

**The chromosomal passenger complex:
Regulation of chromosome segregation in
kinetoplastids**

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Summary

An intricate network of kinases and phosphatases safeguards the fidelity and timeliness of chromosome segregation in eukaryotes. As the catalytic subunit of the chromosomal passenger complex (CPC), the Aurora B kinase plays a pivotal role within this network by destabilizing improper kinetochore-microtubule attachments and relaying the attachment status to the spindle assembly checkpoint. Intriguingly, Aurora B is conserved even in kinetoplastids, an evolutionarily divergent group of eukaryotes, who lack a canonical spindle checkpoint, and whose kinetochores are composed of a unique set of structural and regulatory proteins. It remains unknown how chromosome segregation and mitotic exit are controlled in kinetoplastids, and whether the CPC contributes to these processes as in other eukaryotes. A deeper understanding of the mitotic regulatory circuitry in kinetoplastids is bound to provide evolutionary insights into fundamental principles of cell division in eukaryotes and can lead to the discovery of druggable targets to combat infectious diseases caused by kinetoplastid parasites (e.g. sleeping sickness, Chagas disease, and leishmaniasis).

In my DPhil work presented in this thesis, I studied the mechanism of recruitment of the CPC to the kinetochore and the function of the Aurora B kinase in regulating chromosome segregation in *Trypanosoma brucei*, the causative agent of African sleeping sickness. In the first part of my thesis, I identify two kinetoplastid-specific kinesins, KIN-A and KIN-B, as bona fide CPC proteins in *T. brucei*. I define an assembly pathway for the trypanosome CPC and demonstrate that the KIN-A subunit controls localization of the CPC to kinetochores and to the central spindle during mitosis. By combining ex vivo cross-linking of native protein complexes with structural and cell biological approaches, I reveal that, unlike other eukaryotes that take advantage of histone modifications for centromere recruitment of the CPC, trypanosomes rely on kinetochore proteins to recruit the CPC. I further demonstrate that the kinesin motor activity of KIN-A drives chromosome alignment in prometaphase and translocation of the CPC to the central spindle upon anaphase onset. The data presented in this section is derived from a recent publication entitled 'Dynamic localization of the chromosomal passenger complex is controlled by the orphan kinesin KIN-A in the kinetoplastid parasite *Trypanosoma brucei*' (<https://doi.org/10.7554/eLife.93522.1>), which is currently under revision at *eLife*.

In the second part of my thesis, I employ an analogue-sensitive approach to show that inhibition of Aurora B activity causes defects in kinetochore-microtubule attachments and arrests cells in metaphase, a phenotype that has not been reported in traditional model eukaryotes. Aurora B phosphorylates several kinetoplastid kinetochore proteins in vitro, including the recently identified Bub1/BubR1-like protein KKT14 (Ballmer et al. 2024, *Open Biology*, in press). Depletion of KKT14 overrides the cell cycle arrest caused by Aurora B

inhibition, while overexpression of a non-phosphorylatable KKT14 construct delays anaphase entry, indicating that the CPC and KKT14 are entangled in an unconventional circuitry controlling mitotic progression and error-free chromosome segregation in trypanosomes. Moreover, I demonstrate using a nanobody-based system that re-targeting the catalytic module of the CPC to the outer kinetochore is sufficient to promote mitotic exit but causes extensive chromosome mis-segregation in anaphase. Finally, I propose a revised description of the unique architecture of the trypanosome kinetochore based on novel TEM data. A paper arising from the data presented in this section is currently under revision at *Journal of Cell Biology* under the title: 'An unconventional regulatory circuitry controls anaphase onset and error-free chromosome segregation in trypanosomes' (doi: 10.1101/2024.01.20.576407).

The third section of this thesis comprises a brief characterization of the Bub1/BubR1-like protein KKT14, showing that KKT14 interacts with the WD40 domain protein KKT15 and promotes accurate chromosome segregation in anaphase. The data presented here are included in a manuscript entitled 'Kinetoplastid kinetochore proteins KKT14-KKT15 are divergent Bub1/BubR1-Bub3 proteins' (<https://doi.org/10.1101/2024.01.04.574194>), which has recently been accepted for publication in *Open Biology*.

Finally, I present some preliminary data on the protein phosphatase PP1-1. We recently found that PP1-1 localizes to kinetochores in trypanosomes, making it the second kinetochore-resident phosphatase to have been identified in *T. brucei* (the other being KKIP7). Unlike KKIP7, PP1-1 is essential for cell growth and error-free chromosome segregation.

In summary, the research presented in this thesis establishes the CPC as a master regulator of mitosis in *T. brucei* and provides important insights into the largely unexplored regulatory machinery governing cell division in kinetoplastids.

Declaration

This thesis contains ~40'000 words, 143 pages and includes 38 figures and 4 tables. The work presented in this thesis was undertaken at the Department of Biochemistry, University of Oxford (October 2020 – March 2023) and at the Wellcome Centre for Cell Biology, University of Edinburgh (April 2023 – March 2024) under the supervision of Bungo Akiyoshi and Lothar Schermelleh.

I hereby declare that this submission is my own work unless stated otherwise (see below) and that I have fully acknowledged the assistance received in completing this work and that it contains no material that has not been formally acknowledged. I have mentioned all source materials used and have cited these in accordance with recognised scientific rules. This thesis has not been submitted for a degree elsewhere.

- AF2 predictions and multiple sequence alignments were run by Bungo Akiyoshi.
- Mass spectrometry was performed by Marjorie Fournier at the Advanced Proteomics Facility at the Department of Biochemistry, University of Oxford, and by Mandy Rettel and Jennifer Schwarz at the Proteomics Core Facility at the EMBL in Heidelberg, Germany.
- Positional scanning peptide array analysis was performed by Hua Jane Lou and Benjamin Turk at the Department of Pharmacology, Yale School of Medicine, USA.

Below is the list of publications arising from my DPhil:

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I am the first author on all the published papers listed above and am re-using material from these publications in my DPhil thesis. I have authored all the text and figures reproduced in this thesis unless otherwise stated. I have received written confirmation from all co-authors that I wrote/produced the material presented in this thesis unless otherwise stated and have their permission to include the material here.

A handwritten signature in blue ink, consisting of a series of loops and a long horizontal stroke extending to the right.

Daniel Ballmer

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List of abbreviations

1K1N	1 Kinetoplast 1 Nucleus
APC/C	Anaphase Promoting Complex/Cyclosome
ATP	Adenosine TriPhosphate
AUK1	Aurora Kinase 1
BIR	Baculovirus Inhibitor of apoptosis protein Repeat
bp	base pair
BRB80	Brinkley Renaturing Buffer 80
BSA	Bovine Serum Albumin
Bub1	Budding uninhibited by benzimidazoles 1
Cas9	CRISPR associated protein 9
CCAN	Constitutive Centromere-Associated Network
CD1	Conserved Domain 1
CENP	CENtromere Protein
CH	Calponin Homology
CL	cross-linking
CPC	Chromosomal Passenger Complex
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAPI	4,5-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FAZ	Flagellum Attachment Zone
FRAP	Fluorescence Recovery After Photobleaching
FRET	Förster Resonance Energy Transfer
GFP	Green Fluorescent Protein
GLEBS	GLE2-Binding Sequence
GPI	GlycosylPhosphatidyInositol
H3T3	Histone H3 Threonine 3
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High Performance Liquid Chromatography
INCENP	INner CENtromere Protein
IP	immunoprecipitation
KARD	Kinase Attachment Regulatory Domain

kb	kilo base
kDa	kilo Dalton
KKIP	KKT-Interacting Protein
KKT	Kinetoplastid Kinetochore Protein
KMN	KNL1/Mis12 complex/Ndc80 complex
KnI1	Kinetochore scaffold 1
KT	kinetochore
lfc	log fold change
LDS	lithium dodecyl sulfate
LMP	Low Melting Point
MAP103	Microtubule Associated Protein 103 kDa
Mklp2	Mitotic kinesin-like protein 2
MS	mass spectrometry
MT	microtubule
NLS	Nuclear Localization Signal
NMR	Nuclear Magnetic Resonance
OA	Okadaic Acid
PAE	Predicted Aligned Error
PAGE	PolyAcrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	phosphodeficient
pLDDT	predicted Local Distance Difference Test
PM	phosphomimetic
PP1	Protein Phosphatase 1
ppm	parts per million
RNA	ribonucleic acid
RNAi	RNA interference
RT	room temperature
SAC	Spindle Assembly Checkpoint
SAH	Single Alpha Helical
SDS	sodium dodecyl sulphate
SEC	Size Exclusion Chromatography
SLiM	Short Linear Motif
TCEP	tris(2-carboxyethyl)phosphine
TEM	Transmission Electron Microscopy

WB	Western Blot
WPGMA	Weighted Pair Group Method with Arithmetic Mean
WT	wild type
YFP	Yellow Fluorescent Protein

1. Introduction

1.1 Basic principles of chromosome segregation

In eukaryotes, genetic material is packaged into chromosomes, which during cell division must be faithfully replicated and distributed from mother to daughter cells. To ensure this, the duplicated chromosome pairs (sister chromatids) that are held together by cohesin complexes need to form stable end-on attachments with microtubules emanating from opposite spindle poles, a process referred to as bi-orientation (Musacchio and Desai, 2017). The interaction of sister chromatids with spindle microtubules is mediated by macromolecular protein complexes known as kinetochores, which assemble onto specialized regions on the chromosomes called centromeres and are under tight control by a complex regulatory circuitry. Defects in centromere or kinetochore function can lead to aneuploidy and loss of genomic integrity, resulting in various disease phenotypes (Holland and Cleveland, 2009).

1.1.1 Centromeres

Centromeres can be classified into different types based on their relative length, organization, and positioning on chromosomes. Most species studied to date have 'monocentric' chromosomes, in which a centromere is assembled at a single genetic locus. Such centromeres can be further classified into 'point centromeres' or 'regional centromeres'. Point centromeres were first discovered in the budding yeast *Saccharomyces cerevisiae* and consist of a precise centromeric DNA sequence that is both necessary and sufficient for kinetochore assembly (McGrew et al., 1986; Carbon and Clarke, 1984; Clarke and Carbon, 1983, 1980). Most model organisms, however (including humans, *Drosophila melanogaster*, *Arabidopsis thaliana* and the fission yeast *Schizosaccharomyces pombe*), possess regional centromeres that are typically composed of a stretch of repetitive DNA, that by itself contributes to but is not sufficient for centromere function (Polizzi and Clarke, 1991; Copenhaver et al., 1999; Sun et al., 2003; Fukagawa and Earnshaw, 2014). A third type of centromere is found in some nematodes (including the model organism *Caenorhabditis elegans*), as well as in some insect and plant species, which assemble a diffuse centromere spanning the entire length of the chromosome (Guerra et al., 2010). Such centromeres are referred to as 'holocentromeres', and the corresponding chromosomes are said to be 'holocentric'.

1.1.2 Kinetochore assembly

In most studied eukaryotes, kinetochore assembly is scaffolded by a centromere-specific histone H3 variant, CENP-A (Westhorpe and Straight, 2013; Maddox et al., 2012; Hori and Fukagawa, 2012; Allshire and Karpen, 2008; Black and Cleveland, 2011), and is thus specified epigenetically. A collection of ‘inner kinetochore’ protein complexes called the constitutive centromere-associated network (CCAN) interacts with centromeric CENP-A chromatin and provides a platform for recruitment of the ‘outer kinetochore’ (Cheeseman et al., 2006; Okada et al., 2006; Izuta et al., 2006; Foltz et al., 2006), which captures spindle microtubules during mitosis. A brief description of the composition of the inner and outer kinetochore in humans is given below.

The human CCAN is composed of 16 proteins, which can be subdivided into five modules or subcomplexes: CENP-C, CENP-LN, CENP-HIKM, CENP-OPQUR and CENP-TWSX (Fig. 1.1, A and B). The CCAN is connected to the CENP-A nucleosome via CENP-C and CENP-N (Falk et al., 2015; Kato et al., 2013; Carroll et al., 2010, 2009). Based on recent cryo-EM data (Yatskevich et al., 2022; Pesenti et al., 2022), CENP-LN forms a channel, flanked by CENP-OPQUR and CENP-HIKM lobes (Fig. 1.1B), through which centromeric DNA is threaded through. CENP-C is a largely disordered protein, which interacts directly with the CENP-A nucleosome, as well as with CENP-LN and CENP-HIKM (Klare et al., 2015; Yatskevich et al., 2022; Pentakota et al., 2017; Pesenti et al., 2022; Przewloka et al., 2011). The outer kinetochore is recruited through direct interaction with CENP-C and CENP-T (Przewloka et al., 2011; Screpanti et al., 2011; Gascoigne et al., 2011; Malvezzi et al., 2013; Nishino et al., 2013b).

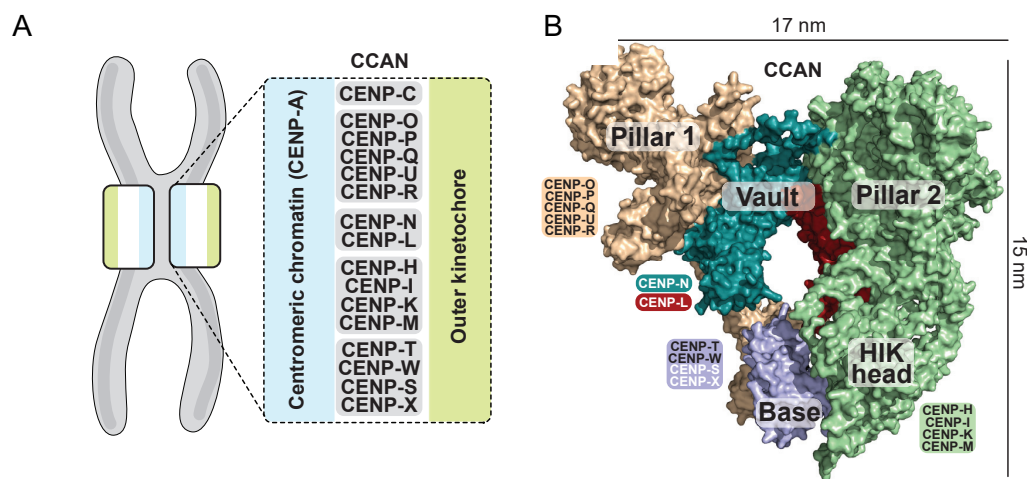


Figure 1.1 Organization of the human CCAN. (A) Schematic illustration of kinetochore organisation including CCAN subcomplexes. (B) Surface model showing the structure of the human CCAN. This figure was adapted from (Pesenti et al., 2022).

With exception of CENP-C, the composition of the CCAN varies dramatically across eukaryotes (van Hooff et al., 2017). For example, most of the CCAN components discussed above are absent in the model eukaryotes *D. melanogaster* and *C. elegans* (McKinley and Cheeseman, 2016). By contrast, key components of the outer kinetochore appear to be highly conserved. The core of the outer kinetochore consists of the KNL1/Mis12 complex/Ndc80 complex (KMN) network (Musacchio and Desai, 2017) (Fig. 1.2). The Mis12 complex (composed of Dsn1, Mis12, Nsl1, and Pmf1) connects the outer kinetochore with the CCAN (Petrovic et al., 2016, 2010; Przewlaka et al., 2011; Screpanti et al., 2011). The Mis12 and Nsl1 subunits interact with Knl1 (Petrovic et al., 2014). Most of the Knl1 protein is composed of a disordered region, which contains a binding site for the PP1 phosphatase and multiple Met–Glu–Leu–Thr (MELT) motifs, which, upon phosphorylation by the Mps1 kinase, provide a platform for recruitment of the spindle assembly checkpoint (SAC) proteins (discussed in chapter 1.1.6) (Krenn et al., 2012; Ghongane et al., 2014; Lara-Gonzalez et al., 2021). Thus, Knl1 acts as primary assembly hub for regulators that signal the attachment status of the kinetochore. The primary receptor for microtubules within the KMN network is the Ndc80 complex, an elongated dumbbell-shaped heterotetramer comprising dimers of Ndc80:Nuf2, and Spc24:Spc25 (Cheeseman and Desai, 2008; Ciferri et al., 2008; Musacchio and Desai, 2017; Cheeseman et al., 2006) (Fig. 1.2). At the centromere-facing end, Spc24-Spc25 form Ring–WD40repeat–DEAD-box helicase (RWD) domains domains that anchor the Ndc80 complex to the kinetochore by interacting with the Mis12 complex and CENP-T (Nishino et al., 2013a; Huisin’T Veld et al., 2016). At the other end, Ndc80 and Nuf2 form calponin homology (CH) domains that directly interact with the microtubule protofilaments (Wei et al., 2007; Ciferri et al., 2005, 2008; Cheeseman et al., 2006). Both Mis12 and Knl1 complexes contribute to microtubule binding by synergistically increasing the microtubule-binding affinity of the Ndc80 complex (Cheeseman et al., 2006).

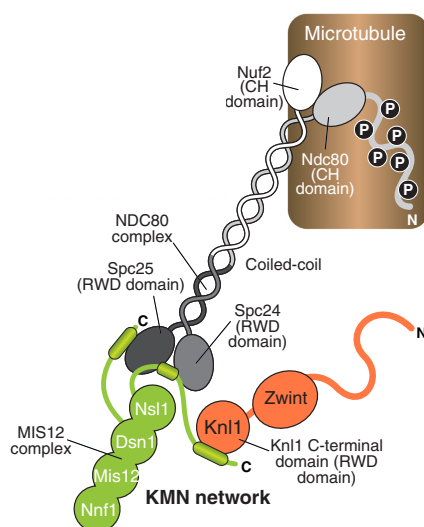


Figure 1.2. Organization of the human KMN network. Schematic illustration of the KMN network, composed of the Knl1 (orange), Mis12 complex (green) and the Ndc80 complex (grey). This figure was adapted from (Pesenti et al., 2016).

Upon association of the KMN network with spindle microtubules, additional protein complexes may assemble at the kinetochore-microtubule (KT-MT) interface in some species to further stabilize the interaction of the outer kinetochore with the spindle apparatus. In metazoans, this role is taken up by the SKA complex (composed of Ska1, Ska2 and Ska3) which binds to curved protofilaments, a distinct feature of growing and shrinking microtubule tips (Abad et al., 2014; Jeyapragash et al., 2012; Schmidt et al., 2012; Auckland et al., 2017). In budding yeast, the unrelated Dam1 complex forms a ring around microtubules, which is physically coupled to the Ndc80 complex (Jenni and Harrison, 2018; Miranda et al., 2005; Ramey et al., 2011; Westermann et al., 2005; Li et al., 2002).

To ensure fidelity and timeliness of chromosome segregation, the KT-MT interface is under a tight regulatory control by a complex circuitry of kinases and phosphatases. The key principles of this regulatory network are discussed in the following chapters.

1.1.3 Regulation of chromosome segregation

To ensure genomic integrity, the sister chromatids need to position themselves on the mitotic spindle in such a manner that they can be equally segregated from mother to daughter cell after the cohesion holding them together is dissolved at the metaphase-anaphase transition. In a process called 'bi-orientation', kinetochores establish stable end-on attachments with microtubules emanating from opposite spindle poles. Such KT-MT attachments are also referred to as 'amphitelic' attachments. In practice, kinetochores engage in a variety of erroneous interactions with microtubules during (pro)metaphase prior to achieving proper bi-orientation (Krenn and Musacchio, 2015) (Fig. 1.3, A and B). Such erroneous attachments can be 'monotelic' (only one of two sister kinetochores bound to microtubules), 'syntelic' (both sister kinetochores bound to microtubules from the same spindle pole), and/or 'merotelic' (single kinetochore bound to microtubules emanating from both spindle poles). Kinetochores can also form 'lateral' attachments to the microtubule lattice before converting them into 'end-on' attachments. In a kind of trial-and-error process referred to as 'error correction', a network of kinases and phosphatases engage in a feedback mechanism that selectively destabilizes erroneous KT-MT attachments. At the heart of this network is the Aurora B Ser/Thr kinase, the catalytic subunit of the chromosomal passenger complex (CPC) (Honda et al., 2003; Gassmann et al., 2004; Sampath et al., 2004; Kim et al., 1999; Nakajima et al., 2009) and primary focus of this DPhil thesis.

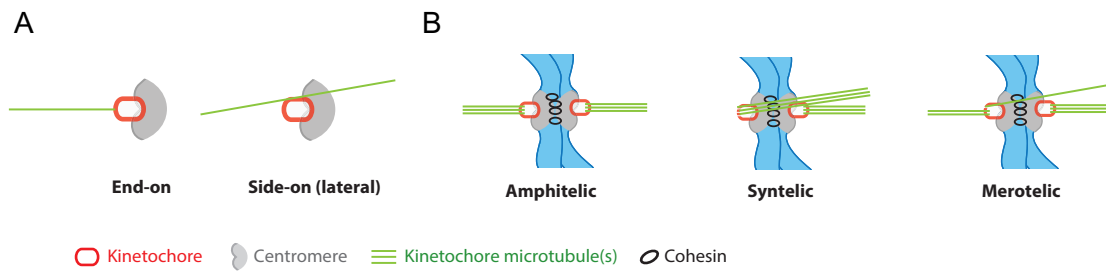


Figure 1.3. KT-MT attachment states. (A) Kinetochores can form end-on attachments with microtubules or bind along the sides of microtubules (lateral attachment). (B) End-on attachments of each sister kinetochore to spindle microtubules emanating from opposite spindle poles is called amphitelic, while the attachment of both sisters to microtubules from the same pole is called syntelic. Merotelic attachments refer to KT-MT attachments, where at least one of the sister kinetochores is attached to microtubules from both poles. This figure was adapted from (McAinsh and Marston, 2022).

1.1.4 Composition and cellular localization of the chromosomal passenger complex

The Aurora B kinase undergoes dynamic localization changes throughout mitosis to enable the spatially restricted phosphorylation of substrates involved in chromosome alignment, chromosome bi-orientation, spindle assembly checkpoint (SAC) signalling and cytokinesis (Carmena et al., 2012). In early mitosis, Aurora B is first detected on chromosome arms and during prometaphase becomes enriched at centromeres, where it destabilizes incorrect kinetochore-microtubule attachments (Krenn and Musacchio, 2015). Upon anaphase onset, Aurora B translocates to the spindle midzone, and during cytokinesis associates with the equatorial cortex to regulate cell abscission (Trivedi and Stukenberg, 2016; Cooke et al., 1987; Adams et al., 2000).

The dynamic localization pattern of the Aurora B kinase is in part achieved through its association with a scaffold comprised of inner centromere protein (INCENP), Borealin and Survivin (Adams et al., 2001, 2000; Gassmann et al., 2004; Romano et al., 2003; Sampath et al., 2004; Vader et al., 2006; Wheatley et al., 2001). Together, these proteins form a tetrameric complex referred to as the chromosomal passenger complex (CPC). The CPC can be partitioned into two functional modules: The ‘catalytic module’ and the ‘localization module’ (Fig. 1.4). The catalytic module is composed of Aurora B in complex with the IN-box at the INCENP C-terminus, which is required for activation of the Aurora B kinase (Bishop and Schumacher, 2002). Full activation of the kinase further requires the Aurora B-mediated phosphorylation of the Thr-Ser-Ser (TSS) motif within the IN-box and auto-phosphorylation of

Aurora B on Thr232 (T232) (Bishop and Schuniacher, 2002; Sessa et al., 2005). These phosphorylation events likely occur in trans, which may explain why increases in the local concentration of the CPC through various clustering mechanisms promote Aurora B activation (Tseng et al., 2010; Kelly et al., 2007a). The localization module of the CPC comprises Borealin, Survivin and the N-terminus of INCENP, which are connected to one another via a three-helical bundle (Jeyaprakash et al., 2007, 2011; Klein et al., 2006). The two modules are linked by the central region of INCENP, composed of an intrinsically disordered domain and a single alpha helical (SAH) domain. INCENP harbours microtubule-binding domains within the N-terminus and the central SAH domain, which play key roles for CPC localization and function (Samejima et al., 2015; Kang et al., 2001; Noujaim et al., 2014; Cormier et al., 2013; Wheatley et al., 2001; Nakajima et al., 2011; Fink et al., 2017; Wheelock et al., 2017; van der Horst et al., 2015; Mackay et al., 1993).

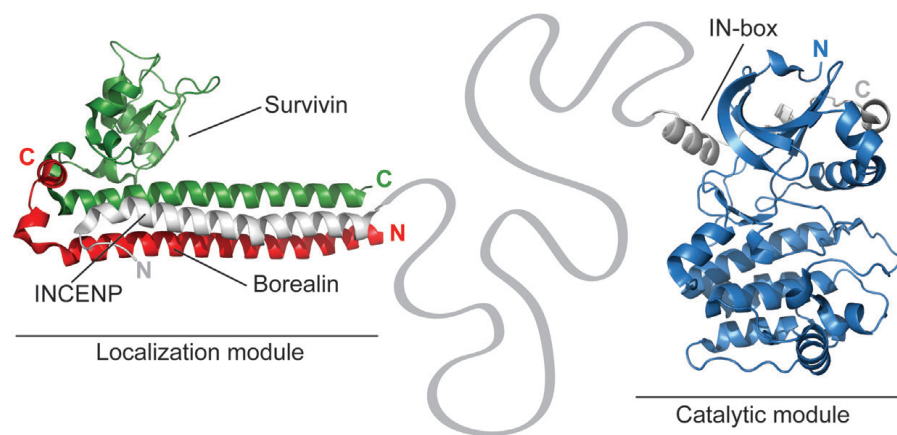


Figure 1.4 Organization of the CPC. Cartoon representation of the CPC, composed of Aurora B (blue), INCENP (grey), Survivin (green) and Borealin (red). The localization module (PDB ID 2QFA) (Jeyaprakash et al., 2007) and the catalytic module (PDB ID 2BFX) (Sessa et al., 2005) of the CPC are linked by the central domain of INCENP. This figure was adapted from (Krenn and Musacchio, 2015).

In vertebrates, recruitment of the CPC to centromeric chromatin depends on two pathways, involving the Haspin and Bub1 kinases. Haspin phosphorylates histone H3 on Thr3 (H3T3ph), which is recognized by the baculovirus IAP repeat (BIR) domain of Survivin (Kelly et al., 2010; Wang et al., 2010; Yamagishi et al., 2010). H3T3ph is initially found along the entire length of chromosomes between sister chromatids but becomes enriched at the inner centromere (the space between sister kinetochores) during late prophase. In contrast, the kinetochore associated Bub1 kinase phosphorylates histone H2A on Thr120 (H2AT120ph)

(Kawashima et al., 2010). H2AT120ph recruits Shugoshin-like proteins (Sgo1 and Sgo2), which in turn are bound by Borealin (Tsukahara et al., 2010; Yamagishi et al., 2010). Recently, Sgo1 has also been demonstrated to interact with the BIR domain of Survivin through an N-terminal histone H3-like motif (Abad et al., 2022; Jeyaprakash et al., 2011). The interactions of Borealin and Survivin with Sgo1 form the basis for a kinetochore-proximal pool of the CPC which is distinct from the main inner centromere pool (Broad et al., 2020; Hadders et al., 2020; Liang et al., 2020).

1.1.5 Error correction by the chromosomal passenger complex

The CPC concentrates at centromeres during early mitosis, where it releases improper KT-MT attachments that lack tension by phosphorylating outer kinetochore proteins, thereby reducing their affinity for microtubules (Musacchio and Desai, 2017). A classic example is the phosphorylation of the disordered N-terminal tail of Ndc80, which neighbors its microtubule-interacting CH domain. The N-terminal tail of Ndc80 has been proposed to act as a 'rheostat', which, depending on the number of sites phosphorylated by Aurora B, can finetune the affinity of the Ndc80 complex for spindle microtubules (Zaytsev et al., 2015; Ciferri et al., 2008). In addition to Ndc80, Aurora B has also been shown to destabilize KT-MT attachments by phosphorylating the Dam1 complex in *S. cerevisiae* and the SKA complex in higher eukaryotes (Cheeseman et al., 2002; Chan et al., 2012).

The error correction process is thought to depend on the ability of the CPC to detect inter- and intra-kinetochore tension, which is associated with stable KT-MT attachments. Indeed, Akiyoshi et al. demonstrated that the application of tension on purified budding yeast kinetochores using a laser trap is by itself sufficient to stabilize KT-MT attachments (Akiyoshi et al., 2010). This parallels the classic experiments performed by Nicklas and colleagues, who used microneedles to impose mechanical tension on meiotic chromosomes in grasshopper spermatocytes (Nicklas and Ward, 1994; Nicklas and Koch, 1969; Ault and Nicklas, 1989).

Various models have been proposed to explain the tension-dependent stabilization of KT-MT attachments in relation to CPC function and localization. One such model is the 'spatial separation' model, which relies on a change in distance between outer kinetochore proteins and the inner centromere, where the bulk of Aurora B is concentrated (Fig. 1.5, A and B). As tension builds up across the inter-sister kinetochore axis, indicative of stably bi-oriented kinetochores, the ability of centromeric Aurora B to reach its substrates is expected to slowly decrease, allowing a progressive stabilization of the KT-MT interface (Liu et al., 2009; Tanaka et al., 2002). The spatial separation model is in part derived from experiments using Förster resonance energy transfer (FRET) sensors (Liu et al., 2009; Fuller et al., 2008). In these experiments, intramolecular FRET between two fluorescent proteins depends on changes in phosphorylation of a linker peptide harbouring an Aurora B substrate consensus motif. The

FRET sensor shows constitutive, tension-independent phosphorylation by Aurora B when targeted to the centromere-kinetochore interface through fusion with CENP-B, but tension-sensitive phosphorylation when positioned further away from the main pool of the CPC at the inner centromere (e.g. through fusion with a subunit of the Mis12 complex at the outer kinetochore). Based on the rapid turnover times of the CPC at centromeres ($t_{1/2} < 1$ min) determined by photobleaching experiments (Ahonen et al., 2009; Beardmore et al., 2004), it has been hypothesized that a population of CPC is released from the centromere, creating a diffusion gradient of active Aurora B (Fig. 1.5A).

An alternative hypothesis for Aurora B function poses that the functionally relevant pool of Aurora B resides not at the inner centromere but near or at kinetochores. This is supported by the observation that the active form of Aurora B (monitored using an antibody recognizing phosphorylated T232) is detectable at kinetochores rather than at centromeres (Posch et al., 2010; Caldas et al., 2013; DeLuca et al., 2011; Santaguida and Musacchio, 2009). Indeed, an inner centromeric pool of PP2A-B56 bound to Shugoshin-1 (Sgo1) has been shown to counteract Aurora B T-loop phosphorylation (Meppelink et al., 2015a). Moreover, activation of Aurora B requires the outer kinetochore component Knl1 (Caldas et al., 2013), further emphasizing the importance of kinetochores rather than centromeres for CPC activity. How the CPC is recruited to the kinetochores and by which mechanism this pool of Aurora B generates the observed phosphorylation gradient within kinetochores warrants further investigation. As alternative to the ‘diffusible gradient model’ (Fig. 1.5A), Santaguida and Musacchio suggested that the central SAH domain of INCENP may act as a flexible ‘dog leash’, allowing the Aurora B kinase to reach its substrates at the outer kinetochore during low intra-kinetochore stretch conditions while being anchored to the base of the kinetochore by means of the CPC localization module (Santaguida and Musacchio, 2009) (Fig. 1.5B). This model is compatible with the sharp confines of Aurora B activity observed within the ~100 nm length scale of the kinetochore. In agreement with the ‘dog leash’ model, truncation of the SAH domain impairs the ability of Aurora B to phosphorylate Dsn1 (Mis12 complex) at the outer kinetochore (Samejima et al., 2015).

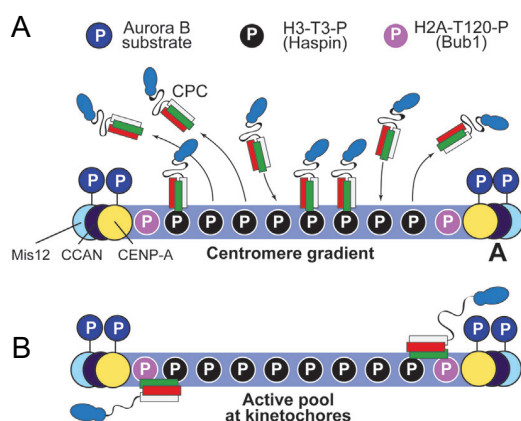


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Figure 1.5 Models for Aurora B function. (A) ‘Diffusible gradient model’: Rapid turnover of CPC’s recruited to the inner centromere generates a gradient of CPC concentration and substrate phosphorylation. Upon proper bi-orientation, Aurora B targets at the outer kinetochore are spatially separated from the peak of this phosphorylation gradient in a tension-dependent manner. (B) ‘Dog leash model’: The active pool of the CPC is tethered at the kinetochore or the kinetochore-proximal chromatin via its localization module. The central region of INCENP acts as a flexible ‘leash’, allowing the catalytic module to phosphorylate its targets within a spatially restricted zone. Depending on intra-kinetochore tension, outer kinetochore proteins will either lie within or outside of this phosphorylation zone. This figure was adapted from (Krenn and Musacchio, 2015).

The classic spatial separation model has been challenged by the observations that the inner centromere localization of Aurora B is dispensable for chromosome bi-orientation in various model organisms (Hengeveld et al., 2017; Campbell and Desai, 2013; Yue et al., 2008). For instance, in budding yeast, deletion of the N-terminal domain of the INCENP^{Sli15} abrogates its association with the Survivin^{Bir1} subunit that targets the CPC to centromeres but fully supports error-free chromosome segregation (Campbell and Desai, 2013). In light of these findings, it was proposed that clustering of CPCs either on microtubules or chromatin is sufficient to activate Aurora B, and that discrimination between improper and proper KT-MT attachments is an intrinsic property of the kinetochore. So-called ‘substrate conformation’ models pose that kinetochore proteins undergo conformational changes when the kinetochore comes under tension, such that they become insensitive to Aurora B-dependent phosphorylation (de Regt et al., 2022; Campbell and Desai, 2013; Hengeveld et al., 2017; Uchida et al., 2009). According to De Regt et al., intra-kinetochore tension may cause key substrate residues to become sterically blocked or cause substrate peptides to stretch, thereby preventing them from being threaded into the active site of the Aurora B kinase. Such models will need to undergo further experimental testing in future studies.

In summary, the CPC recognizes and destabilizes erroneous KT-MT attachments, thereby creating unattached kinetochores, which are granted another opportunity to form amphitelic attachments. Importantly, unattached kinetochores also activate the spindle assembly checkpoint (SAC), a feedback control system that delays the onset of anaphase until all chromosomes have achieved proper bi-orientation (Foley and Kapoor, 2012; Musacchio, 2015; Sacristan and Kops, 2015). The role of the CPC in signaling to the SAC is discussed in the following section.

1.1.6 Role of the chromosomal passenger complex in the spindle assembly checkpoint

SAC components include the kinases Mps1 and Bub1, as well as BubR1 (Mad3), Bub3, Mad1, Mad2, and Cdc20, which are widely conserved among eukaryotes (Kops et al., 2020). It is thought that unattached kinetochores catalyze the production of a diffusible 'wait anaphase' signal, the mitotic checkpoint complex (MCC; composed of Mad2, Cdc20, BubR1 and Bub3), which inhibits the anaphase-promoting complex/cyclosome (APC/C) (Herzog et al., 2009; Izawa and Pines, 2015; Alfieri et al., 2016; Yamaguchi et al., 2016; Sudakin et al., 2001; Chao et al., 2012). The APC/C is a multi-subunit E3 ubiquitin ligase that promotes anaphase onset and sister chromatid separation by marking securin and cyclin B for proteasomal degradation, thereby triggering sister chromatid separation and mitotic exit (Pines, 2011; Alfieri et al., 2017).

The mechanisms through which Aurora B regulates the SAC are interlinked with those mediating error correction. Aurora B activity is needed for recruitment of the Mps1 kinase, which acts upstream of the remaining SAC proteins. Mps1 localizes to kinetochores by binding to the Ndc80 complex, whereby 'naked' Ndc80 complexes (e.g. unattached to microtubules) are preferentially bound by Mps1 (Ji et al., 2015; Hiruma et al., 2015). The precise role of Aurora B in the recruitment of Mps1 is not fully understood, but likely involves phosphorylation of Ndc80 (Zhu et al., 2013; Ji et al., 2015). Mps1 activity triggers a cascade leading to MCC production by phosphorylating multiple MELT motifs on Knl1 to control recruitment of Bub1:Bub3 and BubR1:Bub3 subcomplexes, and subsequently of Mad1, Mad2 and CDC20 (London et al., 2012; Sheppard et al., 2012; Yamagishi et al., 2012). Both Bub1 and its paralog BubR1 (Mad3 in yeast) harbour an ABBA motif, that is required for interaction with CDC20 (Larsen et al., 2007; Diaz-Martinez et al., 2015; DiFiore et al., 2015; Tromer et al., 2016). Interestingly, the kinase activity of Bub1 is largely dispensable for its spindle checkpoint function (Sharp-Baker and Chen, 2001; Kawashima et al., 2010). However, through catalyzing phosphorylation of H2AT120, which promotes CPC recruitment, Bub1 and the CPC are engaged in a positive feedback loop at unattached kinetochores.

To enable rapid changes in phosphorylation status of kinetochore proteins, the activity of kinetochore-resident kinases (e.g. Aurora B, Mps1, Bub1) is carefully balanced by the activity of counteracting phosphatases. The protein phosphatases PP2A-B56 and PP1 are recruited onto kinetochores via binding to BubR1 and Knl1, respectively, and silence the SAC by dephosphorylating the MELT repeats on Knl1, thereby antagonizing Mps1 and CPC activity (Lara-Gonzalez et al., 2021). Importantly, Aurora B-dependent phosphorylation of the RV[S/T]F PP1-docking motif on Knl1 prevents kinetochore targeting of PP1 via Knl1 (Liu et al., 2010; Rosenberg et al., 2011a).

In summary, the timing of anaphase onset in metazoa and yeast is controlled by the rate of MCC production, which depends on the phosphorylation status of the KMN network at each kinetochore, governed by the local activity of Aurora B, the checkpoint kinase Mps1, and antagonizing phosphatases. Upon anaphase onset, the CPC translocates to the central spindle, where it continues to destabilize KT-MT attachments on lagging kinetochores (Sen et al., 2021; Orr et al., 2021; Papini et al., 2021) and is degraded as cells enter G1 (Cooke et al., 1987).

1.2 Chromosome segregation in kinetoplastids

Since key components of the CCAN (such as CENP-A and CENP-C), the KMN network and the SAC are present in nearly all sequenced eukaryotes (Drinnenberg and Akiyoshi, 2017; Meraldi et al., 2006; Tromer et al., 2019; van Hooff et al., 2017), it was long assumed that key principles of chromosome segregation must be shared among eukaryotes. This paradigm was challenged upon discovery of an entirely different set of kinetochore proteins along with the absence CENP-A, CCAN, KMN network and bona fide SAC proteins in the kinetoplastid lineage (Akiyoshi, 2020; Akiyoshi and Gull, 2014; Nerusheva and Akiyoshi, 2016; Nerusheva et al., 2019; D'Archivio and Wickstead, 2017).

1.2.1 The kinetoplastid parasite *Trypanosoma brucei*

The kinetoplastid phylum is an evolutionary divergent group of both free-living and parasitic flagellated protists (Kostygov et al., 2021). The name of this group originates from a specialized organelle, the 'kinetoplast', a mitochondrial DNA cluster located at the base of the flagellum. The parasitic Trypanosomatida (e.g. *T. brucei*, *T. cruzi* and *Leishmania* spp.) pose a serious threat to public health and prosperity in the developing world (Stuart et al., 2008). Collectively, these vector-borne diseases affect more than a billion people worldwide, with an estimated 30'000 deaths occurring annually (WHO, 2017). Vaccine development against kinetoplastids has proven challenging, owing to efficient immune-evasion mechanisms employed by these parasites, and existing drug therapies are unsatisfactory due to poor efficacy, difficulties in administration, prohibitively high costs, severe side effects, or increasing drug resistance (Field et al., 2017; Zofou et al., 2014). Sadly, the development of new drugs against these 'diseases of the poor' has been stagnant, largely due a lack of public interest and economic incentive within the pharmaceutical industry (Mackey et al., 2014; Morens et al., 2004). Especially target-based approaches have seen limited success (Rao et al., 2019), highlighting the need for fundamental research into the molecular cell biology of kinetoplastids.

T. brucei is the causative agent of African sleeping sickness in humans and nagana in domestic animals. During its complex life cycle which involves many morphological and metabolic changes, the parasite transitions between the blood stream of the mammalian host and the alimentary tract and salivary glands of its insect vector, the tsetse fly. *T. brucei* has emerged as an important model organism to study fundamental cell biological processes. Several biological phenomena including antigenic variation (Cross, 1977), glycosyl-phosphatidylinositol (GPI) anchoring of membrane proteins (Ferguson et al., 1988), RNA editing (Blum et al., 1990), and trans-splicing (Sutton and Boothroyd, 1986) were initially discovered in trypanosomes. The genome of *T. brucei* includes ~9000 protein-coding genes and can be readily modified using reverse genetic approaches such as homologous recombination and CRISPR-Cas9 technology (Beneke et al., 2017).

Since the 2014 discovery of unconventional kinetochore proteins in *T. brucei* (Akiyoshi and Gull, 2014), the parasite has also caught the attention of the chromosome segregation field. *T. brucei* possesses 11 homologous pairs of large chromosomes (called megabase chromosomes, 1–6 Mb in size), which harbour regional centromeres (20–120 kb of typically AT-rich repetitive sequences), and an additional ~100 smaller chromosomes (minichromosomes, 50–150 kb; intermediate chromosomes, 200–700 kb), without canonical centromeres (Obado et al., 2007; Ersfeld, 2011; Daniels et al., 2010; Akiyoshi and Gull, 2014). Segregation of both megabase chromosomes and minichromosomes depends on a mitotic spindle, which forms within the nucleus during a closed mitosis (Ogbadoyi et al., 2000).

1.2.2 The trypanosome kinetochore

A unique set of ~30 proteins called kinetoplastid kinetochore proteins (KKTs) and KKT-interacting proteins (KKIPs) are present in *T. brucei*, and homologs thereof have also been identified in other kinetoplastid species (Akiyoshi and Gull, 2014; Nerusheva and Akiyoshi, 2016; Nerusheva et al., 2019; D'Archivio and Wickstead, 2017). Six of these KKT proteins (KKT2, KKT3, KKT4, KKT20, KKT22 and KKT23) localise to centromeres throughout the cell cycle and are thought to form the foundation of the kinetoplastid kinetochore (Akiyoshi and Gull, 2014; Nerusheva and Akiyoshi, 2016). KKT2 and KKT3 localize to centromeres using unique zinc-binding central domains and have divergent polo boxes required for localization of other kinetochore proteins (Marciano et al., 2019; Ishii et al., 2022). They also possess kinase domains classified as unique among known eukaryotic kinase subfamilies (Parsons et al., 2005; Akiyoshi and Gull, 2014). Of note, kinase domains are not present in constitutively localized kinetochore proteins in other eukaryotes, highlighting these protein kinases as a unique feature of kinetoplastid kinetochores. KKT2 and KKT3 are required for recruitment of KKT4, the only known microtubule-binding kinetochore protein in *T. brucei* (Marciano et al.,

2019; Llauro et al., 2018). Another set of proteins including KKT1, KKT5, KKT6, KKT7, KKT16, KKT17, KKT18, KKT24, KKT25, and KKIP1 is detectable at kinetochores from S phase until the end of anaphase (Akiyoshi and Gull, 2014; Nerusheva et al., 2019). Such proteins may play important roles for microtubule binding and/or recruitment of regulatory factors. Another group of proteins comprising KKT8, KKT9, KKT10, KKT11, KKT12 and KKT19, localizes at kinetochores from S phase until metaphase, suggesting that they may be involved in regulating anaphase onset. Indeed, depletion or inhibition of the KKT10 and KKT19 kinases (also known as CLK1 and CLK2, respectively), two functionally redundant paralogs with identical cdc2-like kinase domains (Akiyoshi and Gull, 2014; Ishii and Akiyoshi, 2020; Parsons et al., 2005), results in a prominent delay in the metaphase-anaphase transition (Ishii and Akiyoshi, 2020; Saldivia et al., 2020). Two proteins, KKT14 and KKT15, are unique among kinetoplastid kinetochore proteins in that they localize to kinetochore from G2 to anaphase (Akiyoshi and Gull, 2014). KKT14 and KKT15 have recently been identified to be divergent Bub1/BubR1 and Bub3 proteins, respectively, and are thought to play a crucial role in regulating KT-MT attachments and/or timing of anaphase entry (Ballmer et al., 2024a).

Direct protein–protein interactions among KKT proteins have been demonstrated for KKT16:KKT17:KKT18 (KKT16 complex), KKT8:KKT9:KKT11:KKT12 (KKT8 complex), KKT7:KKT10, KKT2:KKT1, KKT1:KKT6, and KKT3:KKT22:KKT23 (Tromer et al., 2021; Ishii and Akiyoshi, 2020; Ishii et al., 2022) (Ludzia et al., unpublished data). Furthermore, the structure of several KKT protein domains have been solved using X-ray crystallography. These include the C-terminal pseudokinase domain of KKT14 (Ballmer et al., 2024a), the central domain and divergent polo-box domain of KKT2 (Marcianò et al., 2021; Ishii et al., 2022), the polo-box domain of KKT3 (Ishii et al., 2022), the coiled-coil and BRCT domains of KKT4 (Ludzia et al., 2021), and the acetyltransferase domain of KKT23 (Ludzia et al., unpublished data).

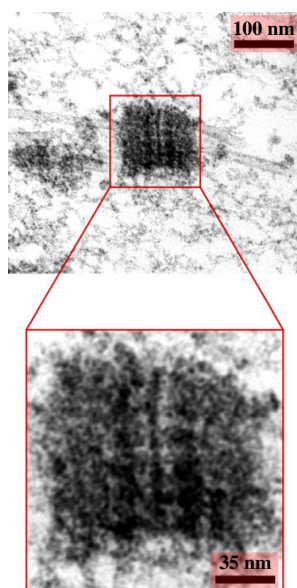
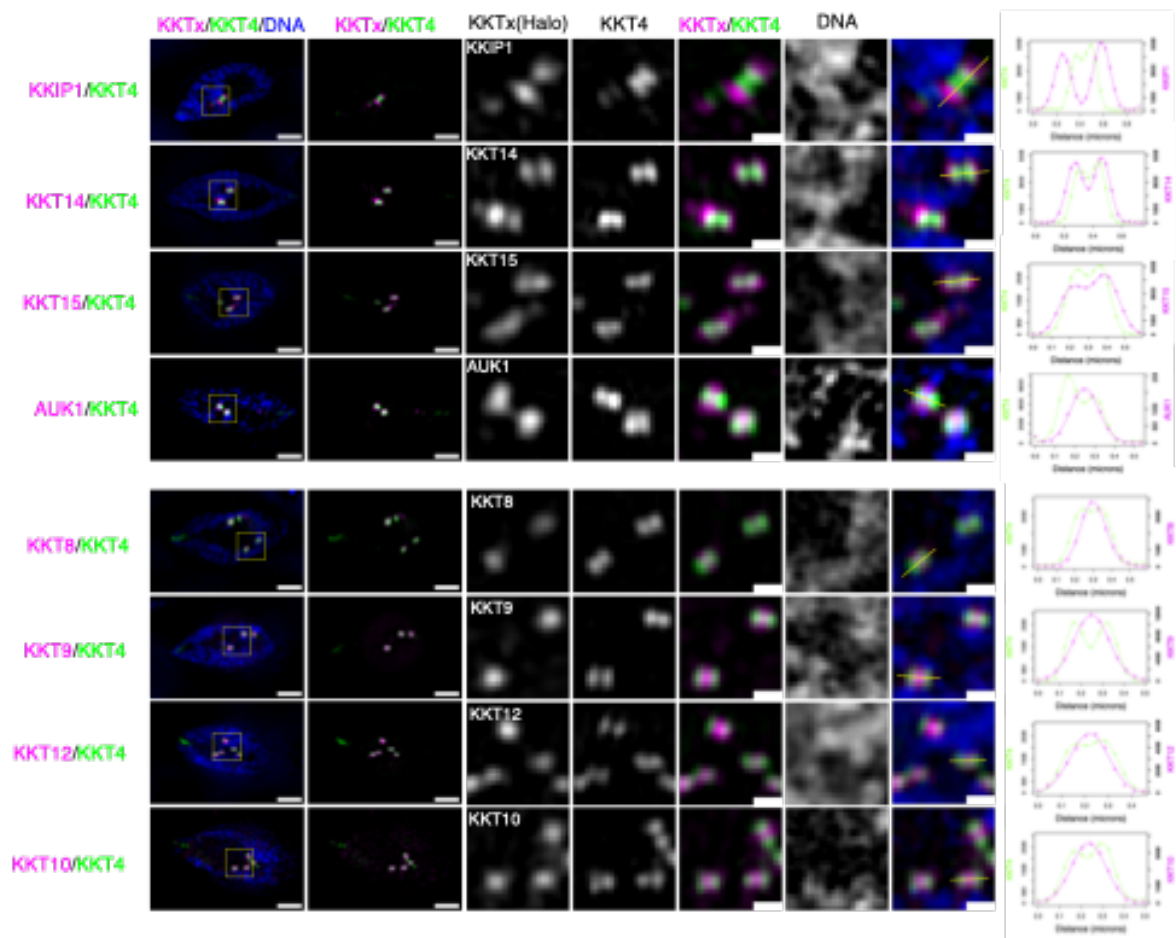


Figure 1.6 The trypanosome kinetochore. Electron micrograph of the trypanosome kinetochore in metaphase, attached to spindle microtubules from opposite poles. Sister kinetochores appear to be positioned in a ‘back-to-back’ configuration without any clear space (e.g. inner centromere) inbetween. Adapted from (Ogbadoyi et al., 2000; Tromer et al., 2021).

A



B

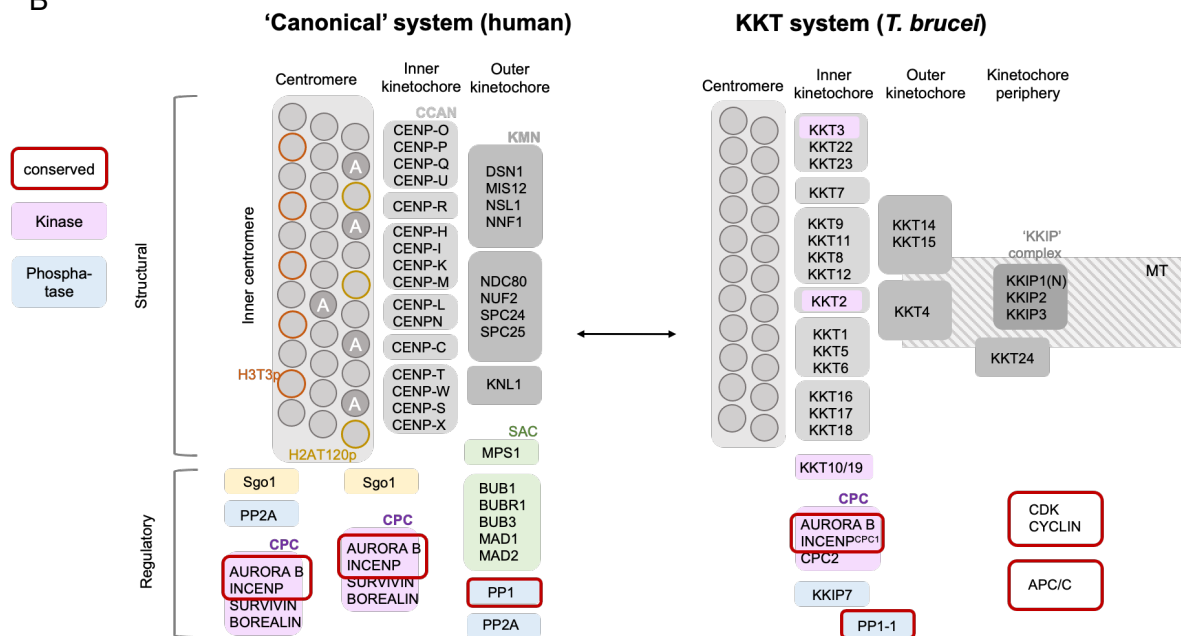


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Figure 1.7 Organization of the trypanosome kinetochore. (A) Representative 3D-SIM fluorescence micrographs showing localization of indicated kinetochore proteins along the inter-sister kinetochore axis (Hayashi and Akiyoshi, unpublished data). (B) Schematic representation of the trypanosome kinetochore showing the localization of KKT and KKIP proteins to the inner kinetochore, outer kinetochore, or kinetochore periphery. A comparison with the human kinetochore is presented on the left.

Based on the finding that some KKT proteins have similarities to components of synaptonemal complexes (zipper-like structures that assemble between homologous chromosomes and promote genetic exchange during meiosis) or the homologous recombination machinery, it has been hypothesized that a kinetoplastid ancestor repurposed parts of its meiotic machinery to assemble unique kinetochores (Tromer et al., 2021). Indeed, like synaptonemal complexes, sister kinetochores are closely paired in trypanosomes (Ogbadoyi et al., 2000), which stands in sharp contrast with canonical kinetochores that have a significant space ($\sim 1 \mu\text{m}$) in between sister kinetochores (called inner centromeres) (Bloom, 2014) (Fig. 1.6). Due to the proximity between sister kinetochores, most of YFP-tagged KKT proteins appear as single dots (rather than pairs of dots) under conventional fluorescence microscopes (Akiyoshi and Gull, 2014), while N-terminally YFP-tagged KKT24 and many KKIP proteins appear as pairs of dots that are separated by $\sim 340 \text{ nm}$ in metaphase (Brusini et al., 2021; Nerusheva et al., 2019). A recent study using 3D-SIM super-resolution microscopy has revealed pairs of dots for KKT4, KKT14, and KKT15 that are separated by $\sim 140 \text{ nm}$, while other tested KKT proteins and Aurora B^{AUK1} still appear as diffraction-limited spots (Hayashi and Akiyoshi, unpublished data) (Fig. 1.7A). Thus, in addition to differences in temporal dynamics of kinetochore localization, KKT and KKIP proteins may also be categorized into ‘inner’ and ‘outer’ structures based on their localization across the inter-sister kinetochore axis. In traditional model eukaryotes, the term ‘outer kinetochore’ refers to the KMN network that engages in end-on interactions with spindle microtubules (Musacchio and Desai, 2017). In trypanosomes, this term was previously used to refer to the KKIP1 protein based on weak similarity of its coiled-coil regions to the outer kinetochore proteins Ndc80/Nuf2 (D’Archivio and Wickstead, 2017; Brusini et al., 2021). However, AlphaFold-based structural predictions do not support the possibility that KKIP1 is a divergent Ndc80/Nuf2 homologue, and currently there is no evidence that KKIP1 has microtubule-binding activity. Instead, KKT4 remains the only kinetochore protein that has been shown to bind microtubules and maintain load-bearing end-on attachments to both growing and shrinking microtubule tips (Llauró et al., 2018). The Akiyoshi lab therefore proposed recently that the term ‘outer kinetochore’ is used for KKT4, KKT14 and KKT15 proteins, ‘kinetochore periphery’ for those (e.g. KKIP1, KKIP2, KKIP3,

KKT24) whose N- or C-terminal ends are located farther away from KKT4, and 'inner kinetochore' for those proteins that appear as single dots at the resolution of 3D-SIM (Ballmer et al., 2024b) (Hayashi and Akiyoshi, unpublished data). The organization of KKT proteins into 'inner kinetochore', 'outer kinetochore' and 'kinetochore periphery' zones is summarized in Fig. 1.7B.

1.2.3 Regulation of chromosome segregation in trypanosomes

Kinetoplastids possess the CDK/cyclin system, cohesin complex, separase, condensin complex, a minimal APC/C, and proteasomes, indicating that the most basic cell-cycle machinery is conserved (Akiyoshi and Gull, 2013). Despite the absence of canonical kinetochore proteins, the Aurora B kinase, too, is conserved in kinetoplastids (Tu et al., 2006; Li et al., 2008a). Whether Aurora B is involved in error correction and/or some form of mitotic checkpoint signaling in trypanosomes is not understood.

Intriguingly, trypanosomes are unable to halt their cell cycle in response to spindle defects and can undergo cytokinesis without any noticeable delay despite absence of nuclear division (Robinson et al., 1995; Ploubidou et al., 1999; Hayashi and Akiyoshi, 2018). Moreover, key components of the SAC appear to be absent, apart from a Mad2 homolog that localizes near basal bodies (Akiyoshi, 2020) and a CDC20 homologue, which lacks a well-conserved Mad2 interaction motif (Akiyoshi and Gull, 2013). These observations lead to the notion that kinetoplastids do not possess a mitotic checkpoint or at least not a canonical SAC system. Nevertheless, despite the large number of chromosomes in *T. brucei*, their mis-segregation rate is very low (~1%, e.g. comparable to human cells, (Ishii and Akiyoshi, 2020; Santaguida and Amon, 2015; Wickstead et al., 2003)), suggesting that trypanosomes do in fact possess an efficient system for correcting improper KT-MT attachments. As described above, many KKT proteins have kinase activity, and it is conceivable that these KKT kinases, together with Aurora B and currently unknown phosphatases, constitute a non-canonical regulatory network at the kinetochore in kinetoplastids. The molecular details of this regulatory circuitry remain to be investigated.

1.2.4 The chromosomal passenger complex in trypanosomes

Early studies suggested that the Aurora B homolog AUK1 (Aurora B^{AUK1}) in *T. brucei* forms a complex with chromosomal passenger complex proteins 1 and 2 (CPC1 and CPC2) and plays a crucial role in mitosis and cytokinesis (Tu et al., 2006; Li et al., 2008a). CPC1 was later found to be a divergent INCENP homolog (hereafter referred to as INCENP^{CPC1}) based on the presence of a conserved C-terminal IN-box (Hu et al., 2014). However, INCENP^{CPC1} lacks the

central SAH domain and N-terminal residues, which in other eukaryotes interact with Survivin and Borealin. In addition, two orphan kinesins, KIN-A and KIN-B, have been proposed to transiently associate with Aurora B^{AUK1} during mitosis (Li et al., 2008a; Li, 2012). Knockdown of Aurora B^{AUK1}, INCENP^{CPC1}, CPC2, KIN-A or KIN-B prevents cells from completing nuclear division (Tu et al., 2006; Li et al., 2008b; Ballmer and Akiyoshi, 2024a), suggesting that the CPC functions as a key regulator of mitosis in *T. brucei*. Yet, in the absence of canonical substrates, the molecular principles and mode of action of the trypanosome CPC remain elusive.

Interestingly, although homologs of the ‘localization module’ proteins Survivin and Borealin have not been identified in kinetoplastids (Komaki et al., 2022), the trypanosome CPC displays a dynamic localization pattern similar to that of the metazoan CPC (Li et al., 2008a): Aurora B^{AUK1}, INCENP^{CPC1} and CPC2 localize to kinetochores in early mitosis and then translocate to the central spindle upon anaphase onset. From late anaphase onwards, an additional population of CPC proteins is detectable at the tip of the new flagellum attachment zone (FAZ), the point of cytokinesis initiation in *T. brucei*. It is presently not understood how the CPC assembles in these evolutionarily divergent eukaryotes nor how its localization dynamics are regulated during the cell cycle.

1.3 Aims of this thesis

Using procyclic (insect) form *T. brucei* as a model system, I aim to reveal the mechanism of recruitment of the CPC to the kinetoplastid kinetochore as well as the function of the Aurora B^{AUK1} kinase in regulating chromosome segregation in kinetoplastids.

2. Materials and Methods

2.1 Cloning

All primers, plasmids, bacmids, and synthetic DNA used in this study as well as their source or construction details are described in Supplemental Tables S1 – S4 (Appendix). All constructs have been sequence verified.

2.2 Trypanosome culture

All trypanosome cell lines used in this study were derived from *T. brucei* SmOxP927 procyclic form cells (TREU 927/4 expressing T7 RNA polymerase and the tetracycline repressor to allow inducible expression; Poon *et al.*, 2012) or from PCF1339 procyclic form cells (TREU 927/4 expressing T7 RNA polymerase, tetracycline repressor and the Cas9 nuclease, Beneke *et al.*, 2017) and are described in Supplemental Table S4 (Appendix). Cells were grown at 28°C in SDM-79 medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 7.5 µg/ml hemin (Brun and Schönenberger, 1979), and appropriate selection drugs. Cell growth was monitored using a CASY cell counter (Roche). PCR products or plasmids linearized by NotI were transfected into cells by electroporation (Biorad). Transfected cells were selected by the addition of 30 µg/ml G418 (Sigma), 25 µg/ml hygromycin (Sigma), 5 µg/ml phleomycin (Sigma), or 10 µg/ml blasticidin S (Insight biotechnology). To obtain endogenously tagged clonal strains, transfected cells were selected by the addition of appropriate drugs and cloned by dispensing dilutions into 96-well plates. Endogenous YFP tagging was performed using the pEnT5-Y vector (Kelly *et al.*, 2007b) or a PCR-based method (Dean *et al.*, 2015). Endogenous tdTomato tagging was performed using pBA148 (Akiyoshi and Gull, 2014) and its derivatives. All constructs for ectopic expression of GFP fusion proteins include a short nuclear localization signal (NLS) (Marchetti *et al.*, 2000). For doxycycline inducible expression of head-to-head (pBA3-based) and hairpin (pBA310-based) RNAi constructs, GFP-NLS (pBA310-based) and GFP-NLS-LacI fusion proteins (pBA795-based), the linearized plasmids were integrated into 177-bp repeats on minichromosomes. Expression of GFP-NLS or GFP-NLS-LacI fusions was induced by the addition of 10 ng/mL doxycycline for 24 h. RNAi was induced by the addition of 1 µg/ml doxycycline. LacO–LacI tethering experiments were carried out as described previously using the LacO array inserted at the rDNA locus (Landeira and Navarro, 2007; Ishii and Akiyoshi, 2020).

2.3 Immunoprecipitation followed by mass spectrometry (IP-MS)

For standard IPs, 400-ml cultures of asynchronously growing cells were grown to ~5 – 10 million cells/ml. Cells were pelleted by centrifugation (800 g, 10 min), washed once with PBS, and extracted in PEME (100 mM Pipes-NaOH, pH 6.9, 2 mM EGTA, 1 mM MgSO₄, and 0.1 mM EDTA) with 1% NP-40, protease inhibitors (10 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml E-64, and 0.2 mM PMSF) and phosphatase inhibitors (1 mM sodium pyrophosphate, 2 mM Na-β-glycerophosphate, 0.1 mM Na₃VO₄, 5 mM NaF, and 100 nM microcystin-LR) for 5 min at RT, followed by centrifugation at 1,800 g for 15 min. Samples were kept on ice from this point on. The pelleted fraction containing kinetochore proteins was resuspended in modified buffer H (BH0.15: 25 mM Hepes, pH 8.0, 2 mM MgCl₂, 0.1 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0, 1% NP-40, 150 mM KCl, and 15% glycerol) with protease and phosphatase inhibitors. Samples were sonicated to solubilize kinetochore proteins (12 s, three times with 1-min intervals on ice). 12 µg of mouse monoclonal anti-GFP antibodies (11814460001; Roche) pre-conjugated with 60 µl slurry of Protein-G magnetic beads (10004D; Thermo Fisher Scientific) with dimethyl pimelimidate (Unnikrishnan et al., 2012) were incubated with the extracts for 2.5 h with constant rotation, followed by four washes with modified BH0.15 containing protease inhibitors, phosphatase inhibitors and 2 mM DTT. Beads were further washed three times with pre-elution buffer (50 mM Tris- HCl, pH 8.3, 75 mM KCl, and 1 mM EGTA). Bound proteins were eluted from the beads by agitation in 60 µl of elution buffer (0.1% RapiGest [186001860; Waters] and 50 mM Tris-HCl, pH 8.3) for 25 min at RT. Eluates were then incubated at 100°C for 5 min. Proteins were reduced with 5 mM DTT at 37°C for 30 min and alkylated with 10 mM iodoacetamide at 37°C for 30 min. The reaction was quenched by adding 10 mM DTT at 37°C for 30 min, and 100 µl of 20 mM Tris-HCl (pH 8.3) was added. Proteins were digested overnight at 37°C with 0.2 µg trypsin (Promega). Formic acid was then added to 2% and the samples were incubated at 37°C for 30 min to cleave RapiGest, followed by centrifugation for 10 min. The supernatant was desalted over a C18 column and analyzed by electrospray tandem mass spectrometry over a 60-min gradient using Q-Exactive (Thermo Fisher Scientific) at the Advanced Proteomics Facility (University of Oxford). For IP-MS of GFP-KKT14^{NTR}, YFP-KKT14 and YFP-KKT22, IP was conducted as described above up to the elution step. Bound proteins were eluted from the beads by agitation in 60 µl of elution buffer (50 mM Tris-HCl, 0.3% SDS, pH 8.3) for 25 min at RT. Reduction of disulfide bridges in cysteine-containing proteins was performed with 10 mM DTT dissolved in 50 mM HEPES, pH 8.5 (56°C, 30 min). Reduced cysteines were alkylated with 20 mM 2-chloroacetamide dissolved in 50 mM HEPES, pH 8.5 (room temperature, in the dark, 30 min). Samples were prepared using the SP3 protocol (Hughes et al., 2019), and trypsin (Promega) was added in the 1:50 enzyme to protein ratio for overnight digestion at 37°C. Next day, peptide recovery was done by collecting supernatant on magnet and combining with second elution of beads with 50 mM HEPES, pH 8.5. For a further sample clean up, an OASIS HLB

μ Elution Plate (Waters) was used. The samples were dissolved in 10 μ L of reconstitution buffer (96:4 water: acetonitrile, 1% formic acid and analyzed by LC-MS/MS using QExactive (Thermo Fisher) in the proteomics core facility at EMBL Heidelberg (<https://www.embl.org/groups/proteomics/>).

Peptides were identified by searching tandem mass spectrometry spectra against the *T. brucei* protein database with MaxQuant (version 2.0.1) with carbamidomethyl cysteine set as a fixed modification and oxidization (Met), phosphorylation (Ser, Thr, and Tyr), and acetylation (Lys) set as variable modifications. Up to two missed cleavages were allowed. The first peptide tolerance was set to 10 ppm. Results were filtered to remove contaminants and reverse hits. Differential enrichment analysis of phosphopeptides was performed using the DEP package in R (Zhang et al., 2018). Reverse hits and contaminants were removed, and results were filtered for peptides that were identified in all replicates of at least one condition. The data was background corrected and normalized by variance stabilizing transformation (vsb). Missing values were imputed using the k-nearest neighbour approach (knn). Differential enrichment analysis of YFP-KKT14 vs YFP-KKT22 was performed on iBAQ values using the DEP package in R (Zhang et al., 2018). Reverse hits and contaminants were removed, and results were filtered for proteins that were identified in all replicates of at least one condition. The data was background corrected and normalized by variance stabilizing transformation (vsb). Missing values were imputed using the k-nearest neighbor approach (knn). Potential interactors were determined using t-tests, with threshold values set to $\log_2$ = 2 and α = 0.05. The volcano plot shown in Fig. 5.1 was constructed using the EnhancedVolcano package (Blighe et al., 2023).

All raw mass spectrometry files and custom database files used in this study have been deposited with the ProteomeXchange Consortium via the PRIDE partner repository (Deutsch et al., 2020; Perez-Riverol et al., 2019) with the dataset identifiers PXD045987 and PXD047806.

2.4 Ex vivo cross-linking of the native CPC and kinetochore complexes (IP-CLMS)

For cross-linking IP-MS experiments, cell cultures were scaled up to 1,600 mL. Cell cultures were treated with 10 μ M MG132 for 4 h to enrich for cells in metaphase. Cell lysis and immunoprecipitation steps were carried out as described above. After four washes with modified BH0.15 containing protease inhibitors, phosphatase inhibitors and 2 mM DTT, beads were washed three times with 25 mM HEPES pH7.5, 150 mM NaCl. Proteins were then cross-linked on beads with 0.4 mM BS³ (bis(sulfosuccinimidyl)suberate) (Thermo Fisher Scientific) for 30 min at RT with agitation, followed by three washes in 25 mM HEPES pH7.5, 150 mM NaCl and a further three washes in 0.1 M ammonium bicarbonate. Samples were then

incubated in 8 M urea dissolved in 0.1 M ammonium bicarbonate for 10 min at RT with agitation. Proteins were reduced with 10 mM TCEP for 20 min and alkylated with 10 mM iodoacetamide for 40 min at RT. Proteins were then pre-digested with 0.4 µg LysC for 2 h at 37°C. The urea concentration in the sample was brought down to < 1 M by addition of 0.1 M ammonium bicarbonate before adding CaCl₂ (to 2 mM) and 0.7 µg of trypsin for overnight digestion at 37°C. Formic acid was then added to 2% and the samples were frozen. The cross-linked samples were further processed and analysed at the proteomics core facility at EMBL Heidelberg. Digested peptides were concentrated and desalted using an OASIS HLB µElution Plate (Waters) according to manufacturer instructions. Crosslinked peptides were enriched using size exclusion chromatography (Leitner et al., 2012). In brief, desalted peptides were reconstituted with SEC buffer (30% (v/v) ACN in 0.1% (v/v) TFA) and fractionated using a Superdex Peptide PC 3.2/30 column (GE) on a 1200 Infinity HPLC system (Agilent) at a flow rate of 0.05 ml/min. Fractions eluting between 50-70 µl were evaporated to dryness and reconstituted in 30 µl 4% (v/v) ACN in 1% (v/v) FA. Collected fractions were analyzed by liquid chromatography (LC)-coupled tandem mass spectrometry (MS/MS) using an UltiMate 3000 RSLC nano LC system (Dionex) fitted with a trapping cartridge (µ-Precolumn C18 PepMap 100, 5µm, 300 µm i.d. x 5 mm, 100 Å) and an analytical column (nanoEase M/Z HSS T3 column 75 µm x 250 mm C18, 1.8 µm, 100 Å, Waters). Trapping was carried out with a constant flow of trapping solvent (0.05% trifluoroacetic acid in water) at 30 µl/min onto the trapping column for 6 minutes. Subsequently, peptides were eluted and separated on the analytical column using a gradient composed of Solvent A (3% DMSO, 0.1% formic acid in water) and solvent B (3% DMSO, 0.1% formic acid in acetonitrile) with a constant flow of 0.3 µl/min. The outlet of the analytical column was coupled directly to an Orbitrap Fusion Lumos (Thermo Scientific, SanJose) mass spectrometer using the nanoFlex source. The peptides were introduced into the Orbitrap Fusion Lumos via a Pico-Tip Emitter 360 µm OD x 20 µm ID; 10 µm tip (CoAnn Technologies) and an applied spray voltage of 2.1 kV, instrument was operated in positive mode. The capillary temperature was set at 275°C. Only charge states of 4-8 were included. The dynamic exclusion was set to 30 sec. and the intensity threshold was 5e⁴. Full mass scans were acquired for a mass range 350-1700 m/z in profile mode in the orbitrap with resolution of 120000. The AGC target was set to Standard and the injection time mode was set to Auto. The instrument was operated in data dependent acquisition (DDA) mode with a cycle time of 3 sec between master scans and MSMS scans were acquired in the Orbitrap with a resolution of 30000, with a fill time of up to 100 ms and a limitation of 2e⁵ ions (AGC target). A normalized collision energy of 32 was applied. MS2 data was acquired in profile mode. RAW MS files were searched by the pLink2 software (Chen et al., 2019), with carbamidomethyl cysteine set as a fixed and oxidization (Met) set as variable modifications.

Up to two missed cleavages were allowed. Precursor tolerance was set to 10 ppm. Cross-links were plotted using xiView (Graham et al., 2019).

2.5 Fluorescence microscopy

Cells were washed once with PBS, settled onto glass slides, and fixed with 4% paraformaldehyde in PBS for 5 min as described previously (Nerusheva and Akiyoshi, 2016). Cells were then permeabilized with 0.1% NP-40 in PBS for 5 min and embedded in mounting media (1% wt/vol 1,4-diazabicyclo[2.2.2]octane, 90% glycerol, 50 mM sodium phosphate, pH 8.0) containing 100 ng/ml DAPI. Images were captured on a Zeiss Axioimager.Z2 microscope (Zeiss) installed with ZEN using a Hamamatsu ORCA-Flash4.0 camera with 63× objective lenses (1.40 NA). Typically, ~20 optical slices spaced 0.24 μm apart were collected. Images were analyzed in ImageJ/Fiji (Schneider et al., 2012). The mean intensity of segmented kinetochore foci for each cell was calculated as follows using a custom macro (Supplemental File). First, metaphase cells were manually selected from the images. Then, nuclei regions were segmented in 3D with a default threshold value in the ImageJ threshold tool at the focus plane. Kinetochores were detected in the nuclei regions by the 3D object counter tool in ImageJ with the threshold determined by the default threshold value at the focus plane. Total fluorescent signal intensity of the kinetochore protein in a nucleus was calculated by summing up the intensity detected by 3D object counter, which was standardized using z-scores. For analysis of kinetochore bi-orientation, images were captured on a DeltaVision OMX V3 or SR housed in the Oxford Micron facility. Fluorescent images were captured with using 60x objective lenses (1.42 NA) (typically 16–21 z sections at 0.25-μm steps) and deconvoluted using softWoRx. Bi-oriented kinetochores were scored manually by quantifying the number of kinetochores (marked by YFP-Aurora B^{AUK1}) that were double positive for tdTomato-KKIP2. Images shown in the figure are central slices. The distance between KKIP2 dots was measured in ImageJ using the 'plot profile' function across the inter-sister kinetochore axis and measuring the distance between the two peaks.

2.6 Transmission electron microscopy (TEM)

Whole cells were fixed in 2% glutaraldehyde, 2% formaldehyde in PEME buffer (see above) for 1 h at RT. Fixed samples were then centrifuged at 800 g for 10 min at RT and the pellet was resuspended in PIPES buffer (0.1 M PIPES at pH 7.2). After several washes in PIPES buffer (5 changes of fresh buffer, each followed by 5 min incubation at RT, rotating), a quenching step (50 mM glycine in PIPES buffer for 15 min at RT, rotating), and a final wash (10 min incubation at RT, rotating), samples were post-fixed with 1% osmium tetroxide and

1.5 % potassium ferrocyanide in PIPES buffer for 1 h at 4°C. Samples were washed 5 times with Milli-Q H₂O and embedded in 4% LMP agarose. The agarose-embedded samples were kept at 4°C for 15 min to allow the agarose to set and were then cut into small blocks, which were then stained with 0.5% aqueous uranyl acetate at 4°C in the dark o/n. Following several wash steps in H₂O, samples were dehydrated through an ethanol series and gradually infiltrated with Agar low viscosity resin. The samples were then transferred into embedding capsules, and the resin was polymerized at 60°C for 24 h. Ultrathin (90 nm) sections were taken with a Diatome diamond knife on a Leica UC7 ultramicrotome and mounted onto 200 mesh copper grids, which were then post-stained in lead citrate for 5 min at RT. Grids were imaged in a Tecnai FEI T12 transmission electron microscope (TEM) operated at 120 kV with a Gatan Oneview digital camera. For detailed visualization of kinetochores and MTs, cells were extracted in 1% NP-40 in PEME for 5 min at RT, and centrifuged at 1800 g for 15 min at RT. The pellet was resuspended in fixing buffer containing 4% glutaraldehyde and 1% tannic acid in PEME. All subsequent steps were performed as described above.

2.7 In silico structure and interaction predictions

Structures and interactions were predicted with AlphaFold2-Multimer-v2 (Evans et al., 2022; Jumper et al., 2021) through ColabFold using MMseqs2 (UniRef+Environmental) and default settings (Mirdita et al., 2022). Structural predictions of KIN-A/KIN-B, KIN-A³¹⁰⁻⁸⁶²/KIN-B³¹⁷⁻⁶²⁴, CPC1/CPC2/KIN-A³⁰⁰⁻⁵⁹⁹/KIN-B 317-624, and KIN-A⁷⁰⁰⁻⁸⁰⁰/KKT9/KKT11 were performed using ColabFold version 1.3.0 (AlphaFold-Multimer version 2), while those of AUK1/CPC1/CPC2/KIN-A¹⁻⁵⁹⁹/KIN-B, KKT7¹⁻²⁶¹/KKT9/KKT11/KKT8/KKT12, KKT9/KKT11/-KKT8/KKT12, KKT7¹⁻²⁶¹/KKT9/KKT11 and KKT14²⁻¹²⁵:KKT15 were performed using ColabFold version 1.5.3 (AlphaFold-Multimer version 2.3.1) using default settings, accessed via <https://colab.research.google.com/github/sokrypton/ColabFold/blob/v1.3.0/AlphaFold2.ipynb> and <https://colab.research.google.com/github/sokrypton/ColabFold/blob/v1.5.3/AlphaFold2.ipynb>. All structure figures were made using PyMOL version 2.5.2 (Schrödinger, LLC). The following command was used to map pLDDT score onto the AF2 predicted structure models: spectrum b, rainbow_rev, maximum=100, minimum=50.

2.8 Protein purification from *E. coli* and insect cells

Recombinant 6HIS-tagged KKT4 fragments (pBA1413: KKT4²⁻¹¹⁴, pBA1065: KKT4¹¹⁵⁻³⁴³, pBA1641: KKT4³⁰⁰⁻⁴⁸⁸, pBA1513: KKT4⁴⁶³⁻⁶⁴⁵), KKT14 fragments (pBA2704: KKT14²⁻³⁵⁷, pBA2353: KKT14³⁵⁸⁻⁶⁸⁵) and CPC fragments (pBA2439: MBP-CPC2¹²¹⁻²⁵⁰, pBA2458: MBP-CPC2, pBA2519: KIN-A²⁻³⁰⁹, pBA2513: KIN-B²⁻³¹⁶, pBA2574) were expressed in *E.*

coli BL21(DE3) cells and purified and eluted from TALON beads as described previously (Llauró et al., 2018; Ishii and Akiyoshi, 2020). Recombinant 6HIS-KKT10 (pBA234: (Ishii and Akiyoshi, 2020)), 6HIS-KKT16/17/18 (pBA202: (Tromer et al., 2021)) and 6HIS-KKT8/9/11/12 (pBA457: (Ishii and Akiyoshi, 2020)) and derivatives were expressed in Rosetta 2(DE3)pLys *E. coli* cells (Novagen) and purified using the same protocol. Briefly, cells were grown in 2xTY media at 37°C to an OD₆₀₀ of ~0.8, at which point protein expression was induced by 0.1 mM IPTG, and then incubated overnight at 20°C. Cells were pelleted at 3,400 g at 4°C and pellets were resuspended in P500 buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 5 mM imidazole and 10% glycerol) supplemented with protease inhibitors (20 µg/ml leupeptin, 20 µg/ml pepstatin, 20 µg/ml E-64, 0.4 mM PMSF) and 1 mM TCEP, and were sonicated on ice. Lysates were treated with benzonase nuclease (500 U/1 l culture) and spun at 48,000 g at 4°C for 30 min. Supernatant was incubated with TALON beads (Takara Clontech) for 1 h at 4°C, rotating. The beads were washed three times with lysis buffer and proteins were then eluted with elution buffer (P500 buffer containing 250 mM imidazole with 1 mM TCEP). For microtubule co-sedimentation assays, 6HIS-KIN-A²⁻³⁰⁹ and 6HIS-KIN-B²⁻³¹⁶ were buffer exchanged into BRB80 (80 mM Pipes-KOH, pH 6.9, 1 mM EGTA, and 1 mM MgCl₂) with 100 mM KCl using a Zeba columns (Thermo Fisher) and flash-frozen in liquid nitrogen for -80°C storage. Polyacrylamide gels were stained with SimplyBlue SafeStain (Invitrogen).

pACEBac plasmids (Bieniossek et al., 2009) containing 3Flag-KKT3 (pBA315), 3Flag-KKT2 (pBA314), KKT3 (pBA882), 3Flag-KKT6/1 (pBA819), SNAP-6HIS-3FLAG-KKT4 (pBA925), 3FLAG-KKT7 (pBA1531), 3Flag-KKT14 (pBA334), 3Flag-Aurora B^{AUK1}/INCENP^{CPC1} (pBA1084) and 3Flag-Aurora B^{AUK1 K58R}/INCENP^{CPC1} (pBA2396) were transformed into DH10EmBacY cells to make bacmids, which were purified and used to transfect Sf9 cells using Cellfectin II transfection reagent (Thermo Fisher). Sf9 cells were grown in Sf-900 II SFM media (Thermo Fisher). Baculovirus was amplified through three rounds of amplification. Recombinant proteins were expressed and purified from Sf9 cells using a protocol described previously (Llauró et al., 2018). Protein concentration was determined by protein assay (Bio-Rad).

2.9 Microtubule co-sedimentation assay

Microtubule co-sedimentation assays were performed as described previously (Ludzia et al., 2021). Briefly, taxol-stabilised microtubules were prepared by mixing 2.5 ml of 100 µM porcine tubulin (Cytoskeleton) resuspended in BRB80 with 1 mM GTP (Cytoskeleton), 1.25 µl BRB80, 0.5 µl of 40 mM MgCl₂, 0.5 µl of 10 mM GTP, and 0.25 µl DMSO, and incubated for 20 min at 37°C. 120 µl of pre-warmed BRB80 containing 12.5 µM Taxol (paclitaxel; Sigma) was added

to the sample to bring the microtubule concentration to $\sim 2 \mu\text{M}$. 20 μl of 6HIS-KIN-A²⁻³⁰⁹ or 6HIS-KIN-B²⁻³¹⁶ (at final concentration of 4 μM) in BRB80 with 100 mM KCl were mixed with 20 μl of microtubules (final, 1 μM) and incubated for 45 min at room temperature. As a control, I incubated 6HIS-KIN-A²⁻³⁰⁹ or 6HIS-KIN-B²⁻³¹⁶ with BRB80 (with 12.5 μM Taxol). The samples were spun at 20,000 g at room temperature for 10 min, and the supernatant was collected. To the tubes containing pelleted fractions, I added 40 μl of chilled BRB80 with 5 mM CaCl_2 and incubated on ice for 5 min to depolymerise microtubules. Following the incubation, samples were boiled for 5 min before SDS-PAGE. Gels were stained with SimplyBlue Safe Stain (Invitrogen). Co-sedimentation assays were performed at least twice with similar results.

2.10 Immunoblotting

Cells were harvested by centrifugation (800 g, 5 min) and washed with 1 ml PBS. The pellet was resuspended in 1 $\times$ LDS sample buffer (Thermo Fisher) with 0.1 M DTT. Denaturation of proteins was performed for 5 min at 95°C. SDS-PAGE and immunoblots were performed by standard methods using the following antibodies: rabbit polyclonal anti-GFP (TP401, 1:5000) and mouse monoclonal TAT1 (anti-trypanosomal-alpha-tubulin, 1:5000, a kind gift from Keith Gull) (Woods et al., 1989). Secondary antibodies used were: IRDye 680RD goat anti-mouse (LI-COR, 926-68070) and IRDye 800CW goat anti-rabbit (LI-COR, 926-32211). Bands were visualized on an ODYSSEY Fc Imaging System (LI-COR).

2.11 In vitro kinase assay

Recombinant kinetochore proteins mixed with active or kinase-dead 3Flag-Aurora B^{AUK1}/INCENP^{CPC1} complexes in kinase buffer (50 mM Tris-HCl pH 7.4, 1 mM DTT, 25 mM β -glycerophosphate, 5 mM MgCl_2 , 5 μCi [³²P]ATP, and 10 μM ATP) were incubated at 30°C for 30 min. The reaction was stopped by the addition of LDS sample buffer (Thermo Fisher). The samples were run on an SDS-PAGE gel, which was stained with Coomassie Brilliant Blue R-250 (Bio-Rad) and subsequently dried and used for autoradiography using a Phosphorimager Screen. The signal was detected by an FLA 7000 scanner (GE Healthcare). The ³²P signal intensity for each protein was quantified in ImageJ/FIJI, normalized to the total protein amount (estimated from measuring the intensity of Coomassie-stained bands). To correct for non-Aurora B^{AUK1}-dependent phosphorylation, the normalized intensities from the kinase-dead controls were subtracted from these values. Normalized and corrected signal intensities are presented relative to Aurora B^{AUK1} auto-phosphorylation in Fig. 3F.

To identify AUK1-dependent phosphorylation sites on KKT14 NTR, [³²P]ATP in the kinase assay was replaced with non-labelled ATP. Gels were stained using SimplyBlue

(Invitrogen) and bands corresponding to the KKT14 NTR were cut out and subjected to in-gel digestion with trypsin. Peptides were extracted from the gel pieces by sonication for 15 minutes, followed by centrifugation and supernatant collection. A solution of 50:50 water: acetonitrile, 1% formic acid (2x the volume of the gel pieces) was added for a second extraction and the samples were again sonicated for 15 minutes, centrifuged and the supernatant pooled with the first extract. The pooled supernatants were processed using speed vacuum centrifugation. The samples were dissolved in 10 µL of reconstitution buffer (96:4 water: acetonitrile, 1% formic acid and analyzed by LC-MS/MS using an Orbitrap Fusion Lumos mass spectrometer (Thermo) at the proteomics core facility at EMBL Heidelberg. Peptides were identified by searching tandem mass spectrometry spectra against the *T. brucei* protein database with MaxQuant as described above. Following S/T sites were mutated to A or D/E in KKT14^{NTR} PD and PM constructs, respectively: S25, T104, S107, S113, T115, S173/S174, T299/S300/S301/S302/S303, T332/T333, S347/T348.

2.12 Positional scanning peptide array analysis

AUK1 phosphorylation site specificity was analyzed using a positional scanning peptide array consisting of 198 peptide mixtures with the general sequence Y-A-x-x-x-x-S/T-x-x-x-x-A-G-K-K(biotin). For each mixture, eight of the “x” positions was a random mixture of 17 amino acids (all natural amino acids except Ser, Thr and Cys), with the remaining one fixed as one of the 20 unmodified amino acids, phosphothreonine, or phosphotyrosine. Peptides (50 µM) were arrayed in 1536-well plates in 2 µl of library buffer (50 mM HEPES, pH 7.4, 1 mM EGTA, 0.4 mM EDTA, 5 mM MgCl₂, 2.5 mM β-glycerophosphate, 1 mM DTT, 0.1% Tween 20) containing 0.1 mg/ml BSA, 0.67 µM PKI, and 50 µM [γ-³²P]ATP (10 µCi/mL). Reactions were initiated by adding AUK1/CPC1 to 37.5 µg/mL, and plates were sealed and incubated at 30°C for 2 hr. Aliquots were then transferred to SAM2 biotin capture membrane (Promega), which was washed, dried, and exposed to a phosphor screen as described (Hutti et al., 2004). Radiolabel incorporation was visualized on a Molecular Imager FX phosphorimager (Bio-Rad) and quantified using QuantityOne Software (Bio-Rad version 11.0.5). Quantified data are the average normalized data from two independent experiments. The sequence logo for Aurora B^{AUK1} was generated using the logomaker package in Python (Tareen and Kinney, 2020). The height of every letter is the ratio of its value to the median value of that position. The serine and threonine heights in position ‘0’ were set to the ratio between their favorability. For improved readability, negative values were adjusted so that the sum does not exceed -10.

2.13 Multiple sequence alignment

Protein sequences and accession numbers for Aurora B^{AUK1}, INCENP^{CPC1}, CPC2, KIN-A and KIN-B used in this study were retrieved from the TriTryp database (Aslett et al., 2010), UniProt (Bateman, 2019), or a published study (Butenko et al., 2020). Searches for homologous proteins were done using BLAST in the TriTryp database (Aslett et al., 2010) or using hmmsearch using manually prepared hmm profiles (HMMER version 3.0; Eddy, 1998). The top hit in each organism was considered as a true ortholog only if the reciprocal BLAST search returned the query protein as a top hit in *T. brucei*. Multiple sequence alignment was performed with MAFFT (L-INS-i method, version 7) (Kato et al., 2019) and visualised with the Clustalx colouring scheme in Jalview (version 2.10) (Waterhouse et al., 2009).

3. Dynamic localization of the chromosomal passenger complex is controlled by the orphan kinesins KIN-A and KIN-B in the kinetoplastid parasite *Trypanosoma brucei*

3.1 Introduction

The chromosomal passenger complex (CPC) is an important regulator of cell division, which shows dynamic subcellular localization throughout mitosis, including kinetochores and the spindle midzone. In traditional model eukaryotes such as yeasts and humans, the CPC consists of the catalytic subunit Aurora B kinase, its activator INCENP, and the localization module proteins Borealin and Survivin. Intriguingly, Aurora B and INCENP as well as their localization pattern are conserved in kinetoplastids, an evolutionarily divergent group of eukaryotes that possess a unique set of kinetochore proteins and lack homologs of Borealin or Survivin. It is not understood how the kinetoplastid CPC assembles or how it is targeted to its subcellular destinations during the cell cycle.

In this chapter, I employed biochemical, structural and cell biological approaches to investigate how the Aurora B homologue AUK1 (hereafter referred to as Aurora B^{AUK1}) is recruited to kinetochores and to the central spindle in the kinetoplastid parasite *T. brucei*.

3.2 Results

3.2.1 KIN-A and KIN-B are bona fide CPC proteins in trypanosomes.

To identify additional interactors of the CPC in trypanosomes, I performed immunoprecipitation followed by liquid chromatography tandem mass spectrometry (IP-MS) of endogenously YFP-tagged Aurora B^{AUK1} (Fig. 3.2A). Besides Aurora^{AUK1}, INCENP^{CPC1} and CPC2, I observed notable enrichment of two orphan kinesins, KIN-A and KIN-B (Wickstead and Gull, 2006), as reported previously (Li et al., 2008a). Both KIN-A and KIN-B were also highly enriched in immunoprecipitates of ectopically expressed GFP-INCENP^{CPC1}. Vice versa, IP-MS against GFP-tagged KIN-A and KIN-B identified Aurora B^{AUK1}, INCENP^{CPC1} and CPC2 as top hits (Fig. 3.1A).

I next assessed the localization dynamics of fluorescently tagged KIN-A and KIN-B over the course of the cell cycle (Fig. 3.1, B – E). *T. brucei* possesses two DNA-containing organelles, the nucleus ('N') and the kinetoplast ('K'). The kinetoplast is an organelle found uniquely in kinetoplastids, which contains the mitochondrial DNA and replicates and

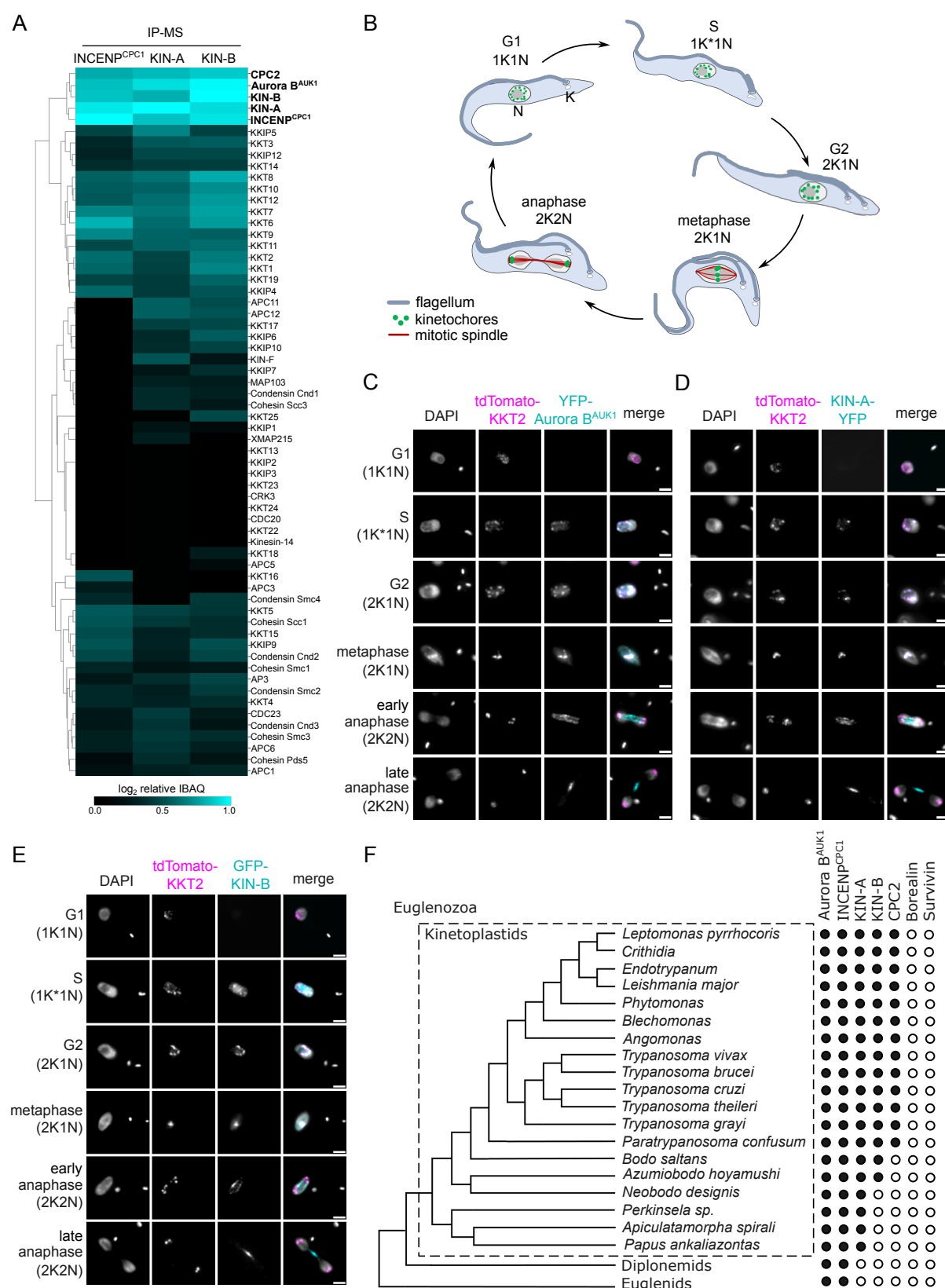


Figure 3.1 (figure legend on following page)

Figure 3.1. KIN-A and KIN-B are bona fide CPC proteins in *T. brucei*. (A) Clustered heatmap showing enrichment (log2 intensity based absolute quantification (IBAQ)) of mitotic proteins co-purifying with ectopically expressed GFP-INCENP^{CPC1}, GFP-KIN-A and GFP-KIN-B. The heatmap was generated using the Python Seaborn library using WPGMA clustering. Cell lines: BAP2190, BAP2286, BAP2288. Immunoprecipitation was performed using anti-GFP antibodies. (B) Cartoon depicting the kinetoplast (K) / nucleus (N) configuration throughout the cell cycle in procyclic *T. brucei*, with K* denoting an elongated kinetoplast. The kinetoplast is an organelle found uniquely in kinetoplastids, which contains the mitochondrial DNA and replicates and segregates prior to nuclear division. The KN configuration serves as a cell cycle marker (Woodward and Gull, 1990; Siegel et al., 2008). (C) to (E) Representative fluorescence micrographs showing the dynamic localization of YFP-Aurora B^{AUK1} (C), KIN-A-YFP (D) and GFP-KIN-B (E) over the course of the cell cycle. Kinetochores are marked with tdTomato-KKT2. DNA was stained with DAPI. Cell lines: BAP1515, BAP3066, BAP2288. Scale bars, 2 μ m. (F) Phylogenetic tree of kinetoplastids, diplomonids and euglenids along with the presence (black dots) / absence (white dots) patterns of CPC components. The phylogenetic tree of Euglenozoa is based on (Butenko et al., 2020).

segregates prior to nuclear division. The 'KN' configuration serves as a good cell cycle marker (Woodward and Gull, 1990; Siegel et al., 2008). To my surprise, KIN-A-YFP and GFP-KIN-B exhibited a CPC-like localization pattern identical to that of Aurora B^{AUK1}: Both kinesins localized to kinetochores from S phase to metaphase, and then translocated to the central spindle in anaphase (Fig. 3.1, C – E). Moreover, like Aurora B^{AUK1}, a population of KIN-A and KIN-B localized at the new FAZ tip from late anaphase onwards (Fig. 3.2, B and C). This was unexpected, because KIN-A and KIN-B were previously reported to localize to the spindle but not to kinetochores or the new FAZ tip (Li et al., 2008a). These data suggest that KIN-A and KIN-B are bona fide CPC proteins in trypanosomes, associating with Aurora B^{AUK1}, INCENP^{CPC1} and CPC2 throughout the cell cycle.

A bioinformatic search for homologs of CPC proteins within Euglenozoa revealed that both KIN-A and KIN-B are present in trypanosomatids and bodonids, with KIN-A homologs detectable even in prokinetoplastids (Fig. 3.1F) (Materials and Methods). CPC2, on the other hand, was detectable only within trypanosomatids. Aurora B^{AUK1} and INCENP^{CPC1} are present in kinetoplastids as well as in diplomonids and euglenids (sister groups of kinetoplastids). Interestingly, homologs of Borealin, Survivin, KIN-A, or KIN-B were not detectable in diplomonids or euglenids, raising the possibility that these organisms may also possess 'non-

canonical' CPC proteins. I conclude that the KIN-A and KIN-B kinesins are highly conserved within kinetoplastids, constituting integral components of the CPC in this evolutionary divergent group of eukaryotes.

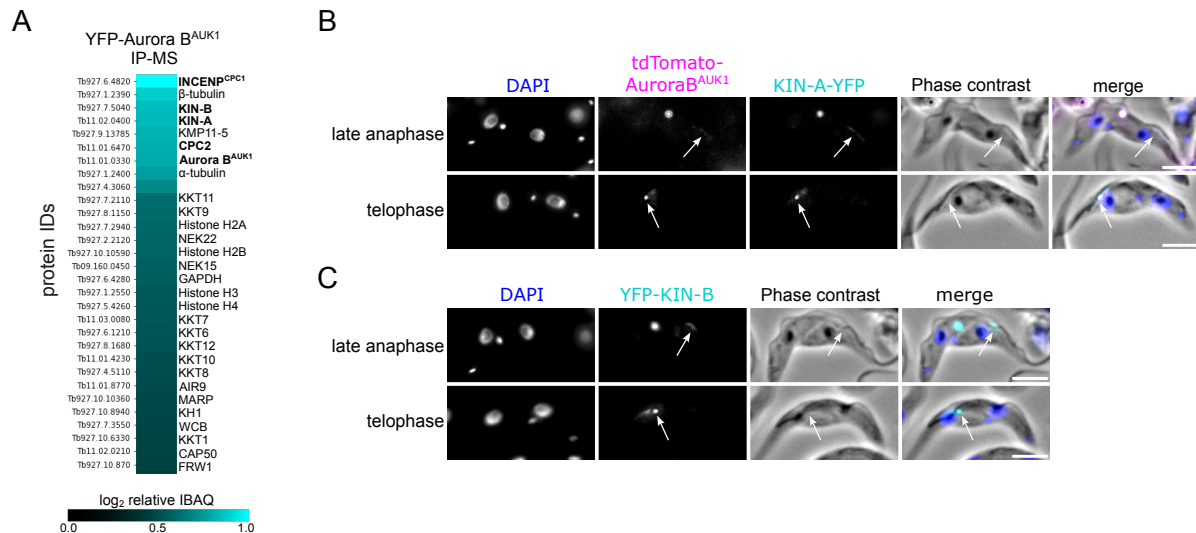


Figure 3.2. KIN-A and KIN-B are bona fide CPC proteins in *T. brucei*. (A) Heatmap showing enrichment (log₂ intensity based absolute quantification (IBAQ)) of the top 30 proteins, excluding ribosomal proteins, co-purifying with YFP-Aurora B^{AUK1}. Cell line: BAP73. Immunoprecipitation was performed using anti-GFP antibodies. (B) and (C) Representative fluorescence micrographs showing the localization of tdTomato-Aurora B^{AUK1} and KIN-A-YFP (B) and YFP-KIN-B (C) in late anaphase and telophase cells. Arrows indicate the population of CPC proteins localized to the new FAZ tip. DNA was stained with DAPI. Cell lines: BAP3067, BAP2528. Scale bars, 2 μm.

3.2.2 KIN-A and KIN-B promote kinetochore localization of the CPC.

To investigate which subunits of the CPC are responsible for its kinetochore targeting, I performed a series of RNAi experiments (Fig. 3.3, A – E). Because CPC2 is poorly conserved among kinetoplastids (Fig. 3.1F) and depletion of CPC2 using two different hairpin RNAi constructs (Supplemental Table S1, Appendix) was inefficient, I did not include CPC2 in these experiments. As previously reported (Li et al., 2008a), depletion of Aurora B^{AUK1}, INCENP^{CPC1}, KIN-A or KIN-B resulted in a prominent growth defect (Fig. 3.4A) with cells arresting in G2/M (2K1N) (Fig. 3.4B). Knockdown of Aurora B^{AUK1} did not affect the kinetochore localization of YFP-tagged INCENP^{CPC1}, KIN-A or KIN-B (Fig. 3.3, A and C – E). Knockdown of INCENP^{CPC1}

caused delocalization of Aurora B^{AUK1} but not of KIN-A or KIN-B (Fig. 3.3, A and B, D and E). In contrast, both Aurora B^{AUK1} and INCENP^{CPC1} were delocalized upon depletion of KIN-A or KIN-B (Figs. 3.3, A-C). RNAi against KIN-A disrupted KIN-B localization and vice versa (Fig. 3.3, A, D and E). Moreover, total protein levels of KIN-B were strongly affected by depletion of KIN-A (Fig. 3.4C), suggesting that the interaction with KIN-A is required to stabilize KIN-B.

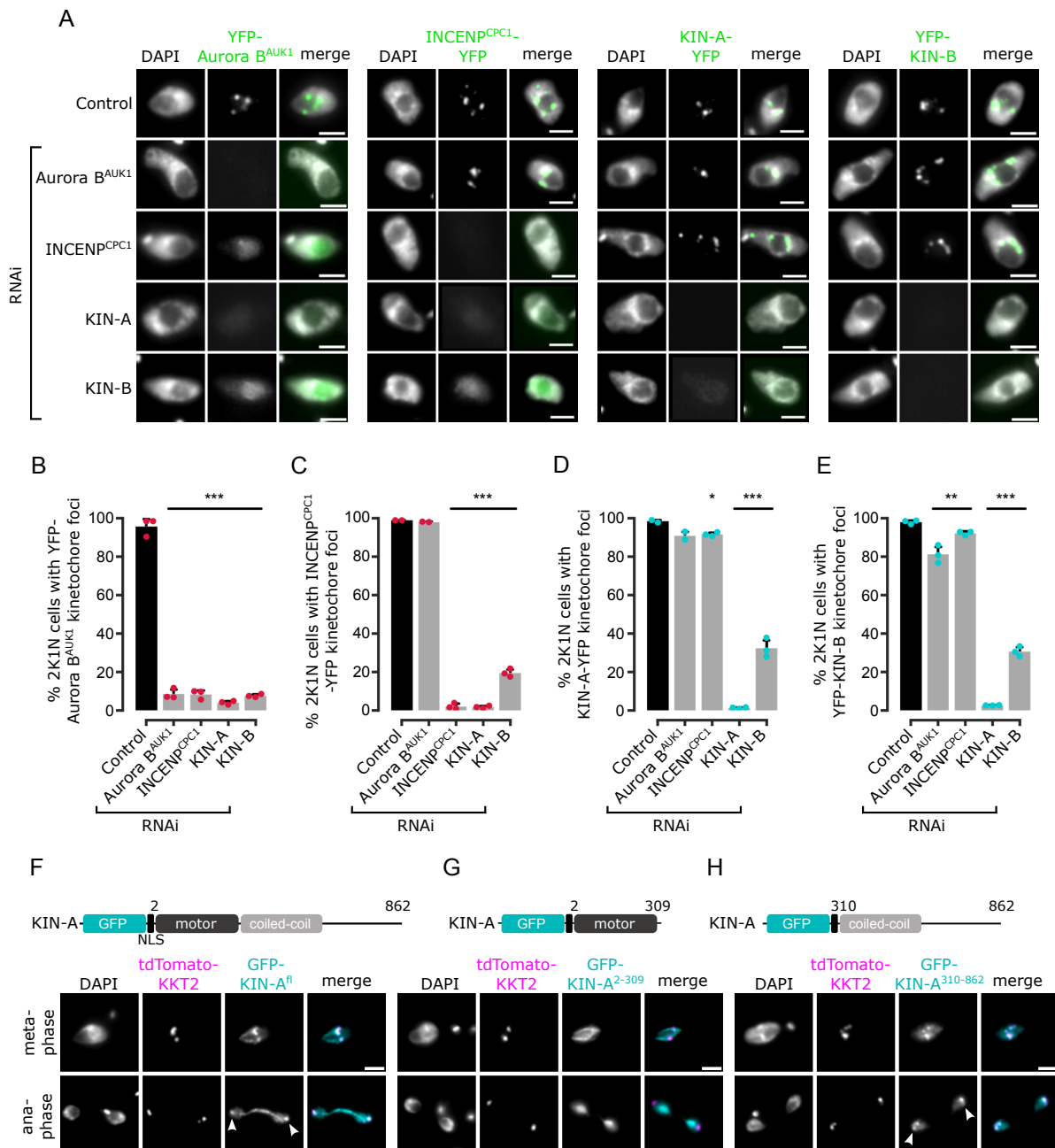


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Figure 3.3. Kinetochore localization of the CPC depends on KIN-A and KIN-B. (A) Representative fluorescence micrographs showing the localization of YFP-tagged Aurora B^{AUK1}, INCENP^{CPC1}, KIN-A and KIN-B in 2K1N cells upon RNAi-mediated knockdown of indicated CPC subunits. Note that nuclear close-ups are shown here. CPC proteins were not detected in the cytoplasm. RNAi was induced with 1 µg/mL doxycycline for 24 h (KIN-B RNAi) or 16 h (all others). Cell lines: BAP3092, BAP2552, BAP2557, BAP3093, BAP2906, BAP2900, BAP2904, BAP3094, BAP2899, BAP2893, BAP2897, BAP3095, BAP3096, BAP2560, BAP2564, BAP3097. Scale bars, 2 µm. (B) to (E) Quantification of 2K1N cells that have kinetochore-like dots of YFP-tagged Aurora B^{AUK1} (B), INCENP^{CPC1} (C), KIN-A (D) and KIN-B (E) upon RNAi-mediated depletion of indicated CPC components. All graphs depict the means (bar) ± SD of at least two replicates (shown as dots). A minimum of 100 cells per replicate were quantified. *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001 (two-sided, unpaired t-test). (F) to (H) Representative fluorescence micrographs showing the localization of ectopically expressed GFP-KIN-A^{fl} (F), -KIN-A²⁻³⁰⁹ (G) and -KIN-A³¹⁰⁻⁸⁶² (H). Expression of GFP fusion proteins was induced with 10 ng/mL doxycycline for 24 h. Kinetochores are marked with tdTomato-KKT2. Arrowheads indicate KIN-A^{fl} and KIN-A³¹⁰⁻⁸⁶² signals at kinetochores. KIN-A²⁻³⁰⁹ is found at mitotic spindle during metaphase. Cell lines: BAP2286, BAP2297, BAP2287. Scale bars, 2 µm

I next ectopically expressed GFP-tagged truncations of KIN-A and KIN-B to assess which domains promote their kinetochore targeting. Both KIN-A and KIN-B contain an N-terminal kinesin motor domain followed by several predicted coiled-coil motifs (Li et al., 2008a), albeit KIN-B is predicted to be an inactive motor (Wickstead and Gull, 2006). In addition, KIN-A has a long, disordered C-terminal tail (see below). Unlike full-length KIN-B, KIN-B²⁻³¹⁶ (inactive motor domain) failed to enrich at kinetochores and was instead found in the nucleolus (Fig. 3.4, D and E). KIN-A²⁻³⁰⁹ (motor domain) was also primarily detected in the nucleolus, although we observed additional spindle and weak kinetochore-like signal in some metaphase cells (Fig. 3.3G). By contrast, both KIN-A³¹⁰⁻⁸⁶² (coiled-coil domain + C-terminal disordered tail) and KIN-B³¹⁷⁻⁶²⁴ (coiled-coil domain) clearly localized to kinetochores from S phase to metaphase (Fig. 3.3H; Fig. 3.4F). Intriguingly, unlike endogenously YFP-tagged KIN-A, ectopically expressed GFP fusions of both full-length KIN-A and KIN-A³¹⁰⁻⁸⁶² clearly localized at kinetochores even in anaphase (Fig. 3.3, F and H). Weak anaphase kinetochore signal was also detectable for KIN-B³¹⁷⁻⁶²⁴ (Fig. 3.4F). GFP fusions of the central coiled-coil domain or the C-terminal disordered tail of KIN-A did not localize to kinetochores (data not shown). These results show that kinetochore localization of the CPC is mediated by KIN-A

and KIN-B and requires both the central coiled-coil domain as well as the C-terminal disordered tail of KIN-A.

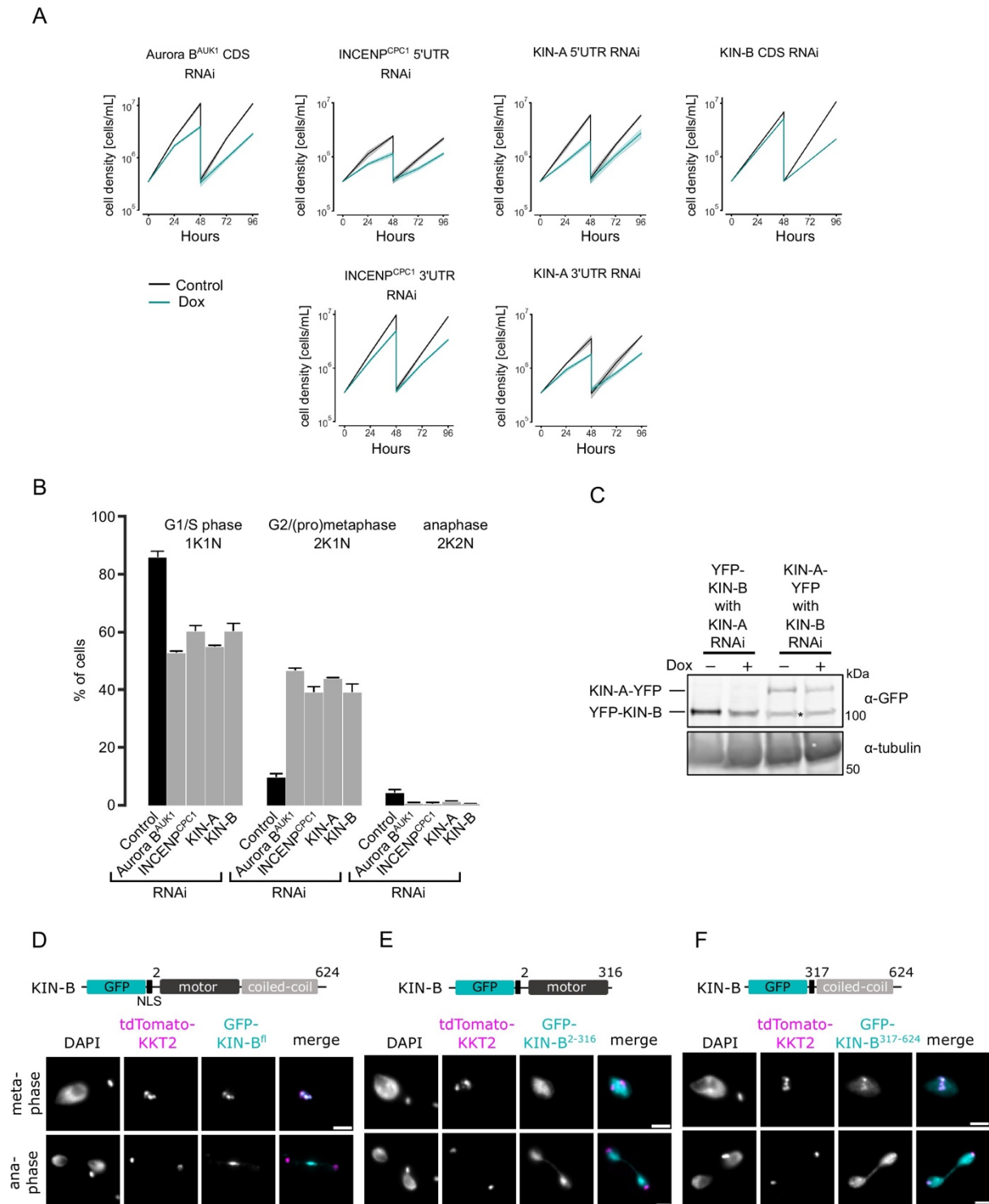


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Figure 3.4. Depletion of CPC proteins causes growth defects and cell cycle arrest.

(A) Growth curves upon RNAi-mediated knockdown of indicated CPC subunits. Data are presented as the mean $\pm$ SD of at least three replicates. RNAi was induced with 1 μ g/mL doxycycline and cultures were diluted at day 2. CDS stands for coding sequence, UTR for untranslated region. Cell lines: BAP941, BAP2250, BAP2251, BAP2557, BAP2558, BAP3076. (B) Cell cycle profiles upon knockdown of indicated CPC subunits. RNAi was induced with 1 μ g/mL doxycycline and cells were fixed at 16h (for Aurora B^{AUK1}, INCENP^{CPC1} and KIN-A RNAi) or 24 h (CPC2 and KIN-B RNAi). All graphs depict the means (bar) $\pm$ SD of at least two replicates. A minimum of 150 cells per replicate were quantified. Cell lines: BAP3092, BAP2554, BAP2557, BAP3093. (C) Western Blot showing levels of YFP-tagged KIN-A or KIN-B upon RNAi-mediated knockdown of KIN-B and KIN-A, respectively. RNAi was induced with 1 μ g/mL doxycycline for 16 h (KIN-A) or 24 h (KIN-B). Proteins were detected using anti GFP-antibodies. Asterisk indicates unspecific band. Cell lines: BAP3095, BAP2564. (D) to (F) Representative fluorescence micrographs showing the localization of ectopically expressed GFP-KIN-B^{fl} (D), -KIN-B²⁻³¹⁶ (E) and -KIN-B³¹⁷⁻⁶²⁴ (F). Expression of GFP fusion proteins was induced with 10 ng/mL doxycycline for 24 h. Kinetochores are marked with tdTomato-KKT2. Cell lines: BAP2288, BAP2289, BAP2290. Scale bars, 2 μ m.

3.2.3 Structural model of the trypanosome CPC

To gain structural insights into the trypanosome CPC, AlphaFold2 (AF2) (Jumper et al., 2021; Mirdita et al., 2022) was used to predict the overall structure of the trypanosome CPC by testing combinations of full-length Aurora B^{AUK1}, INCENP^{CPC1}, CPC2, KIN-A and KIN-B, and truncations thereof (Fig. 3.5, A – D). AF2 confidently predicted parallel coiled coils between KIN-A and KIN-B, with the main region of interaction contained within the central region of KIN-A (residues ~310–550) and the C-terminal region of KIN-B (residues ~320–580). The C-terminal tail (residues ~550–862) of KIN-A is predicted to be intrinsically disordered (pLDDT scores <20, Fig. 3.5, A and B). The first ~55 residues of INCENP^{CPC1}, predicted to form two α -helices (residues ~7–20 and ~36–55), interact with the coiled coils of KIN-A:KIN-B in close proximity to the kinesin motors. A flexible central linker in INCENP^{CPC1} bridges the N-terminus of INCENP^{CPC1} and the catalytic module of the CPC (Aurora B^{AUK1} + INCENP^{CPC1} IN-box). Similarly to INCENP^{CPC1}, an N-terminal α -helical region in CPC2 (residues ~19–75) interacts with the KIN-A:KIN-B coiled coils immediately downstream of the INCENP^{CPC1} binding site. Consistent with these predictions, GFP-tagged INCENP^{CPC1} 2-147 or CPC2²⁻¹²⁰ displayed normal localization dynamics indistinguishable from the corresponding full-length constructs

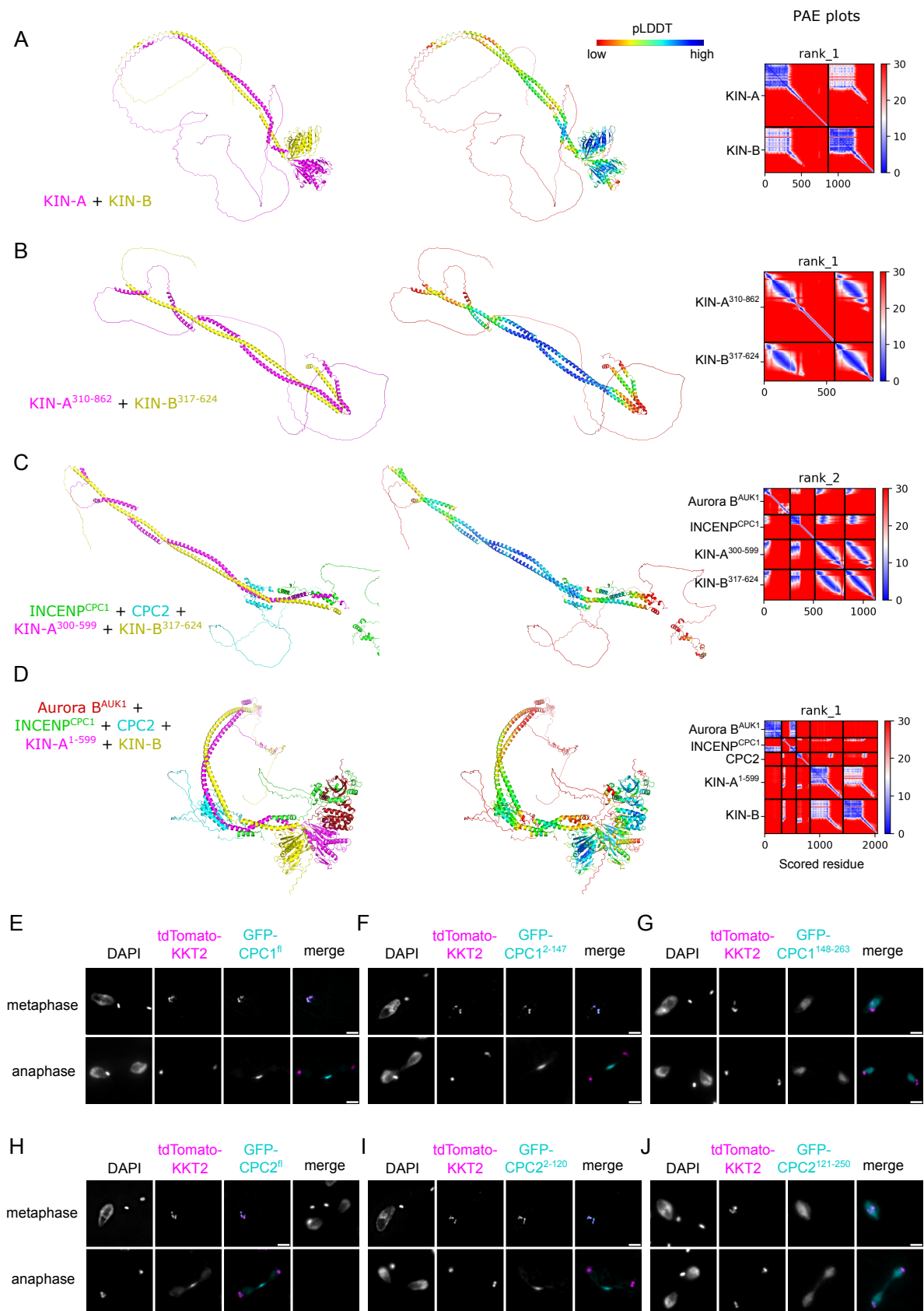


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Figure 3.5. AlphaFold2 models of CPC subcomplexes and localization of CPC1 and CPC2 truncations. (A) to (D) Cartoon representations coloured by protein (left) and pLDDT (center), and PAE plots (right) of AlphaFold2 predictions for indicated CPC subcomplexes. (E) to (J) Representative fluorescence micrographs showing the localization of ectopically expressed GFP-INCENP^{CPC1 fl} (E), -INCENP^{CPC1 2-147} (F), -INCENP^{CPC1 2-147} (G), -CPC2^{fl} (H), -CPC2²⁻¹²⁰ (I) and -CPC2¹²¹⁻²⁵⁰ (J) in different cell cycle stages. Expression of GFP fusion proteins was induced with 10 ng/mL doxycycline for 24 h. Kinetochores are marked with tdTomato-KKT2. Cell lines: BAP2190, BAP2191, BAP2193, BAP2194, BAP2195, BAP2196. Scale bars, 2 μ m.

(Fig. 3.5, E – F and H – I). In contrast, deletion of the N-terminal domains of INCENP^{CPC1} or CPC2 impaired kinetochore localization (Fig. 3.5, G and J). Together, these data suggest that Aurora B^{AUK1} forms a subcomplex with the C-terminus of INCENP^{CPC1}, and that INCENP^{CPC1} and CPC2 interact with the coiled-coil domain of KIN-A:KIN-B via their N-terminal domains.

To validate these findings biochemically, I performed bis(sulfosuccinimidyl) suberate (BS³) cross-linking of native CPC complexes isolated by immunoprecipitation of endogenously tagged YFP-Aurora B^{AUK1} from cells arrested prior to anaphase, followed by mass spectrometry analysis (IP-CLMS) (Fig. 3.6A). Indeed, my IP-CLMS data revealed high-score crosslinks between the predicted coiled-coil domains of KIN-A and KIN-B, suggesting that the two kinesins form a parallel heterodimer. As expected, Aurora B^{AUK1} formed contacts with the C-terminal IN-box of INCENP^{CPC1}, consistent with these two proteins constituting the catalytic module of the CPC. The N-terminal region of INCENP^{CPC1} interacted mainly with KIN-B and to a lesser degree with KIN-A, with most contacts confined to the N-terminal ends of the predicted coiled-coil domains of the kinesins close to their motor domains. The N-terminus of CPC2, on the other hand, formed crosslinks with the coiled-coil domain of KIN-A. We used PyXLinkViewer (Schiffrin et al., 2020) to map the IP-CLMS data onto the assembled AF2 model of the trypanosome CPC (Fig. 3.6B). Using a Euclidian distance cut-off of 30 Å, ~85% of crosslinks were compatible with the model, providing confidence in the AF2 predictions. The few crosslinks that violated the distance constraints mainly represent intra-protein contacts between the INCENP^{CPC1} N- and C-terminal domains or inter-protein contacts between the INCENP^{CPC1} C-terminal domain and the kinesin motor domain of KIN-A. The central domain of INCENP^{CPC1} is predicted to be unstructured (pLDDT scores <20, Fig. 3.5, C – D) and may act as a flexible linker, permitting multiple orientations of the catalytic module relative to the KIN-A:KIN-B scaffold (see Discussion). Taken together, these data indicate that KIN-A and KIN-B interact via their coiled-coil domains, which serve as a scaffold for the assembly of the remaining CPC subunits.

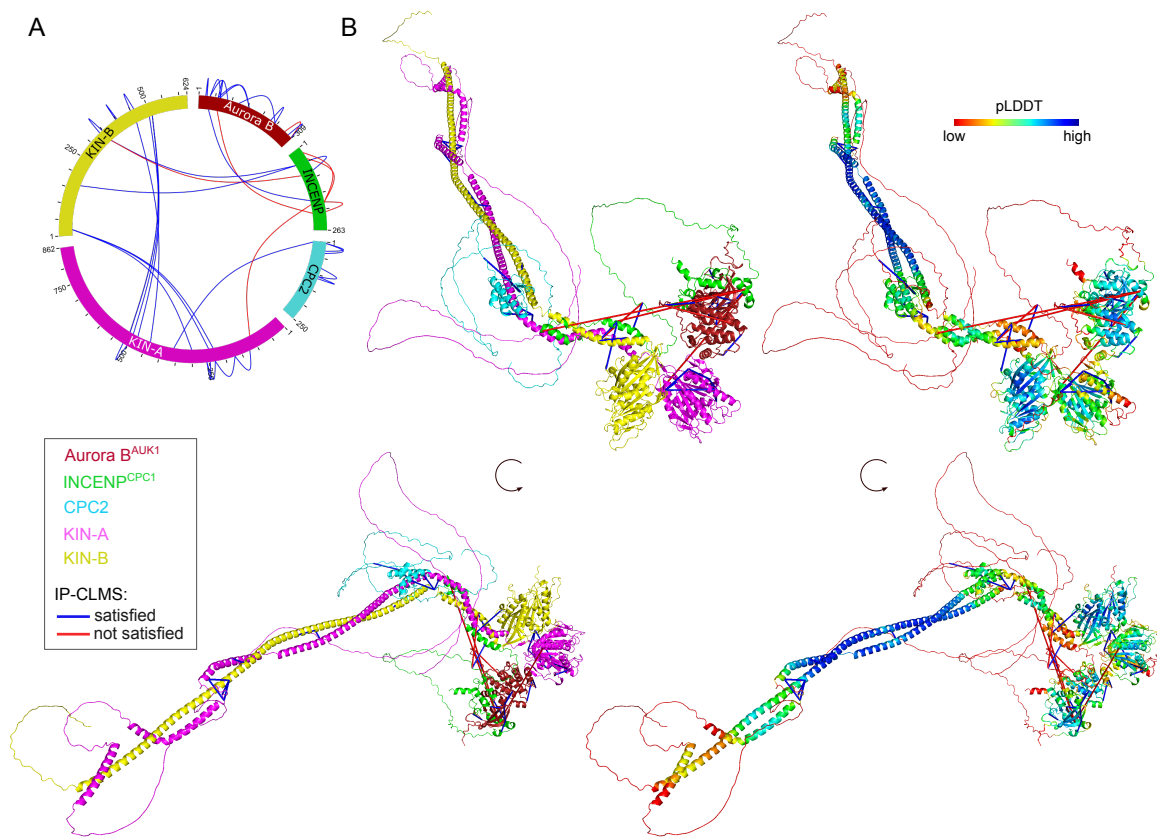


Figure 3.6. Structural model of the trypanosome CPC. (A) Circular view of the BS³ crosslinks observed between the subunits of the trypanosome CPC, obtained from native complexes isolated by immunoprecipitation of YFP-Aurora B^{AUK1} (Cell line: BAP2198b). pLink2 (Chen et al., 2019) was used to obtain crosslinks from mass spectrometry data. xiView (Graham et al., 2019) was used for data visualization. Only crosslinks with a score better than E^{-3} are shown. (B) Cartoon representation showing two orientations of the trypanosome CPC, coloured by protein on the left (Aurora B^{AUK1}: crimson, INCENP^{CPC1}: green, CPC2: cyan, KIN-A: magenta, and KIN-B: yellow) or according to their pLDDT values on the right, assembled from AlphaFold2 predictions shown in Figure S3. The pLDDT score is a per-residue estimate of the confidence in the AlphaFold prediction on a scale from 0 – 100. pLDDT > 70 indicates a reasonable accuracy of the model, while pLDDT < 50 indicates a low accuracy and often reflects disordered regions of the protein (Jumper et al., 2021). BS³ crosslinks in (B) were mapped onto the model using PyXlinkViewer (blue = distance constraints satisfied, red = distance constraints violated, C α -C α Euclidean distance threshold = 30 Å) (Schiffrin et al., 2020).

3.2.4 The CPC is recruited to kinetochores through the KKT7 – KKT8 complex pathway.

Core components of the Haspin-H3T3ph and Bub1-H2AT120ph-Sgo1 pathways that control CPC recruitment to the centromere in other model eukaryotes are not found in kinetoplastids (Berriman, 2005), and so far, no centromere-specific histone modifications and/or histone variants have been uncovered in *T. brucei*. I reasoned that the centromere receptor(s) of the trypanosome CPC may lie within the repertoire of unconventional kinetochore proteins present in kinetoplastids. My IP-CLMS approach failed to detect crosslinks between CPC subunits and other kinetochore components, possibly due to the transient nature of these interactions. Nevertheless, several KKT proteins were commonly enriched in the immunoprecipitates of Aurora B^{AUK1}, INCENP^{CPC1}, KIN-A and KIN-B, the most abundant ones being KKT6, KKT7, KKT8, KKT9, KKT10, KKT11 and KKT12 (Fig. 3.1A; Fig. 3.2A). KKT7 is detected at kinetochores from S phase until the end of anaphase and recruits the KKT8 complex (comprising KKT8, KKT9, KKT11 and KKT12) (Ishii and Akiyoshi, 2020; Akiyoshi and Gull, 2014). The KKT8 complex localizes at kinetochores from S phase and dissociates at the metaphase-anaphase transition.

Using previously validated RNAi constructs (Akiyoshi and Gull, 2014; Marcianò et al., 2021; Llauro et al., 2018), I found that knockdown of KKT7 or KKT9 resulted in dispersal of YFP-Aurora B^{AUK1} from kinetochores in ~70% of cells (Fig. 3.7, A – D). In contrast, depletion of KKT1, KKT2, KKT3, KKT4, KKT6, KKT10/19, KKT14 and KKT16 had little or no effect on YFP-Aurora B^{AUK1} localization (Fig. 3.8, A and B). KIN-A-YFP was also lost from kinetochores upon RNAi-mediated depletion of KKT8 complex subunits (Fig. 3.8, C and D). We next tested whether KKT7 or the KKT8 complex were able to recruit Aurora B^{AUK1} to an ectopic locus using the LacI-LacO system (Landeira and Navarro, 2007). For these experiments, I expressed GFP tagged KKT7²⁻²⁶¹ or KKT8 fused to the Lac repressor (LacI) in trypanosomes containing an ectopic Lac operator (LacO) array stably integrated into rDNA repeats. KKT7 lies upstream of the KKT8 complex (Ishii and Akiyoshi, 2020). Indeed, GFP-KKT7²⁻²⁶¹-LacI recruited tdTomato-KKT8, -KKT9 and -KKT12 (Fig. 3.8E). Expression of both GFP-KKT7²⁻²⁶¹-LacI and GFP-KKT8-LacI resulted in robust recruitment of tdTomato-Aurora B^{AUK1} to LacO foci in S phase (Fig. 3.7, E and F). Intriguingly, we also noticed that, unlike endogenous KKT8 (which is not present in anaphase), ectopically expressed GFP-KKT8-LacI remained at kinetochores during anaphase (Fig. 3.7F). This resulted in a fraction of tdTomato-Aurora B^{AUK1} being trapped at kinetochores during anaphase instead of migrating to the central spindle (Fig. 3.7F). I observed a comparable situation upon ectopic expression of GFP-KIN-A, which is retained on anaphase kinetochores together with tdTomato-KKT8 (Fig. 3.8F). In contrast, Aurora B^{AUK1} was not recruited to LacO foci marked by GFP-KKT7²⁻²⁶¹-LacI in anaphase (Fig. 3.7E).

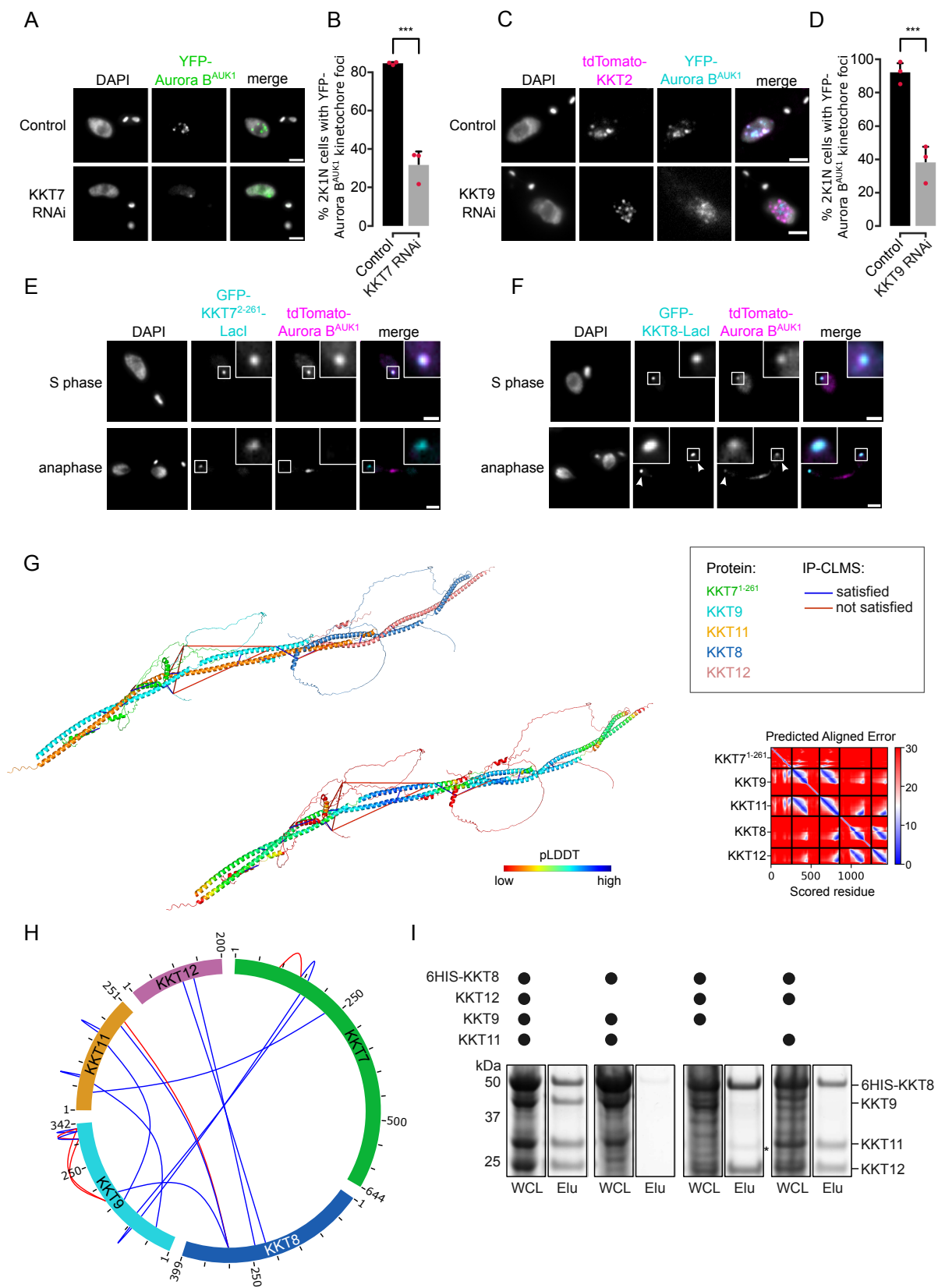


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Figure 3.7. The CPC is recruited to kinetochores via the KKT7 – KKT8 complex pathway.

(A) Representative fluorescence micrographs showing the localization of YFP-Aurora B^{AUK1} upon RNAi-mediated knockdown of KKT7. RNAi was induced with 1 µg/mL doxycycline for 24 h. Cell line: BAP577. Scale bars, 2 µm. (B) Quantification of 2K1N cells that have kinetochore-like dots of YFP-Aurora B^{AUK1} upon knockdown of KKT7. All graphs depict the means (bar) ± SD of three replicates (shown as dots). A minimum of 50 cells per replicate were quantified. ***P ≤ 0.001 (two-sided, unpaired t-test). (C) Representative fluorescence micrographs showing the localization of YFP-Aurora B^{AUK1} upon RNAi-mediated knockdown of KKT9. RNAi was induced with 1 µg/mL doxycycline for 24 h. Kinetochores are marked with tdTomato-KKT2. Cell line: BAP2276. Scale bars, 2 µm. (D) Quantification of 2K1N cells that have kinetochore-like dots of YFP-Aurora B^{AUK1} upon knockdown of KKT9. All graphs depict the means (bar) ± SD of three replicates (shown as dots). A minimum of 50 cells per replicate were quantified. ***P ≤ 0.001 (two-sided, unpaired t-test). (E) and (F) Representative micrographs of cells in S phase and anaphase showing recruitment of tdTomato-Aurora B^{AUK1} to LacO foci marked by ectopically expressed GFP-KKT7²⁻²⁶¹-LacI (E) or -KKT8-LacI (F). The insets show the magnification of the boxed region. Expression of LacI fusion proteins was induced with 10 ng/mL doxycycline for 24 h. Arrowheads in (F) indicate anaphase kinetochore localization of GFP-KKT8-LacI and tdTomato-Aurora B^{AUK1}. Anaphase kinetochore localization of tdTomato-Aurora B^{AUK1} was observed in 75% of anaphase cells expressing GFP-KKT8-LacI (n = 28). Cell lines: BAP1395, BAP2640. Scale bars, 2 µm. Of note, LacI fusions with INCENP^{CPC1}, KIN-A and KIN-B constructs robustly localized to kinetochores like their endogenous counterparts and failed to form distinct LacI foci and could therefore not be used to assess ectopic recruitment of KKT proteins. (G) AlphaFold2 model of the KKT7 – KKT8 complex, coloured by protein (KKT7¹⁻²⁶¹: green, KKT8: blue, KKT12: pink, KKT9: cyan and KKT11: orange) (left) and by pLDDT (center). BS³ crosslinks in (H) were mapped onto the model using PyXlinkViewer (Schiffrin et al., 2020) (blue = distance constraints satisfied, red = distance constraints violated, Cα-Cα Euclidean distance threshold = 30 Å). Right: Predicted Aligned Error (PAE) plot of model shown on the left (rank_2). The colour indicates AlphaFold's expected position error (blue = low, red = high) at the residue on the x axis if the predicted and true structures were aligned on the residue on the y axis (Jumper et al., 2021). (H) Circular view of the BS³ crosslinks observed among KKT7 and KKT8 complex subunits, obtained from native complexes isolated by immunoprecipitation of YFP-tagged KKIP1 (Cell line: BAP710a). pLink2 (Chen et al., 2019) was used to obtain crosslinks from mass spectrometry data and xiView (Graham et al., 2019) was used for data visualization. Only crosslinks with a score better than E⁻³ are shown. (I) Indicated combinations of 6HIS-tagged KKT8 (~46 kDa), KKT9 (~39 kDa), KKT11 (~29 kDa) and KKT12 (~23 kDa) were co-expressed in *E. coli*, followed by metal affinity chromatography and SDS-PAGE. The asterisk indicates a common contaminant.

3.2.5 KKT7 recruits the KKT8 complex via the KKT9:KKT11 subcomplex.

To gain further insight into the structure and assembly hierarchy within the KKT7 – KKT8 complex pathway, I performed CLMS on native complexes isolated by immunoprecipitation of endogenously YFP-tagged kinetochore proteins and mapped the detected crosslinks onto AF2 structure predictions (Fig. 3.7, G and H) (Materials & Methods). AF2 confidently predicted coiled coils between KKT8 and KKT12 and between KKT9 and KKT11 (Figs. 3.7G; and 3.8, G and H), suggesting that KKT8:KKT12 and KKT9:KKT11 each form distinct subcomplexes. To validate these findings, I co-expressed combinations of 6HIS-KKT8, KKT9, KKT11 and KKT12 in *E. coli* and performed metal affinity chromatography (Fig. 3.7I). 6HIS-KKT8 efficiently pulled down KKT9, KKT11 and KKT12, as shown previously (Ishii and Akiyoshi, 2020). In the absence of KKT9, 6HIS-KKT8 still pulled down KKT11 and KKT12. Removal of either KKT9 or KKT11 did not impact formation of the KKT8:KKT12 subcomplex. In contrast, 6HIS-KKT8 could not be recovered without KKT12, indicating that KKT12 is required for formation of the full KKT8 complex. These results support the idea that the KKT8 complex consists of KKT8:KKT12 and KKT9:KKT11 subcomplexes. The two subcomplexes appear to be connected to each other through interactions between the C-terminal end of the KKT8:KKT12 coiled coils and the C-terminus of KKT11. Two alpha helices in KKT7²⁻²⁶¹ (residues ~149–181) are predicted to interact with KKT9:KKT11. Using a distance cut-off of 30 Å, ~70% of crosslinks were compatible with the model (Fig. 3.7, G and H). Of the crosslinks that failed to meet the distance criteria, ~90% involved unstructured regions within KKT7 or the C-terminal tail of KKT8. Collectively, these results reveal that KKT7 recruits the KKT8 complex through interaction with the KKT9:KKT11 subcomplex.

I next examined the localization dependency of KKT8 complex components in cells. Using RNAi constructs against individual subunits of the KKT8 complex (Ishii and Akiyoshi, 2020; Akiyoshi and Gull, 2014; Marcianò et al., 2021), I assessed localization of endogenously YFP-tagged KKT8, KKT9 and KKT12 (Fig. 3.8, I – L). We found that knockdown of any subunit of the KKT8 complex affected protein levels and kinetochore localization of the other subunits, indicating that presence of all subunits is required to stabilize the full complex. YFP-KKT9 was least affected and was still detectable at kinetochores in ~50% of cells depleted of KKT8, KKT11 or KKT12 (Fig. 3.8L). Thus, KKT9:KKT11 may lie upstream of KKT8:KKT12. Indeed, the IP-CLMS data suggest that the KKT9:KKT11 subcomplex directly interacts with the N-terminus of KKT7 (Fig. 3.7H). KKT7 also formed robust crosslinks with the KKT10/19 kinases (data not shown), supporting our previous finding that KKT7 and KKT10 form a stable complex (Ishii and Akiyoshi, 2020).

Together, these data indicate that the KKT7 – KKT8 complex pathway serves as the main CPC recruitment arm in trypanosomes.

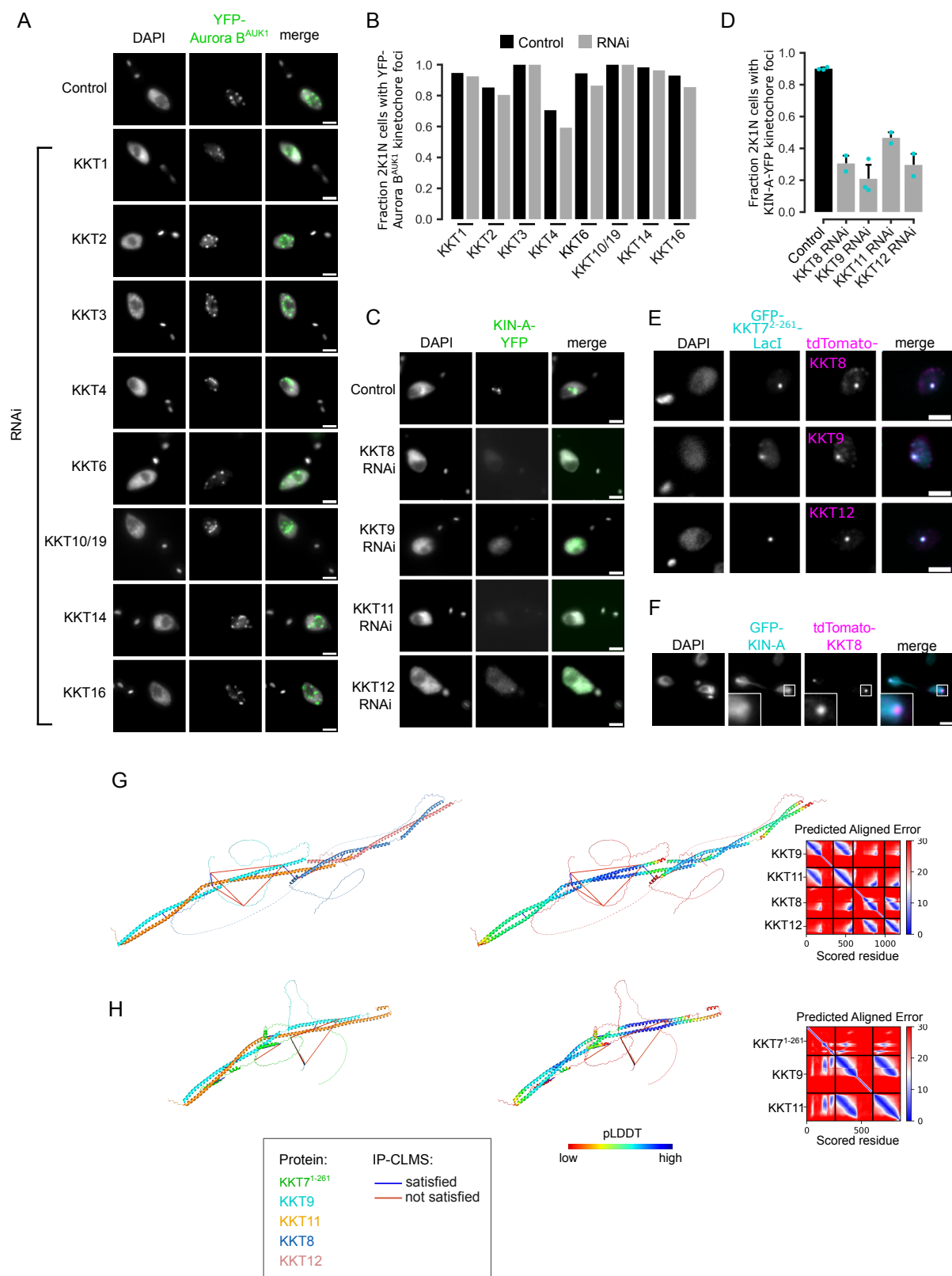


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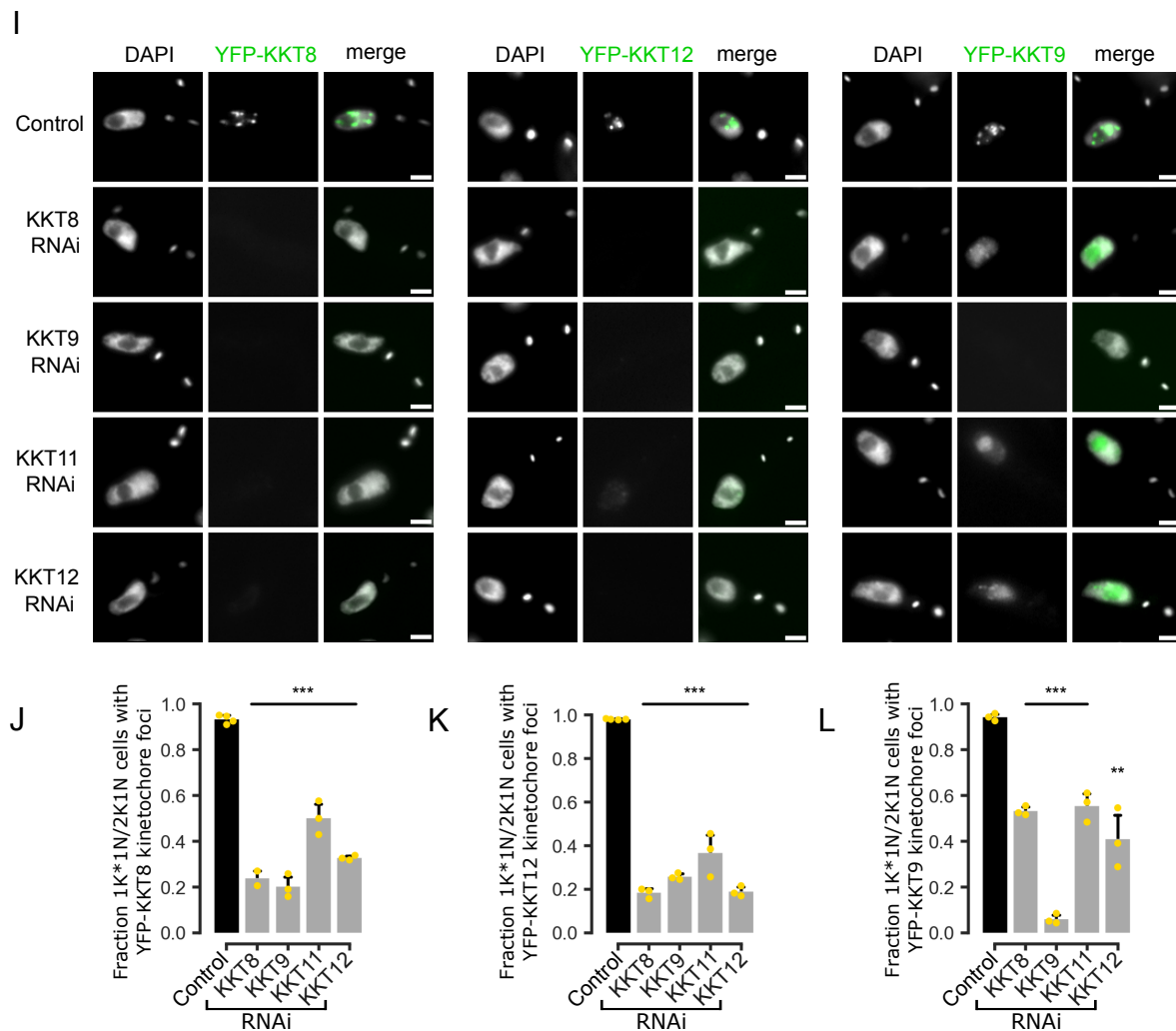


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3.2.6 The KIN-A C-terminal tail interacts with the KKT8 complex through a conserved domain.

By IP-CLMS I failed to detect reliable crosslinks between the CPC and the KKT7 – KKT8 complex or, in fact, any kinetochore proteins. This suggests that the IP-CLMS approach, although well-suited for characterizing stable protein complexes, may not be sensitive enough to detect transient or lower affinity interactions. To overcome this, we used AF2 to probe for potential interactions between the KKT8 and CPC (sub)complexes. AF2 did not predict interactions between the KKT8 complex and INCENP^{CPC1}, CPC2 or KIN-B (data not shown). Intriguingly, AF2 predicted with high confidence interactions between KKT9:KKT11 and a conserved region (residues ~722–741) within the KIN-A C-terminal tail, which I termed conserved domain 1 (CD1) (Figs. 3.9, A and B; and 3.10A, Alignment S1 (Appendix)). This interaction involves a triple helix formed of KIN-A CD1, KKT9 and KKT11 in a region close to

Figure 3.8. Kinetochore localization of Aurora B^{AUK1} and KIN-A depends on the KKT7 – KKT8 complex pathway. (A) Representative fluorescence micrographs showing the localization of YFP-Aurora B^{AUK1} upon RNA-mediated knockdown of indicated KKT proteins. RNAi was induced with 1 µg/mL doxycycline for 24 h. Cell lines: BAP3132, BAP576, BAP2551, BAP2550, BAP3133, BAP1019, BAP2549, BAP2446. Scale bars, 2 µm. (B) Quantification of 2K1N cells that have kinetochore-like dots of YFP-Aurora B^{AUK1} upon knockdown of indicated KKT proteins. A minimum of 40 cells per replicate were examined. (C) Representative fluorescence micrographs showing the localization of KIN-A-YFP upon RNA-mediated knockdown of KKT8 complex subunits. RNAi was induced with 1 µg/mL doxycycline for 24 h. Cell lines: BAP2970, BAP2968, BAP2969, BAP2971. Scale bars, 2 µm. (D) Quantification of 2K1N cells that have kinetochore-like dots of KIN-A-YFP upon knockdown of indicated KKT8 complex subunits. All graphs depict the means (bar) ± SD of at least two replicates (shown as dots). A minimum of 50 cells per replicate were quantified. (E) Representative micrographs showing recruitment of tdTomato-tagged KKT8, KKT9 and KKT12 to LacO foci marked by ectopically expressed GFP-KKT7²⁻²⁶¹-LacI. Expression of GFP fusion proteins was induced with 10 ng/mL doxycycline for 24 h. Cell lines: BAP871, BAP873, BAP874. Scale bars, 2 µm. (F) Representative fluorescence micrograph of an anaphase cell showing localization of tdTomato-KKT8 and ectopically expressed GFP-KIN-A. The insets show the magnification of the boxed region. Expression of GFP-KIN-A was induced with 10 ng/mL doxycycline for 24 h. Cell line: BAP3080. Scale bars, 2 µm. (G) and (H) Cartoon representations coloured by protein (left) and pLDDT (center), and PAE plots (right) of AlphaFold2 predictions for indicated protein complexes. (I) Representative fluorescence micrographs showing localization of YFP-tagged KKT8, KKT12 and KKT9 upon RNAi-mediated knockdown of indicated KKT8 complex subunits. RNAi was induced with 1 µg/mL doxycycline for 24 h. Cell lines: BAP2954, BAP2952, BAP2953, BAP2955, BAP2966, BAP2964, BAP2965, BAP2967, BAP2958, BAP2956, BAP2957, BAP2959. Scale bars, 2 µm. (J) to (L) Quantification of 1K*1N and 2K1N cells that have kinetochore-like dots of YFP-tagged KKT8 (J), KKT12 (K) and KKT9 (L) upon knockdown of indicated KKT8 complex subunits. All graphs depict the means (bar) ± SD of at least three technical replicates (shown as dots) from one experiment. A minimum of 100 cells per replicate were quantified. **P ≤ 0.01, ***P ≤ 0.001 (two-sided, unpaired t-test).

the KKT7 binding site. pLDDT scores improved significantly for KIN-A CD1 in complex with KKT9:KKT11 (>80) compared to KIN-A CD1 alone (~20) (Fig. 3.5, A and B), suggesting that

CD1 forms a helical structure upon binding to KKT9:KKT11. Sequence alignment revealed the presence of a second conserved domain (residues ~816–862) within the C-terminal tail of KIN-A, hereafter referred to as CD2 (Fig. 3.9B; Alignment S1, Appendix). To assess the contributions of CD1 and CD2 for kinetochore recruitment of KIN-A in vivo, I ectopically

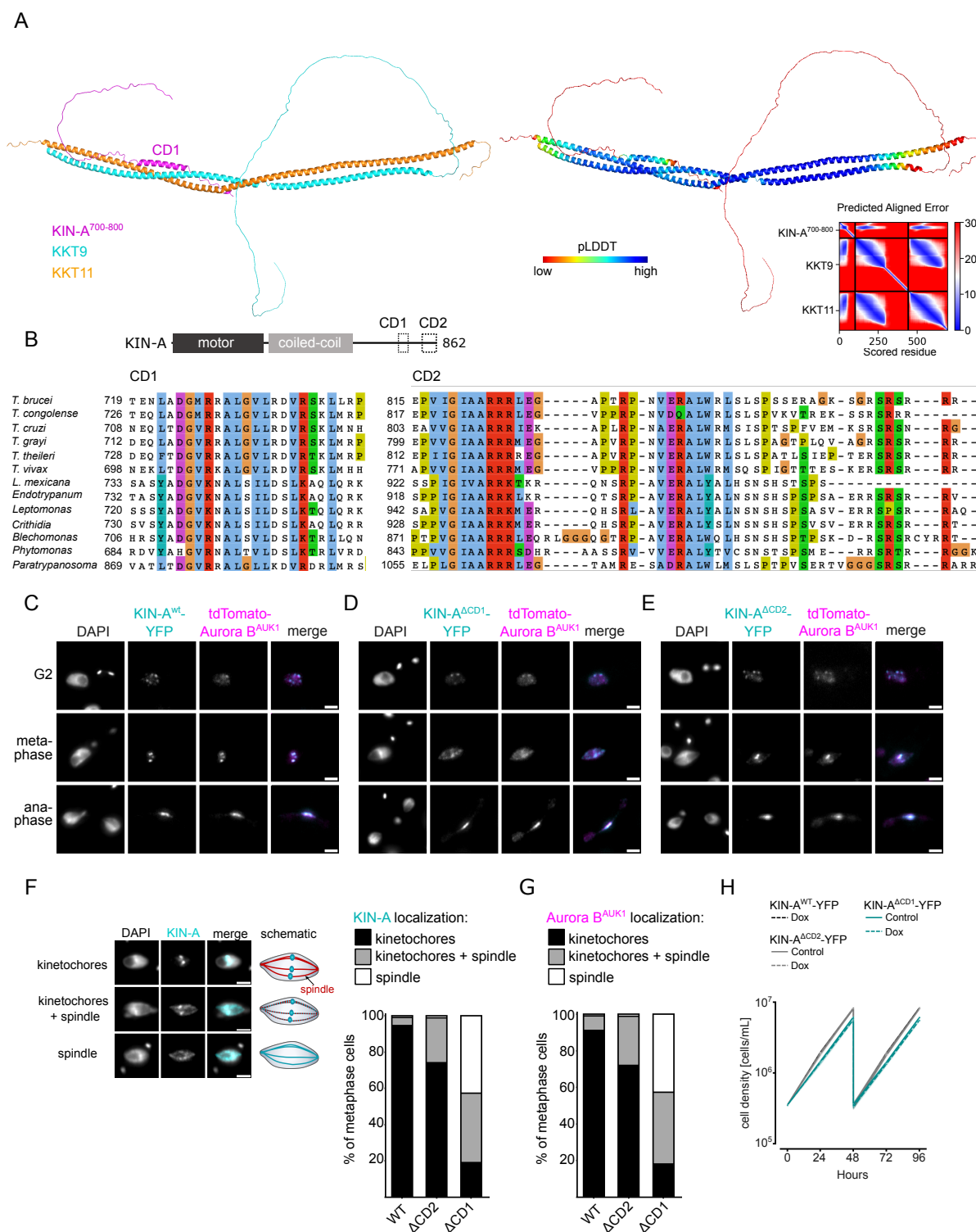


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Figure 3.9. Two conserved domains within the C-terminal tail of KIN-A promote kinetochore recruitment of the CPC. (A) Left: AlphaFold2 model of KKT9:KKT11 in complex with KIN-A⁷⁰⁰⁻⁸⁶². Cartoon representations are coloured by protein (KKT9: cyan, KKT11: orange, KIN-A: magenta) (left) or according to their pLDDT values (blue = high confidence, red = low confidence) (center). Right: Predicted Aligned Error (PAE) plot of model (rank_1) predicted by AlphaFold2 (blue = high confidence, red = low confidence in the relative positions of the domains to one another). CD1 of KIN-A was predicted to interact with KKT9:KKT11 in all five AlphaFold2 models (rank_1 to rank_5). (B) Multiple sequence alignment of KIN-A CD1 and CD2 showing conservation. (C) to (E) Representative fluorescence micrographs showing the localization of tdTomato-Aurora B^{AUK1} and YFP-tagged KIN-A^{wt} (C), KIN-A^{ΔCD1 (717-743)} (D) and KIN-A^{ΔCD2 (816-862)} (E). RNAi was induced with 1 μg/mL doxycycline for 24 h to deplete the untagged KIN-A allele. Cell lines: BAP3067, BAP3128, BAP3127. Scale bars, 2 μm. (F) Stacked bar charts showing the percentage of YFP-tagged KIN-A^{wt}, KIN-A^{ΔCD1} and KIN-A^{ΔCD2} on kinetochores, kinetochores + spindle and spindle only in metaphase cells. Examples and schematic illustrations of the three categories used for scoring are presented on the left. A minimum of 50 cells per condition were quantified. (G) Stacked bar charts showing the percentage of tdTomato-Aurora B^{AUK1} on kinetochores, kinetochores + spindle and spindle only in metaphase cells upon rescue with YFP-tagged KIN-A^{wt}, KIN-A^{ΔCD1} or KIN-A^{ΔCD2}. A minimum of 50 cells per condition were quantified. (H) Growth curves for indicated cell lines and conditions. RNAi was induced with 1 μg/mL doxycycline for to deplete the untagged KIN-A allele in the knockdown conditions and cultures were diluted at day 2. Cell lines: BAP3067, BAP3128, BAP3127.

expressed GFP fusions of the central coiled coils and C-terminal tail of KIN-A (residues ~310–862) lacking either CD1, CD2, or both (Fig. 3.10, B – E). GFP-KIN-A^{310-862 ΔCD2} showed a moderate reduction in kinetochore localization in metaphase and was completely lost from kinetochores in anaphase (Fig. 3.10, C and F). By contrast, GFP-KIN-A^{310-862 ΔCD1} was largely dispersed in metaphase but reappeared at kinetochores in anaphase (Fig. 3.10, D and F). GFP-KIN-A³¹⁰⁻⁷¹⁶ lacking both CD1 and CD2 failed to enrich at kinetochores both in metaphase and anaphase (Fig. 3.10, E and F). These data suggest that CD1 and CD2 synergistically promote kinetochore localization of KIN-A, with CD1 interacting with KKT9:KKT11 and CD2 possibly interacting with another receptor at the kinetochore.

I next tested the relevance of KIN-A CD1 and CD2 for CPC localization and function by replacing one allele of KIN-A with C-terminally tagged wild-type or mutant constructs lacking either CD1 or CD2 and performed RNAi against the 3'UTR of KIN-A to deplete the untagged

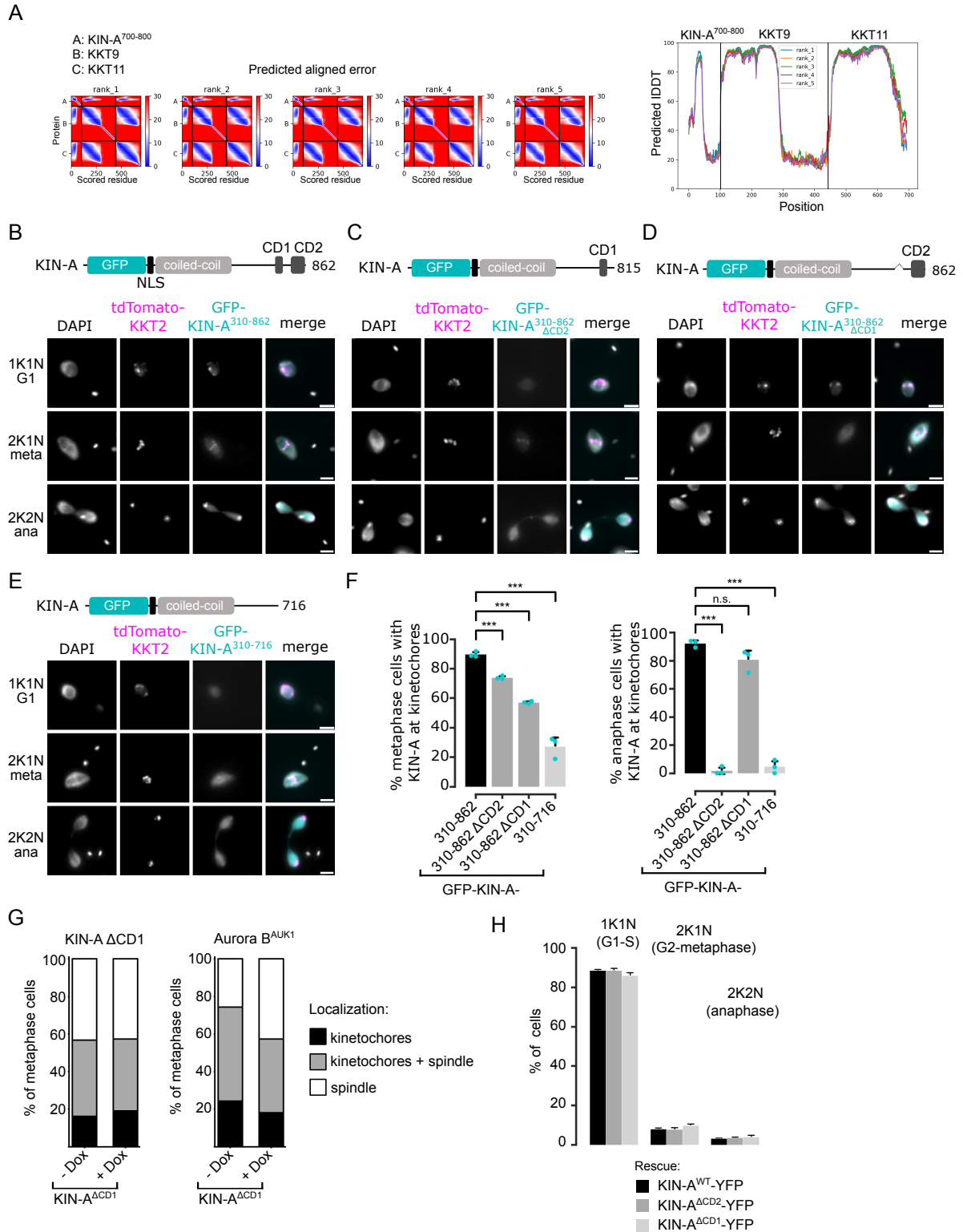


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allele. I was unable to obtain a rescue cell line lacking both CD1 and CD2 as the double-mutant protein was not properly expressed. Wild-type KIN-A-YFP along with Aurora B^{AUK1} robustly localized to kinetochores from S phase until anaphase onset (Figs. 3.1, C and D; and

Figure 3.10. CD1 and CD2 contribute synergistically to kinetochore localization of KIN-A. (A) PAE (left) and pLDDT plots (right) of AlphaFold2 predictions rank1 to rank5 for KIN-A⁷⁰⁰⁻⁸⁰⁰ in complex with KKT9 and KKT11. (B) to (E) Representative fluorescence micrographs showing the localization of ectopically expressed GFP-KIN-A³¹⁰⁻⁸⁶² (B), -KIN-A³¹⁰⁻⁸⁶² Δ CD2 (C) and -KIN-B³¹⁰⁻⁸⁶² Δ CD1 (D) and -KIN-A³¹⁰⁻⁷¹⁶ (E). Expression of GFP fusion proteins was induced with 10 ng/mL doxycycline for 24 h. Kinetochores are marked with tdTomato-KKT2. Cell lines: BAP2287, BAP2948, BAP2949, BAP2947. Scale bars, 2 μ m. (F) Quantification of metaphase (left) and anaphase (right) cells that have kinetochore-like dots of indicated GFP fusion proteins. All graphs depict the means (bar) $\pm$ SD of three replicates (shown as dots). A minimum of 150 cells per replicate were quantified. ***P $\leq$ 0.001 (two-sided, unpaired t-test). (G) Stacked bar charts showing the percentage of KIN-A ^{Δ CD1}-YFP (left) or tdTomato-Aurora B^{AUK1} (right) on kinetochores, kinetochores + spindle and spindle in KIN-A ^{Δ CD1} metaphase cells with and without KIN-A RNAi. A minimum of 70 cells per condition were quantified. Cell line: BAP3128. (H) Cell cycle profiles for the indicated cell lines and conditions. RNAi was induced with 1 μ g/mL doxycycline to deplete the untagged KIN-A allele in the knockdown conditions and cells were fixed after 24 h. All graphs depict the means (bar) $\pm$ SD of three replicates. A minimum of 500 cells per replicate were quantified. Cell lines: BAP3067, BAP3127, BAP3128.

3.9C). KIN-A ^{Δ CD1}-YFP was detectable at kinetochores in G2 but predominantly localized to the mitotic spindle from (pro)metaphase onwards (Fig. 3.9, D and F), indicating that removal of CD1 severely weakens the affinity of KIN-A for kinetochores and instead shifts the balance towards microtubule binding. Interestingly, expression of KIN-A ^{Δ CD1}-YFP similarly affected the localization of Aurora B^{AUK1} (Figs. 3.9, D and G; and 3.10G). We also detected partial spindle localization of Aurora B^{AUK1} in a small population (~25%) of metaphase cells expressing KIN-A ^{Δ CD2}-YFP (Fig. 3.9, E – G). Central spindle localization of KIN-A in anaphase was unaffected by deletion of either CD1 or CD2 (Fig. 3.9, D and E). Remarkably, despite a substantial loss of Aurora B^{AUK1} from kinetochores in metaphase, Δ CD1 cells exhibited normal cell cycle profiles (Fig. 3.10H) and showed only a modest decrease in proliferation rates (Fig. 3.9H). This parallels the situation in budding yeast, in which error-free chromosome segregation can be sustained even when inner centromere localization of Aurora B is largely abolished (Campbell and Desai, 2013; García-Rodríguez et al., 2019).

3.2.7 CPC targeting to the central spindle in anaphase depends on KIN-A ATPase activity.

Finally, I asked how translocation of the CPC to the spindle midzone in anaphase is achieved in trypanosomes. In mammalian cells, dephosphorylation of INCENP and the kinesin Mklp2 upon anaphase onset allows formation of a transient CPC-Mklp2 complex (Kitagawa et al., 2014; Hümmer and Mayer, 2009; Serena et al., 2020; Gruneberg et al., 2004). MKLP2 activity then drives plus-directed movement of this complex along microtubules of the anaphase spindle. I therefore speculated that anaphase translocation of the kinetoplast CPC to the central spindle may involve the kinesin motor domain of KIN-A. KIN-B is unlikely to be a functional kinesin based on the absence of several well-conserved residues and motifs within

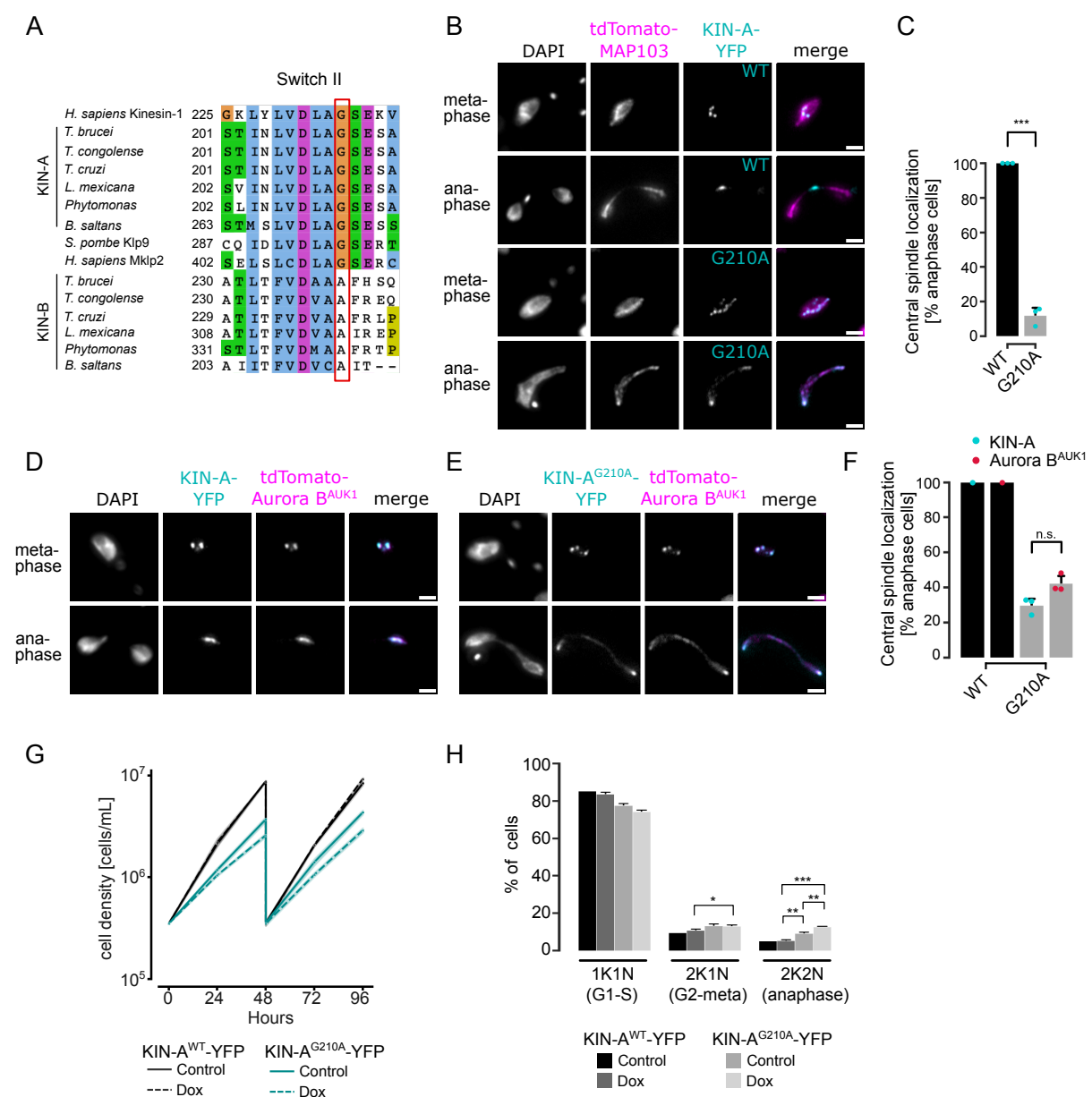


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Figure 3.11. KIN-A ATPase activity is required for central spindle localization of the CPC in anaphase. (A) Multiple sequence alignment showing conservation of Switch II region in KIN-A and KIN-B, with key glycine residue (G210 in *T. brucei*) targeted for rigor mutation highlighted in red. (B) Representative fluorescence micrographs showing the localization of tdTomato-MAP103 (spindle marker) and YFP-tagged KIN-A^{wt} or KIN-A^{G210A} (rigor mutant). RNAi was induced with 1 µg/mL doxycycline for 24 h to deplete the untagged KIN-A allele. Cell lines: BAP3068, BAP3071. Scale bars, 2 µm. (C) Quantification showing the percentage of anaphase cells that have YFP-tagged KIN-A^{wt} or KIN-A^{G210A} localized at the central spindle. All graphs depict the means (bar) ± SD of three replicates (shown as dots). A minimum of 40 cells per replicate were quantified. ***P ≤ 0.001 (two-sided, unpaired t-test). (D) and (E) Representative fluorescence micrographs showing the localization of tdTomato-Aurora B^{AUK1} and YFP-tagged KIN-A^{wt} (D) or KIN-A^{G210A} (E). RNAi was induced with 1 µg/mL doxycycline for 24 h to deplete the untagged KIN-A allele. Cell lines: BAP3067, BAP3070. Scale bars, 2 µm. (F) Quantification showing the percentage of anaphase cells that have tdTomato-Aurora B^{AUK1} localized at the central spindle upon rescue with YFP-tagged KIN-A^{wt} or KIN-A^{G210A}. Graphs for the KIN-A^{G210} rescue conditions (grey) depict the means (bar) ± SD of three replicates (shown as dots). A minimum of 30 cells per replicate were quantified. (G) Growth curves for indicated cell lines and conditions. RNAi was induced with 1 µg/mL doxycycline for to deplete the untagged KIN-A allele in the knockdown conditions and cultures were diluted at day 2. Cell lines: BAP3064, BAP3065. (H) Cell cycle profiles for the indicated cell lines and conditions. RNAi was induced with 1 µg/mL doxycycline to deplete the untagged KIN-A allele in the knockdown conditions and cells were fixed after 24 h. All graphs depict the means (bar) ± SD of at least two replicates. A minimum of 300 cells per replicate were quantified. Cell lines: BAP3064, BAP3065. ***P ≤ 0.001 (two-sided, unpaired t-test).

the motor domain, which are fully present in KIN-A (Li et al., 2008a). These include the P-loop, switch I and switch II motifs, which form the nucleotide binding cleft, and many conserved residues within the α4-L12 elements, which interact with tubulin (Alignment S2 (Appendix)) (Endow et al., 2010). Consistent with this, the motor domain of KIN-B, contrary to KIN-A, failed to localize to the mitotic spindle when expressed ectopically (Fig. 3.4E) and did not co-sediment with microtubules in our in vitro assay (Fig. 3.12A).

Ectopically expressed GFP-KIN-A and -KIN-A²⁻³⁰⁹ partially localized to the mitotic spindle but failed to concentrate at the midzone during anaphase (Fig. 3.4, F and G), suggesting that N-terminal tagging of the KIN-A motor domain may interfere with its function.

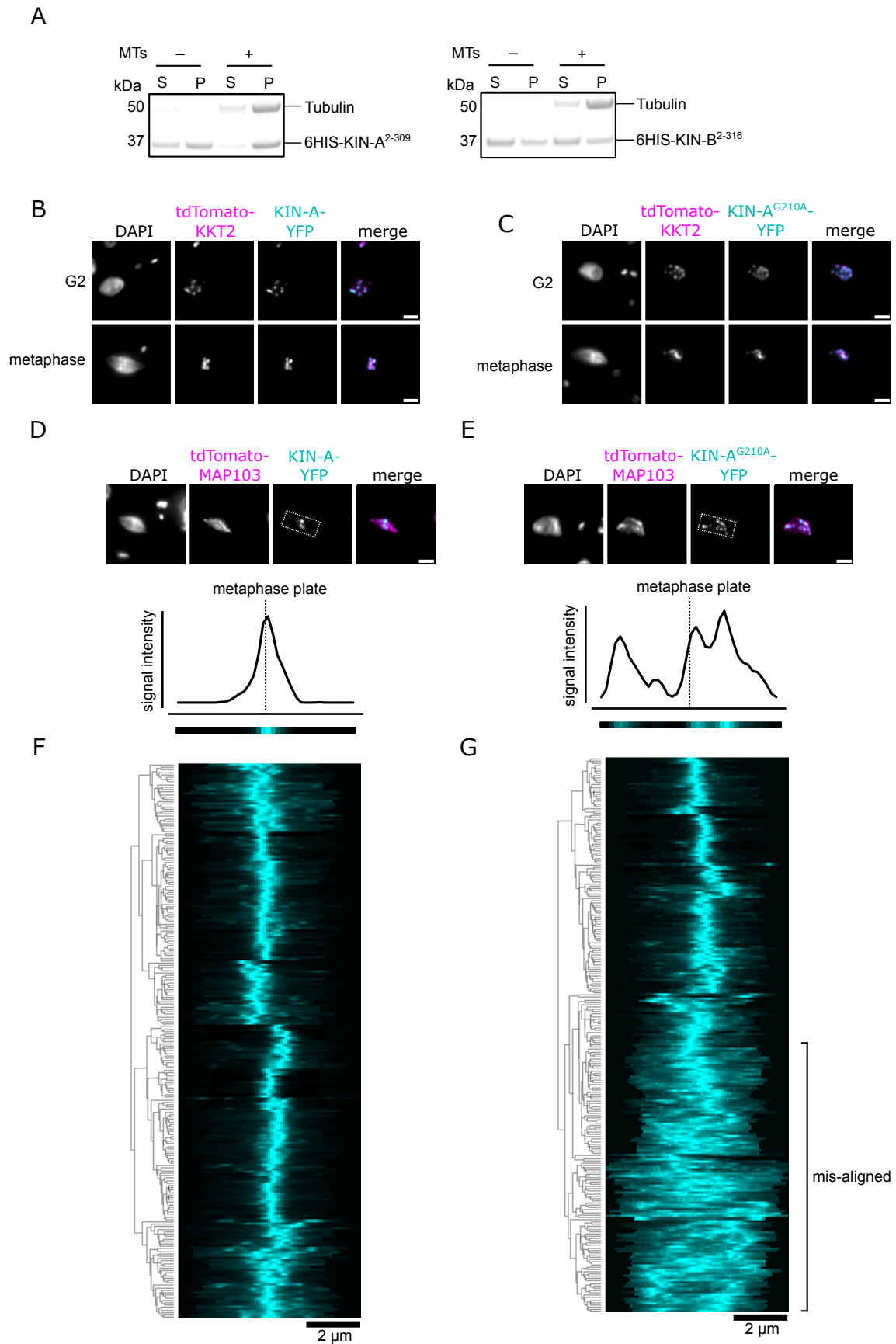


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Figure 3.12. ATPase activity of KIN-A promotes kinetochore alignment at the metaphase plate. (A) Microtubule co-sedimentation assay with 6HIS-KIN-A²⁻³⁰⁹ (left) and 6HIS-KIN-B²⁻³¹⁶ (right). S and P correspond to supernatant and pellet fractions, respectively. Note that both constructs to some extent sedimented even in the absence of microtubules. Hence, lack of microtubule binding for KIN-B may be due to the unstable non-functional protein used in this study. (B) and (C) Representative fluorescence micrographs showing localization of YFP-tagged KIN-A (B) and KIN-A^{G210A} (rigor mutant) (C) in G2 and metaphase. Kinetochores are marked with tdTomato-KKT2. Cell lines: BAP3066, BAP3069. Scale bars, 2 μ m. (D) and (E) Upper: Representative fluorescence micrographs showing localization of YFP-tagged KIN-A (D) and KIN-A^{G210A} (rigor mutant) (E) in metaphase. The mitotic spindle is marked with tdTomato-MAP103. Bounding boxes indicate area used for generation of intensity profiles in FIJI/Image J. Cell lines: BAP3068, BAP3071. Scale bars, 2 μ m. Lower: Example of 1D intensity profiles and corresponding heatmaps from the example cells shown above. (F) and (G) Kinetochore alignment profiles for YFP-tagged KIN-A (F) and KIN-A^{G210A} (G). RNAi was induced with 1 μ g/mL doxycycline for 24 h to deplete the untagged KIN-A allele and cells were treated with 10 μ M MG132 for 4 h prior to fixing to prevent anaphase entry (Hayashi and Akiyoshi, 2018). WPGMA clustering was performed using the Python Seaborn library. Cell lines: BAP3064, BAP3065.

To address whether the ATPase activity of KIN-A is required for central spindle localization of the CPC, I replaced one allele of KIN-A with a C-terminally YFP-tagged G210A ATP hydrolysis-defective rigor mutant (Fig. 3.11A) (Rice et al., 1999) and used an RNAi construct directed against the 3'UTR of KIN-A to deplete the untagged allele. The rigor mutation did not affect recruitment of KIN-A to kinetochores (Fig. 3.12, B and C). However, KIN-A^{G210A}-YFP marked kinetochores were misaligned in ~50% of cells arrested in metaphase, suggesting that ATPase activity of KIN-A promotes chromosome congression to the metaphase plate (Fig. 3.12, D – G). In anaphase, the KIN-A rigor mutant failed to concentrate at the central spindle and instead widely decorated the mitotic spindle, with increased signal observed at spindle poles possibly due to poleward flux (Fig. 3.11, B and C). Expression of the KIN-A^{G210A} rigor mutant prevented Aurora B^{AUK1} from translocating to the central spindle and caused lagging chromosomes (Fig. 3.11, D – F). The KIN-A rigor mutation also slowed cell proliferation even in the presence of wild-type protein and caused accumulation of cells in anaphase (Fig. 3.11, G and H). I conclude that central spindle

localization of the CPC depends on KIN-A motor activity and is required for proper chromosome segregation.

3.3 Discussion

Here, I identified two orphan kinesins, KIN-A and KIN-B, as bona fide CPC proteins in *Trypanosoma brucei*, the causative agent of African sleeping sickness. By employing biochemical, structural, and cell biological approaches, I demonstrated that KIN-A and KIN-B serve as the scaffold for the assembly of the remaining CPC subunits. Kinetochore localization of the CPC depends on the KKT7 – KKT8 complex pathway, with the C-terminal unstructured tail of KIN-A serving as a key interaction partner for the KKT8 complex. Although I provide compelling evidence supporting the interaction of KIN-A with the KKT8 complex based on IP-MS, AF2 modelling and targeted mutations in vivo, this interaction still needs to be validated in vitro using recombinant proteins. In vitro interaction assays could also be used to address the relevance of Aurora B^{AUK1}- and/or CRK3-mediated phosphorylation of the KIN-A C-terminal tail for its binding to the KKT8 complex (see main Discussion).

My data suggests that, unlike other eukaryotes that take advantage of histone modifications for centromere recruitment, trypanosomes rely on kinetochore proteins to recruit the CPC onto kinetochores. However, I cannot exclude that centromere-specific histone marks are present in trypanosomes, which may be recognized by CPC proteins. Moreover, there may be kinetochore proteins outside of the KKT7 – KKT8 complex pathway, which could serve as binding partners for the CPC to synergistically promote robust targeting of the CPC to the inner kinetochore. Future work should also address the importance of CPC subunits other than KIN-A for kinetochore recruitment, in particular CPC2. For example, apart from its N-terminus, which interacts with KIN-A:KIN-B, most of the CPC2 protein appears to be disordered according to AF2. It is conceivable that CPC2 plays a role in the regulation of Aurora B^{AUK1} activity, stability of the CPC and/or targeting to the kinetochore. I did not assess these possibilities in my thesis, as I was unable to efficiently deplete CPC2 using RNAi and CPC2 is poorly conserved in kinetoplastids. Instead, I decided to focus on KIN-A, mainly due to its obvious importance for recruiting the CPC to kinetochores and the fact that KIN-A possesses a kinesin motor domain, which was predicted to be functional. Indeed, I showed that the ATPase activity of KIN-A promotes chromosome alignment in prometaphase and CPC translocation to the central spindle upon anaphase onset. Whether or not KIN-A truly acts as a motor protein and how it cooperates with KIN-B in controlling the localization dynamics of the CPC in trypanosomes, will should be addressed in future research using in vitro systems.

In summary, KIN-A constitutes a unique 'two-in-one' CPC localization module in complex with KIN-B, which directs the CPC to kinetochores (from S phase until metaphase) via its C-terminal tail, and to the central spindle (in anaphase) via its N-terminal kinesin motor domain. My findings highlight the evolutionary diversity of CPC proteins and raise the possibility that kinesins may have served as the original transport vehicles for Aurora B kinases in early eukaryotes.

4. An unconventional regulatory circuitry involving Aurora B controls anaphase onset and error-free chromosome segregation in trypanosomes

4.1 Introduction

Accurate chromosome segregation during mitosis requires that all chromosomes establish stable bi-oriented attachments with the spindle apparatus. Kinetochore form the interface between chromosomes and spindle microtubules and as such are under tight control by complex regulatory circuitry. As part of the chromosomal passenger complex (CPC), the Aurora B kinase plays a central role within this circuitry by destabilizing improper kinetochore-microtubule attachments and relaying the attachment status to the spindle assembly checkpoint, a feedback control system that delays the onset of anaphase by inhibiting the anaphase-promoting complex/cyclosome. Intriguingly, Aurora B is conserved even in kinetoplastids, an evolutionarily divergent group of eukaryotes, whose kinetochores are composed of a unique set of structural and regulatory proteins. Kinetoplastids do not have a canonical spindle checkpoint and it remains unclear how their kinetochores are regulated to ensure the fidelity and timing of chromosome segregation.

In this chapter, I aimed to investigate the role of Aurora B^{AUK1} in regulating chromosome segregation and cell cycle progression in *T. brucei* using cell biological and biochemical approaches.

4.2 Results

4.2.1 Aurora B^{AUK1} controls the metaphase-anaphase transition in trypanosomes.

As reported previously (Jones et al., 2014; Tu et al., 2006; Ballmer and Akiyoshi, 2024a), siRNA-mediated depletion of Aurora B^{AUK1} caused a pronounced cell cycle defect, with cells arresting in G2/M phase after 16 h (Fig. 4.1, A and B). These cells exhibited elongated and aberrantly shaped nuclei (Fig. 4.1C) that were positive for cyclin B^{CYC6} (data not shown), indicating that they were unable to progress into anaphase. The distance between the segregated kinetoplasts ('K') can be used to estimate the progression of cytoplasmic/flagellar

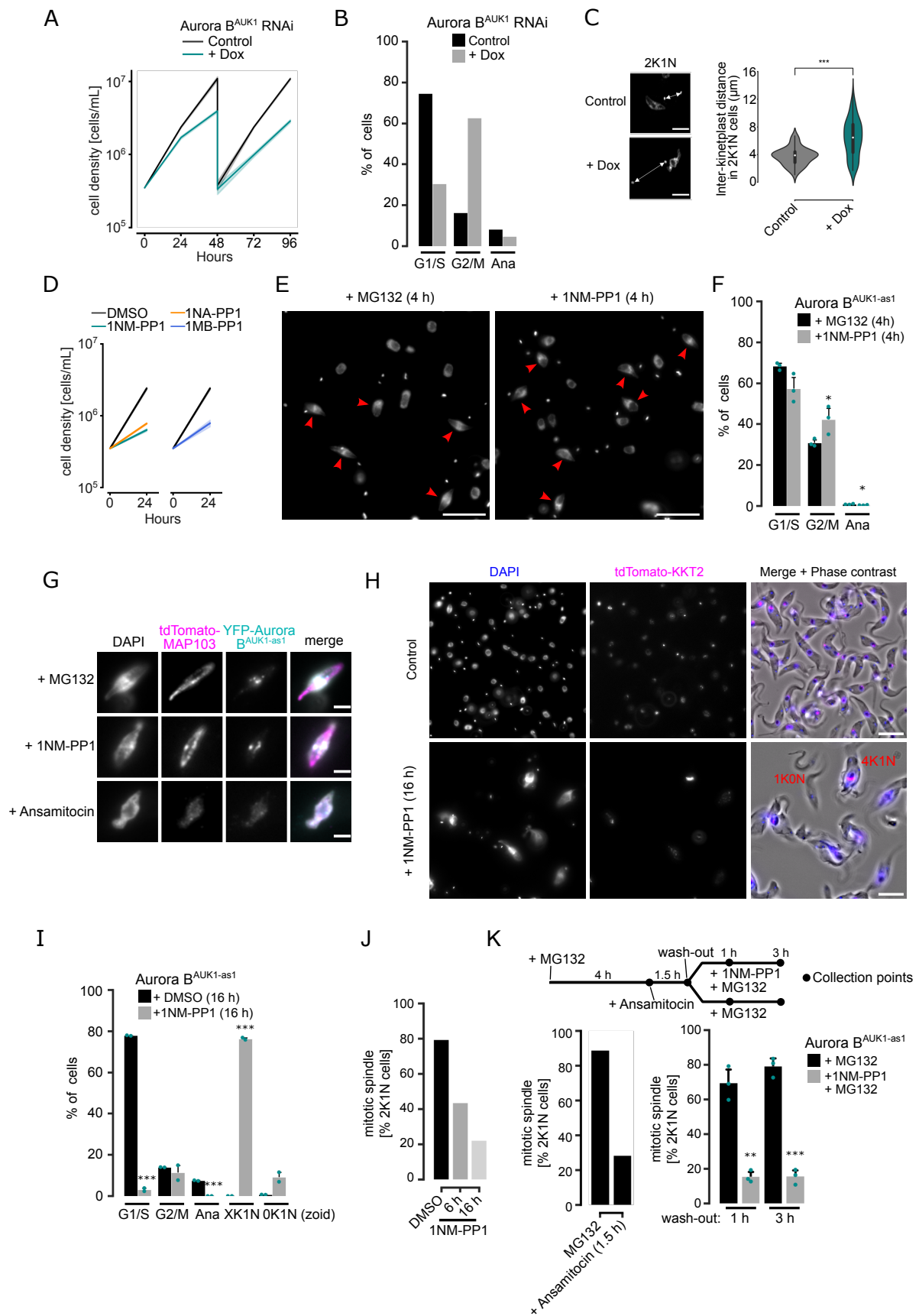


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Figure 4.1. Aurora B^{AUK1} activity is required for mitotic exit and spindle stability. (A) Growth curves upon RNAi-mediated knockdown of Aurora B^{AUK1}. RNAi was induced with 1 µg/mL doxycycline and cultures were diluted at day 2. Data are presented as the mean $\pm$ SD of three replicates. Cell line: BAP941. (B) Cell cycle profile upon knockdown of Aurora B^{AUK1}. RNAi was induced with 1 µg/mL doxycycline and cells were fixed at 16 h. A minimum of 350 cells per condition were quantified. (C) Quantification of the distance between kinetoplasts in 2K1N cells upon depletion of Aurora B^{AUK1} for 16 h. A minimum of 50 cells per condition were quantified. Cell line: BAP2129. *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001 (Mann-Whitney U). (D) Growth curves upon treatment of Aurora B^{AUK1-as1} cells with 2 µM 1NM-PP1, 1NA-PP1 or 1MB-PP1. Data are presented as the mean $\pm$ SD of three replicates. Cell line: BAP2198. (E) Representative fluorescence micrographs showing cell cycle distribution upon treatment of Aurora B^{AUK1-as1} cells with 10 µM MG132 or 2 µM 1NM-PP1 for 4 h. DNA was stained with DAPI. Red arrowheads indicate 2K1N cells. Cell line: BAP2357. Scale bars, 10 µm. (F) Cell cycle profile for indicated conditions as in (E). All graphs depict the means (bar) $\pm$ SD of three replicates (dots). A minimum of 500 cells per replicate were quantified. *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001 (two-sided, unpaired t-test). (G) Representative fluorescence micrographs showing the localization of the spindle marker tdTomato-MAP103 and YFP-Aurora B^{AUK1} upon treatment of Aurora B^{AUK1-as1} cells with 10 µM MG132, 2 µM 1NM-PP1 or 5 nM ansamitocin for 4 h. Cell line: BAP2281. Scale bars, 2 µm. (H) Representative fluorescence micrographs showing an overview of Aurora B^{AUK1-as1} cells treated with DMSO (Control) or 2 µM 1NM-PP1 for 16 h. Examples of a 1K0N (zoid) and a 4K1N cell are labelled in red. Cell lines: BAP2924. Scale bars, 10 µm. (I) Cell cycle profile for indicated conditions as in (H). All graphs depict the means (bar) $\pm$ SD of at least two replicates (dots). A minimum of 200 cells per condition were quantified. *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001 (two-sided, unpaired t-test). (J) Quantification of 2K1N Aurora B^{AUK1-as1} cells that possess a mitotic spindle upon treatment with DMSO or 2 µM 1NM-PP1 for 6 h and 16 h. A minimum of 50 cells per condition were quantified. (K) Upper: Schematic describing experimental design. Aurora B^{AUK1-as1} cells were treated with 10 µM MG132 for 4 h to enrich for cells in metaphase, followed by a 1.5 h treatment with 5 nM ansamitocin to depolymerize the mitotic spindle. Ansamitocin was then washed-out, and cells were allowed to recover and re-form a spindle with or without 2 µM 1NM-PP1. Collection points are indicated with black dots. Lower: Quantification of 2K1N Aurora B^{AUK1-as1} cells that possess a mitotic spindle under indicated conditions. All graphs depict the means (bar) $\pm$ SD of three replicates (dots). A minimum of 90 cells per replicate were quantified. *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001 (two-sided, unpaired t-test). SD of three replicates. Cell line: BAP941. (B) Cell cycle profile upon knockdown of Aurora B^{AUK1}.

division cycle, which is uncoupled from nuclear division ('N') in *T. brucei* (Hayashi and Akiyoshi, 2018; Ploubidou et al., 1999; Robinson et al., 1995). The average inter-kinetoplast distance in 2K1N cells significantly increased upon knock-down of Aurora B^{AUK1} (Fig. 4.1C), consistent with a delay in the metaphase-anaphase transition in the nucleus.

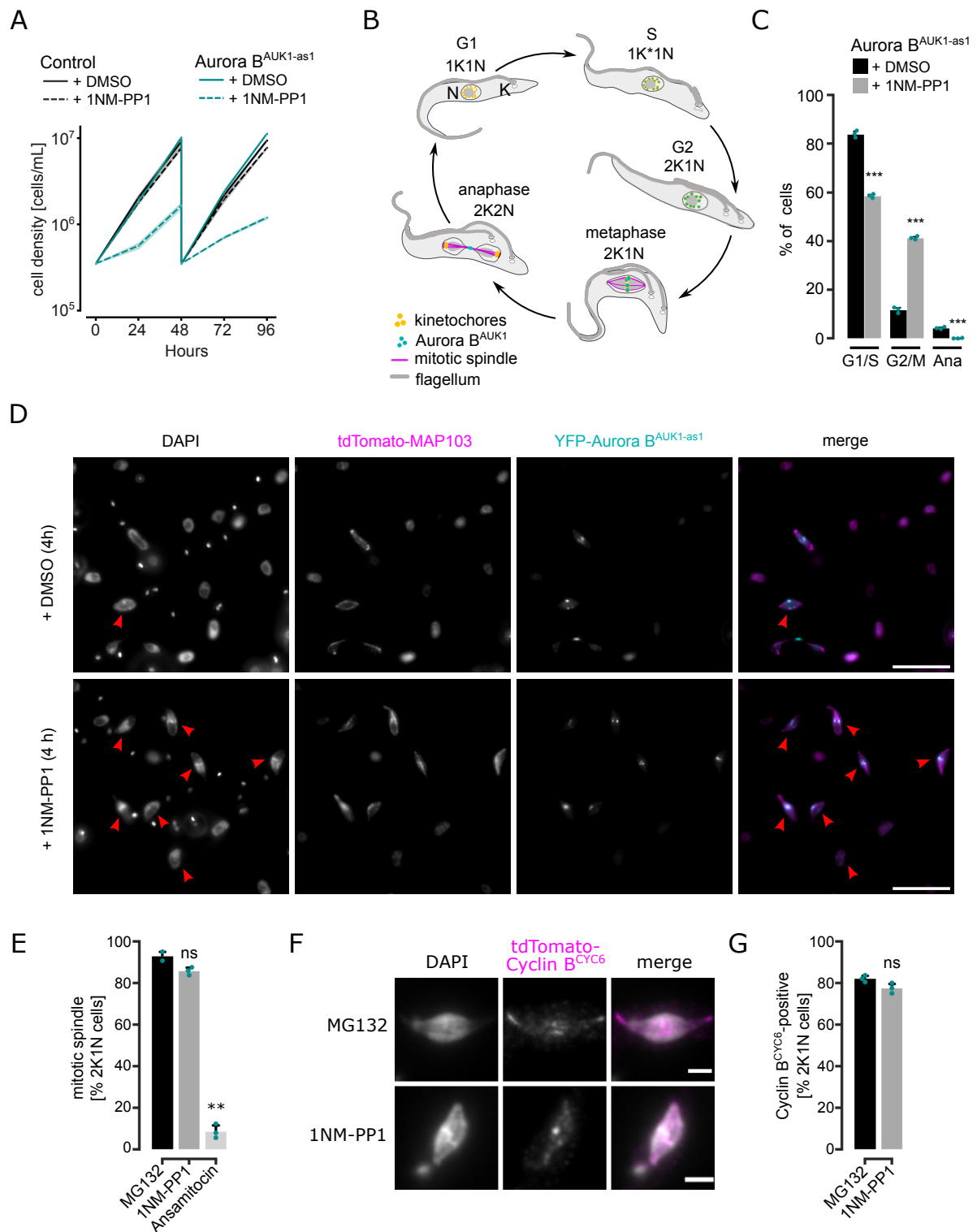


Figure 4.2 (legend on following page)

Figure 4.2. Inhibition of Aurora B^{AUK1} using an analogue-sensitive approach arrests cells in metaphase. (A) Growth curves upon treatment of control and Aurora B^{AUK1-as1} cells with 2 μ M 1NM-PP1 or an equal volume of DMSO. The control cell line is heterozygous for the Aurora B^{AUK1-as1} allele. Cultures were diluted at day 2. Data are presented as the mean $\pm$ SD of three replicates. Cell lines: BAP2169, BAP2198. (B) Cartoon depicting the kinetoplast (K) / nucleus (N) configuration throughout the cell cycle in procyclic *T. brucei*, with K* denoting an elongated kinetoplast (adapted from (Ballmer and Akiyoshi, 2024a)). The kinetoplast is an organelle found uniquely in kinetoplastids, which contains the mitochondrial DNA. It replicates and segregates prior to nuclear division, so the KN configuration serves as a cell cycle marker (Woodward and Gull, 1990; Siegel et al., 2008). Aurora B^{AUK1} localizes to kinetochores from S phase until metaphase and translocates to the central spindle in anaphase. (C) Cell cycle profile of Aurora B^{AUK1-as1} cells upon treatment with 2 μ M 1NM-PP1 or an equal volume of DMSO for 4 h. All graphs depict the means (bar) $\pm$ SD of three replicates. A minimum of 450 cells per replicate were quantified. Cell line: BAP2281. (D) Representative fluorescence micrographs showing YFP-Aurora B^{AUK1-as1} cells expressing tdTomato-MAP103 (spindle marker) treated with 2 μ M 1NM-PP1 or an equal volume of DMSO for 4 h. DNA was stained with DAPI. Red arrowheads indicate 2K1N cells. Cell line: BAP2281. Scale bars, 10 μ m. (E) Quantification of 2K1N Aurora B^{AUK1-as1} cells that possess a mitotic spindle (marked by tdTomato-MAP103) upon treatment with 10 μ M MG132, 2 μ M 1NM-PP1 or 5 nM ansamitocin for 4 h. All graphs depict the means (bar) $\pm$ SD of at least two replicates (shown as dots). A minimum of 40 cells per replicate were quantified. (F) Representative fluorescence micrographs showing the localization of tdTomato-cyclin B^{CYC6} in Aurora B^{AUK1-as1} cells arrested in metaphase upon treatment with 2 μ M 1NM-PP1 or 10 μ M MG132 for 4 h. Scale bars, 2 μ m. (G) Quantification of Aurora B^{AUK1-as1} 2K1N cells that are positive for tdTomato-cyclin B^{CYC6} upon treatment with 2 μ M 1NM-PP1 or 10 μ M MG132 for 4 h. All graphs depict the means (bar) $\pm$ SD of two replicates (shown as dots). A minimum of 35 cells per replicate were quantified. *P < 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001 (two-sided, unpaired t-test).

To test whether the kinase activity of Aurora B^{AUK1} regulates entry into anaphase and to implement a more rapid loss-of-function system, I generated cell lines harboring analogue-sensitive Aurora B^{AUK1} alleles (Aurora B^{AUK1-as1}) (Bishop et al., 2000). Treatment of Aurora B^{AUK1-as1} cells with 2 μ M of ATP analogues (1NM-PP1, 1NA-PP1, or 1MB-PP1) resulted in a prominent growth defect (Figs. 4.2A and 4.1D). Remarkably, after just 4 h of treatment with 1NM-PP1 (corresponding to half a cell cycle), 40% of cells were in a 2K1N state (Fig. 4.2, C

and D), which is comparable to the cell cycle arrest observed upon treatment with the proteasome inhibitor MG132 (Fig. 4.1, E and F) or expression of non-degradable cyclin B^{CYC6} (Hayashi and Akiyoshi, 2018). Morphologically, these cells possessed elongated nuclei with a mitotic spindle (marked by tdTomato-MAP103) and were positive for cyclin B^{CYC6}, indicative of a metaphase arrest (Figs. 4.2, D – G, and 4.1G). After 16 h (~ two cell cycles) of Aurora B^{AUK1} inhibition, most cells had reached a 4K1N state (Fig. 4.1, H and I), consistent with two rounds of kinetoplastid replication having occurred in the absence of nuclear division. Thus, inhibition of Aurora B^{AUK1} kinase activity using our analogue-sensitive system efficiently halts anaphase entry within the first cell cycle. These results are consistent with a previous study showing cell cycle arrest of *T. brucei* upon treatment with high doses of a small-molecule Aurora kinase inhibitor (Li et al., 2009).

Even though spindle assembly was observed upon 4 h inhibition of Aurora B^{AUK1} activity, I noticed that the fraction of metaphase cells with an intact spindle progressively declined upon prolonged 1NM-PP1 treatment (Fig. 4.1J). To test whether Aurora B^{AUK1} is required for spindle stability, I arrested cells in metaphase by MG132 treatment followed by a brief pulse of ansamitocin to depolymerize the mitotic spindle, and then monitored spindle reformation in the presence of 1NM-PP1 or MG132 as a control (Fig. 4.1K). We found that spindle reformation was inefficient in 1NM-PP1 treated cells, suggesting that Aurora B^{AUK1} activity is important for spindle assembly and/or stability. This could explain why the mitotic spindle was previously reported to be lost in trypanosomes depleted for CPC components for 2 days (Li et al., 2008a). Together, these data show that Aurora B^{AUK1} activity controls the metaphase-to-anaphase transition in trypanosomes and is required for preserving the integrity of the mitotic spindle.

4.2.2 Aurora B^{AUK1} activity is required for the establishment of stable KT-MT attachments.

I next aimed to examine the bi-orientation status after inhibiting Aurora B^{AUK1} activity. Kinetochore periphery proteins (e.g. KKIP2, KKIP3) appear as two dots in metaphase cells even under a conventional microscope (Brusini et al., 2021). I therefore reasoned that they may serve as a bi-orientation marker in trypanosomes. Indeed, the number of metaphase kinetochores double positive for KKIP2 was significantly reduced when spindle microtubules were disrupted or Aurora B^{AUK1} activity was inhibited for 4 h (Fig. 4.3, A and B). Similar defects were observed even after 1 h inhibition of Aurora B^{AUK1}. Furthermore, the distance between KKIP2 foci labelling bi-oriented kinetochores was markedly decreased in 1NM-PP1 treated cells (Fig. 4.3C), indicative of reduced level of tension across the inter-sister kinetochore axis.

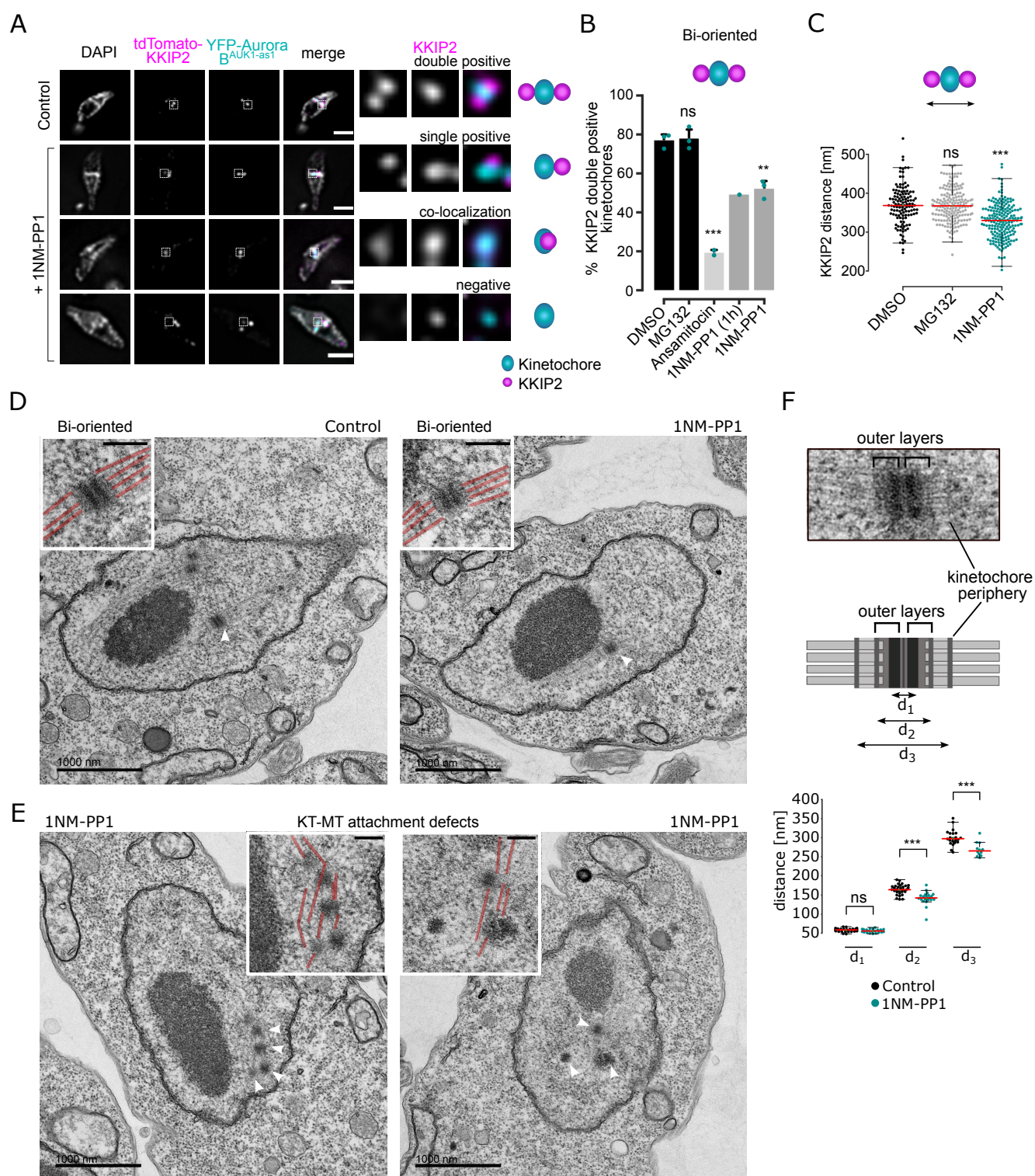


Figure 4.3 (figure legend on following page)

Figure 4.3. Aurora B^{AUK1} activity is required for the establishment of stable KT-MT attachments. (A) Representative fluorescence micrographs showing the configuration of tdTomato-KKIP2 (kinetochore periphery, magenta) and YFP-Aurora B^{AUK1} (inner kinetochore, cyan) in Aurora B^{AUK1-as1} cells arrested in metaphase upon treatment with 2 μ M 1NM-PP1 or 10 μ M MG132 (Control) for 4 h, with a schematic guide for each configuration. Note that the kinetochore periphery component KKIP2 undergoes displacement upon bi-orientation and forms two foci across the inter-sister kinetochore axis ('double positive'). The insets show the magnification of the boxed region. Scale bars, 2 μ m. Cell line: BAP2312. (B) Quantification of bi-oriented kinetochores (as defined in (A)) in Aurora B^{AUK1-as1} cells in metaphase. Cells were treated with 2 μ M 1NM-PP1, 10 μ M MG132, 5 nM ansamitocin or DMSO for 4 h unless otherwise stated. *P < 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001 (two-sided, unpaired t-test). (C) Quantification of the distance between tdTomato-KKIP2 foci at bi-oriented kinetochores in Aurora B^{AUK1-as1} cells arrested in metaphase upon treatment with DMSO (black), 10 μ M MG132 (grey) or 2 μ M 1NM-PP1 (cyan) for 4 h. At least 120 kinetochores (shown as dots) from three replicates were analysed per condition. The median is indicated in red. *P < 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001 (Mann-Whitney U). (D) and (E) Representative transmission electron micrographs showing bi-oriented (D) and improperly attached (E) kinetochores in Aurora B^{AUK1-as1} cells arrested in metaphase upon treatment with 10 μ M MG132 or 2 μ M 1NM-PP1 for 4 h. Scale bars, 1 μ m. White arrowheads indicate the kinetochores shown in magnified insets (scale bars, 200 nm). Microtubules are marked in red in insets. Cell line: BAP2198. (F) Quantification of the distance between d₁, d₂ and d₃ at bi-oriented kinetochores in Aurora B^{AUK1-as1} cells arrested in metaphase upon treatment with 10 μ M MG132 (Control, black) or 2 μ M 1NM-PP1 (cyan) for 4 h. At least 20 kinetochores (shown as dots) from two replicates were analysed per condition. The median is indicated in red. *P < 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001 (Mann-Whitney U).

These results show that Aurora B^{AUK1} activity is important for chromosome bi-orientation in trypanosomes.

YFP-tagged Aurora B^{AUK1-as1} localized at kinetochores in the presence of 1NM-PP1, suggesting that its kinase activity is not essential for targeting the CPC at the centromeric region (Fig. 4.3D). Moreover, Aurora B^{AUK1} inhibition had only a moderate impact on the recruitment of most KKT proteins (Fig. 4.4A). In general, inner kinetochore proteins showed a modest increase in signal intensity at metaphase kinetochores, whereas outer kinetochore and kinetochore periphery proteins were largely unaffected in 1NM-PP1-treated cells, except for KKIP3 whose levels were significantly reduced.

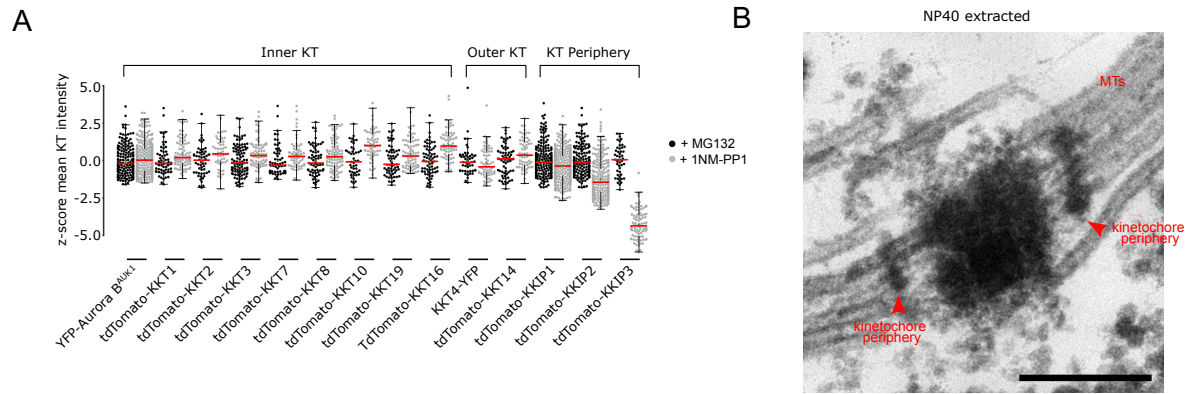


Figure 4.4. Aurora B^{AUK1} activity not required for recruitment of inner and outer kinetochore proteins. (A) Quantification of mean signal intensities presented as z-scores of indicated inner and outer kinetochore and kinetochore periphery components in Aurora B^{AUK1-as1} cells arrested in metaphase by treatment with 10 μ M MG132 (Control) or 2 μ M 1NM-PP1 for 4 h. Kinetochores were segmented in ImageJ/FIJI using a custom macro (See Methods). At least 45 cells (shown as dots) were analysed per condition. The median is indicated in red *P < 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001 (Mann-Whitney U). (B) Transmission electron micrograph of an NP40-extracted sample fixed with a combination of glutaraldehyde and tannic acid, which improves contrast of certain subcellular structures such as microtubules (Ogbadoyi et al., 2000; Fujiwara and Linck, 1982). Kinetochore peripheries are indicated with red arrowheads. Scale bar, 200 nm.

To gain further insights into the ultrastructure of kinetochores and the mode of KT-MT attachments upon Aurora B^{AUK1} inhibition, we performed transmission electron microscopy (TEM) on glutaraldehyde-fixed samples. As described previously (Ogbadoyi et al., 2000), metaphase kinetochores in *T. brucei* appear as electron-dense plaques that contain two ‘outer layers’ (Fig. 4.3, D and F). Spindle microtubules appear to terminate in these outer layers, therefore possibly representing the outer kinetochore in trypanosomes. In addition, another electron-dense structure was detected distal to the outer kinetochore, which was particularly visible in detergent-extracted samples fixed with a combination of glutaraldehyde and tannic acid, which improves contrast of certain subcellular structures such as microtubules (Fig. 4.4B) (Ogbadoyi et al., 2000; Fujiwara and Linck, 1982). Because the position of this structure corresponds to that of the N-terminus of kinetochore periphery proteins, I propose to call it the ‘kinetochore periphery’ (Fig. 4.3F).

I detected at least one bi-oriented kinetochore in 27 out of 36 (75%) imaged metaphase nuclei, defined by their elongated spindle-like shape, in the MG132 treatment condition. In contrast, only 14 out of 39 (~36%) metaphase nuclei had clearly identifiable bi-oriented kinetochores in 1NM-PP1-treated samples. Fig. 4.3E shows two examples of apparent KT-

MT attachment defects upon inhibition of Aurora B^{AUK1}. Moreover, I found a moderate reduction in the distance between the edges of outer layers (d2) and that between the kinetochore peripheries (d3) (Fig. 4.3F) on bi-oriented kinetochores in Aurora B^{AUK1}-inhibited cells, suggesting that tension is indeed reduced across the inter-sister kinetochore axis. In summary, I conclude that Aurora B^{AUK1} activity is required for the establishment of stable bi-oriented KT-MT attachments.

4.2.3 Profiling Aurora B^{AUK1} targets at the kinetoplastid kinetochore

I next aimed to identify CPC targets at the trypanosome kinetochore. Substrates of Aurora B kinases in other eukaryotes typically conform to the consensus [RK]-[RK]-X-[ST] (where X is any residue) (Meraldi et al., 2004). The substrate-recognition motif of *T. brucei* Aurora B^{AUK1} was determined by performing a positional scanning peptide array analysis (Hutti et al., 2004) using recombinant Aurora B^{AUK1} bound to its activator INCENP^{CPC1} (Figs. 4.5, A – C, and 4.6A). The consensus motif for trypanosome Aurora B^{AUK1} closely matched that of its homologs in other eukaryotes: Selectivity for basic residues N-terminal to the phosphorylation site (with a particularly strong preference for Arg at the -2 position) and a preference for Ser over Thr as the phosphorylation site residue. There also appears to be a unique, though modest, preference for basic residues (Arg, Lys, His) at the +2 position, which has not been observed in any of the human Aurora kinases (Johnson et al., 2023). Moreover, Aurora B^{AUK1} strongly deselected peptides containing Pro at position +1.

I next performed *in vitro* kinase assays using active or kinase-dead (K58R, (Li and Wang, 2006)) Aurora B^{AUK1}/INCENP^{CPC1} complexes on recombinant CPC or kinetochore proteins as substrates (Figs. 4.5, D and E, and 4.6B). Among CPC components, we detected strong auto-phosphorylation of Aurora B^{AUK1} and moderate phosphorylation of INCENP^{CPC1} and CPC2. Interestingly, the C-terminal unstructured tail of KIN-A, which directs kinetochore targeting of the CPC in *T. brucei* (Ballmer and Akiyoshi, 2024a), was heavily phosphorylated (Fig 4.6B), raising the possibility that Aurora B^{AUK1} activity may finetune the affinity of KIN-A for its kinetochore receptor(s). The motor domain of KIN-A was weakly phosphorylated. By contrast, the motor domain of KIN-B was not phosphorylated by Aurora B^{AUK1}.

Aurora B^{AUK1} also phosphorylated various KKT proteins *in vitro* (Fig. 4.5, D – F), including the inner kinetochore members KKT1, KKT7 and KKT8. KKT7 and KKT8 are components of the KKT7-KKT8 complex, which serve as the main kinetochore receptor of the CPC in *T. brucei* (Ballmer and Akiyoshi, 2024a). The outer kinetochore protein KKT4, the only microtubule tip-coupling protein so far identified at the kinetoplastid kinetochore (Llauró et al., 2018), was also phosphorylated. Among the KKT4 fragments tested, KKT4¹¹⁵⁻³⁴³ was most strongly phosphorylated by Aurora B^{AUK1} (Fig. 4.6C). The fact that KKT4¹¹⁵⁻³⁴³ contains the microtubule-

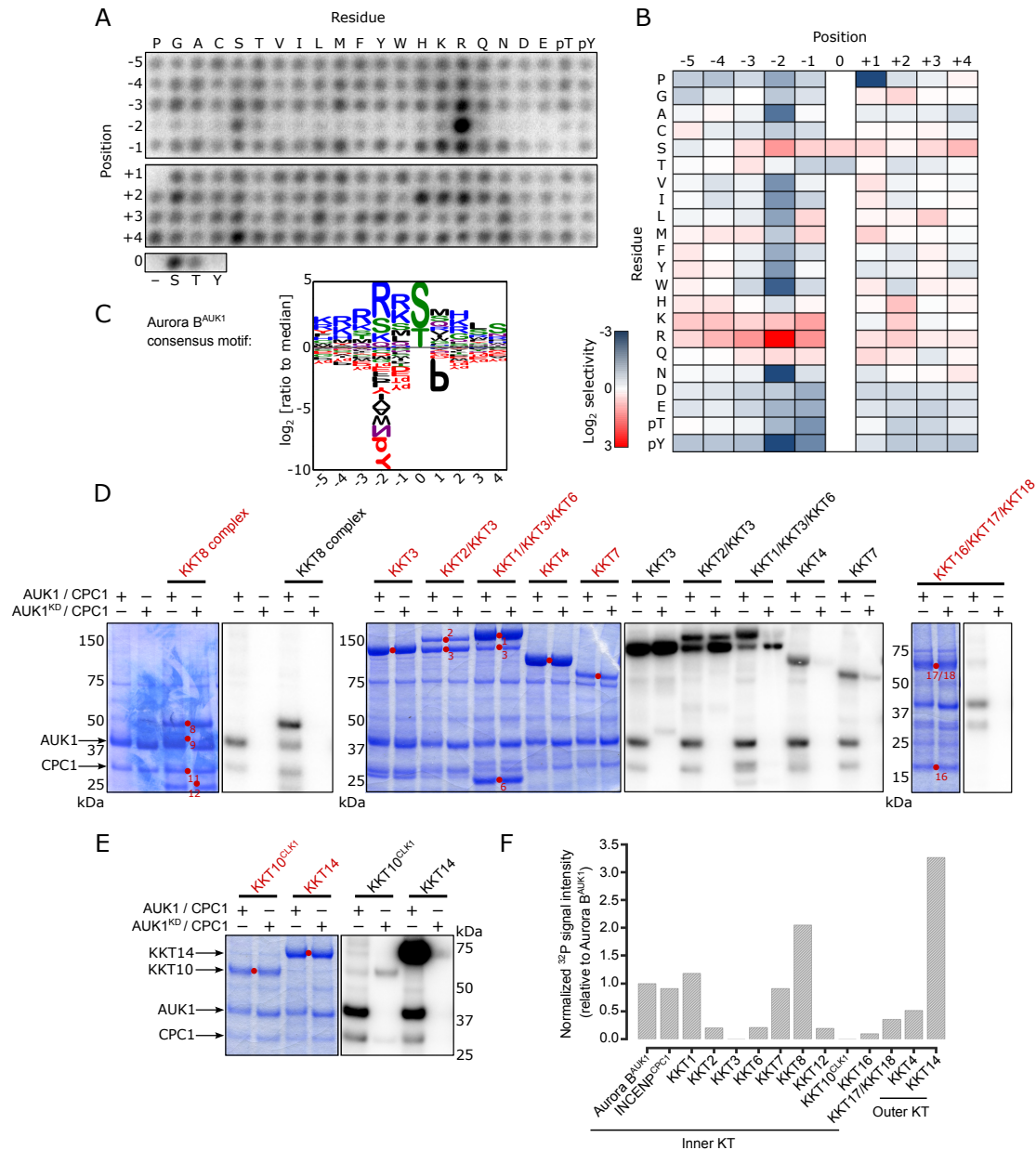


Figure 4.5. Profiling Aurora B^{AUK1} in vitro substrates. (A) Positional scanning peptide array image of recombinant 3FLAG-Aurora B^{AUK1}/INCENP^{CPC1}. Darker spots indicate preferred residues. The second run is shown in Figure S3. (B) Quantification of (A). Spot intensities were normalized so that the average value within a position was equal to one. The heatmap shows the log₂ transformed data (averaged from the two separate runs) with positive selections shown in red and negative selections shown in blue. (C) Aurora B^{AUK1} substrate motif logo. (D) and (E) Aurora B^{AUK1} in vitro kinase assay using the indicated recombinant kinetochore proteins as substrates. The left panel (input) shows the Coomassie Brilliant Blue staining. Substrates are marked with red dots. Phosphorylation was detected by autoradiography. (F) Normalized ³²P signal intensities for indicated proteins relative to Aurora B^{AUK1} auto-phosphorylation.

Figure 4.6. In vitro phosphorylation of CPC and KKT4 fragments by Aurora B^{AUK1}. (A) Positional scanning peptide array image of recombinant 3FLAG-Aurora B^{AUK1}/INCENP^{CPC1}. Darker spots indicate preferred residues. (B) and (C) Aurora B^{AUK1} in vitro kinase assay using the indicated recombinant CPC (B) and KKT4 (C) constructs as substrates. The left panel (input) shows the Coomassie Brilliant Blue staining. Substrates are marked with red dots. Phosphorylation was detected by autoradiography.

Figure 4.6. In vitro phosphorylation of CPC and KKT4 fragments by Aurora B^{AUK1}. (A) Positional scanning peptide array image of recombinant 3FLAG-Aurora B^{AUK1}/INCENP^{CPC1}. Darker spots indicate preferred residues. (B) and (C) Aurora B^{AUK1} in vitro kinase assay using the indicated recombinant CPC (B) and KKT4 (C) constructs as substrates. The left panel (input) shows the Coomassie Brilliant Blue staining. Substrates are marked with red dots. Phosphorylation was detected by autoradiography.

4.2.4 Phosphorylation of KKT14 by Aurora B^{AUK1} promotes anaphase entry.

I next tested the possibility that KKT14 is a key substrate of Aurora B^{AUK1} that controls the metaphase-to-anaphase transition and/or chromosome segregation in trypanosomes. Interestingly, KKT14 depletion partially rescued the cell cycle arrest caused by Aurora B^{AUK1} inhibition, with some cells progressing into anaphase (Fig. 4.7, A and B). Nevertheless, these

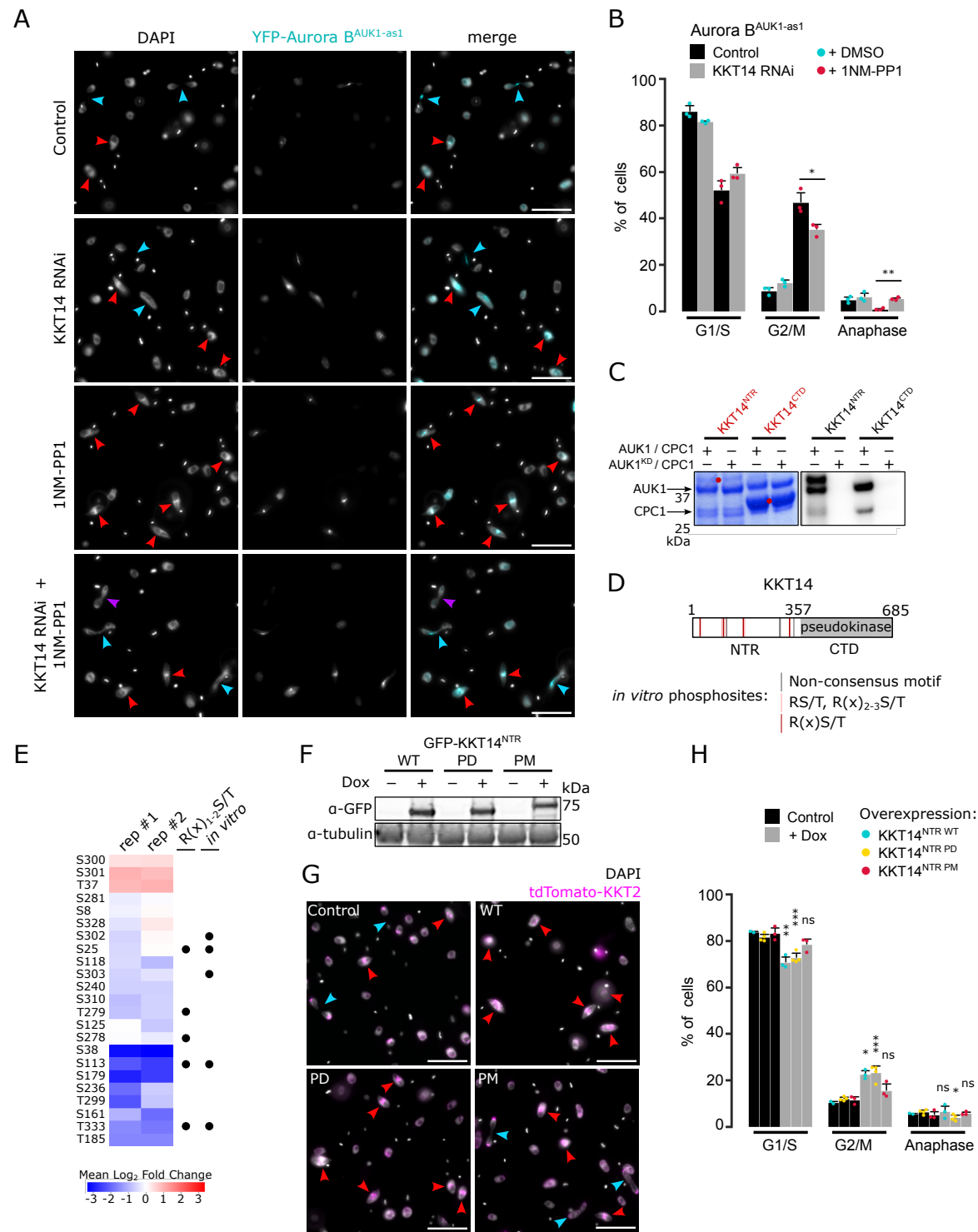


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Figure 4.7. Phosphorylation of KKT14 by Aurora B^{AUK1} regulates anaphase entry. (A) Representative fluorescence micrographs showing Aurora B^{AUK1-as1} cells treated with 2 μ M 1NM-PP1 or an equal volume of DMSO for 4 h. RNAi-mediated knockdown of KKT14 was induced with 1 μ g/mL doxycycline for 20 h. DNA was stained with DAPI. Arrowheads indicate 2K1N (red) and 2K2N (light blue) cells. Purple arrowhead indicates a 2K2N cell that is negative for Aurora B^{AUK1}, suggesting a re-entry into G1 despite failure to complete nuclear division. Cell line: BAP2469. Scale bars, 10 μ m. (B) Cell cycle profile for indicated conditions as in (A). All graphs depict the means (bar) $\pm$ SD of three replicates. A minimum of 300 cells per replicate were quantified. Cell line: BAP2469. (C) Aurora B^{AUK1} in vitro kinase assay using the indicated recombinant KKT14 constructs as substrates. The left panel (input) shows the Coomassie Brilliant Blue staining. Substrates are marked with red dots. Phosphorylation was detected by autoradiography. (D) Schematic representation of KKT14 showing NTR and C-terminal pseudokinase domain. NTR phosphorylation sites detected by mass spectrometry are indicated by lines (grey: Non-consensus motif, orange: RS/T or R(x)₂₋₃S/T, red: R(x)S/T). Following sites were phosphorylated in vitro by 3FLAG-Aurora B^{AUK1}/INCENP^{CPC1}: S25, T104, S107, S113, T115, S173/S174, S302/S303, T333, T348. (E) Quantification of phospho-sites detected in IP-MS analysis of GFP-KKT14^{NTR} from Aurora B^{AUK1-as1} cells treated 2 μ M 1NM-PP1 or 10 μ M MG132 as a control for 4 h (two replicates each, rep #1 and #2). The heatmap shows the log2 fold change in the 1NM-PP1-treated samples compared to the control, with positive values shown in red and negative values shown in blue. Black dots indicate whether phospho-sites match the R(x)₁₋₂S/T consensus motif and whether they were detected in vitro. Cell line: BAP2505. (F) Western blot showing protein levels of indicated GFP-KKT14^{NTR} constructs (WT: Wild-type, PD: Phosphodeficient, PM: Phosphomimetic), induced with 1 μ g/mL doxycycline for 24 h. Tubulin was used as a loading control. Cell lines: BAP2924, BAP2925, BAP2928. (G) Representative fluorescence micrographs showing cell cycle distribution upon overexpression of indicated KKT14^{NTR} constructs, induced with 1 μ g/mL doxycycline for 24 h. TdTomato-KKT2 marks kinetochores and DNA was stained with DAPI. Arrowheads indicate 2K1N (red) and 2K2N (light blue) cells. Cell lines: BAP2924, BAP2925, BAP2928. Scale bars, 10 μ m. (H) Cell cycle profile for indicated conditions as in (G). All graphs depict the means (bar) $\pm$ SD of three replicates. A minimum of 300 cells per replicate were quantified. Cell lines: BAP2924, BAP2925, BAP2928. *P < 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001 (two-sided, unpaired t-test).

anaphase cells displayed lagging chromosomes, and some were negative for Aurora B^{AUK1} (Fig. 4.7A), suggesting that they re-entered G1 despite being unable to complete mitosis.

KKT14 consists of an N-terminal region (NTR) harboring an ABBA motif and a C-terminal pseudokinase domain (Ballmer et al., 2024a). I found that Aurora B^{AUK1} strongly phosphorylated the NTR (KKT14²⁻³⁵⁷) but not the pseudokinase domain (KKT14³⁵⁸⁻⁶⁸⁵) *in vitro* (Fig. 4.7C). 7 out of 11 phospho-sites identified by mass spectrometry (MS) match the consensus motif for Aurora B^{AUK1} (e.g. Arg in position -1, -2 or -3) (Fig. 4.7D). To test whether these sites were also phosphorylated *in vivo*, I performed immunoprecipitation coupled to MS analysis (IP-MS) of GFP-KKT14^{NTR} in Aurora B^{AUK1-as1} cells treated with 1NM-PP1 or MG132 as a control. Many phospho-sites in the NTR were downregulated upon Aurora B^{AUK1} inhibition, including three residues (T333, S25 and S113) which were also phosphorylated *in vitro* and matched the consensus motif for Aurora B^{AUK1} (Fig. 4.7E).

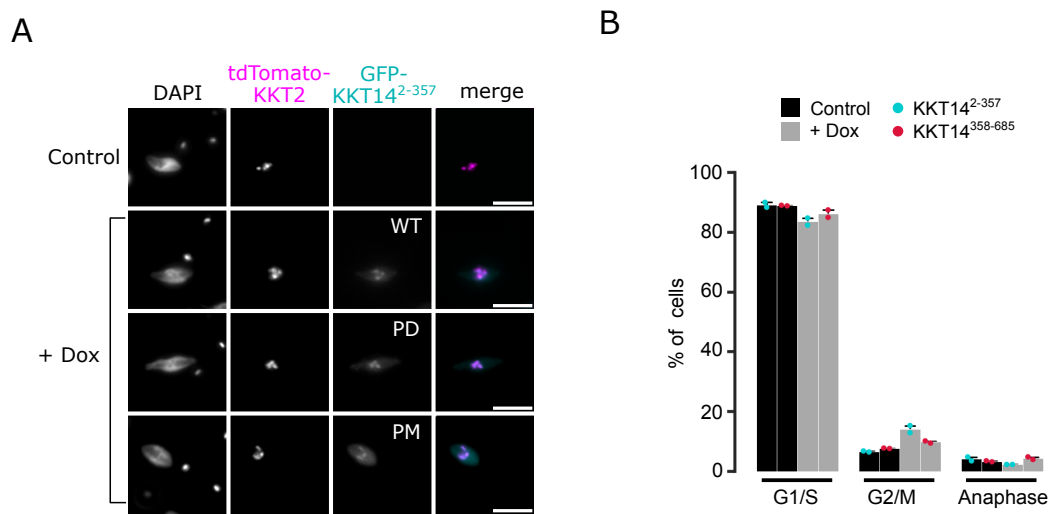


Figure 4.8. Ectopic expression of KKT14 constructs. (A) Representative fluorescence micrographs showing localization of indicated GFP-KKT14^{NTR} constructs (WT: Wild-type, PD: Phosphodeficient, PM: Phosphomimetic) and tdTomato-KKT2 (kinetochore marker). Expression of fusion proteins was induced with 1 μg/mL doxycycline and cells were fixed at 24 h. Cell lines: BAP2924, BAP2925, BAP2928. Scale bars, 2 μm. (B) Cell cycle profiles upon overexpression of GFP-KKT14²⁻³⁵⁷ and GFP-KKT14³⁵⁸⁻⁶⁸⁵. Cell lines: BAP2386, BAP2387. All graphs depict the means (bar) ± SD of two replicates (dots). A minimum of 350 cells per replicate were quantified. *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001 (two-sided, unpaired t-test).

To examine the importance of the CPC-dependent phosphorylation of KKT14, I overexpressed wild-type (WT), phosphodeficient (PD; S/T to A) or phosphomimetic (PM; S/T to D/E) KKT14^{NTR} constructs fused with GFP and monitored cell cycle distribution. All three constructs were expressed at similar levels (Fig. 4.7F) and localized to kinetochores (Fig.

4.8A). Intriguingly, overexpression of WT and PD constructs caused a significant increase in 2K1N cells (Fig. 4.7, G and H), and thus partially phenocopied Aurora B^{AUK1} inhibition. In contrast, expression of KKT14^{NTR} PM or the C-terminal pseudokinase domain did not delay anaphase onset (Figs. 4.7, G and H, and 4.8B). Taken together, these data suggest that phosphorylation of the KKT14 NTR by the CPC modulates the timing of anaphase entry in trypanosomes.

4.2.5 Localization of Aurora B^{AUK1} to the inner kinetochore is important for error-free chromosome segregation.

The prevailing model explaining how Aurora B recognizes and corrects improper KT-MT attachments relies on proximity between outer kinetochore proteins and centromeric pools of Aurora B, which become spatially separated as tension builds up across the inter-sister kinetochore axis in properly bi-oriented kinetochores (Liu et al., 2009; Tanaka et al., 2002). However, this spatial separation model has been challenged by the observations that the inner centromere localization of Aurora B is dispensable for chromosome bi-orientation in various model organisms (Hengeveld et al., 2017; Campbell and Desai, 2013; Yue et al., 2008). A revised spatial separation model posits that localization of Aurora B at centromeres or inner kinetochores is required for bi-orientation (Fischböck-Halwachs et al., 2019; García-Rodríguez et al., 2019). Trypanosome kinetochores offer an interesting opportunity to test this model because trypanosomes intrinsically lack inner centromeres (Tromer et al., 2021) and their CPC localizes primarily via inner kinetochore proteins (Ballmer and Akiyoshi, 2024a). To test the importance of its spatial regulation in trypanosomes, I established a nanobody-based assay to target Aurora B to the inner or outer kinetochore (Fig. 4.9, A and B). I fused the catalytic module of the CPC (Aurora B^{AUK1} + INCENP^{CPC1 148-263}) to tdTomato and a nanobody recognizing GFP or YFP (VhhGFP4, (Ishii and Akiyoshi, 2022; Saerens et al., 2005)) (CPC^{cat}-tdTomato-vhhGFP4) (Fig. 4.9B), enabling the fusion protein to be tethered to YFP-tagged inner (KKT3, KKT9) or outer (KKT4, KKT14) kinetochore components. Importantly, CPC^{cat}-tdTomato-VhhGFP4 itself is not expected to localize at inner kinetochores because it lacks the N-terminal domain of INCENP^{CPC1} that binds the KIN-A:KIN-B scaffold (Ballmer and Akiyoshi, 2024a). Indeed CPC^{cat}-tdTomato-VhhGFP4 failed to localize at kinetochores in the absence of YFP-tagged kinetochore proteins (Fig. 4.9C). In contrast, CPC^{cat}-tdTomato-VhhGFP4 co-localized with YFP-tagged inner or outer KKT proteins (Fig. 4.9, D – G). By incorporating this system into our Aurora B^{AUK1-as1} background cell lines, I selectively inhibited the endogenous kinase, ensuring that the only active Aurora B^{AUK1} molecule derived from the fusion protein (Fig. 4.9B). Following induction of the fusion constructs, we treated cells with 1NM-PP1 or DMSO for 4 h and scored cell cycle distribution and lagging kinetochores in

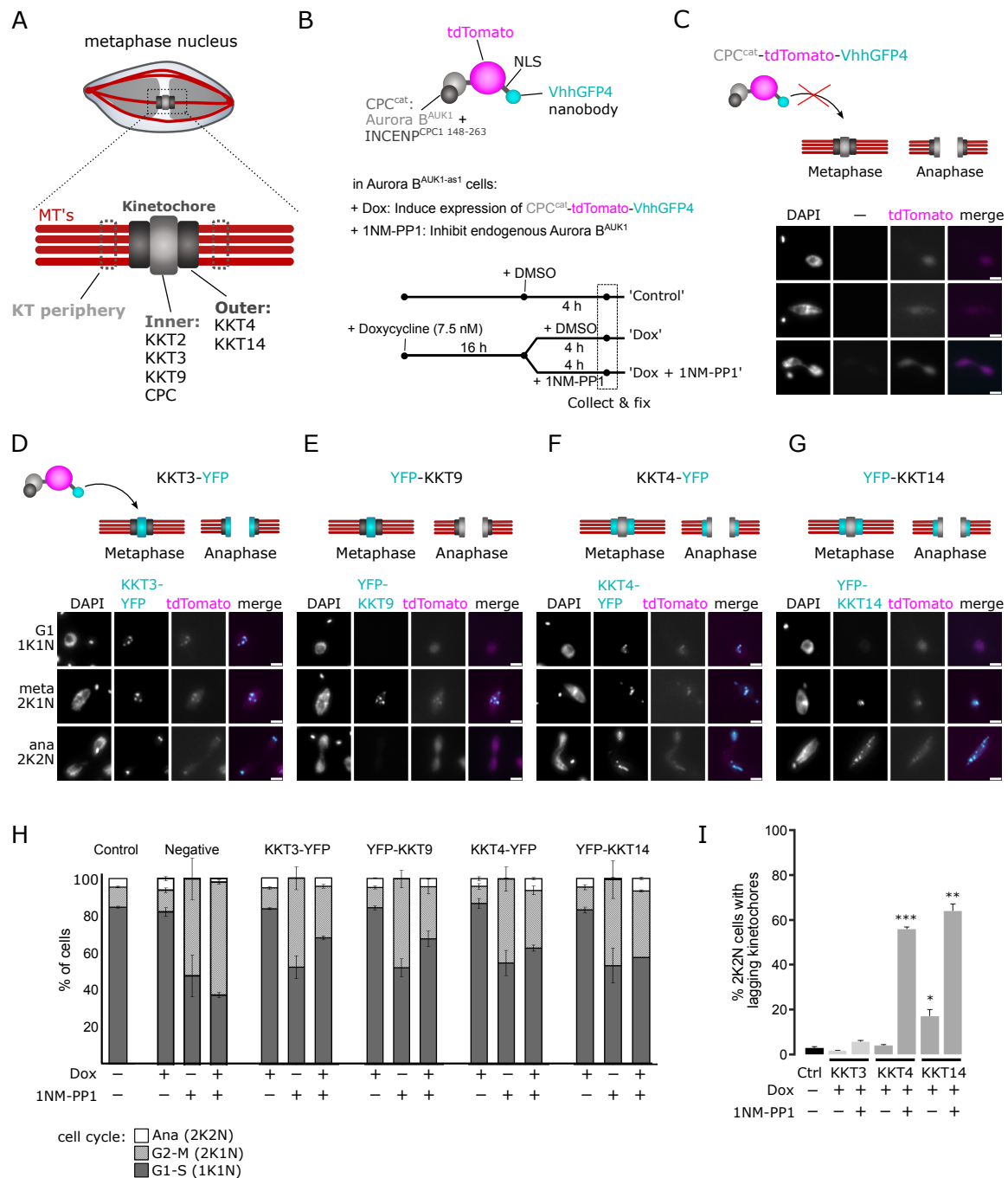


Figure 4.9 (Figure legend on following page)

anaphase (Fig. 4.9B). As expected, untethered CPC^{cat}-tdTomato-VhhGFP4 failed to rescue the effects of Aurora B^{AUK1} inhibition (Fig. 4.9H), while tethering the fusion protein to the inner kinetochore proteins KKT3 or KKT9 (a key interaction partner of the CPC (Ballmer and Akiyoshi, 2024a)) did (Fig. 4.9H). Intriguingly, targeting CPC^{cat}-tdTomato-VhhGFP4 to the outer kinetochore components KKT4 or KKT14 partially restored cell cycle progression but

Figure 4.9. Nanobody-based targeting Aurora B^{AUK1} to the inner or outer kinetochore.

(A) Schematic illustration of the trypanosome kinetochore, indicating proteins localizing to the inner or outer kinetochore. (B) Top: Schematic illustration of CPC^{cat}-tdTomato-VhhGFP4. Aurora B^{AUK1} is fused to the C-terminal domain of INCENP^{CPC1}, which binds to Aurora B^{AUK1} and contains the IN-box required for full Aurora B^{AUK1} activity, but lacks the regions required to interact with endogenous KIN-A:KIN-B at the inner kinetochore (Ballmer and Akiyoshi, 2024a). The fusion construct also contains a nuclear localization signal (NLS) between tdTomato and VhhGFP4. Bottom: Schematic of Aurora B^{AUK1} targeting experiment. Expression of CPC^{cat}-tdTomato-VhhGFP4 was induced for 16 h using 7.5 nM doxycycline in cell lines harboring YFP-tagged inner (KKT3, KKT9) or outer (KKT4, KKT14) kinetochore proteins, followed by addition of either DMSO (Control) or 2 μ M 1NM-PP1 for 4 h to inhibit the endogenous Aurora B^{AUK1} kinase. Cells were then fixed and cell cycle distribution and lagging kinetochores were scored. (C) Fluorescence micrographs showing diffuse nuclear localization of CPC^{cat}-tdTomato-VhhGFP4 induced with 7.5 nM doxycycline in a cell line lacking YFP-tagged kinetochore proteins. Cell line: BAP2671. Scale bars, 2 μ m. (D) to (G) Representative fluorescence micrographs showing the co-localization of CPC^{cat}-tdTomato-vhhGFP4 with YFP-tagged KKT3 (D), KKT9 (E), KKT4 (F) and KKT14 (G). The localization dynamics of the YFP-tagged kinetochore proteins (marked in cyan) in metaphase and anaphase are schematically depicted on top. Cell lines: BAP2673, BAP2990, BAP2991, BAP2992. Scale bars, 2 μ m. (H) Cell cycle profiles for indicated treatment regimes. 'Control' cells were treated with DMSO for 4 h. 'Negative' control corresponds to a cell line that does not express any YFP-tagged protein (as shown in (C)). All graphs depict the means (bar) $\pm$ SD of at least two replicates. A minimum of 500 cells per replicate were quantified. (I) Quantification of lagging kinetochores in 2K2N cells under indicated treatment regimes. 'Control' cells were treated with DMSO for 4 h. Note that lagging kinetochores could not be assessed in the cell line expressing YFP-KKT9, because KKT9 is not present at kinetochores in anaphase. All graphs depict the means (bar) $\pm$ SD of at least two replicates (dots). A minimum of 35 cells per replicate were quantified. *P < 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001 (two-sided, unpaired t-test).

resulted in a massive increase of lagging chromosomes in anaphase (Fig. 4.9, H and I). This is unlikely to be an artifact of impairing KT-MT attachments due to steric hindrance (e.g. by physically blocking access of MTs to binding sites at the kinetochore), because tethering of CPC^{cat}-tdTomato-VhhGFP4 to these outer kinetochore proteins without inhibiting the endogenous Aurora B^{AUK1} did not affect cell cycle progression and sustained error-free chromosome segregation (Fig. 4.9, H and I). I conclude that shifting Aurora B^{AUK1} activity

towards the outer kinetochore is deleterious for proper KT-MT attachments. In contrast, localized CPC activities from either the inner or outer kinetochores are sufficient to promote entry into anaphase.

4.3 Discussion

Here, I showed in *Trypanosoma brucei*, the kinetoplastid parasite that causes African sleeping sickness, that inhibition of Aurora B using an analogue-sensitive approach arrests cells in metaphase, with a reduction in properly bi-oriented kinetochores. Aurora B phosphorylates several kinetochore proteins *in vitro*, including the N-terminal region of the divergent Bub1-like protein KKT14.

KKT14 contains a putative ABBA motif in its N-terminal region, which in other eukaryotes has been shown to interact with CDC20 (see main Introduction). However, I have been unable to purify recombinant *T. brucei* CDC20 protein to test whether it binds KKT14 in an ABBA-dependent manner. When I overexpressed KKT14^{NTR} with the ABBA motif mutated to 'AAAAAA' (ABBA 6A mutant, data not shown), I found that the mutant had the same effect on cell cycle progression as the wild-type control, suggesting that the delay is not dependent on the functional ABBA motif at least in this assay (I cannot exclude the possibility that the mutant KKT14 protein still binds CDC20 via different motifs). I failed to derive an ABBA 6A line targeting the endogenous locus, which could indicate that this motif is essential for cell division. Additional tools (both *in vivo* and *in vitro*) will have to be established to study the functional importance of this motif. Given that my preliminary results were negative, and currently do not have any robust data connecting Aurora B^{AUK1} activity to CDC20-binding through the KKT14 ABBA motif, I did not include any experiments characterizing the function of the ABBA motif in this thesis.

Intriguingly, depletion of KKT14 partially overrides the cell cycle arrest caused by Aurora B inhibition, while overexpression of a non-phosphorylatable KKT14 protein results in a prominent delay in the metaphase-to-anaphase transition. This suggests that CPC-dependent phosphorylation of KKT14 plays a key role in regulating the timing of anaphase onset. However, when Aurora B^{AUK1} activity is not inhibited, overexpression of phosphomimetic (PM) KKT14, unlike phosphodeficient (PD) KKT14, does not noticeably affect cell cycle progression (although it causes lagging kinetochores in anaphase) and induction of KKT14 PM only partially rescues the cell cycle defect caused by Aurora B^{AUK1} inhibition. The absence of a full rescue upon depletion of KKT14 or expression of the phosphomimetic mutant suggests that Aurora B^{AUK1} phosphorylates additional kinetochore proteins to promote error-

free entry into anaphase and/or that continued activity of Aurora B^{AUK1} at the anaphase central spindle is required for completion of nuclear division.

Finally, I demonstrated using a nanobody-based system that re-targeting the catalytic module of the CPC to the outer kinetochore is sufficient to promote mitotic exit but causes massive chromosome mis-segregation in anaphase. However, it is unclear whether the observed increase in lagging kinetochores upon tethering CPC^{cat}-tdTomato-VhhGFP4 to KKT14 or KKT4 is truly a result of Aurora B^{AUK1} activity at the outer kinetochore or simply due to the fusion protein impairing MT-binding activity of the outer kinetochore. However, the fact that lagging kinetochores are only observed when we simultaneously inhibit the endogenous Aurora B^{AUK1} kinase, suggests that this phenotype is caused by shifting the bulk of Aurora B^{AUK1} activity away from the inner kinetochore to the outer kinetochore. I could address this issue more accurately by employing a catalytically inactive Aurora B^{AUK1}.

In summary, my results indicate that the CPC and KKT14 are involved in an unconventional pathway controlling mitotic exit and error-free chromosome segregation in trypanosomes.

5. Functional characterization of the Bub1/BubR1-like protein KKT14

5.1 Introduction

Available evidence suggests that *T. brucei* lacks components of the spindle checkpoint and is unable to delay cell cycle progression in response to spindle defects. It therefore remains poorly understood how kinetoplastids regulate cell cycle progression and ensure the fidelity of chromosome segregation. In a recent study, the Akiyoshi lab reported a crystal structure of the C-terminal domain of KKT14 from the kinetoplastid *Apiculatamorphia spiralis* (subclass Prokinetoplastina) and demonstrated that it is a pseudokinase (Ballmer et al., 2024a). Its structure bears high similarity to the kinase domain of Bub1, a key component of the spindle checkpoint in other eukaryotes. In addition, KKT14 in *T. brucei* (and other trypanosomatids) harbours a putative ABBA motif, a CDC20 interaction motif that is present in Bub1 and its paralog BubR1. By contrast, KKT14 orthologues lack several other motifs present in Bub1 or BubR1, including the Gle2-binding sequence (GLEBS) and the kinetochore attachment regulatory domain (KARD) motifs, which are required for interaction with the WD40 domain protein Bub3 or the PP2A-B56 phosphatase, respectively (Xu et al., 2013; Varadkar et al., 2017; Vallardi et al., 2019; Hertz et al., 2016; Wang et al., 2001; Taylor et al., 1998). It therefore remains unclear whether KKT14 functions as a Bub1/BubR1-like protein in regulating error-free chromosome segregation in trypanosomes. Here, I performed IP-MS to identify potential interaction partners of KKT14 and RNAi-mediated depletion to functionally characterize KKT14.

5.2 Results

5.2.1 KKT14 interacts with KKT15.

KKT14 and KKT15 localize to kinetochores from G2 to anaphase (Akiyoshi and Gull, 2014), a localization pattern which differs from the rest of transiently-localized kinetoplastid kinetochore proteins that start to localize at kinetochores already from S phase onwards. Moreover, both KKT14 and KKT15 localize to the outer kinetochore rather than the inner kinetochore, where most KKT proteins reside (Hayashi and Akiyoshi, unpublished observations). This suggests that KKT14 and KKT15 may directly interact with each other. To test this possibility, I performed IP-MS against YFP-tagged KKT14 and identified KKT15 as top hit (Fig. 5.1A). Vice versa, KKT14 was highly enriched in the immunoprecipitate of YFP-

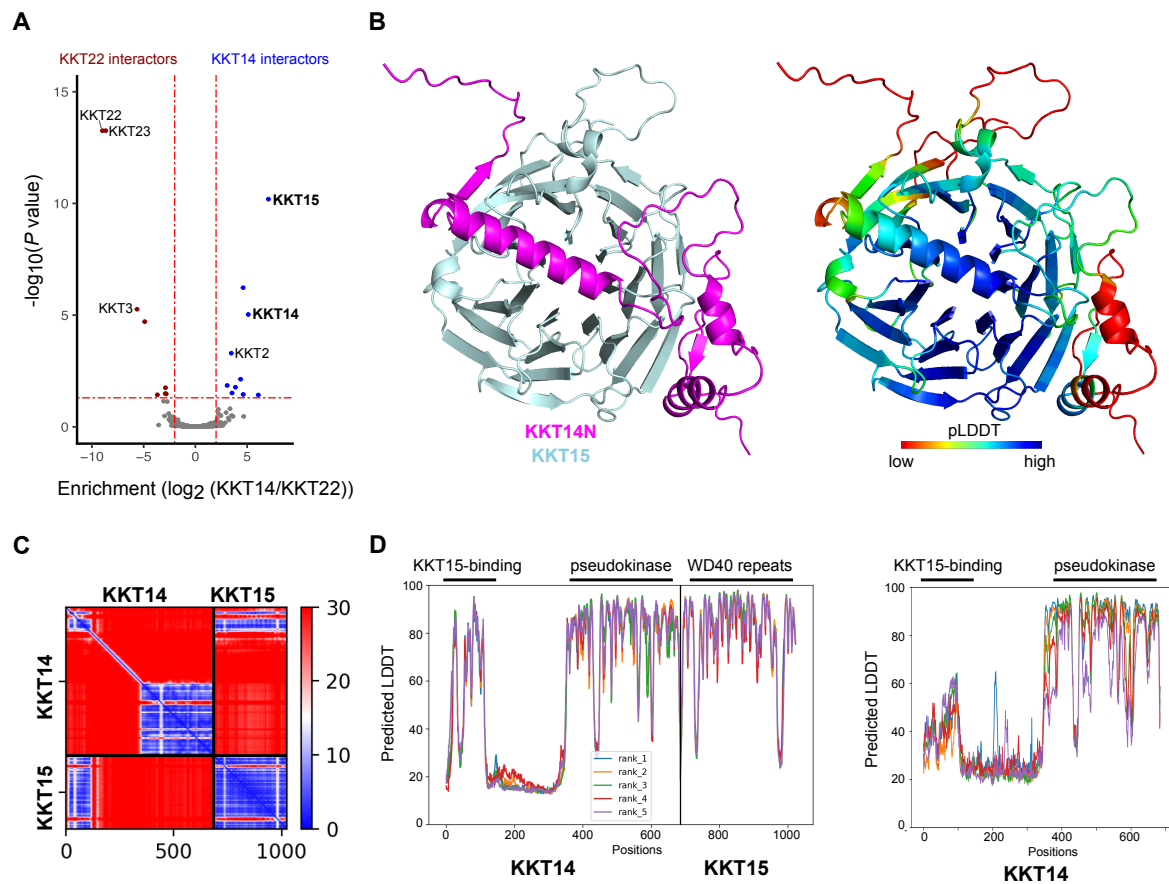


Figure 5.1. KKT14 is predicted to interact with KKT15. (A) A volcano plot showing relative enrichment and significance values between the immunoprecipitates of YFP-KKT14 and YFP-KKT22 (n = 4 each). The kinetochore protein KKT22, which mainly co-purifies with KKT23 and KKT3 (Nerusheva et al., 2019), was used as a comparison. Cell lines: BAP2373, BAP2945. (B) AlphaFold2 predictions of the KKT14²⁻¹²⁵-KKT15 complex in cartoon representation. (C) The Predicted Aligned Error (PAE) plots for the rank 1 model for KKT14-KKT15, predicting interactions via the N-terminal part of KKT14. (D) Predicted local distance difference test (pLDDT) plots for KKT14-KKT15 (left) and KKT14 (right). Figure adapted from (Ballmer et al., 2024a).

KKT15 (data not shown). Despite the absence of the GLEBS motif, which is required for the binding of the N-terminus of Bub1/BubR1 to Bub3 (Primorac et al., 2013; Larsen et al., 2007) AlphaFold2 predicted an interaction between the N-terminal region of KKT14 and KKT15 (Fig. 5.1, B – D). The region of KKT14 predicted to interact with KKT15 is highly conserved among trypanosomatids (residues ~2–111 in *T. brucei*) (data not shown). Interestingly, the confidence score (predicted local distance difference test: pLDDT) for this region improved for KKT14 in complex with KKT15, compared to KKT14 by itself, implying that this region of KKT14 is likely

to form a secondary structure (α -helices) upon interacting with KKT15. These results strongly support the notion that KKT14 and KKT15 are divergent Bub1/BubR1 and Bub3 proteins, albeit they might have adopted a distinct interaction mode.

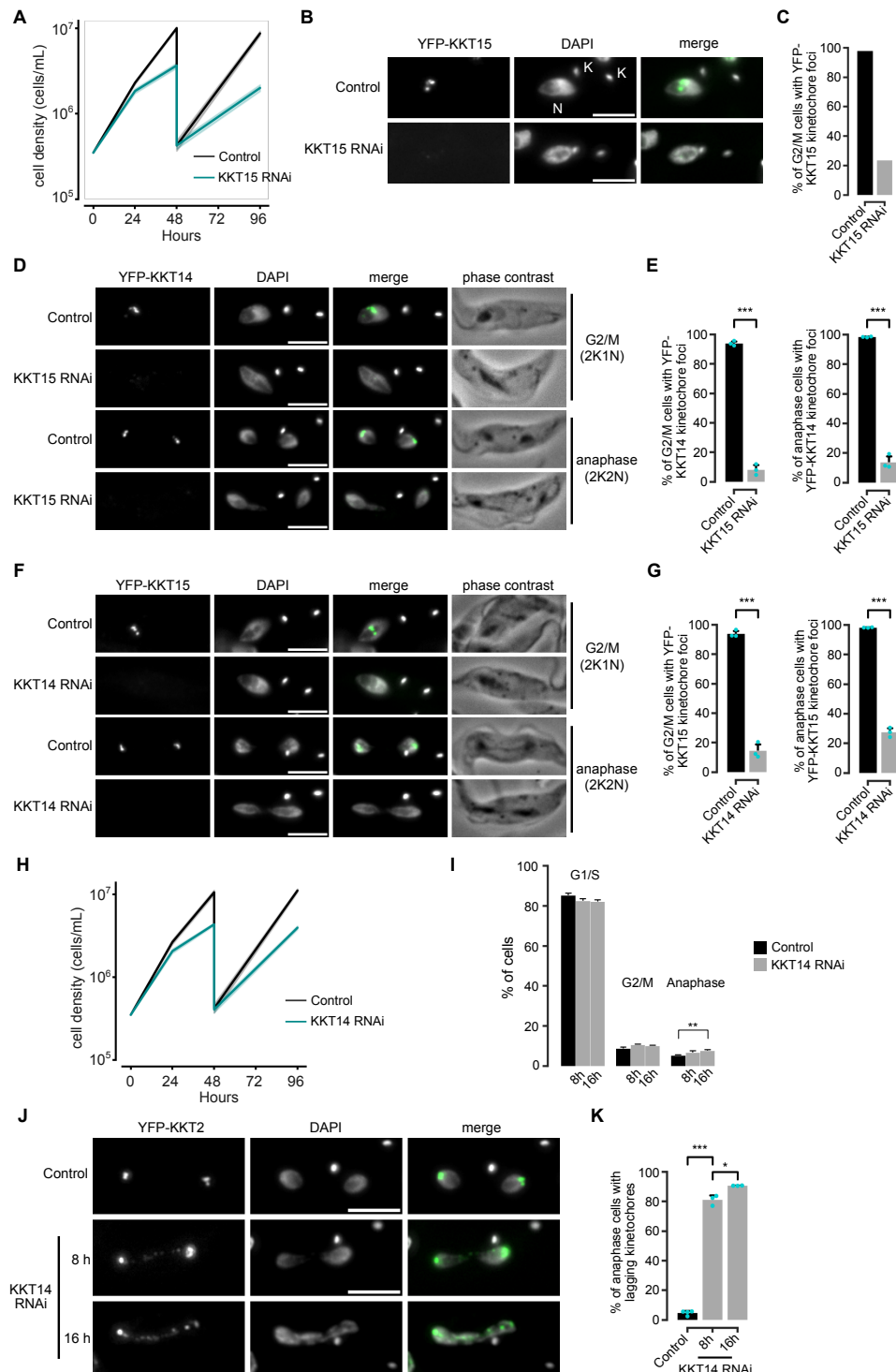


Figure 5.2 (figure legend on following page)

Figure 5.2. KKT14 is essential for accurate chromosome segregation in trypanosomes. (A) Growth curve upon RNAi-mediated knockdown of KKT15 using an RNAi construct against its 3' UTR. Data are presented as the mean $\pm$ SD of three replicates. RNAi was induced with 1 μ g/mL doxycycline and cultures were diluted at day 2. Cell line: BAP2533. (B) and (C) Validation of KKT15 knockdown. RNAi was induced with 1 μ g/mL doxycycline and cultures were diluted at day 2. K and N stand for the kinetoplast and nucleus, respectively. Cell line: BAP2535. At least 130 cells per condition were quantified. (D) Representative fluorescence micrographs showing the localization of YFP-KKT14 upon RNAi-mediated knockdown of KKT15 in G2/M (2K1N) and anaphase (2K2N) cells. RNAi was induced with 1 μ g/mL doxycycline for 24 h. Cell line: BAP2533. (E) Quantification of 2K1N and 2K2N that have kinetochore-like dots of YFP-KKT14 upon RNAi-mediated depletion of KKT15. All graphs depict the means (bar) $\pm$ SD of three replicates (shown as dots). (F) Representative fluorescence micrographs showing the localization of YFP-KKT15 upon RNAi-mediated knockdown of KKT14 in 2K1N and 2K2N cells. RNAi was induced with 1 μ g/mL doxycycline for 24 h. Cell line: BAP2534. (G) Quantification of 2K1N and 2K2N that have kinetochore-like dots of YFP-KKT15 upon RNAi-mediated depletion of KKT14. All graphs depict the means (bar) $\pm$ SD of three replicates (shown as dots). A minimum of 50 cells per replicate were quantified in each condition. (H) Growth curve upon RNAi-mediated knockdown of KKT14. Data are presented as the mean $\pm$ SD of three replicates. RNAi was induced with 1 μ g/mL doxycycline and cultures were diluted at day 2. Cell line: BAP2534. (I) Cell cycle profile upon knockdown of KKT14. RNAi was induced with 1 μ g/mL doxycycline and cells were fixed at 8 h or 16 h. All graphs depict the means (bar) $\pm$ SD of at three replicates. A minimum of 700 cells per replicate were quantified. Cell line: BAP680. (J) Representative fluorescence micrographs showing lagging kinetochores (marked by tdTomato-KKT2) in anaphase cells upon KKT14 knockdown at 8 and 16 h post-induction. RNAi was induced with 1 μ g/mL doxycycline. Cell line: BAP680. (K) Quantification of lagging kinetochores in anaphase cells upon KKT14 knockdown. All graphs depict the means (bar) $\pm$ SD of three replicates (shown as dots). A minimum of 50 cells per replicate were quantified in each condition. * $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ (two-sided, unpaired t-test). Figure adapted from (Ballmer et al., 2024a).

5.2.2 KKT14 and KKT15 are required for accurate chromosome segregation.

I next performed an RNAi-mediated knockdown of KKT14 and KKT15 to assess their function for chromosome segregation. The RNAi construct for KKT14 is previously described (Marcianò et al., 2021), while that for KKT15 was established here (Fig. 5.2, A – C). I found that kinetochore localization of KKT14 and KKT15 is mutually co-dependent (Fig. 5.2, D – G),

further supporting the idea that they form a complex. Although KKT14 depletion caused severe growth defects (Fig. 5.2H) (Marcianò et al., 2021), there was no obvious change in cell cycle profile at 8 or 16 hr after induction of KKT14 RNAi, apart from a moderate increase in anaphase cells (Fig. 5.2I). In contrast, I observed lagging kinetochores in almost all anaphase cells even at 8 hr post-induction (Fig. 5.2, J and K). These results demonstrate that KKT14 is essential for accurate chromosome segregation and cell growth.

5.3 Discussion

IP-MS combined with AlphaFold2 predictions suggest that the N-terminal region of Bub1/BubR1-like protein KKT14 interacts with KKT15, whose WD40 repeat beta-propeller is phylogenetically closely related to Bub3 (Ballmer et al., 2024a). These findings indicate that KKT14 and KKT15 are divergent orthologs of Bub1/BubR1 and Bub3, respectively. KKT14 and KKT15 display identical localization dynamics (e.g. at kinetochores from G2 onwards) and are co-dependent in their recruitment to kinetochores. Together, these findings strongly suggest that KKT14 and KKT15 are direct binding partners, although this remains to be validated in vitro using recombinant proteins.

Depletion of KKT14 causes lagging chromosomes in anaphase. As discussed in the previous chapter, KKT14 appears to be a key target of the Aurora B^{AUK1} kinase involved in regulating the metaphase-anaphase onset in trypanosomes. A thorough investigation of this protein using both in vivo and in vitro approaches (see main Discussion) is bound to provide important insights into the mechanisms controlling anaphase onset in kinetoplastids.

6. The protein phosphatase PP1-1 regulates chromosome segregation in *Trypanosoma brucei*

6.1 Introduction

A dynamic interplay between kinase and phosphatase activities at kinetochores underlies chromosome segregation in eukaryotes. Aurora B-dependent phosphorylation of outer kinetochore substrates destabilizes improper kinetochore-microtubule attachments, thus 'resetting' the kinetochore to provide a new opportunity for bi-orientation. Once this has occurred, outer kinetochore components need to be rapidly dephosphorylated to stabilize correct attachments. Aurora B and the other kinases involved in error correction and the spindle assembly checkpoint (e.g. Mps1, Bub1) are in an antagonistic relationship with protein phosphatases, most notably those of the protein phosphatase 1 (PP1) and 2A (PP2A-B56) families, which are responsible for most of the Ser/Thr dephosphorylation in eukaryotic cells (Brautigan and Shenolikar, 2018; Heroes et al., 2013). Phosphatase activity of PP1 and PP2A proteins is thought to be relatively promiscuous in vitro (Ingebritsen and Cohen, 1983). However, specificity is achieved by localizing the phosphatases in close proximity to their substrates through interaction with short linear motifs (SLiMs) on partner proteins. Well-characterized SLiMs for PP1, for instance, are the RVxF and SILK motifs, both of which are present within the outer kinetochore protein Knl1 (Espeut et al., 2012; Hendrickx et al., 2009; Rosenberg et al., 2011b; Liu et al., 2010). By contrast, distinct B56 regulatory subunits localize the PP2A holoenzyme to the inner centromere and the kinetochore through interaction with Sgo1/2 and the KARD domain of BubR1, respectively (Meppelink et al., 2015b; Liu et al., 2013; Tanno et al., 2010; Kitajima et al., 2006; Suijkerbuijk et al., 2012). Interestingly, PP1 and PP2A-B56-binding motifs are themselves phosphorylated, with opposite phospho-dependencies (e.g. inhibitive for PP1 and enhancing for PP2A recruitment) allowing differential signalling between these phosphatases despite overlapping localizations and substrates (Smith et al., 2019).

Little is known about the role of phosphatases in regulating chromosome segregation in kinetoplastids. The TriTryp phosphatome identified a large number (>50; compared to only 39 in humans) of Ser/Thr phosphatases in trypanosomatids, including clearly identifiable PP1 and PP2A clades (Brenchley et al., 2007). Simultaneous knockdown of 7 PP1s or the PP2A catalytic subunit in procyclic form *T. brucei* caused proliferation defects, and, in the case of the PP1s, a moderate increase in G2/M cells (Li et al., 2006). By contrast, treatment of trypanosomes with okadaic acid (OA), a well characterized inhibitor of PP1 and PP2A

activities, had no effect on nuclear division but prevented kinetoplast replication and cytokinesis, yielding multinucleate cells with a single kinetoplast (Li et al., 2006; Das et al., 1994). Thus, it is likely that other OA-sensitive Ser/Thr phosphatases are present *T. brucei*.

So far, the only phosphatase that has been shown to localize at the kinetochore in trypanosomes is the kinetoplastid-specific Ser/Thr phosphatase KKIP7 (D'Archivio and Wickstead, 2017). KKIP7 is present only on metaphase kinetochores, suggesting that it may play a key role in opposing Aurora B^{AUK1} or the other five kinases at the trypanosome kinetochore (KKT2, KKT3, KKT10, KKT19 and CRK3) to stabilize KT-MT attachments and ensure fidelity of chromosome segregation. However, RNAi-mediated depletion of KKIP7 had no effect on cell growth (unpublished observations). Thus, other kinetochore-resident phosphatases may be present in trypanosomes, which perhaps perform redundant functions. In this chapter, my aim was to characterize the localization dynamics of PP1-1 and test its functional relevance by performing RNAi experiments.

6.2 Results

6.2.1 PP1-1 localizes at kinetochores from late interphase until anaphase.

A BLAST search of PP1^{Glc7} from *S. cerevisiae* against *T. brucei* proteins identified PP1-1 (Tb927.11.8090) as top hit. Indeed, mNG-tagged PP1-1 forms kinetochore-like punctae within the nucleus according to the TrypTag database (Billington et al., 2023). To test the possibility that PP1-1 localizes at the kinetochore, I assessed the co-localization of YFP-tagged PP1-1 with tdTomato-KKT2, a constitutive component of the inner kinetochore (Fig. 6.1). Both YFP-PP1-1 and PP1-1-YFP localized to basal bodies in the cytoplasm and formed foci within the nucleus throughout the cell cycle. These nuclear foci clearly co-localized with tdTomato-KKT2 from G2 phase onwards until anaphase. In addition, PP1-1 showed spindle-like localization in some metaphase cells. Over the course of anaphase, PP1-1 was progressively lost from kinetochores and no longer overlapped with KKT2 in G1 cells. In summary, these data indicate that PP1-1 localizes to kinetochores in *T. brucei*.

6.2.2 Kinetochore localization of PP1-1 partially depends on KKT7.

KKT7 harbours a conserved RSVTF motif within its N-terminus (Akiyoshi and Gull, 2014), as well as a SILK motif in some kinetoplastid species (Fig. 6.2A). Indeed, AlphaFold2 confidently predicted an interaction between KKT7 and PP1-1, which involves the RSVTF motif (data not shown). To assess whether KKT7 is responsible for kinetochore recruitment of PP1-1, I

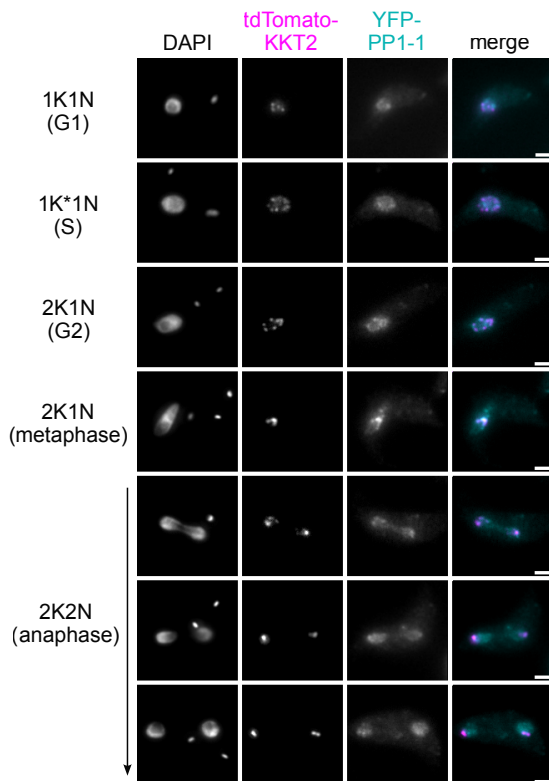


Figure 6.1. PP1-1 localizes to kinetochores from G2 until anaphase. Representative fluorescence micrographs showing the localization of YFP-PP1-1 and tdTomato-KKT2 (kinetochore marker) at indicated cell cycle stages. Cell line: BAP2914.

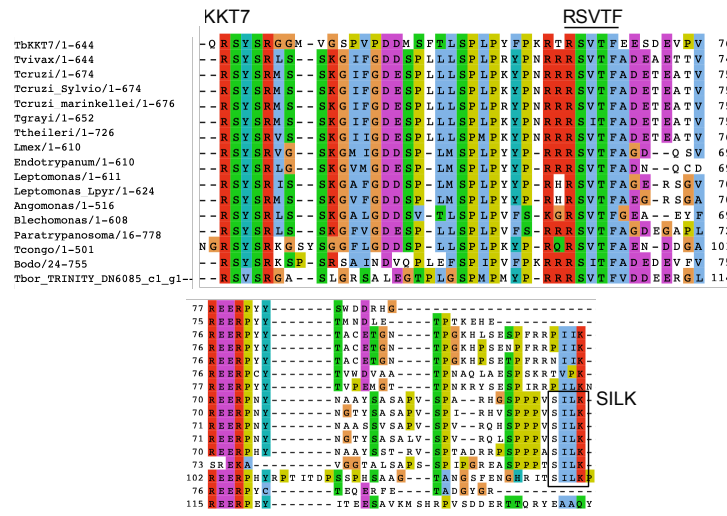
depleted KKT7 using a previously validated shRNA construct directed against the 5'UTR of KKT7 (Ishii and Akiyoshi, 2020) and assessed the co-localization of YFP-PP1-1 or PP1-1-YFP with tdTomato-KKT2. Although, PP1-1 localization was clearly affected, kinetochore enrichment was nonetheless observable in ~60% of 2K1N (G2/M phase) cells (Fig. 6.2, B – D). Thus, additional PP1-1 receptors are likely present at the kinetoplastid kinetochore.

6.2.3 Depletion of PP1-1 causes chromosome mis-segregation.

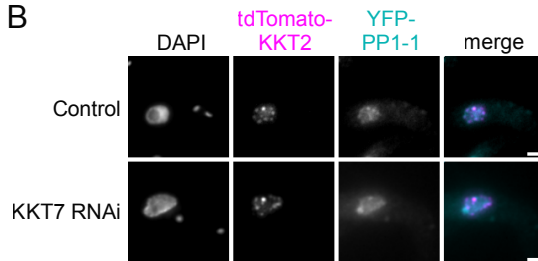
To assess the function of PP1-1, I first tested two shRNA constructs directed against the 5' or the 3' UTR of PP1-1. Both constructs caused an ~80% reduction of PP1-1 protein levels 24 h post induction (Fig. 6.3, A – D). Knockdown of PP1-1 resulted in a prominent growth defect without a major change in cell cycle distribution (Fig. 6.3, E – H). Importantly, ~50% of anaphase cells displayed lagging kinetochores (Fig. 6.3, I – L), suggesting that PP1-1 regulates KT-MT attachments.

So far, the outer kinetochore protein KKT4 remains the only component of the kinetoplastid kinetochore, which has been shown to bind end-on to spindle microtubules and maintain load-bearing attachments to both growing and shrinking microtubule tips (Llauró et al., 2018). KKT4 is phosphorylated in vitro by KKT10/19 and Aurora B^{AUK1} kinases (Ishii and Akiyoshi, 2020; Ballmer et al., 2024b). In analogy to the tension-dependent phosphorylation of Ndc80 in traditional model eukaryotes, the microtubule-binding affinity of KKT4 may thus

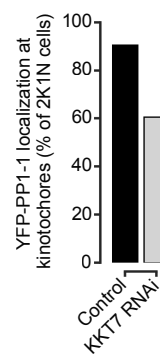
A



B



C



D

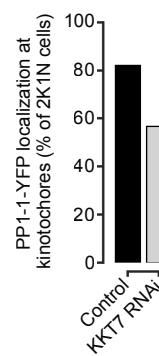


Figure 6.2. Kinetochore recruitment of PP1-1 depends on KKT7. (A) Multiple sequence alignment of KKT7 orthologs in kinetoplastids. The RSVTF and SILK motifs may act as SLiMs for PP1-1 docking. (B) Representative fluorescence micrographs showing the localization of YFP-PP1-1 upon RNAi-mediated knockdown of KKT7. Kinetochores are marked with tdTomato-KKT2. RNAi was induced with 1 μ g/mL doxycycline for 20 h. Cell line: BAP2688. (C) and (D) Quantification of 2K1N cells that have clearly detectable YFP-PP1-1 (C) and PP1-1-YFP (D) signal at kinetochores. At least 60 cells were quantified in each condition. Cell lines: BAP2688, BAP2689.

be subject to phospho-dependent regulation. In support of this notion, I detected an electrophoretic mobility shift of 3FLAG-tagged KKT4 by Western blot in cells depleted for PP1-1 (Fig. 6.4A). By contrast, the band corresponding to phosphorylated PP1-1 was almost entirely lost upon knockdown of the KKT10 and KKT19 kinases (Fig. 6.4A). These data suggest that PP1-1 antagonizes KKT10/19 activity in regulating KKT4.

Intriguingly, 3FLAG-KKT4 was hyper-phosphorylated in cells depleted for the Bub1/BubR1-like protein KKT14 (Fig. 6.4A), indicating that KKT14 may be involved in

recruiting PP1-1. Indeed, PP1-1 localization at the metaphase plate was lost in ~50% of metaphase cells depleted for KKT14 (Fig. 6.4, B – C). Whether KKT14 contributes directly or indirectly to the recruitment of PP1-1 or other phosphatases will be addressed in future studies.

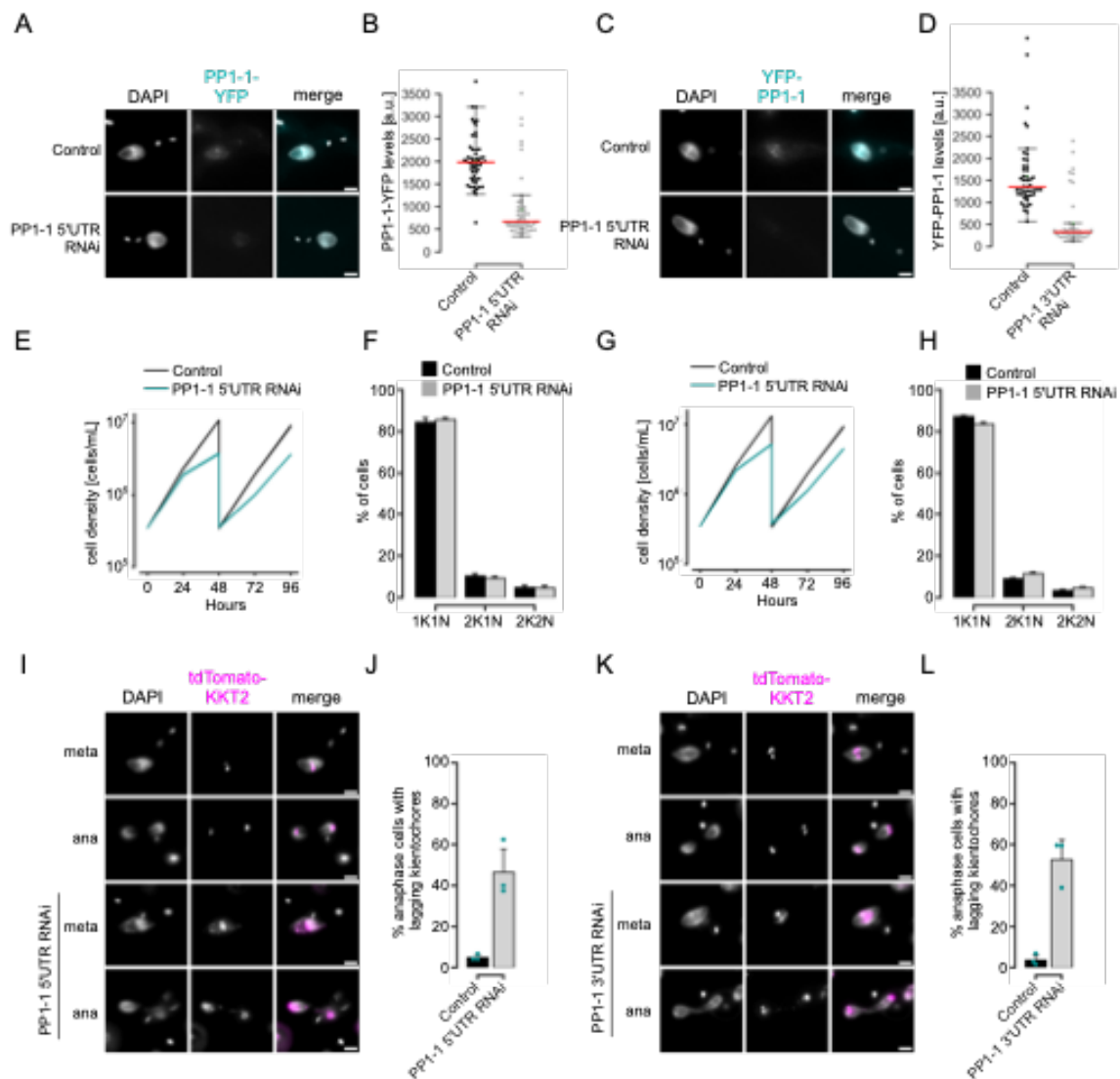


Figure 6.3 (figure legend on following page)

6.3 Discussion

Here, I showed that PP1-1 localizes to kinetochores from G2 until anaphase. Kinetochore localization of PP1-1 is in part dependent on KKT7, which contains a conserved PP1 binding motif. However, it is likely that other PP1-1 receptors are present at the kinetochore since disruption of PP1-1 recruitment to kinetochores was incomplete upon KKT7 RNAi.

Figure 6.3. PP1-1 promotes error-free chromosome segregation in trypanosomes.

(A) Representative fluorescence micrographs showing PP1-1-YFP in control and knockdown conditions. RNAi against the 5' UTR of PP1-1 was induced with 1 µg/mL doxycycline for 24 h. Cell line: BAP2912. (B) Quantification of PP1-1-YFP levels in the nucleus in control and knockdown conditions as in (A). A minimum of 50 cells per replicate were quantified in each condition. (C) Representative fluorescence micrographs showing YFP-PP1-1 in control and knockdown conditions. RNAi against the 3' UTR of PP1-1 was induced with 1 µg/mL doxycycline for 24 h. Cell line: BAP2911. (D) Quantification of YFP-PP1-1 levels in the nucleus in control and knockdown conditions as in (C). A minimum of 50 cells per replicate were quantified in each condition. (E) Growth curve upon RNAi-mediated knockdown of PP1-1 using an shRNA construct against its 5' UTR. Data are presented as the mean $\pm$ SD of three replicates. RNAi was induced with 1 µg/mL doxycycline and cultures were diluted at day 2. Cell line: BAP2914. (F) Cell cycle profile upon knockdown of PP1-1 using an shRNA construct against its 5' UTR. RNAi was induced with 1 µg/mL doxycycline and cells were fixed at 24 h. All graphs depict the means (bar) $\pm$ SD of at three replicates. A minimum of 400 cells per replicate were quantified. (G) Growth curve upon RNAi-mediated knockdown of PP1-1 using an shRNA construct against its 3' UTR. Data are presented as the mean $\pm$ SD of three replicates. RNAi was induced with 1 µg/mL doxycycline and cultures were diluted at day 2. Cell line: BAP2915. (H) Cell cycle profile upon knockdown of PP1-1 using an shRNA construct against its 3' UTR. RNAi was induced with 1 µg/mL doxycycline and cells were fixed at 24 h. All graphs depict the means (bar) $\pm$ SD of at three replicates. A minimum of 400 cells per replicate were quantified. (I) Representative fluorescence micrographs showing lagging kinetochores (marked by tdTomato-KKT2) in anaphase cells upon PP1-1 knockdown using an shRNA construct against its 5' UTR. RNAi was induced with 1 µg/mL doxycycline for 24 h. Cell line: BAP2914. (J) Quantification of lagging kinetochores in anaphase cells upon PP1-1 knockdown as described in (I). All graphs depict the means (bar) $\pm$ SD of three replicates (shown as dots). A minimum of 25 cells per replicate were quantified in each condition. (K) Representative fluorescence micrographs showing lagging kinetochores (marked by tdTomato-KKT2) in anaphase cells upon PP1-1 knockdown using an shRNA construct against its 3' UTR. RNAi was induced with 1 µg/mL doxycycline for 24 h. Cell line: BAP2915. (J) Quantification of lagging kinetochores in anaphase cells upon PP1-1 knockdown as in (K). All graphs depict the means (bar) $\pm$ SD of three replicates (shown as dots). A minimum of 35 cells per replicate were quantified in each condition. * $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ (two-sided, unpaired t-test).

Depletion of PP1-1 caused a slow growth phenotype and extensive mis-segregation of chromosomes in anaphase. Moreover, loss of PP1-1 resulted in hyper-phosphorylation of the outer kinetochore protein KKT4, the primary microtubule-binding protein of the kinetoplastid kinetochore, and a key target of the KKT10/19 and Aurora B^{AUK1} kinases. Thus, phospho-dependent regulation of microtubule-interacting outer kinetochore components may be a conserved mechanism controlling faithful chromosome segregation in eukaryotes.

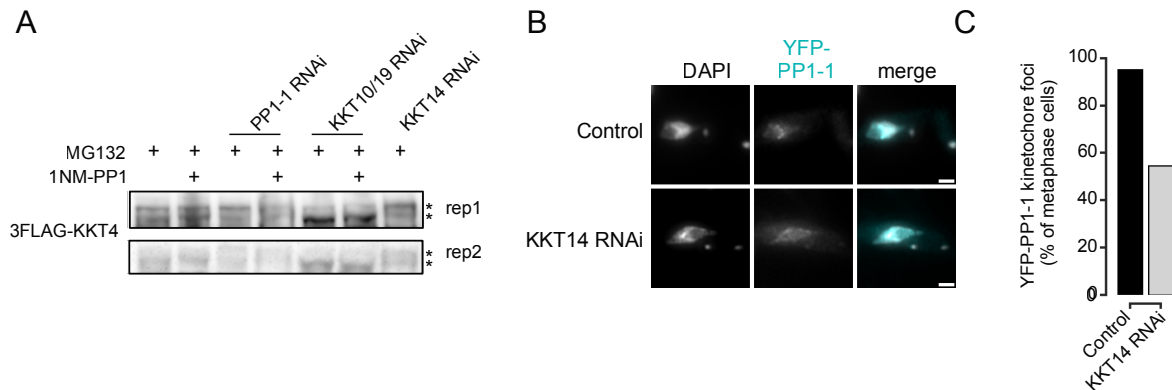


Figure 6.4. PP1-1 dephosphorylates KKT4. (A) Western blot of 3FLAG-KKT4 under indicated treatment and RNAi conditions. Asterisks mark bands corresponding to phosphorylated and dephosphorylated KKT4. Cell lines: BAP3050, BAP3051, BAP3052. (B) Representative fluorescence micrographs showing the localization of YFP-PP1-1 in metaphase upon RNAi-mediated knockdown of KKT14. RNAi was induced with 1 μ g/mL doxycycline for 24 h. Cell line: BAP3077. (C) Quantification of metaphase cells that have clearly detectable YFP-PP1-1 foci at the metaphase plate. At least 200 cells were quantified in each condition. Cell lines: BAP3077.

7. Discussion

How is the CPC targeted to kinetochores and the central spindle in *T. brucei*?

Whereas astonishing diversity in kinetochore composition is seen among eukaryotes (Tromer et al., 2019; Hooff et al., 2017; Komaki et al., 2022), the proteins of the regulatory circuitry underlying chromosome segregation, such as the APC/C, SAC and CPC, are more widely conserved. Homologs of the CPC proteins Aurora B kinase and its associated partner INCENP have been detected in almost all sequenced eukaryotes, including kinetoplastids. The dynamic localization pattern exhibited by the CPC (e.g. transferring from centromeres to the central spindle upon metaphase-anaphase transition) is likewise highly conserved across eukaryotes but appears to be achieved through a variety of different mechanisms. For instance, while CPC recruitment to the centromeres in higher eukaryotes is governed by two histone phosphorylation marks (Haspin-mediated H3T3ph and Bub1-mediated H2AT120ph), budding yeasts employ a combination of histone modifications (Bub1-mediated H2AT121ph) and kinetochore proteins as CPC receptors. For example, Borealin^{Bir1} not only recognizes Sgo1 but also interacts with the CBF3 complex through Ndc10 (Yoon and Carbon, 1999; Cho and Harrison, 2011). Furthermore, INCENP^{Sli15}/Aurora B^{Ipl1} interact with the Ctf19 subunit of the COMA complex at kinetochores (Fischböck-Halwachs et al., 2019). The proteins that form the localization module of the CPC in different species appear to mirror the diversity in centromeric CPC receptors. In fact, many phyla lack Borealin or Survivin homologs (Komaki et al., 2022). Komaki et al. recently identified two functionally redundant CPC proteins in *Arabidopsis*, Borealin Related Interactor 1 and 2 (BORI1 and 2), which engage in a triple helix bundle with INCENP and Borealin using a conserved helical domain but employ an FHA domain instead of a BIR domain to read H3T3ph (Komaki et al., 2022).

During my DPhil, I have identified KIN-A and KIN-B as components of the CPC in trypanosomes and delineated a novel pathway for centromeric recruitment of the CPC in this evolutionary divergent group of eukaryotes. In agreement with this work, an early study on the CPC in *T. brucei* found that KIN-A and KIN-B co-purified with Aurora B^{AUK1}, INCENP^{CPC1} and CPC2 based on a pull-down of an Aurora B^{AUK1}-PTP fusion protein followed by mass spectrometry analysis (Li et al., 2008a). However, HA-tagged KIN-A and KIN-B were not detected at kinetochores (from late interphase until metaphase) nor at the new FAZ tip (from late anaphase), and hence were not interpreted as CPC proteins. Contrary to this report, my data clearly show that KIN-A and KIN-B are constitutive components of the CPC in *T. brucei*. First, YFP-tagged KIN-A and KIN-B co-localize with Aurora B^{AUK1}, INCENP^{CPC1} and CPC2 throughout the cell cycle. Second, KIN-A and KIN-B are readily detected within native CPC complexes isolated by immunoprecipitation of CPC subunits from cells arrested prior to

anaphase and form robust crosslinks with the other CPC subunits. Finally, YFP-Aurora B^{AUK1} and INCENP^{CPC1}-YFP are crucially dependent on KIN-A and KIN-B for localizing both to metaphase kinetochores and to the anaphase central spindle. Thus, the KIN-A:KIN-B subcomplex constitutes the localization module of the trypanosome CPC.

Biochemical, cell biological and in silico modelling approaches indicate that the kinesins KIN-A and KIN-B form coiled coils between their central and C-terminal domains, respectively, which then serve as a scaffold onto which INCENP^{CPC1} and CPC2 assemble via their N-terminal α -helical domains. The catalytic module of the trypanosomes CPC, consisting of Aurora B^{AUK1} bound to the C-terminal IN-box of INCENP^{CPC1}, is connected to the KIN-A:KIN-B scaffold through a flexible linker in INCENP^{CPC1}. While the on-beads cross-linking of native CPCs suggests that the catalytic module is positioned in close proximity to the kinesin heads, this may not necessarily be true in vivo. For example, the catalytic module may exist in both 'locked' and 'open' conformations with regard to its association with the kinesin motor domains. I speculate that the interaction of the KIN-A motor domain with microtubules from prometaphase onwards may cause the catalytic module to disengage from its kinesin head-associated state (Fig. 7.1). In analogy to the SAH domain of INCENP in other model eukaryotes which has been proposed to function as a dog leash (Samejima et al., 2015; Santaguida and Musacchio, 2009), the INCENP^{CPC1} flexible linker in trypanosomes (~100 amino acids long) may then permit Aurora B^{AUK1} to roam across a larger but nevertheless spatially constrained target area to phosphorylate its substrates while still being anchored to the kinetochore via the KIN-A:KIN-B subcomplex, which can interact both with kinetochore components and spindle microtubules. Importantly, this mechanism would allow the CPC to act as an intrinsic 'sensor' of KT-MT attachments. Such models dealing with alternative conformations of the CPC in various cellular context will require further testing in the future.

I propose that multiple weak interactions of the KIN-A C-terminal unstructured tail with kinetochore components act in synergy to stabilize the CPC at kinetochores. Critically, these interactions need to be of transient and reversible nature to permit the dynamic release of the CPC upon anaphase onset. Three mechanisms are likely to play a role in this context (Fig. 7.1). First, removal of the KKT8 complex (the 'CD1 receptor') at the metaphase-anaphase transition effectively eliminates one of the key CPC-kinetochore interfaces. Secondly, the affinity of the KIN-A C-terminal tail for its binding partners at the kinetochore may be further finetuned through reversible post-translational modifications. In support of this, the KIN-A C-terminal tail harbours many putative CRK3 sites (10 sites matching the minimal S/T-P consensus motif for CDKs) and is also heavily phosphorylated by Aurora B^{AUK1} in vitro (Ballmer et al., 2024b). Finally, I speculate that the interaction of KIN-A motor domain with microtubules, coupled to the force generating ATP hydrolysis and possibly plus-end directed motion, eventually outcompetes the weakened interactions of the CPC with the kinetochore and

facilitates the extraction of the CPC from chromosomes onto spindle microtubules during anaphase. Indeed, deletion of the KIN-A motor domain or impairment of its motor function through N-terminal GFP tagging causes the CPC to be trapped at kinetochores in anaphase. Central spindle localization is additionally dependent on the ATPase activity of the KIN-A motor domain as illustrated by the KIN-A rigor mutant.

It remains to be investigated whether KIN-A truly functions as a plus-end directed motor. The role of the KIN-B in this context is equally unclear. Because KIN-B does not possess a functional kinesin motor domain, I deem it unlikely that the KIN-A:KIN-B heterodimer moves hand-over-hand along microtubules as do conventional (kinesin-1 family) kinesins. Rather, the KIN-A motor domain may function as a single-headed unit and drive processive plus-end directed motion using a mechanism similar to the kinesin-3 family kinesin KIF1A (Okada and Hirokawa, 1999). Formation of transient complexes between the CPC and kinesins or other microtubule plus-end tracking proteins upon anaphase onset appears to be a common theme underlying the central spindle translocation of the CPC. For instance, the CPC interacts with MKLP2 or Bim1^{EB1} in human and yeast cells, respectively (Gruneberg et al., 2004; Zimniak et al., 2012). Thus, deeper understanding of the CPC regulation in trypanosomes is bound to provide evolutionary insights into fundamental principles of chromosome segregation in eukaryotes and can lead to the discovery of druggable targets to combat kinetoplastid diseases (Saldivia et al., 2020).

What is the role of Aurora B^{AUK1} in regulating chromosome segregation in *T. brucei*?

From yeast to humans, Aurora kinases impart fidelity to cell division by regulating various processes including kinetochore assembly, chromosome bi-orientation, and SAC signaling (Krenn and Musacchio, 2015). The results presented in this thesis include the first in-depth analysis of the role of Aurora B^{AUK1} in regulating chromosome segregation in kinetoplastids, a group of flagellated protists proposed to be among the earliest branching eukaryotes (Allen et al., 2008; Cavalier-Smith, 2010; Akiyoshi and Gull, 2013). Like in other eukaryotes, kinetochores fail to form proper bi-oriented attachments upon Aurora B^{AUK1} inhibition. In vitro, Aurora B^{AUK1} strongly phosphorylated the MT-binding domain of KKT4, and MT co-sedimentation assays using a phosphomimetic KKT4 mutant found reduced affinity for MTs (data not shown). This suggests that the trypanosome CPC may be involved in an error correction process analogous to that described in other eukaryotes. Consistent with this notion, artificial targeting of Aurora B^{AUK1} to the inner kinetochore (but not to outer inner kinetochore) allowed proper chromosome segregation in anaphase. Thus, the overall mechanistic principles of error correction, e.g. tension-dependent regulation of 'outer' MT-binding proteins that are spatially separated from a main 'inner' pool of the CPC, may be evolutionary conserved.

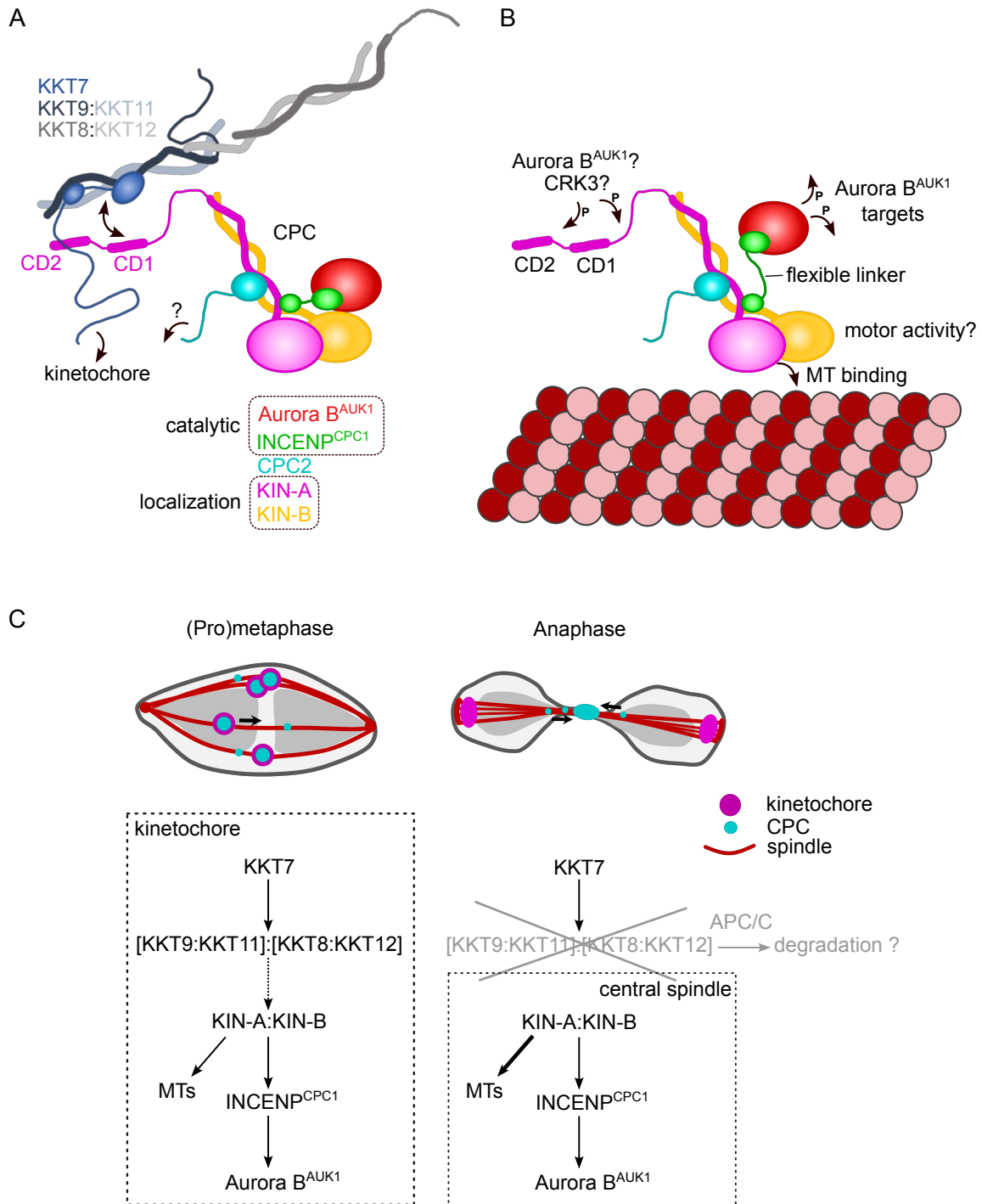


Figure 7.1 (figure legend on following page)

Remarkably, inhibition of Aurora B^{AUK1} in *T. brucei* arrests cells in metaphase, a phenotype that has not been reported in traditional model eukaryotes (Biggins and Murray, 2001; Hauf et al., 2003). My data indicates that the divergent Bub1-homologue KKT14 is a main target of Aurora B^{AUK1}. The KKT14 NTR contains an ABBA motif (a conserved CDC20-

Figure 7.1. Model for CPC localization and function in trypanosomes. (A) KIN-A (magenta) and KIN-B (yellow) interact via their coiled-coil domains and form a scaffold for the assembly of CPC2 (cyan) and the catalytic module of the CPC, composed of Aurora B^{AUK1} (red) and INCENP^{CPC1} (green). During interphase, the catalytic module is positioned close to the kinesin head domains of KIN-A and KIN-B. CPC recruitment to the inner kinetochore is mediated through multiple weak interactions between the C-terminal unstructured tail of KIN-A (magenta), containing CD1 and CD2, with the coiled-coil domain of KKT9:KKT11 (dark blue:light blue) and possibly the N-terminus of KKT7 (blue). The KKT8 complex, comprising KKT9:KKT11 and KKT8:KKT12 (dark grey:light grey) subcomplexes, is connected to the other kinetochore proteins through KKT7. Additional kinetochore targeting domains of the CPC may reside within the C-terminus of KIN-B and/or CPC2. I propose that the KIN-A:KIN-B subcomplex represents the main localization module of the trypanosome CPC. As illustrated in (B), the affinity of the KIN-A C-terminal tail for its receptor(s) at the kinetochore may be further modulated through phosphorylation by the CDK1 homologue CRK3 and the Aurora B^{AUK1} kinase itself (Ballmer et al., 2024b). Interaction of the N-terminal motor domain of KIN-A with spindle microtubules (MTs) from prometaphase onwards causes the catalytic module to disengage from its kinesin head-associated state. The ~100 amino acid long flexible linker within INCENP^{CPC1} would then permit Aurora B^{AUK1} to phosphorylate its substrates within a larger but nevertheless spatially constrained target area while still being anchored to the kinetochore via KIN-A:KIN-B. The motor domain of KIN-A could thus act as built-in sensor for KT-MT attachments. (C) I propose that the trypanosome CPC is recruited to kinetochores via the KKT7-KKT8 complex pathway (dashed arrow) and that motor activity of KIN-A promotes congression of kinetochores to the metaphase plate during early mitosis. The KKT8 complex dissociates from kinetochores at the metaphase-to-anaphase transition and is possibly degraded in an APC/C-dependent manner. Elimination of the KKT8 complex, the primary kinetochore receptor of the CPC, coupled to MT binding and motor activity of the KIN-A motor domain strips the CPC off kinetochores and facilitates its translocation to the central spindle in anaphase.

interaction motif) (Ballmer et al., 2024a), suggesting that this domain might be involved in regulating APC/C activity. Although direct binding to CDC20 and/or APC/C subunits remains to be demonstrated, I speculate that the KKT14 NTR in its unphosphorylated state might prevent premature APC/C activation by sequestering CDC20 or certain subunits of the APC/C, and that this inhibition could be relieved by Aurora B^{AUK1} activity. Alternatively, the NTR in its phosphorylated state may act as a scaffold that promotes APC/C-CDC20 interaction and its

subsequent activation. A third possibility is that kinetoplastids evolved an APC/C- or CDC20-independent mechanism to regulate the metaphase-anaphase transition.

Apart from KKT14, Aurora B^{AUK1} also phosphorylated several CPC subunits in vitro, including its activator INCENP^{CPC1} and the C-terminal tail of KIN-A, which plays a key role in targeting the CPC to kinetochores by interacting with the KKT8 complex at the inner kinetochore. I speculate that Aurora B^{AUK1}-dependent phosphorylation of KIN-A may modulate its kinetochore-binding affinity. Importantly, inhibition of Aurora B^{AUK1} using my analogue sensitive approach did not impair kinetochore localization of the CPC. Rather, phosphorylation of the KIN-A C-terminal tail could serve to weaken the association of KIN-A with its kinetochore receptors and facilitate the release of the CPC from kinetochores onto spindle microtubules upon metaphase-anaphase transition. An intriguing possibility is that the interaction of the KIN-A motor domain with microtubules in (pro)metaphase, coupled to the establishment of proper kinetochore-microtubule attachments, may trigger conformational changes within the CPC (such as tension-dependent stretch of the KIN-A C-terminus) and/or full activation of Aurora B^{AUK1}, thereby allowing temporally and spatially regulated phosphorylation of the KIN-A C-terminal tail. Whether timely release of KIN-A from kinetochores may be a prerequisite for mitotic exit in addition to KKT14 phosphorylation will require further experimental testing in the future.

In addition to the outer kinetochore proteins KKT4 and KKT14, Aurora B^{AUK1} also phosphorylated several inner kinetochore components in vitro, including KKT1, KKT7 and KKT8. All these proteins, apart from KKT14, are also substrates of the two functionally redundant KKT10/19 kinases (also called CLK1/2) (Ishii and Akiyoshi, 2020). Interestingly, KKT10 and KKT19 localize to the inner kinetochore by binding to the N-terminus of KKT7, which in conjuncture with the KKT8 complex also serves as the main recruitment arm for the trypanosome CPC (Ishii and Akiyoshi, 2020; Ballmer and Akiyoshi, 2024a). Moreover, co-depletion of KKT10/19 causes a delay in the metaphase-anaphase progression and lagging kinetochores in anaphase, raising the possibility that these kinases may be involved in some form of error correction process and/or form part of the regulatory circuitry controlling anaphase onset. Contrary to Aurora B^{AUK1}, however, KKT10/19 phosphorylate the C-terminal domain of KKT4 rather than its MT-binding domain (Ishi and Akiyoshi 2020), and the mitotic spindle appears to be hyper-stabilized rather than destabilized in these mutants (unpublished observations). Thus, it is conceivable that KKT10/19 and Aurora B^{AUK1} may play opposing roles for spindle stability, despite their close spatial association.

Re-targeting the CPC from kinetochores onto the mitotic spindle in metaphase was still able to support entry into anaphase (Ballmer and Akiyoshi, 2024b). This was also the case upon tethering of a CPC^{cat}-tdTomato-VhhGFP4 fusion protein to the outer kinetochore while inhibiting endogenous Aurora B^{AUK1-as1}, which localizes at the inner kinetochore. Together,

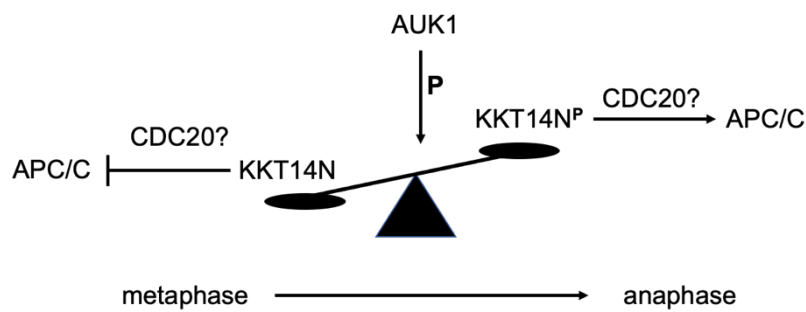


Figure 7.1. Model for CPC- and KKT14-dependent regulation of metaphase-anaphase transition. Phosphorylation of the KKT14 NTR by Aurora B^{AUK1} promotes activation of the APC/C. Hence, timing of anaphase onset is determined by the ration of phosphorylated vs. unphosphorylated KKT14.

these results imply that the ability of Aurora B^{AUK1} to trigger mitotic exit is not strictly dependent on its recruitment to the inner kinetochore and hence may not be spatially regulated. Rather, the trypanosome CPC may sense conformational changes when the kinetochore comes under tension upon forming stable, bi-oriented attachments to the spindle, which would allow conditional phosphorylation of substrates (such as KKT14) involved in the metaphase-anaphase transition. Identification and functional characterization of additional CPC substrates at kinetochores/centromeres will be key to understanding how anaphase onset is controlled in kinetoplastids.

Inhibition of Aurora B^{AUK1} also causes a spindle instability phenotype: In Aurora B^{AUK1-as1} cells treated with 1NM-PP1 the mitotic spindle begins to deteriorate rapidly after >4 h of treatment, fails to reform upon wash-out of the microtubule depolymerizing agent ansamitocin, and spindles are on average shorter compared to those observed in untreated or MG132-treated cells in metaphase (data not shown). How the trypanosome CPC controls spindle formation and/or stability remains unknown. It is possible that the apparent instability of the spindle results from a destabilization of KT-MT attachments in the absence of Aurora B^{AUK1} activity at kinetochores. Another possibility is that Aurora B^{AUK1} regulates the activity of microtubule depolymerases at the kinetochore, similarly to the CPC-dependent phosphorylation of MCAK observed in other eukaryotes. Across model eukaryotes, various defects in spindle formation (such as reduction in spindle MTs, defects in centrosome separation/spindle bi-polarity etc.) have also been reported upon loss-of-function of Aurora A, which localizes to the spindle poles (reviewed in (Magnaghi-Jaulin et al., 2019)) However, trypanosomes lack such an ‘Aurora A-like’ Aurora kinase. Thus, it is conceivable that Aurora

B^{AUK1} integrates both Aurora B- and Aurora A-like functionalities, including regulation of spindle stability.

My results indicate that Aurora B^{AUK1} may regulate three key processes at the kinetochore: The stability of KT-MT attachments at each kinetochore, the stability of the mitotic spindle and the entry into anaphase. Thus, the CPC acts as a master regulator of chromosome segregation in *T. brucei*. I envisage that the CPC may be involved in various pathways at the kinetoplastid kinetochore. Disentangling the contributions of Aurora B^{AUK1} to chromosome segregation in kinetoplastids and exploring its regulatory crosstalk with the other kinetochore-localized kinases (KKT10/19, KKT2, KKT3, CDK^{CRK3}) will prove to be a challenging task but is bound to provide important insights into the evolution of the mitotic circuitry governing eukaryotic cell division.

8. Concluding remarks

Kinetoplastid diseases continue to affect the poorest in society and as our planet warms and globalization advances, the insect vectors responsible for transmission will inevitably roam beyond current habitats, expanding the burden of disease. Research into the molecular cell biology of kinetoplastids is a crucial step in the development of new antiparasitics, which are urgently needed to improve global health and prosperity. I aim to contribute to this cause by revealing key regulatory principles behind kinetoplastid kinetochores using cell biological, biochemical, and structural approaches. During my DPhil in the Akiyoshi lab, I was able to show that two orphan kinesins, KIN-A and KIN-B, are constitutive components of the CPC in trypanosomes and are primarily responsible for targeting the CPC to kinetochores and the central spindle. I further demonstrated that the activity of the Aurora B kinase, the catalytic subunit of the CPC, acts as the main switch controlling metaphase-to-anaphase transition and that Aurora B is involved in an unconventional regulatory circuitry with KKT14, a recently identified Bub1/BubR1-like protein. Further work will be aimed at dissecting the molecular details of this novel regulatory pathway.

9. References

- Abad, M.A., T. Gupta, M.A. Hadders, A. Meppelink, J.P. Wopken, E. Blackburn, J. Zou, A. Gireesh, L. Buzuk, D.A. Kelly, T. McHugh, J. Rappsilber, S.M.A. Lens, and A.A. Jeyaprakash. 2022. Mechanistic basis for Sgo1-mediated centromere localization and function of the CPC. *Journal of Cell Biology*. 221. doi:10.1083/JCB.202108156/213318.
- Abad, M.A., B. Medina, A. Santamaria, J. Zou, C. Plasberg-Hill, A. Madhumalar, U. Jayachandran, P.M. Redli, J. Rappsilber, E.A. Nigg, and A.A. Jeyaprakash. 2014. Structural basis for microtubule recognition by the human kinetochore Ska complex. *Nature Communications* 2014 5:1. 5:1–14. doi:10.1038/ncomms3964.
- Adams, R.R., H. Maiato, W.C. Earnshaw, and M. Carmena. 2001. Essential Roles of Drosophila Inner Centromere Protein (Incenp) and Aurora B in Histone H3 Phosphorylation, Metaphase Chromosome Alignment, Kinetochore Disjunction, and Chromosome Segregation. *Journal of Cell Biology*. 153:865–880. doi:10.1083/JCB.153.4.865.
- Adams, R.R., S.P. Wheatleya, A.M. Gouldsworthy, S.E. Kandels-Lewis, M. Carmena, C. Smythe, D.L. Gerloff, and W.C. Earnshaw. 2000. INCENP binds the Aurora-related kinase AIRK2 and is required to target it to chromosomes, the central spindle and cleavage furrow. *Current Biology*. 10. doi:10.1016/S0960-9822(00)00673-4.
- Ahonen, L.J., A.M. Kukkonen, J. Pouwels, M.A. Bolton, C.D. Jingle, P.T. Stukenberg, and M.J. Kallio. 2009. Perturbation of Incenp function impedes anaphase chromatid movements and chromosomal passenger protein flux at centromeres. *Chromosoma*. 118:71–84. doi:10.1007/S00412-008-0178-0.
- Akiyoshi, B. 2020. Analysis of a Mad2 homolog in Trypanosoma brucei provides possible hints on the origin of the spindle checkpoint. *bioRxiv*.
- Akiyoshi, B., and K. Gull. 2013. Evolutionary cell biology of chromosome segregation: insights from trypanosomes. *Open Biol.* 3. doi:10.1098/rsob.130023.
- Akiyoshi, B., and K. Gull. 2014. Discovery of Unconventional Kinetochores in Kinetoplastids. *Cell*. 156. doi:10.1016/j.cell.2014.01.049.
- Akiyoshi, B., K.K. Sarangapani, A.F. Powers, C.R. Nelson, S.L. Reichow, H. Arellano-Santoyo, T. Gonen, J.A. Ranish, C.L. Asbury, and S. Biggins. 2010. Tension directly stabilizes reconstituted kinetochore-microtubule attachments. *Nature*. 468. doi:10.1038/nature09594.
- Alfieri, C., L. Chang, Z. Zhang, J. Yang, S. Maslen, M. Skehel, and D. Barford. 2016. Molecular basis of APC/C regulation by the spindle assembly checkpoint. *Nature*. 536. doi:10.1038/nature19083.

- Alfieri, C., S. Zhang, and D. Barford. 2017. Visualizing the complex functions and mechanisms of the anaphase promoting complex/cyclosome (APC/C). *Open Biol.* 7. doi:10.1098/RSOB.170204.
- Allen, J.W.A., A.P. Jackson, D.J. Rigden, A.C. Willis, S.J. Ferguson, and M.L. Ginger. 2008. Order within a mosaic distribution of mitochondrial c-type cytochrome biogenesis systems? *FEBS J.* 275:2385–2402. doi:10.1111/J.1742-4658.2008.06380.X.
- Allshire, R.C., and G.H. Karpen. 2008. Epigenetic regulation of centromeric chromatin: old dogs, new tricks? *Nat Rev Genet.* 9. doi:10.1038/nrg2466.
- Aslett, M., C. Aurrecochea, M. Berriman, J. Brestelli, B.P. Brunk, M. Carrington, D.P. Depledge, S. Fischer, B. Gajria, X. Gao, M.J. Gardner, A. Gingle, G. Grant, O.S. Harb, M. Heiges, C. Hertz-Fowler, R. Houston, F. Innamorato, J. Iodice, J.C. Kissinger, E. Kraemer, W. Li, F.J. Logan, J.A. Miller, S. Mitra, P.J. Myler, V. Nayak, C. Pennington, I. Phan, D.F. Pinney, G. Ramasamy, M.B. Rogers, D.S. Roos, C. Ross, D. Sivam, D.F. Smith, G. Srinivasamoorthy, C.J. Stoeckert, S. Subramanian, R. Thibodeau, A. Tivey, C. Treatman, G. Velarde, and H. Wang. 2010. TriTrypDB: a functional genomic resource for the Trypanosomatidae. *Nucleic Acids Res.* 38. doi:10.1093/NAR/GKP851.
- Auckland, P., N.I. Clarke, S.J. Royle, and A.D. McAinsh. 2017. Congressing kinetochores progressively load Ska complexes to prevent force-dependent detachment. *J Cell Biol.* 216:1623–1639. doi:10.1083/JCB.201607096.
- Ault, J.G., and R.B. Nicklas. 1989. Tension, microtubule rearrangements, and the proper distribution of chromosomes in mitosis. *Chromosoma.* 98:33–39. doi:10.1007/BF00293332.
- Ballmer, D., and B. Akiyoshi. 2024a. Dynamic localization of the chromosomal passenger complex is controlled by the orphan kinesin KIN-A in the kinetoplastid parasite *Trypanosoma brucei*. *Elife.* 13. doi:10.7554/ELIFE.93522.
- Ballmer, D., and B. Akiyoshi. 2024b. Dynamic localization of the chromosomal passenger complex in trypanosomes is controlled by the orphan kinesins KIN-A and KIN-B. *Elife.* 13. doi:10.7554/ELIFE.93522.
- Ballmer, D., W. Carter, J.J.E. van Hooff, E.C. Tromer, M. Ishii, P. Ludzia, and B. Akiyoshi. 2024a. Kinetoplastid kinetochore proteins KKT14-KKT15 are divergent Bub1/BubR1-Bub3 proteins. *bioRxiv.* 2024.01.04.574194. doi:10.1101/2024.01.04.574194.
- Ballmer, D., H.J. Lou, M. Ishii, B.E. Turk, and B. Akiyoshi. 2024b. An unconventional regulatory circuitry involving Aurora B controls anaphase onset and error-free chromosome segregation in trypanosomes. *bioRxiv.* 2024.01.20.576407. doi:10.1101/2024.01.20.576407.
- Bateman, A. 2019. UniProt: a worldwide hub of protein knowledge. *Nucleic Acids Res.* 47:D506–D515. doi:10.1093/NAR/GKY1049.

- Beardmore, V.A., L.J. Ahonen, G.J. Gorbsky, and M.J. Kallio. 2004. Survivin dynamics increases at centromeres during G2/M phase transition and is regulated by microtubule-attachment and Aurora B kinase activity. *J Cell Sci.* 117:4033–4042. doi:10.1242/JCS.01242.
- Beneke, T., R. Madden, L. Makin, J. Valli, J. Sunter, and E. Gluenz. 2017. A CRISPR Cas9 high-throughput genome editing toolkit for kinetoplastids. *R Soc Open Sci.* 4:1–16. doi:10.1098/RSOS.170095.
- Berriman, M. 2005. The Genome of the African Trypanosome *Trypanosoma brucei*. *Science* (1979). 309. doi:10.1126/science.1112642.
- Bieniossek, C., Y. Nie, D. Frey, N. Olieric, C. Schaffitzel, I. Collinson, C. Romier, P. Berger, T.J. Richmond, M.O. Steinmetz, and I. Berger. 2009. Automated unrestricted multigene recombineering for multiprotein complex production. *Nat Methods.* 6:447–450. doi:10.1038/NMETH.1326.
- Biggins, S., and A.W. Murray. 2001. The budding yeast protein kinase Ipl1/Aurora allows the absence of tension to activate the spindle checkpoint. *Genes Dev.* 15:3118. doi:10.1101/GAD.934801.
- Billington, K., C. Halliday, R. Madden, P. Dyer, A.R. Barker, F.F. Moreira-Leite, M. Carrington, S. Vaughan, C. Hertz-Fowler, S. Dean, J.D. Sunter, R.J. Wheeler, and K. Gull. 2023. Genome-wide subcellular protein map for the flagellate parasite *Trypanosoma brucei*. *Nat Microbiol.* 8:533–547. doi:10.1038/S41564-022-01295-6.
- Bioconductor - EnhancedVolcano.
- Bishop, A.C., J.A. Ubersax, D.T. Pøtsch, D.P. Matheos, N.S. Gray, J. Blethrow, E. Shimizu, J.Z. Tsien, P.G. Schultz, M.D. Rose, J.L. Wood, D.O. Morgan, and K.M. Shokat. 2000. A chemical switch for inhibitor-sensitive alleles of any protein kinase. *Nature.* 407:395–401. doi:10.1038/35030148.
- Bishop, J.D., and J.M. Schumacher. 2002. Phosphorylation of the Carboxyl Terminus of Inner Centromere Protein (INCENP) by the Aurora B Kinase Stimulates Aurora B Kinase Activity. *Journal of Biological Chemistry.* 277. doi:10.1074/jbc.C200307200.
- Bishop, J.D., and J.M. Schuniacher. 2002. Phosphorylation of the carboxyl terminus of inner centromere protein (INCENP) by the Aurora B Kinase stimulates Aurora B kinase activity. *J Biol Chem.* 277:27577–27580. doi:10.1074/JBC.C200307200.
- Black, B.E., and D.W. Cleveland. 2011. Epigenetic Centromere Propagation and the Nature of CENP-A Nucleosomes. *Cell.* 144. doi:10.1016/j.cell.2011.02.002.
- Bloom, K.S. 2014. Centromeric Heterochromatin: The Primordial Segregation Machine. *Annu Rev Genet.* 48. doi:10.1146/annurev-genet-120213-092033.

- Blum, B., N. Bakalara, and L. Simpson. 1990. A model for RNA editing in kinetoplastid mitochondria: “guide” RNA molecules transcribed from maxicircle DNA provide the edited information. *Cell*. 60:189–198. doi:10.1016/0092-8674(90)90735-W.
- Brautigan, D.L., and S. Shenolikar. 2018. Protein Serine/Threonine Phosphatases: Keys to Unlocking Regulators and Substrates. *Annu Rev Biochem*. 87:921–964. doi:10.1146/ANNUREV-BIOCHEM-062917-012332.
- Brenchley, R., H. Tariq, H. McElhinney, B. Szöör, J. Huxley-Jones, R. Stevens, K. Matthews, and L. Tabernero. 2007. The TriTryp phosphatome: analysis of the protein phosphatase catalytic domains. *BMC Genomics*. 8. doi:10.1186/1471-2164-8-434.
- Broad, A.J., K.F. DeLuca, and J.G. DeLuca. 2020. Aurora B kinase is recruited to multiple discrete kinetochore and centromere regions in human cells. *J Cell Biol*. 219. doi:10.1083/JCB.201905144.
- Brun, R., and M. Schönenberger. 1979. Cultivation and in vitro cloning or procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. *Acta Trop*. 36:289–292.
- Brusini, L., S. D’Archivio, J. McDonald, and B. Wickstead. 2021. Trypanosome KKIP1 Dynamically Links the Inner Kinetochore to a Kinetoplastid Outer Kinetochore Complex. *Front Cell Infect Microbiol*. 11. doi:10.3389/fcimb.2021.641174.
- Butenko, A., F.R. Opperdoes, O. Flegontova, A. Horák, V. Hampl, P. Keeling, R.M.R. Gawryluk, D. Tikhonenkov, P. Flegontov, and J. Lukeš. 2020. Evolution of metabolic capabilities and molecular features of diplomonads, kinetoplastids, and euglenids. *BMC Biology* 2020 18:1. 18:1–28. doi:10.1186/S12915-020-0754-1.
- Caldas, G. V., K.F. DeLuca, and J.G. DeLuca. 2013. KNL1 facilitates phosphorylation of outer kinetochore proteins by promoting Aurora B kinase activity. *J Cell Biol*. 203:957–969. doi:10.1083/JCB.201306054.
- Campbell, C.S., and A. Desai. 2013. Tension sensing by Aurora B kinase is independent of survivin-based centromere localization. *Nature* 2013 497:7447. 497:118–121. doi:10.1038/nature12057.
- Carbon, J., and L. Clarke. 1984. Structural and functional analysis of a yeast centromere (CEN3). *J Cell Sci Suppl*. 1:43–58. doi:10.1242/JCS.1984.SUPPLEMENT_1.4.
- Carmena, M., M. Wheelock, H. Funabiki, and W.C. Earnshaw. 2012. The chromosomal passenger complex (CPC): from easy rider to the godfather of mitosis. *Nat Rev Mol Cell Biol*. 13. doi:10.1038/nrm3474.
- Carroll, C.W., K.J. Milks, and A.F. Straight. 2010. Dual recognition of CENP-A nucleosomes is required for centromere assembly. *J Cell Biol*. 189:1143–1155. doi:10.1083/JCB.201001013.

- Carroll, C.W., M.C.C. Silva, K.M. Godek, L.E.T. Jansen, and A.F. Straight. 2009. Centromere assembly requires the direct recognition of CENP-A nucleosomes by CENP-N. *Nat Cell Biol.* 11:896–902. doi:10.1038/NCB1899.
- Cavalier-Smith, T. 2010. Kingdoms Protozoa and Chromista and the eozoan root of the eukaryotic tree. *Biol Lett.* 6:342–345. doi:10.1098/RSBL.2009.0948.
- Chan, Y.W., A.A. Jeyaprakash, E.A. Nigg, and A. Santamaria. 2012. Aurora B controls kinetochore-microtubule attachments by inhibiting Ska complex-KMN network interaction. *Journal of Cell Biology.* 196:563–571. doi:10.1083/JCB.201109001/VIDEO-5.
- Chao, W.C.H., K. Kulkarni, Z. Zhang, E.H. Kong, and D. Barford. 2012. Structure of the mitotic checkpoint complex. *Nature.* 484:208–213. doi:10.1038/NATURE10896.
- Cheeseman, I.M., S. Anderson, M. Jwa, E.M. Green, J. Kang, J.R. Yates, C.S.M. Chan, D.G. Drubin, and G. Barnes. 2002. Phospho-Regulation of Kinetochore-Microtubule Attachments by the Aurora Kinase Ipl1p. *Cell.* 111. doi:10.1016/S0092-8674(02)00973-X.
- Cheeseman, I.M., J.S. Chappie, E.M. Wilson-Kubalek, and A. Desai. 2006. The Conserved KMN Network Constitutes the Core Microtubule-Binding Site of the Kinetochore. *Cell.* 127. doi:10.1016/j.cell.2006.09.039.
- Cheeseman, I.M., and A. Desai. 2008. Molecular architecture of the kinetochore-microtubule interface. *Nat Rev Mol Cell Biol.* 9:33–46. doi:10.1038/NRM2310.
- Chen, Z.L., J.M. Meng, Y. Cao, J.L. Yin, R.Q. Fang, S.B. Fan, C. Liu, W.F. Zeng, Y.H. Ding, D. Tan, L. Wu, W.J. Zhou, H. Chi, R.X. Sun, M.Q. Dong, and S.M. He. 2019. A high-speed search engine pLink 2 with systematic evaluation for proteome-scale identification of cross-linked peptides. *Nature Communications* 2019 10:1. 10:1–12. doi:10.1038/s41467-019-11337-z.
- Cho, U.S., and S.C. Harrison. 2011. Ndc10 is a platform for inner kinetochore assembly in budding yeast. *Nat Struct Mol Biol.* 19:48–56. doi:10.1038/NSMB.2178.
- Ciferri, C., J. De Lucall, S. Monzani, K.J. Ferrari, D. Ristic, C. Wyman, H. Stark, J. Kilmartin, E.D. Salmon, and A. Musacchio. 2005. Architecture of the human ndc80-hec1 complex, a critical constituent of the outer kinetochore. *J Biol Chem.* 280:29088–29095. doi:10.1074/JBC.M504070200.
- Ciferri, C., S. Pasqualato, E. Screpanti, G. Varetto, S. Santaguida, G. Dos Reis, A. Maiolica, J. Polka, J.G. De Luca, P. De Wulf, M. Salek, J. Rappsilber, C.A. Moores, E.D. Salmon, and A. Musacchio. 2008. Implications for Kinetochore-Microtubule Attachment from the Structure of an Engineered Ndc80 Complex. *Cell.* 133:427–439. doi:10.1016/j.cell.2008.03.020.

- Clarke, L., and J. Carbon. 1980. Isolation of a yeast centromere and construction of functional small circular chromosomes. *Nature*. 287:504–509. doi:10.1038/287504A0.
- Clarke, L., and J. Carbon. 1983. Genomic substitutions of centromeres in *Saccharomyces cerevisiae*. *Nature*. 305:23–28. doi:10.1038/305023A0.
- Cooke, C.A., M.M.S. Heck, and W.C. Earnshaw. 1987. The inner centromere protein (INCENP) antigens: movement from inner centromere to midbody during mitosis. *J Cell Biol*. 105:2053–2067. doi:10.1083/JCB.105.5.2053.
- Copenhaver, G.P., K. Nickel, T. Kuromori, M.I. Benito, S. Kaul, X. Lin, M. Bevan, G. Murphy, B. Harris, L.D. Parnell, W.R. McCombie, R.A. Martienssen, M. Marra, and D. Preuss. 1999. Genetic definition and sequence analysis of *Arabidopsis* centromeres. *Science*. 286:2468–2474. doi:10.1126/SCIENCE.286.5449.2468.
- Cormier, A., D.G. Drubin, and G. Barnes. 2013. Phosphorylation regulates kinase and microtubule binding activities of the budding yeast chromosomal passenger complex in vitro. *J Biol Chem*. 288:23203–23211. doi:10.1074/JBC.M113.491480.
- Cross, G.A.M. 1977. Antigenic variation in trypanosomes. *Am J Trop Med Hyg*. 26:240–244. doi:10.4269/AJTMH.1977.26.240.
- Daniels, J.-P., K. Gull, and B. Wickstead. 2010. Cell Biology of the Trypanosome Genome. *Microbiol Mol Biol Rev*. 74:552. doi:10.1128/MMBR.00024-10.
- D'Archivio, S., and B. Wickstead. 2017. Trypanosome outer kinetochore proteins suggest conservation of chromosome segregation machinery across eukaryotes. *Journal of Cell Biology*. 216. doi:10.1083/jcb.201608043.
- Das, A., M. Gale, V. Carter, and M. Parsons. 1994. The protein phosphatase inhibitor okadaic acid induces defects in cytokinesis and organellar genome segregation in *Trypanosoma brucei*. *J Cell Sci*. 107 (Pt 12):3477–3483. doi:10.1242/JCS.107.12.3477.
- Dean, S., J. Sunter, R.J. Wheeler, I. Hodgkinson, E. Gluenz, and K. Gull. 2015. A toolkit enabling efficient, scalable and reproducible gene tagging in trypanosomatids. *Open Biol*. 5. doi:10.1098/RSOB.140197.
- DeLuca, K.F., S.M.A. Lens, and J.G. DeLuca. 2011. Temporal changes in Hec1 phosphorylation control kinetochore–microtubule attachment stability during mitosis. *J Cell Sci*. 124:622. doi:10.1242/JCS.072629.
- Deutsch, E.W., N. Bandeira, V. Sharma, Y. Perez-Riverol, J.J. Carver, D.J. Kundu, D. García-Seisdedos, A.F. Jarnuczak, S. Hewapathirana, B.S. Pullman, J. Wertz, Z. Sun, S. Kawano, S. Okuda, Y. Watanabe, H. Hermjakob, B. Maclean, M.J. Maccoss, Y. Zhu, Y. Ishihama, and J.A. Vizcaíno. 2020. The ProteomeXchange consortium in 2020: enabling 'big data' approaches in proteomics. *Nucleic Acids Res*. 48:D1145. doi:10.1093/NAR/GKZ984.

- Diaz-Martinez, L.A., W. Tian, B. Li, R. Warrington, L. Jia, C.A. Brautigam, X. Luo, and H. Yu. 2015. The Cdc20-binding Phe box of the spindle checkpoint protein BubR1 maintains the mitotic checkpoint complex during mitosis. *J Biol Chem.* 290:2431–2443. doi:10.1074/JBC.M114.616490.
- DiFiore, B., N.E. Davey, A. Hagting, D. Izawa, J. Mansfeld, T.J. Gibson, and J. Pines. 2015. The ABBA motif binds APC/C activators and is shared by APC/C substrates and regulators. *Dev Cell.* 32:358–372. doi:10.1016/J.DEVCEL.2015.01.003.
- Drinnenberg, I.A., and B. Akiyoshi. 2017. Evolutionary Lessons from Species with Unique Kinetochores. *Prog Mol Subcell Biol.* 56:111–138. doi:10.1007/978-3-319-58592-5_5/TABLES/1.
- Eddy, S.R. 1998. Profile hidden Markov models. *Bioinformatics.* 14:755–763. doi:10.1093/BIOINFORMATICS/14.9.755.
- Endow, S.A., F.J. Kull, and H. Liu. 2010. Kinesins at a glance. *J Cell Sci.* 123:3420. doi:10.1242/JCS.064113.
- Ersfeld, K. 2011. Nuclear architecture, genome and chromatin organisation in *Trypanosoma brucei*. *Res Microbiol.* 162:626–636. doi:10.1016/J.RESMIC.2011.01.014.
- Espeut, J., D.K. Cheerambathur, L. Krenning, K. Oegema, and A. Desai. 2012. Microtubule binding by KNL-1 contributes to spindle checkpoint silencing at the kinetochore. *Journal of Cell Biology.* 196:469–482. doi:10.1083/JCB.201111107/VIDEO-4.
- Evans, R., M. O'Neill, A. Pritzel, N. Antropova, A. Senior, T. Green, A. Židek, R. Bates, S. Blackwell, J. Yim, O. Ronneberger, S. Bodenstein, M. Zielinski, A. Bridgland, A. Potapenko, A. Cowie, K. Tunyasuvunakool, R. Jain, E. Clancy, P. Kohli, J. Jumper, and D. Hassabis. 2022. Protein complex prediction with AlphaFold-Multimer. doi:10.1101/2021.10.04.463034.
- Falk, S.J., L.Y. Guo, N. Sekulic, E.M. Smoak, T. Mani, G.A. Logsdon, K. Gupta, L.E.T. Jansen, G.D. Van Duyne, S.A. Vinogradov, M.A. Lampson, and B.E. Black. 2015. Chromosomes. CENP-C reshapes and stabilizes CENP-A nucleosomes at the centromere. *Science.* 348:699–703. doi:10.1126/SCIENCE.1259308.
- Ferguson, M.A., S.W. Homans, R.A. Dwek, and T.W. Rademacher. 1988. The glycosylphosphatidylinositol membrane anchor of *Trypanosoma brucei* variant surface glycoprotein. *Biochem Soc Trans.* 16:265–268. doi:10.1042/BST0160265.
- Field, M.C., D. Horn, A.H. Fairlamb, M.A.J. Ferguson, D.W. Gray, K.D. Read, M. De Rycker, L.S. Torrie, P.G. Wyatt, S. Wyllie, and I.H. Gilbert. 2017. Anti-trypanosomatid drug discovery: an ongoing challenge and a continuing need. *Nat Rev Microbiol.* 15:217–231. doi:10.1038/NRMICRO.2016.193.

- Fink, S., K. Turnbull, A. Desai, and C.S. Campbell. 2017. An engineered minimal chromosomal passenger complex reveals a role for INCENP/Sli15 spindle association in chromosome biorientation. *J Cell Biol.* 216:911–923. doi:10.1083/JCB.201609123.
- Fischböck-Halwachs, J., S. Singh, M. Potocnjak, G. Hagemann, V. Solis-Mezarino, S. Woike, M. Ghodgaonkar-Steger, F. Weissmann, L.D. Gallego, J. Rojas, J. Andreani, A. Köhler, and F. Herzog. 2019. The COMA complex interacts with Cse4 and positions Sli15/Ipl1 at the budding yeast inner kinetochore. *Elife.* 8. doi:10.7554/ELIFE.42879.
- Foley, E.A., and T.M. Kapoor. 2012. Microtubule attachment and spindle assembly checkpoint signalling at the kinetochore. *Nature Reviews Molecular Cell Biology* 2013 14:1. 14:25–37. doi:10.1038/nrm3494.
- Foltz, D.R., L.E.T. Jansen, B.E. Black, A.O. Bailey, J.R. Yates, and D.W. Cleveland. 2006. The human CENP-A centromeric nucleosome-associated complex. *Nat Cell Biol.* 8. doi:10.1038/ncb1397.
- Fujiwara, K., and R.W. Linck. 1982. The Use of Tannic Acid in Microtubule Research. *Methods Cell Biol.* 24:217–233. doi:10.1016/S0091-679X(08)60657-3.
- Fukagawa, T., and W.C. Earnshaw. 2014. The Centromere: Chromatin Foundation for the Kinetochore Machinery. *Dev Cell.* 30:496. doi:10.1016/J.DEVCEL.2014.08.016.
- Fuller, B.G., M.A. Lampson, E.A. Foley, S. Rosasco-Nitcher, K. V. Le, P. Tobelmann, D.L. Brautigan, P.T. Stukenberg, and T.M. Kapoor. 2008. Midzone activation of aurora B in anaphase produces an intracellular phosphorylation gradient. *Nature* 2008 453:7198. 453:1132–1136. doi:10.1038/nature06923.
- García-Rodríguez, L.J., T. Kasciukovic, V. Denninger, and T.U. Tanaka. 2019. Aurora B-INCENP Localization at Centromeres/Inner Kinetochores Is Required for Chromosome Bi-orientation in Budding Yeast. *Current Biology.* 29:1536-1544.e4. doi:10.1016/J.CUB.2019.03.051.
- Gascoigne, K.E., K. Takeuchi, A. Suzuki, T. Hori, T. Fukagawa, and I.M. Cheeseman. 2011. Induced ectopic kinetochore assembly bypasses the requirement for CENP-A nucleosomes. *Cell.* 145:410–422. doi:10.1016/J.CELL.2011.03.031.
- Gassmann, R., A. Carvalho, A.J. Henzing, S. Ruchaud, D.F. Hudson, R. Honda, E.A. Nigg, D.L. Gerloff, and W.C. Earnshaw. 2004. Borealin: a novel chromosomal passenger required for stability of the bipolar mitotic spindle. *Journal of Cell Biology.* 166. doi:10.1083/jcb.200404001.
- Ghongane, P., M. Kapanidou, A. Asghar, S. Elowe, and V.M. Bolanos-Garcia. 2014. The dynamic protein Knl1 - a kinetochore rendezvous. *J Cell Sci.* 127:3415–3423. doi:10.1242/JCS.149922.

- Graham, M., C. Combe, L. Kolbowski, and J. Rappsilber. 2019. xiView: A common platform for the downstream analysis of Crosslinking Mass Spectrometry data. *bioRxiv*. 561829. doi:10.1101/561829.
- Gruneberg, U., R. Neef, R. Honda, E.A. Nigg, and F.A. Barr. 2004. Relocation of Aurora B from centromeres to the central spindle at the metaphase to anaphase transition requires MKlp2. *J Cell Biol*. 166:167–172. doi:10.1083/JCB.200403084.
- Guerra, M., G. Cabral, M. Cuacos, M. González-García, M. González-Sánchez, J. Vega, and M.J. Puertas. 2010. Neocentrics and holokinetics (holocentrics): chromosomes out of the centromeric rules. *Cytogenet Genome Res*. 129:82–96. doi:10.1159/000314289.
- Hadders, M.A., S. Hindriksen, M.A. Truong, A.N. Mhaskar, J. Pepijn Wopken, M.J.M. Vromans, and S.M.A. Lens. 2020. Untangling the contribution of Haspin and Bub1 to Aurora B function during mitosis. *Journal of Cell Biology*. 219. doi:10.1083/JCB.201907087/133700.
- Hauf, S., R.W. Cole, S. LaTerra, C. Zimmer, G. Schnapp, R. Walter, A. Heckel, J. van Meel, C.L. Rieder, and J.-M. Peters. 2003. The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore–microtubule attachment and in maintaining the spindle assembly checkpoint. *Journal of Cell Biology*. 161. doi:10.1083/jcb.200208092.
- Hayashi, H., and B. Akiyoshi. 2018. Degradation of cyclin B is critical for nuclear division in *Trypanosoma brucei*. *Biol Open*. doi:10.1242/bio.031609.
- Hendrickx, A., M. Beullens, H. Ceulemans, T. Den Abt, A. Van Eynde, E. Nicolaescu, B. Lesage, and M. Bollen. 2009. Docking motif-guided mapping of the interactome of protein phosphatase-1. *Chem Biol*. 16:365–371. doi:10.1016/J.CHEMBIOL.2009.02.012.
- Hengeveld, R.C.C., M.J.M. Vromans, M. Vleugel, M.A. Hadders, and S.M.A. Lens. 2017. Inner centromere localization of the CPC maintains centromere cohesion and allows mitotic checkpoint silencing. *Nat Commun*. 8. doi:10.1038/NCOMMS15542.
- Heroes, E., B. Lesage, J. Görnemann, M. Beullens, L. Van Meervelt, and M. Bollen. 2013. The PP1 binding code: a molecular-lego strategy that governs specificity. *FEBS J*. 280:584–595. doi:10.1111/J.1742-4658.2012.08547.X.
- Hertz, E.P.T., T. Kruse, N.E. Davey, B. López-Méndez, J.O. Sigurðsson, G. Montoya, J. V. Olsen, and J. Nilsson. 2016. A Conserved Motif Provides Binding Specificity to the PP2A-B56 Phosphatase. *Mol Cell*. 63:686–695. doi:10.1016/J.MOLCEL.2016.06.024.
- Herzog, F., I. Primorac, P. Dube, P. Lenart, B. Sander, K. Mechtler, H. Stark, and J.M. Peters. 2009. Structure of the anaphase-promoting complex/cyclosome interacting with a mitotic checkpoint complex. *Science*. 323:1477–1481. doi:10.1126/SCIENCE.1163300.

- Hiruma, Y., C. Sacristan, S.T. Pachis, A. Adamopoulos, T. Kuijt, M. Ubbink, E. Von Castelmur, A. Perrakis, and G.J.P.L. Kops. 2015. CELL DIVISION CYCLE. Competition between MPS1 and microtubules at kinetochores regulates spindle checkpoint signaling. *Science*. 348:1264–1267. doi:10.1126/SCIENCE.AAA4055.
- Holland, A.J., and D.W. Cleveland. 2009. Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. *Nat Rev Mol Cell Biol*. 10:478–487. doi:10.1038/NRM2718.
- Honda, R., R. Körner, and E.A. Nigg. 2003. Exploring the Functional Interactions between Aurora B, INCENP, and Survivin in Mitosis. *Mol Biol Cell*. 14. doi:10.1091/mbc.e02-11-0769.
- Hooff, J.J., E. Tromer, L.M. Wijk, B. Snel, and G.J. Kops. 2017. Evolutionary dynamics of the kinetochore network in eukaryotes as revealed by comparative genomics. *EMBO Rep*. 18. doi:10.15252/embr.201744102.
- van Hooff, J.J., E. Tromer, L.M. van Wijk, B. Snel, and G.J. Kops. 2017. Evolutionary dynamics of the kinetochore network in eukaryotes as revealed by comparative genomics. *EMBO Rep*. 18:1559–1571. doi:10.15252/EMBR.201744102.
- Hori, T., and T. Fukagawa. 2012. Establishment of the vertebrate kinetochores. *Chromosome Research*. 20. doi:10.1007/s10577-012-9289-9.
- van der Horst, A., M.J.M. Vromans, K. Bouwman, M.S. van der Waal, M.A. Hadders, and S.M.A. Lens. 2015. Inter-domain Cooperation in INCENP Promotes Aurora B Relocation from Centromeres to Microtubules. *Cell Rep*. 12:380–387. doi:10.1016/J.CELREP.2015.06.038.
- Hu, H., Z. Yu, Y. Liu, T. Wang, Y. Wei, and Z. Li. 2014. The Aurora B kinase in *Trypanosoma brucei* undergoes post-translational modifications and is targeted to various subcellular locations through binding to TbCPC1. *Mol Microbiol*. 91. doi:10.1111/mmi.12458.
- Hughes, C.S., S. Moggridge, T. Müller, P.H. Sorensen, G.B. Morin, and J. Krijgsveld. 2019. Single-pot, solid-phase-enhanced sample preparation for proteomics experiments. *Nat Protoc*. 14:68–85. doi:10.1038/S41596-018-0082-X.
- Huisin'T Veld, P.J., S. Jeganathan, A. Petrovic, P. Singh, J. John, V. Krenn, F. Weissmann, T. Bange, and A. Musacchio. 2016. Molecular basis of outer kinetochore assembly on CENP-T. *Elife*. 5. doi:10.7554/ELIFE.21007.
- Hümmer, S., and T.U. Mayer. 2009. Cdk1 Negatively Regulates Midzone Localization of the Mitotic Kinesin Mklp2 and the Chromosomal Passenger Complex. *Current Biology*. 19:607–612. doi:10.1016/J.CUB.2009.02.046.

- Hutti, J.E., E.T. Jarrell, J.D. Chang, D.W. Abbott, P. Storz, A. Toker, L.C. Cantley, and B.E. Turk. 2004. A rapid method for determining protein kinase phosphorylation specificity. *Nature Methods* 2004 1:1. 1:27–29. doi:10.1038/nmeth708.
- INGEBRITSEN, T.S., and P. COHEN. 1983. The protein phosphatases involved in cellular regulation. 1. Classification and substrate specificities. *Eur J Biochem.* 132:255–261. doi:10.1111/J.1432-1033.1983.TB07357.X.
- Integrating Neglected Tropical Diseases into Global Health and Development: Fourth World Health Organization Report on Neglected Tropical Diseases (WHO, 2017). 2017.
- Ishii, M., and B. Akiyoshi. 2020. Characterization of unconventional kinetochore kinases KKT10/19 in *Trypanosoma brucei*. *J Cell Sci.* doi:10.1242/jcs.240978.
- Ishii, M., and B. Akiyoshi. 2022. Targeted protein degradation using deGradFP in *Trypanosoma brucei*. *Wellcome Open Res.* 7:175. doi:10.12688/WELLCOMEOPENRES.17964.2.
- Ishii, M., P. Ludzia, G. Marciànò, W. Allen, O.O. Nerusheva, and B. Akiyoshi. 2022. Divergent polo boxes in KKT2 bind KKT1 to initiate the kinetochore assembly cascade in *Trypanosoma brucei*. *Mol Biol Cell.* 33. doi:10.1091/MBE.E22-07-0269-T.
- Izawa, D., and J. Pines. 2015. The mitotic checkpoint complex binds a second CDC20 to inhibit active APC/C. *Nature.* 517:631–634. doi:10.1038/NATURE13911.
- Izuta, H., M. Ikeno, N. Suzuki, T. Tomonaga, N. Nozaki, C. Obuse, Y. Kisu, N. Goshima, F. Nomura, N. Nomura, and K. Yoda. 2006. Comprehensive analysis of the ICEN (Interphase Centromere Complex) components enriched in the CENP-A chromatin of human cells. *Genes to Cells.* 11. doi:10.1111/j.1365-2443.2006.00969.x.
- Jenni, S., and S.C. Harrison. 2018. Structure of the DASH/Dam1 complex shows its role at the yeast kinetochore-microtubule interface. *Science.* 360:552–558. doi:10.1126/SCIENCE.AAR6436.
- Jeyaprakash, A.A., C. Basquin, U. Jayachandran, and E. Conti. 2011. Structural Basis for the Recognition of Phosphorylated Histone H3 by the Survivin Subunit of the Chromosomal Passenger Complex. *Structure.* 19:1625–1634. doi:10.1016/J.STR.2011.09.002.
- Jeyaprakash, A.A., U.R. Klein, D. Lindner, J. Ebert, E.A. Nigg, and E. Conti. 2007. Structure of a Survivin–Borealin–INCENP Core Complex Reveals How Chromosomal Passengers Travel Together. *Cell.* 131. doi:10.1016/j.cell.2007.07.045.
- Jeyaprakash, A.A., A. Santamaria, U. Jayachandran, Y.W. Chan, C. Benda, E.A. Nigg, and E. Conti. 2012. Structural and functional organization of the Ska complex, a key component of the kinetochore-microtubule interface. *Mol Cell.* 46:274–286. doi:10.1016/J.MOLCEL.2012.03.005.

- Ji, Z., H. Gao, and H. Yu. 2015. CELL DIVISION CYCLE. Kinetochore attachment sensed by competitive Mps1 and microtubule binding to Ndc80C. *Science*. 348:1260–1264. doi:10.1126/SCIENCE.AAA4029.
- Johnson, J.L., T.M. Yaron, E.M. Huntsman, A. Kerelsky, J. Song, A. Regev, T.Y. Lin, K. Liberatore, D.M. Cizin, B.M. Cohen, N. Vasan, Y. Ma, K. Krismer, J.T. Robles, B. van de Kooij, A.E. van Vlimmeren, N. Andrée-Busch, N.F. Käufer, M. V. Dorovkov, A.G. Ryazanov, Y. Takagi, E.R. Kasthuber, M.D. Goncalves, B.D. Hopkins, O. Elemento, D.J. Taatjes, A. Maucuer, A. Yamashita, A. Degterev, M. Uduman, J. Lu, S.D. Landry, B. Zhang, I. Cossentino, R. Linding, J. Blenis, P. V. Hornbeck, B.E. Turk, M.B. Yaffe, and L.C. Cantley. 2023. An atlas of substrate specificities for the human serine/threonine kinome. *Nature*. 613:759–766. doi:10.1038/S41586-022-05575-3.
- Jones, N.G., E.B. Thomas, E. Brown, N.J. Dickens, T.C. Hammarton, and J.C. Mottram. 2014. Regulators of Trypanosoma brucei cell cycle progression and differentiation identified using a kinome-wide RNAi screen. *PLoS Pathog*. 10. doi:10.1371/JOURNAL.PPAT.1003886.
- Jumper, J., R. Evans, A. Pritzel, T. Green, M. Figurnov, O. Ronneberger, K. Tunyasuvunakool, R. Bates, A. Židek, A. Potapenko, A. Bridgland, C. Meyer, S.A.A. Kohli, A.J. Ballard, A. Cowie, B. Romera-Paredes, S. Nikolov, R. Jain, J. Adler, T. Back, S. Petersen, D. Reiman, E. Clancy, M. Zielinski, M. Steinegger, M. Pacholska, T. Berghammer, S. Bodenstein, D. Silver, O. Vinyals, A.W. Senior, K. Kavukcuoglu, P. Kohli, and D. Hassabis. 2021. Highly accurate protein structure prediction with AlphaFold. *Nature* 2021 596:7873. 596:583–589. doi:10.1038/s41586-021-03819-2.
- Kang, J.S., I.M. Cheeseman, G. Kallstrom, S. Velmurugan, G. Barnes, and C.S.M. Chan. 2001. Functional cooperation of Dam1, Ipl1, and the inner centromere protein (INCENP)-related protein Sli15 during chromosome segregation. *J Cell Biol*. 155:763–774. doi:10.1083/JCB.200105029.
- Kato, H., J. Jiang, B.R. Zhou, M. Rozendaal, H. Feng, R. Ghirlando, T.S. Xiao, A.F. Straight, and Y. Bai. 2013. A conserved mechanism for centromeric nucleosome recognition by centromere protein CENP-C. *Science*. 340:1110–1113. doi:10.1126/SCIENCE.1235532.
- Katoh, K., J. Rozewicki, and K.D. Yamada. 2019. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief Bioinform*. 20:1160–1166. doi:10.1093/BIB/BBX108.
- Kawashima, S.A., Y. Yamagishi, T. Honda, K.I. Lshiguro, and Y. Watanabe. 2010. Phosphorylation of H2A by Bub1 prevents chromosomal instability through localizing shugoshin. *Science (1979)*. 327:172–177. doi:10.1126/SCIENCE.1180189/SUPPL_FILE/KAWASHIMA-SOM.REVISION.1.PDF.

- Kelly, A.E., C. Ghenoïu, J.Z. Xue, C. Zierhut, H. Kimura, and H. Funabiki. 2010. Survivin reads phosphorylated histone H3 threonine 3 to activate the mitotic kinase Aurora B. *Science*. 330:235–239. doi:10.1126/SCIENCE.1189505.
- Kelly, A.E., S.C. Sampath, T.A. Maniar, E.M. Woo, B.T. Chait, and H. Funabiki. 2007a. Chromosomal enrichment and activation of the aurora B pathway are coupled to spatially regulate spindle assembly. *Dev Cell*. 12:31–43. doi:10.1016/J.DEVCEL.2006.11.001.
- Kelly, S., J. Reed, S. Kramer, L. Ellis, H. Webb, J. Sunter, J. Salje, N. Marinsek, K. Gull, B. Wickstead, and M. Carrington. 2007b. Functional genomics in *Trypanosoma brucei*: A collection of vectors for the expression of tagged proteins from endogenous and ectopic gene loci. *Mol Biochem Parasitol*. 154. doi:10.1016/j.molbiopara.2007.03.012.
- Kim, J., J. Kang, and C.S.M. Chan. 1999. Sli15 Associates with the Ipl1 Protein Kinase to Promote Proper Chromosome Segregation in *Saccharomyces cerevisiae*. *Journal of Cell Biology*. 145. doi:10.1083/jcb.145.7.1381.
- Kitagawa, M., S.Y.S. Fung, U.F.S. Hameed, H. Goto, M. Inagaki, and S.H. Lee. 2014. Cdk1 coordinates timely activation of MKlp2 kinesin with relocation of the chromosome passenger complex for cytokinesis. *Cell Rep*. 7:166–179. doi:10.1016/J.CELREP.2014.02.034.
- Kitajima, T.S., T. Sakuno, K.I. Ishiguro, S.I. Iemura, T. Natsume, S.A. Kawashima, and Y. Watanabe. 2006. Shugoshin collaborates with protein phosphatase 2A to protect cohesin. *Nature*. 441:46–52. doi:10.1038/NATURE04663.
- Klare, K., J.R. Weir, F. Basilico, T. Zimniak, L. Massimiliano, N. Ludwigs, F. Herzog, and A. Musacchio. 2015. CENP-C is a blueprint for constitutive centromere-associated network assembly within human kinetochores. *Journal of Cell Biology*. 210:11–22. doi:10.1083/JCB.201412028.
- Klein, U.R., E.A. Nigg, and U. Gruneberg. 2006. Centromere targeting of the chromosomal passenger complex requires a ternary subcomplex of Borealin, Survivin, and the N-terminal domain of INCENP. *Mol Biol Cell*. 17:2547–2558. doi:10.1091/MBC.E05-12-1133.
- Komaki, S., E.C. Tromer, G. De Jaeger, N. De Winne, M. Heese, and A. Schnittger. 2022. Molecular convergence by differential domain acquisition is a hallmark of chromosomal passenger complex evolution. *Proc Natl Acad Sci U S A*. 119. doi:10.1073/PNAS.2200108119/-/DCSUPPLEMENTAL.
- Kops, G.J.P.L., B. Snel, and E.C. Tromer. 2020. Evolutionary Dynamics of the Spindle Assembly Checkpoint in Eukaryotes. *Curr Biol*. 30:R589–R602. doi:10.1016/J.CUB.2020.02.021.

- Kostygov, A.Y., A. Karnkowska, J. Votýpka, D. Tashyreva, K. Maciszewski, V. Yurchenko, and J. Lukeš. 2021. Euglenozoa: taxonomy, diversity and ecology, symbioses and viruses. *Open Biol.* 11. doi:10.1098/rsob.200407.
- Krenn, V., and A. Musacchio. 2015. The Aurora B Kinase in Chromosome Bi-Orientation and Spindle Checkpoint Signaling. *Front Oncol.* 5. doi:10.3389/FONC.2015.00225.
- Krenn, V., A. Wehenkel, X. Li, S. Santaguida, and A. Musacchio. 2012. Structural analysis reveals features of the spindle checkpoint kinase Bub1–kinetochore subunit Knl1 interaction. *Journal of Cell Biology.* 196:451–467. doi:10.1083/JCB.201110013.
- Landeira, D., and M. Navarro. 2007. Nuclear repositioning of the VSG promoter during developmental silencing in *Trypanosoma brucei*. *Journal of Cell Biology.* 176. doi:10.1083/jcb.200607174.
- Lara-Gonzalez, P., J. Pines, and A. Desai. 2021. Spindle assembly checkpoint activation and silencing at kinetochores. *Semin Cell Dev Biol.* 117:86–98. doi:10.1016/J.SEMCDB.2021.06.009.
- Larsen, N.A., J. Al-Bassam, R.R. Wei, and S.C. Harrison. 2007. Structural analysis of Bub3 interactions in the mitotic spindle checkpoint. *Proc Natl Acad Sci U S A.* 104:1201. doi:10.1073/PNAS.0610358104.
- Leitner, A., R. Reischl, T. Walzthoeni, F. Herzog, S. Bohn, F. Förster, and R. Aebersold. 2012. Expanding the chemical cross-linking toolbox by the use of multiple proteases and enrichment by size exclusion chromatography. *Mol Cell Proteomics.* 11. doi:10.1074/MCP.M111.014126.
- Li, Y., J. Bachant, A.A. Alcasabas, Y. Wang, J. Qin, and S.J. Elledge. 2002. The mitotic spindle is required for loading of the DASH complex onto the kinetochore. *Genes Dev.* 16:183. doi:10.1101/GAD.959402.
- Li, Z. 2012. Regulation of the Cell Division Cycle in *Trypanosoma brucei*. *Eukaryot Cell.* 11:1180. doi:10.1128/EC.00145-12.
- Li, Z., J.H. Lee, F. Chu, A.L. Burlingame, A. Günzl, and C.C. Wang. 2008a. Identification of a Novel Chromosomal Passenger Complex and Its Unique Localization during Cytokinesis in *Trypanosoma brucei*. *PLoS One.* 3. doi:10.1371/journal.pone.0002354.
- Li, Z., X. Tu, and C.C. Wang. 2006. Okadaic acid overcomes the blocked cell cycle caused by depleting Cdc2-related kinases in *Trypanosoma brucei*. *Exp Cell Res.* 312:3504–3516. doi:10.1016/J.YEXCR.2006.07.022.
- Li, Z., T. Umeyama, and C.C. Wang. 2008b. The Chromosomal Passenger Complex and a Mitotic Kinesin Interact with the Tausled-Like Kinase in Trypanosomes to Regulate Mitosis and Cytokinesis. *PLoS One.* 3. doi:10.1371/journal.pone.0003814.

- Li, Z., T. Umeyama, and C.C. Wang. 2009. The Aurora Kinase in *Trypanosoma brucei* Plays Distinctive Roles in Metaphase-Anaphase Transition and Cytokinetic Initiation. *PLoS Pathog.* 5. doi:10.1371/journal.ppat.1000575.
- Li, Z., and C.C. Wang. 2006. Changing Roles of Aurora-B Kinase in Two Life Cycle Stages of *Trypanosoma brucei*. *Eukaryot Cell.* 5. doi:10.1128/EC.00129-06.
- Liang, C., Z. Zhang, Q. Chen, H. Yan, M. Zhang, L. Zhou, J. Xu, W. Lu, and F. Wang. 2020. Centromere-localized Aurora B kinase is required for the fidelity of chromosome segregation. *J Cell Biol.* 219. doi:10.1083/JCB.201907092.
- Liu, D., G. Vader, M.J.M. Vromans, M.A. Lampson, and S.M.A. Lens. 2009. Sensing chromosome bi-orientation by spatial separation of aurora B kinase from kinetochore substrates. *Science.* 323:1350–1353. doi:10.1126/SCIENCE.1167000.
- Liu, D., M. Vleugel, C.B. Backer, T. Hori, T. Fukagawa, I.M. Cheeseman, and M.A. Lampson. 2010. Regulated targeting of protein phosphatase 1 to the outer kinetochore by KNL1 opposes Aurora B kinase. *J Cell Biol.* 188:809–820. doi:10.1083/JCB.201001006.
- Liu, H., L. Jia, and H. Yu. 2013. Phospho-H2A and Cohesin Specify Distinct Tension-Regulated Sgo1 Pools at Kinetochores and Inner Centromeres. *Current Biology.* 23:1927–1933. doi:10.1016/J.CUB.2013.07.078.
- Llauró, A., H. Hayashi, M.E. Bailey, A. Wilson, P. Ludzia, C.L. Asbury, and B. Akiyoshi. 2018. The kinetoplastid kinetochore protein KKT4 is an unconventional microtubule tip–coupling protein. *Journal of Cell Biology.* 217. doi:10.1083/jcb.201711181.
- London, N., S. Ceto, J.A. Ranish, and S. Biggins. 2012. Phosphoregulation of Spc105 by Mps1 and PP1 regulates Bub1 localization to kinetochores. *Curr Biol.* 22:900–906. doi:10.1016/J.CUB.2012.03.052.
- Ludzia, P., E.D. Lowe, G. Marcianò, S. Mohammed, C. Redfield, and B. Akiyoshi. 2021. Structural characterization of KKT4, an unconventional microtubule-binding kinetochore protein. *Structure.* 29:1014-1028.e8. doi:10.1016/J.STR.2021.04.004.
- Mackay, A.M., D.M. Eckley, C. Chue, and W.C. Earnshaw. 1993. Molecular analysis of the INCENPs (inner centromere proteins): separate domains are required for association with microtubules during interphase and with the central spindle during anaphase. *J Cell Biol.* 123:373–385. doi:10.1083/JCB.123.2.373.
- Mackey, T.K., B.A. Liang, R. Cuomo, R. Hafen, K.C. Brouwer, and D.E. Lee. 2014. Emerging and Reemerging Neglected Tropical Diseases: a Review of Key Characteristics, Risk Factors, and the Policy and Innovation Environment. *Clin Microbiol Rev.* 27:949–979. doi:10.1128/CMR.00045-14.
- Maddox, P.S., K.D. Corbett, and A. Desai. 2012. Structure, assembly and reading of centromeric chromatin. *Curr Opin Genet Dev.* 22. doi:10.1016/j.gde.2011.11.005.

- Magnaghi-Jaulin, L., G. Eot-Houllier, E. Gallaud, and R. Giet. 2019. Aurora A Protein Kinase: To the Centrosome and Beyond. *Biomolecules*. 9. doi:10.3390/BIOM9010028.
- Malvezzi, F., G. Litos, A. Schleiffer, A. Heuck, K. Mechtler, T. Clausen, and S. Westermann. 2013. A structural basis for kinetochore recruitment of the Ndc80 complex via two distinct centromere receptors. *EMBO J*. 32:409–423. doi:10.1038/EMBOJ.2012.356.
- Marchetti, M.A., C. Tschudi, H. Kwon, S.L. Wolin, and E. Ullu. 2000. Import of proteins into the trypanosome nucleus and their distribution at karyokinesis. *J Cell Sci*. 113 (Pt 5):899–906. doi:10.1242/JCS.113.5.899.
- Marciano, G., M. Ishii, O. Nerusheva, and B. Akiyoshi. 2019. Unconventional kinetochore kinases KKT2 and KKT3 have unique centromere localization domains. *bioRxiv*.
- Marcianò, G., M. Ishii, O.O. Nerusheva, and B. Akiyoshi. 2021. Kinetoplastid kinetochore proteins KKT2 and KKT3 have unique centromere localization domains. *J Cell Biol*. 220. doi:10.1083/JCB.202101022.
- McAinsh, A.D., and A.L. Marston. 2022. The Four Causes: The Functional Architecture of Centromeres and Kinetochore. *Annu Rev Genet*. 56:279–314. doi:10.1146/ANNUREV-GENET-072820-034559.
- McGrew, J., B. Diehl, and M. Fitzgerald-Hayes. 1986. Single base-pair mutations in centromere element III cause aberrant chromosome segregation in *Saccharomyces cerevisiae*. *Mol Cell Biol*. 6:530–538. doi:10.1128/MCB.6.2.530-538.1986.
- McKinley, K.L., and I.M. Cheeseman. 2016. The molecular basis for centromere identity and function. *Nat Rev Mol Cell Biol*. 17:16–29. doi:10.1038/NRM.2015.5.
- Meppelink, A., L. Kabeche, M.J.M. Vromans, D.A. Compton, and S.M.A. Lens. 2015a. Shugoshin-1 balances Aurora B kinase activity via PP2A to promote chromosome bi-orientation. *Cell Rep*. 11:508–515. doi:10.1016/J.CELREP.2015.03.052.
- Meppelink, A., L. Kabeche, M.J.M. Vromans, D.A. Compton, and S.M.A. Lens. 2015b. Shugoshin-1 balances Aurora B kinase activity via PP2A to promote chromosome bi-orientation. *Cell Rep*. 11:508–515. doi:10.1016/J.CELREP.2015.03.052.
- Meraldi, P., R. Honda, and E.A. Nigg. 2004. Aurora kinases link chromosome segregation and cell division to cancer susceptibility. *Curr Opin Genet Dev*. 14:29–36. doi:10.1016/J.GDE.2003.11.006.
- Meraldi, P., A. McAinsh, E. Rheinbay, and P. Sorger. 2006. Phylogenetic and structural analysis of centromeric DNA and kinetochore proteins. *Genome Biol*. 7. doi:10.1186/gb-2006-7-3-r23.
- Miranda, J.J.L., P. De Wulf, P.K. Sorger, and S.C. Harrison. 2005. The yeast DASH complex forms closed rings on microtubules. *Nat Struct Mol Biol*. 12:138–143. doi:10.1038/NSMB896.

- Mirdita, M., K. Schütze, Y. Moriwaki, L. Heo, S. Ovchinnikov, and M. Steinegger. 2022. ColabFold: making protein folding accessible to all. *Nature Methods* 2022 19:6. 19:679–682. doi:10.1038/s41592-022-01488-1.
- Morens, D.M., G.K. Folkers, and A.S. Fauci. 2004. The challenge of emerging and re-emerging infectious diseases. *Nature*. 430:242–249. doi:10.1038/nature02759.
- Musacchio, A. 2015. The Molecular Biology of Spindle Assembly Checkpoint Signaling Dynamics. *Current Biology*. 25. doi:10.1016/j.cub.2015.08.051.
- Musacchio, A., and A. Desai. 2017. A Molecular View of Kinetochore Assembly and Function. *Biology (Basel)*. 6. doi:10.3390/biology6010005.
- Nakajima, Y., A. Cormier, R.G. Tyers, A. Pigula, Y. Peng, D.G. Drubin, and G. Barnes. 2011. Ipl1/Aurora-dependent phosphorylation of Sli15/INCENP regulates CPC-spindle interaction to ensure proper microtubule dynamics. *J Cell Biol.* 194:137–153. doi:10.1083/JCB.201009137.
- Nakajima, Y., R.G. Tyers, C.C.L. Wong, J.R. Yates, D.G. Drubin, and G. Barnes. 2009. Nbl1p: A Borealin/Dasra/CSC-1-like Protein Essential for Aurora/Ipl1 Complex Function and Integrity in *Saccharomyces cerevisiae*. *Mol Biol Cell*. 20. doi:10.1091/mbc.e08-10-1011.
- Nerusheva, O.O., and B. Akiyoshi. 2016. Divergent polo box domains underpin the unique kinetoplastid kinetochore. *Open Biol.* 6. doi:10.1098/rsob.150206.
- Nerusheva, O.O., P. Ludzia, and B. Akiyoshi. 2019. Identification of four unconventional kinetoplastid kinetochore proteins KKT22–25 in *Trypanosoma brucei*. *Open Biol.* 9. doi:10.1098/rsob.190236.
- Nicklas, R.B., and C.A. Koch. 1969. Chromosome micromanipulation. 3. Spindle fiber tension and the reorientation of mal-oriented chromosomes. *J Cell Biol.* 43:40–50. doi:10.1083/jcb.43.1.40.
- Nicklas, R.B., and S.C. Ward. 1994. Elements of error correction in mitosis: microtubule capture, release, and tension. *J Cell Biol.* 126:1241–1253. doi:10.1083/JCB.126.5.1241.
- Nishino, T., F. Rago, T. Hori, K. Tomii, I.M. Cheeseman, and T. Fukagawa. 2013a. CENP-T provides a structural platform for outer kinetochore assembly. *EMBO J.* 32:424. doi:10.1038/EMBOJ.2012.348.
- Nishino, T., F. Rago, T. Hori, K. Tomii, I.M. Cheeseman, and T. Fukagawa. 2013b. CENP-T provides a structural platform for outer kinetochore assembly. *EMBO J.* 32:424–436. doi:10.1038/EMBOJ.2012.348.
- Noujaim, M., S. Bechstedt, M. Wieczorek, and G.J. Brouhard. 2014. Microtubules accelerate the kinase activity of Aurora-B by a reduction in dimensionality. *PLoS One*. 9. doi:10.1371/JOURNAL.PONE.0086786.

- Obado, S.O., C. Bot, D. Nilsson, B. Andersson, and J.M. Kelly. 2007. Repetitive DNA is associated with centromeric domains in *Trypanosoma brucei* but not *Trypanosoma cruzi*. *Genome Biol.* 8:1–14. doi:10.1186/GB-2007-8-3-R37/FIGURES/8.
- Ogbadoyi, E., K. Ersfeld, D. Robinson, T. Sherwin, and K. Gull. 2000. Architecture of the *Trypanosoma brucei* nucleus during interphase and mitosis. *Chromosoma*. 108:501–513.
- Okada, M., I.M. Cheeseman, T. Hori, K. Okawa, I.X. McLeod, J.R. Yates, A. Desai, and T. Fukagawa. 2006. The CENP-H–I complex is required for the efficient incorporation of newly synthesized CENP-A into centromeres. *Nat Cell Biol.* 8. doi:10.1038/ncb1396.
- Okada, Y., and N. Hirokawa. 1999. A processive single-headed motor: Kinesin superfamily protein KIF1A. *Science (1979)*. 283:1152–1157. doi:10.1126/SCIENCE.283.5405.1152.
- Orr, B., F. De Sousa, A.M. Gomes, O. Afonso, L.T. Ferreira, A.C. Figueiredo, and H. Maiato. 2021. An anaphase surveillance mechanism prevents micronuclei formation from frequent chromosome segregation errors. *Cell Rep.* 37. doi:10.1016/J.CELREP.2021.109783.
- Papini, D., M.D. Levasseur, and J.M.G. Higgins. 2021. The Aurora B gradient sustains kinetochore stability in anaphase. *Cell Rep.* 37. doi:10.1016/J.CELREP.2021.109818.
- Parsons, M., E.A. Worthey, P.N. Ward, and J.C. Mottram. 2005. Comparative analysis of the kinomes of three pathogenic trypanosomatids: *Leishmania major*, *Trypanosoma brucei* and *Trypanosoma cruzi*. *BMC Genomics*. 6. doi:10.1186/1471-2164-6-127.
- Pentakota, S., K. Zhou, C. Smith, S. Maffini, A. Petrovic, G.P. Morgan, J.R. Weir, I.R. Vetter, A. Musacchio, and K. Luger. 2017. Decoding the centromeric nucleosome through CENP-N. *Elife*. 6. doi:10.7554/ELIFE.33442.
- Perez-Riverol, Y., A. Csordas, J. Bai, M. Bernal-Llinares, S. Hewapathirana, D.J. Kundu, A. Inuganti, J. Griss, G. Mayer, M. Eisenacher, E. Pérez, J. Uszkoreit, J. Pfeuffer, T. Sachsenberg, Ş. Yilmaz, S. Tiwary, J. Cox, E. Audain, M. Walzer, A.F. Jarnuczak, T. Ternent, A. Brazma, and J.A. Vizcaíno. 2019. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.* 47:D442–D450. doi:10.1093/NAR/GKY1106.
- Pesenti, M.E., T. Raisch, D. Conti, I.R. Vetter, S. Raunser, and A. Musacchio. 2022. Structure of the human inner kinetochore CCAN complex and its significance for human centromere organization. doi:10.1016/j.molcel.2022.04.027.
- Pesenti, M.E., J.R. Weir, and A. Musacchio. 2016. Progress in the structural and functional characterization of kinetochores. *Curr Opin Struct Biol.* 37:152–163. doi:10.1016/J.SBI.2016.03.003.
- Petrovic, A., J. Keller, Y. Liu, K. Overlack, J. John, Y.N. Dimitrova, S. Jenni, S. van Gerwen, P. Stege, S. Wohlgemuth, P. Rombaut, F. Herzog, S.C. Harrison, I.R. Vetter, and A.

- Musacchio. 2016. Structure of the MIS12 Complex and Molecular Basis of Its Interaction with CENP-C at Human Kinetochores. *Cell*. 167:1028-1040.e15. doi:10.1016/J.CELL.2016.10.005.
- Petrovic, A., S. Mosalaganti, J. Keller, M. Mattiuzzo, K. Overlack, V. Krenn, A. DeAntoni, S. Wohlgemuth, V. Cecatiello, S. Pasqualato, S. Raunser, and A. Musacchio. 2014. Modular assembly of RWD domains on the Mis12 complex underlies outer kinetochore organization. *Mol Cell*. 53:591–605. doi:10.1016/J.MOLCEL.2014.01.019.
- Petrovic, A., S. Pasqualato, P. Dube, V. Krenn, S. Santaguida, D. Cittaro, S. Monzani, L. Massimiliano, J. Keller, A. Tarricone, A. Maiolica, H. Stark, and A. Musacchio. 2010. The MIS12 complex is a protein interaction hub for outer kinetochore assembly. *J Cell Biol*. 190:835–852. doi:10.1083/JCB.201002070.
- Pines, J. 2011. Cubism and the cell cycle: the many faces of the APC/C. *Nat Rev Mol Cell Biol*. 12:427–438. doi:10.1038/NRM3132.
- Ploubidou, A., D.R. Robinson, R.C. Docherty, E.O. Ogbadoyi, and K. Gull. 1999. Evidence for novel cell cycle checkpoints in trypanosomes: kinetoplast segregation and cytokinesis in the absence of mitosis. *J Cell Sci*. 112. doi:10.1242/jcs.112.24.4641.
- Polizzi, C., and L. Clarke. 1991. The chromatin structure of centromeres from fission yeast: differentiation of the central core that correlates with function. *J Cell Biol*. 112:191–201. doi:10.1083/JCB.112.2.191.
- Posch, M., G.A. Khoudoli, S. Swift, E.M. King, J.G. DeLuca, and J.R. Swedlow. 2010. Sds22 regulates aurora B activity and microtubule–kinetochore interactions at mitosis. *J Cell Biol*. 191:61. doi:10.1083/JCB.200912046.
- Primorac, I., J.R. Weir, E. Chirolì, F. Gross, I. Hoffmann, S. van Gerwen, A. Ciliberto, and A. Musacchio. 2013. Bub3 reads phosphorylated MELT repeats to promote spindle assembly checkpoint signaling. *Elife*. 2:1030. doi:10.7554/ELIFE.01030.
- Przewlòka, M.R., Z. Venkei, V.M. Bolanos-Garcia, J. Debski, M. Dadlez, and D.M. Glover. 2011. CENP-C is a structural platform for kinetochore assembly. *Curr Biol*. 21:399–405. doi:10.1016/J.CUB.2011.02.005.
- Ramey, V.H., H.W. Wang, Y. Nakajima, A. Wong, J. Liu, D. Drubin, G. Barnes, and E. Nogales. 2011. The Dam1 ring binds to the E-hook of tubulin and diffuses along the microtubule. *Mol Biol Cell*. 22:457–466. doi:10.1091/MBE.10-10-0841/ASSET/IMAGES/LARGE/457FIG6.JPEG.
- Rao, S.P.S., M.P. Barrett, G. Dranoff, C.J. Faraday, C.R. Gimpelewicz, A. Hailu, C.L. Jones, J.M. Kelly, J.K. Lazdins-Helds, P. Mäser, J. Mengel, J.C. Mottram, C.E. Mowbray, D.L. Sacks, P. Scott, G.F. Späth, R.L. Tarleton, J.M. Spector, and T.T. Diagana. 2019. Drug Discovery for Kinetoplastid Diseases: Future Directions. *ACS Infect Dis*. 5:152–157. doi:10.1021/acsinfecdis.8b00298.

- de Regt, A.K., C.J. Clark, C.L. Asbury, and S. Biggins. 2022. Tension can directly suppress Aurora B kinase-triggered release of kinetochore-microtubule attachments. *Nature Communications* 2022 13:1. 13:1–10. doi:10.1038/s41467-022-29542-8.
- Rice, S., A.W. Lin, D. Safer, C.L. Hart, N. Naber, B.O. Carragher, S.M. Cain, E. Pechatnikova, E.M. Wilson-Kubalek, M. Whittaker, E. Pate, R. Cooke, E.W. Taylor, R.A. Milligan, and R.D. Vale. 1999. A structural change in the kinesin motor protein that drives motility. *Nature* 1999 402:6763. 402:778–784. doi:10.1038/45483.
- Robinson, D.R., T. Sherwin, A. Ploubidou, E.H. Byard, and K. Gull. 1995. Microtubule polarity and dynamics in the control of organelle positioning, segregation, and cytokinesis in the trypanosome cell cycle. *Journal of Cell Biology*. 128. doi:10.1083/jcb.128.6.1163.
- Romano, A., A. Guse, I. Krascenicova, H. Schnabel, R. Schnabel, and M. Glotzer. 2003. CSC-1: a subunit of the Aurora B kinase complex that binds to the survivin-like protein BIR-1 and the incenp-like protein ICP-1. *Journal of Cell Biology*. 161. doi:10.1083/jcb.200207117.
- Rosenberg, J.S., F.R. Cross, and H. Funabiki. 2011a. KNL1/Spc105 Recruits PP1 to Silence the Spindle Assembly Checkpoint. *Curr Biol*. 21:942. doi:10.1016/J.CUB.2011.04.011.
- Rosenberg, J.S., F.R. Cross, and H. Funabiki. 2011b. KNL1/Spc105 recruits PP1 to silence the spindle assembly checkpoint. *Curr Biol*. 21:942–947. doi:10.1016/J.CUB.2011.04.011.
- Sacristan, C., and G.J.P.L. Kops. 2015. Joined at the hip: kinetochores, microtubules, and spindle assembly checkpoint signaling. *Trends Cell Biol*. 25:21–28. doi:10.1016/J.TCB.2014.08.006.
- Saerens, D., M. Pellis, R. Loris, E. Pardon, M. Dumoulin, A. Matagne, L. Wyns, S. Muyldermans, and K. Conrath. 2005. Identification of a universal VHH framework to graft non-canonical antigen-binding loops of camel single-domain antibodies. *J Mol Biol*. 352:597–607. doi:10.1016/J.JMB.2005.07.038.
- Saldivia, M., E. Fang, X. Ma, E. Myburgh, J.B.T. Carnielli, C. Bower-Lepts, E. Brown, R. Ritchie, S.B. Lakshminarayana, Y.-L. Chen, D. Patra, E. Ornelas, H.X.Y. Koh, S.L. Williams, F. Supek, D. Paape, R. McCulloch, M. Kaiser, M.P. Barrett, J. Jiricek, T.T. Diagana, J.C. Mottram, and S.P.S. Rao. 2020. Targeting the trypanosome kinetochore with CLK1 protein kinase inhibitors. *Nat Microbiol*. 5:1207–1216. doi:10.1038/s41564-020-0745-6.
- Samejima, K., M. Platani, M. Wolny, H. Ogawa, G. Vargiu, P.J. Knight, M. Peckham, and W.C. Earnshaw. 2015. The Inner Centromere Protein (INCENP) Coil Is a Single α -Helix (SAH) Domain That Binds Directly to Microtubules and Is Important for Chromosome

- Passenger Complex (CPC) Localization and Function in Mitosis. *J Biol Chem.* 290:21460–21472. doi:10.1074/JBC.M115.645317.
- Sampath, S.C., R. Ohi, O. Leismann, A. Salic, A. Pozniakovski, and H. Funabiki. 2004. The Chromosomal Passenger Complex Is Required for Chromatin-Induced Microtubule Stabilization and Spindle Assembly. *Cell.* 118. doi:10.1016/j.cell.2004.06.026.
- Santaguida, S., and A. Amon. 2015. Short- and long-term effects of chromosome mis-segregation and aneuploidy. *Nature Reviews Molecular Cell Biology* 2015 16:8. 16:473–485. doi:10.1038/nrm4025.
- Santaguida, S., and A. Musacchio. 2009. The life and miracles of kinetochores. *EMBO J.* 28:2511–2531. doi:10.1038/EMBOJ.2009.173.
- Schiffrin, B., S.E. Radford, D.J. Brockwell, and A.N. Calabrese. 2020. PyXlinkViewer: A flexible tool for visualization of protein chemical crosslinking data within the PyMOL molecular graphics system. *Protein Sci.* 29:1851–1857. doi:10.1002/PRO.3902.
- Schmidt, J.C., H. Arthanari, A. Boeszoermenyi, N.M. Dashkevich, E.M. Wilson-Kubalek, N. Monnier, M. Markus, M. Oberer, R.A. Milligan, M. Bathe, G. Wagner, E.L. Grishchuk, and I.M. Cheeseman. 2012. The kinetochore-bound Ska1 complex tracks depolymerizing microtubules and binds to curved protofilaments. *Dev Cell.* 23:968–980. doi:10.1016/J.DEVCEL.2012.09.012.
- Schneider, C.A., W.S. Rasband, and K.W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 2012 9:7. 9:671–675. doi:10.1038/nmeth.2089.
- Screpanti, E., A. De Antoni, G.M. Alushin, A. Petrovic, T. Melis, E. Nogales, and A. Musacchio. 2011. Direct binding of Cenp-C to the Mis12 complex joins the inner and outer kinetochore. *Curr Biol.* 21:391. doi:10.1016/J.CUB.2010.12.039.
- Sen, O., J.U. Harrison, N.J. Burroughs, and A.D. McAnish. 2021. Kinetochore life histories reveal an Aurora-B-dependent error correction mechanism in anaphase. *Dev Cell.* 56:3082–3099.e5. doi:10.1016/J.DEVCEL.2021.10.007.
- Serena, M., R.N. Bastos, P.R. Elliott, and F.A. Barr. 2020. Molecular basis of MKLP2-dependent Aurora B transport from chromatin to the anaphase central spindle. *Journal of Cell Biology.* 219. doi:10.1083/JCB.201910059/151730.
- Sessa, F., M. Mapelli, C. Ciferri, C. Tarricone, L.B. Areces, T.R. Schneider, P.T. Stukenberg, and A. Musacchio. 2005. Mechanism of Aurora B activation by INCENP and inhibition by hesperadin. *Mol Cell.* 18:379–391. doi:10.1016/J.MOLCEL.2005.03.031.
- Sharp-Baker, H., and R.H. Chen. 2001. Spindle checkpoint protein Bub1 is required for kinetochore localization of Mad1, Mad2, Bub3, and CENP-E, independently of its kinase activity. *J Cell Biol.* 153:1239–1249. doi:10.1083/JCB.153.6.1239.
- Shepherd, L.A., J.C. Meadows, A.M. Sochaj, T.C. Lancaster, J. Zou, G.J. Buttrick, J. Rappsilber, K.G. Hardwick, and J.B.A. Millar. 2012. Phosphodependent recruitment of

- Bub1 and Bub3 to Spc7/KNL1 by Mph1 kinase maintains the spindle checkpoint. *Curr Biol.* 22:891–899. doi:10.1016/J.CUB.2012.03.051.
- Siegel, T.N., D.R. Hekstra, and G.A.M. Cross. 2008. Analysis of the *Trypanosoma brucei* cell cycle by quantitative DAPI imaging. *Mol Biochem Parasitol.* 160. doi:10.1016/j.molbiopara.2008.04.004.
- Smith, R.J., M.H. Cordeiro, N.E. Davey, G. Vallardi, A. Ciliberto, F. Gross, and A.T. Saurin. 2019. PP1 and PP2A Use Opposite Phospho-dependencies to Control Distinct Processes at the Kinetochore. *Cell Rep.* 28:2206. doi:10.1016/J.CELREP.2019.07.067.
- Stuart, K., R. Brun, S. Croft, A. Fairlamb, R.E. Gürtler, J. McKerrow, S. Reed, and R. Tarleton. 2008. Kinetoplastids: related protozoan pathogens, different diseases. *Journal of Clinical Investigation.* 118. doi:10.1172/JCI33945.
- Sudakin, V., G.K.T. Chan, and T.J. Yen. 2001. Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. *J Cell Biol.* 154:925–936. doi:10.1083/JCB.200102093.
- Suijkerbuijk, S.J.E., M. Vleugel, A. Teixeira, and G.J.P.L. Kops. 2012. Integration of kinase and phosphatase activities by BUBR1 ensures formation of stable kinetochore-microtubule attachments. *Dev Cell.* 23:745–755. doi:10.1016/J.DEVCEL.2012.09.005.
- Sun, X., H.D. Le, J.M. Wahlstrom, and G.H. Karpen. 2003. Sequence analysis of a functional *Drosophila* centromere. *Genome Res.* 13:182–194. doi:10.1101/GR.681703.
- Sutton, R.E., and J.C. Boothroyd. 1986. Evidence for trans splicing in trypanosomes. *Cell.* 47:527–535. doi:10.1016/0092-8674(86)90617-3.
- Tanaka, T.U., N. Rachidi, C. Janke, G. Pereira, M. Galova, E. Schiebel, M.J.R. Stark, and K. Nasmyth. 2002. Evidence that the Ipl1-Sli15 (Aurora Kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole connections. *Cell.* 108:317–329. doi:10.1016/S0092-8674(02)00633-5.
- Tanno, Y., T.S. Kitajima, T. Honda, Y. Ando, K.I. Ishiguro, and Y. Watanabe. 2010. Phosphorylation of mammalian Sgo2 by Aurora B recruits PP2A and MCAK to centromeres. *Genes Dev.* 24:2169–2179. doi:10.1101/GAD.1945310.
- Tareen, A., and J.B. Kinney. 2020. Logomaker: beautiful sequence logos in Python. *Bioinformatics.* 36:2272–2274. doi:10.1093/BIOINFORMATICS/BTZ921.
- Taylor, S.S., E. Ha, and F. McKeon. 1998. The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase. *J Cell Biol.* 142:1–11. doi:10.1083/JCB.142.1.1.
- Trivedi, P., and P.T. Stukenberg. 2016. A centromere-signaling network underlies the coordination among mitotic events. *Trends Biochem Sci.* 41:160. doi:10.1016/J.TIBS.2015.11.002.

- Tromer, E., D. Bade, B. Snel, and G.J.P.L. Kops. 2016. Phylogenomics-guided discovery of a novel conserved cassette of short linear motifs in BubR1 essential for the spindle checkpoint. *Open Biol.* 6. doi:10.1098/RSOB.160315.
- Tromer, E.C., J.J.E. van Hooff, G.J.P.L. Kops, and B. Snel. 2019. Mosaic origin of the eukaryotic kinetochore. *Proc Natl Acad Sci U S A.* 116:12873–12882. doi:10.1073/PNAS.1821945116/SUPPL_FILE/PNAS.1821945116.SD05.XLSX.
- Tromer, E.C., T.A. Wemyss, P. Ludzia, R.F. Waller, and B. Akiyoshi. 2021. Repurposing of synaptonemal complex proteins for kinetochores in Kinetoplastida. *Open Biol.* 11. doi:10.1098/RSOB.210049.
- Tseng, B.S., L. Tan, T.M. Kapoor, and H. Funabiki. 2010. Dual Detection of Chromosomes and Microtubules by the Chromosomal Passenger Complex Drives Spindle Assembly. *Dev Cell.* 18:903. doi:10.1016/J.DEVCEL.2010.05.018.
- Tsukahara, T., Y. Tanno, and Y. Watanabe. 2010. Phosphorylation of the CPC by Cdk1 promotes chromosome bi-orientation. *Nature* 2010 467:7316. 467:719–723. doi:10.1038/nature09390.
- Tu, X., P. Kumar, Z. Li, and C.C. Wang. 2006. An Aurora Kinase Homologue Is Involved in Regulating Both Mitosis and Cytokinesis in *Trypanosoma brucei*. *Journal of Biological Chemistry.* 281. doi:10.1074/jbc.M511504200.
- Uchida, K.S.K., K. Takagaki, K. Kumada, Y. Hirayama, T. Noda, and T. Hirota. 2009. Kinetochore stretching inactivates the spindle assembly checkpoint. *J Cell Biol.* 184:383–390. doi:10.1083/JCB.200811028.
- Unnikrishnan, A., B. Akiyoshi, S. Biggins, and T. Tsukiyama. 2012. An Efficient Purification System for Native Minichromosome from *Saccharomyces cerevisiae*.
- Vader, G., J.J.W. Kauw, R.H. Medema, and S.M.A. Lens. 2006. Survivin mediates targeting of the chromosomal passenger complex to the centromere and midbody. *EMBO Rep.* 7. doi:10.1038/sj.embor.7400562.
- Vallardi, G., L.A. Allan, L. Crozier, and A.T. Saurin. 2019. Division of labour between pp2a-b56 isoforms at the centromere and kinetochore. *Elife.* 8. doi:10.7554/ELIFE.42619.
- Varadkar, P., F. Abbasi, K. Takeda, J.J. Dyson, and B. McCright. 2017. PP2A-B56y is required for an efficient spindle assembly checkpoint. *Cell Cycle.* 16:1210–1219. doi:10.1080/15384101.2017.1325042.
- Wang, F., J. Dai, J.R. Daum, E. Niedzialkowska, B. Banerjee, P.T. Stukenberg, G.J. Gorbsky, and J.M.G. Higgins. 2010. Histone H3 Thr-3 phosphorylation by Haspin positions Aurora B at centromeres in mitosis. *Science.* 330:231–235. doi:10.1126/SCIENCE.1189435.
- Wang, X., J.R. Babu, J.M. Harden, S.A. Jablonski, M.H. Gazi, W.L. Lingle, P.C. De Groen, T.J. Yen, and J.M.A. Van Deursen. 2001. The mitotic checkpoint protein hBUB3 and

- the mRNA export factor hRAE1 interact with GLE2p-binding sequence (GLEBS)-containing proteins. *J Biol Chem*. 276:26559–26567. doi:10.1074/JBC.M101083200.
- Waterhouse, A.M., J.B. Procter, D.M.A. Martin, M. Clamp, and G.J. Barton. 2009. Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics*. 25:1189–1191. doi:10.1093/BIOINFORMATICS/BTP033.
- Wei, R.R., J. Al-Bassam, and S.C. Harrison. 2007. The Ndc80/HEC1 complex is a contact point for kinetochore-microtubule attachment. *Nat Struct Mol Biol*. 14:54–59. doi:10.1038/NSMB1186.
- Westermann, S., A. Avila-Sakar, H.W. Wang, H. Niederstrasser, J. Wong, D.G. Drubin, E. Nogales, and G. Barnes. 2005. Formation of a dynamic kinetochore- microtubule interface through assembly of the Dam1 ring complex. *Mol Cell*. 17:277–290. doi:10.1016/J.MOLCEL.2004.12.019.
- Westhorpe, F.G., and A.F. Straight. 2013. Functions of the centromere and kinetochore in chromosome segregation. *Curr Opin Cell Biol*. 25. doi:10.1016/j.ceb.2013.02.001.
- Wheatley, S.P., A. Carvalho, P. Vagnarelli, and W.C. Earnshaw. 2001. INCENP is required for proper targeting of Survivin to the centromeres and the anaphase spindle during mitosis. *Current Biology*. 11. doi:10.1016/S0960-9822(01)00238-X.
- Wheelock, M.S., D.J. Wynne, B.S. Tseng, and H. Funabiki. 2017. Dual recognition of chromatin and microtubules by INCENP is important for mitotic progression. *J Cell Biol*. 216:925–941. doi:10.1083/JCB.201609061.
- Wickstead, B., K. Ersfeld, and K. Gull. 2003. The mitotic stability of the minichromosomes of *Trypanosoma brucei*. *Mol Biochem Parasitol*. 132:97–100. doi:10.1016/j.molbiopara.2003.08.007.
- Wickstead, B., and K. Gull. 2006. A “Holistic” Kinesin Phylogeny Reveals New Kinesin Families and Predicts Protein Functions □ D. *Mol Biol Cell*. 17:1734–1743. doi:10.1091/mbc.E05-11-1090.
- Woods, A., T. Sherwin, R. Sasse, T.H. MacRae, A.J. Baines, and K. Gull. 1989. Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies. *J Cell Sci*. 93:491–500. doi:10.1242/JCS.93.3.491.
- Woodward, R., and K. Gull. 1990. Timing of nuclear and kinetoplast DNA replication and early morphological events in the cell cycle of *Trypanosoma brucei*. *J Cell Sci*. 95. doi:10.1242/jcs.95.1.49.
- Xu, P., E.A. Raetz, M. Kitagawa, D.M. Virshup, and S.H. Lee. 2013. BUBR1 recruits PP2A via the B56 family of targeting subunits to promote chromosome congression. *Biol Open*. 2:479–486. doi:10.1242/BIO.20134051.

- Yamagishi, Y., T. Honda, Y. Tanno, and Y. Watanabe. 2010. Two histone marks establish the inner centromere and chromosome bi-orientation. *Science*. 330:239–243. doi:10.1126/SCIENCE.1194498.
- Yamagishi, Y., C.H. Yang, Y. Tanno, and Y. Watanabe. 2012. MPS1/Mph1 phosphorylates the kinetochore protein KNL1/Spc7 to recruit SAC components. *Nat Cell Biol*. 14:746–752. doi:10.1038/NCB2515.
- Yamaguchi, M., R. VanderLinden, F. Weissmann, R. Qiao, P. Dube, N.G. Brown, D. Haselbach, W. Zhang, S.S. Sidhu, J.M. Peters, H. Stark, and B.A. Schulman. 2016. Cryo-EM of Mitotic Checkpoint Complex-Bound APC/C Reveals Reciprocal and Conformational Regulation of Ubiquitin Ligation. *Mol Cell*. 63:593–607. doi:10.1016/J.MOLCEL.2016.07.003.
- Yatskevich, S., K.W. Muir, D. Bellini, Z. Zhang, J. Yang, T. Tischer, M. Predin, T. Dendooven, S.H. McLaughlin, and D. Barford. 2022. Structure of the human inner kinetochore bound to a centromeric CENP-A nucleosome. *Science*. 376:844–852. doi:10.1126/SCIENCE.ABN3810.
- Yoon, H.J., and J. Carbon. 1999. Participation of Bir1p, a member of the inhibitor of apoptosis family, in yeast chromosome segregation events. *Proc Natl Acad Sci U S A*. 96:13208–13213. doi:10.1073/PNAS.96.23.13208.
- Yue, Z., A. Carvalho, Z. Xu, X. Yuan, S. Cardinale, S. Ribeiro, F. Lai, H. Ogawa, E. Gudmundsdottir, R. Gassmann, C.G. Morrison, S. Ruchaud, and W.C. Earnshaw. 2008. Deconstructing Survivin: comprehensive genetic analysis of Survivin function by conditional knockout in a vertebrate cell line. *J Cell Biol*. 183:279. doi:10.1083/JCB.200806118.
- Zaytsev, A. V., J.E. Mick, E. Maslennikov, B. Nikashin, J.G. De Luca, and E.L. Grishchuk. 2015. Multisite phosphorylation of the NDC80 complex gradually tunes its microtubule-binding affinity. *Mol Biol Cell*. 26:1829–1844. doi:10.1091/MBC.E14-11-1539.
- Zhang, X., A.H. Smits, G.B.A. Van Tilburg, H. Ovaa, W. Huber, and M. Vermeulen. 2018. Proteome-wide identification of ubiquitin interactions using UbiA-MS. *Nature Protocols* 2018 13:3. 13:530–550. doi:10.1038/nprot.2017.147.
- Zhu, T., Z. Dou, B. Qin, C. Jin, X. Wang, L. Xu, Z. Wang, L. Zhu, F. Liu, X. Gao, Y. Ke, Z. Wang, F. Aikhionbare, C. Fu, X. Ding, and X. Yao. 2013. Phosphorylation of microtubule-binding protein Hec1 by mitotic kinase Aurora B specifies spindle checkpoint kinase Mps1 signaling at the kinetochore. *J Biol Chem*. 288:36149–36159. doi:10.1074/JBC.M113.507970.
- Zimniak, T., V. Fitz, H. Zhou, F. Lampert, S. Opravil, K. Mechtler, P. Stolt-Bergner, and S. Westermann. 2012. Spatiotemporal regulation of Ipl1/Aurora activity by direct Cdk1 phosphorylation. *Curr Biol*. 22:787–793. doi:10.1016/J.CUB.2012.03.007.

Zofou, D., R.B. Nyasa, D.S. Nsagha, F. Ntie-Kang, H.D. Meriki, J.C.N. Assob, and V. Kuete.
2014. Control of malaria and other vector-borne protozoan diseases in the tropics:
enduring challenges despite considerable progress and achievements. *Infect Dis
Poverty*. 3:1. doi:10.1186/2049-9957-3-1.

10. Appendix

Supplemental Table S1. List of plasmids used in this work.

Plasmid/bacmid name	Description	Selection marker for trypanosomes	Source or how the plasmid/bacmid was made
pEnT5-Y (pBA1)	Endogenous YFP tagging, hygromycin	Hygromycin	Kelly et al. (2007)
pBA3	Inducible expression of RNAi constructs (head to head), integrate at 177bp	-	Wickstead et al. (2002)
pBA7	TY-YFP-AUK1, endogenous locus	Hygromycin	AUK1 5'UTR and CDS fragments were amplified from genomic DNA using BA33/BA34 and BA35/BA36 and cloned into pBA1 using XbaI/BamHI
pBA56	AUK1 RNAi (head to head), integrate at 177bp	Phleomycin	AUK1 (166-578bp of CDS) amplified from genomic DNA using BA244/BA245 and cloned into pBA3 cut with SpeI/HindIII
pBA67	TY-YFP-KKT2, endogenous locus	Hygromycin	Akiyoshi and Gull (2014)
pBA68	TY-YFP-KKT8, endogenous locus	Hygromycin	Akiyoshi and Gull (2014)
pBA70	TY-YFP-KKT3, endogenous locus	Hygromycin	Akiyoshi and Gull (2014)
pBA73	TY-YFP-KKT9, endogenous locus	Hygromycin	Akiyoshi and Gull (2014)
pBA74	TY-YFP-KKT10, endogenous locus	Hygromycin	Akiyoshi and Gull (2014)
pBA77	TY-YFP-KKT12, endogenous locus	Hygromycin	Akiyoshi and Gull (2014)
pBA82	KKT2 RNAi (head to head), integrate at 177bp	Phleomycin	Akiyoshi and Gull (2014)
pBA87	KKT7 RNAi (head to head), integrate at 177bp	Phleomycin	Akiyoshi and Gull (2014)
pBA88	KKT9 RNAi (head to head), integrate at 177bp	Phleomycin	Akiyoshi and Gull (2014)
pBA89	KKT10/19 RNAi (head to head), integrate at 177bp	Phleomycin	Akiyoshi and Gull (2014)
pBA96	TY-YFP-KKT16, endogenous locus	Hygromycin	Akiyoshi and Gull (2014)
pBA97	TY-YFP-KKT14, endogenous locus	Hygromycin	Akiyoshi and Gull (2014)
pBA99	TY-YFP-KKT15, endogenous locus	Hygromycin	Akiyoshi and Gull (2014)
pBA148	Endogenous tdTomato tagging, blastcidin	Blasticidin	Akiyoshi and Gull (2014)
pBA162	TY-tdTomato-KKT1, endogenous locus	Blasticidin	Marciano et al. (2021)
pBA164	TY-tdTomato-KKT2, endogenous locus	Blasticidin	Nerusheva and Akiyoshi (2016)
pBA202	6HIS-KKT16, KKT17, KKT18, polycistronic expression in bacteria	-	Tromer et al. (2021)
pBA214	TY-tdTomato-AUK1, endogenous locus	Blasticidin	AUK1 5'UTR and CDS fragment was cut from pBA7 using XbaI/BamHI and subcloned into pBA148
pBA215	TY-tdTomato-MAP103, endogenous locus	Blasticidin	Lauro et al. (2018)
pBA218	TY-tdTomato-KKT14, endogenous locus	Blasticidin	KKT14 5'UTR and CDS fragment was cut from pBA97 using XbaI/BamHI and subcloned into pBA148
pBA234	6HIS-KKT10, expression in bacteria	-	Ishii and Akiyoshi (2020)
pBA310	Inducible expression vector, integrate at 177bp	Phleomycin	Nerusheva and Akiyoshi (2016)
pBA314	3FLAG-KKT2, expression in insect cells	-	Ishii et al. (2022)
pBA315	3FLAG-KKT3, expression in insect cells	-	Ishii et al. (2022)
pBA334	3FLAG-KKT14, expression in insect cells	-	3FLAG-KKT14 was amplified from genomic DNA using BA745/BA746, cloned into pBA305 using EcoRI/PstI
pBA457	Codon optimized 6HIS-KKT8 KKT9 KKT11 KKT12 polycistronic expression in bacteria	-	Ishii and Akiyoshi (2020)
pBA732	Codon optimized 6HIS-KKT8 KKT9 KKT11 polycistronic expression in bacteria	-	pBA457 was cut with AflIII/BspEI followed by Klenow fill in, purification from an agarose gel and self-ligated to remove KKT12
pBA795	Inducible GFP-NLS-LacI expression vector, integrate at 177bp	Blasticidin	Ishii and Akiyoshi (2020)
pBA802	TY-tdTomato-KKT3, endogenous locus	Blasticidin	KKT3 5'UTR and CDS fragment was cut from pBA70 using XbaI/BamHI and subcloned into pBA148
pBA819	3FLAG-KKT6, KKT1, expression in insect cells	-	Ishii et al. (2022)
pBA861	KKT1 RNAi (hairpin)	Phleomycin	Marciano et al. (2021)
pBA864	KKT6 RNAi (hairpin)	Phleomycin	Marciano et al. (2021)
pBA869	KKT14 RNAi (hairpin), integrate at 177bp	Phleomycin	Marciano et al. (2021)
pBA882	KKT3, expression in insect cells	-	KKT3 was amplified from genomic DNA using BA1374/BA689, cloned into pBA306 using XmaI/NheI
pBA891	Inducible GFP-NLS-KKT7(2-261)-LacI expression, integrate at 177bp	Blasticidin	Ishii and Akiyoshi (2020)
pBA892	Endogenous tdTomato tagging, hygromycin	Hygromycin	Ishii and Akiyoshi (2020)
pBA925	SNAP-6HIS-3FLAG-KKT4, expression in insect cells	-	Lauro et al. (2018)
pBA928	TY-YFP-KKIP1, endogenous locus	Hygromycin	Lauro et al. (2018)
pBA1065	6HIS-KKT4 (115–343), expression in bacteria	-	Lauro et al. (2018)
pBA1084	3FLAG-AUK1 CPC1, expression in insect cells	-	3FLAG-AUK1 CPC1 fragment was cut from pBA938 (BAG41) using I-CeuI/BstXI and subcloned into pACEBac2 (pBA306)
pBA1090	TY-tdTomato-KKIP1, endogenous locus	Blasticidin	KKIP1 5'UTR and CDS fragment was cut from pBA928 using XbaI/BamHI and subcloned into pBA148
pBA1093	TY-tdTomato-KKT8, endogenous locus	Hygromycin	KKT8 5'UTR and CDS fragment was cut from pBA68 using XbaI/BamHI and subcloned into pBA892
pBA1095	TY-tdTomato-KKT9, endogenous locus	Hygromycin	KKT9 5'UTR and CDS fragment was cut from pBA73 using XbaI/BamHI and subcloned into pBA892
pBA1098	TY-tdTomato-KKT12, endogenous locus	Hygromycin	KKT12 5'UTR and CDS fragment was cut from pBA77 using XbaI/BamHI and subcloned into pBA892
pBA1194	pPOTv7 (eYFP, Blast), vector for PCR only tagging (POT) of target genes in <i>T. brucei</i>	Blasticidin	Dean et al. (2015)
pBA1200	KKT11 RNAi (hairpin), integrate at 177bp	Phleomycin	Ishii and Akiyoshi (2020)
pBA1201	KKT16 RNAi (hairpin), integrate at 177bp	Phleomycin	BAG62 was cut with HindIII/BamHI and subcloned into pBA310
pBA1226	TY-YFP-KKIP2, endogenous locus	Hygromycin	Nerusheva and Akiyoshi (2019)
pBA1227	TY-YFP-KKIP3, endogenous locus	Hygromycin	Nerusheva and Akiyoshi (2019)
pBA1316	KKT8 3'UTR RNAi (hairpin), integrate at 177bp	Phleomycin	Marciano et al. (2021)
pBA1317	KKT12 3'UTR RNAi (hairpin), integrate at 177bp	Phleomycin	BAG80 was cut with HindIII/BamHI and subcloned into pBA310
pBA1354	Inducible GFP-NLS-KKT9-LacI expression, integrate at 177bp	Blasticidin	KKT9 CDS was amplified from genomic DNA using BA1870/BA1871 and cloned into pBA795 cut with PacI/AscI
pBA1372	TY-tdTomato-KKT10, endogenous locus	Blasticidin	KKT10 5'UTR and CDS fragment was cut from pBA74 using XbaI/BamHI and subcloned into pBA148
pBA1373	TY-tdTomato-KKT19, endogenous locus	Blasticidin	Ishii and Akiyoshi (2020)
pBA1398	KKT4 3'UTR RNAi (hairpin), integrate at 177bp	Phleomycin	Lauro et al. (2018)
pBA1413	6HIS-KKT4 (2–114), expression in bacteria	-	Lauro et al. (2018)
pBA1419	TY-tdTomato-KKT7, endogenous locus	Blasticidin	Ishii and Akiyoshi (2020)
pBA1420	TY-tdTomato-KKT8, endogenous locus	Blasticidin	Ishii and Akiyoshi (2020)
pBA1452	3FLAG-KKT4, endogenous locus	Blasticidin	Ishii and Akiyoshi (2020)
pBA1513	6HIS-KKT4 (463–645), expression in bacteria	-	Lauro et al. (2018)
pBA1522	TY-tdTomato-AUK1, endogenous locus	Hygromycin	KKT12 5'UTR and CDS fragment was cut from pBA7 using XbaI/BamHI and subcloned into pBA892
pBA1531	3FLAG-KKT7, expression in insect cells	-	3FLAG-KKT7 was amplified from genomic DNA using BA2024/BA2028, cloned into pBA306 using XmaI/NheI
pBA1641	6HIS-KKT4 (300–488), expression in bacteria	-	Lauro et al. (2018)
pBA1711	KKT3 3'UTR RNAi (hairpin), integrate at 177bp	Phleomycin	Marciano et al. (2021)
pBA1713	KKT15 3'UTR RNAi	Phleomycin	KKT15 3'UTR fragment (329-836 bp) amplified from genomic DNA using BA555/BA556 and cloned into pBA3 cut with SpeI/HindIII

pBA2156	TY-tdTomato-KKT16, endogenous locus	Blasticidin	KKT16 5'UTR and CDS fragment was cut from pBA96 using XbaI/BamHI and subcloned into pBA148
pBA2158	pJ1339 (1173+Cas9), for CRISPR-Cas9 gene editing	Puromycin	Beneke et al. (2017)
pBA2335	Codon optimized 6HIS-CPC1 AUK1 CPC2, polycistronic expression in bacteria	-	BAG146 subcloned into pST44/pBA155 using XbaI/MluI
pBA2342	6HIS-CPC1, expression in bacteria	-	CPC1 was amplified from genomic DNA using BA3175/BA3176 and cloned into pRSFDuet-1 cut with BamHI/EcoRI
pBA2343	6HIS-CPC2, expression in bacteria	-	CPC2 was amplified from genomic DNA using BA3177/BA3178 and cloned into pRSFDuet-1 cut with BamHI/EcoRI
pBA2345	TY-YFP-AUK1, endogenous locus	Hygromycin	AUK1 CDS (500bp fragment) amplified using BA33/BA3196 and cloned into pBA7 cut with NotI/XbaI
pBA2346	Inducible GFP-NLS-CPC1 expression, integrate at 177bp	Phleomycin	CPC1 CDS was amplified from genomic DNA using BA3201/BA3204 and cloned into pBA310 using AscI/PacI
pBA2347	Inducible GFP-NLS-CPC2 expression, integrate at 177bp	Phleomycin	CPC2 CDS was amplified from genomic DNA using BA3205/BA3208 and cloned into pBA310 using AscI/PacI
pBA2349	Inducible GFP-NLS-CPC1 (2-147) expression, integrate at 177bp	Phleomycin	CPC1 (2-147) was amplified from genomic DNA using BA3201/BA3202 and cloned into pBA310 using AscI/PacI
pBA2350	Inducible GFP-NLS-CPC1 (148-263) expression, integrate at 177bp	Phleomycin	CPC1 (148-263) was amplified from genomic DNA using BA3203/BA3204 and cloned into pBA310 using AscI/PacI
pBA2351	Inducible GFP-NLS-CPC2 (2-120) expression, integrate at 177bp	Phleomycin	CPC2 (2-120) was amplified from genomic DNA using BA3205/BA3206 and cloned into pBA310 using AscI/PacI
pBA2352	Inducible GFP-NLS-CPC2 (121-250) expression, integrate at 177bp	Phleomycin	CPC2 (121-250) was amplified from genomic DNA using BA3207/BA3208 and cloned into pBA310 using AscI/PacI
pBA2353	6HIS-KKT14 (358-685), expression in bacteria	-	KKT14 (358-685) was amplified from genomic DNA using BA3182/BA3183 and cloned into pRSFDuet-1 cut with BamHI/EcoRI
pBA2358	TY-YFP-AUK1as (M106G), endogenous locus	Hygromycin	Made by site-directed mutagenesis of pBA2345 using BA3199/BA3200
pBA2373	TY-YFP-AUK1, endogenous locus	G418	pBA2358 was cut with XbaI/BamHI and subcloned into pBA191
pBA2396	3FLAG-AUK1 (K58R) CPC1, expression in insect cells	-	Mutagenesis of AUK1 K58R introduced into pBA1084 using BA3240/BA3241
pBA2412	MBP-6HIS-CPC1, expression in bacteria	-	MBP-6HIS was amplified from pBA1016 using BA3284/BA3285 and cloned into pBA2342 cut with NcoI/BamHI
pBA2439	MBP-6HIS-CPC2 (1-120), expression in bacteria	-	CPC2 (1-120) was amplified from pBA2343 using BA3320/BA3321 and cloned into pBA2412 cut with BamHI/EcoRI
pBA2454	TY-AUK1as (M106G), endogenous locus	Hygromycin	pBA2358 cut with SpeI and XbaI (to remove YFP) and self-ligated
pBA2455	TY-AUK1as (M106G), endogenous locus	G418	pBA2373 cut with SpeI and XbaI (to remove YFP) and self-ligated
pBA2458	MBP-6HIS-CPC2, expression in bacteria	-	CPC2 was amplified from pBA2335 using BA3177/BA3178 and cloned into pBA2412 cut with BamHI/EcoRI
pBA2487	CPC1 5'UTR RNAi (hairpin), integrate at 177bp	Phleomycin	BAG157 was cut with HindIII/BamHI and subcloned into pBA310
pBA2488	CPC1 3'UTR RNAi (hairpin), integrate at 177bp	Phleomycin	BAG158 was cut with HindIII/BamHI and subcloned into pBA310
pBA2489	CPC2 5'UTR RNAi (hairpin), integrate at 177bp	Phleomycin	BAG159 was cut with HindIII/BamHI and subcloned into pBA310
pBA2490	CPC2 3'UTR RNAi (hairpin), integrate at 177bp	Phleomycin	BAG160 was cut with HindIII/BamHI and subcloned into pBA310
pBA2509	Inducible GFP-NLS-KIN-B expression, integrate at 177bp	Phleomycin	KIN-B CDS was amplified from genomic DNA using BA3413/BA3416 and cloned into pBA310 using AscI/PacI
pBA2510	Inducible GFP-NLS-KIN-B (2-316) expression, integrate at 177bp	Phleomycin	KIN-B (2-316) was amplified from genomic DNA using BA3413/BA3414 and cloned into pBA310 using AscI/PacI
pBA2511	Inducible GFP-NLS-KIN-B (317-624) expression, integrate at 177bp	Phleomycin	KIN-B (317-624) was amplified from genomic DNA using BA3415/BA3416 and cloned into pBA310 using AscI/PacI
pBA2513	6HIS-KIN-B (2-316) expression in bacteria	-	KIN-B (2-316) was amplified from genomic DNA using BA3421/BA3422 and cloned into pRSFDuet-1 cut with BamHI/EcoRI
pBA2515	Inducible GFP-NLS-KIN-A expression, integrate at 177bp	Phleomycin	KIN-A CDS was amplified from genomic DNA using BA3431/BA3432 and BA3433/BA3412 and cloned into pBA310 using AscI/PacI
pBA2516	Inducible GFP-NLS-KIN-A (2-309) expression, integrate at 177bp	Phleomycin	KIN-A (2-309) was amplified from genomic DNA using BA3409/BA3410 and cloned into pBA310 using AscI/PacI
pBA2517	Inducible GFP-NLS-KIN-A (310-862) expression, integrate at 177bp	Phleomycin	KIN-A (310-862) was amplified from genomic DNA using BA3411/BA3412 and cloned into pBA310 using AscI/PacI
pBA2519	6HIS-KIN-A (2-309) expression in bacteria	-	KIN-A (2-309) was amplified from genomic DNA using BA3417/BA3418 and cloned into pRSFDuet-1 cut with BamHI/EcoRI
pBA2555	KIN-A 5'UTR RNAi (hairpin), integrate at 177bp	Phleomycin	BAG164 was cut with HindIII/BamHI and subcloned into pBA310
pBA2556	KIN-A 3'UTR RNAi (hairpin), integrate at 177bp	Phleomycin	BAG165 was cut with HindIII/BamHI and subcloned into pBA310
pBA2567	TY-tdTomato-KKIP2, endogenous locus	Blasticidin	KKIP2 5'UTR and CDS fragments were amplified from pBA1226 using BA3465/BA3466 and cloned into pBA148 using XbaI, BamHI
pBA2568	TY-tdTomato-KKIP3, endogenous locus	Blasticidin	KKIP3 5'UTR and CDS fragments were amplified from pBA1227 using BA3467/BA3468 and cloned into pBA148 using XbaI, BamHI
pBA2574	6HIS-KIN-A (545-836), expression in bacteria	-	KIN-A (545-836) was amplified from genomic DNA using BA3486/BA3484 and cloned into pRSFDuet-1 cut with BamHI/EcoRI
pBA2663	Inducible GFP-NLS-KKT14(2-357) expression, integrate at 177bp	Phleomycin	Ballmer et al. (2024)
pBA2664	Inducible GFP-NLS-KKT14(358-685) expression, integrate at 177bp	Phleomycin	Ballmer et al. (2024)
pBA2704	6HIS-KKT14 (2-357), expression in bacteria	-	KKT14 (2-357) was amplified from genomic DNA using BA3613/BA3614 and cloned into pRSFDuet-1 cut with BamHI/EcoRI
pBA2707	Inducible GFP-NLS-KKT8-LacI expression, integrate at 177bp	Blasticidin	KKT8 CDS was amplified from pBA457 using BA3644/BA3645 and cloned into pBA795 cut with PacI/AscI
pBA2721	Codon optimized 6HIS-KKT8 KKT11 KKT12 polycistronic expression in bacteria	-	pBA457 was cut with EcoRI/HindIII followed by Klenow fill in, purification from an agarose gel and self-ligated to remove KKT9
pBA2722	Codon optimized 6HIS-KKT8 KKT9 KKT12 polycistronic expression in bacteria	-	pBA457 was cut with SacI/KpnI followed by Klenow fill in, purification from an agarose gel and self-ligated to remove KKT11
pBA2768	KKT14(2-357), S/T to A mutations corresponding to AUK1 in vitro phosphosites	-	BAG189
pBA2769	KKT14(2-357), S/T to D/E mutations corresponding to AUK1 in vitro phosphosites	-	BAG190
pBA2782	CPC1(148-263)-AUK1-tdTomato-NLS-VhhGFP4	-	BAG192
pBA2791	Inducible GFP-NLS-KKT14(2-357) PD expression, integrate at 177bp	Phleomycin	Phosphodeficient (PD) KKT14(2-357) amplified from pBA2768 (BAG189) using BA3817/BA3820 and cloned into pBA310 using PacI, AscI
pBA2792	Inducible GFP-NLS-KKT14(2-357) PM expression, integrate at 177bp	Phleomycin	Phosphomimetic (PM) KKT14(2-357) amplified from pBA2769 (BAG190) using BA3817/BA3820 and cloned into pBA310 using PacI, AscI
pBA2795	Inducible CPC1(148-263)-AUK1-tdTomato-NLS-VhhGFP4, integrate at 177bp	Phleomycin	CPC1(148-263)-AUK1-tdTomato-NLS-VhhGFP4 was amplified from pBA2782 (BAG192) using BA3891/BA3892 and cloned into pBA310 using HindIII/BamHI
pBA2800	TY-YFP-PP1-1, endogenous locus	Hygromycin	Targeting sequence was amplified with BA3878/BA3879 from BAG195 and cloned into pBA1 using XbaI and BamHI
pBA2804	PP1-1-YFP-TY, endogenous locus	Hygromycin	Targeting sequence was amplified with BA33880/BA3381 from BAG196 and cloned into pBA1 using XbaI and BamHI
pBA2863	PP1-1 5'UTR (hairpin), integrate at 177bp	Phleomycin	Targeting sequence from BAG193 was subcloned into pBA310 using HindIII and BamHI
pBA2864	PP1-1 3'UTR (hairpin), integrate at 177bp	Phleomycin	Targeting sequence from BAG194 was subcloned into pBA310 using HindIII and BamHI
pBA2937	Inducible GFP-NLS-KIN-A (310-716) expression, integrate at 177bp	Phleomycin	KIN-A (310-716) was amplified from genomic DNA using BA4011/BA4013 and cloned into pBA310 using AscI/PacI
pBA2938	Inducible GFP-NLS-KIN-A (310-815) expression, integrate at 177bp	Phleomycin	KIN-A (310-815) was amplified from genomic DNA using BA4011/BA4014 and cloned into pBA310 using AscI/PacI
pBA2939	Inducible GFP-NLS-KIN-A (310-862, Δ717-743) expression, integrate at 177bp	Phleomycin	KIN-A (310-716) and (744-862) were amplified from genomic DNA using BA4011/BA4015 and BA4016/BA4017 and cloned into pBA310 using AscI/PacI
pBA2940	KIN-A-YFP-TY, endogenous locus	Hygromycin	KIN-A 3'UTR and CDS fragments were amplified from genomic DNA using BA4018/BA4019 and BA4020/BA4021 and cloned into pBA1 using HindIII, SpeI
pBA2986	KIN-A(G210A)-TY-YFP, endogenous locus	Hygromycin	Made by site-directed mutagenesis of pBA2940 using BA4095/BA4096
pBA3091	KIN-B RNAi (head to head), integrate at 177bp	Phleomycin	KIN-B (1342-1741bp of CDS) amplified from genomic DNA using BA4181/BA4182 and cloned into pBA3 cut with SpeI/HindIII
pBA3154	KIN-A(Δ816-862)-YFP, endogenous locus	Hygromycin	Deletion introduced into pBA2940 by PCR using BA4202/BA4203
pBA3155	KIN-A(Δ717-743)-YFP, endogenous locus	Hygromycin	Deletion introduced into pBA2940 by PCR using BA4204/BA4205
pRK793 (pBA1016)	MBP-6HIS-TEV-6R	-	Tropea et al. (2009)
pMig96 (pBA152)	256LacO, integrate at rDNA	Phleomycin	Landeira and Navarro (2007)
pEnT6-Y (pBA191)	Endogenous YFP tagging, G418	G418	kind gift from Dehua Lai
pPOTv7 (pBA1919)	pPOTv7 (eYFP, Hygromycin) vector for PCR only tagging (POT) of target genes in Trypanosoma brucei.	Hygromycin	Dean et al. (2015)
p2T7-177 (pBA3)	Inducible RNAi vector, integrate at 177 bp	Phleomycin	Wickstead et al. (2002)
pACEBac1 (pBA305)	Baculovirus transfer vector, acceptor vector in the MultiBac system	-	Bieniossek et al. (2008), purchased from Geneva Biotech
pACEBac2 (pBA306)	Baculovirus transfer vector, acceptor vector in the MultiBac system	-	Bieniossek et al. (2008), purchased from Geneva Biotech
pRSFDuet-1	For expression of one or two target proteins in bacteria (kanamycin)	-	Novagen, catalog number 71341-3

Supplemental Table S2. List of primers used in this work.

Primer name	sequence 5'-3'
BA33	GATCGATCTCTAGAGGAGCAGGTAGGTCAACTGAGGTCGGGCG
BA34	GATCGATCGCGGCCGCTGTATAAAGCGATCTCAATT
BA244	GATCGATCACTAGTCGCTGAAAAAGGTTGTCATT
BA245	ACTGACTGAAGCTTTGGAAAACTACTGGCGTCC
BA555	GATCGATC ACTAGT CAAAGCTGCTGTGCTCTCAC
BA556	ACTGACTG AAGCTT ATGCAATCCCAGTACCTCG
BA689	GCTAGCTTACACGCCACCGTAGAGCA
BA745	GAATTCATGGATTACAAGGATGACGACGATAAGGATTACAAGGATGACGACGATAA GGATTACAAGGATGACGACGATAAG GAAGAAGTCAATGCGTATT
BA746	ATGCATGCC TGCAGCTAACGAGGAAATCGGAGCG
BA1374	CCCGGGATGATTGGCAGACGTAGAACAAA
BA1821	GTAATGGAGTTTGTGAGGTGCTTGATGAGGAAAAATTCCTCTTCGGAGGAACCAACAGATGCTCTACGGTGGCGTGGGTTCTGGTAGTGGTTCC
BA1822	GAAATGCGACAGCAGACGGAACGGAATAAATAAAGAGAGGGCTATCTGTAATCTTTACGTACATCACTTCCAAATTTGAGAGACCTGTGC
BA1870	GATCTTAATTAAGTCTCTGGTTCTTACGCGGTC
BA1871	GATCGGCGGCCCGGATGTTCTGGAGTCTCGAA
BA2024	CCCGGGATGGATTACAAGGATGACGACGATAAAGGATTACAAGGATGACGACGATAAGGATTACAAGGATGACGACGATAAGAGCGGATATGGCGAATCTA
BA2028	GCTAGCTCACCCGGTCTCCCCCAA
BA2129	TTGGCAATTCCACATTCGTGCAATTTGGTGTCTCTCTTTTAACTAGACACATCACACCCAGGCAACAGCCTAAAAAGAGTATAATGCAGACCTGCTGC
BA2130	CGGGCTCCAAATTTCCCGGTACTATAAGGGGACATATCTCGCTTTCTGTGCGGTGAGGAAAGCTGGAGCCTACCATACTACCCGATCCTGATCC
BA2504	GAAATGGGAGCTCGAACGATCAGGACGTAGTCTGATGTTCTCGAGGAGATGAGTTGTACTGTTCTGCTATAAGCTGTGATGGTTCTGGTAGTGGTTCC
BA2564	GGAGGAAAGCAGCTGATCATATAATACGCAAGCAGCAGTTTCTCGCTCAACCGTACCATACGCACACAAACCCGCCAATTTGAGAGACCTGTGC
BA3175	GTAATTTTCAAGGATCCATGAGCAACCCTTGGAAC
BA3176	CGCCGAGCTGCAATTTCTACCTTTGAATCAAGTTTG
BA3177	GTAATTTTCAAGGATCCATGCAAGTGAAGAAAGATG
BA3178	CGCCGAGCTCGAATTTCTAGAAATGCAGAGGTAAC
BA3182	CGCCGAGCTCGAATTTCTAACGAGGAAATCGGAGC
BA3183	GTAATTTTCAAGGATCCAGGCATATGGTCTGCAGAG
BA3196	GATCGATC GCGGCCGCCAGTTTGATGTTATTGTTG
BA3199	CTCATTGGGGAGCCATGCGAGCAAGCGCATGCTTAC
BA3200	TGGCTCCCCAATGAGATAAATATCAGTTTCGTG
BA3201	CGGGATATCCATATGTTAATTAGCAACCTTGGAAACGCAAG
BA3202	ATCCGTTTAAACTGATCAGGTCATTCGTGGGGCCATGCT
BA3203	CGGGATATCCATATGTTAATTCATATAAGGAAGCGATTG
BA3204	ATCCGTTTAAACTGATCAGGTCACCTTTGAATCAAGTTTGAAC
BA3205	CGGGATATCCATATGTTAATTCAGTGGAAAGAGATGACTTATTCG
BA3206	ATCCGTTTAAACTGATCAGGTTATGCAATTAACGCCCGCTTG
BA3207	CGGGATATCCATATGTTAATTTTAGCAACACAACCTTCG
BA3208	ATCCGTTTAAACTGATCAGGTTAGAAATGCAGAGGTAACTTTC
BA3240	GCGCTCTGAGGCGCTGTCCATCAAGAAGCTCGCTG
BA3241	GACGCTCAGAGCGCACACGGAAGTTCAGCTCCTTC
BA3284	ACTTTAATAAGGAGATATACATGAAACTGAAGAAGGTAAAC
BA3285	CGTTCGAAGGTTGCTCATGATCCATGATGATGATGATGATG
BA3320	TCATCATCATCATCATGGATCCATGCGAGTGGAAAAAGA TGACCTG
BA3321	CTGCAGGCGCGCGGAGCTCGAATTTTATGCAATTCAGACGAC CGCTTG
BA3409	CGCGGGATATCCATATGTTAATTTAGTATTGAGGTTTCATGTACGCCATT CGACC
BA3410	GGATCCGTTTAAACTGATCAGGTCAGAGTACCATAACCGGATC
BA3411	CGCGGGATATCCATATGTTAATTAACATGCCACCGCTTTTCGC
BA3412	GGATCCGTTTAAACTGATCAGGTCACGACGCGCGGAGCG
BA3413	CGCGGGATATCCATATGTTAATTTAGCAAAACGACAAACCC
BA3414	GGATCCGTTTAAACTGATCAGGCTACCGACTAATGGTCTCAGC
BA3415	CGCGGGATATCCATATGTTAATTTATCATCAGGTTCTTATGTTAGTACAC
BA3416	GGATCCGTTTAAACTGATCAGGCTACACAGCGGAGTACC
BA3417	CTGGAACAACTGTATTTTCAAGGATCCAGTATTGAGGTTTCATGTACGCATTCCG ACC
BA3418	ACCTGCAGGCGCGCGGAGCTCGAATTTTATGAGTACCATAACCGGATC
BA3421	CTGGAACAACTGTATTTTCAAGGATCCAGCAAAACGACAAACCC
BA3422	ACCTGCAGGCGCGCGGAGCTCGAATTTTACCGACTAATGGTCTCAGC
BA3431	CGCGGGATATCCATATGTTAATTTAGTATTGAGGTTTCATGTAC
BA3432	GCGGCGGCACCTAATCTCATCTCAATTTGAC
BA3433	GGATGAGATTGAGTGCCGCCGCAACAAAG
BA3465	GGCATGGACGAGCTGTACAAGCTAGAGGAGCAGGTCGGAATTCG
BA3466	GAAAAGCCAACTAAATGGGAGGATCCCTCAAGCACGTAACGGATC
BA3467	GGCATGGACGAGCTGTACAAGCTAGAGGAGCAGGTCGTGGCG
BA3468	GAAAAGCCAACTAAATGGGAGGATCCCTCTGGGCGGATCGAATTAATGTTTTC
BA3475	GACAAAGTAAGCAGGTGGAAGAGCAAGATTTTTCGCTTAAATATACGGGGTCCACAGGAGCAGGGCCGGGCACTGTTGTATAATGCAGACCTGCTGC
BA3476	AGGTGCGGCTGTGATCAACTCTCTGCTCTCCTCAACTTGGCAGCGCAACTCGAAAGGACCGCGTAAGAACCAGGAGACATACTACCCGATCCTGATCCAG
BA3484	CTGCAGGCGCGCGGAGCTCGAATTTTACTCGACGTTAGGTCGTGTG
BA3486	GGAACAACTGTATTTTCAAGGATCCGATCTGCCGAATGTACAAGCGG
BA3613	ACCTGTATTTTCAAGGATCCGAAGAAGTCAATGCTATTACCCC
BA3614	GCGCCGAGCTCGAATTTCTAAGTAGTATAGTGACGGGATGATGA
BA3644	cgggataatcatalgttaataagcGTGCAAGCACCCCTCCGCT
BA3645	tcacatgataccccaaggcgagcccaAACTGCCGGAACCTACCAGT
BA3817	CGGGATATCCATATGTTAATTAAGGAAGAAGTCAATGCGTATTACCC
BA3820	ATCCGTTTAAACTGATCAGGCGGCCCTAAGTAGGTATAGTGACGGGATGATGAAC
BA3878	GCATGGACGAGCTGTACAAG TCTAGA GGAGCAGGTTCTGTA
BA3879	GAAAAGCCAACTAAATGGGCA GGATCC TATGCTCCGGGAGC
BA3880	CGAAAACAGAAACTCAACTGCAACG AAGCTT ATTGGAAGGAGCAG
BA3881	AGCTCCTCGCCCTTGCTCAC ACTAGT ACCTGCTCCTCGAA
BA3891	ACACCAAAAAGTAAATTCACAAACACGTTACCATGGAAC
BA3892	GAAAGACACTTAAGCGCAGCGGTTCTAGAAGGATCCGCTC
BA3927	ATTTCGTGTACCTTTGCATCACAGTCTTTTCGTTTTCGTGTGCTATTGTGTGCGGAAGGGAGTGGGTCGAAGGCTGAAGGTATAATGCAGACCTGCTGC
BA3927	ATTTCGTGTACCTTTGCATCACAGTCTTTTCGTTTTCGTGTGCTATTGTGTGCGGAAGGGAGTGGGTCGAAGGCTGAAGGTATAATGCAGACCTGCTGC
BA3928	AGCAATGACATGCGCCTGATGTAAGTCTGTCAAAACCCGTAGAGGCTTGTAGGTTGAATACGCAATGACTTCTTCCATACTACCCGATCCTGATCCAG
BA3928	AGCAATGACATGCGCCTGATGTAAGTCTGTCAAAACCCGTAGTAGGCTTGTAGGTTGAATACGCAATGACTTCTTCCATACTACCCGATCCTGATCCAG
BA4011	CGCGGGATATCCATATGTTAATTAACATGCCACCGCTTTTC
BA4013	GGATCCGTTTAAACTGATCAGGTTATGAGGCGCTACGTTTGGG
BA4014	GGATCCGTTTAAACTGATCAGGTTATTCCTGTATCTTCGCTGTTATTAC
BA4015	CGTCCAAAGCTGAGGCGCTACGTTTGGG
BA4016	GTAGCGCTCAGCTTTGGGACGTAATGTTCCGAGTG
BA4017	GGATCCGTTTAAACTGATCAGGTCACGACGCGGGAGCG
BA4018	CAGAAACTCAACTGCAACGAAGCTTTTCTGTTACGTTGTGAAG
BA4019	CTGCGGCCGCCCTTAACACGCTCTAAGGAAG
BA4020	CGTGTAAAGGGCGGCCGAGTATTGAGGTTTCATGTACGCAATCGACCAACGC
BA4021	GCTCCTCGCCCTTGCTCACACTAGTTCTGCTCCACGACGCGGGAGCGGCT
BA4095	CTTGC CGCCTCTGAATCCGCATCTCGTGCAAAAC
BA4096	TTTCAAGGCGGCAAGATCCACTAAGTTGATTGTAATC
BA4181	GTGGCGGCCGCCGCTCTAGAAGTAGTACTCAGAAGGTGACACAG
BA4182	CTCGAGGTGACGGTATCGATAAGCTTTTAAAGTACGTCCCGAAG
BA4202	TATAACGCGGAAGATACAGGAAGGAGCAGGAACATAGTGTG
BA4203	TTCTGTATCTTCGCGTTATTAC
BA4204	GGGTCCAAACGTAGCGCTCAGCTTTGGGACGTAATGTTCC
BA4205	TGAGGCGCTACGTTTGGG

Supplemental Table S3. List of DNA synthesis products used in this work.

[illegible]

Supplemental Table S4. List of cell lines used in this work.

Cell name	Description	parental strain	plasmids	Plasmid and primers used in PCR-based tagging or deletion	Source
SmOxP9	TREU 927/4 procyclic cells expressing T7 RNA polymerase and the tetracycline repressor to allow inducible expression	-	-		Poon et al. (2012)
BAP73	TY-YFP-AUK1	SmOxP9	pBA7		this study
BAP122	TY-YFP-KKT2	SmOxP9	pBA67		Akiyoshi and Gull (2014)
BAP123	TY-YFP-KKT8	SmOxP9	pBA68		Akiyoshi and Gull (2014)
BAP127	TY-YFP-KKT9	SmOxP9	pBA73		Akiyoshi and Gull (2014)
BAP131	TY-YFP-KKT12	SmOxP9	pBA77		Akiyoshi and Gull (2014)
BAP163	TY-YFP-KKT14	SmOxP9	pBA97		Akiyoshi and Gull (2014)
BAP165	TY-YFP-KKT15	SmOxP9	pBA99		Akiyoshi and Gull (2014)
BAP308	TY-tdTomato-KKT2	SmOxP9	pBA164		Nerusheva and Akiyoshi (2016)
BAP347	LacO at rDNA	SmOxP9	pBA152		Ishii and Akiyoshi (2020)
BAP576	TY-YFP-AUK1, KKT2 RNAi	BAP73	pBA7, pBA82		this study
BAP577	TY-YFP-AUK1, KKT7 RNAi	BAP73	pBA7, pBA87		this study
BAP664	LacO, GFP-NLS-KKT7(2-261)-LacI	BAP347	pBA152, pBA891		Ishii and Akiyoshi (2020)
BAP680	TY-YFP-KKT2, KKT14 CDS RNAi	BAP122	pBA869		Marciano et al. (2021)
BAP708	YFP-KKIP1	SmOxP9	pBA928		this study
BAP710	TY-YFP-KKIP1	SmOxP9	pBA928		Llauro et al. (2018)
BAP781	YFP-KKIP1, TY-tdTomato-KKT2	BAP708	pBA928, pBA164		this study
BAP871	LacO, TY-tdTomato-KKT8, GFP-NLS-KKT7(2-261)-LacI	BAP664	pBA152, pBA891, pBA1093		this study
BAP873	LacO, TY-tdTomato-KKT9, GFP-NLS-KKT7(2-261)-LacI	BAP664	pBA152, pBA891, pBA1095		this study
BAP874	LacO, TY-tdTomato-KKT12, GFP-NLS-KKT7(2-261)-LacI	BAP664	pBA152, pBA891, pBA1098		this study
BAP941	TY-YFP-KKT2, AUK1 RNAi	BAP122	pBA56, pBA67		this study
BAP1019	TY-YFP-AUK1, KKT10/19 RNAi	BAP73	pBA7, pBA89		this study
BAP1044	TY-tdTomato-AUK1	SmOxP9	pBA214		this study
BAP1395	LacO, TY-tdTomato-AUK1, GFP-NLS-KKT7(2-261)-LacI	BAP664	pBA152, pBA891, pBA1522		this study
BAP1515	TY-YFP-AUK1, TY-tdTomato-KKT2	BAP73	pBA7, pBA164		this study
BAP1955	PCF1339	SmOxP9	pJ1339 (pBA2158)		Beneke et al. (2017)
BAP1965	LacO, TY-tdTomato-AUK1	BAP347	pBA152, pBA1522		this study
BAP2129	YFP-KKIP1, TY-tdTomato-KKT2, AUK1 RNAi	BAP781	pBA56, pBA928, pBA164		this study
BAP2169	TY-YFP-AUK1as (M106G) (heterozygous)	SmOxP9	pBA2358		this study
BAP2190	TY-tdTomato-KKT2, GFP-NLS-CPC1	BAP308	pBA164, pBA2346		this study
BAP2191	TY-tdTomato-KKT2, GFP-NLS-CPC2	BAP308	pBA164, pBA2347		this study
BAP2193	TY-tdTomato-KKT2, GFP-NLS-CPC1 (2-147)	BAP308	pBA164, pBA2349		this study
BAP2194	TY-tdTomato-KKT2, GFP-NLS-CPC1 (148-263)	BAP308	pBA164, pBA2350		this study
BAP2195	TY-tdTomato-KKT2, GFP-NLS-CPC2 (2-120)	BAP308	pBA164, pBA2351		this study
BAP2196	TY-tdTomato-KKT2, GFP-NLS-CPC2 (121-250)	BAP308	pBA164, pBA2352		this study
BAP2198	TY-YFP-AUK1as (M106G) (homozygous)	BAP2169	pBA2358, pBA2373		this study
BAP2250	CPC1 5'UTR RNAi	SmOxP9	pBA2487		this study
BAP2251	CPC1 3'UTR RNAi	SmOxP9	pBA2488		this study
BAP2252	CPC2 5'UTR RNAi	SmOxP9	pBA2489		this study
BAP2253	CPC2 3'UTR RNAi	SmOxP9	pBA2490		this study
BAP2276	TY-YFP-AUK1, TY-tdTomato-KKT2, KKT9 RNAi	BAP1515	pBA7, pBA164, pBA88		this study
BAP2281	TY-YFP-AUK1as (M106G) (homozygous), TY-tdTomato-MAP103	BAP2198	pBA2358, pBA2373, pBA215		this study
BAP2286	TY-tdTomato-KKT2, GFP-NLS-KIN-A	BAP308	pBA2515, pBA164		this study
BAP2287	TY-tdTomato-KKT2, GFP-NLS-KIN-A (310-862)	BAP308	pBA2517, pBA164		this study
BAP2288	TY-tdTomato-KKT2, GFP-NLS-KIN-B	BAP308	pBA2509, pBA164		this study
BAP2289	TY-tdTomato-KKT2, GFP-NLS-KIN-B (2-316)	BAP308	pBA2510, pBA164		this study
BAP2290	TY-tdTomato-KKT2, GFP-NLS-KIN-B (317-624)	BAP308	pBA2511, pBA164		this study
BAP2297	TY-tdTomato-KKT2, GFP-NLS-KIN-A (2-309)	BAP308	pBA2516, pBA164		this study
BAP2312	TY-YFP-AUK1as (M106G) (homozygous), TY-tdTomato-KKIP2	BAP2198	pBA2358, pBA2373, pBA2567		this study
BAP2313	TY-YFP-AUK1as (M106G) (homozygous), TY-tdTomato-KKIP3	BAP2198	pBA2358, pBA2373, pBA2568		this study
BAP2339	TY-AUK1as (M106G) (homozygous)	SmOxP9	pBA2454, pBA2455		this study
BAP2357	TY-AUK1as (M106G) (homozygous), TY-tdTomato-KKIP1	BAP2339	pBA2454, pBA2455, pBA1090		this study
BAP2360	TY-AUK1as (M106G) (homozygous), TY-tdTomato-KKT1	BAP2339	pBA2454, pBA2455, pBA162		this study
BAP2361	TY-AUK1as (M106G) (homozygous), TY-tdTomato-KKT2	BAP2339	pBA2454, pBA2455, pBA164		this study
BAP2362	TY-AUK1as (M106G) (homozygous), TY-tdTomato-KKT14	BAP2339	pBA2454, pBA2455, pBA218		this study
BAP2363	TY-AUK1as (M106G) (homozygous), TY-tdTomato-KKT3	BAP2339	pBA2454, pBA2455, pBA802		this study
BAP2364	TY-AUK1as (M106G) (homozygous), TY-tdTomato-KKT7	BAP2339	pBA2454, pBA2455, pBA1419		this study
BAP2365	TY-AUK1as (M106G), TY-tdTomato-KKT8	BAP2339	pBA2454, pBA2455, pBA1420		this study
BAP2365	TY-AUK1as (M106G) (homozygous), TY-tdTomato-KKT8	BAP2339	pBA2454, pBA2455, pBA1420		this study
BAP2366	TY-AUK1as (M106G) (homozygous), TY-tdTomato-KKT16	BAP2339	pBA2454, pBA2455, pBA2156		this study
BAP2369	TY-YFP-AUK1, TY-tdTomato-KKT16	BAP73	pBA7, pBA2156		this study
BAP2371	TY-AUK1as (M106G) (homozygous), TY-tdTomato-KKT10	BAP2339	pBA2454, pBA2455, pBA1372		this study
BAP2372	TY-AUK1as (M106G) (homozygous), TY-tdTomato-KKT19	BAP2339	pBA2454, pBA2455, pBA1373		this study
BAP2373	TY-AUK1as (M106G), YFP-KKT22	BAP2339	pPOT tagging	BA3927, BA3928, pBA1194	this study
BAP2386	GFP-NLS-KKT14(2-357)	SmOxP9	pBA2663		this study
BAP2387	GFP-NLS-KKT14(358-685)	SmOxP9	pBA2664		this study
BAP2442	TY-YFP-AUK1as (M106G) (homozygous), 3FLAG-KKT4	BAP2198	pBA2358, pBA2373, pBA1452		this study
BAP2446	TY-YFP-AUK1, TY-tdTomato-KKT16, KKT16 RNAi	BAP2369	pBA7, pBA2156, pBA1201		this study
BAP2469	TY-YFP-AUK1as (M106G) (homozygous), KKT14 RNAi	BAP2198	pBA2358, pBA2373, pBA869		this study
BAP2504	TY-AUK1as (M106G) (homozygous), KKT4-YFP	BAP2339	pBA2454, pBA2455	BA2504/BA2564, pBA1194	this study
BAP2505	TY-AUK1as (M106G) (homozygous), GFP-NLS-KKT14(2-357)	BAP2339	pBA2358, pBA2373, pBA2663		this study
BAP2528	YFP-KIN-B (homozygous)	BAP1955		pBA1919 with BA3749/BA3750, BA3751	this study
BAP2533	TY-YFP-KKT14, KKT15 3'UTR RNAi	BAP163	pBA1713		this study
BAP2534	TY-YFP-KKT15, KKT14 CDS RNAi	BAP165	pBA869		this study
BAP2535	TY-YFP-KKT15, KKT15 3'UTR RNAi	BAP165	pBA1713		this study
BAP2549	TY-YFP-AUK1, KKT14 RNAi	BAP73	pBA7, pBA869		this study
BAP2550	TY-YFP-AUK1, KKT4 3'UTR RNAi	BAP73	pBA7, pBA1398		this study
BAP2551	TY-YFP-AUK1, KKT3 3'UTR RNAi	BAP73	pBA7, pBA1711		this study
BAP2552	TY-YFP-AUK1, CPC1 5'UTR RNAi	BAP73	pBA7, pBA2487		this study
BAP2553	TY-YFP-AUK1, CPC1 3'UTR RNAi	BAP73	pBA7, pBA2488		this study
BAP2554	TY-YFP-AUK1, CPC2 5'UTR RNAi	BAP73	pBA7, pBA2489		this study
BAP2555	TY-YFP-AUK1, CPC2 3'UTR RNAi	BAP73	pBA7, pBA2490		this study
BAP2557	TY-YFP-AUK1, KIN-A 5'UTR RNAi	BAP73	pBA7, pBA2555		this study
BAP2558	TY-YFP-AUK1, KIN-A 3'UTR RNAi	BAP73	pBA7, pBA2556		this study
BAP2559	YFP-KIN-B, CPC1 5'UTR RNAi	BAP2528	pBA2487		this study
BAP2560	YFP-KIN-B, CPC1 3'UTR RNAi	BAP2528	pBA2488		this study
BAP2561	YFP-KIN-B, CPC2 5'UTR RNAi	BAP2528	pBA2489		this study
BAP2562	YFP-KIN-B, CPC2 3'UTR RNAi	BAP2528	pBA2490		this study
BAP2564	YFP-KIN-B, KIN-A 5'UTR RNAi	BAP2528	pBA2555		this study

BAP2565	YFP-KIN-B, KIN-A 3'UTR RNAi	BAP2528	pBA2556		this study
BAP2640	LacO, TY-tdTomato-AUK1, GFP-NLS-KKT8-LacI	BAP1965	pBA152, pBA1522, pBA2707		this study
BAP2642	KIN-A-YFP (homozygous)	BAP1955		pBA1919 with BA3786/BA3787, BA3792	this study
BAP2643	CPC1-YFP (homozygous)	BAP1955		pBA1919 with BA3788/BA3789, BA3793	this study
BAP2645	TY-YFP-PP1-1	SmOxP9	pBA2800		this study
BAP2646	PP1-1-YFP-TY	SmOxP9	pBA2804		this study
BAP2647	TY-YFP-PP1-1, tdTomato-KKT2	BAP308	pBA164, pBA2800		this study
BAP2648	PP1-1-YFP-TY, tdTomato-KKT2	BAP308	pBA164, pBA2804		this study
BAP2671	TY-AUK1as (M106G) (homozygous), CPC1(148-263)-AUK1-tdTomato-NLS-VhhGFP4	BAP2339	pBA2454, pBA2455, pBA2795		this study
BAP2673	TY-AUK1as (M106G) (homozygous), KKT4-YFP, CPC1(148-263)-AUK1-tdTomato-NLS-VhhGFP4	BAP2504	pBA2454, pBA2455, pBA2795	BA2504/BA2564, pBA1194	this study
BAP2688	TY-YFP-PP1-1, tdTomato-KKT2, KKT7 5'UTR RNAi	BAP2647	pBA164, pBA2800, pBA1554		this study
BAP2689	PP1-1-YFP-TY, tdTomato-KKT2, KKT7 5'UTR RNAi	BAP2648	pBA164, pBA2804, pBA1554		this study
BAP2693	KIN-A-YFP, CPC1 5'UTR RNAi	BAP2642	pBA2487		this study
BAP2694	KIN-A-YFP, CPC1 3'UTR RNAi	BAP2642	pBA2488		this study
BAP2695	KIN-A-YFP, CPC2 5'UTR RNAi	BAP2642	pBA2489		this study
BAP2696	KIN-A-YFP, CPC2 3'UTR RNAi	BAP2642	pBA2490		this study
BAP2697	KIN-A-YFP, KIN-A 5'UTR RNAi	BAP2642	pBA2555		this study
BAP2699	KIN-A-YFP, AUK1 RNAi	BAP2642	pBA56		this study
BAP2900	CPC1-YFP, CPC1 5'UTR RNAi	BAP2643	pBA2487		this study
BAP2902	CPC1-YFP, CPC2 5'UTR RNAi	BAP2643	pBA2489		this study
BAP2903	CPC1-YFP, CPC2 3'UTR RNAi	BAP2643	pBA2490		this study
BAP2904	CPC1-YFP, KIN-A 5'UTR RNAi	BAP2643	pBA2555		this study
BAP2905	CPC1-YFP, KIN-A 3'UTR RNAi	BAP2643	pBA2556		this study
BAP2906	CPC1-YFP, AUK1 RNAi	BAP2643	pBA56		this study
BAP2911	TY-YFP-PP1-1, PP1-1 3'UTR RNAi	BAP2645	pBA2800, pBA2864		this study
BAP2912	PP1-1-YFP-TY, PP1-1 5'UTR RNAi	BAP2646	pBA2804, pBA2863		this study
BAP2914	TY-tdTomato-KKT2, PP1-1 5'UTR RNAi	BAP308	pBA164, pBA2863		this study
BAP2915	TY-tdTomato-KKT2, PP1-1 3'UTR RNAi	BAP308	pBA164, pBA2864		this study
BAP2924	TY-AUK1as (M106G) (homozygous), TY-tdTomato-KKT2, GFP-NLS-KKT14(2-357) PD	BAP2361	pBA2454, pBA2455, pBA164, pBA2791		this study
BAP2925	TY-AUK1as (M106G) (homozygous), TY-tdTomato-KKT2, GFP-NLS-KKT14(2-357) PM	BAP2361	pBA2454, pBA2455, pBA164, pBA2792		this study
BAP2928	TY-AUK1as (M106G) (homozygous), TY-tdTomato-KKT2, GFP-NLS-KKT14(2-357)	BAP2361	pBA2454, pBA2455, pBA164, pBA2663		this study
BAP2943	TY-AUK1as (M106G) (homozygous), KKT3-YFP	BAP2339	pBA2454, pBA2455	BA1821/BA1822, pBA1194	this study
BAP2944	TY-AUK1as (M106G) (homozygous), YFP-KKT9	BAP2339	pBA2454, pBA2455	BA3475/BA3476, pBA1194	this study
BAP2945	TY-AUK1as (M106G) (homozygous), YFP-KKT14	BAP2339	pBA2454, pBA2455	BA3927/BA3928, pBA1194	this study
BAP2945	TY-AUK1as (M106G), YFP-KKT14	BAP2339	pPOT tagging	BA2129, BA2130, pBA1194	this study
BAP2947	TY-tdTomato-KKT2, GFP-NLS-KIN-A (310-716)	BAP308	pBA164, pBA2937		this study
BAP2948	TY-tdTomato-KKT2, GFP-NLS-KIN-A (310-815)	BAP308	pBA164, pBA2938		this study
BAP2949	TY-tdTomato-KKT2, GFP-NLS-KIN-A (310-862, Δ717-743)	BAP308	pBA164, pBA2939		this study
BAP2952	TY-YFP-KKT8, KKT9 RNAi	BAP123	pBA68, pBA88		this study
BAP2953	TY-YFP-KKT8, KKT11 RNAi	BAP123	pBA68, pBA1200		this study
BAP2954	TY-YFP-KKT8, KKT8 3'UTR RNAi	BAP123	pBA68, pBA1316		this study
BAP2955	TY-YFP-KKT8, KKT12 3'UTR RNAi	BAP123	pBA68, pBA1317		this study
BAP2956	TY-YFP-KKT9, KKT9 RNAi	BAP127	pBA73, pBA88		this study
BAP2957	TY-YFP-KKT9, KKT11 RNAi	BAP127	pBA73, pBA1200		this study
BAP2958	TY-YFP-KKT9, KKT8 3'UTR RNAi	BAP127	pBA73, pBA1316		this study
BAP2959	TY-YFP-KKT9, KKT12 3'UTR RNAi	BAP127	pBA73, pBA1317		this study
BAP2964	TY-YFP-KKT12, KKT9 RNAi	BAP131	pBA77, pBA88		this study
BAP2965	TY-YFP-KKT12, KKT11 RNAi	BAP131	pBA77, pBA1200		this study
BAP2966	TY-YFP-KKT12, KKT8 3'UTR RNAi	BAP131	pBA77, pBA1316		this study
BAP2967	TY-YFP-KKT12, KKT12 3'UTR RNAi	BAP131	pBA77, pBA1317		this study
BAP2968	KIN-A-YFP, KKT9 RNAi	BAP2642	pBA88		this study
BAP2969	KIN-A-YFP, KKT11 RNAi	BAP2642	pBA1200		this study
BAP2970	KIN-A-YFP, KKT8 3'UTR RNAi	BAP2642	pBA1316		this study
BAP2971	KIN-A-YFP, KKT12 3'UTR RNAi	BAP2642	pBA1317		this study
BAP2990	TY-AUK1as (M106G) (homozygous), KKT3-YFP, CPC1(148-263)-AUK1-tdTomato-NLS-VhhGFP4	BAP2943	pBA2454, pBA2455, pBA2795	BA1821/BA1822, pBA1194	this study
BAP2991	TY-AUK1as (M106G) (homozygous), YFP-KKT9, CPC1(148-263)-AUK1-tdTomato-NLS-VhhGFP4	BAP2944	pBA2454, pBA2455, pBA2795	BA3475/BA3476, pBA1194	this study
BAP2992	TY-AUK1as (M106G) (homozygous), YFP-KKT14, CPC1(148-263)-AUK1-tdTomato-NLS-VhhGFP4	BAP2945	pBA2454, pBA2455, pBA2795	BA3927/BA3928, pBA1194	this study
BAP3037	KIN-A-YFP-TY	SmOxP9	pBA2940		this study
BAP3038	KIN-A(G210A)-YFP-TY	SmOxP9	pBA2986		this study
BAP3047	TY-YFP-AUK1as (M106G) (homozygous), 3FLAG-KKT4, PP1-1 5'UTR RNAi	BAP2442	pBA2358, pBA2373, pBA1452, pBA2863		this study
BAP3048	TY-YFP-AUK1as (M106G) (homozygous), 3FLAG-KKT4, KKT10/19 RNAi	BAP2442	pBA2358, pBA2373, pBA1452, pBA89		this study
BAP3049	TY-YFP-AUK1as (M106G) (homozygous), 3FLAG-KKT4, KKT14 CDS RNAi	BAP2442	pBA2358, pBA2373, pBA1452, pBA869		this study
BAP3064	KIN-A-YFP-TY, KIN-A 3'UTR RNAi	BAP3037	pBA2940, pBA2556		this study
BAP3065	KIN-A(G210A)-YFP-TY, KIN-A 3'UTR RNAi	BAP3038	pBA2986, pBA2556		this study
BAP3066	KIN-A-YFP-TY, TY-tdTomato-KKT2, KIN-A 3'UTR RNAi	BAP3064	pBA2940, pBA2556, pBA164		this study
BAP3067	KIN-A-YFP-TY, TY-tdTomato-AUK1, KIN-A 3'UTR RNAi	BAP3064	pBA2940, pBA2556, pBA214		this study
BAP3068	KIN-A-YFP-TY, TY-tdTomato-MAP103, KIN-A 3'UTR RNAi	BAP3064	pBA2940, pBA2556, pBA215		this study
BAP3069	KIN-A(G210A)-YFP-TY, TY-tdTomato-KKT2, KIN-A 3'UTR RNAi	BAP3065	pBA2986, pBA2556, pBA164		this study
BAP3070	KIN-A(G210A)-YFP-TY, TY-tdTomato-AUK1, KIN-A 3'UTR RNAi	BAP3065	pBA2986, pBA2556, pBA214		this study
BAP3071	KIN-A(G210A)-YFP-TY, TY-tdTomato-MAP103, KIN-A 3'UTR RNAi	BAP3065	pBA2986, pBA2556, pBA215		this study
BAP3072	LacO, TY-tdTomato-AUK1, GFP-NLS-KKT9-LacI	BAP1965	pBA152, pBA1522, pBA1354		this study
BAP3076	KIN-B RNAi	SmOxP9	pBA3091		this study
BAP3077	TY-YFP-PP1-1, KKT14 CDS RNAi	BAP2645	pBA2800, pBA869		this study
BAP3080	TY-AUK1as (M106G), TY-tdTomato-KKT8, GFP-NLS-KIN-A	BAP2365	pBA2454, pBA2455, pBA1420, pBA2515		this study
BAP3092	TY-YFP-AUK1, AUK1 RNAi	BAP73	pBA7, pBA56		this study
BAP3093	TY-YFP-AUK1, KIN-B RNAi	BAP73	pBA7, pBA3091		this study
BAP3094	CPC1-YFP, KIN-B RNAi	BAP2643	pBA3091		this study
BAP3095	KIN-A-YFP, KIN-B RNAi	BAP2642	pBA3091		this study
BAP3096	YFP-KIN-B, AUK1 RNAi	BAP2528	pBA56		this study
BAP3097	YFP-KIN-B, KIN-B RNAi	BAP2528	pBA3091		this study
BAP3117	TY-tdTomato-AUK1, KIN-A(Δ816-862)-YFP	BAP1044	pBA214, pBA3154		this study
BAP3118	TY-tdTomato-AUK1, KIN-A(Δ717-743)-YFP	BAP1044	pBA214, pBA3155		this study
BAP3127	TY-tdTomato-AUK1, KIN-A(Δ816-862)-YFP, KIN-A 3' UTR RNAi	BAP3117	pBA214, pBA3154, pBA2556		this study
BAP3128	TY-tdTomato-AUK1, KIN-A(Δ717-743)-YFP, KIN-A 3' UTR RNAi	BAP3118	pBA214, pBA3155, pBA2556		this study
BAP3132	TY-YFP-AUK1, KKT1 RNAi	BAP73	pBA7, pBA861		this study
BAP3133	TY-YFP-AUK1, KKT6 RNAi	BAP73	pBA7, pBA864		this study

Alignment S1. Multiple sequence alignment of KIN-A.



Alignment S2. Multiple sequence alignment of KIN-A and KIN-B from different kinetoplastids, human Kinesin-1, human Mklp2, and yeast Klp9.

