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A Role for Aurora A Kinase in Timely Nuclear Envelope Breakdown

A Dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biomedical Sciences

by

Nathan Charles Portier

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2007
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Chair

University of California, San Diego

2007
Dedication

I would like to dedicate this thesis to my parents for their never-ending support and love.
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NEBD – Nuclear envelope breakdown
RNAi – RNA-mediated interference
PCM – Pericentriolar material
CNN - Centrosomin
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Abstract of the Dissertation

A Role for Aurora A kinase in Timely Nuclear Envelope Breakdown

by

Nathan Charles Portier

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2007

Professor Karen Oegema, Chair

The Aurora kinases are a family of mitotic kinases that are responsible for a variety of functions within the cell. Depletion of the centrosome-associated family member, Aurora A, results several phenotypes, including defects in centrosome maturation, centrosome separation, robust aster and spindle nucleation, and loss of cell polarity. We have used depletions of the Aurora A homolog, AIR-1, in C. elegans early embryonic divisions to discover a novel role for the kinase in timely nuclear envelope breakdown (NEBD). Here, we describe the generation of an assay used to determine the extent of chromosome
condensation using fluorescence time-lapse images as input. This assay allowed us to determine that depletion of AIR-1 does not affect the onset or rate of chromosome condensation, rather a specific delay in NEBD is seen. AIR-1 depletion also affects the progression of nuclear envelope permeabilization once it has begun, with a coordinate slowing of lamin and nuclear pore component protein loss. Further, protein depletions that result in a centrosome maturation defect demonstrate an intermediate NEBD delay with respect to $air-1(RNAi)$ embryos.
Chapter 1
Introduction

The Aurora family of kinases

The Aurora kinases are a family of kinases that have been shown to be involved in a variety of mitotic events including centrosome maturation, spindle assembly, chromosome segregation, and the completion of cytokinesis. Originally discovered in *Drosophila melanogaster* by Glover and coworkers, it was noted the *aur* gene encoded a 47 kD serine/threonine kinase that, when mutated, resulted in sterility (Glover *et. al.* 1995). In addition, embryos derived from *aur* mothers underwent abnormal mitosis in that there were many monopolar spindles present, thought to be a consequence of a defect in centrosome separation. In these embryos two closely spaced centrosomes were observed at the center of the monopolar spindles using Rb188, an antibody directed against centrosomes (Glover *et. al.* 1995).

The *Saccharomyces cerevisiae* homolog ipl1, for “increase in ploidy”, had been previously discovered, but was uncharacterized, in a screen looking for proteins required for proper chromosome segregation (Chan and Botstein, 1993). Identification of the *Saccharomyces pombe* homolog occurred later, during a screen for genes that suppressed the harmful effects of overexpressing *Xenopus*
laevis Aurora A in *S. pombe*, and was termed Ark1 for Aurora-related kinase 1 (Petersen *et. al.* 2001, Leverson *et. al.* 2002).

Another Aurora family member was found to be required for the completion of cytokinesis (reviewed in Crane *et. al.*, 2003, Carmena and Earnshaw, 2003). In *C. elegans* embryos depleted of an Aurora paralog, chromosomes replicated and condensed normally, but failed to be segregated due to a catastrophic failure of cytokinesis (Schumaker *et. al.* 1998). Close monitoring of cytokinesis revealed that the cytokinetic furrow ingressed in these cells, then regressed resulting in multinucleate cells.

Thus, it appears that yeast contain one Aurora gene, Ipl1. *C. elegans* and *X. laevis* contain 2 paralogs, and humans express three Aurora family members. Though the nomenclature became unwieldy, the names of the Aurora family members was simplified to Aurora –A, -B, and –C. This classification system is based largely on protein localization. Aurora family members localize to the centrosomes throughout the cell cycle and are responsible for centrosome maturation, formation of a proper bipolar spindle, and microtubule aster stability. Aurora B is a member of the chromosomal passenger complex that includes INCENP and survivin, and it localizes to chromatin prior to anaphase of mitosis, whereupon it is found at the spindle midzone (Adams *et. al.* 2001). Aurora B is primarily responsible for proper chromosome segregation and completion of cytokinesis. Less is known about the third family member, Aurora C. It appears to be expressed strongly in the testis, and localizes to the centrosomes in late mitosis, anaphase to telophase (reviewed in Carmena and Earnshaw, 2003).
Aurora A and cancer

A link between Aurora A function and cancer has been well established. Aurora A is found at the gene locus 20q13.2, which is frequently amplified in a variety of cancer types (Bar-Shira et al. 2002). Overexpression of Aurora A in NIH3T3 cells results in transformation, as these cells become tumor-forming in nude mice and form colonies when cultured in soft agar (Bischoff et al. 1998, Littlepage et al. 2002, Wang et al. 2006, Zhou et al. 1998). In addition to being overexpressed in many cancer types, including breast, colorectal, and gastric cancers, certain polymorphisms found in Aurora A are predictive for cancer and poor prognosis (Andrews, 2005, Crane et al. 2004, Marumoto et al. 2005, Meraldi et al. 2004).

How disruption of Aurora A function results in transformation and tumorgenesis is less well understood. It is known that Aurora A interacts with the tumor suppressor p53. Overexpressing Aurora A in HeLa cells, which are defective for p53 function, results in aneuploidy, while addition of p53 to these cells suppresses this phenotype (Littlepage et al. 2002, Meraldi et al. 2002). It has also been shown that human p53 and Aurora A interact by both two hybrid and in vitro binding experiments (Chen et al. 2002). p53 appears to bind the N-terminus of Aurora A in in vitro experiments, and inhibits its kinase activity. Overexpression of Aurora A may be able to overcome endogenous p53 activity, leading to a disruption of normal cell cycle progression, aneuploidy, and cancer.
Another hallmark of Aurora A overexpression is the presence of supernumerary centrosomes. Just as it is important for there to be two and only two copies of each chromosome as cells enter mitosis, it is just as important for there to be only two centrosomes present. The presence of only one centrosome results in monopolar spindles that are unable to segregate chromosomes, while the presence of more than one centrosome results in multipolar spindles that are unable to accurately divide the sister chromatids between the daughter cell. It has been postulated that the amplification and subsequent overexpression of Aurora A may overcome the spindle checkpoint, inhibit cytokinesis, and result in the formation of tetraploid cells (Meraldi et. al., 2002, Anand et. al. 2003). This effect seems particularly strong in cells lacking functional p53. Further work is required to determine the mechanism of Aurora A induced tumorigenesis.

Activation of Aurora A

In order to be effective as a mitotic kinase, Aurora A must be activated and deactivated in a timely fashion. A great deal of work has been performed investigating the mechanism of Aurora A activation, and it has been found to involve a number of proteins working in concert to promote timely Aurora A function.

Three phosphorylation sites on Aurora A have been described (Littlepage et. al. 2002). One of these threonine residues (Thr295 in X laevis, Thr288 in humans, Thr260 in yeast) is contained within the activation T-loop of Aurora A
and is essential for kinase activity (Walter et al. 2000). This residue is contained within the consensus sequence for both PKA, and also consensus sequence worked out for the yeast Aurora homolog Ipl1 (Cheeseman et al. 2002). Walter and co-workers have demonstrated in vitro that PKA is able to phosphorylate and activate Aurora A (Walter et al. 2000). The activating threonine is also found within the Ipl1 consensus site, ({KR} X {TS} {ILV}) and has been shown to be an autophosphorylation site in S. cerevisiae (Cheeseman et al. 2002). Currently, autophosphorylation appears to be the primary mechanism of Aurora A activation. This being the case, keeping the kinase in an inactive state until mitosis relies on a slightly complicated mechanism. To date, the best-studied activator of Aurora A is TPX2.

During interphase, the phosphatase PP1 associates with Aurora A and is able to keep the activating phosphorylation from being maintained. In addition, TPX2 is kept sequestered from Aurora A through interaction with an importin α/β complex. During mitosis, the presence of RanGTP frees TPX2 from the inhibitory complex, allowing TPX2 to interact with Aurora A kinase. TPX2 then antagonizes PP1, causing a conformational change that keeps the threonine required for activation in a position that is inaccessible to the phosphatase (Bayliss et al. 2003, Eyers et al. 2003). This coordination between Aurora A activation and the presence of RanGTP also plays a role in the establishment of a proper bipolar spindle. In addition, recent studies in C. elegans have identified a homolog of Tpx2, named TPXL-1, which shares sequence homology with Tpx2 only within the N-terminus of the protein (Ozlu et al. 2005). Interestingly, TPXL-
1 was shown to be responsible for only a subset of the Aurora A functions. Depletion of TPX1 by RNAi resulted in defects in spindle assembly and aster formation, while centrosome maturation (as judged by recruitment of centrosomal \( \gamma \)-tubulin), cell polarity, and chromosome segregation (as judged by spindle elongation), appeared unaffected (Ozlu et al. 2005).

Additional activators of Aurora A have been found in various systems, including Ajuba and Bora. Ajuba was found to interact with Aurora A in HeLa cells and serves a role in the proper timing of mitotic entry (Hirota et al., 2003). Bora was discovered in Drosophila, and mutations result in centrosome maturation defects and difficulties in asymmetric cell division (Hutterer et al. 2006). However, whether the activation pathways for these proteins is conserved is debatable, as no Ajuba homologs have been found in lower organisms such as nematodes, and the Bora homolog is not required for embryonic viability in C. elegans.

**Aurora A and centrosome maturation**

In order to fulfill their roles in bipolar spindle assembly, centrosomes increase in size and microtubule nucleating capacity several fold leading up to mitosis. Mitotic centrosomes isolated from mitotic Chinese hamster ovary (CHO) cells were larger and able to nucleate far more microtubules in an *in vitro* nucleation assay than their interphase counterparts (Kuriyama and Borisy, 1981). In addition, the increase in centrosomal size has been documented by tagging
the pericentriolar material (PCM) component $\gamma$-tubulin with GFP in PtKG cells and monitoring centrosomal accumulation (Khodjakov and Rieder, 1999). Other PCM components such as centrosomin (CNN) have been shown to accumulate at the centrosome during mitosis as well (Megraw et al. 1999).

Aurora A appears to be responsible for the accumulation of PCM components at the centrosome. Depletions of Aurora A result in a diminished accumulation of $\gamma$-tubulin and centrosomin at the centrosome during mitosis, suggesting a role in centrosome maturation. In embryos depleted of AIR-1, the C. elegans Aurora A homolog, the amount of $\gamma$-tubulin present at centrosomes is decreased 7-8 fold compared to control embryos. The amount of $\alpha$-tubulin is decreased 3-fold as well (Hannak et al. 2001). How depletion of AIR-1 results in this phenotype is currently not fully understood.

In Drosophila cells, it is known that recruitment of centrosomin, a protein required for $\gamma$-tubulin accumulation at the centrosome, is dependent upon interaction with Aurora A (Terada et al. 2003). Recent work has identified an activator of Aurora A, called Bora, which is required for proper centrosome maturation (Hutterer et al. 2006). However, although this protein is conserved in other organisms, depletion of the C. elegans homolog by RNAi does not appear to result in mitotic phenotypes (Kamath et al. 2005, Sonnichsen et al 2005).

How Aurora A phosphorylation results in mature centrosomes is currently under investigation. Inroads are being made into the mechanism however, as downstream Aurora targets with possible functions in centrosome maturation are being identified. One such protein is Lats2, a serine/threonine kinase that is a
phosphorylation target of Aurora A both in vitro and in vivo (Toji et. al. 2004). Phosphorylation by Aurora A is required to target Lats2 to the centrosome during mitosis. Further, depletion of Lats2 in HeLa cells resulted in a diminished accumulation of \( \gamma \)-tubulin at the centrosomes (Abe et. al. 2006). Lats2 was also found to interact with the Aurora A activator Ajuba by yeast two-hybrid, indicating that there may be an additional level of regulation remaining to be worked out.

Another protein that requires phosphorylation by Aurora A for its centrosomal localization is NDEL1. A mutation that mimics the phosphorylated form of NDEL1 is able to rescue several of the phenotypes associated with Aurora A depletion in HeLa cells, including \( \gamma \)-tubulin recruitment, delays in mitotic entry, and localization of TACC3 (Mori et. al. 2003). It is becoming increasingly clear that Aurora A does have a function in the accumulation of pericentriolar material during mitosis, although the mechanism of this accumulation remains elusive.

**Aurora A and microtubule nucleation**

Cells that have mutated or depleted their stores of Aurora A show far fewer microtubules nucleated from their centrosomes during mitosis. In C. elegans embryos, depletion of AIR-1 by RNAi results in a 3-fold decrease in the amount of \( \alpha \)-tubulin found at the centrosome (Hannak et. al. 2001). This phenotype may be the result of an indirect effect, as Aurora A depletion also inhibits centrosome maturation, lowering the concentration of microtubule nucleating factors such as \( \gamma \)-tubulin as much as 8-fold (Hannak et. al. 2001).
However, several microtubule-associated proteins (MAPs) are targets of Aurora A, and may suggest a more direct role for the kinase in microtubule nucleation.

Aurora A phosphorylates a family of highly coiled-coil proteins called TACCs (for transforming acidic coiled-coil containing), at a highly conserved consensus sequence contained within the protein (Barros et al. 2005, Kinoshita et al. 2005, Peset et al. 2005). This phosphorylation by Aurora A, at least in the case of the Drosophila TACC family member, of D-TACC is required for its centrosomal localization (Giet et al. 2002). TACC family proteins then interact with the XMAP215 family of proteins at the centrosome, which are loaded onto microtubules and aid in stabilization (Lee et al. 2001, Gergely et al. 2003, Kinoshita et al. 2005). TACC proteins and XMAP215 act to stabilize microtubules at the centrosome by antagonizing the microtubule-destabilizing action of the kinesin MCAK (Kinoshita et al., 2001, Kinoshita et al. 2005). This mechanism of microtubule stabilization has not been fully established, but does provide an alternative to microtubule nucleation defects simply being the result of compromised centrosome maturation in Aurora A deficient cells.

Aurora A in spindle assembly and centrosome separation

When originally characterized in Drosophila embryos, mutation of Aurora A resulted in the appearance of monopolar spindles (Glover et al. 1995). Depletions of Aurora A kinase in C. elegans embryos and Xenopus egg extracts have confirmed this phenotype (Hannak et al. 2001, Liu and Ruderman 2006, Roghi et al. 1998). Additionally, treatment of HeLa cells with siRNA resulted in
monopolar spindles exhibiting two closely spaced centrosomes (Marumoto et. al. 2003). These results suggest a role for Aurora A in both bipolar spindle assembly and centrosome separation. It has been shown that Aurora A is not required for centrosome duplication, just separation (Meraldi et. al. 2002).

Two non-exclusive models have been proposed for the assembly of a bipolar spindle. One, the search and capture model, postulates that microtubules nucleated at opposite poles interact with kinetochores, become stabilized, and form the basic bipolar spindle structure. The other model proposes that the RanGTP gradient established at chromosomes through the action of RanGEFs like RCC-1 results in local nucleation of microtubules that grow out and are resolved into poles (Tsai et. al. 2003). As noted above, RanGTP acts to release the Aurora A activating protein Tpx2 from the importin α/β complex, allowing for the stabilization of microtubules (reviewed in Carmena and Earnshaw, 2003). This is one mechanism through which Aurora A acts to establish a bipolar spindle.

Recent work has elucidated a role for Aurora A kinase in bipolar spindle assembly even in the absence of centrosomes (Tsai and Zheng, 2005). Using a Xenopus extract system, they have shown that magnetic beads coated in Aurora A are able to form bipolar spindles even in the absence of chromosomes. This is not an indirect effect due to the presence of microtubule stabilizing factors. In the same system, XMAP215-coated beads are able to nucleate microtubules, but they do not resolve into bipolar spindles (Tsai and Zheng 2005).
The study of bipolar spindle assembly by and microtubule nucleation Aurora A may reveal that another phenotype associated with Aurora A depletion, centrosome separation, may be an indirect effect. Initial characterization of the Aurora A mutation phenotype noted that two centrosomes were closely spaced and monopolar spindles were present (Glover, et. al. 1995). It was proposed that Aurora A played a role in centrosome separation. Additional argument for Aurora A involvement in this process comes from the discovery that the kinesis Eg5 a substrate of the kinase (Giet et. al. 1999). It has been postulated that Eg5 can maintain centrosome separation by acting to slide antiparallel microtubules away from one another, maintaining centrosome distance. Currently, there is no evidence that the Aurora phosphorylation is required for Eg5 function.

Most of the studies showing two closely spaced centrosomes rely on a snapshot of two centrosome-staining dots contained within the center of a monopolar spindle by immunofluorescence. A better understanding of the dynamic effects of Aurora A depletion are shown by Hannak and coworkers (Hannak et. al. 2001). The C. elegans Aurora A homolog AIR-1 is depleted using RNAi, and time-lapse images are taken. In them, one can see that the centrosomes separate normally, taking up positions on opposing sides of the paternal pronucleus. Only after nuclear envelope breakdown (NEBD) do the centrosomes collapse into one another (Hannak et. al. 2001). Thus, it seems that AIR-1 is required not for centrosomal separation per se, but rather the maintenance of the separation. This phenotype could be the result of other defects that arise when AIR-1 is depleted, such as the inability to nucleate the
proper number of microtubules, or an inability to properly assemble a bipolar spindle. In conclusion, a complicating factor in understanding the functions of Aurora A within a cell is the fact that some phenotypes are likely to be caused by direct effects of the kinase, and others are likely to be indirect effects.

**A role for Aurora A in oocyte maturation**

Functions of Aurora A are not restricted to regulation of mitotic events. A seemingly independent role for the kinase has been discovered, in which phosphorylation by Aurora A influences the completion of meiotic events resulting in oocyte maturation.

In *Xenopus* development, the oocyte remains in an immature state with the cell cycle arrested at the G2/M transition in meiosis I. Stimulation with progesterone breaks the arrest, allowing for the completion of meiosis I and creating a mature oocyte that is arrested at metaphase of meiosis II until fertilization occurs. Addition of progesterone prepares the oocytes for maturation by stimulating two independent signaling cascades within the cell that converge to activate cyclin B/cdc2 (Reviewed in Stanford et. al. 2003). In one, an inhibitor of cyclin B/cdc2 (Myt1) is inhibited, in the other, cdc25B, an activator of cyclin B/cdc2 is activated (reviewed in Stanford et. al. 2003). The former pathway is dependent upon Aurora A kinase activity.

Progesterone stimulated oocyte maturation is translationally regulated. Prior to progesterone stimulation, the mRNA of an upstream protein in the
signaling cascade leading to Myt1 inhibition, Mos, is maintained as a non-polyadenylated state (Andresson and Ruderman, 1998). Mos is kept from being translated by a cytoplasmic polyadenylation element binding protein (CPEB) that binds the cytoplasmic polyadenylation element (CPE) in the 3’ UTR of the mRNA. Aurora A phosphorylates CPEB on Ser174, converting it from a translational repressor to a translational activator (Mendez et. al. 2000). Overexpression of Aurora A in this system shortens the time before newly synthesized Mos protein is detected, and lowers the concentration of progesterone required to induce maturation of the oocytes. Even in the absence of progesterone, injection of a constitutively active Aurora A results in CPEB phosphorylation, Mos translation, and the downstream activation of cdc2/cyclin B (Ma et. al. 2003).

Aurora A involvement in cell polarity

Because many of the phenotypes associated with depletion and mutation of Aurora A kinase involve mis-segregation of chromatin and abnormal bipolar spindle formation, Schumaker and co-workers investigated whether embryonic cells were able to maintain their identities in the absence of functional Aurora A. To do this, they investigated the localization of P-granules and PIE-1 proteins in the early C. elegans embryonic divisions (Schumaker et. al. 1998).

P-granules are particles that contain RNA and RNA associated-proteins. In wild-type embryos, they localize to the posterior half of the embryo in the first
C. elegans cell division, and remain posteriorized each subsequent division, This allows for the maintenance of germ-line identity. In embryos depleted of Aurora A, P-granules were found in both throughout the embryo. In addition, it was found that another germ-line factor, PIE-1 was not localized properly in embryos depleted of Aurora A kinase. In wild-type embryos, PIE-1 localizes to the posterior centrosome of the embryo, which is destined to become the germ-line. During mitosis, PIE-1 present at the centrosome of the somatic daughter cell is lost. In air-1(RNAi) embryos, PIE-1 is found in the germ cells, although the centrosome localization is lost in about half the embryos (Schumaker et. al. 1998). This defect in maintenance of polarity cues would likely result in severe developmental defects, and embryonic lethality.

Pharmacological agents directed against Aurora A

Aurora A kinase has been shown to be overexpressed in breast, colorectal, and gastric cancers, among others. In addition, it has been demonstrated that forced overexpression of Aurora A in NIH3T3 and Rat1 cells results in transformation, allowing them to form tumors in nude mice as well as grow in soft agar (Bischoff et. al. 1998). This close association with cancer and tumorigenesis has led to a concerted effort to discover small molecule inhibitors that disrupt Aurora A function. Several studies have been published detailing the effects and efficacies of these molecules.
One hurdle in the development of small molecule inhibitors directed against Aurora A is specificity for the kinase. Early attempts focused on molecules such as hesperadin and the small molecule ZM447439, which mimicked Aurora depletion phenotypes in mammalian cells (Ditchfield et. al. 2003, Hauf et. al. 2003). Structural studies performed on Aurora A have solved the catalytic region of the kinase (Cheetham et. al. 2002, Nowakowski, et. al. 2002). However, within this region a strong sequence similarity between both Aurora A and B exist, making targeting difficult. To date, many of the molecules directed against the Aurora kinases have been unable to preferentially inhibit Aurora A over Aurora B, with the exception of VX-680 (now MK-0457) (Harrington et. al. 2004). Study of this drug is currently in its infancy, but it has been shown be effective against acute myelogenous leukaemia (AML). Treatment with VX-680 inhibits colony formation in primary leukemia cells, as well as prevents tumor formation in nude mice challenged with primary cells (Doggrell, 2004), although the mechanism of this inhibition is not fully understood. Aurora A remains a hot target for molecular therapeutics. In the coming years several more inhibitors are likely to hit the market, shedding more light on the function of Aurora A within cells.

**Aurora A and mitotic entry**

Proper progression through the cell cycle requires the coordinated effort of a number of proteins, including cyclin B, Cdk1, and polo kinase, among others. Increasing evidence points toward a role for Aurora A kinase in the G2/M
transition. Many cell cycle regulators localize to the centrosome, and it has been
shown that pools of activated cyclin B/Cdk1 appear at the centrosome prior to
later mitotic events (DeSouza et. al. 2000, Jackman et. al. 2003). Thus, it is
possible that its role in assembling a proper, mature centrosome may result in
the defect in cell cycle progression. As Aurora A has a demonstrated role in
centrosome maturation, this mitotic entry phenotype may be an indirect effect.
However, in the absence of centrosomes, the cells eventually undergo mitosis,
arguing against a centrosomal requirement for cell cycle progression. Several
studies point toward a more direct role for Aurora A in this process.

Recently, Liu and Ruderman have shown that in cycling Xenopus extracts,
both depletion of endogenous Aurora A and addition of a kinase dead version of
the protein result in a delay, but not arrest of cell cycle progression. This effect
was independent of the presence of centrosomes and chromosomes, arguing for
a direct role for Aurora A in this process (Liu and Ruderman, 2006). In HeLa
cells, depletion of Aurora A by siRNA resulted in a lengthy delay in the onset of
mitosis in synchronized cells (Hirota et. al. 2003). In this system, Aurora A was
responsible for the centrosomal targeting of cyclin B/Cdk1. Thus, it appears that
both Aurora A and centrosome function play roles in proper mitotic entry.

In C. elegans, it was noted that timing of nuclear envelope breakdown,
(NEBD), appeared slightly delayed with respect to the completion of
chromosome condensation in embryos depleted of AIR-1 (Hannak et. al. 2001).
This observation was the basis for the work contained in chapters 2 and 3 of this
dissertation.
Overview of centrosome structure

The centrosome consists of a pair of orthogonally oriented centrioles surrounded by an electro-dense cloud of protein called the pericentriolar material (reviewed in Blagden and Glover, 2003). There are two centrosomes present in each cell during mitosis that serve as the primary microtubule nucleating and organizing centers. During mitosis, centrosomes increase in size due to accumulation of PCM during a process called centrosome maturation. This functions to increase the microtubule nucleating capacity of centrosomes, which is necessary for proper bipolar spindle formation (Kuriyama and Borisy, 1981).

Centrioles are composed of a symmetrical array of nine singlet microtubules in C. elegans (Delattre and Gonczy, 2004). Within each centrosome there are two centrioles, one mother and one daughter oriented orthogonally at the center of the pericentriolar material. It has been established that centrioles determine centrosome number (Sluder and Rieder, 1985). In addition, centriolar proteins are responsible for the regulation of centrosome size. In C. elegans, partial depletions of many centriole-associated proteins result in asymmetric recruitment of pericentriolar material around the centriole (Kirkham et al., 2003). This assembly defect occurs at newly formed centrioles, sperm derived centrioles are able to recruit normal levels of PCM. Thus, it is the function of the centrioles to organize and specify the location of the PCM. How the centrioles are able to organize a structure ten times their size is currently not understood.
In 1998, the existence of a centrosomal scaffolding structure termed the centromatrix was hypothesized (Schnackenberg et al. 1999). The centromatrix was observed after extracting mature, isolated centrosomes with potassium iodide and examining the insoluble remains by 3D EM tomography (Schnackenberg et al., 1998). These extracted centrosomes were unable to nucleate microtubules; however incubating the scaffolds with mitotic extracts or column fractions containing the $\gamma$-tubulin Ring Complex ($\gamma$-TuRC) restored their MT nucleating ability (Schnackenberg et al., 1998). This result indicated that an insoluble scaffold that can bind to the $\gamma$-TuRC forms around the centrioles. Interestingly, upon potassium iodide extraction, the centrioles were lost, and did not appear to reform after extract incubation, suggesting that after its assembly the PCM retains its structural integrity and ability to nucleate microtubules in the absence of centrioles (Schnackenberg et al., 1998).

**C. elegans as a model system**

*Caenorhabditis elegans* is a small soil-dwelling nematode that is an ideal system in which to investigate cell division, development, and many other cellular and organismal processes. Worms are easy to maintain and have a short life cycle in which the worm grows from an embryo into an adult in 4 days. Second, robust RNA interference (RNAi) machinery coupled with an assembly-line like gonad structure allows for reproducibly high levels of protein depletion (>95%) by RNAi (Montgomery and Fire, 1998). Because the mRNA is targeted in the
gonad, and existing protein is packaged into embryos and expelled through egg laying, protein depletion by RNAi is independent of protein turnover rates, and embryos can be acquired that are almost completely depleted of a target protein. Third, fertilization in the gonad triggers the first mitotic division. Therefore, the roles of proteins essential for mitotic divisions, such as most centrosomal components, can be studied in the first mitosis attempted by the depleted cell (fertilized zygote). These early embryonic cell divisions in *C. elegans* are highly stereotypical, allowing for quantitative characterization and comparison of mutant and RNAi phenotypes.

*C. elegans* embryos are amenable to live imaging due to their accessibility and transparency; nuclei, centrosomes, and other sub-cellular structures are clearly visible by transmitted light microscopy. Transgenic *C. elegans* strains can be created by ballistic bombardment, allowing for the study of embryos expressing fluorescently tagged proteins that can be used to monitor the behavior of any specific protein during mitosis (Wilm *et al.*, 1999). Furthermore, large-scale biochemical analysis can be performed and are aided by the existence of a comprehensive library of *C. elegans* peptides against which mass spectrometry results are checked.

**References**


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Chapter 2

Molecular Analysis of Mitotic Chromosome Condensation Using a Quantitative Time-Resolved Fluorescence Microscopy Assay

Abstract

Chromosomes condense during mitotic entry to facilitate their segregation. Condensation is typically assayed in fixed preparations, limiting analysis of contributing factors. Here, we describe a quantitative method to monitor condensation kinetics in living cells expressing green fluorescent protein (GFP) fused to a core histone. We demonstrate the utility of this method by using it to analyze the molecular requirements for the condensation of holocentric chromosomes during the first division of the C. elegans embryo. In control embryos, the fluorescence intensity distribution for nuclear GFP:histone changes during two distinct time intervals separated by a plateau phase. During the first interval, primary condensation converts diffuse chromatin into discrete linear chromosomes. After the plateau, secondary condensation compacts the curvilinear chromosomes to form shorter bar-shaped structures. We quantitatively compare the consequences on this characteristic profile of depleting the condensin complex, the mitosis-specific histone H3 kinase Aurora
B, the centromeric histone CENP-A, and CENP-C, a conserved protein required for kinetochore assembly. Both condensin and CENP-A play critical but distinct roles in primary condensation. In contrast, depletion of CENP-C slows but does not prevent primary condensation. Finally, Aurora B inhibition has no effect on primary condensation, but slightly delays secondary condensation. These results provide new insights into the process of condensation, help resolve apparent contradictions from prior studies, and indicate that CENP-A chromatin has an intrinsic role in the condensation of holocentric chromosomes that is independent of its requirement for kinetochore assembly.

**Introduction**

As cells enter mitosis, replicated chromosomes, consisting of two identical DNA molecules called sister chromatids, are compacted to facilitate segregation by the mitotic spindle. The packaging of each chromosome into a folded rod-shaped structure, often thousands of times shorter than the DNA molecule itself, is called condensation (Gassman et. al. 2004, Hirano 2006, Huang et. al. 2005, Nasmyth and Haering, 2005). Condensation not only resolves different chromosomes, but the two sister chromatids, which form morphologically distinct rods that remain connected along one side by sister chromatid cohesion.

Two related protein complexes, condensins I and II, together with the topoisomerase family of DNA decatenating enzymes are critical for proper condensation (Gassman et. al. 2004, Hirano 2006, Huang et. al. 2005, Nasmyth and Haering, 2005). Inhibition of condensin blocks the ability of mitotic Xenopus
extracts to reorganize sperm chromatin into linear rod-shaped chromosomes (Hirano et. al. 1997, Hirano and Mitchison, 1994), suggesting an essential role in chromosome compaction and resolution. However, inhibiting condensin in Drosophila (Coelho et. al. 2003, Steffensen et. al. 2001), C. elegans (Hagstrom et. al. 2002), and vertebrate cells (Hirota et. al. 204, Hudson et. al. 2003) delays, but does not prevent, compaction. The compacted chromosomes that form after condensin inhibition are sensitive to hypotonic fixation conditions and exhibit defects during alignment and segregation that suggest compromised structural integrity (Gassman et. al. 2004).

Concurrent with condensation, kinetochores assemble on centromeric chromatin to provide a chromosomal attachment site for spindle microtubules. Interactions between kinetochores and spindle microtubules play a central role in chromosome alignment and segregation (Kline-Smith et. al. 2005, Maiato et. al. 2004). Two distinct chromosome architectures are prevalent among metazoans: monocentric, in which kinetochore assembly is restricted to a localized region of each chromatid, and holocentric, in which diffuse kinetochores assemble along the entire chromatid length (Maddox et. al. 2004). Both monocentric and holocentric chromosomes assemble kinetochores on specialized chromatin containing the histone H3 variant CENP-A (Blower and Karpen, 2001, Buchwitz et. al. 1999, Howman et. al. 2000, Oegema et. al. 2001). Following condensation in vertebrates (monocentric), CENP-A containing chromatin is positioned on the poleward surface of each sister chromatid in a localized chromosomal region called the primary constriction. Holocentric chromosomes lack a primary
constriction. Instead, a stripe of centromeric chromatin runs along the entire length of each mitotic chromatid.

Analysis of mitotic chromosome remodeling has been limited, with few exceptions (Kimura and Cook, 2001, Manders et al. 1999, Swedlow et al. 1993, by the methods available to monitor this dynamic process. Here, we describe a simple but powerful quantitative analysis method that can be used to monitor the kinetics of chromosome condensation in timelapse sequences of cells expressing GFP fused to a core histone. We demonstrate the utility of the method by combining it with RNA-interference mediated depletion in the C. elegans embryo to quantitatively analyze the molecular requirements for the condensation of holocentric chromosomes.

Results

A Method to Quantitatively Monitor Chromosome Condensation

Our goal was to develop an analysis method to extract quantitative kinetic information on the progress of chromosome condensation from timelapse sequences. We decided to use the C. elegans embryo as a model system because of its stereotypical first mitotic division. Analyzing the consequences of depleting essential chromosome components is also straight-forward because RNA interference (RNAi) can be used to generate oocytes reproducibly >95% depleted of targeted proteins (Oegema and Hyman, wormbook). During the first division following fertilization, mitotic prophase initiates when the oocyte and sperm-derived pronuclei are on opposite sides of the embryo, and continues as
the pronuclei migrate towards each other. After the pronuclei meet, the nuclear envelopes become permeable (Nuclear Envelope Breakdown, NEBD) and the condensed chromosomes interact with spindle microtubules to align and segregate. These dynamic events can be monitored in embryos co-expressing GFP:histone H2b and GFP:g-tubulin (Oegema et. al. 2001). The latter fusion protein marks the centrosomes, has no nuclear signal, and serves as an independent timer of cell cycle progression.

We imaged the GFP:histone by using spinning disc confocal optics to collect time-lapse z-series containing the nucleus. Maximum intensity projections were generated for each timepoint, and the largest square region that fit within the sperm pronucleus was cut out for further analysis (Fig. 1A). Condensation was analyzed in the sperm pronucleus to facilitate comparison between control embryos and embryos depleted of proteins by RNAi. After fertilization, the sperm chromatin decondenses, is replicated, and then condenses during prophase of the first mitotic division. In contrast, the chromosomes in the oocyte pronucleus complete two rounds of meiotic segregation prior to entering the S-phase that precedes the first mitotic division. Failure of meiotic segregation when proteins required for chromosome structure are depleted can result in defects in the oocyte chromatin that preclude analysis of their mitotic condensation. The chromatin in the sperm-derived pronucleus does not inherit meiotic defects because the meiotic divisions that produce the sperm occur at a developmental stage prior to initiation of the RNAi. The images of the chromatin in the sperm pronucleus were individually scaled, setting the minimum intensity to 0 and the
maximum to 255 (Fig. 1B). Individual scaling ensures that the shape of the fluorescence intensity distribution at each time point is independent of fluctuations due to photobleaching or changes in illumination intensity.
Figure 2.1. A quantitative method to monitor chromosome condensation in living cells

(A) Embryos coexpressing GFP-histone and GFP-α-tubulin were imaged by using spinning disk confocal optics. A five-plane z-series was collected every 10 s, and a maximum intensity projection was generated for each time point. The largest square region that fit within the sperm pronucleus was cut out for further analysis. The change in centrosomal α-tubulin (arrowheads) serves as a chromatin-independent marker of cell cycle progression. (B) Representative images of a single nucleus after scaling (setting the minimum pixel intensity in each image to 0 and the maximum to 255). Times are with respect to NEBD. The time intervals corresponding to primary and secondary condensation are labeled (the pause is indicated by a dashed line). (Scale bar: 5 μm.) (C) Examples of the fluorescence intensity distribution for GFP:histone in individual nuclei at different time points. Vertical bars mark the thresholds used to measure the progressive change in the shape of the fluorescence intensity distribution that accompanies condensation (cyan, 80% of the image maximum; pink, 65%; green, 50%; red, 35%; blue, 20%). (D) Scaled images of nuclear GFP-histone (left column) were partitioned by using five different thresholds. In each row, the same image is repeated with the pixels below the indicated threshold in color. (Scale bar: 5 μm.) (E) Kinetic plot of the percentage of pixels below each threshold (the condensation parameter) as a function of time. The average values of the condensation parameters for each threshold were measured from 12 sequences time-aligned with respect to NEBD (error bars  SE). The intervals corresponding to primary (between 450 and 325 s; dark gray) and secondary (between 200 and 0 s; light gray) condensation are indicated.
Chromosome condensation is accompanied by a progressive change in the shape of the fluorescence intensity distribution of the nuclear GFP:histone signal (Fig. 1C). Prior to condensation, the signal is relatively homogeneous and, after scaling, the majority of pixels are distributed around a central peak at ~65% of the image maximum (scaled intensity = 166; Fig. 1C, -450s panel). As the chromatin condenses, the GFP:histone fluorescence concentrates in a smaller area of the image at the expense of signal elsewhere in the nucleus, progressively increasing the percentage of pixels further away from the image maximum (Fig. 1C). To quantify this shift in the shape of the fluorescence intensity distribution, we monitored the flux of pixels across a grating of thresholds set at 80%, 65%, 50%, 35% and 20% of the maximum intensity of the image (scaled intensities = 204, 166, 127, 89 and 51, respectively; Fig. 1C-E). Kinetic profiles to compare control and specifically perturbed embryos were generated by plotting the percentage of pixels below each threshold (the condensation parameter) as a function of time. Times were calculated relative to NEBD, defined as the timepoint when diffusion out of the nucleus resulted in equilibration of the free nuclear GFP:histone signal with the cytoplasm. For every condition, the condensation parameters measured from time-aligned sequences of 10-20 different embryos were averaged and plotted.

When condensation initiated, the intensity of the majority of pixels was already less than the high threshold values (80% and 65%). Consequently, the kinetic profiles for these thresholds saturated early in the timecourse (Fig. 1D,E; cyan and pink traces). In contrast, the percentage of pixels less than the 50 and
35% thresholds progressively increased throughout condensation. The percentage of pixels with intensities less than the 20% threshold was nearly constant during the early stages of condensation, but changed dramatically during the later stages of compaction. Two advantages of an intensity-distribution based method over approaches based on segmenting the nucleus to estimate chromosome volume are: (1) the ability to monitor early compaction prior to the establishment of defined chromosomes on which accurate measurements of length and diameter can be made and, (2) sensitive detection of changes late in condensation, accompanied by relatively small changes in chromosome volume, from relatively low z-resolution image stacks. The later is true because small increases in the peak chromosomal signal generate a differential that pushes the pixels in the remainder of the nuclear below progressively lower thresholds. The analysis method is simple and robust and can be used to analyze data acquired using wide-field (Fig. 3), as well as confocal optics.

**Chromosome Condensation is Temporally Biphasic in C. elegans**

Quantitative analysis revealed that condensation is temporally biphasic in control *C. elegans* embryos. Detectable changes in the shape of the fluorescence intensity distribution were confined to two distinct time intervals. Condensation was first detected ~450 seconds prior to NEBD (Fig. 1B,E). Over the subsequent 125s interval, a consistent increase in the condensation parameters was observed for thresholds between 35 and 65%. We term this
initial phase “primary condensation”. Qualitatively, primary condensation corresponds to the organization of diffuse chromatin into distinct linear chromosomes (Fig. 1B). Primary condensation was followed by a ~125 second pause during which there was no statistically significant change in the percentage of pixels falling below any threshold (Fig. 1E), indicating that the shape of the fluorescence intensity distribution for the nuclear GFP:histone signal did not change. Distinct linear chromosomes with a high degree of curvature were observed throughout this plateau (Fig. 1B). After the pause, a second shift in the shape of the intensity distribution, which we term “secondary condensation”, was detected over the 200s interval immediately preceding NEBD. Secondary condensation, which corresponds to a change from elongated, highly curved chromosomes to compact, bar-shaped chromosomes, was characterized by a consistent increase in the condensation parameters for thresholds between 20 and 50% (Fig. 1B,E). Thus, our analysis method revealed that condensation occurs in two temporally distinct phases. During a primary phase (lasting ~125s), diffuse chromatin is compacted to form distinct chromosomes. In a subsequent secondary phase (lasting ~ 200s) initiated after a pause, curved linear chromosomes are further compacted to generate bar-shaped mitotic chromosomes.

**The Condensin Subunit SMC-4 is Required for Primary Condensation**

The related condensin I & II complexes play critical roles in chromosome condensation (Hirano, 2006). The core of both condensin complexes consists of
the same two essential SMC (structural maintenance of chromosome) family ATPase subunits. In *C. elegans*, condensin II contributes to condensation, while a condensin I like complex is thought to have been adapted for sex chromosome dosage compensation (Hagstrom and Meyer, 2003). To examine the role of condensin, we depleted SMC-4, which is predicted to abolish condensin function. We used the three most informative thresholds (20, 35 and 50%) for this analysis. In SMC-4 depleted embryos, no condensation was detected coincident with the primary and plateau phases in wild-type (Fig. 2B). However, approximately 60s prior to NEBD, when secondary condensation is well underway in controls, the percentage of pixels falling below the 50 and 35% thresholds began to increase. Visual inspection revealed that this was due to a precipitous clumping of chromatin. Distinct chromosomes were not observed at any stage (Fig. 2G). Thus, consistent with prior work (Hagstrom *et. al.* 2002, Hirota *et. al.* 2004, Hudson *et. al.* 2003), our results indicate that condensin has a critical role in the timely compaction of chromatin during prophase. Although some compaction occurs in condensin depleted embryos, the chromatin aggregates into a tangled meshwork and distinct chromosomes are not observed.
Figure 2.2. Kinetic analysis of chromosome condensation in embryos depleted of conserved chromosomal proteins

Shown are plots of the average value of the condensation parameters vs. time for three thresholds (A–F) (green, 50%; red, 35%; blue, 20%) and images from representative time-lapse sequences (G). The time intervals when primary (dark gray) and secondary (light gray) condensation occurs in control embryos are marked on all graphs for reference. Condensation kinetics in control embryos (A; n 12) and embryos depleted of SMC-4 (B; n 18), HCP-6 (C; n 12), CeCENP-A (D; n 15), CeCENP-C (E; n 10), and AIR-2 (F; n 12). In E and F, control traces (solid lines) are superimposed to facilitate comparison. (Scale bar in G: 5 m.) (H) Schematic comparing the organization of holocentric chromosomes to the region of the primary constriction of monocentric chromosomes. (I) Speculative model for the formation of mitotic C. elegans chromosomes. In WT, condensin (orange circles) and chromatin containing CENP-A (green) are both required for primary condensation. We speculate that unknown compaction factor(s) (blue triangles) drive secondary condensation. In condensin-depleted embryos, primary condensation fails, but compaction into a disorganized meshwork still occurs. CENP-A-containing chromatin is present and functions to attach the disorganized chromatin meshwork to the mitotic spindle (9). In CENP-A-depleted embryos, primary condensation fails. The chromatin ultimately compacts into discrete masses because of the action of condensing and other unknown factors but is unable to assemble kinetochores that can attach to spindle microtubules (18).
The Condensation of Holocentric Chromosomes is Highly Aberrant Following Depletion of CENP-A

In the region of the primary constriction of mitotic monocentric chromosomes, histone H3 containing “inner-centromeric chromatin” is sandwiched between chromatin containing the histone H3 variant CENP-A that forms the structural base for the kinetochore. Analysis of the role of CENP-A chromatin in the assembly of mitotic chromosomes is limited by the fact that the primary constriction is only a small proportion of total chromatin. Characterizing the bulk properties of chromatin in holocentrics, where an architecture analogous to the primary constriction extends along the entire length of each chromosome (Fig. 2H), may therefore be useful to investigate the structural properties of CENP-A chromatin. Consistent with a prior qualitative observation (Chan et. al. 2004), we found that depletion of CeCENP-A dramatically altered condensation kinetics. In CeCENP-A depleted embryos, as in SMC-4 depleted embryos, no condensation was detected coincident with the primary or plateau phases in controls. However, coincident with secondary condensation in controls, the chromatin in CeCENP-A depleted embryos abruptly compacted (Fig. 2D). The condensation defect in CeCENP-A depleted embryos was kinetically distinct from that in condensin depleted embryos; condensation initiated earlier and progressed farther, reaching a wild-type extent of compaction prior to NEBD. In addition to this kinetic difference, visual inspection revealed spatially distinct chromosomes by the end of prophase, in contrast to the disorganized chromatin meshwork in condensin-depleted embryos (Fig. 2G). All CeCENP-A depleted
embryos exhibited a kinetochore-null phenotype (Oegema et al. 2001), and quantitative immunoblotting confirmed >97% depletion (data not shown), indicating that the less severe condensation defect is not due to poor protein depletion. We conclude that in C. elegans centromeric chromatin is required for the timely compaction and resolution of chromosomes in early prophase. However, the differences in compaction kinetics and final chromosome morphology between CeCENP-A and condensin-depleted embryos indicate that condensin-mediated compaction occurs in the absence of centromeric chromatin.

The Role of CENP-A Chromatin in the Condensation of Holocentric Chromosomes is Independent of its Role in Directing Kinetochore Assembly

To determine if the role of CENP-A in condensation is linked to its role in kinetochore assembly, we characterized embryos depleted of the kinetochore structural component CENP-C. Depletion of CeCENP-C blocks the recruitment of all known kinetochore components except for CeCENP-A (Oegema et al. 2001, Moore and Roth, 2001). Condensation in CeCENP-C depleted embryos was qualitatively similar to controls in that curved linear chromosomes, and subsequently shorter bar-shaped chromosomes, are formed (Fig. 2G). However, the rate of primary condensation was slowed relative to controls and condensation continued coincident with the plateau phase in wild-type (Fig. 2E). The extent of chromosome condensation in CeCENP-C depleted embryos quantitatively resembled that in comparable control embryos ~110s prior to
NEBD, roughly when the chromatin in CeCENP-A depleted embryos first begins to compact (compare Fig. 2D and E). The conditions used for this experiment result in >95% depletion of CeCENP-C (Desai et. al. 2003), and a clear kinetochore-null phenotype was evident in all embryos filmed.

The fact that depletion of CeCENP-C slows primary condensation is interesting in light of previous work demonstrating a role for CeCENP-C in sister kinetochore resolution, which normally occurs after primary condensation is complete (Moore, et. al. 2005; our unpublished data). These results suggest that either successful resolution of sister kinetochores depends on the timely completion of primary condensation, or that depletion of CeCENP-C inhibits an upstream process required for both a normal rate of primary condensation and sister kinetochore resolution. Further work will be needed to distinguish between these possibilities. More importantly, the effect of depleting CeCENP-C on chromosome condensation is clearly much less severe than depleting CeCENP-A. We therefore conclude that CeCENP-A containing chromatin has a critical role in the condensation of holocentric chromosomes that is independent of its requirement for kinetochore assembly.
**Figure 2.3. Kinetics analysis of chromosome condensation in time-lapse sequences acquired using widefield optics**

Images of embryos coexpressing GFP:histone H2B and GFP:g-tubulin were collected on a Nikon E800 upright microscope (Nikon Instruments, Melville, NY) using a 60 \* 1.4 N.A. Plan Apo objective lens (no auxiliary magnification) and an Orca ER CCD camera (Hamamatsu Photonics, Bridgewater, NJ) without binning.  

(A) Representative images of GFP:histone in a single nucleus before scaling. Times are with respect to nuclear envelope breakdown (NEBD). (Scale bar: 5 mm.) (B) Kinetic plot generated by plotting the percentage of pixels below each of the indicated thresholds (the condensation parameter) as a function of time. For each condition, the average value of the condensation parameter for each threshold measured from nine sequences time-aligned with respect to NEBD is plotted (error bars = SE). Primary condensation (dark gray), secondary condensation (light gray), and the intervening pause (white) are indicated.
HCP-6 Depletion Results in a Condensation Defect Kinetically Identical to Depletion of SMC-4

HCP-6 is the *C. elegans* homolog of CAP-D3, a non-SMC subunit of condensin II (Chan *et. al.* 2004, Hirano 2005, Stear and Roth, 2002). In contrast to the SMC subunits, SMC-4 and MIX-1 (Hagstrom *et. al.* 2002, Chan *et. al.* 2004), the accumulation of HCP-6 on metaphase chromosomes requires CENP-A (Chan *et. al.* 2004, Stear and Roth, 2002). In contrast, during meiotic prophase, when condensin activity restructures the recombined bivalent chromosomes, HCP-6 localizes to chromosomes independently of CENP-A (Chan *et. al.* 2004). Whether HCP-6 requires CENP-A to target to chromosomes during mitotic prophase is not clear. The signals obtained with antibodies to condensin subunits during prophase are weak. In addition, since it is difficult to determine whether proteins are present on chromosomes prior to their condensation to form distinct units, the condensation delay in CENP-A depleted embryos complicates analysis of HCP-6 targeting. In light of our finding that the condensation defect in SMC-4 depleted embryos is more severe than that in CENP-A depleted embryos, there are two possibilities for how HCP-6 contributes to mitotic chromosome formation: (1) HCP-6 is an integral subunit of condensin that targets to prophase chromosomes in the absence of CENP-A and contributes to their condensation, or (2) HCP-6 is a specific adaptor that targets a subset of condensin to chromosomes in a CENP-A dependent fashion during prophase, possibly accounting for the condensation defect in CENP-A depleted embryos. To distinguish between these possibilities, we analyzed HCP-6
depleted embryos to determine if condensation resembled that following depletion of SMC-4 or CeCENP-A. Kinetic analysis revealed that the condensation profile following depletion of HCP-6 is identical to that following depletion of SMC-4 (Fig. 2B,C), and more severe than that resulting from depletion of CeCENP-A. This result suggests that during mitotic prophase HCP-6 is an integral subunit of condensin that targets to chromosomes independently of CENP-A.

**Depletion of Aurora B Delays Secondary Condensation**

During mitotic entry the mitotic kinase Aurora B phosphorylates histone H3 (Hsu et. al. 2000) and promotes removal of cohesin and remodeling complexes from chromatin (Losada et. al. 2002, MacCallum et. al. 2002). However, the role of Aurora B in chromosome condensation is less clear. In budding yeast, vertebrate cells, and *Xenopus* extracts Aurora B does not have a significant role in recruiting condensin to the chromosome arms or in prophase condensation (Losada et. al. 2002, MacCallum et. al. 2002, Lavoie et. al. 2004, Ono et. al. 2004). However, in human cells, Aurora B is required to recruit condensin to the region of the primary constriction and for the proper morphology of this chromosomai region (Ono et. al. 2004). In *C. elegans*, where the entire chromosome has an architecture similar to that of the primary constriction in vertebrate cells, previous work has shown that the two SMC subunits of condensin fail to localize to metaphase chromosomes in embryos depleted of Aurora B (Hagstrom et. al. 2002, Kaitna et. al. 2002). Nevertheless, qualitative
inspection did not suggest a major condensation defect (Hagstrom et al. 2002, Oegema et al. 2001, Kaitna et al. 2002).

To better understand its role in the condensation of holocentric chromosomes, we used our assay to characterize Aurora-B depleted embryos. Consistent with prior work, primary condensation proceeded with normal kinetics (Fig. 2F). However, Aurora B depleted embryos exhibited a delay in secondary condensation. In particular, the pronounced increase in the percentage of pixels below the 20% threshold, a hallmark of secondary condensation, was delayed by about 65s relative to controls.

The relatively mild condensation defect in Aurora-B depleted embryos seemed at odds with a role for Aurora-B in targeting condensin to chromosomes. To re-examine this issue, we performed immunofluorescence using antibodies against SMC-4 in fixed embryos, and used spinning disc confocal microscopy to examine the localization of a GFP fusion with F55C5.4, the C. elegans homolog of the hCAP-G2 subunit of condensin (Ono et al. 2003), in living embryos. By both methods, condensin localized to chromosomes beginning in prophase and persisting through metaphase in control as well as Aurora-B depleted embryos (Sup. Fig. 2). We conclude that condensin targets to holocentric C. elegans chromosomes in Aurora-B depleted embryos, consistent with their relatively normal condensation.
Figure 2.4. Condensin targets to chromosomes during prophase in Aurora B-depleted embryos

(A) Control and AIR-2-depleted prophase embryos fixed and stained for DNA, the condensin subunit SMC-4, and AIR-2. AIR-2 is absent from chromosomes in the depleted embryos, but SMC-4 is detected on chromosomes. (B) Selected stills from time-lapse sequences of control and AIR-2-depleted embryos expressing a GFP fusion with the *Caenorhabditis elegans* homolog of the hCAP-G2 subunit of condensin. (Scale bars: 5 mm.)
Discussion

A Quantitative Live Imaging Assay for Chromosome Condensation

Live cell imaging, in combination with specific functional perturbations, is a powerful approach for the mechanistic dissection of cellular processes (Rieder and Khodjakov, 2003,, Tsien, 2005). However, extracting meaningful kinetic measurements remains challenging, and methods to quantify large-scale changes in cellular architecture visible in live imaging data would enhance the utility of this approach. Here, we describe a simple, robust strategy to monitor the redistribution of nuclear GFP-histone signal during chromosome condensation. This method, in which the percentage of pixels falling below a series of thresholds is quantified to monitor progressive changes in the shape of the fluorescence intensity distribution of a GFP marker, could also prove useful to analyze other dynamic processes, such as redistribution of the Golgi apparatus during the cell cycle or rearrangement of cortical components during assembly of the cytokinetic furrow.

Chromosome Condensation is Temporally Biphasic in C. elegans

Our kinetic analysis revealed that C. elegans chromosomes condense with biphasic kinetics, suggesting that compaction occurs in at least two discrete steps. Primary condensation converts diffuse chromatin into discrete linear chromosomes and secondary condensation further compacts these chromosomes to shorter bar-shaped structures. Consistent with this idea, a recent study using EM and immunofluorescence to characterize prophase in
fixed vertebrate cells reported two prominent classes of cells: (1) “middle prophase” cells containing well-defined chromosomes ~0.4-0.5 µm in diameter, and (2) “late prophase” cells containing shorter chromosomes ~0.8-1.0 µm in diameter (39). These two classes likely correspond to cells that have completed primary and secondary condensation, respectively, suggesting that biphasic kinetics will be a conserved aspect of condensation that is not limited to organisms with holocentric chromosomes.

**Condensin is Required to Form Distinct Chromosomes During the First Embryonic Division of the C. elegans Embryo**

Primary condensation fails in condensin-depleted *C. elegans* embryos and, although some late compaction occurs, discrete chromosomes of normal structure are never observed. An early role for condensin is consistent with previous studies showing that compaction is delayed in the absence of condensin (Hagstrom et al. 2002, Hirota et al. 2004, Hudson et al. 2003), but inconsistent with the proposal that condensin is only required in late prophase, when it is first observed to concentrate along the chromatid axis (Kireeva et al. 2004). The fact that individual chromosomes are not observed in depleted embryos suggests that condensin has a critical role in coupling the untangling of interphase chromatin to compaction in this system. This result is in contrast to recent studies in vertebrate cultured cells in which individual compacted chromosomes with reduced structural integrity were able to form following condensin depletion (Hirota et al. 2004, Hudson et al. 2003), but is similar to
results previously reported following depletion of condensin in Xenopus extracts (Hirano et al. 1997). One explanation for these differences could be the nature of the remodeling that takes place in distinct experimental systems. In both Xenopus extracts and in the C. elegans embryo, the ability of sperm chromatin to be remodeled into mitotic chromosomes is being assayed. In contrast, in vertebrate somatic cells, the ability of previously formed chromosomes to continue undergoing cycles of condensation and decondensation is examined. It is possible that these two processes have differing requirements for condensin function. For example, the chromosomes in vertebrate somatic cells could possess a scaffold that provides them with a structural memory to direct their compaction in the absence of condensin (Gassman et al. 2004). Such a scaffold could be lacking when chromosomes first attempt to reform from sperm chromatin.

**Centromeric Chromatin has a Critical Role in the Condensation of Holocentric Chromosomes**

The amplification of the structural role of centromeric chromatin in holocentric chromosomes allowed us to use our method to examine the role of CENP-A chromatin in condensation. Like condensin, CeCENP-A is required for primary condensation; chromatin compaction is delayed in CeCENP-A depleted embryos and well-formed linear chromosomes are not observed at any point. However, the defect resulting from CeCENP-A depletion is markedly less severe that that following depletion of condensin; compaction initiates earlier and
spatially distinct chromatin masses, although abnormal in morphology, are formed. Interestingly, depletion of CeCENP-C, which abolishes the targeting of all known kinetochore components except CeCENP-A, resulted in only minor condensation defects. The comparison between the two depletion phenotypes strongly argues that CeCENP-A containing chromatin has an intrinsic role in condensation that is independent of its role in kinetochore assembly.

How could CeCENP-A containing chromatin, which is only a small fraction of total chromatin (~5-10% based on our unpublished results), exert such a dramatic effect on primary condensation? One possibility is that centromeric chromatin locally concentrates condensin to initiate condensation. Alternatively, CENP-A containing chromatin may have an organizational role that in cooperation with condensin helps to structure the chromosome to ensure timely compaction (Fig. 2I). An enticing hypothesis is that the contribution of CENP-A containing chromatin to condensation may serve to ensure its final placement on opposing surfaces of sister chromatids, a necessary condition for attachment of sister kinetochores to opposite spindle poles and accurate segregation of the genome. Centromeric chromatin-mediated condensation may also provide structural integrity at the base of the kinetochore, allowing the efficient translation of forces generated by kinetochore-spindle interactions into chromosome movement.

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**Methods**

**Live Imaging**

Embryos were imaged using a spinning disk confocal (McBain Instruments, Los Angeles, CA) mounted on a Nikon TE2000e inverted microscope (Nikon Instruments, Melville, NY). Images were acquired using a 60x 1.4NA Plan Apo objective lens with 1.5x auxiliary magnification using an Orca ER CCD camera (Hamamatsu Photonics, Bridgewater, NJ) with 2x2 binning. Acquisition parameters, shutters and focus were controlled by MetaMorph software (Universal Imaging, Downingtown, PA). The condensation parameter was measured using 3 custom macros (available upon request).

**RNAi**
L4 hermaphrodites of the *C. elegans* strain TH32 (expressing GFP-histone H2B and GFP-g-tubulin) were injected with dsRNAs (Table 1), incubated for 48 hours at 20°C, and dissected to obtain recently fertilized depleted embryos. All depletions were confirmed by immunofluorescence and/or immunoblotting as described (26).
### Table 2.1. dsRNAs used for RNA interference

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<th>RNAi Target</th>
<th>Template used for dsRNA Production</th>
<th>Oligonucleotides used for dsRNA Production</th>
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<td>Genomic DNA</td>
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<td>Genomic DNA</td>
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</tr>
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**BOLD**: T3/T7 Promoter Sequences
References


Chapter 3

A microtubule-independent role for Aurora A and centrosomes in nuclear envelope breakdown

Summary

Aurora A kinase localizes to centrosomes and is required for centrosome maturation and spindle assembly. Here, we describe a microtubule-independent role for Aurora A and centrosomes in nuclear envelope breakdown (NEBD) during the first mitotic division of the *C. elegans* embryo. Aurora A depletion does not alter the onset or kinetics of chromosome condensation, but dramatically lengthens the interval between the completion of condensation and NEBD. Inhibiting centrosome assembly by other means also lengthens this interval, albeit to a lesser extent than Aurora A depletion. By contrast, centrosomally nucleated microtubules and the nuclear envelope-associated motor dynein are not required for timely NEBD. These results indicate that mitotic centrosomes generate a diffusible factor, which we propose is activated Aurora A, that promotes NEBD. A positive feedback loop, in which an Aurora A-dependent increase in centrosome size promotes Aurora A activation, may temporally couple centrosome maturation to NEBD during mitotic entry.
Introduction

Aurora A is a mitotic kinase that choreographs events during mitotic entry. Interest in Aurora A has been stimulated by its connection to tumorigenesis. Aurora A resides in a genomic region often amplified in tumors (Bar-Shira et al., 2002) and its overexpression can transform cells in culture and in vivo (Bischoff et al., 1998; Wang et al., 2006; Zhou et al., 1998). Aurora A is overexpressed in a high proportion of breast, colorectal and gastric cancers and a specific allele of Aurora A, F31I, has been linked to increased cancer susceptibility in humans (Andrews, 2005; Crane et al., 2004; Marumoto et al., 2005; Meraldi et al., 2004).

Several demonstrated functions of Aurora A are connected to centrosomes (Crane et al., 2004; Ducat and Zheng, 2004; Dutertre et al., 2002; Marumoto et al., 2005). Centrosomes consist of a pair of centrioles surrounded by pericentriolar material that promotes microtubule assembly. During cell division, centrosomal microtubule asters contribute to the formation and positioning of the mitotic spindle. In preparation for these functions, centrosomes “mature” during mitotic entry, recruiting additional pericentriolar material to increase ~5-fold in size and nucleating capacity (Palazzo et al., 2000). Aurora A localizes to the pericentriolar material and is required for maturation (Berdnik and Knoblich, 2002; Blagden and Glover, 2003; Brittle and Ohkura, 2005; Hannak et al., 2001). Centrosomal Aurora A is in dynamic equilibrium with a cytoplasmic pool, turning over rapidly (half-life of ~3s in human cells; Stenoien et al., 2003). This rapid turnover indicates that Aurora A has a signaling rather than structural
role in centrosome assembly, and that events at centrosomes have the potential to influence the state of the cytoplasmic pool of Aurora A.

In addition to centrosome maturation, Aurora A has been implicated in regulating cell cycle progression. In cycling *Xenopus* extracts, depletion of Aurora A delays both the activation of Cdk1 and chromosome condensation (Liu and Ruderman, 2006). A delay in Cdk1 activation has also been documented following RNAi-mediated depletion of Aurora A in human cells (Hirota et al., 2003). The connection between the role of Aurora A in centrosome assembly and cell cycle progression is less clear. Although postulated to be interconnected in human cells (Hirota et al., 2003), the effect of depleting Aurora A on Cdk1 activation is independent of the presence of centrosomes in *Xenopus* extracts (Liu and Ruderman, 2006).

Subsequent to its involvement in Cdk1 activation and centrosome maturation, both of which occur prior to NEBD, Aurora A promotes spindle assembly in conjunction with its activator TPX2. TPX2 is regulated by the Ran pathway after NEBD, and inhibition of TPX2 blocks spindle assembly without apparent effects on centrosome structure or cell cycle progression (Crane et al., 2004; Ducat and Zheng, 2004; Eyers and Maller, 2003; Garrett et al., 2002; Kufer et al., 2003; Özlü et al., 2005).

Here, we capitalize on the highly stereotypical first division of the *C. elegans* embryo to explore the role of Aurora A in the coordination of mitotic events during the period leading up to NEBD. We show that following Aurora A inhibition chromosomes initiate and complete condensation with normal timing,
suggesting that Cdk1 is activated normally. However, Aurora A depleted embryos exhibit a specific delay between the completion of chromosome condensation and NEBD. Inhibition of centrosome assembly via other means also delays NEBD, but to a lesser extent than depletion of Aurora A. By contrast, inhibition of microtubule assembly or depletion of dynein does not alter NEBD timing, indicating that the role of centrosomes and Aurora A is not mediated by centrosomal microtubules. Our results demonstrate an important role for centrosomes and Aurora A in NEBD, and suggest a model in which positive feedback between increasing centrosome size and Aurora A activation temporally couples centrosome maturation to NEBD.

Results

Aurora A Is Required for Centrosome Maturation and the Proper Timing of Events During Mitotic Entry

To explore the functions of Aurora A during the period leading up to NEBD, we took advantage of the stereotypical first mitotic division of the C. elegans embryo. After fertilization, the chromosomes in the oocyte nucleus complete their meiotic segregation, generating the oocyte pronucleus and two polar bodies. Subsequently, the embryo enters its first mitotic division. During prophase, the replicated chromosomes condense as the oocyte and sperm pronuclei migrate towards each other. Concurrently, the centrosomes associated with the sperm pronucleus increase ~5-fold in size. After the pronuclei meet, the nuclear envelopes become permeable and the chromosomes interact with
spindle microtubules to align and segregate. (Oegema and Hyman, 2005; prophase events are illustrated in Fig. 1B). Nuclear envelope permeabilization can be followed by monitoring the diffusion of free GFP:histone out of the nucleus. We refer to the timepoint when the free nuclear GFP:histone fluorescence has equilibrated with the cytoplasm as NEBD (nuclear envelope breakdown). In previous work, we established RNAi conditions that reduce Aurora A protein levels by >90% and analyzed the consequences of this depletion (Hannak et al., 2001; Fig. 1A). This analysis revealed that Aurora A is required for the increase in centrosome size during mitotic entry. In addition, mitotic entry appeared to take longer in Aurora A-depleted embryos (Hannak et al., 2001; Fig. 1A), although the lack of quantifiable cell cycle reference points precluded further analysis of this defect.
Figure 3.1. Aurora A Is Required for Centrosome Maturation and for Proper Timing of Events during Mitotic Entry

(A) Selected stills from time-lapse sequences of control (top) and Aurora A depleted (air-1(RNAi); bottom) embryos expressing GFP:histone. Times are seconds after the first panel of each sequence. Schematics above each panel also illustrate the previously characterized (Hannak et al., 2001) effect of Aurora A depletion on centrosome (red) maturation. Bar, 10 µm. (B) Schematics illustrate the stages between the onset of chromosome condensation and NEBD of the first mitotic division that follows fertilization. (B-1) Outline of the image analysis method (Maddox et al., 2006) used to quantitatively monitor the kinetics of chromosome condensation. (B-2) Plot of the averaged condensation parameter versus time for control (red squares; n=12 embryos) and smc-4(RNAi) (purple triangles; n=8 embryos) embryos. The average value of the condensation parameter was calculated after aligning the sequences with respect to NEBD. Traces are displayed with the onset of condensation in controls as t=0 (arrow marks NEBD in both data sets). Error bars are SEM.
A

Control

0s 510s 600s 760s

air-1(RNAi)

0s 780s 1130s 1270s

B

1. Quantify Condensation Kinetics Relative to NEBD

2. Display Averaged Plot Aligned with Respect to Condensation Onset

Acquire and project 3D timelapse images of GFP:histone

Calculate extent of chromosome condensation in sperm pronucleus (% pixels < 50% image max)
Depletion of Aurora A Results in a Specific Delay between the Completion of Chromosome Condensation and Nuclear Envelope Breakdown

In *Xenopus* extracts, depletion of Aurora A results in parallel delays in Cdk1 activation and chromosome condensation (Liu and Ruderman, 2006), a process downstream of Cdk1 activation (Liu and Ruderman, 2006; Potapova et al., 2006; Shimada et al., 1998). To test whether the delay in mitotic entry in Aurora A depleted *C. elegans* embryos is due to a defect in Cdk1 activation, we used a recently developed image analysis method (outlined in Fig. 1B; Maddox et al., 2006) to compare chromosome condensation kinetics in control and Aurora A depleted embryos. In this method, the progressive shift of the fluorescence intensity distribution of the GFP:histone signal that accompanies condensation is monitored by measuring the percentage of pixels with intensities less than 50% of the image maximum (the condensation parameter) in individually scaled projections of 3D time-lapse sequences. The condensation parameter increases monotonically as chromosomes condense and can be used to quantitatively compare condensation kinetics between control and perturbed embryos (Fig. 1B; Maddox et al., 2006). For every tested perturbation, the average of the condensation parameter at each time point was calculated from values measured in 6-12 embryos time-aligned with respect to NEBD. To simplify presentation, the plots of the condensation parameter are displayed aligned with the onset of condensation. No increase in the condensation parameter was detected during prophase in embryos depleted of SMC-4 (Fig. 1B), one of the ATPase subunits of the condensin complex (Hagstrom et al.,
2002), and intermediate effects are clearly detected following depletions of other chromosomal proteins (Maddox et al., 2006), validating the method.

In Aurora A depleted embryos, chromosomes condensed with kinetics identical to those in control embryos, attaining a state of maximum condensation over a period of ~500s (Fig. 2A). However, there was a striking delay between the end of condensation and NEBD (Fig. 2A,B,D). To determine if the onset of condensation was delayed, we used the independent timer of pronuclear size. We filmed embryos starting at the onset of anaphase of meiosis II, and found that pronuclear diameter steadily increased ~2-fold between the end of meiosis II and NEBD in both control and Aurora A-depleted embryos. The average pronuclear diameter when Aurora A depleted embryos initiated condensation (7.0 ± 0.1 µm) was indistinguishable from controls (7.1 ± 0.1 µm), indicating that the timing of condensation onset was not altered by Aurora A depletion (Fig. 2C, D). However, in contrast to control embryos where NEBD occurred ~60s after chromosome condensation was complete, chromosomes remained in the condensed state for an additional 7 minutes before NEBD in Aurora A depleted embryos.
Figure 3.2. Depletion of Aurora A Specifically Delays Nuclear Envelope Breakdown

Aurora A depleted embryos condense chromosomes with normal kinetics but exhibit a dramatic delay between the completion of condensation and NEBD. (A) Plot of the average of the condensation parameter versus time for control (red squares; n=12) and Aurora A depleted embryos (air-1(RNAi); blue circles; n=8). The average value of the condensation parameter was calculated after aligning the sequences with respect to NEBD. Traces are displayed aligned with respect to the onset of condensation. Error bars are SEM. (B) Representative images of the sperm pronucleus in a control and an Aurora A depleted embryo. Images are at 60s intervals. The onset (arrowheads) and completion (asterisks) of condensation defined by the analysis in (A) are indicated, as is the timing of NEBD (vertical bars). Scale bar is 5 µm. (C) Plot of the average diameter of the sperm pronucleus vs. time after the onset of anaphase of meiosis II in control (red squares; n=5) and Aurora A depleted (air-1(RNAi); blue circles; n=5) embryos. The average pronuclear diameter at the onset of condensation calculated from the data in (A) is indicated for control (red arrow) and Aurora A depleted (blue arrow) embryos. (D) Schematic summarizing the timing of events during the first mitotic division in control and Aurora A-depleted embryos. Chromosomes initiate and complete condensation with identical timing relative to anaphase of meiosis II in both types of embryos. However, nuclear envelope permeabilization is dramatically delayed by Aurora A depletion.
The normal onset and kinetics of chromosome condensation indicates that Aurora A depletion does not result in a global defect in Cdk1 activation. Consistent with this conclusion, the interval between the onset of meiosis II anaphase and regression of the pseudocleavage furrow, a cortical event analogous to the relaxation of surface contractile waves that accompany Cdk1 activation in *Xenopus* embryos (Rankin and Kirschner, 1997), was also not altered by Aurora A depletion (12.9 ± 0.3 min in controls vs. 13.4 ± 0.5 min in Aurora A depleted embryos; n=9 for both). Given that wild-type embryos take only 20 min to progress from the end of meiosis II to NEBD, and an additional 4 minutes to progress from NEBD to the onset of cytokinesis, the magnitude of the NEBD delay following Aurora A depletion (~7 min) indicates that Aurora A makes a major contribution to timely NEBD.

**Depletion of Aurora A Slows the Progression of Nuclear Envelope Breakdown in Addition to Delaying its Onset**

Permeabilization of the nuclear envelope has been shown to proceed in two phases (Lenart et al., 2003; Terasaki et al., 2001). During the first phase, the peripheral components of the nuclear pores are dismantled, rendering the envelope permeable to macromolecules up to the size of the open pore, ~40 nm in diameter. In the second phase, the nuclear pores are completely removed, concomitant with the fenestration of the nuclear envelope which allows larger particles to enter the nuclear space (Lenart et al., 2003). To determine whether depletion of Aurora A slows the process of permeabilization in addition to
delaying its onset, we monitored the interval between the sequential entry into the nuclear space of two different-sized cytoplasmic markers (Fig. 3A,B). As a marker for the first phase, we used a Texas-Red labeled 70 kD dextran with a predicted hydrodynamic radius of 36 nm (Lenart et al., 2003). As a marker for the second phase, we used a GFP fusion with the heavy chain of myosin II (GFP:NMY-2; Nance et al., 2003), which is expected to have a hydrodynamic radius larger than 40 nm (Citi and Kendrick-Jones, 1987). In control embryos (n=10), GFP:NMY-2 entered the nucleus 42 ± 4 s after the 70kD dextran. In Aurora A depleted embryos (n=7), this interval was increased to 60 ± 9 s (p<0.0004), indicating that, in addition to a dramatic delay in the onset of permeabilization, depletion of Aurora A slows the progressive permeabilization of the nuclear envelope once it initiates.

Next, we examined the dynamics of nuclear pores and the nuclear lamina using strains co-expressing RFP-histone and either a GFP fusion with NUP-155, a stable component of the pore wall (Franz et al., 2005), or a YFP fusion with LMN-1, the single C. elegans B-type lamin (Liu et al., 2000; Riemer et al, 1993). This analysis revealed no significant difference between control and Aurora A-depleted embryos in the localization of NUP-155 or LMN-1 at the time when the nuclear envelope became permeable to free RFP-histone. This indicates that depletion of Aurora A coordinately delays the onset of nuclear envelope permeabilization, nuclear pore removal, and lamin disassembly by ~ 7 minutes.
Figure 3.3. Aurora A Depletion Slows the Progression of Nuclear Envelope Breakdown in Addition to Dramatically Delaying its Onset

(A) Selected stills from time-lapse sequences of nuclei in control and Aurora A depleted embryos that are expressing GFP:NMY-2 and contain Texas Red conjugated 70kD dextran introduced by injection. Images are shown at 24s intervals. Times are in seconds relative to entry of 70kD dextran into the nuclear space. (B) Bar graph of the average interval between the entry of 70kD dextran and GFP:NMY-2 into the nuclear space (indicated by the double-headed arrows in A, measured in 10 control and 7 air-1(RNAi) embryos. Error bars are the 95% confidence interval. (C,D) Selected stills from time-lapse sequences of nuclei in control and Aurora A depleted embryos co-expressing RFP:mCherry:histone and either GFP:NUP-155 (C) or YFP:LMN-1 (D). Times are in seconds relative to exit of free RFP:histone from the nucleus. Bars, 5 µm. (E) Graph plotting the average intensity of YFP:LMN-1 associated with the nuclear periphery as a function of time in seconds relative to loss of free RFP:histone from the nucleus (n=6 for each condition; error bars are the 80% confidence interval).
We next monitored the progressive loss of pores and the lamina following permeabilization onset. Consistent with the slowing of permeabilization observed in the dextran-myosin analysis (Fig. 3A), the progression of nuclear pore removal (Fig. 3C) and disassembly of the lamin meshwork (Fig. 3D,E) were also slowed by Aurora A depletion. We conclude that after permeabilization initiates, pore removal, fenestration of the envelope, and lamin disassembly all progress more slowly in Aurora A-depleted embryos than in controls.

The Aurora A Activator TPXL-1 is Not Required for Timely Nuclear Envelope Breakdown

TPX2 is a well-characterized Aurora A activator that plays an important role in spindle assembly (Crane et al., 2004; Ducat and Zheng, 2004; Eyers and Maller, 2003; Kufer et al., 2003). To determine if TPX2 contributes to Aurora A-dependent timely NEBD, we analyzed embryos depleted of the C. elegans TPX2-related protein, TPXL-1. Centrosomes mature and separate normally in TPXL-1 depleted embryos. However, after NEBD, the two centrosomal microtubule asters collapse into the chromosomes (Supplemental Fig. 1A; Özlü et al., 2005). There was no significant difference between control and TPXL-1 depleted embryos in the length of the interval between the completion of chromosome condensation and NEBD (Supplemental Figure 1). This result is consistent with the emerging picture of genetically separable functions for Aurora A before and after NEBD (Crane et al., 2004; Ducat and Zheng, 2004; Eyers and Maller, 2003; Garrett et al., 2002; Kufer et al., 2003; Özlü et al., 2005). After NEBD, Aurora A
acts in concert with TPX2-like proteins and the Ran pathway to promote spindle assembly. Prior to NEBD, a different activator(s) mediates the critical functions of Aurora A in centrosome maturation and nuclear envelope permeabilization.
Figure 3.S1. The Aurora A Activator TPXL-1 Is Not Required for Timely Nuclear Envelope Breakdown

(A) Selected stills from time-lapse sequences of control and tpnl-1(RNAi) embryos expressing GFP:histone and GFP:γ-tubulin. Times are with respect to permeabilization of the nuclear envelope to GFP:histone (NEBD). Bar, 10µm. (B) Plot comparing condensation kinetics in control (n=10; red squares) and tpnl-1(RNAi) (n=10; brown circles) embryos. The average value of the condensation parameter was calculated after aligning the sequences with respect to NEBD. Traces are displayed aligned with respect to the onset of condensation. Arrows mark the timing of NEBD for each condition. Error bars are SEM. (C) Representative images of the sperm pronucleus in a control and an TPXL-1 depleted embryo. Images are at 60s intervals. The onset (arrowheads) and completion (asterisks) of condensation defined by the analysis in (B) are indicated, as is the timing of NEBD (vertical bars). Bar, 5 µm.
Centrosomal Microtubules and Nuclear Envelope-Associated Dynein are Not Critical for the Timing of Nuclear Envelope Permeabilization

Previous work in human cells suggested that centrosomal microtubules interact with dynein on the nuclear envelope to generate tension that accelerates NEBD (Beaudouin et al., 2002; Salina et al., 2002). Because Aurora A is required for the increase in centrosome size and nucleating capacity that normally occurs prior to NEBD, such a mechanism could explain the NEBD delay in Aurora A-inhibited embryos. To investigate this possibility, we characterized embryos depleted of dynein or treated with nocodazole to depolymerize centrosomal microtubules. Depletion of dynein resulted in the expected phenotypes (Gönczy et al., 1999) — multiple oocyte pronuclei as a consequence of defects in female meiosis (Fig. 4A; arrowheads), and failure of centrosome separation and pronuclear migration. However, the interval between the completion of chromosome condensation and breakdown of the sperm pronuclear envelope was not significantly different from controls (Fig. 4B, E). Nocodazole treatment, which eliminated detectable microtubule polymers, prevented pronuclear migration, and caused the centrosomes to dissociate from the nuclei (Fig. 4A), also did not cause a significant delay (Fig. 4C, E). To more rigorously test the consequences of disrupting centrosome-nucleus attachment, we analyzed embryos depleted of ZYG-12, a hook domain-containing protein required for the association of centrosomes with the nuclear envelope (Malone et al., 2003). Prior to NEBD, the centrosomes in ZYG-12 depleted embryos are randomly positioned within the embryo (Fig. 4A). Although the interval between
the completion of chromosome condensation and NEBD that we measured in ZYG-12 depleted embryos was ~50s longer than that in controls (Fig. 4D, E), this delay is an order of magnitude less than that following depletion of Aurora A (Fig. 2A) and we cannot be certain if this difference is significant as it is close to the typical error for this measurement (between ~10-40s). We conclude that a defect in mechanical interactions between centrosomally nucleated microtubules and the nuclear envelope cannot account for the dramatic delay in nuclear envelope breakdown caused by Aurora A depletion.
Figure 3.4. Centrosomal Microtubules and Nuclear Envelope-Associated Dynein are Not Critical for the Timing of Nuclear Envelope Permeabilization

(A) Selected stills from time-lapse sequences of control, dynein depleted (dhc-1(RNAi)), nocodazole treated, and zyg-12(RNAi) embryos expressing GFP:histone and GFP:γ-tubulin. The sperm pronucleus (orange asterisks), extra maternal pronuclei resulting from failure of meiotic segregation in the DHC-1 depleted embryo (yellow arrowheads), and the centrosomes in the ZYG-12 depleted embryo (blue arrows) are indicated. Times are with respect to the exit of free GFP histone from the nucleus. Bar, 10 µm. (B-D) Plots comparing condensation kinetics in control embryos (n=10; red squares) with those in dhc-1(RNAi) (B; brown circles, n=6), nocodazole treated (C; purple circles, n=7), and zyg-12(RNAi) (D; cyan circles, n=10) embryos. Arrows mark the timing of NEBD for each condition. Error bars are SEM. (E) Representative images of the sperm pronucleus in a control, a dhc-1(RNAi), a nocodazole-treated, and a zyg-12(RNAi) embryo. Images are at 60s intervals. The onset (arrowheads) and completion (asterisks) of condensation defined by the analysis in (B-D) are indicated, as is the timing of NEBD (vertical bars). Bar, 5 µm.
Inhibiting Centrosome Assembly Delays Nuclear Envelope Breakdown, but to a Lesser Extent than Depletion of Aurora A

The NEBD delay resulting from inhibition of Aurora A, while independent of centrosomal microtubules, could be a consequence of its effect on centrosome structure. To explore this possibility, we analyzed embryos in which centrosome assembly was perturbed by depletion of SPD-2 or SPD-5. Like Aurora A, SPD-2 localizes to centrosomes and is required for their mitotic maturation. Depletion of SPD-2 results in mitotic centrosomes that are much smaller than in wild-type (Fig. 5A,B; Kemp et al., 2004; Pelletier et al., 2004). SPD-5 is the major scaffold protein for centrosome assembly in *C. elegans*. Depletion of SPD-5 completely inhibits the recruitment of pericentriolar material by the centrioles; in SPD-5 depleted embryos no foci of the centrosomal marker γ-tubulin or centrosomal microtubule asters are observed at any cell cycle stage (Fig. 5A,B; Hamill et al., 2002). Chromosomes in embryos depleted of SPD-2 (Fig. 5C, E) or SPD-5 (Fig. 5D, E) condensed with kinetics similar to controls. However, both perturbations increased the interval between the completion of chromosome condensation and NEBD by ~3 min. This delay, while significant, is less than the 7 min delay observed for Aurora A depletion under identical conditions. Simultaneous depletion of SPD-5 and Aurora A resulted in an NEBD delay identical to that in embryos depleted of Aurora A alone (Supplemental Figure 2). We conclude that centrosomes play an important role in promoting timely NEBD. This function is intrinsic to the pericentriolar material and independent of centrosomally-nucleated microtubules. In Aurora A depleted embryos, the centrosomes that
remain do not accelerate NEBD. However, cytoplasmic Aurora A can accelerate NEBD, albeit less effectively, in the absence of centrosomes.
Figure 3.5. Inhibiting Centrosome Assembly Delays Nuclear Envelope Breakdown, but to a Lesser Extent than Depletion of Aurora A

(A) Prophase control, air-1(RNAi), spd-2(RNAi) and spd-5(RNAi) embryos just prior to or after NEBD were fixed and stained for DNA and microtubules (red and green; top panels) and γ-tubulin (lower panels). Bar, 5 µm. Insets are magnified 2.5-fold. (B) Schematic illustrating the consequences of depleting each of the indicated proteins on centrosome structure. (C,D) Plots of the condensation parameter versus time comparing control (n=12) and SPD-2 depleted (C; n=10), or SPD-5 depleted (D; n=6), embryos. The timing of NEBD in control (red arrows), SPD-2 depleted (yellow arrow), and SPD-5 depleted (green arrow) embryos is indicated. For comparison, the timing of NEBD in embryos depleted of Aurora A (blue arrows; replotted from Fig. 2) is also shown. Error bars are SEM. (E) Representative images of the sperm pronucleus from a control, an air-1(RNAi), a spd-2(RNAi) and a spd-5(RNAi) embryo. Images are at 60s intervals. The onset (arrowheads) and completion (asterisks) of condensation defined by the analysis in (C, D) are indicated, as is the timing of NEBD (horizontal bars). Scale bar is 5 µm.
Centrosomes Generate a Diffusible Factor that Promotes Nuclear Envelope Permeabilization

Our results demonstrate that centrosomes promote nuclear envelope permeabilization and that it is the presence of centrosomes, and not mechanical interactions between centrosomal microtubules and motor proteins on the nuclear envelope, that is critical for timely NEBD. Cumulatively, these findings suggest that mitotic centrosomes generate a diffusible factor that promotes NEBD. If this hypothesis is correct, then increasing the distance between the centrosome and the nucleus should delay nuclear envelope permeabilization by the amount of time it takes the signal to diffuse the additional distance. We tested this idea by measuring the interval between permeabilization of the oocyte-derived and sperm-derived pronuclei in embryos that fail to undergo pronuclear migration (Fig. 6A,B). Migration of the oocyte-derived pronucleus towards the sperm-pronucleus is mediated by interactions between microtubules (nucleated by the centrosomes associated with the sperm pronucleus), and dynein (on the envelope of the oocyte-derived pronucleus). Consequently, pronuclei fail to migrate in nocodazole-treated embryos as well as in embryos in which centrosome assembly (spd-5(RNAi)) or dynein is inhibited (dhc-1(RNAi)). In nocodazole-treated embryos, the sperm pronucleus, which is immediately adjacent to the centrosomes, became permeable to GFP:histone ~80s before the oocyte pronucleus, which is on the other side of the embryo (Fig. 6A,B). A similar asynchrony in the permeabilization of the sperm and oocyte pronuclei was observed in dynein-depleted embryos. This asynchrony is absent in SPD-5
depleted embryos (which lack functional centrosomes) and in ZYG-12 depleted embryos (in which functional centrosomes are present but not in preferential proximity to either pronucleus). We conclude that increasing the distance between the centrosomes and the maternal pronucleus by preventing pronuclear migration can delay its permeabilization by as much as 80s. In the absence of centrosomes, both pronuclei breakdown synchronously about ~200s after the pronuclei in controls. Cumulatively, these data strongly argue that centrosomes generate a diffusible factor that promotes NEBD.

To determine if an ~80s delay is consistent with diffusion of a cytoplasmic signal across the embryo, we introduced a 10 kD photoactivatable dextran by gonad injection into embryos. 10 kD dextran has an effective hydrodynamic diameter of 10.6 nm (Lenart et al., 2003), similar to that of a globular protein complex of ~250 kDa. A signal produced by the sperm centrosomes in the nocodazole/dynein-inhibition experiment (Fig 6A,B) was simulated by using a pulse of UV light to photoactivate the dextran on one side of the embryo (Supplemental Fig. 3A). Diffusion of the activated dextran was monitored by plotting the equilibrium ratio (Supplemental Fig. 3B, C), which declines from 1 to 0 as the activated dextran comes to diffusional equilibrium. The equilibrium ratio dropped by 50%, over ~50s. This value is similar to the ~80s asynchrony between the breakdown of the centrosome proximal and distal pronuclei, indicating that diffusion of a centrosomally-generated cytoplasmic signal is a feasible mechanism for explaining this asynchrony.
The fact that depletion of Aurora A delays NEBD to a significantly greater extent than completely inhibiting centrosome assembly, leads us to speculate that the diffusible factor generated by centrosomes that promotes NEBD is activated Aurora A. This idea is consistent with the rapid turnover of Aurora A previously documented in human cells (half-life = 3s; Stenoien et al., 2003). To confirm that centrosomal Aurora A also turns over rapidly in the C. elegans embryo, we monitored the fluorescence recovery after photobleaching of centrosomal GFP:AIR-1 prior to NEBD (Fig. 6C,D). Centrosomal GFP:AIR-1 recovered to 95 ± 24% of its initial value, with a half time of 11.8 ± 2.1 seconds (n=8; errors are 95% confidence interval). We conclude that the centrosomal and cytoplasmic populations of Aurora A are in rapid equilibrium, making Aurora A an excellent candidate for the diffusible centrosomally-generated signal that promotes NEBD.
Figure 3.6. Centrosomes Generate a Diffusible Factor that Promotes Nuclear Envelope Permeabilization

(A) Images of the oocyte and sperm pronuclei in representative embryos for each condition. Schematics above each set of images illustrate the effect of the perturbations on the relative positions of the centrosomes and nuclei. (B) Graph of the interval between permeabilization of the oocyte and sperm-derived pronuclei to GFP:histone (time of oocyte pronuclear permeabilization - time of sperm pronuclear permeabilization) in control embryos, and embryos that fail to undergo pronuclear migration. In contrast to control embryos (n=12), permeabilization of the oocyte and sperm derived pronuclei is asynchronous in nocodazole-treated (n=5) and dynein depleted (dhc-1(RNAi); n=5) embryos, with the oocyte pronucleus breaking down later than the sperm pronucleus. This asynchrony is not observed in embryos in which pronuclear migration fails due to lack of functional centrosomes (spd-5(RNAi); n=5) or when centrosomes do not remain in proximity to the sperm pronucleus (zyg-12(RNAi); n=10). Error bars are the 80% confidence interval. (C) Selected still images from two representative experiments in which the recovery of centrosomal GFP:AIR-1 fluorescence was monitored after photobleaching. Times in seconds after the bleach. Bar, 1 µm. (D) Graph shows the average fraction of GFP fluorescence recovered as a function of time in seconds after the photobleach (n=8; error bars are the 80% confidence interval).
DISCUSSION

A Role For Aurora A in Accelerating the Timing and Kinetics of NEBD

Previous work demonstrated that Aurora A depletion appears to lengthen mitotic entry during the first division of the C. elegans embryo (Hannak et al., 2001). However, the lack of quantifiable cell cycle reference points prevented determination of the nature of this delay. Here, we use a recently developed method to monitor the kinetics of chromosome condensation (Maddox et al., 2006) to show that depletion of Aurora A specifically lengthens the interval between the completion of chromosome condensation and NEBD. By contrast, the onset and kinetics of chromosome condensation and regression of the pseudocleavage furrow, a cortical event analogous to the Cdk1-stimulated relaxation of surface contractile waves in Xenopus embryos (Rankin and Kirschner, 1997), were not altered by Aurora A depletion. These results indicate that the delay in nuclear envelope permeabilization that we describe here is distinct from the delay in global Cdk1 activation observed following depletion of Aurora A in human cells and Xenopus extracts (Hirota et al., 2003; Liu and Ruderman, 2006). It is important to note that our data do not rule out the possibility that Aurora A might also have a role in Cdk1 activation in C. elegans. When the dsRNA against Aurora A is introduced into L4 stage hermaphrodites, embryo production ceases after ~26 hours. By contrast, control hermaphrodites (as well as hermaphrodites in which other essential cell division proteins are depleted) continue embryo production for more than 48 hours. We therefore analyzed Aurora A depleted embryos produced between 22 and 26 hours after
dsRNA injection. These embryos are ~90% depleted of Aurora A by western blotting (Hannak et al., 2001), but are hypomorphic and not null for Aurora A function. As RNAi of the C. elegans homolog of Cdk1 also leads to cessation of embryo production (Boxem et al., 1999), further work will be needed to determine if the sterility following depletion of Aurora A reflects an additional role for this kinase in Cdk1 activation during oocyte maturation.
Figure 3.7. Model for the Role of Aurora A in Coordinating Centrosome Maturation with NEBD

(A) We propose that the mitotic centrosome scaffold harbors an Aurora A activator. Activated Aurora A promotes NEBD, the first step of which is permeabilization of the envelope due to loss of peripheral components of the nuclear pores (Lenart et al., 2003; Terasaki et al., 2001). The role of Aurora A in pore opening could be via direct phosphorylation of pore components or mediated by as yet unknown intermediates. (B) In wild-type embryos, a positive feedback loop in which the Aurora A-dependent increase in centrosome size increases the rate of Aurora A activation, generates a gradient of active Aurora A with the maximum concentration near the centrosomes. When centrosomes are mature and the local concentration of active Aurora A is sufficiently high, the nuclear envelope becomes permeable. In the absence of centrosomes, the activation of Aurora A and permeabilization of the nuclear envelope are delayed. In Aurora A depleted embryos an even greater delay is observed.
Centrosomes Generate a Diffusible Factor that Promotes NEBD

Our results provide three lines of evidence supporting the idea that centrosomes generate a diffusible factor that promotes nuclear envelope permeabilization. First, analysis of nocodazole-treated and dynein depleted embryos indicates that it is the presence of the mitotic centrosome scaffold rather than mechanical interactions between centrosomal microtubules and the nuclear envelope that is critical for timely NEBD. Second, depletion of ZYG-12, which causes the centrosomes to dissociate from the nuclear envelope and be randomly positioned within the embryo, does not delay NEBD to the same extent as completely inhibiting centrosome assembly. This result indicates that centrosomes can act at a distance to promote NEBD. Third, analysis of the asynchrony in NEBD between the sperm and oocyte nuclei when pronuclear migration is inhibited indicates that the breakdown of the oocyte nucleus, which is further from the centrosomes, is significantly delayed relative to breakdown of the sperm nucleus, which is proximal to the centrosomes. A comparison with the rate of diffusion of photoactivated 10 kD dextran indicates that the magnitude of this delay is consistent with the time required for diffusion of a centrosomally-generated factor that promotes NEBD.

As depletion of Aurora A delays NEBD to a significantly greater extent than completely inhibiting centrosome assembly, we speculate that the diffusible factor generated by centrosomes that promotes NEBD is activated Aurora A. We propose that the pericentriolar material of the centrosome catalyzes Aurora A activation (Fig. 7A) and activated Aurora A in turn, either directly or indirectly,
promotes NEBD. The idea that events at mitotic centrosomes could affect the cytoplasmic pool of Aurora A is supported by the rapid turnover of Aurora A at centrosomes. Such a mechanism requires a centrosomally-localized Aurora A activator. TPXL-1, the well-characterized Aurora A activator, is important for spindle assembly after NEBD, but does not contribute to the functions of Aurora A prior to NEBD (Özlü et al., 2005; this study). In human cells, the LIM protein ajuba is proposed to activate Aurora A during mitotic entry (Hirota et al., 2003). However, homologs of ajuba have not been identified outside of vertebrates. A recently described Aurora A activator, Bora, does not localize to centrosomes (Hutterer et al., 2006) and its inhibition in multiple genome-wide RNAi screens is reported to not affect C. elegans embryo viability (Kamath et al., 2003; Rual et al., 2004; Sönnichsen et al., 2005). We therefore suspect that an unidentified activator on the centrosome scaffold is important for Aurora A function prior to NEBD.

Overall, our analysis supports the emerging picture in which the functions of Aurora A prior to and after NEBD are mediated by distinct sets of activators/effectors. It will be interesting to see if the same is true for the different events that require Aurora A in the period leading up to NEBD: Cdk1 activation (Hirota et al., 2003; Liu and Ruderman, 2006), centrosome maturation (Berdnik and Knoblich, 2002; Blagden and Glover, 2003; Brittle and Ohkura, 2005; Hannak et al., 2001), the establishment of cell polarity (Berdnik and Knoblich, 2002; Hutterer et al., 2006; Schumacher et al., 1998), and nuclear envelope breakdown itself (this study).
Figure 3.S2. Simultaneous Depletion of SPD-5 and Aurora A Results in a Delay in NEBD Identical to that in Embryos Depleted of Aurora A Alone

(A) Plots comparing condensation kinetics in embryos depleted of SPD-5 (spd-5(RNAi); green diamonds; n=6) or AIR-1 alone (air-1(RNAi); blue circles; n=7) with those in embryos simultaneously depleted of both proteins (spd-5 & air-1(RNAi); orange triangles, n=6). Control embryos (n=10) were imaged in parallel, but for clarity the condensation kinetics for the controls are not plotted. The timing of NEBD in control (red arrow), SPD-5 depleted (green arrow), AIR-1 depleted (blue arrow) and AIR-1/SPD-5 depleted (orange arrow) embryos are indicated. Error bars are SEM. (B) Representative images of the sperm pronucleus from a control, a spd-5(RNAi), an air-1(RNAi), and a spd-5 & air-1(RNAi) embryo. Images are at 60s intervals. The completion (asterisks) of condensation defined by the analysis in (A) is indicated, as is the timing of NEBD (vertical bars). Bar, 5 μm. Note that although relative extent of the delays in the SPD-5 and AIR-1 depleted embryos is the same, their absolute magnitude is reduced relative to the experiment shown in Figs. 2 and 5. This is likely explained by variation in experimental conditions, primarily the temperature of the microscope room (which has a strong effect on timing and is difficult to precisely control over the six month period separating the early and new experiments) and possibly also the injection protocol, extent of compression, and light exposure. To control for variation in experimental conditions, we collected data on all perturbations as well as controls at the same time under the same conditions and compared these data sets. The lack of a synergistic defect if AIR-1 and SPD-5 are co-depleted is clearly evident in the results.
Possible Roles for Aurora A in the Control of NEBD Timing

Depletion of Aurora A delays the onset of nuclear envelope permeabilization, lamin disassembly, and nuclear pore removal to an essentially identical extent (Fig. 3). Although it is possible that Aurora A regulates all three events independently, the coordinate delay suggests that Aurora A might regulate one event that is a prerequisite for the other two. Since nuclear pore disassembly is accompanied by phosphorylation of a subset of the nucleoporins (Belgareh et al., 2001; Favreau et al., 1996; Ganeshan and Parnaik, 2000; Macaulay et al., 1995; Onischenko et al., 2005), an attractive possibility is that Aurora A either directly or indirectly triggers phosphorylation of nuclear pore components, promoting envelope permeabilization and subsequent pore removal and lamin disassembly. Alternatively, the coordinate regulation by Aurora A could be explained by its control of an upstream event required for all three aspects of nuclear envelope breakdown. One mechanism is suggested by the observation that cyclin B1 accumulates in the nucleus after chromosome condensation but prior to changes in nuclear envelope permeability (Hagting et al., 1999, Terasaki et al., 2003). This nuclear accumulation is regulated by phosphorylation (Hagting et al., 1998, 1999; Toyoshima et al., 1998; Yang et al., 1998) and may reflect a role for nuclear-localized Cdk1-cyclin B1 in triggering NEBD, although direct evidence for this idea is currently lacking. Assuming that the translocation of Cdk1-cyclin B1 into the nucleus is important for NEBD, Aurora A could promote NEBD by regulating the balance of Cdk1-cyclin B1
import and export during prophase. Distinguishing between these and other possibilities will require future work.

Aurora A Coordinates Multiple Events During Mitotic Entry

Mitotic progression is directed by the sequential activation of cyclin dependent kinases. However, how the many events that occur during mitotic entry are coordinated remains an important question. Aurora and polo family kinases refine the broad strokes of Cdk regulation to ensure that mitotic events occur in their proper sequence (Barr et al., 2004; Crane et al., 2004; Ducat and Zheng, 2004; Dutertre et al., 2002; Lowery et al., 2005; Marumoto et al., 2005). The fact that Aurora A is important for both centrosome maturation and NEBD suggests the existence of a positive feedback loop between an Aurora A-dependent increase in centrosome size during mitotic entry and the cellular pool of activated Aurora A that, either directly or indirectly, promotes NEBD. This idea provides an attractive explanation for how the temporal coupling between centrosome maturation, (an event that occurs in preparation for chromosome segregation on the spindle), and NEBD, (which provides centrosomal microtubules access to the replicated chromosomes), is achieved. Our findings also lend support to the emerging idea that the centrosome acts as a signaling scaffold (Doxsey et al., 2005), coordinating the progression of mitotic events independently of its role as a microtubule nucleating and organizing center.
Figure 3.S3. Monitoring the Kinetics of Diffusion of Photoactivated 10 kD Dextran Across the Embryo

(A) Selected stills from a sequence in which the diffusion of 10 kD dextran across the embryo was monitored. Caged rhodamine dextran was photoactivated on the far right of the embryo by a brief pulse of UV light, and the subsequent diffusion of the fluorescent dextran was monitored. Times are seconds after photoactivation. Greyscale (left) and pseudocolored images (right) of the photoactivated dextran are shown. Bar, 10 µm. See supplemental movie 8. (B) The equilibration ratio is equal to the total intensity in a box on the activated side of the embryo (red) box minus the total intensity in a box on the unactivated side of the embryo (cyan) at time t, divided by the same difference immediately after photoactivation. (C) Plot of the average of equilibration ratio vs. time in seconds after photoactivation. Error bars are the standard deviation (n=5).
A

-5s

0s

50s

B

Equilibrium Ratio

$(A-U)_t / (A-U)_0$

C

Equilibrium Ratio

1.0

0.5

0.0

Time (seconds after dextran photoactivation)

0

25

50

75

100

10 KDa dextran
Experimental Procedures

Strains and Culture Conditions

*C. elegans* strains expressing GFP:histone (AZ212; Praitis et al., 2001), GFP:NMY-2 (JJ1473; Nance et al., 2003), and co-expressing GFP:γ-tubulin and GFP:histone (TH32; Cheeseman et al., 2004) were maintained at 20°C. The strains OD139, co-expressing RFP<sup>mCherry</sup>:histone H2B and YFP:LMN-1 (unc-119(ed3) III; ltls37[pAA64; unc-119(+)] pie-1/RFP<sup>mCherry</sup>::HIS-58]; qals3507 [unc-119(+)] pie-1/YFP::LMN-1], and OD141, co-expressing RFP<sup>mCherry</sup>:histone H2B and GFP:NUP-155 (unc-119(ed3) III; ltls37[pAA64; unc-119(+)] pie-1/RFP<sup>mCherry</sup>::HIS-58]; [unc-119(+); pie-1/GFP::NUP-155]), were generated by mating previously described strains expressing fluorescent fusions with LMN-1 (Galy et al., 2003), NUP-155 (Franz et al., 2005), and histone H2B (McNally et al., 2006) and were maintained at 25°C. The strain OD142 expressing a GFP fusion with AIR-1 was generated by cloning the spliced AIR-1 coding sequence into the Spe I site of pIC26 (Cheeseman et al., 2004), and integrating the construct into DP38 (*unc-119* (ed3)) by ballistic bombardment (Praitis et al., 2001).

RNA Interference

dsRNA was prepared as described (Oegema et al., 2001). DNA templates were generated using the following gene-specific primers: *smc-4*
(TAATACGACTCACTATAGGCTCCAAAACAAGCCGAACCTT,
AATTAACCCCTACTAAAG-GTGACATCTTCTTTTTCTACATCA),  \textit{air-1}
(TAATACGACTCACTATAGGGCCTCTCGGAAA-AGGAAAGT,
AATTAACCCCTACTAAAGGCCTTGATTCTGGCGATCAAT),  \textit{spd-2} (AATTA-
ACCCTCACTAAAGGTGCATGCGAATAAGACGAAG,
TAATACGACTCACTATAGGTT-GCGGACACAGAAGAAAACAAA),  \textit{spd-5}
(AATTAACCCTCATAAGGGTGTCGCAACCA-GTTCTGAAT,
TAATACGACTCACTATAGGATGGAGGCAATTGTTGCTG),  \textit{dhc-1}
(AATTAACCCTCACTAAAGGGGAAGGAGGAGGCTCAACGACA,
TAATACGACTCACTATA-GGCCCTTTCTTCTGGGTCTTC),  \textit{zyg-12}
(AATTAACCCTCATAAGGGACGGCTGG-CTTGAACAATG,
TAATACGACTCACTATAGGGCAACTGAGCAATCCCATTT) to amplify regions
of genomic N2 DNA. For depletion of Aurora A, the previously described RNAi
conditions that led to >90% depletion were used (Hannak et al., 2001). For
depletion of DHC-1 and ZYG-12, worms were incubated at 20°C for 24-28 hours
post injection. For all other depletions, injected L4 larvae were incubated at 20°C
for 48 hours prior to analysis of their embryos.

\textbf{Imaging of Embryos Expressing GFP:histone and Analysis of Chromosome
Condensation}

Embryos expressing GFP:histone were imaged at 20°C using a Nikon
E800 upright microscope (Nikon Instruments, Melville, NY) equipped with a 60x
1.4NA Plan Apo objective lens and an Orca ER CCD camera (Hamamatsu
Photonics, Bridgewater, NJ) without binning. For condensation analysis, embryos were dissected and mounted in M9 (22 mM KH$_2$PO$_4$, 42 mM Na$_2$HPO$_4$, 86 mM NaCl, 1 mM MgSO$_4$) on a 2% agarose pad under a coverslip. At 10s intervals, 5 z-sections at 2 µm steps were acquired using a 250 ms exposure and a 12.5% transmission neutral density filter. Presented images are maximum intensity projections of the z-series for the indicated time points. Condensation kinetics were analyzed as described previously (Maddox et al., 2006). The average of the condensation parameter at each time point was calculated from values measured in 6-12 different embryos (as indicated) time-aligned with respect to NEBD. Averaged condensation profiles are displayed aligned with respect to the onset of chromosome condensation. For measurement of pronuclear diameter, embryos were mounted prior to anaphase of meiosis II without compression (Monen et al., 2005). Prior to anaphase of meiosis II, 3 fluorescence z-sections at 2 µm steps were collected at 30 s intervals. After anaphase of meiosis II, 5 DIC (Differential Interference Contrast) z-sections at 2 µm steps were collected at 10s intervals until pronuclear meeting. After pronuclear meeting, 5 fluorescence z-sections at 2 µm steps were collected at 10s intervals until metaphase of mitosis.

**Imaging of Embryos Expressing YFP:LMN-1 and GFP:NUP-155**

Embryos from the strains OD139 and OD141 were imaged using a spinning disk confocal (McBain Instruments, Los Angles, CA) mounted on a Nikon TE2000e inverted microscope (Nikon Instruments, Melville, NY). Images
were acquired using a 60x 1.4NA Plan Apo objective lens with 1.5x auxiliary magnification using an Orca ER CCD camera (Hamamatsu Photonics, Bridgewater, NJ) with 2x2 binning. Acquisition parameters, shutters and focus were controlled by MetaMorph software (Universal Imaging, Downingtown, PA). YFP:Lamin at the nuclear periphery was quantified by averaging the total fluorescence intensity measured in six separate 5x5 pixel regions at the periphery of the sperm pronucleus for each timepoint.

**Monitoring Nuclear Entry of 70kDa dextran and GFP:NMY-2**

Texas-Red conjugated, lysine fixable 70kD dextran (Molecular Probes) at 0.08 mg/mL in injection buffer (1 mM potassium citrate, 6.7 mM KPO4, pH 7.5, 0.67% PEG) was injected into the gonads of control and air-1(RNAi) worms expressing GFP:NMY-2. After 4 hours, dextran-containing embryos were dissected from the mothers and imaged using a spinning disk confocal as described for imaging of embryos from the strains OD139 and OD141 except 4x4 binning was used. Entry was defined as the point when the nuclear and cytoplasmic fluorescence of the probe had equilibrated.

**Nocodazole Treatment**

Nocodazole was introduced into embryos following chitinase treatment. 3 worms were dissected in 8µL egg salts (48mM NaCl, 118 mM KCl) on a 24x60 coverslip on which a drop of 4µL polylysine (1 mg/mL) had been dried in an oven for 10 minutes. The buffer was removed with a mouth pipet and replaced with
8µL of 9:1 ddH2O:bleach by volume. After 2 minutes, the bleach solution was replaced with 8µL egg salts buffer, followed sequentially by 8µL L-15 blastomere culture medium (Edgar, 1995) and 8µL chitinase (5U/mL in L-15 blastomere culture medium). After 4 minutes, the chitinase solution was replaced with 8µL L-15 blastomere culture medium containing 10µg/mL nocodazole. Embryos were filmed as described above for measurement of pronuclear size except 5 fluorescence sections were acquired at 2µm z steps every 10s.

**Measuring Turnover of Centrosomal Aurora A**

For photobleaching of centrosomal GFP:AIR-1, embryos were mounted without compression (Monen et al., 2005) in L-15 blastomere culture medium containing 10µg/mL nocodazole to minimize centrosome movement. Embryos were imaged using a spinning disk confocal (McBain Instruments, Los Angles, CA) mounted on a Nikon TE2000e inverted microscope (Nikon Instruments, Melville, NY). Images were acquired by using a 60 x1.4 N.A. Plan Apo objective lens with a Xion electron multiplication back-thinned CCD camera (Andor technologies) with no binning. Photobleaching was performed by selecting the 488nm line from a Krypton-Argon mixed gas 2.5W water cooled laser (Spectra-Physics, Mountain View, CA) which was steered into a custom modified epi-fluorescence port creating a single diffraction limited spot at the field diaphragm which is projected to the objective focal plane (full width half max at the focal point ~800nm). Exposure times for bleaching were 1-2 seconds. Acquisition parameters, shutters, and focus were controlled by MetaMorph software.
(Universal Imaging, Downingtown, PA). The fluorescence intensity of centrosomal GFP:AIR-1 was calculated by measuring the total intensity in a box containing the centrosome and subtracting the camera background. The signal at the first postbleach timepoint (typically 15-25% the pre-bleach value) was subtracted from all postbleach measurements and the fraction of fluorescence recovered at each timepoint was calculated by dividing by the difference between the prebleach and first postbleach measurements. Kaleidagraph software (Synergy Software, Reading PA) was used to fit the data to an equation of the form $y = m(1 - \exp(-kt))$ where $m$ is the maximum fractional recovery and the half time for recovery $t_{1/2} = -\ln(0.5)/k$.

**Monitoring the Kinetics of Diffusion of Photoactivated 10 kD Dextran Across the Embryo**

CMNCBZ-caged 5-(and-6)-carboxy-Q-rhodamine dextran (10,000 MW) (Molecular Probes) was re-suspended to 5 mg/mL in injection buffer and injected into the gonad of adult worms expressing a GFP labeled plasma membrane marker (OD58, Audhya et al., 2005). After 5 hours of recovery at 20°C, the dextran-containing embryos were dissected from the mothers, mounted on a 2% agarose pad under a coverslip in M9, and imaged using a DeltaVision microscope (Applied Precision) equipped with a CoolSnap CCD camera (Roper Scientific) and a 60x 1.4 NA Olympus U-PlanApo objective. To selectively photo-activate a small population of the cytoplasmic caged-dextran, the field diaphragm was closed until only a small portion of embryo was in view; this region of the
embryo was illuminated with a 2-second pulse of unattenuated UV excitation through the DAPI filter to release the caging moiety. Immediately following photo-activation, the field diaphragm was opened to image the entire embryo and 100 ms exposures with 2x2 binning were collected at 5 second intervals for 5 minutes.

Simultaneous Depletion of SPD-5 and Aurora A

The protocol described below was used to control for the fact that mixing an RNA with a second RNA reduces its effectiveness. In addition, whereas SPD-5 can be essentially completely depleted over a time course of ~48 hours without causing sterility of the injected parent, worms injected with RNA targeting AIR-1 that are incubated at 20°C become sterile within 22-26 hours. To obtain SPD-5 depleted embryos, L4 hermaphrodites were injected with an RNA targeting SPD-5 48 hours prior to imaging and then again with an equal concentration (µg/ml) mixture of SPD-5 RNA and control RNA (an RNA against the yeast gene CTF13, which has no C. elegans homologs) 24 hours prior to imaging. AIR-1 depleted embryos were obtained by injecting L4 hermaphrodites with control RNA 48 hours prior to imaging, and then again with an equal concentration (µg/ml) mixture of control and AIR-1 RNA 24 hours prior to imaging. The AIR-1 & SPD-5 double depleted embryos were obtained by injecting L4 hermaphrodites with SPD-5 RNA 48 hours prior to imaging, and then again with an equal concentration (µg/ml) mixture of SPD-5 and AIR-1 RNA 24 hours prior to imaging. All incubations were at 20°C.
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Abbreviations

NEBD- Nuclear Envelope Breakdown; RNAi- RNA mediated interference
References


Chapter 4
Conclusions and Future Directions

Conclusions

Between its links to cancer, tumorigenesis, and general mitotic effects, the study of Aurora A kinase has been increasing recently. Already the regulatory sequences within the protein have been elucidated, mechanisms for activation and degradation have been established, and interacting proteins are being found all the time. However, a better understanding of its primary functions is needed, as well as an increased knowledge of how the interacting proteins help to carry out their roles in mitosis. Distinguishing between direct and indirect functions of Aurora A requires an increased understanding of its interacting proteins and positive feedback loops. In addition, as seen in this dissertation, novel functions of the kinase are still being discovered.

*C. elegans* has been an ideal system for the studies undertaken in this dissertation. The short lifespan of the organism shortened the time interval between the concept of an experiment and the acquisition of data. The ease of maintaining large quantities of worms meant that there were always ample organisms for experiments. The efficacy and ease of RNAi experiments, both in the generation of the dsRNA itself, introduction of the RNA into the animals, and the greater than 95% knock-down of the target protein proved invaluable for the initial characterization of the AIR-1 depletion phenotype.
Having solved the protein structure of the catalytic domain allows for the development of directed small molecule inhibitors, a process that is just now getting off the ground. VX-680, (MK-0457) is a promising start, improving upon earlier inhibitors such as hesperadin and ZM447439, that were shown not to be as specific for Aurora A as would be required for therapeutic trials. The strong sequence similarity within the catalytic cleft of the Aurora family proteins, specifically Aurora B kinase, will make specificity a constant struggle. The focus on Aurora A as an anti-cancer target should yield additional small molecule inhibitors in the coming years. These pharmacological agents can be used as tools that should increase our understanding Aurora A function.

In this work, a novel function of Aurora A kinase has been discovered. In addition, a new method for monitoring the dynamics of chromosome condensation has been developed. This method for condensation analysis is semi-automated, and allows for the rapid analysis of any time-lapse fluorescence data. In addition to being simple to use and adjustable, through threshold manipulation, for a variety of acquisition methods, the assay can be applied to any system that consists of a fluorescent tag that changes in either shape or intensity over the duration of the experiment. It has already been used to monitor the transition from dispersed membrane networks into more tubular structures, other uses are sure to be uncovered.

The creation of the chromosome condensation assay allowed for the characterization of the nuclear envelope breakdown delay that results from a depletion of the Aurora A homolog AIR-1 in C. elegans embryos. Monitoring
chromosome dynamics showed that both the onset and rate of chromosome condensation in control and air-1(RNAi) embryos were identical. However, the chromosomes persist in a fully condensed state for many minutes before NEBD occurs. Additionally, it was found that the rate of nuclear envelope permeabilization was slowed in the air-1(RNAi) embryos. This was evidenced by the increased time between entry of two differently sized fluorescent markers into the nuclear space, as well as analysis of the localization of various nuclear envelope-associated proteins in the AIR-1 depleted embryos. The fact that all of the nuclear envelope proteins analyzed showed a delayed departure from the nucleus suggests that AIR-1 may influence the activity of a master regulator of NEBD. Cyclin B/Cdk1 complex is an attractive target for Aurora A activity, future experiments will be performed to see if there is a link between the mitotic kinases.

**Future Directions**

Having demonstrated that depletion of Aurora A kinase in the early C. elegans embryo results in a specific delay in nuclear envelope breakdown, several follow-up experiments can build on this story and provide further insight into the mechanism of this phenotype. To begin, it will be important to determine whether or not the kinase activity of Aurora A is required for this effect. Work has already begun on this question, thus far with limited results. Attempts to introduce small-molecule Aurora inhibitors into the early C. elegans embryo have not yet met with success. The chitinase treatment method used to treat the
embryos with nocodazole (Portier et. al. 2007) was unsuccessful in generating any of the published phenotypes associated with the drugs. This may be because the drugs are not able to pass through the vitelline membrane, which remains intact after chitinase treatment, or the molecules may be inactive against the *C. elegans* Aurora kinases. Experiments are currently being performed to determine the efficacy of the drugs against Aurora A kinase activity *in vitro*.

I expect to find that the kinase activity of AIR-1 is required for the NEBD delay phenotype. If so, the question becomes what are the activator and the target for AIR-1 in this process? Currently, the only known activator of AIR-1 is the Tpx2 homolog TPXL-1 (Ozlu et. al. 2005). However, the nuclear envelope broke down with similar timing in both control and *tpxl-1(RNAi)* embryos (Portier et. al. 2007). This suggests that there is another activator that is required for the NEBD functions of AIR-1. In addition, whether AIR-1 acts to promote timely NEBD directly or indirectly, there must be a phosphorylation target. Two possibilities exist; one, that AIR-1 directly phosphorylates a nuclear pore component, leading to disassembly of the pore and nuclear permeabilization. This is an attractive hypothesis, as many nuclear pore components are known to be phosphorylated in the events leading up to NEBD. Alternatively, AIR-1 may influence the activity of a master regulator of NEBD, such as cyclin B/Cdk1 activity. This seems more likely, due to the fact that all nuclear envelope components examined were delayed in leaving the nuclear membrane in *air-1(RNAi)* embryos. If the target of AIR-1 phosphorylation was a nuclear pore component, it seems likely that the loss of NUP-155, a nuclear pore marker,
would be delayed with respect to the lamins. However, a more precise timing of events must be undertaken before such assertions can be proven.

Finally, many of the functions of Aurora A are closely tied to centrosome function. Indeed, one of the functions of Aurora A is to ensure that centrosomes fully mature prior to mitosis. How Aurora A recruits pericentriolar material in a timely fashion has not been fully determined. Further studies into Aurora A interactors, both in vitro and in vivo are planned, with the goal of answering this question. Once again, *C. elegans* is an ideal model system for answering this question, as several genome-wide screens have been RNAi to knock down protein levels of the approximately 19,000 genes present in the early embryo (reviewed in Grant and Wilkinson, 2003). Of these genes, approximately 1600 were shown to result in embryonic lethality (www.wormbase.org). Upon closer inspection, depletion of three proteins (SPD-2, SPD-5, and Aurora-A kinase (AIR-1)) by either RNAi or mutagenesis was shown to have severe defects in the recruitment of PCM as defined by γ-tubulin recruitment (Hamill et. al. 2002, Hannak et. al. 2001, Kemp et. al. 2004, Pelletier et. al. 2004, Schumaker et. al. 1998). This recruitment defect results in centrosomes that are unable to nucleate the proper number of microtubules and ultimately experience catastrophic mitotic defects as proper mitotic spindles do not form. Understanding the interactions between these proteins that all result in centrosome maturation phenotypes will go a long way toward elucidating the function of AIR-1 in this process.
References


