CELL CYCLE REGULATION OF THE CENTRIOLAR PROTEIN ANA2

By

TAYLOR RAYMOND DIBBLE

A Thesis Submitted to the Honors College

In Partial Fulfillment of the Bachelors degree
With Honors in

Molecular and Cellular Biology

THE UNIVERSITY OF ARIZONA

MAY 2 0 1 5

Approved by:

Dr. Gregory C. Rogers
Department of Cellular and Molecular Medicine

Abstract

The centrosome functions to nucleate microtubule growth and organize the mitotic spindle during cell division. The centrosome normally duplicates once per cell cycle, ensuring a bipolar spindle that divides sister chromatids equally between two daughter cells during mitosis. However, improper duplication or over-duplication of centrosomes can lead to chromosomal instability, a hallmark of cancer. Two barrel-shaped structures called centrioles function as the duplication factors for centrosomes. Mutations in important centriole structural proteins can cause either down-regulation or amplification of centriole duplication. One of these proteins, Ana2, is required for duplication and mutations in its human orthologue, STIL, can cause disorders in neurological development. Normally, Ana2 localizes to an existing 'mother' centriole during S-phase and plays an essential role in the assembly of the procentriole that will become a mature 'daughter' during G2. In this study, we identified changes in total cellular levels of Ana2 by arresting S2 *Drosophila* cells in different phases of the cell cycle and immunoblotting for Ana2. We found that levels of both endogenous Ana2 and transiently overexpressed Ana2 are low during G1 and increase during S-phase. Endogenous Ana2 levels were highest in G2, consistent with centriole maturation during this phase of the cell cycle.

Introduction

The centrosome is an organelle that orients the mitotic spindle and anchors the microtubule cytoskeleton (Brownlee & Rogers, 2013). Its role in maintaining cell polarity is critical during development and somatic cell division (Bornens, 2012). Within the centrosome are two barrel-shaped structures containing microtubules called centrioles. Centrioles play a role in recruiting an agglomeration of proteins called pericentriolar material (PCM), which nucleates new microtubules from the centrosome (Brownlee & Rogers, 2013).

The two centrioles, termed ‘mother’ and ‘daughter,’ are structurally and temporally distinct and have functional differences as well. In motile cells, the mother centriole can act as a basal body, from which grow cilia and eukaryotic flagella (Bornens, 2012). The most significant distinction between the mother and daughter centrioles is found in their role as duplication factors for the centrosome (Wong & Stearns, 2003). Following mitosis, the mother and daughter centriole disengage from one another, a process shown to be required for duplication (Tsou & Stearns, 2006). The two centrioles now serve as mothers, each acting as a scaffold for the formation of a pro-centriole, which later becomes the daughter (Brownlee & Rogers, 2013). The cell will then enter mitosis with two centrosomes and two pairs of centrioles. This semi-conservative fashion of duplication is similar in some ways to DNA replication and occurs during S-phase (Brownlee & Rogers, 2013).

Disruptions in the life-cycle of a centrosome and its constituent machinery are of interest to our lab due to their numerous implications for cancer and neurological development. Mitosis is a crucial event in the cell cycle, as the duplicated genome must be divided equally between the two daughter cells. In embryonic cells, this process is disrupted by “relaxed” cell-cycle checkpoints (Ambartsumyan & Clark, 2008). In somatic cells, mitosis is facilitated normally by two centrosomes at opposite ends of the cell. Centrosome amplification, the presence of more than the normal two centrosomes in one cell, can lead to chromosomal fragmentation and aneuploidy, two indicators of chromosomal instability (Gordon, Resio, & Pellman, 2012). One proposed mechanism involves a multipolar spindle “intermediate” leading to a bipolar mitotic spindle with extra centrosomes (Ganem, Godinho, & Pellman, 2009); this results in the presence of lagging chromosomes and subsequent mis-segregation of sister chromatids (Gordon et al., 2012). In breast tumors, centrosome amplification and increased centriole size have been found to be positively correlated with chromosomal instability (Lingle et al., 2002). Down-regulation of centrosome duplication can

also lead to chromosomal instability, a hallmark of cancer (Brownlee & Rogers, 2013). The development of primary microcephaly, a neurological development disorder, and other ciliopathies can be caused by both centrosome amplification and disruption of duplication (Bettencourt-Dias, Hildebrandt, Pellman, Woods, & Godinho, 2011). These numerous links between centrosome duplication and human disease make it an exciting area of research.

The focus of this study is the *Drosophila melanogaster* centriolar protein Anastral Spindle 2 (Ana2), a protein shown to be required for centriole duplication (Stevens, Dobbelaere, Brunk, Franz, & Raff, 2010). Overexpression of Ana2 or Asterless, another crucial centriolar protein, can lead to the formation of *de novo* centriole-like structures in unfertilized eggs (Stevens et al., 2010a). In somatic cells, Ana2 overexpression causes centriole amplification, where a mother centriole is surrounded by daughter centrioles in a 'rosette' phenotype (Brownlee & Rogers, 2013). During duplication, Ana2 localizes specifically to the growing pro-centriole forming off of the proximal end of the mother (Stevens et al., 2010a). Ana2, along with its binding partner Sas6, is incorporated into the inner 'cartwheel' structure of the early daughter centriole (Stevens, Roque, & Raff, 2010). Loading of these two proteins onto the daughter centriole promotes duplication and maintenance of the mother-daughter connection called 'engagement' (Stevens et al. 2010b). Recent research has found that the regulator enzyme Polo-like kinase 4 (Plk4) phosphorylates Ana2 in a conserved region of the protein called the STAN domain, allowing it to bind Sas6 and localize correctly to the daughter centriole (Dzhindzhev et al, 2014). STIL, the human orthologue of Ana2, is also necessary for centriole duplication and co-localizes with SAS-6 to the early daughter centriole (Arquint, Sonnen, Stierhof, & Nigg, 2012). Mutations in STIL have been shown to cause primary microcephaly (Kitagawa et al., 2011a).

The asymmetric localization of Ana2 to the daughter centriole and the role of Plk4 in its targeting indicate that the regulation of Ana2 is tightly controlled. Cell-cycle dependent

regulation of Ana2 is consistent with the restriction of centrosome duplication to once per cell-cycle, during S-phase (Wong & Stearns, 2003). Previous research showed that cyclin-dependent kinase 2 (Cdk2) activity is required for centrosome duplication (Matsumoto, Hayashi, & Nishida, 1999; Lacey, Jackson, & Stearns, 1999). Cdk2 overexpression in cells arrested in G1 phase was sufficient to reverse the inhibition of centrosome duplication (Matsumoto et al., 1999). After duplication, Plk1 activity is necessary to promote centriole 'maturation' (Fig.1), leading to recruitment of PCM and 'priming' centrioles for duplication in next cell cycle (Brownlee & Rogers, 2013). During mitosis, Cdk1 triggers a decrease in centrosomal-associated STIL (Arquint & Nigg, 2014). Subsequently, activation of the anaphase-promoting complex (APC) leads to degradation of cytoplasmic STIL and centriole disengagement (Arquint & Nigg, 2014). Ana2/STIL levels stay low during G1 phase until they rise again during S-phase, concurrent with an increase in Cdk2 activity (Matsumoto et al., 1999). However, recent research has shown that a small population of Ana2 and Sas6 localizes to the mother centriole and begins procentriole formation during early G1 (Dzhindzhev et al, 2014). In this study, we analyze how cellular Ana2 levels change throughout the cell cycle and whether these findings are consistent with previous models of Ana2 and STIL regulation.

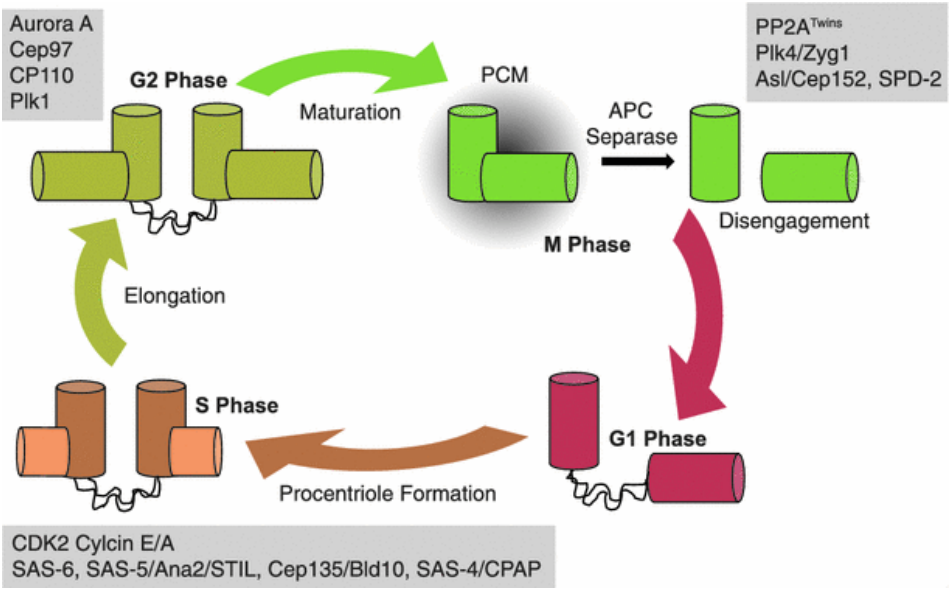

Figure 1

Life cycle of a centriole. Two centrosomes and their accompanying pairs of centrioles split to form bipolar spindle during mitosis. During anaphase, APC triggers centriole disengagement. Plk4 is required for procentriole formation, which occurs later during S-phase. Sas6 and Ana2 localize to the procentriole, which elongates to form the daughter centriole during G2 phase, a process promoted by Plk1. The two pairs of duplicated centrioles then split to form the bipolar spindle again in mitosis.

Results

Based on previous models of Ana2 regulation, total cellular levels of Ana2 should be low during the G1 phase of the cell cycle, increase during S-phase as Cdk2 is activated and centrioles begin duplication, and be highest during G2 phase, when the daughter centriole elongates (Brownlee & Rogers, 2013). A sharp drop in Ana2 should occur during mitosis with activation of the APC complex and subsequent degradation of Ana2 by the proteasome (Arquint & Nigg, 2014). To test this model, we cultured in a six-well plate four different samples of S2 fruit fly cells, each arrested in a different stage of the cell cycle. One sample of untreated, asynchronous cells was also cultured to serve as a reference for a non-arrested cell population. We incubated one of the samples with dsRNA against the transcription factor E2F to arrest the cells in G1 phase. Another sample was incubated with dsRNA against the phosphatase String (Stg) to arrest the cells in G2 phase. To confirm successful knockdown of these two proteins and adequate cell-cycle arrest, flow cytometry was used to analyze levels of chromosomal DNA using a propidium iodide (PI) stain (Fig. 2A). Compared to a cell-cycle profile of asynchronous S2 cells, E2F-knockdown cells show a strong peak corresponding to cells in G1, whereas Stg-knockdown cells show a strong peak corresponding to G2, with very few cells in G1 or dead. This indicated successful cell-cycle arrest in G1 and G2.

To block cells in S-phase and in mitosis, we treated one sample with the drugs aphidicolin and hydroxyurea and another sample with the drug colchicine. Aphidicolin and hydroxyurea arrest cells in S-phase by inhibiting DNA replication, whereas colchicine blocks cells in mitosis by inhibiting microtubule polymerization. We confirmed mitotic arrest by comparing levels of phospho-histone H3, a mitotic marker, in asynchronous cells versus cells treated with colchicine for 12 hours (Fig. 2B).

We first analyzed endogenous Ana2 levels in non-transfected cells. Western blotting for Ana2 showed that Ana2 levels increased from G1 to S-phase to G2, where levels were the highest (Fig. 2C). This is consistent with the model that Ana2 is stabilized by an increase in Cdk2 activity during S-phase, when centriole duplication occurs. Interestingly, Ana2 levels were not the highest for colchicine-treated cells, which theoretically should have blocked them in mitosis before the onset of anaphase and APC-mediated degradation of Ana2. This result is yet to be explained by current research.

Next, we investigated Ana2 levels in cells transfected with Ana2 tagged with a N-terminal V5 marker to look at post-transcriptional regulation only. Cells were transfected on Day 0 and expression was induced concurrently with dsRNA or drug treatment. Immunoblotting for V5 showed exogenous Ana2 levels increasing from G1 to S-phase, as expected (Fig. 2D). However, Ana2 levels were nearly gone in Stg dsRNA-treated cells, inconsistent with the model of a rise in Ana2 during S-phase and G2. This could be an indirect result of inactivating a Cdk by knocking down Stg; however, this result is not the same for endogenous Ana2, indicating perhaps an important effect of transcriptional control.

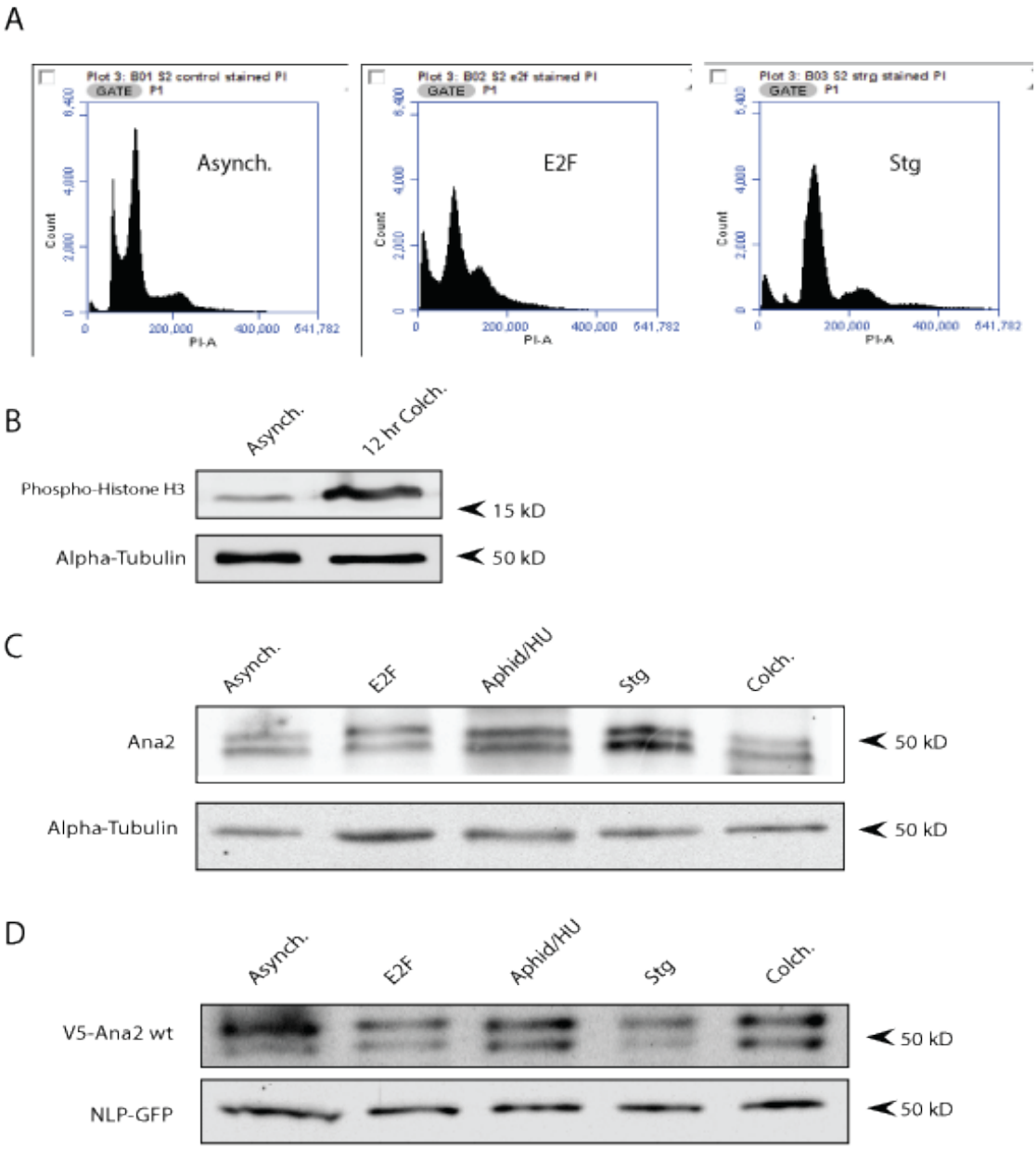

Figure 2

Levels of both endogenous Ana2 and transiently overexpressed V5-Ana2 increase from G1 to S-phase. (A) Flow cytometric analysis of asynchronous, E2F dsRNA-treated, and Stg dsRNA-treated S2 cells shows an asynchronous cell cycle profile, G1 arrest, and G2 arrest, respectively. (B) Western blotting shows an increase in phospho-histone H3 (mitotic marker) for S2 cells treated with colchicine for 12 hours compared to an asynchronous cell population. Alpha-tubulin served as a loading control. (C) Western blotting shows an increase in endogenous Ana2 levels from cells treated with E2F dsRNA, to cells treated with aphidicolin and hydroxyurea, to cells treated with Stg dsRNA. Alpha-tubulin served as a loading control. (D) Western blotting

shows an increase in V5-Ana2 levels from cells treated with E2F dsRNA to cells treated with aphidicolin and hydroxyurea and a sharp decrease in cells treated with Stg dsRNA. Cells were co-transfected with V5-Ana2 wt and Nlp-GFP, a transfection and loading control. Expression was induced 24 hours after transfection.

Discussion

In this study, we demonstrated that endogenous Ana2 levels rise from G1 to S-phase to G2, consistent with the model that Ana2 is degraded following mitosis and rises again during S-phase. Mitotic Ana2 levels were similar to those in asynchronous cells, but the reason for this unexpected result has not yet been discovered. Transiently overexpressed exogenous Ana2 also increased from G1 to S-phase, but its levels went down in G2, most likely due indirectly to Cdk1 inactivation. For reasons yet to be understood, transcriptionally –controlled endogenous Ana2 did not show this phenotype; rather, its levels were high in G2, as expected.

Research in our lab continues to focus on how Ana2 is regulated at these specific points during the cell cycle and what cellular components contribute to this regulation. Plk4 has been shown to directly phosphorylate Ana2, allowing it to bind Sas6 and correctly localize to a spot on the mother centriole (Dzhindzhev et al, 2014). The phosphatase PP2A, which stabilizes Plk4 during mitosis, has also been shown to play a role in the regulation of SAS-5, the distant orthologue of Ana2 in *Caenorhabditis elegans* (Kitagawa et al., 2011b). The catalytic and structural subunits of PP2A are necessary for centriole formation in *C. elegans* and interact directly with the SAS-5/SAS-6 complex to target it correctly to centrioles (Kitagawa et al., 2011b). However, current research from our lab has shown that the PP2A inhibitor okadaic acid stabilizes Ana2, suggesting that PP2A perhaps counteracts the effects of a stabilizing cyclin-dependent kinase. Further research could shed light on which Ana2 residues PP2A acts upon, and whether these residues coincide with

phosphorylation activity by another kinase. This would elucidate the regulatory mechanisms acting upon Ana2 and their differences from those in *C. elegans*.

This study helps confirm how total cell levels of Ana2 change throughout the cell cycle and identifies transcriptional regulation as perhaps an important component in Ana2 stabilization during the G2 stage of the cell cycle.

Materials and Methods

Cell Culture, RNAi, and Drug Treatment

Drosophila S2 cells were cultured in Sf900 II serum-free media (Life Technologies) in six-well plates. RNAi was performed over the course of five days, with 10μg dsRNA added to 1mL of media each day. Cells were maintained at 60-90% confluency and media was replaced every other day. dsRNA synthesis was performed as previously described (Rogers & Rogers, 2008). In short, dsRNA was synthesized from cDNA gene sequences with the T7 promoter sequence 5′-TAATACGACTCACTATAGGG-3′. Control dsRNA was made from a DNA template amplified from a sequence of the pEGFP-N1 vector (Takara Bio Inc.) using the primers 5′-CGCTTTTCTGGATTCATCGAC-3′ and 5′-TGAGTAACCTGAGGCTATG-3′ with the same T7 promoter sequence. Cell-cycle arrest in S-phase was accomplished by treating cells with 1 μM hydroxyurea and 10 μM aphidicolin (1 mL media total) for 24 hours. Arrest in M-phase was accomplished by treating cells with 30 μM colchicine (1 mL media total) for 12 hours.

Cell Transfection

Full-length cDNA of wild-type *Drosophila* Ana2 was cloned into the pMT vector with an upstream in-frame V5 tag and an inducible metallothionein promoter. All transfections were performed by mixing 1.8 μg V5-Ana2 wt in pMT expression plasmid with 0.2 μg of Nlp-

EGFP in the pMT plasmid as a transfection control. Transient transfections of cells was performed using the Nucleofector II (Lonza) and induced the subsequent day with a final concentration of .1 mM copper sulfate in 1 mL total of media. Drug treatment began at the same time as induction.

Western Blotting

S2 cell lysates were produced by lysing cells in PBS and 0.1% Triton X-100. Laemmli sample buffer was added to lysates, and the samples were then boiled for 5 min. Bradford protein assays (Bio-Rad Laboratories) were used to measure protein concentration of the lysates, per manufacturer's instructions. Samples of equal amounts of protein were run on SDS-PAGE, transferred to a nitrocellulose membrane, blocked with 5% milk in PBS and 0.1% tween, probed with primary and secondary antibodies, and scanned on an Odyssey imager (LI-COR Biosciences). Transfected Nlp-EGFP (as previously described in Rogers et al., 2009) was used as a loading control for transfected samples, and alpha-tubulin was used as a loading control for all other experiments. Antibodies used were mouse anti-α-tubulin monoclonal DM1A (Sigma-Aldrich), mouse anti-GFP monoclonal JL8 (Takara Bio Inc.), mouse anti-V5 monoclonal (Life Technologies), rabbit anti-phospho-histone H3 monoclonal (Millipore Life Sciences), and rabbit anti-Ana2 polyclonal (Rogers laboratory) at 1:1,500 dilutions. Secondary antibodies (LI-COR Biosciences) were prepared according to manufacturer's instructions at 1:3,000 dilutions.

Flow Cytometry

S2 cells were pelleted at 1,000 g for 5 min, resuspended in 0.5 ml PBS, and vortexed while intermittently adding 0.5 ml of cold 100% ethanol. Fixed cells were incubated on ice for 20 min, pelleted (1,000 g for 5 min), and resuspended in a 0.5 ml propidium iodide (PI)-RNase

solution (50 µg/ml PI + 100 µg/ml RNase Type1 I-A [QIAGEN] in PBS). After 20 min, cells were passed through a 12 × 75–mm flow cytometry tube (Falcon; Thermo Fisher Scientific). Cytometric analysis was performed in the Arizona Cancer Center in the laboratory of Dr. Anne Cress using a BD Accuri C6 flow cytometer equipped with an air-cooled 15-mW argon ion laser tuned to 488nm. List mode data files consisting of 10,000 cells gated on forward scatter versus side scatter were acquired and analyzed using CFlow Plus software. An unstained control was used to compare to cells stained with PI.

References

Ambartsumyan, G. & Clark, A.T. 2008. Aneuploidy and early human embryo development. *Hum*. *Mol*. *Genet*. 17:R10-15.

Arquint, C., & Nigg, E.A. 2014. STIL microcephaly mutations interfere with APC/C-mediated degradation and cause centriole amplification. *Curr*. *Biol*. 24:351-360.

Arquint, C., Sonnen, K.F., Stierhof, Y.D. & Nigg, E.A. 2012. Cell-cycle-regulated expression of STIL controls centriole number in human cells. *J*. *Cell Sci*. 125:1342-1352.

Bettencourt-Dias, M., Hildebrandt, F., Pellman, D., Woods, G. & Godinho, S.A. 2011. Centrosomes and cilia in human disease. *Trends Genet*. 27:307-315.

Bornens, M. 2012. The centrosome in cells and organisms. *Science*. 335:422-426.

Brownlee, C.W. & Rogers, G.C. 2013. Show me your license, please: deregulation of centriole duplication mechanisms that promote amplification. *Cell Mol*. *Life Sci*. 70:1021-1034.

Delattre, M., Leidel, S., Wani, K., Baumer, K., Bamat, J., Schnabel, H., Feichtinger, R., Schnabel, R. & Gonczy, P. 2004. Centriolar SAS-5 is required for centrosome duplication in C. elegans. *Nat*. *Cell Biol*. 6:656-664.

Dzhindzhev, N. S., Tzolovsky, G., Lipinszki, Z., Schneider, S., Lattao, R., Fu, J., Debski, J., Dadlez, M., & Glover, D.M. 2014. Plk4 phosphorylates Ana2 to trigger Sas6 recruitment and procentriole formation. *Curr*. *Biol*. 24:2526-2532.

Ganem, N.J., Godinho, S.A. & Pellman, D. 2009. A mechanism linking extra centrosomes to chromosomal instability. *Nature*. 460:278-282.

Gordon, D.J., Resio, B. & Pellman, D. 2012. Causes and consequences of aneuploidy in cancer. *Nat*. *Rev*. *Genet*. 13:189-203.

Kitagawa, D. *et al*. 2011a. Spindle positioning in human cells relies on proper centriole formation and on the microcephaly proteins CPAP and STIL. *Journal of cell science* 124:3884–93.

Kitagawa, D., Fluckiger, I., Polanowska, J., Keller, D., Reboul, J. & Gonczy, P. 2011b. PP2A phosphatase acts upon SAS-5 to ensure centriole formation in C. elegans embryos. *Dev*. *Cell* 20:550- 562.

Lacey, K.R., Jackson, P.K. & Stearns, T. 1999. Cyclin-dependent kinase control of centrosome duplication. *Proc*. *Natl*. *Acad*. *Sci*. *USA* 96:2817-2822.

Lingle, W.L., Barrett, S.L., Negron, V.C., D'Assoro, A.B., Boeneman, K., Liu, W., Whitehead, C.M., Reynolds, C. & Salisbury, J.L. .2002. Centrosome amplification drives chromosomal instability in breast tumor development. *Proc*. *Natl*. *Acad*. *Sci*. *USA* 99:1978-1983.

Matsumoto, Y., Hayashi, K. & Nishida, E. 1999. Cyclin-dependent kinase 2 (Cdk2) is required for centrosome duplication in mammalian cells. *Curr*. *Biol*. 9:429-432.

Rogers, G.C., Rusan, N.M., Roberts, D.M., Peifer, M., & Rogers, S.L. 2009. The SCF^{Slimb} ubiquitin ligase regulates Plk4/Sak levels to block centriole reduplication. *J*. *Cell Biol*. 184:225-239.

Rogers, S.L. & Rogers, G.C. 2008. Culture of Drosophila S2 cells and their use for RNAi-mediated loss-of-function studies and immunofluorescence microscopy. *Nat*. *Protoc*. 3:606-611.

Stevens, N.R., Dobbelaere, J., Brunk, K., Franz, A. & Raff, J.W. 2010a. Drosophila Ana2 is a conserved centriole duplication factor. *J*. *Cell Biol*. 188:313-323.

Stevens, N.R., Roque, H. & Raff, J.W. 2010b. DSas-6 and Ana2 coassemble into tubules to promote centriole duplication and engagement. *Dev*. *Cell*. 19:913-919.

Tsou, M.F. & Stearns, T. 2006. Mechanism limiting centrosome duplication to once per cell cycle. *Nature*. 442:947-951.

Wong, C. & Stearns, T. 2003. Centrosome number is controlled by a centrosome-intrinsic block to reduplication. *Nat*. *Cell Biol*. 5:539-544.