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RESEARCH ARTICLE

# Casein kinase II is required for proper cell division and acts as a negative regulator of centrosome duplication in *Caenorhabditis elegans* embryos

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## ABSTRACT

Centrosomes are the primary microtubule-organizing centers that orchestrate microtubule dynamics during the cell cycle. The correct number of centrosomes is pivotal for establishing bipolar mitotic spindles that ensure accurate segregation of chromosomes. Thus, centrioles must duplicate once per cell cycle, one daughter per mother centriole, the process of which requires highly coordinated actions among core factors and modulators. Protein phosphorylation is shown to regulate the stability, localization and activity of centrosome proteins. Here, we report the function of Casein kinase II (CK2) in early *Caenorhabditis elegans* embryos. The catalytic subunit (KIN-3/CK2α) of CK2 localizes to nuclei, centrosomes and midbodies. Inactivating CK2 leads to cell division defects, including chromosome missegregation, cytokinesis failure and aberrant centrosome behavior. Furthermore, depletion or inhibiting kinase activity of CK2 results in elevated ZYG-1 levels at centrosomes, restoring centrosome duplication and embryonic viability to *zyg-1* mutants. Our data suggest that CK2 functions in cell division and negatively regulates centrosome duplication in a kinase-dependent manner.

**KEY WORDS: Casein kinase II, CK2, *Caenorhabditis elegans*, Centrosome, KIN-3, ZYG-1**

## INTRODUCTION

Control of proper centrosome number is crucial for the fidelity of cell division (Gönczy, 2015). In animal cells, centrosomes organize microtubules to direct the formation of bipolar mitotic spindles that contribute to accurate segregation of genomic content. Centrosomes comprise two orthogonally arranged centrioles surrounded by a dense network of proteins termed pericentriolar material (PCM). Centrioles must duplicate exactly once per cell cycle to provide daughter cells with the correct number of centrosomes. Cells with abnormal centrosome number are prone to errors in DNA segregation and cytokinesis, leading to genomic instability and tumorigenesis (Godinho and Pellman, 2014).

Genetic analyses in *C. elegans* have elucidated a core set of five conserved factors required for centriole duplication, including the Plk4-related kinase ZYG-1 (O'Connell et al., 2001), the coiled coil protein SPD-2 (Kemp et al., 2004; Pelletier et al., 2004), SAS-5 (Delattre et al., 2004), SAS-6 (Leidel et al., 2005) and SAS-4 (Kirkham et al., 2003; Leidel and Gönczy, 2003). SPD-2 and ZYG-1 localize early to the site of nascent centriole formation and are required for the sequential recruitment of the SAS-5/SAS-6 complex and SAS-4 (Delattre et al., 2006; Pelletier et al., 2006). In human cells, increased activity of centriole factors (Plk4, STIL or HsSAS6) leads to centrosome amplification (Arquint and Nigg, 2014; Kleylein-Sohn et al., 2007; Strnad et al., 2007), suggesting that regulating the levels and activity of those factors is essential for precise control of centrosome number.

Post-translational modifications have also been implicated in centrosome assembly. For example, protein phosphorylations regulate the localization and stability of core centriole regulators. In humans and flies, the kinase Plk4 is required for recruiting STIL/Ana2/SAS-5 and SAS-6 to nascent centrioles via phosphorylation (Dzhindzhev et al., 2014; Kratz et al., 2015). Plk4 levels are controlled through autophosphorylation followed by proteosomal degradation (Cunha-Ferreira et al., 2013; Guderian et al., 2010; Holland et al., 2010; Klebba et al., 2013). Many other protein kinases are also shown to control centrosome assembly and function, including PLK-1 (Decker et al., 2011; Lane and Nigg, 1996), Aurora A Kinase (Hannak et al., 2001), and CDK2/Cyclin E (Hinchcliffe et al., 1999).

Conversely, Protein phosphatase 2A (PP2A) plays a key role in centrosome duplication, and such role appears to be conserved in humans, flies and nematodes (Brownlee et al., 2011; Kitagawa et al., 2011; Song et al., 2011), underlying the coordinated kinase/phosphatase action in regulating centrosome duplication. The *C. elegans* catalytic subunit of PP2A, LET-92, was identified by proteomic analysis of the RNA-binding protein SZY-20 that negatively regulates a core centriole factor ZYG-1 (Song et al., 2008, 2011). It has been shown that PP2A positively regulates centrosome duplication by promoting the stability and/or localization of ZYG-1 and SAS-5 in the *C. elegans* embryo (Kitagawa et al., 2011; Song et al., 2011). However, the counteracting kinase(s) to PP2A in centrosome duplication has not yet been identified. Interestingly, the proteomic approach that identified SZY-20 associating factors has listed both positive and negative regulators of centrosome duplication (Song et al., 2011; Stubenvoll et al., 2016). Thus, it is possible that one or more kinases interacting with SZY-20 might counteract PP2A in centrosome assembly.

One of the SZY-20 interacting factors we identified is the *C. elegans* Casein kinase 2 holoenzyme (CK2), an evolutionarily conserved serine/threonine protein kinase (Hu and Rubin, 1990). In general, CK2 acts as a tetrameric holoenzyme comprising two catalytic (CK2α) and two regulatory (CK2β) subunits (Niefind et al., 2009). CK2 targets a large number of substrates that are

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involved in cellular proliferation (Meggio and Pinna, 2003). Aberrant CK2α activity has been shown to result in centrosome amplification in mammalian cells (St-Denis et al., 2009). Consistently, elevated CK2 activities are frequently observed in many human cancers (Guerra and Issinger, 2008; Trembley et al., 2010). The *C. elegans* genome contains a single catalytic and regulatory subunit encoded by *kin-3* and *kin-10*, respectively (Hu and Rubin, 1990, 1991). In *C. elegans*, CK2 is also shown to function in a broad range of cellular processes, including cell proliferation in the germ line (Wang et al., 2014), Wnt signaling (de Groot et al., 2014), male sensory cilia (Hu et al., 2006) and microRNA targeting (Alessi et al., 2015). However, while genome-wide RNAi screening indicated that CK2 is required for the viability of *C. elegans* embryos (Fraser et al., 2000; Sönnichsen et al., 2005), the function of CK2 during embryonic development remains largely undefined. In this study, we investigated the role of *C. elegans* CK2 during cell division in early embryos. We show that protein kinase CK2 is required for proper cell division and cytokinesis, and negatively regulates centrosome duplication.

## RESULTS

We have identified KIN-3/CK2α, the catalytic subunit of protein kinase CK2 through mass spectrometry of co-immunoprecipitated materials with anti-SZY-20. The conserved RNA-binding protein SZY-20 is known to negatively regulate centrosome assembly by opposing ZYG-1 at the centrosome (Kemp et al., 2007; Song et al., 2008). Mass spectrometry analysis identified three KIN-3 peptides (11% coverage; Table S1) that were present in SZY-20 co-precipitates but absent in IgG control. Consistently, we also show that KIN-3::GFP co-precipitates with SZY-20 (10.4% coverage) and the regulatory subunit of CK2, KIN-10/CK2β (5.1% coverage) (Table S1). Further, we confirmed the physical interaction between KIN-3 and SZY-20 using immunoprecipitation followed by immunoblot (Fig. S1). Together, our results suggest that the protein kinase CK2 holoenzyme physically associates with SZY-20 *in vivo*.

### CK2 acts as a negative regulator of centrosome duplication

Given that *szy-20* is a known genetic suppressor of *zyg-1* (Kemp et al., 2007; Song et al., 2008), we asked if CK2 functions in centrosome assembly using *zyg-1(it25)* mutants. *zyg-1(it25)* is a temperature-sensitive (ts) mutation that, at the restrictive temperature of 24°C, results in a failure of centrosome duplication, leading to monopolar spindles at the second mitosis and 100% embryonic lethality (O'Connell et al., 2001). At the semi-restrictive temperature 22.5°C, *kin-3(RNAi)* significantly increased (25±25%) embryonic viability to *zyg-1(it25)* mutants compared to control RNAi (8.0±13%, $P<0.001$) (Fig. 1A, Table S2), while *kin-3 (RNAi)* produced embryonic lethality (47±10%, $n=2433$) in wild-type animals (Table S2). In contrast, at the restrictive temperature of 24°C, *kin-3(RNAi)* had no significant effect on embryonic viability of *zyg-1(it25)* mutants (Fig. 1A) although *kin-3(RNAi)* had a similar effect on embryonic lethality of wild-type animals (44±16%, $n=3724$) (Table S2). Thus, knocking down KIN-3 partially restores embryonic viability in *zyg-1(it25)* mutants, suggesting that *kin-3* acts as a genetic suppressor of *zyg*-1, perhaps through ZYG-1 activity to regulate centrosome assembly.

As *zyg-1* is essential for centrosome duplication, it is likely that *kin-3(RNAi)* restores embryonic viability to *zyg-1(it25)* by rescuing centrosome duplication. To test this, we examined *zyg-1(it25); kin-3 (RNAi)* embryos that formed bipolar mitotic spindles at the second mitosis to quantify the event of successful centriole duplication during the first cell cycle. Compared to control RNAi, *kin-3(RNAi)* led to a significant fold increase in bipolar spindle formation to *zyg-1(it25)* embryos at all temperatures examined (Fig. 1B,C). However, we observed no signs of monopolar spindle formation in *kin-3 (RNAi)* treated wild-type embryos. Therefore, *kin-3(RNAi)* restores embryonic viability to *zyg-1(it25)* through restoration in centriole duplication. We then questioned if KIN-3 regulates centrosome duplication as part of the CK2 holoenzyme or as a free subunit, independently of the holoenzyme. If the holoenzyme CK2 functions in centrosome regulation, we should observe similar effects on *zyg-1 (it25)* mutants by depleting KIN-10, the sole regulatory subunit (CK2β) of CK2 in *C. elegans* (Fig. 1B,C). Compared to control, *kin-10(RNAi)* resulted in significantly increased levels of embryonic viability and bipolar spindle formation to *zyg-1(it25)* embryos at the semi-restrictive temperature, 22.5°C. Thus, it is likely that the protein kinase CK2 holoenzyme has a role in centrosome duplication as a negative regulator.

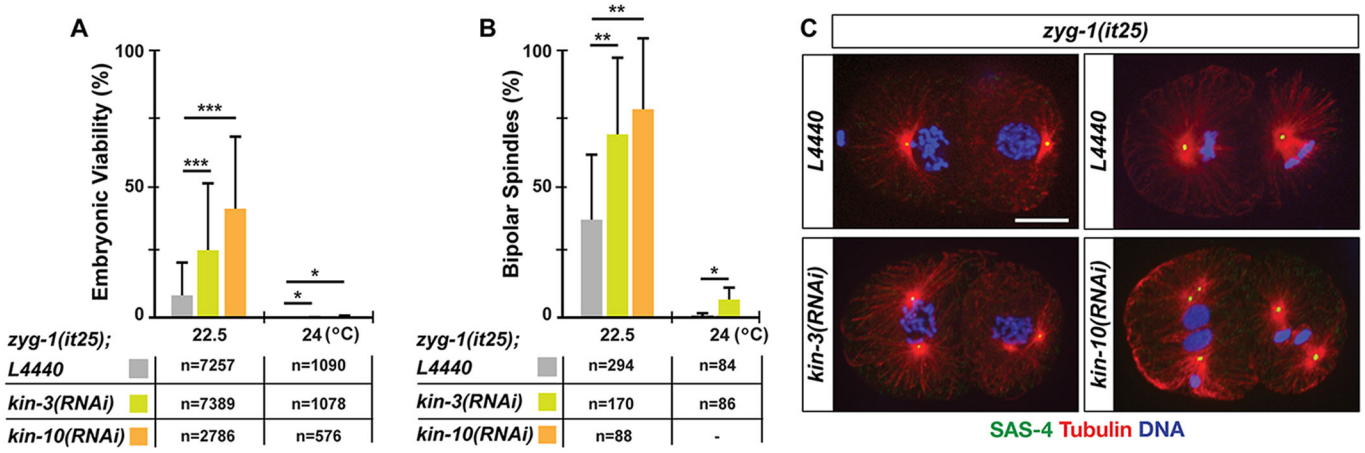


**Fig. 1. Knocking down CK2 partially restores embryonic viability and bipolar spindle formation to *zyg-1(it25)*.** Depleting CK2 subunits by either *kin-3 (RNAi)* or *kin-10(RNAi)* leads to an increase in both (A) embryonic viability and (B) bipolar spindle formation to *zyg-1(it25)* mutants, which were examined at restrictive (24°C) and semi-restrictive temperatures (22.5°C). (A,B) Mean values are presented. Error bars are standard deviation (s.d). n is given as the number of embryos (A) or the number of blastomeres (B) at second mitosis. ***$P<0.001$, **$P<0.01$, *$P<0.05$ (two-tailed *t*-test). (C) Immunofluorescence of *zyg-1(it25)* embryos raised at 22.5°C illustrates mitotic spindles at second mitosis. *kin-3(RNAi)* or *kin-10(RNAi)* restores bipolar spindles to *zyg-1(it25)* embryos, but control embryos display monopoles. SAS-4 was used as a centriole marker. Scale bar: 10 µm.

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As KIN-3 physically interacts with SZY-20, we asked whether *kin-3* and *szy-20* genetically interact as well. We tested how co-depletion of KIN-3 and SZY-20 affects bipolar spindle formation in *zyg-1(it25)* embryos (Fig. S2). At the restrictive temperature, 24°C, the *szy-20(bs52)* mutation restores bipolar spindle formation to *zyg-1(it25)* embryos (49±12%) as reported previously (Song et al., 2008). Strikingly, depleting KIN-3 in *zyg-1(it25);szy-20(bs52)* double mutants led to nearly 100% bipolar spindle formation to *zyg-1(it25)*, whereas *kin-3(RNAi)* alone exhibits a much lower level (13±8%) of bipolar spindle formation in *zyg-1(it25)*. Thus, co-depleting *kin-3* and *szy-20* further enhances bipolar spindle formation to *zyg-1(it25)* mutants, suggesting that *kin-3* genetically interacts with *szy-20* to negatively regulate centrosome assembly.

### CK2 negatively regulates centrosomal ZYG-1 levels

How does protein kinase CK2 holoenzyme influence centrosome duplication? Given that protein phosphorylations are known to regulate the stability, localization and activity of centrosome factors (Brownlee et al., 2011; Cunha-Ferreira et al., 2013; Guderian et al., 2010; Holland et al., 2010; Klebba et al., 2013; Song et al., 2011), we first examined whether KIN-3 affected ZYG-1 localization to centrosomes in wild-type embryos. We immunostained embryos using anti-ZYG-1 (Stubenvoll et al., 2016), and quantified centrosomal levels of ZYG-1 in *kin-3(RNAi)* embryos compared to controls. We measured the fluorescence intensity of centrosome-associated ZYG-1 at the first anaphase where ZYG-1 levels at centrosomes are highest (Song et al., 2008). In embryos depleted of KIN-3, we observed a significant increase in centrosomal ZYG-1 levels (1.7±0.7 fold; $P<0.0001$) compared to controls (Fig. 2A,B). We continued to examine the effect of KIN-3 knockdown on other centriole factors. By immunostaining embryos for SAS-4 (Kirkham et al., 2003; Leidel and Gönczy, 2003) and SAS-5 (Delattre et al., 2004), we quantified the levels of centrosome-associated SAS-5 and SAS-4 at first anaphase (Fig. 2A,B). However, we observed no significant changes in centrosomal levels of SAS-4 or SAS-5 between *kin-3(RNAi)* and control embryos.

SPD-2 localizes to both PCM and centrioles (Kemp et al., 2004; Pelletier et al., 2004) and is required for localization of ZYG-1 (Delattre et al., 2006; Pelletier et al., 2006). Increased levels of ZYG-1 at centrosomes could result from elevated SPD-2. To address this, we acquired time-lapse movies of KIN-3 depleted embryos expressing GFP::SPD-2 and GFP::histone (Kemp et al., 2004). Quantifying the fluorescence intensity of GFP::SPD-2 at first metaphase centrosomes showed no significant changes (0.88±0.32 fold, $P=0.3$) in centrosomal SPD-2 levels in *kin-3(RNAi)* embryos compared to control (Fig. 2A,B). Thus, elevated ZYG-1 levels in *kin-3(RNAi)* centrosomes are unlikely due to SPD-2 activity. We also examined another PCM factor TBG-1 (γ-tubulin; Hannak et al., 2001). By quantitative immunofluorescence using anti-TBG-1, we observed no significant change in centrosomal TBG-1 levels between *kin-3(RNAi)* and control embryos. Further, quantitative immunoblot analyses revealed that overall levels of centrosome factors tested were unaffected by KIN-3 depletion (Fig. 2C).

Together, our results suggest that CK2 specifically regulates ZYG-1 levels at the centrosome. We then asked if increased ZYG-1 levels at first anaphase centrosomes resulted from a cell cycle shift by loss of KIN-3. To address cell cycle dependence, we recorded 4D time-lapse movies using embryos expressing GFP::ZYG-1-C-term that contains a C-terminal fragment (217-706 aa) of ZYG-1 and localizes to centrosomes (Peters et al., 2010; Shimanovskaya et al., 2014), starting from pronuclear migration through visual separation of centriole pairs at anaphase during the first cell cycle (Fig. 2D, Movie 1). Throughout the first cell cycle in *kin-3(RNAi)* embryos, we observed a 1.5-fold increase in PCM-associated GFP::ZYG-1 levels relative to control (Fig. 2E), but no significant difference in cytoplasmic levels between *kin-3(RNAi)* and control embryos (Fig. 2E), suggesting that KIN-3 influences centrosome-associated ZYG-1 levels throughout the cell cycle. However, we cannot exclude the possibility that KIN-3 might regulate overall ZYG-1 levels, and thereby influence ZYG-1 levels at centrosomes. While direct measurement of endogenous levels of ZYG-1 will address this question, we were unable to assess overall levels of ZYG-1 due to technical limits to the detection of endogenous levels of ZYG-1, largely owing to low abundance of ZYG-1 in *C. elegans* embryos.

### Depletion of CK2 restores ZYG-1 levels at *zyg-1* mutant centrosomes

Elevated levels of centrosomal ZYG-1 by depleting CK2 might lead to the restoration of bipolar spindles and embryonic viability to *zyg-1* mutants. It has been shown that several genetic suppressors of *zyg-1* regulate ZYG-1 levels at centrosomes (Peel et al., 2012; Song et al., 2011; Stubenvoll et al., 2016). We show that centrosomes in *zyg-1(it25)* embryos exhibit a significantly reduced level (~60%) of ZYG-1 compared to wild-type controls at first anaphase (Fig. 3A,B). In fact, depleting CK2 by *kin-3(RNAi)* or *kin-10(RNAi)* resulted in significantly increased ZYG-1 levels at *zyg-1(it25)* centrosomes (Fig. 3A,B). Consistently, *kin-10(RNAi)* led to increased centrosomal ZYG-1 in wild-type embryos (Fig. S3), comparable to the effect by *kin-3 (RNAi)* (Fig. 2A,B). Together, our results suggest that inhibiting the holoenzyme CK2 restores centrosome duplication to *zyg-1 (it25)*, at least in part, by increasing centrosome-associated ZYG-1 levels at centrosomes.

### CK2 is required for proper cell division in *C. elegans* embryos

Prior studies in *C. elegans* have shown that CK2 is required for embryonic viability (Fraser et al., 2000; Sönnichsen et al., 2005) and that CK2 functions in stem cell proliferation in germ line development (Wang et al., 2014). However, the specific role of CK2 in *C. elegans* embryos has not been examined in detail. As homozygous mutant animals for *kin-3* or *kin-10* arrest at late larval stages, we treated L4 stage worms by RNAi-feeding to knockdown the catalytic subunit (KIN-3/CK2α) of CK2, and examined the knockdown effect in early embryos. As reported previously (Alessi et al., 2015), animals exposed to *kin-3(RNAi)* for 24 h produced no significant embryonic lethality, although these animals exhibited other phenotypes such as sterility and protruding vulva (wormbase.org; Wang et al., 2014). Strikingly, when L4 worms were exposed for an extended period (36-48 h) to *kin-3(RNAi)*, these animals produced significant and reproducible embryonic lethality (Fig. 4A, Table S2) and a reduced number of progeny produced for a 24 h period (Fig. 4B). We also observed that *kin-10(RNAi)* led to a similar level of embryonic lethality (Fig. 4A, Table S2), suggesting that protein kinase holoenzyme CK2 is required for *C. elegans* embryogenesis.

To understand the cause of embryonic lethality by CK2 depletion, we examined early embryos expressing GFP::β-tubulin, mCherry::γ-tubulin and mCherry::histone (Toya et al., 2010) using confocal live imaging (Fig. 4C, Movies 2 and 3). During mitotic cell division, *kin-3(RNAi)* resulted in various cell cycle defects, including aberrant chromosome segregation (Fig. 4Ca, Movie 2), detached centrosomes where centrosomes lose association with the nucleus (Fig. 4Cb), abnormal PCM morphology (Fig. 4Cc), and

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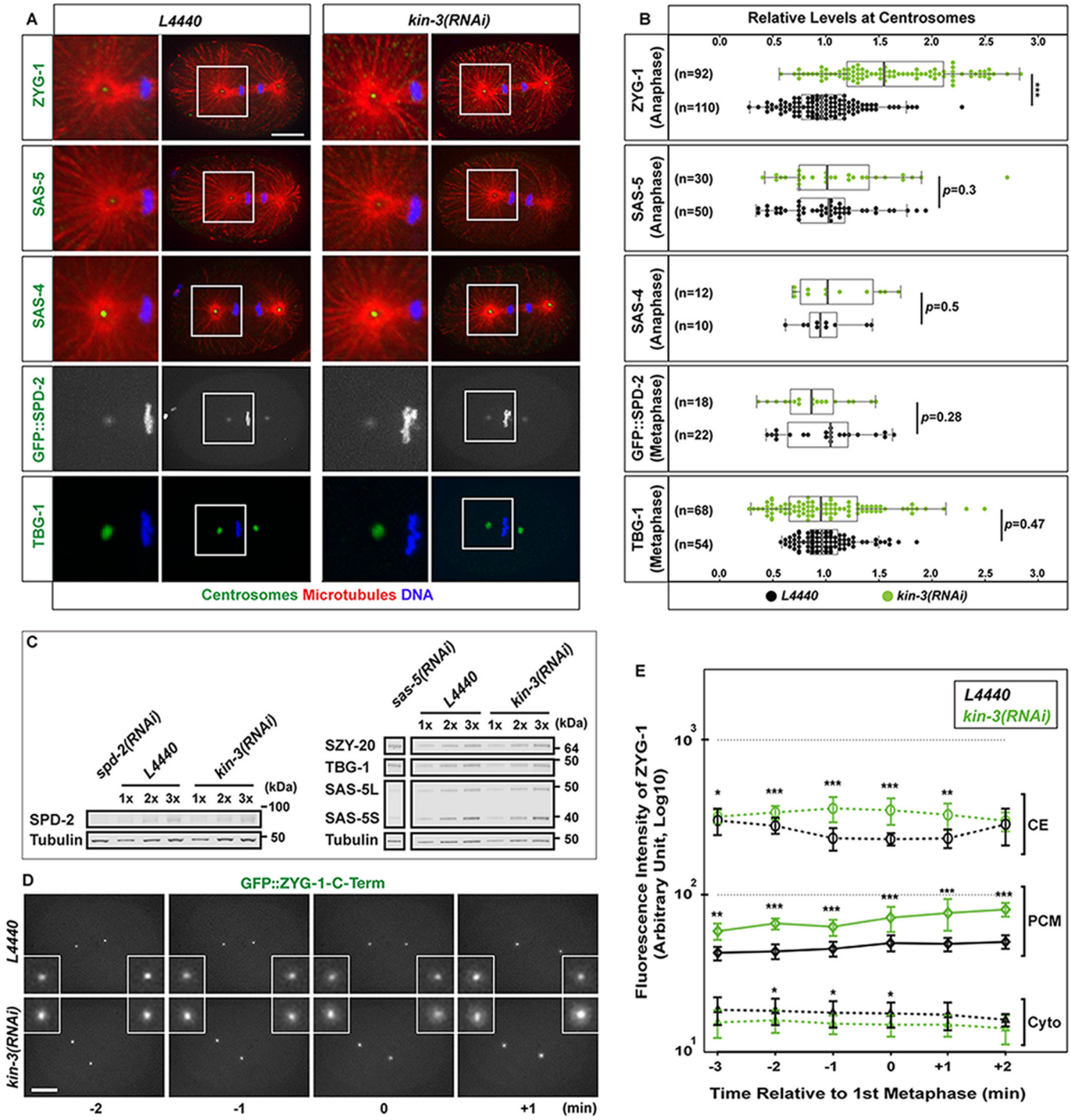


**Fig. 2. KIN-3 depletion leads to increased levels of centrosomal ZYG-1.** (A) Wild-type embryos stained for centrosome factors (ZYG-1, SAS-5, SAS-4, TBG-1: green), microtubules and DNA illustrate centrosomal localization of each factor. For SPD-2, shown are still-images from time-lapse movies of embryos expressing GFP::SPD-2 and GFP::Histone. (B) Quantification of centrosomal levels of each factor in *kin-3(RNAi)* (green dots) relative to controls (black dots). Each dot represents a centrosome. Box ranges from the first through third quartile of the data. Thick bar indicates the median. Solid horizontal line extends 1.5 times the inter-quartile range or to the minimum and maximum data point. ***$P$<0.001 (two-tailed $t$-test). (C) Quantitative immunoblot analyses of embryonic lysates reveal that KIN-3 knockdown leads to no significant changes in overall levels of SPD-2 (0.92±0.41-fold, $n$=4), SZY-20 (1.03±0.3, $n$=7), TBG-1 (1.06±0.35, $n$=10), both isoforms of SAS-5L (404aa: 1.01±0.21, $n$=16) and SAS-5S (288aa: 1.04±0.42, $n$=15) relative to controls. $n$ indicates the number of biological replicates. Tubulin was used as a loading control. (D) Still images from time-lapse movies of embryos expressing GFP::ZYG-1-C-term. Depleting KIN-3 results in elevated levels of centrosomal GFP::ZYG-1 throughout the first cell cycle. Time (min) is given relative to first metaphase (t=0). (E) Quantification of GFP::ZYG-1-C-term levels in *kin-3(RNAi)* (green lines) and control embryos (black lines). Mean fluorescence intensity is plotted ($n$=10 centrosomes in 5 embryos). CE, Centriole; PCM, Centrosome; Cyto, Cytoplasm. Error bars are s.d. ***$P$<0.001, **$P$<0.01, *$P$<0.05 (two-tailed $t$-test). (A,D) Insets highlight centrosomes magnified 4-fold. Scale bar: 10 µm.

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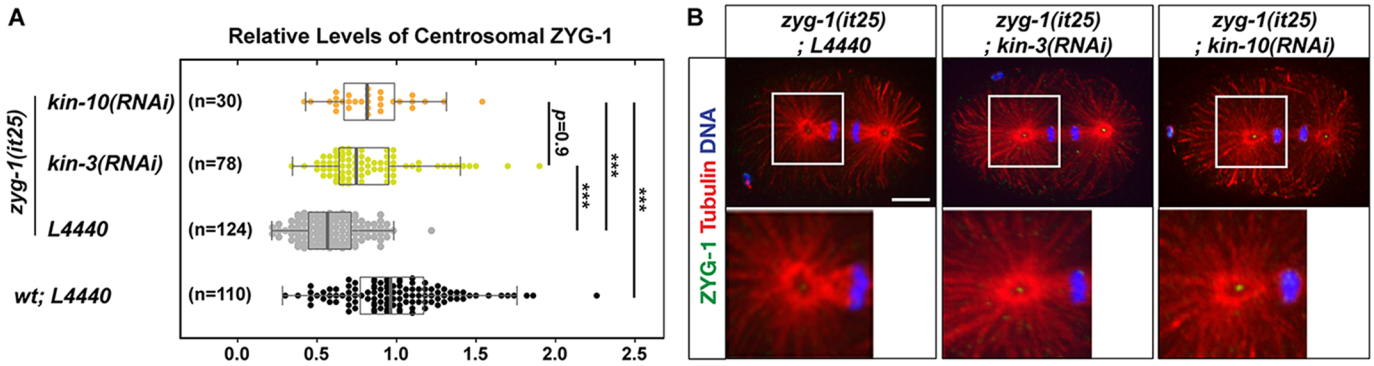


**Fig. 3. Depleting CK2 restores centrosomal ZYG-1 levels in *zyg-1(it25)* embryos.** (A) Quantification of centrosomal ZYG-1 levels at first anaphase in *zyg-1 (it25)* embryos exposed to *kin-3(RNAi)*, *kin-10(RNAi)* or *L4440*. Values are relative to wild-type centrosomes treated with control RNAi (*L4440*). Shown is the same wild-type control data presented in Fig. 2B (for ZYG-1). At the semi-restrictive temperature 22.5°C, *zyg-1(it25)* embryos exhibit reduced ZYG-1 levels at centrosomes (0.59±0.32) compared to the wild-type. *kin-3(RNAi)* or *kin-10(RNAi)* in *zyg-1(it25)* mutants restores centrosomal ZYG-1 levels to near wild-type levels (0.83±0.50 and 0.84±0.42, respectively). Each dot represents a centrosome. Box ranges from the first through third quartile of the data, and thick bar represents the median. Solid horizontal line extends 1.5 times the inter-quartile range or to the minimum and maximum data point. ***$P$<0.001 (two-tailed $t$-test). Note that data for control RNAi-treated wild-type embryos are also presented in Fig. 2B, but included for quantitative analysis. (B) *zyg-1(it25)* embryos stained for ZYG-1 illustrate centrosome-associated ZYG-1 localization. Inset illustrates centrosomal regions magnified 4-fold. Scale bar: 10 µm.

cytokinesis failure (Fig. 4Cd, Movie 3) resulting from incomplete cytokinetic furrow formation (Fig. 4D, Movie 3). We also observed an error in meiotic division, which likely results in polar body extrusion failure and thereby extra DNA in embryos (Fig. 4Cc, Fig. S4). Furthermore, 4D time-lapse imaging revealed a significant cell cycle delay in *kin-3(RNAi)* embryos (Fig 4E, Fig. S5, Movies 2, 3). Together, our results indicate that CK2 is required for proper cell divisions and is thus essential for *C. elegans* embryogenesis.

### KIN-3, the catalytic subunit of CK2 exhibits dynamic changes in subcellular localization during the cell cycle

To determine how CK2 might function in the early cell cycle, we examined the subcellular localization of KIN-3 in early embryos. Live imaging of embryos expressing KIN-3::GFP revealed dynamic localization patterns during cell cycle progression (Fig. 5, Movie 4). During interphase and early mitosis, KIN-3 is enriched at nuclei. Upon nuclear envelope breakdown (NEBD), KIN-3 appears to localize to the metaphase spindle and remain associated with spindle microtubules and centrosomes throughout mitosis (Fig. S6). During later stages of cell division, KIN-3 is highly enriched in the cytokinetic midbody (Fig. 5A,B), which is evidenced by co-localization of KIN-3::GFP and mCherry-tagged plasma membrane (Green et al., 2013; Kachur et al., 2008) (Fig. 5B). KIN-3::GFP localizes within a spherical area associated with the cytokinetic furrow that is surrounded by the plasma membrane supporting that KIN-3 localizes to the midbody. *kin-3(RNAi)* nearly abolished KIN-3::GFP expression (Fig. 5A), suggesting that KIN-3::GFP expression represent localization patterns specific to KIN-3.

Given our finding on KIN-3 localization at the midbody and its related role in cytokinesis, we asked if KIN-3 functions at the midbody to regulate cell division. The Aurora/Ipl1p-related kinase AIR-2 regulates multiple steps of cell division including chromosome segregation and cytokinesis (Schumacher et al., 1998). In particular AIR-2 plays a key role in cytokinesis through ZEN-4, both functioning at the midbody (Kaitna et al., 2000; Severson et al., 2000). To address the possible role of CK2 at the midbody, we tested a genetic interaction between *kin-3* and *air-2* or *zen-4* (Fig. S7, Table S2). *kin-3(RNAi)* led to an increase in embryonic lethality of *air-2(or207)* or *zen-4(or153)* (Severson et al., 2000), suggesting that *kin-3* has a positive genetic interaction with *air-2* and *zen-4*, two genes associated with the midbody. Thus, it seems likely that KIN-3 function in early cell cycle as a component of the midbody structure.

### CK2 dependent phosphorylation likely functions in centrosome duplication and the cell cycle

To further support the CK2 holoenzyme-dependent regulation of *zyg-1*, we tested whether chemical inhibition of CK2 could restore embryonic viability to *zyg-1(it25)*. We used the highly selective chemical inhibitor of CK2, 4,5,6,7-tetrabromobenzotriazole (TBB) that competes for binding at the ATP-binding site of CK2 (Sarno et al., 2001; Szyszka et al., 1995). It has been reported that TBB specifically abolishes CK2-dependent phosphorylation through *in vitro* kinase assay without affecting the expression levels of CK2 subunits (Alessi et al., 2015; Pagano et al., 2008; Sarno et al., 2001; Szyszka et al., 1995; Wang et al., 2014; Yde et al., 2008). Compared to DMSO controls, TBB treatment partially restores embryonic viability and bipolar spindle formation to *zyg-1(it25)* animals (Fig. 6A,C,D). Consistent with CK2 depletion by RNAi, we also observed that TBB-treated embryos possess increased levels of ZYG-1 at centrosomes (1.48±0.57-fold; $P$<0.001) compared to the DMSO control (Fig. 6E,F). While TBB produced no significant effect in embryonic viability, TBB treated animals produced a significantly reduced number of progeny (Fig. 6B) that was also observed by RNAi knockdown (Fig. 4B). Cytological analysis further confirmed that embryos treated with TBB exhibit cell cycle defects including detached centrosomes, DNA missegregation and abnormal PCM morphology (Fig. 6G). Taken together, our data suggest that the protein kinase CK2 holoenzyme functions in centrosome duplication and cell division, likely through CK2 holoenzyme-dependent phosphorylation.

## DISCUSSION

### *C. elegans* protein kinase CK2 functions in cell division during embryogenesis

In this study, we have investigated the function of the holoenzyme CK2 in early *C. elegans* embryos. Our data suggest that both the catalytic (KIN-3/CK2α) and regulatory (KIN-10/CK2β) subunits of CK2 are required for the survival of *C. elegans* embryos, which is consistent with prior genome-wide studies (Fraser et al., 2000; Sönnichsen et al., 2005). Cytological analyses reveal that RNAi-

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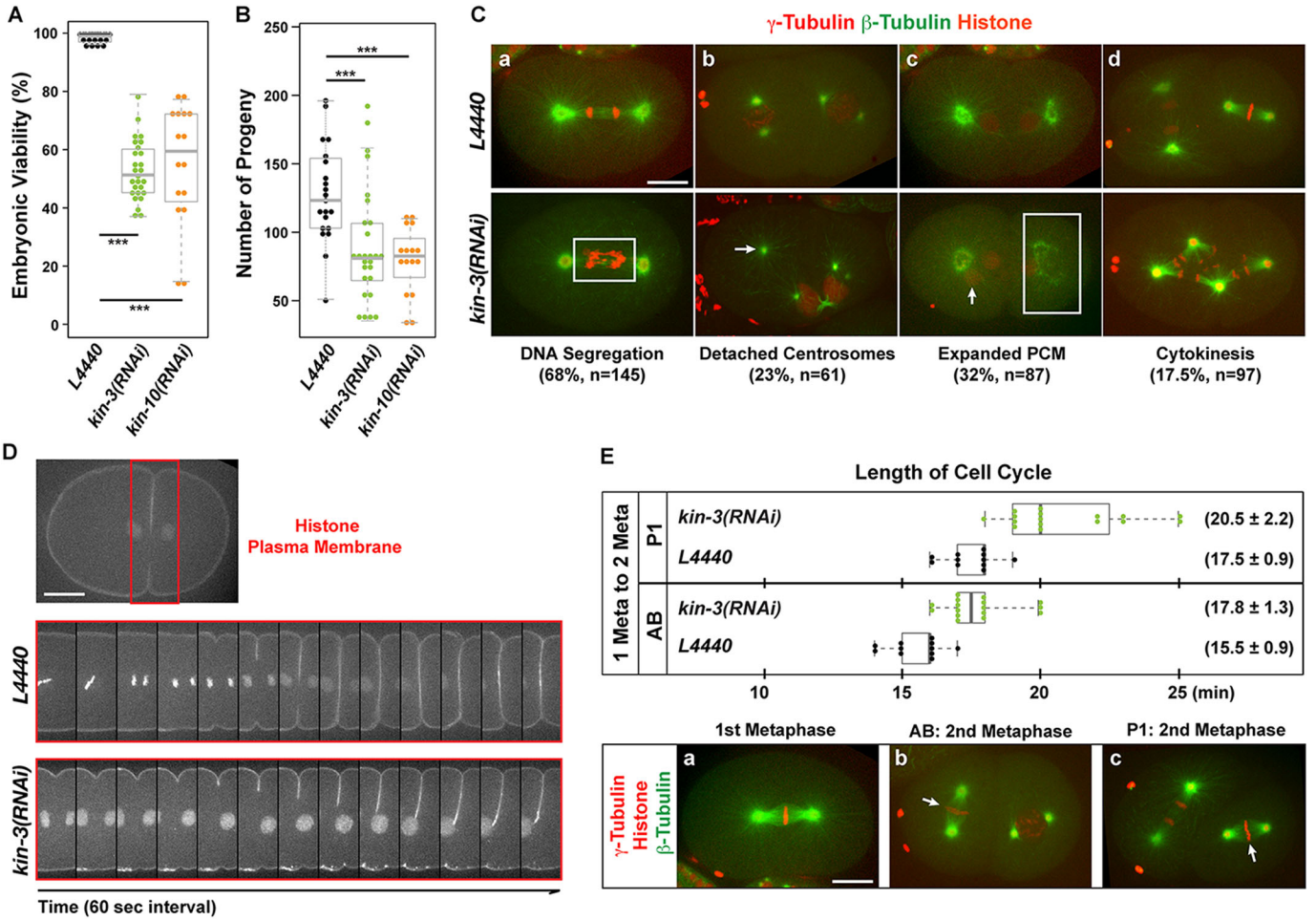


**Fig. 4. CK2 is required for early cell divisions in *C. elegans* embryos.** (A) Knockdown of KIN-3 or KIN-10 by RNAi results in embryonic lethality (Mean±s.d.: 47±10% and 45±21%, respectively). (B) Knockdown of KIN-3 or KIN-10 by RNAi leads to a significant reduction in the number of progeny produced for 24 h (90±43 and 79±24, respectively) compared to controls (133±43). For A and B, each dot represents an animal. (C) Wild-type embryos expressing GFP::β-tubulin, mCherry::γ-tubulin and mCherry:histone: *kin-3(RNAi)* results in defective cell divisions such as (a) lagging DNA (box, Movie 2), (b) detached centrosomes (arrow), (c) abnormal PCM morphology (box) and extra DNA (arrow), and (d) cytokinesis failure (Movie 3). (D) Embryos expressing mCherry::histone and mCherry:: plasma membrane. Boxed regions highlight cytokinetic furrow shown in bottom panels. Time-lapse recordings of cleavage furrow formation and ingression in L4440 and *kin-3(RNAi)* embryos. (E) CK2 depletion leads to a delay in cell cycle progression. Top: measurement of cell cycle lengths (average±s.d.) from first metaphase to second metaphase in AB or P1 cell. Each dot represents an embryo. Bottom: wild-type embryo representing cell cycle stages used for quantification. Note that second metaphase of the anterior blastomere (b, arrow) initiates before second metaphase of the posterior blastomere (c, arrow). (A,B,E) Box ranges from the first through third quartile of the data, and thick bar represents the median. Dashed line extends 1.5 times the inter-quartile range or to the minimum and maximum data point. ***$P$<0.001 (two-tailed $t$-test). Scale bar: 10 µm.

based depletion of CK2 results in abnormal cell divisions, including chromosome missegregation, a delay in cell cycle progression, cytokinesis failure, as well as aberrant centrosome behaviors. Furthermore, treating embryos with TBB, a chemical inhibitor of CK2, produces similar effects on early cell divisions to those by RNAi knockdown. TBB inhibits CK2 kinase activity via competitive binding to the ATP-binding site of CK2 (Sarno et al., 2001) and it has been reliably utilized for the specific inhibition of CK2 function in the *C. elegans* system (Alessi et al., 2015; Wang et al., 2014). Thus, it seems likely that protein kinase activity of the CK2 holoenzyme is responsible for proper cell division.

The pleiotropic phenotypes caused by CK2 depletion are likely a combined outcome of multiple substrates targeted by CK2 (Meggio and Pinna, 2003). The most prevalent phenotype we observed is abnormal chromosome segregation (Fig. 4C, 68%, $n$=145), including chromosome misalignment at metaphase, lagging DNA at anaphase, and extra DNA during early cell divisions in *C. elegans* embryos. Such roles appear to be evolutionarily conserved in yeast and human cells (Peng et al., 2011; St-Denis et al., 2009). For example, CK2 is known to phosphorylate kinetochore factors (Ndc10, Mif2/CENP-C) in budding yeast (Peng et al., 2011), and the microtubule plus-end tracking protein CLIP-170 that ensures kinetochores attachment to mitotic spindles in human cells (Li et al., 2010). In addition, CK2 targets the chromosome passenger complex (survivin/BIR-1) that functions in chromosome segregation and cytokinesis in mammalian cells (Barrett et al., 2011; Kitagawa and Lee, 2015) and phosphorylates Mad2 to regulate the spindle assembly checkpoint in yeast (Shimada et al., 2009). While we do not know specific cell cycle regulators phosphorylated by *C. elegans* CK2 in early cell division, it appears that *C. elegans* CK2 functions in chromosome segregation and cell cycle progression, possibly through multiple factors targeted by protein kinase CK2.

The subcellular localization of KIN-3, the catalytic subunit of *C. elegans* CK2, appears to correlate well with the function of CK2 in cell division. Our confocal live imaging reveal that KIN-3::GFP

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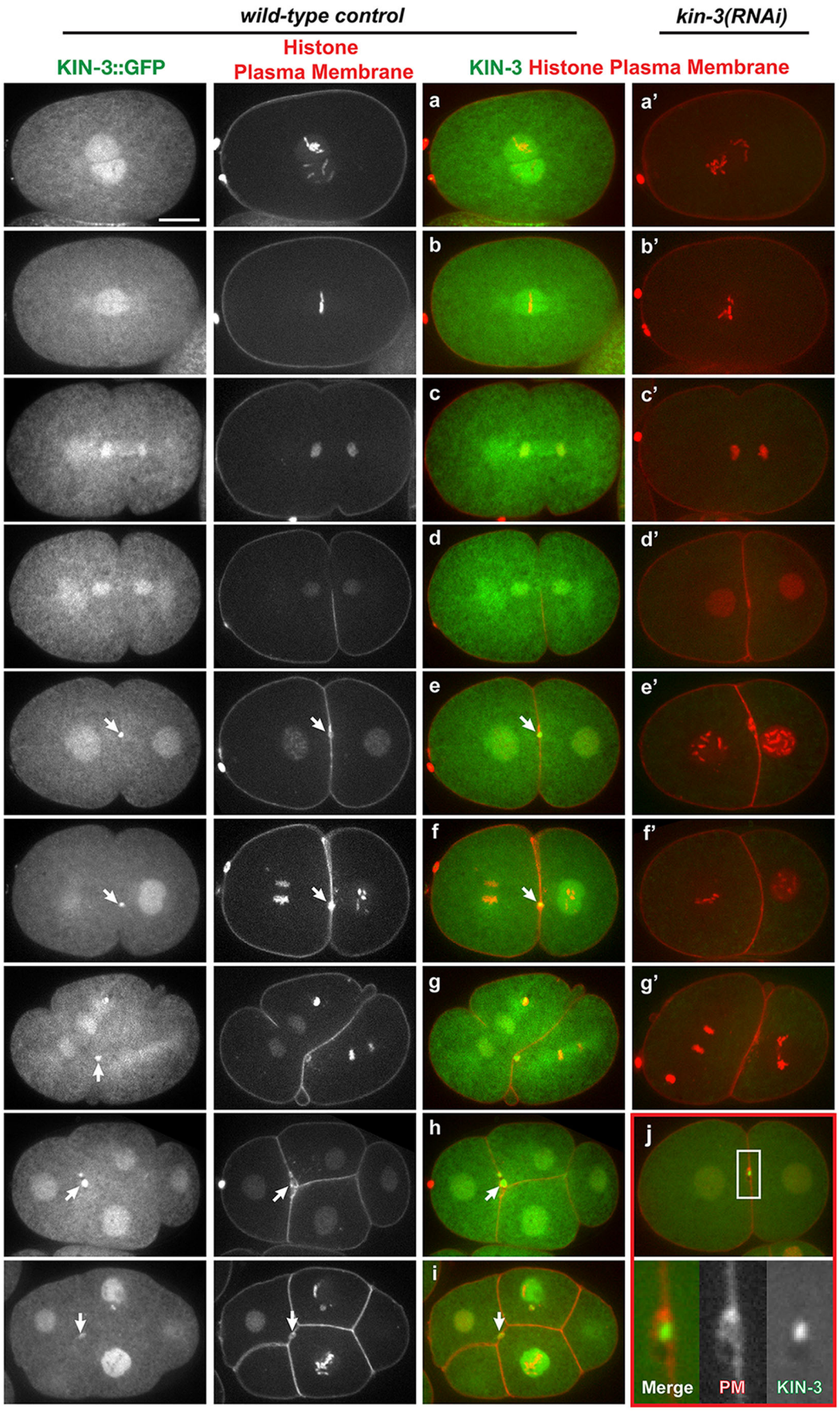


**Fig. 5.** See next page for legend.

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**Fig. 5. Subcellular localization of KIN-3::GFP.** Still images of embryos expressing KIN-3::GFP, mCherry::histone and mCherry::plasma membrane, illustrating that KIN-3 is enriched in nuclei at prophase (a), localizes to the mitotic spindle and centrosomes at mitosis including metaphase (b), anaphase (c) and telophase (d) (see Fig. S6). At completion of the first cytokinesis, KIN-3 becomes highly enriched at the midbody-associated structure (e-i, arrows). KIN-3 localization at the nuclei, mitotic spindles and midbody can be observed during second mitosis and later cell cycle stages (f-i). All subcellular localizations of KIN-3::GFP are abolished by depletion of KIN-3 by RNAi (a'-g'). The midbody localization of KIN-3::GFP is highlighted ( j) by co-localization of plasma membrane (PM). Insets are magnified 4-fold. Scale bar: 10 µm.

exhibits highly dynamic patterns of subcellular localization during the cell cycle. KIN-3 localizes to nuclei during interphase and early mitosis, and associates with mitotic spindles and centrosomes at mitosis. At later steps of cell division, KIN-3 becomes highly enriched as a distinct focus at the midbody. Our observations are consistent with subcellular localization of the catalytic subunit CK2α described in human cells, where CK2α localizes to nuclei (Penner et al., 1997), midbodies (Salvi et al., 2014), microtubules (Lim et al., 2004) and centrosomes (Faust et al., 2002). In the *C. elegans* germ line, the regulatory subunit KIN-10 is also shown to localize at centrosomes in mitotic cells (Wang et al., 2014). Many known midbody components are known to be required for proper cytokinesis (Green et al., 2012). A role for CK2 at the midbody is further supported by a proteomic survey of the mammalian midbody, where the regulatory subunit CK2β was identified (Skop et al., 2004). Our mass spectrometry analysis also suggests that KIN-3 physically associates with several known midbody proteins including ZEN-4 and CYK-4 (Table S1) (Jantsch-Plunger et al., 2000; Raich et al., 1998). In addition, we show that *kin-3* exhibits a positive genetic interaction with *air-2* and *zen-4* that encode midbody proteins regulating cytokinesis (Kaitna et al., 2000; Severson et al., 2000), suggesting that CK2 functions as a part of the midbody structure. Therefore, the holoenzyme CK2 is required for early cell division in *C. elegans* embryos, which appears to be conserved between nematodes and mammals.

### CK2 negatively regulates centrosome duplication in the *C. elegans* embryo

Protein kinase CK2 was identified as part of the SZY-20 immunocomplex. Given that *szy-20* is a genetic suppressor of *zyg-1* (Kemp et al., 2007; Song et al., 2008), we speculated that CK2 might have a role in centrosome assembly. Our results show that inhibiting either subunit of CK2 restores centrosome duplication and embryonic viability to *zyg-1(it25)* mutants at the semi-restrictive temperature, suggesting the *C. elegans* CK2 holoenzyme functions in centrosome assembly as a negative regulator. In contrast, CK2 depletion did not restore embryonic viability to *zyg-1(it25)* mutants at the restrictive temperature, suggesting that CK2 is not a bypass suppressor but requires ZYG-1 activity. Furthermore, *kin-3* appears to exhibit a positive genetic interaction with *szy-20* in regulating centrosome duplication, consistent with their physical association. Inhibiting CK2 kinase activity with TBB leads to restoration of centrosome duplication and embryonic viability to *zyg-1(it25)* mutants, indicating that CK2-dependent phosphorylation plays a critical role in centrosome duplication. While it has been shown that aberrant CK2α activity leads to centrosome amplification in mammalian cells (St-Denis et al., 2009), it remains unclear how CK2 function is linked to centrosome assembly. Given that KIN-3 localizes at centrosomes, CK2 might influence centrosome assembly via phosphorylation of centrosome-associated factors, although it is also possible that CK2 targets centrosome regulators in the cytoplasm before they are recruited to centrosomes.

Our work suggests that *C. elegans* CK2 might function in centrosome duplication by targeting ZYG-1. Both RNAi and TBB mediated inhibition of CK2 function led to elevated levels of ZYG-1 at centrosomes, suggesting that CK2-dependent phosphorylation regulates ZYG-1 by controlling either localization or stability. ZYG-1 phosphorylation by CK2 might interfere with ZYG-1 recruitment to centrosomes. Alternatively, CK2-dependent phosphorylation of ZYG-1 may be a targeting signal for proteasomal degradation. Thus, inhibiting CK2 activity prevents ZYG-1 from degradation, increasing overall ZYG-1 abundance and thereby centrosomal levels. The latter is consistent with previous studies showing that phosphorylation is required for proteasomal degradation of the ZYG-1 homolog Plk4 in the mammalian system (Cunha-Ferreira et al., 2013; Guderian et al., 2010; Holland et al., 2010; Klebba et al., 2013). In either case, increased ZYG-1 levels at centrosomes by inhibiting CK2 activity, at least partially, explains how reducing CK2 activity restores centrosome duplication to *zyg-1(it25)* embryos. It has been known that CK2 is a constitutively active Ser/Thr kinase that favors a conserved target motif including acidic amino acid residues near the phosphorylated residue (Salvi et al., 2009). Although it is beyond the scope of our current study, identifying substrates and specific amino acid residues targeted by CK2 will help in understanding how CK2 regulates centrosome duplication, in particular, how protein kinase CK2 influences ZYG-1 levels at centrosomes in *C. elegans* embryos. In any event, our data suggest that the holoenzyme CK2 functions to influence ZYG-1 levels at centrosomes through its kinase activity and thus, we report the protein kinase CK2 as a negative regulator of centrosome duplication.

In this study, we investigated the role of the conserved protein kinase CK2 in early *C. elegans* embryos, and show that CK2 acts as a negative regulator of centriole duplication and is required for proper cell cycle progression and cytokinesis.

## MATERIALS AND METHODS

### *C. elegans* strains and genetic analysis

The *C. elegans* strains used in this study were obtained from were obtained from the Caenorhabditis Genetics Center (CGC) (and indicated in Table S3) and maintained on MYOB plates seeded with *E. coli* OP50. All strains were derived from the wild-type Bristol N2 strain using standard genetics (Brenner, 1974; Church et al., 1995). Strains were maintained at 16 or 19°C unless otherwise indicated. A full list of strains used in this study is listed in Table S3. The KIN-3::GFP::3xFLAG strain (MTU5) was generated by standard particle bombardment (Praitis et al., 2001). The KIN-3::GFP::3XFLAG construct was acquired from TransgenOme (Sarov et al., 2012, construct number: 6236103120536928 D08), which contains 22kbp of the *kin-3* 5′UTR and 9kbp of the *kin-3* 3′UTR (Sarov et al., 2012). RNAi feeding was performed as previously described (Kamath et al., 2003), and the L4440 empty feeding vector was used as a negative control (Kamath et al., 2003).

For embryonic viability and progeny number assays, individual L4 animals were transferred to new plates and allowed to self-fertilize for 24 h at the temperatures indicated. For extended RNAi treatments (36-48 h), animals were transferred to a new plate in 24 h, and allowed to self-fertilize for an additional 24 h before removal. Progeny were allowed at least 24 h to complete embryogenesis before counting the number of hatched larvae and unhatched (dead) eggs.

### Cytological analysis

For immunostaining, the following primary antibodies were used at 1:2000-3000 dilutions: α-Tubulin: DM1a (Sigma, St-Louis, MO, USA), α-GFP: $IgG_1κ$ (Roche, Indianapolis, IN, USA), α-ZYG-1 (Stubenvoll et al., 2016), α-TBG-1 (Stubenvoll et al., 2016), α-SAS-4 (Song et al., 2008), α-SAS-5 (this study; Fig. S8B) and α-SZY-20 (Song et al., 2008). Alexa Fluor 488

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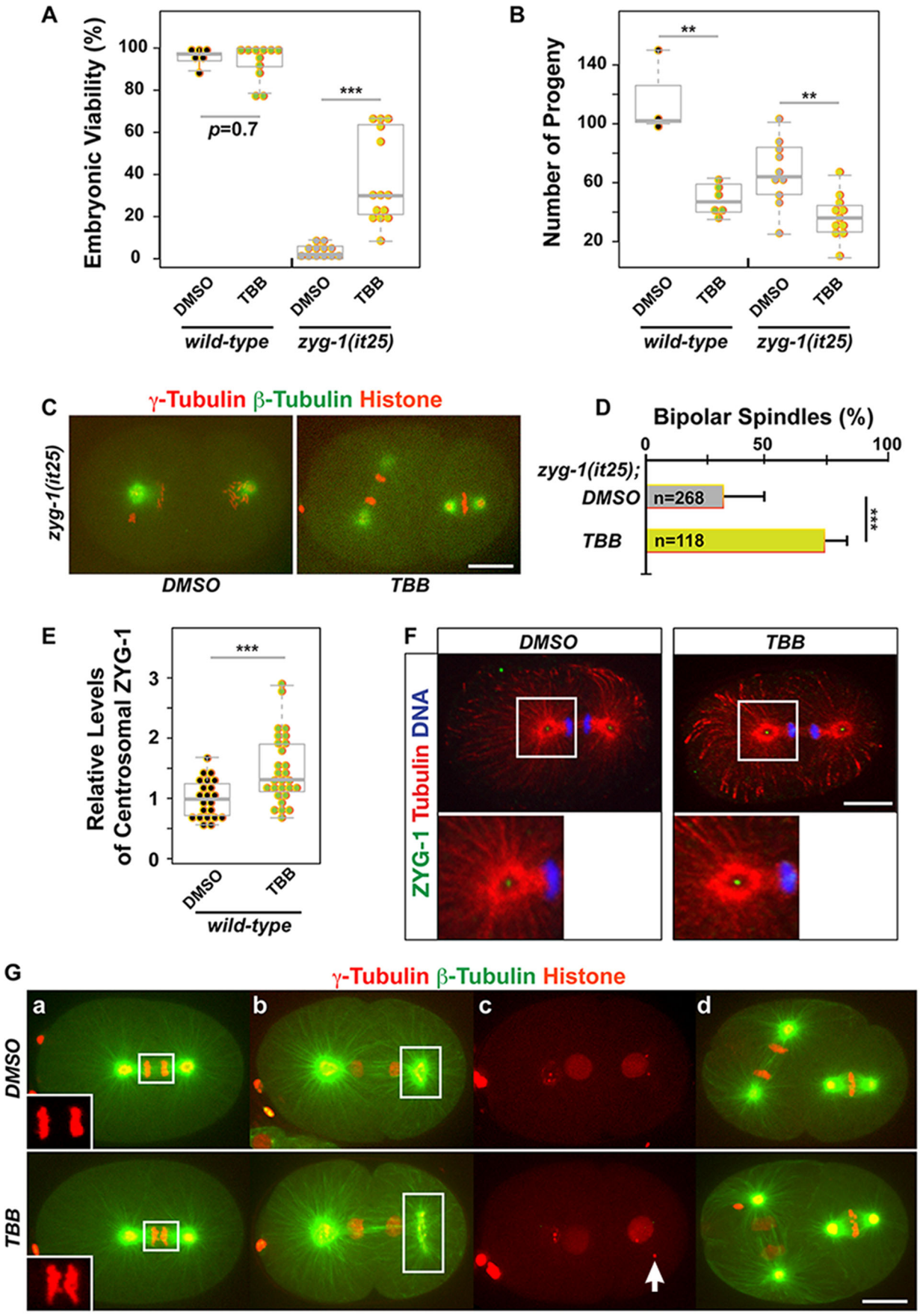


**Fig. 6.** See next page for legend.

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**Fig. 6. TBB, the chemical inhibitor of CK2, phenocopies CK2 depletion.** (A) TBB treatment restores embryonic viability to *zyg-1(it25)* animals at 22.5°C (DMSO control: 3±3%; TBB: 37±22%), but TBB had a mild effect on wild-type worms (DMSO: 95±6%; TBB: 92±8%). (B) TBB treatment leads to a significant reduction in the number of progeny produced by L4 animals allowed to self-fertilize for 24 h [wild-type (DMSO: 117±28; TBB: 49±11), *zyg-1(it25)* (DMSO: 67±23; TBB: 39±13)]. For A and B, each dot represents an animal. (C) TBB-treated *zyg-1(it25)* embryo exhibits bipolar spindles, but DMSO control embryo with monopolar spindles. (D) TBB treatment enhances bipolar spindle formation in *zyg-1(it25)* embryos (DMSO: 32±16%; TBB: 73±9.5%, *n*=blastomeres). Error bars are s.d. (E) Quantification of centrosomal ZYG-1 levels in TBB-treated wild-type embryos. Each dot represents a centrosome. (A,B,E) Box ranges from the first through third quartile of the data. Thick bar represents the median. Dashed line extends 1.5 times the inter-quartile range or to the minimum and maximum data point. (F) TBB-treated wild-type embryo shows more intense ZYG-1 focus at centrosomes. Insets illustrate centrosomal regions magnified 4-fold. (G) Wild-type embryos treated with TBB exhibit defective cell divisions that are similar to RNAi-mediated depletion of CK2: (a) lagging DNA (inset boxes are 2-fold magnifications), (b) expanded PCM (boxes), (c) detached centrosomes (arrow), (d) cell cycle delay. Note cell cycle stages in AB (left) relative to P1 (right) cell. The AB cell (left) is at second anaphase in DMSO controls, but at later mitosis (second telophase) in TBB-treated embryos, whereas the P1 cell (right) is at second metaphase in both TBB and DMSO treated embryos, suggesting that cell cycle in P1 is delayed in TBB-treated embryos. Shown are still images from live confocal imaging. ***$P$<0.001, **$P$<0.01 (two-tailed $t$-test). Scale bar: 10 µm.

and 561 (Invitrogen, Carlsbad, CA, USA) were used as secondary antibodies. Affinity-purified rabbit polyclonal antibody for KIN-3 was generated (YenZym, South San Franscisco, CA, USA) against the following peptides (aa361-373): Ac-VEDSSDHEEDVVV-amide. Immunostaining was performed as described previously (Song et al., 2008).

Confocal microscopy was performed as described in (Stubenvoll et al., 2016) using a Nikon Eclipse Ti-U microscope equipped with a Plan Apo 60×1.4 NA lens, a Spinning Disk Confocal (CSU X1) and a Photometrics Evolve 512 camera. Images were acquired using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). MetaMorph was used to draw and quantify regions of fluorescence intensity and Adobe Photoshop CS6 was used for image processing. To quantify centrosomal signals (SPD-2::GFP, TBG-1), the average intensity within a 25-pixel (1 pixel=0.151 µm) diameter region was measured within an area centered on the centrosome and the focal plane with the highest average intensity (corresponding to the centrosome) was recorded. The average fluorescence intensity within a 25-pixel diameter region drawn outside of the embryo was used for background subtraction. Centriolar signals (ZYG-1, SAS-5, SAS-4) were quantified in the same manner, except that 8-pixel diameter regions were used.

## Immunoprecipitation

Embryos were extracted by bleaching gravid worms in a hypochlorite solution [1:2:1 ratio of M9 buffer, bleach (5.25% sodium hypochlorite) and 5 M NaCl], washed with M9 buffer, flash-frozen in liquid nitrogen and stored at −80°C until use. For α-GFP immunoprecipitation experiments, 20 µl of Mouse-α-GFP magnetic beads (MBL, Naka-ku, Nagoya, Japan) were used per reaction. Beads were prepared by washing twice for 15 min in PBST (PBS; 0.1% Triton-X), followed by a third wash in 1× lysis buffer [50 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$, 200 mM KCl, and 10% glycerol (v/v)] (Cheeseman et al., 2004). Embryos were ground in microcentrifuge tubes containing an equal amount of 1× lysis buffer supplemented with complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and MG132 (Tocris, Avonmouth, Bristol, UK) and briefly sonicated prior to centrifugation. Samples were spun in a desktop centrifuge at 12,000 ***g*** twice for 20 min, collecting the supernatant after each spin. Protein quantification was then determined using a NanoDrop spectrophotometer (Thermo-Fisher, Hanover Park, IL, USA) and adjusted such that the same amount of total protein was used for each reaction. Beads were then added to the microcentrifuge tubes containing embryonic lysates. Samples were incubated and rotated for one hour at 4°C, and subsequently washed three times with PBST. Samples were then resuspended in 20 µl of a solution containing 2× Laemmli Sample Buffer (Sigma, St-Louis, MO, USA) and 10% β-mercaptoethanol (v/v) and boiled for five minutes. Mass spectrometry analysis was performed as described previously (Song et al., 2011).

## Western blotting

For western blotting experiments, samples were sonicated for 5 min and boiled in a solution of 2× Laemmli Sample Buffer and 10% β-mercaptoethanol before being fractionated on a 4-12% NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA, USA). The iBlot Gel Transfer system (Invitrogen, Carlsbad, CA, USA) was then used to transfer samples to a nitrocellulose membrane. The following antibodies were used at 1:3000-10,000 dilutions: α-Tubulin: DM1a (Sigma, St-Louis, MO, USA), α-GFP: $IgG_1κ$ (Roche, Indianapolis, IN, USA), α-SPD-2 (Song et al., 2008), α-SZY-20 (Song et al., 2008), α-TBG-1 (Stubenvoll et al., 2016), α-SAS-5 (Song et al., 2011) and α-KIN-3 (this study; Fig. S8A). IRDye secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) were used at a 1:10,000 dilution. Blots were imaged using the Odyssey infrared scanner (LI-COR Biosciences, Lincoln, NE, USA), and analyzed using Image Studio software (LI-COR Biosciences, Lincoln, NE, USA). Affinity-purified rabbit polyclonal antibody for KIN-3 was generated (YenZym, South San Franscisco, CA, USA) against the following peptides (aa338-356): Ac-CQADGQGASNSASSQSSDAK-amide.

## TBB treatment

MYOB plates were first seeded with OP50 bacteria and allowed to dry overnight. The media was then supplemented with 0.5 mM TBB (Tocris, Avonmouth, Bristol, UK) dissolved in a solution of 50% DMSO. TBB was added to the surface of plates, such that the final concentration of TBB was 15 µM based on the volume of media, and allowed to soak and diffuse through media overnight. Final TBB concentrations were derived from (Wang et al., 2014). An equal volume of solution containing 50% DMSO was added to plates and used as a control.

## Statistical analysis

All $P$-values were calculated using two-tailed $t$-tests assuming equal variance among sample groups. Statistics are presented as mean±s.d. unless otherwise specified. Data were independently replicated at least three times for all experiments and subsequently analyzed for statistical significance.

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### Competing interests

The authors declare no competing or financial interests.

### Author Contributions

J.C.M. and M.H.S. designed the experiments and wrote the manuscript. J.C.M. and M.H.S. performed quantifications of confocal imaging and protein levels from western blots. J.C.M., M.M.K., M.D.S., L.E.D. and M.H.S. performed experiments and provided data.

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### Supplementary information

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