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Functional Analysis of DdINCENP, a chromosomal passenger protein, in *Dictyostelium*

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Functional analysis of DdINCENP, a chromosomal passenger protein, in *Dictyostelium*

by

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Dedication

To my parents and my wife

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Functional analysis of DdINCENP, a chromosomal passenger protein, in *Dictyostelium*

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Dictyostelium DdINCENP is a chromosomal passenger protein associated with centromeres, the spindle midzone and poles during mitosis and the cleavage furrow during cytokinesis. Disruption of the single DdINCENP gene revealed important roles for this protein in mitosis and cytokinesis. DdINCENP null cells lack a robust spindle midzone and are hypersensitive to microtubule depolymerizing drugs suggesting that their spindles may not be stable. Furthermore DdCP224, a protein homologous to the microtubule-stabilizing protein TOGp/XMAP215, was absent from the spindle midzone of DdINCENP null cells. Overexpression of DdCP224 rescued the weak spindle midzone defect of DdINCENP null cells. While not required for the localization of the myosin II contractile ring and subsequent formation of a cleavage furrow, DdINCENP is important for the abscission of daughter cells at the end of cytokinesis.

The localization of DdINCENP at the cleavage furrow is modulated by myosin II. Loss of myosin II restricted the localization of DdINCENP to a narrow zone at the cleavage furrow. Kif12, a homolog of mitotic kinesin like protein (MKLP), was essential

for relocalization of DdINCENP from the central spindle to the cleavage furrow. Furthermore, Kif12 was also localized at the cortex of the cleavage furrow and its localization during cytokinesis closely resembled that of DdINCENP, suggesting a possible interaction between them. The correct localization of DdINCENP during cytokinesis also required its N-terminal sequence. $DdINCENP_{1-500}$ was found at the cleavage furrow and interacted with the actin cytoskeleton. Domain analysis of DdINCENP also revealed that its $DdINCENP_{1-500}$ was sufficient to rescue the weak spindle defect of DdINCENP null cells.

Table of Contents

List of Figures

List of Tables

Chapter 1: Introduction

1.1 CHROMOSOMAL PASSENGER PROTEINS

Chromosomal passenger proteins are a group of proteins which have a dynamic localization during cell division. They typically localize in the nucleus during interphase and transfer to the chromosomes during prophase. By prometaphase, these passenger proteins begin to concentrate at the inner-centromeric regions of sister chromatids, where they reside throughout metaphase. At the onset of anaphase, these proteins transfer to the central spindle where they remain there through telophase. During cytokinesis, they are found at the midbody and the cortex region of the cleavage furrow (Cooke et al. 1987; Adams et al. 2001). Several chromosomal-passenger proteins have been discovered, including inner centromere protein (INCENP), Aurora B, Survivin, TD-60, and Dasra/Borealin (Mollinari et al. 2003; Gassmann et al. 2004; Sampath et al. 2004). INCENP, Aurora B and Survivin have been shown to interact with each other by forming a chromosomal passenger complex (Kang et al. 2001; Bolton et al. 2002; Cheeseman et al. 2002). This protein complex also interacts with Dasra/Borealin in both Xenopus and human cells (Gassmann et al. 2004; Sampath et al. 2004).

Chromosomal passenger proteins are found in such diverse organisms as yeast, nematode, fruit fly, and vertebrates, which indicates their importance for cell survival. The homologs of INCENP, Aurora B and Survivin have been found in almost every model system. By contrast, the homolog of neither Borealin nor TD-60 has been identified in lower eukaryotic systems such as yeast and *Dictyostelium*.

1.2 CHROMOSOMAL PASSENGER PROTEINS ARE ESSENTIAL FOR MITOSIS

Considering their dynamic distribution during mitosis, it is not surprising that chromosomal-passenger proteins are emerging as critical regulators of mitosis. They have important functions in such diverse aspects of mitosis including chromosome condensation, metaphase chromosome congression, kinetochore bipolar spindle attachment, proper chromosome segregation and checkpoint control (Mackay et al. 1998; Kim et al. 1999; Giet et al. 2001; Cheeseman et al. 2002; Tanaka et al. 2002; Gassmann et al. 2004; Sampath et al. 2004). Here, I will focus on the functions of Aurora B kinase and INCENP during mitosis.

Studies in the last several years have clearly demonstrated that Aurora B is one of the most important kinases regulating mitosis. All the aforementioned functions of chromosomal passenger proteins need the activity of Aurora B kinase. Its substrates include itself, INCENP and many other proteins, which play vital roles during mitosis. For example, Ipl1, its homolog in *S. cerevisiae*, phosphorylates many kinetochore proteins including Dam1p, Ndc80p and Spc34p (Cheeseman et al. 2002). In vertebrate systems, both the centromeric localization and the microtubule depolymerizing activity of MCAK (mitotic centromere-associated kinesin) are regulated by Aurora B phosphorylation (Andrews et al. 2004; Lan et al. 2004). The activity of MCAK at centromeres is essential for establishing the bi-oriented attachment of kinetochore during metaphase. The substrates of Aurora B kinase are not limited to the kinetochore proteins, but also include histone H3 (Hsu et al. 2000; Adams et al. 2001; Giet and Glover 2001). Recently, it has been shown that the phosphorylation of H3 serine 10 could release heterochromatin protein 1 (HP1) from H3 during mitosis (Fischle et al. 2005).

In contrast to Aurora B, the role played by INCENP during mitosis is not as well understood. Nevertheless, INCENP is clearly an essential protein for mitosis as

demonstrated by the loss of function studies in *S. cerevisiae*, *Drosophila* and *C. elegans* (Kim et al. 1999; Kaitna et al. 2000; Adams et al. 2001). Like Aurora B, INCENP has been found in a wide range of species from yeast to mammals. The most significant homology among these INCENP proteins is the C-terminal IN-box domain, which binds to Aurora B kinase (Kang et al. 2001; Bolton et al. 2002). One function of INCENP we do know is that it is both a substrate and stimulator of Aurora B kinase (Kang et al. 2001; Bishop et al. 2002; Honda et al. 2003). Additionally, INCENP targets Aurora B to centromeres and the central spindle (Adams et al. 2000; Kaitna et al. 2000). However, INCENP clearly has other mitotic functions. For example, a recent study has demonstrated that INCENP also recruits Polo kinase, another important mitotic kinase, to centromeres (Goto et al. 2006).

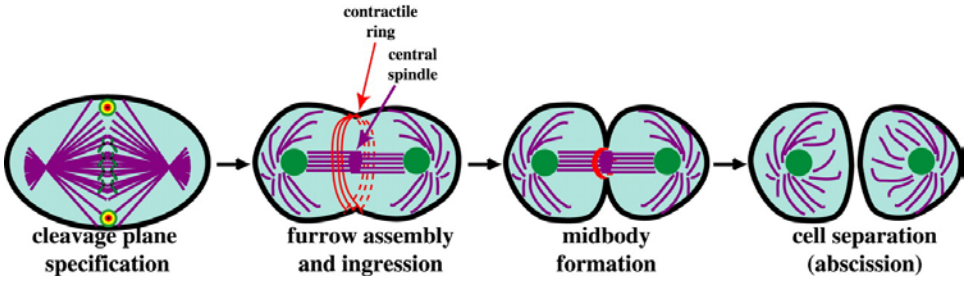
1.3 CHROMOSOMAL PASSENGER PROTEINS AND CYTOKINESIS

Before discussing the functions of chromosomal passenger proteins during cytokinesis, I will briefly review cytokinesis and the cytokinetic machinery.

1.3.1 Overview of cytokinesis

Cytokinesis is the last stage of cell division, when the daughter cells physically separate from each other after the segregation of chromosomes. It usually starts with the initiation of the cleavage furrow at the equator of the dividing cell during anaphase and ends with the abscission of the cytoplasmic bridge connecting the two daughter cells (Figure 1.1). The first stage of cytokinesis is the initiation. It is essential to begin cytokinesis only after the complete segregation of the duplicated chromosomes. Furthermore, the cleavage furrow has to be positioned at the correct place to separate the daughter cells equally. The second stage of cytokinesis involves the ingression of the cleavage furrow. The driving force of this process is the actin myosin contractile ring

(Glotzer 2005). Additionally, the central spindle plays an essential role at this stage (D'Avino et al. 2005). The third and final stage occurs when the two daughter cells are separated by abscission of the cytoplasmic bridge connecting them. At this final stage, the central spindle is condensed and bundled by the cleavage furrow to form a condensed structure called the midbody, which also plays an important role at the abscission stage (Mullins et al. 1982; Skop et al. 2004). However, the midbody structure is present only in metazoans but not in *Dictyostelium* or yeast.



(© by Glotzer, M., et al. 2001)

Figure 1.1: Overview of cytokinesis. Cytokinesis usually initiates during anaphase at the equator of the dividing cells. The assembly of the actomyosin contractile ring and the central spindle will be coupled with the ingression of the cleavage furrow. A midbody structure is formed before the two daughter cells become separated in a process called abscission (Glotzer 2001).

1.3.2 Induction of cytokinesis

Two of the key remaining questions to be answered about cytokinesis are: what is the signal that induces cytokinesis and where does it come from? Although there is still much debate on the first question, it is becoming clear that the mitotic spindle is the source of the signal (D'Avino et al. 2005). This conclusion is clearly supported by the findings that initiation of the cleavage furrow could still occur after the removal of either

the chromosomes or the asters in grasshopper spermatocytes (Zhang et al. 1996; Alsop et al. 2003). The presence of residual microtubules was enough to induce cytokinesis in those cells. Khodjahov et al. made a similar finding in mammalian cells. After the spindle was assembled during metaphase, cytokinesis would initiate even after one centrosome was removed (Khodjakov et al. 2001). This model could also explain the formation of Rappaport furrows (Figure 1.2) (Rappaport 1961). In this classical experiment conducted on dividing sand dollar eggs, ectopic furrows could be induced between two asters even without the presence of chromosomes between them (Figure1.2).

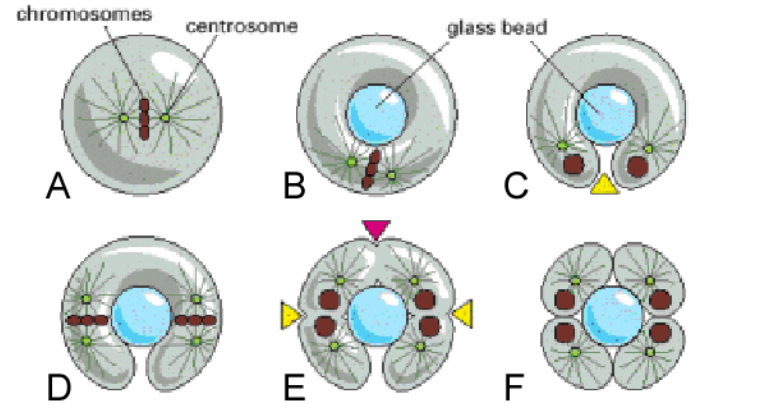


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Figure 1.2 A Rappaport furrow can be induced between two neighboring centrosomes. A bi-nucleate egg cell was induced by pushing a glass bead into the large egg during first division (A-C). In the next cell division (D-F), the cleavage furrows initiated between both the centrosomes linked by a central spindle (yellow arrow head) and the two centrosomes simply adjacent (red arrow head). The latter furrow is also called Rappaport furrow (Alberts et al. 2002).

1.3.3 Central spindle bound kinesins and their roles in cytokinesis

Both astral microtubules and the central spindle are important for cytokinesis. Nevertheless, I will focus on the functions of the central spindle, because that is where chromosomal passenger proteins are found. The central spindle is formed by the bundling of the interdigitating microtubules from the opposing spindle poles during anaphase. It is also called the spindle midzone. Many molecules localized at the central spindle are essential for cytokinesis, including kinesins, microtubule-associated proteins (MAP), and chromosomal passenger proteins. Among them, kinesins play a particularly important role, because they bundle anti-parallel microtubules to form the central spindle, thus providing a scaffold for the other proteins (Nislow et al. 1992).

Kinesins are microtubule plus end directed motor proteins. Some are localized at the central spindle and midbody during cytokinesis, including KIF4 and mitotic kinesin like proteins (MKLP) (Sellitto et al. 1988; Hill et al. 2000; Zhu et al. 2005). These kinesins are important for both the assembly of the central spindle and cytokinesis (Raich et al. 1998; Hill et al. 2000; Kurasawa et al. 2004; Zhu and Jiang 2005). They also interact with MAPs and kinases on the central spindle. For example, KIF4 interacts directly with PRC1, a MAP, and recruits it to the central spindle (Zhu and Jiang 2005). PRC1 has the ability to stabilize the central spindle and is essential for cytokinesis (Jiang et al. 1998; Mollinari et al. 2002). Another example is Polo kinase, an important kinase for cytokinesis, associates with MKLPs at the central spindle (Lee et al. 1995; Neef et al. 2003).

The importance of kinesins during cytokinesis is also demonstrated by the finding of the centralspindlin protein complex. The centralspindlin complex is composed of MKLP1 together with MgcRacGAP, a Rho-family GTPase-activating protein (Mishima et al. 2002; Mishima et al. 2004). MgcRacGAP is also essential for cytokinesis in both

mammalian cells and *C.elegans* (Jantsch-Plunger et al. 2000; Hirose et al. 2001). Although it is not clear which Rho-family protein is regulated by MgcRacGAP during cytokinesis, there is data suggesting that MgcRacGAP may become a RhoA GAP after its phosphorylation by Aurora B kinase (Minoshima et al. 2003). (For the function of RhoA, see the section below) Moreover, MgcRacGAP interacts with ECT2, a RhoA GEF, another essential protein for cytokinesis (Tatsumoto et al. 1999; Mishima et al. 2002; Yuce et al. 2005) (Figure 1.3).

1.3.4 The actin myosin contractile ring and cytokinesis

Another important component of the cytokinetic machinery is the actin myosin contractile ring. Myosin II is an essential component of the ring, demonstrated by the cytokinesis defect caused by either the loss of myosin II gene or the inhibition of myosin II activity (De Lozanne et al. 1987; Straight et al. 2003). There are also other actin binding proteins found on the contractile ring, such as formin, profilin and cofilin. They also are essential for cytokinesis (Balasubramanian et al. 1994; Gunsalus et al. 1995; Chang et al. 1997).

RhoA, a small Rho family GTPase and a key regulator of the contractile ring, is localized at the cleavage furrow (Kishi et al. 1993; Yamochi et al. 1994; Tatsumoto et al. 1999; D'Avino et al. 2005). There are several pathways that RhoA is involved in regulating the contractile ring as shown in Figure 1.3. One key pathway involves regulating the activity of citron kinase and Rho kinase (ROK/CIT-K) (Kosako et al. 2000; D'Avino et al. 2004). Both kinases can phosphorylate myosin regulatory light chain to regulate the contractile ring (Kosako et al. 2000; Yamashiro et al. 2003).

Besides actin and myosin II, two other cytoskeleton proteins, septin and anillin, are also important for cytokinesis. Septins are a conserved family of GTPases implicated in various cellular processes (Schmidt et al. 2004). The septin ring is found at the division

site of budding yeast (Longtine et al. 1996). However, their function during cytokinesis is not as well understood in mammalian cells. Anillin is an actin- and myosin II- binding protein, highly enriched at the cleavage furrow (Field et al. 1995). However, its function at the contractile ring is unknown (Field et al. 2005).

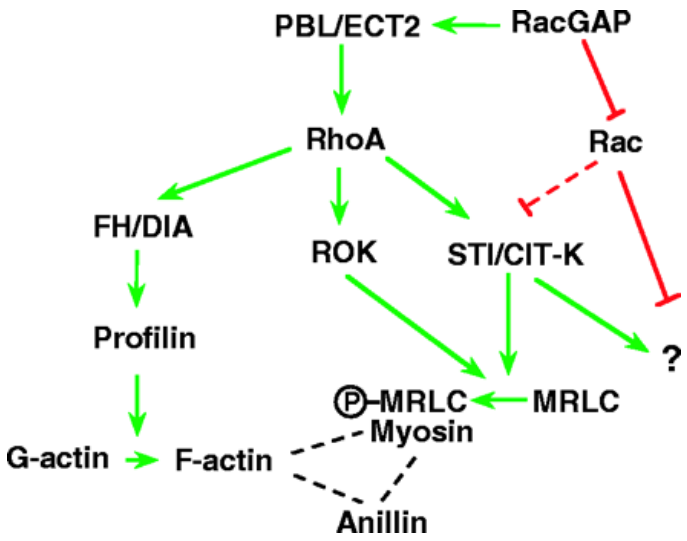


Figure 1.3 RhoA is the key regulator of the actin myosin contractile ring. The different pathways RhoA uses to regulate the actomyosin contractile ring are shown. (D'Avino et al. 2005).

1.3.5 The function of chromosomal passenger proteins during cytokinesis

Chromosomal-passenger proteins are localized at the central spindle and the cleavage furrow, both important components of the cytokinetic machinery. They play a prominent role during cytokinesis (Adams et al. 2001). Their importance was demonstrated by the early finding that two dominant-negative mutants of INCENP caused cytokinesis defects in mammalian cells (Eckley et al. 1997; Mackay et al. 1998). Additionally, in both *C. elegans* embryos and *Drosophila* cultured cells, depletion of

INCENP or Aurora B causes a cytokinesis defect (Schumacher et al. 1998; Kaitna et al. 2000; Giet and Glover 2001).

Again, the role of Aurora B kinase played in cytokinesis has been the best characterized. Both components of the centralspindlin complex, MgcRacGAP and MKLP1, are substrates of Aurora B kinase. Interestingly, phosphorylation of MgcRacGAP promotes its GAP activity towards RhoA (Minoshima et al. 2003). The phosphorylated MKLP-1 promotes the completion of cytokinesis, but the mechanism is unknown (Guse et al. 2005). Aurora B does not regulate the central spindle localization of MKLP1/2 (Gruneberg et al. 2004). However, Aurora B kinase does affect the localization of ZEN-4, the *C.elegans* homolog of MKLP, at the cleavage furrow (Kaitna et al. 2000; Severson et al. 2000). Nevertheless, the functions of the other chromosomal passenger proteins remain unknown including INCENP.

1.4 *DICTYOSTELIUM*

1.4.1 Overview of *Dictyostelium*

Dictyostelium discoideum is a social amoeba that resides in the soil. Although *Dictyostelium* cells usually live as independent amoeba, they can develop into a multi-cellular organism when being challenged in a nutrition-depleted environment. *Dictyostelium* has been used to study a wide range of topics such as cytokinesis, cell motility, chemotaxis, membrane trafficking and development (De Lozanne and Spudich 1987; O'Halloran et al. 1992; Kimmel et al. 2004; Franca-Koh et al. 2006).

The genome of *Dictyostelium* has been fully sequenced. It has 34 mega base pairs and there are 12,500 predicted genes distributed among six chromosomes and one rDNA extra-chromosomal palindromic element (Sucgang et al. 2003; Eichinger et al. 2005). This is consistent with the finding of six complementation groups by genetic linkage

studies (Loomis et al. 1995). However, cytological studies have repeatedly found seven chromosome-like bodies during mitosis (Robson et al. 1977; Welker et al. 1980). The discrepancy is likely explained by the finding that the extra-chromosomal rDNA palindromes condense into chromosome-like structures during mitosis (Sucgang et al. 2003). The DNA sequence of *Dictyotelium* centromeres has not been defined. A possible candidate is the single clustered repeats of the DIRS (*Dictyostelium* Intermediate Repeat Sequence) at one end of each chromosome (Eichinger et al. 2005). It is known that the *Dictyostelium* chromosomes are telocentric (Cappello et al. 1984; Loomis et al. 1995).

1.4.2 The cell cycle of *Dictyostelium*

When *Dictyostelium* cells are grown on bacteria as a food source, they double every 3-4 hours. In comparison, they double every 8-10 hours when grown in axenic media. *Dictyostelium* growing cells do not have a G1 phase, have a very short S phase of 30 minutes or less, and a lengthy G2 phase, which lasts 6 hours or more (Weijer et al. 1984). The M phase usually takes about 10 minutes and is divided into two equal halves by the start of anaphase (Roos et al. 1981).

Dictyostelium cells have closed mitosis and the nuclear envelope does not disassemble during cell division (Moens 1976). The spindle dynamics of *Dictyostelium* cells during mitosis is very similar to that of higher eukaryotic cells (Roos and Camenzind 1981; Roos et al. 1984). During interphase, the centrosome is adjacent to but separate from the nucleus in *Dictyostelium* (Moens 1976). When cells enter prophase, most cytoplasmic microtubules are disassembled and the centrosomes become docked into the nuclear envelope. The duplication of centrosomes also occurs at this stage. At the transition from prophase to prometaphase, the two daughter centrosomes begin to separate from each other (Ueda et al. 1999). At the same time, the mitotic spindle assembles between the two centrosomes and appears as a short rod within the nucleus

(Roos et al. 1984). During metaphase, chromosomes converge on a circle around the metaphase plate with their centromeric ends pointed inward. At the same time, the mitotic spindle elongates and the astral microtubules become more abundant (Roos et al. 1984; McIntosh et al. 1985). During anaphase and telophase, *Dictyostelium* cells have a very prominent central spindle (McIntosh et al. 1985). The central spindle is disassembled before the end of cytokinesis and there is no midbody structure found in *Dictyostelium* (Roos et al. 1984; Neujahr et al. 1998).

1.5 CYTOKINESIS OF *DICTYOSTELIUM*

There are several advantages of using *Dictyostelium* as a model system to study cell biology. First, it is inexpensive to maintain the cultured cells, which can be grown in axenic media or on bacteria. Because of their relatively short doubling time (8-10shours) the cells can be grown in a large-scale suspension culture to harvest a large number of cells in a short period of time. Secondly, *Dictyostelium* is a genetically tractable system. The high frequency of homologous recombination makes it relatively easy to either knock out endogenous genes or insert exogenous gene into the genome. It is also possible to create a library of *Dictyostelium* strains containing random plasmid insertions in their genomes by Restriction Enzyme-Mediated Integration (REMI) (Kuspa et al. 1992). Furthermore, a method has been developed for complementation screens of mutant strains using a cDNA-expression library (Robinson et al. 2000).

Dictyostelium is a particularly suitable system to study cytokinesis for several reasons. First, cytokinesis of *Dictyostelium* cells is similar to that of animal cells on their dependence on the actomyosin contractile ring. In *Dictyostelium*, the contractile ring is assembled during metaphase and gradually constricts with the ingression of the cleavage furrow (Fukui 1990). Secondly, it has also been shown that *Dictyostelium* cells cannot grow in suspension culture if they have cytokinesis defects (De Lozanne and Spudich

1987; Larochelle et al. 1996). Combined with REMI, this finding makes it possible to conduct a large scale screen for the cytokinesis mutants in *Dictyostelium*. In fact, several cytokinesis mutants have been isolated through this approach in our lab including *racE* and *lvsA* mutants (Larochelle et al. 1996; Kwak et al. 1999). Lastly, a large number of *Dictyostelium* cytokinesis mutants have been identified. The analysis of these mutants reveals that there are a variety of proteins involved in cytokinesis in *Dictyostelium*. They include not only actin cytoskeleton bound proteins such as coronin, cortexillin I/II and dynacortin (de Hostos et al. 1993; Faix et al. 1996; Robinson and Spudich 2000), but also membrane bound proteins such as dynamin, clathrin and LvsA (Niswonger et al. 1997; Kwak et al. 1999; Wienke et al. 1999). The availability of a wide range of cytokinesis mutants would greatly facilitate the research on cytokinesis in *Dictyostelium*.

1.6 SIGNIFICANCE

Cytokinesis is one of most fundamental biological process, which is still poorly understood. Emerging evidence suggests that there is a close coordination between mitosis and cytokinesis. Chromosomal passenger proteins have a dynamic localization and play many important roles during both mitosis and cytokinesis. Therefore, they are emerging as possible key players of the crosstalk between mitosis and cytokinesis. However, their functions during cytokinesis are not well studied. There are many questions that remain open. Are they involved in the signal pathway inducing the initiation of cytokinesis? What proteins do they interact with at the central spindle? What mechanism targets them to the cleavage furrow? Finally, do they interact with the actin myosin contractile ring? In this thesis I have tried to address some of these questions by studying the function of INCENP using *Dictyostelium* as a model system.

Chapter 2: Materials and Methods

2.1 Cloning of DdINCENP cDNA and construction of GFP-DdINCENP

A full-length cDNA of DdINCENP was constructed by the Polymerase Chain Reaction (PCR) in two separate fragments as follows. A 0.7kb N-terminal cDNA fragment of the gene was amplified from a *Dictyostelium* cDNA library using primers AO420 (5'-ATGGATTTTATTAAAAAAAATACAATTAATAGAATCAATGGGTTACC-3') and AO441 (5'-GTGAAACAGCAATTGATTGAAACTCATGATAAAC-3'). A 3.2kb C-terminal segment of the gene was generated from AX2 genomic DNA using primers AO442 (5'-GTTTATCATGAGTTTCAATCAATTGCTGTTTCAC-3') and AO387 (5'-CTCGAGTTATTTTTTATTAACAATGATTGGATTAGTAAAACCC-3'). These two segments were ligated to generate the full-length cDNA of DdINCENP. The GFP-DdINCENP construct was generated by cloning the DdINCENP cDNA downstream of the GFP sequence in the pTXGFP plasmid. pTXGFP is a generous gift from Dr. Tom Egelhoff (Levi et al. 2000).

2.2 Cell culture, transformation and constructs

Three different parental cell lines were used in these experiments, AX2, NC4A2 and ORF+. The cells were cultured with HL-5 medium in Petri dishes at 19^{o}C. When the cells were tested for sensitivity to the microtubule-depolymerizing drugs, they were grown in 50 ml flasks on a shaker at 200 rpm. The cultures all started at 2x10^{5} cells/ml.

The GFP-DdCP224 construct is a generous gift from Dr. Graf (Graf et al. 2000). The fusion protein is driven by the constitutive actin 15 promoter. The GFP-α-tubulin construct is a gift from Dr. Gerisch (Kimble et al. 2000). The GFP-myosin construct was a gift from Dr. Spudich. All these three plasmids carry a G418-resistance cassette. The constructs were introduced into the cells with electroporation and the transformants were

selected in the HL-5 medium by 10 μg/ml G418. Both thiabendazole and nocodazole are from Sigma (Sigma Chemical Co., St. Louis, MO).

2.3 Disruption of the DdINCENP gene by homologous recombination

The kinase domain dead mutant of Aurora B was contructed by Hui Li in our laboratory. DdINCENP null cells were generated by homologous recombination between a DdINCENP knockout construct and the DdINCENP coding sequence on the genome. The knockout construct consisted of a blasticidin-resistance (BSR) cassette flanked by 5' and 3' fragments of the DdINCENP gene. The 1 kb 5' fragment was amplified by AO386 (5'-GAGCTCGGTATTGCAAAGCCAACACCACTTAC-3') and AO402 (5'-GGTACCGTTGCTGATGCTGTATTAGCAGC-3'), and the 1 kb 3' fragment was amplified by AO403 (5'-AAGCTTGACGTTAATCAAAGTACAAAAGATAAATC-3') and AO387 (5'-CTCGAGTTATTTTTTATTAACAATGATTGGATTAGTAAAACCC-3'). Both fragments were cloned into plasmid pSP72-Bsr (Wang et al. 2002). The construct was linearized by SacI and XhoI before its introduction into AX2, NC4A2 or ORF+ cells via electroporation. The transformants were then cultured on 96 well plates. Individual clones were screened by PCR and confirmed by western blotting with rabbit anti-INCENP antibodies. A total of six DdINCENP null cell lines were identified. All the phenotypes of the DdINCENP null cells described in the paper were verified by examining at least two of the knockout cell lines.

2.4 Western blot analysis

A GST-DdINCENP$_{676-852}$ fusion protein was expressed in *E. coli* and purified according to previous protocols (Kwak et al. 1999). The purified fusion protein was injected into rabbits to raise polyclonal anti-DdINCENP antibodies (Cocalico Biologicals, Reamstown, PA). For the western blot analysis of DdINCENP, whole cell lysates of

$1x10^6$ cells were loaded in each lane and separated on a 6% SDS-PAGE gel (Koonce et al. 1990).

2.5 Microscopy of live cells

For live microscopy of mitotic cells, cells in log phase growth were plated on a small Petri dish with a cover slip on the bottom (MatTek, Ashland, MA). After the cells attached to the plate, the medium was removed and replaced with low-fluorescence (LF) medium for at least 30 minutes before observations (Bretschneider et al. 2002). The live imaging of the cells was conducted by using a Nikon Eclipse TE200 microscope (Nikon Instruments, Dallas, TX) equipped with a 100 × 1.4 NA PlanFluor Objective, shuttered illumination, and a Quantix 57 camera (Roper Scientific, Tucson, AZ) controlled by Metamorph (Universal Imaging Corp., West Chester, PA). The exposure time for the GFP fluorescence was 50 ms with the interval time being at least 10 s.

2.6 Immunostaining and microscopy of fixed cells

The cells were allowed to attach to the acid-cleaned cover slips overnight in a 8.5cm Petri dish prior to fixation. Then they were fixed according to the protocol published by Dr. McIntosh (Koonce and McIntosh 1990). Briefly, the cells were fixed at room temperature with 2.5% formaldehyde in a PIPES-EGTA buffer for 3 minutes followed by 1% formaldehyde in dehydrated methanol at -10^oC for 5 minutes. The anti-alpha-tubulin antibody is a monoclonal antibody from Sigma (Sigma Chemical Co., St. Louis, MO). Anti-DdCP224 antibody is a gift from Dr. Graf. The secondary antibody is a Texas-red conjugated goat-anti-mouse antibody (Molecular Probes, Eugene, OR). The protocol for DAPI-staining was described previously (Gerald et al. 2001).

The protocol for staining F-actin was modified from the one previously described by Gerald et. al (Gerald et al. 1998). Briefly, 2 $x10^5$ cells were plated on a coverslip.

Then, the cells were fixed with 3.7% formaldehyde in PBS for 30 minutes at room temperature before being permeabilized by 0.2% Triton X-100 in PBS for 5 minutes. The coverslip was washed 3 times in a fixing chamber with PBS buffer before the cells were stained with TxRed conjugated Phalloidin (Molecular Probes, Eugene, OR) in PBS for 30 min in a covered humid chamber at room temperature. The coverslip was washed three times in PBS, and rinsed briefly in dH_2O before being mounted onto a glass slide.

2.7 Mutagenesis of DdCP224

Point mutations in the DdCP224 gene were introduced using the Quikchange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The cDNA sequence of DdCP224 (6.5 Kb) was first cleaved out of the expression vector SpexG418 (Graf et al. 2000) to be ligated into pSP72 vector by using its flanking digestion sites of Sac I and BamH I. SpexG418 is a construct based on the GFP fusion vector pB15-GFP (6 Kb), which is bigger than the 2.9Kb pSP72 vector. This smaller vector facilitated the amplification reactions in the process of mutagenesis. All the following amplification reactions were done using PSP72-DdCP224. Four primers were used to mutate each of the potential Aurora B phosphorylation sites, S368 and S1268. To mutate S368 to alanine, I used primers, AO599 (5'- GAGAAAAAGACAGCTGTTTTACAATC AGTTCATACTACAATGG-3') and AO600 (5'- CCATTGTAGTATGAACTGA TTGTAAAACAGCTGTCTTTTTCTC-3') to amply DdCP224 gene sequence. To mutate S368 to glutamic acid, I used primers, AO601 (5'- GAGAAAAAGACAGAAGTTTTA CAATCAGTTCATACTACAATGG-3') and AO602 (5'- CCATTGTAGTATGAACT GATTGTAAAACTTCTGTCTTTTTCTC-3'). To mutate S1268 to alanine, two primers AO603 (5'- CAAACAGATCTTTAAACAAGCGATTCAACAATTGGAAGAAC-3') and AO604 (5'- GTTCTTCCAATTGTTGAATCGCTTGTTTAAAGATCTGTTTG-3') were used. To mutate S1268 to glutamic acid, AO605 (5'- CAAACAGA

TCTTTAAACAAGAGATTCAACAATTGGAAGAAC-3') and AO606 (5'-GTTCTTCCAATTGTTGAATCTCTTGTTTAAAGATCTGTTTG-3') were used. After the mutagenesis, the cDNA sequence of DdCP24 was sequenced to determine that the mutations were introduced at the desired sites and that there were no secondary mutations. The mutated cDNAs of DdCP224 were re-ligated to pB15-GFP vector to be expressed in *Dictyostelium* cells.

2.8 Construction of truncated DdINCENP mutants

The C-terminal truncated mutants of DdINCENP were made by utilizing internal restriction endonuclease sites in the DdINCENP gene. $DdINCENP_{1-273}$ was made by cutting one of the internal EcoRI site closest to the N-terminus of the DdINCENP sequence. $DdINCENP_{1-500}$ was made by cutting the only PvuII site in the gene. $DdINCENP_{1-1013}$ was made by cutting the only BamHI site in the gene. In contrast, $DdINCENP_{488-1321}$ was amplified with the primers AO386 and AO387 (described above). All four truncated mutants were cloned downstream of the GFP sequence in the pTXGFP expression vector.

2.9 TAP purification

$DdINCENP_{1-500}$ was cloned downstream of the TAP-GFP sequence in the pTX-TAPGFP sequence. pTX-TAPGFP was constructed by Joe Mireles in our laboratory. The procedure for purifying the TAP tagged protein in *Dictyostelium* was modified from the one described by Rigaut (Rigaut et al. 1999) and that by Koch (Koch et al. 2006). For large scale purification, 500 ml of cells expressing either TAP-GFP or TAP-GFP-$DdINCENP_{1-500}$ were cultured in a 2 liter flask on a shaker at 200 RPM. The cells were harvested when the density reached $5x10^6$ cells/ml. The cells were centrifuged at 5000 rpm for 5 minutes and washed twice with PDF buffer. The pellet was resuspended in 40

ml of lysis buffer (50 mM Tris-Cl, 50 mM KCl, 2mM $MgCl_2$, 0.5 mM DTT, 150mM NaCl, PH 8.0) with protease inhibitors (5 μg/ml Leupeptin, 0.1 mM PMSF, 1 mM TAME, 1 μg/ml Trypsin Inhibitors, 0.8 μM Aproptinin, 1 mM Benzamidine, 0.1 mM AEBSF, 10 μM E-64, 1 μg/ml Pepstatin, 40 μM Bebstatin) (Sigma Chemical Co., St. Louis, MO). The suspension was passed 3-4 times through a nebulizer on ice. To ensure the total lysis of the cells, 10ul of the lysate were examined on a hemocytometer with a light microscope. The cell lysate was centrifuged at 14,000 g for 30 minutes. At the same time, 200 μl IgG agarose beads (Amersham Biosciences, Uppsala, Sweden) were pre-equilibrated and washed with 15ml IPP150 buffer (10 mM Tris-Cl, 150 mM NaCl, 0.1 % NP40, pH 8.0). The IgG beads were added to the supernatant of the cell lysis and they were incubated on a rotator for 2 h at 4°C in a 50 ml tube to allow the TAP tagged proteins bind to the beads. The suspension was packed into a 15 ml column (BIO-RAD, Hercules, CA, USA). The packed beads were washed with 30 ml IPP150 buffer followed by 10 ml of TEV cleavage buffer [10 mM Tris-Cl, 150 mM NaCl, 0.1% NP40, 0.5 mM EDTA, 1 mM DTT (added immediately before use), pH 8.0]. Then, 150 units (15μl) of AcTEV protease (Invitrogen, Carlsbad, CA) were added with 1 ml TEV cleavage buffer to the column. The suspension was incubated at 4°C in the column on a rocker for overnight to allow the thorough digestion of the proteins from the beads.

The flow through (1 ml) from the column was collected and a small aliquot was examined by SDS-PAGE gel. Although the standard protocol called for a 2-step affinity purification (Rigaut et al. 1999), the first step was found to be very efficient in eliminating unspecific binding proteins. There were surprisingly few unspecific proteins found in the sample prepared from the tagged proteins, after comparing it with that from the vector only control. Another 1 ml of TEV cleavage buffer was added to the beads and the two elutes were combined. 6 ml calmodulin binding buffer (CBB) [10 mM Tris-

Cl, 150 mM NaCl, 0.1% NP40, 10 mM â-mercaptoethanol (add just before use), 1 mM Mg-Acetate, 1 mM imidazole, 2 mM CaCl2, pH 8.0] and 6µl 1 M CaCl2 were added to the eluate in a 15 ml column. 200 µl of calmodulin beads (Stratagene, La Jolla, CA), washed beforehand with 10 ml of CBB were added to the column. The suspension was rotated for 1 h at 4°C to allow the binding of the tagged proteins to the beads. Then, the beads were washed with 30 ml of CBB. To elute the proteins from the beads, 200 ul of hot reduced sampling buffer (RSB) were added to the beads and the beads were boiled at 95°C for 5 minutes. The eluate was loaded on a SDS-gel for analysis. Otherwise, the proteins were eluted with 1 ml of CBB containing 20 mM EGTA and were precipitated with trichloroacetic acid (10% final concentration) for 30 minutes at 4°C. The pellet was washed twice with cold acetone and resuspended in 100 ul RSB for analysis.

Mass spectrometry identification of the proteins was done by the core facilities of Institute for Cell and Molecule Biology at the University of Texas at Austin.

2.10 Streaming Assay

To study the localization of GFP-DdINCENP1-500 in the starved cells undergoing chemotactic aggregation, cells in log phase growth were plated on a small Petri dish with a cover slip on the bottom (MatTek, Ashland, MA). When the cells grew to near confluency on the plate, the media was replaced with PDF buffer (2 mM KCl, 1.1 mM K2HPO4, 1.32 mM KH2PO4, 0.1 mM CaCl2, 0.25 mM MgSO4, pH 6.7) and the cells were starved in the buffer for about 12 hours at 18°C. After aggregation of the cells was observed on the plate, live fluorescence microscopy was used to follow the movement of the cells.

2.11 Triton X-100 exaction of the actin cytoskeleton

For this experiment, all the following steps were done on ice to avoid the degradation of proteins. $2x10^7$ cells were harvested and washed with cold 100 mM MES buffer (PH 6.8) once. Then the cells were resuspended in 1 ml lysis buffer (100 mM MES, 1 mM $MgCl_2$, 0.5% Triton X-100, 2.5 mM EGTA, 0-200 mM NaCl, PH6.8) with 1% protease inhibitors cocktail (Sigma Chemical Co., St. Louis, MO). The suspension was centrifuged for 1 minute at 4^oC. The supernatant was removed carefully and the pellet was washed with the lysis buffer twice. The pellet was then re-suspended with 1 ml lysis buffer for analysis. Both the supernatant and the pellet suspension were mixed with RSB buffer (1:1) before being analyzed by SDS-PAGE gel.

Chapter 3: *Dictyostelium* DdINCENP, a protein with an important role in mitosis and cytokinesis

3.1 INTRODUCTION

Chromosomal passenger proteins are emerging as important molecules regulating different stages of cell division. Their dynamic localization enables them to play essential roles during both mitosis and cytokinesis. Among this group of molecules, the function of inner centromere protein (INCENP) is poorly understood. INCENP is known to bind to Aurora B kinase through its C-terminal IN box and promote the kinase activity of Aurora B. Additionally, INCENP is also a substrate of Aurora B (see Chapter1). However, it may have other unknown functions particularly during cytokinesis.

INCENP was the first identified chromosomal passenger protein. Its name originated from the finding that it is localized at the region between the centromeres of the two sister chromatids during prometaphase and metaphase. It was discovered in a screen for chromosome scaffold proteins by a group led by Dr. William Earnshaw in 1987 (Cooke et al. 1987). Even since, its unique dynamic localization during cell division has attracted a lot of interests to learn more about its functions. One of the earliest studies showed that the expression of a truncated mutant of chicken INCENP, $INCENP_{1-405}$, caused chromosome segregation defects (Mackay et al. 1998). This finding demonstrated that INCENP plays an important role during mitosis.

The finding of Aurora B kinase and its association with INCENP contributed greatly to uncover the function of INCENP during mitosis. The first orthologue of Aurora B kinase, Ipl1, was identified in a screen for chromosome-gain and increase-in-ploidy mutants in *S. cerevisiae*, long before the discovery that it was a chromosomal passenger protein (Chan et al. 1993). Later, Ipl1 was found to be a protein kinase essential for mitosis (Francisco et al. 1994). The name Aurora came from the *Drosophila* mutant of

this kinase (Glover et al. 1995). Although there is only one Aurora kinase in budding yeast, there are at least two such kinases in each metazoan. One such kinase in *C. elegans*, AIR-2, was found to be a chromosomal passenger protein and essential for cytokinesis (Schumacher et al. 1998). Based on this finding, all the Aurora kinases, which have similar localization and function, are designated as Aurora B. Soon after, it was found that INCENP can form a protein complex with Aurora B (Adams et al. 2000; Kaitna et al. 2000). This was confirmed in *S. cerevisiae* by the discovery that Sli15 is a homolog of INCENP, which has physical and genetic interactions with Ipl1 (Kim et al. 1999; Adams et al. 2000; Kaitna et al. 2000).

All these findings greatly facilitated the effort to uncover the function of INCENP during mitosis. More recent studies showed that INCENP and Aurora B are co-dependent on their localization at the centromeres and the central spindle (Adams et al. 2001; Giet and Glover 2001). Additionally, INCENP was found to stimulate the kinase activity of Aurora B (Kang et al. 2001; Bishop and Schumacher 2002). The discovery of Survivin and Borealin also revealed that INCENP may be a central component of the chromosomal passenger protein complex, because it interacts with every proteins in the complex while Aurora B does not (Gassmann et al. 2004; Klein et al. 2006).

In this study, I identified and characterized the *Dictyostelium* INCENP homolog, DdINCENP. I studied the localization of this protein during mitosis and found it to be a bona-fide chromosomal passenger protein. I also studied the function of this protein during mitosis and cytokinesis by constructing DdINCENP null cell lines. I found that these mutant cells have defects in chromosome segregation, central spindle stability and cytokinesis.

3.2 RESULTS

3.2.1 Identification of the INCENP homolog in *Dictyostelium*

Since the IN-box motif is the only sequence conserved among INCENP proteins from different species, I used this motif to search the *Dictyostelium* genome sequence for potential INCENP homologs. I found that *Dictyostelium* contains only one gene (entry DDB0189463 in http://dictybase.org) that encodes a protein similar to other INCENP proteins. This gene, encodes a predicted protein, named DdINCENP, of 1320 amino acids and 152 kDa. DdINCENP has the conserved C-terminal IN-box domain (Figure 3.1), known to be involved in binding to Aurora B kinase (Kang et al. 2001). This domain includes two highly conserved serine residues that are phosphorylated by Aurora B (Bishop and Schumacher 2002; Cheeseman et al. 2002) (asterisks, Figure 3.1). Within this region, DdINCENP is 44% identical to fission yeast INCENP and 21% identical to vertebrate INCENP. DdINCENP also has a coil-coiled domain found in all other INCENP proteins. The function of this domain is not clear since a truncated chicken INCENP protein without the coil-coiled domain still localizes properly (jMackay et al. 1993). Besides these two conserved domains, no other sequence similarity can be discerned when comparing sequences of INCENP proteins from distant species.

To confirm that DdINCENP is in fact an INCENP homolog, I determined whether it displayed the dynamic distribution characteristic of chromosomal passenger proteins during mitosis. For this purpose, DdINCENP tagged with GFP at the N-terminus was introduced into wild type cells and the movement of GFP-DdINCENP during mitosis was studied by live fluorescence microscopy. To correlate the localization of GFP-DdINCENP with different stages of mitosis, I also stained these cells with DAPI, since *Dictyostelium* cells have a very distinct morphology of condensed DNA during different stages of mitosis (Roos et al. 1984). After examining a large number of mitotic cells

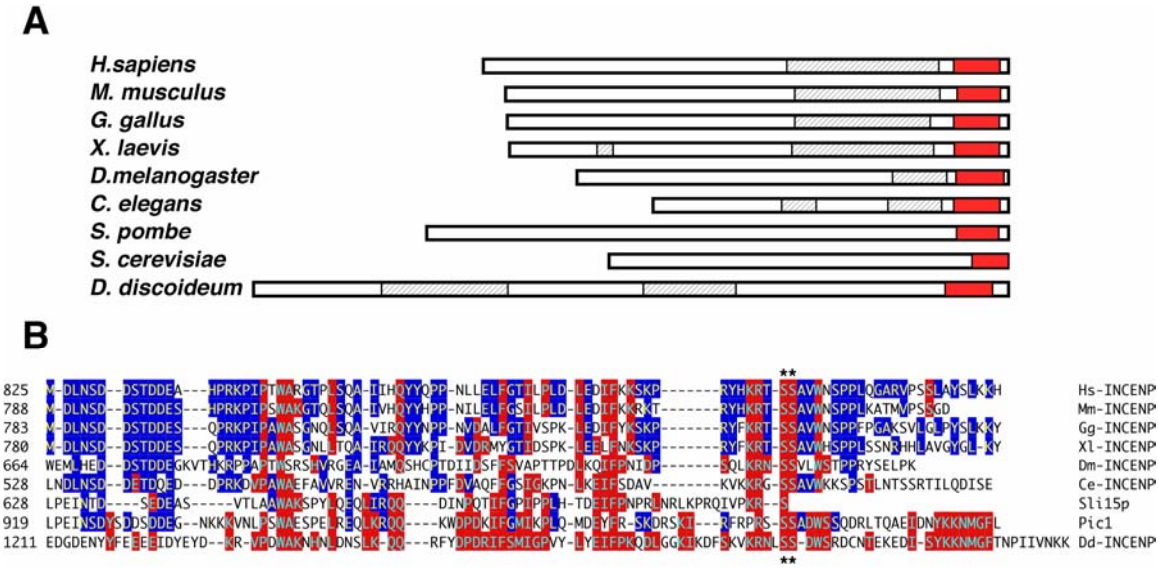


Figure 3.1: Domain organization of INCENP proteins and alignment of the IN-Box domain. (A) Diagram indicating the relative size of INCENP proteins from several organisms. The shaded box near the C-terminus of all INCENPs represents the conserved IN-Box domain. The hatched portions indicate regions with a high likelihood to form a coiled-coil domain as shown by the Protean Software (Lasergene). (B) Alignment of the IN-Box domain of INCENPs from different organisms. Residues highlighted in red indicate those that are identical with the *Dictyostelium* DdINCENP sequence. Residues highlighted in blue indicate those that are conserved in at least 4 species other than *Dictyostelium*. Asterisks indicate the serine residues phosphorylated by Aurora B (Bishop and Schumacher 2002).

expressing GFP-DdINCENP, I concluded that DdINCENP has a pattern of localization during mitosis similar to that of other INCENP proteins.

During prometaphase, GFP-DdINCENP concentrated at a few dots adjacent to the condensed chromosomes (Figure 3.2 A, B). Occasionally, a dot of GFP-DdINCENP could be seen at one end of an isolated chromosome (Figure 3.2 A, B). Since *Dictyostelium* chromosomes are known to be telocentric, I interpreted these images as representing the centromeric localization of GFP-DdINCENP. At metaphase, GFP-DdINCENP was concentrated at a single dot in the middle of the spindle and was surrounded by the chromosomes aligned on the metaphase plate (Figure 3.2 C, D). This interpretation was confirmed by our collaborator W. Nellen (University of Kassel, Germany). His group demonstrated that HcpA, a homolog of heterochromatin protein 1 (HP1) in *Dictyostelium*, was colocalized at centromeres together with histone 3 lysine 9 dimethylation (H3K9Me2) during mitosis (Kaller et al. 2006). They found that GFP-DdINCENP was colocalized with HcpA and H3K9Me2 during metaphase (Figure 3.3 A, C).

After the onset of anaphase, GFP-DdINCENP moved to the spindle midzone and the spindle poles (Figure 3.2 E). During anaphase, GFP-DdINCENP did not localize to the daughter chromosome centromeres still labeled by HcpA and H3K9Me2 (Figure 3.3 B"', D"'). GFP-DdINCENP remained at the spindle midzone and spindle poles through late telophase (Figure 3.2 F, G). When the cleavage furrow was initiated, GFP-DdINCENP began to concentrate increasingly at the furrow area, especially at the cortex region (Figure 3.4). As the furrow progressed, the cortical localization of GFP-INCENP at the furrow became more prominent (Figure 3.4). By the abscission stage of cytokinesis, GFP-INCENP was highly concentrated in the thin cytoplasmic bridge connecting the two daughter cells (Figure 3.4). Therefore, the dynamic localization of

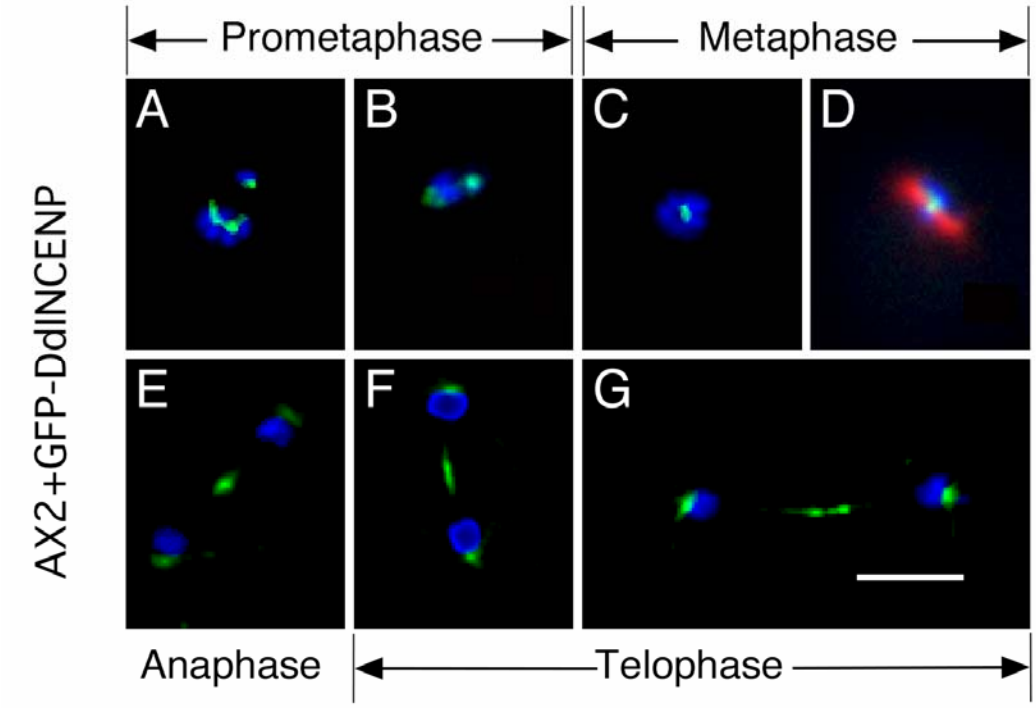


Figure 3.2: DdINCENP displays dynamic localization during mitosis. (*A-G*) Fluorescence images of AX2 cells expressing GFP-DdINCENP (green). DNA is shown in blue and tubulin is shown in red. (*A, B*) During prometaphase, GFP-DdINCENP concentrated at a few dots adjacent to the condensed chromosomes. These dots were consistent with a centromeric localization of DdINCENP. (*C, D*) During metaphase, GFP-DdINCENP concentrated in a single dot, which was surrounded by the chromosomes aligned on the metaphase plate. The dot may represent the conglomerate of the kinetochores at metaphase. The cell in C was imaged from one pole of the mitotic spindle. The cell in D was imaged from the side of the spindle. Notice that DdINCENP was not found at the spindle poles at this stage. (*E-G*) From anaphase to telophase, GFP-DdINCENP localized at the spindle poles and the spindle midzone. Bar, 5 μm.

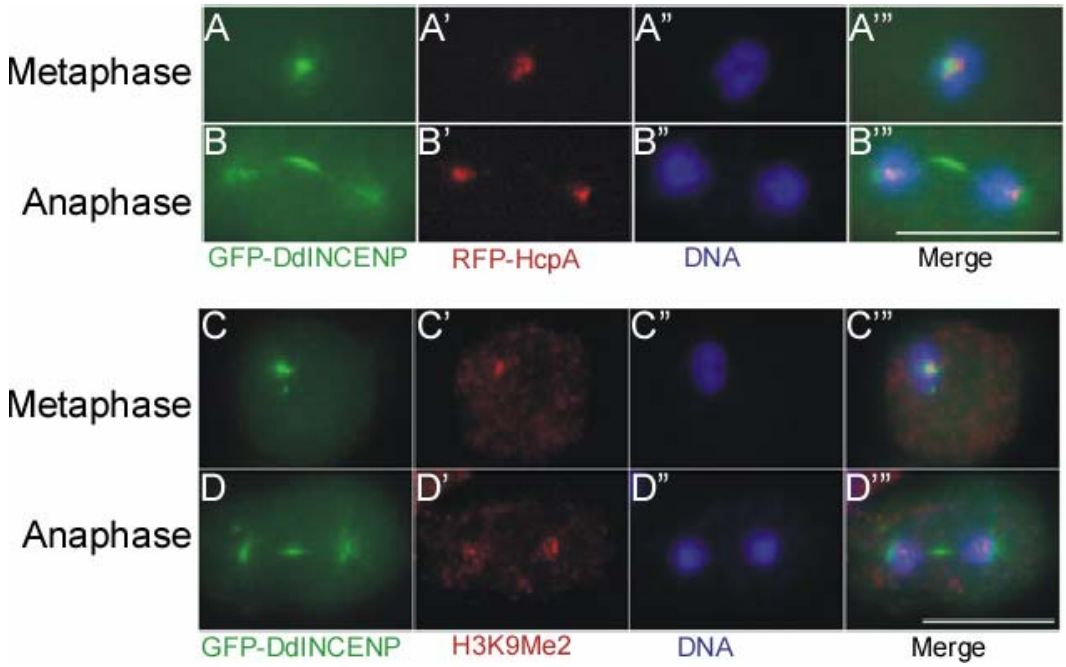


Figure 3.3: DdINCENP localizes to the centromeres during metaphase and then transfers to the central spindle and the spindle pole bodies. GFP-DdINCENP colocalized with two centromeric markers, RFP tagged HcpA or histone H3 lysine 9 dimethylation (H3K9Me2). (A-A"', B-B"') Shown are wild type cells expressing both GFP-DdINCENP (green) and RFP-HcpA (red). The cells were fixed and stained with DAPI to shown DNA (blue). (C-C"', D-D"') Shown are wild type cells expressing GFP-DdINCENP. The cells were fixed and stained with DAPI and anti-H3K9Me2 antibody (red). During metaphase, GFP-DdINCENP was found at the same loci with HcpA and H3K9Me2 in the center of the metaphase plate (A"', C"'). During anaphase, GFP-DdINCENP was found on the central spindle and the spindle pole bodies (A, D). At this stage, the centromeres, as labeled by HcpA or H3K9Me2, have moved next to the spindle poles (B"', D"'). Bar, 10um. These images were kindly provided by Kaller and Nellen (Kassel University, Germany).

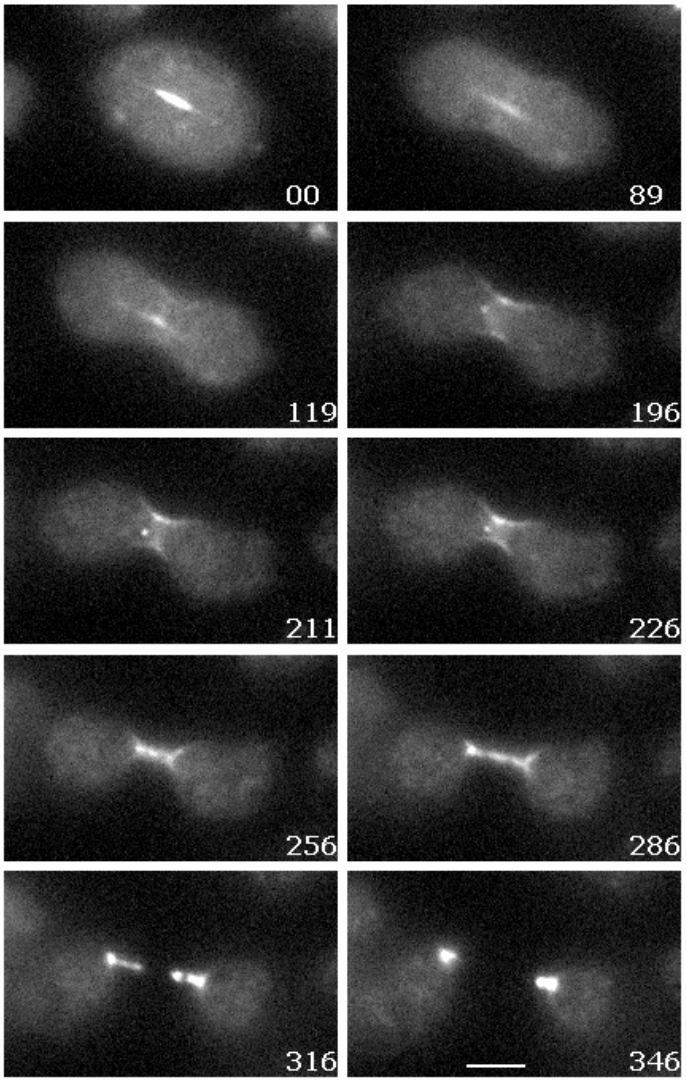


Figure 3.4: DdINCENP localizes at the cleavage furrow during cytokinesis. An AX2 cell expressing GFP-DdINCENP was followed by time-lapse video microcopy from early telophase. GFP-DdINCENP was especially concentrated in the cortex region of the furrow and the intracellular bridge during cytokinesis. The numbers indicate time in seconds. Bar, 5 μm.

GFP-DdINCENP during mitosis and cytokinesis is typical of a chromosomal passenger protein.

Further evidence that DdINCENP is a bona fide INCENP protein also came from the study of the association of DdINCENP with Aurora kinase. The *Dictyostelium* genome contains a single Aurora-like kinase gene (Genebank Accession XP_641803). My co-worker Hui Li has generated antibodies against this predicted protein and used them to immunoprecipitate DdAurora from *Dictyostelium* extracts. He found that DdINCENP co-immunoprecipitated with DdAurora indicating that both form part of a chromosomal passenger complex.

Interestingly, I found that GFP-DdINCENP localized to several discrete foci in the nucleus during interphase (Figure 3.5 A-D). Careful analysis of these cells revealed that these foci were distributed around the periphery of the nucleus, closely associated with the nuclear envelope. Quantification of many cells revealed that each nucleus had an average of seven GFP-DdINCENP foci (n=111, Figure 3.5E). Since *Dictyostelium* has six chromosomes plus one extra chromosomal element (see chapter 1), I postulated that these foci represented the localization of centromeres during interphase. However, Kaller et al. found that the centromeres, labeled by both RFP-HcpA and H3K9Me2, are localized in one single cluster during interphase (Kaller et al. 2006). Additionally, they found that none of the foci labeled by GFP-DdINCENP overlapped with the cluster recognized by HcpA and H3K9Me2 (Figure 3.5). Therefore, it appears that GFP-DdINCENP, like metazoan INCENPs, does not bind to centromeres during interphase. Unfortunately, I do not know yet what is the identity of the GFP-DdINCENP labeled interphase foci. In chapter 5, I will show that some of those foci may be found inside the nucleoli (Discussed in Chapter 5).

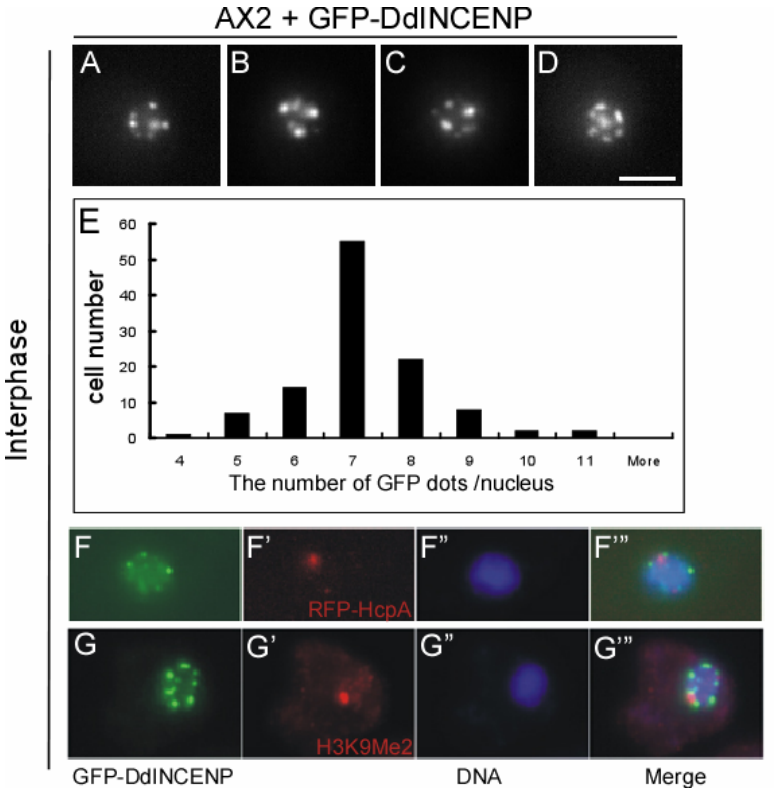


Figure 3.5: DdINCENP does not localize to the centromeres during interphase. (A-D) During interphase, GFP-DdINCENP localized in several discrete foci in nucleus. The fluorescence images of four wild type cells expressing GFP-DdINCENP are shown. (E) The average number of the foci labeled by GFP-DdINCENP in each nucleus is seven (n=111, standard deviation=1.2, mean=7.2). However, GFP-DdINCENP did not colocalize with the centromeric makers, HcpA or H3K9Me2. (F-F''') Wild type cell expressing both GFP-DdINCENP (green) and RFP-HcpA (red). The cell was fixed and stained with DAPI to shown DNA (blue). (G-G''') Wild type cell expressing GFP-DdINCENP. The cell was fixed and stained with DAPI and anti-H3K9Me2 antibody (red). Bar, 5um. Images F-F''' and G-G''' were kindly provided by Kaller and Nellen (Kassel University, Germany).

3.2.2 DdINCENP is important for mitosis

To determine the function of DdINCENP in *Dictyostelium*, I disrupted the DdINCENP gene by targeted gene knockout and characterized the phenotype of the DdINCENP null cells. The loss of DdINCENP in these cells was confirmed by PCR (data not shown) and western blot analysis (Figure 3.6 A). DdINCENP null cells derived from different parental cell lines (AX2, NC4A2 and ORF+) shared a common phenotype: all DdINCENP null cells divided at much slower rate than wild type cells (Figure 3.6 B), and a high percentage of mutant cells became multinucleate (39%, n=395) (Figure 3.6 C). Furthermore, 33% (n=395) of the mutant cells had an enlarged nuclei, a sign of failed mitosis. This phenotype suggested that the DdINCENP null cells harbored defects in both mitosis and cytokinesis. To further investigate this phenotype, I introduced a GFP-histone 2B construct into DdINCENP null cells to monitor their ability to segregate chromosomes. I found that some DdINCENP null cells had serious chromosome segregation defects; some of them were arrested at metaphase (data not shown), while others had lagging chromosomes (Figure 3.7). The behavior of chromosomes in the DdINCENP null cells contrasted sharply with that of wild-type cells which always displayed normal segregation of chromosomes (Graf et al. 2003) (Figure 3.7). The mitotic and the cytokinetic defects displayed by the DdINCENP null cells were consistent with the observation in other organisms that INCENP plays important roles during mitosis (Kaitna et al. 2000; Adams et al. 2001).

3.2.3 DdINCENP regulates the spindle localization of microtubule stabilizing protein DdCP224

To investigate the mitotic defect of DdINCENP null cells in more detail, I decided to compare the structure of the mitotic spindle of wild type and mutant cells. Like fungi, the nuclear envelope of *Dictyostelium* cells does not dismantle during mitosis and the

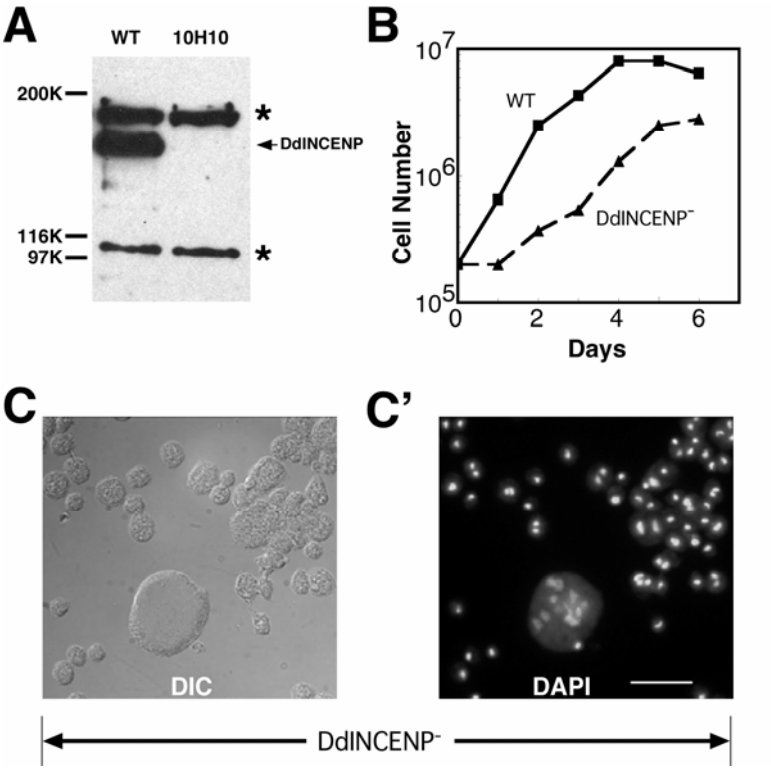


Figure 3.6: DdINCENP null cells have mitosis and cytokinesis defects. (*A*) Immunoblot analysis of whole lysates of wild type (WT) and DdINCENP null cells (10H10) probed with anti-DdINCENP antibodies. The arrow points to the position of the DdINCENP protein (140K Da) and * indicates the positions of two unspecific bands recognized by the polyclonal antibodies. (*B*) Growth curve of the wild cells (square solid line) and DdINCENP null cells (triangle dash line) in suspension cultures. DdINCENP null cells grew much more slowly than wild type cells. (*C-C'*) A typical population of DdINCENP null cells. The cells were visualized by Differential Interference Contrast (DIC) microscopy (C) and stained with DAPI to visualize their nuclei (*C'*). While some cells contained a single normal nucleus, many cells had multiple or enlarged nuclei. Bar, 15 μm.

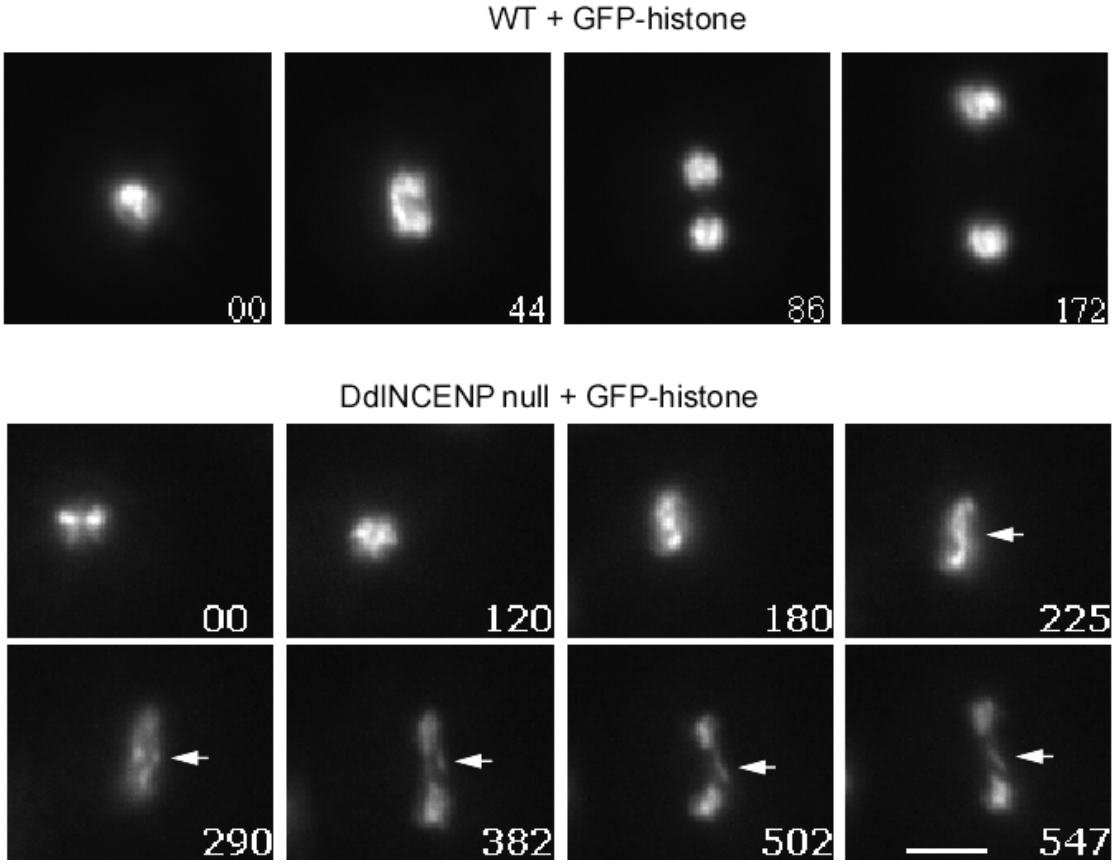


Figure 3.7: DdINCENP null cells have a chromosome segregation defect. Time-lapse video microscopy of wild type and DdINCENP null cells during mitosis, both of which expressed GFP-histone 2B. The micrographs were taken by fluorescence microscopy to show the movement of chromosomes (as indicated by GFP-histone 2B) from metaphase to anaphase in these two cells. The times are indicated in seconds. As shown, the wild type cell segregated its chromosomes normally in a short amount of time (86 sec.). However, the DdINCENP null cell displayed lagging chromosomes (arrows) during the segregation and stalled at anaphase. Other cells were arrested at metaphase for the entire observation period. Bar, 5 μm.

spindle is assembled inside the nucleus. Wild type cells display robust spindles that have a defined midzone region of overlapping microtubules (Roos et al. 1984; McIntosh et al. 1985) (Figure 3.8 A, B). In contrast, the spindle in most DdINCENP null cells was very thin and the midzone region was difficult to identify (Figure 3.8 D). Some DdINCENP null cells in anaphase or telophase had no perceptible central spindle (Figure 3.8 C, E). This phenotype indicated that DdINCENP is critical for the assembly or maintenance of the spindle midzone during mitosis.

My results agree with previous studies showing a role for the chromosomal passenger protein complex in the stability of the mitotic spindle in yeast (Buvelot et al. 2003; Pereira et al. 2003). In vertebrate systems, the chromosomal passenger protein complex regulates the localization and activity of MCAK, a microtubule-depolymerizing protein crucial for spindle assembly (Lan et al. 2004; Sampath et al. 2004) (Also see chapter 1). The activity of MCAK is antagonized *in vivo* by XMAP215/TOGp, a microtubule-stabilizing protein (Cassimeris et al. 2004; Holmfeldt et al. 2004). Thus, I considered the possibility that the spindle defect of our DdINCENP null cells may be due to misregulation of these microtubule regulatory proteins.

Although the protein MCAK has not been characterized in *Dictyostelium*, the *Dictyostelium* homolog of XMAP215, DdCP224, is a centrosome-localized protein known to be crucial for the stability of microtubules during interphase. During mitosis, DdCP224 localizes at both spindle pole bodies and the spindle midzone (Figure 3.9 A, B) (Graf et al. 2000; Graf et al. 2003). I found that DdCP224 localized normally to the interphase centrosomes in the DdINCENP null cells. However, while DdCP224 still localized at the spindle pole bodies of these cells, it was absent from their spindle midzone (Figure 3.9 C, D). The absence of DdCP224 at the spindle midzone could underlie the spindle defect of DdINCENP null cells. To investigate this possibility I

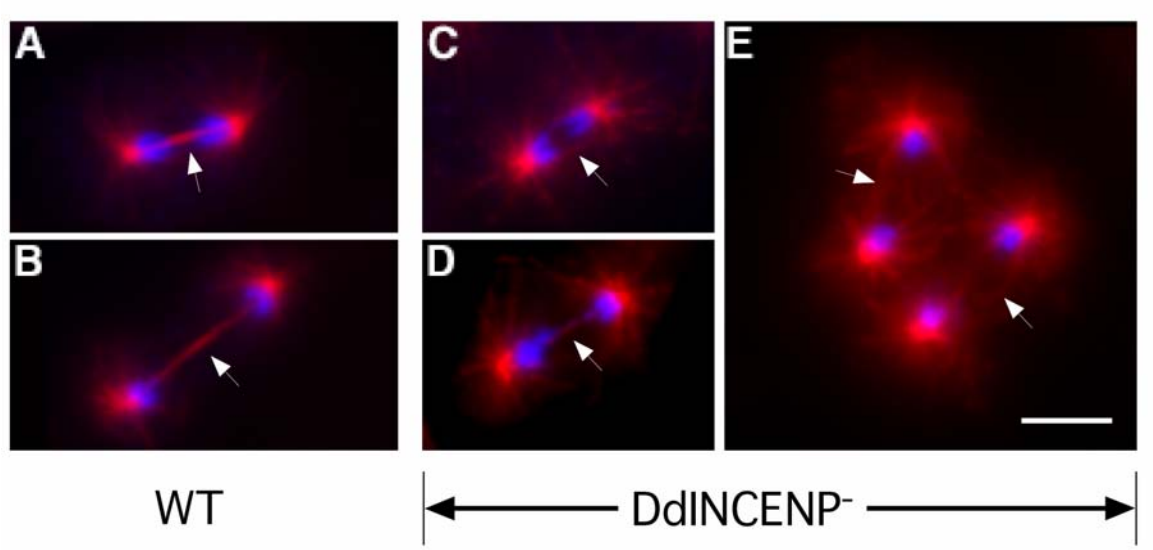


Figure 3.8: DdINCENP null cells have a defective spindle midzone. (A-E) Merged immunofluorescence images of wild type (A, B) and DdINCENP null cells (C-E) during anaphase, showing DNA in blue and tubulin in red. The arrows point to the position of the spindle midzone in these cells. The mitotic wild type cells had robust spindle midzone during anaphase. However, the DdINCENP null cells showed weak spindle midzone (D) and sometimes the spindle midzone was imperceptible (C, E). Bar, 5 μm.

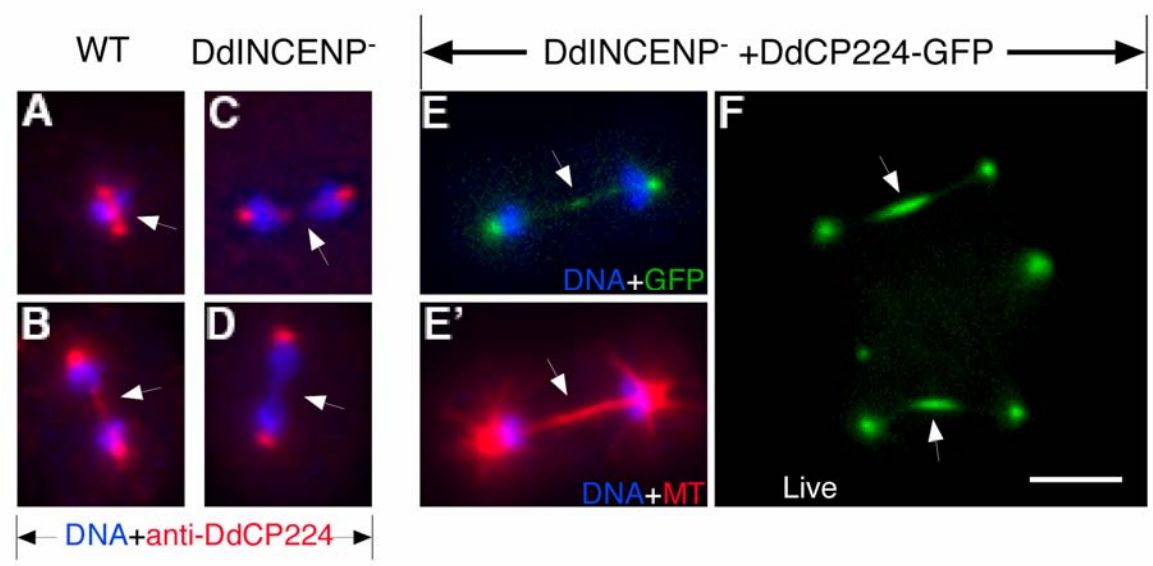


Figure 3.9: DdINCENP is essential for the localization of DdCP224, an XMAP215/TOGp homolog, to the spindle midzone. (A-D) Merged immunofluorescence images of wild type cells (A, B) and DdINCENP null cells (C, D) during anaphase. DdCP224 localized at the spindle poles and the spindle midzone during mitosis in wild type cells. However, DdCP224 was absent from the spindle midzone in the DdINCENP null cells. DNA is shown in blue and DdCP224 is shown in red. Arrows point to the position of the spindle midzone. (E-F) Overexpression of DdCP24 rescued the spindle midzone defect of DdINCENP null cells. (E, E') The immunofluorescence pictures of a DdINCENP null cell over-expressing DdCP224-GFP (green), showing DNA in blue and tubulin in red. Arrows point to the position of the spindle midzone. DdCP224-GFP was localized on the spindle midzone (E). As a result, these cells had a much more robust spindle midzone (E') than DdINCENP null cells (Figure 3.8). (F) The fluorescence image of a live DdINCENP null cell expressing DdCP224-GFP. The cell was at anaphase. Over-expressed DdCP224-GFP was clearly localized in the spindle midzone. Bar, 5 μm.

tested whether enhanced expression of DdCP224 could stabilize the spindle midzone of DdINCENP null cells. I introduced into the mutant cells a vector for the overexpression of GFP-tagged DdCP224 (Graf et al. 2000), and found that this protein was localized on both the spindle pole bodies and the spindle midzone of dividing cells (Figure 3.9 E, F). Furthermore, the spindle midzone of these DdINCENP null cells was now as robust (9/10 cells) as those observed in wild type cells (Figure 3.9 E'). Therefore, DdINCENP is essential for the localization of DdCP224 at the spindle midzone and DdCP224 is a likely downstream target of the chromosome passenger protein complex for stabilizing the spindle midzone.

3.2.4 Searching of the potential phosphorylation sites of DdCP224 by Aurora B

To further investigate the possibility that DdCP224 is a substrate of Aurora B kinase, I wanted to examine its localization in the Aurora B null cells. However, the depletion of Aurora B appeared to be lethal in *Dictyostelium* (Hui Li, personal communication). Therefore, I decided to test whether overexpression of the kinase dead domain of Aurora B (AurB-KD) (constructed by Hui Li) would affect the mitotic localization of DdCP224. Surprisingly, I found that DdCP224 still localized to the central spindle in cells expressing GFP-AurB-KD (Figure 3.10 B).

Another way to test my hypothesis was to find the potential phosphorylation sites in the sequence of DdCP224. Because I expected these sites to be conserved among the different members of DdCP224 family proteins, I decided to search for them in the two conserved domains of DdCP224 (Graf et al. 2000). The conserved amino acid sequence of the phosphorylation sites by Aurora B kinases has been identified as [RK]X[T/S][ILV] (X represents any amino acid) (Cheeseman et al. 2002). The search returned one potential phosphorylation site in each of the conserved domains of DdCP224. Both of them are serine sites, S368 in the motif [KTSV] and S1268 in [KQSI]. S368 was a

particularly interesting one, because it was conserved in the homolog of DdCP224 in *S. cerevisiae*, *S. pombe* and *C. elegans* (Figure 3.10 A). S1268 is in the conserved region 2, which is only found in DdCP224 and its *C.elegans* homolog, Zyg-9 (Figure 3.10 A). Nevertheless, a corresponding site could still be found in Zyg-9. Therefore, I decided to test the role of these two potential phosphorylation sites by changing these two serines to either alanines (A) to mimic the unphosphorylated status, or glutamic acids (E) to mimic the phosphorylated status. The two mutants were named DdCP224-AA and DdCP224-EE.

To test whether the mutations affect the mitotic localization of DdCP224, I examined whether these two mutants, tagged with GFP, were able to localize to the central spindle. I found that the distribution of both mutants at the central spindle was indistinguishable from the wild-type protein (Figure 3.10 C, D). In addition, overexpression of the wild-type or mutant proteins did not appear to have a dominant negative effect on the growth of wild type cells (Figure 3.10 E). Interestingly, I found that cells overexpressing wild-type DdCP224-GFP were hypersensitive to the microtubule depolymerizing drug thiabendazole (TBZ). In contrast, overexpression of either DdCP224-AA-GFP or DdCP224-EE-GFP did not cause hypersensitivity to thiabendazole (Figure 3.10 F). It was previously shown that overexpression of DdCP224 causes the formation of supernumerary centrosomes (Graf et al. 2000). It is likely that the sensitivity of these cells to TBZ is the result of the abnormal centrosome number.

It is an interesting discovery that the mutant DdCP224 proteins can localize normally but do not cause sensitivity to TBZ. This finding suggests that, while the localization of DdCP224 may not be controlled by the phosphorylation, its activity on the centrosomes and spindle may be controlled by Aurora phosphorylation. Since the depletion of DdCP224 was lethal (Ralph Graf, personal communication), I was not able

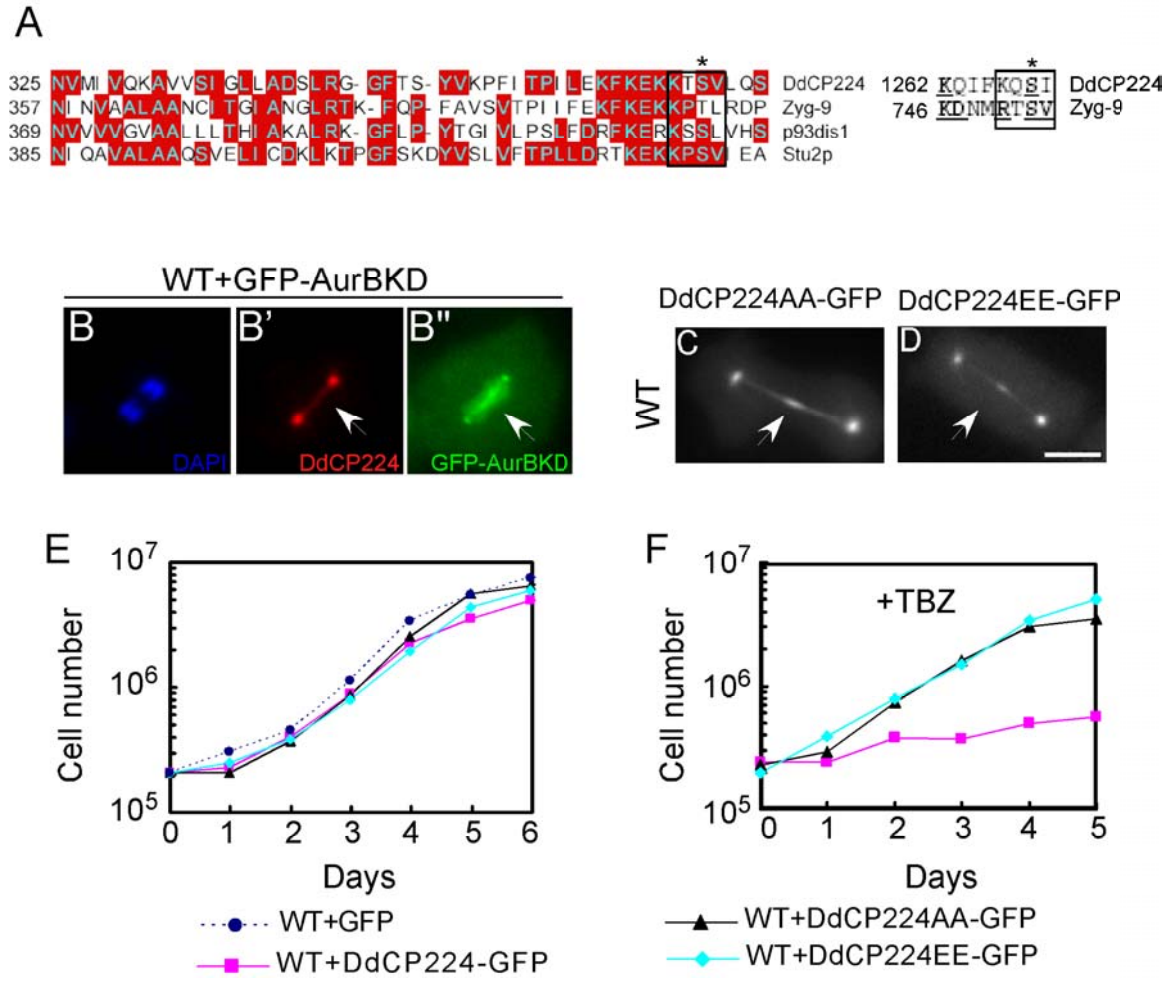


Figure 3.10 (see next page for the legend)

Figure 3.10: Identification of potential phosphorylation sites of DdCP224 by Aurora B kinase. (A) There are two potential phosphorylation sites (*) by Aurora B. The first serine site is in the conserved region 1 of DdCP224 (left) and appears to be conserved among the DdCP224 homolog of *S. pombe* (P93dis1), *S. cerevisiae* (Stu2p) and *C. elegans* (Zyg-9). Residues highlighted in red indicate those that are conserved in at least 2 species and the box indicates the consensus Aurora B phosphorylation site. The second serine site is in the conserved region 2 of DdCP224 (right). P93dis1 and Stu2p do not share this region (Graf et al. 2000). (B, B', B") The expression of kinase domain dead Aurora B (AurB-KD) in wild type (WT) cells did not disrupt the localization of DdCP224 at the central spindle. Immunofluorescence micrographs of wild type cells expressing GFP-AurB-KD are shown. DNA is shown in blue. GFP-AurB-KD is shown in green. The localization of DdCP224 was determined by immunofluorescence, shown in red. Arrows point to the central spindle. (C-D) DdCP224-AA and DdCP224-EE localized to the central spindle (arrows). The fluorescence images of wild type cells expressing DdCP224-AA-GFP (C) and DdCP224-EE-GFP (D) are shown. Bar, 5um. (E) The over-expression of DdCP224-AA and DdCP224-EE did not cause growth defects in wild type cells. Wild type cells expressing a GFP vector control grew at the same rate as those cells expressing either of the two mutants tagged with GFP and those expressing the wild type DdCP224 tagged GFP. (F) The cells expressing DdCP224-GFP were hypersensitive to TBZ. In contrast, those expressing either DdCP-AA-GFP or DdCP-EE-GFP were not. The cells were grown in suspension in the presence of 1ug/ml TBZ (thiabendazole).

to test whether the two mutants could rescue the defect caused by the loss of the endogenous DdCP224.

3.2.5 DdINCENP is important for the stability of the mitotic spindle

While my data suggested that DdINCENP helps recruit DdCP224 to stabilize the midzone spindle, I suspected that this was not the sole function of DdINCENP during mitosis. This was supported by the fact that DdINCENP mutant cells expressing DdCP224-GFP divided at a much slower rate than wild type cells (Figure 3.11). To explore the mitosis defect of the DdINCENP null cells in more detail, I introduced GFP-tubulin into these cells to analyze their microtubule behavior during mitosis. Surprisingly, all the DdINCENP null cells expressing GFP-tubulin died shortly after transformation. Although GFP-tubulin has been widely used in *Dictyostelium* for live microscopy of microtubule structures (Kimble et al. 2000), it has been shown that GFP-tubulin can slightly affect microtubule dynamics in vivo (Kimble et al. 2000). This raised the possibility that the microtubule organization in the DdINCENP mutant cells might be unstable relative to that in wild type cells, thus making such cells hypersensitive to the destabilization of microtubules as a result of expressing GFP-tubulin. Therefore, I decided to examine the sensitivity of the mutant cells to microtubule depolymerizing drugs.

I found that DdINCENP null cells were highly sensitive to microtubule depolymerizing drugs such as nocodazole and thiabendazole compared to wild type cells (Figure 3,12 A, B). I considered two possible explanations for this hypersensitivity. First, DdINCENP null cells could have an unstable microtubule organization during interphase, which would indicate that DdINCENP was essential for a stable microtubule network. However, this hypothesis was not supported by our data. I found that the depolymerization of interphase microtubules by nocodazole and thiabendazole was

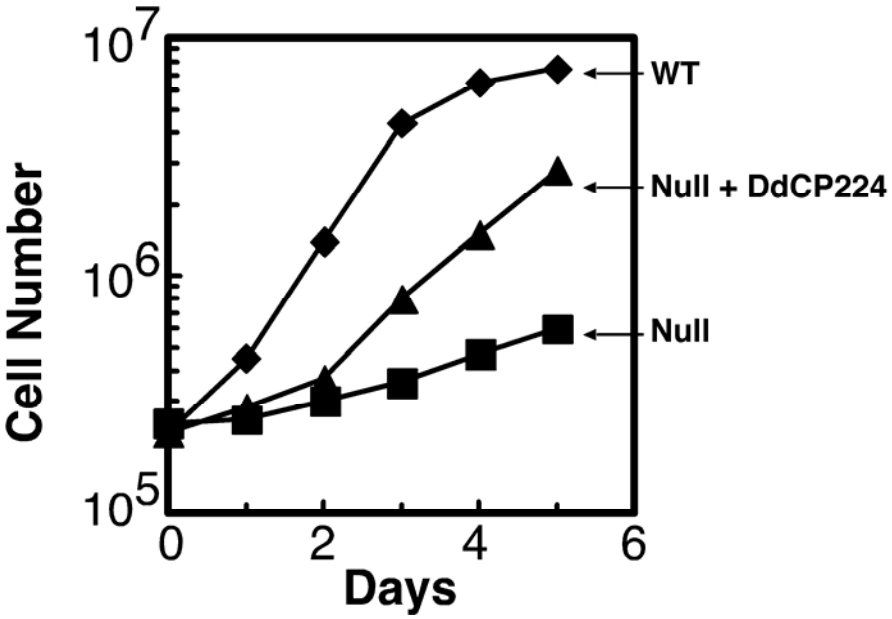


Figure 3.11: Overexpression of DdCP224, a TOGp/XMAP215 homolog, only partially rescues the growth defect of DdINCENP null cells. AX2 (diamond), DdINCENP null (square) and DdINCENP null expressing DdCP224-GFP (triangle) cells were placed in suspension cultures and their cell titers were monitored daily. Although overexpression of DdCP224-GFP made the DdINCENP null cells grow faster, these cells still lagged behind the wild type (AX2) cells in growth.

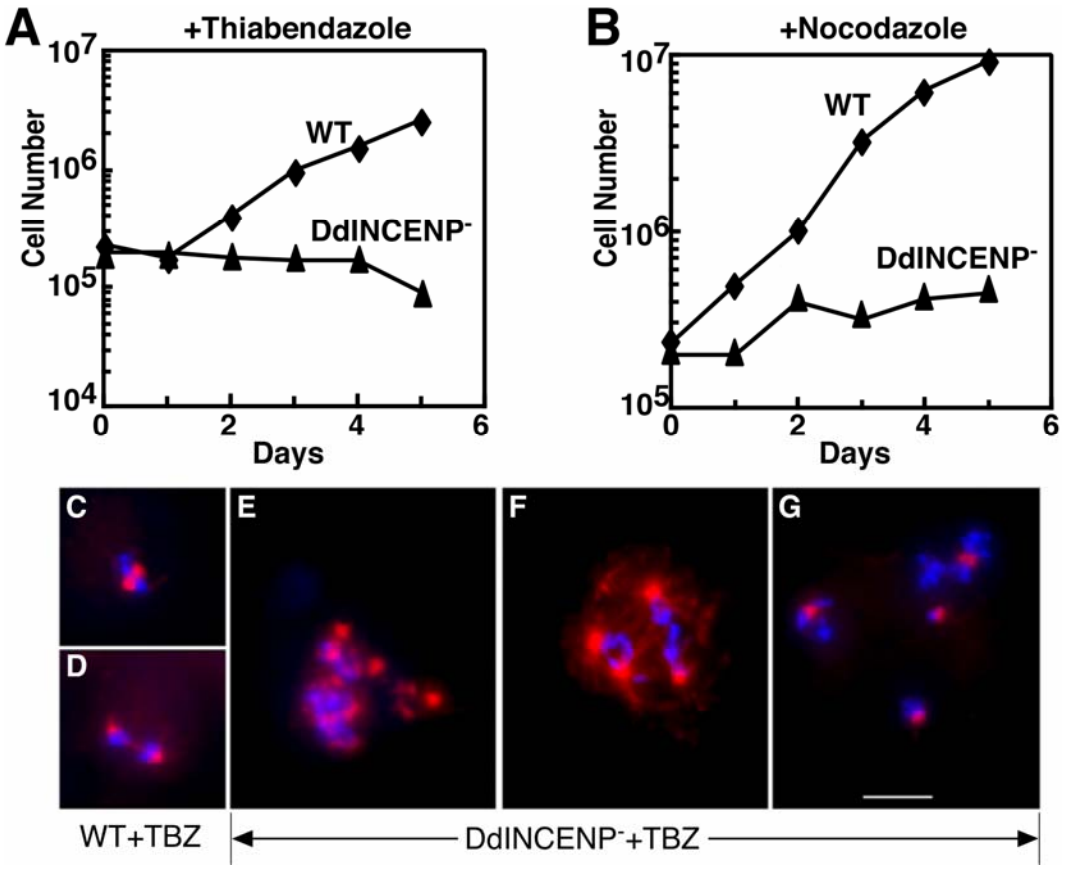


Figure 3.12: DdINCENP null cells are hypersensitive to microtubule-depolymerizing drugs. (A, B) Growth curve of wild type cells (diamond) and DdINCENP null cells (triangle) in the presence of 2ug/ml of thiabendazole (A) or 2ug/ml of nocodazole (B). (C-G) Immunofluorescence images of mitotic wild type cells (C, D) and DdINCENP null cells (E-G) in the presence of 2ug/ml thiabendazole (TBZ). DNA is shown in blue and tubulin is shown in red. Wild type cells displayed normal metaphase spindles (C) and anaphase spindles with a well defined spindle midzone (D). In contrast, DdINCENP null cells were blocked at prometaphase by the drug treatment and contained either multipolar (E, F) or monopolar spindles (G, a single multinucleate mitotic cell is shown). Bar, 5 μm.

indistinguishable in wild type and mutant cells (data not shown). Similarly the recovery of interphase microtubules after drug washout was indistinguishable in both cell lines (data not shown). A second possibility was that the depletion of DdINCENP impaired the ability of the mutant cells to establish a proper mitotic spindle structure. To test this, I examined whether wild type and DdINCENP null cells showed differences in assembling the mitotic spindles in the presence of microtubule-depolymerizing drugs. I found that wild type cells continued to establish normal bipolar spindles in the presence of 2 μg/ml of thiabendazole, although their spindle midzones were not as robust as those formed in the absence of the drugs (Figure 3.12 C, D). In contrast, DdINCENP null cells exposed to 2 μg/ml thiabendazole could not form bipolar spindles. Instead 100% of the mitotic cells had aberrant spindle structures and were blocked at prometaphase, which was evident from the condensed but scattered chromosomes (Figure 3.12 E-G). As a result, these mutant cells died within 2 days of exposure to the drugs. In addition, the treated mutant cells displayed microtubule organizing centers of different sizes indicating a problem with spindle pole body duplication or separation (Figure 3.12 E-G). The formation of abnormal spindles occurred both in multinucleate and mononucleate DdINCENP null cells. Taken together, these data suggest that DdINCENP plays a vital role in the establishment and/or maintenance of a bipolar spindle.

3.2.6 DdINCENP plays an important role in the completion of cytokinesis but not in the formation of normal or ectopic cleavage furrows

The localization of GFP-DdINCENP at the cleavage furrow throughout cytokinesis and the formation of multinucleate cells in the absence of DdINCENP indicate that DdINCENP plays a key role in this process. Since INCENP also localizes to ectopic, or Rappaport, furrows in vertebrate cells (Eckley et al. 1997), it has been suggested that INCENP may play a role in the formation and stabilization of cleavage furrows. To

explore this role in more detail, I imaged DdINCENP null cells undergoing cytokinesis (Figure 3.13). All DdINCENP null cells were able to form a cleavage furrow that constricted normally until the abscission stage, when the two daughter cells were only connected by a thin cytoplasmic bridge. In wild type cells, the bridge was short and the abscission stage took no more than 2 minutes (Figure 3.13). In contrast, most DdINCENP null cells had a prolonged abscission stage that lasted as long as 13 minutes. About half of the cytokinesis events I observed failed (13 out of 24) because the cleavage furrow eventually regressed and the daughter cells fused back with each other to become a multinucleate cell (Figure 3.13). The cytokinesis defect of these mutant cells was rescued by the re-introduction of GFP-DdINCENP.

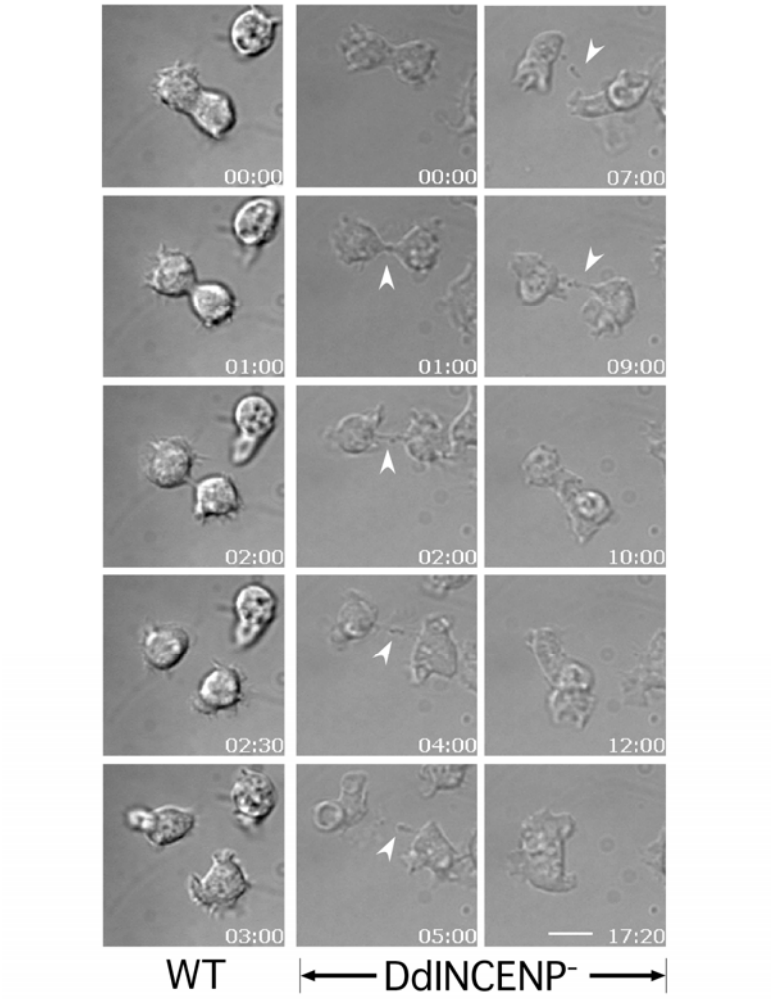


Figure 3.13: DdINCENP null cells have a late cytokinesis defect. Time lapse video microscopy of a wild type cell and a DdINCENP null cell undergoing cytokinesis. Cytokinesis of the wild type cell was completed in about 3 minutes. In contrast, DdINCENP null cell failed in cytokinesis due to the inability to break the thin cytoplasmic bridge connecting the daughter cells (arrow head). The times are indicated in minutes: seconds. Bar, 5 μm.

3.3 DISCUSSION

I have identified the *Dictyostelium* protein DdINCENP as a new member of the INCENP protein family. In addition to conserved domains, DdINCENP also displays the dynamic behavior of other chromosomal passenger proteins but with some additional unique characteristics. DdINCENP localizes to the centromeres from prophase to metaphase, and then transfers to the spindle midzone and spindle poles at the onset of anaphase. This is different from metazoan cells where INCENP translocates from the centromeres to the spindle midzone but is not found at the spindle poles.

DdINCENP associates with the cleavage furrow during cytokinesis and concentrates in the intercellular bridge connecting daughter cells. In metazoans INCENP is also known to localize to the midbody. However, in *Dictyostelium* the central spindle breaks well before the abscission stage of cytokinesis and a microtubule-containing midbody is not formed in this organism (Roos et al. 1984; McIntosh et al. 1985; Kimble et al. 2000). This suggests that the accumulation of INCENPs at the intercellular cytoplasmic bridge is not dependent on a midbody.

3.3.1 DdINCENP and spindle assembly

Considering the localization of INCENP in mitosis, it is not surprising that it plays important roles in several mitotic events (Mackay et al. 1998; Giet and Glover 2001; Tanaka et al. 2002; Sampath et al. 2004). Analysis of our DdINCENP null cell lines confirmed a role for this protein in spindle assembly and chromosome segregation. Our results further suggest a role for DdINCENP in spindle stability and localization of proteins at the spindle midzone.

The hypersensitivity of DdINCENP null cells to microtubule depolymerizing drugs revealed the importance of DdINCENP for the stability of the bipolar spindle. In the presence of these drugs mitotic DdINCENP null cells failed to establish a normal

bipolar spindle structure. It seems likely that DdINCENP exerts this role by controlling the activity of proteins that modulate microtubule stability. For example, the localization of metazoan MKLP1, a kinesin essential for central spindle formation, is dependent on INCENP function (Zhu et al. 2005). Similarly, the activity of vertebrate MCAK, a microtubule depolymerizing protein, is regulated through its phosphorylation by Aurora B kinase (Andrews et al. 2004; Lan et al. 2004). While a *Dictyostelium* homolog of MCAK has yet to be identified, the homolog of MCAK's antagonist, XMAP215/TOGp, has been characterized as the *Dictyostelium* DdCP224 protein. In contrast to MCAK, XMAP215/TOGp is known to stabilize mitotic spindles (Holmfeldt et al. 2004). I found that DdCP224 is absent from spindles of DdINCENP null cells and that DdCP224 overexpression enhances the ability of DdINCENP null cells to build spindles.

While these results do not demonstrate a direct interaction between DdINCENP and DdCP224, they suggest that DdINCENP may regulate the localization and/or activity of DdCP224. One possible model is that DdCP224, like MCAK, is directly phosphorylated by the INCENP-Aurora B protein complex during mitosis. I tested this model by two different approaches. First, I expressed AurB-KD, a kinase domain dead version of Aurora B, in the wild type cells and found DdCP224 was still localized at the central spindle in these cells. However, this could be due to the presence of endogenous Aurora, which may be sufficient to phosphorylate DdCP224. Secondly, I found two potential Aurora B phosphorylation sites in the two conserved regions of DdCP224. I replaced the serine in each site with alanine or glutamic acid and made DdCP224-AA and DdCP224-EE. However, these two mutants did not show any mislocalization during mitosis, nor did they have any dominant negative effect on the growth rate of the cells expressing them. Interestingly, I found the cells over-expressing DdCP224-GFP were hypersensitive to TBZ, which is similar to DdINCENP null cells. Additionally, Graf et al.

observed that almost half of these cells have more than one centrosome per nucleus, possibly a result of failed mitosis (Graf et al. 2000), Since DdCP224 was a microtubule stabilizing protein, this finding suggested that the stability of microtubules need to be tightly controlled to ensure successful mitosis. In contrast, the cells over-expressing DdCP224-AA-GFP or DdCP224-EE-GFP didn't show such a phenotype. This finding suggested that the mutations may have decrease the activity of DdCP224 during mitosis, so that the dominant negative effect of wild type DdCP224 was eliminated.

3.3.2 DdINCENP and cytokinesis

DdINCENP begins to localize at the cortex region of the cleavage furrow during late telophase. When cells reach late cytokinesis, DdINCENP is highly concentrated at the cortical region of the furrow area, suggesting an important role for DdINCENP during cytokinesis. Indeed, DdINCENP null cells have a severe cytokinesis defect: at the end of cytokinesis the mutant cells are impaired in severing the thin cytoplasmic bridge connecting two daughter cells. Similarly, the mammalian cells expressing a dominant negative INCENP mutant also had a late cytokinesis defect (Mackay et al. 1998). However, since *Dictyostelium* does not have a spindle midzone or midbody structure during late cytokinesis (Roos et al. 1984; McIntosh et al. 1985), the cytokinesis defect of DdINCENP null cells cannot be attributed solely to their defective spindle midzone. Additionally, it is also unlikely that their cytokinesis defect is due to a malfunction of the actin-myosin ring, since myosin II localizes normally at the furrow area in the mutant cells (see Chapter 4, Figure 4.3). Instead, I postulate that the cytokinesis defect of DdINCENP null cells may be due to a failure in the fusion of the membranes of the cytoplasmic bridge. Several membrane trafficking proteins have been shown to be required late in cytokinesis including dynamin, clathrin, LvsA, syntaxin, rabs and others (Wessels et al. 2000). Interestingly, the depletion of dynamin in *Dictyostelium* caused a

very similar late cytokinesis defect as the daughter cells were often connected by a thin cytoplasmic bridge for prolonged period (Wienke et al. 1999).

Chapter 4: The molecules required for the correct localization of DdINCENP at the cleavage furrow

4. 1 INTRODUCTION

Chromosomal passenger proteins have a dynamic localization during mitosis and cytokinesis. At the transition from metaphase to anaphase, DdINCENP transfers from centromeres to the central spindle. Then, at the beginning of cytokinesis, DdINCENP is targeted to the cortex of the cleavage furrow when the central spindle begins to disassemble. It is relatively unknown how the chromosomal-passenger protein complex (CPC) is able to move so precisely and timely at different stages of mitosis and cytokinesis.

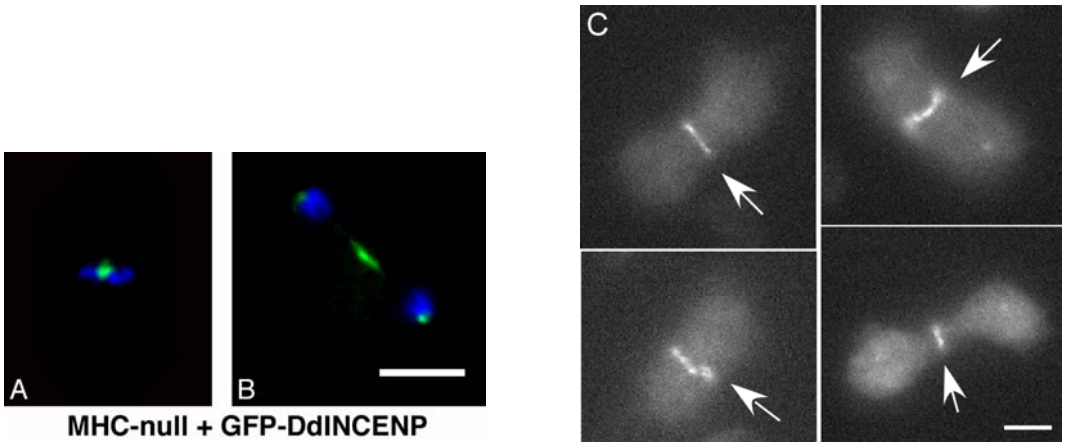
One of the first studies to address this question is the domain analysis of chicken INCENP (Ainsztein et al. 1998). Although that study revealed the N-terminal 68 amino acids of INCENP as the sequence required for its targeting to the centromeres and the midbody, the molecular mechanism driving the dynamic movement of INCENP during mitosis remains elusive. There is data suggesting that components of the CPC are interdependent on their mitotic localization. For example, studies in *Drosophila* have shown that Aurora B kinase and INCENP are dependent on each other to localize to the central spindle (Adams et al. 2001). However, this may not be the case in other organisms. For example, the mitotic spindle localization of Ipl1, the budding yeast homolog of Aurora B, does not require Sli15, the homolog of INCENP (Kang et al. 2001). Additionally, the post-translation modification of CPC proteins plays a role in the regulation of their dynamic localization. For example, in *S. cerevisiae*, the dephosphorylation by CDC14 phosphatase regulates the transfer of Ipl1 and Sli15 to the central spindle (Pereira and Schiebel 2003). Moreover, mitotic kinesin like protein MKLP-2 is essential for the relocation of Aurora B and INCENP from centromeres to the

central spindle (Gruneberg et al. 2004). However, nothing is known about how the CPC proteins transfer from the central spindle to the cortex of the cleavage furrow during cytokinesis. Here, I addressed this question by investigating the localization of DdINCENP during cytokinesis in different mutants with cytokinesis defects. By doing that, I hoped to determine some of the requirements for the targeting of DdINCENP to the cortex of the cleavage furrow.

4. 2 RESULTS

4.2.1 The localization of DdINCENP at the cleavage furrow is influenced by myosin II but it occurs by a different mechanism

The localization of DdINCENP at the cleavage furrow is very similar to Myosin II, a component of the contractile ring. Therefore, I first tested the idea that DdINCENP localized to the cleavage furrow through its interaction with the acto-myosin contractile ring. Thus, I determined whether DdINCENP could localize to the cleavage furrow in the absence of a contractile ring in myosin II null cells. It has been well known that myosin II plays an important role in cytokinesis in *Dictyostelium* (De Lozanne and Spudich 1987; Neujahr et al. 1997). However, myosin II null cells are still able to form cleavage furrows by an attachment-mediated process, cytokinesis B (Uyeda et al. 2000), that is not dependent on a contractile ring. I expressed GFP-DdINCENP in myosin II null cells and examined its localization in dividing cells attached to a substrate. The localization of GFP-DdINCENP to the spindle midzone and spindle poles was completely normal in the myosin II null cells (Figure 4.1A, B). However, when these cells entered into cytokinesis B, GFP-DdINCENP localized to a very narrow band at the equator of the cells (Figure 4.1C, D). This distribution is remarkably different from the broad equatorial distribution of GFP-DdINCENP in wild type cells (see chapter 3, Figure 3.4).



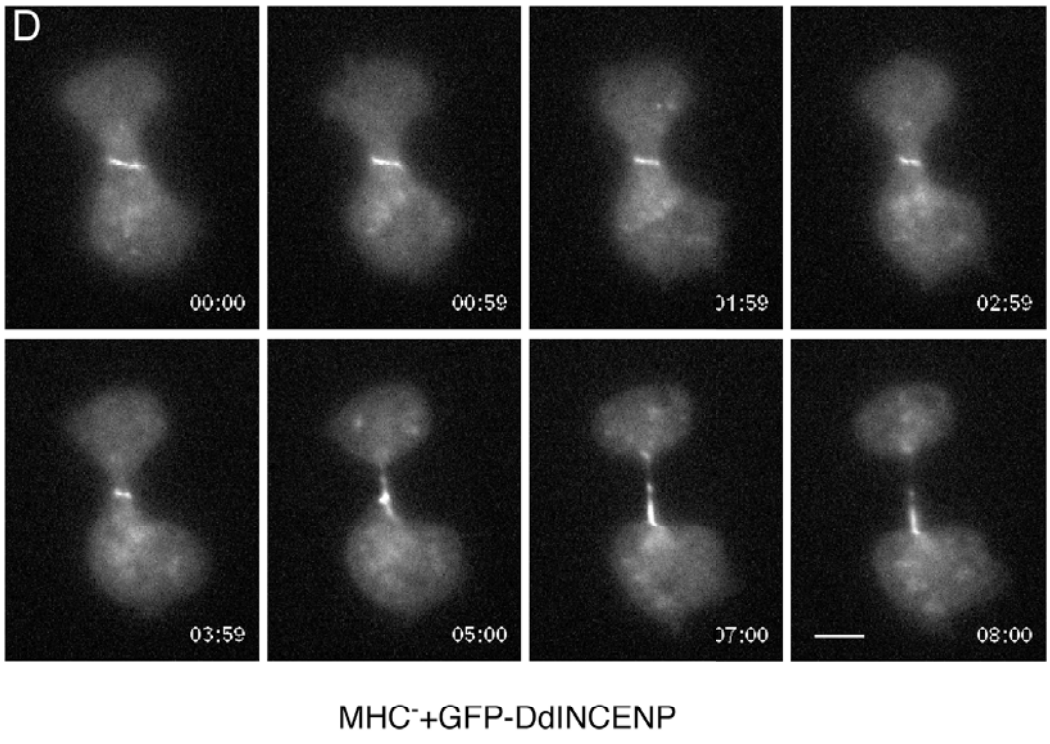


Figure 4.1 (see next page for the legend)

Figure 4.1: The distribution of DdINCENP in the cleavage furrow area was modulated by Myosin II. (A-B) The mitotic spindle localization of DdINCENP was not dependent on Myosin II. Fluorescent microscopy images of GFP-DdINCENP expressed in myosin II heavy chain null cells. DNA is shown in blue and GFP-DdINCENP is shown in green. As in wild type cells, DdINCENP localized in the middle of chromosome congregation during metaphase (A), and was found on both central spindle and spindle pole bodies at anaphase (B). (C) In contrast to the cortical distribution of GFP-DdINCENP in wild type cells, GFP-DdINCENP formed a narrow zone (arrows) at the equatorial plane of the dividing cell. Fluorescent microcopy pictures of four live myosin II null cells expressing GFP-DdINCENP were shown during their cytokinesis. (D) Time lapse video microscopy was performed on a myosin heavy chain null cell expressing GFP-DdINCENP. The band of GFP-DdINCENP decreased in size as the cleavage furrow ingressed. The times are indicated in minutes: seconds. Bar, 5 μm.

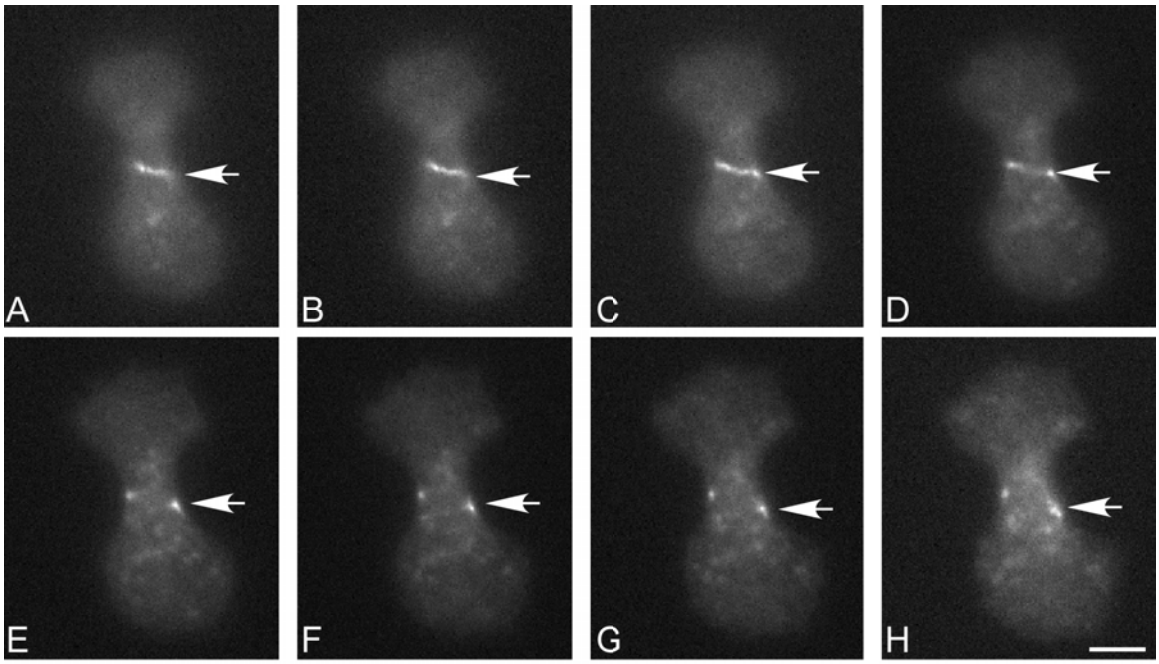


Figure 4.2: DdINCENP was localized at a very narrow band at the equator of the cleavage furrow during cytokinesis in the Myosin II null cells. Z-series fluorescence imaging of GFP-DdINCENP in a myosin heavy chain null cell undergoing traction-mediated cytokinesis (cytokinesis B). This Z-series starts at a focal plane near the top of the cell (A) and moves toward the cell-substrate contact (H). GFP-DdINCENP formed a very narrow band at the equator of the cell, most prominent at the top of the cell and absent at the bottom of the cell. This distribution contrasted sharply with the broad cortical localization of DdINCENP in the cleavage furrow of wild-type cells. Optical sections were imaged every 0.5 μm. Bar, 5 um.

optical sectioning of multiple cells at this stage revealed that GFP-DdINCENP was found as a hemicircle on the top surface of the cell (Figure 4.2). This diameter of this hemicircle decreased concomitantly with the constriction of the cleavage furrow (Figure 4.1).

I also determined whether the localization of myosin II to the cleavage furrow was dependent on DdINCENP. By expressing GFP-myosin II heavy chain (MHC) in DdINCENP null cells, I found that myosin II localized normally to the cleavage furrow of these cells (Figure 4.3). Furthermore, the acto-myosin ring was able to contract to the point that there was only a thin cytoplasmic bridge connecting the two daughter cells before the cleavage furrow regressed (Figure 4.3). At that stage, GFP-myosin II still highly concentrated at the cleavage site. This experiment confirmed that DdINCENP null cells did not fail to initiate actomyosin contractile ring at the beginning of cytokinesis (also see Chapter 3). Thus, while myosin and INCENP do not depend on each other to localize to the cleavage furrow, myosin II strongly influences the organization of INCENP at the furrow.

Finally, I determined the localization of DdINCENP in dividing binucleate cells to ascertain its role in the formation of ectopic Rappaport furrows. *Dictyostelium* is a good system to explore this question. *Dictyostelium* cells undergo closed mitosis and form an intranuclear spindle, yet can induce the formation of ectopic furrows between adjacent asters (Neujahr et al. 1997). Therefore, the localization of DdINCENP at the ectopic furrows could be easily examined. Myosin II is known to localize to ectopic as well as equatorial furrows suggesting that its localization is controlled by the position of astral microtubules (Figure 4.4). Surprisingly, I found that DdINCENP localized only on two of the four cleavage furrows (Figure 4.4). These results suggest that the localization of

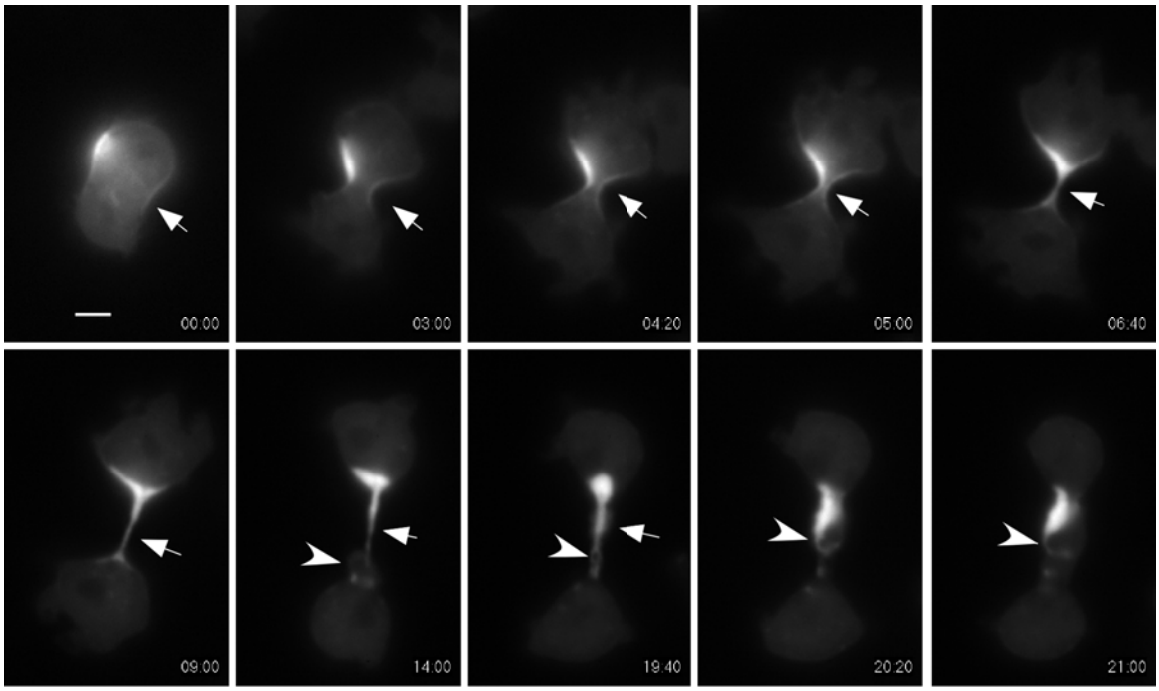


Figure 4.3: Myosin II can still localize at the cleavage furrow without DdINCENP. A time lapse series of DdINCENP null expressing GFP-MHC (myosin heavy chain) during cytokinesis is shown. In this DdINCENP null cell, GFP-MHC still localized to the cortex of the cleavage furrow (arrows) and the localization pattern was unchanged as the furrow progressed normally. However, when the two daughter cells were only connected by a thin cytoplasmic bridge, the bridge was not cleaved for a extended period of time. There were bulges (arrow head) appearing on both sides of the cytoplasmic bridge. Eventually, the cytokinesis failed as the two daughter cells fused. Times are indicated in minutes: seconds. Bar, 5 um.

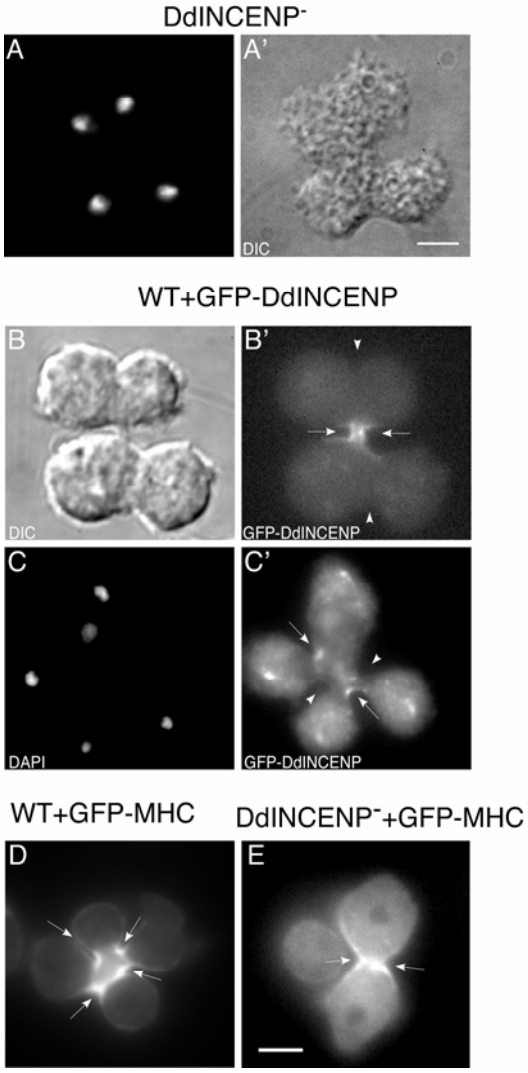


Figure 4. 4: DdINCENP is not essential for the formation of equatorial or ectopic cleavage furrows during cytokinesis. (A, A') Live images of a DdINCENP null cell forming Rappaport (ectopic) furrows during 4-way cytokinesis. As shown, DdINCENP null cells are able to make normal and ectopic cleavage furrows. (B, B', C, C') GFP-DdINCENP localized only to one pair of cleavage furrows in a four-way cell division. Arrows indicate the position of the presumptive equatorial cleavage furrows and arrowheads indicate the position of presumptive ectopic furrows. (B, B') Live DIC and fluorescent images of an AX2 cell expressing GFP-DdINCENP ongoing a 4-way division. (C, C') Fluorescent image of a wild type cells expressing GFP-DdINCENP. The cell was stained by DAPI to better illustrate the 4-way cytokinesis. (D) Live fluorescent image of an AX2 cell expressing GFP-MHC (myosin II heavy chain) during a 4-way cytokinesis. Myosin II localized to both normal and ectopic furrows (arrows). (E) Myosin II localizes normally at the cleavage furrow of DdINCENP null cell. The fluorescent microscopy picture of a DdINCENP null cell expressing GFP-Myosin II heavy chain. The absence of DdINCENP does not affect the localization of myosin II at the cleavage furrow (arrows). Bar, 5 μm

DdINCENP at the furrow is controlled by the position of the central spindle and therefore occurs by a mechanism distinct from that driving myosin II localization.

4.2.2 The transfer of DdINCENP from the central spindle to the cortex of the cleavage furrow is dependent on KIF12

DdINCENP was localized at the central spindle before transferring to the cortex of the cleavage furrow at the beginning of cytokinesis. Therefore, it was natural to suspect that some proteins on the central spindle are involved in this process of targeting DdINCENP to the cleavage furrow. A recent study has showed that the *Dictyostelium* kinesin-6 protein Kif12, an MKLP1 homolog, is important for cytokinesis (Lakshmikanth et al. 2004). Interestingly, Kif12 null cells also have a late cytokinesis defect, which is very similar to the cytokinesis defect found of the DdINCENP null cells (Chapter 3). Furthermore, Kif12 localized at the spindle midzone during anaphase and telophase, where DdINCENP also resides. Therefore, I decided to test whether Kif12 is required for the translocation of DdINCENP to the cleavage furrow.

I examined the localization of GFP-DdINCENP in Kif12 null cells during cell division. GFP-DdINCENP was still at the spindle pole bodies and the central spindle during anaphase and telophase as it was in the wild type cells (Figure 4.5). Remarkably, it did not translocate to the cortex of the cleavage furrow in the Kif12 null cells (Figure 4.5). Furthermore, GFP-DdINCENP did not concentrated at the cytoplasmic bridge at the end of cytokinesis in these mutant cells. Instead, GFP-DdINCENP was found on the central spindle throughout cytokinesis. Even after the disassembly of the central spindle, it still localized to the remnants of the spindle, which separated into the two daughter cells (arrows, Figure 4.5).

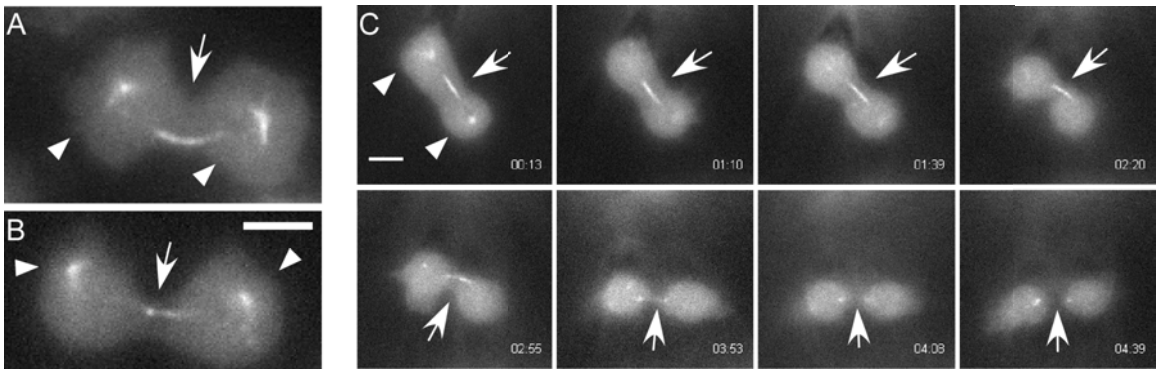


Figure 4.5: Kif12 was essential for the transfer of DdINCENP from the central spindle to the cortex of the cleavage furrow. (A-B) DdINCENP was absent from the cortex of the cleavage furrow (arrows) in the cleavage furrow. Two Kif112 null cells expressing GFP-DdINCENP were shown during cytokinesis. GFP-DdINCENP still localized to the central spindle and the spindle pole bodies (arrow heads) when the cleavage furrow ingressed significantly. In contrast, in wild type cells, GFP-DdINCENP transferred to the cortex of the cleavage furrow when the furrow just began to ingress. (C) DdINCENP failed to transfer to the cortex of the cleavage furrow in the absence of Kif12. A time lapse series of a Kif12 null cell expressing GFP-DdINCENP during cytokinesis is shown. Arrows point to the cleavage furrow. Noticeably, GFP-DdINCENP was also absent from the cytoplasmic bridge at the end of cytokinesis, while it highly concentrated on the bridge in the wild type cells. Times are indicated in minutes: seconds. Bar, 5 um.

My observations suggested a possible role of Kif12 in the delivery of DdINCENP from the central spindle to the cortex of the cleavage furrow. This predicted that KIF12 should also be found at the cortex of the cleavage furrow, which had not been shown previously. Therefore, I decided to carefully examine the localization of KIF12 during cytokinesis. Surprisingly, I found that, in addition to its localization at the central spindle and spindle pole bodies, GFP-KIF12 was also found at the cortex of the cleavage furrow (Figure 4.6) Like DdINCENP, GFP-Kif12 also highly concentrated at the cytoplasmic bridge (Figure 4.6). This finding supports the idea that Kif12 transports DdINCENP from the spindle midzone to the cortex of cleavage furrow.

In contrast, the localization of GFP-Kif12 was not changed in the mitotic DdINCENP null cells. It still localized to the central spindle and the cortex of the cleavage furrow during cell division (Figure 4.7).

4.2.3 Kif12 may have a role in the centromeric localization of DdINCENP

GFP-DdINCENP localized to the spindle pole bodies and the spindle midzone during anaphase in Kif12 null cells (Figure 4.5). Therefore, it appeared that Kif12 was not involved in the transfer of DdINCENP to the central spindle and the spindle pole bodies at the transition from metaphase to anaphase. However, it remained a possibility that Kif12 was involved in the targeting of DdINCENP to centromeres during prophase/prometaphase. Therefore, I examined the localization of GFP-DdINCENP during prometaphase and metaphase in the Kif12 null cells. In wild type cells, GFP-DdINCENP was usually found at several discrete loci close to the condensed chromosomes during prometaphase (see Chapter 3, Figure 3.2). In comparison, in Kif12 null cells during prometaphase, GFP-DdINCENP was excluded from the condensed chromosomes and was dispersed homogenously in the remaining space of the nucleoplasm (Figure 4.8).

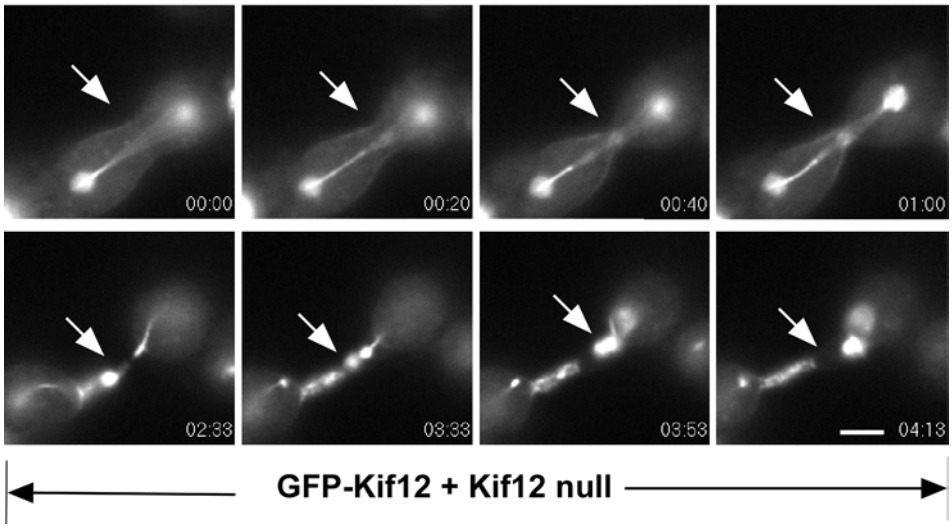


Figure 4.6: Kif12 was localized on the central spindle during mitosis and at the cleavage furrow during cytokinesis. Shown are time lapse fluorescence images of a Kif12 null cell expressing GFP-Kif12 during cytokinesis. GFP-Kif12 was found on the central spindle. Moreover, it localized to the cortex of the cleavage furrow (white arrow) during cytokinesis and was clearly seen at the remnant of the bridge after the daughter cells separated. Times are indicated in minutes: seconds. Bar, 5um.

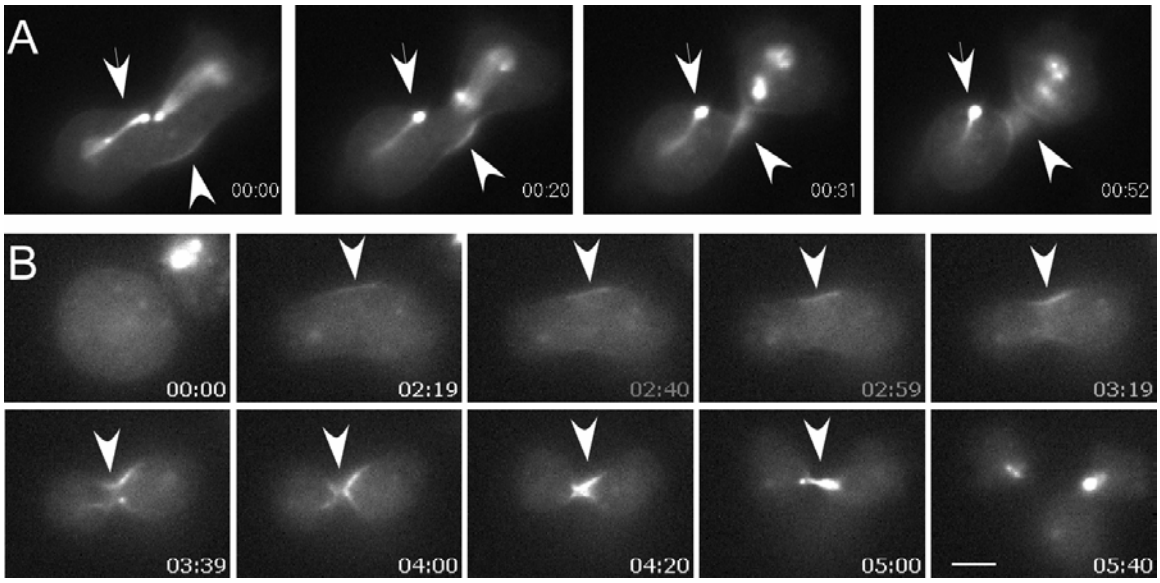


Figure 4.7: The localization of Kif12 during mitosis and cytokinesis did not dependent on DdINCENP. The time lapse fluorescence micrographs of DdINCENP null cells expressing GFP-Kif12 are shown. (A) GFP-Kif12 still localized at the central spindle (arrows) in the DdINCENP null cell. The cell was entering cytokinesis when the central spindle dissembled and the GFP-Kif12 began to accumulate at the cortex of the cleavage furrow (arrow heads). (B) In the DdINCENP null cells, GFP-Kif12 was found at the cortex of the cleavage furrow (arrow heads) at the early stage of cytokinesis and continued to build up at the furrow area. At the end of the cytokinesis, GFP-Kif12 was highly enriched at the cytoplasmic bridge. Times are indicated in minutes: seconds. Bar, 5 um.

This finding implied that DdINCENP may not localize to the centromeres in Kif12 null cells during prometaphase.

However, two findings suggested that DdINCENP was still transferred to the centromeres in Kif12 null cells. First, GFP-DdINCENP was found at a single spot, surrounded by chromosomes, in Kif12 null cells during metaphase (Figure 4.8). That was similar to its localization during metaphase in wild type cells, where I showed the spot corresponded to the agglomerated centromeres (Chapter 3, Figure 3.2 and Figure 3.3). Secondly, if the loss of Kif12 led to a defect in DdINCENP binding to the centromeres, I expected to see that Kif12 null cell have mitosis defects. It has been shown that INCENP and Aurora B are essential for the bi-orientation attachment of kinetochore by spindles (Cheeseman et al. 2002; Tanaka et al. 2002). Accordingly, I have shown that DdINCENP null cells had a chromosome segregation defect (Chapter 3, Figure 3.7). I did found that Kif12 null cells often had enlarged nuclei, which could be a result of mitosis defects (Figure 4.9). If DdINCENP was absent from the centromeres in Kif12 null cells, I expected that these cells had a similar chromosome segregation defect. Therefore, I followed the movement of the chromosomes, labeled by GFP-histone H2B, in Kif12 null cells during anaphase (Figure 4.9). Surprisingly, I did not find any lagging chromosomes or metaphase arrest in Kif12 null cells (n=5). Additionally, I found the central spindle was normal in Kif12 null cells (Figure 4.9 B, C). Therefore, it is possible that the association of DdINCENP with the centromeres was simply delayed in Kif12 null cells from prophase or prometaphase to metaphase.

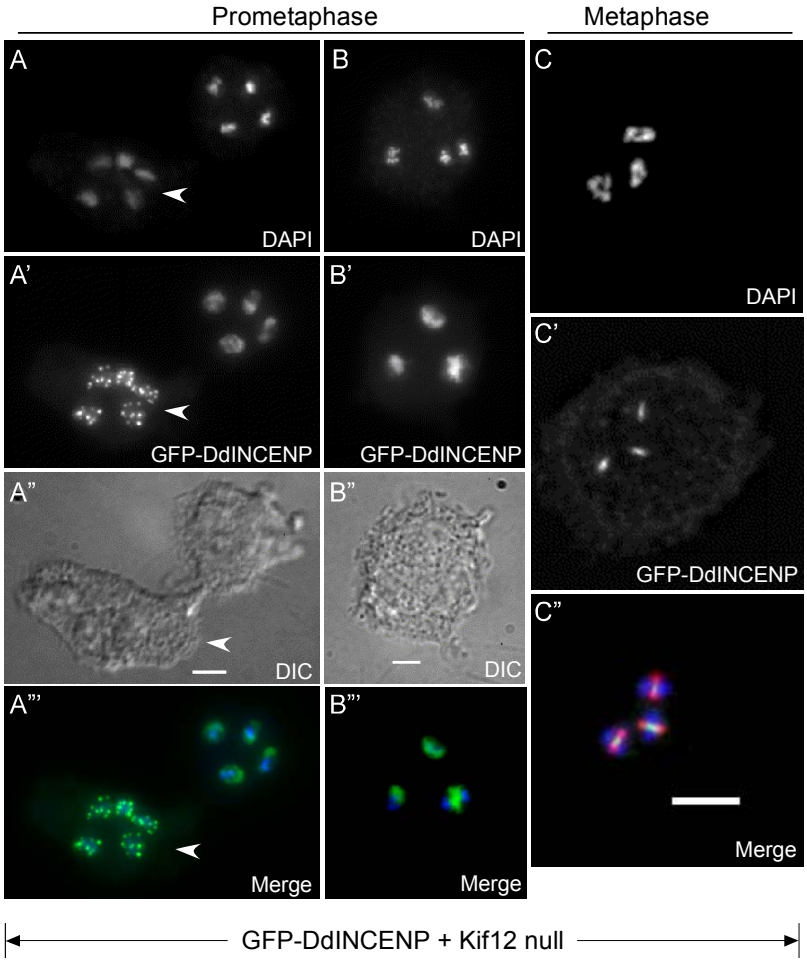


Figure 4.8: The localization of DdINCENP at the centromeres during prometaphase depended on Kif12. (A-B) The micrographs of Kif12 null cells expressing GFP-DdINCENP during prometaphase. GFP-DdINCENP localized to the punctate foci in the nuclei of Kif12 null cell during interphase (A', arrow head). However, in prometaphase Kif12 null cells, GFP-DdINCENP was diffused in the nuclei (A', B'). In contrast, GFP-DdINCENP localized to the discrete centromeres in the prometaphase nucleus of wild type cells. Bar, 10 um. (C) GFP-DdINCENP localized to the central spindle during metaphase in the Kif12 null cells. As in the wild type cells, GFP-DdINCENP still localized in the middle of the metaphase plate and the spindle. Bar, 5 um.

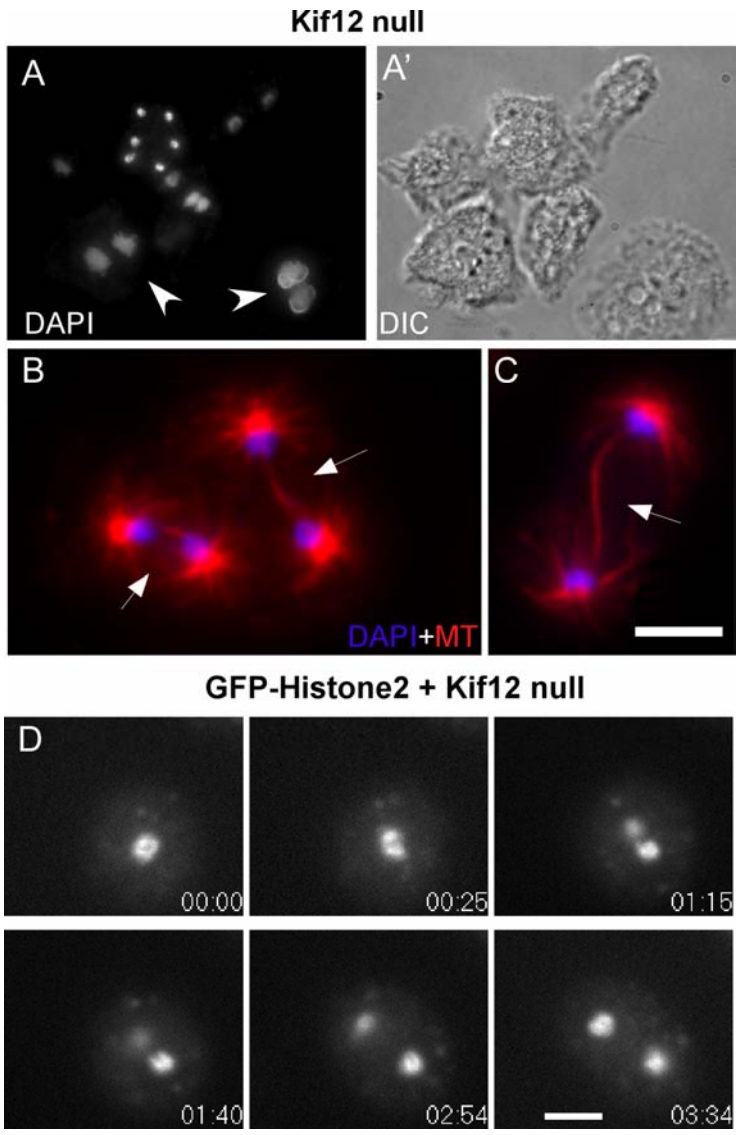


Figure 4.9: Kif12 null cells did not have either the midzone spindle or chromosome segregation defect. Kif12 null cells often had enlarged nuclei (A, arrow heads), which could be a sign of a mitosis defect. However, there was no midzone spindle defect found in Kif12 null cells (B-C, arrows). (D) There was no lagging chromosome during anaphase in Kif12 null cells. A time lapse fluorescence images of a Kif12 null cell expressing GFP-histone H2B during mitosis was shown. Times are indicated in minutes: seconds. Bars, 5 um.

4.2.3 LvsA, RacE and clathrin are not involved in the localization of DdINCENP to the cleavage furrow

Our lab has isolated several mutants of *Dictyostelium* cells with cytokinesis defect, including RacE null, Large Volume sphere A (LvsA) null and Clathrin null mutants (Larochelle et al. 1996; Niswonger and O'Halloran 1997; Kwak et al. 1999). One common defect of these mutants is that they were all able to initiate cleavage furrow formation, but only to have the furrow regress later. This phenotype reassembles the late cytokinesis defect of DdINCENP null cells to some extent (Figure 3.13). The similarity of their phenotype made me decide to test the possibility that the localization of DdINCENP during cytokinesis may be affected in some of these mutants. Unlike RhoA, RacE does not play a role in the actomyosin contractile ring. The exact role that RacE plays during cytokinesis remain unknown, although RacE null cells appear to have low cortical tension around the cell cortex (Gerald et al. 1998). Since DdINCENP localized to the cortex of the cleavage furrow, it is a possible downstream target of RacE. To test whether RacE plays a role in recruiting DdINCENP to the cleavage furrow, I examined the localization of GFP-DdINCENP in RacE null cells during cytokinesis. I found that GFP-DdINCENP still localized to the cleavage furrow in RacE null cells (Figure 4.10 A). Therefore, RacE was not involved in the targeting and organization of DdINCENP at the cortex of the cleavage furrow.

LvsA protein has homology to the mouse beige protein and the human Chediak-Higashi syndrome protein (CHS), both of which play important role in the process of membrane trafficking (Kwak et al. 1999). LvsA null mutant cells have similar cytokinesis defect as clathrin null cells have (Niswonger and O'Halloran 1997; Kwak et al. 1999). Both mutant cells can initiate cleavage furrow formation, before the furrow regresses and cytokinesis fails. To examine the role that these two proteins may play in targeting

DdINCENP to the cleavage furrow, I examined the localization of GFP-DdINCENP in both LvsA null and clathrin null cells during cytokinesis. I found that GFP-DdINCENP still localized to the cleavage furrow in both mutant cells (Figure 4.10 B, C, C'). Therefore, neither LvsA nor clathrin played a role in the localization of DdINCENP at the furrow.

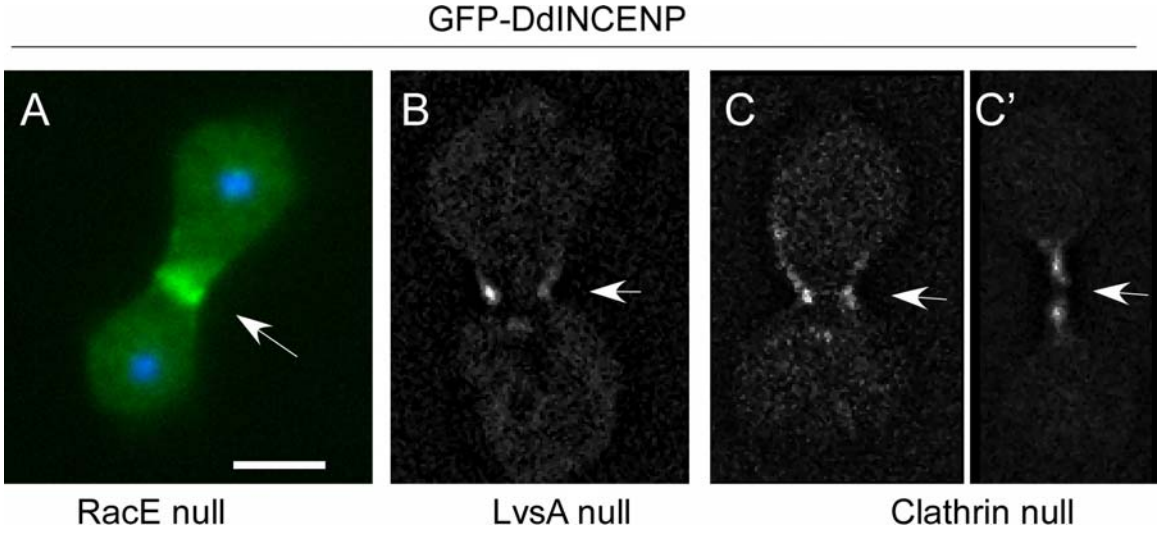


Figure 4.10: RacE, LvsA and clathrin were not involved in the localization of DdINCENP at the cleavage furrow. (A) A fluorescence micrograph of a RacE null cell expressing GFP-DdINCENP during cytokinesis. The cell was fixed and stained with DAPI to show DNA (blue). (B) A live fluorescence micrograph of a LvsA null cell expressing GFP-DdINCENP during cytokinesis. (C, C') Live fluorescence micrographs of clathrin null cells expressing GFP-DdINCENP. GFP-DdINCENP localized to the cortex of the cleavage furrow (C), before concentrated at the cytoplasmic bridge (C'). Arrows, the cleavage furrow. Bar, 5um.

4.3 DISCUSSION

4.3.1 The mechanism involved in the localization of DdINCENP at the cleavage furrow is different from that controlling the formation of the contractile ring

I found that the localization of DdINCENP at the cleavage furrow was strongly influenced by myosin II. While not required for the furrow localization of DdINCENP, myosin II is involved in broadening the distribution of DdINCENP at the furrow. These results highlight for the first time a potential interaction between INCENP and the actomyosin contractile ring. The nature of this interaction and its significance for INCENP function will be described in more detail in the next chapter (see Chapter 5).

A general assumption in the field of cytokinesis has been that a common mechanism must be involved in the determination of the plane of division and the recruitment of the different components of the cleavage furrow. Our results clearly indicate that the mechanism involved in the equatorial localization of DdINCENP is different from that regulating the distribution of myosin II at the cleavage furrow. This distinction cannot be made in normal mononucleate cells undergoing mitosis because there is a single plane of division. However, the formation of ectopic, or Rappaport furrows in binucleate cells provides a unique system to clearly distinguish these two localization mechanisms. The myosin II contractile ring is always formed at both equatorial furrows and ectopic furrows in *Dictyostelium* and other systems. These results strongly suggest that the contractile ring must be positioned wherever microtubules of opposite polarities meet the cortical cytoskeleton. In contrast, DdINCENP localized only at two of the four cleavage furrows, presumably at the equator. This equatorial

localization of DdINCENP was found in both wild type and myosin II mutant cells. Thus, the distribution of DdINCENP is not controlled by the encounter of antiparallel microtubules, but must be regulated by the position of the spindle midzone. Since the *Dictyostelium* spindle midzone is no longer surrounded by the nuclear envelope in late anaphase and telophase, it is possible that DdINCENP transfers directly from the spindle midzone, where it is highly concentrated during mitosis, to the cortex of the cell.

4.3.2 Kif12 is required for the translocation of DdINCENP to the cleavage furrow

Kif12 belongs to the KIF-6 family of kinesin-related proteins (Kollmar et al. 2003; Lakshmikanth et al. 2004). It is the only member of this kinesin family in *Dictyostelium*, while there are three KIF-6 kinesins in human including MKLP1, MKLP-2 and M-phase phosphoprotein 1 (MPP1) (Miki et al. 2001; Kollmar and Glockner 2003). All three of them play important roles during cytokinesis (Hill et al. 2000; Kuriyama et al. 2002; Abaza et al. 2003). Among the three, MKLP1 and MKLP2 have been well studied. Both of them localize to the central spindle from anaphase on and highly concentrate in the midbody during cytokinesis. Additionally, they both can crosslink the anti-parallel microtubules to help assemble the central spindle (Nislow et al. 1992; Matuliene et al. 2002; Neef et al. 2003). However, they play different roles during cytokinesis. MKLP-1 interacts with MgcRacGAP to form a centralspindlin complex, which interact with RhoA (Mishima et al. 2002; Mishima et al. 2004). In comparison, MKLP-2, also known as Rab6 kinesin, was identified as a kinesin interacting with Rab6 (Echard et al. 1998). It is required for the translocation of both Aurora B and Polo kinase from the centromeres to the central spindle (Neef et al. 2003; Gruneberg et al. 2004).

ZEN-4 is the only kinesin-6 family protein found in *C. elegans* and there are two kinesin-6 family proteins found in *Drosophila*, Pavarotti and KLP67A (Kollmar and Glockner 2003). Loss of either Zen-4 or Pavarotti led to a late cytokinesis defect where the cleavage furrow would initiate but eventually regress (Adams et al. 1998; Raich et al. 1998). Kif12 null cells have a similar cytokinesis defect (Lakshmikanth et al. 2004). The phenotype of these mutants is reminiscent of the late cytokinesis defect found in INCENP depleted cells in *Drosophila*, *C.elegans* and *Dictyostelium* (Kaitna et al. 2000; Adams et al. 2001; Giet and Glover 2001).

Here I demonstrated that Kif12 is essential for the transfer of DdINCENP to the cortex of the cleavage furrow. The absence of DdINCENP from the cleavage furrow in Kif12 null cells was not due to a defective central spindle, because the central spindle of these cells appeared to be normal and DdINCENP was still localized at the central spindle in these cells. It was not a result of these mutant cells failing to initiate the cleavage furrow, either. Lakshmikanth et al. observed that some Kif12 null cells can still initiate the cleavage furrow before the furrow ingresses, although myosin II is not as enriched at the contractile ring in these cells as it is in wild type cells (Lakshmikanth et al. 2004). Consistent with his finding, I found Kif12 null cells could initiate the cleavage furrow, although not asymmetrically (Figure 4.5). However, DdINCENP was still absent from the actively contracting furrow in these Kif12 null cells.

I also showed that Kif12 and DdINCENP had an almost identical localization from anaphase to cytokinesis. They were both found at the central spindle and the spindle pole bodies before targeting to the cortex of the cleavage furrow. Additionally, DdINCENP was likely a downstream target of Kif12 during cytokinesis, because the loss of DdINCENP did not affect the localization of Kif12 at the cleavage furrow. Based on these data, an appealing model is that Kif12, a plus end directed kinesin, transports

DdINCENP to the cortex of the cleavage furrow along microtubules. To test this model, I will investigate whether Kif12 directly interacts with DdINCENP. Additionally, I will examine the localization of Kif12 in a 4-way cell division. I have shown DdINCENP depended on the central spindle for its localization at the cleavage furrow and was not found at ectopic furrows. Therefore, it would be interesting to examine whether Kif12 is also absent from the ectopic furrows. I will also investigate whether the localization of Kif12 during cytokinesis, in Myosin II null cells, is similar to the narrow zone distribution of DdINCENP in these cells. This experiment would further test whether DdINCENP and Kif12 are closely associated during cytokinesis.

4.3.3 Kif12 was not essential for the localization of DdINCENP at the centromeres and the central spindle

In Kif12 null cells, DdINCENP was still found at the centromeres during metaphase. However, the centromeric localization of DdINCENP during prometaphase was not as prominent as it was in the wild type cells. This finding suggested that Kif12 may be also involved in transferring DdINCENP to the centromeres after the cells entered mitosis. The mechanism of such a process still remains unknown. Therefore, it would be interesting to examine the localization of INCENP during prometaphase in either MKLP1 or MKLP2 depleted human cells, where it is relatively easy to investigate the localization of INCENP at the inner-centromere.

I also showed that the loss of Kif12 did not prevent DdINCENP from transferring to the central spindle. However, my co-worker Hui Li showed that DdAurB, the *Dictyostelium* homolog of Aurora B kinase, was absent from the central spindle in Kif12 null cells. This is surprising, given that INCENP and Aurora B usually co-exist in a protein complex during cell division in higher eukaryotic systems (Adams et al. 2000; Kaitna et al. 2000). One possible explanation was that there were two different

mechanisms involved in the localization of DdINCENP and DdAurB after the onset of anaphase. Therefore, in the absence of Kif12, DdINCENP could still transfer to the central spindle through a different pathway from the one that DdAurB uses. This model was supported by the finding that DdAurB, unlike DdINCENP, is not found on the cortex of the cleavage furrow during early cytokinesis. Instead, DdAurB concentrates on the intercellular bridge near the end of cytokinesis (Hui Li, personal communication).

4.3.4 RacE, LvsA and Clathrin are not required for the localization of DdINCENP at the cleavage furrow

RacE belongs to the Rho-family of GTPases. This group of protein includes RhoA, which plays a key in regulating the actin myosin contractile ring through its activity towards ho-KinaseROK and citron kinase during cytokinesis (Chapter 1) (Larochelle et al. 1997). There is no homolog of RhoA in *Dictyostelium* and RacE is the only member of Rho-family proteins known to play an important role during cytokinesis. However, the loss of RacE does not affect the assembly of the actomyosin contractile ring. Additionally, I found that DdINCENP still localized to the cleavage furrow RacE null cells. Therefore, the cytokinesis defect of RacE null cells was not due to the incorrect localization of DdINCENP.

Both LvsA and clathrin are likely to play a role in the membrane trafficking of cytokinesis, which is an essential component of the cytokinetic machinery (Albertson et al. 2005). Moreover, LvsA localizes to contractile vacuole membrane compartment and is important for its proper function (Gerald et al. 2002). The cytokinesis defect of LvsA null and clathrin null cells is the regression of the cleavage furrow after the furrow initiates (Kwak et al. 1999). My data indicated that this defect was not due to the absent of DdINCENP from the cleavage furrow. Additionally, clathrin null cells have a defect in accumulating myosin II to the cleavage furrow during cytokinesis (Niswonger and

O'Halloran 1997). The discrepancy between the localization of Myosin II and DdINCENP in clathrin null cells concurred with our previous conclusion that there are two different mechanisms to recruit myosin II and DdINCENP separately to the cleavage furrow.

Chapter 5 Functional Domain Analysis of DdINCENP

5.1 INTRODUCTION

Although the functions of INCENPs from different species are highly conserved, their sequences are not. Besides the small IN-box domain at their C-termini and coiled-coil segments, no other sequence conservation is discernible among them. Nonetheless, most INCENP proteins share the ability to localize to centromeres, spindles, and the cleavage furrow. Therefore, it is important to determine which segments of DdINCENP impact its different properties. Comparison of the functional domains of DdINCENP with those from other species may help elucidate the common mechanism of their actions.

The domain structure of chicken INCENP has been studied in the greatest detail. Surprisingly, the N-terminal 68-amino acid sequence of INCENP is essential for its localization at the centromeres (Ainsztein et al. 1998). This sequence is also required for the transfer of INCENP from the centromeres to the central spindle (Ainsztein et al. 1998). There are two conserved motifs in this 68-amino acid sequence among INCENPs from chicken, frog and mouse (Ainsztein et al. 1998). One is for the centromere localization of INCENP, while the other one is necessary for the midzone spindle localization. Furthermore, this 68-amino acid domain is sufficient to form a ternary complex with Survivin and Borealin to localize to the centromeres (Klein et al. 2006). However, this domain of INCENP has not been found in either invertebrates or unicellular organisms. Instead, domain analysis of Sli15, the budding yeast INCENP homolog, found that its middle portion is essential for the localization at the central spindle, while the N-terminal sequence is not necessary for either its spindle localization nor its interaction with Ipl1, the Aurora B homolog (Kang et al. 2001). More recently, it

was found that Thr 59 and Thr 388 of vertebrate INCENP are phosphorylated by Cdk1 and the phosphorylation of Thr 388 is critical for the recruitment of Polo kinase to centromeres (Goto et al. 2006).

DdINCENP is by far the largest member of the INCENP family. I am particularly interested in defining the domain required for the transfer of INCENP to the cortex of the cleavage furrow at the beginning of cytokinesis, which has not been studied any other vertebrate system. This will help us further understand the mechanism by which INCENP targets to the cleavage furrow. *Dictyostelium* is a particularly suitable system to study this question. INCENP localizes to both the central spindle and the cortex of the cleavage furrow during cytokinesis in vertebrate systems. The proximity of the central spindle and cleavage furrow in these systems makes it difficult to determine whether a truncated INCENP localizes to only one of these two sites or both sites. In contrast, the central spindle is disassembled early during cytokinesis in *Dictyostelium* and DdINCENP is only found at the cortex of the cleavage furrow. Therefore, I can easily determine which truncated INCENP localizes to the cleavage furrow and which does not.

Here, I made a series of DdINCENP truncated mutants tagged with GFP. The localization of these chimeras during cytokinesis was followed by fluorescence microscopy and I found that the N-terminal 488-amino acid sequence of DdINCENP was essential and sufficient for its localization at the cortex of the cleavage furrow. Interestingly, this N-terminal sequence of DdINCENP was also found to be associated with the cell cortex and pinocytic cups during interphase. Therefore, I propose that DdINCENP can associate with the actin cytoskeleton through its N-terminal domain.

5.2 RESULTS

5.2.1 The N-terminal 488-amino acids of DdINCENP are necessary and sufficient for its cleavage furrow localization

First, I wanted to test whether the IN-box domain is necessary for the localization of DdINCENP at the cortex of the cleavage furrow and whether the N-terminal sequence of DdINCENP is essential in this process. To answer these two questions, I generated two truncated mutants, DdINCENP$_{1\text{-}1013}$ and DdINCENP$_{488\text{-}1320}$ (Figure 5.1). Both truncated mutants were tagged with GFP and expressed in both wild type and DdINCENP cells. I would only present my findings in DdINCENP null cells below, which was identical to what I found in wild type cells.

Remarkably, I found that GFP-DdINCENP$_{1\text{-}1013}$ had essentially the same localization at the cleavage furrow as the full length DdINCENP (Figure 5.2). GFP-DdINCENP$_{1\text{-}1013}$ was targeted to the cortex of the cleavage furrow early in cytokinesis and concentrated on the cytoplasmic bridge. In contrast, GFP-DdINCENP$_{488\text{-}1320}$ was distributed throughout the cell during cytokinesis, although it became more concentrated in the nucleus at the end of cytokinesis. This protein was clearly absent from the cortex of the cleavage furrow and the cytoplasmic bridge connecting the two daughter cells (Figure 5.2). To rule out the possibility that GFP-DdINCENP$_{488\text{-}1320}$ was not folded properly, I examined its localization during mitosis. Although the fusion protein mostly diffused in the cytoplasm throughout mitosis, particularly during metaphase (Figure 5.3), careful analysis revealed that it could still associate with the spindle pole and the spindle midzone during anaphase (Figure 5.4). This was very similar to the mitotic localization of GFP-DdINCENP$_{1\text{-}1013}$, although the IN-box-less mutant did show a much stronger affinity to the spindle pole bodies and the central spindles (Figure 5.4). Therefore, I

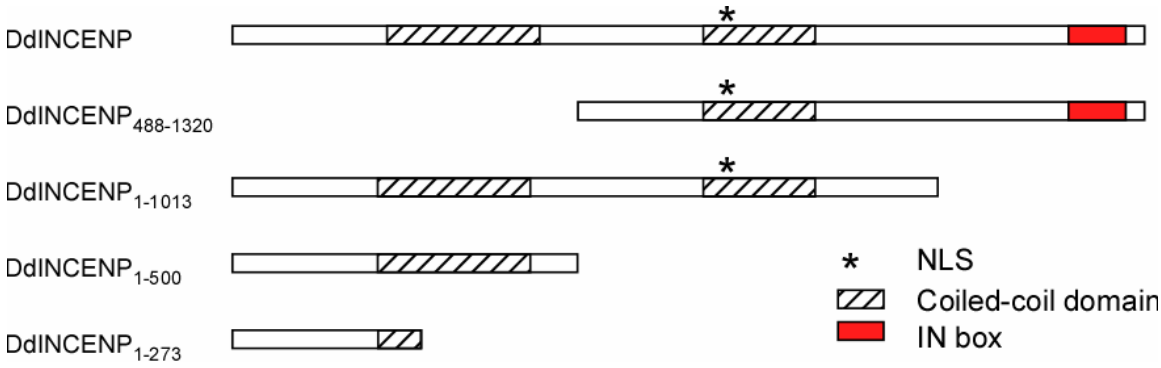


Figure 5.1: Domain analysis of DdINCENP. Show are the four truncated mutants of DdINCENP used in this study. * represent where the putative nucleus localization (NLS) signal is.

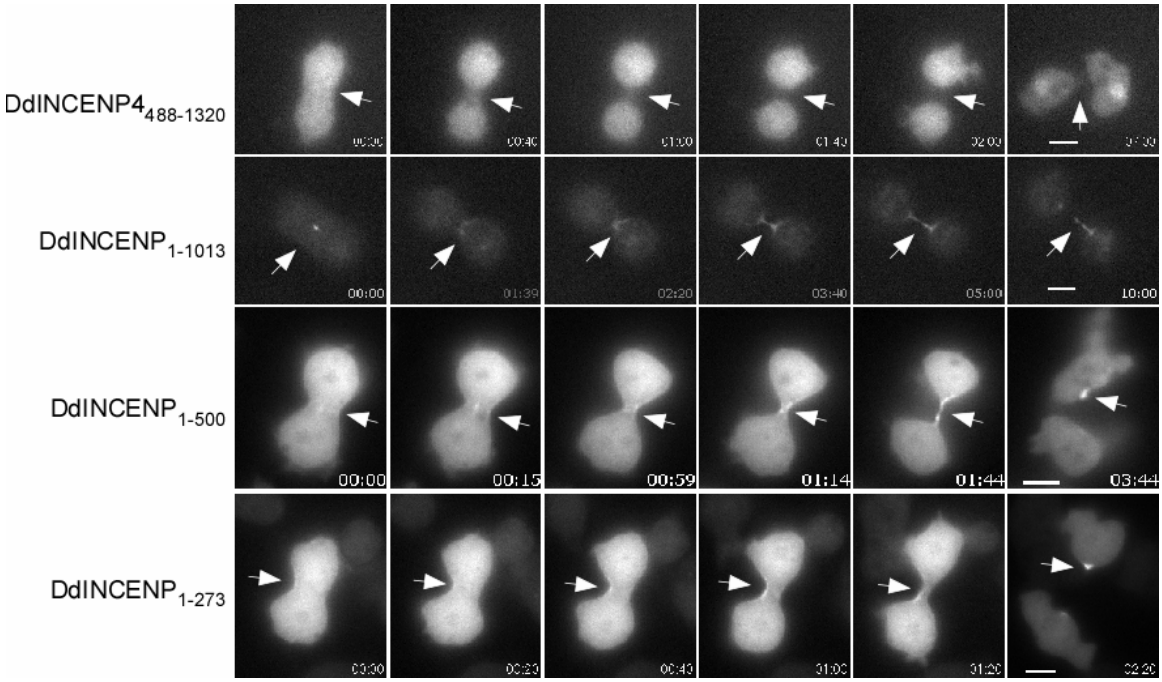


Figure 5.2: The N-terminal of DdINCENP is essential for its translocation to the cleavage furrow during cytokinesis. The four different truncated mutants of DdINCENP were all tagged with GFP and expressed in DdINCENP null cells. The time lapse fluorescence micrographs of these cells during cytokinesis were shown. GFP-$DdINCENP_{488-1320}$ was absent from the cortex of the cleavage furrow (arrows) and was homogenously distributed in the cytoplasm during cytokinesis. On the contrary, the three chimeras were all found at the cortex of the cleavage furrow during cytokinesis. Times are indicated in minutes: seconds. Bar, 5 um.

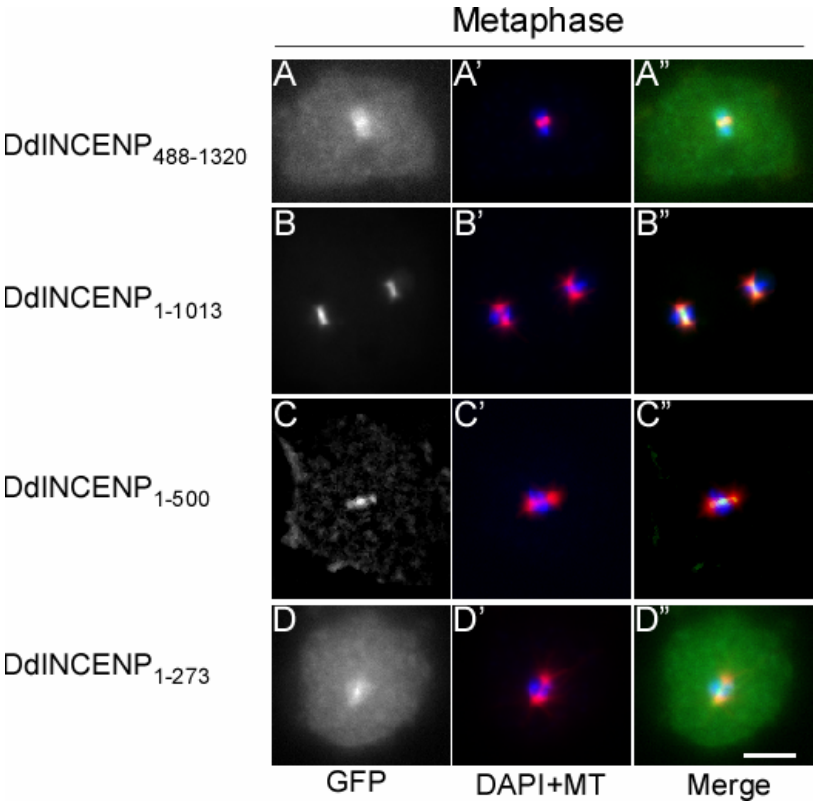


Figure 5.3: Neither IN box nor N-terminal sequence of DdINCENP is essential for its correct localization during mitosis. The GFP-tagged truncated DdINCENP mutants were expressed in DdINCENP null cells. Immunofluorescence microscopy images of these cells during metaphase are shown. (A-D) GFP fluorescence signal is shown. (A'-D') Merged immunofluorescence images to show DNA in blue and microtubules in red. During metaphase, *Dictyostelium* cells have a very short bi-polar spindle and the chromosomes are congregated (Chapter 1). (A"-D") Merged fluorescence micrographs to show GFP in green, DNA in blue and microtubules in red. All four truncated mutants localized to the centromeres, which conglomerated during metaphase and were in the middle of the spindle (Chapter 3, figure 3.3). GFP-DdINCENP$_{1-1013}$ showed a stronger affiliation to the spindle and the centromeres than the others. Bars, 5 um.

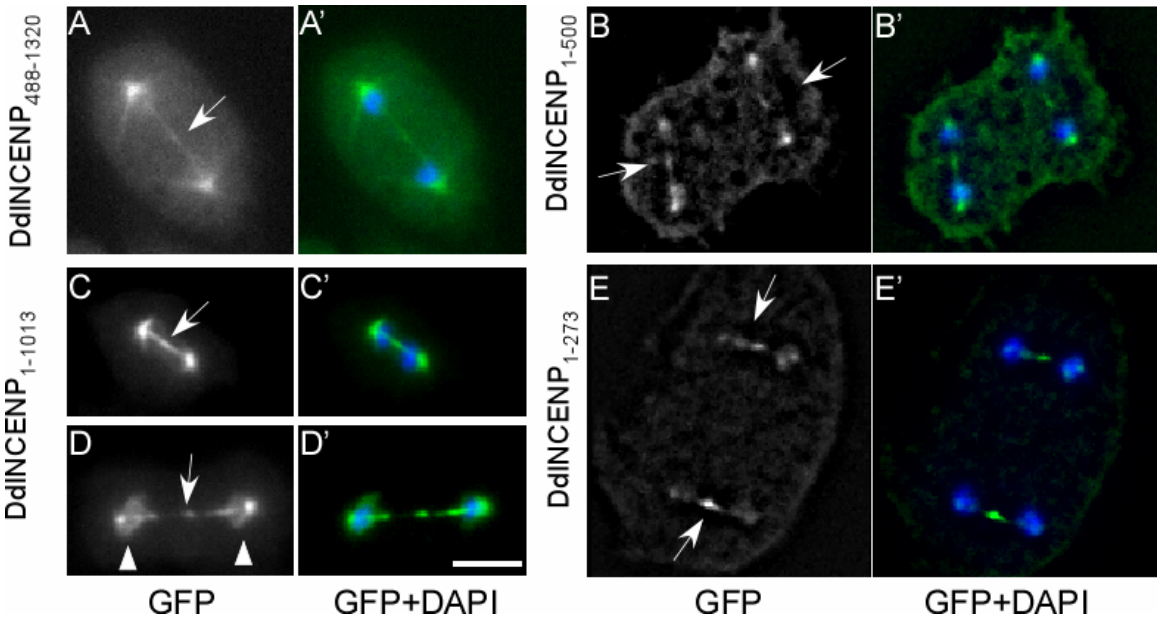


Figure 5.4: The localization of DdINCENP at the central spindle does not depend on either the IN box or its N-terminal fragment. The GFP-tagged mutants were expressed in DdINCENP null cells. Fluorescence microscopy images of these cells during anaphase are shown. (A-E) The GFP fluorescence signal is shown (A'-E') Merged fluorescence micrographs to show DNA (blue) and GFP fluorescence (green). All four DdINCENP mutants localized to the spindle pole bodies and the central spindles (arrows) during anaphase. Again, GFP-DdINCENP1-1013 had the strongest affinity to the central spindle compared to the others (C, C', D, D'). Additionally, GFP-DdINCENP1-1013 localized to the nuclear envelope (arrow heads) during telophase (D). Bar, 5 um.

concluded that the N-terminal 488-amino acid fragment was essential for the proper localization of DdINCENP during cytokinesis. However, it was still unknown whether the N-terminal fragment was sufficient to localize to the cortex of the cleavage furrow. To address that, I decided to make another truncated mutant, DdINCENP$_{1\text{-}500}$.

GFP-DdINCENP$_{1\text{-}500}$ was expressed in the DdINCENP null cells. Interestingly, I found that GFP-DdINCENP$_{1\text{-}500}$ still localized at the cortex of the cleavage furrow during cytokinesis (Figure 5.2). Additionally, its mitotic localization was very similar to that of DdINCENP$_{1\text{-}1013}$ (Figure 5.3 and Figure 5.4), although it showed strong cytoplasmic localization possibly due to its lack of a nuclear localization signal (Figure 5.1). The sequence of DdINCENP$_{1\text{-}500}$ included one of the two coiled-coil domains of the full length DdINCENP (Figure 5.1). A logical hypothesis would be that the coiled-coil domain was necessary to recruit DdINCENP to the cleavage furrow. Therefore, I also made DdINCENP$_{1\text{-}273}$, which had no coil-coiled domain, to test whether the coiled-coil domains of DdINCENP were crucial for its localization during cytokinesis. GFP tagged DdINCENP$_{1\text{-}273}$ was expressed in the DdINCENP null cells. Surprisingly, I found that GFP-DdINCENP$_{1\text{-}273}$ was still able to localize to the cleavage furrow and cytoplasmic bridge during cytokinesis (Figure 5.2) and to the mitotic spindle during mitosis (Figure 5.3 and Figure 5.4). Therefore, I concluded that the N-terminal 273-amino acid fragment of DdINCENP was sufficient for its localization at the cleavage furrow and the central spindle.

I have shown previously that both Kif12 and myosin II were involved in targeting of DdINCENP to the cortex of the cleavage furrow (see Chapter 4). Logically, I expected to see that the localization of DdINCENP$_{1\text{-}500}$ at the furrow would be affected by the loss of either one of these two proteins. Additionally, DdINCENP$_{488\text{-}1320}$ should still be absent from the furrow as it was in wild type cells. As expected, GFP-DdINCENP$_{1\text{-}500}$ was

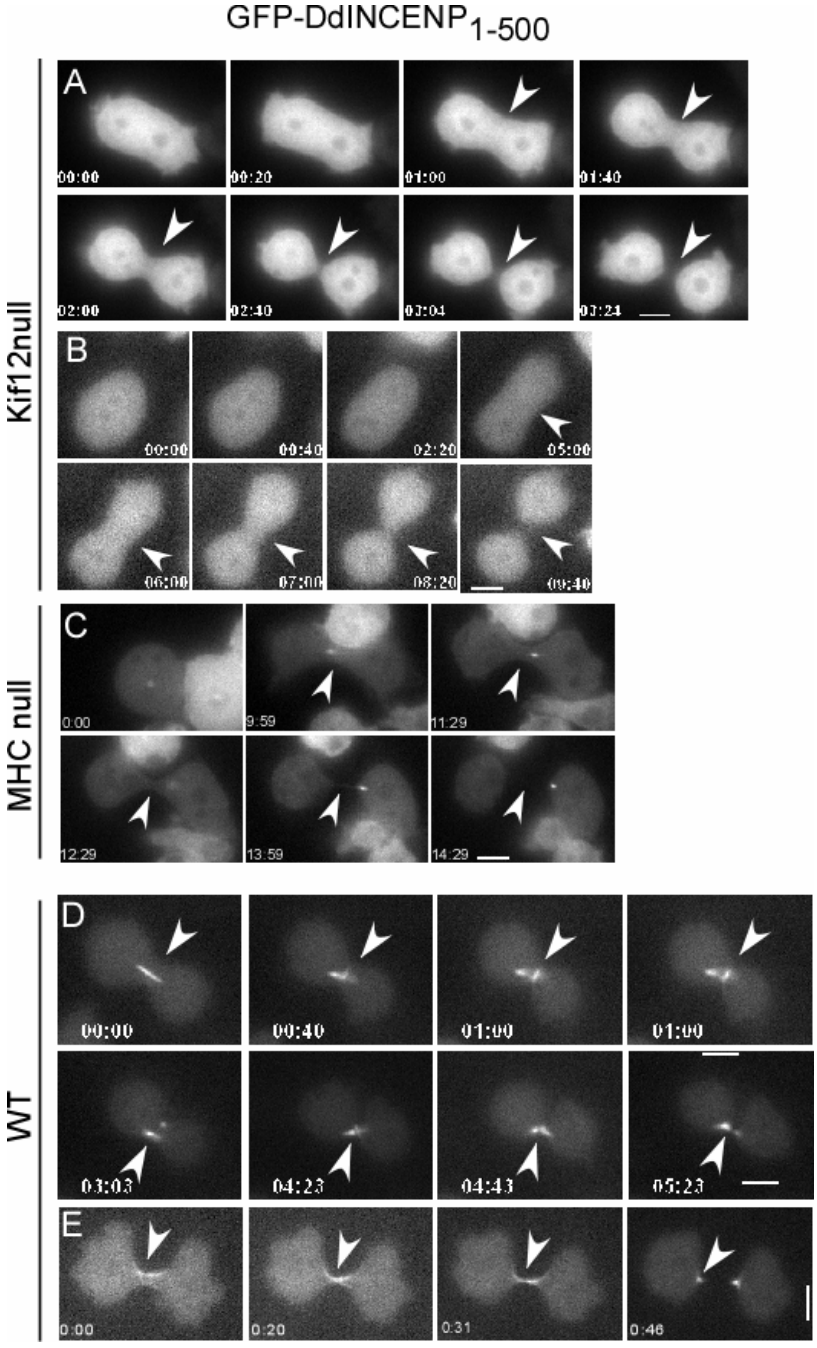


Figure 5.5 (see next page for the legend)

Figure 5.5: Both Kif12 and Myosin II are essential for the localization of DdINCENP1-500 at the cleavage furrow. GFP- DdINCENP1-500 was expressed in wild type, myosin II null, and Kif12 null cells separately. Time lapse fluorescence micrographs of these cells during cytokinesis are shown. Arrow heads point to the cleavage furrow. The GFP- DdINCENP1-500 localized to the cortex of the cleavage furrow in wild type cells (D, E). However, it was not found at the cleavage furrow in either Kif12 null cell (A, B) or myosin II null cells (C). Interestingly, GFP-DdINCENP1-500 was not detected at the central spindle in kif12null cells, either. On the contrary, it labeled the central spindle prominently in wild type cells (D). Times are indicated in minutes: seconds. Bars, 5 um.

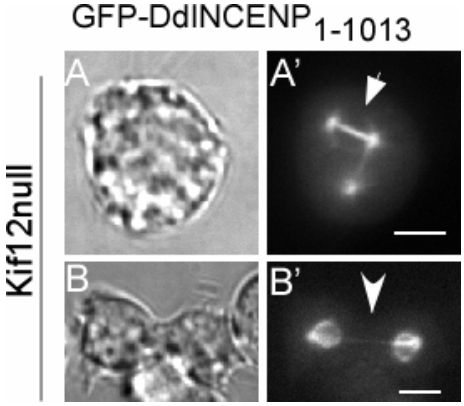


Figure 5.6: GFP-DdINCENP1-1013 also depends on Kif12 to transfer to the cleavage furrow. The live fluorescence microscopy images of Kif12 null cell expressing GFP-DdINCENP1-1013 are shown. Although the GFP tagged protein localized to the central spindle (arrow) in Kif12null cells (A'), it failed to transfer to the cleavage furrow (arrow head) during cytokinesis (B'). DIC images of the cells were also shown in A and B. Bars, 5 um.

absent from the cortex of the cleavage furrow in Kif12 null cells (Figure 5.5A-B). Surprisingly, the protein was not found at the central spindle of Kif12 null cells (Figure 5.5A-B). In contrast, it localized prominently on the central spindle in wild type cells (Figure 5.5D). Similar to DdINCENP$_{1-500}$, GFP-DdINCENP$_{1-1013}$ failed to target to the cleavage furrow in kif12null cells (Figure 5.6). These results confirmed my previous finding that Kif12 was essential for DdINCENP to localize to the cleavage furrow.

Previously, I have also shown that DdINCENP localized to a very narrow band at the equator of dividing myosin II null cells. Since DdINCENP$_{1-500}$ contains the portion necessary for the cleavage furrow localization, I expected that this fragment would localize to a similar band during cytokinesis in myosin II null cells. Surprisingly, GFP-DdINCENP$_{1-500}$ was totally absent from the cleavage furrow of myosin II null cells (Figure 5.5C). To explain this result, I postulated a direct interaction between the N-terminal 500 amino acids of DdINCENP and the acto-myosin contractile ring. Such an interaction would enable DdINCENP$_{1-500}$ to target to the cortex of the cleavage furrow. Subsequent experiments provided further evidence for this interaction, which would be described later in this chapter.

5.2.2 The N-terminal 500 amino acids of DdINCENP are sufficient to rescue the midzone spindle defect of DdINCENP null cells

I also investigated whether these truncated DdINCENP mutants would rescue the mitotic defects of DdINCENP null cells. One defect of DdINCENP null cells was their weak central spindle (Chapter 3, Figure 3.8). Given that both DdINCENP$_{1-500}$ and DdINCENP$_{488-1320}$ were found on the central spindle during mitosis, I was interested in determining if one of them could rescue the central spindle defect. Therefore, I examined the central spindle of DdINCENP null cells expressing either GFP-DdINCENP$_{1-500}$ or GFP-DdINCENP$_{488-1320}$. I expected that DdINCENP$_{488-1320}$ would rescue the defect

because it has the IN-box sequence. Surprisingly, the expression of GFP-DdINCENP$_{1-500}$ clearly rescued the weak spindle midzone defect of DdINCENP null cells (Figure 5.7 A, B), indicating that the N-terminal 500 amino acid segment of DdINCENP was sufficient for assembling a robust spindle midzone. In contrast, the expression of GFP-DdINCENP$_{488-1320}$ did not fully restore the intensity of microtubules on the central spindle (Figure 5.7 C, D). The central spindles of these cells were similar to those found in the DdINCENP null cells. However, I could not determine whether the failure to assemble a robust spindle was due to the missing 500 amino acids in this construct or due to its lower level of expression (Figure 5.7 E).

The mitotic and cytokinesis defects of DdINCENP null cells resulted in much slower growth rate compared to wild type cells (Chapter 3, Figure 3.6). This defect could be fully rescued by the expression of GFP-DdINCENP in the mutant cells. Remarkably, the expression of GFP-DdINCENP$_{1-500}$ also significantly improved the growth rate of the mutant cells, although the rate was still slightly lower than that of the cells expressing GFP-DdINCENP (Figure 5.7 F). In contrast, the expression of GFP-DdINCENP$_{488-1320}$ had no effect on the growth of the DdINCENP null cells (Figure 5.7 F). This finding, together with the results described above, demonstrated the important role that the N-terminal 500 amino acids of DdINCENP played during mitosis.

The other defect found in DdINCENP null cells was their hypersensitivity to microtubule destabilizing drugs such as thiabendazole (TBZ) and nocodazole. DdINCENP null cells could not grow in the presence of 1ug/ml of TBZ. Such a low concentration of TBZ had almost no effect on the growth of wild type cells (Chapter 3, Figure 3.11). I have shown that this phenotype was a result of the unstable mitotic spindle of DdINCENP null cells (Chapter 3, figure 3.11). The expression of GFP-DdINCENP

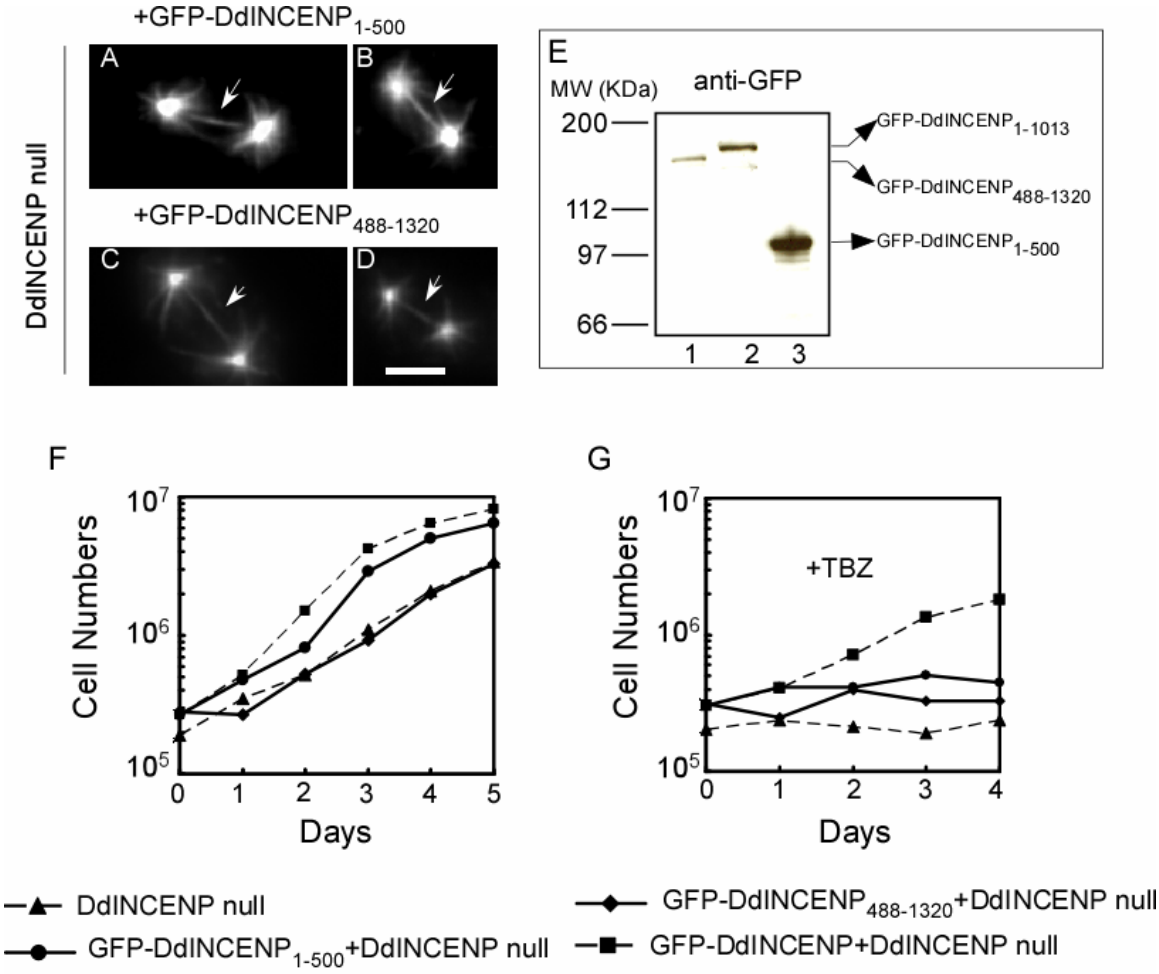


Figure 5.7 (see next page for the legend)

Figure 5.7: N-terminal 500 amino acids of DdINCENP are important for its function during mitosis. (A, B, C, D) The immunofluorescence microscopy pictures of DdINCENP null cells stained with anti-tubulin antibody, expressing either GFP-DdINCENP1-500 (A, B) or GFP-DdINCENP488-1320 (C, D). Arrows point to the central spindle. The expression of GFP-DdINCENP1-500 fully rescued the midzone spindle defect, while the expression of GFP-DdINCENP488-1320 only partially restored the intensity of the central spindle. (E) The expression level of the DdINCENP truncated mutants varied from each other. The total lysis of the DdINCENP null cells expressing GFP-DdINCENP488-1320 (lane 1), GFP-DdINCENP1-1013 (lane 2) and GFP-DdINCENP1-500 (lane 3) were individually loaded on the gel. 2x105 cells were loaded in each lane. The membrane was blotted with anti-GFP antibody. The expression level of GFP-DdINCENP1-500 was the highest among the three, while the amount of GFP-DdINCENP488-1320 was the lowest. (F) The expression of GFP-DdINCENP488-1320 also rescued the growth defect of DdINCENP null cells. The growth curves of the four cell lines were shown. DdINCENP null cells expressing GFP-DdINCENP488-1320 (solid line, diamond) grew as slow as the DdINCENP null cells (dash line, triangle). In the contrary, DdINCENP null cells expressing GFP-DdINCENP1-500 (solid line, circle) grew almost as fast as those cells expressing GFP-DdINCENP (dash line, quadrangle). (G) However, the expression of neither DdINCENP1-500 nor DdINCENP488-1320 could rescue the hypersensitivity to thiabendazole (TBZ) of the DdINCENP null cells, while the full length DdINCENP did. The four cell lines were grown in suspension with the presence of 1ug/ml TBZ.

could partially rescue this phenotype, although the cells still grew slower than wild type cells (Figure 5.7G). However, neither the expression of GFP-DdINCENP$_{1\text{-}500}$ nor GFP-DdINCENP$_{488\text{-}1320}$ could rescue TBZ sensitivity of DdINCENP null cells (Figure 5.7G). Therefore, both N-terminal and C-terminal portions of DdINCENP were necessary to stabilize the mitotic spindle.

5.2.3 IN-box is important for the function of DdINCENP during mitosis and cytokinesis

The data presented above mainly demonstrated the importance of the N-terminal sequence of DdINCENP to its functions. This is a bit surprising, given the commonly held view that INCENP mainly acts as a regulator of Aurora B kinase though the IN box. Nevertheless, I still thought that the IN box should be an essential part of DdINCENP, considering how conservative the sequences are at this domain across the species. DdINCENP$_{1\text{-}1013}$, which lacks the IN box (Figure 5.1), provided an excellent tool to explore this hypothesis. Therefore, I studied the mitosis of the DdINCENP null cells expressing GFP-DdINCENP$_{1\text{-}1013}$. In these mitotic cells, GFP- DdINCENP$_{1\text{-}1013}$ labeled the central spindle and the spindle pole bodies (Figure 5.4C). Additionally, as I described later in this chapter later, I found this chimera decorated the inner membrane of nuclear envelope, particularly during anaphase and telophase (Figure 5.4D). This made it relatively easy to follow the live mitosis in these cells, because both the movement of the nuclei and the spindle could be tracked by fluorescence microscopy.

As expected, the expression of GFP- DdINCENP$_{1\text{-}1013}$ did not fully rescue the mitosis and cytokinesis defect of the DdINCENP null cells. The most common defect found in these cells was mitotic failure during anaphase (Figure 5.8A, B). These cells clearly progressed through metaphase, judged from the appearance of the central spindle. However, they stalled in anaphase for an extended period of time. Sometimes, the central

spindle bended and forced the two daughter nuclei to come together (Figure 5.8A). While cell division in wild type cells usually takes no more than 10 minutes, the mutant cells did not progress to cytokinesis even after more than 15 minutes. Additionally, some cells were arrested in metaphase after the formation of a short bipolar spindle structure (Figure 5.8C). Both of these two phenotypes have been described for the DdINCENP null cells (see Chapter 3, Figure 3.7). There, I showed that the anaphase arrest in DdINCENP null cells was often caused by lagging chromosomes. Therefore, my data suggested that the DdINCENP IN box is required for the transition from metaphase to anaphase and anaphase to telophase.

Not all DdINCENP null cells failed in mitosis. Similarly, some DdINCENP null cells expressing GFP- GFP- DdINCENP$_{1\text{-}1013}$ progressed through anaphase and were then able to initiate cytokinesis (Figure 5.8D). The initiation of cytokinesis was clearly preceded by the separation of the two daughter nuclei and the disassembly of the central spindle. However, cytokinesis frequently failed and the two daughter cells merged to become a binucleate cell. These observations demonstrated that the IN-Box of DdINCENP was also important for successful cytokinesis, although the IN-box was not required for the localization of DdINCENP at the cleavage furrow.

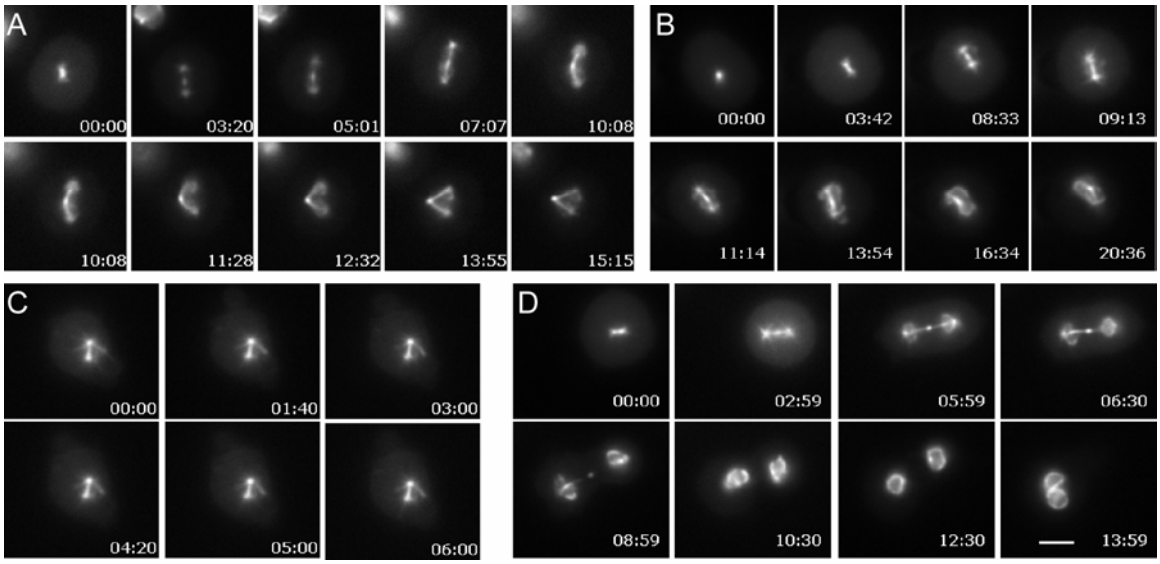


Figure 5.8: The IN box motif of DdINCENP is important for its function during both mitosis and cytokinesis. The time lapse fluorescence micrographs of the DdINCENP null cells expressing GFP- DdINCENP1-1013 were shown. The most common defect found in the DdINCENP null cells was mitotic failure during anaphase (A, B). In these cells, the central spindle either bent (A) or did not elongate (B). Additionally, some cells were arrested in metaphase (C). Sometimes, the cells were able to initiate cytokinesis before the cytokinesis failed and the two daughter cells fused with each other (D). Times are indicated in minutes: seconds. Bar, 5 um.

5.2.4 DdINCENP$_{1-500}$ localizes to the cortex of cell and interacts with the actin cytoskeleton during interphase

GFP-DdINCENP$_{1-500}$ was a cytosolic protein during interphase due to its lack of a nucleus localization signal (Figure 5.1). Upon careful examination, I found that GFP-DdINCENP$_{1-500}$ was associated with the cortex of wild type cell during interphase. In particular, GFP-DdINCENP$_{1-500}$ prominently localized to the bottom of the pinocytic cup (Figure 5.9A). Staining with phalloidin revealed extensive co-localization of F-actin and GFP-DdINCENP$_{1-500}$ at the pinocytosis sites (Figure 5.9B). These observations raised the possibility that DdINCENP$_{1-500}$ may interact directly with the actin cytoskeleton during interphase. As I mentioned earlier, this possibility was also suggested by the finding that Myosin II modulates the cleavage furrow localization of full length DdINCENP and DdINCENP$_{1-500}$ during cytokinesis. These results suggested that DdINCENP perhaps interacted with the actin cytoskeleton by directly binding to F-actin through its first 500 amino acids. To test this hypothesis, I determined the localization of GFP- DdINCENP$_{1-500}$ in polarized moving cells. When *Dictyostelium* cells are starved, they become polarized and begin directed moving. During this process, F-actin localizes to the leading edge of the streaming cells. I found that GFP-DdINCENP$_{1-500}$ was not enriched in either the front or the rear of polarized streaming cells (Figure 5.10A). Therefore, it was unlikely that DdINCENP$_{1-500}$ interacted directly with F-actin during interphase. Intriguingly, I found that GFP- DdINCENP$_{1-500}$ was enriched in the rear of myosin II null moving cells (Figure 5.10B).

I further examined the possible interaction between DdINCENP$_{1-500}$ and the actin cytoskeleton using a biochemical assay. When *Dictyostelium* cells are lysed by Triton X-100 and centrifuged to separate the pellet and supernatant, the actin cytoskeleton is enriched in the pellet while other cytosolic proteins remain in the supernatant.

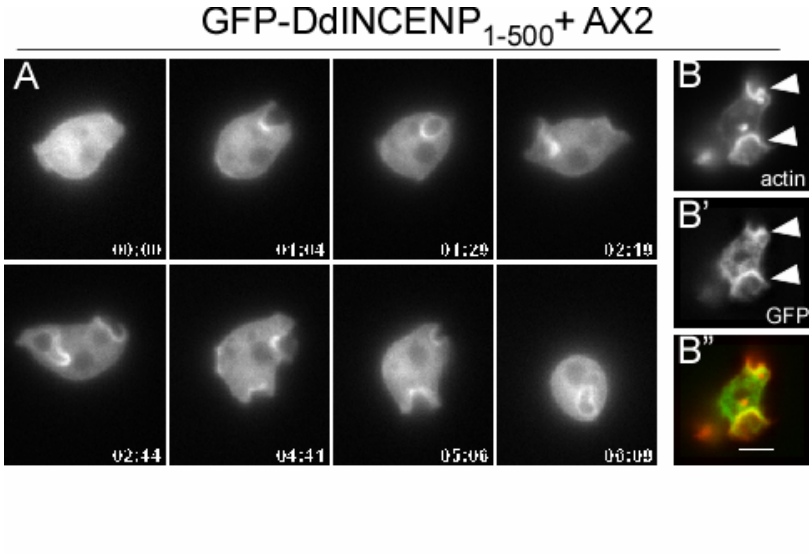


Figure 5.9: DdINCENP1-500 localizes to the cell cortex and the pinocytic cup during interphase. (A) The time lapse fluorescence micrographs of wild type cells (AX2) expressing GFP- DdINCENP1-500 are shown. The protein was enriched at the bottom of the pinocytic cup during pinocytosis. (B, B', B") AX2 cells expressing GFP-DdINCENP1-500 were fixed and immunostained with Texas Red labeled pholloidin for F-actin (B). The chimera protein was co-localized with F-actin on the pinocytic cups (arrow heads). Times are indicated in minutes: seconds. Bar, 5 um.

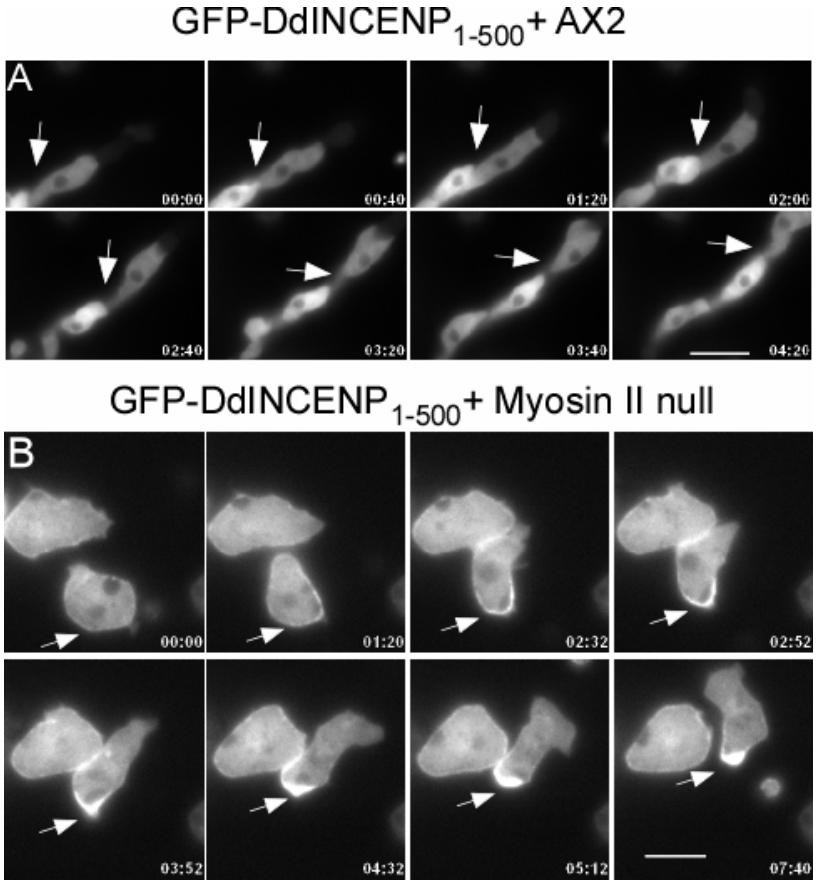


Figure 5.10: DdINCENP1-500 is not directly associated with F-actin in the moving cells. (A) GFP- DdINCENP1-500 did not show any enrichment in either the front or the rear of the moving wild type cells. AX2 cells expressing GFP- DdINCENP1-500 were starved and forced to move toward the aggregation center. The time lapse fluorescence micrographs of these moving cells are shown. Arrows point to the rear of the moving cells. Times are indicated in minutes: seconds. Bar, 10 um. (B) In the contrary, the chimera was greatly enriched in the rear of the moving Myosin II null cells. The time lapse fluorescence micrographs of the myosin II nulls expressing GFP- DdINCENP1-500 are shown. Arrows point to the rear of the moving cells. Times are indicated in minutes: seconds. Bar, 10 um.

As revealed by commassie blue staining, actin and myosin II were the most prominent proteins in the pellet while most other proteins remained in the supernatant (Figure 5.11A'). In this experiment, GFP-DdINCENP$_{1-500}$ almost equally distributed between the pellet and supernatant (Figure 5.11A). I also increased the salt concentration in the lysis buffer in order to decrease low affinity, unspecific interactions between proteins and the actin cytoskeleton. I found that some GFP-DdINCENP$_{1-500}$ still remained in the pellet, even when the concentration of salt was 200 mM (Figure 5.11A). This data strengthened the hypothesis that DdINCENP$_{1-500}$ may have the ability to interact with the actin cytoskeleton.

To precisely identify the proteins that associated with DdINCENP$_{1-500}$ in interphase cells, I constructed a TAP (Tandem Affinity Purification) tagged GFP-DdINCENP$_{1-500}$, which had similar localization as the untagged protein. Using the two-step affinity purification method, I purified the proteins associated with TAP-GFP-DdINCENP$_{1-500}$. As a control, I also purified proteins associated with TAP-GFP. By comparing the profiles of these two purifications (Figure 5.12), I found 4 proteins that co-purified specifically with DdINCENP$_{1-500}$ and not with GFP. I identified these proteins by Mass Spectrometry. They were actin, ABP-50, Hsp-70 and myosin II heavy chain. This experiment further confirmed the interaction between DdINCENP$_{1-500}$ and the actin cytoskeleton.

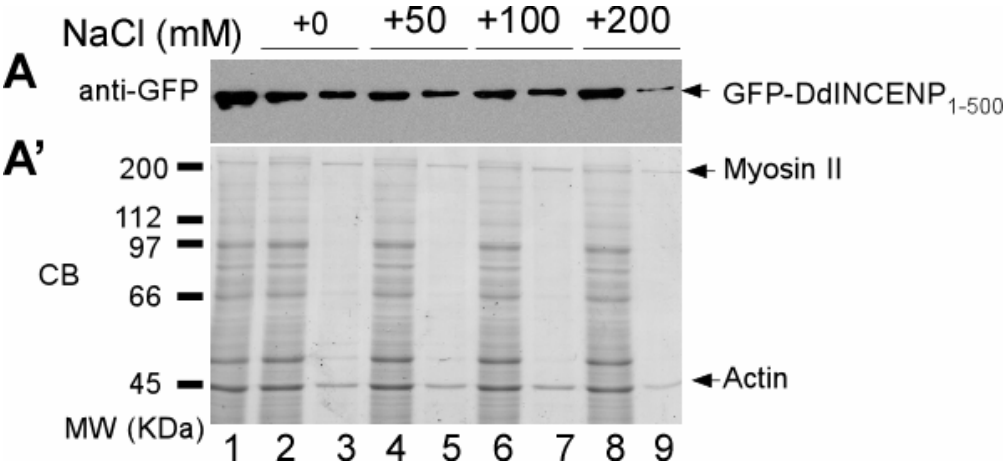


Figure 5.11: DdINCENP1-500 is associated with actin cytoskeleton. (A, A') GFP-DdINCENP1-500 was enriched in the Triton X-100 pellet of actin cytoskeleton. AX2 cells expressing GFP-DdINCENP1-500 was lysised by Triton X-100 lysis buffer with different concentrations of NaCl from 0 to 200 mM. Lane 1, total lysis. Lane (3, 5, 7, 9), the pellets. Lane (2, 4, 6, 8), the supernatants. As the commassie blue stained gel (A') showed, most proteins from the total lysis were found in the supernatant. Only the actin cytoskeleton and its associated proteins were enriched in the pellets, as myosin II and actin (arrows) were the two most prominent constituents. With the incremented concentration of salt in the lysis buffer, less proteins were found in the pellets. GFP-DdINCENP1-500 was enriched in the pellets as the anti-GFP western blot (A) showed. Almost 50% of the fusion protein was found in the pellet when the concentration of NaCl was low (lane 2-7), although its association with the actin cytoskeleton was significantly reduced in the high concentration of salt (lane 8-9).

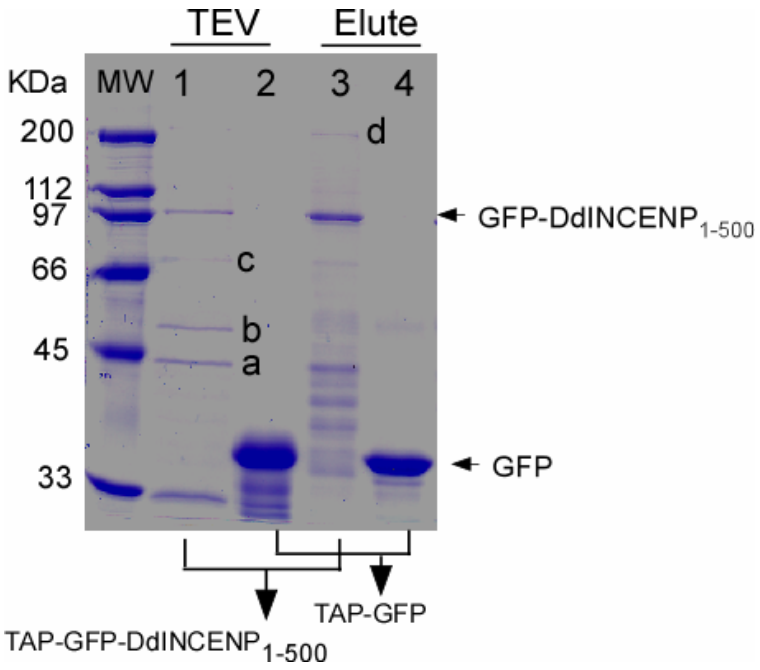


Figure 5.12 Using TAP tagged GFP-DdINCENP1-500, components of the actin cytoskeleton were purified through the affinity purification. TAP-GFP-DdINCENP1-500 and TAP-GFP were expressed separately in wild type cells. 2.5x109 cells of each cell line were harvested for the TAP purification. The protein samples purified with TAP-GFP-DdINCENP1-500 (lane 1, 3) and TAP-GFP (lane 2, 4) were loaded on the gel, which was stained by commassie blue. Lane 1, 2: the protein sample after the TEV cleavage from the IgG beads. Lane 3, 4: the eluted protein sample from the Calmodulin beads after the second affinity purification. From the prominence of GFP-DdINCENP1-500 in the samples, it was evident that the TAP purification was successful. Similarly, the control protein GFP was greatly enriched in the control samples. By comparing the proteins pulled by two TAP tagged proteins, I found that 4 protein bands were specific in the samples from TAP-GFP-DdINCENP1-500. These four samples were all identified by Mass Spectrometry. a, actin; b, ABP-50; c, Hsp-70; d, myosin II heavy chain.

5.2.5 Truncated mutants of DdINCENP localizes to the nucleolus during interphase

An unusual property of GFP-DdINCENP is its distribution on several discrete foci in the nucleus of interphase cells (Figure 5.13 A-B). I showed previously that those foci did not colocalize with centromeres and thus, their identity remained unknown (See Chapter 3). The availability of the truncated DdINCENP mutants provided a new tool to explore this issue in more detail. Therefore, I investigated the interphase localization of all four truncated DdINCENP mutants in the DdINCENP null cells (Figure 5.13).

Although GFP-DdINCENP$_{488\text{-}1320}$ was homogenously distributed in the nuclei of most cells, it was also found on punctate foci in cells that highly expressed the fusion protein (Figure 5.13C). These foci were similar to those labeled by full length GFP-DdINCENP (Figure 5.13A, B). Therefore, the depletion of the N-terminal 500 amino acids of DdINCENP likely decreased its affinity to those discrete foci in the nucleus. In comparison, GFP-DdINCENP$_{1\text{-}1013}$ stained the inner membrane of nuclear envelope in most cells, although it was also found in discrete foci in the nucleolus (Figure 5.13D, E). This was in stark contrast with the localization of either the full length DdINCENP or DdINCENP$_{488\text{-}1320}$. Remarkably, both GFP-DdINCENP$_{1\text{-}500}$ and GFP-DdINCENP$_{1\text{-}273}$ were similarly found at a small spot within each of the two nucleoli (Figure 13F, G, H, I). Thus, it is likely that at least two of those foci labeled by DdINCENP during interphase were in the nucleoli. My data also suggested that N-terminal 273 amino acids of DdINCENP were sufficient for its unique distribution pattern in the nucleoli. However, the nature of such a possible nucleoli structure and the significance of such nucleoli distribution remain unknown.

Additionally, I found that the GFP-DdINCENP$_{1\text{-}500}$ localized to the centrosomes, labeled by either anti-tubulin or anti-DdCP224 antibody, during interphase (Figure 5.13F, G). This was not surprising, given its localization at the spindle poles during mitosis. The

lack of centrosomal localization in interphase by either GFP-DdINCENP or GFP-DdINCENP$_{1-1013}$ could be explained by the fact that they were localized in the nucleus. By contrast, DdINCENP$_{1-500}$ did not have the putative nucleus localization signal (Figure 5.1) and appeared to be largely a cytosolic protein. Interestingly, GFP-DdINCENP$_{1-273}$ did not localize to the centrosomes (Figure 5.13H, I), although it was found in the cytoplasm during interphase. However, GFP-DdINCENP$_{1-273}$ was still found in the spindle pole bodies during mitosis (Figure 5.4E). Thus, the centrosome-interacting motif of DdINCENP appeared to be the N-terminal coiled-coil domain (Figure 5.1), which was not necessary for the localization of DdINCENP at the spindle poles.

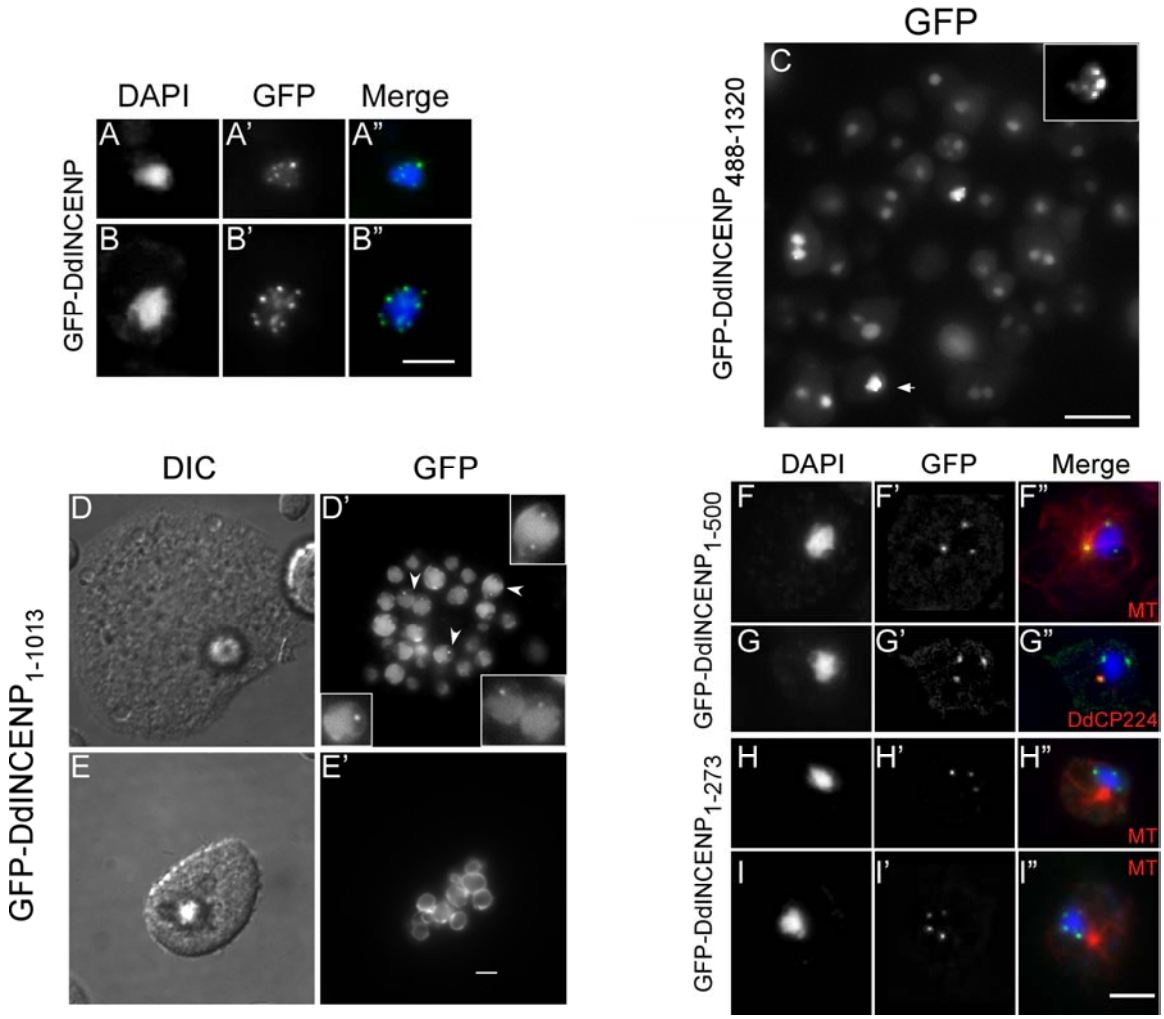


Figure 5.13 (see next page for the legend)

Figure 5.13: The localization of the DdINCENP truncated mutants during interphase. (A-B) The fluorescence microscopy images of wild type (AX2) cells expressing GFP-DdINCENP. GFP-DdINCENP was found in discrete foci in the nucleus during interphase. DNA is shown in blue. GFP fluorescence is shown in green. Bar, 5um. (C) The fluorescence microscopy images of live AX2 cells expressing GFP-DdINCENP488-1320. Although the GFP fluorescence was homogenous in the nuclei of most cells, sometimes it was found at punctate foci in those cells highly expressing the fusion protein (arrow, insert). Bar, 10um. (D-E) The fluorescence microscopy images of live AX2 cells expressing GFP-DdINCENP1-1013. The GFP fluorescence most commonly painted the nuclear envelope (E'). However, it was also found at the discrete foci in the nucleolus (arrow heads, inserts). Bar, 5um. (F-G) The immunofluorescence microscopy images of AX2 cells expressing GFP-DdINCENP1-500. The chimera was usually found at discrete foci in the nucleolus in addition to its localization at the centrosomes (F", G"). The cells were stained with either anti-tubulin (F", red) or anti-DdCP224 (G", red) antibodies to show the interphase centrosomes. (H-I) The immunofluorescence microscopy images of AX2 cells expressing GFP-DdINCENP1-273. Similar to GFP-DdINCENP1-500, this fluorescence of the chimera was found at the discrete foci in the nucleolus in wild type cells during interphase. However, it did not

show co-localization with the centrosomes stained by anti-tubulin antibody (H" and I", red). Bar, 5 um.

5.3 DISCUSSION

5.3.1 The localization of DdINCENP to the cleavage furrow is dependent on its N-terminal 500 amino acids

INCENP can interact with Aurora B with its C-terminal IN-box domain to act as both a substrate and regulator of Aurora B kinase (Bishop and Schumacher 2002). Additionally, they appear to be interdependent for their localization during mitosis (Adams et al. 2001). However, my data suggested that the interaction with Aurora B kinase may not be essential for targeting DdINCENP to the cleavage furrow. I made three IN-box-less C-terminal truncated DdINCENP mutants, including $DdINCENP_{1-1013}$, $DdINCENP_{1-500}$ and $DdINCENP_{1-273.}$ All three mutants were found at the cleavage furrow. In contrast, the N-terminal truncated mutant $DdINCENP_{488-1320}$ lacking the IN-box was not found at the furrow.

All three C-terminal truncated mutants were able to localize on the central spindle before transferring to the central spindle. Therefore, it could be argued that the spindle localization was a prerequisite for DdINCENP to target to the cleavage furrow. Indeed, the kinesin Kif12 protein, essential for the localization of DdINCENP at the cleavage furrow, was also essential for these C-terminal truncated mutants of DdINCENP to localize to the furrow.

Until this work, there had been no report on the essential motifs required for the transfer of INCENP from the central spindle to the cleavage furrow. However, it has been reported that the N-terminal 68 amino acids of chicken INCENP are essential for its localization to the centromeres, the spindle midzone and the midbody (Ainsztein et al.

1998). Therefore, it appears that the N-terminal sequences of INCENP are functionally conserved from *Dictyostelium* to human, although they are not conserved at the sequence level. It seems also likely that the N-terminal 68 amino acids of vertebrate INCENP may be essential for its localization at the cleavage furrow, similar to its homolog in *Dictyostelium*.

The N-terminal 500 amino acids of DdINCENP were essential not only for its dynamic localization during cell division, but also for its mitotic function. This was demonstrated by the fact that the expression of GFP-DdINCENP$_{1-500}$ alone could rescue both the weak central spindle and the growth defect of DdINCENP null cells. One likely scenario is that the N-terminal sequence of DdINCENP could recruit additional proteins, such as other chromosomal passenger proteins or the Polo kinase, to the spindle midzone to coordinate mitosis and cytokinesis. In fact, INCENP was found to be essential for the centromere localization of Polo kinase in vertebrate system (Goto et al. 2006). An important question raised by my truncation experiments is whether the different DdINCENP fragments are able to interact with the endogenous Aurora kinase. Although the IN-box has been defined as an important interaction domain of INCENP with Aurora B, it is not necessarily the only way in which INCENP and Aurora interact. It is possible, for example, that the N-terminus of INCENP interacts with Aurora through other chromosomal passenger proteins. My colleague Hui Li is currently exploring the possible interaction between the different DdINCENP constructs and the *Dictyostelium* Aurora homolog.

5.3.2 Possible Interaction between DdINCENP and the actin cytoskeleton

I have shown that myosin II modulates the localization of DdINCENP at the cleavage furrow (chapter 4). GFP-DdINCENP was found on a narrow band at the equator of the cleavage furrow in myosin II null cells, compared with the much broader cortex

distribution of DdINCENP in wild type cells. Furthermore, I found here that the localization of GFP-DdINCENP$_{1-500}$ to the cleavage furrow depended on myosin II. This raised the possibility that DdINCENP interacts with the actin cytoskeleton at the cortex of the cleavage furrow during cytokinesis. However, this interaction appeared to be transient during cytokinesis and difficult to demonstrate. In contrast, the interaction between DdINCENP$_{1-500}$ and the actin cytoskeleton was more stable and easier to study. In fact, I found that almost half of GFP- DdINCENP$_{1-500}$ was found in a Triton X-100 cytoskeletal fraction. Furthermore, while this truncated mutant was localized at the cleavage furrow during cytokinesis, it was also found at the cell cortex and enriched at pinocytic cups during interphase. Macropinocytosis is an actin-dependent process that allows *Dictyostelium* cells to engulf the nutrient medium by forming a pinocytic cup. This process is known to involve many actin associated proteins such as coronin, talin and Profilin (Cardelli 2001). Some of those proteins, which localize to the pinocytic sites, are also found at the cleavage furrow and have important functions during both pinocytosis and cytokinesis (Konzok et al. 1999; Cardelli 2001; Rivero et al. 2002).

To better understand the interaction of GFP-DdINCENP$_{1-500}$ with the cytoskeleton, I also investigated its localization in streaming cells during chemotaxis. F-actin is usually concentrated in the front end of streaming cells while myosin II is mostly at the rear. Since GFP-DdINCENP$_{1-500}$ did not show any particular preference on its distribution in streaming cells, I concluded that it was unlikely that DdINCENP$_{1-500}$ was directly associated with either F-actin or myosin II. It seems likely that an intermediate molecule recruits DdINCENP$_{1-500}$ to the cell cortex. This hypothesis is consistent with the finding that GFP-DdINCENP$_{1-500}$ was enriched at the rear of the moving myosin II null cells. It is possible that these cells have compensated the loss of myosin II with another cytoskeletal molecule to help the contraction at the rear of the cell.

To define more clearly the interactions between GFP-DdINCENP$_{1-500}$ and the actin cytoskeleton, I decided to tag GFP-DdINCENP$_{1-500}$ with tandem affinity purification (TAP) tag and identify its associated proteins by affinity purification. TAP tag has been widely used for purifying protein complexes, because it eliminates most of the nonspecific proteins through a two-step affinity purification procedure (Puig et al. 2001). Using this approach, I identified four proteins that copurified with TAP-GFP-DdINCENP$_{1-500}$ as actin, myosin II heavy chain, Hsp70 and ABP-50. Of these four, it seems unlikely that DdINCENP$_{1-500}$ binds directly to either actin or myosin II for the reason described above. Although *Dictyostelium* Hsp70 is a chaperone protein, it also binds to the F-actin capping protein cap32/34 and regulates their activity, through its N-terminal ATPase domain (Haus et al. 1993). ABP50, also called elongation factor 1 alpha, binds F-actin and can bundle actin filament (Dharmawardhane et al. 1991). During chemotaxis, ABP-50 colocalizes with F-actin, which makes it unlikely to be the link between DdINCENP$_{1-500}$ and the actin cytoskeleton. However, the abundance of these proteins in the pool of the proteins pulled down did provide evidence that DdINCENP$_{1-500}$ was associated with the actin cytoskeleton. Due to the limited ability to sequence the purified proteins with low abundance, I did not identify any novel interacting partner of DdINCENP.

5.3.3 Distribution of DdINCENP Truncated Mutants during Interphase

During interphase in vertebrate cells, INCENP is found diffused throughout the nucleus (Cooke et al. 1987). Therefore, I found it intriguing that DdINCENP was associated with seven nuclear foci during interphase. Our colleagues Kaller & Nellen (Germany) helped us determine that these foci were not the centromeres of *Dictyostelium*, as they did not colocalize with either Hcp A or lysine 3 di-methyl histone H3, both of which are centromere markers (Kaller et al. 2006). Additionally, the

centromeres of *Dictyostelium* are congregated on a single area adjacent to the nuclear envelope during interphase, which is similar to what occurs in yeast cells (Funabiki et al. 1993; Kaller et al. 2006).

Although the nature of the DdINCENP labeled foci is still not known, they likely are not protein aggregations. First, the number of the foci per nucleus was seven, which is exactly the number of condensed bodies visible at metaphase in chromosome spread experiments (Robson and Williams 1977; Welker and Williams 1980). In situ hybridization experiments have shown that these seven condensed bodies represent the six *Dictyostelium* chromosomes and the extrachromosomal rRNA element (Sucgang et al. 2003). Secondly, two truncated mutants of DdINCENP also localized to punctate foci in the nucleus during interphase, although they were only found at discreet foci within the nucleolus. The nucleolus is a nuclear substructure where most ribonucleoprotein complexes are assembled including ribosomes, tRNAs and telomerase. *Dictyostelium* has 2-4 nucleoli during interphase, all of which are close to the nuclear envelope (Maclean et al. 1984). One common feature among the interphase localization of DdINCENP, $DdINCENP_{1-500}$ and $DdINCENP_{1-1013}$ was that they all distributed at the proximity of the nuclear envelope. In higher eukaryotic systems, a portion of heterochromatin localizes close to the inner nuclear membrane during interphase (Mathog et al. 1984; Blobel 1985). Heterochromatin 1 (HP1) links the heterochromatin to lamin B receptor (LBR) on the inner nuclear envelope (Ye et al. 1996; Ye et al. 1997). Interestingly, HP1 has been shown to interact with the N-terminal of INCENP (Ainsztein et al. 1998). Therefore, it seems likely that DdINCENP binds to a heterochromatin structure close to the nuclear membrane through its interaction with HP1. The *Dictyostelium* homologs of HP1 are HcpA, HcpB and HcpC (Kaller et al. 2006). Although the majority of HcpA and HcpB localizes to one single foci during interphase (Kaller et al. 2006), they also localize to

some minor sites in the nucleus. It is possible that those minor sites at the proximity of the nuclear membrane represent the DdINCENP-labeled foci.

I also found that GFP-DdINCENP$_{1-500}$ localized to the centrosomes during interphase. Interestingly, although DdINCENP$_{1-273}$ did not localize to centrosomes during interphase, it was found at the spindle pole bodies during mitosis. Therefore, the domain required for binding to the spindle pole bodies was not the same one for binding to the interphase centrosome.

Chapter 6: Plasmids and cell lines

Table 6.1 Plasmids and cell lines used in Chapter 3:

Plasmids:	**Descriptions:**
pTXGFP-DdINCENP	GFP tagged DdINCENP at the NH_2-terminal, in pTXGFP vector, G418 resistance, under actin 15 promoter.
GFP-DdCP224	GFP tagged DdCP224 at the Carbonyl-terminal, in pB15-GFP vector (Graf et al. 2000), G418 resistance, under actin 15 promoter. The version with blasticidin-resistance is also available.
GFP-tubulin	GFP-α-tubulin fusion under the actin15 promoter, in pDEXRH vector (Neujahr et al. 1998). The version with blasticidin-resistance is also available.
GFP-histone H2B	GFP tagged histone H2B at the NH_2-terminal, in pDEXRH vector, G418 resistance, under actin 15 promoter (Graf et al. 2003).
GFP-DdCP224-AA	GFP tagged DdCP224, whose S368 and S1268 are replaced by alanines.
GFP-DdCP224-EE	GFP tagged DdCP224, whose S368 and S1268 are replaced by glutamic acids.
GFP-AurBKD	GFP tagged DdAurB, whose K139 is replaced with arginine, in pTXGFP vector.
Cell lines:	**Descriptions:**
10B6, 10H10	DdINCENP null cells, blasticidin-resistance, based on AX2 cells.
KO2-3	DdINCENP null cells, blasticidin-resistance, based on NC4A2 cells.

Table 6.2 Plasmids and cell lines used in Chapter 4:

Plasmids:	**Descriptions:**
GFP-MHC	GFP tagged myosin heavy chain, G418-resistance, in pBigGFP vector (Moores et al. 1996). G418-resistance.
GFP-Kif12	GFP tagged Kif12, G418-resistance, in pTXGFP vector (Lakshmikanth et al. 2004). G418-resistance.
Cell lines:	**Descriptions:**
MHC null	Myosin heavy chain null cells, blasticidin-resistance, based on AX2 cells.
kif12 null	Kif12 null cells, blasticidin-resistance, based on AX2 cells (Lakshmikanth et al. 2004).
racE null	RacE null cells, based on DH1 cells.
lvsA null	LvsA null cells, based on DH1 cells.
5E2	Clathrin heavy chain null cells, blasticidin-resistance, based on AX2 cells.

Table 6.3 Plasmids and cell lines used in Chapter 5:

Plasmids:	**Descriptions:**
GFP-DdINCENP$_{1\text{-}1013}$	GFP tagged DdINCENP$_{1\text{-}1013}$ at the NH_2-terminal, in pTXGFP vector, G418-resistance.
GFP-DdINCENP$_{1\text{-}500}$	GFP tagged DdINCENP$_{1\text{-}500}$ at the NH_2-terminal, in pTXGFP vector, G418-resistance.
GFP-DdINCENP$_{1\text{-}273}$	GFP tagged DdINCENP$_{1\text{-}273}$ at the NH_2-terminal, in pTXGFP vector, G418-resistance.
TAP- GFP-DdINCENP$_{1\text{-}500}$	GFP tagged DdINCENP$_{1\text{-}500}$ at the NH_2-terminal, in pTXiTAPGFP vector, G418-resistance.
GFP-DdINCENP$_{488\text{-}1320}$	GFP tagged DdINCENP$_{488\text{-}1320}$ at the NH_2-terminal, in pTXGFP vector, G418-resistance.

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