

Research Proposal - Control of Centrosome Duplication

SPECIFIC AIMS

The goal of the cell division cycle is to produce two genetically identical cells from one. To do this, cells must duplicate their chromosomes and centrosomes once, and only once, and then separate the duplicated chromosomes into two daughter cells. The segregation of chromosomes is achieved by the bipolar mitotic spindle, which is generated by the duplicated centrosomes. So far, the mechanism of centrosome duplication is not well understood. In this research proposal, we have two specific aims:

1. To investigate whether γ -tubulin and centrin are required for centrosome duplication. **Hypothesis** - The pericentriolar components, γ -tubulin and centrin, are critically important for the duplication of centrosomes.
2. To investigate the role of phosphorylation in the regulation of centrosome duplication by Cdk2-cyclin E. **Hypothesis** - Cdk2-cyclin E regulates centrosome duplication via the phosphorylation of centrosomal components.

The experiments proposed to test these two hypotheses will illustrate the basic mechanisms of cell division and may promote better understanding of the diseases that result from abnormal cell division.

INTRODUCTION

The centrosome, a unique cytoplasmic domain that is localized in the central part of interphase cells, functions as the major microtubule organizing center (MTOC) in animal cells. It regulates the polarity, number, and spatial arrangement of microtubules.¹ Together with the microtubule cytoskeleton, the centrosome plays important roles in a number of cellular events, including the segregation of chromosomes at mitosis, the organization and transport of cytoplasmic organelles, morphogenesis, and determination of cell shape.² Furthermore, through its well-established role in the assembly of microtubule arrays and impact on other structures such as actin cytoskeleton, the centrosome is crucial for cell motility, proliferation and differentiation.³

The Centrosome: Structure and Function

The centrosome is composed of a pair of centrioles, short cylindrical structures, surrounded by dense amorphous pericentriolar material. The two centrioles, assembled from α - and β - tubulins, are positioned at right angles to each other. They are not directly related with the nucleation of microtubules, rather, they form a scaffold for the assembly of pericentriolar material.¹ Interestingly, plants do not have centrioles, and centrioles are also missing in some animal cell types.⁴ Therefore, the pericentriolar material, which contains elements that monitor the nucleation and organization of microtubules, is the most

fundamental part of the centrosome.¹ Its best characterized component is the γ -tubulin ring complex, which provides a direct template to nucleate microtubules.⁵ Another component, centrin, is a Ca^{2+} -modulated protein that participates in centrosome organization.⁶ In addition, pericentrin, a large coiled-coil protein, has also been localized to the pericentriolar matrix. This protein constitutes a reticular lattice that may play a role in linking the γ -tubulin ring complex to the matrix.⁷ Despite the identification of these components, the overall structure and function of the pericentriolar material remains largely unknown.

The Centrosome Duplication Cycle

The centrosome duplicates only once per cell cycle. Through the bipolar mitotic spindle that forms from it, the centrosome helps segregate the replicated chromosomes into the two daughter cells. Because the centrosome determines the strict bipolarity of the cell division process, it is critically important for the cell to precisely couple centrosome duplication to the whole cell cycle so as to guarantee that the cell contains only two centrosomes at the beginning of mitosis.¹ The process of centrosome duplication is initiated at the G_1 -S transition, at about the same time that DNA replication begins. At late G_1 , the two centrioles lose their orthogonal pattern and separate from each other by a small distance. Then, two new daughter centrioles appear adjacent to each parental centriole. The duplication event processes through S and G_2 , and into mitosis. As the cell comes into prophase, the two duplicated centrosomes, including both the pair of newly replicated centrioles and the associated pericentriolar material, move in opposite directions to form the poles of the mitotic spindle. Following mitosis, each daughter cell retains a single centrosome from its mother.¹ Thus, centrosome duplication, like DNA replication, is semi-conservative in that one centriole is new and the other is inherited from the parental centrosome.¹ Nevertheless, little is known of how the centrosome is able to duplicate and how the multiple steps in the centrosome cycle are so orderly regulated.

Figure 1. The centrosome duplication cycle

What will Happen if Centrosome Duplication has Errors?

The major function of the centrosome is its ability to nucleate the growth of microtubules, which form the bipolar mitotic spindle to direct chromosome segregation during cell division. Failure in centrosome duplication can cause the formation of monopolar spindles that are incapable of segregating chromosomes; consequently, the cell division process will be blocked. Excessive rounds of centrosome duplication, on the other hand, can result in the formation of multipolar spindles and ultimately, chromosome missegregation (aneuploidy) and genomic instability, which are characteristic of many malignant tumors. Recently, abnormally high numbers of centrosomes have been found in many kinds of tumor cells.⁸ These tumor cells include those that have deficient or mutant tumor suppressor proteins p53, Brca1 or Brca2,^{9,10,11} and those overexpressing SKT15/aurora 2 kinase.^{12,13} Therefore, elucidation of the molecular mechanisms that

regulate centrosome duplication can not only promote better understanding of this fundamental cellular event, but can also shed light on its role in cell transformation and malignancy, and lead to new strategies for cancer diagnosis and therapy.

How is Centrosome Duplication Controlled?

Two precisely coordinated duplication events occur in each eukaryotic cell cycle: one is the replication of chromosomal DNA and the other is the duplication of the centrosome. The tight concurrence of centrosome duplication and DNA replication raises the question: does the centrosome contain nucleic acid, the duplication of which is controlled by the same machinery that controls DNA replication? This idea has been around for many years, but so far all investigations have failed to find nucleic acid components associated with centrioles or pericentriolar material. Another difference between centrosome duplication and DNA replication is that DNA replication has a strict control that involves a mechanism termed “licensing”. The “licensing” of DNA replication depends on selective access of replication factors to the DNA, thereby ensuring that DNA replication occurs only once per cell cycle.¹ In contrast, centrosome duplication is less tightly controlled, as in both embryonic and somatic systems it is possible to have multiple rounds of centrosome duplication within one cycle if the time spent in S phase is extended artificially.^{14,15,16} Thus, the centrosome must employ some other mechanism of duplication.

Over the past years, many kinds of regulatory proteins, such as kinases, phosphatases, ubiquitin enzymes and cyclins, have been localized in the centrosome,² but their specific role in the centrosome cycle and their connections to the cell cycle are not clear. The precise synchronization between centrosomal and nuclear events during the cell cycle suggests the existence of a regulatory mechanism that controls the coordination of these two cycles. The periodic activation of the cyclin dependent kinases (Cdks) is a potential regulatory mechanism since it is the major factor that directs the progression of the cell cycle.¹⁷ Studies of sea urchin and *Xenopus* have demonstrated repeated centrosome duplication under conditions where the mitotic cell cycle has been arrested in S phase by blocking protein synthesis.^{15,18} Thus, centrosome duplication cannot be regulated by those Cdk-cyclin complexes whose activities are dependent on the synthesis of their cyclin subunits. In this regard, the mitotic Cdk-cyclin complexes, Cdk1-cyclin A and Cdk1-cyclin B, should be ruled out as the force driving centrosome duplication since their activities are highly dependent on the translation of their cyclins.¹⁵ The Cdk2-cyclin E complex, however, is a potential candidate for driving centrosome duplication at least in early *Xenopus* embryos, since Cdk2-cyclin E activity is independent of the synthesis of cyclin E before the mid-blastula transition.¹⁹ Furthermore, Cdk2-cyclin E plays a key role in DNA replication,¹⁷ which occurs almost parallel to centrosome duplication in the cell cycle. In addition, Cdk2-cyclin E activity reaches its peak at the G₁-S transition in somatic mammalian cells,¹⁷ at the very time both DNA replication and centrosome duplication begin.

Recent studies of mammalian somatic cells have confirmed the importance of Cdk2 activity in regulating centrosome duplication. By treating CHO cells with hydroxyurea (HU) or mimosine, two cell cycle inhibitors that arrest cells at S phase and late G₁ phase respectively, Matsumoto et al.²⁰ found that centrosome duplication was not blocked by HU, as previously reported,¹⁶ but was blocked by mimosine, and the Cdk2 activity was much

lower in cells treated with mimosine than those treated with HU. In addition, overexpression of the Cdk2 inhibitor p21 prevented repeated centrosome duplication in HU-treated CHO cells. Furthermore, Cdk2 overexpression overcame the inhibition of centrosome duplication by mimosine treatment. These results, though unable to reveal whether cyclin E or cyclin A is crucial to drive the centrosome cycle, have provided strong evidence for the requirement of Cdk2 activity for centrosome duplication, revealing an important clue for further studies.

Summary - The centrosome, composed of two centrioles and pericentriolar material, is the major microtubule organizing center (MTOC) in animal cells. It plays a central role in organizing the mitotic spindle to separate the chromosomes at mitosis. The centrosome duplicates once and only once per cell cycle in a semi-conservative manner. Errors in this process can lead to the formation of aberrant spindles and ultimately chromosome missegregation. How centrosome duplication is regulated and coordinated with the mitotic cell cycle has been a mystery. Recent studies in mammalian cells have shown that the Cdk2-cyclin complex might play an important role in regulating centrosome duplication, but the precise mechanism remains to be investigated further.

SPECIFIC AIMS – Preliminary Data

We have presented evidence that Cdk2-cyclin complexes might play an important part in regulating centrosome duplication. This grant is designed to investigate the following questions: (1) which kind of cyclin is crucial for the regulation of centrosome duplication by Cdk2? (2) how does the Cdk2-cyclin complex regulate centrosome duplication? (3) what are the substrates and partners of the Cdk2-cyclin complex that control the centrosome duplication? (4) does the Cdk2-cyclin complex promote the initiation of centrosome duplication via the phosphorylation of centrosome components? The following preliminary data support these specific aims.

The mechanism of centrosome duplication has been studied by both *in vivo* and *in vitro* experiments over the past years. Lacey et al.²¹ treated *Xenopus* embryos with cycloheximide, which stops the cell cycle but supports repeated centrosome duplication. To test whether Cdk2-cyclin E drives centrosome duplication, they injected individual blastomeres in these arrested embryos with the Cdk inhibitor p21, which bound to and inhibited the activity of either cyclin A or cyclin E. They found that injected cells had two centrosomes whereas uninjected cells had eight centrosomes (Figure 2A). In addition, the inhibition of centrosome duplication by p21 was dose-dependent. Injection of p21 at a concentration of 1 μ M resulted in an average of 3.1 centrosomes whereas p21 at 3 μ M resulted in 1.9 centrosomes (Table 1). In contrast, cells injected with a control solution had an average of 6.4 centrosomes compared with 7.3 centrosomes in uninjected cells (Table 1).

To study whether the effect of p21 injection was a result of Cdk2-cyclin E inhibition, Lacey et al.²¹ co-injected p21 with a 2-fold molar excess of cyclin E. They found that the co-injected cells were able to undergo repeated centrosome duplication with the same pattern as the uninjected cells (Figure 2B and B'). The cells injected with p21 and cyclin E

had 7.7 centrosomes, similar to the number in uninjected cells (Table 1). Thus, excess cyclin E was able to alleviate the inhibition of centrosome duplication by p21.

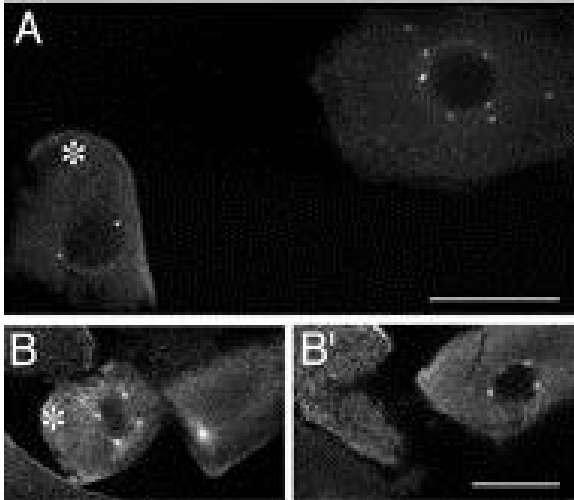


Figure 2. The cdk inhibitor p21 blocks centrosome duplication in frog embryos. Asterisks mark the injected cells, as visualized by coinjection of a fluorescein-conjugated dextran. (A) p21. (B and B') p21 and cyclin E (two different sections are shown to view all centrosomes). (A) -Tubulin staining. (B and B') -Tubulin staining. The difference in cell size is due to the variation in cell stage at the time of injection. (Bar = 100 μ m.)

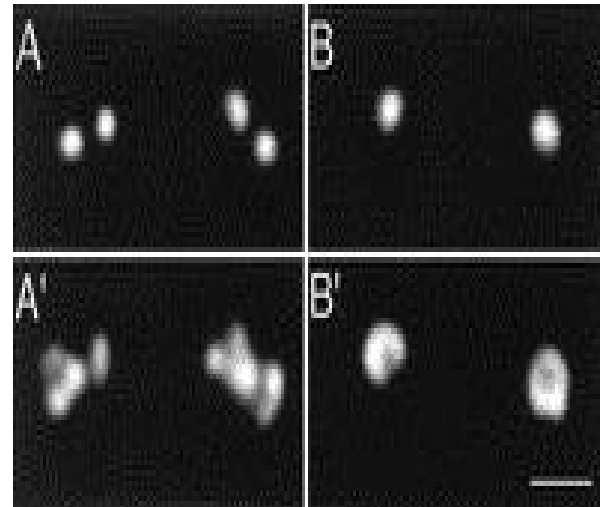


Figure 3. An in vitro centriole separation assay. Deconvolution images of centriole doublets and singlets. (A and A') These doublets represent the 0-h starting point of the assay. (B and B') At the 1-h endpoint, the majority of centrosomes are singlets, as depicted. (A and B) - Tubulin staining. (A' and B') -Tubulin staining. (Bar = 1 μ m.)

Solution injected	Average number of centrosomes*		Ratio, I/U†
	Injected cells	Uninjected cells	
Control‡	6.4 \pm 0.8 n = 18	7.3 \pm 0.6 n = 31	0.88
p21, 3 μ M	1.9 \pm 0.3 n = 20	6.0 \pm 0.6 n = 20	0.32§
p21, 2 μ M	2.1 \pm 0.2 n = 36	10.4 \pm 0.6 n = 38	0.20§
p21, 1.5 μ M	2.5 \pm 0.2 n = 32	5.5 \pm 0.4 n = 23	0.45§
p21, 1 μ M	3.1 \pm 0.3 n = 52	5.6 \pm 0.5 n = 51	0.55§
p21 + cyclin E	7.7 \pm 0.5 n = 40	7.5 \pm 0.5 n = 34	1.03

Table 1. Summary of in vivo injection experiments

n is the number of cells counted for each condition.

*The average number of centrosomes is shown \pm SEM.

†The ratio is centrosome number for injected cells divided by centrosome number for uninjected cells.

‡The control solution used was PBS.

§For these experiments, the average number of centrosomes in injected cells vs. uninjected cells is significantly different within a 99% confidence level.

||Cyclin E was added at twice the molarity of p21.

Lacey et al.²¹ confirmed the requirement of Cdk2-cyclin E activity for centrosome duplication by studying the separation of centrioles, an early step during the centrosome duplication cycle, in an *in vitro* system. This system involves seeding extracts from cycloheximide-treated *Xenopus* zygotes with centrosomes from *Xenopus* XTC cells. They determined whether centriole pairs resolve into singlets by staining for α -tubulin to visualize centrioles and staining for γ -tubulin to visualize the pericentriolar material (Figure 3). At the starting point, most centrioles appeared as doublets (Figure 3A and A'). By 1 hour, most centrioles appeared as singlets (Figure 3B and B'). The percent conversion of starting doublet centrioles to nondoublet forms was 90% (Table 2). To test the requirement for Cdk2-cyclin E in the centriole separation assay, Lacey et al. added centrosomes to *Xenopus* egg extracts that had been incubated with p21. They found that p21 blocked centriole separation with a conversion percentage of only 12% (Table 2).

The role of Cdk2-cyclin E for centrosome duplication was further investigated. Lacey et al.²¹ observed that centriole separation in this *in vitro* system was blocked by adding Suc1 beads, which deplete Cdks and associated proteins, and addition of Cdk2-cyclin E could reverse the inhibition of centriole separation (Figure 4A). These results further confirmed the requirement of Cdk2-cyclin E for centrosome duplication. Lacey et al. also found that the active mitotic state inhibited the process of centrosome duplication (Figure 4B), similar to the previous observation in sea urchin embryos.¹⁴ These results indicate that there should exist an intrinsic centrosome duplication machinery that is active during S phase but inhibited during mitosis. The requirement of Cdk2-cyclin E to drive the centrosome duplication process and the mitotic state to prevent it may function together in embryonic cells; consequently, the centrosome duplication is coordinated with the mitotic cycle, thereby ensuring that the centrosome duplicates only once in each cell cycle.

Condition	Percentage*			Conversion percentage†
	oo	o	o o	
0 h	77	23	—	—‡
1 h	8	77	15	90
p21	68	26	6	12

Table 2. Centriole separation assay data

p21 was used at a final concentration of 15 μ M.

*Approximately 500 centrosomes were counted, categorized, and expressed as a percentage. The three categories depict doublets (D), singlets (S), and separated doublets (SD), respectively.

†This represents the percentage of doublets that was converted to singlets or separated doublets, using the formula: $[(S + SD) - (S_0)]/D_0$; where $S_0 = 23$ and $D_0 = 77$. If the calculated conversion percentage was 0, a minimum value ($<1/500 = <0.2$) was used.

‡The conversion value at time 0 is set to zero.

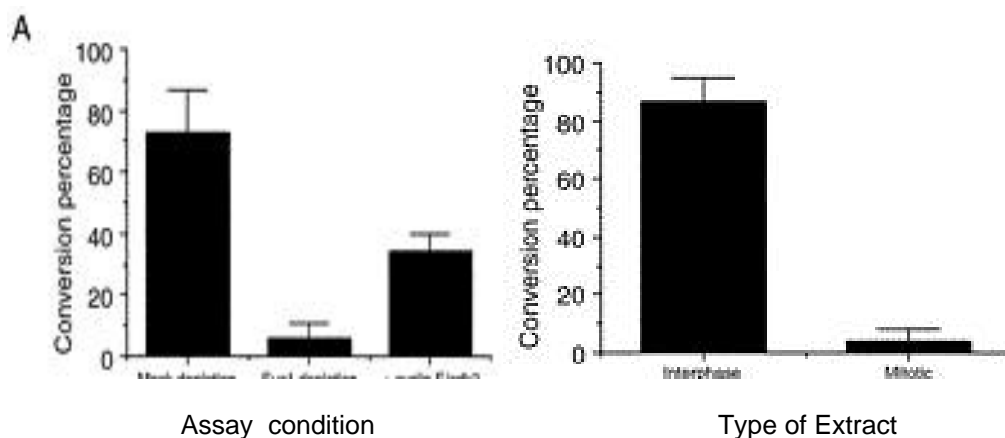


Figure 4. The dependence of centriole separation on cyclin-dependent kinases.

The graphs express centriole separation activity as conversion percentages, as described in Table 2. (A) Depletion and rescue of centriole separation activity with Suc1 and Cdk2-cyclin E. (B) The mitotic state inhibits centriole separation.

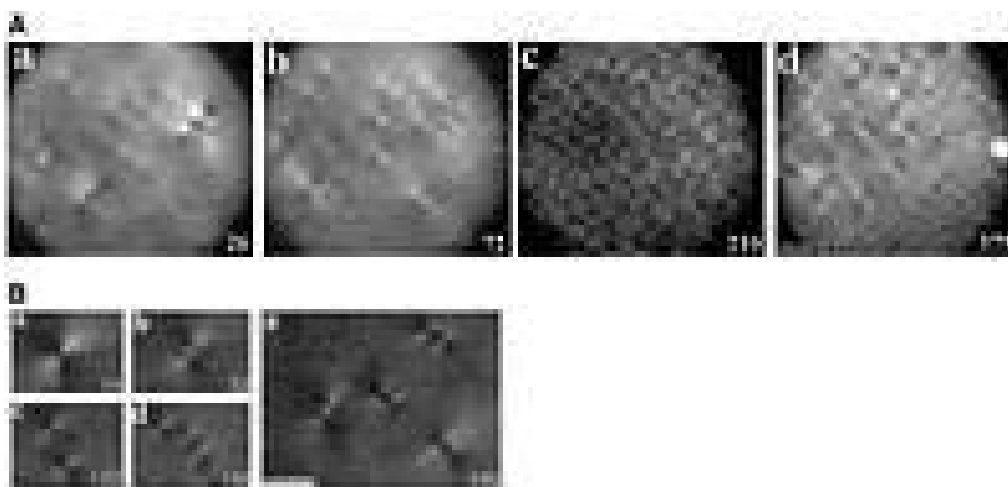


Figure 5. (A) Repeated rounds of aster doubling in an aphidicolin-treated extract.

Frames from a time-lapse video sequence, showing the increase in aster number over time in a microscope field. The decrease in aster number in (d) is due to the migration of asters from the plane of focus and field of view. Minutes after addition of sperm nuclei are seen in the lower right corner of each frame.

(B) Time-lapse sequence showing an individual aster from another aphidicolin-treated extract undergoing three rounds of doubling.

(a) The aster at the start of the time-lapse sequence. (b and c) Doubling of this aster and separation of the daughter asters. (d) Doubling of these daughters. (e) Third round of doubling yields eight asters. Minutes after addition of sperm nuclei are seen in the lower right corner of each frame.

Round of duplication	Starting number of asters at each round	Number of asters lost*	Number that remain	Number and percentage that duplicate
First	62	2	60	57 (95%)
Second	114	19	95	91 (96%)
Third	182	76	106	70 (66%)
Fourth	140	37	103	4 (4%)

*This is the number of asters that moved out of the plane of focus or off the field of view before they doubled.

Table 3. Aphidicolin-treated extracts followed for 6 hours.

Hinchcliffe et al.²² took another approach to investigate the importance of Cdk2-cyclin E in centrosome duplication. Based on cycling *Xenopus* egg extracts that had been arrested in S phase with aphidicolin, they established an *in vitro* system in which repeated centrosome duplication occurs, represented by multiple rounds of aster doubling. They used time-lapse photography and polarization optics to observe the overall increase in centrosome numbers over time (Figure 5). Demembrated sperm nuclei, instead of purified centrosomes, were added into the egg extracts to provide basal bodies for the first round of centrosome duplication. Because *Xenopus* extracts do not spontaneously assemble microtubule asters or centrosomes in the absence of added sperm nuclei, this increase in aster number indicates centrosome doubling. They analyzed aster doubling in a given field in aphidicolin-treated extracts and scored those that had at least one first, second, or third generation daughter aster visible for the duration of 6-hour experiment (Table 3). Thus, this *in vitro* system allowed them to observe the complete centrosome duplication cycle over time.

To test the hypothesis that Cdk2-cyclin E was required for centrosome duplication, Hinchcliffe et al.²² selectively inactivated Cdk2-cyclin E by adding 34Xic-1, a modified form of the *Xenopus* Cdk inhibitor that selectively inhibits Cdk2-cyclin E activity but not Cdk1-cyclin A or Cdk1-cyclin B activities at the concentration used. They found that repeated centrosome duplication in *Xenopus* extracts was inhibited by 34Xic-1 (Figure 6A and Table 4). Furthermore, purified Cdk2-cyclin E was able to restore multiple rounds of centrosome duplication (Figure 6B and Table 4). These observations indicate that the Cdk2-cyclin E complex plays a critical role in promoting centrosome duplication.

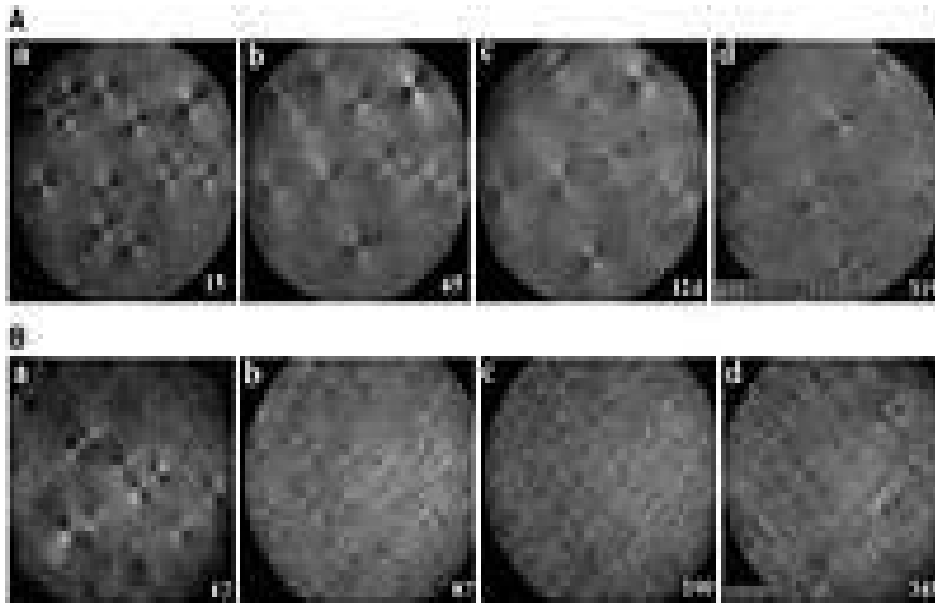


Figure 6. (A) Inhibition of Cdk2-cyclin E blocks repeated centrosome reproduction.

Time-lapse sequence showing one round of aster doubling in an aphidicolin-treated extract containing 175 nM 34Xic1. Minutes after addition of sperm nuclei are seen in the lower right corner of each frame.

(B) Restoration of repeated centrosome duplication by Cdk2-cyclin E.

Time-lapse sequence showing multiple rounds of aster doubling in an aphidicolin treated extract containing 175 nM 34Xic1 plus 245 nM Cdk2-E. Minutes after addition of sperm nuclei are seen in the lower right corner of each frame.

Round of duplication	Starting number of asters at each round	Number of asters lost*	Number that remain	Number and percentage that duplicate
		<i>Xic1-C</i>		
First	34	0	34	34 (100%)
Second	68	15	53	53 (100%)
Third	106	33	73	46 (63%)
		$\Delta 34Xic1$		
First	64	8	56	53 (95%)
Second	106	20	86	10 (12%)
Third	20	1	19	0 (0%)
		$\Delta 34Xic1 + Cdk2-E$		
First	56	0	56	56 (100%)
Second	112	12	100	91 (91%)
Third	182	46	136	43 (68%)

*This is the number of asters that moved out of the plane of focus or off the field of view before they doubled.

Table 4. Aphidicolin-treated extracts followed for 6 hours.

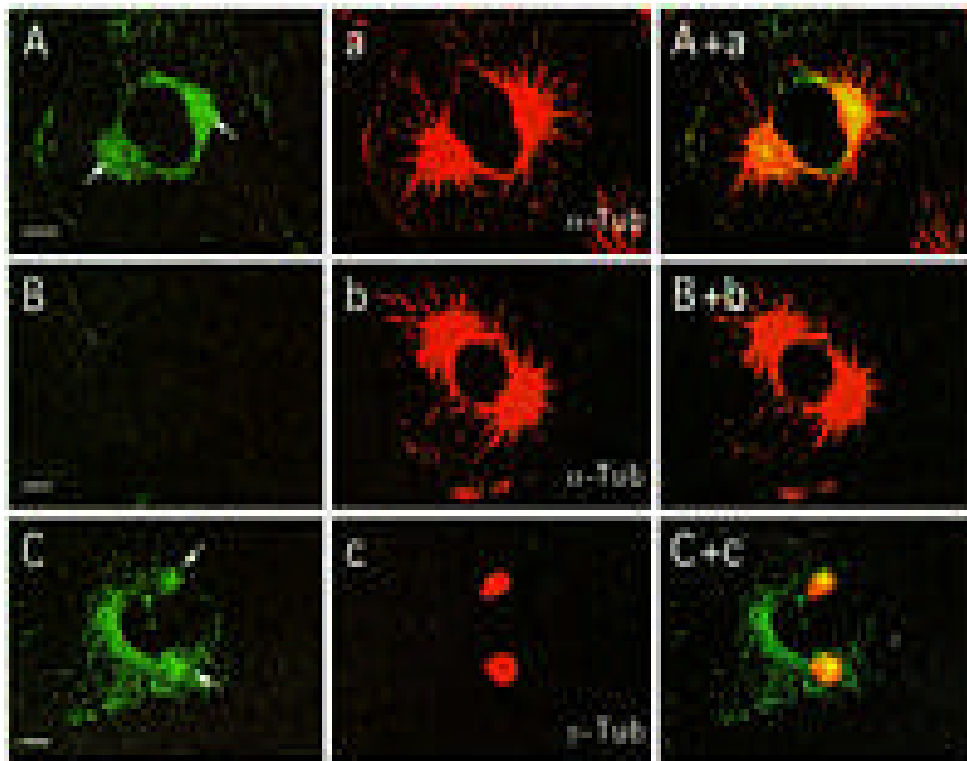


Figure 7. Localization of cyclin E in *Xenopus* embryonic blastomeres by immunofluorescence microscopy. Shown are three separate cells double immunostained with antibody to cyclin E (A through C) and antibody to α -tubulin (α -Tub) (a and b) or antibody to α -tubulin (α -Tub) (c). In (B), the antibody to cyclin E was preabsorbed to the initial cyclin E antigen. A + a, B + b, and C + c represent merged images. Centromeres are marked by arrows (A and C). Bars in (A), (B), and (C), 10 μ m.

Hinchcliffe et al.²² also found that inhibition of Cdk2-cyclin E activity did not block the first round of centrosome duplication, but did block further rounds of duplication (Figure 6A and Table 4). These results suggest that a “licensing” event exists at S phase for the centrosome reduplication and that the Cdk2-cyclin E complex may act as the “licensing” factor, without which the centrosome is not permitted to begin the second round of duplication.

Taken together, these preliminary data have provided evidence for the importance of the Cdk2-cyclin E complex in centrosome duplication, linking the centrosome cycle to the mitotic cell cycle. However, the exact mechanism of how the centrosome duplication process is controlled remains to be investigated. For example, how does the Cdk2-cyclin E complex promote the initiation of the duplication process? What are the substrates and partners of the Cdk2-cyclin E complex? In the above experiments, centrosome duplication and DNA replication both depend on the presence of the Cdk2-cyclin E complex in early embryos. As all of the components required for centrosome duplication are stored in the egg, initiation of the duplication process must depend on the phosphorylation of some embryonic components by Cdk2-cyclin E or its downstream targets. This raises a question: which molecules involved in initiating centrosome duplication are controlled by the Cdk2-cyclin E complex? So far, very little information is known about the potential downstream factors of the Cdk2-cyclin E complex. - Tubulin and centrin seem to be involved in the very early steps of centrosome duplication,^{23,24} so it may be helpful to look at the behavior of these two proteins during the centrosome duplication process. Interestingly, by confocal immunofluorescence microscopy, Hinchcliffe et al.²² found that cyclin E was distributed throughout the cytoplasm but showed maximal concentration at the centrosome in *Xenopus* embryonic blastomeres (Figure 7). Therefore, Cdk2-cyclin E may trigger centrosome duplication by the phosphorylation of centrosomal components, especially the pericentriolar material. Investigation of the centrosomal proteins upon activation and inactivation of Cdk2-cyclin E might help elucidate the above questions.

Specific Aim 1 – To investigate the role of the pericentriolar components, -tubulin and centrin, in centrosome duplication

Rationale - For centrosome duplication, the link to the cell cycle has been established with the discovery of the requirement for Cdk2-cyclin E kinase activity, but little is known of the precise mechanism that controls the duplication process. The localization of cyclin E and other regulatory proteins to the pericentriolar matrix suggests that the pericentriolar components might be critically important for centrosome duplication. Recently, Ruiz et al.²³ have revealed the requirement of -tubulin for the duplication of basal bodies, the equivalent of centrosomes in *Paramecium*, and centrin has also been implicated in the duplication of centrosomes.²⁴ Therefore, it is fascinating to study whether -tubulin and centrin are required for centrosome duplication. To investigate this question, initially we will develop an *in vitro* centrosome duplication system based on that of Hinchcliffe et al.²² that carries out repeated centrosome duplication. The *in*

in vitro assay has unique advantages over other methods; for example, with the S phase-arrested egg extracts we can monitor how various substances affect centrosome duplication without worrying about the normal blocks to the duplication as the cell cycle progresses. In addition, with the *in vitro* system we can examine the centrosome duplication process in more detail.

In Vitro Study - Felix et al.²⁵ have demonstrated that the centrioles of demembrated *Xenopus* sperm can reconstitute functional centrosomes *in vitro* by recruiting pericentriolar material from *Xenopus* egg extracts. This allows us to selectively deplete γ -tubulin or centrin from the *Xenopus* egg extracts by immunodepletion as described.^{25,26} Then, we will ask whether the depletion of γ -tubulin or centrin can block centrosome duplication; both morphological observation and quantification assay, as described by Hinchcliffe et al.²², will be carried out to examine the effect of such depletions. It is possible that centrosome duplication is not inhibited by depletion of γ -tubulin or centrin, however, this is very unlikely, since the *Paramecium* basal body fails to duplicate after depletion of gamma-tubulin,²³ and in yeast duplication of the spindle pole body, the counterpart of animal centrosomes, requires the centrin homolog *cdc31p*.²⁴

In this experiment, mostly likely, depletion of γ -tubulin or centrin will block centrosome duplication in the egg extracts. This means that these two pericentriolar components or proteins associated with them are necessary for duplication of the centrosomes *in vitro*. To examine this, we will add back bacteria-expressed γ -tubulin or centrin into the depleted *Xenopus* egg extracts; the requirement of γ -tubulin or centrin for centrosome duplication will be confirmed by the restoration of the duplication event. Production of fusion protein of γ -tubulin or centrin in bacteria is better than purification by biochemical methods because the concentrations of these two components are very low in *Xenopus*, making it difficult to get enough purified proteins to use. After we confirm the requirement of γ -tubulin and/or centrin for centrosome duplication, we will investigate which of their functional domains play the key role in the duplication of centrosomes. This will be studied by adding recombinant proteins that contain different domains of γ -tubulin or centrin into the depleted egg extracts to observe their effect on centrosome duplication. Another, more subtle, approach is to use an array of point-mutants previously established in the genetic system, such as yeast, for this *in vitro* centrosome duplication assay.

In Vitro Centrosome Duplication Assay - To make S phase-arrested *Xenopus* egg extracts, first, cycling *Xenopus* egg extracts are prepared as described by Murray et al.²⁹ and then added with aphidicolin. Demembrated *Xenopus* sperm nuclei prepared by the method of Blow et al.³⁰ is added to the extracts to act as seeds for centrosome duplication. H1 kinase assay and Time-lapse videomicroscopy are used to confirm that the extracts are arrested in S phase. Then, rhodamine-labeled α - and β - tubulins are added to the aphidicolin-treated extracts to visualize the centrioles and the astral microtubules under fluorescence microscopy. Time-lapse imaging and polarization optics are used to directly observe aster doubling and hence centrosome duplication over

6 hours as described by Hinchcliffe et al.²² The pattern of centrosome duplication in aphidicolin treated extracts is quantified by analyzing the rounds of aster doubling in a given field during the 6-hour experiment.

Protein Depletion from Egg Extracts- Depletion of γ -tubulin or centrin from the *Xenopus* egg extracts is performed essentially as described by Ohba et al.²⁶ Briefly, 5 μ l of affinity-purified antibodies to *Xenopus* γ -tubulin or centrin is mixed with 3 μ l of protein A-sepharose beads, and the mixture is incubated for 2 hours with rotation. After incubation with antibodies, the beads are washed and mixed with 10 μ l of *Xenopus* egg extracts, and the mixture is incubated for 30 minutes on ice. Then, the beads are pelleted by centrifugation, and the cytosol is removed and reincubated with fresh beads as described above. In the end, Western-blot analysis is performed to evaluate the depletion of the pericentriolar component. In control groups, both aphidicolin-treated *Xenopus* egg extracts without further manipulation and those eluted with IgG-bound beads are used.

Pitfalls & Alternative Approach – The *in vitro* experiment proposed here is designed to investigate the importance of the pericentriolar components, γ -tubulin and centrin, in centrosome duplication. In this experiment, we plan to express γ -tubulin and centrin in bacteria. However, it is possible that bacteria-expressed γ -tubulin or centrin is insoluble; therefore, they might not work in *Xenopus* egg extracts. In this case, we have to rely on alternative methods, such as expressing them in insects. In addition, although this *in vitro* centrosome duplication assay is likely to test our hypothesis that γ -tubulin and centrin are required for the duplication of centrosomes, it will be very useful to see correlation with another system, preferably *in vivo*. This will help rule out the possibility that the centrosomes formed from the sperm basal bodies in the *in vitro* assay are subject to different rules for duplication than centrosomes found in normally dividing cells.

In Vivo Study – Previous studies have shown that both cycloheximide treatment of *Xenopus* embryos²¹ and aphidicolin treatment of sea urchin zygotes¹⁴ block cell division and DNA replication, but allow many rounds of centrosome duplication. Thus, to study centrosome duplication *in vivo*, we can establish an *in vivo* system like above²¹, using cells from *Xenopus* embryos, that supports repeated centrosome duplication. Then, by injecting antisense mRNA that is specific to γ -tubulin or centrin into these cells, before cycloheximide treatment, to block the function of γ -tubulin or centrin, we will be able to test our hypothesis that γ -tubulin and centrin are required for centrosome duplication. The role of these two pericentriolar components in centrosome duplication can be further investigated by methods similar to those used in the *in vitro* studies as discussed above.

Specific Aim 2 – To investigate the role of phosphorylation in the regulation of centrosome duplication by Cdk2-cyclin E

Rationale - Recent studies have provided evidence that the Cdk2-cyclin E complex is critical for centrosome duplication,^{20,21,22} but little else is known about the regulation of

this process. Excitingly, cyclin E has been localized to the centrosomal region,²² indicating that Cdk2-cyclin E might initiate the centrosome duplication process by the phosphorylation of centrosomal components, especially the pericentriolar materials. Thus, investigation of the centrosomal components upon activation and inactivation of Cdk2-cyclin E might help elucidate this question.

First, we will employ the above *in vitro* system that supports repeated centrosome duplication in aphidicolin-treated *Xenopus* egg extracts. To investigate the role of phosphorylation in the regulation of centrosome duplication by Cdk2-cyclin E, we will selectively inactivate Cdk2-cyclin E by the addition of recombinant 34Xic1, a *Xenopus* Cdk inhibitor that is specific to Cdk2-cyclin E at low concentrations²², or dominant-negative Cdk2 which completely blocks the activity of cdk2-cyclin E.²⁷ The *Xenopus* egg extracts will be prepared as described above, added with [³²P]-ATP and then divided into two parts. To the first part (Extract I), C-Xic1, the C-terminal half of Xic1, will be added as a control because it does not affect the activity of Cdk2-cyclin E *in vitro*. To the second part, we will add 34Xic1 and then split this sample into two further parts. Then, we will add buffer to the first of them (Extract II) and different dosages of bacteria-expressed, purified active Cdk2-cyclin E complex to the second (Extract III). The pattern of aster doubling under these three conditions will be visualized by fluorescence microscopy as discussed in the first specific aim. From our preliminary data, we can predict that both Extract I and Extract II will be able to support repeated centrosome duplication whereas Extract III will not.

After we observe the results of centrosome duplication experiments, we will perform sucrose density-gradient centrifugation to collect the protein components from the above three reaction mixtures. Then, different centrosomal components will be separated by SDS-polyacrylamide gel electrophoresis. Using autoradiography, the components, which are phosphorylated during the Cdk2-cyclin E complex regulated centrosome duplication, will light up. We will then excise these proteins from the SDS gel and determine their sequences followed by protein data base searches. This approach has been successfully used by Li et al. to separate and identify trace-level membrane proteins from *H. influenzae*.²⁸ Through this experiment, we will be able to identify the proteins downstream to Cdk2-cyclin E which are directly or indirectly phosphorylated during centrosome duplication. If they are unknown proteins, we will clone their cDNAs based on the protein sequences. If they are known proteins, this step will be skipped. The role of phosphorylation of these proteins during centrosome duplication will be further investigated. For example, with time-lapse immunofluorescence microscopy, we can study the localization of these proteins during the centrosome duplication process. This method will also help reveal whether these proteins are original centrosomal proteins or cytoplasmic proteins recruited to the centrosome upon the initiation of centrosome duplication. In addition, we can perform an *in vitro* phosphorylation experiment to differentiate between proteins phosphorylated directly by Cdk2-cyclin E and those phosphorylated by this complex's downstream targets.

Pitfalls & Alternative Approach – Through our experiment, we can identify proteins phosphorylated during the Cdk2-cyclin E regulated centrosome duplication process.

However, it will be hard to distinguish centrosome components from DNA replication machinery and general cytoplasmic factors that are also phosphorylated by Cdk2-cyclin E. This might be able to overcome by immunodepletion and add-back experiment similar to that we discussed in the first specific aim. In addition, we can conduct an alternative experiment to fractionate *Xenopus* egg extracts to define the proteins necessary and sufficient for the reconstitution of centrosome duplication *in vitro*. Then, we can investigate the interaction between Cdk2-cyclin E and these proteins to elucidate the mechanism of centrosome duplication in more detail.

Fractionation of Xenopus Egg Extracts – The method for fractionating *Xenopus* egg extracts is derived from that of Liu et al.³¹; by this method, they have succeeded in the identification of several important components, such as cytochrome C and Apaf-1, in the apoptotic cell death. In brief, egg extracts are applied to a 10 ml phenyl sepharose column equilibrated with buffer N (buffer for preparing demembrated *Xenopus* sperm nuclei) containing 50% ammonium sulfate. The column is washed with two bed volumes of buffer N containing 50% ammonium sulfate and eluted with buffer N containing 1 M ammonium sulfate. The eluate is loaded onto a Superdex-200 gel filtration column (300 ml) equilibrated with buffer N and eluted with the same buffer. Fractions of 10 ml are collected and used for *in vitro* centrosome duplication assay. The active fractions from the gel-filtration column are pooled and loaded onto a Mono Q 5/5 and a Mono S 5/5 column connected together. The columns are pre-equilibrated with buffer N. After loading, the columns are disconnected, and the Mono S column is washed with 5 ml of buffer N containing 0.1 M NaCl and the fractions required for centrosome duplication are eluted from the column with a 20-ml 0.1 M–0.3 M linear NaCl gradient. Fractions of 1 ml are collected and used for *in vitro* centrosome duplication assay. The active fractions are subjected to SDS-polyacrylamide gel electrophoresis followed by silver staining. The protein factors required for centrosome duplication will be observed.

SUMMARY – Centrosomes, which organize the mitotic spindle required for the segregation of chromosomes at mitosis, duplicate once, and only once, during the cell cycle. Recent studies have provided evidence for the necessity of Cdk2-cyclin E in centrosome duplication, but how this duplication process is regulated and coordinated with the mitotic cell cycle remains largely unknown. This research proposal is to investigate whether the two pericentriolar components, γ -tubulin and centrin, are required for centrosome duplication, to investigate the role of phosphorylation in the regulation of centrosome duplication by Cdk2-cyclin E, and to identify the protein factors required for this important event. Elucidation of these questions can not only promote better understanding of the centrosome duplication process as well as the whole cell cycle, but can also provide important clues for the diagnosis and therapy of diseases that result from abnormal cell division.

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