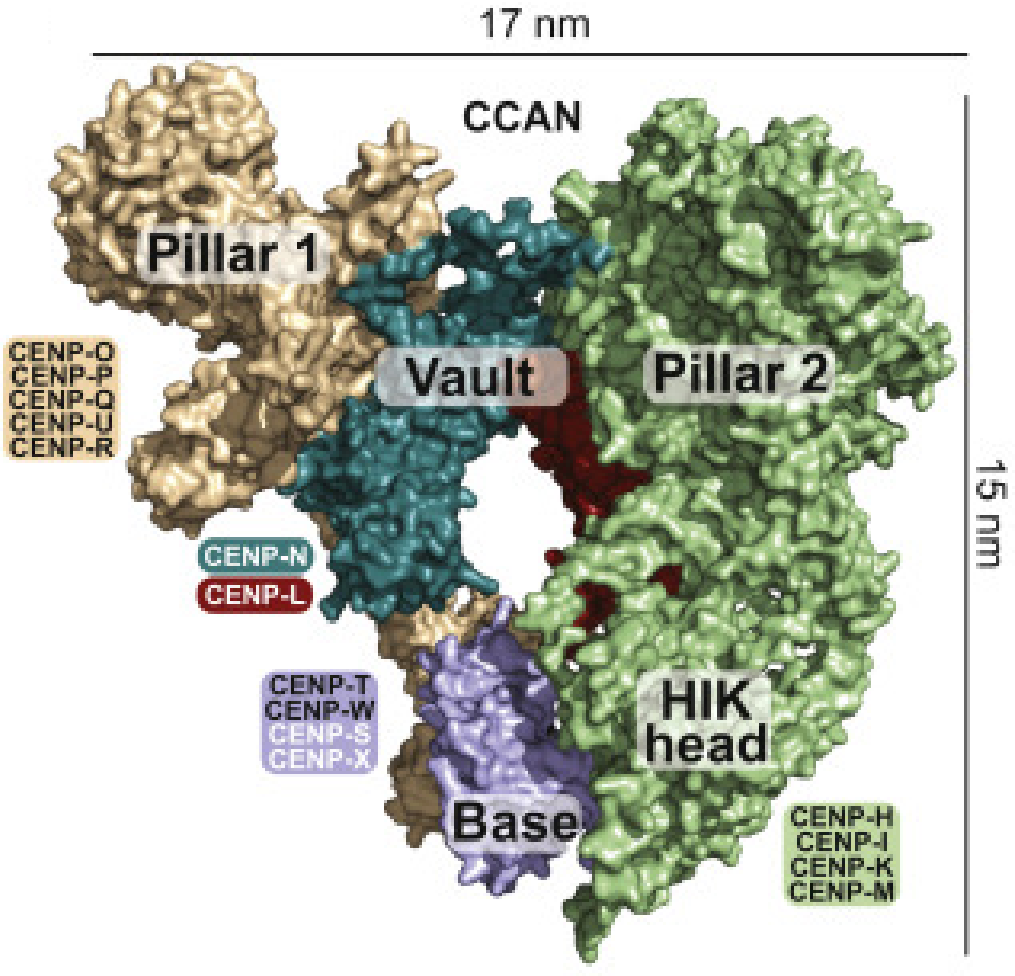


Figure 1-9 Surface model of CCAN
Surface model of CENP-16 complex colored to identify distinct sub-modules which contain CENP-L/N, CENP-H/I/K/M, CENP-T/W/S/X, CENP-O/P/Q/U/R.

Figure adapted from Pesenti et al., 2022

4.3 Structure of CCAN in complex with CENP-A nucleosome

Recently, a significant advance has been made in understanding the structure of the CCAN (constitutive centromere-associated network) in complex with the CENP-A nucleosome. This breakthrough provides valuable insights into the organization and function of key components involved in centromere assembly. In this structure, the CCAN, a critical part of centromere architecture, is composed of several interdependent CENP (centromere protein) modules and the CENP-A nucleosome, on the other hand, wrap with a 171 bp α-satellite sequence. It has been observed that the CCAN tightly interacts with the linker α-satellite DNA of the CENP-A nucleosome. The positively charged CCAN channel, formed by CENP-L/N, CENP-H/I/K/M, and CENP-T/W/S/X, acts as a grip for the linker α-satellite DNA, and the stability of CCAN association with the CENP-A nucleosome is significantly influenced by the α-satellite linker DNA. The CENP-C interacts with the CENP-L/N and CENP-H/I/K/M modules within the CCAN. Additionally, CENP-C directly binds to the CENP-A within the CENP-A nucleosome, and this interaction imparts specificity to the assembled CCAN for CENP-A mono-nucleosomes (Yatskevich et al., 2022) (**Figure 1-10**).

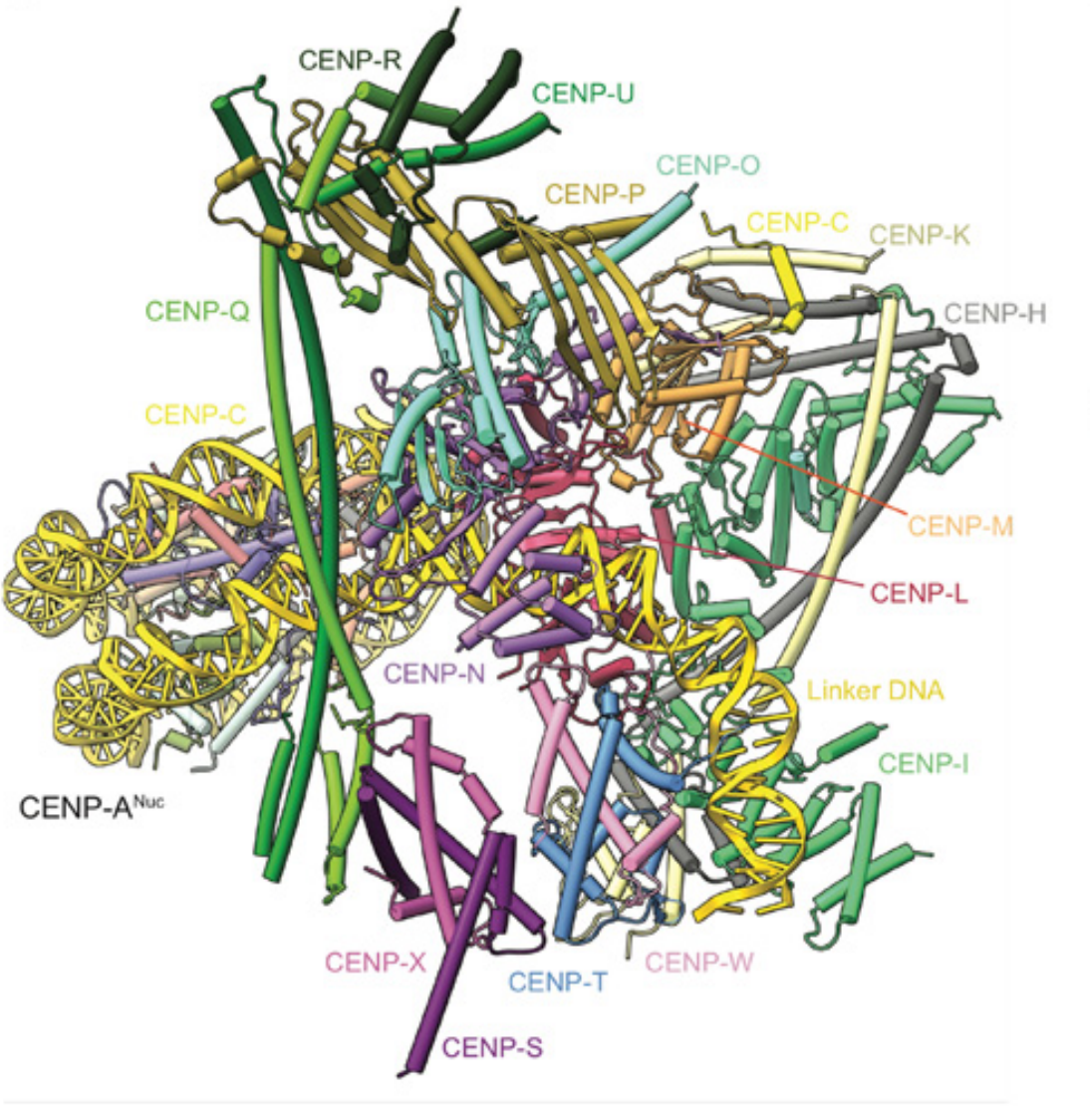


Figure 1-10 Atomic model of the CCAN in complex with CENP-A nucleosome
In this structure, the CCAN is assembled from a network of interdependent CENPs modules, and the CENP-A nucleosome is reconstituted with 171 bp α-satellite sequence. The positively charged CCAN channel, which consists of CENP-L/N, CENP-H/I/K/M and CENP-T/W/S/X, grips linker α-satellite DNA of CENP-A nucleosome. And the α-Satellite linker DNA provides a crucial determinant of stable CCAN association with CENP-A nucleosome. CENP-C interact with CENP-L/N and CENP-H/I/K/M modules and directly bind the CENP-A in CENP-A nucleosome confers selectivity of assembled CCAN for CENP-A mono-nucleosomes.
Figure adapted from Yatskevich et al., 2022

4.4 Relationship between CCAN and CENP-A incorporation

Initially, the requirement of CENP-I (Mis6) for CENP-A incorporation was found in yeast (Takahashi et al., 2000). Upon the identification of vertebrate CENP-I (Nishihashi et al., 2002), its requirement alongside CENP-H/K/M in CENP-A incorporation have been shown (Okada et al., 2006). Subsequently, investigations revealed that artificial new centromere formation, accompanied by existing CENP-A, could be induced by ectopically tethering CENP-I and CENP-C in chicken cells (Hori et al., 2013). At a molecular level, the CENP-C C-terminus was initially reported to directly engage the CENP-A specific chaperone HJURP, for CENP-A loading in human cells (Tachiwana et al., 2015). However, it was subsequently discovered that the induction of CENP-A incorporation through tethering CENP-I or CENP-C in human cells relies on the Mis18 complex (Shono et al., 2015). In Xenopus egg extract systems, a direct recruitment of HJURP for CENP-A incorporation by CENP-C was clearly demonstrated, independent of the Mis18 complex-mediated CENP-A incorporation pathway (Flores Servin et al., 2023; French et al., 2017). Consequently, the relationship between the constitutive centromere-associated network (CCAN) and CENP-A incorporation remains a topic of ongoing debate.

5. Genome project

5.1 Overview

The genome, an intricate repository of an organism's genetic material, encompasses both its coding genes and non-coding DNA sequences, collectively serving as the architectural blueprint governing cellular structure, function, and regulation. Universally present across the spectrum of living organisms, ranging from uncomplicated single-celled bacteria to intricate multicellular entities like humans, genomes primarily consist of the genetic molecule, DNA, characterized by its double-stranded helical structure. Genes, integral constituents of genomes, represent specific segments within the DNA, encapsulating the vital instructions necessary for the construction of an organism. These instructions dictate an organism's traits, characteristics, and physiological functions. Notably, genomes exhibit substantial diversity in terms of size and complexity, exhibiting dramatic variations across different species. For instance, humans possess relatively expansive and intricately structured genomes, while certain simpler organisms exhibit significantly more concise genetic blueprints. Crucially, a considerable proportion of the genome is comprised of non-

coding DNA, playing a multifaceted role in gene regulation, providing structural support, and fulfilling various other functions. Genome sequencing, the process of meticulously determining the precise order of DNA nucleotides within a given genome, has experienced marked acceleration and increased affordability, facilitating the sequencing of genomes across a diverse array of organisms. Understanding genomes stands as a foundational tenet of genetics and biology, offering profound implications for numerous practical applications in domains such as medicine, agriculture, and biotechnology.

The Genome Project-read is an international research endeavor that aims to decipher the chemical sequence of genetic material across all organisms, encompassing their entire genome. One of the most notable endeavors within this project is the human genome project-read, which strives to identify the approximately 50,000 to 100,000 genes within the human genome. This initiative also provides researchers with valuable tools for analyzing genetic information comprehensively. Furthermore, the project seeks to generate physical and genetic maps of the human genome. In a remarkable achievement, an accurate and complete human genome sequence was successfully completed in 2003, two years ahead of the original schedule set by the Human Genome Project. Moreover, the project was accomplished at a cost significantly lower than the initial estimated budget. Currently, a new initiative called the genome project-write is underway, which entails the synthesis of entire genomes across various organisms. It is anticipated that the genome project-write will yield significant benefits similar to those of the genome project-read.

5.2 Chromosome synthesis

Synthesized chromosome or artificial chromosomes (ACs) are laboratory-engineered DNA constructs that mimic natural chromosomes in their behavior. They have proven to be valuable tools for studying the structure and function of chromosomes and for introducing and controlling new DNA in cells. ACs can be constructed with properties such as centromeres, telomeres, origins of replication, and specific sequences required for their stable maintenance within the cell as autonomous, self-replicating chromosomes. By employing circular alphoid input DNA, researchers were able to circumvent the necessity for telomeres during the synthesis of artificial chromosomes (Ebersole et al., 2000). The potential of ACs has attracted significant research attention in various fields (Boeke et al., 2016). ACs offer unique advantages such as the ability to overcome challenges associated with traditional gene delivery methods and the capacity to carry larger genes and regulatory elements. These features enable precise and controlled gene expression in targeted cells, making ACs a promising avenue for future research and application.

5.2.1 Centromere seeding

To ensure accurate chromosome segregation during mitosis and maintain chromosome stability, microtubules must attach to the centromere through the kinetochore complex. The centromere is crucial for proper cell division, as it is responsible for the faithful division of sister chromatids into daughter cells. Importantly, the centromere is typically defined epigenetically, with CENP-A serving as a key epigenetic marker. This epigenetic nature makes it challenging to seed the centromere by synthesizing specific DNA sequences. To overcome this difficulty, our group employed an epigenetic approach to successfully seed the centromere for a given chromosome. Specifically, our group targeted the chicken chromosome Z and inserted LoxP sites around the native centromere, while placing a LacO-array at the end of the p arm. Additionally, our group expressed a fusion protein of LacI and a centromere inducer in cells in the presence of IPTG. After IPTG removal, the LacI-fused inducer could be tethered at the LacO-array. Our group anticipated that the centromere epigenetic marker CENP-A would incorporate at the LacO-array. Following activation of Cre-recombinase, the native centromere could be eliminated, allowing us to isolate surviving cells with an artificially seeded new centromere at the LacO-array site (**Figure 1-11**).

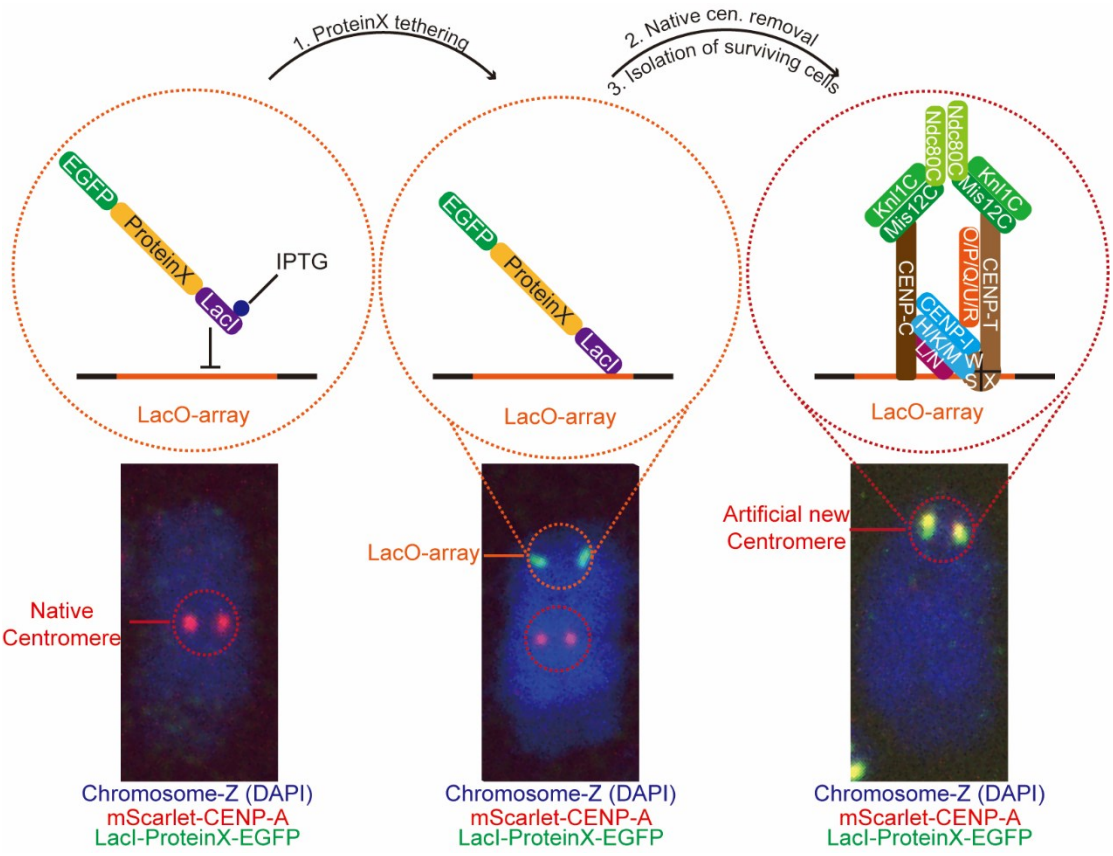


Figure 1-11. Centromere seeding
(A) An experimental design to generate a new centromere at a non-centromere locus (inserted -LacO-array) on the chicken chromosome Z, following the removal of the native centromere. Cells are maintained in the presence of IPTG to inhibit the interaction between LacO and LacI fused proteinX. Upon removal of IPTG, a LacIfused proteinX was allowed to bind to the LacO-array and initiate the incorporation of CENP-A. Consequently, additional kinetochore proteins were assembled on the LacO locus. Finally, the activation of Cre-recombinase was employed to delete the native centromere of chromosome Z, then the artificial kinetochore was confirmed to be functional. Once CENP-A is incorporated, the artificial kinetochore is functional even in the presence of IPTG.

5.2.1.1 Inducer for centromere seeding

Through the utilization of an epigenetic approach, our group embarked on a comprehensive investigation to determine how to seed a centromere at a non-centromere locus for a specific chromosome. Our study involved screening numerous factors, including components of the CENP-A incorporation machinery, inner kinetochore components, and outer kinetochore components. Eventually, our group identified four key factors that successfully induced the seeding of a new centromere (Hori et al., 2013). These factors include HJURP, which is recognized as the CENP-A specific chaperone, Knl2, the primary component of the Mis18 complex responsible for HJURP recruitment, as well as CENP-I and CENP-C, which serve as inner kinetochore components (**Figure 1-12**).

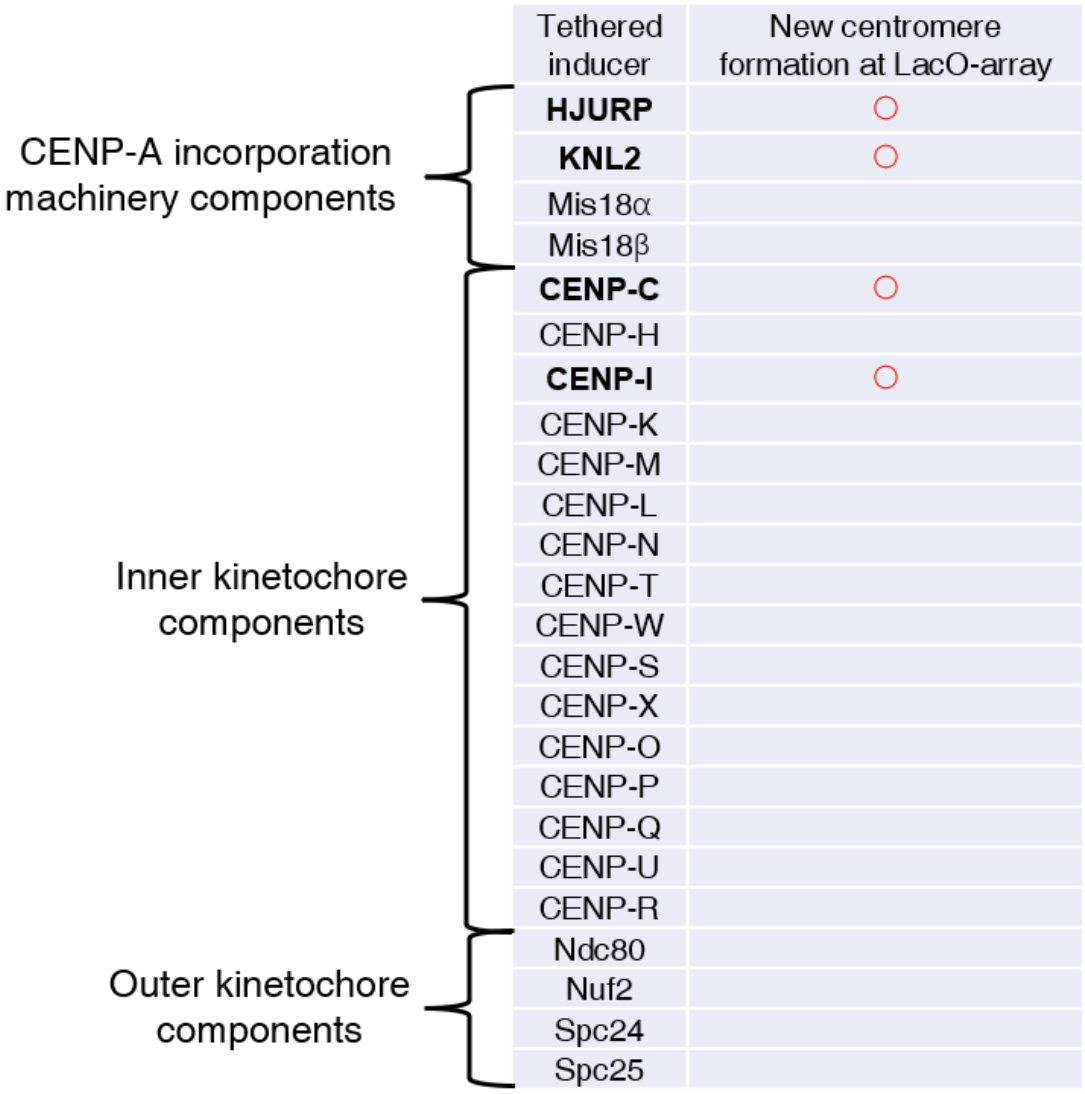


Figure 1-12. Inducers for centromere seeding
CENP-A incorporation machinery components, inner kinetochore components, outer kinetochore components are screened for centromere seeding. Four of them (HJURP, Knl2, CENP-I and CENP-C) are responsible for centromere seeding, as tethering for them causes new centromere formation at non-centromere locus.

5.2.1.1.1 HJURP

As previously mentioned, the discovery of the CENP-A specific chaperone, HJURP, elucidated its crucial role in the direct incorporation of CENP-A into chromatin (Dunleavy et al., 2009; Foltz et al., 2009). In parallel, research in fission yeast revealed that Scm3 (HJURP) was essential for maintaining sub-kinetochore chromatin integrity and facilitating stable CENP-A assembly into centromeric chromatin (Pidoux et al., 2009; Williams et al., 2009). It was established that HJURP forms a binding interaction with CENP-A through a highly conserved N-terminal domain, orchestrating its

deposition at centromeres (Shuaib et al., 2010). Furthermore, it was reported that the inheritance of CENP-A nucleosomes during DNA replication is also contingent upon HJURP activity (Zasadzińska et al., 2018).

Remarkably, tethering only the N-terminal region of HJURP is sufficient to induce robust CENP-A incorporation at a non-centromere locus and recruit a functional kinetochore (Hori et al., 2013).

5.2.1.1.2 Knl2

Initially, the requirement of Mis18 (the homologue of Mis18 complex containing Knl2) for CENP-A loading and histone deacetylation at centromeres in fission yeast was documented (Hayashi et al., 2004). Subsequently, the essential role of Knl2, a Myb domain-containing protein, in the assembly of CENP-A chromatin in C. elegans was identified (Maddox et al., 2007). At the same time, the recognition of the Mis18 complex in human cells, which accumulates specifically at the telophase-G1 centromere and plays a pivotal role in the subsequent recruitment of newly synthesized CENP-A (Fujita et al., 2007). In humans, Knl2 localizes to the centromere during the G1 phase, which corresponds to the timing of CENP-A incorporation (Fujita et al., 2007; Maddox et al., 2007). In contrast, chicken Knl2 localizes to the centromere throughout the cell cycle by directly interacting with existing CENP-A nucleosomes (Hori et al., 2017; Jiang et al., 2023). The Mis18α/β subunits of the Mis18 complex then recruit HJURP through the N-terminal region of HJURP for CENP-A loading (Nardi et al., 2016; Pan et al., 2017, 2019).

Importantly, tethering Knl2 alone is sufficient to induce CENP-A incorporation at a non-centromere locus and recruit a functional kinetochore in chicken cells (Perpelescu et al., 2015).

5.2.1.1.3 CENP-I

Reportedly, Mis6 (the equivalent of CENP-I) is crucial for the localization of a CENP-A-like protein in budding yeast (Takahashi et al., 2000). In fission yeast, Ctf3 (CENP-I) forms a complex with Mcm16 (CENP-H) and Mcm22 (CENP-K) (Measday et al., 2002). The CENP-I was initially recognized as a homologue of fission yeast Mis6 in vertebrate cells (Nishihashi et al., 2002). In human cells, CENP-I plays a pivotal role in specifying the localization of CENP-F, MAD1, and MAD2 to kinetochores, thereby being essential for mitosis (S. T. Liu et al., 2003). Subsequent research revealed that the CENP-H/I/K/M complex is integral for the efficient incorporation of newly synthesized CENP-A into centromeres (Okada et al., 2006). Moreover, this complex collaborates with FACT and CHD1 to facilitate the deposition of CENP-A at centromeres (Okada et al., 2009). CENP-I, serving as a stable centromeric component, is incorporated via a "loading-only" mechanism during the S phase (Hemmerich et al., 2008). Interestingly, the intricate balance between SENP6 and RNF4 governs CENP-I assembly through SUMO-targeted destabilization of inner plate components (Mukhopadhyay et al., 2010).

The CENP-H/I/K/M complex has been identified as capable of generating a functional spindle assembly checkpoint (SAC) when Aurora B activity is compromised in mammalian cells (Matson et al., 2012). Furthermore, the dual activities of Aurora B and CENP-I establish a molecular switch that sustains a robust spindle checkpoint signal at prometaphase kinetochores until they achieve mature attachments to microtubules (Matson & Stukenberg, 2014). Additionally, it has been observed that Ctf3 (CENP-I) serves as a docking site for the desumoylase Ulp2 at the kinetochore (Quan et al., 2021).

Notably, CENP-I forms a highly cohesive complex with CENP-H/K/M proteins in vertebrates. This interaction involves the N-terminal Heat-repeat of CENP-I binding to the C-terminal coil-coil region of CENP-H/K and the C-terminal Heat-repeat of CENP-I binding to the N-terminal coil-coil region of CENP-H/K. (Hinshaw et al., 2019; Okada et al., 2006; Pesenti et al., 2022; Tian et al., 2022; Yan et al., 2019; Yatskevich et al., 2022; Zhang et al., 2020).

Intriguingly, the tethering of CENP-I alone is sufficient to induce CENP-A incorporation at a non-centromere locus and recruit a functional kinetochore in chicken cells (Hori et al., 2013).

5.2.1.1.4 CENP-C

As mentioned before, the CENP-C was firstly identified along with CENP-A and CENP-B by sera from patient who have the symptoms of scleroderma CREST (W. Earnshaw et al., 1986; W. C. Earnshaw & Rothfield, 1985). Initially, researchers identified CENP-C as a DNA-binding protein harboring a unique DNA-binding motif (Sugimoto et al., 1994). Subsequently, it was determined that the DNA-binding domain of CENP-C coincided with its centromere targeting domain (Yang et al., 1996). Now, CENP-C is widely recognized as a pivotal protein involved in the organization of the centromere-kinetochore-microtubule attachment in human cells. Its multifunctional properties allow for direct interactions with the C-terminal region of CENP-A nucleosomes, recruitment of other inner kinetochore components via its middle region, and engagement of outer kinetochore components through its N-terminal region. Recently, it has been demonstrated that the outer kinetochore recruitment mediated by the CENP-C N-terminus and the CENP-A nucleosome interaction mediated by the CENP-C motif at the CENP-C C-terminus are not essential for cell proliferation (Hara et al., 2018, 2023). This discovery has sparked curiosity regarding the genuine role of CENP-C at the centromere. Notably, it has been reported that the C-terminal region of CENP-C can also directly interact with HJURP, facilitating new CENP-A loading in human cells (Tachiwana et al., 2015). Similar observations have been made in Xenopus, where Xenopus Knl2 and CENP-C recruit HJURP independently for CENP-A incorporation (Flores Servin et al., 2023; French et al., 2017). In contrast, in chicken cells, Knl2-dependent CENP-A incorporation appears to be the predominant mechanism for CENP-A incorporation at the native centromere, as knockout of CENP-C only rarely affects new CENP-A incorporation.

Intriguingly, tethering CENP-C alone is sufficient to induce CENP-A

incorporation at a non-centromere locus and recruit a functional kinetochore in chicken cells (Hori et al., 2013).

5.2.1.2 Chicken DT40 cells

The chicken DT40 cell line is a highly regarded and extensively utilized model system in various cellular research areas, owing to its exceptional efficiency in homologous gene targeting. Over the past two decades, the DT40 cell line has emerged as a prominent model system for investigating DNA repair and immunoglobulin diversification. It originated from a bursal lymphoma in a female domestic layer chicken that was infected with avian leukosis virus (ALV) (Baba et al., 1985; Baba & Humphries, 1984). The remarkable capability of efficient homologous gene targeting in DT40 facilitates gene disruptions and sequence manipulations, rendering it an exceptional choice for genetic investigations (Buerstedde & Takeda, 1991).

5.2.1.3 LacI-LacO

The LacI-LacO system is a genetic regulatory system that controls the expression of genes involved in lactose metabolism in E.coli bacteria (Jacob & Monod, 1961). The system consists of two components, the LacI repressor protein and the LacO operator sequence. The LacI protein binds to the operator sequence and prevents RNA polymerase from transcribing genes involved in lactose metabolism. When lactose is present, it binds to LacI and causes a conformational change that prevents it from binding to the operator sequence. This allows RNA polymerase to transcribe genes involved in lactose metabolism. Nowadays, this LacI-LacO interaction is used to tether the quired protein to LacO-array integrated into specific genomic site by genome editing. And the Isopropyl β-d-1-thiogalactopyranoside (IPTG), the molecular mimic of allolactose, is developed for inhibition of LacI-LacO interaction.

5.2.1.4 Cre-LoxP

Cre-LoxP is a site-specific recombinase technology that allows for deletions, insertions, translocations, and inversions at specific sites in the DNA of cells (Sauer, 1987; Sauer & Henderson, 1988). It is used to modify genes in vivo and in vitro. The Cre-LoxP system consists of two components: Cre recombinase and LoxP sites. Cre recombinase is an enzyme that recognizes LoxP sites and catalyzes recombination

between them. The LoxP sites are short DNA sequences that are recognized by Cre recombinase. The Cre-LoxP system has been widely used in genetic engineering to create conditional gene knockouts, tissue-specific gene expression, and lineage tracing.

Chapter 2: Induction of CENP-A incorporation at non-centromere locus

1. Introduction

The Genome Project-read, as previously mentioned, is an initiative aimed at sequencing the entire genome of various organisms. One of the notable achievements under this project is the completion of the Human Genome Project-read in 2004. Currently, researchers are venturing into the next phase known as the Genome Project-write, which involves the synthesis of entire genomes (Boeke et al., 2016). The Genome Project-write holds great potential for yielding significant benefits, similar to those seen in the Genome Project-read. Synthesis of chromosomes, a key aspect of the Genome Project-write, has the potential to bring about substantial advancements in several areas. Firstly, it can contribute to a deeper understanding of chromosome structure and function, shedding light on the intricate mechanisms governing genetic information. Additionally, the synthesis of chromosomes can enable the delivery of designed genes for gene therapy, opening up new possibilities for targeted treatments and disease management. Ultimately, the ultimate goal of this project is to engineer life itself. By embarking on the Genome Project-write, researchers anticipate unlocking new avenues of knowledge and innovation that can revolutionize various fields, ultimately benefitting society at large.

The presence of a functional centromere is essential for ensuring chromosome stability during cell division. To achieve faithful chromosome segregation in mitosis, microtubules must attach to the centromere through the kinetochore complex, enabling the equal distribution of sister chromatids into daughter cells. Therefore, when synthesizing a chromosome, it becomes crucial to establish a properly seeded centromere to maintain stability during cell division.

The centromere is primarily defined through epigenetic mechanisms. A key epigenetic marker of the centromere is the presence of CENP-A (Histone H3 variant) containing nucleosomes, which are exclusively found at the centromere region. These CENP-A nucleosomes containing chromatin serve as a crucial platform for recruiting inner kinetochore proteins (**C**onstitutive **C**entromere **A**ssociated **N**etwork-CCAN) throughout the cell cycle. Subsequently the CCAN recruit outer kinetochore proteins (KMN network) specifically during the M phase of the cell cycle providing the attachment site for microtubules. Due to this epigenetic nature, seeding a centromere by synthesizing a specific DNA sequence becomes challenging.

To overcome the challenge of seeding a centromere using specific DNA sequences, our group employed a chromosomal engineering approach that led to successful

centromere seeding for the chicken chromosome-Z through epigenetic way. We achieved this by inserting a LacO-array into the chromosome-Z, thereby providing a site for centromere seeding. In addition, our group expressed a centromere inducer fused with LacI in chicken cells. Following the removal of the native centromere from the chromosome-Z, our group isolated a functional centromere that formed at the LacO locus and recruited a functional kinetochore. The successful centromere inducers used in this process included HJURP, a CENP-A specific chaperone, Knl2, the main component of the Mis18 complex responsible for HJURP recruitment, and CENP-I and CENP-C, which are inner kinetochore (CCAN) components. Notably, upon tethering these centromere inducers, our group observed the incorporation of the centromere epigenetic marker, CENP-A, at the LacO locus, which seems to be the trigger for new centromere formation.

The centromere seeding project led to an intriguing observation suggesting that CCAN components might play a role in the CENP-A incorporation process. In chicken cells, the CENP-A incorporation at the native centromere is well-established to depend on Knl2 within the Mis18 complex. Knl2 plays a crucial role in recognizing the existing CENP-A nucleosomes, facilitating subsequent recruitment of the Mis18a/b subunit. This subunit, in turn, recruits HJURP, the CENP-A specific chaperone, for CENP-A loading. This provides a clear understanding of how tethering HJURP and Knl2 can induce CENP-A incorporation. However, the current CENP-A incorporation model lacks information about the involvement of CCAN in the CENP-A incorporation machinery at the native centromere. As a result, the mechanisms through which CCAN components, such as CENP-I or CENP-C, induce CENP-A incorporation remain intriguing and warrant further investigation.

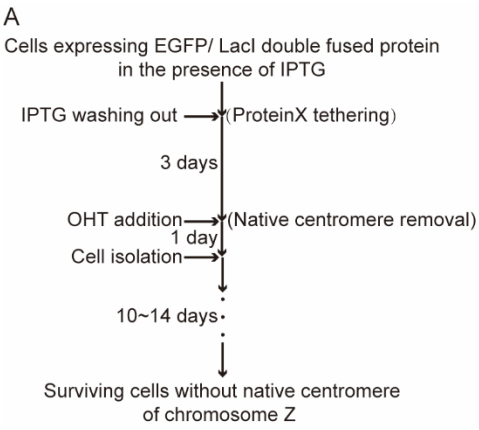
2. Results

2.1 Ectopic tethering of inner kinetochore proteins causes efficient new centromere formation with full kinetochore recruitment at this ectopic locus

Previously, our group conducted a comprehensive screening of various factors, including CENP-A incorporation machinery components, inner kinetochore (CCAN) components, and outer kinetochore components, with the aim of identifying inducers capable of successfully seeding new centromeres at ectopic loci. After an extensive investigation, our group identified four such inducers: the CENP-A specific chaperone HJURP, main components of the Mis18 complex Knl2, and inner kinetochore (CCAN)

components CENP-I and CENP-C (Hori et al., 2013). These inducers demonstrated the ability to induce new centromere formation at the LacO locus (**Figure 1-7**). By focusing on chromosome Z of chicken DT40 cells, our group strategically inserted 256 copies of the LacO sequence in close proximity to the end of chromosome Z. Additionally, our group introduced LoxP sequences surrounding the native centromere of chromosome Z. Moreover, our group expressed LacI/EGFP double-fused inducers in the presence of IPTG (**Figure 1-6**). This experimental design allowed us to precisely target the chromosome Z and investigate the potential of the identified inducers to seed new centromeres at the designated LacO locus.

To initiate the centromere seeding process, I first washed out IPTG from the cell culture medium to enable the localization of the LacI/EGFP double-fused inducers at the LacO locus. The presence of IPTG would inhibit the LacI-LacO interaction. After three days of incubation, hydroxytamoxifen (OHT) was introduced to the cell culture to activate Cre-recombinase, which facilitated the recombination of the LoxP site, resulting in the removal of the native centromere of chromosome Z. Subsequently, the cells were plated into a 96-well plate one day after the removal of the native centromere of chromosome Z. Given that chromosome Z contains essential genes for cell proliferation, maintaining chromosome Z is crucial for cell survival (**Figure 2-1A**). Through this methodology, I successfully confirmed that the ectopic tethering of HJURP, particularly HJURP N-terminus (aa 1-400), Knl2, CENP-I, and CENP-C, specifically CENP-C C-terminus (aa 601-864), significantly increased the number of surviving cells after the removal of the native centromere of chromosome Z (**Figure 2-1B**).



B

Tethered Protein	Number of surviving cells (/10^5 cells)
HJURP(1-400)	25±5
Knl2	25±9
CENP-I	46±21
CENP-C	71±23
CENP-C(601-864)	147±33
None	3±1

Figure 2-1 Assessment of artificial new centromere formation rate

A.

The experimental time course for Figure 1-6. The chicken DT40 cell line expressing EGFP/ LacI fused proteins were cultured in the presence of IPTG, and the assay began after the removal of IPTG (Day 0). Following a 3-days incubation, 4-hydroxytamoxifen (OHT) was added to activate Cre-recombinase. After an additional 1-day incubation, the cells were plated and grown for 10 to 14 days to isolate the surviving cells.

B.

The number of surviving colonies per 10^5 cells, when EGFP/LacI double fused HJURP (1-400), KnlL2, CENP-I, CENP-C, CENP-C (601-864) were localized to the LacO locus after removing a native centromere on chromosome Z. Each assay was conducted twice, and the presented results depict the mean ± standard deviation (SD).

The tethering of EGFP/LacI fused with no additional components at the LacO locus resulted in a consistent finding of around 3 surviving cells per 10^5 cells (**Figure 2-1B**), corroborating our previous results (Shang et al., 2013). Upon removal of the native centromere of chromosome Z, I observed the formation of a new centromere marked by mScarlet-tagged CENP-A. Notably, this new centromere emerged at the central position of chromosome Z, proximal to the original centromere position and distant from the LacO locus where the tethering occurred, which is also consistent with our previous results (**Figure 2-2A**) (Shang et al., 2013). Furthermore, cells with EGFP/LacI fusion without additional tethering showed normal proliferation regardless of the presence or absence of IPTG, indicating that the formation and function of this new centromere, which is proximal to the original centromere position, are independent of EGFP-LacI tethering (**Figure 2-2B**).

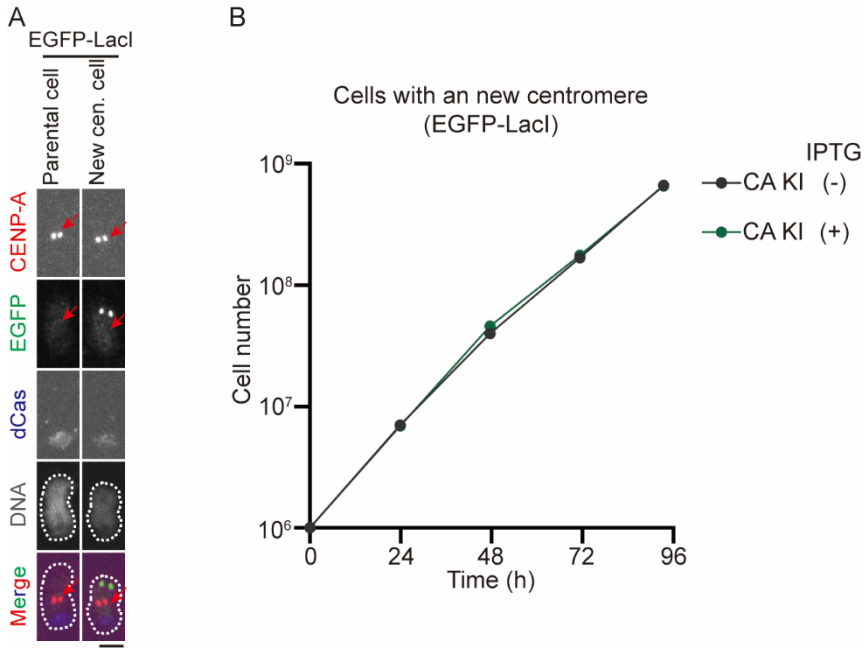


Figure 2-2 Automated new centromere formation

A.

The visualization of chromosome Z in cells expressing EGFP-LacI before and after the new centromere formation. The centromere position was indicated by mScarlet tagged CENP-A (red arrow). A Halo tagged dCas protein labels the chromosome Z specific satellite sequence at end of q arm. The merging of mScarlet tagged CENP-A, EGFP-LacI and Halo tagged dCas with outline of chromosome Z was shown in the bottom. The centromere was highlighted by a red arrow. The new centromere was not formed at the LacO locus. Bar, 2.5 μm.

B.
The proliferation of cells with a new centromere shown in (A) in the absence or presence of IPTG.

As a negative control, cells expressing the EGFP/LacI double-fused CENP-T(1-530) exhibited regular proliferation in the absence of IPTG. However, upon the introduction of IPTG, proliferation ceased. These indicates that CENP-T(1-530) induces an artificial kinetochore which relies on the tethering action of LacI at the LacO-array just as our group showed previously (Hori et al., 2013). (**Figure 2-3A**).

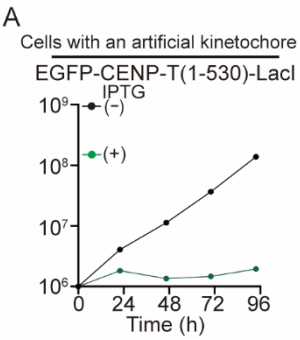


Figure 2-3 Artificial kinetochore formation
A.
The proliferation of cells with an artificial new centromere tethered by CENP-T N-terminus (1-530) is shown in the absence or presence of IPTG.

Consistent with our expectations, the tethering of LacI/EGFP double-fused CENP-A incorporation machinery components (HJURP N-terminus (aa 1-400), Knl2) to the LacO locus resulted in a significant increase in the number of surviving cells, to around 10 times (**Figure 2-1B**). Notably, I observed the formation of new centromeres, marked by mScarlet-tagged CENP-A, at the LacO locus in close proximity to the chromosome end, where inner kinetochore localization (marked by CENP-T) and our kinetochore localization (marked by Dsn1) were also detected. This observation was a result of tethering LacI/EGFP double-fused HJURP N-terminus (aa 1-400), Knl2 (**Figure 2-4A**). Importantly, these newly formed centromeres exhibited normal proliferation even in the presence of IPTG, indicating their functional independence from the tethered LacI/EGFP double-fused inducers. This functional autonomy may be attributed to the presence of the centromere epigenetic marker CENP-A, which plays a crucial role in maintaining the integrity and functionality of the newly formed centromere (**Figure 2-4B**).

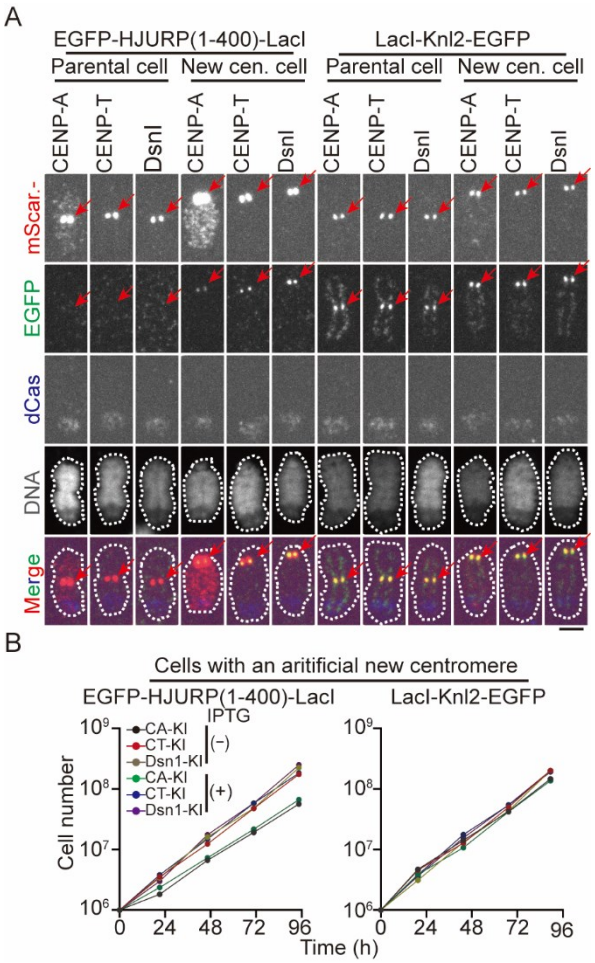


Figure 2-4 CENP-A incorporation machinery induced artificial new centromere formation with full kinetochore recruitment

A.

The visualization of chromosome Z in cells expressing EGFP/LacI double fused HJURP(1-400), Knl2 before and after the artificial new centromere formation. mScarlet tagged CENP-A, CENP-T, and Dsn1 were visualized. A Halo tagged dCas protein labels the chromosome Z specific satellite sequence at end of q arm. A new centromere was formed at the LacO locus and inner and outer kinetochore proteins were localized at a new centromere. The merging of mScarlet tagged protein, EGFP/ LacI double fused protein and Halo tagged dCas with outline of chromosome Z was shown in the bottom. The centromere was highlighted by a red arrow. Bar, 2.5 μm.

B.

The proliferation of cells with an artificial new centromere formed at the LacO locus on chromosome Z shown in (A) in the absence or presence of IPTG. Data is for cells with an artificial new centromere formed at the LacO locus induced by EGFP/ LacI double fused HJURP (1-400), Knl2 tethering, respectively, were shown.

Surprisingly, the tethering of LacI/EGFP double-fused inner kinetochore components (CENP-I, CENP-C or CENP-C C-terminus (aa 601-864)) to the LacO

locus also resulted in a significant increase in the number of surviving cells, ranging from 20 to 70 times (**Figure 2-1B**). As with the previous approach, I observed the formation of new centromeres, marked by mScarlet-tagged CENP-A, at the LacO locus in close proximity to the chromosome end, where inner kinetochore localization (marked by CENP-T) and our kinetochore localization (marked by Dsn1) were also detected. This observation was a result of tethering LacI/EGFP double-fused CENP-I, CENP-C or CENP-C C-terminus (aa 601-864) (**Figure 2-5A**). Remarkably, the newly formed centromeres demonstrated functional autonomy and normal proliferation, even in the presence of IPTG. Once again, the recruitment of the centromere epigenetic marker CENP-A by inner kinetochore components (CENP-I or CENP-C) likely contributed significantly to the establishment and functionality of the newly formed centromeres (**Figure 2-5B**).

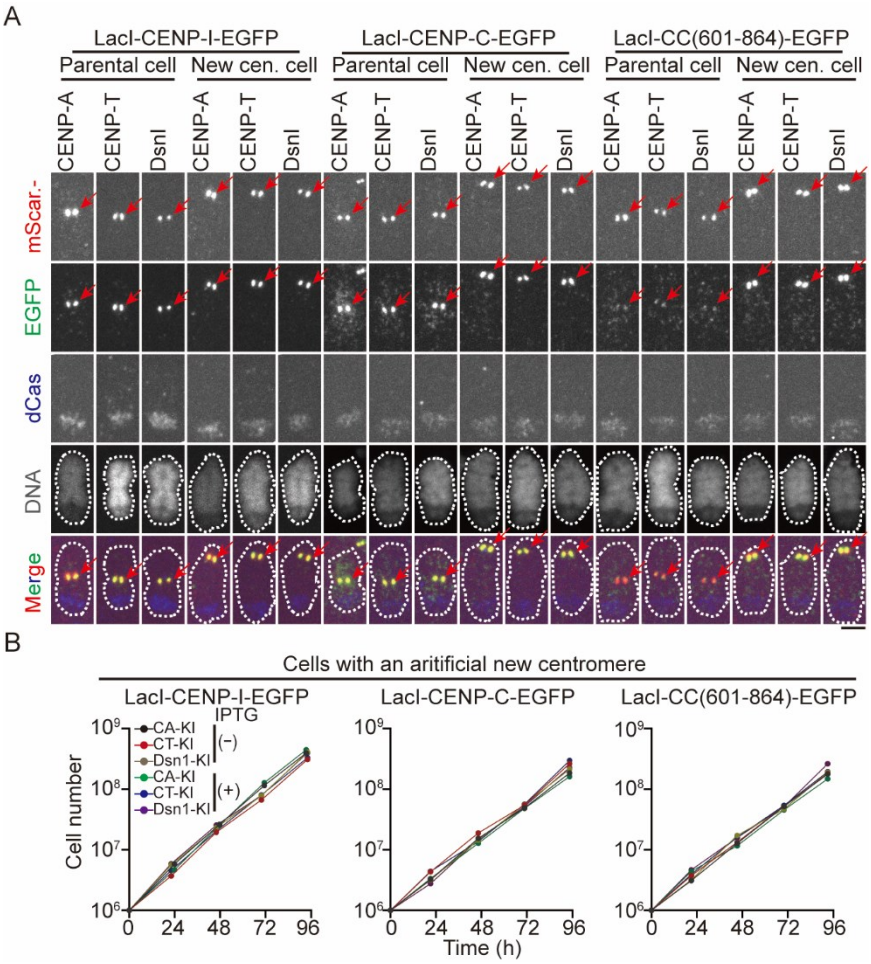


Figure 2-5 CCAN induced artificial new centromere formation with full kinetochore recruitment

A.

The visualization of chromosome Z in cells expressing EGFP/ LacI double fused CENP-I, CENP-C, CENP-C (601-864) before and after the artificial new centromere formation. mScarlet tagged CENP-A, CENP-T, and Dsn1 were visualized. A Halo

tagged dCas protein labels the chromosome Z specific satellite sequence at end of q arm. A new centromere was formed at the LacO locus and inner and outer kinetochore proteins were localized at a new centromere. The merging of mScarlet tagged protein, EGFP/ LacI double fused protein and Halo tagged dCas with outline of chromosome Z was shown in the bottom. The centromere was highlighted by a red arrow. Bar, 2.5 μm.
B.
The proliferation of cells with an artificial new centromere formed at the LacO locus on chromosome Z shown in (A) in the absence or presence of IPTG. Data is for cells with an artificial new centromere formed at the LacO locus induced by EGFP/ LacI double fused CENP-I, CENP-C, or CENP-C (601-864) tethering, respectively, were shown.

2.2 CENP-A incorporation occurs at the LacO locus following the tethering of HJURP N-terminus, Knl2, CENP-I, CENP-C and CENP-C C-terminus

In contrast to the CENP-C N-terminus and CENP-T N-terminus tethering, which serve as the bridge to mediate microtubule attachment in the absence of CENP-A incorporation (Hori et al., 2013), our investigation revealed that artificial new centromere induction by HJURP N-terminus, Knl2, CENP-I, CENP-C, and CENP-C C-terminus is independent of the tethered inducer. This independence is achieved through the acquisition of the centromere epigenetic marker CENP-A (**Figure 2-4A, Figure 2-5A**). Thus, the successful new centromere inducers appear to induce CENP-A incorporation as a crucial factor in the formation of new centromeres at non-centromere loci.

To verify this, I cultured cells containing the LacO array on chromosome Z, expressing mScarlet tagged CENP-A, and LacI/EGFP double fused HJURP N-terminus, Knl2, CENP-I, CENP-C or CENP-C C-terminus in the presence of IPTG. After washing out the IPTG from the cell culture medium, I sampled cells each day during a 3-day incubation period for chromosome spread to observe chromosome Z (**Figure 2-6A**).

Following the removal of IPTG, proper localization of LacI/EGFP double fused HJURP N-terminus, Knl2, CENP-I, CENP-C, CENP-C C-terminus or none at the LacO locus was confirmed (**Figure 2-6B, D**). Compared to the tethering of EGFP-LacI alone, CENP-A incorporation was observed when tethering LacI/EGFP double fused HJURP N-terminus, Knl2, CENP-I, CENP-C or CENP-C C-terminus (**Figure 2-6B, C**). For Knl2, CENP-I, CENP-C and CENP-C C-terminus tethering, CENP-A incorporation was clearly observed on day 3 after IPTG removal (**Figure 2-6B, C**), the time point at which previously I removed the native centromere of chromosome Z and screened the

surviving cells (**Figure 2-1A**). For HJURP N-terminus (aa 1-400) tethering, although the tethered EGFP/ LacI double-fused HJURP (1-400) signal at the LacO locus was extremely weaker compared to other LacI/EGFP double fused proteins (approximately one-tenth), the CENP-A incorporation induced by HJURP N-terminus was the strongest (approximately ten times higher than other LacI/EGFP double-fused proteins) (**Figure 2-6B, C**). And strong CENP-A incorporation was observed from day 1 after IPTG removal by HJURP N-terminus tethering (**Figure 2-6B, C**), likely due to the direct mediation of CENP-A incorporation by the CENP-A specific chaperone HJURP. While not as strong as HJURP tethering, considering Knl2, CENP-I, CENP-C or CENP-C C-terminus tethering induces new centromere formation with centromere epigenetic marker CENP-A (**Figure 2-4A, Figure 2-5A**), CENP-A incorporation on day 3 after IPTG removal, depending on Knl2, CENP-I, CENP-C or CENP-C C-terminus tethering, was sufficient and responsible for the formation of new centromeres at non-centromere loci.

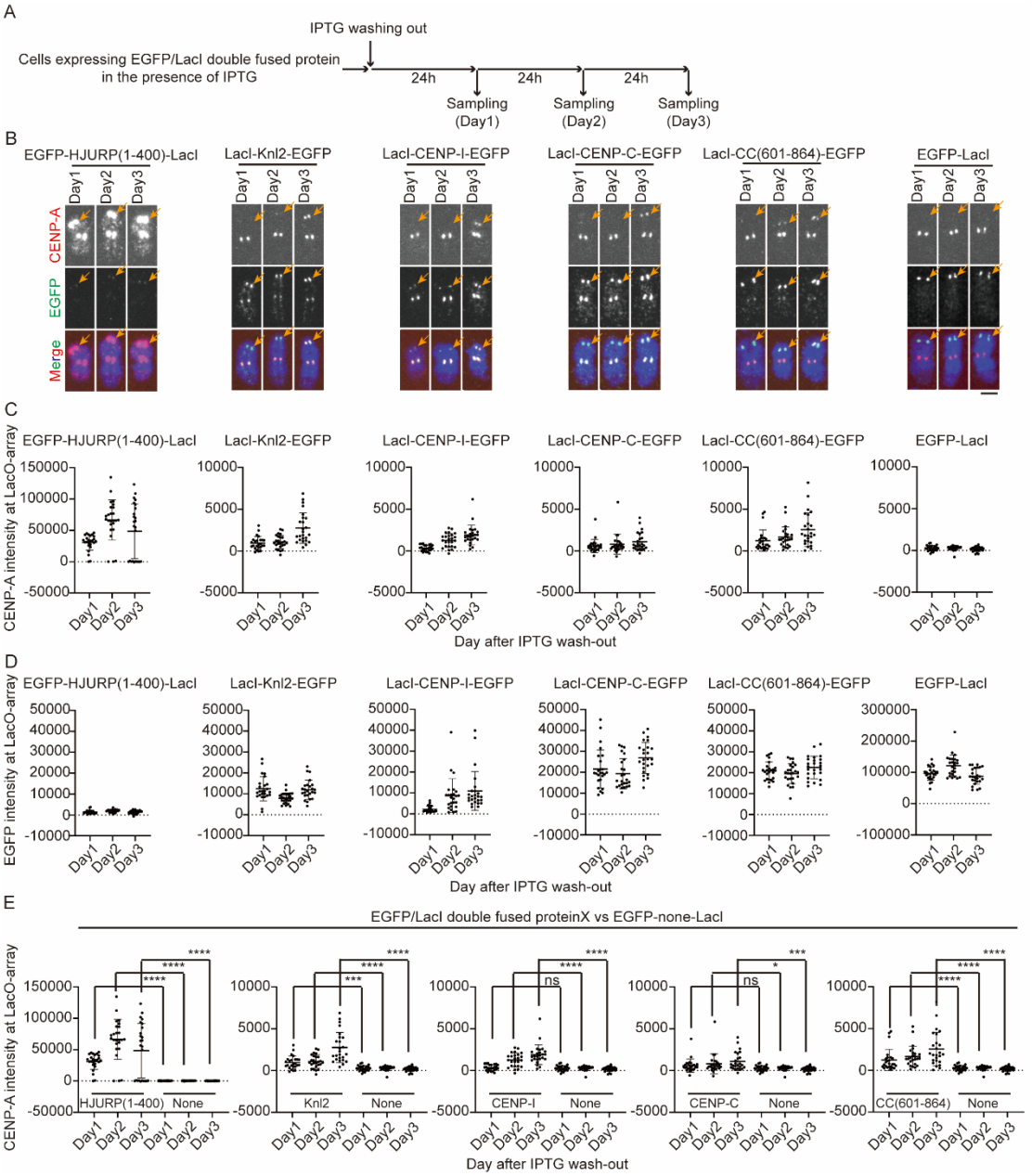


Figure 2-6 Assessment of CENP-A incorporation at LacO locus

A.

The experimental scheme to visualize centromeric proteins at the LacO locus. The chicken DT40 cell line expressing EGFP/ LacI double fused proteins were cultured in the presence of IPTG, and the assay began after the removal of IPTG (Day 0). For 3 days, chromosome spreads were prepared to observe CENP-A and other centromeric proteins at LacO locus at each day.

B.

The visualization of mScarlet tagged CENP-A and EGFP/ LacI double fused HJURP (1-400), KNL2, CENP-I, CENP-C or CENP-C C-terminus at the LacO locus on the chromosome-Z at Day1, 2, and 3 in the scheme of (A). The merging of mScarlet tagged CENP-A, EGFP/ LacI double fused protein and DAPI stained chromosome Z was

shown in the bottom. The LacO locus was highlighted by an orange arrow. Bar, 2.5 μm.
C.
The intensities of mScarlet tagged CENP-A at the LacO locus shown in (B). The mean ± standard deviation (SD) of the intensity values was shown. n=25.
D.
The intensities of EGFP/ LacI double-fused proteins at the LacO locus in (B). The mean ± standard deviation (SD) of the intensity values was shown. n=25.
E.
Significance of CENP-A incorporation by protein tethering shown in (B) and (C). The mean ± standard deviation (SD) of the intensity values is shown. (n = 25; ****, $P < 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, $P > 0.05$; unpaired t-test, two tailed).

In EGFP-HJURP(1-400)-LacI tethering cells, I observed a robust occurrence of CENP-A incorporation not only at the LacO locus but also at the native centromere of chromosome Z, a surprising finding. This was unexpected given that HJURP is known to localize to the centromere during the G1 phase, reliant on its C-terminal region responsible for recruitment by the Mis18 complex (Pan et al., 2019; Perpelescu et al., 2015; Zasadzińska et al., 2013). To delve deeper, I investigated the localization of EGFP-HJURP(1-400)$^{\text{N-terminus}}$-LacI and discovered that the HJURP N-terminal region also localizes to the centromere during interphase (**Figure 2-7A**). This suggests the existence of an unknown mechanism for HJURP recruitment.

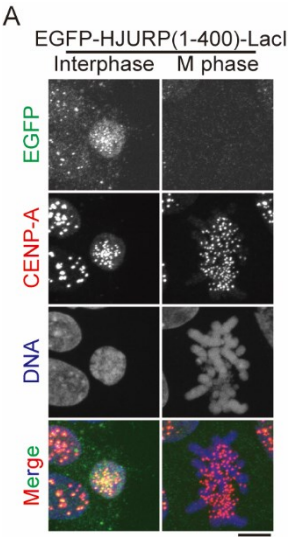


Figure 2-7 HJURP N-terminus Localize to centromere in interphase
A. Localization profile of EGFP-LacI double fused with HJURP (1-400) in interphase and mitotic cells expressing mScarlet CENP-A. EGFP-LacI fused HJURP (1-400) localized to interphase endogenous centromere but not mitotic centromeres. Bar, 12 μm.

2.3 Tethering of CENP-I or CENP-C C-terminus induces CENP-A incorporation in the absence of Knl2

Based on the current CENP-A incorporation machinery proposed in chicken cells, which involves Knl2 in the Mis18 complex recognizing existing CENP-A nucleosomes and the recruitment of Mis18α/β subunits, followed by the recruitment of HJURP (CENP-A-specific chaperones) for loading new CENP-A (Hori et al., 2017; Jiang et al., 2023), it is reasonable to expect that HJURP and Knl2 tethering could induce CENP-A incorporation at the LacO locus. However, the mechanism underlying CENP-A incorporation induced by CCAN components, CENP-I and CENP-C particularly CENP-C C-terminus, remains unclear. To address this question, I utilized cells containing a LacO array in chromosome Z and expressed mScarlet tagged CENP-A along with LacI/EGFP double fused Knl2, CENP-I, or CENP-C C-terminus respectively, in which the queried protein was replaced with an Auxin-Inducible-Degron (AID) tagged version (Nishimura et al., 2009). For the assay, I initiated tethering by washing out IPTG from the cell culture medium and induced queried protein knock-out by adding IAA into the cell culture medium. Subsequently, I sampled the cells for chromosome spread each day during a 3-day incubation period (**Figure 2-8A**).

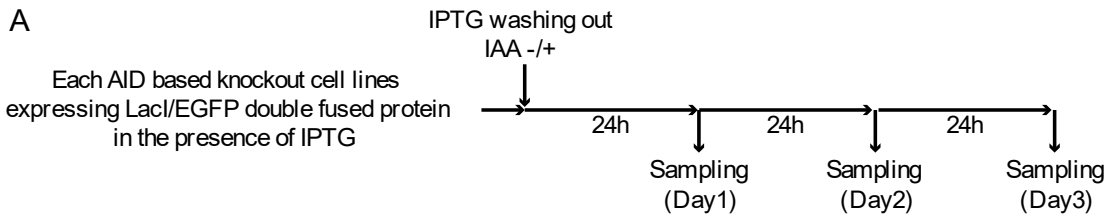


Figure 2-8 Assessment of CENP-A incorporation at LacO locus under AID based protein knockout condition
A.
The experimental scheme to visualize CENP-A and other centromeric proteins at the LacO locus in Auxin-Inducible-Degron (AID) based knockout cells. Cells were cultured in the presence of IPTG, and the assay commenced after the removal of IPTG (Day 0), in the absence or presence of IAA. For 3 days, chromosome spreads were prepared to observe CENP-A and other centromeric proteins at the LacO locus at each day.

Upon HJURP knock-out, the tethering of Knl2, CENP-I, and CENP-C C-terminus fails to induce CENP-A incorporation at the LacO locus, emphasizing the critical role of HJURP (the CENP-A specific chaperone) in the final step of CENP-A incorporation (**Figure 2-9A, B, C**). These results suggest that all CENP-A inducible tethering proteins

ultimately rely on HJURP for successful CENP-A incorporation.

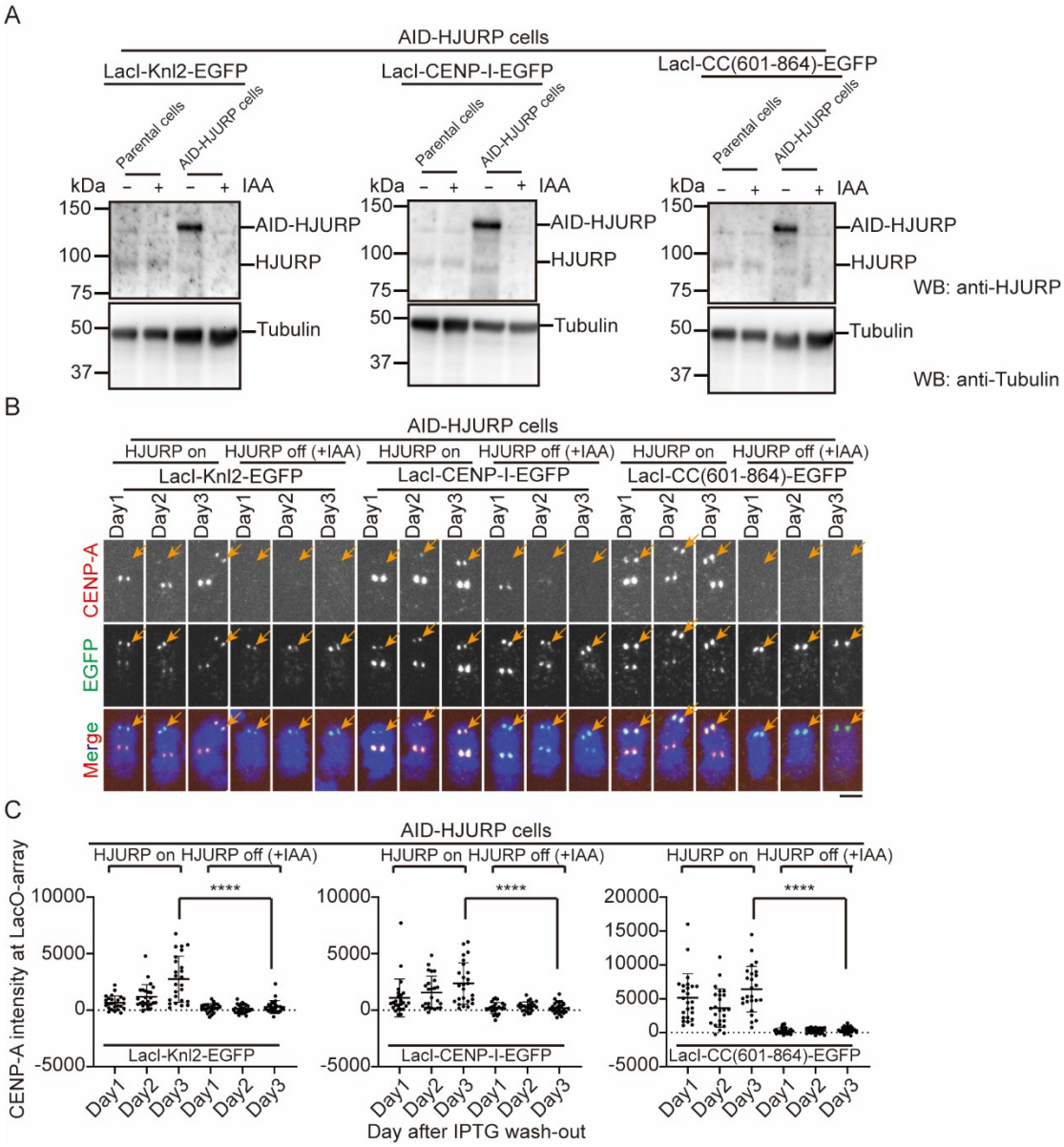


Figure 2-9 HJURP is required for Knl2, CENP-I and CENP-C C-terminus tethering dependent CENP-A incorporation

A.

Confirmation of protein degradation of AID tagged HJURP in cells expressing LacI / EGFP double fused Knl2, CENP-I or CENP-C C-terminus (601-864). The cells were cultured either without IAA (-IAA) or with IAA (+IAA) for 5 hours.

B.

The visualization of mScarlet tagged CENP-A and LacI/EGFP double fused Knl2, CENP-I, CENP-C (601-864) at the LacO locus on the chromosome Z at Day1, 2, and 3 in the scheme of **Figure 2-8A** in AID-HJURP cells in the absence (HJURP On) or presence (HJURP Off) of IAA. The merging of mScarlet tagged CENP-A, LacI/EGFP double fused protein and DAPI stained chromosome Z was shown in the bottom. The

LacO locus was highlighted by an orange arrow. Bar, 2.5 μm.
C.
The intensity of mScarlet tagged CENP-A at the LacO locus shown in (B) in AID-HJURP cells in the absence (HJURP On) or presence (HJURP Off) of IAA. The mean ± standard deviation (SD) of the intensity values was shown. (n = 25; ****, $P < 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, $P > 0.05$; unpaired t test, two tailed).

However, interestingly, in the case of Knl2 knockout, both CENP-I and CENP-C C-terminus tethering still manage to induce CENP-A incorporation at the LacO locus. Moreover, Knl2 knockout under CENP-I tethering leads to hyper-activated CENP-A incorporation (**Figure 2-10A, B, C**), a phenomenon that warrants further investigation. These results indicate that CCAN (CENP-I or CENP-C C-terminus) dependent CENP-A incorporation can occur even in the absence of Knl2.

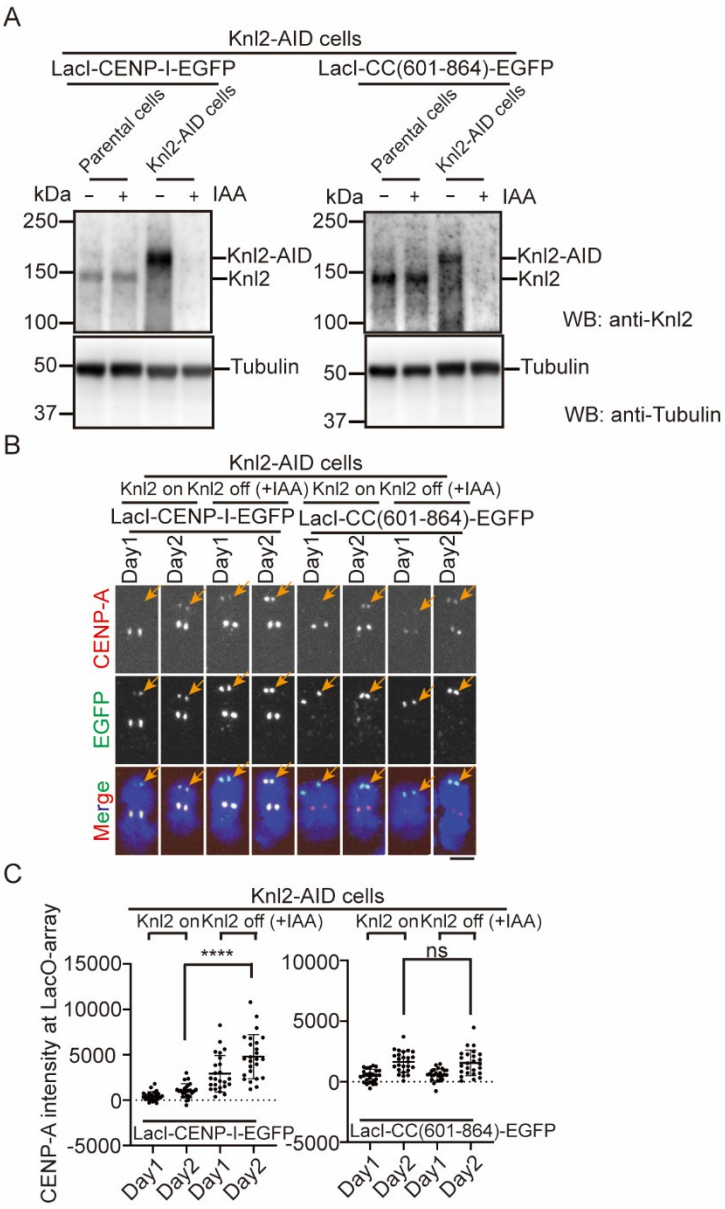


Figure 2-10 Tethering of CENP-I or CENP-C C-terminus induces the CENP-A incorporation at LacO locus independent with Knl2

A.

Confirmation of protein degradation of AID tagged Knl2 in cells expressing LacI/EGFP double fused CENP-I or CENP-C C-terminus (601-864). The cells were cultured either without IAA (-IAA) or with IAA (+IAA) for 5 hours.

B.

The visualization of mScarlet tagged CENP-A and LacI/EGFP double fused CENP-I and CENP-C (601-864) at the LacO locus on the chromosome Z at Day1, and 2 (cells almost died in day3) in the scheme of **Figure 2-8A** in Knl2-AID cells in the absence (Knl2 On) or presence (Knl2 Off) of IAA. The merging of mScarlet tagged CENP-A, LacI/EGFP double fused protein and DAPI stained chromosome Z was shown in the bottom. The LacO locus was highlighted by an orange arrow. Bar, 2.5 μm.

C.

The intensity of mScarlet tagged CENP-A at the LacO locus shown in (B) in Knl2-AID cells in the absence (Knl2 On) or presence (Knl2 Off) of IAA. The mean ± standard deviation (SD) of the intensity values was shown. (n = 25; ****, P < 0.0001; ***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05; ns, P > 0.05; unpaired t test, two tailed).

Additionally, I conducted an assay involving the knock-out of Mis18α under the CENP-C C-terminus tethering condition. While the incorporation of CENP-A was impacted following the knock-out of Mis18α, it was still observable at the LacO locus (**Figure 2-11A, B, C**). When considering the collective outcomes presented in **Figure 2-9**, it appears that, at least in the context of CENP-C C-terminus-dependent CENP-A incorporation, the Mis18 complex (comprising Mis18α/β subunits and Knl2) is not an essential requirement.

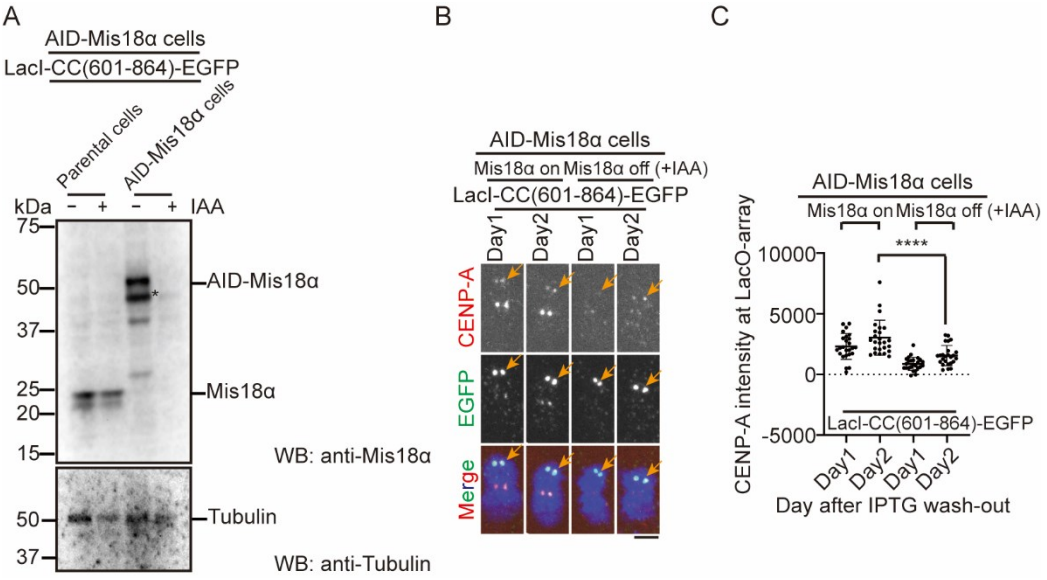


Figure 2-11 Mis18α is required for CENP-C C-terminus tethering dependent CENP-A incorporation

A.

Confirmation of AID-Mis18α degradation in cells expressing the LacI/EGFP double-fused CENP-C C-terminus (601–864). Cells were cultured without IAA (-IAA) or with IAA (+IAA) for 5 h. The asterisk (*) indicates a nonspecific band.

B.

(H) Visualization of mScarlet-tagged CENP-A and LacI/EGFP double-fused with CENP-C (601–864) at the LacO locus on chromosome-Z at Day1, and 2 (cells almost died in day3) in the scheme of **Figure 2-8A** in AID-Mis18α cells in the absence (Mis18α On) or presence (Mis18α Off) of IAA. Merged images of mScarlet tagged CENP-A, LacI/EGFP double fused CENP-C (601–864) and DAPI stained chromosome Z are shown in the bottom. The LacO locus is indicated by an orange arrow. Bar, 2.5 μm.

C.

The intensity of mScarlet-tagged CENP-A at the LacO locus is shown in AID-Mis18α

cells in the absence (Mis18α On) or presence (Mis18α Off) of IAA is shown. The mean ± standard deviation (SD) of the intensity values is shown. (n = 25; ****, $P < 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, $P > 0.05$; unpaired t test, two tailed).

Furthermore, under CENP-C knock-out conditions, Knl2 tethering remains effective in inducing CENP-A incorporation at the LacO locus, whereas CENP-I tethering loses its ability to do so (**Figure 2-12A, B, C**). As our group initially hypothesized, Knl2 tethering appears to form the Mis18 complex with Mis18α/β subunits and subsequently recruits HJURP for CENP-A loading, a process in which CCAN is not required. The finding that CENP-I tethering dependent CENP-A incorporation necessitates CENP-C recruitment suggests that after CENP-I tethering, CENP-C becomes essential for CENP-A incorporation.

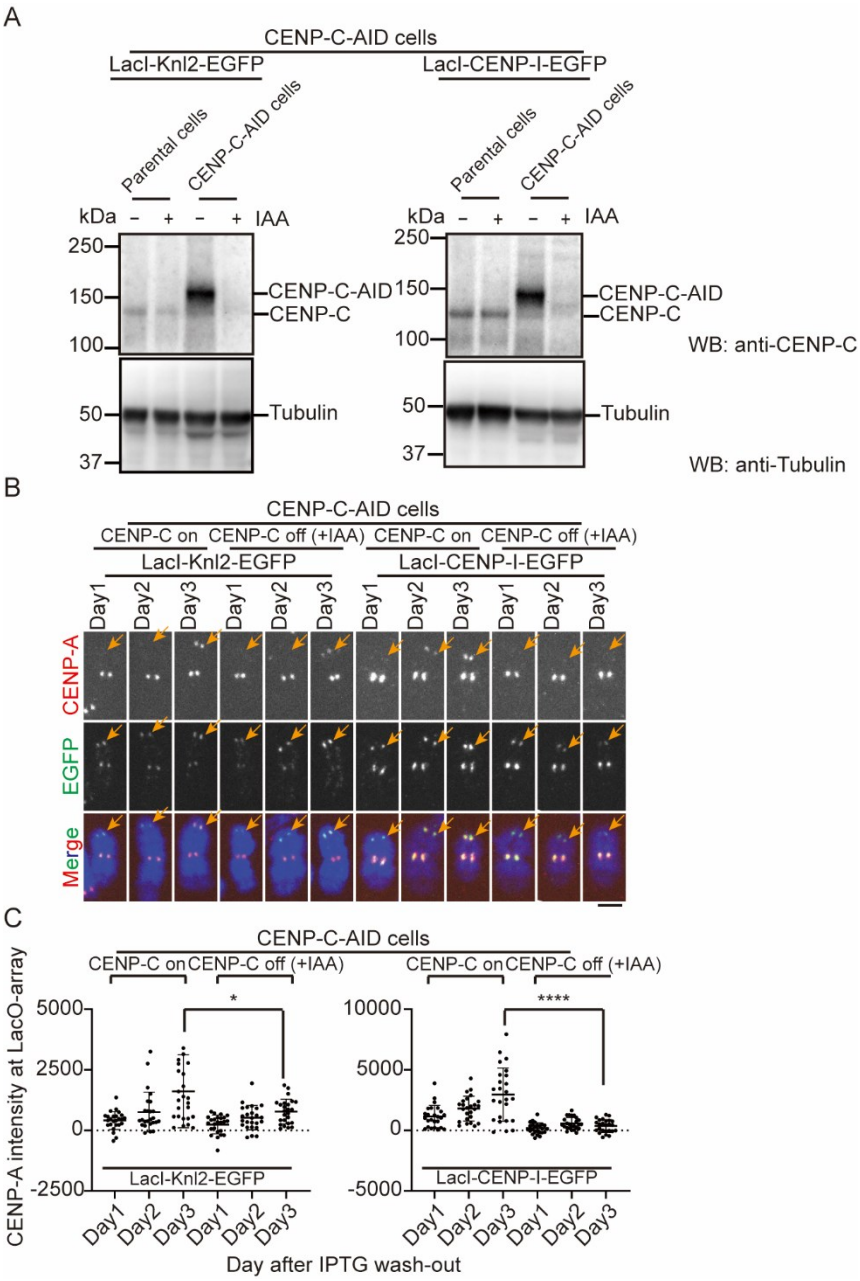


Figure 2-12 CENP-C is required for CENP-I tethering dependent CENP-A incorporation but not for Knl2 tethering dependent CENP-A incorporation

A.

Confirmation of protein degradation of AID tagged CENP-C in cells expressing LacI/EGFP double fused Knl2, CENP-I or CENP-C C-terminus (601-864). The cells were cultured either with without IAA (-IAA) or IAA (+IAA) for 5 hours.

B.

The visualization of mScarlet tagged CENP-A and LacI/EGFP double fused Knl2 and CENP-I at the LacO locus on the chromosome-Z at Day1, 2, and 3 in the scheme of **Figure 2-8A** in CENP-C-AID cells in the absence (CENP-C On) or presence (CENP-C Off) of IAA. The merging of mScarlet tagged CENP-A, LacI/EGFP double fused protein and DAPI stained chromosome Z are shown in the bottom. The LacO locus was

highlighted by an orange arrow. Bar, 2.5 μm.
C.
The intensity of mScarlet tagged CENP-A at the LacO locus shown in (B) in CENP-C-AID cells in the absence (CENP-C On) or presence (CENP-C Off) of IAA. The mean ± standard deviation (SD) of the intensity values was shown. (n = 25; ****, $P < 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, $P > 0.05$; unpaired t test, two tailed).

I observed that the AID based HJURP knock-out cell line exhibited an enhancement in CENP-A incorporation at the LacO locus induced by Knl2, CENP-I, or CENP-C C-terminus tethering (**Figure 2-9B, C**) when compared to other AID-based protein knock-out cell lines (**Figure 2-10B, C; Figure 2-11B, C; Figure 2-12B, C**). This enhancement might be attributed to the overexpression of HJURP (**Figure 2-9A**). Further, I assessed CENP-A incorporation under Knl2, CENP-C or Mis18α knock-out conditions while simultaneously overexpressing HJURP. The results revealed that HJURP overexpression does enhance CENP-A incorporation at the LacO locus; however, it does not rescue the CENP-A incorporation deficits caused by specific protein knockout. This suggests that HJURP overexpression does not significantly affect the overall conclusions drawn from our study (**Figure 2-13A, B, C, D, E, F, G**).

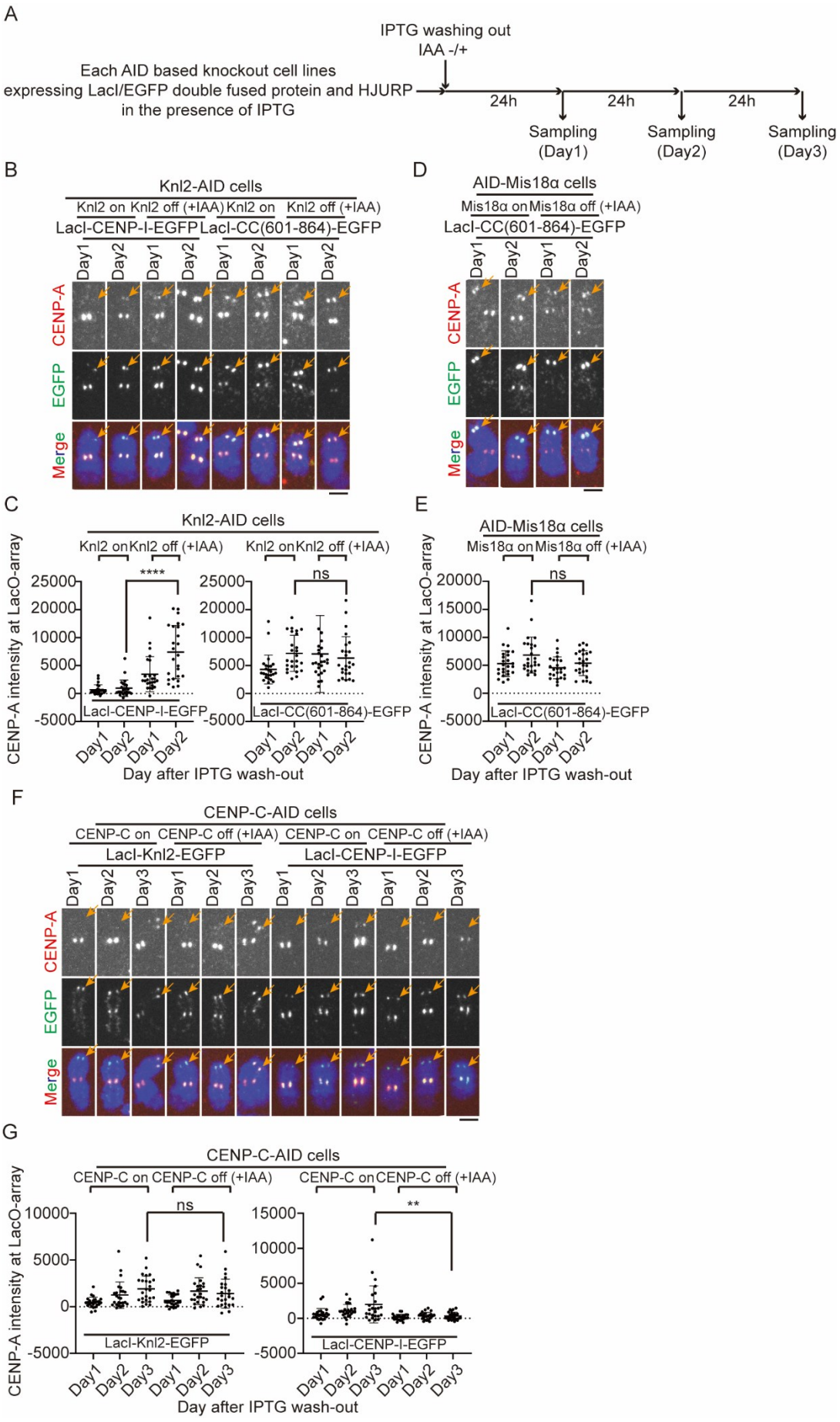

A
IPTG washing out
IAA -/+
Each AID based knockout cell lines
expressing LacI/EGFP double fused protein and HJURP
in the presence of IPTG
24h
24h
24h
Sampling
(Day1)
Sampling
(Day2)
Sampling
(Day3)
B
Knl2-AID cells
Knl2 on
Knl2 off (+IAA)
Knl2 on
Knl2 off (+IAA)
LacI-CENP-I-EGFP
LacI-CC(601-864)-EGFP
Day1
Day2
CENP-A
EGFP
Merge
D
AID-Mis18α cells
Mis18α on
Mis18α off (+IAA)
LacI-CC(601-864)-EGFP
C
Knl2-AID cells
****
ns
CENP-A intensity at LacO-array
25000
20000
10000
5000
0
-5000
LacI-CENP-I-EGFP
LacI-CC(601-864)-EGFP
Day after IPTG wash-out
E
AID-Mis18α cells
15000
F
CENP-C-AID cells
CENP-C on
CENP-C off (+IAA)
LacI-Knl2-EGFP
LacI-CENP-I-EGFP
Day3
G
**
LacI-Knl2-EGFP

Figure 2-13 HJURP overexpression enhances the CENP-A incorporation at LacO locus but not rescues CENP-A incorporation at LacO locus caused by specific protein knockout

A.

The experimental scheme to visualize CENP-A and other centromeric proteins at the LacO locus in Auxin-Inducible-Degron (AID) based knockout cells. Cells simultaneously express HJURP. Cells were cultured in the presence of IPTG, and the assay commenced after the removal of IPTG (Day 0), in the absence or presence of IAA. For 3 days, chromosome spreads were prepared to observe CENP-A and other centromeric proteins at the LacO locus at each day.

B.

The visualization of mScarlet tagged CENP-A and LacI/EGFP double fused CENP-I or CENP-C (601-864) at the LacO locus on the chromosome Z at Day1, and 2 (cells almost died in day3) in the scheme of (A) in Knl2-AID cells in the absence (Knl2 On) or presence (Knl2 Off) of IAA. The merging of mScarlet tagged CENP-A, LacI/EGFP double fused protein and DAPI stained chromosome Z was shown in the bottom. The LacO locus was highlighted by an orange arrow. Bar, 2.5 μm.

C.

The intensity of mScarlet tagged CENP-A at the LacO locus shown in (B) in Knl2-AID cells in the absence (Knl2 On) or presence (Knl2 Off) of IAA. The mean ± standard deviation (SD) of the intensity values was shown. (n = 25; ****, $P < 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, $P > 0.05$; unpaired t test, two tailed).

D.

Visualization of mScarlet-tagged CENP-A and LacI/EGFP double-fused CENP-C (601–864) at the LacO locus on chromosome Z on Day1, and 2 (cells almost died in day3) in the scheme of (A) in AID-Mis18α cells in the presence (Mis18α Off) or absence (Mis18α On) of IAA. Merged images of mScarlet tagged CENP-A, LacI/EGFP double fused protein and DAPI stained chromosome Z are shown in the bottom. The LacO locus is indicated by an orange arrow. Bar, 2.5 μm.

E.

The intensity of mScarlet-tagged CENP-A at the LacO locus in AID-Mis18α cells in the absence (Mis18α On) or presence (Mis18α Off) or of IAA is shown. The mean ± standard deviation (SD) of the intensity values is shown. (n = 25; ****, $P < 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, $P > 0.05$; unpaired t test, two tailed).

F.

The visualization of mScarlet tagged CENP-A and LacI/EGFP double fused Knl2 or CENP-I at the LacO locus on the chromosome Z at Day1, 2, and 3 in the scheme of (A) in CENP-C-AID cells in the absence (CENP-C On) or presence (CENP-C Off) of IAA. The merging of mScarlet tagged CENP-A, LacI/EGFP double fused protein and DAPI stained chromosome Z was shown in the bottom. The LacO locus was highlighted by an orange arrow. Bar, 2.5 μm.

G.

The intensity of mScarlet tagged CENP-A at the LacO locus shown in (D) in CENP-C-

AID cells in the absence (CENP-C On) or presence (CENP-C Off) of IAA. The mean ± standard deviation (SD) of the intensity values was shown. (n = 25; ****, $P < 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, $P > 0.05$; unpaired t test, two tailed).

In the context of CCAN dependent CENP-A incorporation, it has been observed that CENP-I tethering requires the presence of CENP-C for successful CENP-A incorporation (**Figure 2-12B, C; Figure 2-13F, G**), which piques our curiosity about the reverse that does CENP-C, particularly CENP-C C-terminus tethering, necessitate CENP-I for CENP-A incorporation? However, a major obstacle hindering our exploration of this question is that CENP-I knockout induces severe mitotic arrest, whereas CENP-A incorporation predominantly occurs during the G1 phase, requiring cells to exit mitosis. This inherent problem renders it theoretically challenging to assess CENP-A incorporation under CENP-I knockout conditions. Consequently, I devised a strategy to overcome this issue by utilizing mitotic arrest inactivation. To conduct our experiment, I employed cells containing LacO array in chromosome Z, where mScarlet tagged CENP-A and LacI/EGFP double-fused CENP-C C-terminus were expressed. In this cell line, endogenous CENP-I was replaced with an AID-tagged version, and HJURP was overexpressed to enhance CENP-A incorporation. To initiate the assay, I added nocodazole to the cell culture to arrest cells at mitosis, and IAA was introduced to induce CENP-I knockout. After 5 hours of incubation, IPTG was washed out to initiate LacI/EGFP double fused CENP-C C-terminus tethering. Following 3 hours of incubation, I sampled some cells for chromosome spread before exiting mitosis. Next, I introduced reversine to the cell culture to trigger mitosis exit, as reversine acts as a spindle checkpoint inhibitor (Santaguida et al., 2010). After an additional 3 hours of incubation, I removed reversine to allow cells to arrest at the subsequent mitosis. Finally, following 10 hours of incubation, I sampled cells for chromosome spread to examine CENP-A incorporation within one complete cell cycle (**Figure 2-14A**).

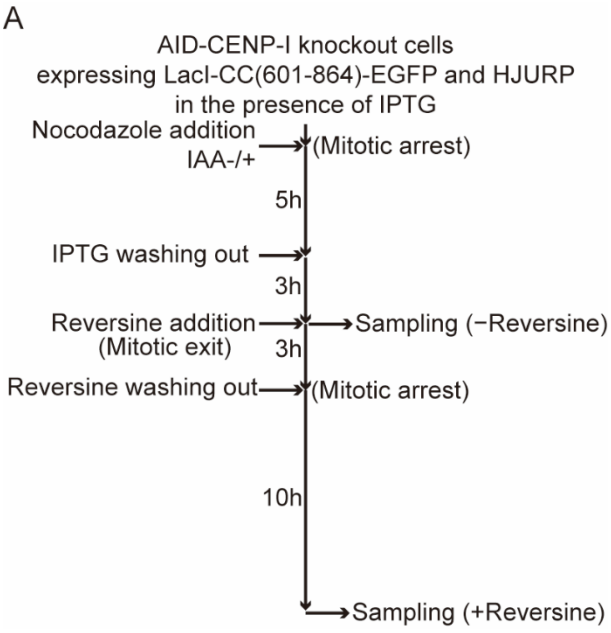


Figure 2-14 Assessment of CENP-A incorporation at LacO locus under AID based protein knockout condition depending on CENP-C C-terminus tethering within one cell cycle

A.

The experimental scheme to visualize CENP-A and other centromeric proteins at the LacO locus in AID-CENP-I cells expressing LacI/EGFP double fused CENP-C (601-864). These cell lines were cultured in the presence of IPTG and the assay commenced with the addition of Nocodazole in either the absence or presence of IAA. After 5 hours incubation, IPTG was washed out. Following an additional 3 hours incubation, chromosome spreads were prepared using some cells (-Reversine). The remaining cells were treated with Reversine for 3 hours, followed by a washout of Reversine. After a subsequent 10-hours incubation, chromosome spreads were prepared (+Reversine).

Previously, our research demonstrated that CENP-A incorporation, induced by CENP-C C-terminus tethering, relies on the presence of HJURP but not Knl2 or Mis18α (**Figure 2-9B, C; Figure 2-10B, C; Figure 2-11B, C; Figure 2-13B, C, D, E**). I employed this improved experimental approach and validated this conclusion comprehensively within one complete cell cycle (**Figure 2-15A, B**). Our findings consistently support our previous observation.

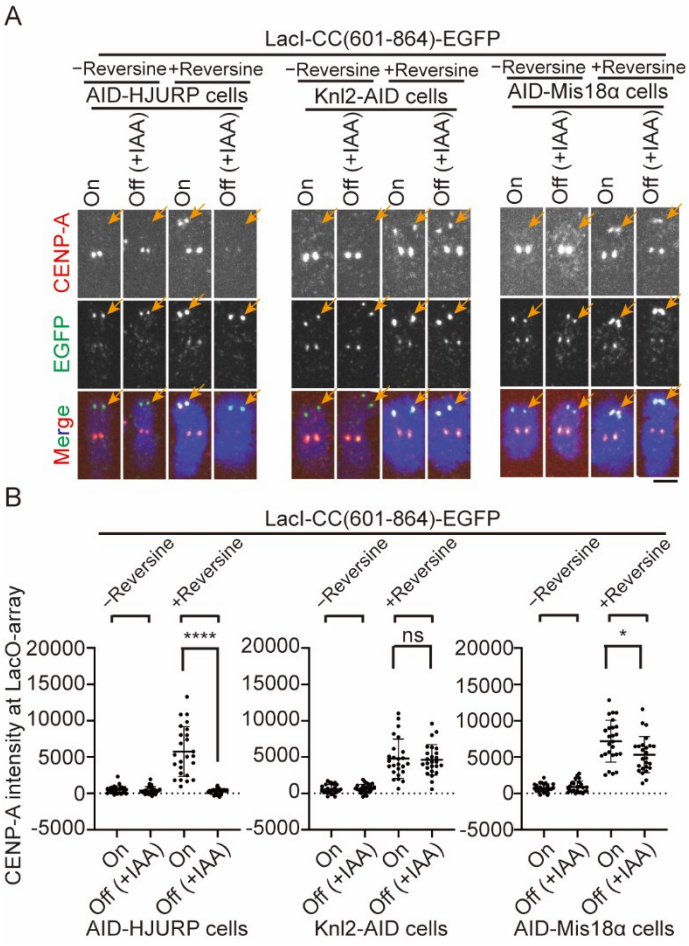


Figure 2-15 CENP-C C-terminus dependent CENP-A incorporation within one cell cycle requires HJURP but not Knl2 or Mis18α

A.

The visualization of mScarlet tagged CENP-A and LacI/EGFP double fused CENP-C (601-864) at the LacO locus in AID-based knockout cells for HJURP, Knl2 or Mis18α. Experiments were performed, as shown in **Figure 2-14A**. Observation was conducted both before and after the addition of reversine, in the absence or presence of IAA. The merging of mScarlet tagged CENP-A, LacI/EGFP double fused CENP-C (601-864) and DAPI stained chromosome Z was shown in the bottom. The LacO locus was highlighted by an orange arrow. Bar, 2.5 μm.

B.

The intensity of mScarlet tagged CENP-A at the LacO locus shown in (A). The mean ± standard deviation (SD) of the intensity values was shown. (n = 25; ****, $P < 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, $P > 0.05$; unpaired t test, two tailed).

Subsequently, I investigated CENP-A incorporation during CENP-C C-terminus tethering under conditions of CENP-I knockout. Notably, before mitosis exit, CENP-A incorporation did not occur, irrespective of the presence or absence of CENP-I. However, after mitosis exit, I observed CENP-A incorporation, independent of CENP-I (**Figure 2-16A, B, C**), thereby indicating that CENP-C C-terminus dependent CENP-A incorporation does not necessitate CENP-I.

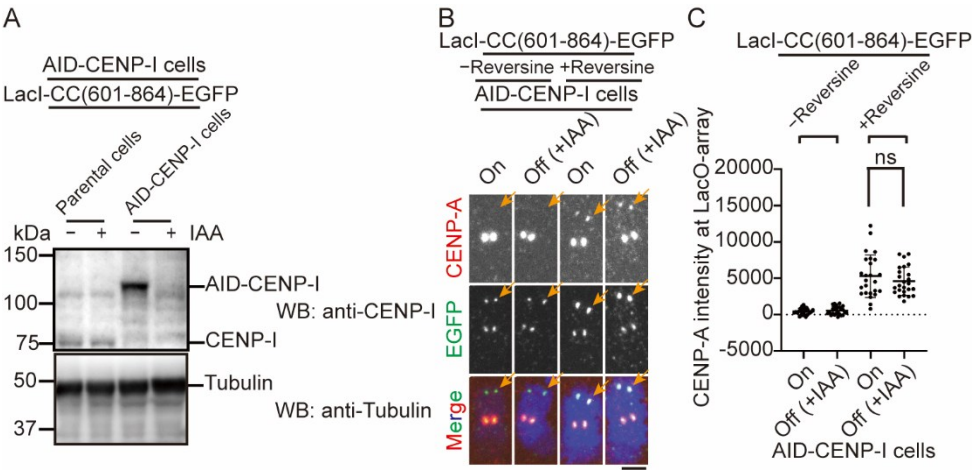


Figure 2-16 CENP-C C-terminus dependent CENP-A incorporation within one cell cycle doesn't require CENP-I

A.

Confirmation of protein degradation of AID tagged CENP-I in cells expressing LacI/EGFP double-fused CENP-C C-terminus (601-864). The cells were cultured either without IAA (-IAA) or with IAA (+IAA) for 5 hours.

B.

The visualization of mScarlet tagged CENP-A and LacI/EGFP double fused CENP-C

(601-864) at the LacO locus in AID-CENP-I cells. This visualization was performed before and after the addition of Reversine, in the absence or presence of IAA, according to **Figure 2-14A**. The merging of mScarlet tagged CENP-A, LacI/EGFP double fused CENP-C (601-864) and DAPI stained chromosome Z was shown in the bottom. The LacO locus was highlighted by an orange arrow. Bar, 2.5 μm.
C.
The intensity of mScarlet tagged CENP-A at the LacO locus shown in (B) in AID-CENP-I cells in the absence (CENP-I On) or presence (CENP-I Off) of IAA. The mean ± standard deviation (SD) of the intensity values was shown. (n = 25; ****, $P < 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, $P > 0.05$; unpaired t test, two tailed).

Furthermore, I sought to explore CENP-A incorporation during CENP-C C-terminus tethering under CENP-C knockout conditions. Considering that the CENP-C C-terminus harbors a dimerization domain, it was essential to rule out the possibility that it recruits another full-length CENP-C protein for CENP-A incorporation. Our results demonstrate that even in the absence of full-length CENP-C, CENP-A incorporation persists after mitosis exit (**Figure 2-17A, B, C**), strongly implying that the CENP-C C-terminus itself is sufficient for inducing CENP-A incorporation.

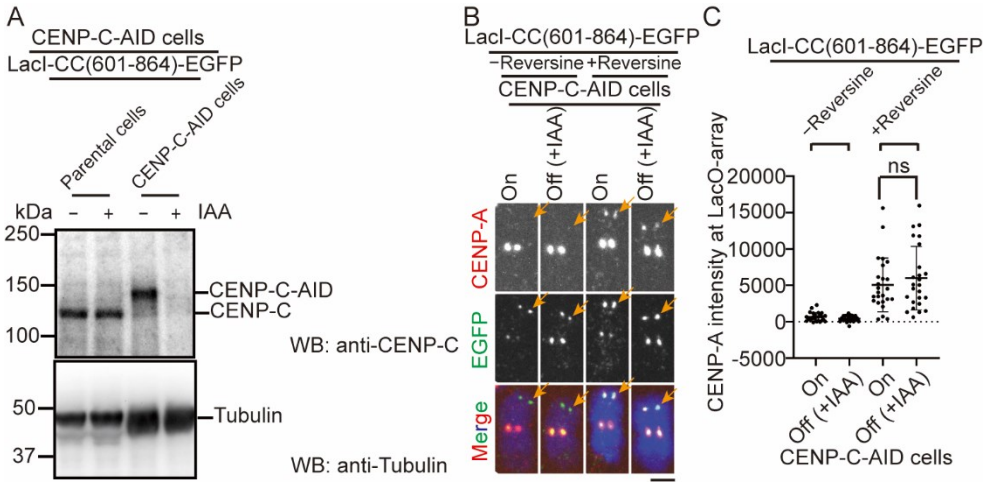


Figure 2-17 CENP-C C-terminus dependent CENP-A incorporation within one cell cycle doesn't require CENP-C
A.
Confirmation of protein degradation of AID tagged CENP-C in cells expressing LacI/EGFP double fused CENP-C C-terminus (601-864). The cells were cultured either with without IAA (-IAA) or IAA (+IAA) for 5 hours.
B.
The visualization of mScarlet tagged CENP-A and LacI/EGFP double fused CENP-C (601-864) at the LacO locus in AID-based knockout cells for CENP-C. Experiments were performed, as shown in **Figure 2-14A**. Observation was conducted both before and after the addition of reversine, in the absence or presence of IAA. The merging of mScarlet tagged CENP-A, LacI/EGFP double fused CENP-C (601-864) and DAPI

stained chromosome Z was shown in the bottom. The LacO locus was highlighted by an orange arrow. Bar, 2.5 μm.
B.
The intensity of mScarlet tagged CENP-A at the LacO locus shown in (A). The mean ± standard deviation (SD) of the intensity values was shown. (n = 25; ****, P < 0.0001; ***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05; ns, P > 0.05; unpaired t test, two tailed).

In conclusion, our comprehensive analysis revealed that in the absence of HJURP, none of the tethering of other factors induced CENP-A incorporation at the LacO locus. As anticipated, Knl2 effectively induced CENP-A incorporation at the LacO locus even in the absence of CENP-C. Interestingly, both CENP-C C-terminus and CENP-I exhibited the ability to induce CENP-A incorporation at the LacO locus independently of the Mis18 complex component Knl2. Moreover, the CENP-C C-terminus exhibits the capacity to trigger CENP-A incorporation at the LacO locus even in the absence of the Mis18 complex component, Mis18α. Furthermore, while both CENP-C C-terminus and CENP-I could trigger CENP-A incorporation, CENP-C C-terminus-induced CENP-A incorporation occurred in the absence of CENP-I, whereas the reverse was not observed, indicating that CENP-I based CENP-A incorporation relies on the presence of CENP-C (**Figure 2-18A**).

A

Knocked out protein	Tethered protein	CENP-A incorporation at the LacO-array
HJURP	Knl2	−
	CENP-I	−
	CENP-C(601-864)	−
Knl2	CENP-I	+
	CENP-C(601-864)	+
Mis18α	CENP-C(601-864)	+
CENP-C	Knl2	+
	CENP-I	−
CENP-I	CENP-C(601-864)	+

Figure 2-18 Summary for CENP-A incorporation dependency based on various inducer tethering
A.
Summary of requirement for CENP-A incorporation at the LacO locus induced by Knl2, CENP-I or CENP-C C-terminus tethering.

2.4 CENP-A recruitment by CENP-I tethering requires a proper CCAN formation

CENP-I typically forms a closely-knit complex with CENP-H/K/M, which constitutes one of the subcomplexes within the CCAN. Recently, the Cryo-EM structure of the human CCAN has been successfully resolved, encompassing the complete assembly of the CENP-H/I/K/M complex (Pesenti et al., 2022; Tian et al., 2022; Yatskevich et al., 2022). By employing the human structure as a reference, I generated a homology model of the chicken CENP-H/I/K/M complex. Within one CENP-H/I inter-surface of this homology model, we found a potential interaction site, in which W212 of CENP-H appears to insert into a hydrophobic pocket formed by CENP-I residues V136, V139, L175, and I178. Notably, a sequence alignment analysis of the corresponding CENP-H/I region among various vertebrate species revealed a high degree of conservation in these hydrophobic residues (**Figure 2-19A, B**).

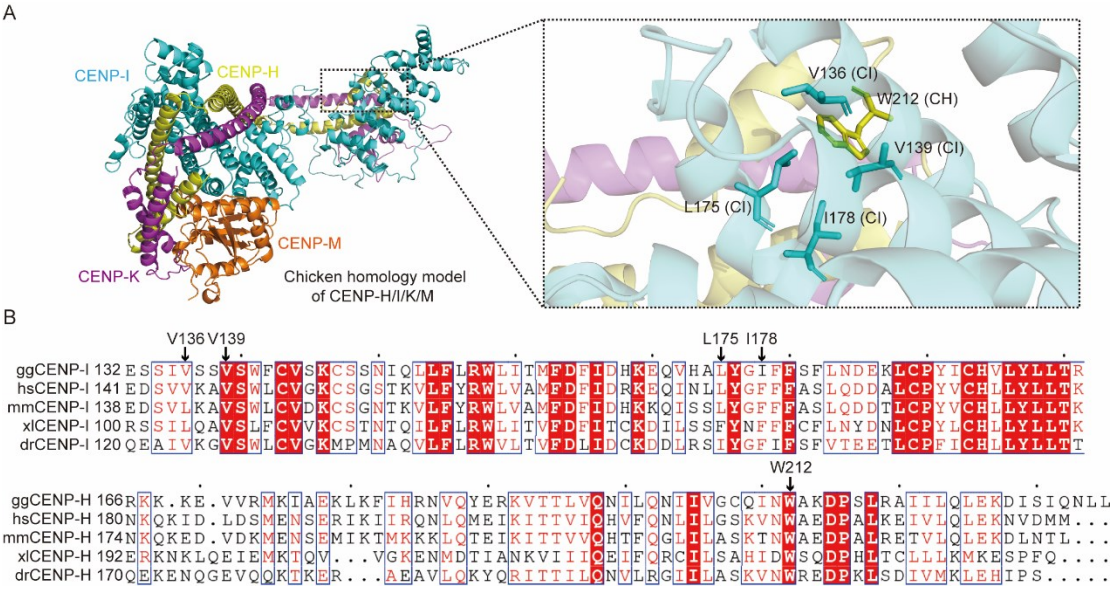


Figure 2-19 Interaction site within CENP-H/I inter-surface

A.

A homology model of chicken CENP-H/I/K/M. A magnified view shows hydrophobic interaction site between CENP-H and CENP-I in the CENP-I N-terminal Heat-repeat, with critical residues represented for clarity.

B.

Alignment of CENP-I N-terminal and CENP-H C-terminal sequences in various species. These regions are expected involving a hydrophobic interaction between CENP-H and CENP-I. Predicted critical residues involved in this interaction are depicted.

To delve deeper into this interaction, I introduced alanine mutations into CENP-I at residues V136, V139, L175, and I178, creating a CENP-I mutant termed CENP-I(4A). Subsequently, I expressed CENP-I(4A) tagged with EGFP in a Tet-responsive CENP-I condition knock out cell line, allowing CENP-I expression to be controlled by tetracycline (Tet) treatment. After 12 hours of Tet addition, western blot analysis revealed the absence of CENP-I in the cells, in contrast to the constant expression of CENP-I(WT)-EGFP or CENP-I(4A)-EGFP (**Figure 2-20A**). Notably, mere CENP-I knock-out caused cell proliferation to cease after 48 hours, leading to subsequent cell death. However, expressing CENP-I(WT)-EGFP successfully rescued cell proliferation, while expressing CENP-I(4A)-EGFP failed to do so (**Figure 2-20B**), indicating that CENP-I(4A) is not functional. To assess CENP-H/I/K/M complex formation, I conducted immunoprecipitation with anti-GFP after CENP-I knock-out. The results revealed that CENP-H, CENP-K, CENP-M, and even CENP-C could be immunoprecipitated with CENP-I(WT)-EGFP, whereas these CENP proteins could not be immunoprecipitated with CENP-I(4A)-EGFP (**Figure 2-20C**), which indicates CENP-I(4A) disrupts the CENP-H/I/K/M complex formation and affects the CCAN formation. This disruption may explain why CENP-I(4A) lacks functionality. Moreover, I examined the localization of CENP-I(4A)-EGFP in cells after CENP-I knockout. While CENP-I(WT)-EGFP was expected to properly localize at the centromere, CENP-I(4A) lost its chromatin localization, indicating its inability to accurately target the centromere (**Figure 2-20D**).

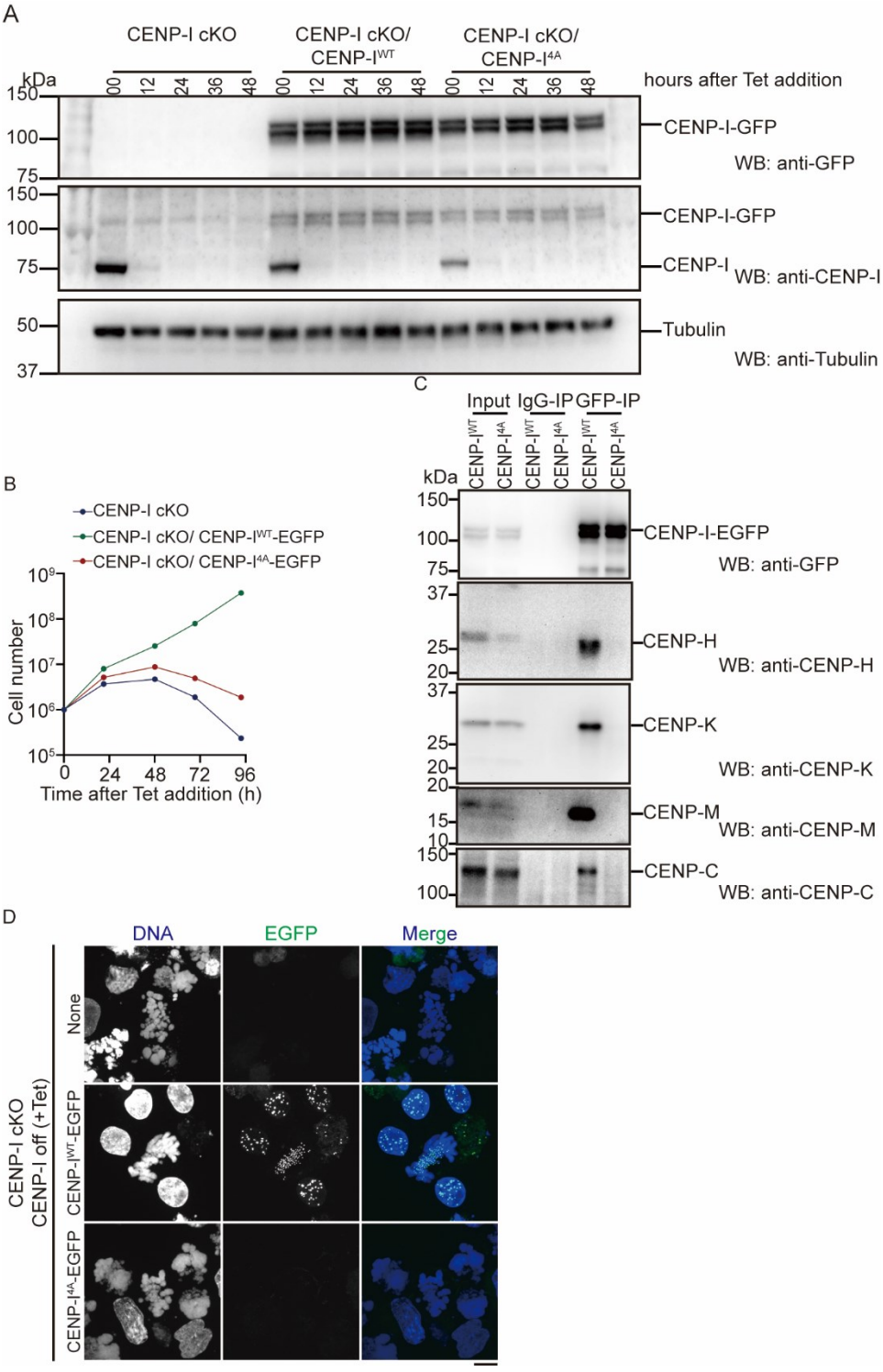


Figure 2-20 CENP-I(4A) disrupts CCAN formation

A.

Expression of EGFP fused CENP-I (WT or 4A mutant) in cKO-CENP-I DT40 cells. The indicated times mean incubation period after Tet addition. Turn-off of CENP-I transgene expression upon Tet addition was confirmed by Immunoblot analysis with an anti-CENP-I antibody.

B.

Proliferation record of cKO-CENP-I cells expressing GFP-fused ggCENP-I WT or 4A mutant, at indicated time points after Tet addition.
C.
Immunoprecipitation (IP) with an anti-GFP antibody for cKO-CENP-I cells expressing either CENP-I^{WT}-EGFP or CENP-I^{4A}-EGFP in the presence of Tet for 12 hours. Immunoprecipitated samples were detected by specific antibodies for (CENP-H, CENP-K, CENP-M, and CENP-C.
D.
Localization of EGFP tagged ggCENP-I WT or 4A expressed in cKO-CENP-I cells in the presence of Tet. Bar, 10μm.

Having established that CENP-I(4A) disrupts CENP-H/I/K/M complex formation, and consequent CCAN assembly, I aimed to investigate whether CENP-I(4A) can induce CENP-A incorporation at the LacO locus independently of the CENP-H/I/K/M complex. To explore this, I utilized cells containing the LacO array on chromosome Z, where I expressed mScarlet tagged CENP-A along with either LacI/EGFP double fused CENP-I(WT) or CENP-I(4A). Additionally, I expressed Halo tagged CENP-C in these cells. Following IPTG washing out to initiate tethering, I sampled the cells for chromosome spread daily during a three-day incubation period. Our observations revealed a stark contrast between CENP-I(WT) and CENP-I(4A) tethering. While CENP-I(WT) tethering induced CENP-A incorporation and recruited CENP-C to the LacO locus, CENP-I(4A) tethering failed to induce CENP-A incorporation or recruit CENP-C at the LacO locus (**Figure 2-21A, B, C, D**). Collectively, these results strongly suggest that CENP-I alone might not possess the ability to induce CENP-A incorporation. The formation of the CENP-H/I/K/M complex appears to be crucial for CENP-C recruitment, which in turn is essential for inducing CENP-A incorporation at the LacO locus.

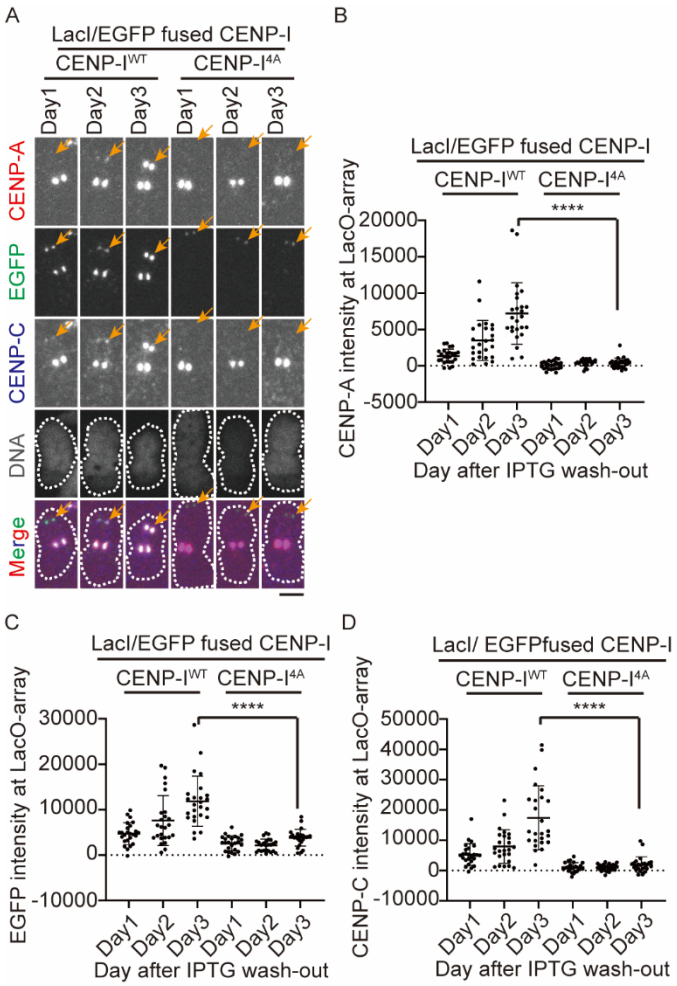


Figure 2-21 Tethering CENP-I(4A) doesn't induce CENP-A at LacO locus

A.

The visualization of mScarlet tagged CENP-A, LacI/EGFP double fused CENP-I (WT or 4A) and Halo tagged CENP-C at the LacO locus on the chromosome-Z at Day1, 2, and 3 after removal of IPTG in the scheme of **Figure 2-6A**. The merging of mScarlet tagged CENP-A, LacI/EGFP double fused CENP-I (WT or 4A) and Halo tagged CENP-C with outline of chromosome Z was shown in the bottom. The LacO locus was highlighted by an orange arrow. Bar, 2.5 μm.

B

(G) The intensity of mScarlet tagged CENP-A at the LacO locus in cells with LacO array on chromosome Z expressing LacI/EGFP double fused CENP-I (WT or 4A), shown in (A). The mean ± standard deviation (SD) of the intensity values was shown. (n = 25; ****, $P < 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, $P > 0.05$; unpaired t test, two tailed).

C

The intensity of EGFP at the LacO locus in cells with LacO array on chromosome Z expressing LacI/EGFP double fused CENP-I (WT or 4A), shown in (A). The mean ± standard deviation (SD) of the intensity values was shown. (n = 25; ****, $P < 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, $P > 0.05$; unpaired t test, two tailed).

D
The intensity of Halo taggedCENP-C at the LacO locus in cells with LacO array on chromosome Z expressing LacI/EGFP double fused CENP-I (WT or 4A), shown in (A). The mean ± standard deviation (SD) of the intensity values was shown. (n = 25; ****, P < 0.0001; ***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05; ns, P > 0.05; unpaired t test, two tailed).

2.5 CENP-C-CENP-I interaction is essential for CENP-I dependent CENP-A incorporation at the LacO locus

As demonstrated in the previous findings, CENP-I tethering alone failed to induce CENP-A incorporation at the LacO locus in the absence of CENP-C (**Figure 2-12B, C; Figure 2-13F, G**). Additionally, the CENP-I(4A) mutant, which disrupts the CENP-H/I/K/M complex formation, exhibited the inability to recruit CENP-C and induce CENP-A incorporation at the LacO locus (**Figure 2-21A, B, C, D**). Based on these results, I can postulate that CENP-I tethering necessitates CENP-C for CENP-A incorporation at the LacO locus, and the CENP-C-CENP-I interaction plays a vital role in this process. Notably, I found that the C-terminus of CENP-C, containing the CENP-C motif and Cupin domain, is responsible for ectopically inducing CENP-A incorporation (Hori et al., 2013). Furthermore, in middle region of chicken CENP-C, an CCAN-Binding-Domain (CBD) (aa 166-324) exists, which plays a crucial role in interacting with the CENP-H/I/K/M complex (Hara et al., 2023; Nagpal et al., 2015) (**Figure 2-22A**).

Figure 2-22 Domain organization of chicken CENP-C
A.
Diagram depicting the domain organization of chicken CENP-C. The CCAN-binding domain (CBD) spans amino acids 166 to 324, the CENP-C motif at amino acids 655 to 675 and the Cupin-domain at amino acids 761 to 850.

To investigate the necessity of the interaction between CENP-C and CENP-I for CENP-I dependent CENP-A incorporation at the LacO locus, I conducted an experiment using cells containing the LacO array on chromosome Z. In this setup, I expressed mScarlet tagged CENP-A along with LacI/EGFP double fused CENP-I. I introduced an AID-tagged CENP-C to replace endogenous CENP-C and additionally

expressed either Halo tagged CENP-C(WT) or CENP-C(ΔCBD) mutant. Following IPTG washing out to initiate LacI/EGFP double fused CENP-I tethering, I added IAA for AID tagged CENP-C knockout, then sampled the cells for chromosome spread each day over a three days incubation period. As previously mentioned, CENP-C knockout inhibits CENP-A incorporation at the LacO locus through CENP-I tethering (**Figure 2-12B, C; Figure 2-13F, G**). Notably, under CENP-C knockout conditions, additionally expressed Halo tagged CENP-C(WT) could be recruited to the LacO locus, effectively rescuing CENP-A incorporation at LacO locus. Conversely, additionally expressed Halo tagged CENP-C(ΔCBD) mutant could not be recruited to the LacO locus and failed to rescue CENP-A incorporation at LacO locus (**Figure 2-23A, B, C, D, E**). This finding indicates that the CCAN Binding Domain (CBD) of CENP-C is indispensable for CENP-I dependent CENP-A incorporation, suggesting that CENP-C recruitment by CENP-I tethering via the CBD of CENP-C is critical for CENP-A incorporation at the LacO locus.

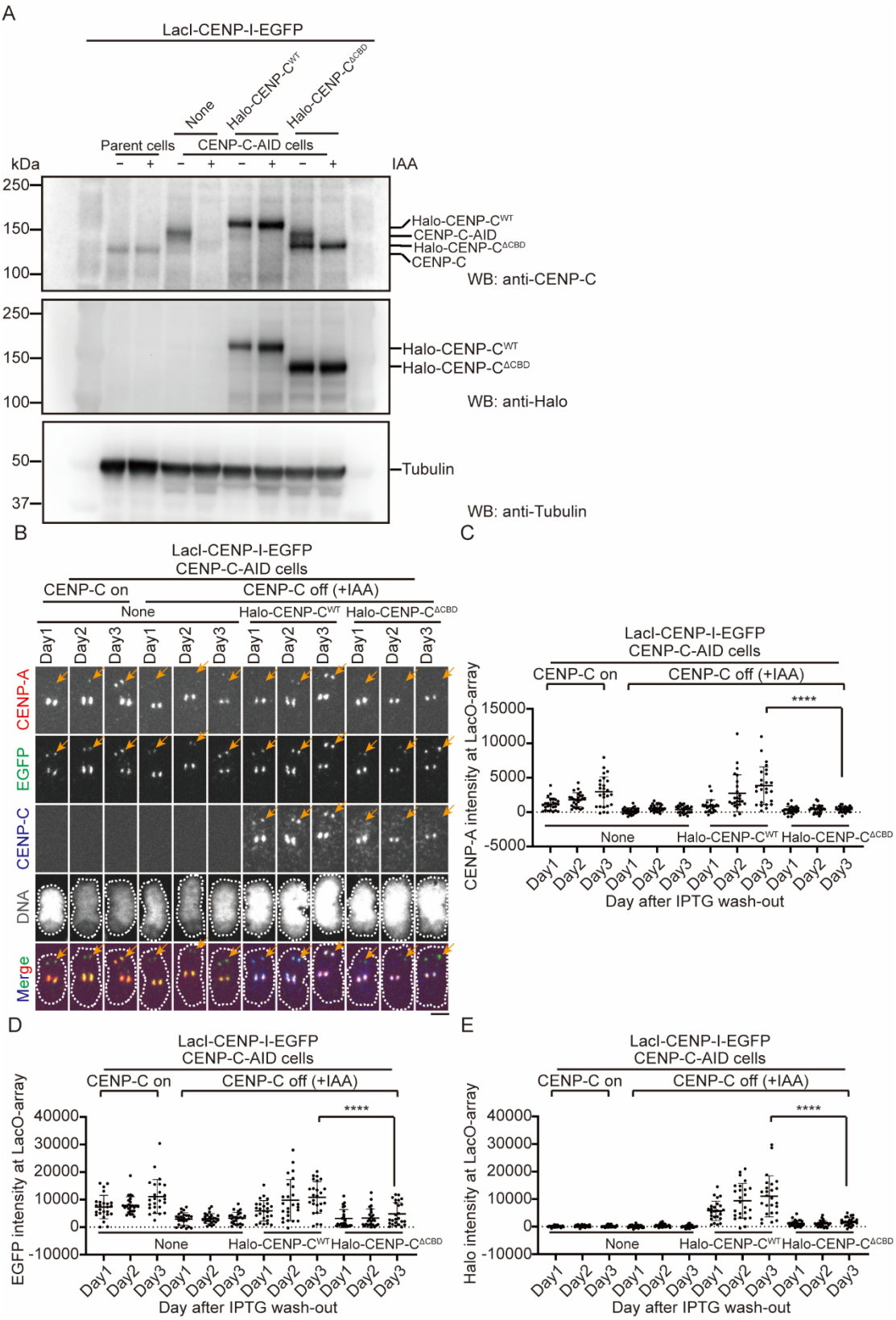


Figure 2-23 CBD of CENP-C is required for CENP-I dependent CENP-A incorporation

A.

Expression of Halo tagged CENP-C (WT or ΔCBD mutant) in CENP-C-AID cells expressing LacI/EGFP double fused CENP-I. The cells were cultured either without IAA (-IAA) or with IAA (+IAA) for 5 hours. Both anti-Halo and anti-CENP-C antibodies were used.
B.
The visualization of mScarlet tagged CENP-A and LacI/EGFP double fused CENP-I, and Halo tagged CENP-C (WT or ΔCBD) at the LacO locus on the chromosome Z at Day1, 2, and 3 in the scheme of **Figure 2-8A** in CENP-C-AID cells expressing Halo tagged CENP-C (WT or ΔCBD) in the presence of IAA. The merging of mScarlet tagged CENP-A, LacI/EGFP double fused CENP-I and Halo tagged CENP-C (WT or ΔCBD) with outline of chromosome Z was shown in the bottom. The LacO locus was highlighted by an orange arrow. Bar, 2.5 μm.
C
The intensity of mScarlet tagged CENP-A at the LacO locus shown in (B) in CENP-C-AID cells expressing Halo tagged CENP-C (WT or ΔCBD) in the presence of IAA. The mean ± standard deviation (SD) of the intensity values was shown. (n = 25; ****, $P < 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, $P > 0.05$; unpaired t test, two tailed).
D
The intensity of LacI/EGFP double fused CENP-I at the LacO locus shown in (B) in CENP-C-AID cells expressing Halo-tagged CENP-C (WT or ΔCBD) in the presence of IAA. The mean ± standard deviation (SD) of the intensity values was shown. (n = 25; ****, $P < 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, $P > 0.05$; unpaired t test, two tailed).
E
The intensity of Halo tagged CENP-C at the LacO locus shown in (B) in CENP-C-AID cells expressing Halo-tagged CENP-C (WT or ΔCBD) in the presence of IAA. The mean ± standard deviation (SD) of the intensity values was shown. (n = 25; ****, $P < 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, $P > 0.05$; unpaired t test, two tailed).

2.6 CENP-C C-terminus recruits HJURP directly for loading CENP-A at the LacO locus

As previously demonstrated, CENP-I dependent CENP-A incorporation necessitates the presence of the CCAN Binding Domain (CBD) of CENP-C, enabling it to interact with CENP-H/I/K/M (**Figure 2-23A, B, C, D, E**). Additionally, I have found that the C-terminus of CENP-C is responsible for inducing CENP-A incorporation (Hori et al., 2013) (**Figure 2-6B, C**), and remarkably, this CENP-C C-terminus induced CENP-A incorporation occurs even in the absence of Knl2 (**Figure**

2-10A, B, C; Figure 2-13A, B, C; Figure 2-15A, B), Mis18α (**Figure 2-11A, B, C; Figure 2-13D, E; Figure 2-15A, B)**, CENP-I (**Figure 2-16A, B, C**) and CENP-C(FL) **Figure 2-17A, B, C**) but not in absence of HJURP (**Figure 2-9A, B, C; Figure 2-15A, B**). These compelling findings prompt us to consider the possibility that the CENP-C C-terminus may directly interact with HJURP to induce CENP-A incorporation at the LacO locus, operating independently of the well-known Mis18 complex-HJURP dependent CENP-A loading pathway.

At first, to investigate the specific region of HJURP responsible for interacting with CENP-C, I transiently overexpressed GFP tagged CENP-C(FL) along with Flag tagged HJURP(FL), HJURP N-terminus (aa 1-254), and HJURP C-terminus (aa 255-end) in chicken DT40 cells. Subsequently, I performed immunoprecipitation using anti-GFP to assess the protein-protein interactions. Remarkably, I observed that the N-terminus of HJURP, but not the C-terminus, co-immunoprecipitated with CENP-C (**Figure 2-24A**). This finding clearly indicates that the N-terminus of HJURP is the key region responsible for the interaction with CENP-C, and it differs from the Mis18 complex interaction region (aa 255-500 of chicken HJURP) (Perpelescu et al., 2015).

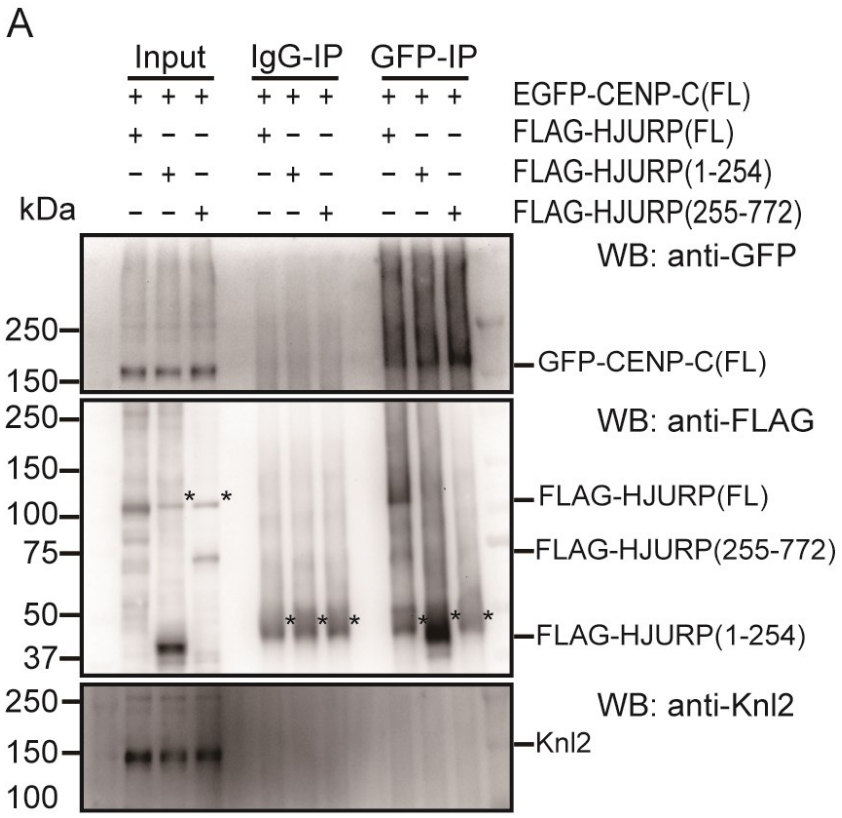


Figure 2-24 HJURP N-terminus is responsible for interaction with CENP-C
A.
Co-immunoprecipitation (Co-IP) with an anti-GFP antibody in cells transiently expressing GFP-CENP-C (FL) and various FLAG-HJURP (FL, 1-254, or 255-772). Immunoprecipitated samples were detected by specific antibodies for GFP, FLAG or Knl2. An asterisk (*) indicates a non-specific band.

As previously demonstrated, our findings underscore the role of CENP-C C-terminus in initiating CENP-A incorporation at the LacO locus. This strongly suggests that the CENP-C C-terminus likely holds the key to interacting with HJURP, thus triggering the process of CENP-A incorporation. To investigate the interaction between CENP-C C-terminus and HJURP, I conducted a detailed dissection of the CENP-C C-terminus (aa 601-864). This region comprises a CENP-C motif, responsible for direct interaction with the CENP-A nucleosome (Ariyoshi et al., 2021; Watanabe et al., 2019), and a Cupin domain, responsible for CENP-C dimerization (Hara et al., 2023). Within the Cupin domain, Y799 and H843 are crucial residues that localize at the dimer interface and are involved in CENP-C dimerization (Hara et al., 2023). Even though the CENP-C C-terminus mutant (Y799A, H843A) retains its dimeric form (unpublished data), as additional dimer hooks within the CENP-C C-terminus also contribute to the dimerization process (Hara et al., 2023). In our study, I divided the CENP-C C-terminus into two regions: the CENP-C motif containing region (aa 601-720) and the Cupin domain containing region (aa 721-864). I analyzed these CENP-C C-terminus truncation mutants combined with CENP-C C-terminus (aa 601-864, Y799A, H843A) mutant additionally to gain insights into how the CENP-C C-terminus interacts with HJURP (**Figure 2-25A**).

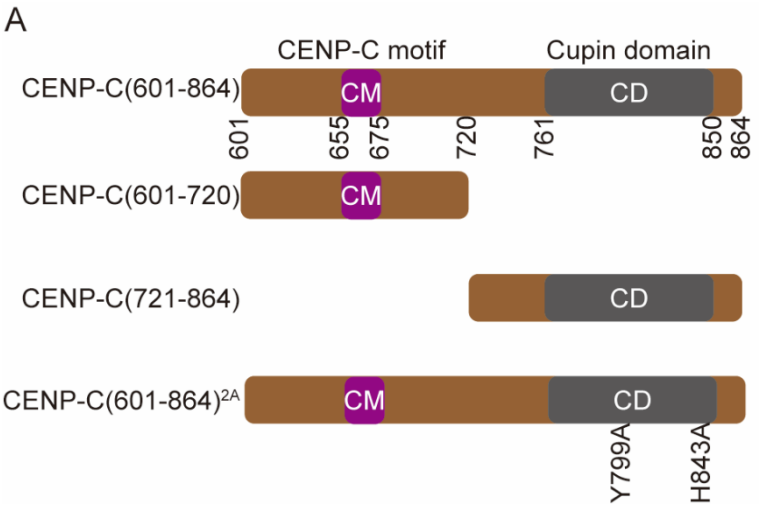


Figure 2-25 Illustration of chicken CENP-C C-terminus mutants
A.
The schematic representation illustrates the mutants of the chicken CENP-C C-terminus (601-864) used for Co-immunoprecipitation (IP) experiments. The CENP-C motif containing region encompasses amino acids 601-720, while the Cupin domain containing region spans amino acids 721-864. The CENP-C C-terminus (601-864)2A mutants involve the replacement of Y799 and H843 with alanine.

Similarly, I conducted transient overexpression of the EGFP tagged CENP-C C-terminus mutants mentioned above, along with Flag tagged HJURP(FL), in chicken

DT40 cells. Subsequently, I performed immunoprecipitation using anti-GFP to assess the protein-protein interactions. I found that neither the CENP-C motif containing region (aa 601-720) nor the Cupin domain containing region (aa 721-864) alone could co-immunoprecipitate HJURP. In contrast, the CENP-C C-terminus mutant (aa 601-864, Y799A, H843A) exhibited a robust co-immunoprecipitation with HJURP, akin to the wild-type CENP-C C-terminus (aa 601-864) (**Figure 2-26A**). These findings indicate that both the CENP-C motif and the Cupin domain are essential for the interaction between CENP-C and HJURP. The dimeric form of CENP-C also appears to be critical for this interaction, as demonstrated by the CENP-C C-terminus mutant (aa 601-864, Y799A, H843A), which retains the dimeric structure and retains its ability to interact with HJURP.

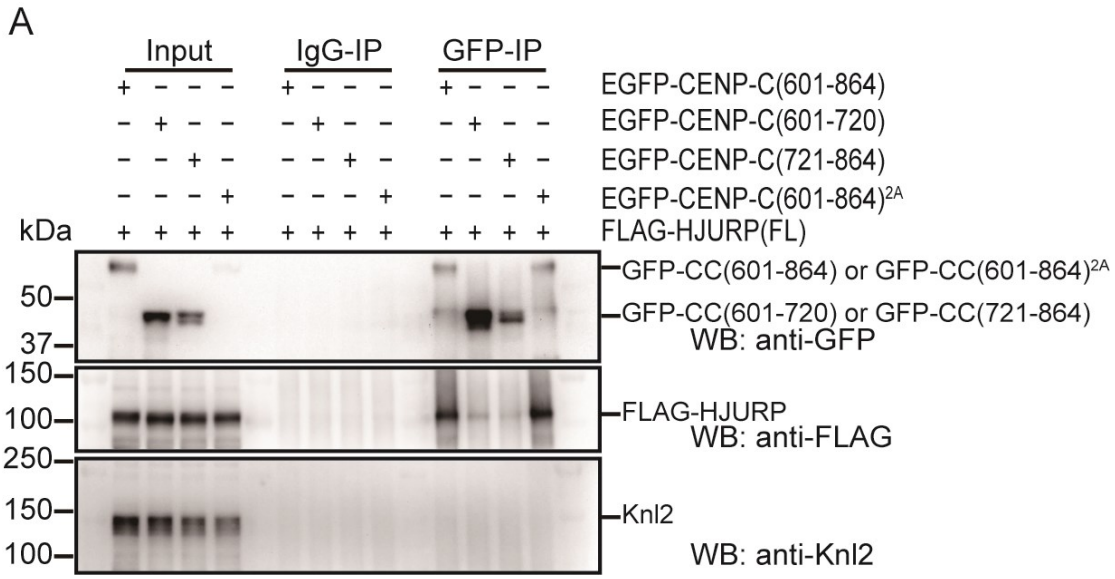


Figure 2-26 Interaction between CENP-C C-terminus and HJURP requires CENP-C motif, Cupin domain and dimer form
A.
Co-immunoprecipitation (Co-IP) with an anti-GFP antibody in cells transiently expressing various GFP tagged CENP-C mutants shown in **Figure 2-22A** and FLAG tagged HJURP. Immunoprecipitated samples were detected by specific antibodies for GFP, FLAG or Knl2.

Next, I sought to determine whether the CENP-C C-terminus mutants could induce CENP-A incorporation at the LacO locus. To this end, I utilized cells containing the LacO array on chromosome Z, expressing mScarlet tagged CENP-A, and LacI/EGFP double fused with the various CENP-C C-terminus mutants mentioned above **(Figure 2-25A)**. Following IPTG washing out to initiate tethering, I sampled cells for chromosome spread daily during a three days incubation period. As I expected, I observed that both the CENP-C motif containing region (aa 601-720) and the Cupin domain containing region (aa 721-864), which failed to interact with HJURP, were unable to induce CENP-A incorporation at the LacO locus. Conversely, some colonies

expressing the CENP-C C-terminus (aa 601-864, Y799A, H843A) mutant did exhibit CENP-A incorporation at the LacO locus, albeit with reduced intensity compared to the wild-type CENP-C C-terminus. These findings indicate that the proper interaction between the CENP-C C-terminus and HJURP is indispensable for CENP-C C-terminus induced CENP-A incorporation at the LacO locus (**Figure 2-27A, B**).

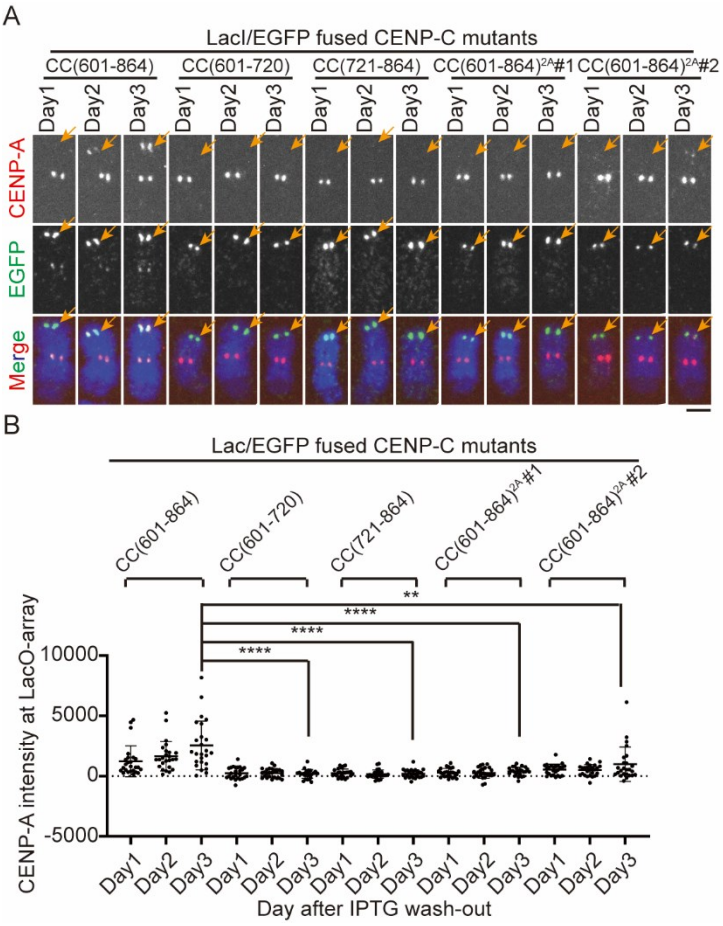


Figure 2-27 Proper interaction between CENP-C C-terminus and HJURP is required for CENP-C C-terminus induced CENP-A incorporation at LacO locus
A.
The visualization of mScarlet tagged CENP-A and LacI/EGFP double fused various CENP-C mutants at the LacO locus on the chromosome Z at Day1, 2, and 3 in the scheme of **Figure 2-6A**. The merging of mScarlet tagged CENP-A, LacI/EGFP double fused CENP-C mutants and DAPI stained chromosome Z was shown in the bottom. The LacO locus was highlighted by an orange arrow. Bar, 2.5 μm.
B.
The intensities of mScarlet tagged CENP-A at the LacO locus shown in (A). The mean ± standard deviation (SD) of the intensity values was shown. (n = 25; ****, $P < 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, $P > 0.05$; unpaired t test, two tailed).

Finally, I investigated the timing of HJURP recruitment by tethered CENP-C C-

terminus. As the recruitment of HJURP at native centromeres is a tightly regulated process that occurs only during early G1 phase (Bodor et al., 2013; Jansen et al., 2007), immediately following mitosis exit, I was curious to understand when the recruitment takes place in our ectopic tethering system. To address this, I employed cells containing the LacO array on chromosome Z and expressing mScarlet tagged CENP-A, along with LacI/EGFP double fused CENP-C C-terminus. I additionally expressed Halo tagged HJURP in this cell line. I washed out IPTG to initiate CENP-C C-terminus tethering. Four hours later, I sampled the cells for observation. Remarkably, I observed that HJURP recruitment at the LacO locus by CENP-C C-terminus occurred exclusively during the interphase, especially G1 phase as HJURP localizing to native centromere, while no recruitment was detected during the M phases. This result indicates that tethered CENP-C C-terminus selectively recruits HJURP during the G1 phase, mirroring the timing observed for HJURP recruitment at native centromeres (**Figure 2-28A**).

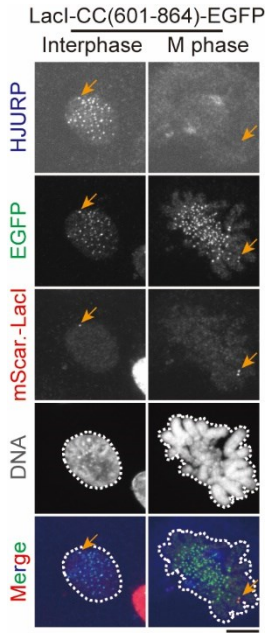


Figure 2-27 Tethered CENP-C C-terminus recruits HJURP to LacO locus at G1 phase exclusively

A.

Representative images of the localization of Halo tagged HJURP and LacI/EGFP double-fused CENP-C C-terminal (601–864) at the LacO locus. LacO locus was identified by mScarlet-LacI, which was transiently expressed. Merged images of mScarlet-LacI, LacI/EGFP double fused CENP-C C-terminus and Halo tagged HJURP with outline of nucleus are shown. The LacO locus was indicated by an orange arrow. Images of interphase and mitotic cells at 4 h after removing IPTG are shown. Bar, 12μm.

2.7 Working hypothesis

Our group developed an epigenetic approach to induce CENP-A incorporation at designated loci (LacO locus) by concomitantly expressing LacI fused CENP-A incorporation inducers to seed new centromeres in a given chromosome (chicken chromosome Z). Through our investigations, our group successfully identified several proteins that could serve as effective CENP-A incorporation inducers, including HJURP, specifically its N-terminus, Knl2, CENP-I, and CENP-C, particularly its C-terminus. HJURP is a known CENP-A specific chaperone, and the N-terminal region of HJURP (aa 1-55) has the ability to directly interact with the CENP-A/H4 heterodimer (Hori et al., 2020), facilitating its incorporation into chromatin to form a CENP-A containing nucleosome. In native centromeres, HJURP localization is tightly regulated to occur exclusively during the G1 phase, limiting CENP-A incorporation. Therefore, direct tethering of HJURP resulted in robust CENP-A incorporation at the LacO locus, as the LacI fused HJURP remained localized to the LacO locus throughout all phases of the cell cycle (**Figure 2-29A**). When Knl2 was tethered, it formed the Mis18 complex, and the Mis18α/β subunits recruited HJURP for CENP-A incorporation during the G1 phase, mirroring the process observed at native centromeres (**Figure 2-29B**). As I demonstrated, when the C-terminus of CENP-C was tethered, it could directly recruit HJURP during the G1 phase for CENP-A loading (**Figure 2-29C**). In the case of CENP-I tethering, it first formed the CENP-H/I/K/M complex, followed by the recruitment of CENP-C through the CBD of CENP-C. Subsequently, the recruited CENP-C used its C-terminus to directly recruit HJURP during the G1 phase, leading to CENP-A incorporation, akin to the direct tethering of the CENP-C C-terminus alone (**Figure 2-29D**).

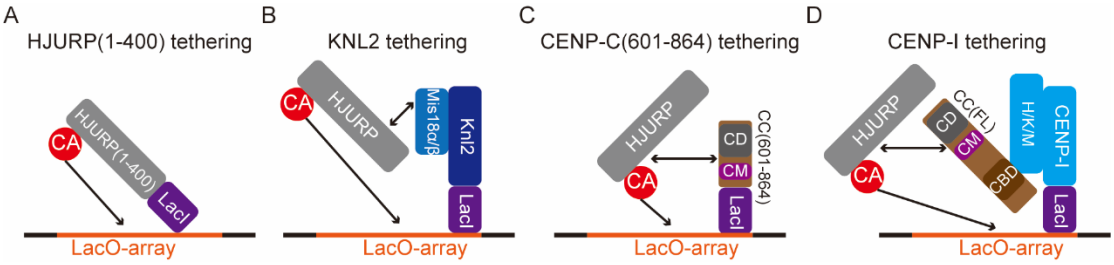


Figure 2-29 Model of artificial new centromere formation induced by HJURP, Knl2, CENP-C C-terminus or CENP-I

A.

I propose a model by which distinct mechanisms govern the artificial new centromere formation by induction of CENP-A to the non-centromere LacO locus. Since the CENP-A specific chaperone HJURP directly binds to CENP-A via its Scm-domain (1-55),

HJURP tethering might directly recruit CENP-A to the LacO locus.
B.
Knl2 forms the Mis18 complex with Mis18α/β, and therefore, the Mis18 complex should be formed on the LacO locus by Knl2 tethering. Thus, Knl2 tethering causes CENP-A incorporation at the LacO locus via HJURP-Mis18 complex interaction.
C.
CENP-C C-terminus (601-864) directly binds to HJURP independent on the Mis18 complex and induces CENP-A incorporation at the LacO locus via HJURP-CENP-C interaction.
D.
CENP-I tethering causes formation of the CENP-H/I/K/M complex at the LacO locus, which recruits CENP-C through its CCAN-binding domain. Then, CENP-C recruits HJURP and induces CENP-A incorporation like CENP-C tethering.

3. Discussion

In accordance with our prior investigation (Hori et al., 2013; Perpelescu et al., 2015), I have confirmed that tethering HJURP N terminus (aa 1-400), Knl2, CENP-I, and CENP-C C terminus (aa 601-864) leads to CENP-A incorporation at the LacO locus. HJURP, a specific chaperone for CENP-A, interacts with the Mis18 complex containing Knl2, which suggests that these components collectively recruit CENP-A to the LacO locus (**Figure 2-29**). However, the mechanism through which CENP-I or CENP-C C terminus (aa 601-864) induces CENP-A incorporation at the LacO locus remained unclear, prompting us to investigate this aspect in the present study. Our findings demonstrate that CENP-I or CENP-C C terminus (aa 601-864) can induce CENP-A incorporation at the LacO locus even in the absence of Knl2, while CENP-I alone is unable to do so without CENP-C (**Figure 2-10; Figure 2-12; Figure 2-13**). Moreover, I observed that CENP-C C-terminus can associate with HJURP independently of Knl2 (**Figure 2-26**). Based on these observations, I propose that CENP-C C-terminus directly binds to HJURP, facilitating CENP-A incorporation, and that CENP-I recruits CENP-A through the CENP-C-HJURP interaction to the LacO locus in chicken DT40 cells (**Figure 2-29**). This newly identified CENP-C-HJURP pathway operates independently of the Mis18 complex-HJURP pathway in inducing CENP-A incorporation.

Surprisingly, our findings revealed that tethering the CCAN components (CENP-I, CENP-C, and CENP-C (601-864)) at the LacO locus resulted in a higher rate of efficient new centromere formation compared to tethering the CENP-A incorporation machinery components (HJURP (1-400) and Knl2) with the same induction time of 3 days (**Figure 2-1B**). This observation can be attributed to two main aspects. Firstly, the centromere's crucial function is to recruit CCAN for the outer kinetochore recruitment, in turn the microtubules' attachment. Tethering CCAN components at the LacO locus may lead to the recruitment of other CCAN proteins, partially fulfilling CCAN

functions before subsequent CENP-A incorporation. This accelerated process reduces the time required for the artificial new centromere to become fully functional. Secondly, HJURP, as the CENP-A specific chaperone, is normally recruited exclusively to native centromeres during G1 phase. However, tethering HJURP leads to its continuous localization at the LacO locus throughout the cell cycle. This caused hyper CENP-A incorporation at the LacO locus and a relatively low proliferation rate (**Figure 2-4A, B; Figure 2-6B, C**). These results indicate that an overload of CENP-A could be detrimental to cells. Regarding the lower new centromere formation rate observed with Knl2 tethering at the LacO locus, I currently lack a clear explanation. However, considering that tethering Knl2 (Mis18BP1) alone could not induce CENP-A incorporation in human cells (Shono et al., 2015), I believe that tethering Knl2 alone may not fully mimic its native centromere function. Consequently, our results suggest that tethering the CCAN components (CENP-I, CENP-C, and CENP-C (601-864)) might be a more favorable option than tethering CENP-A incorporation machinery components (HJURP and Knl2) when seeding a new centromere for a given chromosome.

Interestingly, our investigation revealed that the knockout of Knl2 results in a hyperactivated CENP-A incorporation at the LacO locus, a phenomenon that is dependent on CENP-I tethering **(Figure 2-10 B, C; Figure 2-13 B, C)**. Despite this intriguing observation, the underlying reasons for this phenomenon remain elusive. It appears that Knl2 might play a role in suppressing CENP-A incorporation in some manner. Notably, when Knl2 is knocked out, CENP-A incorporation still occurs at the LacO locus, albeit without hyperactivation, under the CENP-C C-terminus tethering condition **(Figure 2-10 B, C; Figure 2-13 B, C)**. An interesting observation is that Knl2 seems to rely on its interaction with CENP-I, rather than CENP-C, to execute its function of suppressing CENP-A incorporation. Moreover, it is worth noting that the phenomenon of Knl2 mediated suppression of CENP-A incorporation was exclusively observed at the LacO locus and not at the native centromere (data not shown). This observation led us to speculate that Knl2's role in suppressing CENP-A incorporation might specifically apply to non-centromeric loci. It is conceivable that Knl2's function involves restraining CENP-A incorporation at non-centromeric sites, ensuring that the centromeric epigenetic marker CENP-A is exclusively inherited at native centromeres.

Our findings demonstrate that CENP-A incorporation can still occur at the LacO locus even in the absence of Knl2 when CENP-C C-terminus is tethered. However, I did observe that the intensity of CENP-A at the LacO locus is slightly diminished following Mis18α knockout **(Figure 2-15 A, B)**. This intriguing result suggests that Mis18 complex might play a role in bolstering the stability of CENP-A on chromatin. This role could potentially be attributed to Knl2's direct interaction with CENP-A nucleosomes through a CENP-C like motif resembling that of Knl2 (Hori et al., 2017; Jiang et al., 2023).

Our results clearly illustrate that tethering CENP-I(4A) fails to bring about the recruitment of CENP-C to the LacO locus and subsequent induction of CENP-A incorporation **(Figure 2-21 A, B, D)**. Interestingly, the level of tethered CENP-I(4A) remained consistently steady, contrasting with the gradual increase observed in the case

of tethered CENP-I(WT) after initiation of tethering **(Figure 2-21 A, C)**. This phenomenon could potentially be attributed to the fact that the incorporated CENP-A, induced by CENP-I(WT), further facilitates the recruitment of additional CENP-I(WT) independently of tethering. Given that CENP-I(4A) lacks functionality, the quantity of CENP-I(4A) at the LacO locus appears to depend on the extent of tethering that occurs.

I have already established that CENP-I tethering is contingent upon CENP-C for CENP-A incorporation at the LacO locus. Particularly noteworthy is the requirement for the interaction between CENP-C and CENP-H/I/K/M, which is indispensable for enabling CENP-I tethering mediated CENP-A incorporation **(Figure 2-23B, D, E)**. Given that CENP-C C-terminus can directly engage HJURP to load CENP-A **(Figure 2-26A; Figure 2-27A, B; Figure 2-28A)**, it becomes conceivable that, subsequent to CENP-I tethering, the initial step involves the formation of the CENP-H/I/K/M complex, which subsequently recruits CENP-C through its interaction with the CBD of CENP-C. Following this, the recruited CENP-C employs its C-terminal domain to directly engage HJURP, thereby orchestrating the loading of CENP-A (**Figure 2-29D**).

Our investigation has revealed that under artificial conditions, tethering of the CENP-C C-terminus is a pivotal factor in inducing CENP-A incorporation at the LacO locus. Intriguingly, our observation of the interaction between the HJURP N-terminus and CENP-C C-terminus in chicken cells occurs under more general circumstances **(Figure 2-24A; Figure 2-26A)**. This suggests that the CENP-C C-terminus possesses a broad capability to interact with HJURP, transcending specific experimental setups. The localization of EGFP-HJURP$^{N\text{-terminus}}$-LacI at native centromeres during interphase (G1) **(Figure 2-7A)** aligns with the timing observed for HJURP recruitment by CENP-C C-terminus tethering at LacO louc **(Figure 2-28A)**. Notably distinct from the impact of HJURP knock-out, as evidenced by compromised CENP-A levels solely at the native centromere of chromosome Z, the depletion of Knl2, Mis18α, or CENP-C **(Figure 2-10 B, C; Figure 2-11 B, C; Figure 2-12 B, C; Figure 2-13 B, C, D, E, F, G)** underscores a significant likelihood of CENP-C C-terminus-dependency in HJURP recruitment within the natural context. Recent reports also indicate that the interaction between CENP-C C-terminus and HJURP N-terminus is conserved in other organisms, such as Xenopus (Flores Servin et al., 2023; French et al., 2017), in addition to previously observed interactions in humans (Tachiwana et al., 2015). This growing body of evidence prompts an essential question: Does the interaction between CENP-C C-terminus and HJURP N-terminus indeed occur within native centromeres, and if so, what characteristic does it play in HJURP recruitment and the loading of new CENP-A? However, the situation becomes more intricate due to the reported dependence of CENP-I (partial via CENP-C) and CENP-C tethering on Mis18BP1 (Knl2) for CENP-A incorporation in human cells (Shono et al., 2015). The complexity deepens as I contemplate the potential implications of CENP-C-HJURP-dependent CENP-A incorporation, given that the conventional pathway for CENP-A incorporation heavily relies on the Mis18 complex-HJURP mechanism. This prompts further inquiries into the potential interplay between these two distinct pathways and their respective contributions to CENP-A deposition.

Materials and Methods

Chicken DT40 cells

I used the chicken DT40 CL18 cell line as the wild-type (Buerstedde et al., 1990). The cells were cultured at 38.5°C in DMEM supplemented with 10% FBS, 1% chicken serum, and Penicillin-Streptomycin (referred to as DT40 culture medium).

In our previous studies (Hori et al., 2013), I created a cell line in which chromosome Z was engineered. In this cell line, a LacO-array (256× repeat) was inserted at the p-arm and two LoxP sites were introduced across the native centromere of chromosome Z. To establish a chromosome Z engineered cell lines expressing EGFP-HJURP (1-400)-LacI, LacI-KnlNL2-EGFP, LacI-CENP-I-EGFP, LacI-CENP-C(FL)-EGFP, or LacI-CENP-C (601-864)-EGFP under control of phosphoglycerate kinase (PGK1) promoter, I co-transfected a plasmid encoding each LacI/EGFP fused protein with a hygromycin-resistant gene and a pX335-PGK1 plasmid encoding sg RNA for targeting into PGK1 gene locus and Cas9 (D10A) using electroporation. The transfected cells were selected in the DT40 culture medium containing 2.5 mg/ml hygromycin and 20 μM IPTG (for suppression of LacI-LacO interaction). Subsequently, the cells expressing mScarlet tagged CENP-T or mScarlet tagged DsnI under the endogenous promoter were established by a CRISPR/Cas9 mediated gene targeting method.

To establish a chromosome Z engineered cell line expressing mScarlet tagged CENP-A or CENP-A under control of the β-actin promoter, a plasmid encoding mScarlet fused ggCENP-A or ggCENP-A was co-transfected with an EcoGPT gene and pX335-β-actin encoding sgRNA targeting into β-actin gene locus and Cas9 (D10A) using electroporation. The transfected cells were selected in the DT40 culture medium containing 25 μg/ml mycophenolic acid and 125 μg/ml xanthine. Using this cell line, plasmids encoding EGFP-LacI, EGFP-HJURP (1-400)-LacI, LacI-KnlNL2-EGFP, LacI-CENP-I^{WT}-EGFP, LacI-CENP-I^{4A} (V136A, V139A, L175A, I178A)-EGFP, LacI-CENP-C (FL)-EGFP, LacI-CENP-C (601-864)-EGFP, LacI-CENP-C (601-720)-EGFP, LacI-CENP-C (721-864)-EGFP, or LacI-CENP-C $(601\text{-}864)^{2A}$ (Y799A, H843A)-EGFP with hygromycin resistant genes were co-transfected with pX335-PGK1 encoding Cas9 (D10A). The transfected cells were selected in the DT40 culture medium containing 2.5 mg/ml hygromycin and 20 μM IPTG.

To create AID (Auxin-induced-degron) based knockout lines for HJURP, Knl2, CENP-I, CENP-C or Mis18α expressing LacI-Knl2-EGFP, LacI-CENP-I-EGFP, or LacI-CENP-C (601-864)-EGFP, a linearized pAID plasmid containing HJURP, Knl2, CENP-I, CENP-C or Mis18α cDNA and pX330 containing sgRNA (which targets the chicken HJURP, KnlNL2, CENP-I or CENP-C endogenous gene locus) and Cas9 gene, were co-transfected into each line expressing LacI-Knl2-EGFP, LacI-CENP-I-EGFP, or LacI-CENP-C (601-864)-EGFP (Nishimura & Fukagawa, 2017). The transfected cells were then selected in the DT40 culture medium containing 1 mg/ml L-Histidinol

dihydrochloride and 20 μM IPTG. To verify the successful knockout of the AID fusion protein after indole-3-acetic acid (IAA) addition, immunoblot analyses were performed to confirm complete replacement of the endogenous target protein with AID fused one and the knockout of AID fused proteins. To degrade AID fused protein, 500 μM IAA (Sigma) was used. To facilitate CENP-A incorporation in the AID based Knl2, CENP-I, CENP-C knockout cells, a plasmid encoding Halo tagged HJURP with a puromycin resistance gene was transfected using electroporation. The Halo-HJURP integrated cells were then selected in the DT40 culture medium containing 0.5 μg/ml puromycin and 20 μM IPTG.

To conduct Figure 4 experiments, I generated LacI-CENP-I^{WT}-EGFP or LacI-CENP-I^{4A} (V136A, V139A, L175A, I178A)-EGFP cell line expressing mScarlet-CENP-A and Halo-CENP-C. I introduced plasmids encoding Halo-CENP-C (WT) with a puromycin resistance gene into each LacI/EGFP double fused CENP-I expressing cells. Cells were selected using 0.5 μg/ml puromycin and 20 μM IPTG in the DT40 culture medium.

To investigate the replacement of CENP-C by its mutant under CENP-I tethering (Figure 5), I utilized the CENP-C-AID line expressing mScarlet tagged CENP-A and LacI-CENP-I-EGFP. To introduce plasmids encoding Halo-CENP-C^{WT} or Halo-CENP-C$^{(\Delta 166\text{-}324:\ \Delta CBD)}$ with a puromycin resistance gene, electroporation was employed. The resulting Halo-CENP-C^{WT} or Halo-CENP-C$^{\Delta CBD}$ integrated cells were selected using 0.5 μg/ml puromycin and 20 μM IPTG in DT40 culture medium.

The ggCENP-I conditional knockout (cKO) cell line has been previously described (Nishihashi et al., 2002). To suppress expression of CENP-I, 2 μg/ml Tet. (Sigma) was added to the culture medium. To generate a cKO-ggCENP-I cell line expressing either CENP-I^{WT}-EGFP or CENP-I^{4A} (V136A, V139A, L175A, I178A)-EGFP under PGK1 promoter, a plasmid encoding the desired protein along with a hygromycin resistance gene and pX335-PGK1 was co-transfected into the cells using electroporation. Following transfection, cells were selected in DT40 culture medium containing 2.5 mg/ml hygromycin (**Figure 3-1A**).

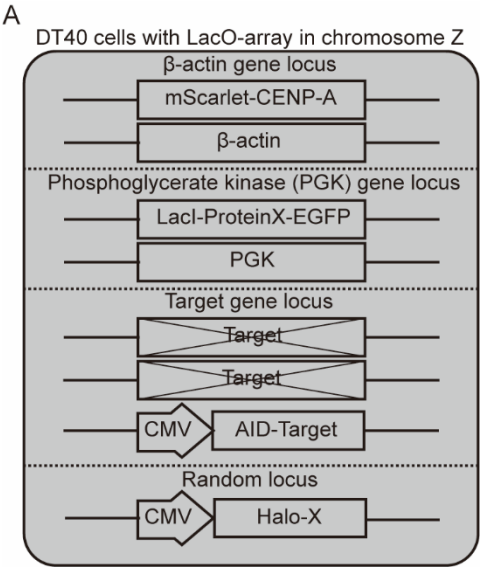


Figure 3-1 Generation of each cell lines
Schematic representation for generation of each cell lines. An mScarlet tagged CENP-A expression cassette was integrated into one of β-actin allele. A LacI/EGFP double fusion expression cassette containing target gene was integrated into one of Phosphoglycerate kinase (PGK) gene allele. For generation of AID-based knockout cell lines, endogenous alleles of a target gene were disrupted by using CRISPR-Cas9 gene editing, and a construct containing AID tagged cDNA of a target gene was randomly integrated into genome. Additionally, Halo tagged protein expression cassette was randomly integrated into genome.

Cell counting

To quantify the number of DT40 cells, a 10 μl sample of cultured medium was mixed with same volume of 0.4 wt/vol% solution of Trypan Blue (Wako) and assessed using a Countess II automated cell counter (Thermo Fisher).

Plasmid constructions

To generate a construct for integration of mScarlet fused CENP-A into one β-actin allele using CRISPR/Cas9 genome editing, the mScarlet, ggCENP-A, IRES2, and EcoGPT sequences were amplified using PCR and cloned into the pBluescript SK vector, which contains a 2-kb β-actin genome region surrounding exon 1 by an In-Fusion method (Takara). A sgRNA sequence surrounding the start of β-actin exon 1 for targeting was designed using Cas-Designer and was cloned into the pX335 plasmid (Addgene 54233), which encodes SpCas9 nickase (D10A) (Cong et al., 2013). The final plasmid is called pX335-β-actin.

To generate the constructs for integration of EGFP/LacI double-fusion proteins into one PGK1 allele using CRISPR/Cas9 genome editing, the following components were amplified using PCR: EGFP, LacI, protein gene (HJURP (1-400), KnlNL2, CENP-I^{WT}, CENP-I^{4A} (V136A, V139A, L175A, I178A), CENP-C (FL), CENP-C (601-864), CENP-C (601-720), CENP-C (721-864), CENP-C (601-864)^{2A} (Y799A, H843A)), the SV40 promoter, and the hygromycin resistant gene sequence. These components were then cloned into the pBluescript-KS(+) vector, which contains a 2-kb PGK1 genome region surrounding exon 1 by an In-Fusion method (Takara). A sgRNA sequence surrounding the start of β-actin exon 1 for targeting was designed using Cas-Designer and was cloned into the pX335 plasmid (Addgene 54233) (Cong et al., 2013). The final plasmid is called pX335-PGK1.

I generated constructs for integration of EGFP fused CENP-I^{WT} and CENP-I^{4A} (V136A, V139A, L175A, I178A) into one PGK1 allele using CRISPR/Cas9 genome editing. I amplified the EGFP, CENP-I^{WT} or CENP-I^{4A} (V136A, V139A, L175A, I178A) sequences, along with the SV40 promoter and hygromycin resistant gene sequence, using PCR and cloned them into the pBS-KS(+) vector containing the 2 kb PGK1

genome region surrounding exon1 by an In-Fusion method (Takara).

To generate a construct for random integration of AID tagged HJURP, Knl2, CENP-I, CENP-C or Mis18α along with TIR1 into DT40 cells, PCR was used to amplify the HJURP, Knl2, CENP-I, CENP-C or Mis18α sequence respectively and cloned into the pAID vector, which containing TIR1 sequence and a L-Histidinol dihydrochloride resistant gene sequence {Nishimura, 2017 #5681} by an In-Fusion method (Takara). The sgRNA for targeting endogenous HJURP, Knl2, CENP-I, CENP-C or Mis18α respectively were designed using Cas-Designer and was cloned into the pX330 plasmid (Addgene 42330). The final plasmid is called pX330 -HJURP, -Knl2, -CENP-I, -CENP-C or -Mis18α respectively.

To generate constructs for random integration of Halo tagged HJURP, CENP-C^{WT}, or CENP-$C^{\Delta CBD}$ into DT40 cells, PCR was used to amplify the Halo tag respective proteins (HJURP, CENP-C^{WT}, or CENP-$C^{\Delta CBD}$) sequence along with the SV40 promoter and a puromycin resistant gene sequence. The amplified fragments were then cloned into the pEGFP-N1 vector, which lacks the EGFP sequence by an In-Fusion method (Takara).

To generate constructs for transient expression of Flag-tagged HJURP variants (FL, 1-254, and 255-772) and EGFP-tagged CENP-C variants (FL, 601-864, 601-720, 721-864, and 601-864) within DT40 cells, individual HJURP fragments underwent PCR amplification and subsequent cloning into the p3xFLAG-CMVTM-10 vector using the In-Fusion method (Takara). Concurrently, PCR amplification of the CENP-C fragments was performed, followed by their insertion into the pEGFP-C1 vector, also utilizing the In-Fusion method (Takara).

To generate constructs for transient expression of mScarlet-tagged LacI, PCR was used to amplify the mScarlet and LacI sequence. The amplified fragments were then cloned into the pEGFP-N1 vector, lacking the EGFP sequence.

Immunoblotting

To prepare whole-cell samples, DT40 cells were harvested, washed with PBS, and suspended in 1× SDS-PAGE sampling buffer (250mM Tris-HCl pH6.8, 10% SDS, 50% Glycerol, 5%2-Mercaptoethanol, 0.5% Bromophenol blue) (at a final concentration of 5×10^4 cells/μl) prior to sonication and heating for 5 minutes at 96°C. Protein samples were separated on a SuperSep Ace, 5-20% (Wako) gel and then transferred to an Immobilon-P membrane (Merck) using a HorizeBlOT system (ATTO).

The primary antibodies used in this study were as follows: rabbit anti-ggCENP-C (Fukagawa et al., 1999), rabbit anti-ggCENP-H (Fukagawa et al., 2001), rabbit anti-ggCENP-I (Nishihashi et al., 2002), rabbit anti-ggCENP-K {Okada, 2006 #5699}, rabbit anti-ggCENP-M (Okada et al., 2006), rabbit anti-ggKnl2 (Hori et al., 2017), rabbit anti-ggMis18α (Hori et al., 2017), rabbit anti-ggHJURP (Perpelescu et al., 2015), rabbit anti-ggCENP-A (Hori et al., 2020), mouse anti-Halo (PROMEGA), mouse anti-FLAG (Sigma), rabbit anti-GFP (MBL), and mouse anti-α-tubulin (Sigma). The secondary antibodies used were HRP-conjugated anti-rabbit IgG (Jackson ImmunoResearch), HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch), and

HRP-conjugated anti-rabbit IgG true-blot (Rockland). To enhance sensitivity and specificity, Signal Enhancer Hikari (Nacalai Tesque) was used for all antibodies. The membranes were blocked in 5% skim milk dissolved in TBST (20mM Tris-HCl pH7.5; 150mM NaCl; 0.1% Tween-20) if necessary. The membranes were incubated with the 0.02% primary antibodies (0.01% for rabbit anti-ggCENP-C antibody; 0.1% for rabbit anti-ggCENP-I anti-body) for 1 hour at room temperature or overnight at 4°C, and with the 0.005% secondary antibodies (0.003% secondary antibody when rabbit anti-ggCENP-C antibody was used as primary antibody) for 1 hour at room temperature. For antibody stripping, the membranes were incubated in stripping buffer (2% SDS, 62.5mM Tris-HCl, 0.7% 2-Mercaptoethanol) for 20min at 60°C. The proteins that reacted with the antibodies were detected using ECL Prime (GE Healthcare), visualized and quantified with a ChemiDoc Touch system (Bio-Rad). The acquired images were processed using Image Lab 6.1.0 (Bio-Rad) and Illustrator CC (Adobe).

Immunoprecipitation

To immunoprecipitate CENP-I^{WT}-GFP or CENP-I^{4A} (V136A, V139A, L175A, I178A)-EGFP using anti-GFP, I harvested cKO-ggCENP-I cells expressing the respective constructs, which were cultured in the presence of 2 μg/ml Tet. for 12 hours. After cells were washed with PBS, cells were sequentially resuspended in TMS buffer (0.25M sucrose, 10mM Tris-HCl pH7.5, 5mM $MgCl_2$) and Buffer-A (15mM Hepes-NaOH pH7.4, 15mM NaCl, 60mM KCl, 0.34M sucrose, 0.5mM spermidine, 0.15mM spermine, 1mM DTT, and 100ng/ml TSA, supplemented with 1× complete EDTA-free proteinase inhibitor (Roche)). Following centrifugation, the cell pellet was quick-frozen in liquid nitrogen and suspended in Buffer-A again to a final density of 2×10^8 cells/ml. I then added $CaCl_2$ (final: 3mM) and micrococcal nuclease (NEB) (240 gel units/ml at final concentration) and rotated the suspension at room temperature for 1 hour. To stop the reaction, I added EDTA (final: 10mM), followed by NaCl (300mM at final concentration) to the digested chromatin fraction. The solubilized fraction was incubated with either anti-GFP antibody (MBL) or control rabbit IgG (Sigma)-bound Protein-G Dynabeads (Thermo Fisher) at 4 °C for 2 hours. The proteins precipitated with antibody-bound beads were washed with PBS three times and eluted by adding 2× sampling buffer and heating at 96 °C for 5 min.

To perform immunoprecipitation of GFP-CENP-C (FL), GFP-CENP-C (601-864), GFP-CENP-C (601-720), GFP-CENP-C (721-864), and GFP-CENP-C (601-$864)^{2A}$(Y799A, H843A) using anti-GFP, I transiently expressed these constructs along with FLAG-HJURP (FL), FLAG -HJURP (1-254) or FLAG-HJURP (255-772) in wild-type CL18 DT40 cells by transfection of plasmids (40μg plasmids in 150μl suspension buffer containing 3×10^6 cells). The cells were harvested at 24 hours after transfection, washed with PBS, and suspended in lysis buffer (25 mM Tris-HCl pH7.5, 275 mM NaCl, 5 mM $MgCl_2$, 0.1% NP40, 1 mM DTT, 0.5 μM okadaic acid, 5 mM NaF, 0.3 mM Na_3VO_4, 10 mM β-glycerophosphate-$5H_2O$, 50 U/ml Turbo-nuclease). The suspension was incubated on ice for 2 hours followed by incubation at 37°C for 10 min. After centrifugation, the solubilized fraction was incubated with anti-GFP antibody (MBL)-

or control rabbit IgG (Sigma)-bound Protein-G Dynabeads (Thermo Fisher) at 4°C for 2 hours. The proteins precipitated with antibody beads were washed thrice with lysis buffer and twice with PBS, and eluted by adding 2× SDS-PAGE sampling buffer and heating at 96°C for 5 min.

Chromosome observation

DT40 cells were cyto-spun onto glass slides. The cells were fixed with 4% PFA in PBS for 10 minutes, permeabilized in 0.5% NP-40 in PBS for 10 minutes, and stained with 1 μg/ml DAPI in PBS for 10 minutes. The stained samples were washed with PBS and mounted with VECTASHIELD mounting medium (Vector Laboratories).

To observe chromosome spread, DT40 cells were treated with Nocodazole for one hour before cyto-spinning onto glass slides. The cells were expanded in hypotonic buffer (40 mM KCl, 20 mM HEPES-NaOH pH7.5, 0.5 mM EDTA) for 10 minutes at 37°C, before fixation.

To detect chromosome Z, a CasFISH method was used. For CasFISH detection, the samples after permeabilization and before DAPI staining were washed with PBS three times and then incubated with the CasFISH reaction complex (25 nM dCas tagged Halo conjugated with JF646 (Janelia Fluor), 100 nM sgRNA for targeting a chromosome Z specific satellite sequence, 20 mM Hepes-NaOH pH7.4, 100 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 5% BSA, 5 mM DTT, 0.5% Tween20) for 30 minutes at 37°C, followed by washing with PBS three times.

For detection of Halo tagged proteins on chromosome Z, DT40 cells were stained with 200nM JF646 (Janelia Fluor) before cyto-spinning onto glass slides.

Evaluation of efficiency for artificial centromere formation

DT40 cells containing a LacO-array (256×repeat) at end of the p-arm and LoxP-sequences across the native centromere of chromosome Z, and expressing each EGFP/LacI fused target protein were used in this assay. The cells were maintained in medium containing 20 μM IPTG. IPTG was washed with fresh medium twice at 0 day. After three days, 4-hydroxytamoxifen (OHT, Sigma) was added to activate Cre-recombinase. Then, native centromeres were removed by recombination between two LoxP sites. After 16 hours of treatment of OHT, cells were washed with fresh medium twice, and cells were plated into 96-well plates at several dilutions. After incubation for 8 hours, zeocin-containing medium (final concentration at 1 mg/ml) was added to the plates. Then, after further incubation for 16 hours, Fialuridine (FIAU, Sigma)-containing medium (final concentration at 0.5 μM) was added to select surviving cells. After two weeks of selection, numbers of the survival colonies on the plates were counted.

Structure modelling

To generate a homology model of the chicken CENP-H/I/K/M complex, I utilized

the SWISS-MODEL workspace with the cryo-EM structure of the human CENP-H/I/K/M complex (Protein Data Bank (PDB) number: 7QOO) as the template. To visualize the resulting models, I employed the PyMOL Molecular Graphics System, version 2.5.2 (Schrödinger) and generated ribbon presentations of the structures.

Quantification and statistical analysis

The fluorescence signal intensities of EGFP fused proteins, mScarlet fused proteins, or Halo tagged proteins conjugated with JF646 (Janelia Fluor) on the LacO locus were quantified using Imaris software (Bitplane). I measured fluorescence signals of two sister LacO arrays and obtained mean of two sister signals. Twenty-five of LacO signals in each cell were measured and background signals in regions without signals were subtracted, respectively. The plot was made by GraphPad Prism8. In each graph, means of 25 cells and standard deviation were shown. The unpaired two tail t tests were done for corresponding groups.

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Achievements

Publications:

1. Hori, T., Cao, J., Nishimura, K., Ariyoshi, M., Arimura, Y., Kurumizaka, H., & Fukagawa, T. “Essentiality of CENP-A Depends on Its Binding Mode to HJURP”. *Cell reports* (Article), (2020) 33(7), 108388.

2. JingHui Cao, Tetsuya Hori, Mariko Ariyoshi, and Tatsuo Fukagawa. “Artificial tethering of CCAN induces CENP-A deposition without Knl2 in DT40 cells”. *Journal of Cell Science* (Article), (In press)

Poster presentation:

1. Jinghui Cao, Tetsuya Hori, Mariko Ariyoshi, Tatsuo Fukagawa; “Domain analysis of chicken CENP-A using in vitro reconstitution”; FBS Retreat; 12; Awaji Yumebutai, International Conference Center; May 23 (Thu)- May 24 (Fri), 2019

2. Jinghui Cao, Yasuhiro Arimura, Mariko Ariyoshi, Hitoshi Kurumizaka, Tetsuya Hori, and Tatsuo Fukagawa; “Interaction mode between CENP-A and HJURP in vitro”; The 42nd Annual Meeting of the Molecular Biology Society of Japan; 4P-0202; Fukuoka International Congress Center, Fukuoka Sunpalace Hotel & Hall, Marine Messe Fukuoka; Date: 2019.12.3 (Tue) -2019.12.6 (Fri)

3. Jinghui Cao, Yasuhiro Arimura, Mariko Ariyoshi, Hitoshi Kurumizaka, Tetsuya Hori, and Tatsuo Fukagawa; “Biochemical analysis of interaction between CENP-A/H4 and HJURP”; The 43nd Annual Meeting of the Molecular Biology Society of Japan; 3P-0121; online; Date: 2020.12.2 (Wed) -2020.12.4 (Fri)

4. Jinghui Cao, Tetsuya Hori and Tatsuo Fukagawa; “Analysis for the CENP-I dependent CENP-A incorporation pathway”; The 44nd Annual Meeting of the Molecular Biology Society of Japan; 1P-0219; Pacifico Yokohama; Date: 2021.12.1 (Wed) -2021.12.3 (Fri)

5. Jinghui Cao, Tetsuya Hori and Tatsuo Fukagawa; “Analysis for the CENP-I dependent CENP-A incorporation into centromeres”; The 45nd Annual Meeting of the Molecular Biology Society of Japan; 1P-239; Makuhari Messe; Date: 2022.11.30 (Wed) -2022.12.2 (Fri)

6. Jinghui Cao, Tetsuya Hori, Mariko Ariyoshi and Tatsuo Fukagawa; “Analysis for the CCAN dependent CENP-A incorporation”; The 46nd Annual Meeting of the Molecular Biology Society of Japan; 1P-353; Kobe International Conference Center; Date:

2023.12.6 (Wed) -2023.12.8 (Fri)

Award:

1. Monbukagakusho Honors Scholarship for Privately-Financed International Students (2018)

2. Next-Generation Challenging Researcher Fostering Project for Promoting Interdisciplinary Integration and Responsible for Social Implementation (2021-2023)