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Characterization of a Novel Role for the Tousled- like Kinase in Kinetochore Assembly and Function in Caenorhabditis elegans

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A

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By

Jessica De Orbeta, MS

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DEDICATION

To my family, who loved and supported me through the distance. For all their sacrifices, dedication, and encouragement throughout this journey.

To my husband Ricardo, for showing me endless love and support.

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Characterization of a Novel Role for the Tousled-like Kinase in Kinetochore Assembly and Function in *Caenorhabditis elegans*

Publication No._____

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Chromosome segregation is a critical step during cell division to avoid aneuploidy and promote proper organismal development. Correct sister chromatid positioning and separation during mitosis helps to achieve faithful transmission of genetic material to daughter cells. This prevents improper chromosome partitioning that can potentially result in extrachromosomal fragments, increasing the tumorigenic potential of the cells. The kinetochore is a protenaicious structure responsible for the initiation and orchestration of chromosome movement during mitosis. This highly conserved structure among eukaryotes is required for chromosome attachment to the mitotic spindle and failure to assemble the kinetochore results in aberrant chromosome segregation. Thus elucidating the mechanism of kinetochore assembly is important to have a better understanding of the regulation that controls chromosome segregation. Our previous work identified the *C. elegans* Tousled-like kinase (TLK-1) as a mitotic kinase and depletion of TLK-1 results in embryonic lethality, characterized by nuclei displaying poor mitotic chromosome alignment, lagging chromosome, and chromosome bridges during anaphase. Additionally, previous studies from our group revealed that TLK-1 is

phosphorylated independently by Aurora B at serine 634, and by CHK-1 at threonine T610. The research presented herein reveals that both phosphorylated forms of TLK-1 associate with the kinetochore during mitosis. Moreover, by systematic depletion of kinetochore proteins, I uncovered that pTLK-1 is *bona fide* kinetochore component that is located at the outer kinetochore layer, influencing the microtubule-binding interface. I also demonstrated that TLK-1 is necessary for the kinetochore localization of the microtubule interacting proteins CLS-2 and LIS-1 and I show that embryos depleted of TLK-1 presented an aberrant twisted kinetochore pattern. Furthermore, I established that the inner kinetochore protein KNL-2 is an *in vitro* substrate of TLK-1 indicating a possible role of TLK-1 in regulating centromeric assembly. Collectively, these results suggest a novel role for the Tousled-like kinase in regulation of kinetochore assembly and microtubule dynamics and demonstrate the necessity of TLK-1 for proper chromosome segregation in *C. elegans.*

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CHAPTER I: Introduction

Caenorhabditis elegans: A model experimental system

In 1974, after observing that the current success of molecular biology was due in part to the use of model organisms, Sydney Brenner introduced the nematode *Caenorhabditis elegans* (*C. elegans*) as a model organism for pursuing research in neurology, developmental and molecular biology. Brenner recognized *C. elegans* as a useful tool due to its physiological simplicity and genetic tractability (Brenner, 1974). *C. elegans* is a free-living nematode that is about 1 millimeter in length and is transparent, which aids live imaging of embryonic development. These features make it a great system for studying questions related to development, cell biology and neurology (Altun, 2002-2012). *C. elegans* has five pairs of autosomes and one sex chromosome. It has two sexes, self-fertilizing hermaphrodites (XX) and males (XO). Hermaphrodites can self-fertilize, and are capable of producing 300-350 genetically identical progeny. Hermaphrodites can also mate with males, but cannot fertilize one another (Altun, 2002-2012; Brenner, 1974). In nature, hermaphrodites are the most common sex. Males arise spontaneously at a low frequency (0.1%) via nondisjunction of the X chromosome during oogenesis. When hermaphrodites mate with males, 50% of the progeny will be males and 50% will be hermaphrodites. In the laboratory, self-fertilization of hermaphrodites or crossing with males can be manipulated to produce progeny with the desired genotypes (Altun, 2002-2012).

Conserved mechanisms of chromosome segregation

In eukaryotic organisms the cell cycle is a process whereby cells transmit genetic material from a mother cell to two daughter cells. The cell cycle is composed of four stages that are each defined by the involvement of different regulatory proteins that promote faithful chromosome segregation. Deregulation of chromosome segregation during the cell cycle results in aneuploidy, which is defined as an unequal number of chromosomes segregated to each daughter cell. In humans and other organisms, aneuploidy can result in embryonic lethality, developmental defects, and tumor formation (Nagaoka et al., 2012). Thus, it is fundamental to understand the molecular mechanisms regulating mitotic proteins and how they affect chromosome segregation.

An important step that precedes chromosome segregation is the packing of the DNA into a highly compacted structure known as chromatin. In eukaryotes, 147 base pairs of DNA are wrapped around a single nucleosome. Nucleosomes are formed by an octamer of histones consisting of two copies of histone H2A, H2B, H3, and H4 (Eitoku et al., 2008; Margueron and Reinberg, 2010). The resulting chromatin not only provides a packaging structure, but is also a dynamic configuration that is able to respond to extra- and intra-cellular regulatory cues. Amino-terminal tails project from each core histone and are subject to different post-translational modifications (PTMs), such as phosphorylation, methylation, acetylation, and monoubiquitylation (Berger et al., 2009; Margueron and Reinberg, 2010; Trojer and Reinberg, 2006). These modifications are called “the histone code” and are able to change the chromatin structure and confer new functions and dynamics to its configuration. In addition to these modifications, chromatin is also characterized by the presence of histone variants, the position of the

chromatin within the nucleus, and nucleosome spacing (Eitoku et al., 2008; Margueron and Reinberg, 2010; Westermann et al., 2006). An example of the role of a histone variant in chromosome segregation is the deposition of the histone H3 variant CENP-A^{HCP-3} (Centromere Associated Protein A). Localization of CENP-A^{HCP-3} at a specific region of chromosomes establishes the site of the centromere and subsequent assembly of the kinetochore, a key mitotic structure required for microtubule binding and chromosome segregation (Sullivan, 2001; Van Hooser et al., 2001). Although there are some variations among species, histones and PTMs are evolutionary conserved, which indicates the importance of understanding histone function and chromatin structure.

DNA is synthesized during the S-phase of the cell cycle. The newly replicated chromosomes or sister chromatids are then physically joined. The cohesin complex mediates their cohesion by forming a ring-like structure that catenates the DNA. This catenation prevents the newly replicated chromosomes from undergoing premature segregation and thereby prevents aneuploidy. Therefore, together with DNA packaging into chromatin, DNA replication and cohesion are critical cell cycle processes that ensure correct chromosome segregation (Blow and Tanaka, 2005).

Early in the mitotic phase, replicated chromosomes are extended and unstructured. This lack of morphology can potentially result in chromosome catenation and missegregation (Stear and Roth, 2002). To avoid segregation defects during mitosis, chromosomes undergo dramatic structural changes mediated by condensation. Chromosome condensation results in ever more compacted sister chromatids, allows the establishment of the kinetochore, and facilitates proper chromosome alignment and orientation during metaphase (Hirano, 2005; Stear and Roth, 2002).

During mitosis, microtubules are nucleated from centrosomes positioned at opposite poles of the cell and a population of these microtubules attach to kinetochores. Microtubules are formed by the polymerization of tubulin dimers, which are highly conserved across species. Microtubules are highly dynamic structures that can be regulated by post-translational modifications or by binding of microtubule-associated proteins (MAPs). The interaction between microtubules and kinetochores ultimately generates bi-oriented sister chromatids. The formation of appropriate, bipolar kinetochore-microtubule (K-MT) attachments activates the molecular cues that lead to the separation and movement of sister chromatids to opposite poles of the cell in anaphase (Cheeseman and Desai, 2008).

The summary above briefly highlights important processes that are required during the cell cycle for proper chromosome segregation. Disrupting the function of any of these pathways can have detrimental consequences with respect to human development and health. Chromosome segregation errors can result in developmental defects such as Down syndrome, and can also increase the tumorigenic potential of cells (Baker et al., 2005; Rao et al., 2009; Thompson et al., 2010). In the last decade, molecular studies confirmed that cancer cells and tumors are highly aneuploid. The genetic instability that arises from errors in chromosome segregation is now viewed as a driver of cancer development (Rao et al., 2009). The following sections of this dissertation will explain in greater detail the regulation of the mitotic spindle and the processes required for accurate chromosome segregation. The main focus will be on highly conserved components of the kinetochore and their interplay with microtubules, with an emphasis on my results obtained studying mitosis in *Caenorhabditis elegans*.

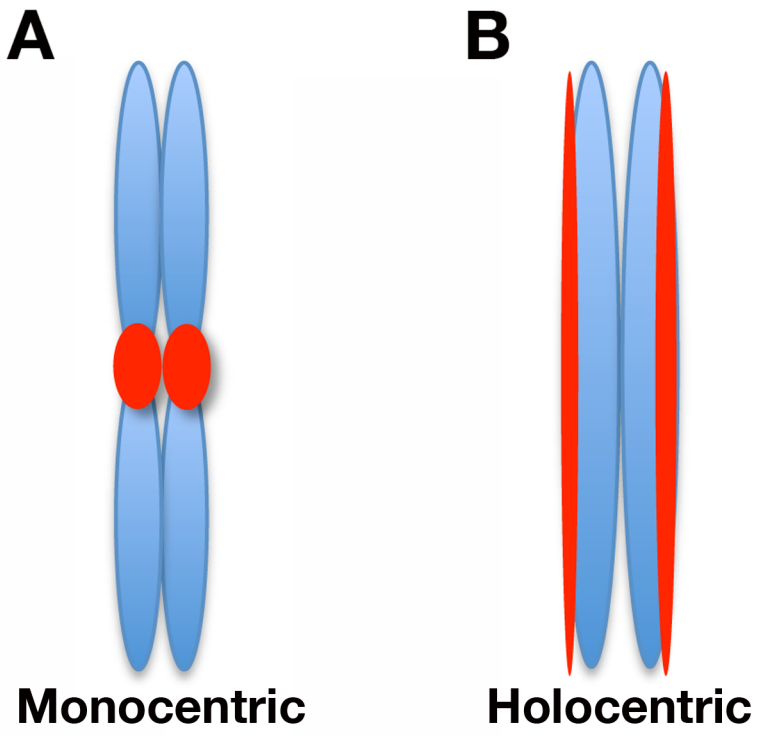
Kinetochore assembly and function

Kinetochore assembly, which is a prerequisite for the attachment of centromeric DNA to spindle microtubules, is a critical step during chromosome segregation. To avoid chromosome loss, kinetochores must be efficient at capturing microtubules emanating from the two spindle poles and at converting initial transient contacts into stable end-coupled attachments capable of resisting the forces that drive chromosome alignment. Failure to assemble the kinetochore disables chromosome attachment to the mitotic spindle, resulting in defective chromosome segregation. Therefore, kinetochore assembly and kinetochore-microtubule attachment must be carefully regulated (Cheeseman and Desai, 2008).

Electron microscopy studies of mammalian cells revealed that the kinetochore is a multi-layered structure consisting of: 1) an electron dense inner plate which appears to be in contact with the centromeric chromatin, 2) an outer plate composed of proteins that contact microtubules, and 3) a layer in between, often referred to as the inner space (Brinkley and Stubblefield, 1966; Rattner and Bazett-Jones, 1989). In the absence of microtubule attachments, an additional structure, known as the fibrous corona, extends from the outer plate (McEwen et al., 1998). The majority of the kinetochore is not present during interphase and becomes apparent only after the cell has entered mitosis and when interaction with microtubules is necessary. In mammalian cells the kinetochore is built in a monocentric fashion, meaning that the kinetochore is assembled at a specific region on each chromosome (Kitagawa, 2009). In contrast, *C. elegans* chromosomes are holocentric and the kinetochore is built along the entire length of each mitotic chromosome (Maddox et al., 2004) (Figure 1).

Figure 1. Structural representation of the kinetochore region

A) Structural representation of monocentric kinetochores. Sister chromatids appear in blue and the kinetochore region in red as a discreet region on the chromosomes. B) Structural representation of holocentric kinetochores. Sister chromatids appear in blue and the kinetochore region in red, distributed along the chromosomes.


A
B
Monocentric
Holocentric

Despite being holocentric, the *C. elegans* kinetochore is also a multi-layered structure when analyzed by electron microscopy, suggesting that *C. elegans* kinetochores are essentially extended versions of mammalian kinetochores (Albertson and Thomson, 1982). In the past decade, research in mammalian cells and *C. elegans* has revealed that the kinetochore is a macromolecular complex formed by more than 90 proteins and that most of these proteins are highly conserved across species. These studies also showed that the kinetochore is assembled in a hierarchical manner and that the proper function of the kinetochore directly depends on the interaction between the proteins and subcomplexes within the larger structure (Cheeseman and Desai, 2008).

The inner kinetochore plate: Specifying the site of kinetochore assembly

A key step that ensures accurate chromosome segregation during mitosis is the assembly of the kinetochore at a specific site on each sister chromatid, at a region known as the centromere. If two microtubule-binding kinetochores are built on a single sister chromatid, this will generate a dicentric chromosome, which results in chromosome breakage at anaphase and increased genomic instability (Pathak and Gagos, 1994). Consequently, the location of the centromere on the chromosome has to be well specified and maintained during the cell cycle (Carroll and Straight, 2006). Different properties mark the centromere as the site for kinetochore establishment including chromatin structure, transcriptional activity, and the presence of CENP-A (Centromere Associated Protein A) (Dernburg, 2001).

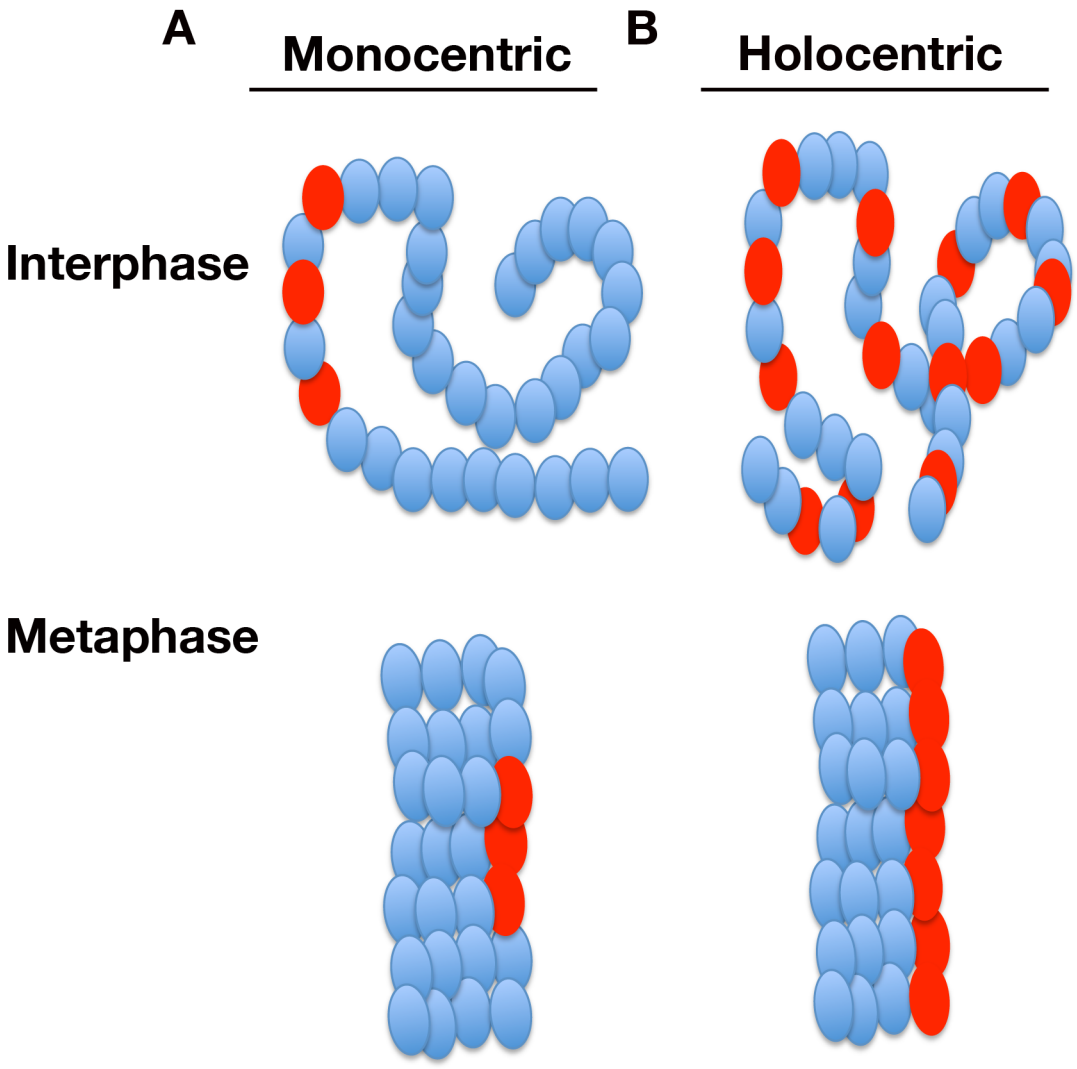
Incorporation of CENP-A, a histone H3 variant, into centromeric nucleosomes is an epigenetic marker of centromere identity and is required for kinetochore assembly. In

C. elegans, HCP-3 (Holocentric Protein 3) is the CENP-A homolog and is present in nucleosomal arrays distributed along the entire length of each chromosome (Dernburg, 2001). It is thought that the higher levels of chromatin compaction achieved during mitosis brings together the CENP-A^{HCP-3} molecules and allows them to act as a single element (Figure 2), consistent with the idea that *C. elegans* holocentric kinetochores are extended versions of single kinetochores (Maddox et al., 2004). In *C. elegans* CENP-A^{HCP-3} is required for kinetochore assembly, and embryos depleted of CENP-A^{HCP-3} do not undergo chromosome segregation (Buchwitz et al., 1999).

Although CENP-A is sufficient to drive kinetochore formation in *Drosophila* in other organisms the presence of CENP-A is not sufficient for complete kinetochore assembly (Mendiburo et al., 2011). Additional proteins that are constitutive components of the centromere are required for proper kinetochore establishment. One example is the Mis16-Mis18 complex in *S. pombe*, one of the first molecular components to be implicated in CENP-A deposition at centromeres (Hayashi et al., 2004). Experiments with *mis16* and *mis18* mutants revealed an increased number of chromosome segregation defects due to a reduction of centromeric Cnp1 (the CENP-A homolog in *S. pombe)*. Additionally, these experiments revealed an increased level of histone H3 and H4 acetylation, suggesting that post-translational modification of core histones plays a role in promoting the correct chromatin structure necessary for CENP-A deposition (Hayashi et al., 2004; Zhang et al., 1999). Studies in mammalian cells that revealed that RBAP46 and RBAP48, the human orthologs of Mis16, are part of the NuRD histone deacetylase complex further support this model (Zhang et al., 1999).

Figure 2. The repeat subunit model of kinetochore assembly

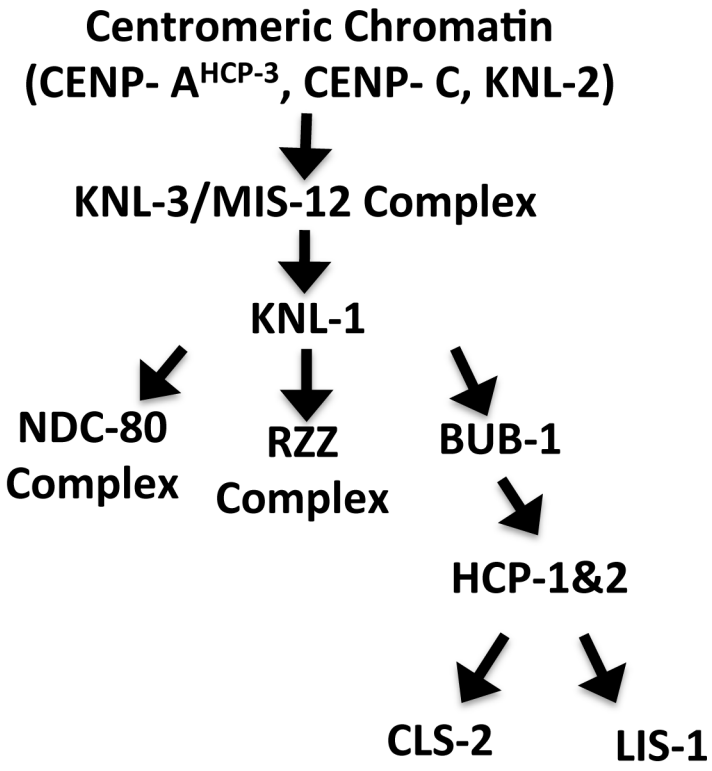
Kinetochore assembly and function during mitosis requires a specialized chromatin structure at centromeres and incorporation of the CENP-A^{HCP-3} histone variant. CENP-A^{HCP-3} exists in repetitive nucleosomal patches (red) interspersed by nucleosomes containing canonical H3 (blue). A) During interphase, CenH3 nucleosomes in monocentric species are confined to a specific chromosomal region. B) In holocentric chromosomes CENP-A^{HCP-3} localizes throughout the chromosomes. After mitotic chromosome condensation tethers, CENP-A^{HCP-3} subunits are threaded together to establish the centromere base for kinetochore assembly.


A
Monocentric
B
Holocentric
Interphase
Metaphase

Sequence homology searches resulted in the identification of two human homologs of the Mis18 protein: MIS18α and MIS18β. Interestingly, pull down experiments confirmed the interaction of both MIS18α and MIS18β with the human Mis16 homologs RBAP46 and RBAP48 (Zhang et al., 1999). An additional uncharacterized protein, named MIS18 binding protein (MIS18BP), was also found to interact with MIS18α and MIS18β (Fujita et al., 2007). Simultaneously, RNAi screening in *C. elegans* uncovered a MIS18BP homolog dubbed KNL-2 (Maddox et al., 2007). Depletion of KNL-2 results in a kinetochore null phenotype (KNL) in which chromosomes fail to congress and align at the spindle equator, resulting in severe chromosome segregation defects. Additional studies revealed that KNL-2 is indispensable for CENP-A centromere targeting (Maddox et al., 2007). These studies confirmed that centromeric CENP-A is localized to the base of the kinetochore and established KNL-2 as a CENP-A centromeric loading factor (Figure 3). CENP-A loading is also conserved in budding and fission yeast. In budding yeast, *SCM3* was identified as the CENP-A^{CSE4p} assembly factor, acting as a functional homolog of *C. elegans* KNL-2 (Pidoux et al., 2009). Furthermore, *Sc*Scm3p binds Ndc10p, a component of the Cbf3p kinetochore complex, and is required for centromeric localization of CENP-A^{CSE4p} (Camahort et al., 2007; Stoler et al., 2007). In a similar fashion, CENP-A^{Cnp1} incorporation at centromeres in fission yeast proceeds in cooperation with Mis16 and Mis18 (Pidoux et al., 2009).

Figure 3. Molecular dependency of the kinetochore hierarchy in *C. elegans*

Schematic of the kinetochore assembly hierarchy in *C. elegans* based on phenotypic analysis, targeting dependencies, and biochemical data.


Centromeric Chromatin
(CENP- A HCP-3, CENP- C, KNL-2)
KNL-3/MIS-12 Complex
KNL-1
NDC-80 Complex
RZZ Complex
BUB-1
HCP-1&2
CLS-2
LIS-1

Establishing the core microtubule binding sites: Linking the centromere and microtubule-interacting complexes

Functional and proteomic analysis of human cells and *C. elegans* embryos have identified additional CENP-A interacting proteins that are present at centromeres during different stages of the cell cycle. Proteomic approaches have identified a group of 15 proteins acting downstream of CENP-A known as the CCAN (Constitutive Centromere Associated Network), which includes CENP-C, CENP-H, CENP-I, and CENP-K (Foltz et al., 2006). Functional analyses suggested that in addition to providing an appropriate environment for CENP-A incorporation, CCAN components also play a structural role in establishing and reinforcing a stable foundation for kinetochore assembly (Foltz et al., 2006).

In *C. elegans,* both CENP-A^{HCP-3} and CENP-C^{HCP-4} localize to the centromere suggesting that CENP-C^{HCP-4} may have a role in linking centromeric chromatin to the kinetochore holocomplex. Embryos depleted of CENP-C^{HCP-4} displayed a characteristic "kinetochore null" (KNL) phenotype, very similar to CENP-A^{HCP-3} depletion (Moore and Roth, 2001). In CENP-C^{HCP-4} depleted embryos, chromosomes failed to align to the metaphase plate and anaphase bridges were present, suggesting that chromosome segregation was defective (Moore et al., 2005). Further studies revealed that when CENP-A^{HCP-3} is depleted, CENP-C^{HCP-4} does not localize to chromosomes. In contrast, CENP-C^{HCP-4} depletion does not affect CENP-A^{HCP-3} chromosome association (Desai et al., 2003; Oegema et al., 2001). Unlike its human counterpart, *C. elegans* CENP-C^{HCP-4} is not found at chromosomes during interphase (Moore and Roth, 2001). This difference may be in part due to differences in the peri-centromeric structure. Human monocentric chromosomes are surrounded by constitutive heterochromatin that remains condensed

during the cell cycle; in contrast, holocentric chromosomes are not embedded in heterochromatin and may require that chromosome condensation occurs prior to CENP-C^{HCP-4} kinetochore recruitment. Despite the temporal differences observed, these experiments revealed a conserved and ordered assembly pathway for kinetochore establishment in *C. elegans*.

In *C. elegans*, anaphase chromosome movement is mediated by the generation of cortical pulling forces that induce the separation of the spindle poles towards opposite poles of the embryo (Grill et al., 2001; Oegema et al., 2001). Prior to anaphase, these cortical pulling forces are counteracted by functional kinetochore-microtubule interactions. Thus, the timing of spindle pole separation is an indirect measurement of the quality of kinetochore-microtubule attachments (Oegema et al., 2001). CENP-C^{HCP-4} depleted embryos also displayed a premature separation of the spindle poles suggesting that CENP-C^{HCP-4} is required for the formation of functional kinetochore-microtubule attachments (Oegema et al., 2001).

A novel, evolutionarily conserved kinetochore protein, KNL-1, was identified in a *C. elegans* RNA-interference screen for additional genes that display a "kinetochore null" phenotype (Oegema et al., 2001; Sonnichsen et al., 2005). While KNL-1 depletion, like that of CENP-A^{HCP-3} or CENP-C^{HCP-4}, leads to a "kinetochore-null" phenotype, KNL-1 depletion does not affect the centromere localization of these proteins. In contrast, the depletion of CENP-A^{HCP-3} or CENP-C^{HCP-4} prevented KNL-1 localization to kinetochores. Consistent with KNL-1 being downstream of CENP-A^{HCP-3} and CENP-C^{HCP-4} in the kinetochore assembly hierarchy, well-condensed chromosomes with two resolved "lines" of CENP-A^{HCP-3} staining were present in KNL-1-depleted embryos,

suggesting that the kinetochore null phenotype observed in KNL-1 depleted embryos is not due to a failure at the "base" of the centromere, but rather is due to defects in the adjacent layers and the MT-binding ability of these kinetochores. In further support of this model, KNL-1 co-immunoprecipitates with NDC-80 and NUF2^{HIM-10}, two subunits of the highly conserved kinetochore-microtubule binding complex known as NDC80 (Cheeseman and Desai, 2005; Desai et al., 2003).

Tandem affinity purification of KNL-1 from *C. elegans* embryo extracts identified numerous KNL-1 interacting proteins (Cheeseman and Desai, 2005; Cheeseman et al., 2004). This approach resulted in the identification of 11 additional kinetochore proteins, including KNL-3, KPB-1-KBP-5 (KNL Binding Protein), and MIS-12 (Cheeseman et al., 2004). Two of the identified proteins, MIS-12 and KBP-3, have homologs in other species. MIS-12 is homologous to human MIS12 and KBP-3 is similar to *Sc*Spc25p, a kinetochore component that associates with *Sc*Ncd80p and *Sc*Nuf2p (Cheeseman et al., 2004; McCleland et al., 2004). Moreover, immunofluorescence characterization of all KNL-1-interacting proteins revealed that they exhibit similar kinetochore localization, supporting a model whereby they function together in the middle layer of the kinetochore hierarchy (Cheeseman et al., 2004).

Further efforts to characterize the function of various kinetochore components showed that NDC-80 depletion resulted in a weaker chromosome segregation phenotype than the kinetochore-null phenotype displayed by *knl-1* or *knl-3(RNAi)* embryos. Interestingly, KBP-3 depletion phenocopied NDC-80 depletion, both of which resulted in embryos with disorganized metaphase plates and delayed sister chromatid separation. The similarity between the NDC-80 and KBP-3 depletion phenotypes extended to the

quantitative analysis of spindle elongation; each phenotype displayed a similar premature spindle elongation phenotype indicative of a defect in kinetochore-microtubule attachments (Cheeseman et al., 2006; Cheeseman et al., 2004). Pairwise depletion of KNL-1, NDC-80, and MIS-12 revealed a delay in the targeting of outer kinetochore proteins and placed KNL-1 upstream of NDC-80 and MIS-12 in the kinetochore assembly hierarchy. Additional biochemical analysis with purified-kinetochore complexes identified two synergistic microtubule-binding sites in the *C. elegans* kinetochore, one in KNL-1 and the other in the NDC-80 complex. Altogether, this protein super-complex was dubbed the KMN (**K**NL-1, **M**IS-12, and **N**DC-80) network and constitutes the core of the microtubule-binding site of the kinetochore (Figure 3) (Cheeseman et al., 2006).

Downstream of the KMN network: KNL-1 as a branching point in the kinetochore hierarchy

Systematic depletion of *C. elegans* kinetochore components has been used jointly with biochemical and cytological techniques to elucidate the assembly and function of the kinetochore. The screening of mouse monoclonal antibodies isolated from hybridoma cell lines lead to the identification of *C. elegans* HCP-1 and revealed that HCP-1 co-localizes with the bona fide kinetochore component CENP-A^{HCP-3} (Moore et al., 1999). HCP-1 harbors multiple coiled-coil domains and shares 74% sequence homology with human CENP-F (Moore et al., 1999). CENP-F is a conserved protein that links the kinetochore with microtubules via its interaction with the NDEL1/NDE1/LIS1/Dynein microtubule motor complex (Vergnolle and Taylor, 2007). HCP-1 displays a dynamic localization pattern throughout mitosis; in prophase, HCP-1

is distributed in a punctate manner along chromosomes and from late prometaphase to early anaphase as holocentric, kinetochore "stripes" (Moore et al., 1999). Kinetochore localization of HCP-1 occurs downstream of KNL-1 and BUB-1, but does not depend on the NDC-80 complex (Desai et al., 2003).

Genome homology searches in *C. elegans* aided the identification of HCP-2, which shares 54% sequence homology with HCP-1 (Moore et al., 1999). HCP-2 co-localizes with HCP-1 during mitosis and immunoprecipitation experiments revealed that they physically interact, suggesting that they work together in a complex (Cheeseman et al., 2005; Moore et al., 1999). Functionally, the independent depletion of HCP-1 or HCP-2 does not reduce viability nor induce chromosome defects in *C. elegans* embryos. In contrast, co-depletion of both HCP-1 and -2 results in ~100% embryonic lethality and induces chromosome segregation defects and aneuploidy (Cheeseman et al., 2005). More extensive characterization of HCP-1&-2 depletion in *C. elegans* embryos revealed that while mitotic spindle assembly occurred, the midzone microtubules normally present between separating chromatids during anaphase were absent (Encalada et al., 2005). In addition, the distance from each centrosome to the metaphase plate was reduced, and centrosome separation was premature (Cheeseman et al., 2005). Together, these findings indicate that HCP-1 and -2 have redundant roles in the regulation of microtubule dynamics.

BUB-1 is a component of the Spindle Assembly Checkpoint (SAC) and is required to induce SAC-dependent mitotic delays upon microtubule disruption or other errors in spindle assembly (Musacchio, 2011). In *C. elegans,* BUB-1 localizes to kinetochores from prophase to anaphase and its kinetochore localization depends on

CENP-A^{HCP-3}, CENP-C^{HCP-4}, and KNL-1 (Encalada et al., 2005; Oegema et al., 2001; Sonnichsen et al., 2005). Depletion of BUB-1 causes embryonic lethality characterized by the presence of unaligned chromosomes at metaphase and lagging sister chromatids at anaphase (Sonnichsen et al., 2005). However, BUB-1 depletion does not cause any obvious defects in spindle microtubule formation or function. Contrary to what is observed in HCP-1&-2 depleted embryos, the spindle midzone forms normally, and centrosome kinetics are similar to those in control embryos (Encalada et al., 2005). Hence the mitotic defects observed in BUB-1 depleted embryos are different from those observed in HCP-1&-2 depleted embryos, even though BUB-1 is required for HCP-1&-2 kinetochore localization. This discrepancy may be due to BUB-1's dual roles in mitotic progression and SAC signaling (Kitagawa, 2009). Taken together, these results place HCP-1&-2 in the kinetochore hierarchy downstream of KNL-1 and BUB-1 and parallel to NDC-80 (Figure 3).

Biochemical analysis revealed that CLASP^{CLS-2} is associated with HCP-1&-2 . CLS-2 is the *C. elegans* homolog of CLASP2 (Cytoplasmic Linker-Associated Protein 2). CLASP^{CLS-2} is a microtubule binding protein required for microtubule polymerization, correct spindle formation, and chromosome segregation during mitosis (Lansbergen et al., 2006; Markus et al., 2009; Mimori-Kiyosue et al., 2005; Mimori-Kiyosue et al., 2006). CLASP^{CLS-2} localizes to kinetochores in an HCP-1&-2 dependent manner from prometaphase to early anaphase (Cheeseman et al., 2004). Similar to HCP-1&-2, CLASP^{CLS-2} depletion causes premature centrosome separation and a reduction in the distance from the centrosome to the metaphase plate (Cheeseman and Desai, 2008). Given the similarities between the HCP-1&-2, and CLASP^{CLS-2} depletion phenotypes, it

was proposed that a major function of HCP-1&-2 is to act as a scaffold to recruit CLASP^{CLS-2} to kinetochores and facilitate CLASP^{CLS-2} interaction with microtubules (Cheeseman et al., 2005; Kitagawa, 2009).

The HCP-1/2 human homolog, CENP-F, is also responsible for the kinetochore localization of LIS1 (Cheeseman et al., 2005). LIS1 is encoded by the gene responsible for lissencephaly, a neurological diseases characterized by lack of cell proliferation and cell migration (Shu et al., 2004). Depletion of the LIS1 homolog from *C. elegans* embryos resulted in pronuclear migration, chromosome segregation, and spindle positioning defects (Cockell et al., 2004). In early embryos, *C. elegans* LIS-1 is distributed throughout the cytoplasm, but it is enriched at the nuclear periphery during late prophase, at the kinetochore during metaphase, and translocates to the microtubule asters that emanate radially from the centrosomes as well as the cell cortex during telophase (Yan et al., 2003). LIS-1 localization to the nuclear periphery, centrosomes, microtubule asters, and cell cortex is dependent on the dynein heavy chain DHC-1 (Cockell et al., 2004; Yan et al., 2003). Interestingly, LIS-1 kinetochore localization is independent of DHC-1, but is dependent on CENP-C^{HCP-4}, KNL-1 and CENP-F (Cockell et al., 2004). Although, the exact role of LIS-1 at the kinetochore remains elusive it has been proposed that LIS-1 kinetochore localization may modulate dynein and/or dynactin to impact microtubule dynamics and chromosome movement (Cockell et al., 2004).

In addition to NDC-80 and BUB-1, recruitment of the conserved ROD/ZWILCH/ZW10 (RZZ) complex is also dependent on KNL-1 (Cheeseman et al., 2004)(Figure 3). The RZZ complex was first studied in *Drosophila,* which revealed that this complex is required for the kinetochore recruitment of dynein/dynactin (Starr et al.,

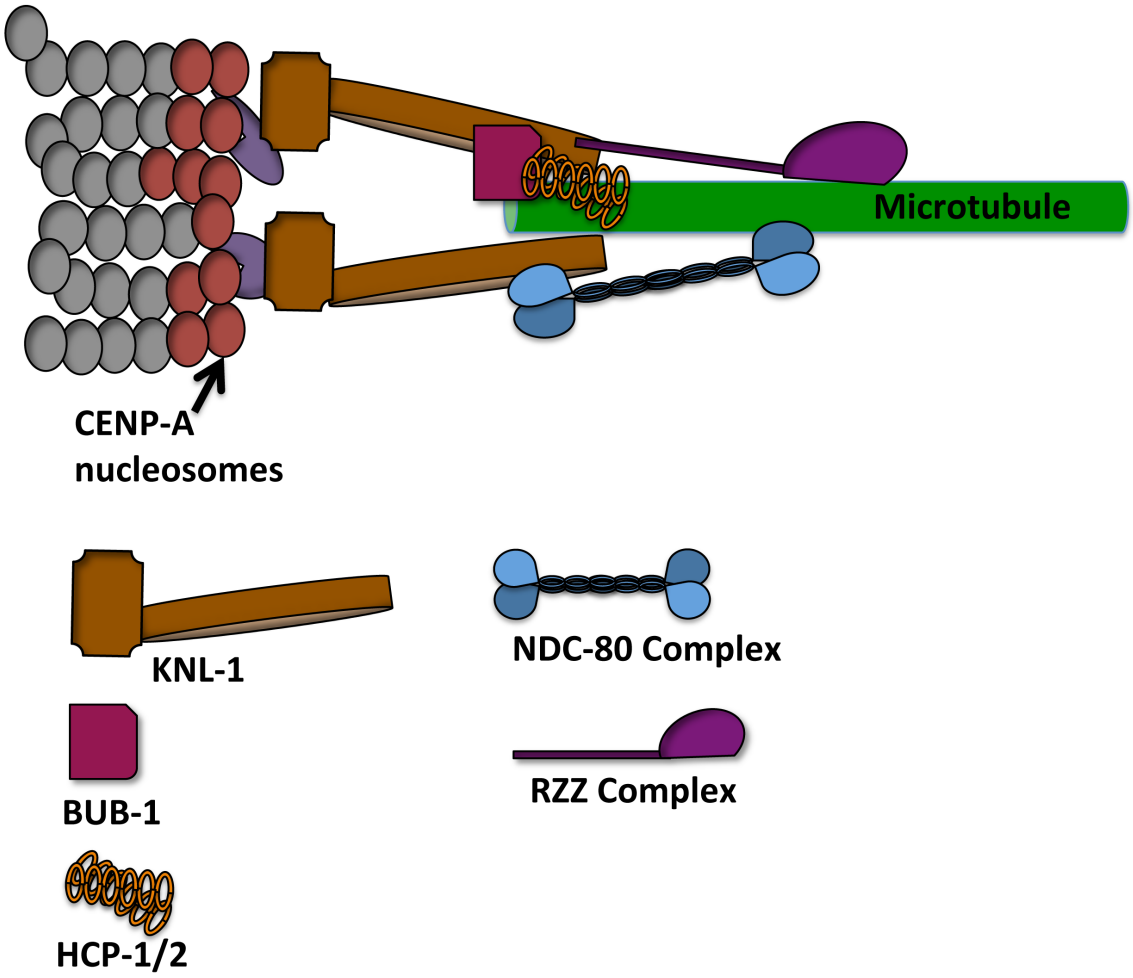
1997; Williams et al., 1992; Williams et al., 2003). After nuclear envelope breakdown, kinetochores make monoriented and lateral microtubule attachments that are ultimately resolved into end-on attachments that promote chromosome segregation to opposite poles at anaphase onset. This separation of sister chromatids to opposite poles is the result of dynein movement towards the minus-end of kinetochore microtubules (Vorozhko et al., 2008). Depletion of any of the RZZ complex subunits results in the premature dynein-induced poleward movement of sister chromatids during early prometaphase, resulting in severe chromosome segregation defects (Savoian et al., 2000). Studies in *C. elegans* revealed that each of the RZZ subunits are interdependent for their kinetochore localization, but are independent of NDC-80 and BUB-1 (Gassmann et al., 2008). Furthermore, depletion of individual RZZ subunits had no effect on the localization of KNL-1, KNL-2, KNL-3, MIS-12, NDC-80, KBP-3, BUB-1, HCP-1, or $CLASP^{CLS-2}$. These experiments placed the RZZ complex as an independent branch downstream of KNL-1(Gassmann et al., 2008) (Figure 3). Lastly, the RZZ complex is responsible for the kinetochore recruitment of the dynein and dynactin binding protein, SPDL-1 (Gassmann et al., 2008).

Kinetochore-microtubule interactions

The ultimate function of the kinetochore during cell division is to act as a molecular machine that interacts with spindle microtubules to achieve bipolar spindle attachment and promote accurate chromosome segregation. As explained above, the NDC-80 complex, RZZ complex, and BUB-1 define three independent branches downstream of KNL-1 that work together to form a microtubule-binding interface at kinetochores (Figure 4).

Figure 4. Model for the components of the microtubule-binding interface at kinetochores

Three different microtubule-binding branches dependent on KNL-1 are present in the model. The BUB-1 and HCP-1 & 2 branch, the NDC- 80 complex, and the RZZ complex. These components connect KNL-1 with microtubules and synergize in their microtubule binding activites. The microtubule-binding interface assembles on a specialized chromatin region specified by CENP-A nucleosomes.


Microtubule
CENP-A
nucleosomes
KNL-1
NDC-80 Complex
BUB-1
RZZ Complex
HCP-1/2

Interestingly, a separate class of proteins can bind to spindle microtubules independently of kinetochore proteins but can also interact with conserved kinetochore proteins. One of the best characterized of these is Dam1p. In *S. cerevisiae,* Dam1p functions in a complex of ten subunits, requires Ndc80p for its kinetochore localization, and interacts with microtubules *in vitro* (Cheeseman et al., 2001; Westermann et al., 2006).

Ultrastructural analysis of recombinant Dam1p complexes *in vitro* revealed that Dam1p oligomerizes to form ring-like structures around microtubules (Westermann et al., 2005). This ring-like structure is considered to be important for Dam1p's interaction with microtubules. The Dam1p complex slides along microtubules and couples microtubule plus-end depolymerization with chromosome segregation at anaphase (Westermann et al., 2006). In addition to the ring-like structure, smaller Dam1p oligomers (of one to four Dam1p complexes) have been identified *in vivo* and *in vitro* (Gao et al., 2010; Gestaut et al., 2008). Although their smaller size would prevent ring formation around microtubules, these smaller structures or "patches" slide along microtubules and track their depolymerizing plus-ends (Welburn et al., 2009). Sequenced-based comparisons failed to identify *DAM1* homologs in other organisms but recent studies uncovered the SKA1 complex as a likely functional homolog in higher organisms (Gaitanos et al., 2009). Like the Dam1p complex, the SKA complex oligomerizes, binds microtubules, and couples chromosome segregation to microtubule depolymerization. However, SKA1 oligomers do not form rings and ring formation is not required for its function (Welburn et al., 2009). In mammals, the SKA1 complex and the KMN network complement each other to establish functional kinetochore-microtubule attachments (Gaitanos et al., 2009).

Key microtubule motor proteins that localize to the kinetochore are CENP-E and dynein (Schmidt et al., 2005; Yen et al., 1991). CENP-E is a plus-end directed motor protein that regulates various steps during mitosis, including the lateral sliding of mono-oriented chromosomes along kinetochore microtubules. These movements promote the ultimate bi-orientation of sister chromatids and complete chromosome alignment at metaphase (Schaar et al., 1997). Live imaging studies of human CENP-E revealed that CENP-E localizes to microtubule plus-ends, and that this localization was essential for spindle elongation at anaphase. Altogether, these results indicate that CENP-E functions at the kinetochore-microtubule interface, and plays a key role in chromosome alignment and sister chromatid separation (Sardar et al., 2010).

There are two independent pools of dynein in cells, intraflagellar transport (IFT) dynein and cytoplasmic dynein (Kardon and Vale, 2009). IFT dynein functions to move cargo along the axoneme towards the basal body. In contrast, cytoplasmic dynein carries out minus end-directed microtubule transport as well as other mitotic functions such as centrosome separation bipolar spindle formation, nuclear envelope breakdown, and Spindle Assembly Checkpoint silencing (Schmidt et al., 2005). Cytoplasmic dynein exists as a 1.5 Megadalton complex that consists of approximately 12 smaller proteins, including multiple non-catalytic subunits that function as attachment points and regulators of dynein cargo (Kardon and Vale, 2009; Schmidt et al., 2005). Some of the best characterized of these non-catalytic subunits are dynactin, the components of the RZZ complex (ROD/ZWILCH/ZW10), SPINDLY, and LIS1 (Kardon and Vale, 2009). Dynein localizes to kinetochores during prometaphase and generates the pulling forces that promote chromosome movement and alignment to a metaphase plate (Yang et al.,

2007). Studies in budding yeast revealed that Lis1p is required for the localization of dynein to kinetochores and microtubule plus-ends. LIS1 binds directly to dynein and it is proposed that once sister chromatids are bi-oriented, this interaction allows dynein to transport components of the SAC from kinetochores to the microtubule-minus ends embedded in the centrosomes, thereby silencing the SAC and promoting anaphase onset (Howell et al., 2001; Wojcik et al., 2001).

Kinases during the cell cycle

The cell cycle is highly organized process that is steered by the activity of a diverse group of kinases (Nurse, 1990). Tyrosine receptor kinases are responsible for the cellular recognition and response to mitogenic cues, hormone receptor coupled kinases for transcriptional regulation during development, and serine/threonine kinases for entering and progressing through the cell cycle (Hunter, 1995). Given the importance of kinases at different levels of the cell cycle, much research has been devoted towards the understanding of kinase-dependent regulation of these cellular processes (Nigg, 2001). One of the best-characterized groups of kinases is the cyclin dependent kinases (CDKs), which are known to drive important cell cycle transitions *via* a conserved mechanism in eukaryotes (Nurse, 1990).

CDK1 drives the G2/M transition. CDK1 regulates this critical transition by binding the cyclically expressed A- and B- type cyclins (Dunphy, 1994). Upon binding to either A- or B-type cyclins, CDK1 promotes centrosome duplication, spindle assembly, chromosome condensation, and anaphase onset (Malumbres and Barbacid, 2005). The CDK1-Cyclin complexes remain inactive until DNA conditions are appropriate to initiate mitosis. Once DNA conditions are optimal, CDK1 is

dephosphorylated by the action of the CDC25 phosphatase and mitosis ensues (Dunphy, 1994). To avoid premature mitotic entry, specific checkpoints supervise the cell cycle machinery at different stages (Weinert and LH., 1988). For example, during G1 and G2, DNA damage caused by ionizing radiation induces cell cycle arrest (Rieder, 2011; Shiloh, 2006) and replication fork defects result in S phase arrest (Branzei and Foiani, 2010). During mitosis, the spindle assembly checkpoint (SAC) prohibits anaphase entry in the presence of unattached kinetochores or lack of tension across attached kinetochores (Kops et al., 2005).

Chk1 kinase

One of the key effectors of the cell cycle is the conserved CHK1 kinase. The principal role of CHK1 is to mediate the signaling network triggered by the DNA damage checkpoint (Tapia-Alveal et al., 2009). DNA damage or replicative stress activates the "sensor" kinases ATR (ataxia and *RAD3* related) and ATM (ataxia telangiectasia mutated), and results in the phosphorylation and activation of CHK1 (Bartek and Lukas, 2003). Interestingly, CHK1 is also implicated in chromatin assembly *via* its interaction with the Tousled-like kinase (TLK1) during S phase (Groth et al., 2003). The initial characterization of TLK1 in mammalian cells revealed that TLK1 kinase activity peaks during S phase (Barr et al., 2004; Sillje et al., 1999). However, TLK1 kinase activity was found to be reduced after exposure to DNA damaging agents or replication inhibiting drugs. This inhibitory effect was restricted to S phase, suggesting that the regulation of TLK1 activity is cell cycle dependent (Sillje et al., 1999). TLK1 inhibition upon DNA damage directly correlated with CHK1 activation by ATM and CHK1 was found to directly phosphorylate TLK1 at serine 695. Further

experiments confirmed that TLK1(S695) phosphorylation was necessary for the inhibition of TLK1 kinase activity (Groth et al., 2003). Altogether, these data revealed that CHK1 is necessary for an ATM mediated inhibition of TLK1 kinase activity. Since CHK1 was previously thought to be an exclusive substrate of ATR and not ATM, these studies also revealed that there is functional crosstalk between ATM and CHK1 in response to DNA damage.

Intriguingly, a function for CHK1 in the spindle assembly checkpoint (SAC) has also been unveiled. The SAC prevents premature chromosome segregation by monitoring kinetochore-microtubule attachments and delaying chromosome movement until the bipolar attachment of all sister chromatids is achieved (Kops et al., 2005). The principal components of the SAC pathway are MAD1, MAD2 and BUBR1 (also known as Mad3), BUB1, and BUB3 (Kops et al., 2010). In the presence of unattached kinetochores or lack of kinetochore tension, the MAD and BUB proteins are recruited to the kinetochore and this recruitment inhibits the activation of the anaphase promoting complex (APC), thus delaying mitotic exit (Skoufias et al., 2001). CHK1 is also required to delay mitotic progression after DNA damage, and to promote this delay in a MAD2-dependent manner. Consistent with this idea, CHK1-depleted cells exhibit increased levels of spontaneous chromosome missegregation, indicating that CHK1 is required to avoid chromosomal instability (Zachos et al., 2007).

Additional analysis of CHK1 revealed that CHK1 localizes to kinetochores and that in CHK1-depleted cells, BUBR1, an important kinetochore component in mammals, fails to localize to the kinetochore (Zachos et al., 2007). BUBR1 kinetochore localization is indispensable for the activation of the spindle checkpoint in response to

misaligned chromosomes (Elowe, 2011). Moreover, in the presence of unstable microtubules, CHK1 phosphorylates the Aurora B kinase, increasing Aurora B activity to sustain SAC activation (Zachos et al., 2007). Taken together, these results indicate that CHK1 regulates multiple cell cycle checkpoints by interacting with a variety of different kinases.

Aurora kinases

The Aurora kinases are a family of highly conserved serine/threonine kinases essential for meiotic and mitotic processes (Carmena and Earnshaw, 2003). The initial member, *S. cerevisiae* Ipl1p, was identified in a genetic screen for gene products required to maintain accurate chromosome segregation (Chan and Botstein, 1993). This screen identified two mutant alleles of *ipl* that resulted in increased aneuploidy. Subsequently, a mitotic mutant that failed to assemble a bipolar spindle was identified in *Drosophila*, and was dubbed *aurora* (Glover et al., 1995). The molecular identification of *aurora* revealed that it is homologous to Ipl1p. The identification of these two kinases lead to the identification of *Aurora* orthologs in other species including AurA and AurB in *Xenopus* (Giet and Prigent, 2000), AIR-1 and AIR-2 in *C. elegans* (Schumacher et al., 1998a; Schumacher et al., 1998b), *Aurora B* in *Drosophila* (Giet and Glover, 2001) and three paralogs in mammals, Aurora A, B, and C (Carmena and Earnshaw, 2003). The Aurora kinases fall into two distinct subclasses, Aurora A and B, that display highly specific subcellular localizations and have very distinct functions during the cell cycle. Aurora A kinases are localized to the centrosomes and peri-centrosomal microtubules and play roles in centrosome maturation and bipolar spindle assembly (Hannak et al., 2001; Schumacher et al., 1998a). In contrast, Aurora B kinases associate with

chromosome arms in early prophase, and are specifically localized to the inner-centromere in prometaphase and metaphase. At anaphase onset, they translocate to the spindle midzone (Carmena and Earnshaw, 2003; Schumacher et al., 1998b). Aurora C is restricted to mammals, and is closely related to Aurora B (Slattery et al., 2008). Aurora C expression appears to be highest in the male and female germ line where it may influence chromosome segregation and cytokinesis during meiosis (Adams et al., 2001; Yang et al., 2010).

The overexpression of Aurora kinases has been implicated in tumor development and high levels of Aurora B have been detected in several cancer cell lines (Adams et al., 2001; Lin et al., 2010). In *C. elegans* embryos, depletion of the Aurora B homolog AIR-2 results in embryonic lethality and severe meiotic and mitotic defects (Schumacher et al., 1998b). In all species studied thus far, Aurora B is a component of the chromosomal passenger complex (CPC). The CPC is composed of Aurora B, INCENP, Survivin, and Borealin, and all members display the same cell-cycle dependent localization pattern as well very similar loss-of-function phenotypes (Liu et al., 2009). As noted above, Aurora B and other CPC members localize to chromosome arms during prophase and accumulate at the inner centromere between sister kinetochores during prometaphase and metaphase, where the complex aids in the correction of aberrant kinetochore-microtubule (K-MT) attachments (Liu et al., 2009). Upon chromosome separation at anaphase, the CPC transfers to the spindle midzone microtubules and during telophase concentrates at the cytokinesis midbody (Liu et al., 2009). In organisms with monocentric kinetochores each of the CPC components is interdependent for complex formation and kinetochore localization (Ruchaud et al., 2007). Surprisingly, in *C.*

elegans, AIR-2/Aurora B is not required for the association and localization of the other components (Romano et al., 2003). However, since *C. elegans* kinetochores are distributed along the length of chromosomes, there is a larger surface for microtubule attachment, and, hence, a likely concomitant increase in the formation of incorrect K-MT attachments. Thus it is probable that AIR-2 is subject to additional levels of regulation than its counterparts in other species.

The spatial and functional differences of the Aurora kinases are controlled in part by their ability to interact with different substrates activators (Carmena et al., 2009). For instance, prior to entering mitosis, Aurora A binds and phosphorylates the AJUBA LIM domain protein. This interaction allows for the recruitment of CDK1-cyclin B to centrosomes and promotes mitotic entry (Hirota et al., 2003). In a similar fashion, the microtubule-associated protein TPX2 is a substrate and activator of Aurora A that is required for localization of active Aurora A to mitotic spindle microtubules (Kufer et al., 2002; Ozlu et al., 2005).

One of the many roles of AIR-2/Aurora B during mitosis is to destabilize incorrect K-MT attachments that do not generate tension between sister kinetochores (Cheeseman et al., 2002). Aurora B acts as the catalytic component of the CPC and is subject to different means of regulation. Aurora B activity is regulated by the fellow CPC component INCENP, as Aurora B mediated phosphorylation of the INCENP C-terminus increases Aurora B kinase activity ten-fold (Bishop and Schumacher, 2002; Ehsan et al., 2004; Rosasco-Nitcher et al., 2008). Also, Aurora B is regulated by an interaction with the CPC- associated protein TD-60 (Rosasco-Nitcher et al., 2008) and in mammals, via phosphorylation of Borealin by the Mps1 kinase (Jelluma et al., 2008).

Additionally, our group identified the Tousled-like kinase TLK-1 as an additional substrate activator of *C. elegans* AIR-2/Aurora B (Han et al., 2005). The significance of the interaction between AIR-2 and TLK-1 and its impact on mitosis will be discussed in detail in the next section.

The precise mechanism by which Aurora B controls K-MT interactions remains elusive. Studies of Aurora substrates in *S. cerevisiae* revealed that Ipl1p phosphorylates the kinetochore and microtubule-associated complexes Dam1p and Ndc80p (Cheeseman et al., 2002). These experiments suggest that Ndc80p phosphorylation by Ipl1p decreases Ndc80p microtubule binding affinity (Cheeseman et al., 2006). Similarly, phosphorylation of Dam1p by Ipl1p results in the dissociation of Dam1p from microtubules and kinetochores (Cheeseman et al., 2002). A proposed model for Aurora B regulation of kinetochore-microtubule attachment is that the timely relocation of the CPC from the centromere to the central spindle prevents the physical interaction between the CPC and its substrates at the outer kinetochore, allowing for microtubule stabilization and anaphase onset (Fuller et al., 2008; Liu et al., 2009).

Tousled-liked kinases

The Tousled kinase (TSL) was first identified in *Arabidopsis thaliana* as a gene responsible for proper flower and leaf development (Roe et al., 1993). Mutations in the *TSL* gene resulted in delayed flowering and leaf morphological defects, implicating TSL in organ development (Roe et al., 1997; Roe et al., 1993). Molecular characterization of TSL revealed that it is a serine/threonine kinase composed of a C-terminal catalytic domain, an N-terminal nuclear localization domain,, and two alpha-helical regions that promote protein oligomerization (Roe et al., 1997). Molecular cloning and

bioinformatics searches were utilized to identify two human homologs of TSL, *TLK1* and *TLK2* (Sillje et al., 1999). The human proteins share 50% amino acid sequence homology with Arabidopsis TSL and 84% similarity with each other (Yamakawa et al., 1997). Additionally, human TLK1 and TLK2 also showed conservation with Arabidopsis TSL at a functional level. Both the plant and human kinases undergo autophosphorylation, oligomerize, and exhibit nuclear localization. In addition, their highest activity is linked to ongoing DNA replication during S-phase (Sillje et al., 1999; Yamakawa et al., 1997).

Only a handful of TLK1 substrates have been identified, the first reported being the chromatin assembly factor ASF1, suggesting a role for TLK1 in chromatin remodeling (Carrera et al., 2003; Ehsan et al., 2004; Groth et al., 2003; Han et al., 2005; Han et al., 2003; Pilyugin et al., 2009; Sillje and Nigg, 2001). Although, the functional consequence of ASF1 phosphorylation by TLK1 remains elusive, it was recently found that TLK1 phosphorylation of ASF1 increases ASF1 protein stability (Pilyugin et al., 2009). Additional TLK1 substrates include the RAD9 DNA repair protein (Sunavala-Dossabhoy and De Benedetti, 2009), the DEAD-box RNA helicase p68 (Kodym et al., 2005) , and histone H3 serine 10 (H3S10) (Ehsan et al., 2004; Li et al., 2001).

As described above, UV-induced DNA damage, DNA double-stranded breaks, and inhibition of ongoing DNA replication strongly inhibit TLK kinase activity (Groth et al., 2003; Krause et al., 2003). CHK1 phosphorylation of TLK1 results in TLK1 inactivation in an ATM-dependent manner; a pathway independent of the CHK1 canonical activator, ATR. NBS1, the Nijmegen Breakage Syndrome protein, is also required for TLK1 inactivation in the same DNA damage and DNA replication defective

conditions (Krause et al., 2003) . Intriguingly, overexpression of TLK1 in mammalian cells enhanced the repair of UV-induced double stranded breaks and conferred resistance to ionizing radiation independently of TLK1 kinase activity (Li et al., 2001; Sen and De Benedetti, 2006; Sunavala-Dossabhoy et al., 2005; Sunavala-Dossabhoy and De Benedetti, 2009). Other studies suggest that TLK1 may play a role in the regulation of transcription. In *Arabidopsis*, TSL is necessary to maintain transcriptional gene silencing (Wang et al., 2007), and in *C. elegans,* TLK-1 is required to maintain post-translational modifications associated with transcriptional elongation, such as phosphorylation of the RNA polymerase II (RNAPII) C-terminal domain at serine 2 and the methylation of histone H3 at lysine 36 (Han et al., 2003).

In human cells, TLK1 phosphorylates histone H3 serine 10 (H3S10), but studies in other systems suggest a non-direct mechanism of H3S10 phosphorylation. For instance, in *C. elegans* TLK-1 does not phosphorylate H3S10, instead TLK-1 acts as a substrate and activator of Aurora B/AIR-2, itself a H3S10 kinase (Han et al., 2005). In our laboratory, TLK-1 was identified in a yeast two-hybrid screen for Aurora B/AIR-2 interacting proteins (Han et al., 2005). Our studies revealed that Aurora B/AIR-2 phosphorylates TLK-1 at Serine 634 (TLK-1 pS634) *in vitro*, an event that creates a positive feedback loop that increases AIR-2 activity. Moreover, this AIR-2 activation is independent of TLK-1 kinase activity, but dependent on the interaction between AIR-2 and INCENP/ICP-1 *in vitro* (Riefler et al., 2008). Immunolocalization experiments in *C. elegans* revealed that TLK-1 is localized to the nucleus during interphase and prophase, and is faintly visible around metaphase chromosomes. In contrast, a phospho-specific antibody that recognizes pTLK-1(S634) revealed that pTLK-1(S634) is associated with

kinetochores in prophase and kinetochores and kinetochore microtubules (K-MTs) at metaphase (Han et al., 2005). These results suggest that S634 phosphorylation may recruit TLK-1 from chromatin to the kinetochore and K-MTs (Han et al., 2005).

Consistent with TLK-1 having a potential role at the kinetochore, loss of TLK-1 results in metaphase chromosome alignment defects, mitotic delays, and anaphase chromosome bridges (Han et al., 2005). In addition, depletion of TLK-1 from embryos harboring a temperature-sensitive (ts) allele of *aurora B/air-2*, exacerbated the *air-2* ts phenotype, resulting in severe chromosome segregation defects at the permissive temperature (Han et al., 2005). Altogether, these results indicate that *C. elegans* TLK-1 plays an essential role in mitosis. Studies in other organisms suggest that such a role is likely to be evolutionarily conserved. In *Trypanosoma brucei,* the TLK-1 homolog, *Tb*TLK1 localizes to centrosomes in an Aurora kinase dependent manner and loss of *Tb*TLK1 or Aurora B results in severe chromosome segregation defects (Li et al., 2007). Similarly, in *Drosophila,* co-depletion of *tlk* and *mars*, a protein required for chromosome segregation, results in severe chromosome segregation defects that appear to be due to an inability to polymerize mitotic microtubules (Li et al., 2009). Also, in epithelial breast cell lines, expression of catalytically inactive Tlk1b results in the formation of multiple mitotic spindles in single cells and leads to severe chromosome segregation defects (Sunavala-Dossabhoy et al., 2003).

Despite its apparent conservation among disparate species, the mechanism by which Tousled-like kinases regulate chromosome segregation remains to be understood. In this dissertation, I present evidence that TLK-1 has a critical role in the regulation of kinetochore assembly and function. In particular, TLK-1 impacts the recruitment of

essential kinetochore-associated proteins and impacts the stability of kinetochore-microtubule attachments and chromosome segregation in *C. elegans* embryos.

CHAPTER II: TLK-1 phospho-isoforms localize to the kinetochore affecting kinetochore assembly and microtubule interactions

Introduction

The Tousled Like Kinase (TLK-1) is an evolutionarily conserved serine and threonine protein kinase implicated in multiple cellular process such as DNA replication and repair (Groth et al., 2003; Krause et al., 2003), transcription (Han et al., 2003) and mitotic chromosome segregation (Han et al., 2005) . Depletion of TLK-1 in *C. elegans* embryos *via* mutation or RNAi results in embryonic lethality at the 50-100-cell stage of embryonic development. Prior to embryonic arrest, TLK-1 depleted embryos display aberrant mitoses, characterized by defects in chromosome alignment during metaphase, delayed mitotic progression, and anaphase chromosome bridges. Interestingly, previous studies from our group revealed that in addition to Aurora B-dependent phosphorylation at serine 634, TLK-1 is also phosphorylated by CHK-1 at threonine T610 (Deyter, 2010)

In my work, I discovered that pTLK-1(T610), like pTLK-1(S634), is localized to kinetochores in prophase and kinetochores and K-MTs in metaphase. Here, I present evidence that pTLK-1(T610) and (S634) are associated with the outer kinetochore and kinetochore microtubules in a CENP-F^{HCP-1} dependent manner. Furthermore, TLK-1 is required for the kinetochore association of at least two microtubule-binding proteins to the kinetochore, which is consistent with the phenotypic defects of TLK-1 depleted embryos. Altogether, this dissertation reveals a novel role for TLK-1 in kinetochore function and microtubule dynamics and the regulation of mitotic spindle assembly by an integrated network of conserved protein kinases.

Results

Phosphorylation of TLK-1 at T610 is dependent on CHK-1 but not the Polo kinase PLK-1

Previous studies from our laboratory found that in addition to S634, TLK-1 is also phosphorylated at T610. Since T610 lies in a consensus site for the CHK-1 kinase, *in vitro* kinases assays with an active form of human CHK1 and recombinant TLK- proteins were performed (Deyter, 2010). This analysis revealed that TLK-1 is directly phosphorylated by CHK1 *in vitro*, and that T610 is a primary site for CHK1 phosphorylation (Deyter, 2010). To confirm that CHK1 is necessary for this phosphorylation *in vivo*, an affinity-purified phospho-specific antibody raised against a pT610 peptide was used to immunostain embryos isolated from wild-type *C. elegans* hermaphrodites as well as hermaphrodites heterozygous for a deletion allele of *chk-1*. Twenty-five percent of the embryos from the *chk-1 -/+* mothers should be homozygous for the *chk-1* mutant allele, and should display reduced or absent pTLK-1(T610) immunostaining. Indeed, immunostaining revealed that, pTLK-1(T610) immunostaining was markedly reduced in a portion of the embryos from *chk-1*-/+ mothers, but not those from wild-type mothers (Deyter, 2010). Together, these results suggest that CHK-1 directly phosphorylates TLK-1(T610) *in vivo*.

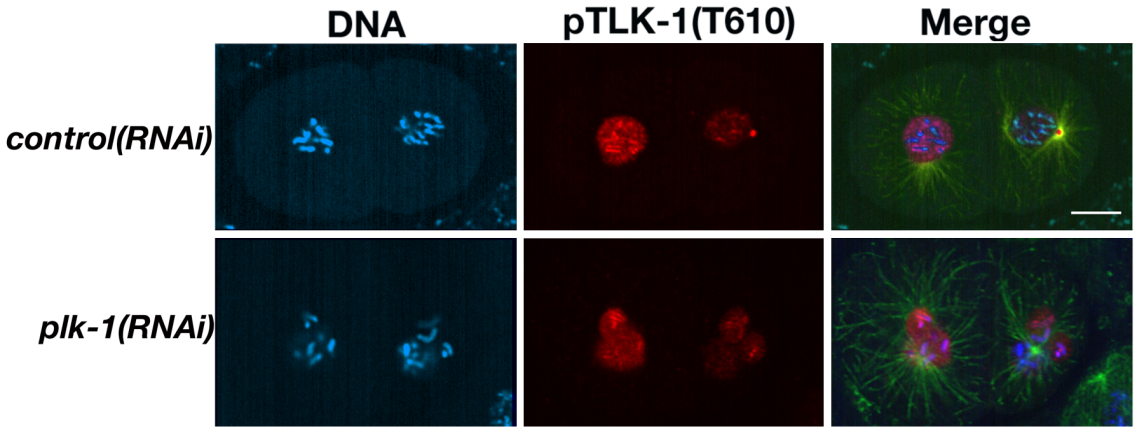
Although these data suggest that CHK-1 is sufficient for TLK-1 phosphorylation *in vitro* and is necessary for TLK-1 phosphorylation *in vivo*, the TLK-1(T610) site (E-E-T_{610}-D-L-S) also matches the consensus phosphorylation site for the Polo kinase family

(Nakajima et al., 2003). In humans, PLK1 localizes to kinetochores from prophase to metaphase, where it has a prominent role in chromosome separation (Kyung et al., 2008) . Upon anaphase onset, PLK1 relocates to the central spindle during anaphase where it plays a conserved role in cytokinesis (Barr et al., 2004). Cells depleted of PLK1 display a variety of defects including delays in mitotic entry, chromosome alignment and segregation errors, delays in the metaphase-to-anaphase transition, and cytokinesis failure (Goto et al., 2006; Lens et al., 2010).

Given that TLK-1(T610) resides in a PLK-1 consensus site and the kinetochore localization and functions of PLK-1 and TLK-1 partially overlap, I tested whether, in addition to CHK-1, TLK-1(T610) phosphorylation and localization may also be affected by PLK-1. Hence, wild-type hermaphrodites were fed dsRNA corresponding to *C. elegans* PLK-1 and a control dsRNA (as described in Materials and Methods, Chapter V). The resulting control and *plk-1(RNAi)* embryos were co-immunostained with affinity purified pTLK-1(T610) and tubulin-specific antibodies. pTLK-1 (T610) levels were not reduced in *plk-1(RNAi)* versus *control(RNAi)* embryos (Figure 5). These results indicate that PLK-1 does not affect the phosphorylation of TLK-1(T610) nor the localization of the pTLK-1(T610) protein, and support a model whereby TLK-1(T610) phosphorylation is dependent on the CHK-1 kinase.

Figure 5. PLK-1 depletion does not affect TLK-1 phosphorylation at T610 in *C. elegans* embryos

control(RNAi) and *plk-1(RNAi)*-depleted embryos were fixed and stained with DAPI (DNA)(blue), and α-tubulin (green) and pTLK-1(T610) (red) antibodies. Two-cell embryos are shown (Scale bar = 10 μm).

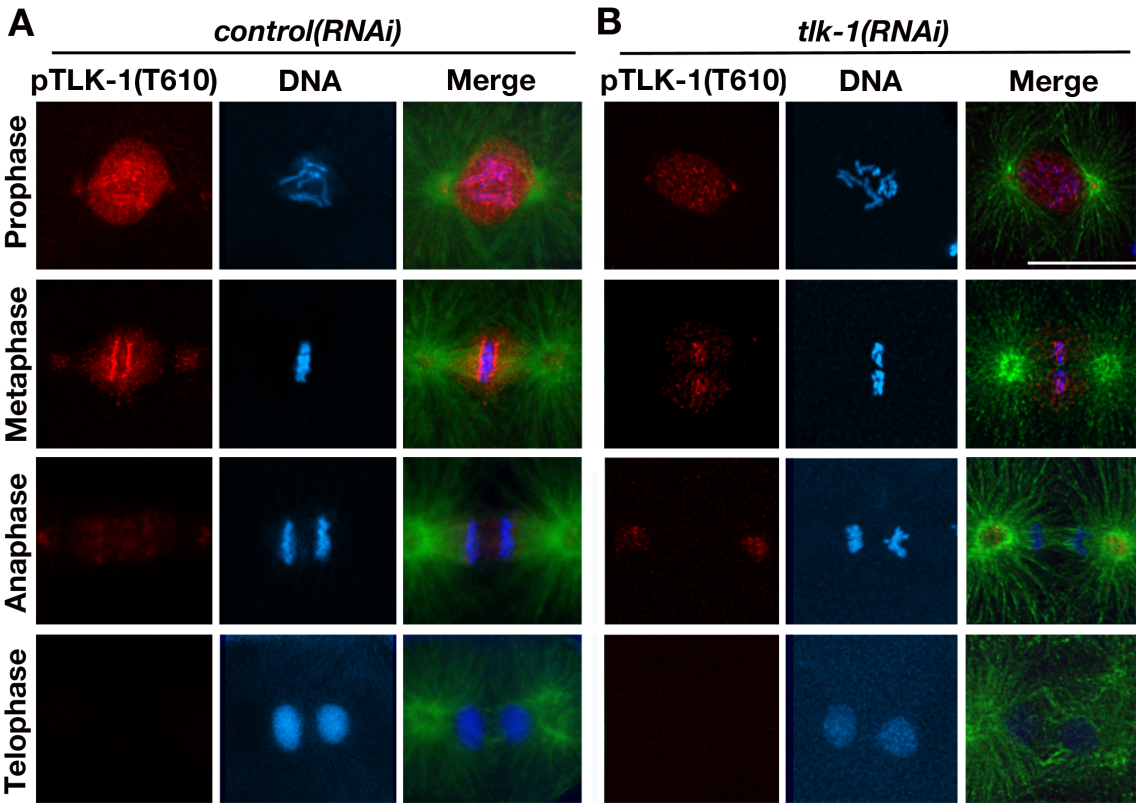

DNA
pTLK-1(T610)
Merge
control(RNAi)
plk-1(RNAi)

TLK-1 pT610 localizes to kinetochores and kinetochore microtubules during mitosis

Previous immunolocalization studies from our laboratory utilizing an affinity-purified polyclonal antibody raised against a full-length recombinant TLK-1 protein (α-TLK-1) revealed that *C. elegans* TLK-1 is apparent in interphase and prophase nuclei (Han et al., 2003). In contrast, the pTLK-1 (T610) antibody revealed nuclear localization exclusively in prophase, and was organized in two parallel kinetochore-like stripes surrounding each chromosome as the cells proceeded into late prophase (prior to nuclear envelope breakdown (NEB) (Figure 6A). These stripes persisted after NEB, through prometaphase and metaphase (Figure 6A). Additionally, once NEB occurred, pTLK-1(T610) staining was also detected on K-MTs (Figure 6A and data not shown). Depletion of TLK-1 by RNAi consistently reduced pTLK-1(T610) staining, thereby confirming antibody specificity (Figure 6B). In addition to the kinetochore and K-MT staining, centrosomal pTLK-1(T610) was often present, appearing during prophase, and persisting through anaphase (Figure 6 and data not shown). However, the centrosomal reactivity of the pTLK-1 (T610) antibody was still present in *tlk-1(RNAi)* embryos suggesting that the centrosomal staining is not specific (Figure 6 and data not shown). In summation, my finding that pTLK-1(T610) is localized to kinetochores and K-MTs during mitosis lead to the hypothesis that the TLK-1 kinase has a functional role at the kinetochore and is regulated by phosphorylation at T610.

Figure 6. pTLK-1(T610) localizes to kinetochores and kinetochore microtubules

Embryos excised from hermaphrodites treated with A) *control(RNAi)* and B) *tlk-1(RNAi)* were fixed and stained with DAPI to visualize DNA (blue), an α-tubulin antibody (green), and affinity-purified pTLK-1(T610) antibody (red). Data presented here is from one-cell embryos (Scale bar = 10 μm).


A
control(RNAi)
B
tlk-1(RNAi)
pTLK-1(T610)
DNA
Merge
pTLK-1(T610)
DNA
Merge
Prophase
Metaphase
Anaphase
Telophase

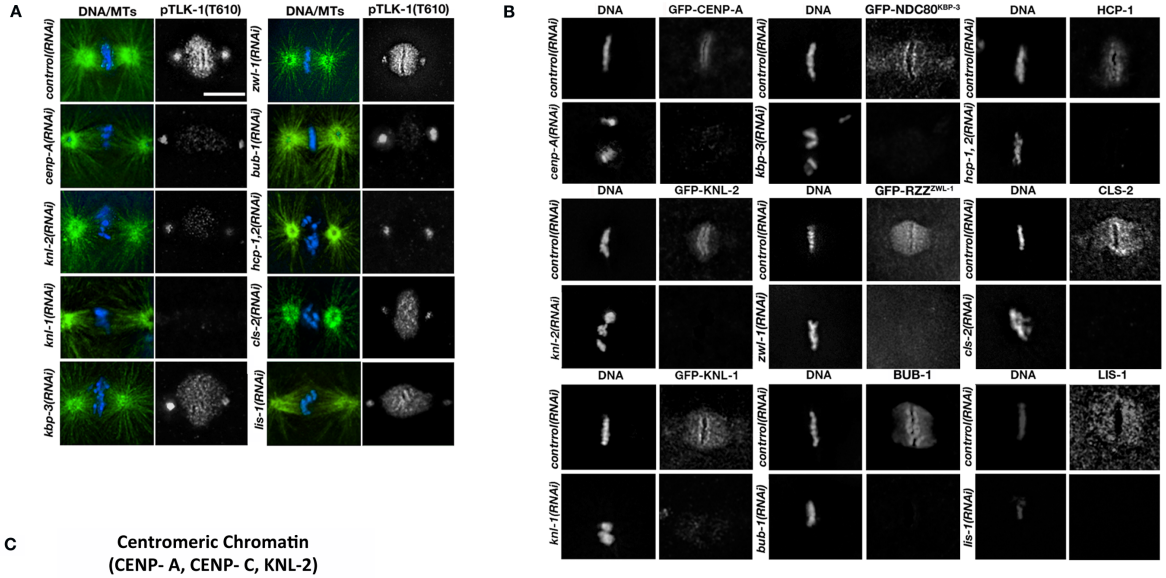
pTLK-1(T610) kinetochore localization is downstream of KNL-1

The remarkable difference in subcellular localization of pTLK-1(T610) and the non-phosphorylated form of TLK-1 (Han et al., 2003) suggest that pTLK-1(T610) may influence kinetochore function. As described above, the kinetochore is a proteinacious multilayer structure that is assembled in a hierarchical fashion (Oegema et al., 2001). The position of a kinetochore component within the hierarchy reveals the potential role of particular proteins in the kinetochore functions. For instance, placement at the centromere "base" of the kinetochore would suggest a role in centromere establishment or stability whereas localization to the outer kinetochore would indicate a functional role in microtubule binding and/or dynamics. To establish the placement of pTLK-1(T610) in the kinetochore hierarchy, I systematically performed RNAi depletion of known kinetochore components at different levels of the hierarchy.

Firstly, I depleted the inner kinetochore proteins CENP-A^{HCP-3} and KNL-2. CENP-A^{HCP-3} is a centromere-specific variant of histone H3 that specifies the centromere while centromeric KNL-2 is required for CENP-A^{HCP-3} deposition (Maddox et al., 2007). Previous studies revealed that CENP-A^{HCP-3} and KNL-2 kinetochore localization are interdependent and work together to initiate kinetochore assembly. In CENP-A^{HCP-3} and KNL-2-depleted embryos, pTLK-1(T610) failed to localize to kinetochores, indicating that pTLK-1(T610) is a bona fide kinetochore protein that lies downstream of centromere establishment (Figure 7A). To determine the efficiency of RNAi, I depleted CENP-A^{HCP-3} and KNL-2 side-by-side in embryos expressing GFP::CENP-A^{HCP-3} or GFP::KNL-2 and immunostained for GFP to assess the level of RNAi depletion (Figure 7B).

Figure 7. Molecular dependency of pTLK-1(T610) kinetochore localization

A) Embryos depleted of the indicated kinetochore proteins were fixed and stained with DAPI (Blue), and α-tubulin (green) and pTLK-1(T610) (red) -specific antibodies. Embryos at the one- and two-cell stage are shown. Scale bar = 2 μm. B) Embryos isolated from wild-type and the specified GFP-transgenic lines were depleted of the indicated kinetochore proteins and were fixed and stained with a GFP-specific antibody (for GFP fusion proteins) or with antibodies specific for the depleted proteins. (*i.e.,* BUB-1, HCP-1, CLS-2, and LIS-1). Embryos at the one- and two-cell stage are shown. C) Schematic representation of the *C. elegans* kinetochore hierarchy demonstrating pTLK-1(T610) placement in red.


A
DNA/MTs
pTLK-1(T610)
DNA/MTs
pTLK-1(T610)
control(RNAi)
zwl-1(RNAi)
cenp-A(RNAi)
bub-1(RNAi)
knl-2(RNAi)
hcp-1,2(RNAi)
knl-1(RNAi)
cls-2(RNAi)
kbp-3(RNAi)
lis-1(RNAi)
B
DNA
GFP-CENP-A
DNA
GFP-NDC80KBP-3
DNA
HCP-1
control(RNAi)
cenp-A(RNAi)
kbp-3(RNAi)
hcp-1, 2(RNAi)
DNA
GFP-KNL-2
DNA
GFP-RZZZWL-1
DNA
CLS-2
control(RNAi)
knl-2(RNAi)
zwl-1(RNAi)
cls-2(RNAi)
DNA
GFP-KNL-1
DNA
BUB-1
DNA
LIS-1
control(RNAi)
knl-1(RNAi)
bub-1(RNAi)
lis-1(RNAi)
C
Centromeric Chromatin
(CENP- A, CENP- C, KNL-2)
KNL-3/MIS-12 Complex
KNL-1
NDC-80KBP-3 Complex
RZZZWL-1 Complex
BUB-1
HCP-1&2
pTLK-1 (T610)
CLS-2
LIS-1

Since pTLK-1(T610) lies downstream of CENP-A^{HCP-3} and KNL-2 in the kinetochore hierarchy, I investigated whether pTLK-1(T610) localization is dependent on KNL-1. KNL-1 is downstream of CENP-A^{HCP-3} and KNL-2 and its role is to establish a functional kinetochore platform for microtubule binding (Desai et al., 2003). KNL-1 is considered a branching point in the kinetochore hierarchy as it is required for the recruitment of the NDC-80 complex, the RZZ complex, and BUB-1 (Cheeseman et al., 2004; Desai et al., 2003; Essex et al., 2009). I found that when KNL-1 was depleted pTLK-1(T610) no longer localized to kinetochores (Figure 7A). As above, I confirmed the efficiency of *knl-1(RNAi)* by assaying the extent of GFP::KNL-1 depletion in parallel (Figure 7B). This experiment revealed that pTLK-1(T610) is downstream of KNL-1 in the kinetochore hierarchy and thus indicates that TLK-1 may have a role in regulating kinetochore-microtubule attachment and/or dynamics.

Despite the fact that the NDC-80 complex, the RZZ complex, and BUB-1 are all downstream of KNL-1 in the kinetochore hierarchy (Figure 3), their kinetochore localization is independent of one another. To establish whether pTLK-1 lies within one of these branches, I singly depleted components of each branch. To assess the NDC-80 complex, I depleted KBP-3, an NDC-80 member whose depletion causes chromosome misalignment and missegregation (Cheeseman et al., 2006). Upon KBP-3 depletion, embryos presented the characteristic KBP-3 phenotype of misaligned metaphase chromosomes (Figure 7A). In addition, GFP immunostaining of GFP::KBP-3 embryos confirmed the efficacy of *kbp-3(RNAi)* depletion (Figure 7B). However, despite the loss of KBP-3, pTLK-1(T610) kinetochore localization was not affected, suggesting that the association of pTLK-1(T610) with kinetochores is likely to be independent of the NDC-

80 complex. Similar results were obtained when I depleted another component of the complex, NDC-80. Embryos depleted of NDC-80 displayed chromosome misalignment and missegregation defects but did not prevent pTLK-1(T610) kinetochore localization (data not shown).

To examine the RZZ complex, I depleted embryos of the RZZ component ZWL-1. Similar to the results obtained for KBP-3 depletion, pTLK-1(T610) kinetochore localization was not affected by ZWL-1 depletion (Figure 7A). In addition, pTLK-1(T610) localization was also not affected by the loss of Spindly, a downstream component of the RZZ complex (data not shown). These results indicate that pTLK-1(T610) kinetochore localization is independent of the RZZ complex. Lastly, I tested whether pTLK-1(T610) kinetochore localization was impacted by BUB-1 depletion. In BUB-1 depleted embryos, pTLK-1(T610) was no longer localized to kinetochores (Figure 7A), suggesting that pTLK-1(T610) lies downstream of BUB-1 in the kinetochore hierarchy. This result indicates that pTLK-1(T610) is an outer kinetochore protein that may function at the kinetochore/microtubule interface.

BUB-1 is required for the kinetochore localization of the CENP-F orthologs HCP-1 and -2 (Encalada et al., 2005). HCP-1 and -2 are outer kinetochore proteins that interact with microtubules and aid in the establishment of functional kinetochore-microtubule attachments (Cheeseman et al., 2005). To test if HCP-1 and -2 are required for pTLK-1(T610) kinetochore recruitment, I co-depleted embryos of HCP-1 and HCP-2 (as described in Materials and Methods). In embryos depleted of HCP-1 and -2 pTLK-1 (T610) failed to localize to kinetochores indicating that pTLK-1(T610) is downstream of HCP-1 and -2 in the kinetochore hierarchy. Previous studies revealed that *C. elegans*

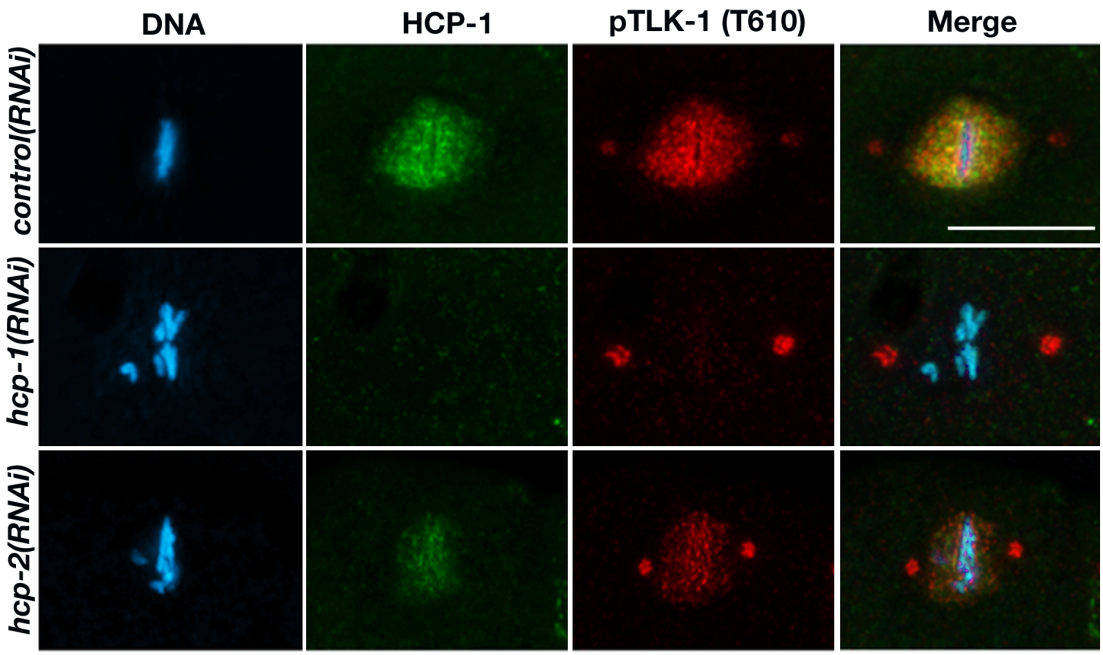
HCP-1 and HCP-2 are redundant, since their co-depletion led to a strong embryonic lethal phenotype, while their individual depletion did not result in strong phenotypes (Cheeseman et al., 2005). To determine if pTLK-1(T610) kinetochore localization was dependent on both HCP-1 and HCP-2, I singly depleted HCP-1 and HCP-2 from embryos. In HCP-1-depleted embryos pTLK-1(T610) staining was not present at kinetochores (Figure 8). Surprisingly, in HCP-2-depleted embryos, pTLK-1(T610) kinetochore localization was intact (Figure 8). These results revealed that pTLK-1(T610) kinetochore localization is primarily dependent on HCP-1, and not HCP-2, and establishes a non-redundant, independent role for HCP-1 in the kinetochore recruitment of pTLK-1(T610). Due to the lack of an HCP-2 antibody, *hcp-2(RNAi)* effectiveness was confirmed by the presence of misaligned metaphase chromosomes and other mitotic defects (Figure 8 and data not shown).

To further assess the placement of pTLK-1(T610) in the kinetochore hierarchy, I examined embryos depleted of CLASP2/CLS-2. CLS-2 is involved in microtubule polymerization and requires HCP-1 and -2 for its kinetochore recruitment (Cheeseman et al., 2005). *cls-2(RNAi)* resulted in undetectable α-CLS-2 immunostaining and chromosome alignment defects (Figure 7B), but did not affect the kinetochore localization of pTLK-1(T610) (Figure 7A). In addition, I examined embryos depleted of LIS-1. LIS-1 is a microtubule interacting protein whose kinetochore localization is also dependent on CENP-F/HCP-1,-2 (Cockell et al., 2004; Vergnolle and Taylor, 2007). In *lis-1(RNAi)* embryos, LIS-1 immunostaining was greatly reduced and gross chromosome defects were apparent (Figure 7B), but the association of pTLK-1(T610) with kinetochores was not affected (Figure 7A). Altogether, these results confirm the

placement of pTLK-1(T610) at the outer kinetochore and indicates that TLK-1 lies downstream of HCP-1, but is independent of CLS-2 and LIS-1.

Figure 8. HCP-1, but not HCP-2, is required for pTLK-1(T610) kinetochore localization

Embryos from *control(RNAi), hcp-1(RNAi)* and *hcp-2(RNAi)* -treated hermaphrodites were fixed and stained with DAPI (Blue), and HCP-1 (green) and pTLK-1(T610) (red) antibodies. Embryos at the one- cell stage are shown. (Scale bar = 5 μm)


DNA
HCP-1
pTLK-1 (T610)
Merge
control(RNAi)
hcp-1(RNAi)
hcp-2(RNAi)

TLK-1 kinetochore localization is necessary for the kinetochore recruitment of microtubule interacting proteins

As described above, molecular dependency experiments placed pTLK-1(T610) downstream of HCP-1 and independent or upstream of CLS-2 and LIS-1 in the kinetochore hierarchy. The placement of pTLK-1(T610) at the outer kinetochore suggests that pTLK-1(T610) may have a role in microtubule binding and/or dynamics. Since CLS-2 and LIS-1 are HCP-1 and -2-dependent outer kinetochore proteins that regulate microtubule dynamics I sought to determine if TLK-1 affects the kinetochore localization of CLS-2 and LIS-1. In this particular experiment my analysis focused on multicellular embryos (32-50 cells) since we have previously determined that the *tlk-1(RNAi)* phenotype is most penetrant at this stage of development (Han et al., 2005).

Multicellular *control* and *tlk-1(RNAi)* embryos were fixed and stained with CLS-2 and LIS-1 antibodies as well as an α-tubulin antibody to assess microtubules. In control embryos, CLS-2 immunostaining staining revealed two kinetochore stripes that appeared in metaphase but disappeared at anaphase onset (Figure 9A and data not shown). In contrast, CLS-2 kinetochore staining was greatly reduced in TLK-1-depleted embryos (Figure 9A). Similar results were obtained for LIS-1 immunostaining except that LIS-1 immunostaining was also present in the cytoplasm in addition to kinetochores. In the absence of TLK-1, LIS-1 was no longer associated with kinetochores (Figure 9A). Together, these experiments suggest that the localization of CLS-2 and LIS-1 to kinetochores is dependent on TLK-1, effectively placing TLK-1 in a linear pathway downstream of HCP-1 and upstream of these two microtubule-binding proteins.

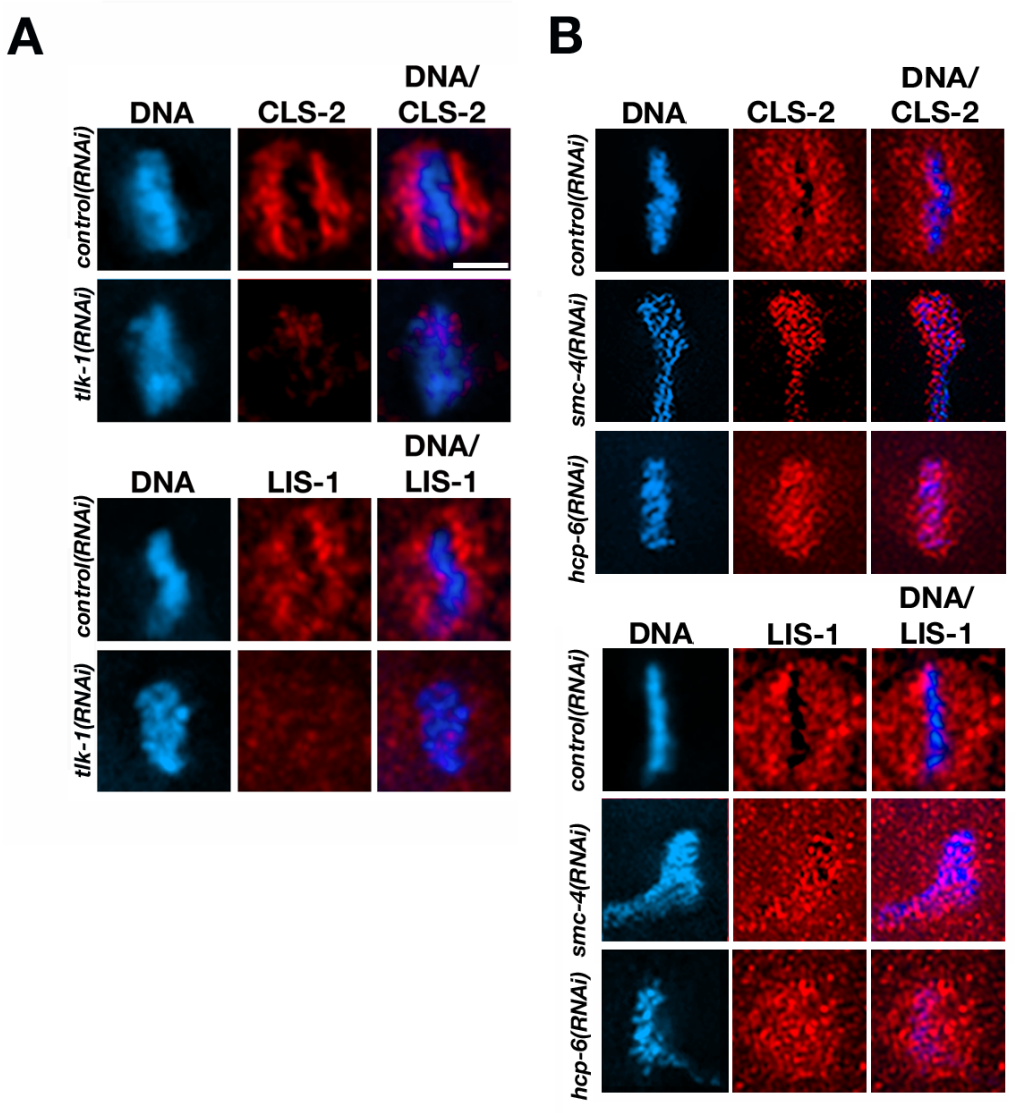
A characteristic phenotype of *tlk-1(RNAi)*- treated embryos is the presence of misaligned chromosomes during metaphase as well as wider than normal metaphase plates (Han et al., 2005). Misaligned chromosomes and wide metaphase plates are phenotypes that can be indicative of chromosomes condensation defects (Chan et al., 2004). Therefore, it can be argued that the loss of CLS-2 and LIS-1 in *tlk-1(RNAi)*-treated embryos is due to a reduction in chromosome condensation rather than a direct effect of TLK-1 on kinetochores. To address this concern I assessed CLS-2 and LIS-1 localization in embryos depleted of the condensation proteins SMC-4 and HCP-6.

SMC-4 or Structural Maintenance of Chromosomes protein 4 is a core subunit of the condensin I and II complexes which are required for mitotic chromosome condensation (Hagstrom et al., 2002). SMC-4 exhibits a chromosomal localization and this chromosomal localization remains unaffected in embryos depleted of CENP-C$^{HCP-4,}$ indicating that SMC-4 chromosome localization is independent of kinetochore assembly (Hagstrom et al., 2002). In SMC-4 depleted embryos, gross defects in chromosome condensation were apparent (Figure 9B). However, CLS-2 and LIS-1 still exhibited chromosomal localization, which contrasted with the complete loss of chromosome or kinetochore-associated CLS-2 and LIS-1 observed in TLK-1-depleted embryos (Figure 9). Similar results were obtained with embryos depleted of HCP-6. HCP-6 is also part of the condensin complex but unlike SMC-4, HCP-6 requires kinetochore assembly for its localization to chromosomes (Stear and Roth, 2002). As in *smc-4(RNAi)* embryos, HCP-6 depletion resulted in gross chromosome condensation and segregation defects, but did not prevent CLS-2 or LIS-1 chromosomal localization (Figure 9B). These

results indicate that the loss of CLS-2 and LIS-1 from chromosomes in TLK-1-depleted embryos is not a secondary consequence of accompanying chromosome condensation defects. Instead, these results suggest that CLS-2 and LIS-1 failed to localize to chromosomes in *tlk-1(RNAi)* embryos because TLK-1 is an integral component of the outer kinetochore.

Figure 9. TLK-1 is required for CLS-2 and LIS-1 kinetochore localization

A) Multicellular embryos treated with *control(RNAi)* and *tlk-1(RNAi)* were fixed and stained with DAPI to visualize DNA (Blue), an α-tubulin antibody (green), and anti-CLS-2 or anti-LIS-1 antibodies (red) B) Multicellular embryos treated with *control(RNAi), smc-4(RNAi)* and *hcp-6(RNAi)* were fixed and stained with DAPI to visualize DNA (Blue), an α-tubulin antibody (green), and anti-CLS-2 or anti-LIS-1 antibodies (red). (Scale bar = 5 μm).


A
DNA
CLS-2
DNA/
CLS-2
control(RNAi)
tlk-1(RNAi)
DNA
LIS-1
DNA/
LIS-1
control(RNAi)
tlk-1(RNAi)
B
DNA
CLS-2
DNA/
CLS-2
control(RNAi)
smc-4(RNAi)
hcp-6(RNAi)
DNA
LIS-1
DNA/
LIS-1
control(RNAi)
smc-4(RNAi)
hcp-6(RNAi)

pTLK-1(T610) kinetochore localization is independent of microtubule binding

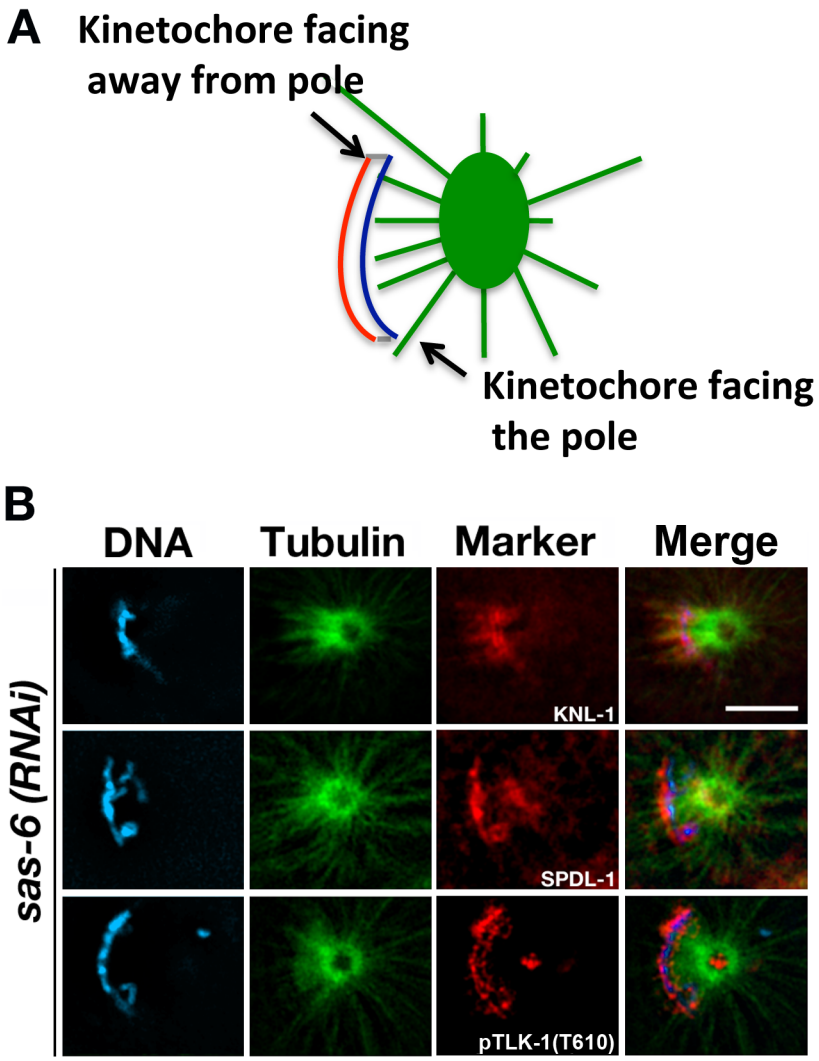
Effective sister chromatid separation is highly dependent on proper kinetochore assembly. The experiments above placed pTLK-1(T610) in the outer kinetochore layer at the core of the microtubule-binding interface. To better understand pTLK-1(T610) kinetochore function I addressed whether pTLK-1(T610) kinetochore localization is dependent on microtubule attachment. To answer this question I took advantage of the *sas-6(RNAi)* phenotype. SAS-6 is a centriolar protein required for centriole duplication and maturation (Dammermann et al., 2004; Leidel et al., 2005). Depletion of SAS-6 results in monopolar spindles where only the kinetochore facing the pole is attached to spindle microtubules. The kinetochore on the sister chromatid facing away from the pole remains unattached (Figure 10A).

The kinetochore protein KNL-1 localizes to the kinetochore independently of microtubule attachments (Yamamoto et al., 2008). Hence, embryos expressing GFP::KNL-1 were used as a control in this experiment. To this end, GFP::KNL-1 expressing embryos depleted of SAS-6 were immunostained for GFP, revealing that, as expected, KNL-1 was localized to both kinetochores (Figure 10B). As a positive control for a protein whose localization is dependent on microtubule attachments, I used embryos expressing GFP::SPDL-1. SPINDLY/SPDL-1 is essential for the recruitment of the SAC protein MAD-3 to unattached kinetochores (Yamamoto et al., 2008). Once kinetochore-microtubule attachments are made, SPDL-1 is released from kinetochores. Hence, SPDL-1 only binds to unattached kinetochores (Yamamoto et al., 2008). In GFP::SPDL-1 expressing embryos depleted of SAS-6, SPDL-1 was localized to the

unattached kinetochore and not the centrosome-proximal, attached kinetochore (Figure 10B). Finally, SAS-6-depleted embryos immunostained with our pTLK-1(T610) antibody revealed that pTLK-1(T610), like KNL-1, localized to both the attached and unattached kinetochore faces, indicating that pTLK-1(T610) kinetochore localization is independent of kinetochore-microtubule attachment (Figure 10B). These results indicate that TLK-1, unlike SPDL-1, is not sensitive to kinetochore-microtubule attachment and suggest that TLK-1 likely plays a role in recruiting microtubule-binding proteins and may promote the establishment of appropriate kinetochore-microtubule attachments.

Figure 10. pTLK-1(T610) kinetochore localization is not regulated by microtubule attachment

A) Schematic representation of kinetochore-microtubule attachments upon monopolar spindle induction via *sas-6(RNAi)*. The red kinetochore represents the unattached kinetochore, while the blue represents the attached, poleward facing kinetochore. B) Embryos from *sas-6(RNAi)*-treated hermaphrodites were fixed and stained with DAPI (blue), a tubulin-specfic antibody (green) and a GFP-specific antibody to visualize GFP::KNL-1 and GFP::SPDL-1 (red), or pTLK-1(T610) (red) antibody. Embryos at the two-cell stage are shown (Scale bar = 5 μm).


A
Kinetochore facing away from pole
Kinetochore facing the pole
B
DNA
Tubulin
Marker
Merge
sas-6 (RNAi)
KNL-1
SPDL-1
pTLK-1(T610)

TLK-1 is required for proper kinetochore architecture

As described above, I established that pTLK-1(T610) is a *bona fide* kinetochore protein that is localized to the outer kinetochore layer, at the kinetochore-microtubule binding interface. These results were obtained by depleting different kinetochore components, and assessing pTLK-1(T610) localization in their absence. However, it is well established that some kinetochore proteins exhibit co-dependency with other kinetochore components with respect to their kinetochore localization (Cheeseman et al., 2004). To determine if pTLK-1(T610) affects the localization of other kinetochore proteins I examined the localization of several kinetochore proteins in TLK-1-depleted embryos.

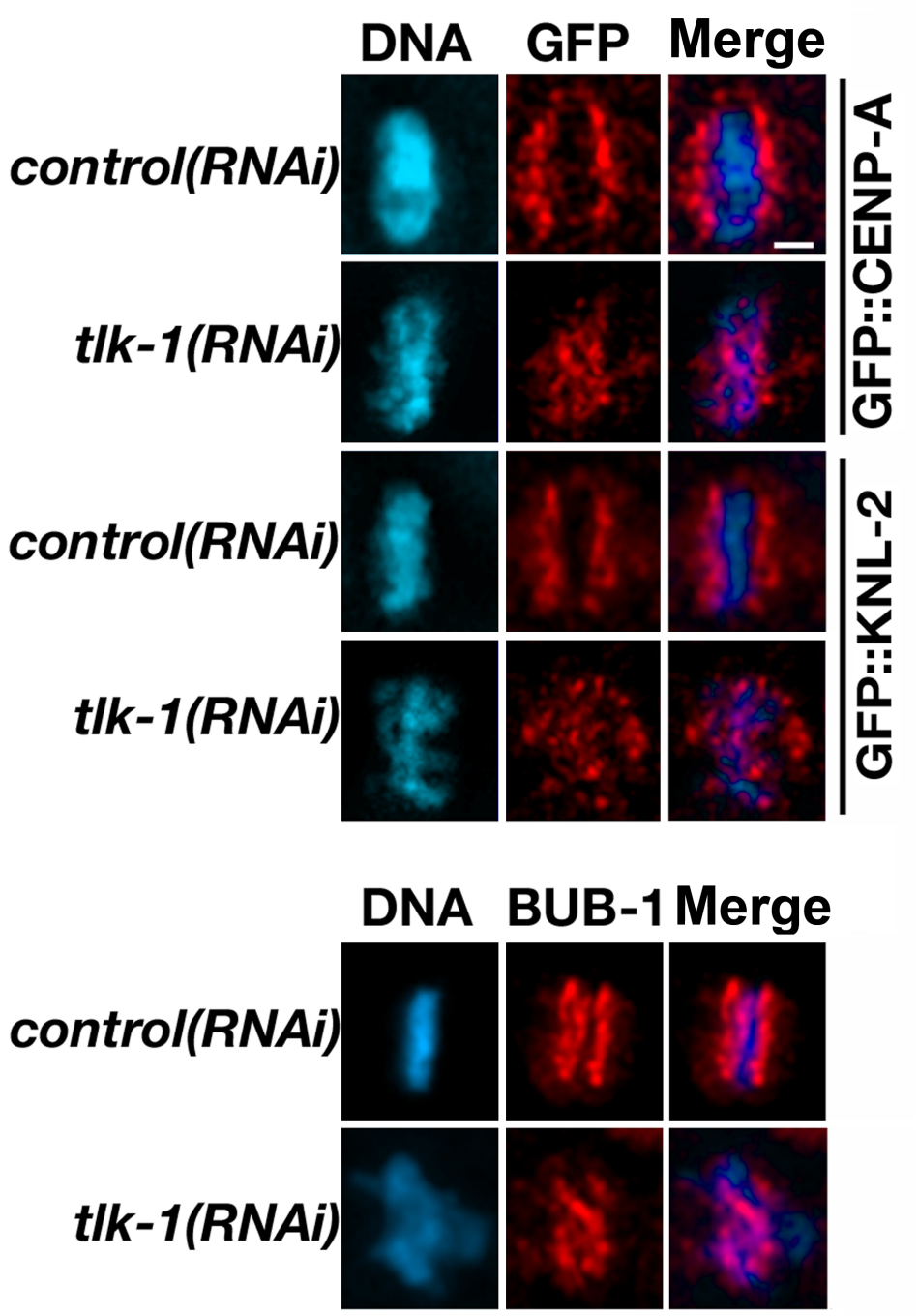
For this analysis I focused on multicellular embryos (32-50 cells) where *tlk-1(RNAi)* is the most penetrant (Han et al., 2003). To this end, strains expressing GFP::CENP-A^{HCP-3} and GFP::KNL-2 were treated with *control* and *tlk-1(RNAi)* and the resultant embryos were fixed and stained with GFP and α-tubulin specific antibodies. Visual analysis of GFP::CENP-A^{HCP-3} and GFP::KNL-2 localization in TLK-1-depleted embryos did not reveal a difference in staining intensity compared to control embryos. However, when examining the subcellular localization of GFP::CENP-A^{HCP-3} and GFP::KNL-2, I found that kinetochore architecture was aberrant in *tlk-1(RNAi)* embryos vs. control embryos (Figure 11). In control embryos, GFP::CENP-A^{HCP-3} and GFP::KNL-2 localized to metaphase kinetochores in two defined kinetochore "stripes" (Figure 11). In TLK-1 depleted embryos, these proteins still localized to kinetochores but the kinetochores appeared to be "twisted" (Figure 11). These results suggest that

while CENP-A^{HCP-3} and KNL-2 kinetochore localization is independent of TLK-1, TLK-1 plays a critical role in maintaining proper kinetochore structure.

To establish the influence of TLK-1 on other kinetochore proteins, I analyzed the localization of BUB-1 in *control* and *tlk-1(RNAi)*-depleted embryos, as assayed by immunostaining with a BUB-1-specific antibody. As with GFP::CENP-A^{HCP-3} and GFP::KNL-2, BUB-1 was still localized to kinetochores in TLK-1-depleted cells, but displayed a twisted-like pattern, indicative of aberrant kinetochore architecture (Figure 11). All together these results revealed while TLK-1 does not affect the recruitment of CENP-A^{HCP-3}, KNL-2, and BUB-1 to the centromere or kinetochore respectively, it is required to maintain the correct architecture of these structures.

Figure 11. TLK-1 depletion affects kinetochore architecture

Embryos treated with *control(RNAi)* and *tlk-1(RNAi)* were fixed and stained with DAPI (blue), GFP (red, top panels), BUB-1 (red, bottom panel) and α-tubulin (green) antibodies. Cells from multi-cellular embryos are shown. (Scale bar = 5 μm).


DNA
GFP
Merge
control(RNAi)
tlk-1(RNAi)
control(RNAi)
tlk-1(RNAi)
GFP::CENP-A
GFP::KNL-2
DNA
BUB-1
Merge
control(RNAi)
tlk-1(RNAi)

TLK-1 influences kinetochore –microtubule attachments

Establishment of proper kinetochore-microtubule attachments is critical for proper chromosome segregation during mitosis (Cheeseman and Desai, 2008). Kinetochore-microtubule attachments are regulated by outer kinetochore proteins that create a microtubule binding interface which stabilize these attachments, preventing premature sister chromatid separation. Here, I present evidence that one function of TLK-1 is the localization of CLS-2 and LIS-1 to kinetochores. While loss of LIS-1 by *lis-1(RNAi)* leads to a very strong phenotype characterized by severe chromosome segregation defects (Cockell et al., 2004; Dawe et al., 2001), loss of CLS-2 presents milder defects that can also be modulated by the amount of dsRNA fed to *C. elegans* hermaphrodites. As described previously, *tlk-1(RNAi)* does not result in chromosome segregation defects in early embryos (Han et al., 2005). To genetically assess the interaction between TLK-1 and CLS-2, I took advantage of these milder phenotypes, and assessed whether co-depletion results in a phenotypic enhancement that would be indicative of a functional interaction between CLS-2 and TLK-1, similar to what we previously reported for TLK-1 and AIR-2 (Han et al., 2005).

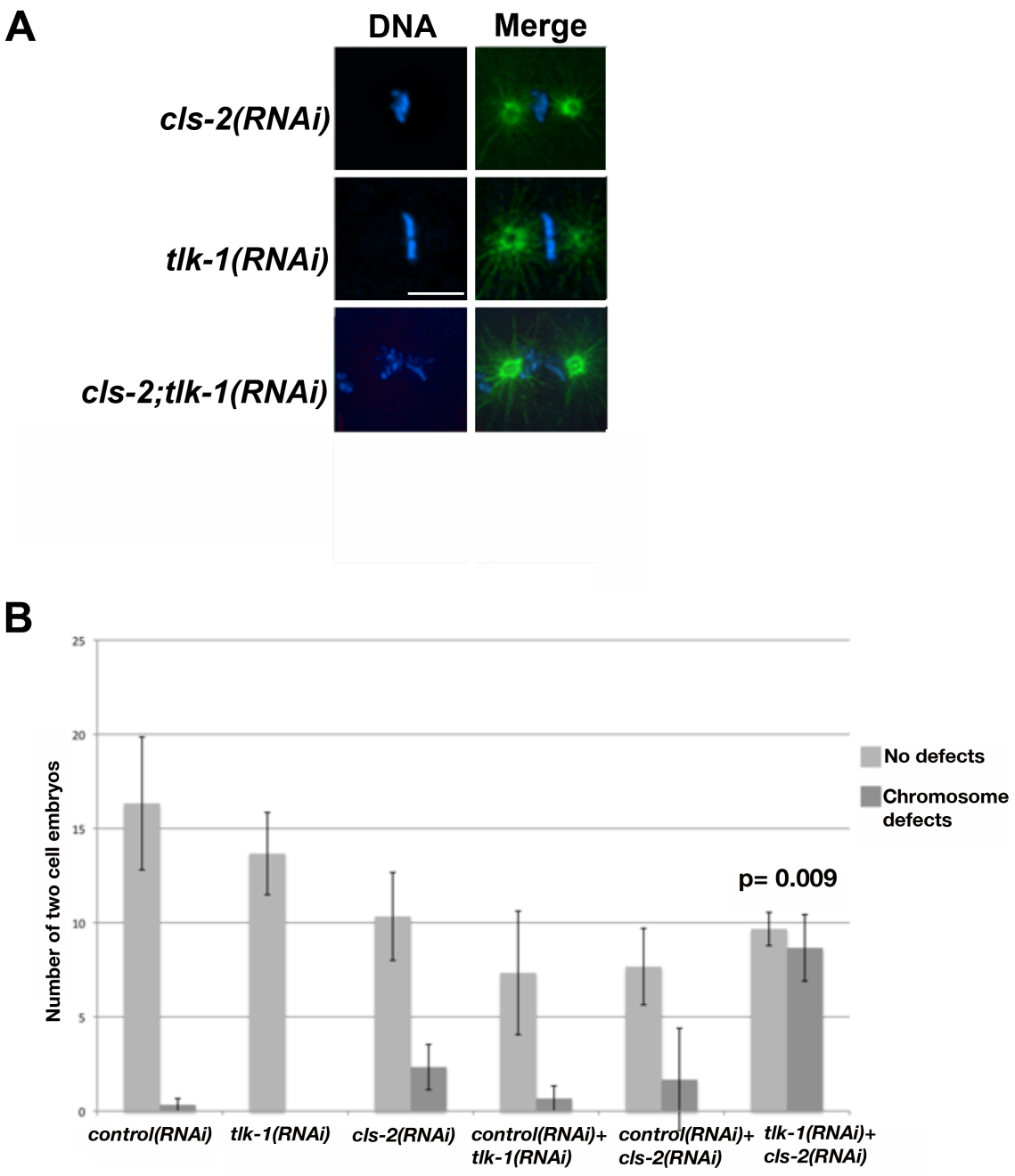
To this end I used the feeding method of *RNAi* to singly deplete and co-deplete CLS-2 and TLK-1. As a control, I also combined *cls-2* dsRNA and *tlk-1* dsRNA with control dsRNA in equal amounts. One- and two-cell embryos were examined for chromosome segregation defects such as metaphase chromosome misalignment and anaphase bridges. Among *control* (n=50) and *tlk-1(RNAi)* (n=41) early embryos, I found no embryos with any observable chromosome segregation defects (Figure 12), although older *tlk-1(RNAi)* embryos displayed the anaphase bridges and wider

metaphase plates characteristic of TLK-1 depletion, indicating the effectiveness of the *tlk-1(RNAi)*(data not shown). Interestingly, 18% of *cls-2(RNAi)* embryos had chromosome segregation defects, yet this was not significantly different than *control(RNAi)* embryos (p=0.18) (Figure 12). Co-depletion of *control(RNAi*) with *cls-2(RNAi)* or *tlk-1(RNAi)* yielded similar results, with less that 10% of embryos displaying mitotic defects. In contrast, analysis of *cls-2*;*tlk-1(RNAi)* co-depleted embryos revealed that 50% of the early embryos displayed mitotic defects (Figure 12), a statistically-significant increase as compared to *control(RNAi)* (p=0.009) and *cls-2;control(RNAi)* co-depletion (p=0.03) (Figure 12). Additionally, in *cls-2*;*tlk-1(RNAi)* co-depleted embryos the chromosomes do not appear to align correctly at the metaphase plate, while in the *cls-2(RNAi*) chromosome congress and align at the metaphase plate. Collectively, these results suggest that TLK-1 and CLS-2 genetically interact and are necessary for proper chromosome alignment during metaphase in *C. elegans* embryos.

Further studies of *cls-2;tlk-1(RNAi)* co-depleted embryos will be necessary to determine how CLS-2 and TLK-1 co-depletion affects K–MT attachments. One assay is live cell imaging of embryos expressing GFP-α-tubulin and mCherry-histone H2B, and to track the timing and rate of mitotic spindle elongation of embryos co-depleted of CLS-2 and TLK-1. If CLS-2 and TLK-1 co-depletion weakens K-MT interactions, this would result in premature spindle elongation due to lack of resistance to cortical pulling forces. In addition, the rate of microtubule polymerization can be assessed since CLS-2 is required for proper microtubule polymerization during mitosis (Lansbergen et al., 2006).

Figure 12. TLK-1 depletion exacerbates the CLS-2 phenotype

A) Embryos treated with the indicated RNAi were fixed and stained with DAPI (blue) and an a-tubulin specific antibody (green). Metaphase cells from two-cell embryos are shown. Scale bar = 5 um B) Quantitation of two-cell embryos displaying metaphase alignment defects for the indicated *(RNAi)* conditions. Error bars: mean +/- standard error of the means (s.e.m.).


A
DNA
Merge
cls-2(RNAi)
tlk-1(RNAi)
cls-2;tlk-1(RNAi)
B
Number of two cell embryos
25
20
15
10
5
0
No defects
Chromosome defects
p= 0.009
control(RNAi)
tlk-1(RNAi)
cls-2(RNAi)
control(RNAi)+ tlk-1(RNAi)
control(RNAi)+ cls-2(RNAi)
tlk-1(RNAi)+ cls-2(RNAi)

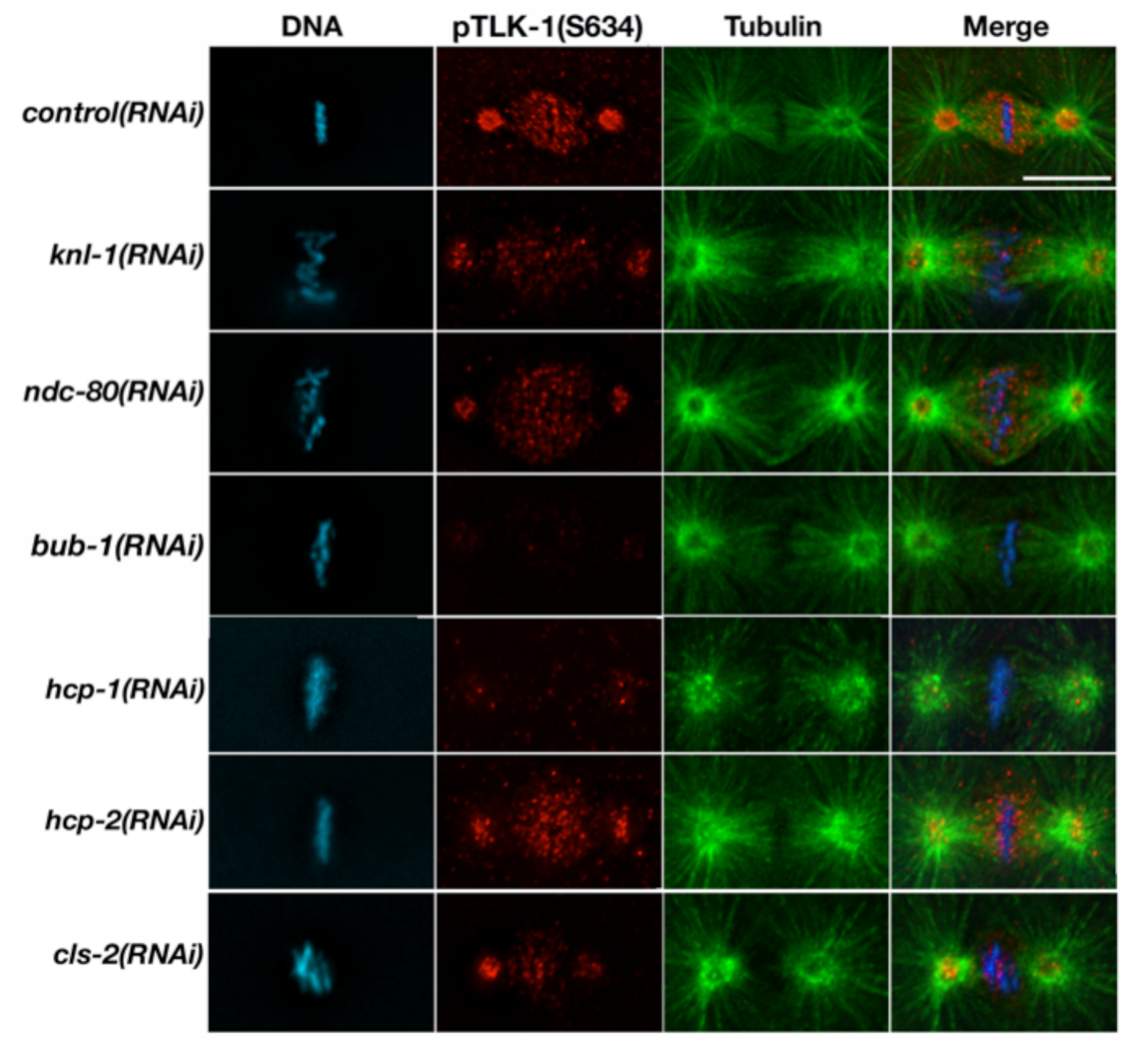
pTLK-1(S634) displays a kinetochore molecular dependency similar to pTLK-1(T610)

The function of cell cycle-associated kinases such as those of the Polo and Aurora families are intimately-associated with their subcellular localization (Carmena and Earnshaw, 2003). As mentioned previously, Aurora B/AIR-2 mediated phosphorylation of TLK-1 at serine 634 creates an activation feedback loop that results in increased AIR-2 activity *in vivo* (Han et al., 2005). The localization of pTLK-1(S634) was originally characterized with limited microscopy resolution and without the advantage of deconvolution (Han et al., 2005). This initial characterization revealed that pTLK-1(S634) was significantly reduced in embryos treated with *air-2(RNAi)* indicating that pTLK-1(S634) is an *in vivo* substrate of AIR-2 (Han et al., 2005). Despite the low resolution, the expression pattern and subcellular localization of pTLK-1(S634) was substantially different from the unmodified form of TLK-1 (Han et al., 2005). In these experiments pTLK-1(S634) was found to be associated with chromosomes from prophase to metaphase (Han et al., 2005). During metaphase, pTLK-1 (S634) localization was described as a "halo" around the chromosomes (Han et al., 2005). Using higher microscopy resolution and deconvolution, our lab discovered that pTLK-1(S634) is localized to kinetochores from prophase to metaphase, as well as KT-MTs during metaphase (J.M.S., unpublished). To determine whether pTLK-1(S634) displays the same dependence as pTLK-1(T610), I undertook a similar series of depletion experiments to determine the placement of pTLK-1(S634) with respect to the kinetochore hierarchy (as in Figure 7). These experiments revealed that pTLK-1(S634) behaves identically to pTLK-1(T610) indicating that both TLK-1 phosphoisoforms are

localized to the outer kinetochore downstream of HCP-1 and upstream of CLS-2 and LIS-1 (Figure 13 and data not shown. Furthermore, the kinetochore localization of pTLK-1(S634), like pTLK-1(T610) is not affected by the presence or absence of kinetochore-microtubule attachment (data not shown).

Figure 13. Molecular dependency of pTLK-1(S634) kinetochore localization

Embryos depleted of the indicated kinetochore proteins were fixed and stained with DAPI (Blue), and α-tubulin (green) and pTLK-1(S634) (red)-specific antibodies. Cells from one- or two-cell embryos are shown. Scale bar = 10 um


DNA
pTLK-1(S634)
Tubulin
Merge
control(RNAi)
knl-1(RNAi)
ndc-80(RNAi)
bub-1(RNAi)
hcp-1(RNAi)
hcp-2(RNAi)
cls-2(RNAi)

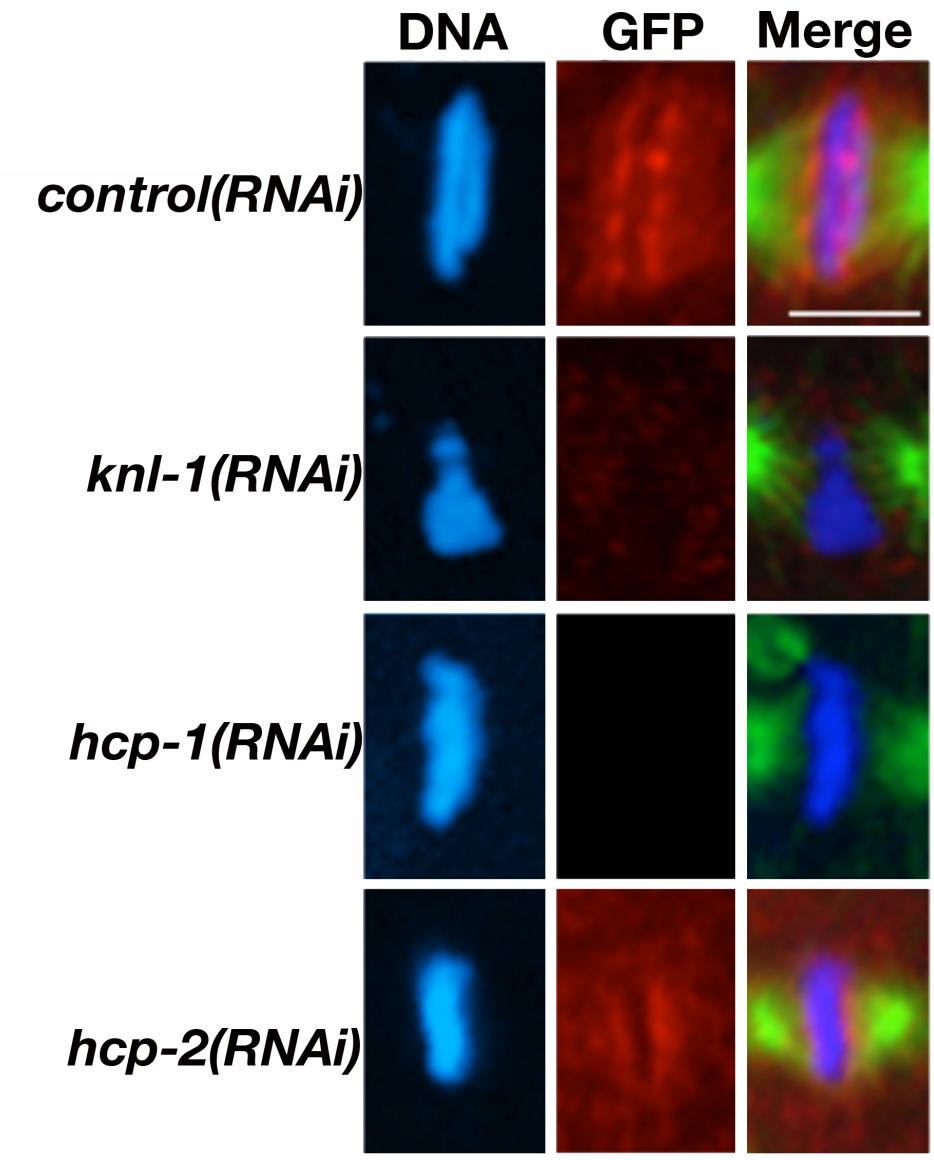
Exogenous GFP::TLK-1 exhibits kinetochore localization

After discovering the remarkable change in TLK-1 subcellular localization upon phosphorylation it was important to generate a tool to study TLK-1 behavior *in vivo*. Towards this end our laboratory generated a strain expressing a GFP::TLK-1 fusion protein (Materials and Methods). Analysis of GFP::TLK-1 by GFP immunostaining revealed that like the endogenous protein, GFP::TLK-1 is localized to interphase and prophase nuclei (data not shown). In addition, it displays kinetochore localization during prophase and metaphase and kinetochore and kinetochore-microtubule localization during metaphase (Figure 14). This suggests that GFP-TLK-1 behaves *in vivo* like each pTLK-1 isoform and provides additional evidence to validate the results I obtained with pTLK-1 specific antibodies.

To further characterize GFP::TLK-1 kinetochore localization, I depleted known kinetochore proteins as described above (Figure 7). As observed with both pTLK-1 isoforms, depletion of KNL-1 results in loss of GFP::TLK-1 kinetochore localization (Figure 14). I then depleted proteins downstream of KNL-1, including HCP-1 and HCP-2. As above, depletion of HCP-1, but not HCP-2, diminished GFP-TLK-1 kinetochore localization; suggesting that, as with the pTLK-1 isoforms, GFP::TLK-1 requires HCP-1 for its kinetochore localization (Figure 14). Altogether, these results confirm that TLK-1 associates with the outer kinetochore in an HCP-1 dependent manner. I hypothesize that this GFP::TLK-1 fusion protein, like endogenous TLK-1, is subject to phosphorylation by the CHK-1 and AIR-2 kinases at T610 and S634 respectively, and that these modifications are critical for the kinetochore association and mitotic functions of TLK-1.

Figure 14. GFP::TLK-1 line recapitulates the pTLK-1 kinetochore localization pattern

One- or two-cell Embryos expressing GFP::TLK-1 were depleted of the indicated kinetochore proteins and fixed and stained with DAPI (blue), and α-tubulin (green) and GFP) (red) -specific antibodies. Metaphases plates from one- and two-cell embryos are shown. (Scale bar = 5um).


DNA
GFP
Merge
control(RNAi)
knl-1(RNAi)
hcp-1(RNAi)
hcp-2(RNAi)

Chapter III: The kinetochore protein KNL-2 is an *in vitro* substrate of TLK-1

Introduction

The centromeric protein CENP-A, a histone H3 isoform, is a component of all eukaryotic centromeres and appears to be the primary epigenetic signal for centromere identity and kinetochore establishment. Previous studies revealed that KNL-2, a Myb-domain protein, is necessary for the loading of CENP-A to the centromere (Maddox et al., 2007). In *C. elegans*, both *cenp-A*(*RNAi*) embryos and *knl-2*(*RNAi*) embryos display a kinetochore null phenotype, with no evidence of kinetochore recruitment establishment or chromosome attachment to mitotic spindle microtubules (Maddox et al., 2007). In human cells, the KNL-2 homolog, Mis18Bp, is also required for CENP-A loading at the centromere (Fujita et al., 2007). Although, KNL-2 is required to load CENP-A at the centromere, the mechanism by which KNL-2 is regulated remains unknown.

KNL-2 is an unusual Myb- domain protein since it carries a single copy of this domain, whereas the majority of Myb-proteins have two or more domains. This characteristic is also shared by Tki, a Tousled kinase substrate identified in plants (Ehsan et al., 2004) . In *C. elegans*, the Tousled-like kinase (TLK-1) is involved in many cell cycle events. As shown in the previous chapter TLK-1 phosphorylation localizes TLK-1 to the kinetochore during metaphase in *C. elegans* embryos. Given these similarities and the localization of TLK-1 and KNL-2 at the kinetochore I sought out to determine if KNL-2 is an *in vitro* substrate of TLK-1. In this chapter I present evidence that identified KNL-2 as is an *in vitro* substrate of TLK-1, suggesting that TLK-1 kinase activity plays a role in kinetochore assembly.

Results

TKL-1 phosphorylates the inner kinetochore protein KNL-2 *in vitro*

To determine if KNL-2 is an *in vitro* substrate of TLK-1, the full-length *C. elegans knl-2* cDNA was amplified from a cDNA library and sub-cloned into a GST bacterial expression vector. Upon transformation and IPTG induction, protein extracts were made and the GST-KNL-2 protein purified on glutathione beads (as described in Materials and Methods). Unfortunately, GST-KNL-2 bacterial expression was not optimal, and attempts to use different expression tags yield similar results. To circumvent this problem I created three overlapping GST-tagged fragments of KNL-2, an N-terminal fragment, a Middle-fragment and a C-terminal-fragment, to aid in the identification of potential phosphorylation sites. The N-terminal and the Middle-terminal had an overlapping region of 108 amino acids and the Middle-fragment and the C-terminal fragment had a region of 152 overlapping amino acids (Figure 15A). Fortunately, these protein fragments were highly expressed and readily purified (data not shown).

Kinase active and kinase dead forms of TLK-1 were fused to the maltose binding protein (MBP) and expressed and purified from *E. coli*. *In vitro* kinase assay utilizing the purified GST-KNL-2 fragments and MBP-TLK-1 and MBP-TLK-1KD were performed. This assay revealed TLK-1 dependent phosphorylation of the KNL-2 Middle-fragment, indicating that TLK-1 phosphorylates KNL-2 in this region of 331 amino acids (Figure 15B).

To further map the KNL-2 phosphorylation site in the KNL-2 Middle fragment, I created three non-overlapping GST-KNL-2 sub-fragments of the KNL-2 Middle-

fragment. M-fragment 1 contained the first 107 amino acids of the KNL-2 Middle-fragment, M-fragment 2 comprised the next 104 amino acids and M-fragment 3 contained the last 129 amino acids of the KNL-2 Middle fragment (Figure 16A). After expression and purification of these GST-KNL-2 M-sub-fragments, kinase assays were preformed as described above. These assays revealed a major MBP-TLK-1 phosphorylation site in KNL-2 M-fragment 3 (Figure 16B). (Note that there is also a significant site in Fragment 2).

To aid in the identification of the specific KNL-2 phosphorylation site in the KNL-2 M-fragment 3, I performed an amino acid sequence alignment of this region from *M. musculus* (MmKNL-2), *H. sapiens* (HsMis18), *G. gallus* (GgKNL-2) and *C. elegans* (CeKNL-2). Since TLK-1 is a serine/threonine kinase I focused on conserved serine and threonine residues in this region. This alignment uncovered the following conserved sites in the KNL-2 M-fragment 3: CeKNL-2 S437, S447, S527, and S533 (Figure 17). To test the contribution of these amino acids to KNL-2 phosphorylation I generated a non-phosphorylatable mutant for each site (KNL-2S437A, S447A, S527A, and S533A).

A kinase assay utilizing the KNL-2 non-phosphorylatable mutants revealed that in the presence of active MBP-TLK-1 KNL-2 S533A phosphorylation was reduced compared to the other mutants (Figure 18). These results suggest that KNL-2 is phosphorylated *in vitro* by TLK-1 at S533. These results are remarkable since this is the first evidence of TLK-1 dependent phosphorylation a kinetochore protein and further supports an important role for the TLK-1 kinase in the regulation of the kinetochore.

Figure 15. TLK-1 phosphorylates KNL-2 M- fragment *in vitro*

A) Schematic representation of the GST-KNL-2 overlapping fragments B) Bacterially expressed and purified recombinant MBP-TLK-1 and MBP-TLK-1 kinase-dead (KD) proteins were incubated in kinase reaction buffer with the indicated purified recombinant GST-KNL-2 fragments. The level of [^{32}P] incorporation into the KNL-2 (pKNL-2) fragments were assessed by phosophoimaing. Loading of the GST-KNL-2 fragments was assessed by by Ponceau S staining.

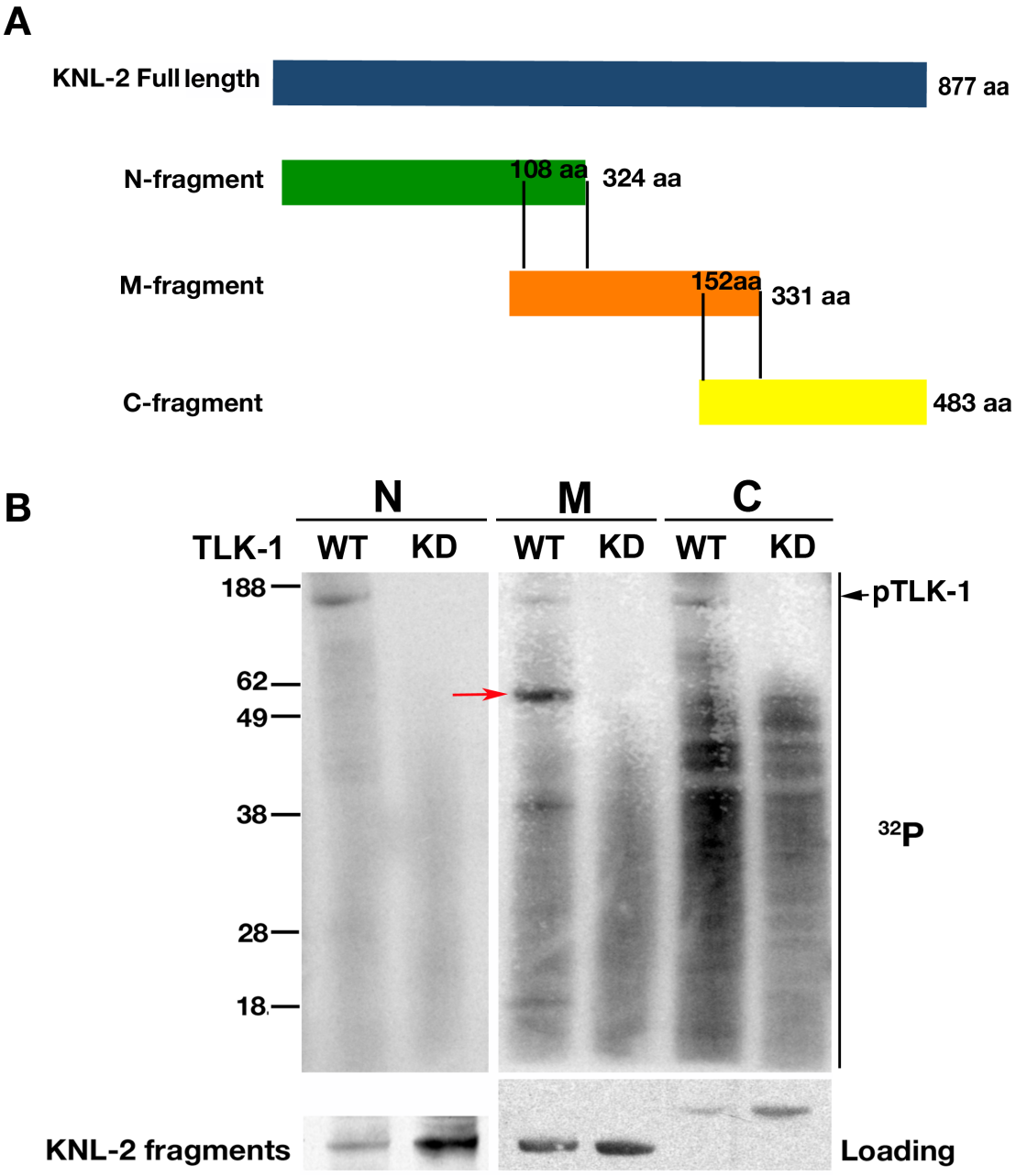

A
KNL-2 Full length
877 aa
N-fragment
108 aa
324 aa
M-fragment
152aa
331 aa
C-fragment
483 aa
B
N
M
C
TLK-1
WT
KD
WT
KD
WT
KD
188
62
49
38
28
18
pTLK-1
32P
KNL-2 fragments
Loading

Figure 16. TLK-1 phosphorylates the KNL-2 Middle-fragment 3 *in vitro*

A) Schematic representation of the GST-KNL-2 Middle fragments 1-3. B) Bacterially expressed recombinant MBP-TLK-1 and MBP-TLK-1 KD were incubated in kinase reaction buffer with the indicated recombinant GST-KNL-2 Middle fragments 1-3. The level of [^{32}P] incorporation into the KNL-2 (pKNL-2) fragments and MBP-TLK-1 autophosphorylation were assessed by phosophoimaing. Loading of the GST-KNL-2 fragments was assessed by by Ponceau S staining.

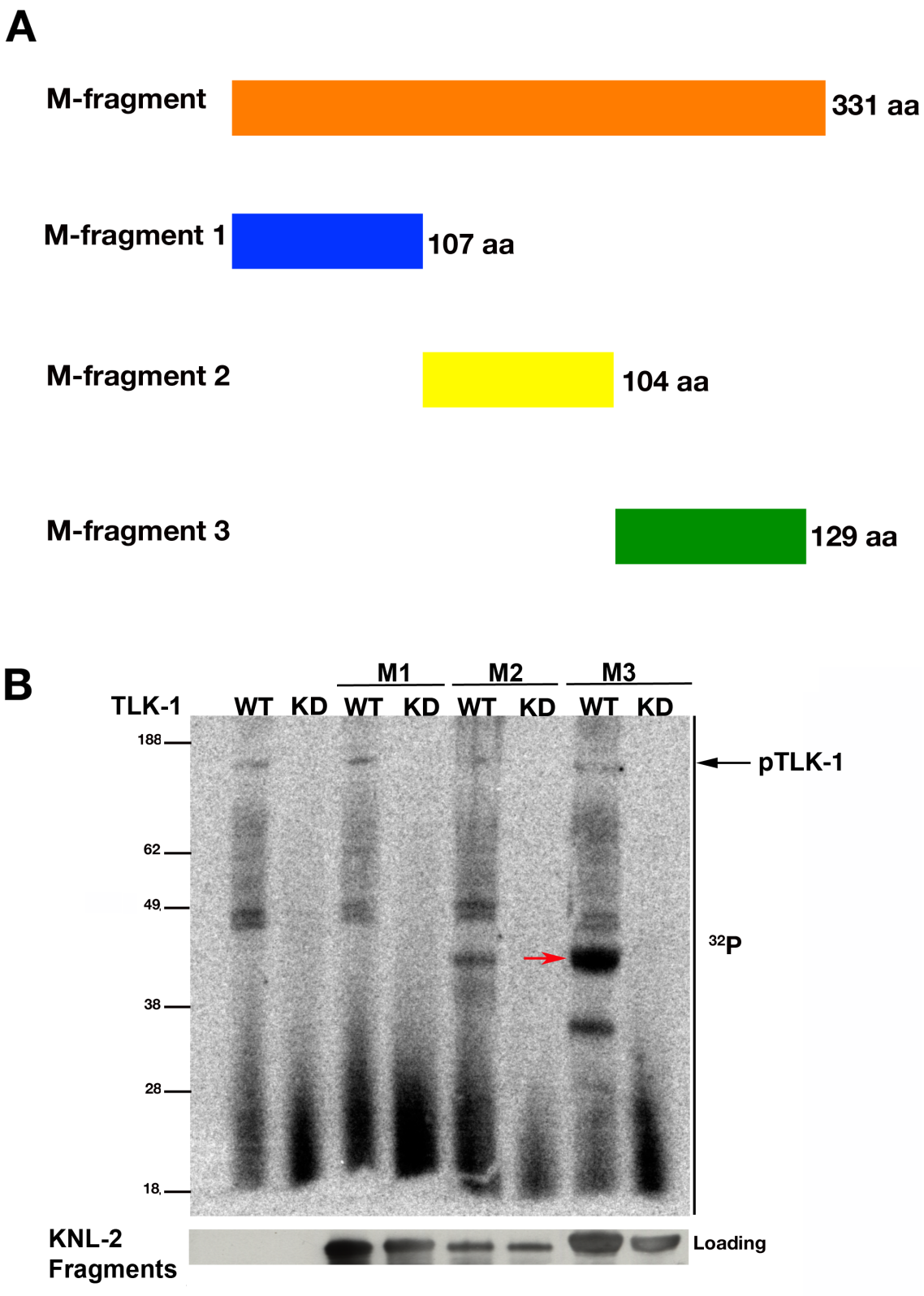

A
M-fragment
331 aa
M-fragment 1
107 aa
M-fragment 2
104 aa
M-fragment 3
129 aa
B
M1
M2
M3
TLK-1
WT KD WT KD WT KD WT KD
188
62
49
38
28
18
pTLK-1
32P
KNL-2
Fragments
Loading

Figure 17. An alignment of the KNL-2 Middle fragments 1-3 in various species

Clustal W alignment of KNL-2 homologs of the Middle fragment region is shown. Blue underline: GST-KNL-2 Middle fragment 1; yellow underline: GST-KNL-2 Middle fragment 2; green underline: GST-KNL-2 Middle fragment 3. Conserved serine or threonine residues in the GST-KNL-2 Middle fragment 3 are boxed in red (completely conserved) and orange (partially conserved) or indicated with a red asterisk (conserved in *C. elegans* and chicken).

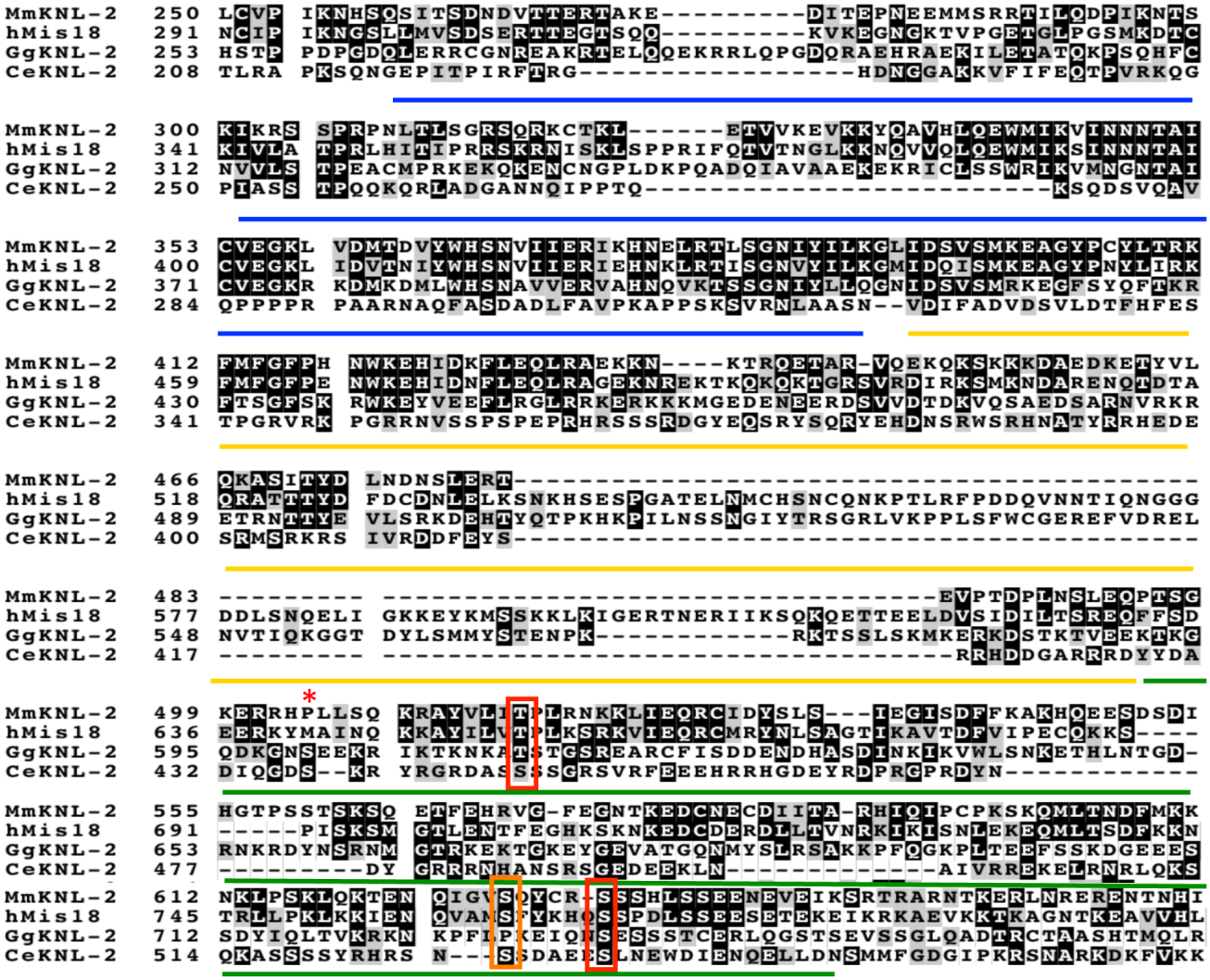
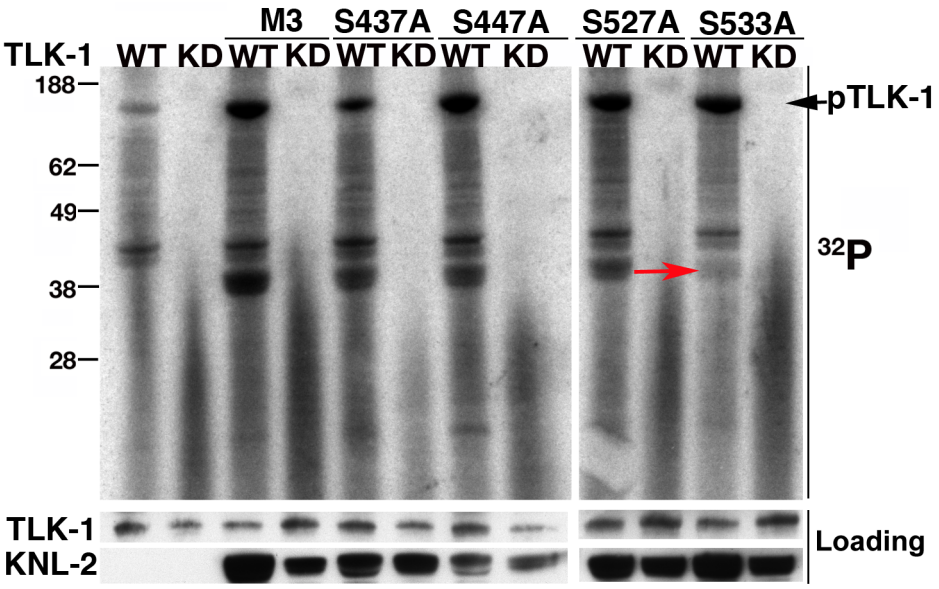

MmKNL-2 250 LCVP IKNHSQSITSDNDVTTERTAKE---------DITEPNEEMMSRRTILQDPIKNTS
hMis18 291 NCIP IKNGSLLMVSDSERTTEGTSQQ---------KVKEGNGKTVPGETGLPGSMKDTC
GgKNL-2 253 HSTP PDPGDQLERRCGNREAKRTELQQEKRRLQPGDQRAEHRAEKILETATQKPSQHFC
CeKNL-2 208 TLRA PKSQNGEPITPIRFTRG----------HDNGGAKKVFIFEQTPVRKQG
MmKNL-2 300 KIKRS SPRPNLTLSGRSQRKCTKL------ETVVKEVKKYQAVHLQEWMIKVINNNTAI
hMis18 341 KIVLA TPRLHITIPRRSKRNISKLSPPRIFQTVTNGLKKNQVVQLQEWMIKSINNNTAI
GgKNL-2 312 NVVLS TPEACMPRKEKQKENCNGPLDKPQADQIAVAAEKEKRICLSSWRIKVMNGNTAI
CeKNL-2 250 PIASS TPQQKQRLADGANNQIPPTQ----------KSQDSVQAV
MmKNL-2 353 CVEGKL VDMTDVYWHSNVIIERIKHNELRTLSGNIYILKGLIDSVSMKEAGYPCYLTRK
hMis18 400 CVEGKL IDVTNIYWHSNVIIERIEHNKLRTISGNVYILKGMIDQISMKEAGYPNYLIRK
GgKNL-2 371 CVEGKR KDMKDMLWHSNAVVERVAHNQVKTSSGNIYLLQGNIDSVSMRKEGFSYQFTKR
CeKNL-2 284 QPPPPR PAARNAQFASDADLFAVPKAPPSKSVRNLAASN--VDIFADVDSVLDTFHFES
MmKNL-2 412 FMFGFPH NWKEHIDKFLEQLRAEKKN----KTRQETAR-VQEKQKSKKKDAEDKETYVL
hMis18 459 FMFGFPE NWKEHIDNFLEQLRAGEKNREKTKQKQKTGRSVRDIRKSMKNDARENQTDTA
GgKNL-2 430 FTSGFSK RWKEYVEEFLRGLRRKERKKKMGEDENEERDSVVDTDKVQSAEDSARNVRKR
CeKNL-2 341 TPGRVRK PGRRNVSSPSPEPRHRSSSRDGYEQSRYSQRYEHDNSRWSRHNATYRRHEDE
MmKNL-2 466 QKASITYD LNDNSLERT----------
hMis18 518 QRATTTYD FDCDNLELKSNKHSESPGATELNMCHSNCQNKPTLRFPDDQVNNTIQNGGG
GgKNL-2 489 ETRNTTYE VLSRKDEHTYQTPKHKPILNSSNGIYTRSGRLVKPPLSFWCGEREFVDREL
CeKNL-2 400 SRMSRKRS IVRDDFEYS----------
MmKNL-2 483 --------- ----------EVPTDPLNSLEQPTSG
hMis18 577 DDLSNQELI GKKEYKMSSKKLKIGERTNERIIKSQKQETTEELDVSIDILTSREQFFSD
GgKNL-2 548 NVTIQKGGT DYLSMMYSTENPK----------RKTSSLSKMKERKDSTKTVEEKTKG
CeKNL-2 417 --------- ----------RRHDDGARRRDYYDA
MmKNL-2 499 KERRHPLLSQ KRAYVLITPLRNKKLIEQRCIDYSLS---IEGISDFFKAKHQEESDSDI
hMis18 636 EERKYMAINQ KKAYILVTPLKSRKVIEQRCMRYNLSAGTIKAVTDFVIPECQKKS----
GgKNL-2 595 QDKGNSEEKR IKTKNKATSTGSREARCFISDDENDHASDINKIKVWLSNKETHLNTGD-
CeKNL-2 432 DIQGDS--KR YRGRDASSSGRSVRFEEEHRRHGDEYRDPRGPRDYN----------
MmKNL-2 555 HGTPSSTSKSQ ETFEHRVG-FEGNTKEDCNECDIITA-RHIQIPCPKSKQMLTNDFMKK
hMis18 691 -----PISKSM GTLENTFEGHKSKNKEDCDERDLLTVNRKIKISNLEKEQMLTSDFKKN
GgKNL-2 653 RNKRDYNSRNM GTRKEKTGKEYGEVATGQNMYSLRSAKKPFQGKPLTEEFSSKDGEEES
CeKNL-2 477 ----------DY GRRRNHANSRSGEDEEKLN----------AIVRREKELRNRLQKS
MmKNL-2 612 NKLPSKLQKTEN QIGVSQYCR-SSSHLSSEENEVEIKSRTRARNTKERLNRERENTNHI
hMis18 745 TRLLPKLKKIEN QVAMSFYKHQSSPDLSSEESETEKEIKRKAEVKKTKAGNTKEAVVHL
GgKNL-2 712 SDYIQLTVKRKN KPFIPKEIQISESSSTCERLQGSTSEVSSGLQADTRCTAASHTMQLR
CeKNL-2 514 QKASSSSYRHRS N---SSDAEESLNEWDIENQELLDNSMMFGDGIPKRSNARKDKFVKK

Figure 18. TLK-1 phosphorylates the KNL-2 Middle-fragment at S533 *in vitro*

Bacterially expressed recombinant MBP-TLK-1 and MBP-TLK-1 KD proteins were incubated in kinase reaction buffer with the indicated recombinant GST-KNL-2 Middle fragments 3 mutant proteins. The level of [^{32}P] incorporation into the KNL-2 (pKNL-2) fragments and MBP-TLK-1 autophosphorylation were assessed by autoradiography phosophoimaing. Loading of MBP-TLK-1, MBP-TLK-KD, and the GST-KNL-2 fragments was assessed by Ponceau S staining.


M3
S437A
S447A
S527A
S533A
TLK-1
WT KD WT KD WT KD WT KD WT KD
188
62
49
38
28
pTLK-1
32P
TLK-1
KNL-2
Loading

CHAPTER V: Discussion and Significance

Discussion

Here, I report a novel role for Tousled-like kinase in kinetochore assembly and function that likely contributes to maintenance of embryonic viability. Additionally, my work revealed that TLK-1 is required to maintain proper kinetochore structure and also determined that KNL-2 is a novel *in vitro* substrate of TLK-1.

During cell division, chromosome segregation must occur accurately in order to maintain cell viability and organismal development. To effectively progress through mitosis it is imperative to correctly assemble the kinetochore structure. The kinetochore is a multilayer protein structure critical for microtubule binding and proper chromosome segregation. I established that pTLK-1(T610) and pTLK-1(S634) are outer kinetochore proteins that are localized to the microtubule binding interface. The kinetochore localization of these TLK-1 phospho-isoforms is dependent on the microtubule binding protein, HCP-1. Interestingly, the MitoCheck consortium has determined that the human TLK-1 homologs, Tlk1 and Tlk2, biochemically purify with the human homolog of HCP-1, CENP-F (Hutchins, 2010). These findings suggest that TLK-1 and HCP-1 physically interact in *C. elegans* through a conserved mechanism. Additionally, loss of TLK-1 in *C. elegans* embryos results in chromosome segregation defects characterized by wide metaphase plates, which is consistent with TLK-1 having a role in kinetochore-microtubule attachment and spindle assembly.

Previous studies from Cheeseman *et al.* demonstrated that HCP-1 is necessary for the kinetochore recruitment of CLS-2. In their study, HCP-1 and CLS-2 depletion displayed a similar mitotic chromosome segregation phenotype, explained by the loss of sister chromatid bi-orientation (Cheeseman et al., 2005). This phenotype was

suppressed by abolishing the astral pulling forces that drive the spindle poles apart, suggesting that CLS-2 is required for sister chromatid bi-orientation when kinetochore-microtubule attachments are subjected to poleward force (Cheeseman et al., 2005). I therefore investigated whether depletion of CLS-2 affects pTLK-1 kinetochore localization. These experiments revealed that in CLS-2 depleted embryos, even when chromosome defects were present, pTLK-1 localized at the kinetochore. However, this kinetochore localization appeared disorganized as compared to control embryos, which which could be explained by the loss of CLS-2 and its effect on kinetochore structure. In contrast to these findings, embryos depleted of TLK-1 displayed a loss of CLS-2 kinetochore localization. These results suggest that pTLK-1 kinetochore localization depends on HCP-1 and is required for CLS-2 kinetochore recruitment, indicating that pTLK-1 plays a role in regulating kinetochore-microtubule attachments.

Interestingly, Vergnolle and Taylor identified in human epithelial cells HCP-1 as an effector of LIS-1 (Vergnolle and Taylor, 2007). LIS-1 is a dynein interacting protein and depletion of LIS-1 causes mitotic delays, likely induced by problems with sister chromatids bi-orientation (Vallee and Tsai, 2006) Given LIS-1 dependency on HCP-1 for its kinetochore localization, I sought to determine if LIS-1 played a role in pTLK-1 kinetochore localization. Similar to CLS-2 depleted embryos, LIS-1 depleted embryos did not display the characteristic kinetochore "two stripes" structure, but rather a disorganized structure probably due to the role of LIS-1 in chromosome alignment. Further experiments aimed to determine the role of TLK-1 in LIS-1 kinetochore localization revealed that in TLK-1 depleted embryos, LIS-1 fails to localize at the kinetochore. All together, these findings indicate that TLK-1 likely controls kinetochore

microtubule dynamics through regulation of LIS-1 localization.

The placement of pTLK-1 upstream of CLS-2 and LIS-1 in the kinetochore hierarchy was surprising since this suggested that depletion of TLK-1 should have a similar phenotype to depletion of either CLS-2 or LIS-1. However, previous characterization of *tlk-1(RNAi)* from our group revealed that *tlk-1(RNAi)* is not fully penetrant in early embryos and the first *tlk-1(RNAi*) chromosome segregation phenotype is visible at the 16-cell embryonic stage. One possible explanation is that TLK-1 is acting in concert with other protein(s) at the same level of the kinetochore hierarchy, functioning redundantly to prevent aneuploidy in early embryos. Another possibility is that *tlk-1(RNAi)* is not completely robust, and TLK-1 protein from the mother may persist in the early embryo and compensate for the loss of embryonic TLK-1. Unfortunately, hermaphrodites harboring a homozygous *tlk-1* deletion allele, *tlk-1(tm2395*), develop into sterile adults, preventing their use in the study of the early embryonic divisions.

Given the experimental limitations in studying TLK-1 in early embryos, I took advantage of older embryos to examine the effect of TLK-1 depletion on other kinetochore components. These experiments revealed that TLK-1 is required to maintain the structure and rigidity of the centromere and kinetochore, despite the localization of TLK-1 to the outer kinetochore.

The changes in the architecture of the centromere (as revealed by CENP-A and KNL-2 immunostaining) were unexpected since TLK-1 is far distant from CENP-A and KNL-2 in the kinetochore hierarchy. One possible explanation for this observation is that TLK-1 functions in the kinetochore hierarchy as a scaffold protein, recruiting

microtubule-stabilizing proteins (*e.g.*, CLS-2 and LIS-1) that are required to establish proper kinetochore-microtubule attachments and for generating microtubule tension during metaphase. Therefore, loss of TLK-1 destabilizes kinetochore-microtubule attachments possibly by the action of depolymerized microtubules, reducing microtubule tension during metaphase, and thus allowing for the distortion of the kinetochore and the underlying centromere.

A similar twisted kinetochore phenotype was reported for embryos depleted of SMC-4, a core subunit of the Condensin II complex. Indeed, mitotic chromosomes appear to be progressively less condensed as *tlk-1(RNAi)* embryos develop. This observation raises the possibility that the absence of CLS-2 and LIS-1 at the kinetochore could be due to a lack of chromosome condensation and not directly due to TLK-1 loss. However, the consendation defects in TLK-1 depleted embryos are much less severe than in condensin-depleted embryos, and I found that in the absence of the condensin components SMC-4 and HCP-6, CLS-2 and LIS-1 still localize to chromosomes and kinetochores. These results suggest that incomplete chromosome condensation is not the primary defect in *tlk-1(RNAi)* embryos. A more direct measurement of the DNA condensation status would be to measure the DNA condensation rate in dividing embryos using live cell imaging. However, tracking DNA condensation in *tlk-1(RNAi)* embryos will require the analysis of older embryos where the *tlk-1(RNAi)* phenotype is most penetrant. This task become increasingly more arduous in older embryos due to the difficulty in precisely identifying and quantitating condensation differences in specific cells in different embryos at identical stages of development.

Another interesting finding from this work was the *in vitro* phosphorylation of

the inner kinetochore protein KNL-2 at S533 by TLK-1. This is a remarkable observation since my work suggests that pTLK-1 is an outer kinetochore protein. Thus, this new finding implies a potential role for TLK-1 in regulating inner kinetochore proteins as well as microtubule binding proteins at the outer kinetochore. A direct interaction between TLK-1 and KNL-2 is consistent with pTLK-1 localization at kinetochores during prophase. Kinetochore localization during early prophase is characteristic of inner kinetochore proteins, while outer kinetochore proteins are not recruited to the kinetochores until metaphase. Given these results, one can hypothesize that KNL-2 phosphorylation by TLK-1 influences KNL-2 activity at the inner centromere and *vice versa* since in *Arabidopsi*s TKI, a protein that share structural similarities with KNL-2, binds to the plant homolog of TLK-1 increasing its kinase activity. Regardless, additional experiments will be required to determine the functional role of KNL-2 phosphorylation *in vivo* and its possible role in regulating kinetochore assembly in *C. elegans*.

As described previously, our group discovered that the *C. elegans* TLK-1 kinase is a substrate of the AIR-2/Aurora B and CHK-1 kinases. These findings suggest that TLK-1 plays a role in integrating two different signaling pathways. AIR-2/Aurora B is the catalytic component of the highly conserved chromosomal passenger complex (CPC) that is localized to the inner centromere and phosphorylates targets at both the inner and outer kinetochore. AIR-2/Aurora B-dependent phosphorylation destabilizes unproductive kinetochore-microtubule attachment, leading to microtubule release and eventual reattachment in the proper orientation. The best studied role of is as a downstream effector of ATR during the DNA damage response.

However, recent studies revealed that CHK-1 depletion in mammalian cells results in chromosome segregation defects and failure to trigger the SAC in response to taxol (Zachos et al., 2007). This suggests a novel role for CHK-1during mitosis, which is particularly interesting since our group identified TLK-1 as a phosphorylation target of CHK-1. A possible explanation for the significance of TLK-1 phosphorylations events is that independent phosphorylation of TLK-1 by AIR-2/Aurora B and CHK-1 targets TLK-1 to the nucleus during early prophase. Additionally, TLK-1 nuclear localization during prophase and prometaphase will allow TLK-1 interaction with KNL-2, possibly affecting KNL-2 activity and influencing the kinetochore establishment. I propose that later during metaphase TLK-1 acts as a scaffold protein recruiting microtubule interacting proteins and stabilizing kinetochore-microtubule attachments. To further support this idea, Gary Deyter from our lab showed that, CYB-3, a dynein regulator binds to TLK-1 on a pT610 dependent manner (Deyter et al. 2010), providing another potential mechanism in which TLK-1 could be regulating kinetochore microtubule dynamics.

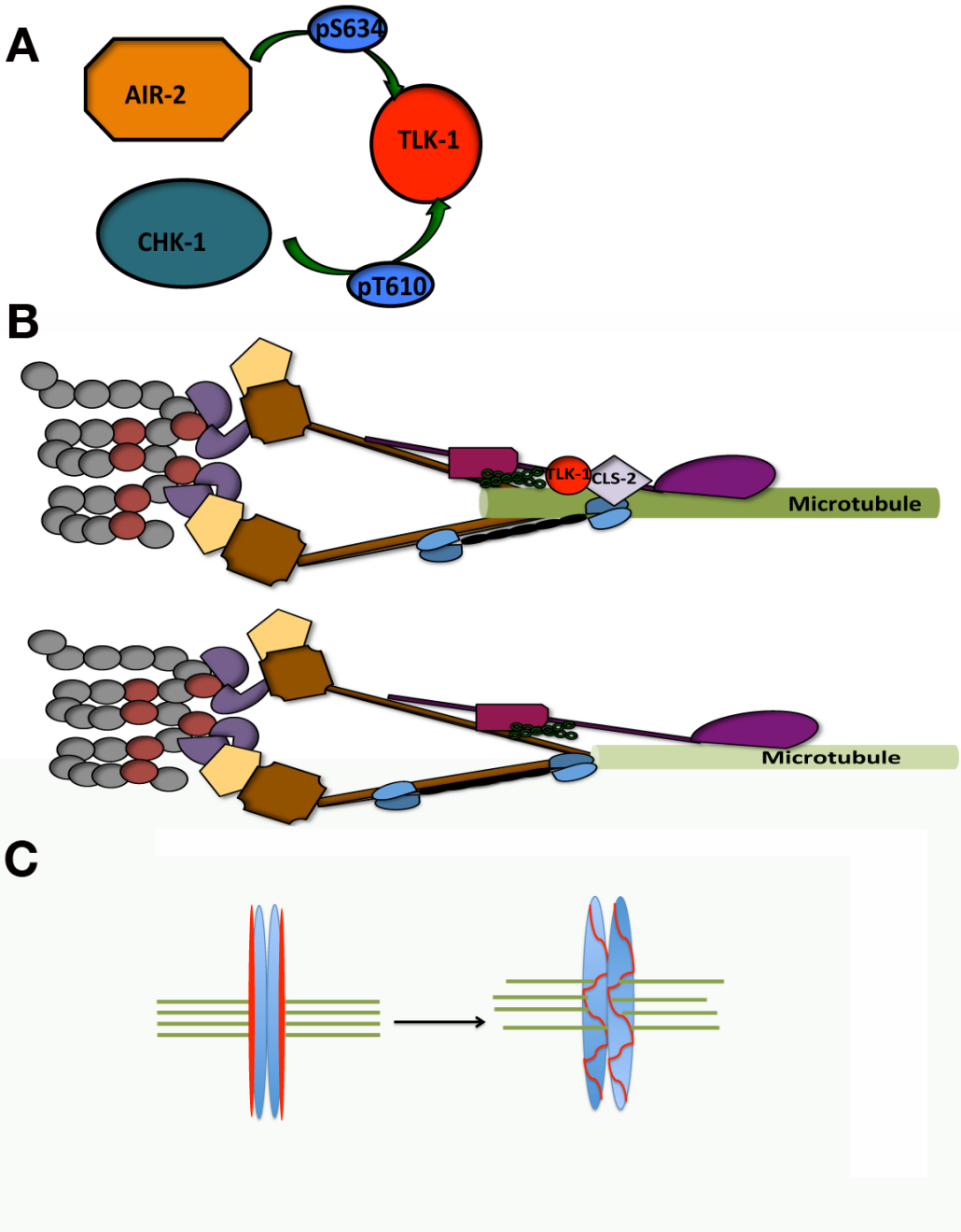
Significance

Initial characterization of plant and human TLK-1 family members suggested that Tousled-like kinase function primarily in S-phase and DNA repair. , While previous research from our group uncovered an important mitotic role for TLK-1 in *C. elegans* (Han et al., 2005), the data I presented in this Dissertation offers specific new insights into this nascent mitotic role for TLK-1. I found that TLK-1 is an essential component of the kinetochore in *C.elegans*, influencing kinetochore structure, assembly, and

function to promote accurate chromosome segregation. In humans, Tlks are also required for the mitotic process (Sunavala-Dossabhoy et al., 2003). TLK activity is rapidly inhibited in the presence of DNA double-stranded breaks and over-expression of either TLK1 or TLK2 results in resistance to ionizing radiation and promotes the repair of double-stranded breaks (Krause et al., 2003). Additionally, expression of kinase-dead human Tlk1B in normal breast epithelial cells resulted in chromosome segregation defects, kinetochore-microtubule attachment failures, and multipolar spindles (Sunavala-Dossabhoy et al., 2003). Furthermore, treatment of breast cancer cell lines with TLK1 siRNA revealed severe defects in the structure of the mitotic spindle characterized by mono-polar spindles and aberrant metaphase chromosome alignment, similar to the metaphase defects observed in *C .elegans tlk-1(RNAi)* embryos (Han et al., 2003). Moreover, our group recently discovered that TLK1 is highly expressed in triple negative breast cancer cell lines, a diverse group that lacks an established targeted chemotherapy (J.M.S., unpublished). Therefore, understanding the mechanisms by which TLKs control different cellular processes is pivotal for the potential development and establishment of new therapies targeting patients that have high expression or mis-regulation of TLK1. In summary, I have uncovered a novel kinetochore-based role for TLK-1 in mitosis, which generates several new testable hypotheses as to how different mitotic kinase pathways are integrated to regulate microtubule dynamics and chromosome segregation, with the ultimate goal of preventing aneuploidy.

Figure 19. Model for TLK-1 role at the kinetochore

A) The independent phosphorylation of TLK-1 by AIR-2 and CHK-1 target TLK-1 to the kinetochore. B) Once at the kinetochore, TLK-1 recruits CLS-2 stabilizing kinetochore-microtubule attachments. In the absence of TLK-1, CLS-2 is not localized to the kinetochore resulting in defect of microtubule polymerization, creating loss and less stable kinetochore-microtubule attachments. C) As a result of these unstable kinetochore-microtubule attachments the kinetochore structure is affected showing a twisted kinetochore phenotype in the absence of TLK-1.


A
AIR-2
pS634
TLK-1
CHK-1
pT610
B
TLK-1
CLS-2
Microtubule
Microtubule
C

CHAPTER IV: Materials and Methods

C. elegans strains and RNAi resistant transgenes:

C. elegans strains were grown and maintained at 20°C as described (Brenner, 1974). *C. elegans* strains used in this study are listed in Table 1: N2 Bristol (wild-type); OD13 *(*Cheeseman et al., 2004); OD31 (Maddox et al., 2007); OD42 (Gassmann et al., 2008); OD101 (Maddox et al., 2007); OD248 (Dumont et al., 2010); RQ279 (Yamamoto et al., 2008) and JS353. OD13 was provided by the *Caenorhabditis* Genetic Center, University of Minnesota; OD31, OD42, OD101, and OD248 were provided by A.Desai, University of California, San Diego, CA; RQ279 was provided by R. Kitagawa, Nationwide Children's Hospital, Columbus, OH.

The JS353 strain was generated by cloning the entire coding region of the *tlk-1* cDNA into the SpeI site of the pIC26 vector, creating an N-terminal GFP-TEV-S –TLK-1 fusion driven by the *pie-1* promoter. The GFP-TLK-1 construct was introduced into *unc-119 (-)* worms using microparticle bombardment (Praitis et al., 2001). After microparticle bombardment, hermaphrodites were screened for rescue of the *unc-119 ed3* deletion. The progeny of the *unc-119 ed3* rescued worms were visually examined for GFP expression in oocytes and early embryonic nuclei. GFP-specific immunostaining was utilized to confirm expression and subcellular localization (Catalog number A11122, Invitrogen, Eugene, OR). Cloning of the *tlk-1* cDNA into the pIC26 vector was performed by Gary Deyter, and microparticle bombardment and screening by Tokiko Furuta.

Table 1. *C. elegans* strains used in this study

Strain name	Genotype
N2	Wild type
OD13	*unc-119(ed3) III; ltIs9 [pIC34; pie-1/GFP-TEV-STag::kbp-3; unc-119 (+)]*
OD31	*unc-119(ed3) III; ltIs22[pPM3; pie-1::GFP-TEV-STag::knl2-2; + unc-119(+)]*
OD42	*unc-119(ed3) III; ltIs31 [pIC70; pie-*1/GFP-*TEV-STag::zwl-1; unc-119 (+)]*
OD101	*unc-119(ed3) III; ltIs21[pAZ132; pie-1::GFP-TEV-STag::hcp-3; + unc-119(+)]*
OD248	*unc-119(ed3) III; ltIs138 [pJD14; pie-1/knl1::GFP; unc-119(+)]*
RQ279	*unc-119(ed3) (III); jzIs41[pRK177(Ppie-1::GFP::spdl-1); unc-119(+)]*
JS353	*unc-119(ed3) (III); vwIs11 [pZB1(Ppie-1::GFP::tlk-1); unc-119(+)]*

RNA-mediated interference

The feeding method of RNAi delivery was used to deplete PLK-1, TLK-1, HCP-3, KNL-2, KNL-1, KBP-3, ZWL-1, BUB-1, HCP-1, HCP-2, CLS-2, LIS-1 and SAS-6, as previously described by Timmons and Fire (Timmons and Fire, 1998). RNAi plasmids for *hcp-3*, *knl-2, knl-1*, *kbp-3*, *bub-1,hcp-1, hcp-2 zwl-1, cls-2, lis-1,sas-6*, smc-4 were obtained from the Geneservice Ltd. *C. elegans* feeding library (Kamath and Ahringer, 2003). The RNAi plasmid to deplete HCP-6 was created by digesting the EST cosmid yk249h7 with EcoRI and XhoI and clone the digestion product into the T7 L4440 vector. Likely, the NDC-80 depletion was achieved by digesting the EST cosmid y1110A7A.1 with with EcoRI and XhoI and clone the digestion product into the T7 L4440 vector To deplete each individual protein, *E. coli* HT115 (DE3) bacteria was transformed with the respective RNAi plasmids. A 1 ml LB + 100 µg/µl ampicillin

liquid culture was inoculated with a single colony of HT115 bacterial transformation and grown overnight at 37°C. The following day these cultures were expanded into 50 ml LB/amp using a 1:100 dilution and grown for six hours at 37°C. After six hours 200 ul per plate were spread onto nematode growth (NG) plates supplemented with 20% β-lactose and placed at 25°C for 72 hours. Subsequently, the plates were seeded with L4-stage hermaphrodites and incubated at 25°C for 24 hours (Arur et al., 2009). The L4440 RNAi vector was used as an RNAi control.

To generate the dilute *cls-2(RNAi)* conditions for the *tlk-1* and *cls-2* co-depletion experiments the RNAi conditions were modified as follows: after expanding the cultures for six hours 10 ul *cls-2(RNAi)* bacteria were mixed with 190 ul *control(RNAi)* or *tlk-1(RNAi)* bacteria. The mixture was plated as above and the plates were seeded with L4-stage hermaphrodites and incubated at 25°C for 24 hours.

Immunostaining

Embryos from adult hermaphrodites were picked onto a 10 µl egg buffer on a Poly-L-Lysine coated glass slide (Sigma, St Louis, MO). To release the embryos a coverslip was placed over the animals and pressure was applied. The slides were subsequently placed on an aluminum plate over dry ice for 1 hour. To crack the embryo's cuticle and aid its permeabilization the coverslip were quickly snapped off. Slides were fixed in -20°C methanol for 20 minutes, followed by sequential re-hydrations; 80:20, 50:50, and 20:80 methanol to 1x PBS with 0.1% Tween (PBST). After hydration samples were block in 1X PBST with 1% BSA for 1 hour at room temperature and then incubated overnight in primary antibody diluted in PBST at 4°C. Primary antibodies used are listed in Table 2: anti-tubulin (1:2000; Sigma, St. Louis,

MO), anti-TLK-1 T610 (1:500) (Deyter, 2010), anti-TLK-1 T634 (1:100) (Han et al., 2005), anti-HCP-1 (1:100) (Moore et al., 1999), anti-BUB-1 (1:1000) (Oegema et al., 2001), anti-CLS-2 (1:200) (Cheeseman et al., 2005) and anti-LIS-1 (1:100) (Cockell et al., 2004). Samples were then washed with PBST and secondary antibodies were applied for 2 hours at room temperature. Secondary antibodies used were: Alexa Fluor 488 goat anti-mouse IgG and (both at 1:1000) (Invitrogen Molecular Probes, Eugene, OR). After incubation with the secondary antibodies the samples were washed with PBST and mounted using ProLong with DAPI (Invitrogen, Eugene, OR). For GFP staining the samples were fixed as described by Seydoux and Dunn (Seydoux and Dunn, 1997) and sainted with anti-GFP (1:200) (rabbit polyclonal, Invitrogen, Eugene, OR). Immunofluorescent images were acquired on a Nikon 2000U inverted microscope equipped with a Photometrics Coolsnap HQ camera. Metamorph software was used with a 60x 1.49NA Plan Apo oil immersion objective and Z sections were acquired at a 0.2um steps.

Table 2. Antibodies used in this study

Antibody	Source	Dilution
anti-tubulin	mouse	1:2000
anti-TLK-1 pT610	rabbit	1:500
anti-TLK-1 pS634	rabbit	1:100
anti-HCP-1	mouse	1:100
anti-BUB-1	rabbit	1:1000
anti-CLS-2	rabbit	1:100

anti-LIS-1	rabbit	1:100
anti-GFP	rabbit	1:200
Alexa 488 anti-mouse IgG	goat	1:1000
Alexa 555 anti-rabbit IgG	goat	1:1000

Generation of recombinant protein expression plasmids

The entire coding sequence of theTLK-1 was subcloned into the XbaI site of the pMAL-2cx vector (New England Biolabs, Beverly,MA). MBP-N-terminal fusions of TLK-1 wild-type and TLK-1 kinase-dead were generated as previously described (Han et al., 2003). The full-length coding region of the *knl-2* cDNA was isolated by PCR amplification from a *C. elegans* cDNA library using the following primers: JD1 (5_-GGATCCATGGGTGATACGGAAATTGTTC-3_) and JD2 (5_-CTCGAGTTAGTAGATGGATGTGTCTTC-3_). The PCR product was subcloned into the BamHI and XhoI sites of the pGEX-6P1 vector (GE Healthcare Life Sciences, Piscataway, NJ) to generate an N-terminally GST-tagged full-length KNL-2 fusion construct.

The GST-KNL-2 fusion protein construct described above was used to create the GST-KNL-2 fragments as follows. N-terminal fragment: the full-length GST-KNL-2 construct was subject to restriction enzyme digestion at the unique BamHI and SalI restriction sites and the products analyzed by gel electrophoresis. A 972 base-pair BamHI and SalI fragment was cloned into the pGEX-6P1 vector. M- and C-terminal fragments: The full-length GST-KNL-2 construct was used as a template to amplify the

corresponding segments. The PCR products were analyzed by gel electrophoresis and subsequently ligated into the pGEX-6P1 vector.

Primers used to generate the GST-KNL-2 Middle and C-terminal fragments:

1. **KNL-2 Middle fragment**

5' BamHI--- KNL-2 Middle fragment (Fwd)

JD7 5_- GGATCCGACATATTCGCAGATGTTGACC-_3

5' XhoI--- KNL-2 Middle fragment (Rvs)

JD8 5_- CTCGAGATTATCCAGAAGCTCTTGGTT-_3

2. **KNL-2 C-terminal fragment**

5' BamHI--- KNL-2 C-terminal fragment (Fwd)

JD3 5_- GGATCCGGCGAGCCAATCACTCCGATAA-_3

5' XhoI--- KNL-2 C-terminal fragment (Rvs)

JD2 5_- CTCGAGTTAGTAGATGGATGTGTCTTC-_3

Non-overlapping GST-KNL-2 Middle fragments 1-3 were generated using the GST-KNL-2 Middle fragment as a template to amplify the corresponding segments. The products were subcloned into the BamHI and XhoI sites of the pGEX-6P1 vector. Site-directed mutagenesis was utilized to generate the GFP-KNL-2 M-fragment 3 S/A mutations at the following sites S437, S447, S527, and S533. All constructs were verified by automated DNA sequencing at the MDACC DNA Analysis Core Facility (M. D.Anderson CCSG Grant, NCI CA-16672.

Primers used to generate the GST-KNL-2 Middle fragments:

1. KNL-2 Middle fragment 1

5' BamHI--- KNL-2 M fragment 1 (Fwd)

JD7 5_- GGATCCGACATATTCGCAGATGTTGACC-_3

5' Xho1--- KNL-2 M fragment 1 (Rvs)

JD9 5_- CTCGAGGACATTCGAAGCTGCTAAGTT-_3

2. KNL-2 Middle fragment 2

5' BamHI--- KNL-2 M fragment 2 (Fwd)

JD11 5_- GGATCCGACATATTCGCAGATGTTGAC-_3

5' XhoI--- KNL-2 M fragment 2 (Rvs)

JD12 5_- CTCGAGATCTCTACGCCTCGCACCATC-_3

3. KNL-2 Middle fragment 3

5' BamHI--- KNL-2 M fragment 3 (Fwd)

JD13 5_- GGATCCCATGACGATGGTGCGAGGCGT-_3

5' XhoI--- KNL-2 M fragment 3 (Rvs)

JD8 5_- CTCGAG ATTATCCAGAAGCTCTTGGTT-_3

Recombinant Protein Purification and Kinase Assays

Escherichia coli strain BL21 (DE3) pLys were transformed with the expression constructs for MBP-TLK-1, MBP-TLK-1 KD and the GST-KNL-2 fragments. After inoculation and growth of 1 ml overnight cultures at room temperature, cultures were expanded to 500 ml LB + 500 uL ampicillin and grown with shaking at room temperature to an OD600 = 0.5. Recombinant protein expression was induced by addition of 1mM IPTG and incubation at room temperature for four hours. Induced

cultures were pelleted and re-suspended in 3 ml lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% Triton-X100, 1 mM PMSF, supplemented with Roche Complete protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN) and sonicated on ice doing three pulses of 30 seconds with 1 minute rest in between pulses at a 40% power level. Lysates corresponding to the GST fusion constructs were clarified by centrifugation, and the supernatants were added to 50 µl glutathione-agarose beads (Sigma-Aldrich) and incubated rocking at 4˚C for 2 hours. The beads were washed in phosphate-buffered saline, 40 mM HEPES, pH 7.6 and resuspended in 50 ul of 40 mM HEPES, pH 7.6.

Lysates corresponding to the MBP-TLK-1 and MBP-TLK-1 KD constructs were clarified by centrifugation, and the supernatants were added to 50 µl amylose-agarose beads (New England Biolabs). Amylose beads were incubated rocking at 4˚C for 2 hours, washed in phosphate-buffered saline, 40 mM HEPES, pH 7.6, and re-suspended in 60 µl elution buffer (40 mM HEPES pH 7.6, 200 mM NaCl, 1mM EDTA, 10 mM maltose). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and stained with Coomassie Brilliant Blue.

Purified and eluted MBP-TLK-1 WT and MBP-TLK-1 KD were re-suspended individually in 20.5 ul kinase buffer (20 mm HEPES, pH 7.6, 5 mm EGTA, 1 mm dithiothreitol, 25 mmβ-glycerophosphate, 7.5 mm magnesium chloride, 10 nm ATP, 30 µCi (3 Ci/µmol) [γ-^{32}P]ATP) and the respective GST-KNL-2 construct bound to the beads was added to each reaction and incubated at room temperature for 20 minutes. Kinase reactions were terminated by adding 10 µl 2× SDS-PAGE loading buffer, separated by SDS-PAGE, and blotted to nitrocellulose. Radioactive phosphate

incorporation was visualized by phosphoimaging and protein loading was determined by staining membranes with Ponceau S and/or by probing with MBP- and GST-specific antibodies.

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