

Graduate School of Biostudies, Kyoto University

**Development of protein-based artificial kinetochores for
preventing age-related chromosome segregation errors
in oocytes**

周 遠卓

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Abstract

Chromosome segregation error in oocytes increases dramatically with maternal age, leading to miscarriages and congenital diseases. A major cause of these age-related errors is premature chromosome separation, which arises from progressive weakening of chromosome cohesion. This weakening produces a loosely connected chromosome architecture that fails to resist microtubule-driven bipolar pulling forces during meiosis I and II. These microtubule pulling forces are transmitted through kinetochores, large macromolecular complexes assembled at centromeres. However, strategies to manipulate kinetochore-microtubule forces to prevent premature chromosome separation remain largely unexplored. Here, I develop a protein-based artificial kinetochore that prevents meiotic errors in aged mouse oocytes. In this system, the NDC80 complex, a core microtubule-binding component of the kinetochore, is tethered to genetically engineered multimeric (GEM) nanoparticles. The artificial kinetochore establishes bipolar microtubule attachments decorated with the kinetochore fiber-associated protein HURP. Competition between HURP-decorated microtubule fibers at artificial kinetochores and endogenous kinetochores weakens kinetochore-microtubule attachments, thereby reducing microtubule pulling forces exerted on chromosomes without disrupting chromosome segregation. In aged oocytes, this force modulation efficiently prevents premature chromosome separation during unperturbed meiosis I and II, facilitating egg euploidy. Overall, this study introduces a biocompatible, protein-based artificial kinetochore as a promising strategy to prevent meiotic errors in aged oocytes by modulating HURP-decorated kinetochore-microtubule attachments.

Abbreviation

ACA	anti-centromere antibodies
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
IBMX	3-isobutyl-1-metyl-xanthine
MTOC	microtubule-organizing center
mRNA	messenger ribonucleic acid
NEBD	nuclear envelope breakdown
PBS	phosphate buffered saline
PBT	phosphate buffered saline 0.1% TritonX-100
PMSG	pregnant mare serum gonadotrophin
WT	wild type strain
FWHM	full width at half maximum
GEM	genetically encoded multimeric nanoparticles
FP	fluorescent protein
FKBP	FK506 binding protein
FRB	FKBP-rapamycin binding
M-plate	Metaphase plate

Chapter 1

Introduction

1.1. Age-related chromosome segregation errors in oocytes

1.1.1. The finite nature of female reproductive potential

Female reproductive potential declines with advancing maternal age, which is one of the most serious challenges in reproductive medicine. Reproductive aging occurs before systemic health aging, originating in the female germ cell: the oocyte. Unlike male germ cells, which are produced continuously in large numbers throughout adulthood, the pool of oocytes is established during the fetal stage and then remains arrested in the cell cycle until ovulation at the adult stage, when oocyte maturation and chromosome segregation occur. This cell cycle arrest lengthens with age, spanning months in mice and several decades in humans. This extremely long arrest is thought to contribute to the age-related increase in chromosome mis-segregation in oocytes. The consequence of chromosome mis-segregation is profound, leading to the production of aneuploid gametes (eggs) containing an incorrect number of chromosomes. Aneuploidy is the leading cause of age-related infertility, recurrent pregnancy loss, and congenital disorders such as Down syndrome.

The incidence of aneuploidy in human eggs follows a characteristic curve that dramatically increases with maternal age ^{1,2}. Large-scale data from preimplantation genetic testing for aneuploidy (PGT-A) and population-based data also paint a clear picture of the age-related risk. In women aged 26-30 years, meiotic error rates range from 20-27%, and this error rate escalates steadily from age 30 to 43. By the time a woman reaches her early 40s, the rate of embryonic aneuploidy exceeds 60% ³⁻⁵. It is reported that over 90% of viable trisomies detected at birth are of maternal meiotic origin, underscoring the oocyte as the primary source of age-related aneuploidy ⁶.

1.1.2. Hypotheses for high error rates in aged oocytes

The high error rate in aged oocytes results from a combination of multiple factors, including weakened chromosome cohesion and increased metabolic and oxidative stress.

Loss of chromosome cohesion: Age-related weakening of chromosome cohesion is one of the most prevalent and well-studied mechanisms. Chromosome cohesion is maintained by the cohesin complex, which connects sister chromatids to ensure chromosome integrity ⁷. The cohesin complex, containing the meiosis-specific subunit REC8, is loaded onto chromosomes only once, during fetal development, and is never functionally replenished ⁸⁻⁹. Over decades of prophase arrest, these cohesin complexes gradually dissociate from chromosomes, resulting in loosely associated bivalent chromosomes. This “cohesin-loss” model explains one of the most common types of meiotic errors: premature separation of homologous chromosomes in meiosis I, leading to aneuploid oocytes at meiosis II. The protective protein Shugoshin-2 (SGO2), which guards centromeric cohesin from cleavage, also declines in aged oocytes, generating prematurely separated sister chromatids in meiosis

II and thereby aneuploid eggs¹⁰⁻¹² (Fig. 1).

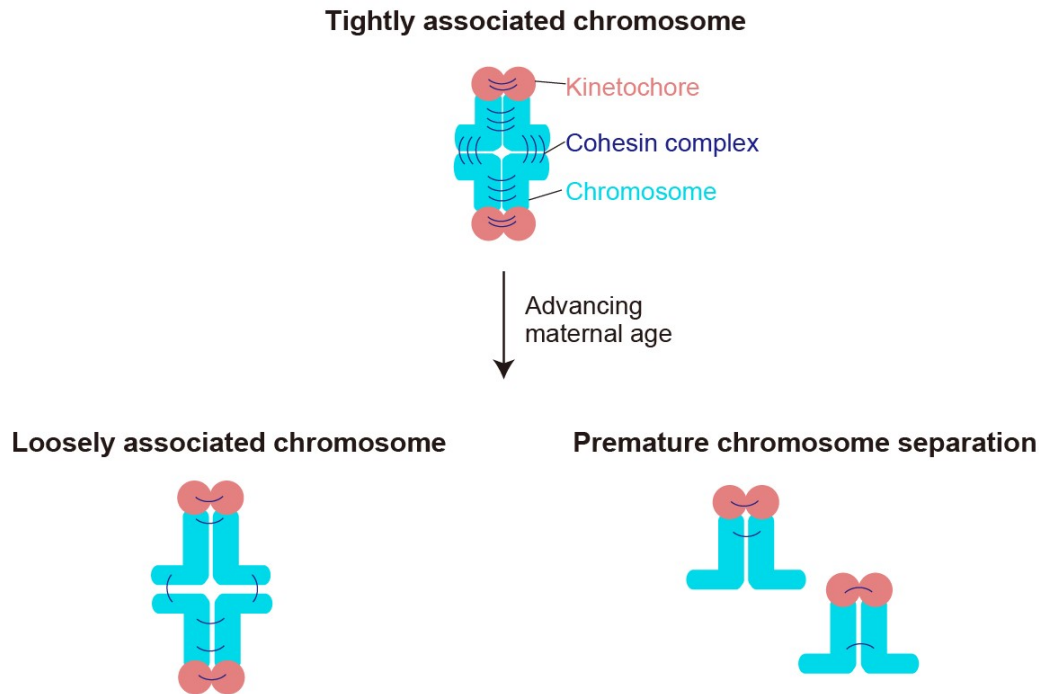


Fig. 1: Age-related chromosome cohesion loss causes premature chromosome separation.

Increased metabolic stress: The metabolite nicotinamide adenine dinucleotide (NAD^+/NADH) is a crucial cofactor and enzyme substrate required for energy metabolism. With advancing maternal age, oocytes undergo a progressive loss of metabolic homeostasis, characterized by a decline in NAD^+ levels and elevated reactive oxygen species (ROS), ultimately leading to the production of aneuploid eggs¹³⁻¹⁵. Oocyte NAD^+ levels drop sharply with maternal aging, and the age-related upregulation of the NADase CD38 further accelerates NAD^+ depletion, impairing spindle assembly and chromosome segregation^{15,16}. ROS, produced primarily in the mitochondria as by-products of oxidative metabolism, damage proteins, lipids, and DNA. This oxidative stress is thought to arise in aged oocytes, contributing to aneuploidy formation^{17,18}. Furthermore, single-cell multi-omics analyses suggest a strong association between mitochondrial dysfunction and defective spindle assembly¹⁹.

Age-related oocyte deterioration is not driven by a single factor but rather by multiple, interdependent processes. Increased oxidative stress leads to the accumulation of DNA damage, while aged oocytes exhibit a reduced capacity for DNA repair, allowing more DNA damage to persist. Age-related cohesion loss further contributes to this imbalance by promoting error prone DNA repair and increasing DNA damage²⁰. Oocytes with DNA damage are frequently arrested in metaphase I and display lagging chromosomes during

anaphase I, resulting in the formation of aneuploid eggs. Thus, this complex cellular fragility underlies the increased incidence of aneuploidy in aged oocytes.

1.1.3. Prevention strategies for age-related oocyte errors

Given the decline of these age-related factors in oocytes, developing strategies to prevent age-related segregation errors has long been a central focus. Previous studies have shown that *de novo* loading of REC8 onto fully grown germinal vesicle (GV) stage mouse oocytes is highly inefficient, indicating that cohesin loading in fully grown oocytes is largely irreversible⁸. Other pharmacological approaches have aimed to improve the overall cellular environment of the oocyte, particularly by counteracting the age-associated metabolic and mitochondrial decline. In line with this strategy, supplementation with the mitochondrial coenzyme Q10 (CoQ10) or the NAD⁺ precursors nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR) has shown moderate success in aged mouse oocytes. These interventions partially restore mitochondrial function, enhance cellular energy production, improve spindle integrity, and reduce aneuploidy rates^{13-15,18,20-23}. A recent study reported that transplanting and culturing aged oocytes within young follicles significantly reduced chromosomal abnormalities and cohesin loss²⁴. However, the lengthy culture protocol limits its future applicability.

1.2. Kinetochore: the key player in meiosis

1.2.1. The sophisticated protein architecture of the kinetochore

The kinetochore is a large, multiprotein complex assembled at the centromeric region of each chromosome. It is organized into a hierarchical architecture comprising three layers: the inner centromere, the inner kinetochore, and the outer kinetochore (Fig. 2).

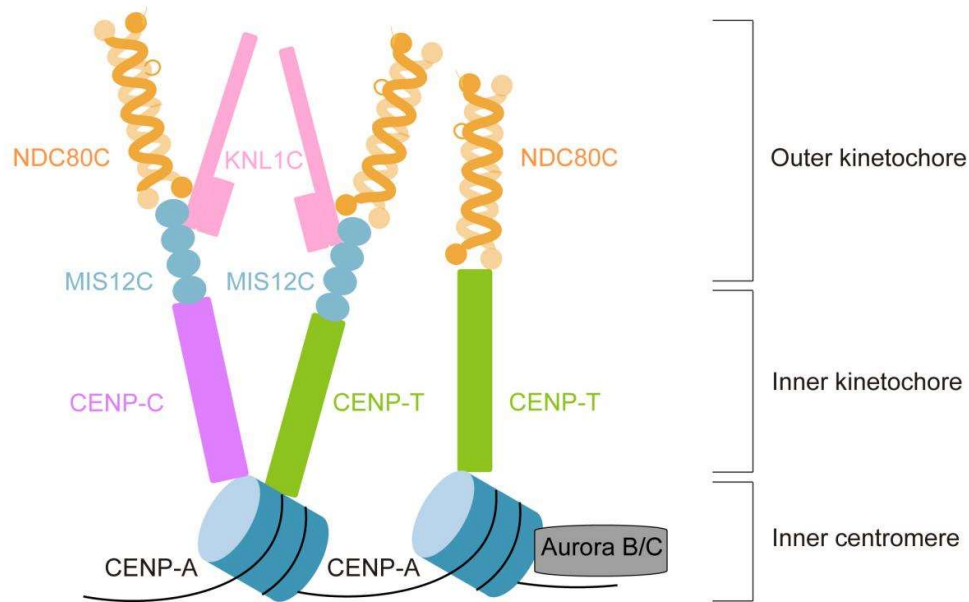


Fig .2: Diagram of kinetochore structure.

Inner centromere: The basal layer of the kinetochore is a specialized chromatin domain defined by the presence of the histone H3 variant, CENP-A. In mammalian oocytes, CENP-A nucleosomes are incorporated during fetal development and persist throughout the entire reproductive lifespan without turnover, thereby providing a stable epigenetic mark that defines centromere identity²⁷. Aurora B/C kinases are also components of the inner centromere and play a key role in the error-correction mechanism of kinetochore-microtubule attachments.

Inner kinetochore: The Constitutive Centromere-Associated Network (CCAN) is built upon the CENP-A-containing nucleosomes. It consists of approximately 16 different proteins, including the critical scaffolding components CENP-C and CENP-T. Unlike the static CENP-A, CENP-C must be dynamically reloaded onto centromeres during the long prophase arrest, where it plays a crucial role in maintaining sister centromere cohesion. Furthermore, CENP-C, together with CENP-T, forms a platform that recruits the outer kinetochore²⁸. Other CCAN components, such as CENP-V, can directly bind and bundle spindle microtubules, providing an additional mechanical link between the chromosome and the spindle²⁹.

Outer kinetochore: The outer kinetochore plays critical roles in microtubule binding and spindle assembly checkpoint (SAC) activation and is also known as the KMN network, which comprises three main complexes: the KNL1 complex, the MIS12 complex, and the NDC80 complex. The MIS12 complex acts as a central linker, bridging the inner kinetochore

(via CENP-C) to the outer components. Oocytes have evolved a unique adaptation within this complex: they express a germline-specific splice isoform of the DSN1 subunit lacking a critical phosphorylation site for Aurora B kinase. This adaptation allows the MIS12 complex to constitutively localize to centromeres, ensuring kinetochore assembly even under conditions of low kinase activity, a critical feature for the long meiotic arrest ³⁰.

KNL1 is a large, intrinsically disordered scaffolding protein that serves as the primary signaling platform. It contains multiple “MELT” motifs that, when phosphorylated by the MPS1 kinase, recruit the checkpoint proteins BUB1/3 and MAD1/2, thereby generating the SAC signal ^{31,32}. KNL1 depletion leads to a severely compromised SAC, resulting in premature anaphase onset and aneuploidy ³¹.

The NDC80 complex is the key microtubule-binding component of the kinetochore. It is a long, rod-shaped heterotetramer consisting of the NDC80/NUF2 heterodimer and the SPC24/SPC25 heterodimer. The SPC24/SPC25 complex connects to the inner kinetochore through both direct binding to the MIS12 complex, recruited by CENP-C and CENP-T, and direct binding to CENP-T. The unstructured N-terminus tail of NDC80 captures microtubule plus ends to establish stable microtubule attachments ³³. The affinity of the NDC80 complex for microtubules is regulated by its N-terminus tail, calponin homology domain (CHD), and loop domain, making it an important mediator of correct kinetochore-microtubule attachments ³⁴⁻³⁷.

1.3. Kinetochore-microtubule attachments in oocytes

1.3.1. Stable, bipolar microtubule attachments

Chromosome biorientation is maintained by stable, bipolar kinetochore-microtubule (KT-MT) attachments, which serves as a prerequisite for correct chromosome segregation. In oocytes, the spindle assembles without centrosomes, forming an acentrosomal spindle, and thus presents unique challenges. In mitotic cells, the duplicated centrosomes separate and move to opposite poles of the cell, where they nucleate and organize the spindle microtubules. By contrast, the acentrosomal spindle is organized by microtubule-organizing centers (MTOCs) that cluster into two focused poles, resulting in an inherently unstable spindle assembly and contributing to the error-prone nature of oocyte meiosis (Fig. 3a).

The formation of bipolar kinetochore-microtubule attachments is essential not only for accurate chromosome segregation but also for proper meiotic spindle organization (Fig. 3b). In oocytes expressing an NDC80 phospho-mutant, which fails to form stable end-on attachments, MTOCs are not properly restrained, leading to abnormal spindle formation and subsequent chromosome segregation errors ^{38,39}. To ensure the correct formation of bipolar microtubule attachments, several mediators and error-correction mechanisms operate to

safeguard this process.

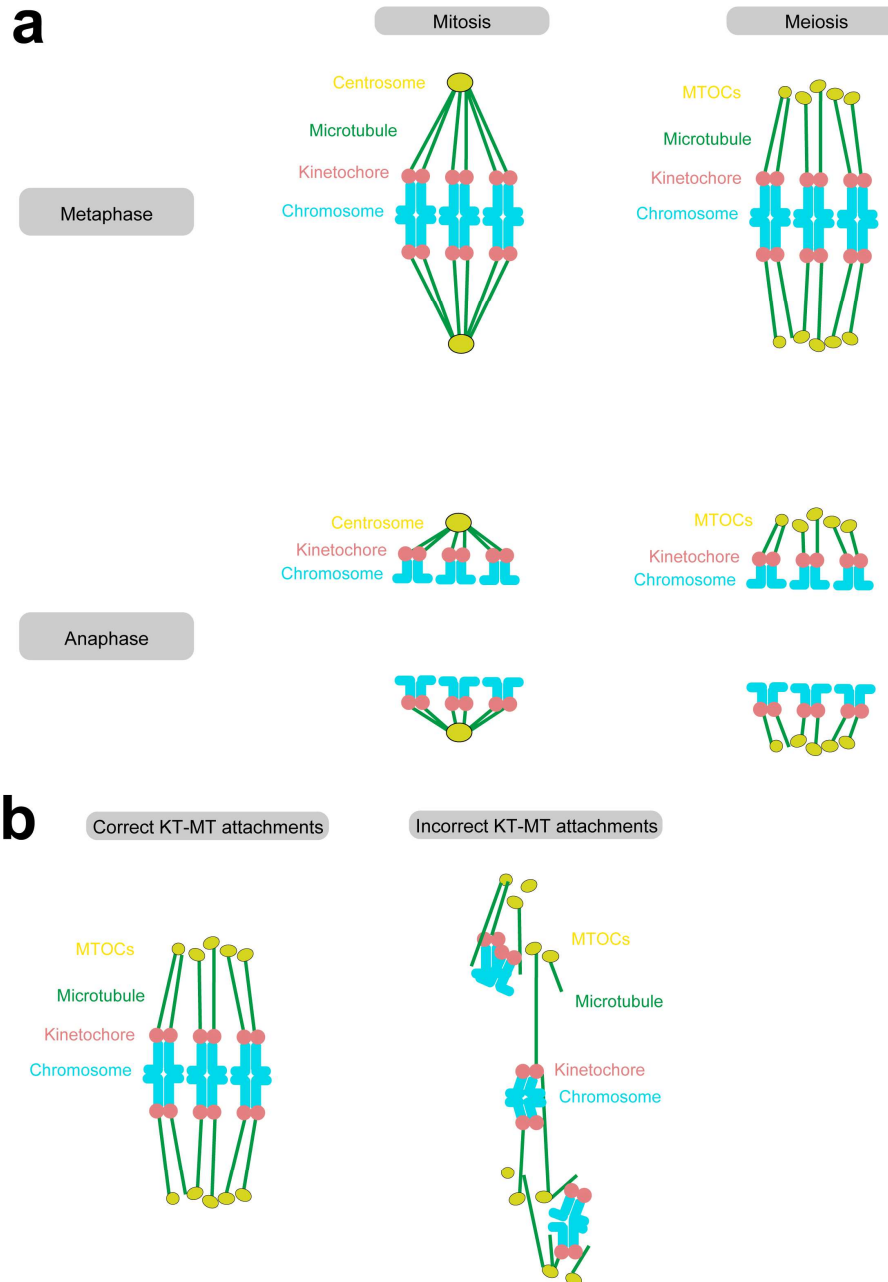


Fig. 3: Correct bipolar microtubule attachment is critical for the fidelity of chromosome segregation.

(a) Model of spindle assembly in mitosis and meiosis.

(b) Kinetochore-microtubule (KT-MT) attachments shape the meiotic spindle assembly.

1.3.2. Error correction of kinetochore-microtubule attachments

The NDC80 complex, the critical microtubule-interacting component of the

kinetochore, captures microtubules upon nuclear envelope breakdown (NEBD) initially forming unstable lateral attachments. It subsequently establishes stable, bipolar end-on microtubule attachments before anaphase I. The formation of bipolar end-on microtubule attachments is essential for SAC inactivation, which ensures correct chromosome segregation. The NDC80 complex is therefore a key regulator of the “attach or detach” decision.

The prevailing model for error correction is the tension model mediated by Aurora B/C-dependent phosphorylation. When a chromosome is correctly attached to both poles (bipolar attachment), pulling forces from opposite spindle poles generate tension across the centromere. This tension physically stretches the kinetochore, increasing the distance between the inner centromere, where Aurora B/C kinases are concentrated, and its substrates in the outer kinetochore, such as NDC80^{40,41}. The Aurora B/C-dependent phosphorylation of NDC80 decreases its affinity for microtubules, resulting in microtubule detachment. In contrast, this spatial separation prevents Aurora B/C kinases from phosphorylating NDC80, thereby stabilizing the microtubule attachment.

Conversely, in incorrect, low-tension attachments (syntelic, lateral, or merotelic), the kinetochore remains relaxed, keeping NDC80 in close proximity to Aurora B/C kinases. Continuous phosphorylation destabilizes microtubule attachments, allowing the kinetochore to reform a correct attachment (Fig. 4). High-resolution imaging in mouse oocytes has revealed that Aurora B/C remains close to the kinetochore-microtubule interface even under tension, suggesting that meiotic attachments are intrinsically less stable than mitotic ones⁴².

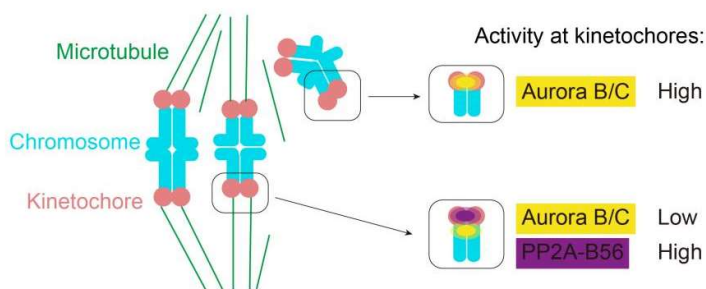


Fig. 4: Aurora B/C activity counteracts with PP2A-B56 to contribute to error correction of kinetochore microtubule attachments.

Once tension is established, protein phosphatases are recruited to stabilize correct microtubule attachments. PP2A-B56, in particular, is targeted to kinetochores, where it counteracts Aurora B/C activity by dephosphorylating NDC80 and other substrates, thereby stabilizing microtubule attachments⁴². The precise spatiotemporal regulation between Aurora B/C and PP2A-B56 is essential for preventing chromosome segregation errors (Fig. 4).

1.3.3. Other regulators of kinetochore-microtubule attachments

Besides the NDC80 complex, a range of proteins has been implicated in forming and stabilizing end-on attachments. The Ska complex (Ska1/Ska2/Ska3) tracks depolymerizing microtubule plus ends in a force-bearing manner, functioning together with the NDC80 complex to stabilize kinetochore-microtubule attachments^{43,44}. Kinesin motor proteins such as CENP-E, a plus-end-directed motor, help capture microtubules and drive chromosome congression by coordinating microtubule attachments, while Kif18A, a plus-end depolymerase, is thought to regulate microtubule dynamics at the kinetochore to control chromosome oscillations at the metaphase plate⁴⁵⁻⁴⁷. Several microtubule-associated proteins (MAPs), including CLASP (Cytoplasmic Linker Associated Protein), ch-TOG, MCAK and HURP (Hepatooma Up-Regulated Protein), have been reported to associate with kinetochore fibers (k-fibers), where they contribute to stabilization during mitosis. However, their precise roles in the meiotic spindle remain unclear⁴⁷⁻⁵¹.

1.4. HURP (DLGAP5): A microtubule-interacting protein

1.4.1. HURP in mitosis

HURP is a bona fide microtubule-associated protein (MAP) and a cargo of the nuclear import receptor importin- β . In the cytoplasm, it remains in an inactive, sequestered state. Upon NEBD, the small GTPase Ran, bound to GTP to form Ran-GTP, becomes highly concentrated on and around chromosome clusters. Ran-GTP binds to importin- β , causing it to release its cargo, including HURP. This release generates a steep gradient of active HURP centered around chromosomes, where it promotes the nucleation and stabilization of kinetochore-microtubule fibers⁵².

HURP binds directly to the microtubule lattice, enhancing microtubule polymerization and stability. HURP-coated microtubules exhibit decreased tubulin turnover and markedly increased lifetimes⁵³. In mitotic cells, HURP is critical for stabilizing the chromosome-proximal ends of kinetochore fibers, thereby maintaining sufficient inter-kinetochore tension required for SAC inactivation. Depletion of HURP results in short, unstable spindles, failure to generate tension, and subsequent chromosome mis-segregation^{47,54}.

1.4.2. The meiosis-specific function of HURP

In oocytes, which lack centrosomes, spindle assembly is primarily driven by the Ran-GTP pathway⁵⁵. HURP is necessary for RanGTP-induced branching microtubule nucleation and for stabilizing the microtubule lattice⁵⁶. Unlike in mitosis, where HURP localizes prominently to k-fibers and contributes to their stabilization, in oocytes HURP is mainly enriched in the central region of the spindle during metaphase I and becomes enriched on k-fibers at late metaphase I^{25,26}. Consequently, the precise function of HURP in the meiotic

spindle remains under investigation.

A recent study reported the construction of artificial kinetochores using microscale magnetic beads tethered to the outer kinetochore component NDC80/NUF2 complex, termed NDC80 beads ^{25,26}. These NDC80 beads achieved a biorientation-like state and established bipolar HURP-positive microtubule attachments. Notably, the dominant central positioning of NDC80 beads on the metaphase plate (M-plate) displaced endogenous chromosomes from the high-tension inner area toward the outer region. This redistribution was associated with a reduction in premature chromosome segregation during prolonged meiosis I in aged mouse oocytes ²⁶.

However, this error-preventing effect was observed under non-physiological conditions and provided only moderate protection. Future studies should dissect the underlying mechanism of NDC80 bead-dependent error prevention, either by verifying the effect of chromosome displacement from the inner M-plate or by directly assessing changes in microtubule pulling forces on chromosomes. Moreover, the non-biodegradable nature of NDC80 beads, together with their disruption of normal chromosome organization on the M-plate, limits their practical applicability as a strategy for preventing chromosome segregation errors in aged oocytes.

In this study, I developed a protein-based artificial kinetochore that functions as a microtubule decoy. This decoy established a biorientation-like state without disrupting chromosome organization and, through its interaction with microtubules, reduced the microtubule pulling forces exerted on endogenous chromosomes. Consequently, this mechanism efficiently prevented premature chromosome separation during unperturbed meiosis I and II in aged mouse oocytes, leading to a reduction in the production of aneuploid eggs. Together, these findings shed light on the potential of biocompatible protein-based decoys as a novel strategy to prevent age-related aneuploidy in oocytes.

Chapter 2

Materials and Methods

2.1. Mice

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at RIKEN Kobe Branch. B6D2F1 (C57BL/6 × DBA/2) female mice were used to obtain oocytes. Mice aged 60-95 weeks were used to obtain aged oocytes. For the remaining experiments, mice aged 8-12 weeks were used.

2.2. Mouse oocyte culture

Mice were intraperitoneally injected with either 0.2 ml (10 IU) of pregnant mare serum gonadotropin (PMSG) or 0.1 ml of CARD HyperOva (KYUDO). Fully-grown germinal vesicle (GV) oocytes were collected 48 hours after PMSG injection. Oocytes were incubated in the M2 medium containing 200 μ M 3-isobutyl-1-methylxanthine (IBMX, Sigma) at 37°C. Meiotic resumption was induced by IBMX washout. When indicated, the following reagents were added immediately after IBMX washout: 10 μ M nocodazole (Sigma), 100 μ M monastrol (Sigma), 2 μ M proTAME (Bio-Techne R&D Systems) or 500 nM rapamycin (Funakoshi). In Fig. 17, 20 μ M monastrol (Sigma) was added to the medium at 5 hours after IBMX washout.

2.3. Plasmids

Plasmids used for *in vitro* mRNA synthesis and subsequent microinjection into oocytes are listed in Table 1. All plasmids were verified by DNA sequencing.

Table 1. Plasmids used for *in vitro* mRNA synthesis

Plasmid Name	Reference or Source
phAG (humanized Azami-Green for Fluoppi)	MBL; AM-VS0801M
pCDNA3.1-pCMV-PfV-GS-Sapphire	Delarue M et al, 2018
pEGFP-FRB	Karginov AV et al, 2010
pGEMHE_hAG-Pfv-Sapphire-GS-FRB	This study
YFP-FKBP (YF)	Inoue T et al, 2005
pGEMHE_NDC80-GFP	Asai et al, 2024
pGEMHE_NDC80-FKBP	This study
pGEMHE- NDC80 ^{ΔSPC} -WT-FKBP	This study
pGEMHE- NDC80 ^{ΔSPC} -DFAA-FKBP	This study
pGEMHE- NDC80 ^{ΔSPC} -9A-FKBP	This study

pGEMHE- NUF2	Asai et al, 2024
pGEMHE- NUF2-HA	Asai et al, 2024
pGEMHE- FKBP-GS-FKBP	This study
pGEMHE- H2B-mCherry	Kitajima et al, 2011
pGEMHE- CENP-T-mCherry	This study
pGEMHE- mCherry	Asai et al, 2024
pGEMHE- EGFP-HURP	This study

Primers used for plasmid construction are listed in Table 2.

Table 2. Primers used for plasmid construction in this study

Primer Name	Sequence (5' → 3')
mHec1-new-XhoI-F	GGGCTCGAGATGAAGCGCAGTTCA
mHec1-new-XmaI-R	CCCCGGGTTTGTCTCGGGAGCCTT
GEM-AgeI-F	GGGGGGACCGGTATGCTCTCAATAAAT
GEM-GS-XhoI-R	TTTCTCGAGGGAGCCTGATCCCGACCCACTACCTTTG TACAATTCATC
GS-FRB-HindIII-F	AAAAAGCTTTCTGGCAGCGGTAGTGGGTCGGGATCA GGCTCCATCCTCTGGCATGAG
FRB-NotI-R	GGGGCGGCCCGCCTTTGAGATTCGTCG
GS-FKBP-AgeI-F	ACCGGTGTCTGGCAGCGGTAGTGGGTCGGGAGGAGT GCAGGTGGAA
FKBP-XhoI-R	CCCCTCGAGTTCCAGTTTTAGAAGCTC
FKBP-AgeI-F	GGGACCGGTATGGGAGTGCAGGTGGAAACC
FKBP-GS-XhoI-R	CCCCTCGAGTCCCGACCCACTACCGCTGCCAGATTCC AGTTTTAGAAG

2.4. RNA microinjection

mRNAs were transcribed and purified using the mMESSAGE mMACHINE T7 kit (Thermo Fisher Scientific). The mRNAs were microinjected into fully-grown GV oocytes, followed by 2 hours incubation at 37°C until IBMX washout. 0.3 pg of NDC80(ΔSPC-WT/ΔSPC-DFAA/FL-WT/FL-9A)-FKBP, 0.3 pg of NUF2, 0.3 pg of NUF2-HA, 0.45 pg of H2B-mCherry, 3.5 pg of hAG-Pfv-Sapphire-GS-FRB, 0.15 pg of FKBP-GS-FKBP, 1 pg of

CENP-T-mCherry, 1 pg of mCherry, and 3 pg of EGFP-HURP were injected. For experiments with microbeads, microinjections were performed with 1 pg of NDC80-GFP, 1 pg of NUF2, 1.5 pg of H2B-mCherry²⁵.

2.5. Bead microinjection

Rat anti-GFP antibody (D153-3, MBL; 1:30 dilution) was immobilized onto protein G-conjugated beads (MonoMag Protein G Beads, 1 μm in diameter MPG1000 or 3 μm in diameter MPG3000, Ocean NanoTech; 1:20 dilution) in PBS for 2.5 hours at 4°C with rotation. The beads were washed and subsequently stored in PBS. The anti-GFP-bound beads were introduced into mRNA-injected GV-stage oocytes through microinjection using a piezo pulse. Four beads of 3 μm in diameter or 4–11 beads of 1 μm in diameter were microinjected into each oocyte. The microinjected oocytes were then cultured at 37°C for 1 hour before meiotic resumption.

2.6. Live imaging

An LSM900 confocal microscope (Carl Zeiss), equipped with a 40×C-Apochromat 1.2NA water immersion objective lens (Carl Zeiss), was controlled using Zen software with a custom ImageJ macro implementing the previously reported autofocus method⁶¹.

For experiments under nocodazole treatment, 81 z-confocal sections (every 0.5 μm) of 512 × 512 pixel xy images were recorded. These images covered a total volume of 79.9 μm × 79.9 μm × 40 μm , and were acquired at 30-minute time intervals. For experiments under monastrol treatment, 29 z-confocal sections (every 1.25 μm) of 512 × 512 pixel xy images were recorded. These images covered a total volume of 39.93 μm × 39.93 μm × 35 μm , and were acquired at 3-minute time intervals. For the other experiments, z-confocal sections (every 1 μm or 1.5 μm) of 512 × 512 pixel xy images were recorded, which were acquired at 10-minute time intervals.

For experiments with microbeads, an LSM780 confocal microscope (Carl Zeiss), equipped with a 40×C-Apochromat 1.2NA water immersion objective lens (Carl Zeiss), was controlled using Zen software with the multi-position autofocus macro PipelineConstructor⁶². For imaging of chromosomes and beads, 25 z-confocal sections (every 1.25 μm) of 512 × 512 pixel xy images were recorded, which covered a total volume of 38.65 μm × 38.65 μm × 30 μm , acquired at 5-minute time intervals for at least 12 hours after the induction of meiotic resumption.

2.7. Immunofluorescence

Oocytes were fixed with a fixation buffer (1.6% formaldehyde in 100 mM PIPES (pH7.0), 1 mM MgCl₂, 0.1% Triton-X100) at room temperature for 30 minutes. After washing four times, oocytes were incubated in PBT (PBS + 0.1% Triton-X100) at 4°C overnight. Oocytes were blocked with 3% BSA-PBT at room temperature for 1 hour and then incubated with primary antibodies in 3% BSA-PBT at 4°C overnight. Oocytes were washed four times in 3% BSA-PBT and incubated with secondary antibodies and 5 µg/ml Hoechst33342 (Invitrogen) in 3% BSA-PBT at room temperature for 2 hours or at 4°C overnight.

The primary antibodies used were mouse anti-HA (BioLegend, MMS-101R; 1:500), rabbit anti-DSN1 (a gift from Dr. H. Shibuya; 1:500), rabbit anti-CENP-C (a gift from Dr. Y. Watanabe; 1:500), mouse anti-mCherry (Clontech, 632543; 1:500), human anti-centromere antibodies (ACA, Antibodies Incorporated, 15–234; 1:500), rabbit anti-HURP (Santa Cruz Biotechnology, sc-98809; 1:500), and rat anti- α tubulin (YL1/2) (Bio-Rad, MCA77G; 1:1500). The secondary antibody used was Alexa Fluor 488 goat anti-Human IgG (H + L) (A11013), Alexa Fluor 555 goat anti-rat IgG (H+L) (A21434), goat anti-mouse IgG (H + L) (A21424), goat anti-rabbit IgG (H + L) (A21429), Alexa Fluor 647 goat anti-Human IgG (H + L) (A21445), goat anti-rat IgG (H + L) (A21247), and donkey anti-rabbit IgG (H+L) (A31573) (1:500, Molecular Probes).

Oocytes were imaged under an LSM880 with Airyscan confocal microscope, equipped with a 40 $\times$ C-Apochromat 1.2NA water immersion objective lens (Carl Zeiss). For experiments under nocodazole treatment, z-confocal sections (every 0.5 µm) of 512 $\times$ 512 pixel xy images were recorded. For the other experiments, z-confocal sections (every 0.2 µm) of 512 $\times$ 512 pixel xy images were recorded.

2.8. 4D analysis

Chromosomes and NDC80-GEM foci were reconstructed and segmented in 3D using Imaris software (Bitplane) with an automatic thresholding method, followed by manual validation. The spindle axis was manually estimated based on chromosome distribution at each time point, and the spindle center was defined as the center of mass of chromosomes. For the time points before the spindle axis was established (e.g. 2 hours after NEBD), the same spindle axis determined at 3 hours after NEBD was applied²⁶. During the alignment analysis, NDC80-GEM clusters located outside of the spindle were manually excluded. The volume of NDC80-GEM clusters was calculated using the algorithm equipped in Imaris. To generate the kymograph of chromosomes and NDC80-GEMs, signals were projected onto the spindle equator for each time point with an in-house macro developed in Fiji⁶³.

2.9. Signal intensity quantification

All measurement of fluorescence signals was performed in Fiji ⁶³. For the full width at half maximum (FWHM) analysis, a line selection was drawn either through the center of the NDC80-GEM cluster or perpendicular to the microtubule bundle at a distance of 0.5 μm from the microtubule tip. The intensity plot profile along the line was obtained and fitted to a Gaussian distribution, which was subsequently used to calculate the FWHM. To measure the signal intensity of NDC80-GEM clusters and kinetochores, ten randomly selected rectangular areas (four pixels each) were measured for each group, and the cytoplasmic background intensity was then subtracted. To measure the signal intensity on NDC80 beads, the mean fluorescence intensity on each bead was measured and subtracted by the mean intensity in an adjacent cytoplasmic region. For the classification of microtubule attachments using immunofluorescence images, the HURP intensity plot profile along a line drawn over the NDC80-GEM cluster was obtained and background-subtracted. On this line profile, the peak HURP intensities at the two sides of the NDC80-GEM cluster were defined as Int_A and Int_C, respectively, while the minimum HURP intensity around the middle of the cluster was defined as Int_B. If Int_B was less than half of both Int_A and Int_C, the attachment was categorized into “bipolar attachment”.

2.10. Statistical analysis

All graphs and statistical analysis were performed in R. Boxplots were generated using `geom_boxplot` in R. The central line represents the median, the box shows the interquartile range (IQR) (spanning from the 25th to the 75th percentile), and the whiskers extend to 1.5 times the IQR. All experiments were performed independently at least three times.

Chapter 3

Results

3.1. Construction of protein-based artificial kinetochores

3.1.1. The design of protein-based artificial kinetochores

The NDC80/NUF2 complex is a key component of the outer kinetochore, directly interacting with microtubules through their N-terminal domains. A recent study constructed an artificial kinetochore by tethering the NDC80/NUF2 complex to 2- μm microbeads, which achieved a biorientation-like state through bipolar microtubule attachments²⁵. In addition, higher-order assembly has been reported to be essential for the functional efficiency of kinetochore proteins⁶⁴. I therefore decided to design protein-based artificial kinetochores based on the concept of higher-order assembly of the NDC80/NUF2 complex. Genetically encoded multimeric nanoparticles (GEMs) are homomultimeric scaffolds fused to a T-Sapphire protein, which self-assemble into a nanoparticle approximately 40 nm in diameter with bright fluorescence⁵⁷. I constructed the NDC80 protein tethered to **FK506 binding protein (FKBP)** domain and the GEM scaffold tethered to the fluorescent protein (FP) T-Sapphire followed by **FKBP-rapamycin binding (FRB)** domain of FKBP12-rapamycin associated protein. The NDC80^{ΔSPC}/NUF2 (NDC80¹⁻⁶¹¹/NUF2) complex lacks the domain interacting with the SPC24/SPC25 complex and therefore fails to localize to the kinetochore. NDC80^{ΔSPC} serves as a proper model to isolate the function of NDC80 without interference of other kinetochore proteins. The interaction between the NDC80^{ΔSPC}/NUF2 complex and GEM was induced via rapamycin-dependent FKBP-FRB interaction, hereafter referred to as NDC80^{ΔSPC}-GEM (Fig. 5).

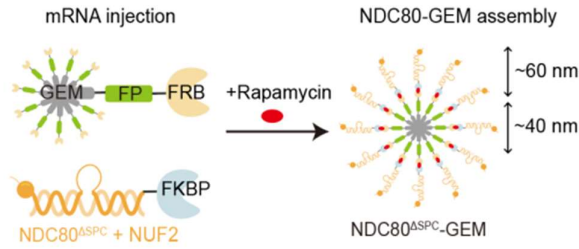


Fig. 5: Construction of NDC80^{ΔSPC}-GEM.

Design of NDC80^{ΔSPC}-GEM. Abbreviations are defined in the main text.

3.1.2. The assembly of NDC80-GEM clusters depends on NDC80-NDC80 interaction

The NDC80/NUF2 complex is a well-established microtubule-interacting factor that is recruited to kinetochores during prometaphase I, coinciding with the initiation of kinetochore-microtubule interactions. To test whether NDC80-GEM assemblies can form independently of microtubules, I removed microtubules by treating oocytes with nocodazole, a microtubule-depolymerizing chemical, before the entry of M-phase of meiosis I marked by NEBD (Fig. 6a). NDC80^{ΔSPC}-GEM clusters were expressed in oocytes and imaged at 4 hours after NEBD, corresponding to metaphase I. Under these conditions, NUF2 signals co-localized with NDC80^{ΔSPC}-GEM clusters (Fig. 6b). These observations suggest that NDC80^{ΔSPC}-GEM assembles into clusters coated with the NDC80/NUF2 complex at metaphase I, even in the absence of microtubules.

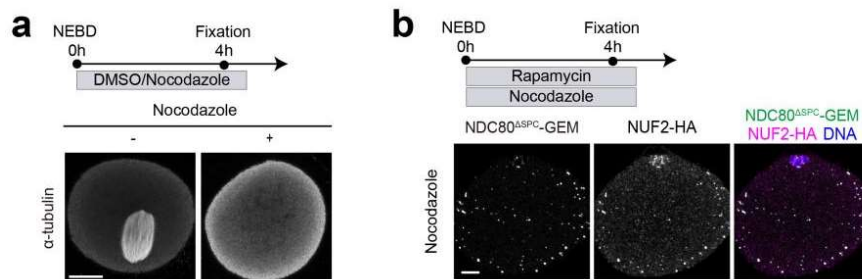


Fig. 6: Assembly of NDC80^{ΔSPC}-GEM clusters in microtubule-free oocytes.

(a) Nocodazole inhibits microtubule polymerization. Oocytes were fixed 4 hours after NEBD and immunostained for α-tubulin (gray). Scale bar, 20 μm.

(b) Co-localization of NUF2 on NDC80^{ΔSPC}-GEM foci. Oocytes were fixed 4 hours after NEBD and immunostained for NUF2-HA (magenta) and DNA (Hoechst33342, blue). Z-projection images are shown. Scale bar, 10 μm.

The loop domain of the NDC80 protein has been reported to promote NDC80-NDC80 interaction⁶⁵, raising the possibility that this interaction contributes to the formation of NDC80^{ΔSPC}-GEM clusters. To test this possibility, I introduced amino acid substitutions that disrupt NDC80-NDC80 interaction, thereby generating the NDC80^{ΔSPC}-DFAA-GEM construct. The original construct, NDC80^{ΔSPC}-GEM, was used as a control and hereafter referred to as NDC80^{ΔSPC}-WT-GEM. At 4 hours after NEBD, NDC80^{ΔSPC}-WT-GEM formed clusters with bright fluorescence, which were evenly distributed throughout the cytoplasm (Fig. 7a). For quantitative analysis, clusters were identified in three dimensions, their number was counted, and their size was estimated using the full width at half maximum (FWHM), measured by drawing a line across the center of each cluster (Fig. 7b, c). This analysis revealed that NDC80^{ΔSPC}-DFAA-GEM formed significantly fewer and smaller clusters compared with NDC80^{ΔSPC}-WT-GEM (Fig. 7a-e). Moreover, when GEM alone was expressed, no clusters were detected, consistent with the previous report that single GEM particles have a diameter of approximately 40 nm⁵⁷, which is below the diffraction limit of conventional light microscopy. These results indicate that the formation of NDC80^{ΔSPC}-GEM clusters depends on NDC80-NDC80 interactions mediated by the NDC80 loop domain.

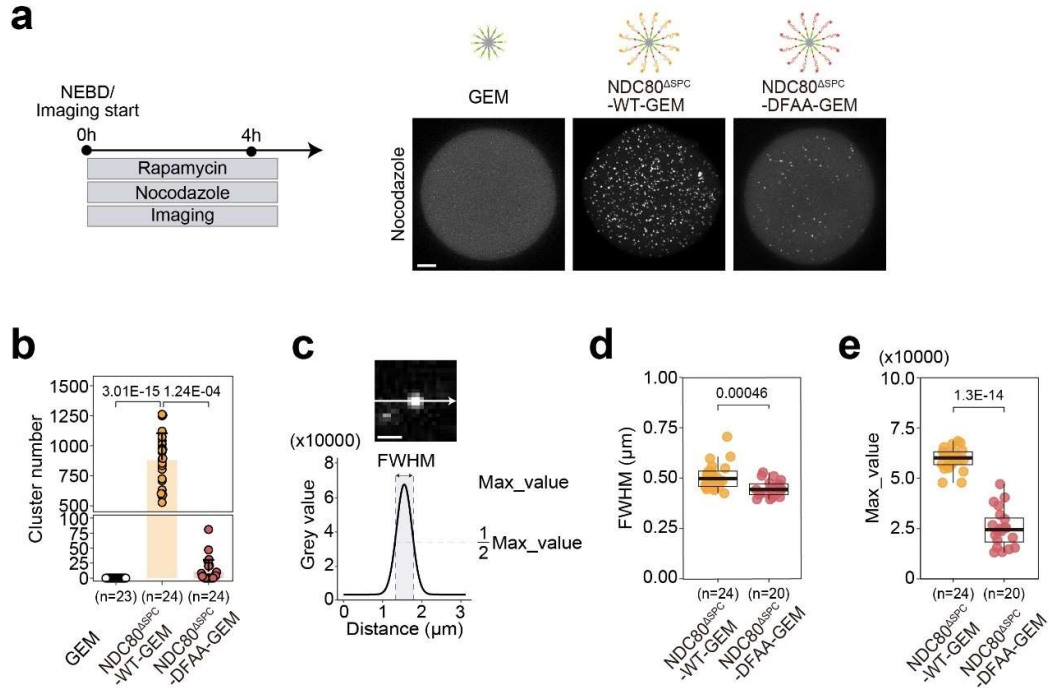


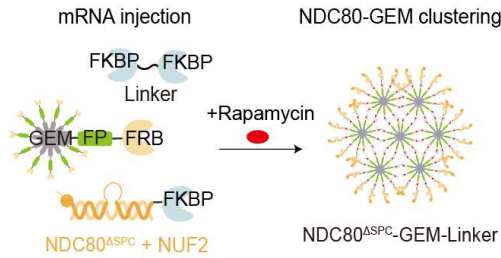
Fig. 7: NDC80^{ASPC}-WT-GEM particles form clusters via the loop domain of NDC80.
 (a) Nocodazole-treated oocytes expressing GEM, NDC80^{ASPC}-WT-GEM, and NDC80^{ASPC}-DFAA-GEM constructs were imaged. Z-projection images of GEM at 4 hours after nuclear envelope breakdown (NEBD, a marker for M-phase entry) are shown in (b). Scale bar, 10 μm.
 (b) The number of NDC80^{ASPC}-GEM foci was counted within a 40-μm thick cytoplasmic region. Each dot represents an oocyte (n = 23, 24, 24 oocytes for GEM, NDC80^{ASPC}-WT-GEM, and NDC80^{ASPC}-DFAA-GEM, respectively, pooled from 3 independent experiments). Bars show the mean + SD. P-values were calculated using the Kruskal-Wallis test followed by Dunn's post-hoc test with Bonferroni correction.
 (c-d) The diameter of individual fluorescent foci was estimated by measuring the full width at half maximum (FWHM) of signal intensity. Each dot represents the mean value averaged from 10 individual foci randomly selected per oocyte (n = 24, 20 oocytes for NDC80^{ASPC}-WT-GEM and NDC80^{ASPC}-DFAA-GEM, respectively, pooled from 3 independent experiments). Box plots: the centre line indicates the median, the box boundaries represent the 25th and 75th percentiles, and the whiskers extend to 1.5 × the interquartile range from the box. P-values were calculated with unpaired two-tailed t-tests.
 (e) Maximum fluorescence intensity of NDC80-GEM foci in (a). Each dot represents the mean value in an oocyte (n = 24, 20 oocytes for NDC80^{ASPC}-WT-GEM and NDC80^{ASPC}-DFAA-GEM, respectively, pooled from 3 independent experiments). Box plots: the centre line indicates the median, the box boundaries represent the 25th and 75th percentiles, and the whiskers extend to 1.5 × the interquartile range from the box. P-values were calculated with unpaired two-tailed t-tests.

3.1.3. Design of larger NDC80^{ASPC}-GEM-Linker clusters

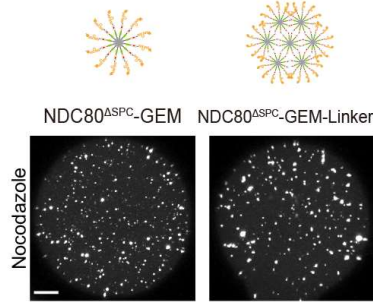
In our previous study, the size of NDC80 beads was shown to be critical for alignment

efficiency: NDC80 beads with a diameter of 2 μm achieved nearly 100% alignment, whereas smaller beads with a diameter of 1 μm showed substantially lower alignment efficiency²⁵. To increase the size of NDC80^{ASPC}-GEM clusters, I introduced a Linker consisting of two copies of the FKBP domain connected by a short, flexible GS linker, thereby generating the NDC80^{ASPC}-GEM-Linker construct (Fig. 8a). In the absence of microtubules, NDC80^{ASPC}-GEM-Linker clusters were larger and exhibited higher fluorescence intensity compared with NDC80^{ASPC}-GEM clusters (Fig. 8b-d). These results indicate that incorporation of the Linker promotes the formation of larger NDC80^{ASPC}-GEM clusters, validating the Linker-based strategy for increasing cluster size.

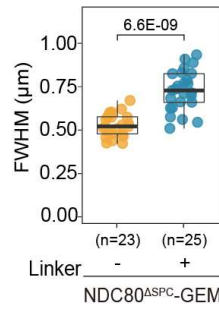
a



b



c



d

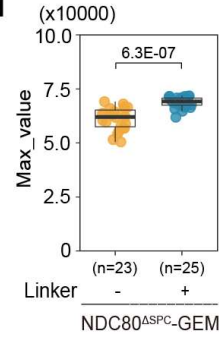


Fig. 8: Construction of NDC80^{ASPC}-GEM-Linker.

(a) Design of enlarged NDC80^{ASPC}-GEM clusters with Linker.

(b-c) NDC80^{ASPC}-WT-GEM clusters are enlarged by Linker. As in (b) and (c), z-projection images of nocodazole-treated oocytes and a plot for measured FWHM values are shown.

Scale bar, 10 μm . Box plots: the centre line indicates the median, the box boundaries represent the 25th and 75th percentiles, and the whiskers extend to $1.5 \times$ the interquartile range from the box. P-values were calculated with unpaired two-tailed t-tests. The number of oocytes is shown in parentheses ($n = 23, 25$ oocytes for NDC80^{ASPC}-GEM and NDC80^{ASPC}-GEM-Linker, respectively, pooled from 3 independent experiments).

(d) Maximum fluorescence intensity of NDC80-GEM foci in (b). Each dot represents the mean value in an oocyte ($n = 23, 25$ oocytes for NDC80^{ASPC}-GEM, NDC80^{ASPC}-GEM-Linker, respectively, pooled from 3 independent experiments). Box plots: the centre line indicates the median, the box boundaries represent the 25th and 75th percentiles, and the whiskers extend to $1.5 \times$ the interquartile range from the box. P-values were calculated with unpaired two-tailed t-tests.

3.2. The large size is required for the alignment of NDC80^{ASPC}-GEM-Linker

clusters

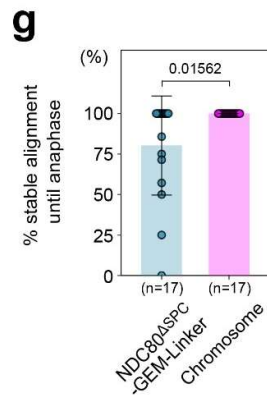
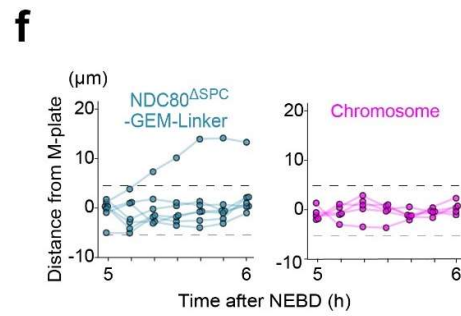
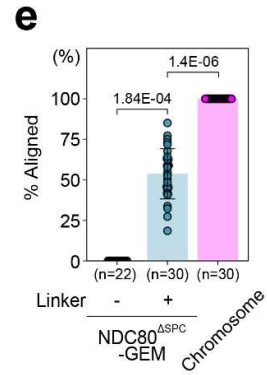
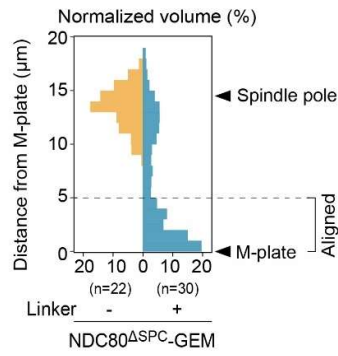
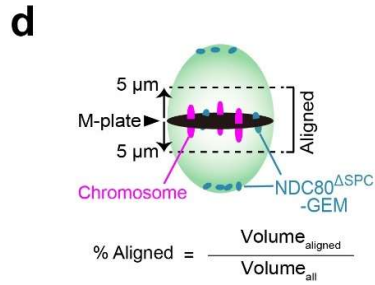
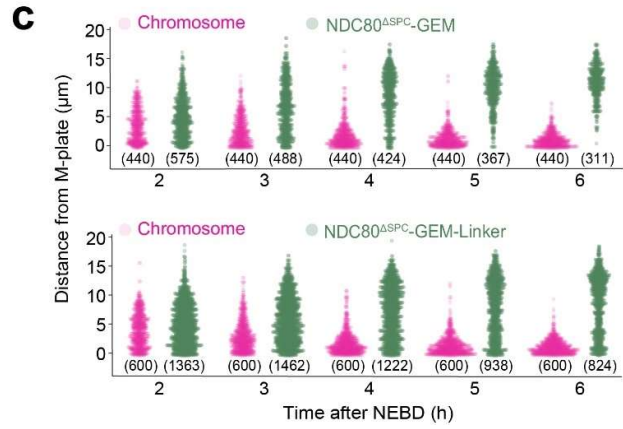
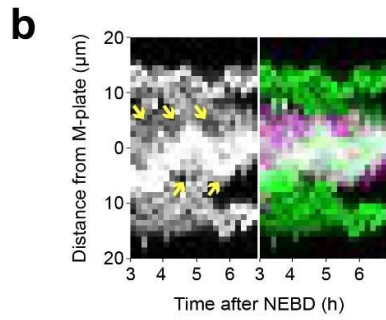
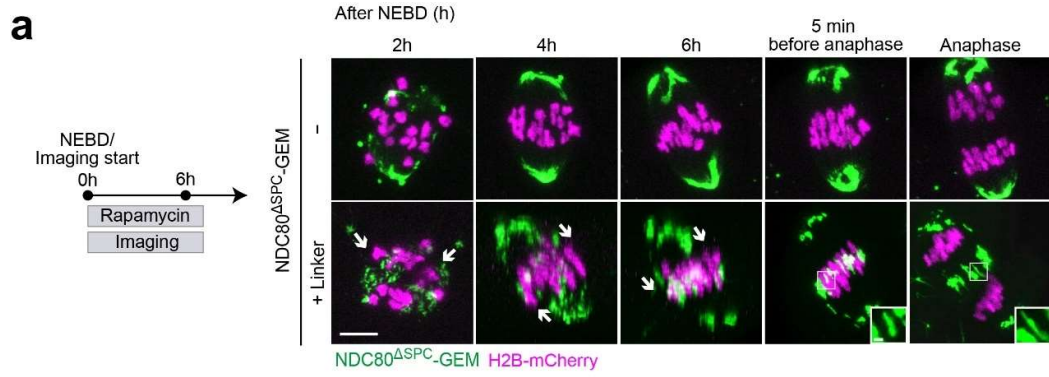
To compare the alignment abilities between small NDC80^{ΔSPC}-GEM clusters and larger NDC80^{ΔSPC}-GEM-Linker clusters, I performed live imaging to monitor their dynamics during meiosis I (Fig. 9a). At 2 hours after NEBD, NDC80^{ΔSPC}-GEM clusters moved from the cytoplasm to the spindle, localizing to the surface of the monopolar spindle in close proximity to chromosomes. From 4 to 6 hours after NEBD, chromosomes became fully aligned at the metaphase plate (M-plate), defined as the region within 5 μm of the M-plate. In contrast, NDC80^{ΔSPC}-GEM clusters remained near the spindle poles throughout metaphase I, similar to CENP-T clusters, which have a diameter of approximately 35 nm⁶⁴.

In contrast, the larger NDC80^{ΔSPC}-GEM-Linker clusters exhibited continuous movement from the spindle poles toward the M-plate during early metaphase I (Fig. 9b). The percentage of aligned NDC80^{ΔSPC}-GEM-Linker clusters increased progressively from 2 to 6 hours after NEBD (Fig. 9c). By late metaphase I, a large proportion of NDC80^{ΔSPC}-GEM-Linker clusters were maintained at the M-plate and exhibited an elongated morphology (Fig. 9a, c). When chromosomes segregated at anaphase I, the aligned NDC80^{ΔSPC}-GEM-Linker clusters retained their plate localization (Fig. 9a), similar to NDC80 beads²⁵. Together, these results suggest that NDC80^{ΔSPC}-GEM-Linker clusters undergo gradual, size-dependent alignment during metaphase I.

To quantitatively compare alignment efficiency, I calculated the percentage of aligned cluster volume relative to the total cluster volume. This analysis revealed a marked improvement in alignment, from 0% for NDC80^{ΔSPC}-GEM clusters to ~50% for NDC80^{ΔSPC}-GEM-Linker clusters, although this value remained lower than that of endogenous chromosomes, which achieved 100% alignment (Fig. 9d-e). These results demonstrate that increased cluster size enables NDC80^{ΔSPC}-GEM-Linker clusters, but not smaller NDC80^{ΔSPC}-GEM clusters, to align at the M-plate during metaphase I.

To assess the stability of alignment, aligned NDC80^{ΔSPC}-GEM-Linker clusters and chromosomes were tracked within the same oocyte for 1 hour. ~80% of NDC80^{ΔSPC}-GEM-Linker clusters remained stably aligned until anaphase, although this proportion was still lower than that observed for chromosomes (Fig. 9f-g). These results indicate that alignment of NDC80^{ΔSPC}-GEM-Linker clusters is largely stable once established.

Endogenous chromosomes undergo stretching during metaphase I as a result of bipolar pulling forces generated by kinetochore-microtubule attachments. Similarly, aligned NDC80^{ΔSPC}-GEM-Linker clusters displayed elongation along the spindle axis at 6 hours after NEBD (Fig. 9h-i), consistent with the presence of microtubule-driven tensile forces. Taken together, these results demonstrate that increased cluster size enables stable alignment of NDC80^{ΔSPC}-GEM-Linker clusters at the M-plate.



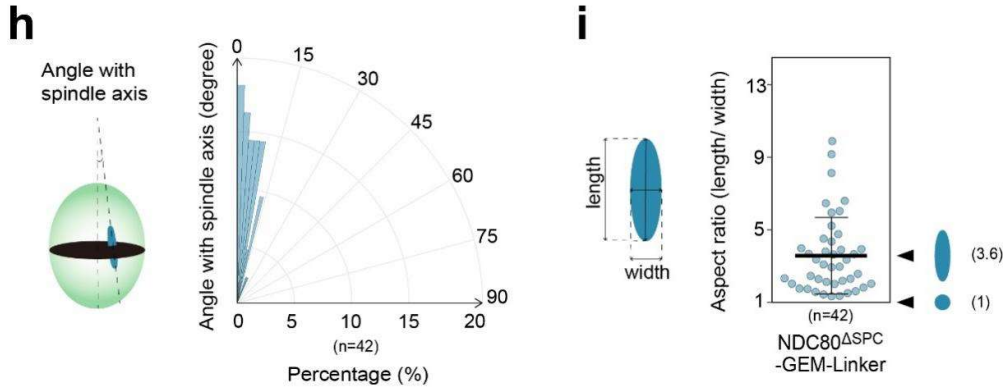


Fig. 9: Size-correlated alignment capacity of NDC80^{ASPC}-GEM clusters.

(a) NDC80^{ASPC}-GEM clusters align with the help of Linker. Live imaging of NDC80^{ASPC}-GEM and NDC80^{ASPC}-GEM-Linker through M-phase of meiosis I. 3D-reconstructed images of chromosomes (H2B-mCherry, magenta) and NDC80^{ASPC}-GEM (green) viewed from the side of the spindle are shown. Signals are interpolated in z. Arrows show the direction of NDC80^{ASPC}-GEM-Linker movement. Scale bar, 10 μ m. Insets show the magnified region (3 independent experiments were repeated with the same results). Scale bar, 1 μ m.

(b) Kymograph of chromosomes (H2B-mCherry, magenta) and NDC80^{ASPC}-GEM (green) along the spindle axis. Arrows show the direction of NDC80^{ASPC}-GEM-Linker movement toward the metaphase plate (M-plate).

(c) Linker improves the alignment of NDC80^{ASPC}-GEM-Linker clusters. Reconstructed 3D images shown in (a) were used to detect the positions of NDC80-GEM clusters and chromosomes. Distance from the metaphase plate (M-plate) was measured in 3D over time. The number of chromosomes or NDC80^{ASPC}-GEM clusters is shown in parenthesis (n = 440 chromosomes and 568 NDC80^{ASPC}-GEM clusters from 22 oocytes, n = 600 chromosomes and 1146 NDC80^{ASPC}-GEM-Linker clusters from 30 oocytes, pooled from 3 independent experiments). All oocytes remained in metaphase I until 6 hours after NEBD.

(d, e) Quantification of alignment. The distance of NDC80^{ASPC}-GEM clusters from the M-plate was measured in 3D at metaphase I (6 hours after NEBD). “Aligned” refers to positions within 5 μ m from the M-plate. In (c), the volume of individual clusters was normalized with their total volume within the oocyte. The number of oocytes is shown in parentheses (n = 22, 30 oocytes for NDC80^{ASPC}-GEM and NDC80^{ASPC}-GEM-Linker, respectively, pooled from 3 independent experiments). In (e), each dot represents an oocyte (n = 22, 30, 30 oocytes for NDC80^{ASPC}-GEM, NDC80^{ASPC}-GEM-Linker and chromosome, respectively, pooled from 3 independent experiments). For this analysis, both NDC80^{ASPC}-GEM-Linker and chromosomes were imaged and analyzed in the same oocytes. P-values were calculated with the Kruskal-Wallis test followed by Dunn’s post-hoc test with Bonferroni correction.

(f) Alignment stability of NDC80^{ASPC}-GEM-Linker clusters. NDC80^{ASPC}-GEM-Linker clusters and chromosomes that were aligned at 5 hours after NEBD were tracked in 3D over time. The temporal change of representative trajectories in distance from the M-plate from 5 to 6 hours after NEBD is shown (n = 7, 5 trajectories for NDC80^{ASPC}-GEM-Linker clusters and chromosomes, respectively, from one single oocyte included in (e)).

(g) Alignment of NDC80^{ASPC}-GEM-Linker clusters is not fully stable. Percentage of NDC80^{ASPC}-GEM-Linker clusters that maintained their positions within 5 μ m from the M-

plate from 5 hours after NEBD until anaphase onset (8.2 hours after NEBD on average). For this analysis, both NDC80^{ASPC}-GEM-Linker and chromosomes were imaged and analyzed in the same oocytes (n = 17 oocytes from 3 independent experiments). P-value was calculated using a Wilcoxon paired signed-rank test, which was selected because NDC80^{ASPC}-GEM-Linker and chromosomes were analyzed as paired samples from the same oocytes.

(h, i) Aligned NDC80^{ASPC}-GEM-Linker clusters exhibit an elongated shape along the spindle axis. Individual clusters were fitted to an ellipsoid. In (g), angle between the longest axis of the ellipsoid and the spindle axis was measured at metaphase I (6 hours after NEBD). In (i), the aspect ratio was calculated. Each dot represents an aligned NDC80^{ASPC}-GEM-Linker cluster. The number of clusters is shown in parentheses (n = 42 NDC80^{ASPC}-GEM-Linker clusters pooled from 9 oocytes from 3 independent experiments).

(d-i) Bars show the mean +/- SD.

3.3. NDC80^{FL}-GEM-Linker clusters, but not NDC80^{ASPC}-GEM-Linker clusters, recruit the inner kinetochore protein CENP-T

Kinetochore assembly is initiated prior to NEBD, but its structure is not yet fully assembled at this stage. Upon NEBD, recruitment of outer kinetochore components is triggered and progressively proceeds during prometaphase I. This stepwise assembly ensures the maturation of a fully functional kinetochore, with the outer kinetochore providing essential platforms for microtubule attachment and spindle checkpoint signaling. The NDC80 complex is recruited to kinetochores via direct binding to the inner kinetochore protein CENP-T and indirect interaction with the MIS12 complex (Fig. 10a).

To examine the protein components associated with NDC80-GEM-Linker clusters, I compared their co-localization with CENP-T (magenta) and DSN1 (magenta), a subunit of the MIS12 complex, between NDC80^{ASPC}-GEM-Linker clusters and NDC80^{FL}-GEM-Linker clusters in nocodazole-treated oocytes (Fig. 10b-c). Oocytes were fixed 4 hours after NEBD (metaphase I), a time point permissive for interactions between the NDC80 complex and other kinetochore components, and stained with antibodies against each component separately. Consistent with expectations, NDC80^{FL}-GEM-Linker clusters, which retain the SPC24/SPC25-interacting domains of NDC80²⁵, showed clear co-localization with CENP-T. In contrast, NDC80^{ASPC}-GEM-Linker clusters, which lack SPC24/SPC25-interacting domains, did not show detectable co-localization with CENP-T (Fig. 10b). Despite the robust overlap between CENP-T and NDC80^{FL}-GEM-Linker clusters, the MIS12 complex protein DSN1 did not co-localize with NDC80^{FL}-GEM-Linker clusters (Fig. 10c). Taken together, these results indicate that NDC80^{FL}-GEM-Linker clusters selectively recruit the inner kinetochore protein CENP-T but not the MIS12 complex, whereas NDC80^{ASPC}-GEM-Linker clusters fail to recruit CENP-T.

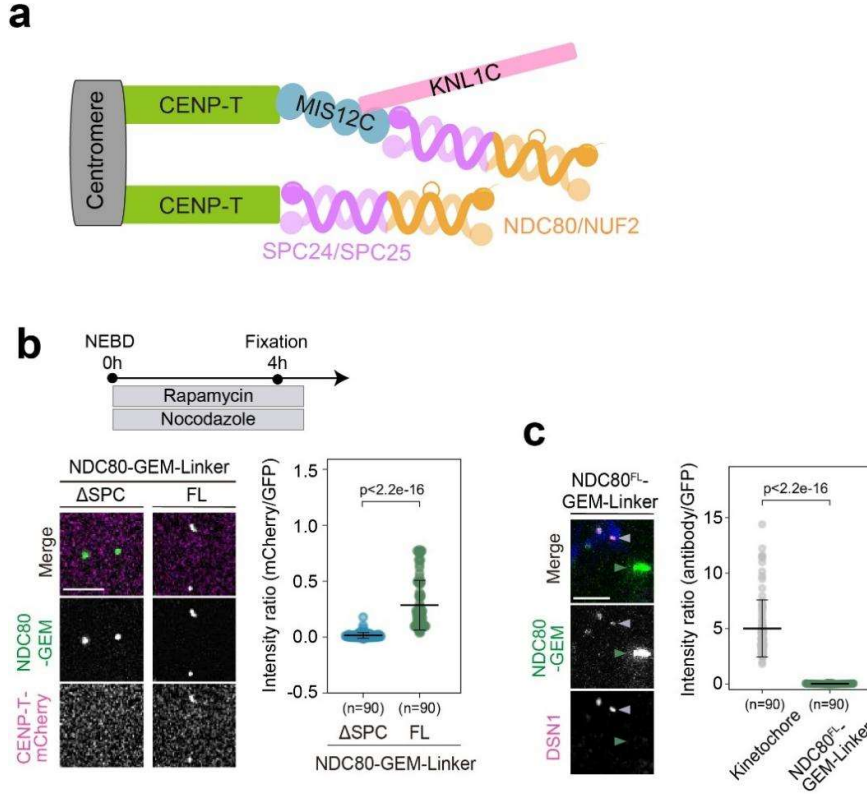


Fig. 10: NDC80^{FL}-GEM-Linker clusters interacted with inner kinetochore protein CENP-T. (a) Diagram of kinetochore organization with the outer kinetochore Ndc80 complex (NDC80-NUF2-SPC24-SPC25), Mis12 complex (MIS12C) and the inner kinetochore protein CENP-T. (b) Co-localization of CENP-T with NDC80^{FL}-GEM-Linker clusters. Nocodazole-treated oocytes were fixed 4 hours after NEBD and immunostained for CENP-T-mCherry (magenta). Z-projection images are shown. Scale bar, 5 μm. P-values were calculated using unpaired two-tailed t-tests (n = 90 NDC80^{ΔSPC}-GEM-Linker clusters from 9 oocytes and n = 90 NDC80^{FL}-GEM-Linker clusters from 9 oocytes, respectively, pooled from 3 independent experiments). Error bars indicate the mean and SD. (c) No detectable recruitment of DSN1 onto NDC80^{FL}-GEM-Linker clusters. Oocytes were fixed 4 hours after NEBD and immunostained for DSN1 (magenta) and DNA (Hoechst33342, blue). Z-projection images are shown. Kinetochores and NDC80^{FL}-GEM-Linker clusters are indicated by gray and green arrowheads, respectively. Scale bar, 5 μm. P-values were calculated using unpaired two-tailed t-tests (n = 90 kinetochores and 90 NDC80^{FL}-GEM-Linker clusters analyzed in the same 9 oocytes, pooled from 3 independent experiments). Error bars indicate the mean +/- SD.

3.4. NDC80^{FL}-GEM-Linker clusters exhibit improved alignment ability

3.4.1. NDC80^{FL}-GEM-Linker assembles into larger clusters

The kinetochore recruitment of the NDC80 complex primarily relies on the inner kinetochore protein CENP-T⁶⁶. Given that NDC80^{FL}-GEM-Linker clusters, but not NDC80^{ΔSPC}-GEM-Linker clusters, recruit CENP-T (Fig. 10b), I asked whether NDC80^{FL}-

GEM-Linker forms larger clusters. To address this question, I compared the cluster sizes between NDC80^{ΔSPC}-GEM-Linker and NDC80^{FL}-GEM-Linker in nocodazole-treated oocytes using the same size analysis as in Fig. 7d. Larger NDC80^{FL}-GEM-Linker clusters with irregularly extended morphology were observed in the cytoplasm (Fig. 11a). FWHM analysis confirmed that NDC80^{FL}-GEM-Linker formed significantly larger clusters with increased fluorescence intensity compared to NDC80^{ΔSPC}-GEM-Linker (Fig. 11b-c). These results indicate that NDC80^{FL}-GEM-Linker assembles into larger clusters than NDC80^{ΔSPC}-GEM-Linker.

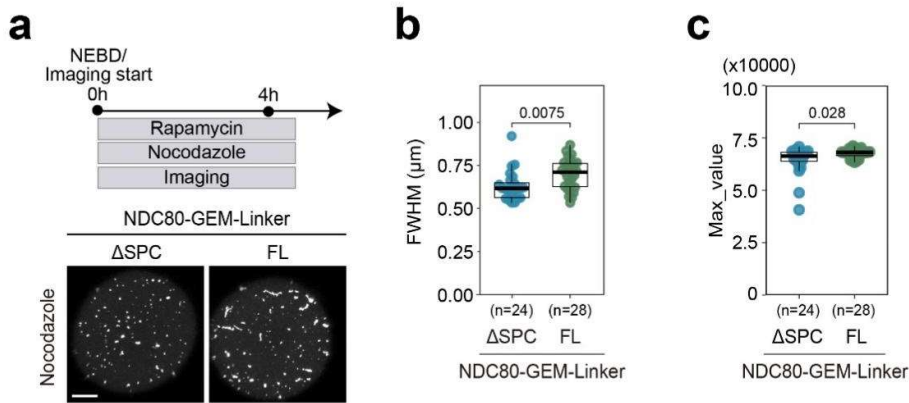


Fig. 11: The SPC domain facilitates NDC80-GEM-Linker clustering.

(a) Assembly of NDC80^{ΔSPC}-GEM-Linker and NDC80^{FL}-GEM-Linker clusters in nocodazole-treated oocytes. Z-projection images of NDC80-GEM-Linker at 4 hours after NEBD. Scale bar, 10 μm.

(b) The SPC domain facilitates NDC80-GEM-Linker clustering. FWHM of NDC80-GEM-Linker fluorescence foci was measured as in Fig. 1d. Each dot represents the mean value averaged from 10 individual foci randomly selected per oocyte (n = 24, 28 oocytes for NDC80^{ΔSPC}-GEM-Linker and NDC80^{FL}-GEM-Linker, respectively, pooled from 3 independent experiments). Box plots: the centre line indicates the median, the box boundaries represent the 25th and 75th percentiles, and the whiskers extend to 1.5 × the interquartile range from the box. P-values were calculated with unpaired two-tailed t-tests.

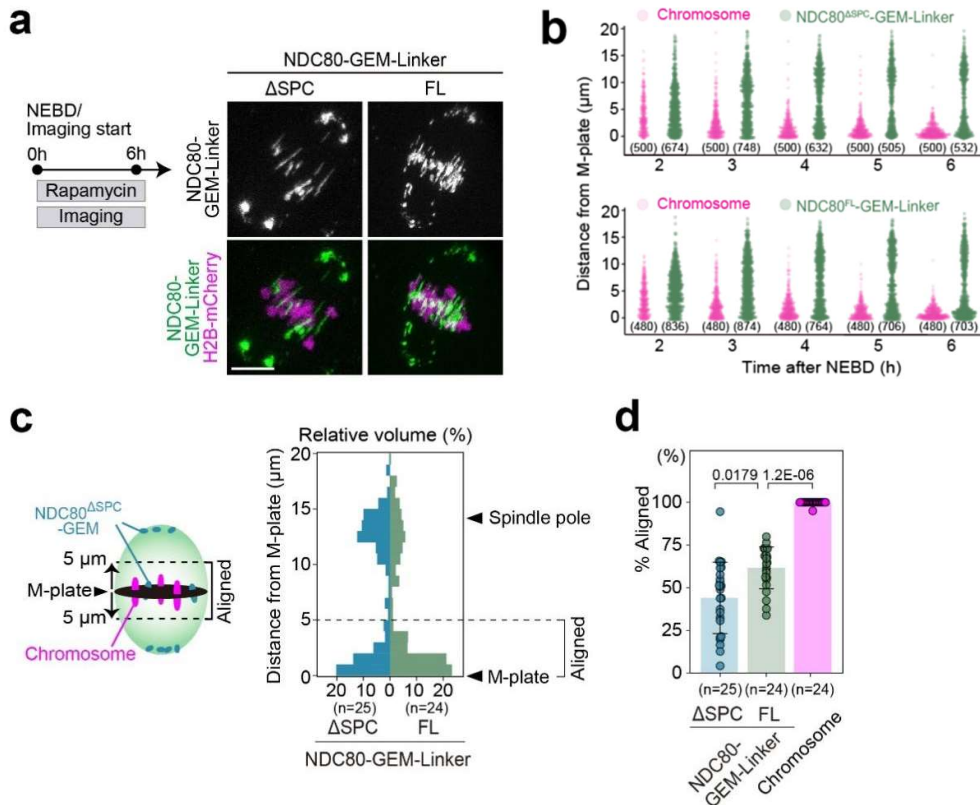
(c) Maximum fluorescence intensity of NDC80-GEM-Linker foci in (a). Each dot represents the mean value averaged from 10 individual foci randomly selected per oocyte (n = 24, 28 oocytes for NDC80^{ΔSPC}-GEM-Linker and NDC80^{FL}-GEM-Linker, respectively, pooled from 3 independent experiments). Box plots: the centre line indicates the median, the box boundaries represent the 25th and 75th percentiles, and the whiskers extend to 1.5 × the interquartile range from the box. P-values were calculated with unpaired two-tailed t-tests.

3.4.2. NDC80^{FL}-GEM-Linker clusters exhibit enhanced alignment on the plate

The large size of NDC80^{ΔSPC}-GEM-Linker clusters is required for their alignment. I therefore asked whether the even larger clusters formed by NDC80^{FL}-GEM-Linker further improve alignment efficiency. To address this question, I performed live imaging in oocytes expressing either NDC80^{ΔSPC}-GEM-Linker clusters or NDC80^{FL}-GEM-Linker clusters (Fig.

12a). NDC80^{FL}-GEM-Linker clusters exhibited overall dynamics similar to those of NDC80^{ΔSPC}-GEM-Linker clusters between 2 to 6 hours after NEBD, gradually aligning on the M-plate, with only a small fraction remaining at the spindle poles (Fig. 12a-b). Quantitative analysis revealed that NDC80^{FL}-GEM-Linker showed a significantly higher proportion of aligned clusters at 6 hours after NEBD, although this proportion remained lower than that observed for endogenous chromosomes (Fig. 12c-d). These results suggest that NDC80^{FL}-GEM-Linker clusters exhibit enhanced alignment efficiency at metaphase I.

To examine whether NDC80^{FL}-GEM-Linker clusters also exhibit enhanced alignment stability, I tracked the behavior of NDC80^{FL}-GEM-Linker clusters and endogenous chromosomes within the same oocyte for 1 hour, starting 5 hours after NEBD. Stable alignment of NDC80^{FL}-GEM-Linker clusters on the M-plate was maintained throughout this period (Fig. 12e). The proportion of NDC80^{FL}-GEM-Linker clusters that remained stably aligned until anaphase was comparable to that of NDC80^{ΔSPC}-GEM-Linker clusters, but lower than that of endogenous chromosomes, which exhibited 100% stability (Fig. 12f). Together, these results indicate that NDC80^{FL}-GEM-Linker assembles into larger clusters with enhanced alignment efficiency compared to NDC80^{ΔSPC}-GEM-Linker clusters, but does not exhibit a clear additional enhancement in alignment stability.



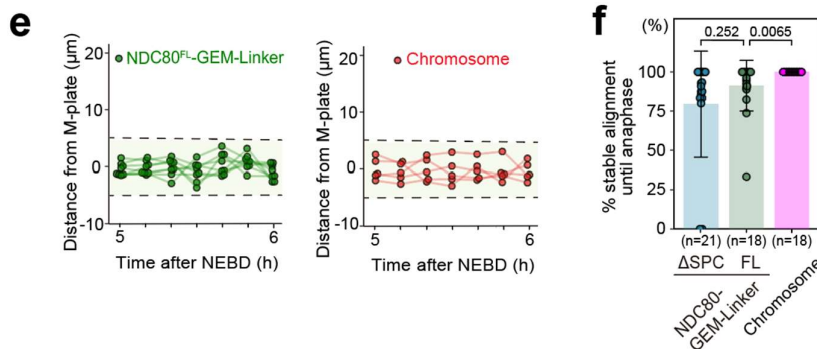


Fig. 12: SPC24-SPC25-binding domain improves NDC80-GEM-Linker cluster alignment.

(a) Live imaging of NDC80^{ΔSPC}-GEM-Linker and NDC80^{FL}-GEM-Linker clusters in nocodazole-free oocytes. Z-projection images of chromosomes (H2B-mCherry, magenta) and NDC80-GEM (green) at 6 hours after NEBD (metaphase I) are shown. Scale bar, 10 μm .

(b) The SPC24-SPC25-binding domain improves NDC80-GEM-Linker cluster alignment. Live imaging dataset of NDC80-GEM-Linker clusters throughout meiosis I (a) were reconstructed into 3D and used to measure the distance of NDC80-GEM-Linker clusters and chromosomes from the metaphase plate. The number of chromosomes or NDC80-GEM-Linker clusters is shown in parenthesis (n = 500 chromosomes and 532 NDC80^{ΔSPC}-GEM-Linker clusters from 25 oocytes, n = 480 chromosomes and 703 NDC80^{FL}-GEM-Linker clusters from 24 oocytes, pooled from 3 independent experiments). All oocytes remained in metaphase I until 6 hours after NEBD.

(c, d) The SPC domain facilitates NDC80-GEM-Linker alignment. Relative volume of NDC80-GEM-Linker clusters along the spindle axis was determined in 3D-reconstructed image datasets in (a). The distance from the spindle equator was quantified. “Aligned” refers to positions within 5 μm from the spindle equator. In (c), the number of oocytes is shown in parentheses (n = 25, 24 oocytes for NDC80^{ΔSPC}-GEM-Linker and NDC80^{FL}-GEM-Linker, respectively, pooled from 3 independent experiments). In (d), each dot represents an oocyte (n = 25, 24, 24 oocytes for NDC80^{ΔSPC}-GEM-Linker, NDC80^{FL}-GEM-Linker clusters and chromosome, respectively, pooled from 3 independent experiments). For this analysis, both NDC80^{FL}-GEM-Linker clusters and chromosomes were imaged and analyzed in the same oocytes. P-values were calculated with the Kruskal-Wallis test followed by Dunn’s post-hoc test.

(e) Alignment stability of NDC80^{FL}-GEM-Linker clusters. Individual NDC80^{FL}-GEM-Linker clusters and chromosomes that were aligned at 5 hours after NEBD were tracked in 3D over time. The temporal change of representative trajectories in distance from the M-plate from 5 to 6 hours after NEBD is shown (n = 8, 5 trajectories for NDC80^{FL}-GEM-Linker clusters and chromosomes, respectively, from one single oocyte included in (d)).

(f) NDC80^{FL}-GEM-Linker cluster alignment is not fully stable. Percentage of NDC80-GEM-Linker clusters and chromosomes that maintained stable alignment from 5 hours after NEBD until anaphase onset. The number of oocytes is shown in parentheses (n = 21, 18, 18 oocytes for NDC80^{ΔSPC}-GEM-Linker clusters, NDC80^{FL}-GEM-Linker clusters and chromosome, respectively, pooled from 3 independent experiments). For this analysis, both NDC80^{FL}-GEM-Linker clusters and chromosomes were imaged and analyzed in the same oocytes. P-values were calculated with the Kruskal-Wallis test followed by Dunn’s post-hoc test.

(d-f) Bars indicate the mean $\pm$ SD.

3.5. Larger sizes of artificial kinetochores prefer the inner position on the plate

3.5.1. NDC80^{FL}-GEM-Linker clusters do not preferentially localize to the inner area of the M-plate

NDC80 beads preferentially localize to the inner area of the M-plate, thereby replacing chromosomes toward the outer area²⁵. The inner area has been described as “high-tension” area, where chromosomes exhibit a higher frequency of premature chromosome separation²⁶. Consistent with this notion, repositioning chromosomes away from the inner area of the M-plate by NDC80 beads suppresses premature chromosome separation²⁶. To test whether NDC80^{FL}-GEM-Linker clusters exhibit a spatial preference similar to that of NDC80 beads, I analyzed live imaging data of NDC80^{FL}-GEM-Linker clusters at 6 hours after NEBD shown in Fig. 12a (Fig. 13a, b). The positions of aligned NDC80^{FL}-GEM-Linker clusters and endogenous chromosomes were extracted and mapped onto the M-plate (Fig. 13c), and their distances from the spindle axis were quantified (Fig. 13d). In contrast to NDC80 beads, NDC80^{FL}-GEM-Linker clusters were predominantly distributed in the outer area of the M-plate rather than the inner area (Fig. 13b-d). Thus, NDC80^{FL}-GEM-Linker clusters do not preferentially localize to the inner area of the M-plate, and the spatial distribution of endogenous chromosomes remains largely unperturbed (Fig. 13a-d).

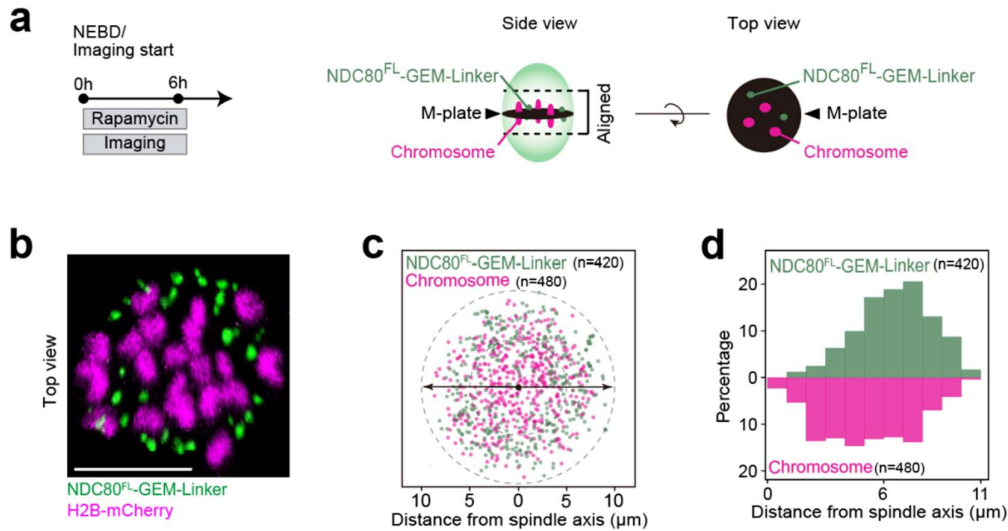


Fig. 13: NDC80^{FL}-GEM-Linker clusters do not prefer the inner position on the plate.

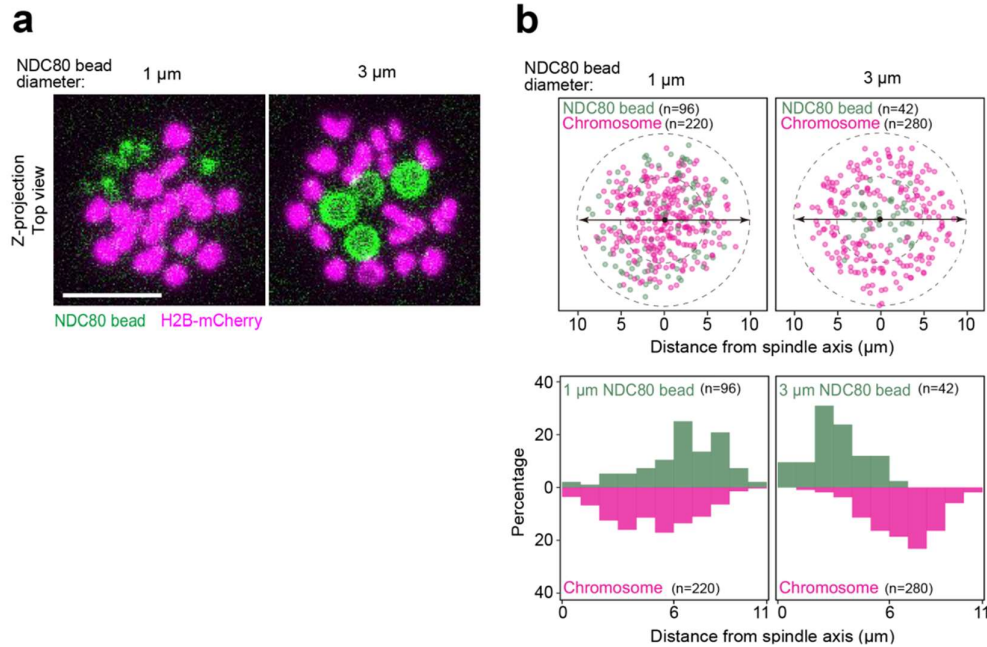
(a) Diagram of aligned NDC80^{FL}-GEM-Linker clusters and chromosomes on the metaphase plate (M-plate) viewed from the side and top of the spindle.

(b) Top view of the M-plate with chromosomes (H2B-mCherry, magenta) and NDC80^{FL}-GEM-Linker clusters (green) at 6 hours after NEBD (metaphase I). Scale bar, 10 μm.

(c, d) NDC80^{FL}-GEM-Linker clusters do not preferentially position in the inner region of the M-plate. In (c), each green dot represents the position of an NDC80^{FL}-GEM-Linker cluster, whereas each magenta dot represents the position of a chromosome, viewed from the top of the spindle. The black dot represents the center of the M-plate. Two dashed circles indicate 5 μm and 10 μm from the M-plate center. The number of measured chromosomes and clusters from 24 oocytes is shown in parentheses (n = 480, 420 from 3 independent experiments).

3.5.2. Size-dependent positioning of NDC80 beads on the M-plate

The diameter of NDC80^{FL}-GEM-Linker clusters was $\sim 0.7 \mu\text{m}$, whereas NDC80 beads had a diameter of $2 \mu\text{m}$ as reported previously²⁵ (Fig. 11b). To investigate whether the size difference between NDC80^{FL}-GEM-Linker clusters and NDC80 beads contributes to their distinct spatial preferences on the M-plate, NDC80 bead injection experiments were performed by Kohei Asai. In these experiments, NDC80 beads of different diameters ($1 \mu\text{m}$ and $3 \mu\text{m}$) were microinjected into prophase I-arrested GV oocytes, and their positions on the M-plate were quantitatively analyzed in this study (Fig. 14a, b). The $3\text{-}\mu\text{m}$ NDC80 beads exhibited robust localization to the inner area of the M-plate, consistent with the previously reported behavior of $2\text{-}\mu\text{m}$ NDC80 beads²⁵. In contrast, $1\text{-}\mu\text{m}$ NDC80 beads did not preferentially localize to the inner area but instead were positioned in the outer area of the M-plate, resembling the spatial distribution observed for NDC80^{FL}-GEM-Linker clusters. Furthermore, neither the alignment efficiency nor the NDC80 fluorescence intensity differed significantly between the $1\text{-}\mu\text{m}$ and $3\text{-}\mu\text{m}$ NDC80 beads (Fig. 14c-e). These results indicate that although smaller NDC80 beads retain the ability to establish a biorientation-like state, they lose the capacity to dominantly occupy the inner area of the M-plate. Together, these observations demonstrate that the size of artificial kinetochores is a key determinant of their spatial positioning on the M-plate, with larger structures preferentially localizing to the inner area.



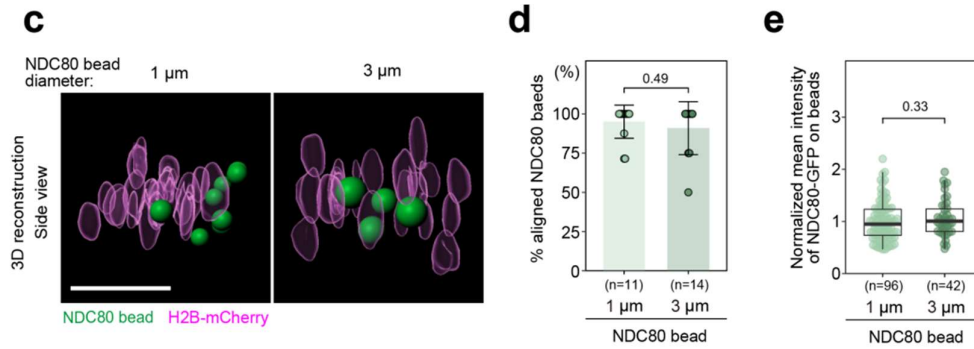


Fig. 14: Size-dependency of the position on the M-plate.

(a) Spatial distribution of NDC80 beads of different sizes on the metaphase plate. Oocytes carrying NDC80 beads of 1- or 3- μ m in diameter (green) and expressing H2B-mCherry (magenta) were imaged at 6 hours after NEBD (metaphase I). Z-projection images are shown. Note that these images provide a view from the top of the spindle, as illustrated in Fig. 13a, showing the distribution of NDC80 beads and chromosomes on the metaphase plate. Scale bar, 10 μ m.

(b) Larger, but not smaller, NDC80 beads preferentially position in the inner region of the metaphase plate. Top: Localization of NDC80 beads and chromosomes in (a) on the metaphase plate. Bottom: Percentage of NDC80 beads and chromosomes from spindle axis in (a). The number of measured chromosomes and NDC80 beads is shown in parentheses ($n = 96$ NDC80 beads and 220 chromosomes from 11 oocytes for 1- μ m beads, and $n = 42$ NDC80 beads and 280 chromosomes from 11 oocytes for 3- μ m beads, pooled from 3 independent experiments).

(c, d) Both larger and smaller NDC80 beads can align at the metaphase plate. Images shown in (a) were reconstructed into 3D and viewed from the side of the spindle. Scale bar, 10 μ m. NDC80 beads were categorized as “aligned” if positioned within 5 μ m from the metaphase plate. The percentage of aligned NDC80 beads was determined in each oocyte. In (d), each dot represents an oocyte ($n = 11, 14$ oocytes from 1- μ m beads, 3- μ m beads, respectively, pooled from 3 independent experiments). P-values were calculated using unpaired two-tailed t-tests. Error bars indicate the mean $\pm$ SD.

(e) Larger and smaller beads have comparable NDC80 density. Images shown in (a) were used to measure NDC80-GFP fluorescence intensity on beads. Box plots: the centre line indicates the median, the box boundaries represent the 25th and 75th percentiles, and the whiskers extend to $1.5 \times$ the interquartile range from the box. P-values were calculated with unpaired two-tailed t-tests. ($n = 96, 42$ beads from 11 oocytes carrying 1- μ m NDC80 beads and 14 oocytes carrying 3- μ m NDC80 beads, respectively, pooled from 3 independent experiments).

3.6. NDC80^{ASPC}-GEM-Linker clusters and NDC80^{FL}-GEM-Linker clusters establish bipolar microtubule attachments

3.6.1. HURP is a microtubule-associated protein enriched on kinetochore fibers in meiosis I

Bipolar kinetochore-microtubule attachments are essential for accurate chromosome segregation. During early meiosis I, microtubules attach to kinetochores and exert pulling

forces that drive chromosome movement toward the M-plate. Stable chromosome alignment is achieved through bipolar attachments from opposite spindle poles, which generates balanced opposing forces required for faithful chromosome segregation during anaphase.

HURP is a microtubule-associated protein that accumulates on kinetochore fibers and contributes to their stabilization during mitosis⁵¹. However, whether HURP associates with kinetochore fibers during oocyte meiosis has remained unclear. To address this, metaphase I oocytes were fixed and immunostained for HURP. Under these conditions, the majority of chromosomes exhibited bipolar microtubule attachments decorated with HURP, indicating that HURP associates with kinetochore fibers during meiosis I (Fig. 15a). Because kinetochore fibers are more resistant to cold-induced depolymerization than non-kinetochore spindle microtubules, cold-treatment is commonly used to selectively visualize kinetochore fibers⁶⁷. Following a 5-minute cold treatment, metaphase I oocytes were fixed and stained for HURP. HURP signals co-localized with cold-stable kinetochore fibers, further supporting the conclusion that HURP is enriched on kinetochore fibers in meiotic oocytes (Fig. 15b).

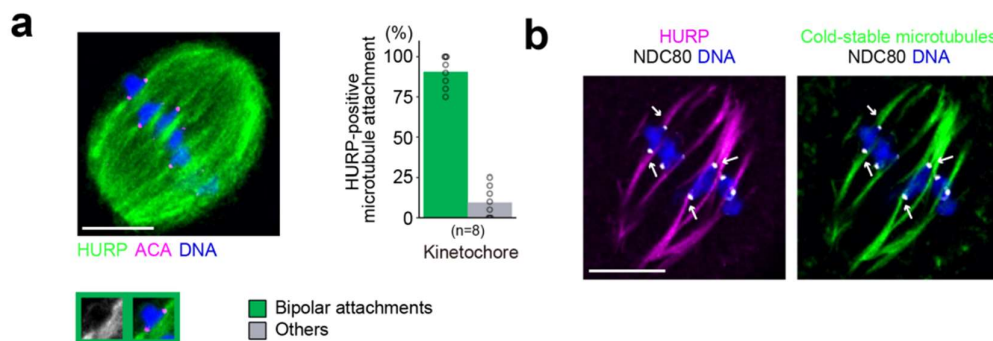


Fig. 15: HURP enriched on kinetochore fibers in meiosis I.

(a) HURP-decorated fibers attach to chromosomal kinetochores. Oocytes were fixed 6 hours after NEBD (metaphase I) and immunostained for HURP (green), kinetochores (ACA, magenta), and DNA (Hoechst33342, blue). Single-slice images are shown. Scale bar, 10 μ m. Attachments of HURP bundles with kinetochores are classified into bipolar attachments and others. The number of oocytes is shown in parentheses (n = 8 oocytes from 3 independent experiments).

(b) Kinetochore-attached HURP fibers overlap with cold-stable microtubules. Oocytes were fixed 6 hours after NEBD (metaphase I) and immunostained for HURP (magenta), α -tubulin (green), kinetochores (ACA, gray), and DNA (Hoechst33342, blue). Single-slice images are shown. Arrows indicate overlapped bipolar microtubule-kinetochore attachments. Scale bar, 10 μ m.

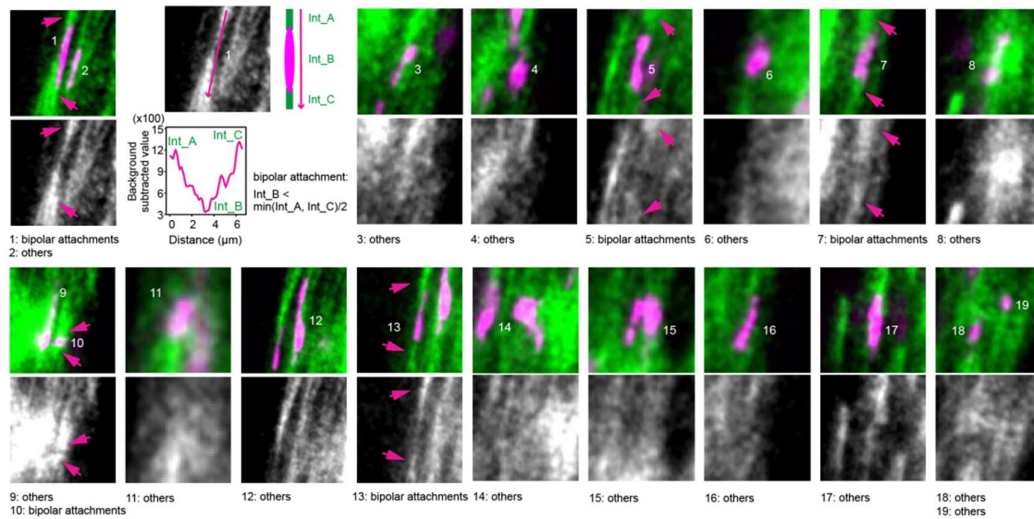
3.6.2. NDC80^{ASPC}-GEM-Linker clusters and NDC80^{FL}-GEM-Linker clusters establish bipolar microtubule attachments

NDC80^{ASPC}-GEM-Linker clusters exhibited stable alignment at the M-plate, and aligned clusters displayed an elongated morphology oriented along the spindle axis during late metaphase I (Fig. 9a, h-i; 12a). I therefore hypothesized that NDC80^{ASPC}-GEM-Linker clusters and NDC80^{FL}-GEM-Linker clusters establish bipolar microtubule attachments, thereby maintaining a biorientation-like state at the M-plate. To examine their microtubule

attachments, oocytes expressing NDC80^{ΔSPC}-GEM-Linker clusters or NDC80^{FL}-GEM-Linker clusters were fixed 6 hours after NEBD (metaphase I) or at 15 hours after NEBD (late metaphase I prolonged by proTAME treatment⁶⁸), and then immunostained for HURP. Microtubule attachments were identified and categorized as bipolar attachments or others based on the defined criteria (Fig. 16a).

At 6 hours after NEBD, NDC80^{FL}-GEM-Linker clusters exhibited a higher proportion of HURP-positive bipolar attachments compared with NDC80^{ΔSPC}-GEM-Linker clusters. However, at 15 hours after NEBD, nearly all NDC80^{FL}-GEM-Linker clusters and NDC80^{ΔSPC}-GEM-Linker clusters established HURP-positive bipolar microtubule attachments (Fig. 16b-e). Moreover, NDC80^{FL}-GEM-Linker clusters formed cold-stable microtubule attachments at 15 hours after NEBD, comparable to those observed on endogenous chromosomes (Fig. 16f, g). Together, these results suggest that aligned NDC80^{ΔSPC}-GEM-Linker clusters and NDC80^{FL}-GEM-Linker clusters establish bipolar microtubule attachments. The higher frequency of bipolar attachments observed for NDC80^{FL}-GEM-Linker clusters is consistent with their enhanced alignment capacity (Fig. 12c-f).

a



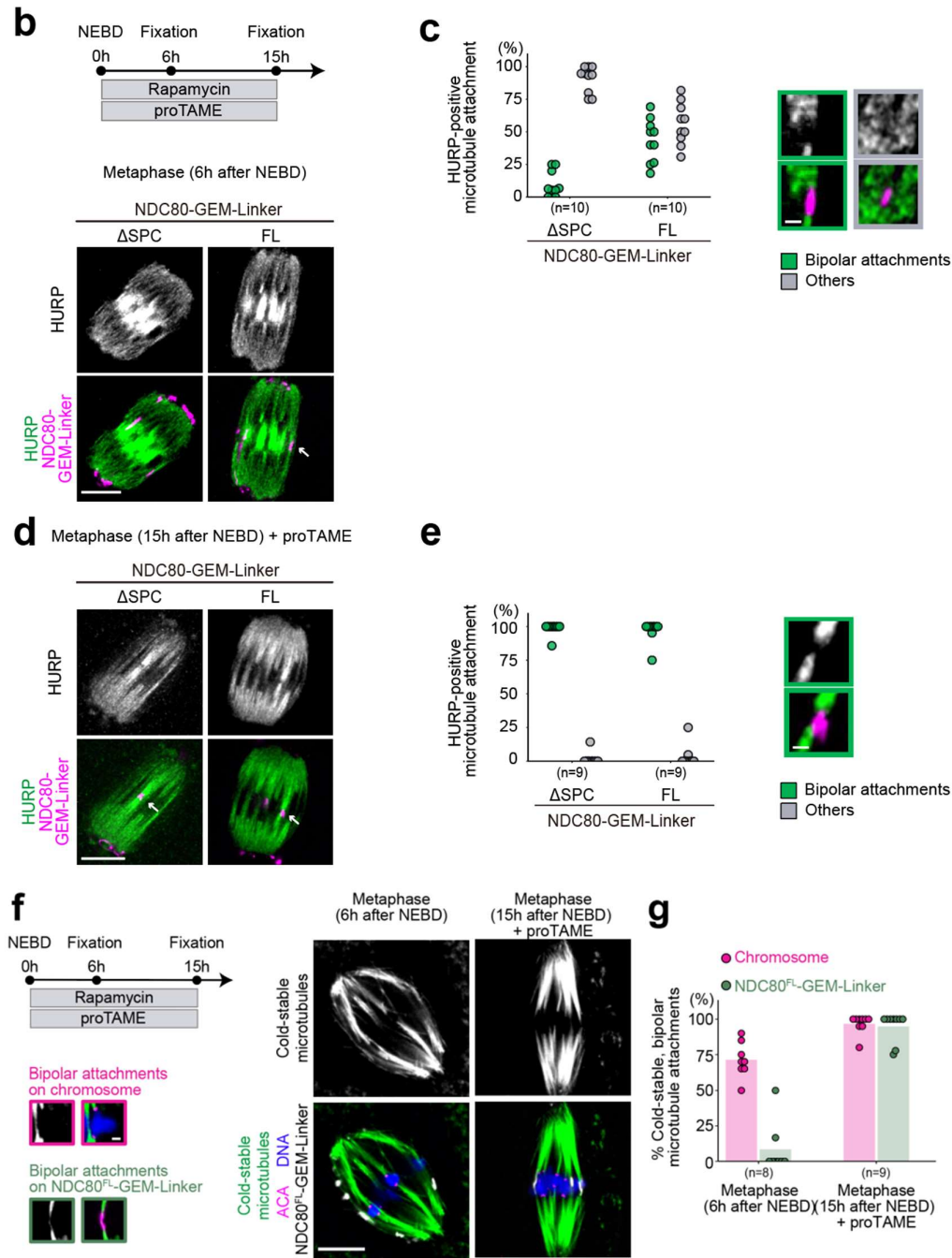


Fig. 16: NDC80-GEM-Linker established bipolar microtubule attachments.

(a) HURP attachments of all individual aligned NDC80^{FL}-GEM-Linker clusters in one oocyte. Single-slice images of NDC80^{FL}-GEM-Linker (magenta)-expressing oocytes immunostained for HURP (green) are shown. Attachments of HURP bundles are classified into bipolar attachments and others. Arrows indicate bipolar HURP attachment. Bipolar attachment is defined by measuring HURP intensity along the NDC80^{FL}-GEM-Linker cluster and is quantitatively assessed using a defined formula (see Materials and Methods).

(b–e) Aligned NDC80-GEM-Linker clusters establish bipolar microtubule attachment. Microtubule attachments of aligned NDC80-GEM-Linker clusters were categorized at 6

hours (metaphase I) (b,c) and 15 hours (prolonged metaphase I) (d,e) after NEBD. To prolong metaphase I, oocytes were treated with proTAME. Single-slice images of NDC80-GEM-Linker (magenta)-expressing oocytes immunostained for HURP (green) are shown. Arrows indicate NDC80-GEM-Linker clusters with bipolar attachment of HURP bundles. Scale bar, 10 μ m. Attachments of HURP bundles with NDC80-GEM-Linker are classified into bipolar attachments (green frame) and others (gray frame). Scale bar, 1 μ m. The number of oocytes is shown in parentheses (n = 10, 10, 9, 9 oocytes from NDC80^{ΔSPC}-GEM-Linker at 6 hours, NDC80^{FL}-GEM-Linker at 6 hours, NDC80^{ΔSPC}-GEM-Linker at 15 hours and NDC80^{FL}-GEM-Linker at 15 hours, respectively, pooled from 3 independent experiments).

(f) Aligned NDC80^{FL}-GEM-Linker clusters establish cold-stable, bipolar microtubule attachment. Microtubule attachments were categorized at 6 hours (metaphase I) and 15 hours (prolonged metaphase I) after NEBD. To prolong metaphase I, oocytes were treated with proTAME. Single-slice images of NDC80^{FL}-GEM-Linker (gray)-expressing oocytes immunostained for α -tubulin (green), kinetochores (ACA, magenta), and DNA (Hoechst33342, blue) are shown. Bipolar attachments of cold-stable microtubules are classified into in chromosome (red frame) and in NDC80^{FL}-GEM-Linker (green frame). Scale bar, 10 μ m.

(g) Quantification of cold-stable, bipolar microtubule attachments. Images in (f) were used. Attachments on chromosomes and NDC80^{FL}-GEM-Linker clusters were analyzed in same oocytes. Each dot indicates an oocyte (n = 8, 9 oocytes from metaphase (6 hours after NEBD) and metaphase (15 hours after NEBD) + proTAME, respectively, pooled from 3 independent experiments). Bars indicate the mean.

3.6.3. Removal of bipolar microtubule attachments reduces the extension degree of NDC80^{FL}-GEM-Linker clusters

Aligned NDC80-GEM-Linker clusters exhibited an elongated morphology oriented along the spindle axis (Fig. 9a, h-i). To verify whether this cluster elongation results from bipolar microtubule pulling forces, oocytes were treated with monastrol, a KIF11/Eg5 inhibitor that disrupts spindle bipolarity⁶⁹. Under these conditions, the extension degree of NDC80^{FL}-GEM-Linker clusters was assessed following the removal of bipolar microtubule attachments. Oocytes expressing NDC80^{FL}-GEM-Linker clusters were allowed to undergo normal meiosis I until 4 hours after NEBD and were then treated with 100 μ M monastrol. Shape changes of NDC80^{FL}-GEM-Linker clusters were monitored by live imaging after monastrol treatment (Fig. 17a). Monastrol treatment resulted in complete disruption of spindle bipolarity and was accompanied by a marked reduction in the extension degree of NDC80^{FL}-GEM-Linker clusters (Fig. 17a, b). Together, these results indicate that the elongation of NDC80^{FL}-GEM-Linker clusters depends on bipolar microtubule attachments that generate pulling forces.

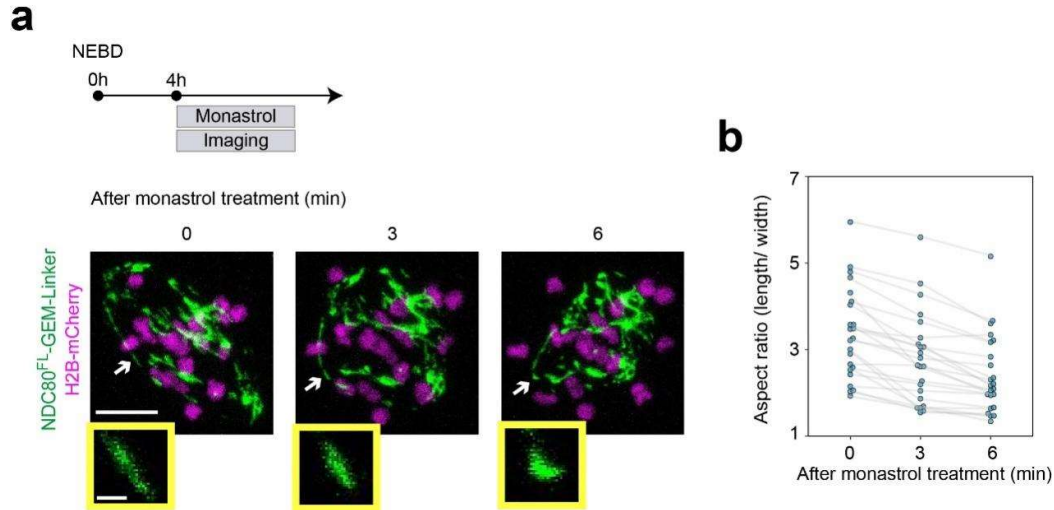


Fig. 17: Removal of bipolar microtubule attachments reduced the extension degree of NDC80^{FL}-GEM-Linker clusters.

(a) The degree of the elongated shape of NDC80^{FL}-GEM-Linker clusters is reduced without bipolar microtubule pulling forces. Live imaging of NDC80^{FL}-GEM-Linker clusters upon monastrol addition is shown. Arrows indicate the magnified NDC80^{FL}-GEM-Linker cluster. Scale bar, 10 μ m. Insets show the magnified region (1 experiment was repeated for the results). Scale bar, 1 μ m.

(b) Quantification of the aspect ratio of NDC80^{FL}-GEM-Linker clusters in (a). Individual clusters were fitted to an ellipsoid. The aspect ratio was calculated. Each dot represents an NDC80^{FL}-GEM-Linker cluster. 23 NDC80^{FL}-GEM-Linker clusters from 3 oocytes in 1 experiment were analyzed.

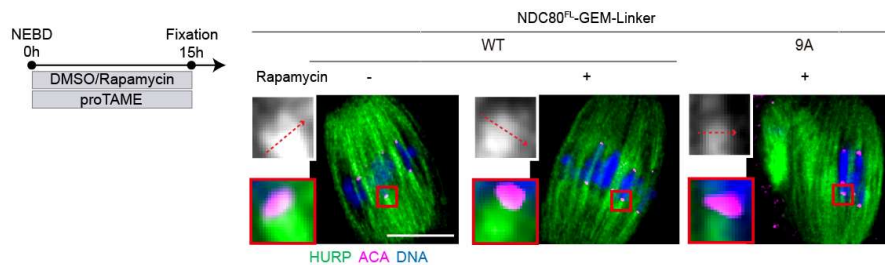
3.7. NDC80^{FL}-GEM-Linker clusters compete with endogenous kinetochores for HURP-decorated microtubule fibers

Kinetochores microtubule fibers are essential for maintaining chromosome biorientation and generating bipolar pulling forces required for faithful chromosome segregation. Because NDC80^{FL}-GEM-Linker clusters form bipolar microtubule attachments in a manner similar to endogenous kinetochores (Fig. 16d, e), I asked whether microtubule fibers associated with NDC80^{FL}-GEM-Linker clusters coexist independently with endogenous kinetochores or instead compete for a limited pool of microtubule associated proteins (MAPs).

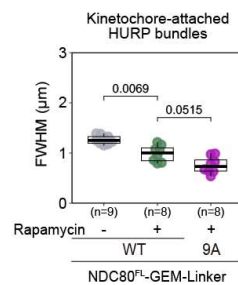
NDC80-9A is a phospho-deficient mutant with increased microtubule-binding affinity^{34, 70}. To examine whether NDC80^{FL}-WT-GEM-Linker clusters compete with endogenous kinetochores for limited MAPs, I compared three groups: GEM, which lacks microtubule-binding affinity; NDC80^{FL}-WT-GEM-Linker, which exhibits moderate microtubule affinity; and NDC80^{FL}-9A-GEM-Linker, which shows enhanced microtubule affinity. The width of endogenous kinetochore fibers was quantified by drawing a line perpendicular to the microtubule bundle at 0.5 μ m from the microtubule tip, followed by FWHM analysis.

A significant reduction in the width of HURP-decorated kinetochore fibers was observed in oocytes expressing NDC80^{FL}-WT-GEM-Linker, and this reduction was further enhanced in oocytes expressing NDC80^{FL}-9A-GEM-Linker (Fig. 18a, b). Notably, the moderate reduction in kinetochore fiber width caused by NDC80^{FL}-WT-GEM-Linker did not impair chromosome alignment, whereas dominant engagement of HURP-decorated microtubules by NDC80^{FL}-9A-GEM-Linker clusters led to chromosome misalignment (Fig. 18c). Together, these results indicate that aligned NDC80^{FL}-GEM-Linker clusters establish their microtubule fibers by competing with endogenous kinetochores for a limited pool of HURP-decorated microtubules. Importantly, a moderate reduction of endogenous kinetochore fiber width does not comprise chromosome alignment or segregation, suggesting the potential of NDC80^{FL}-GEM-Linker as a tool to precisely manipulate kinetochore-microtubule pulling forces (Fig. 18c-e).

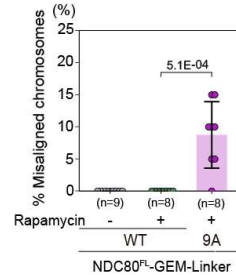
a



b



c



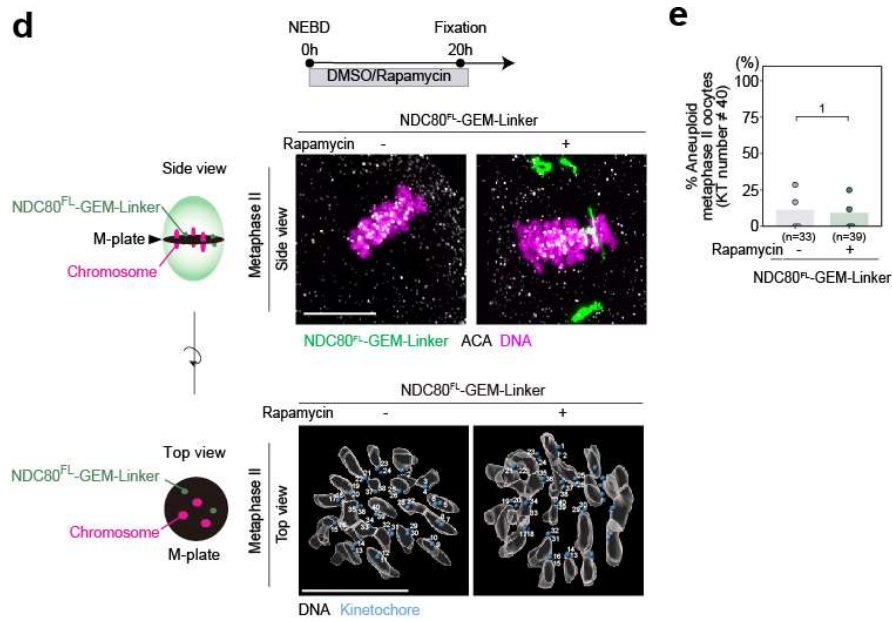


Fig. 18: NDC80^{FL}-GEM-Linker clusters competed with native kinetochores for HURP-decorated microtubule fibers.

(a) NDC80^{FL}-GEM-Linker clusters weaken HURP-decorated kinetochore-microtubule fibers. Oocytes expressing NDC80^{FL}-WT-GEM-Linker clusters and NDC80^{FL}-9A-GEM-Linker clusters were arrested at metaphase I with proTAME, fixed at 15 hours after NEBD, and immunostained for HURP (green), kinetochores (ACA, magenta), and DNA (Hoechst33342, blue). Oocytes expressing the components for NDC80^{FL}-WT-GEM-Linker clusters without rapamycin were used as control. Single-slice images are shown. Insets show the magnified region (3 independent experiments were repeated with the same results). Gray-scale images show magnified HURP signals. Dashed arrows indicate the lines used for FWHM analysis in (f). Scale bar, 10 μ m.

(b) Quantification of the thickness of kinetochore-attached HURP bundles. Images in (a) were used for FWHM analysis as shown in Fig. 7d. Box plots: the centre line indicates the median, the box boundaries represent the 25th and 75th percentiles, and the whiskers extend to $1.5 \times$ the interquartile range from the box. Each dot indicates the mean of 40 kinetochore-attached HURP bundles per oocyte ($n = 9, 8, 8$ oocytes from control (without rapamycin), NDC80^{FL}-WT-GEM-Linker and NDC80^{FL}-9A-GEM-Linker conditions, respectively, pooled from 3 independent experiments). P-values were calculated with the Kruskal-Wallis test followed by Dunn's post-hoc test with p-value adjustment using the Benjamini-Hochberg method.

(c) NDC80^{FL}-WT-GEM-Linker clusters do not interfere with chromosome alignment, whereas NDC80^{FL}-9A-GEM-Linker clusters do. Images in (a) were used to measure the percentage of chromosomes positioned more than 5 μ m from the M-plate. P-values were calculated with the Kruskal-Wallis test followed by Dunn's post-hoc test. The number of oocytes is shown in parentheses ($n = 9, 8, 8$ oocytes from control (without rapamycin), NDC80^{FL}-WT-GEM-Linker and NDC80^{FL}-9A-GEM-Linker conditions, respectively, pooled from 3 independent experiments). Error bars indicate the mean $\pm$ SD.

(d) NDC80^{FL}-GEM-Linker clusters do not increase the aneuploidy rate of metaphase II eggs.

Oocytes carrying NDC80^{FL}-GEM-Linker clusters were matured in vitro and then immunostained for kinetochores (ACA, white) and DNA (Hoechst33342, magenta) at 20 hours after NEBD (metaphase II). The top panel shows z-projection images, providing a view from the side of the spindle. In the bottom panel, images were reconstructed into 3D and viewed from the top of the spindle. Kinetochores are shown as sky blue spots. Note that the oocyte has all 40 kinetochores and thus euploid. Scale bar, 10 μ m.

(e) Percentage of aneuploid eggs. Metaphase II eggs that carried an abnormal number of kinetochores (the normal number is 40) were categorized as “aneuploid”. Bars indicate the means. P-values were calculated using Fisher’s exact tests (two-sided). The number of oocytes is shown in parentheses (n = 33, 39 oocytes from conditions without (–) and with (+) rapamycin, respectively, pooled from 4 independent experiments). Each dot corresponds to the data from one experiment.

3.8. NDC80^{FL}-GEM-Linker clusters reduce chromosome segregation errors

during meiosis I

3.8.1. NDC80^{FL}-GEM-Linker clusters reduce premature chromosome separation during prolonged meiosis I

Aged oocytes exhibit an increased frequency of chromosome mis-segregation, leading to the generation of aneuploid eggs. During meiosis I, bipolar microtubule attachments exert pulling forces on homologous chromosomes, while chromosome cohesion resists these forces, generating tension that stabilizes chromosome biorientation at metaphase I. With advancing age, chromosome cohesion progressively weakens¹⁰⁻¹¹, reducing the ability of chromosomes to withstand microtubule-generated pulling forces. As a consequence, chromosomes become prone to premature separation, resulting in increased aneuploidy. NDC80^{FL}-GEM-Linker clusters offer a potential strategy to precisely modulate kinetochore-microtubule interactions and thereby microtubule pulling forces acting on chromosomes.

In mouse oocytes, the baseline error rate during meiosis I is approximately 10%²⁶, which is considerably lower than that observed in human oocytes^{7, 26}. To increase the frequency of meiotic errors and enable the robust assessment of the effects of NDC80^{FL}-GEM-Linker clusters, aged oocytes were arrested in prolonged metaphase I by treatment with the anaphase inhibitor proTAME⁶⁸. Oocytes were fixed 22 hours after NEBD and stained for kinetochores. Formation of NDC80^{FL}-GEM-Linker clusters was induced by rapamycin treatment, with control and experimental groups defined as – or + rapamycin, respectively. Aged oocytes expressing NDC80^{FL}-GEM-Linker clusters exhibited a significant reduction in premature chromosome separation without disrupting chromosome alignment (Fig. 19a-c). By contrast, rapamycin treatment alone, in the absence of NDC80^{FL}-GEM-Linker clusters, did not reduce premature chromosome separation (Fig. 19d, e). In summary, these results indicate that NDC80^{FL}-GEM-Linker clusters effectively reduce premature chromosome separation during prolonged meiosis I in aged mouse oocytes.

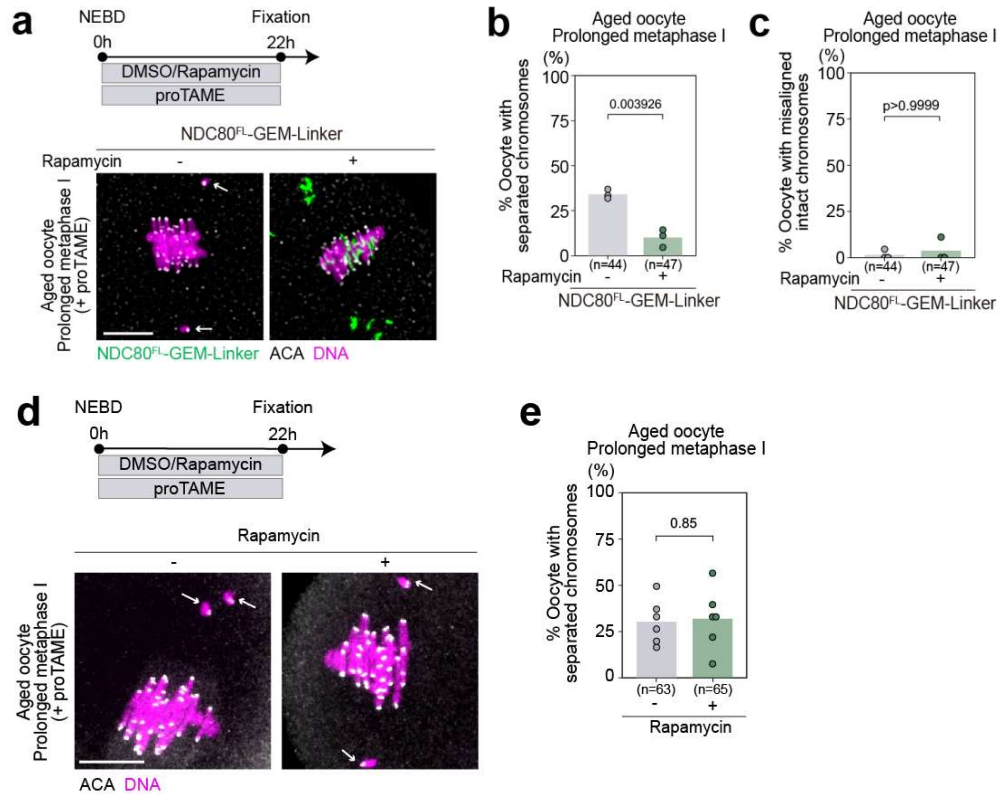


Fig. 19: NDC80^{FL}-GEM-Linker clusters reduced premature chromosome separation in prolonged meiosis I oocytes.

(a) NDC80^{FL}-GEM-Linker clusters prevent premature chromosome separation during prolonged metaphase I. Oocytes derived from aged mice (85.7 ± 2.0 weeks old) were treated with proTAME to prolong metaphase I and immunostained for NDC80^{FL}-GEM clusters (green), kinetochores (ACA, gray), and DNA (Hoechst33342, magenta) at 22 hours after NEBD. Oocytes without rapamycin were used as control. Z-projection images are shown. Arrows indicate prematurely separated chromosomes. Scale bar, 10 μ m.

(b) Percentage of oocytes with one or more prematurely separated chromosomes in (a). P-values were calculated using Fisher's exact tests (two-sided). The number of oocytes is shown in parentheses ($n = 44, 47$ oocytes from conditions without (–) and with (+) rapamycin, respectively, pooled from 3 independent experiments). Each dot corresponds to the data from one experiment.

(c) NDC80^{FL}-GEM-Linker clusters do not interfere with the alignment of intact chromosomes. In this analysis, prematurely separated chromosomes were excluded from oocyte categorization. Percentage of oocytes with one or more misaligned intact chromosomes is shown. P-values were calculated using Fisher's exact tests (two-sided). The number of oocytes is shown in parentheses ($n = 44, 47$ oocytes from conditions without(–) and with (+) rapamycin, respectively, pooled from 3 independent experiments). Each dot corresponds to the data from one experiment.

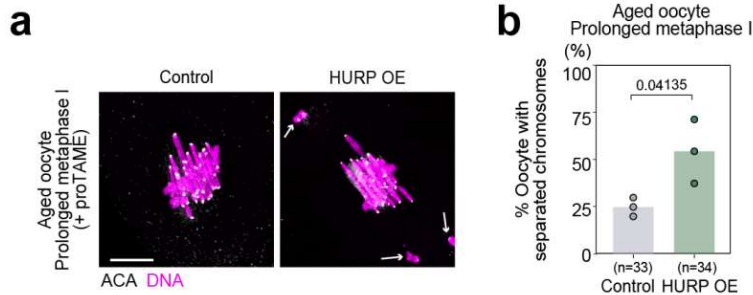
(d, e) Rapamycin treatment does not reduce premature chromosome separation during prolonged meiosis I. Oocytes were collected from aged mice (82.5 ± 7.9 weeks old), treated with or without rapamycin, and cultured in the presence of proTAME. Oocytes were

immunostained for kinetochores (ACA, gray), and DNA (Hoechst33342, magenta) at 22 hours after NEBD. Z-projection images are shown. Arrows indicate prematurely separated chromosomes. Scale bar, 10 μ m. P-values were calculated using Fisher's exact tests (two-sided). The number of oocytes is shown in parentheses (n = 63, 65 oocytes from conditions without (–) and with (+) rapamycin, respectively, pooled from 6 independent experiments). Each dot corresponds to the data from one experiment. (b–e) Bars indicate the means.

3.8.2. Excessive microtubule pulling forces lead to premature chromosome separation in prolonged metaphase I oocytes

Our observations suggest that NDC80^{FL}-GEM-Linker clusters compete with endogenous kinetochores for a limited pool of HURP-decorated microtubules and thereby prevent premature chromosome separation in aged oocytes. If HURP itself is a limiting factor, I reasoned that HURP overexpression should facilitate premature chromosome separation. To test this possibility, I overexpressed HURP in aged oocytes by injecting HURP mRNA into GV-stage oocytes. Oocytes were fixed 22 hours after NEBD, corresponding to prolonged metaphase I induced by proTAME treatment, and stained with ACA (gray) and Hoechst (magenta) (Fig. 20a). The proportion of oocytes exhibiting separated chromosomes increased significantly upon HURP overexpression (Fig. 20b). These results indicate that HURP is a key microtubule-associated protein within a limited pool that affects the balance of kinetochore microtubule fibers regulated by NDC80^{FL}-GEM-Linker clusters.

Monastrol, an Eg5/KIF11 inhibitor, induces the formation of monopolar spindles and thereby abolishes bipolar pulling forces ⁶⁹. In monastrol-treated oocytes, this experimental condition allowed us to directly assess the contribution of microtubule-generated tension to premature chromosome separation. Consistent with this expectation, monastrol treatment significantly reduced the incidence of premature chromosome separation in aged oocytes (Fig. 20c, d). Notably, elimination of bipolar pulling forces did not reduce premature chromosome separation to zero, indicating severely weakened cohesion in severely aged oocytes (81.5 ± 13.3 weeks old). Similarly, in severely aged oocytes (85.7 ± 2.0 weeks old), expression of NDC80^{FL}-GEM-Linker clusters decreased the rate of premature chromosome separation to a level comparable to that observed following monastrol treatment (Fig. 19b, 20b). Together, these results indicate that excessive microtubule pulling forces contribute to premature chromosome separation during prolonged metaphase I in aged oocytes.



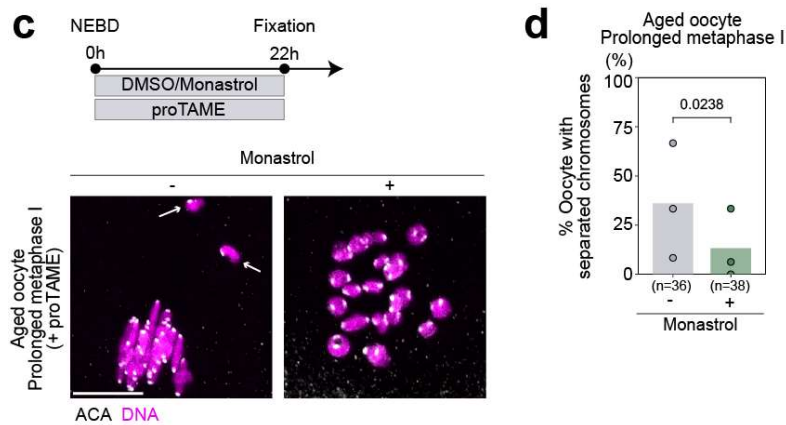


Fig. 20: Excessive microtubule pulling forces lead to premature chromosome separation in prolonged metaphase I oocytes.

(a) HURP overexpression increases premature chromosome separation during prolonged metaphase I. Oocytes derived from aged mice (84.7 ± 3.3 weeks old) were used. HURP was overexpressed through RNA microinjection into prophase-I oocytes (HURP OE). Oocytes microinjected with H₂O were used as control. Oocytes were treated with proTAME to prolong metaphase I and immunostained for kinetochores (ACA, gray) and DNA (Hoechst33342, magenta) at 22 hours after NEBD. Z-projection images are shown. Arrows indicate prematurely separated chromosomes. Scale bar, 10 μ m.

(b) Percentage of oocytes with one or more prematurely separated chromosomes in (d). P-values were calculated using Fisher's exact tests (two-sided). The number of oocytes is shown in parentheses (n = 33, 34 oocytes from control and HURP OE, respectively, pooled from 3 independent experiments). Each dot corresponds to the data from one experiment.

(c, d) Monastrol treatment significantly reduces premature chromosome separation. Oocytes were collected from aged mice (81.5 ± 13.3 weeks old), treated with or without monastrol, and cultured in the presence of proTAME. Oocytes were immunostained for kinetochores (ACA, gray), and DNA (Hoechst33342, magenta) at 22 hours after NEBD. Z-projection images are shown. Arrows indicate prematurely separated chromosomes. Scale bar, 10 μ m. P-values were calculated using Fisher's exact tests (two-sided). The number of oocytes is shown in parentheses (n = 36, 38 oocytes from conditions without (-) and with (+) monastrol, respectively, pooled from 3 independent experiments). Each dot corresponds to the data from one experiment.

3.8.3. NDC80^{FL}-GEM-Linker clusters prevent the formation of aneuploidy during unperturbed metaphase I

To examine whether NDC80^{FL}-GEM-Linker clusters prevent chromosome segregation errors during physiological meiosis I in aged oocytes, metaphase II oocytes were fixed and the number of kinetochores was counted, with a kinetochore count of 40 indicating an euploid egg. Representative top-view images of metaphase II spindles showing all sister kinetochores aligned on the M-plate are shown (Fig. 21a). I found that induction of NDC80^{FL}-GEM-Linker clusters significantly reduced the proportion of oocytes carrying an abnormal number of kinetochores (Fig. 21b), suggesting suppression of chromosome segregation errors during meiosis I. In summary, these results demonstrate that NDC80^{FL}-

GEM-Linker clusters effectively reduce aneuploidy arising from errors during meiosis I in aged oocytes.

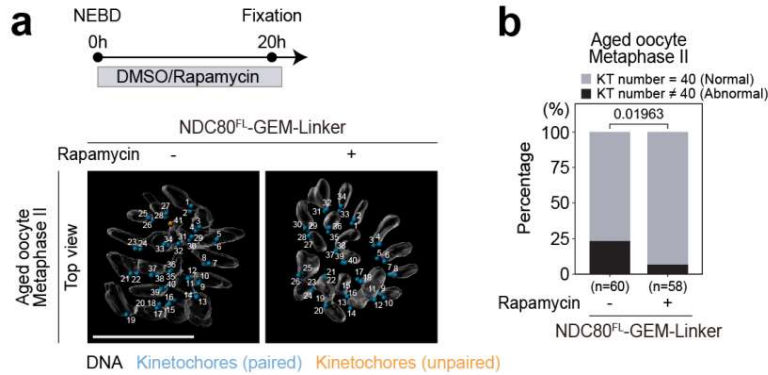


Fig. 21: NDC80^{FL}-GEM-Linker clusters prevented aneuploidy in unperturbed metaphase I oocytes.

(a) NDC80^{FL}-GEM-Linker clusters suppress chromosome mis-segregation during unperturbed meiosis I in aged oocytes (75.4 ± 10.7 weeks old). Metaphase II oocytes at 20 hours after NEBD were immunostained for kinetochores (ACA, gray) and DNA (Hoechst33342, magenta). 3D-reconstructed images viewed from the top of the spindle are shown. Kinetochores of separated chromatids are shown as orange spots, while kinetochores of paired chromatids are shown as sky blue spots. Note that the normal number of kinetochores is 40. Scale bar, 10 μ m.

(b) Percentage of oocytes with an abnormal number of kinetochores in (a). P-values were calculated using Fisher's exact tests (two-sided). The number of oocytes is shown in parentheses (n = 60, 58 oocytes from conditions without (-) and with (+) rapamycin, respectively, pooled from 6 independent experiments).

3.9. NDC80^{FL}-GEM-Linker clusters reduce errors at meiosis II

3.9.1. NDC80^{FL}-GEM-Linker clusters reduce prematurely separated chromatids during unperturbed metaphase II

To examine the error-preventing effect of NDC80^{FL}-GEM-Linker clusters in metaphase II oocytes, mRNAs encoding all essential components of NDC80^{FL}-GEM-Linker clusters were injected into GV-stage aged oocytes, followed by rapamycin treatment to induce the formation of NDC80^{FL}-GEM-Linker clusters. Oocytes were fixed 22 hours after NEBD and stained for kinetochores. In aged metaphase II oocytes, rapamycin-induced formation of NDC80^{FL}-GEM-Linker clusters significantly reduced the number of separated sister chromatids (Fig. 22a, b). By contrast, aged oocytes treated with rapamycin alone, but lacking NDC80^{FL}-GEM-Linker clusters, still exhibited a high incidence of sister chromatid separation (Fig. 22c, d). These findings suggest that NDC80^{FL}-GEM-Linker clusters reduce premature separation of sister chromatids in aged oocytes during meiosis II.

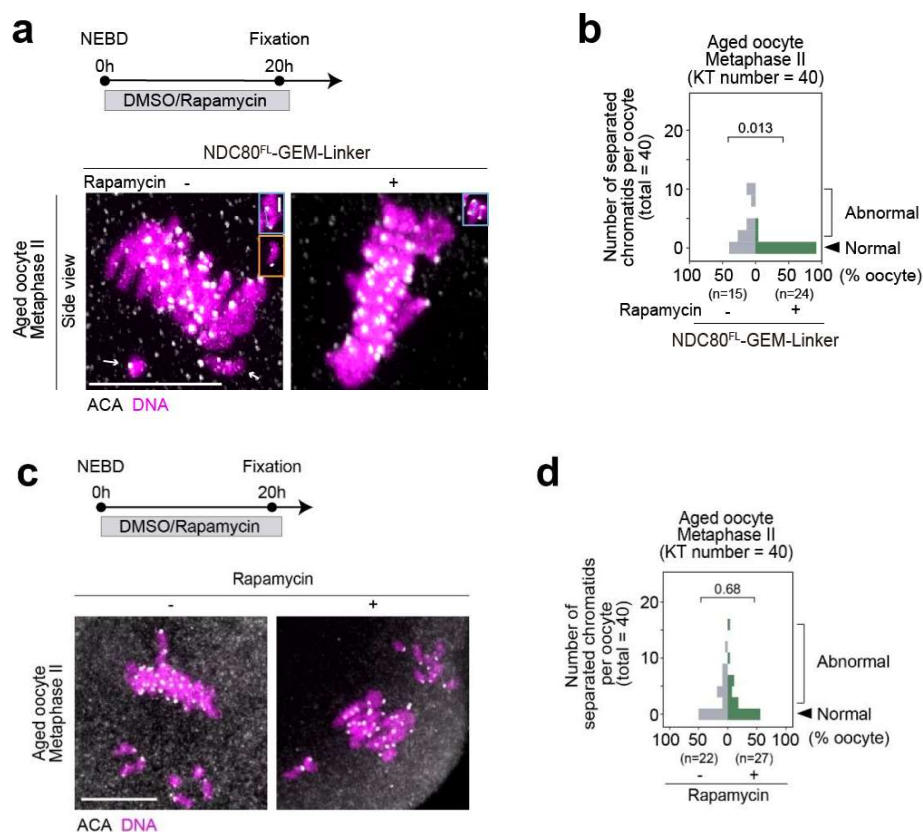


Fig. 22: NDC80^{FL}-GEM-Linker clusters reduced prematurely separated chromatids in unperturbed metaphase II.

(a) NDC80^{FL}-GEM-Linker clusters prevent premature sister chromatid separation during unperturbed metaphase II in aged oocytes (61.2 ± 2.5 weeks old). Metaphase II oocytes at 20 hours after NEBD were immunostained for kinetochores (ACA, gray) and DNA (Hoechst33342, magenta). Z-projection images, which provide a view from the side of the spindle, are shown. Arrows indicate prematurely separated chromatids. Scale bar, 10 μ m. All chromatids were categorized into “prematurely separated chromatids” (magnified in orange frame) or “paired chromatids” (sky blue frame). Scale bar, 2 μ m.

(b) Number of prematurely separated sister chromatids in (a). Oocytes carrying the normal number of kinetochores (40) were used for this analysis. P-values were calculated using unpaired two-tailed t-tests. The number of oocytes is shown in parentheses ($n = 15, 24$ oocytes from conditions without (–) or with (+) rapamycin, respectively, pooled from 2 independent experiments).

(c) Rapamycin treatment does not reduce premature sister chromatid separation during meiosis II. Oocytes were collected from aged mice (77.9 ± 15.1 weeks old), treated with or without rapamycin. Oocytes were immunostained for kinetochores (ACA, gray), and DNA (Hoechst33342, magenta) at 20 hours after NEBD. Z-projection images are shown. Scale bar, 10 μ m.

(d) Number of prematurely separated sister chromatids in (c). P-values were calculated using unpaired two-tailed t-tests. The number of oocytes is shown in parentheses ($n = 22, 27$ oocytes from conditions without (–) and with (+) rapamycin, respectively, pooled from 4 independent

experiments).

3.9.2. NDC80^{FL}-GEM-Linker clusters reduce chromosome tension without disrupting alignment during unperturbed metaphase II

Excessive bipolar pulling forces on aged chromosomes cause premature separation during meiosis I (Fig. 20c-d). To determine whether NDC80^{FL}-GEM-Linker clusters prevent premature separation in meiosis II by reducing bipolar pulling forces, chromosome tension was estimated by measuring inter-kinetochore distance in the sister chromatids shown in Fig. 22a. From top-view spindle images, sister chromatids were categorized as separated chromatids (orange spots) or paired chromatids (sky blue spots)(Fig. 23a). Prematurely separated chromatids were excluded from further analysis, and inter-kinetochore distances were measured only for aligned, paired chromatids. Aged oocytes expressing NDC80^{FL}-GEM-Linker clusters exhibited a significantly decreased inter-kinetochore distance compared with aged control oocytes, without detectable disruption of chromosome alignment (Fig. 23b, c). Although NDC80^{FL}-GEM-Linker clusters reduced chromosome tension in aged oocytes relative to aged controls, inter-kinetochore distances were not fully restored to the levels observed in young oocytes (Fig. 23b). This incomplete rescue is likely attributable to age-associated weakening of cohesion, which results in loosely connected sister chromatids that maintain increased inter-kinetochore distances even when microtubule pulling forces are attenuated. In summary, NDC80^{FL}-GEM-Linker clusters reduce chromosome tension without disrupting chromosome alignment, thereby suppressing premature chromosome separation during unperturbed meiosis II in aged oocytes.

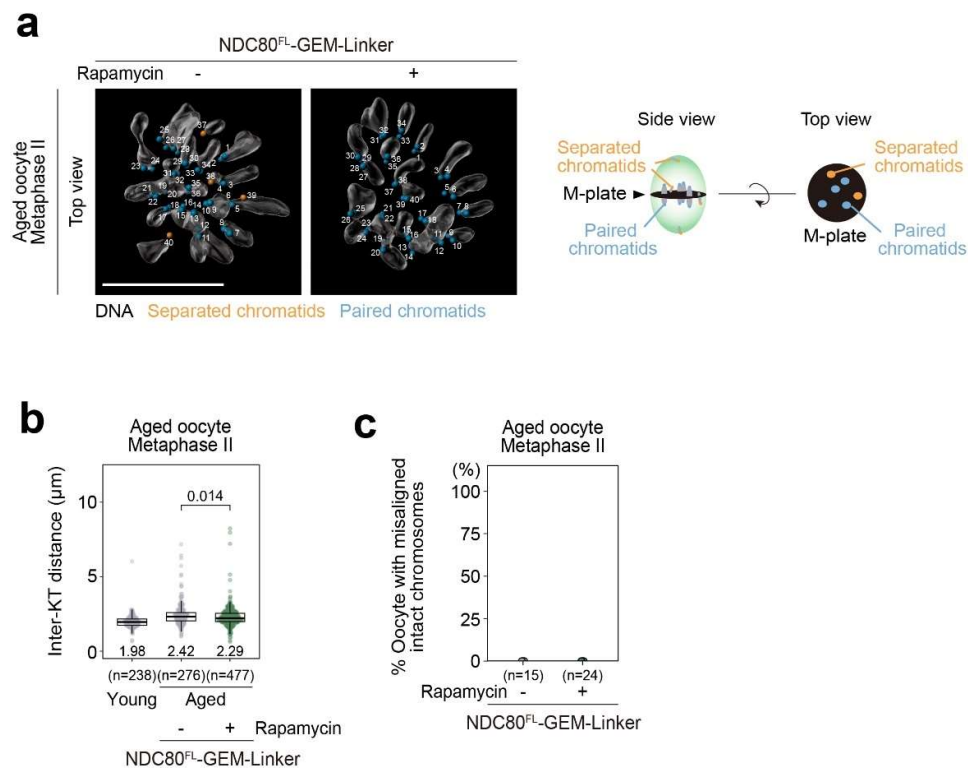


Fig. 23: NDC80^{FL}-GEM-Linker clusters reduced chromosome tension without disrupting alignment in unperturbed metaphase II.

(a) NDC80^{FL}-GEM-Linker clusters prevent premature sister chromatid separation during unperturbed metaphase II in aged oocytes. 3D-reconstructed images viewed from the top of the spindle are shown. Kinetochore of separated chromatids are shown as orange spots, while kinetochores of paired chromatids are shown as sky blue spots. Note that the oocyte has all the 40 kinetochores. Scale bar, 10 μ m.

(b) NDC80^{FL}-GEM-Linker clusters reduce inter-kinetochore distance of paired sister chromatids. Using images shown in (a), distance between paired sister kinetochores was measured. Data from young oocytes (8 weeks old) with no RNA microinjection or rapamycin treatment are shown for reference. Box plots: the centre line indicates the median, the box boundaries represent the 25th and 75th percentiles, and the whiskers extend to 1.5 $\times$ the interquartile range from the box. P-values were calculated using unpaired two-tailed t-tests. The number of chromatid pairs is shown in parentheses (n = 238, 276, 477 chromatid pairs from 12 young oocytes, 15 aged oocytes without rapamycin, and 24 aged oocytes with rapamycin, respectively, pooled from 2 independent experiments).

(c) NDC80^{FL}-GEM-Linker clusters do not interfere with the alignment of paired chromatids. In this analysis, prematurely separated chromatids were excluded from oocyte categorization. Percentage of oocytes with one or more misaligned intact chromosomes is shown. The number of oocytes is shown in parentheses (n = 15, 24 oocytes from conditions without (–) and with (+) rapamycin, respectively, pooled from 2 independent experiments).

To further confirm the effect of NDC80^{FL}-GEM-Linker clusters in severely aged metaphase II oocytes (83.4 ± 0.5 weeks old), oocytes were subjected to the same experimental conditions as those used in Fig. 22-23 (Fig. 24). Significant reductions in premature chromatid separation and chromosome tension were observed in oocytes expressing NDC80^{FL}-GEM-Linker clusters, while chromosome alignment remained intact (Fig. 24b-e). However, the error-preventing effect of NDC80^{FL}-GEM-Linker clusters was limited in severely aged oocytes. After induction of NDC80^{FL}-GEM-Linker clusters, only 23% of severely aged metaphase II oocytes exhibited normal morphology without chromatid separation, despite a marked reduction in the number of separated chromatids (Fig. 24b). These results are consistent with observations in severely aged meiosis I oocytes, where meiotic errors persisted even after the removal of bipolar pulling forces, indicating intrinsic limitations in error prevention in severely aged oocytes. In summary, NDC80^{FL}-GEM-Linker clusters effectively reduce chromosome tension and suppress premature sister chromatid separation in aged metaphase II oocytes, but their protective effect is constrained under conditions of severe maternal aging.

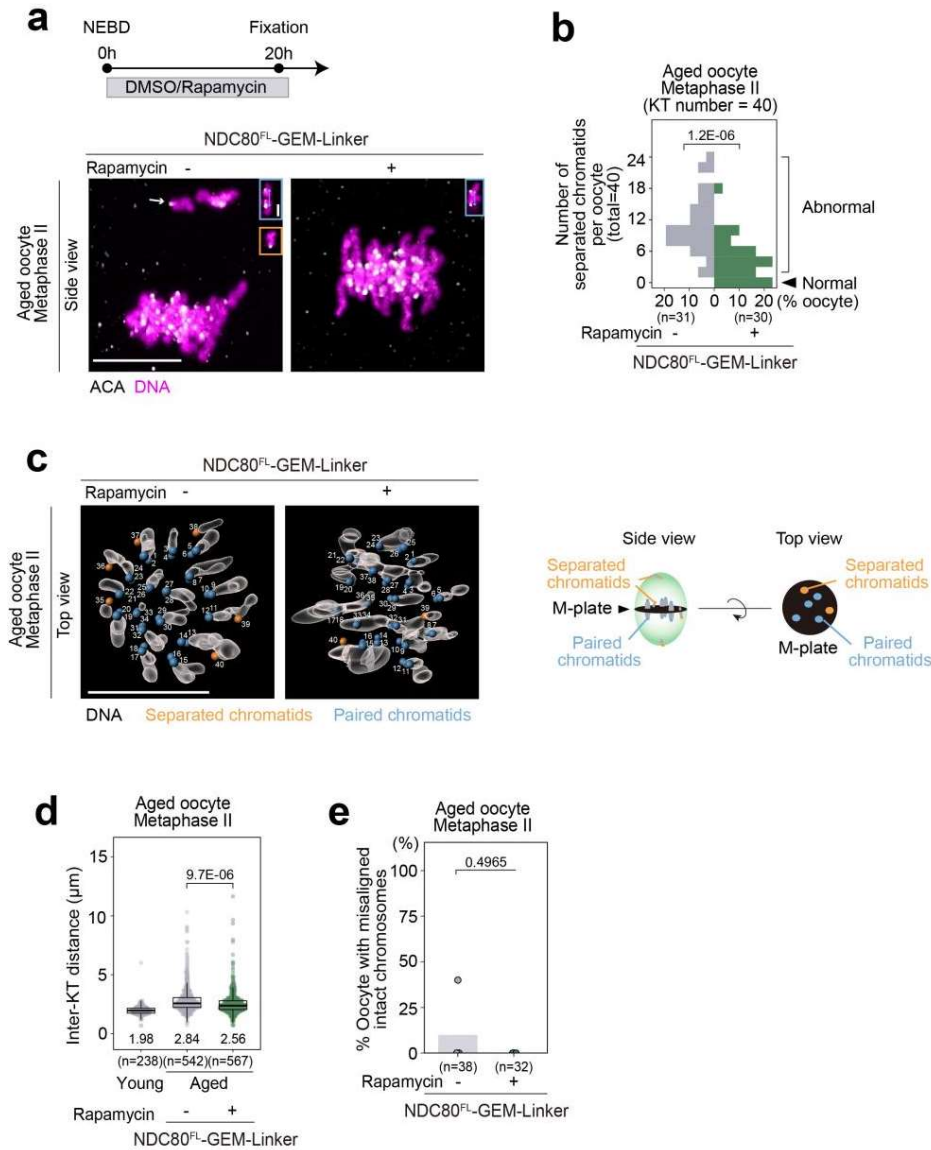


Fig. 24: NDC80^{FL}-GEM-Linker clusters prevent premature sister chromatid separation during meiosis II in severely aged oocytes.

(a) NDC80^{FL}-GEM-Linker clusters prevent premature sister chromatid separation during unperturbed metaphase II in aged oocytes (83.4 ± 0.5 weeks old). Metaphase II oocytes at 20 hours after NEBD were immunostained for kinetochores (ACA, gray) and DNA (Hoechst33342, magenta). Z-projection images, which provide a view from the side of the spindle, are shown. Arrows indicate prematurely separated chromatids. Scale bar, 10 μm. All chromatids were categorized into “prematurely separated chromatids” (magnified in orange frame) or “paired chromatids” (sky blue frame). Scale bar, 2 μm.

(b) Number of prematurely separated sister chromatids in (a). Oocytes carrying the normal number of kinetochores (40) were used for this analysis. P-values were calculated using unpaired two-tailed t-tests (n = 31, 30 oocytes from conditions without (–) and with (+) rapamycin, respectively, pooled from 4 independent experiments).

(c) NDC80^{FL}-GEM-Linker clusters prevent premature sister chromatid separation during

unperturbed metaphase II in aged oocytes. 3D-reconstructed images viewed from the top of the spindle are shown. Kinetochores of separated chromatids are shown as orange spots, while kinetochores of paired chromatids are shown as sky blue spots. Note that the oocyte has all the 40 kinetochores. Scale bar, 10 μ m.

(d) NDC80^{FL}-GEM-Linker clusters reduce inter-kinetochore distance of paired sister chromatids. Using images shown in (a), distance between paired sister kinetochores was measured. Data from young oocytes (8 weeks old) with no RNA microinjection or rapamycin treatment are shown for reference. P-values were calculated using unpaired two-tailed t-tests. The number of chromatid pairs is shown in parentheses (n = 238, 542, 567 chromatid pairs from 12 young oocytes, 18 aged oocytes without rapamycin, 32 aged oocytes with rapamycin, respectively, pooled from 4 independent experiments).

(e) NDC80^{FL}-GEM-Linker clusters do not interfere with the alignment of paired chromatids. In this analysis, prematurely separated chromatids were excluded from oocyte categorization. Percentage of oocytes with one or more misaligned intact chromosomes is shown. P-values were calculated using Fisher's exact tests (two-sided). The number of oocytes is shown in parentheses (n = 38, 32 oocytes from conditions without (–) and with (+) rapamycin, respectively, pooled from 4 independent experiments).

Chapter 4

Discussion

This study engineered NDC80-GEM-Linker clusters as protein-based artificial kinetochores by tethering the outer kinetochore component NDC80/NUF2 complex to microscale clusters of the protein scaffold GEM. These protein-based artificial kinetochores self-assembled in mouse oocytes and established a biorientation-like state on the M-plate with stable microtubule attachments, similar to endogenous chromosomes. While the artificial kinetochores did not perturb the overall arrangement of endogenous chromosomes on the M-plate, NDC80-GEM-Linker clusters competed modestly with endogenous kinetochores for HURP-decorated microtubule fibers. In young oocytes, microtubule pulling forces are properly balanced to ensure accurate chromosome segregation. In aged oocytes, however, age-associated weakening of chromosome cohesion reduces the ability of chromosomes to counteract these excessive forces, predisposing them to premature chromosome separation and, consequently, increased aneuploidy. By interacting with HURP-decorated microtubule fibers from a limited pool, NDC80-GEM-Linker clusters reduced microtubule pulling forces exerted on aged chromosomes, thereby suppressing premature chromosome separation (Fig. 25). Together, these findings demonstrate that protein-based artificial kinetochores provide a promising strategy to prevent meiotic errors in aged oocytes through precise and gentle modulation of kinetochore-microtubule interactions.

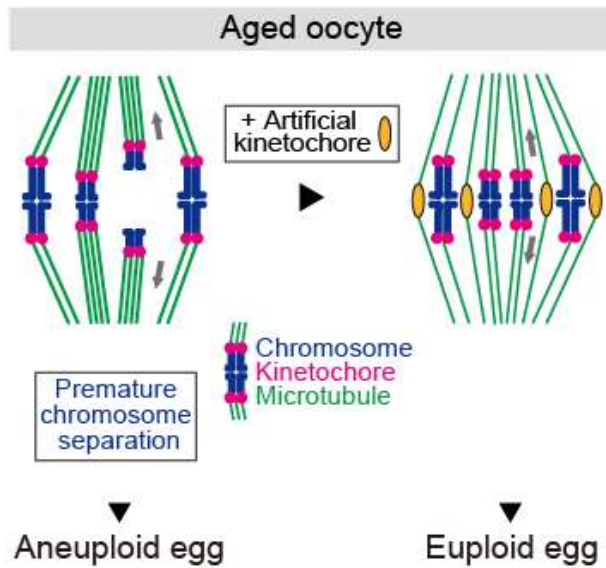


Fig. 25: Model for how artificial kinetochores prevent meiotic errors in aged oocytes. Protein-based artificial kinetochore competes with endogenous kinetochores for limited HURP-decorated microtubule fibers. This competition reduces microtubule pulling forces on chromosomes, thereby preventing premature chromosome separation, a major cause of aneuploidy production in aged oocytes.

The size-dependent spatial distribution of artificial kinetochores

This study revealed that small NDC80-GEM clusters failed to align on the M-plate, whereas larger NDC80-GEM-Linker clusters successfully achieved stable M-plate alignment (Fig. 9a–e). This observation is consistent with a previous study in somatic cells, in which a CENP-T-based artificial kinetochore with a diameter of approximately 35 nm failed to establish spindle biorientation⁶⁴. Together, these findings suggest that artificial kinetochores

below a certain size are insufficient to support stable alignment within the spindle. These results imply that the size of artificial kinetochores is a key determinant of spatial behavior during chromosome segregation and raise the possibility that the characteristic size of endogenous kinetochores contributes to their spatial positioning within the spindle.

Consistent with this idea, increasing the diameter of NDC80 beads from 1 μm to 3 μm altered their preferred position on the M-plate (Fig. 14a, b), revealing a previously unexplored relationship between artificial kinetochore size and spatial positioning within the spindle. Although artificial kinetochores lack native chromatin context, these observations support that size-dependent kinetochore properties can directly influence spatial dynamics.

Chromosome size-dependent spatial distribution of chromosomes

Independent of artificial kinetochore systems, size-dependent spatial distribution has also been reported for endogenous chromosomes, with smaller chromosomes preferentially occupying the inner area of the M-plate ²⁴. In addition, chromosomes within the spindle have been proposed to exhibit a liquid crystal-like properties, which may promote their ordered arrangement on the M-plate ⁷².

In endogenous chromosomes, kinetochores assemble on the core centromeres, whose size is regulated by heterochromatin boundaries through multiple molecular mechanisms ⁷¹. It is therefore plausible that each chromosome establishes a pair of centromeres of a characteristic size, and consequently a pair of kinetochores, which in turn influences its spatial behavior within the spindle. Together, kinetochore size and chromosome-scale physical properties may cooperate to establish the characteristic size-dependent spatial distribution observed during cell division. Future studies will be required to determine how kinetochore heterogeneity and chromosome size are integrated to shape chromosome positioning.

The relationship between HURP-decorated fibers and alignment

Results in this study show that NDC80^{FL}-GEM-Linker, which assembles into larger clusters, exhibits higher alignment efficiency and forms a greater proportion of bipolar, HURP-decorated microtubule attachments compared with NDC80^{ΔSPC}-GEM-Linker (Fig. 11, 12, 16b-c). This correlation suggests that the ability to establish HURP-decorated microtubule fibers contributes to alignment efficiency. Notably, however, bipolar HURP-decorated attachments were observed only on a subset of aligned clusters, indicating that HURP decoration is not an obligatory feature of initial alignment.

Based on these observations, I propose a stepwise model in which initial microtubule attachments guide artificial kinetochores toward the M-plate, followed by HURP decoration that stabilizes these bipolar attachments and supports stable alignment. Future studies will be required to dissect the molecular mechanisms governing both the establishment of initial microtubule attachments and their subsequent stabilization by HURP. In addition, it will also be important to determine whether endogenous kinetochores differ in their capacity to form HURP-decorated microtubule fibers and whether such differences contribute to chromosome-

specific alignment behavior.

Bipolar microtubule attachments on aligned artificial kinetochores

This study demonstrates that NDC80^{FL}-WT-GEM-Linker competes with endogenous kinetochores for a limited pool of HURP-decorated microtubule fibers without perturbing chromosome alignment, whereas NDC80^{FL}-9A-GEM-Linker, which generates stronger competition, induces chromosome misalignment (Fig. 18a-c). These observations suggest that components required for stable kinetochore-microtubule attachments are limited, and that excessive occupation of these components by artificial kinetochores compromises chromosome alignment.

Traditionally, cold-stable microtubules have been used as a hallmark of stable kinetochore-microtubule attachments that support chromosome biorientation^{38, 67}. However, results in this study suggest that this definition may be incomplete. During prolonged metaphase I, aligned NDC80^{FL}-GEM-Linker clusters exhibited both HURP-decorated and cold-stable microtubules, whereas during early metaphase I, only a small proportion of aligned NDC80^{FL}-GEM-Linker clusters were associated with HURP-decorated microtubules. These findings support a model in which kinetochore-microtubule attachments undergo progressive maturation, with HURP decoration and cold-stability representing a later stabilization step rather than a prerequisite for biorientation. Future studies will be required to determine the molecular criteria of functional kinetochore-microtubule attachments and to identify the specific microtubule-associated proteins that confer stability.

Insufficient bipolar microtubule pulling forces on chromosomes

NDC80^{FL}-9A-GEM-Linker generated strong competition with endogenous kinetochores for HURP-decorated microtubule fibers, which resulted in chromosome misalignment. Traditionally, chromosome misalignment has been attributed to unbalanced microtubule pulling forces arising from incorrect kinetochore-microtubule attachments. However, observations in this study suggest a different mechanism. NDC80^{FL}-9A-GEM-Linker did not preferentially compete on one side of the kinetochore; instead, it reduced the overall availability of HURP-decorated microtubule fibers on both sides. This indicates that chromosome misalignment in this context is caused by insufficient bipolar microtubule pulling forces, rather than asymmetric pulling forces. In other words, in addition to balanced force generation, a minimal level of bipolar tension appears to be required to maintain stable chromosome alignment. In future studies, it will be important to determine the threshold of bipolar pulling forces necessary to maintain proper chromosome alignment.

A strategy for error prevention in aged oocytes

The frequency of chromosome mis-segregation in oocytes increases dramatically with maternal age, resulting in miscarriages and congenital disorders. Preventing these segregation errors is therefore of great importance. In this study, the design of a protein-based artificial kinetochore achieved a remarkably effective reduction in meiotic errors in aged mouse oocytes. These findings demonstrate that a protein-based artificial kinetochore can substantially reduce meiotic errors in aged mouse oocytes, highlighting the feasibility of error

prevention during oocyte maturation.

Recently, a reconstituted follicle strategy was reported to reduce aging-associated segregation errors by rejuvenating the follicular microenvironment²⁴. In this approach, aged oocytes were cultured with reconstituted young follicles in an *in vitro* follicle growth system, leading to improved meiotic maturation, developmental competence, and chromosome segregation fidelity. Importantly, this rejuvenation was accompanied by enhanced somatic-oocyte interactions, transcriptomic and metabolomic remodeling, and improved mitochondrial function, suggesting that age-related meiotic defects can be alleviated through long-term, system-level restoration of the oocyte growth environment. Rather than directly targeting the chromosome segregation machinery, this strategy improves meiotic fidelity as a downstream consequence of global cellular and metabolic rejuvenation during folliculogenesis.

In contrast to this long-term (~7 days), environment-based intervention, my artificial kinetochore strategy described in this study acts at a later stage of oocyte maturation and directly targets the mechanical balance of kinetochore-microtubule interactions during meiosis. By functioning as a decoy for HURP-decorated microtubule fibers, NDC80^{FL}-GEM-Linker clusters reduce excessive bipolar pulling forces acting on aged chromosomes, thereby compensating for weakened cohesion during short-term *in vitro* maturation (IVM; <1 day). Given that *in vitro* maturation is widely used in clinical reproductive medicine, this artificial kinetochore strategy provides a biocompatible and potentially practical approach to suppress age-related meiotic errors.

Error prevention in severely aged oocytes

Despite its effectiveness in moderately aged oocytes, the error-preventing effect of the protein-based artificial kinetochore was limited in severely aged oocytes. Even monastrol treatment, which markedly reduces bipolar pulling forces, failed to fully rescue premature chromosome separation (Fig. 20c-d). These results indicate that manipulation of microtubule pulling forces alone is insufficient to correct meiotic errors at advanced maternal age.

Because chromosome cohesion progressively weakens with aging, severely compromised cohesion is likely unable to maintain bivalent integrity from NEBD onward. As a result, severely aged oocytes may become unresponsive to microtubule-based error prevention strategies. In the future, it will be important to explore combined approaches that both strengthen chromosome cohesion and reduce microtubule pulling forces in order to more effectively prevent meiotic errors in severely aged oocytes.

Chapter 5

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