Genetic and RNAi analysis of INCENP (inner centromere protein) and DNA Topoisomerase II in *Drosophila melanogaster*

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I hereby declare that this thesis has been composed by myself and that the work presented is my own, except where stated otherwise

Chih-Jui Chang
20th October 2003

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Abstract:

This thesis is divided into two parts. In part I, I carried out a genetic screen to obtain new *incenp* alleles and interactors. After screening 5000 chromosomes, I isolated 18 mutants, one of them was a new allele of *incenp* that we called $incenp^{3747}$. Two mutants (3322, 4330) were dominant female sterile. All of the mutants were homozygous lethal. Some of the mutants showed defects in eyes, wings, bristle when combined with $incenp^{P(EP)2340}$. The sequence of the coding region of *incenp* in $incenp^{3747}$ homozygous mutants showed a point mutation. A single base change in exon 4 resulted in a stop codon. The predicted molecular weight of the putative truncated gene product would be 61kD. The truncated protein was not detected by Western blot, which could suggest that it is unstable although I cannot rule out the unlikely possibility that our antibody failed to detect the truncated product. The phenotypic analysis of $incenp^{3747}$ showed that the homozygous embryos exhibit chromosome segregation defects. In mixed populations of early embryos ($incenp^{3747}$/CyO-ftz-LacZ) I found chromosome segregation defects as early as cycle 3. Some of the nuclei also failed to follow the globally synchronous mitotic oscillator in the early divisions. My analysis revealed that these early defects resulted from a maternal contribution to the phenotype, due to genetic interaction with the balancer chromosome. Later in development, homozygous mutant embryos showed aberrant neuronal morphology and the homozygous mutant first instar larvae escapers exhibit abnormal behaviour.

In part II, I used dsRNAi in *Drosophila* S2 cells to carry out a detailed functional analysis of the role of Topoisomerase II (Topo II) during mitosis. I found that Topo II was not required for the assembly of a functional kinetochore or the targeting of chromosomal passenger proteins, nonetheless, it was essential for anaphase sister chromatid separation. Surprisingly, in metaphase cells depleted of Topo II, one or more chromosome arms frequently stretched out from the metaphase plate to the vicinity of the spindle pole. This was not kinetochore-based movement, as the centromere of the affected chromosome was located on the plate. The FISH experiments revealed that the protruding chromosome arm could be the X, or either arm of chromosome 3, but apparently not chromosome 2.

Table of contents

List of Figures

List of tables

Common Abbreviations

aa	Amino acid(s)
ATP	Adenosine 5'-triphosphate
bp	Base-pair
°C	Degree Celsius
cDNA	Complementary Deoxyribonucleic Acid
cm	centimeter
Da	Dalton
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymine triphosphate
DMSO	Dimehtyl Sulfate
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced Chemi-Luminescence
EDTA	Ethylenediaminetetraacetic acid
g	gram(s)
h	hour(s)
HEPES	*N-2*-hydroxyethylpiperazine-*N'-2*-ethanesulfonic acid
HRP	Horseradish Peroxidase
kb	kilobase
kDa	kilodalton
kV	kilovolt(s)
l	Litter(s)
LB	Luria Broth (medium)
M	Molar
mA	milliampere(s)
mg	milligram(s)
mM	millimolar
mmol	millimole(s)
min	minute(s)
ml	milliliter(s)

mRNA	Messenger Ribonucleic Acid
ng	nanogram(s)
nm	nanometer(s)
nt	nucleotide
PAGE	Polyacryamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
pmol	picomole(s)
RNA	Ribonucleic Acid
RT	Room Temperature
rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulphate
TAE	Tris/Acetate (buffer)
TBS	Tris-buffered Saline
TEMED	*N,N,N',N'*-tetramethyl-ethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
µg	microgram(s)
µl	microliter(s)
µm	micrometer(s)

Note

In the text, '*Drosophila* should be taken to signify the '*Drosophila melanogaster*' unless otherwise specified. I have used the nomenclature *incenp*^{3747}, *incenp* and INCENP to signify *Drosophila* mutants, genes and proteins respectively.

PART I

Genetic Analysis of INCENP In *Drosophila*

Chapter 1

Introduction

1.1 General introduction

In order to produce two daughter cells that are accurate copies of the parent, a cell must grow and replicate its DNA before division. These events take place during the cell cycle, which can be subdivided into four phases: S phase (the chromosomal DNA is replicated), M phase (two daughter cells are formed), G1 phase (the intervening gap phase between mitosis and DNA replication), and G2 phase (the intervening gap phase between the completion of DNA replication and mitosis). In M phase (mitosis), the replicated DNA of eukaryotes has to be condensed and segregated faithfully for equal partition into the two daughter cells. Here we only focus on the events that take place in mitosis.

1.2 Mitosis and cytokinesis

For the purpose of its study, mitosis has been arbitrarily separated into five phases: prophase, prometaphase, metaphase, anaphase and telophase (Figure 1.1). During prophase, the chromosomes become condensed (Figure 1.1 A). In prometaphase, the nuclear envelope starts to break down and the chromosomes attach to the spindle by the ability of kinetochores to capture the ends of microtubules (Figure 1.1 B, C). When all of the chromosomes have attained bipolar orientations and moved to positions midway between the two spindle poles, the cell is said to be in metaphase (Figure 1.1 D). In anaphase, the sister chromatids disjoin and begin to move toward the poles (Figure 1.1 E, F). In telophase, the chromosomes approach the poles and the nuclear envelope starts to reform (Figure 1.1 G). During cytokinesis, the cell cleaves between the separated nuclei (Figure 1.1 H). The mechanism of cytokinesis requires the function of a contractile ring of actin and myosin that forms just prior to the onset of a cleavage furrow. The contractile ring is arrayed perpendicular to the mitotic spindle and at an equal distance between the two spindle poles.

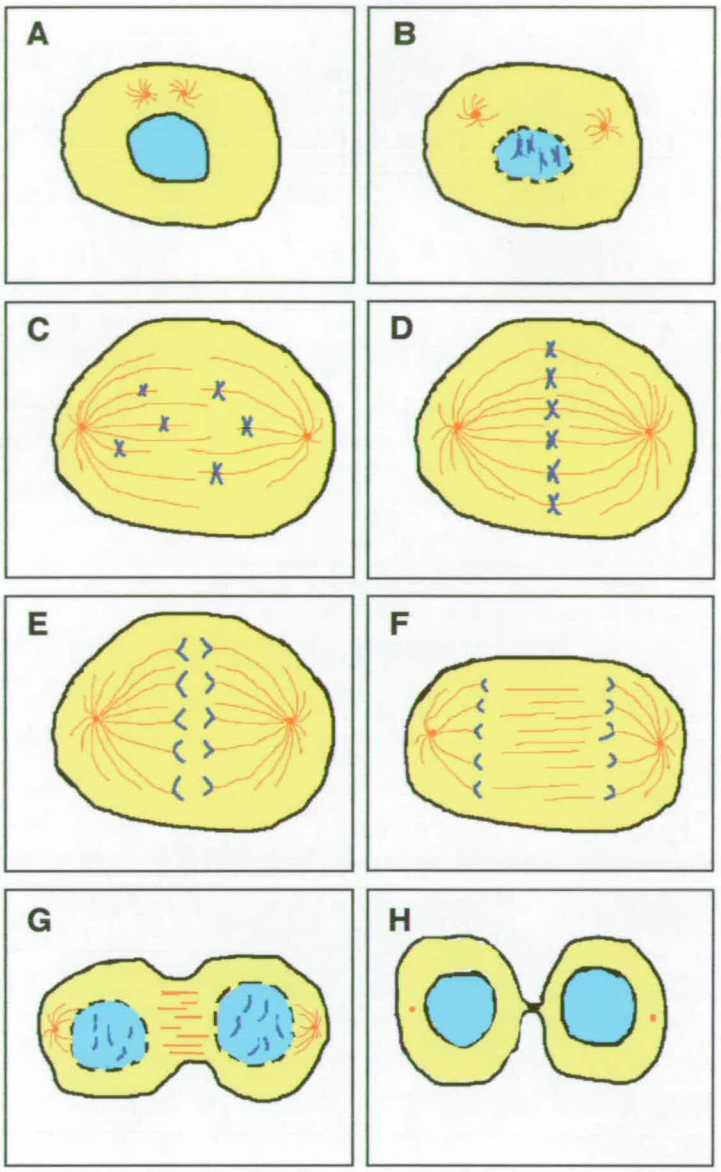


Figure 1.1 Diagrammatic representation of eukaryotic mitosis
(A) Prophase. (B,C) Prometaphase. (D) Metaphase. (E,F) Anaphase. (G) Telophase. (F) Cytokinesis. Chromosomes are shown in blue, microtubules in red.

1.3 The centromere of higher eukaryotes

The centromere is a specialized region on the chromosome that typically contains highly repetitive DNA sequences and a variety of associated proteins (Cleveland et al., 2003). It has three functions: (I) it is the site at which the replicated sister chromatids attach to each other. The cohesion complex is bound all along the chromosome during S phase, then most dissociates during prophase except at the centromere. At the metaphase/anaphase transition, the cohesion between the sister chromatids is released at the centromere. (II) It is the site to which the mitotic and meiotic spindle is attached to the chromosome. (III) It contains a number of motor proteins that are responsible for chromosome movement and checkpoint proteins that are responsible for ensuring the fidelity of the mitotic process.

The protein components of the higher eukaryotic centromere can be classified into three groups. The members of the first group are proteins that are present on the centromere throughout the cell cycle. The members of the second group are checkpoint proteins that are bound to centromeres only until they make proper attachments to the spindle. The proteins in the third group are chromosomal passenger proteins, which localize to different cellular locations during the mitotic cycle. The characterization of the passenger proteins will be discussed below.

1.4 Chromosomal passenger proteins

The success of mitosis depends on the integration of chromosomal and cytoskeletal behavior. In mitosis, the replicated chromosomes must segregate equally to the two daughter cells. Cell cleavage (cytokinesis) should not happen until sister chromatids have been segregated to opposite poles of the cell during anaphase. Therefore, the reorganization of the cytoskeleton that occurs during cytokinesis has to be coordinated with the movements of the chromosomes. A class of proteins termed chromosomal passengers has been proposed to play an important role in the coordination of chromosomal and cytoskeletal functions in mitosis (Earnshaw and Cooke, 1991).

Chromosomal passenger proteins were originally identified by their subcellular localization (Cooke et al., 1987). These proteins associate with chromosomes during the early stages of mitosis and transfer from chromosomes to the central spindle at the onset of anaphase. The details of this behavior will be described in the following section. Four chromosomal passenger proteins have been identified: INCENP (Inner centromere protein) (Cooke et al., 1987), Aurora B (Schumacher et al., 1998), Survivin (Skoufias et al., 2000; Uren et al., 2000) and Telophase disc 60 (Andreassen et al., 1991). Recently, two other proteins have also been characterized as chromosomal passengers, ICIS (inner centromere protein KinI stimulator) (Ohi et al., 2003) and CSC-1 (chromosome segregation defective) (Romano et al., 2003).

1.5 INCENP

Inner **cen**tromere **p**rotein (INCENP), the first chromosomal passenger protein to be described, was discovered in a screen for proteins in the mitotic chromosome scaffold (Cooke et al., 1987). The INCENP proteins were originally identified in chicken as a doublet on SDS-PAGE gels consisting of a shorter 96 kDa INCENP1 and a 101 kDa INCENP2 polypeptide containing a 38 amino acid insertion that arises through differential splicing of a single primary RNA transcript (Mackay et al., 1993). Homologs of INCENP have been identified in *Xenopus* (Stukenberg et al., 1997), mouse (Fowler et al., 1998; Saffery et al., 1999), *C. elegans* (ICP-1) (Kaitna et al., 2000), *Drosophila* (Adams et al., 2001c), and budding yeast (Sli15p) (Kim et al., 1999). The human INCENP gene is a single copy gene found at chromosomal region 11q12. HsINCENP colocalizes with the Aurora B (formerly known as AIRK2) kinase on chromosomes and is overexpressed in tumor cells (Adams et al., 2001b). INCENP has been shown to be a microtubule-binding protein. INCENP can bind to microtubules directly and if microtubule dynamics are suppressed by exposure to taxol, INCENP fails to reach the equatorial cortex during late mitosis (Wheatley et al., 2001b).

When expressed in mammalian cells, chicken INCENP shows an identical cell cycle distribution to the endogenous protein. This suggests conservation of functional domains between species (Mackay et al., 1993). Overexpression of a truncation mutant of chicken INCENP ($INCENP_{1-405}$) and a chimeric CENP-B:INCENP protein in mammalian cell culture resulted in a dominant-negative phenotype, with defects in

prometaphase chromosome alignment and completion of cytokinesis (Eckley et al., 1997; Mackay et al., 1998). The study of INCENP knockout mice shows that INCENP is an essential gene. The homozygous-null embryos die at the 32-64-cells stage. The phenotype of these embryos is characterized by the absence of discernible metaphase or anaphase stages, absence of midbodies, multinucleated cells, multipolar mitotic spindles, micronuclei formation, abnormal microtubule bundling and chromatin bridges (Cutts et al., 1999). However, the early death of the embryos made it difficult to observe a sufficient number of mitotic cells to perform a thorough analysis of the phenotype. RNAi studies in *Caenorhabditis elegans* embryos showed that INCENP was required for the proper segregation of the chromosome and for efficient completion of cytokinesis (Kaitna et al., 2000). INCENP-depleted embryos showed mitotic chromosome segregation defects and failure of completion of cytokinesis (Oegema et al., 2001). An RNAi study in *Drosophila* S2 cells also suggested roles of INCENP in chromosome structure, prometaphase congression, chromosome segregation and completion of cytokinesis (Adams et al., 2001c).

Cell cycle distribution of INCENP

INCENP is dispersed on the chromosome arms in the early stages of mitosis (Figure1.2 A) and concentrates at centromeres during prometaphase and early metaphase (Figure1.2 B) (Earnshaw and Cooke, 1991). At the metaphase-anaphase transition, INCENP detaches from the chromosomes and becomes concentrated on linear arrays that transect the metaphase plate. At anaphase onset, it is found in the spindle midzone and associated with the overlapping antiparallel microtubules of the central spindle (Figure 1.2 C). In late anaphase, a portion of INCENP localizes to the

inner surface of the plasma membrane in the place where the contractile ring is going to form (Eckley et al., 1997). During cytokinesis, INCENP is found at normal and ectopic cleavage furrows formed between spindles (Eckley et al., 1997; Savoian et al., 1999) (Figure 1.2 D).

Molecular analysis of INCENP

Molecular analysis of INCENP reveals that it is a complex multidomain protein. Two highly conserved functional modules have been identified within the first 68 amino terminal residues: one is responsible for transfer to the spindle, the second for targeting to the centromere (Ainsztein et al., 1998). Adjacent to these modules is a region of the protein responsible for binding heterochromatin protein-1 α ($HP1^{Hs\alpha}$). The C-terminal half of INCENP contains a putative coiled-coil region (residues 511-696). The minimal region that displayed a positive interaction with β-tubulin was $INCENP_{48-85}$ (Wheatley et al., 2001b). The IN-BOX is very conserved 40-50-amino acid domain in the COOH-terminal of INCENP, which defines the INCENP family from yeast to humans (Adams et al., 2000) (Figure 1.3). It was showed that Ipl1 kinase binds to and phosphorylates Sli15 in the IN-BOX region. This binding of Sli15 and Ipl1 increased the kinase activity of Ipl1 (Kang et al., 2001).

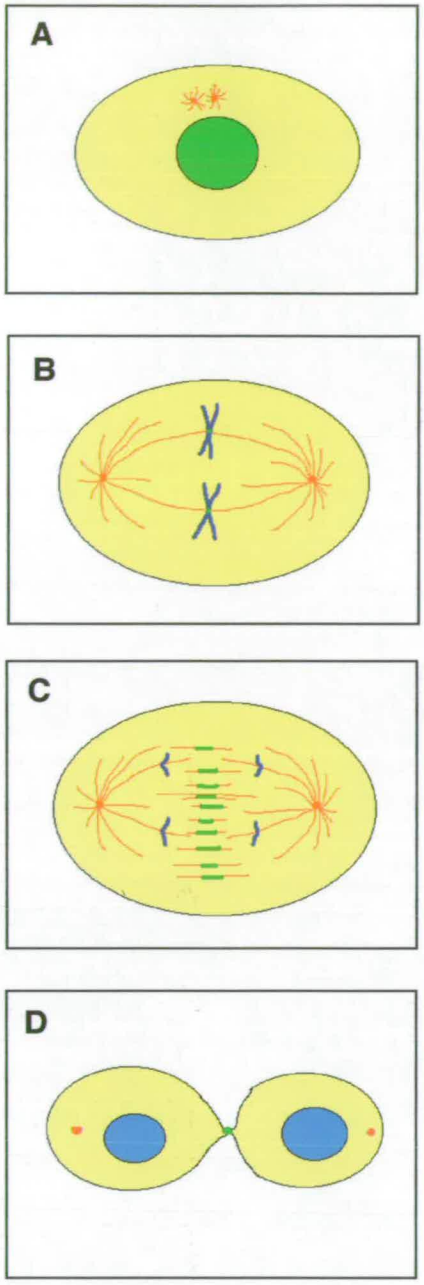


Figure 1.2 Cell cycle distribution of INCENP
(A) Prophase, INCENP is dispersed on the chromosome arms. (B) Metaphase, INCENP concentrates at centromeres. (C) Anaphase, INCENP associats with the overlapping antiparallel microtubules of the central spindle. (D) Cytokinesis, INCENP is found at midbody.

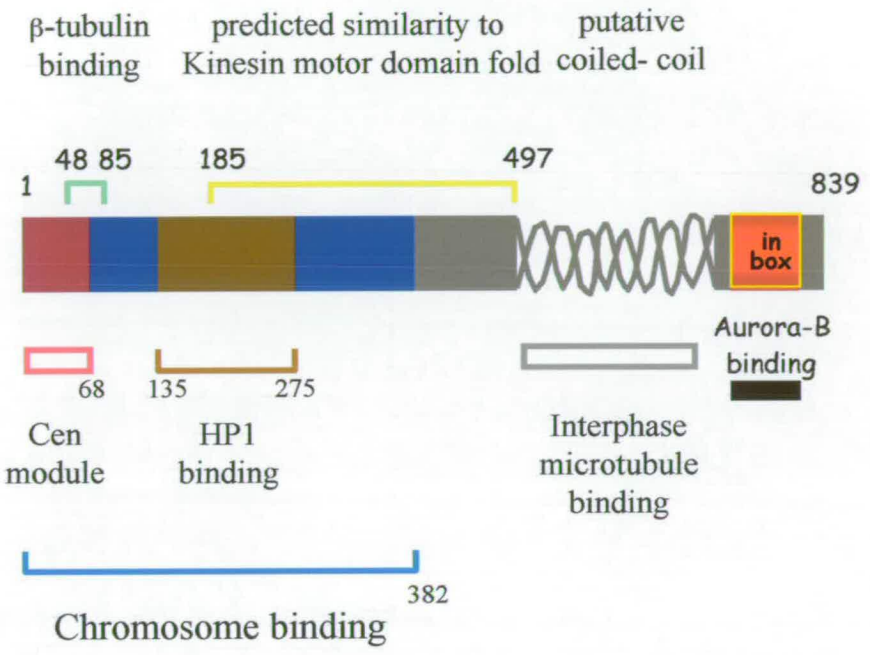


Figure 1.3 Functional domain of chicken INCENP (adapted from Wheatley et al., 2001)

1.6 Aurora B

Aurora kinases are a family of conserved serine/threonine kinases that have been shown to be involved in many aspects of cell cycle, including centrosome separation, bipolar spindle assembly and chromosome segregation (Giet and Prigent, 1999); (Nigg, 2001). In yeast, a single Aurora kinase has been identified (Francisco and Chan, 1994; Petersen et al., 2001). In mammals, there are three Aurora kinase families, Aurora A, Aurora B and Aurora C (Adams et al., 2001a; Carmena and Earnshaw, 2003; Nigg, 2001). They have different localizations and roles in the cell cycle. Aurora A kinase localizes to centrosomes and spindle pole microtubules (Bischoff et al., 1998; Giet et al., 2002). It is implicated in centrosome separation, maturation and spindle assembly (Kufer et al., 2002; Tsai et al., 2003). Aurora C has only been identified in mammals. It localizes to spindle poles and is expressed in testis (Kimura et al., 1999).

Mammalian Aurora B was first cloned in a screen to isolate novel protein kinases that caused growth arrest when overexpressed in *S. cerevisiae* (Terada et al., 1998). Aurora B is the only one of the Aurora kinase family members that behaves as a chromosomal passenger protein. It localizes in the centromere in prometaphase and transfers to the central spindles in anaphase. In telophase, it becomes concentrated at the midbody (Adams et al., 2000; Terada et al., 1998).

In *Saccharomyces cerevisiae*, the Ipl1 kinase is responsible for histone H3 phosphorylation (Biggins et al., 1999; Chan and Botstein, 1993). In *Caenorhabditis*

elegans and *Drosophila melanogaster*, histone H3 is also phosphorylated on serine[10] by the respective Aurora kinase homologoues AIR2 and Aurora B (Adams et al., 2001c; Giet and Glover, 2001; Hsu et al., 2000). CENP-A is also a substrate of Aurora B (Zeitlin et al., 2001). Proteomic analysis of human metaphase chromosomes suggests that topoisomerase IIα is also a substrate of Aurora B kinase (Morrison et al., 2002). Studies in *Caenorhabditis elegans* and humans show that INCENP is a direct substrate of Aurora B kinase. Aurora B kinase AIR-2 specifically phosphorylated the *C. elegans* INCENP ICP-1 at two adjacent serines within the carboxyl terminus (Bishop and Schumacher, 2002; Honda et al., 2003). The essential kinetochore protein Ndc10p was also shown to be a substrate of Ipl1 in yeast (Biggins et al., 1999).

1.7 Survivin

Survivin was first discovered by Altieri and co-workers in 1997 (Ambrosini et al., 1997). It is a member of the IAP family (**in**hibitor of **ap**optosis) of anti-apoptotic proteins. IAPs contain a BIR-domain (baculoviral IAP repeat) (Miller, 1999), which is essential for interaction with pro-apoptotic proteins, such as caspases (Deveraux and Reed, 1999). Survivin exists as a dimer (Verdecia et al., 2000). Altieri and coworkers suggest that survivin is phosphorylated by p34cdc2 kinase (O'Connor et al., 2000). Survivin binds SMAC and stops it from inactivating IAP protein (Song et al., 2003). This may explain anti-apoptotic effects seen in numerous transfection experiments.

Apart from its role in apoptosis, survivin plays an important role during cell division (Fraser et al., 1999; Li et al., 2000a; Uren et al., 1999). Survivin is expressed during G2/M phases and has been shown to behave as a chromosome passenger protein (Skoufias et al., 2000; Uren et al., 2000; Wheatley et al., 2001a). Inhibition of survivin has been associated with cell-cycle defects, including pleiotropic cell division, cell growth and cell morphology (Li et al., 1999). Homologues of Survivin also exist in yeast (BIR1 and BIR2) and *Drosophila* (Deterin) (Jones et al., 2000; Uren et al., 1998).

In fission yeast, BIR1 mutants display a 'cut' phenotype, in which the septum forms in dividing yeast before the chromatin has segregated into the daughter cells. These studies suggest that survivin is involved in cytokinesis and chromosome segregation (Uren et al., 1999). This was also seen when survivin mutants were expressed in human cells (Li et al., 1999).

Survivin is required for a sustained spindle checkpoint arrest in response to lack of tension. Cells lacking survivin enter mitosis with normally and form bipolar spindles, but are delayed in prometaphase. This prometaphase delay is due to activation of the spindle assembly checkpoint (Carvalho et al., 2003; Lens et al., 2003).

1.8 TD-60

TD-60 (**T**elophase **d**isc **60** kDa protein) is a mitosis-specific antigen identified by human autoimmune serum (Andreassen et al., 1991). TD-60 is also a passenger protein based on its distribution in mitosis by immunofluorescence staining. TD-60 has been shown to colocalize with INCENP and Aurora B throughout the cell cycle (Martineau-Thuillier et al., 1998).

Apart from its localization, little was known about the function of this protein until the human gene was cloned (Mollinari et al., 2003). Mollinari and colleagues showed by sequence analysis that TD-60 is a member of the RCC1 family of guanine nucleotide exchange factors (GEFs). They suggest that TD-60 might act as a specific GEF for Rac1 during mitosis by showing that it binds to the small G protein Rac1 in its nucleotide-free form. siRNA suppression showed that TD-60 is required for progression from prometaphase to metaphase.

1.9 Chromosomal passenger Complex

There are several studies that show the chromosomal passenger proteins form a complex to regulate many events of mitosis. It has been shown that Survivin and Aurora B interact directly in a two-hybrid assay and that in an in vitro pull-down assay (Wheatley et al., 2001b). In budding yeast, Sli15p was shown to be synthetically lethal with temperature-sensitive mutants of the Aurora B homolog Ipl1, and these two proteins also formed a complex (Kim et al., 1999). Other biochemical

evidence also showed that Aurora B physically interacts with INCENP. INCENP is stockpiled in *Xenopus* eggs in an 11S complex together with Aurora B kinase (Adams et al., 2000). Stukenberg and colleagues showed that xSurvivin exists in a complex with both xINCENP and xAurora B in interphase and mitotic *Xenopus* extracts. Immunodepletion of xAurora B can completely remove xSurvivin and xINCENP from extracts, suggesting that all of the xSurvivin and xINCENP is physically associated with xAurora B kinase (Bolton et al., 2002). In *Caenorhabditis elegans*, another component of the chromosomal passenger complex was identified, CSC-1 (chromosome segregation and cytokinesis defective). CSC-1 has been shown to interact with AIR-2 (Aurora B), ICP-1 (INCENP) and BIR-1 (Survivin) by genetic and biochemical evidences (Romano et al., 2003). However, no homologous was reported in other organisms.

Biological roles of chromosomal passenger protein complex

dsRNAi experiments in *Drosophila* and *Caenorhabditis elegans* showed that cells lacking Aurora B or INCENP have similar mitotic defects, including missegregation of chromosomes, and failure in cytokinesis. The localization of Aurora B and INCENP is inter-dependent on each other (Adams et al., 2001c). In budding yeast, Ipl1-Sli15 is required for chromosome segregation (Biggins et al., 1999). The complex is required to promote chromosome bi-orientation by altering kinetochore-spindle pole connections (Tanaka et al., 2002).

In *C. elegans*, RNAi experiments showed that elimination of BIR-1/survivin function led to mislocalization of the Aurora B/AIR-2 kinase, defects in histone H3 phosphorylation and abnormal chromosome condensation (Speliotes et al., 2000).

Survivin-null mouse embryos displayed polyploidy and failed cytokinesis (Uren et al., 2000). Both INCENP and Survivin contribute to the localization of the complex and regulate Aurora B kinase (Honda et al., 2003). Recombinant Human Aurora B was strongly activated upon binding of INCENP, but not Survivin. This activation resulted in phosphorylation of INCENP (within its C-terminal IN-Box), and is required for activation of the Aurora B/INCENP complex.

1.10 The use of *Drosophila melanogaster* as model organism for the study of mitosis

Drosophila melanogaster has been used as a model for genetic studies for over a hundred years. In 2000, the completion of the euchromatic *Drosophila* genome sequence made *Drosophila* even more powerful as a model organism. Several characteristics make *Drosophila* an ideal system for the study of mitosis. Firstly, mutational analysis can identify genes essential for this process; secondly, mitosis can be studied in a developmental context; and thirdly, *Drosophila* offers a very favorable cytology for these studies.

In *Drosophila*, maternally provided protein and mRNA are sufficient to drive development during the first thirteen embryonic cycles, a rapid succession of S and M phases without intervening gaps. Homozygous mutants for genes essential in mitosis can therefore reach this stage due to the contribution of maternal gene product in the egg by their heterozygous mothers. Three more divisions (in this case with a G2) take the embryo through cellularisation and gastrulation. Only the cells that are going to form the nervous system keep dividing after that. The time in

development in which the mutant phenotype is first observed depends on the stage at which the maternal contribution is exhausted for null alleles.

Most processes involved in larval development depend on cell growth and endoreduplication. Cell division (in this case with the canonical G1 and G2 gap phases) is restricted to the brain, the imaginal discs and the abdominal histoblasts late in larval stages, so this is when most mitotic mutant phenotypes will first be detected (Gatti and Baker, 1989). This means that *Drosophila* allows us to study many different types of cell cycle, including G_2-regulated cycles (arrest in G_2 phase), G_1-regulated cycles (arrest in G_1 phase) and cycles of DNA synthesis without mitosis (endoreplication, cells become polyploid). Besides, *Drosophila* has four easily identifiable mitotic chromosomes that provide a convenient system in studies of chromosome morphology.

Chapter 2

A genetic screen for new *incenp* alleles and interactors

2.1 Introduction

This part of the project had a dual purpose: on one hand, I wanted to study the function of INCENP in a developing multicellular organism; on the other, I wanted to identify new interactors of INCENP to help dissect the function of the chromosomal passengers in the different stages of cell division. In order to serve these two purposes I carried out a mutagenesis screen to look for new alleles and interactors of DmINCENP. To this aim I made use of a previously identified semi-dominant allele of DmINCENP-(*incenp*$^{P(EP)2340}$, see below). The analysis of the mitotic phenotypes of the newly generated mutant alleles in different developmental stages would provide information about the requirements for DmINCENP in development. Identification of the new genetic interactors would potentially give new insights of the mechanism of action of the chromosomal passengers in cell division.

2.2 *incenp*$^{P(EP)2340}$

incenp$^{P(EP)2340}$ is a mutant allele of DmINCENP, in which a P-element is inserted into the third exon of the *Drosophila incenp* gene (Figure2.1). This mutant was generated in a mis-expression screen (a screen to identify genes that, when over- or mis-expressed in a pattern of interest, give a specific phenotype or modulate an existing mutant phenotype) (Rorth, 1996). It is homozygous lethal and was described originally as a female sterile dominant mutation. Dr. Mar Carmena carried out the initial characterization of *incenp*$^{P(EP)2340}$. Male individuals carrying the insertion show a dominant meiotic phenotype. The defects analyzed include misshapen meiotic spindles, tetrapolar or multipolar spindles and defects in the onion stage consistent with problems in chromosome disjunction and cytokinesis. In order to demonstrate that the P-element insertion in the DmINCENP gene was responsible for the defects, she excised the P-element. The precise excisions reverted the lethality and the dominant phenotype. All the imprecise excisions obtained were lethal and also showed dominant phenotypes.

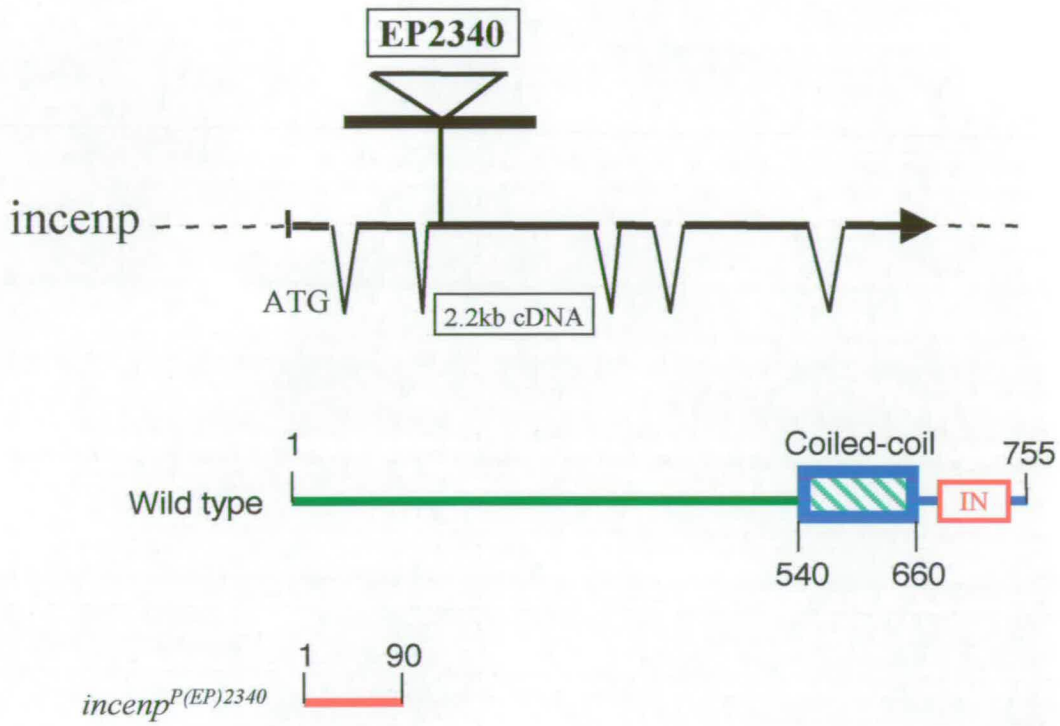


Figure 2.1 Insertion site of the P-element in *incenp*$^{P(EP)2340}$
Schematic representation of the protein encoded by *incenp*$^{P(EP)2340}$. A P-element is inserted into the third exon of the *Drosophila* incenp gene. The putative protein length of DmINCENP gene product of *incenp*$^{P(EP)2340}$ is 90 amino acids.

2.3 Genetic screen

Choice of mutagen: EMS-treatment

In general, there are three different ways to create mutations in *Drosophila*: chemical mutagens, radiation and insertional mutagenesis. The method to be used depends on the type of mutation one wants to obtain. Chemical mutagens are able to create point mutations or small deletions, which is ideal for an allelic series. Additionally, point mutations can generate mutant proteins which are expressed but disrupted in their interactions with other proteins. As I was looking for new alleles and interactors of INCENP, I chose the chemical mutagen Ethylmethane sulfonate (EMS), an alkylating agent, to induce mutations for our screen. Of course, a drawback of this strategy is that the mapping of point mutations can be time-consuming and, in some cases, difficult.

Screen strategy

The screen strategy is shown in Figure 2.2. Male flies (w^{1118}) were mutagenised for two reasons: (1) the mature sperm is more sensitive to mutagens than oocytes; (2) one male will be able to mate with many females to propagate the chromosomes with mutations, making the recovery of mutated chromosomes more efficient.

The INCENP gene is located in 43A04 (on the second chromosome). In order to simplify the screen, I only isolated mutations in the second chromosomes, although some interactors of INCENP could be in the other chromosomes. The mutagenised males were crossed to virgin females carrying a balancer (CyO-Kr-GFP). EMS could

affect gonial cells as well as mature sperm. Mutagenesis of the gonial cells would result in different sperm carrying the same mutation. To avoid isolating the same mutation more than once, the males were removed from the females after mating for four days.

To isolate mutations that interact genetically with *incenp*$^{P(EP)2340}$, the mutagenised males were then individually crossed to virgin females carrying *incenp*$^{P(EP)2340}$ over a balancer chromosome, CyO. The progeny of this cross will have one of the following four genotypes: (1) */CyO (*=mutagenised chromosome); (2) */ *incenp*$^{P(EP)2340}$; (3) *incenp*$^{P(EP)2340}$/CyO-Kr-GFP; (4) CyO-Kr-GFP/CyO: these flies are late embryonic lethal. I sought to isolate and analyze three kinds of mutations from the individual crosses:

(1) Mutations which were lethal over *incenp*$^{P(EP)2340}$.

All of the progeny showed the dominant curly wing phenotype of the balancer chromosomes.

(2) Mutations which showed a visible phenotype over *incenp*$^{P(EP)2340}$.

In this case, some of the straight winged progeny (*/*incenp*$^{P(EP)2340}$) were viable but showed external phenotypes consistent with defects in mitosis. These included rough eyes, nicked wings and/or duplicated bristles.

(3) Mutations which were male sterile over *incenp*$^{P(EP)2340}$.

I tested the fertility of every individual male carrying a mutagenised chromosome over *incenp*$^{P(EP)2340}$ by crossing them to wild type females.

5000 individual mutagenised chromosomes were generated and analyzed. I isolated eighteen homozygous lethal mutations. They are 201, 282, 487, 497, 549, 666, 1608, 1650, 2364, 2374, 2388, 2394, 2519, 3322, 3747, 3959, 4056 and 4330. Two of these were dominant female sterile mutants (3322, 4330).

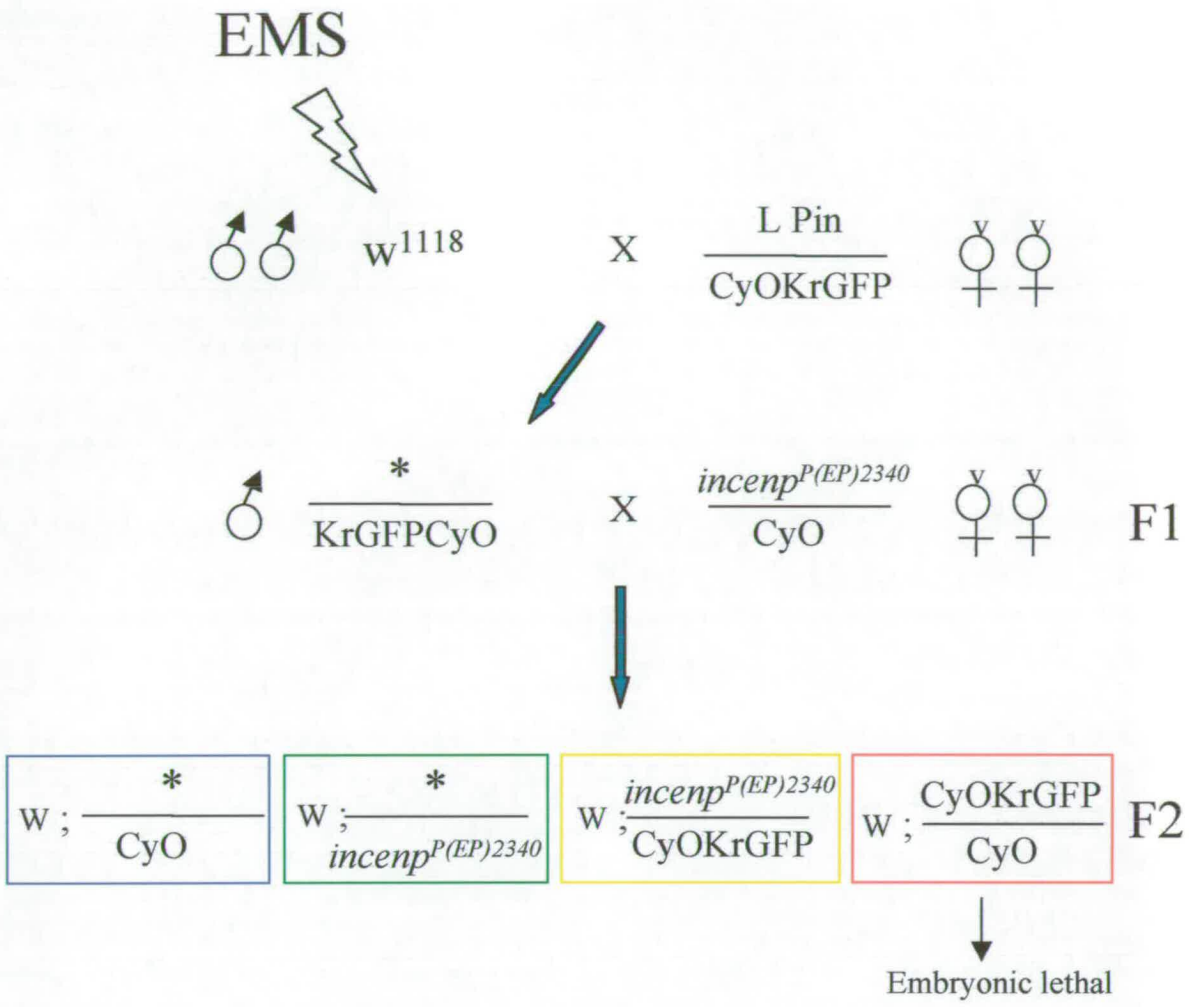


Figure 2.2 Scheme of the screen strategy
The wild type strain w^{1118} was exposed to EMS. The EMS-mutagenised flies were crossed to a strain containing two marked chromosomes; the flies selected for were those that had the balancer CyO-Kr-GFP. These flies were then crossed to *incenp*$^{P(EP)2340}$ to analyze the phenotype. * denotes the EMS generated mutations.

2.4 The female sterile dominants: 3322, 4330

3322 and 4330 are homozygous lethal. They are also lethal when transheterozygous to *incenp*$^{P(EP)2340}$. I was unable to establish a stock by crossing males and females carrying the mutation over balancer chromosome. The reason for this is that these mutations are female sterile dominant. In order to keep these chromosomes, heterozygous males were isolated every generation and out-crossed to *incenp*$^{P(EP)2340}$ /CyO females.

2.5 Initial characterization of the mutants

Complementation analysis

The EMS mutants were crossed to one another to analyze the genetic interactions among them (Table 2.1). The transheterozygotes could be divided into two groups according to their phenotype:(I) Lethal; (II) Sterile.

(I): Lethal interactions:

201/282, 201/487, 201/497, 201/549, 201/1650, 201/2364, 201/2374, 201/4056, 210/3322, 201/4330, 2364/2374, 666/3747, 1650/3959, 1650/2388, 549/3322, 549/4330, 1650/2388, 2519/3747,

(II): Sterile interactions:

3747/1608, 3747/666, 3747/549, 1608/4056.

The results of the analysis showed that 2364 and 2374 were apparently in a single complementation group. The two dominant female sterile mutants 3322 and 4330 also define a single complementation group. 201 interacted with 10 of the 18 mutants. All the mutants have currently been mapped in the lab by deficiency complementation. The results showed that 201 did not complement many deficiencies (much more so than other mutants) along the second chromosome. This meant that 201 unlikely carried more EMS-mutations than other mutant lines, instead this suggested that 201 could be an essential gene that genetically interacts with a large number of other genes. This analysis provided the preliminary results of interactions among those mutations. However, the complementation test was performed before the 'cleaning' of the chromosome (see section 2.7). These genetic interactions needed to be further confirmed after 'cleaning', as these were EMS-induced mutations, and multiple hits on the same chromosome cannot be excluded.

♂ \ ♀	201	487	497	549	666	1608	1650	2364	2374	3747	4056	282	2388	2394	2519	3959	3322	4330
201			●	●			●		●			●					X	X
487	●																X	X
497	●																X	X
549	●																X	X
666																	X	X
1608																	X	X
1650	●												●				X	X
2364	●								●								X	X
2374	●							●									X	X
3747					●												X	X
4056	●																X	X
282	●																X	X
2388																	X	X
2394																	X	X
2519										●							X	X
3959							●										X	X
3322	●			●														X
4330	●			●													X	

Table 2.1 Complementation test of EMS mutants

Black dot: No complementation. X: crosses could not be performed (3322 and 4330 are female dominant sterile).

Lethal stage of mutants

All of the mutants are homozygous lethal. During the screen, I isolated those mutants based on analyzing the phenotype of transheterozygous progeny (*incenp*$^{P(EP)2340}$/mutant). Most of the mutants are lethal when combined with *incenp*$^{P(EP)2340}$ except for 282, 2388, 2394, 2519, 3959, which showed defects in the eyes, bristles, wings (Figure 2.3, Figure 2.4 Figure 2.5). After establishing lines from each mutant, it became possible to analyze the phenotype of transheterozygous *incenp*$^{P(EP)2340}$/mutant combinations on a larger scale. Some of the transheterozygotes turned out to be semilethal (Table 2.2). The lethal stage of the homozygous mutants was also analyzed (by Dr. Sarah Goulding).

Mutant line	Stage of lethality	Lethality over *incenp*$^{P(EP)2340}$
201	EL	L
282	EL	SL
497	L1	SL
549	EL	SL
666	L3	L
1608	EL	V
1650	EL	SL
2364	EL	V
2388	EL	V
2394	EL	V
2519	EL	SL
3747	EL	L
3959	EL	SL
4056	EL	SL

Table 2.2 Lethality of mutants

EL: Embryonic lethal. L1: Viable to first instar larvae. L3: Viable to third instar larvae. V: Viable. L: Lethal. SL: Semi-lethal.

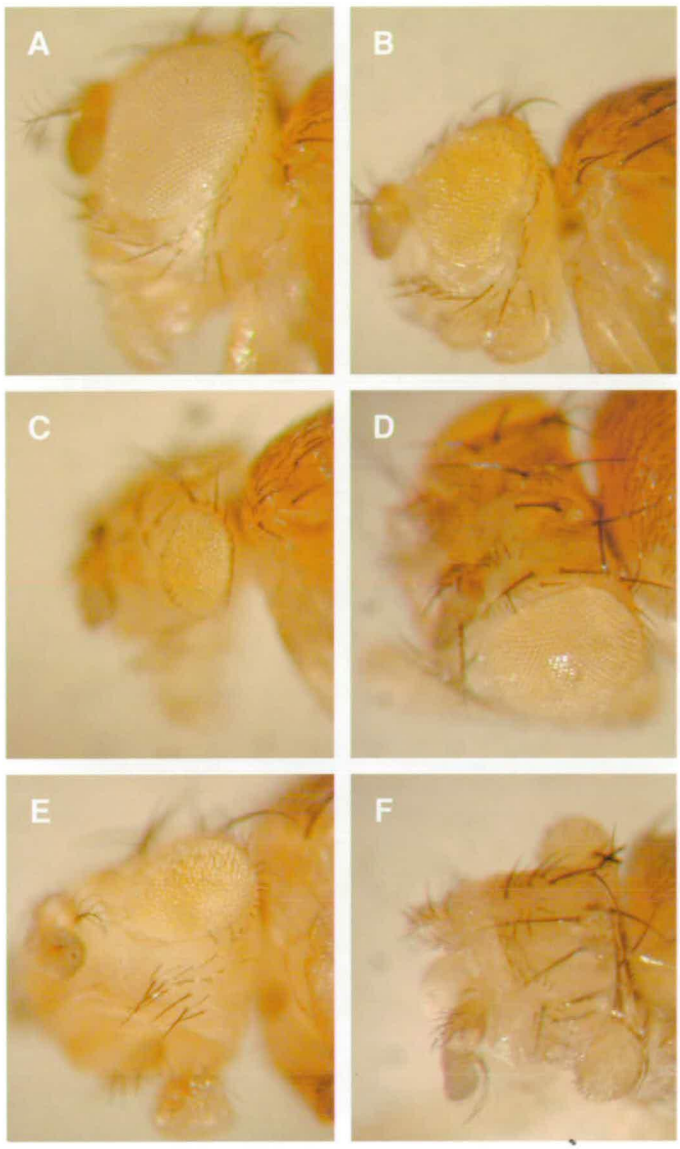


Figure 2.3 Eye phenotypes of EMS-induced mutations over *incenp*$^{P(EP)2340}$

Some mutants showed eye defects when combined with *incenp*$^{P(EP)2340}$. Note the reduced and rough eyes in transheterozygotes. (A) Wild type. (B) Transheterozygous 282/*incenp*$^{P(EP)2340}$. (C) Transheterozygous 2388/*incenp*$^{P(EP)2340}$. (D) Transheterozygous 2394/*incenp*$^{P(EP)2340}$. (E) Transheterozygous 2519/*incenp*$^{P(EP)2340}$. (F) Transheterozygous 3959/*incenp*$^{P(EP)2340}$.

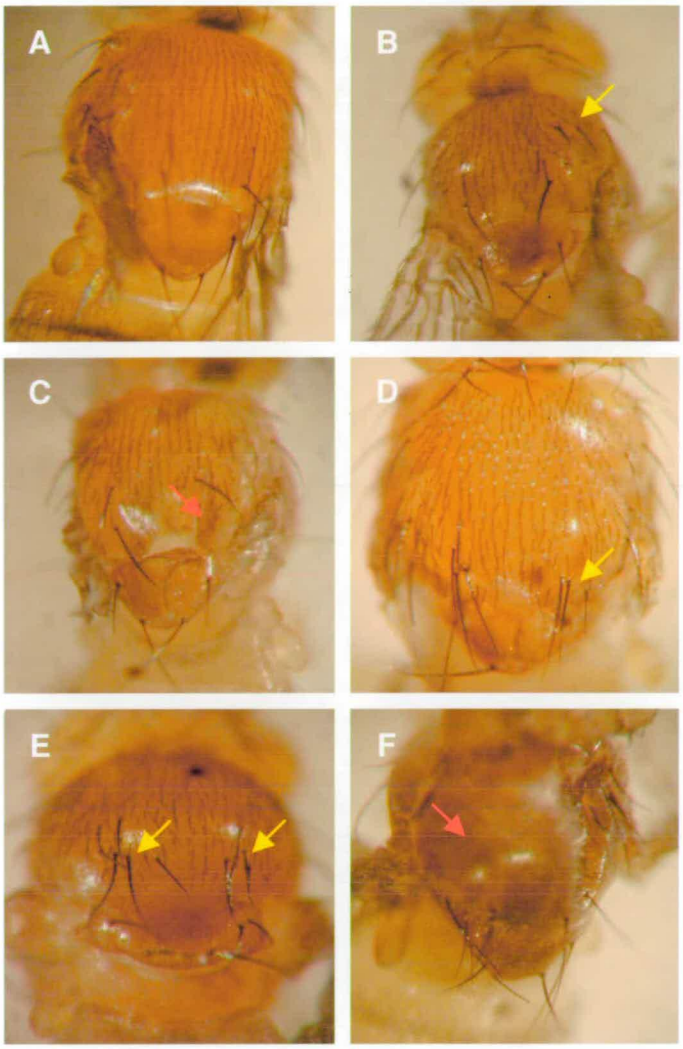


Figure 2.4 Bristle phenotypes of EMS-induced mutations over *incenp*$^{P(EP)2340}$

Some mutants showed bristle defects when combined with *incenp*$^{P(EP)2340}$. Note the missing or misplaced bristles in transheterozygotes. (A) Wild type. (B) Transheterozygous 282/*incenp*$^{P(EP)2340}$. (C) Transheterozygous 2388/*incenp*$^{P(EP)2340}$. (D) Transheterozygous 2394/*incenp*$^{P(EP)2340}$. (E) Transheterozygous 2519/*incenp*$^{P(EP)2340}$. (F) Transheterozygous 3959/*incenp*$^{P(EP)2340}$. The yellow arrows point to the misplaced bristles. The red arrows point to the missing bristles.

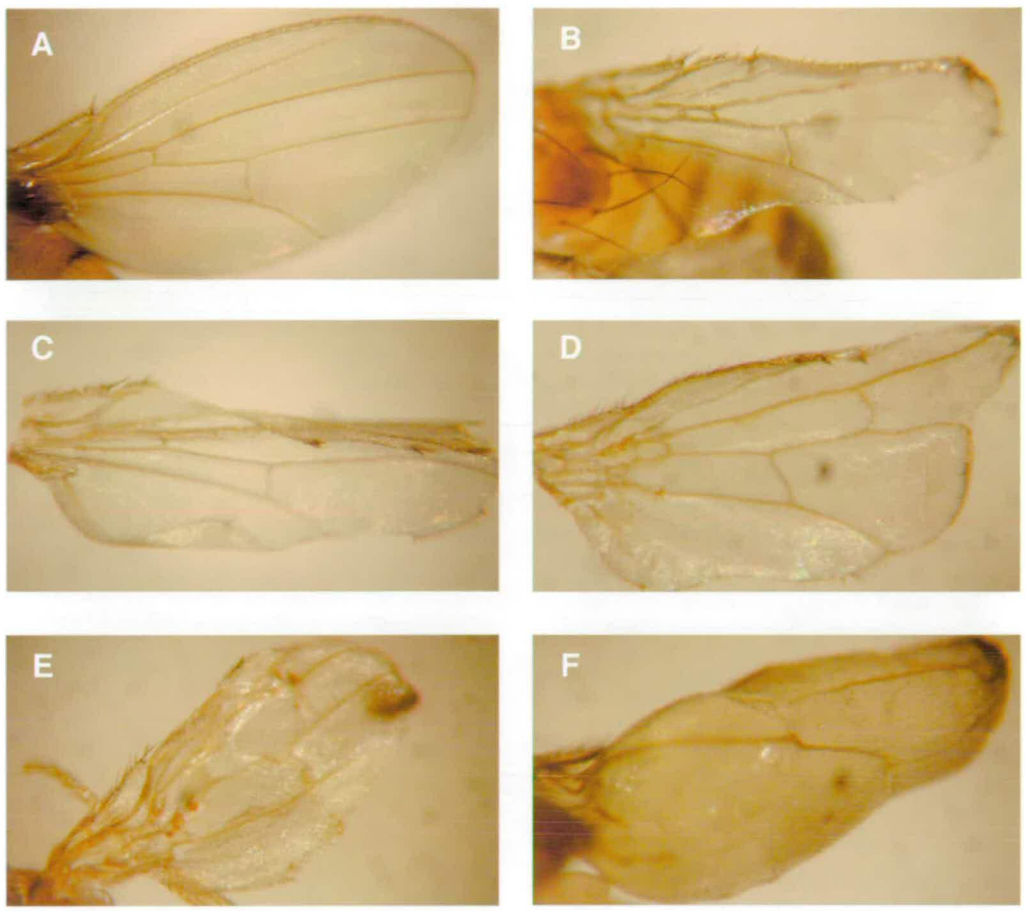


Figure 2.5 Wing phenotypes of EMS-induced mutations over *incenp*$^{P(EP)2340}$

Some mutants showed wing defects when combined with *incenp*$^{P(EP)2340}$. Note the high proportion of irregular wing shapes in transheterozygotes. (A) Wild type. (B) Transheterozygous 282/*incenp*$^{P(EP)2340}$. (C) Transheterozygous 2388/*incenp*$^{P(EP)2340}$. (D) Transheterozygous 2394/*incenp*$^{P(EP)2340}$. (E) Transheterozygous 2519/*incenp*$^{P(EP)2340}$. (F) Transheterozygous 3959/*incenp*$^{P(EP)2340}$.

2.6 A putative new allele of *incenp*

One of the purposes of this screen was to isolate new alleles of *incenp*. To find out if any of our mutations could be a new alleles of *incenp*, the mutants were crossed to Df(2R)pk78k (breakpoints, 42E03-43C03), a chromosomal deficiency that uncovers the *incenp* gene. 3747 was the only mutant which did not complement Df(2R)pk78k and therefore was selected for further characterization as a putative new allele of *incenp*.

The EMS mutants obtained in this screen have been the subject of further study in the lab. However, for the purpose of my PhD, I concentrated almost exclusively on the characterization of the 3747 mutation.

2.7 Chromosome "cleaning"

EMS is a chemical mutagen that affects DNA randomly. This means that there is a possibility that the mutant chromosomes could contain multiple hits. In order to remove these possible background mutations, the chromosome containing 3747 was "cleaned" by genetic recombination. This means substituting as much as the mutagenised chromosome as possible by a formally wild-type chromosome, keeping of course the chromosomal location of interest. I used two strains containing multiple recessive 'inocuous' markers along the second chromosome (Figure 2.6, Table 2.3). DmINCENP maps in 43A04, close to the marker cinnabar (cn) (43E16). I used this marker to follow the 3747 mutation.

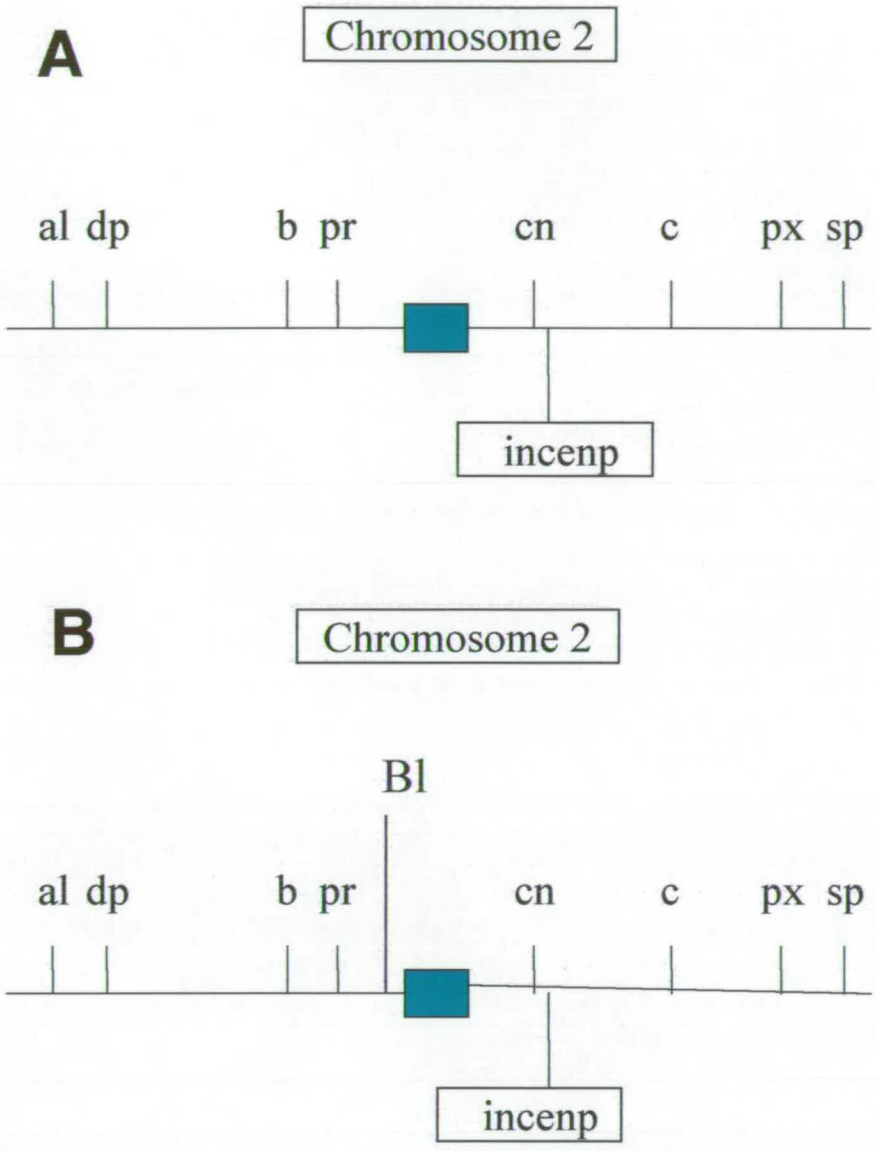


Figure 2.6 Diagram of multiple markers on chromosome 2
Schematic representation of the position of the recessive mutations on the multiply-marked second chromosome. The position of incenp gene is close to cn mutation. (A) The chromosome used for recombination. (B) The chromosome used for analysis of the recombinant lines of *incenp*^{3747}. This chromosome also carried a dominant mutation Bl, which was used as a visible marker to discriminate chromosomes that carried *incenp*^{3747}.

Recessive marker	aristaless (al)	dumpy (dp)	black (b)	purple (pr)	cinnabar (cn)	curve (c)	plexus (px)	speck (sp)
Cytological position	21C3	25A1	34D5	38B3	43E16	52D3-7	58E3-8	60C1-2
Recombinational position	2-0.4	2-13	2-48.5	2-54.5	2-57.5	2-75.5	2-100.5	2-107

Table 2.3 The positions of the recessive mutations on the multiply-marked second chromosome

The strategy is shown in Figure 2.7. First, I obtained females carrying 3747 over a multiple marker chromosome (*al dp b pr cn c px sp*). Meiotic recombination during prophase of meiosis I in these females will produce different exchanged chromosomes. I wished to obtain a chromosome carrying all the recessive markers except cn. This would mean that most of the chromosome was "cleaned", but the region containing 3747 was kept. In order to check the presence or absence of the different markers, the recombinant chromosomes were placed over a chromosome containing the same marker, plus an additional dominant marker (Bristle, *Bl*) for the purpose of sorting the correct genotypes in our crosses (*al dp b pr Bl cn c px sp*). In F2, I was able to identify recombinant chromosome carrying the wild-type allele of *cn*. Table 2.4 showed the different strains obtained in the "cleaning" process. The genotype of the "cleanest" strain obtained was *al dp* b^+ pr^+ cn^+ *c px sp*. It was named 3747-r3. This strain was used for further analysis.

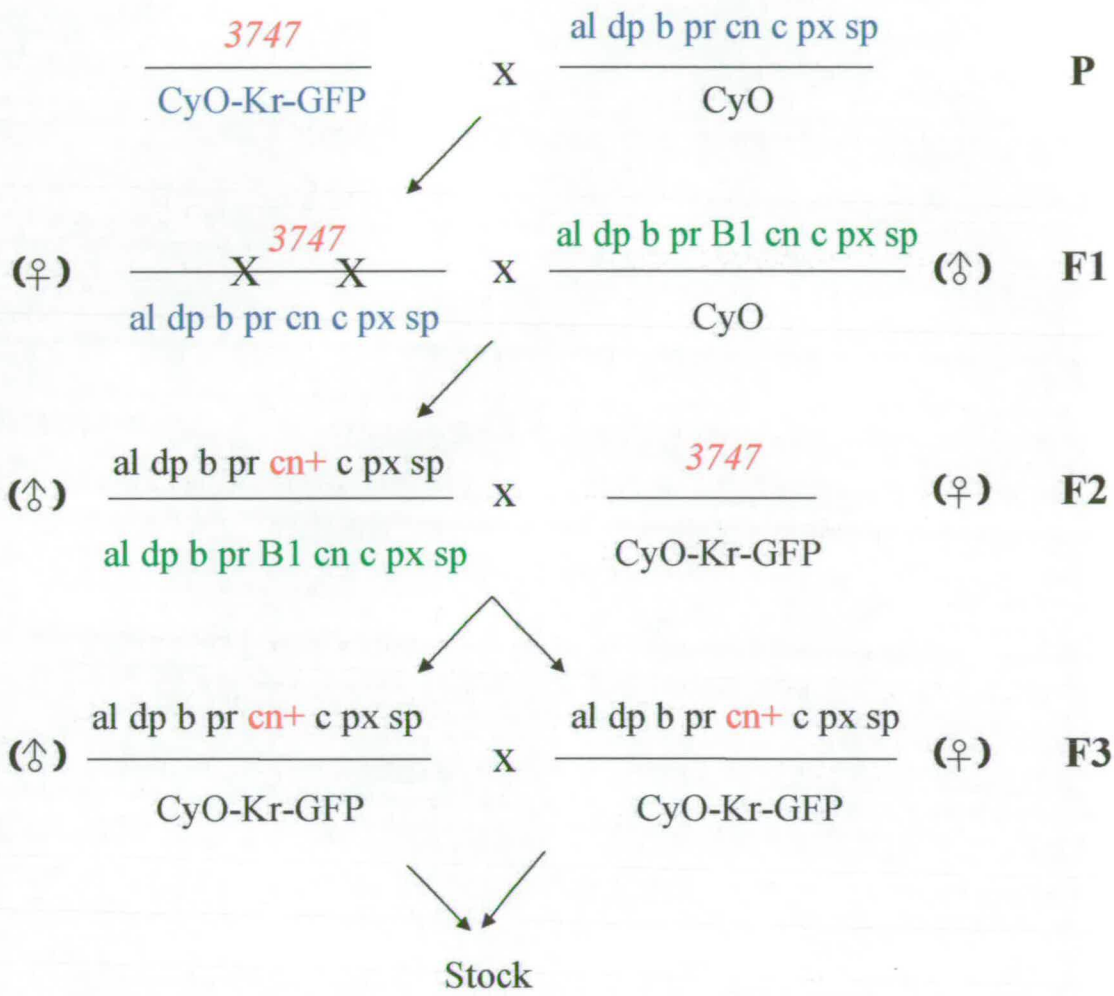


Figure 2.7 Schematic representation of chromosome 'cleaning'
The 3747 mutant strain was subjected to chromosome 'cleaning' by meiotic recombination to remove other EMS hits generated on the same chromosome. The DmINCENP gene mapped close to mutation cn. The recombinant lines that lacked the cn mutation (cn+) also carried mutation 3747.

Strain Number	Genotype of recombinant 3747	Lethality of Homozygous	Lethality over *incenp*$^{P(EP)2340}$
2	al dp b^+ pr^+ cn^+ c px^+ sp	Lethal	Lethal
3	al dp b^+ pr^+ cn^+ c px sp	Lethal	Lethal
5	al dp b^+ pr^+ cn^+ c+ px^+ sp	Lethal	Lethal
6	al dp^+ b^+ pr^+ cn^+ c^+ px^+ sp^+	Lethal	Lethal
7	al dp b^+ pr^+ cn^+ c^+ px^+ sp^+	Lethal	Lethal
8	al^+ dp^+ b^+ pr^+ cn^+ c px sp	Lethal	Lethal
10	al^+ dp^+ b^+ pr^+ cn^+ c px sp	Lethal	Lethal
11	al^+ dp^+ b^+ pr^+ cn^+ c^+ px sp	Lethal	Lethal
13	al dp^+ b^+ pr^+ cn^+ c px sp	Lethal	Lethal
18	al^+ dp^+ b^+ pr^+ cn^+ c^+ px sp	Lethal	Lethal
21	al dp b^+ pr^+ cn^+ c^+ px^+ sp^+	Lethal	Lethal
23	al^+ dp^+ b^+ pr^+ cn^+ c px sp	Lethal	Lethal
24	al dp b^+ pr^+ cn^+ c^+ px^+ sp^+	Lethal	Lethal
26	al^+ dp^+ b^+ pr^+ cn^+ c^+ px sp	Lethal	Lethal
27	al^+ dp^+ b^+ pr^+ cn^+ c^+ px sp	Lethal	Lethal
28	al^+ dp^+ b^+ pr^+ cn^+ c px sp	Lethal	Lethal
29	al dp b^+ pr^+ cn^+ c^+ px^+ sp^+	Lethal	Lethal
30	al dp^+ b^+ pr^+ cn^+ c^+ px sp	Lethal	Lethal

Table 2.4 Recombinant strains of 3747 and their lethality

2.8 Fine deficiency mapping

In order to further refine the mapping of 3747 (and to ensure that I did not lose the mutation after cleaning), the "cleaned" 3747-r3 chromosome was crossed to six strains carrying chromosomal deficiencies with breakpoints in the *incenp* gene region (Table 2.5).

Genotype	Breakpts/Insertion
Df(2R)Dr1^{rv3}, bw^{1}/SM6a	042E01-04 ; 043C03
dac^{9} pk^{sple-3}/CyO, Df(2R)cn-S3	043B01-02 ; 044B07-09
Df(2R)pk78s/CyO	042F;043F08 ; 059F05-08
Df(2R)nap19, cn^{1}bw^{1}/In(2LR)Gla	041E02-F01 ; 043A02-B01
Df(2R)P32a, cn*/CyO	043A03 ; 043F06
Df(2R)ST1, Adh^{n5}pr^{1}cn*/CyO	042B03-05 ; 043E15-18

Table 2.5 Deficiency used in complementation tests for mapping of 3747

The results of the mapping are shown in Figure 2.8. Three deficiency strains did not complement 3747. They are (in abbreviation) Df(2R)Dr1, Df(2R)p32a and Df(2R)ST1. They all uncover the genomic region 43A04 containing the DmINCENP gene. In the contrast, three deficiency strains did complement 3747. They are (in abbreviation) Df(2R)cn-S3, Df(2R)pk78s and Df(2R)nap19. They do not uncover the region 43A04.

The genetic evidence suggested that 3747 could be a new allele of *incenp*. 3747 neither complements $incenp^{P(EP)2340}$ or any of the imprecise excisions generated by the mobilization of the P-element nor does it complement deficiencies uncovering 43A04, the genomic region containing the *incenp* gene. However, 43A04 is still a big region which contains several other genes. I still could not rule out that 3747 could be a mutation in a neighboring gene. Therefore, molecular characterization of the mutant was required to confirm that 3747 was a new allele of *incenp*.

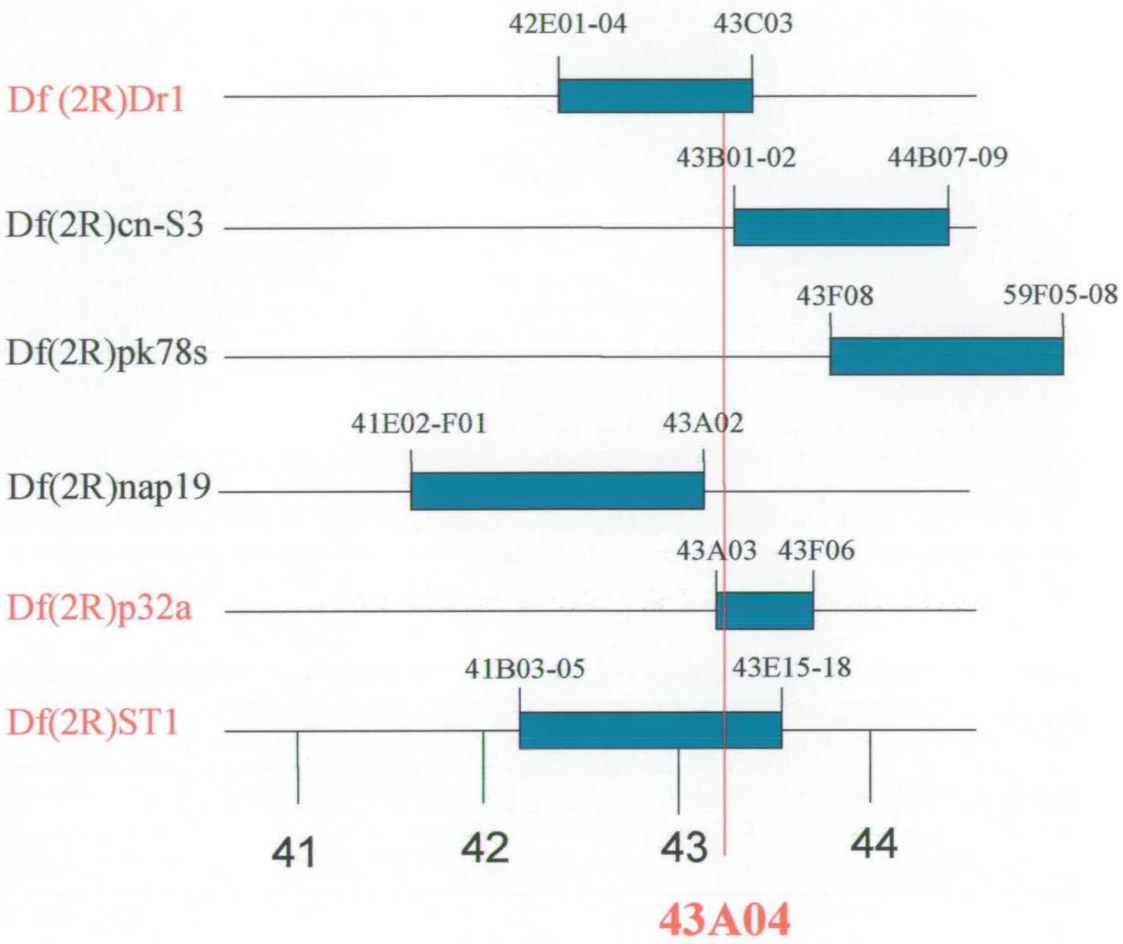


Figure 2.8 Mapping of 3747

Six deficiencies that uncover, or are close to the DmINCENP gene were used to map 3747. Three of the deficiencies (red color) did not complement 3747. Three of the deficiencies (black color) complemented 3747. 3747 was mapped to 43A04, where the DmINCENP gene was located.

Chapter 3

Molecular analysis of 3747

3.1 Introduction

In the previous chapter, I described the isolation and mapping of the 3747 EMS mutation to the region 43A04 on the right arm of chromosome 2, by a series of crosses to deficiency lines with known breakpoints in the region. In this chapter I describe the strategies used for the molecular characterization of the 3747 mutation and its confirmation as a new *incenp* allele.

3.2 Strategy to identify the mutation in 3747

3747 was a mutant generated by EMS, an alkylating agent that produces a high proportion of point mutations, and on occasions produces small deletions. The DmINCENP gene has six exons and five introns encompassing a total of about 3Kb. In order to identify the mutation in 3747, I used PCR to amplify the entire coding region of the *incenp* gene using as template genomic DNA from a single 3747 homozygous embryo. I then sequenced the PCR products directly. I designed eight

primers (incenp1, incenp2, incenp3, incenp4, incenp5, incenp6, incenp7 and incenp8-r) for sequencing reactions (Figure 3.1). The sequences of the primers are listed in a table (see Materials and Methods). Incenp1 and incenp8-r were also used as primers for the PCR reactions.

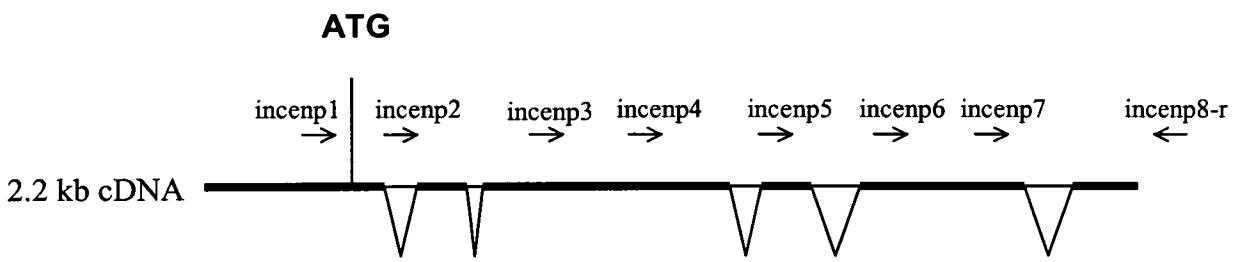


Figure 3.1 Design of sequencing primers and PCR primers
The coding region of the DmINCENP gene is about 3 kb, with 6 exons and 5 small introns. Eight primers were used to sequence the whole coding region. incenp1 and incenp8-r were also used as PCR primers.

3.3 PCR amplification of the coding region of *incenp*

In order to obtain a good quality and quantity of PCR product to use as template for the subsequent sequencing reactions, different conditions for the PCR were tested. In general, increasing the annealing temperature increases the specificity of the primers while reducing the product yield. Trying different annealing conditions, the reaction was optimized to give a clean band and a high yield.

I used genomic DNA from wild type adult flies as template. A gradient annealing temperature from 51°C to 65°C was used for the PCR reaction. The results showed that the primers were highly specific and the PCR reactions produced single products of the expected size (3 Kb) (Figure 3.2A). The results showed that the amount of the products increased with the annealing temperature. This may be due to the secondary structure of genomic DNA (A higher annealing temperature would release the secondary structure).

As 3747 is EMS-induced, it could be a point mutation or a small deletion. Since the PCR product was to be used as template for the sequencing reaction, the accuracy of the PCR reaction was very important. Even a single base PCR error would affect the analysis. To reduce the possibility of error in the PCR, I used a proofreading DNA polymerase (TaKaRa LA Taq™, TaKaRa). Another way to reduce errors is to reduce the number of PCR cycles. However, reducing the cycles also reduces the amount of the product. To find out the minimum number of cycles, which would produce a reasonable amount of product for sequencing, I performed different PCR reactions with different number of cycles (Figure 3.2B). The result showed that a reasonable

amount of product could be obtained when the PCR reaction was performed for 20 cycles.

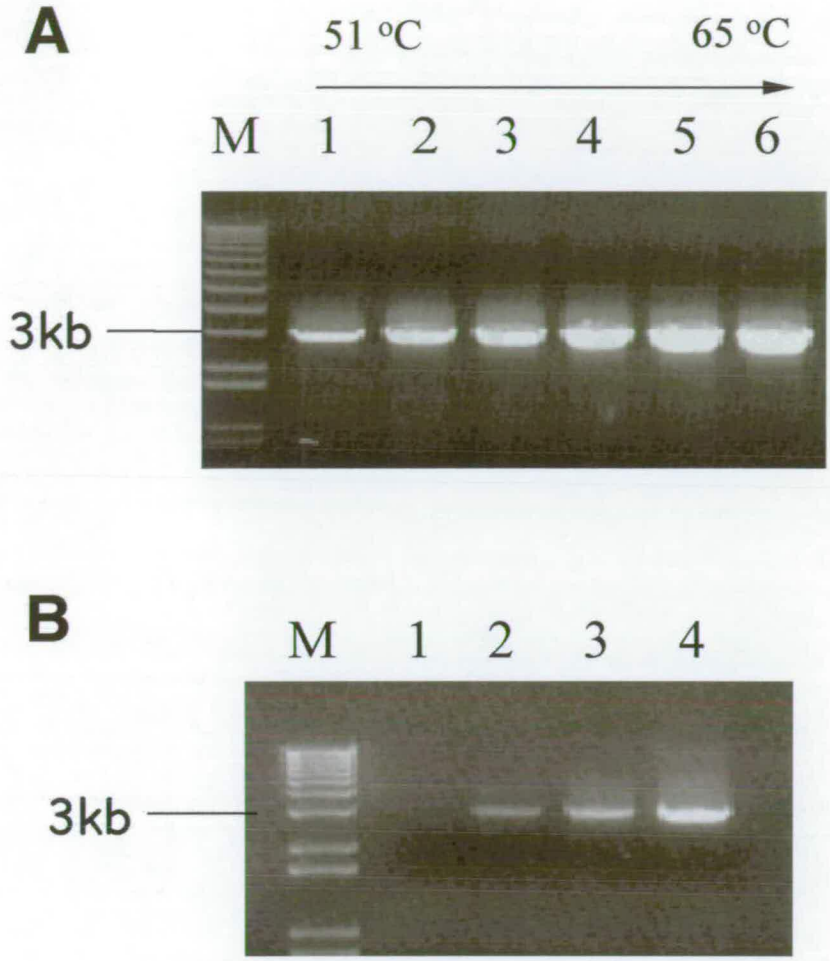


Figure 3.2 PCR conditions for amplifying the DmINCENP gene
Genomic DNA prepared from wild type adult flies was used as template. Incenp1 and incenp8-r were used as primers. (A) Lane 1-6 show PCR products that were generated at different annealing temperatures, from 51°C-65°C. (B) PCR reactions were performed with different cycles. Lane 1, control reaction, no template was added. Lane 2, 20 cycles; Lane 3, 25 cycles; Lane 4, 30 cycles.

3.4 Sequencing from single homozygous 3747 embryos

In order to select homozygous mutant embryos, the balancer chromosome CyO-Kr-GFP was used. This chromosome expresses Green Fluorescent Protein (GFP) under the control of the promoter of the *Kruppel* gene (Kr, see Materials and Methods). This will allow me to select embryos homozygous for the 3747 chromosome, which do not show this specific pattern of expression of GFP.

However, I could not rule out totally the possibility that embryos carrying the GFP balancer fail to express GFP (even though the embryos aged for 12 hours at 18°C should express GFP). If genomic DNA is extracted from more than one embryo, it is formally possible to obtain a mixture of genomic DNA (3747 and wild-type). This would make the sequencing results more difficult to analyze and interpret. Therefore, I extracted 3747 genomic DNA from single 3747 homozygous embryos for each PCR reaction.

Genomic DNA from four single embryos was prepared as template to perform four separate PCRs (Figure 3.3). The PCR products from the genomic DNA of a single embryo were used directly for sequencing reactions. Comparison of the sequence from more than four different sequencing reactions revealed a point mutation in exon 4 of the *incenp* gene (Figure 3.4). This single base change in exon 4 resulted in a stop codon (Figure 3.5). The predicted molecular weight of the putative truncated gene product would be 61 kD. This result confirms 3747 as a new allele of *incenp*. It will therefore be referred as $incenp^{3747}$ from now on.

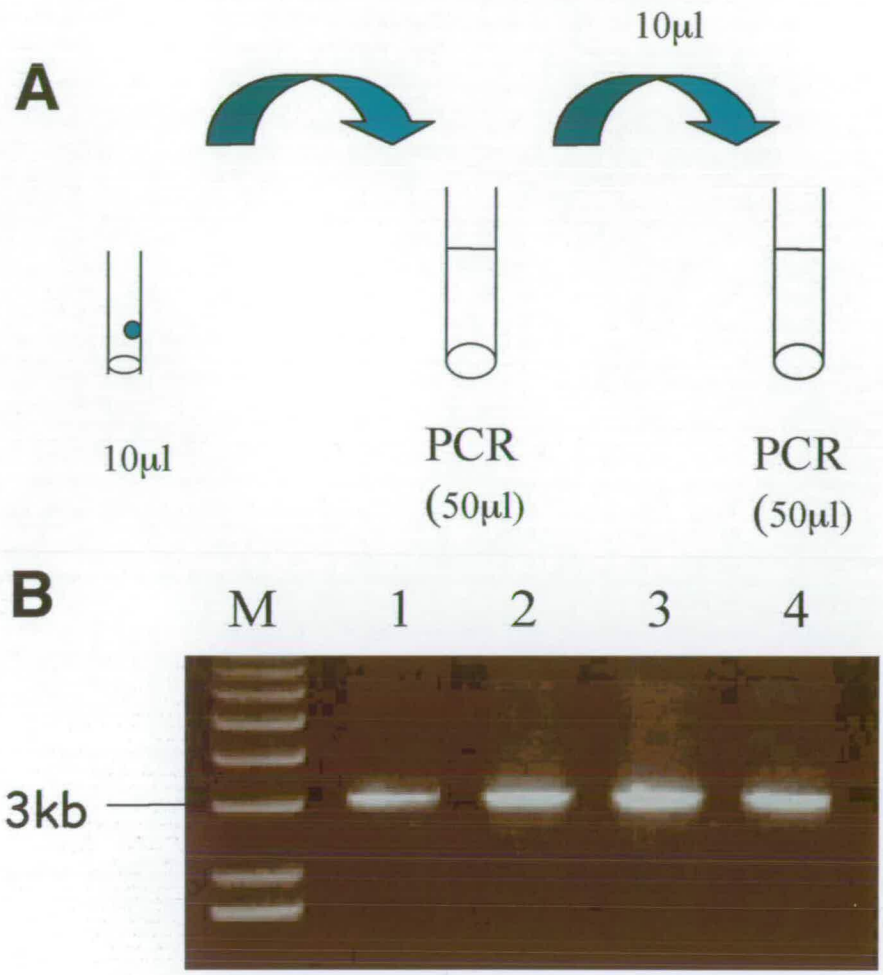


Figure 3.3 Preparation of PCR products from single embryos for sequencing

(A) Schematic representation of PCR strategy. A single embryo (green dot) was homogenized in 10 µl extraction buffer. The whole extraction was used as template for the first PCR reaction. After this, 10 µl was taken as the template for the second PCR reaction. (B) Gel electrophoresis of the second PCR product. Lane 1 to lane 4 shows 2 µl of second PCR product from four individual single embryos.

```
WT      ATGGAGGACATCTTGGGCGTGCTGGGCTCGCTTTCGGACCTGCGCAGGGAGCTCGAGGTC 60
3747    ATGGAGGACATCTTGGGCGTGCTGGGCTCGCTTTCGGACCTGCGCAGGGAGCTCGAGGTC 60
        ************************************************************

WT      CTGCGCAAGGCCCACTTTGAGGAGCTGGACCACCTCTTCTACGGCACAGGCCAGCCGGAG 120
3747    CTGCGCAAGGCCCACTTTGAGGAGCTGGACCACCTCTTCTACGGCACAGGCCAGCCGGAG 120
        ************************************************************

WT      GCGGAGGCCAAACCGAGGGACAGTGCTCCGGCAGCGAAGAGTCAGGAGAACTCTTCGGTG 180
3747    GCGGAGGCCAAACCGAGGGACAGTGCTCCGGCAGCGAAGAGTCAGGAGAACTCTTCGGTG 180
        ************************************************************

WT      ACGCCGCAGCAAACGAAAAAGCGCCCCAAGCGACTCACCTCACTGGCCGAGGATCAGAAT 240
3747    ACGCCGCAGCAAACGAAAAAGCGCCCCAAGCGACTCACCTCACTGGCCGAGGATCAGAAT 240
        ************************************************************

WT      GAGCCCGAGGCTCCAGACGCCACGGCCAACAACACGTCGGCCCGTCAAAGCACTCGGGTC 300
3747    GAGCCCGAGGCTCCAGACGCCACGGCCAACAACACGTCGGCCCGTCAAAGCACTCGGGTC 300
        ************************************************************

WT      AGCAACTCCCAGTTGCTGGCTATCGCCGAGGACGAGCACAACTCCACGGCCTCGCTGATG 360
3747    AGCAACTCCCAGTTGCTGGCTATCGCCGAGGACGAGCACAACTCCACGGCCTCGCTGATG 360
        ************************************************************

WT      CCGCCTCCGCCTGTGCCCGTCTCGGCGGACACCACCCTCGGCTCCGGAAGGCCACAGCGA 420
3747    CCGCCTCCGCCTGTGCCCGTCTCGGCGGACACCACCCTCGGCTCCGGAAGGCCACAGCGA 420
        ************************************************************

WT      GCCGCTAAGCTGAAGACCGAGAAATTACTCAAGGAGCCCTCACTCAACCGTAAGATGCGG 480
3747    GCCGCTAAGCTGAAGACCGAGAAATTACTCAAGGAGCCCTCACTCAACCGTAAGATGCGG 480
        ************************************************************

WT      CGGCCCAGCAGCGAGGAGCTCGTAAAGGTCAAGGTTGAGAGCGAGCAGCGAGTCTCCCAG 540
3747    CGGCCCAGCAGCGAGGAGCTCGTAAAGGTCAAGGTTGAGAGCGAGCAGCGAGTCTCCCAG 540
        ************************************************************

WT      TTCAACAGCTTCACCAGTGCACAGGCGTTAGAGGAAAATAAACTAGCGGAGCCGGAATTA 600
3747    TTCAACAGCTTCACCAGTGCACAGGCGTTAGAGGAAAATAAACTAGCGGAGCCGGAATTA 600
        ************************************************************

WT      AAAGAGCCAGCAGCAGAAACAGCTGAGCAGCAAAAGCCTCCGGAGGAGGCCTCAGTCACA 660
3747    AAAGAGCCAGCAGCAGAAACAGCTGAGCAGCAAAAGCCTCCGGAGGAGGCCTCAGTCACA 660
        ************************************************************

WT      GAGGACGTGAACACCACGAAGACACTCCGCGTGAAGGTAAAGCGCGAGAAACTCAGTACC 720
3747    GAGGACGTGAACACCACGAAGACACTCCGCGTGAAGGTAAAGCGCGAGAAACTCAGTACC 720
        ************************************************************

WT      GAAGCAGTGCCCCCGTTAACCAATGCGGTCTCCACGGCAAATGTGACCACCGTCTCCTCT 780
3747    GAAGCAGTGCCCCCGTTAACCAATGCGGTCTCCACGGCAAATGTGACCACCGTCTCCTCT 780
        ************************************************************

WT      GTAACAACGGAGGCAGCGCGACCGGACGATACGGTGGCCAGCAACACGACGACATCCACG 840
3747    GTAACAACGGAGGCAGCGCGACCGGACGATACGGTGGCCAGCAACACGACGACATCCACG 840
        ************************************************************
```

Figure 3.4 DmINCENP cDNA sequence of 3747

```
WT      GAGGTCTCCAAGAAGGTCCGCAAGAAGAAGAAGGACGTGGAAAGCCACCGACCCATAAAG 900
3747    GAGGTCTCCAAGAAGGTCCGCAAGAAGAAGAAGGACGTGGAAAGCCACCGACCCATAAAG 900
        ************************************************************

WT      GTGGAACGATTCAGCGACCTGGACAAGAGCTCGCCAGTCTCGTCGCGCACGCGCAAGTGT 960
3747    GTGGAACGATTCAGCGACCTGGACAAGAGCTCGCCAGTCTCGTCGCGCACGCGCAAGTGT 960
        ************************************************************

WT      AGCAGCGATTCGCGAACGGTTCAGGAGCGATCCATATACAAGGATGCACTCGAAGATCCG 1020
3747    AGCAGCGATTCGCGAACGGTTCAGGAGCGATCCATATACAAGGATGCACTCGAAGATCCG 1020
        ************************************************************

WT      CCCGTTGCAGAGCAATCAGTGGCTGCAGTTGCTCCGGCTGCTGTCAATGAGACCGTGGCG 1080
3747    CCCGTTGCAGAGCAATCAGTGGCTGCAGTTGCTCCGGCTGCTGTCAATGAGACCGTGGCG 1080
        ************************************************************

WT      ATTTCCAACGCCACCCTCGTACTGGGTCCTGCGCCGACGAGCGATAGTCCAATAGGACCA 1140
3747    ATTTCCAACGCCACCCTCGTACTGGGTCCTGCGCCGACGAGCGATAGTCCAATAGGACCA 1140
        ************************************************************

WT      GCTGGAGATGCCACTTTTGAGGTGCACTCCAGCGACAAGAAACAACCTCTGAGTAAGCAG 1200
3747    GCTGGAGATGCCACTTTTGAGGTGCACTCCAGCGACAAGAAACAACCTCTGAGTAAGCAG 1200
        ************************************************************

WT      GACAGCCTCTTAACGGAGGACGAGTCGGTGGAGGAGAAGCTGCCGACGACCAAGCTGCTC 1260
3747    GACAGCCTCTTAACGGAGGACGAGTCGGTGGAGGAGAAGCTGCCGACGACCAAGCTGCTC 1260
        ************************************************************

WT      TCGAGCATAAAGGCTATAAAACTGCCTACCCGTACACATGAGCTTTTCAACCCACTGCTT 1320
3747    TCGAGCATAAAGGCTATAAAACTGCCTACCCGTACACATGAGCTTTTCAACCCACTGCTT 1320
        ************************************************************

WT      CAGAGTCCAGTGAAAATGCGCGTGGAGGCGTTCGAGAATGTGGCAAACGCACAGAACAGC 1380
3747    CAGAGTCCAGTGAAAATGCGCGTGGAGGCGTTCGAGAATGTGGCAAACGCATAGAACAGC 1380
        ************************************************** ********

WT      ATGCGCCCTAAGCGGGGAAAAGATCTGCAGGGCACGCCAGGATCAAACAACACTCCCAAA 1440
3747    ATGCGCCCTAAGCGGGGAAAAGATCTGCAGGGCACGCCAGGATCAAACAACACTCCCAAA 1440
        ************************************************************

WT      ATTGGCAAGCTGCCTGCTCCGACTGTTGGTCGCTTTTTCACACCGACTCAAACGACAAGC 1500
3747    ATTGGCAAGCTGCCTGCTCCGACTGTTGGTCGCTTTTTCACACCGACTCAAACGACAAGC 1500
        ************************************************************

WT      ACACTACCATTAAGCTCGGCGCAGCCCAAGAAGGGACCTGCCAGCGCGTCTAAGGCCACT 1560
3747    ACACTACCATTAAGCTCGGCGCAGCCCAAGAAGGGACCTGCCAGCGCGTCTAAGGCCACT 1560
        ************************************************************

WT      AGTCTGCTGAAGACGGCCACCGGTACGAACCTAAGGTCGGTCAATTCCACGTCCACAAAA 1620
3747    AGTCTGCTGAAGACGGCCACCGGTACGAACCTAAGGTCGGTCAATTCCACGTCCACAAAA 1620
        ************************************************************

WT      ACGCTGTCGCGCGAGAACAGCGGAGACGACTTCCGCAAGGGCCTGCACAATCTGGCGGAG 1680
3747    ACGCTGTCGCGCGAGAACAGCGGAGACGACTTCCGCAAGGGCCTGCACAATCTGGCGGAG 1680
        ************************************************************
```

Figure 3.4 DmINCENP cDNA sequence of 3747

```
WT      GAGCGCAAGAAGCTGCGTGAGCAAAAGCACCAGCAGGCTGCCCAGCAGCGCGAAGCCAAG 1740
3747    GAGCGCAAGAAGCTGCGTGAGCAAAAGCACCAGCAGGCTGCCCAGCAGCGCGAAGCCAAG 1740
        ************************************************************

WT      GAGCGGGAGCGGGCAGAGCGTATGGCCAAGCTGGCCGCCGAGCGCGCCAAGAAGCAGGAG 1800
3747    GAGCGGGAGCGGGCAGAGCGTATGGCCAAGCTGGCCGCCGAGCGCGCCAAGAAGCAGGAG 1800
        ************************************************************

WT      GAGCGAAAGCGAATTGAAGAGCGCAAACGACAAGAGCTGGAGGAGCTCCAGCGGAAGATG 1860
3747    GAGCGAAAGCGAATTGAAGAGCGCAAACGACAAGAGCTGGAGGAGCTCCAGCGGAAGATG 1860
        ************************************************************

WT      CGCCAGCAAGAGGAAGCCGAGGCGCTCAGGAAGGCCAAGCTCAAGGAGCTGGAGCAGCAG 1920
3747    CGCCAGCAAGAGGAAGCCGAGGCGCTCAGGAAGGCCAAGCTCAAGGAGCTGGAGCAGCAG 1920
        ************************************************************

WT      AAACTGCAGCAGCTAACCGGCGCCAAGCCCAAGAAGATGCTTCCGCCGCCGCCGAAAACC 1980
3747    AAACTGCAGCAGCTAACCGGCGCCAAGCCCAAGAAGATGCTTCCGCCGCCGCCGAAAACC 1980
        ************************************************************

WT      AAGTACACCTGGGAGATGCTGCACGAAGACGACTCGACAGACGACGAGGGAAAGGTCACC 2040
3747    AAGTACACCTGGGAGATGCTGCACGAAGACGACTCGACAGACGACGAGGGAAAGGTCACC 2040
        ************************************************************

WT      CACAAGCGTCCCCCAGCGCCCACCTGGAGTCGAAGCCACGTAAGGGGAGAGGCAATTGCT 2100
3747    CACAAGCGTCCCCCAGCGCCCACCTGGAGTCGAAGCCACGTAAGGGGAGAGGCAATTGCT 2100
        ********************************************************************

WT      ATGCAGAGCCACTGCCCAACCGACATCATCGACAGCTTTTTCTCGGTGGCGCCCACCACA 2160
3747    ATGCAGAGCCACTGCCCAACCGACATCATCGACAGCTTTTTCTCGGTGGCGCCCACCACA 2160
        ************************************************************

WT      CCGGACCTGAAGCAGATATTCCCGAACATCGATCCCAGCCAGCTGAAGCGAAACTCCAGC 2220
3747    CCGGACCTGAAGCAGATATTCCCGAACATCGATCCCAGCCAGCTGAAGCGAAACTCCAGC 2220
        ************************************************************

WT      GTGCTGTGGTCCACGCCGCCGCGCTATTCCGAGCTGCCGAAATACTAGCCAAGTAGTCCT 2280
3747    GTGCTGTGGTCCACGCCGCCGCGCTATTCCGAGCTGCCGAAATACTAGCCAAGTAGTCCT 2280
        ************************************************************

WT      GCTCT 2285
3747    GCTCT 2285
        *****
```

Figure 3.4 DmINCENP cDNA sequence of 3747

DmINCENP has 6 exons. WT: wild type (w^{1118}). Exon 1 is shown in blue; Exon 2 green; Exon 3 purple; Exon 4 orange; Exon 5 pink; Exon 6 black. Note a point mutation (C to T) in exon 4 (1370), which results in a stop codon.

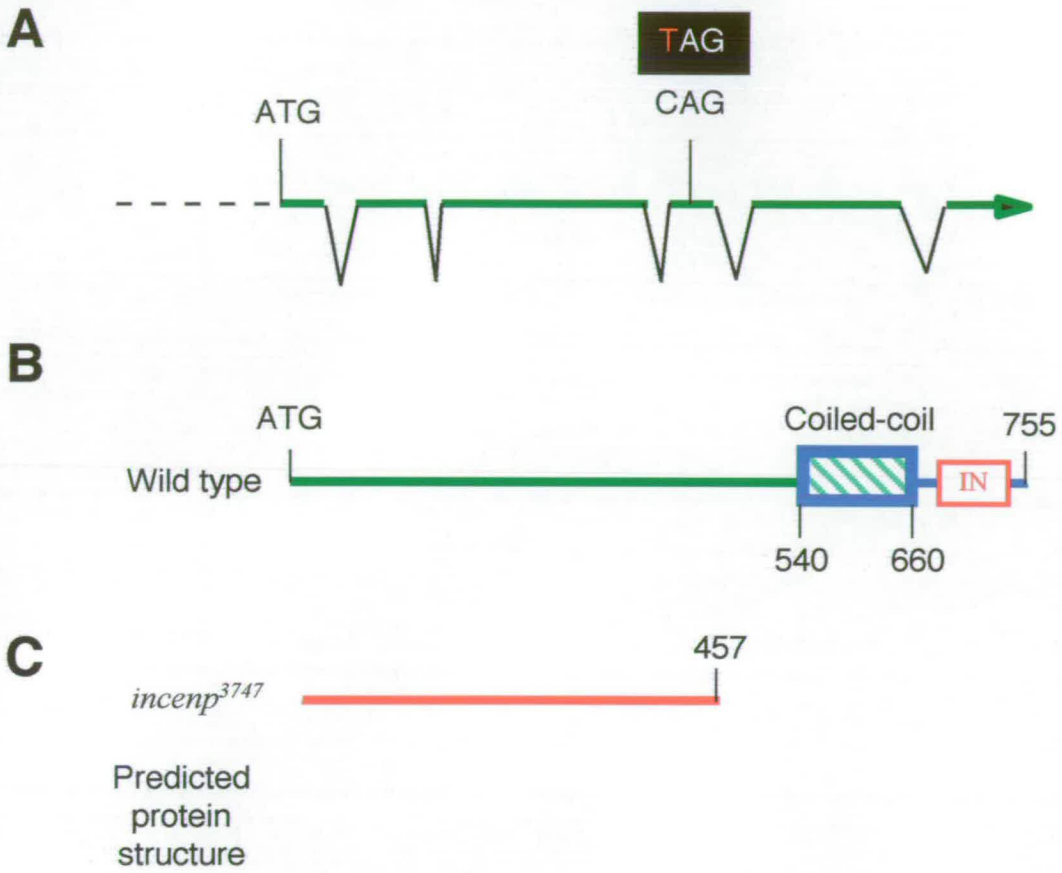


Figure 3.5 DmINCENP gene sequence and predicted protein structure of *incenp*^{3747}

(A) *Incenp*^{3747} carried a point mutation (C to A) in exon 4 of DmINCENP gene. (B) The wild type INCENP protein structure. (C) The predicted protein structure of incenp^{3747}. Incenp^{3747} is a truncated vision of INCENP protein, which lacks the C-terminal region.

3.5 Immunoblotting of the gene product of *incenp*^{3747}

Western blotting was used to analyze the gene product of *incenp*^{3747}. According to the sequence of *incenp*^{3747}, the protein product of this mutated gene would be a truncated version of INCENP. The immunoblot was performed by loading protein from forty homozygous embryos per lane of the following genotype: wild type (w^{1118}, positive control), Df(2R)pk78k (negative control), *incenp*^{3747} and *incenp*$^{P(EP)2340}$. The method used to select the homozygous embryos is described in chapter 8 (Materials and Methods).

The result of this experiment was shown in Figure 3.6. The expected full-length protein with molecular weight of 110kD was detected in wild type, but was absent from the Df(2R)pk78k, *incenp*^{3747} and *incenp*$^{P(EP)2340}$ lanes. No truncated protein product was detected in both *incenp*^{3747} and *incenp*$^{P(EP)2340}$ lanes. The loading control using anti-alpha-tubulin antibody showed that all lanes appear nearly equally loaded. This result reconfirms *incenp*^{3747} as a new allele of *incenp*. However, the truncated protein was not detected in the blot. This could suggest that the predicted truncated protein is unstable and might be degraded very quickly although I cannot rule out the possibility that our antibody Rb801, which is raised against the N-terminal 348 amino acids of the protein (Adams et al., 2001c), failed to detect the truncated protein, which would end at amino acid 457.

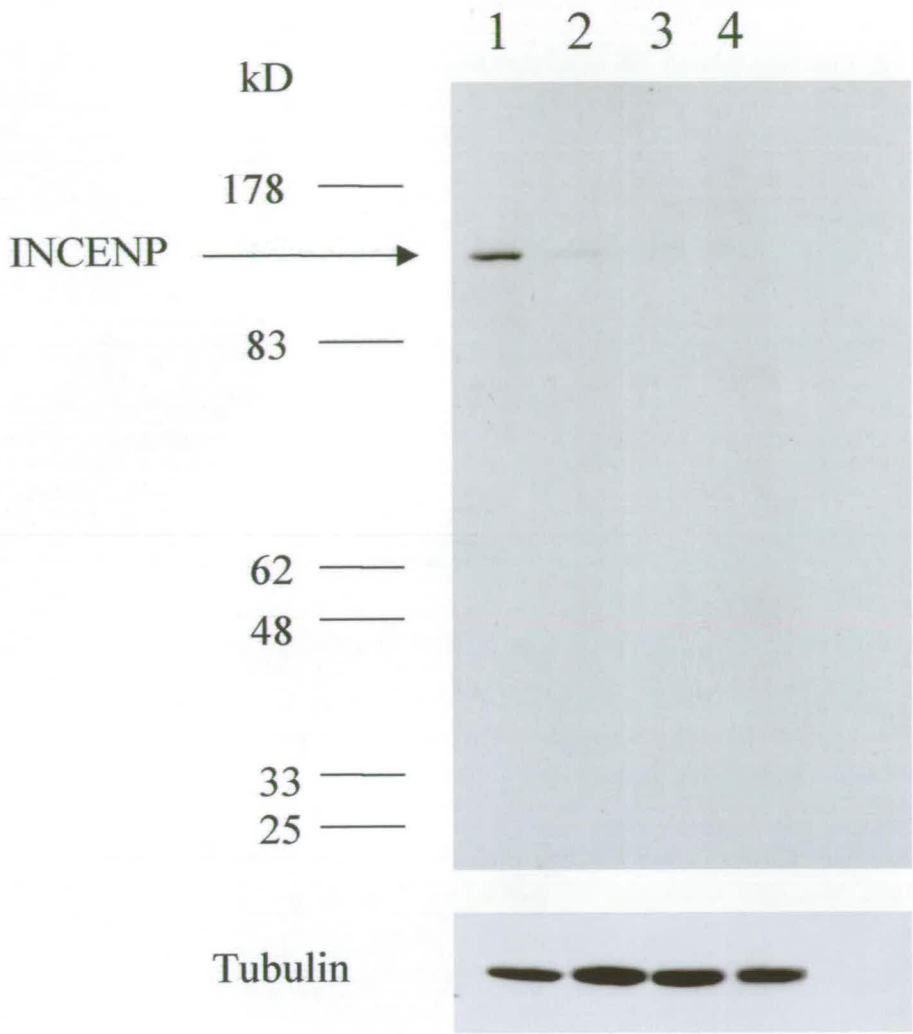


Figure 3.6 Immunoblotting of the gene product of *incenp*^{3747}
Protein was extracted from 40 homozygous embryos of wild type (Lane 1), Df(2R)pk78k (Lane 2), *incenp*^{3747} (Lane 3), *incenp*$^{P(EP)2340}$ (Lane 4). Anti-DmINCENP antibody (Rb801-1) was used for the blot. The full-length of INCENP was detected in wild type, but not in other strains. (Lower panel) Blotting of anti-alpha-tubulin antibody as loading control.

Chapter 4

Phenotypic analysis of *incenp*^{3747}

4.1 Introduction

Several experimental approaches have been tried thus far in order to gain insight in the function of INCENP. Knockdown of the protein level in dsRNAi studies of *Drosophila* cell lines and *Caenorhabditis elegans* showed that INCENP is required for chromosome structure, prometaphase congression, and chromosome segregation. (Adams et al., 2001c; Kaitna et al., 2000). These studies also showed that INCENP is required for the completion of cytokinesis. Analysis of mutants in budding yeast showed that the INCENP/Aurora B complex is required for bi-orientation of the chromosomes by promoting turnover of kinetochore-microtubule connections until biorientation of sister kinetochore between opposite spindle poles creates tension in the surrounding chromatin (Tanaka et al., 2002). Studies of INCENP knockout mice suggested that INCENP is required for multiple processes during mitosis (Cutts et al., 1999). However, the early death of the embryos makes it difficult to obtain a sufficient number of cells to analyze the role of INCENP in mitosis. Analysis of

incenp^{3747} will allow us for the first time to study the consequences of the lack of the protein function in a multicellular developing eukaryote.

4.2 *incenp*^{3747}: Characterization of the embryonic phenotype of homozygous individuals

In order to analyze the phenotype of *incenp*^{3747} embryos at an early stage, I used CyO-ftz-LacZ, a balancer chromosome expressing the LacZ gene under the control of the promoter of the *fushi-tarazu* gene (ftz). Embryos were collected and aged for the adequate amounts of time (see Materials and Methods) and then stained with DAPI to visualize DNA and with anti-beta-galactosidase antibody to distinguish homozygous from heterozygous embryos. Homozygous embryos do not show expression of beta-galactosidase (negative selection).

Most five-hour old embryos are in cycle 14. At this stage, membranes grow inward between the blastoderm nuclei, creating an epithelial monolayer of approximately 6000 blastodermal cells surrounding the multinucleate and polyploid yolk cells. In *incenp*^{3747} homozygous embryos (LacZ negative), most of the nuclei were missing from the surface (Figure 4.1). Masses of DNA of unusual size were observed in some parts of the embryo. This phenotype suggested defects had arisen before cycle14, maybe causing defective nuclei to sink into the interior of the embryo.

To find out at which stage defects first arise, we studied earlier embryos. In 3-hour aged embryos, the syncytial nuclei reach the embryonic surface. Some of the

embryos also started to show defects at this stage. Some of the nuclei were missing from the surface (Figure 4.2). This phenotype is similar to the phenotype described for homozygous *incenp*$^{P(EP)2340}$ embryos (Tseng and Hariharan, 2002).

Study of earlier phenotypes suffers from the handicap of our inability to distinguish heterozygous from homozygous embryos. In mixed populations of earlier embryos we found chromosome segregation defects as early as cycle 3 (see chromosome bridges in figure 4.3B). Some of the nuclei also failed to follow the globally synchronous mitotic oscillator (Figure 4.3C). These early defects suggest a maternal contribution to the phenotype, either due to a partial dominance of the mutation or genetic interaction with some component of the maternal genotype.

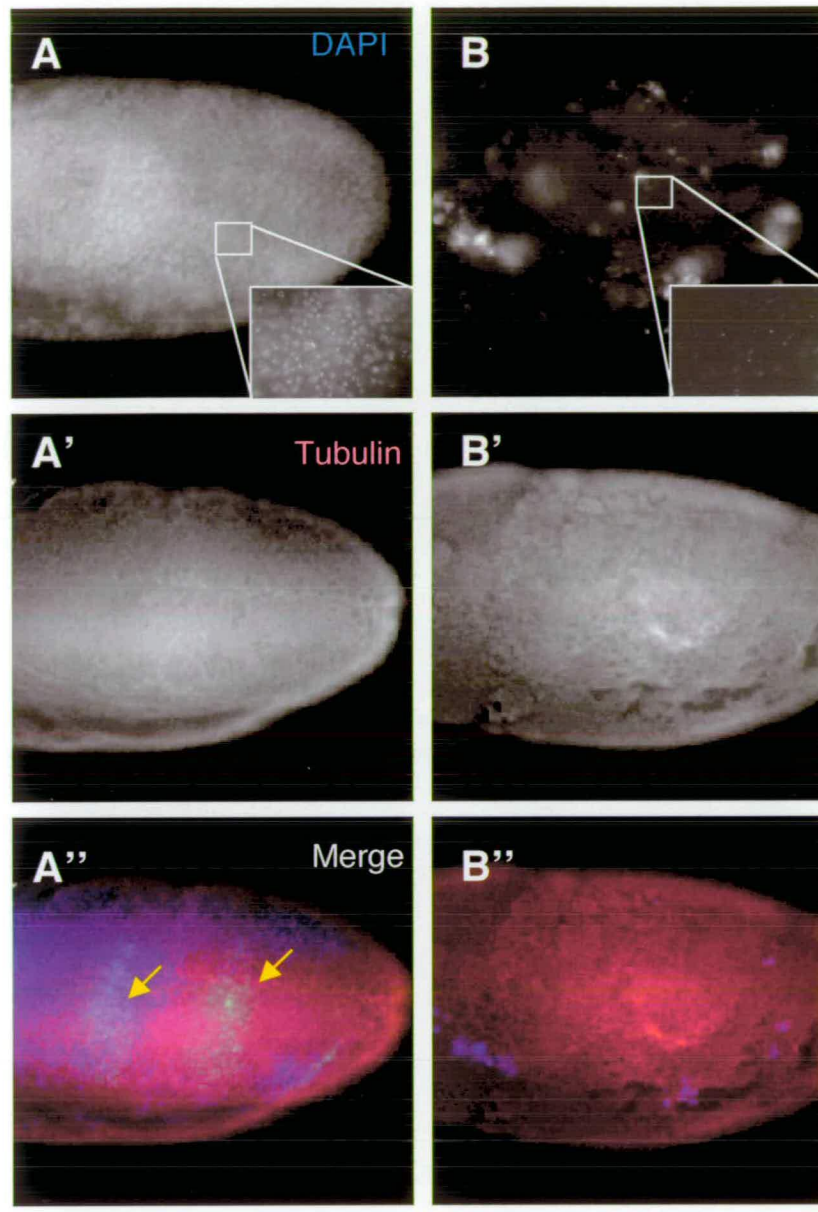


Figure 4.1 Staining of 5-hour-old emryos
(A-A'') *Incenp*^{3747}/CyOftzLacZ embryo. The embryo developed normally to mitotic cycle 14. The arrows in A'' point to the expression of LacZ. (B-B'') *Incenp*^{3747}/*incenp*^{3747}. Most of the nuclei were missing from the periphery and had apparently sunk into the interior of the embryo.

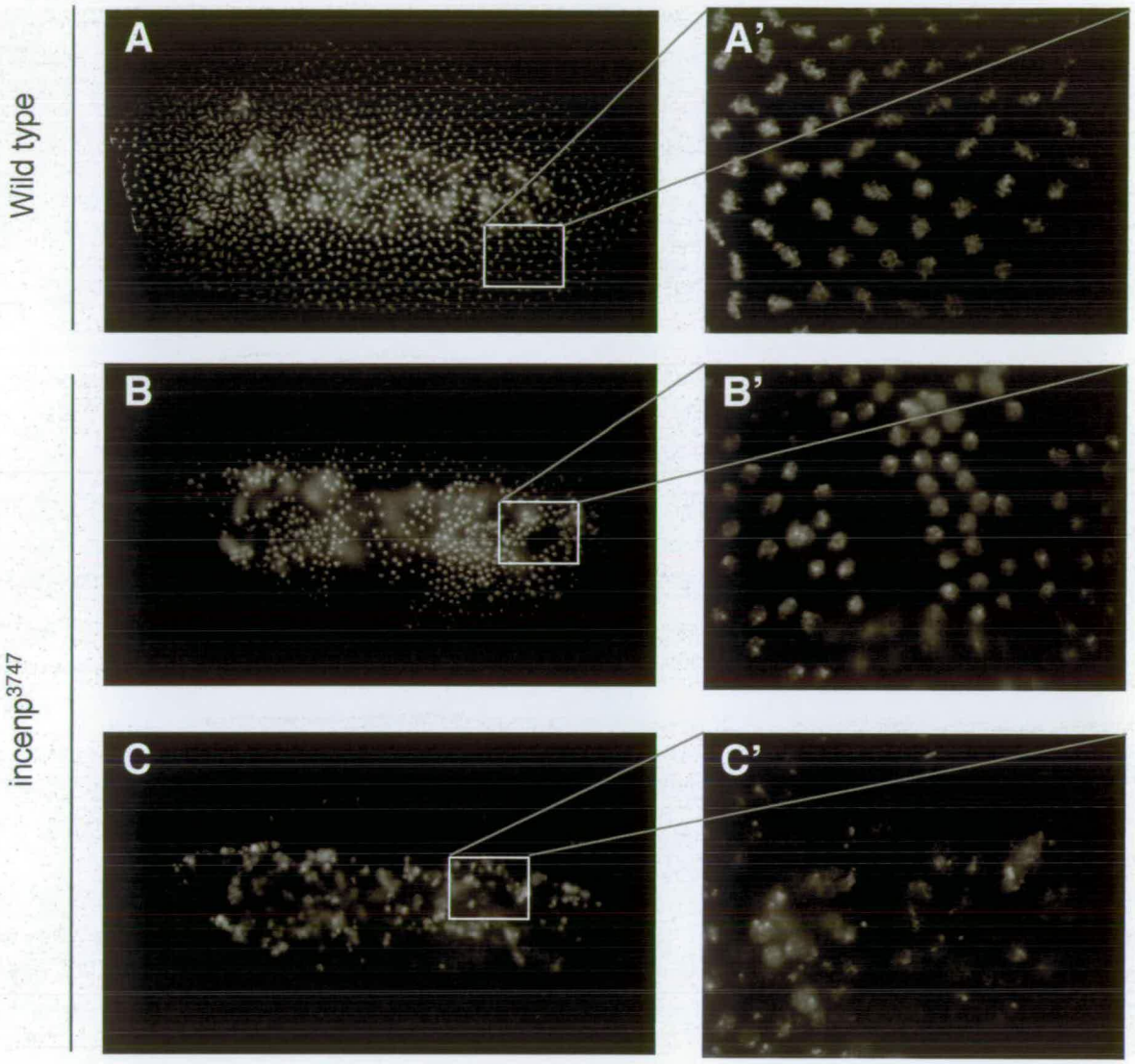


Figure 4.2 DAPI staining of 1.5-hour-old embryos
(A-A') Wild type. The nuclei on the surface of this embryo were in mitosis. (B-B') *incenp*^{3747} embryos. Some of the nuclei were missing from the surface of the embryo and had sunk into the interior of the embryo. (C'-C') Interior focal plan of *incenp*^{3747} embryo. The DNA was clumped in a mass and appeared to be degraded.

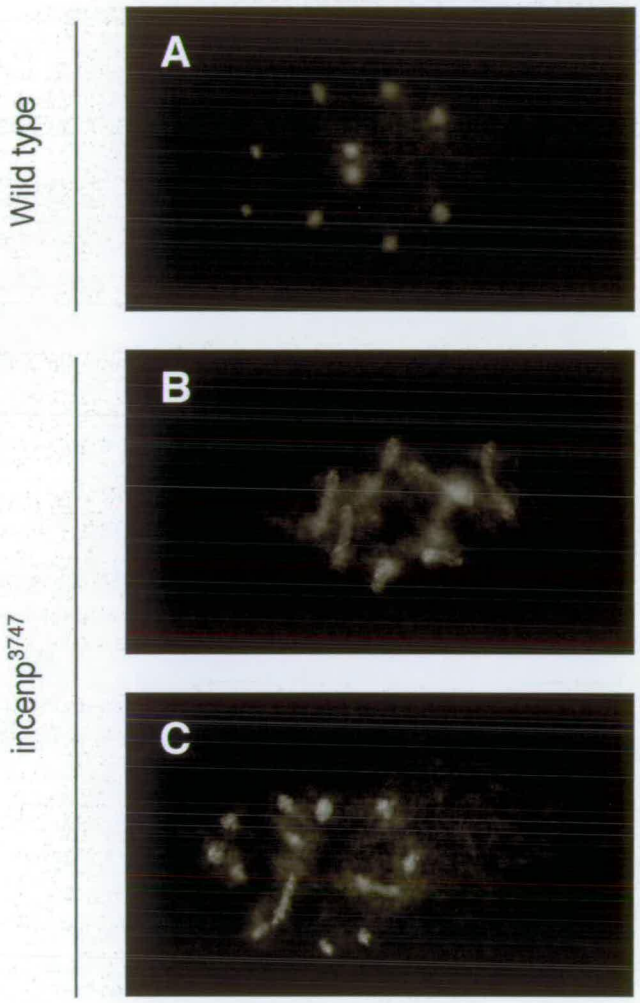


Figure 4.3 DAPI staining of 0.5-hour-old embryos
(A) Wild type. The nuclei were dividing synchronously. (B) *incenp*^{3747}. The nuclei were dividing synchronously, but showed a chromosomal segregation defect. (C) *incenp*^{3747}. The nuclei failed to follow the globally synchronous mitotic oscillator.

4.3 The early phenotype of *incenp*^{3747}

Wieschaus and Sweeton (Wieschaus and Sweeton, 1988) and Merrill (Merrill et al., 1988) used stocks that carried compound chromosomes to study early embryogenesis in *Drosophila*. They found that nullo -X, -2L, -2R, -3L, -3R and -4 embryos progress through mitoses 1-13. These embryos were normal in mitotic timing and morphology and showed the earliest defects in cellularization, gastrulation and mitotic pattern during cycle 14. This means that these processes are under the maternal control and do not require zygotic contribution.

One possible explanation of the early embryonic phenotype in *incenp*^{3747} homozygous embryos is a dominant maternal effect of the mutation. However, all of our initial data suggested that *incenp*^{3747} is a recessive mutation. Heterozygous individuals do not show any defects in mitosis or meiosis, any external phenotypes or any variation in fertility (Mar Carmena and Chih-Jui Chang, unpublished). Another possible explanation is a genetic interaction of the mutation with another genetic aberration in the maternal genotype: in my case, the most likely source would be the balancer chromosome. I decided to perform a detailed analysis of the hatching rates of homozygous embryos from mothers of different genotypes to distinguish between these two possibilities.

The following strains of flies were used in this study: *incenp*^{3747}/CyO-Kr-GFP, *incenp*^{3747}/+ and +/CyO-Kr-GFP. '+' denotes the wild type chromosome. The results are shown in figure 4.4. The hatching rate of embryos from cross (♀(*incenp*^{3747}/+) × w^{1118}♂) is 84.5%, which is not significantly different to the one from cross (♂

(*incenp*^{3747}/+) x w^{1118} ♀)-90%. In these crosses, there is no balancer chromosome in the strain. The results suggested that the hatching rates were similar no matter whether the female was wild type or *incenp*^{3747}/+. This meant that there was no dominant maternal effect of *incenp*^{3747}.

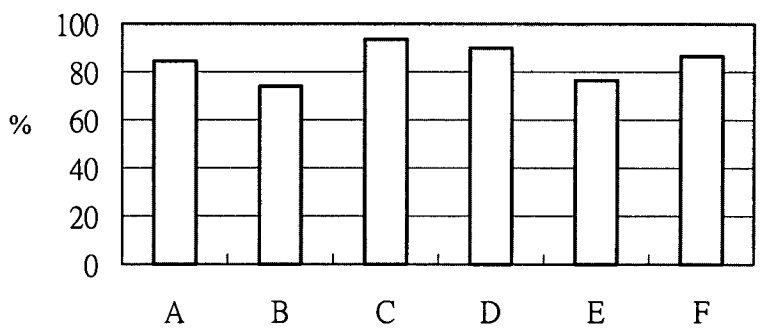
The hatching rate of embryos from cross (♀(+/ CyOKrGFP) x w^{1118} ♂) is 93%, which also shows no significant differences to the one from cross (♂(+/ CyOKrGFP) x w^{1118} ♀). This suggested that the balancer chromosome CyO-Kr-GFP on its own has no dominant effect on the hatching rate. However, the hatching rate of embryos from crosses (♀(*incenp*^{3747}/ CyOKrGFP) x w^{1118} ♂) and (♂(*incenp*^{3747}/ CyOKrGFP) x w^{1118} ♀) were 74% and 76%, which were significantly lower than others. This could be the total effect from *incenp*^{3747} and balancer individually.

The results of the hatching rates showed that *incenp*^{3747} is recessive, and that it has no dominant effect on early embryonic development.

A

Strains	Embryo Counted (hatched/dead)		Percentage (hatched %)		Average Percentage (hatched %)
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	
♀(*incenp*^{3747}/+) x w^{1118}♂	911/117	996/148	82	87	84.5
♀(*incenp*^{3747}/ CyOKrGFP)x w^{1118}♂	998/246	679/318	80	68	74
♀(+/ CyOKrGFP) x w^{1118}♂	825/38	1029/74	95	92	93.5
♂(*incenp*^{3747}/+) x w^{1118}♀	746/75	668/80	90	90	90
♂(*incenp*^{3747}/ CyOKrGFP) x w^{1118}♀	571/166	809/245	77	76	76.5
♂(+/ CyOKrGFP) x w^{1118}♀	770/667	781/180	92	81	86.5

B



A= ♀ (*incenp*^{3747}/+) x w^{1118}♂

B= ♀ (*incenp*^{3747}/ CyOKrGFP) x w^{1118}♂

C= ♀ (+/ CyOKrGFP) x w^{1118}♂

D= ♂ (*incenp*^{3747}/+) x w^{1118}♀

E= ♂ (*incenp*^{3747}/ CyOKrGFP) x w^{1118}

F= ♂ (+/ CyOKrGFP) x w^{1118}♀

Figure 4.4 Hatching rate of *incenp*^{3747}

(A) Hatching rates of different strains of *incenp*^{3747}. About 1000 embryos were scored in each experiment. (+), wild type chromosome. (B) Schematic representation of the hatching rates

For the purposes of the understanding of the early embryonic phenotypic defects, these results suggested the hypothesis that *incenp*^{3747} could interact with the maternal CyO-ftz-LacZ balancer, and this genetic interaction resulted in the early phenotype in homozygous embryos. To test this hypothesis, three different strains, *incenp*^{3747}/+, *incenp*^{3747}/CyO-Kr-GFP, *incenp*^{3747}/CyO-ftz-LacZ, were used to analyze the early embryonic phenotype.

The embryos from those strains were collected for half an hour. After aging for 30 minutes, they were fixed and stained with DAPI to see the DNA (see materials and methods). Only 1% of the embryos from the strain *incenp*^{3747}/+ showed defects in early embryogenesis (< cycle 10). ('+' denotes the wild type chromosome). 6% of the embryos from the strain *incenp*^{3747}/CyO-Kr-GFP showed defects. 12% of embryos from stain *incenp*^{3747}/CyO-ftz-LacZ showed defects (Figure 4.5). The results suggested that the early embryonic defects of *incenp*^{3747} were likely due to a genetic interaction between *incenp*^{3747} and the CyO-ftz-LacZ balancer. Apparently, this interaction caused the maternal dominant effect during early embryogenesis.

A

Strains	Embryo numbers		Early phenotype %
	Normal	Defective	(Cycle 1~10)
♀(*incenp*^{3747}/+) x(*incenp*^{3747}/+)♂	55	1	1 %
♀(*incenp*^{3747}/ CyOKrGFP) x(*incenp*^{3747}/ CyOKrGFP)♂	45	3	6 %
♀(*incenp*^{3747}/CyOftzLacZ) x(*incenp*^{3747}/CyOftzLacZ)♂	43	6	12 %

B

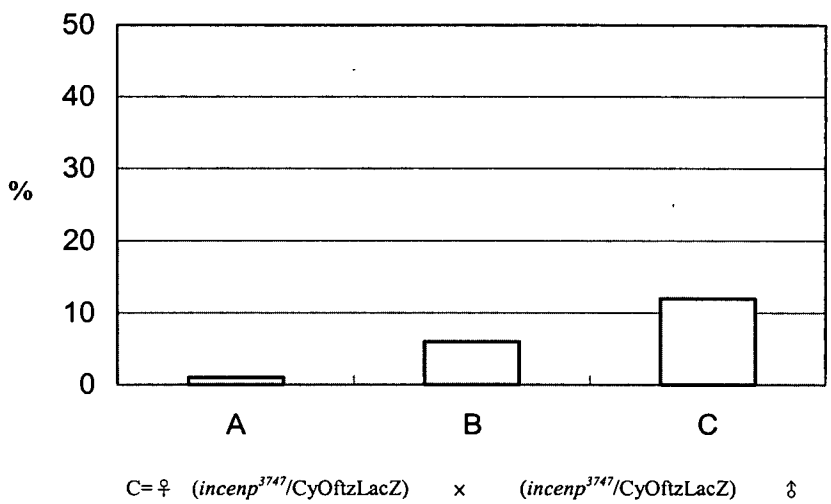


Figure 4.5 Quantification of early embryonic defects in *incenp*^{3747} strains

(A) Number of different *incenp*^{3747} strains embryos that showed early embryonic defects. (+), wild type chromosome. (B) Schematic representation of the percentages of the phenotype.

4.4 *incenp*^{3747} homozygous embryos exhibit aberrant neuronal morphology

The cells that are going to originate the peripheral nervous system (PNS) are the only ones that divide in late embryogenesis after cycle 16 (there is only one exception: one of the mitotic domains goes into cycle 17). The defects in later developmental stages were studied making use of the monoclonal antibody 22C10 (Mab22C10), a molecular probe for neuronal development (Zipursky et al., 1984). This antibody recognizes a protein called Futsch, which is required for neuronal development (Hummel et al., 2000).

Embryos were aged and stained with 22C10 and anti-GFP antibodies. The results showed that there was a reduced number of neurons and clusters of neurons appeared disorganized in *incenp*^{3747} homozygous embryos, compared to *incenp*^{3747}/CyO-Kr-GFP (Figure 4.6, the images were captured by Sarah Goulding and Jayne Miller). This phenotype provided evidence that *incenp*^{3747} homozygous embryos were defective in mitosis in late embryos.

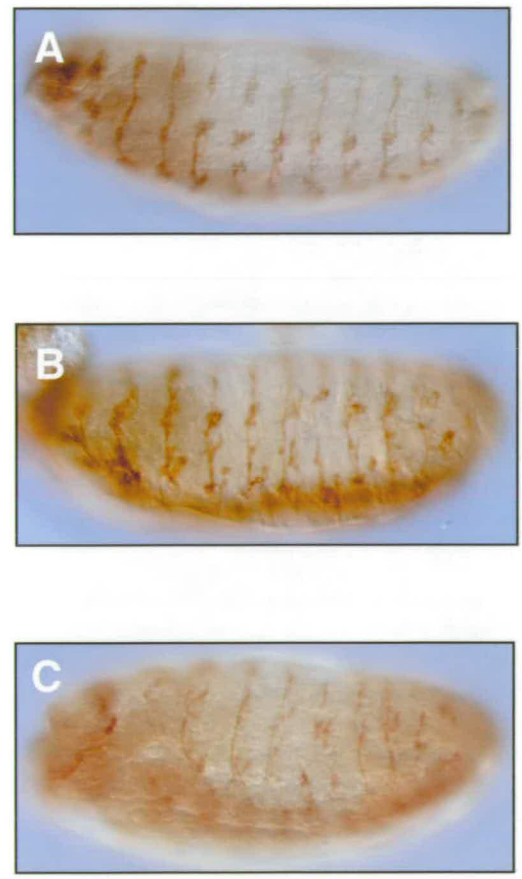


Figure 4.6 Embryonic neuronal staining
Embryos stained with an antibody against the neural epitope, 22C10. (A) The heterozygous embryo *incenp*^{3747}/CyO-Kr-GFP showed no defect in neuronal patterning. (B,C) The homozygous embryo *incenp*^{3747}/*incenp*^{3747}. These embryos showed reduced numbers of neurons and lack of organization of neuron clusters.

4.5 *incenp*^{3747} homozygous larvae exhibit abnormal behaviour

According to the hatching rate analysis, *incenp*^{3747} is homozygous lethal. However, a few *incenp*^{3747} homozygous first instar larva "escapers" could be isolated (<0.1%). If these individuals developed to third instar, I would have the opportunity to study the mitotic phenotype in larval neuroblasts. The homozygous larvae were kept in optimized growing conditions (apple juice agar plate with yeast paste), but none of the homozygous larvae developed any further.

After being put in the plate, heterozygous *incenp*^{3747}/CyO-Kr-GFP larvae moved to the yeast paste after wandering shortly (Figure 4.7A). In contrast, homozygous *incenp*^{3747}/*incenp*^{3747} larvae behaved abnormally, moving in a circular path and away from the yeast paste (Figure 4.7B).

If the larvae were kept on an apple juice agar plate without yeast paste the heterozygous larvae moved randomly all around the plate (Figure 4.7C). In contrast, homozygous larva still moved in circles (Figure 4.7D). This abnormal movement could be result from the abnormal neural development, which was previously shown by the 22C10 staining of homozygous embryos.

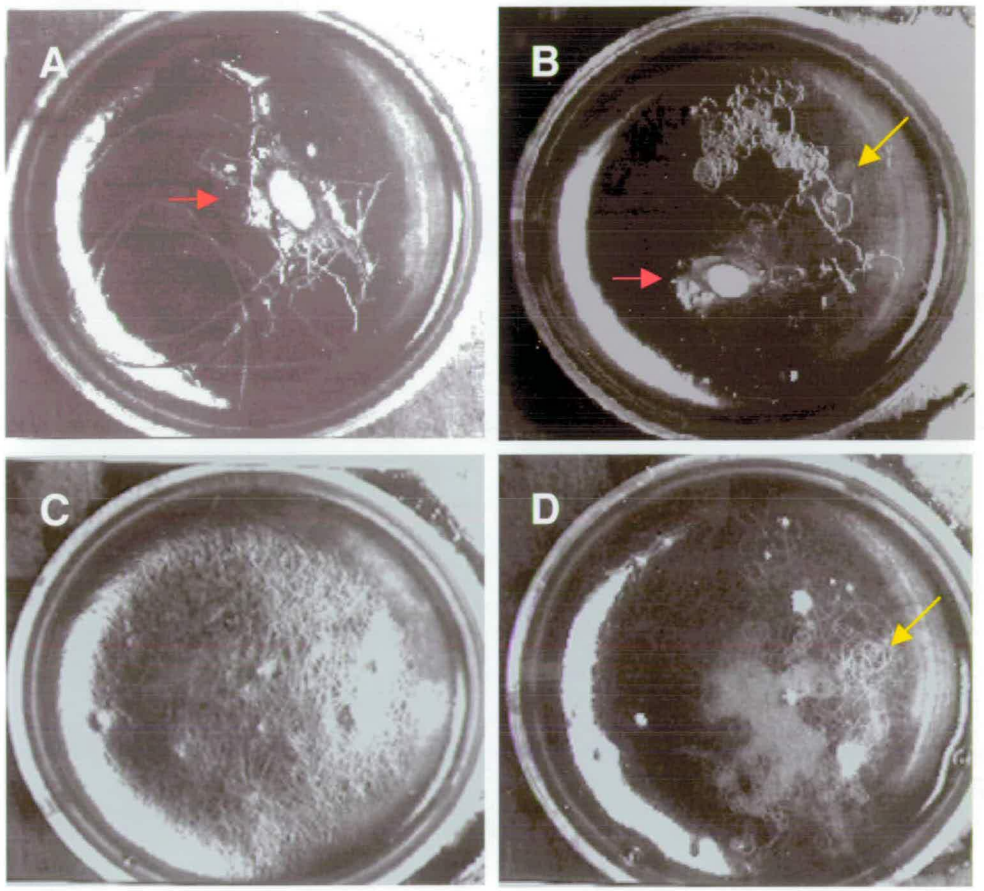


Figure 4.7 *incenp*^{3747}/*incenp*^{3747} first instar larvae show abnormal movement

The paths of larvae on agar plates with yeast paste (A, B), or without yeast paste (C, D). The hatching rate of incenp^{3747} homozygous was less than 0.1%. Few *incenp*^{3747} homozygotes survived to become first instar larvae, which moved abnormally in circles and died before development to second instar larvae. (A, C) *incenp*^{3747}/CyOKrGFP larvae. These larvae moved randomly on the plate (C), then moved to yeast paste (A). (B, D). *incenp*^{3747}/*incenp*^{3747} larvae. They moved in circles. The red arrows point to the yeast paste. The yellow arrows point the circular path of the abnormal larvae.

Chapter 5

Discussion and conclusion

5.1 Overall success of the screen

The aim of this EMS mutagenesis screen was to obtain new alleles and interactors of INCENP. The screen was designed to isolate EMS-induced mutants showing any lethality, male sterility or visible phenotype when trans-heterozygous with *incenp*$^{P(EP)2340}$, a P-element insertion in the *incenp* gene which shows a semi-dominant phenotype in mitosis and meiosis (Mar Carmena and Jayne Miller, unpublished). In a diploid organism, a complementation test is performed by intercrossing heterozygous recessive mutants and observing whether or not the progeny have a wild-type phenotype. If the mutants are in different unrelated genes, the progeny will normally have wild-type function provided by the respective wild-type alleles. However, if the two gene products form a complex or functionally interact to each other, half the dose of each wild-type gene may not result in full functionality and therefore they may not complement. These mutations are called second-site non-complementors (Hays et al., 1989; White-Cooper et al., 1996).

In my case, the system is specially sensitized to mutations in genes interacting with *incenp* due to the partial dominant effect of the mutation used. One possible explanation for the dominant effect observed in the $incenp^{P(EP)2340}$ heterozygous flies is the production of a truncated product. The P-element insertion in $incenp^{P(EP)2340}$ produces a smaller transcript (Sarah Goulding, unpublished), but our antibodies do not detect a smaller protein in Western blots of homozygous or heterozygous individuals. This could be due to differences in secondary structure and/or antigen presentation of the smaller protein. If our hypothesis is correct and this smaller protein is produced, it would be a polypeptide containing the domains required for chromosomal targeting but lacking the IN-BOX, the conserved domain by which INCENP binds Aurora B kinase. In other words, this smaller polypeptide would in theory be able to bind to chromatin/spindle microtubules and therefore compete with the wild-type protein for the chromosomal/microtubule binding sites/factors, but should not target the kinase to its substrates. Additionally, INCENP is required for the activation of Aurora B (Honda et al., 2003) and the truncated polypeptide would not be able to perform this function. In this scenario, with Aurora B partially delocalised from its cellular substrates, the system is in a very precarious equilibrium that can be thrown further out of balance by mutation of a gene coding for an interacting protein.

The screen was labor intensive, but proved very successful. After screening only 5000 chromosomes, I obtained 18 interacting mutations, including one new recessive allele of *incenp* ($incenp^{3747}$) and two dominant female sterile mutants (3322, 4330).

All 18 mutants were homozygous lethal. Preliminary analysis of the phenotypes of some transheterozygous combinations (mutant/*incenp*$^{P(EP)2340}$) showed defects in mitosis and meiosis (Jayne Miller, Lisa Pang, Mar Carmena), which makes them worthy of further characterization. Additionally, this preliminary analysis also showed clear differences in the phenotypes of the transheterozygous combinations. Some show multiple defects in chromosome structure and segregation in mitosis and meiosis, whereas other combinations show a cytokinesis specific phenotype. Further analysis and identification of the mutated genes and characterization of their interaction with INCENP will help dissect out the different functions of the chromosomal passenger complex in mitosis and meiosis.

All the mutants from my screen have now been mapped by deficiency complementation, which showed most of them contained more than one EMS 'hit' (Sarah Goulding, Duncan Sproul and Mar Carmena). The mutagenised chromosomes have been cleaned by meiotic recombination. Future identification of the mutated genes and characterization of their interaction with INCENP will shed new light into the functions of the chromosomal passenger complex. For the purpose of this PhD thesis, I focused on the analysis of one of the mutants, *incenp*^{3747}, a new recessive allele of *incenp*.

5.2 *Incenp*^{3747} is a new allele of *incenp*

3747 was the only mutant isolated in the screen that failed to complement chromosomal deficiencies for the *incenp* locus. The chromosome containing 3747 was cleaned by genetic recombination and the recombinant chromosome 3747-r3 used for further study. Fine deficiency mapping using six deficiencies with breakpoints around the *incenp* gene confirmed that 3747 mapped in the *incenp* genomic region. Furthermore, 3747 did not complement imprecise excisions of *incenp*$^{P(EP)2340}$. Although all the genetic evidence was in favor of 3747 being a new allele of *incenp*, molecular analysis was required to confirm this.

The most straightforward approach was to analyze the sequence of the *incenp* gene in 3747. This revealed the existence of a point mutation, which resulted in a stop codon (in codon 457) before the coiled-coil region of the protein. The predicted molecular weight of the 457 amino acid C-terminal truncated protein product would be about 61 kDa. 3747 was then named *incenp*^{3747}, as this result confirmed it is an allele of *incenp*.

5.3 The protein product of *incenp*^{3747}

Unlike *incenp*$^{P(EP)2340}$ heterozygous individuals, heterozygous *incenp*^{3747} individuals did not show any defects in mitosis or meiosis (Mar Carmena and Jayne Miller, unpublished data). This seems to indicate that *incenp*^{3747} is a recessive allele. The Western blot results showed that not only was wild type INCENP missing in

incenp^{3747} homozygous embryos but I also failed to detect any truncated version of INCENP. There could be two possible explanations: firstly, the truncated protein could be very unstable in vivo and be degraded very quickly. Secondly, the antibody R801-1 (Adams et al., 2001c) might fail to detect the truncated protein.

Polyclonal antibody R801-1 was raised against the N-terminal region of *Drosophila* INCENP ($INCENP_{1-348}$). The putative truncated protein encoded by *incenp*^{3747} ($INCENP_{1-457}$) would therefore carry all the epitopes that the antibody R801-1 was raised against. However, one cannot absolutely rule out that differences in secondary structure of the polypeptide would hinder antibody recognition.

5.4 Is INCENP required in early embryogenesis in *Drosophila*?

The INCENP knockout mouse revealed that INCENP is essential for mouse development (Cutts et al., 1999). The homozygous embryos die at the 32-64-cell stage with disruption of cytokinesis and abnormal bundling of microtubules. *Incenp*^{3747} is also embryonic lethal. In *Caenorhabditis elegans*, dsRNAi showed that INCENP is involved in chromosome segregation and completion of cytokinesis (Kaitna et al., 2000). These studies were aimed to investigate the role of INCENP in a multicellular system. However, the early death of the mouse embryos made it difficult to obtain a significant number of cells to be studied. dsRNAi experiment in *C. elegans* only knockdown the protein levels and the phenotype analysis was reduced to very low numbers of early embryos that fail to progress to the two-cell

stage. *Incenp*^{3747}, by contrast, will allow us to study the function of INCENP in different stages during development.

To analyze the phenotype of INCENP null embryos, I need a marker to discriminate homozygous embryos as early as possible during development. The balancer chromosome CyO-ftz-LacZ was used in the analysis of early embryonic development. This balancer carries an insertion of the lacZ gene (encoding β-galactosidase) expressed under the control of the promoter of the gene *futzi-taratzu* (ftz). The peak of expression of β-galactosidase occurs between 4.5 and 10.5 hours after egg laying (Hiromi et al., 1985). In *incenp*^{3747} homozygous five-hour embryos, most of the nuclei were missing from the surface. This result led me to analyze even earlier stages of embryogenesis. Surprisingly, defective chromosome segregation was observed as early as in mitotic cycle 3. Zygotic gene expression has no effect on cell-cycle progression before cycle 14 in *Drosophila*. Early development is determined by maternal cytoplasmic mRNA and its transcribed product. The early embryonic phenotype suggested that *incenp*^{3747} could be a dominant-negative mutant. However, by using different balancers and wild type chromosomes, I found that the early phenotype was caused by a genetic interaction between the CyO-ftz-LacZ balancer chromosome and *incenp*^{3747}. Future analysis using a non-interacting balancer should allow us to study the early function of INCENP in embryonic development.

5.5 INCENP is required for neural development in *Drosophila*

Incenp^{3747} homozygous twenty-hour old embryos showed an abnormal neuronal phenotype. The immunostaining with anti-GFP antibody (as a marker to recognize homozygous embryos) and Mab22C10 showed a reduction in the number of neurons and lack of organization of the neuronal cluster in *incenp*^{3747} homozygous embryos. This decrease in the number of neurons is consistent with failure of cell division. The defects in mitosis are much severe in peripheral nervous system (PNS) since these cells undergo more division cycles than ectodermal cells during embryo development.

Although *incenp*^{3747} is embryonic lethal, some *incenp*^{3747} homozygous first instar larva "escapers" (<0.1 %) survived. These larvae did not develop further. Consistently with the defective neuronal phenotype observed in of *incenp*^{3747} homozygous embryos, the behavior and movement of these larvae was abnormal. Instead of moving randomly, they moved abnormally in circles.

PART II

RNAi Analysis of Topo II in *Drosophila* cells

Chapter 6

Topo II dsRNAi

6.1 Introduction

The first studies of topoisomerases date from the 1970s when *Escherichia coli* DNA topoisomerase I (Wang, 1971) and DNA gyrase (Gellert et al., 1976) were discovered. DNA topoisomerases are enzymes that catalyze DNA topological transformations by transiently cleaving either a single DNA strand or a pair of complementary DNA strands, in one duplex segment, to form an enzyme-operated gate. DNA topoisomerases fall into two categories, according to their reaction mechanism. Type I Topoisomerases do not require ATP and the DNA strands are transiently broken one at a time. For type II enzymes, the reaction is ATP-dependent, and a pair of strands in the DNA double helix is transiently broken in concert by a dimeric enzyme. DNA topoisomerases play roles in many events relating to DNA metabolism, including replication, transcription and chromosome segregation (Cook et al., 1992; Postow et al., 2001; Uemura et al., 1987).

Topoisomerase II (Topo II)

In nonvertebrate eukaryotes, the type II topoisomerases are homodimers with a monomer molecular weight of about 170 kDa. Only one isoform of Topo II is present in yeast (Goto and Wang, 1984), *Drosophila* (Wyckoff et al., 1989), and *C. elegans* (Berks, 1995). In mammals, there are two isoforms of Topo II, encoded by different genes (α and β (Chung et al., 1989)). Both of them can complement yeast Topo II function (Adachi et al., 1992). The two enzymes show amino acid conservation throughout most of the protein except in the carboxy terminal region (Austin et al., 1993). The enzymatic properties of two isoforms are similar (Austin et al., 1995). However, the two isoforms show differences in cell cycle expression and in their localizations in mitosis. Topoisomerase IIα is expressed in proliferating cells whereas topoisomerase IIβ is expressed at equal levels both in dividing cells and cells withdrawing from cell cycle. Topoisomerase IIα is found to increase during S phase and reach its maximum level in M phase. In contrast, topoisomerase IIβ is expressed at constant a level through the cell cycle (Woessner et al., 1991). Topo IIα associates with chromosomes from metaphase to telophase, whereas Topo IIβ mainly localizes diffusely in the cytoplasm until anaphase onset, when low levels of the protein associate with the chromatids (Christensen et al., 2002).

By using a double mutant (cold-sensitive strains mutated in genes coding for Topo II (cs-topo2) and beta-tubulin (cs-nda3)), Yanagida and colleagues showed that inactivation of Topo II in the absence of the spindle leads to the production of extended chromosomes, and reactivation of Topo II leads to shortened chromosomes. These results suggested that Topo II is required for the final stages of chromosome

condensation in S. pombe (Uemura et al., 1987).

The role of Topo II in chromosome condensation has also been studied using mitotic Xenopus eggs extract (Adachi et al., 1991). HeLa nuclei, which contain high complement of endogenous Topo II, are converted to mitotic chromosomes in Topo II-depleted extracts. In contrast, the chicken erythrocyte nuclei, which contain very low amounts of Topo II (Heck and Earnshaw, 1986) cannot be converted to condensed chromosomes in the depleted extract. The finding that Topo II is a major constituent of the chromosome scaffold suggests that Topo II plays a role in the structural maintenance of chromosomes (Earnshaw et al., 1985; Gasser et al., 1986).

In fission yeast, analysis of top2 ts mutants suggested that Topo II is required for segregation of sister chromatids at mitosis (Uemura and Yanagida, 1984). In vertebrate cells, Topo II activity is required for individualization of chromosomes (the process by which catenated strands of DNA between different chromosomes are resolved and each chromosome is converted into an individual mobile unit (Gimenez-Abian et al., 2000). The decatenation activity of Topo II is required for the formation of distinguishable pairs of sister chromatids in prometaphase (Gimenez-Abian et al., 1995). Some catalytic inhibitors, such as ICRF-193, were used to study the function of Topo II. The chromosome failed to segregate when cells treated with Topo II inhibitor (ICRF-193) (Haraguchi et al., 1997) or the more potent inhibitors ICRF-159 or ICRF-187 (Gorbsky, 1994).

Topoisomerase II (Topo II) interacts with a number of other proteins, including several involved in chromosome condensation. It was reported that the *Drosophila*

condensin subunit Barren and Topo II associate in vitro and colocalize in mitosis (Bhat et al., 1996). Barren also activates Topo II in vitro, although this result could not be reproduced in yeast (Lavoie et al., 2000). In addition, Topo II localization is affected in *S. cerevisiae* YSC4 mutants, the homologue of the condensin subunit XCAPD2 (Lavoie et al., 2002). *Drosophila* Topo II and Barren share chromatin binding sites with Polycomb group proteins and coimmunoprecipitate with one of these proteins, Polyhomeotic (ph). Mutants in the ph gene show defects in chromosome segregation (Lupo et al., 2001). These results raise the possibility that Topo II and condensin components might cooperate in regulating chromosome structure both in mitosis and in interphase.

In this part of study, I used double strand RNA interference to analyze the role of Topo II in cell division in *Drosophil*a. In the absence of Topo II, mitotic chromosomes can condense, although the detailed structures produced are not entirely normal. Thus, Topo II is not essential for mitotic chromosome condensation, but it may have a role in the establishment of chromosome architecture. Topo II is not essential for centromere/kinetochore assembly or function, but it is required for sister chromatid separation at anaphase. Quite surprisingly, Topo II is required for the formation of a compact metaphase plate.

6.2 Introduction to RNA interference (RNAi)

RNA interference (RNAi) is a powerful tool for selective gene silencing, triggered by double-stranded RNAs (dsRNA). The phenomenon was first discovered in the nematode worm *Caenorhabditis elegans*. The injection of dsRNA into *Caenorhabditis elegans* interferes with the expression of specific genes that contain a highly homologous region to the delivered dsRNA (Fire et al., 1998). Subsequently, this phenomenon was observed and experimentally demonstrated in flies (Kennerdell and Carthew, 1998) and vertebrates (Li et al., 2000b).

The initial experiments in *C. elegans* suggested that RNAi occurs at the post-transcriptional level (Fire et al., 1998). The introduction of long dsRNAs induces selective mRNA destruction. The long dsRNAs are recognized and cut to small fragments of 21-25 nucleotides, which were termed short-interfering RNAs (siRNAs) (Bernstein et al., 2001; Zamore et al., 2000). These processes are mediated by the multi-domain Rnase-III enzyme Dicer and the rde-1 (for RNAi defective)/ago-1 (argonaute) family of proteins. The small fragments of dsRNA (siRNAs) generated by the RNAi process contain two perfectly complementary RNA strands (Elbashir et al., 2001; Zamore et al., 2000). The function of the siRNAs is to guide the RNA-induced silencing protein complex (RISC) to target mRNAs, which contain regions homologous identical to these siRNAs. The mRNAs are then cleaved by the nuclease (Hammond et al., 2000). The nuclease that is responsible for this process is still unknown.

6.3 Design and preparation of double stranded RNA (ds RNA)

Two fragments from the 5' end of DmTopo II gene fused to the T7 RNA polymerase promoter were used as PCR primers (Figure 6.1). A random human intronic sequence was used as control dsRNA as described previously (Adams et al., 2001c). The EST clone LD24716 was used as a PCR reaction template. The PCR fragments obtained (about 700bp) were used as template for RNA synthesis using the Megascript kit (Ambion).

6.4 dsRNA interference in S2 cells

The RNAi method causes a gradual depletion of the proteins under study, and the proteins are not necessarily lost from all cells. Therefore, samples were taken for analysis by immunoblotting and immunofluorescence at different time points (24, 48, 72, 96, 120, 144 hours after RNAi). Immunoblotting analysis of cells treated with DmTopo II dsRNA showed that the levels of DmTopo II in cultures were highly reduced by 48 hours and the protein became nearly undetectable after 72 hours (Figure 6.2A). In order to know the detection limit of the antibody, a titration experiment was performed. The result showed that the antibody used in this study could detect Topo II from 5 x 10^4 cells, but not from 1 x 10^4 cells (Figure 6.2 B). It could be concluded that levels of the protein in the culture fall by at least a factor of 20 in response to the dsRNA treatment. The protein did not re-accumulate in the cells even by 144 hours after addition of dsRNA.

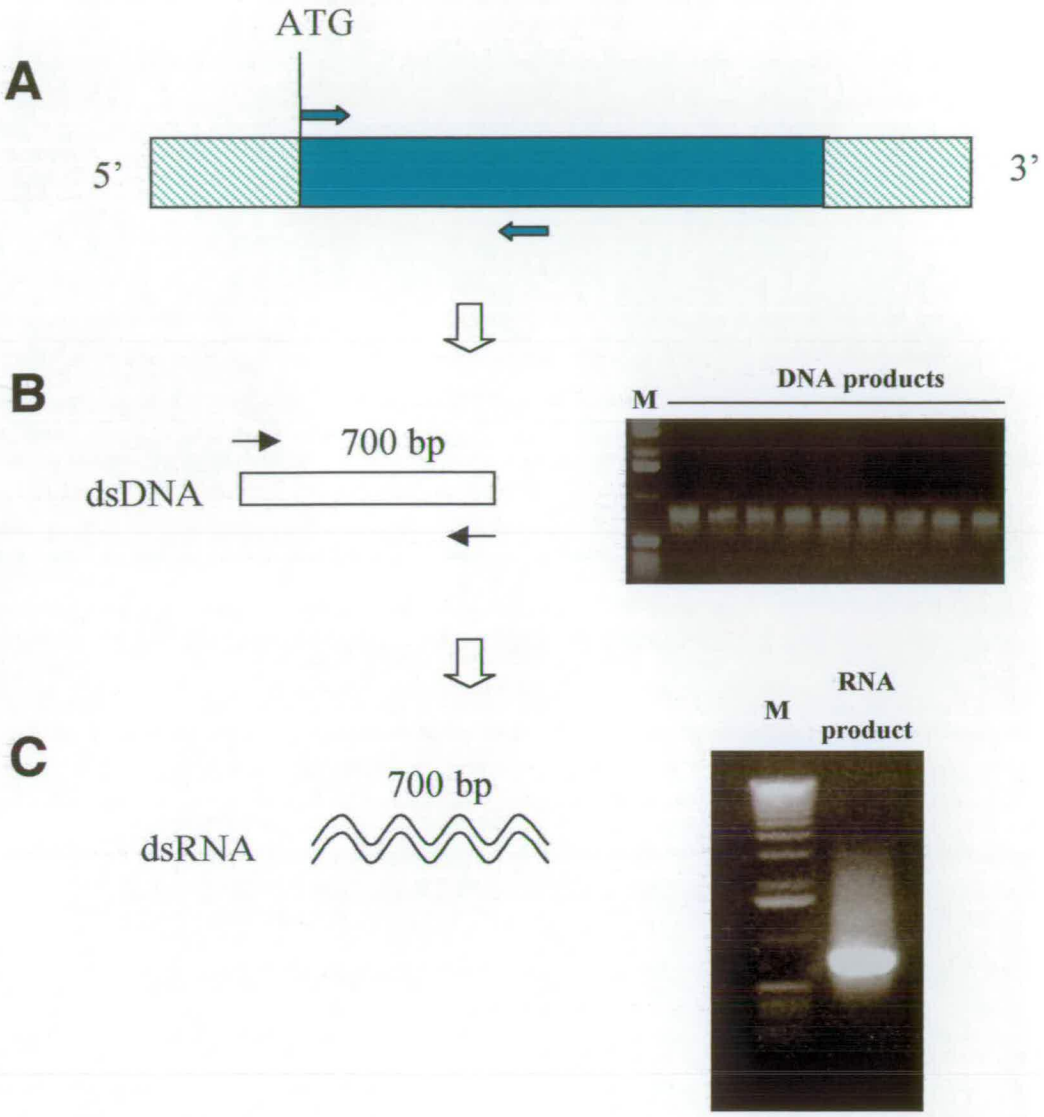


Figure 6.1 Design of RNAi primers and synthesis of double-strand RNA

(A) Design of RNAi primers. Two primers were designed to amplify the N-terminal region of the DmTopo II gene. (B) Gel electrophoresis of the PCR product. Nine reactions were performed to obtain reasonable amount of DNA product for synthesis of double stranded RNA (dsRNA). (C) Gel electrophoresis of dsRNA synthesized from the PCR products.

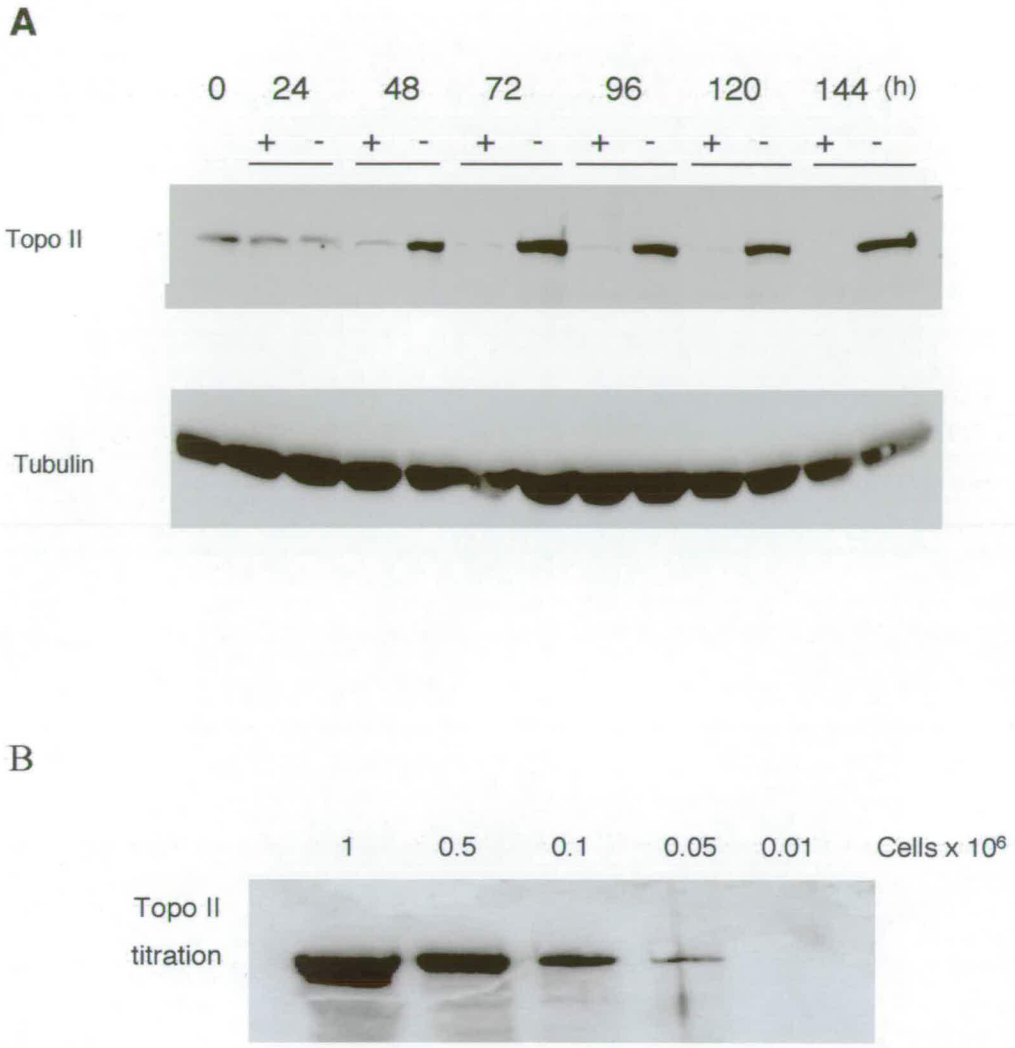


Figure 6.2 Efficient depletion of Topo II in *Drosophila* S2 cells using RNAi

(A) (Upper panel), Topo II levels begin to fail by 48 hours after the addition of dsRNA, and become undetectable by 72 hours. (Lower panel), Loading control (anti-tubulin). (-), control-RNAi. (+), Topo II RNAi-treated. 1 x 10^6 cells were loaded in each lane. (B) Detection limit of anti-Topo II antibody. Anti-Topo II antibody used can detect Topo II in 5 x 10^4 cells, but not 1 x 10^4 cells.

6.5 Effect of Topo II RNAi on cell growth and mitosis

There was no significant difference in mitotic index between cultures treated with control or Topo II dsRNA, although the index in control experiments was always slightly lower than that in RNAi experiments (Figure 6.3D). The growth curves also showed that there was no significant difference between experimental and control cells in the rate of proliferation (Figure 6.3E). Both populations displayed a slight decline in mitotic index over the course of the experiment, presumably because the medium was becoming depleted of essential ingredients. These data are from three independent experiments. In each experiment, more than two thousand cells were scored at every time point.

Detailed analysis of the distribution of mitotic phases in cells following Topo II RNAi revealed surprisingly few differences between the Topo II-depleted cells (Figure 6.3B) and cells treated with a control dsRNA (Figure 6.3A). However, the Topo II-depleted cultures showed a steady increase in anaphase cells. This result suggested that the Topo II-depleted cells might be delayed at anaphase. If prometaphases and metaphases were grouped together (this was because spindles are oriented randomly in these small non-adherent cells, and it is frequently difficult to distinguish between metaphase and prometaphase), no significant differences were observed between experiments and controls (Figure 6.3C). In fact, the only significant difference seen in these experiments was the slight increase in the percentage of anaphase cells in the 96, 120 and 144 hours time points (Figure 6.3B).

These observations reveal that *Drosophila* S2 cells do not have a checkpoint that arrests the cell cycle in response to loss of Topo II function, and that progress through mitosis is not delayed significantly by lack of Topo II activity.

Figure 6.3 Effect of Topo II RNAi on cell growth and mitosis

Scoring of mitotic cells in the different phases of mitosis reveals only slight differences between cells following control RNAi (using dsRNA corresponding to a human intronic sequence – (A)) and Topo II RNAi (B). The Topo II RNAi causes a significant increase in the fraction of anaphase cells seen at later times. (C) There is no significant difference in any of the mitotic phases when control and Topo II RNAi cells (selected because the spindle is viewed from the side and the metaphase plate can be unambiguously identified) are scored at 72 h post addition of dsRNA. (D) There is no significant difference in mitotic index between control and Topo II RNAi –treated cultures over the course of the experiment. (E) The cell growth curves of control and Topo II RNAi-treated cultures. These data are from three independent experiments. In each experiment, more than two thousand cells were scored at every time point. The mitotic index was determined by observing the DNA and spindle staining.

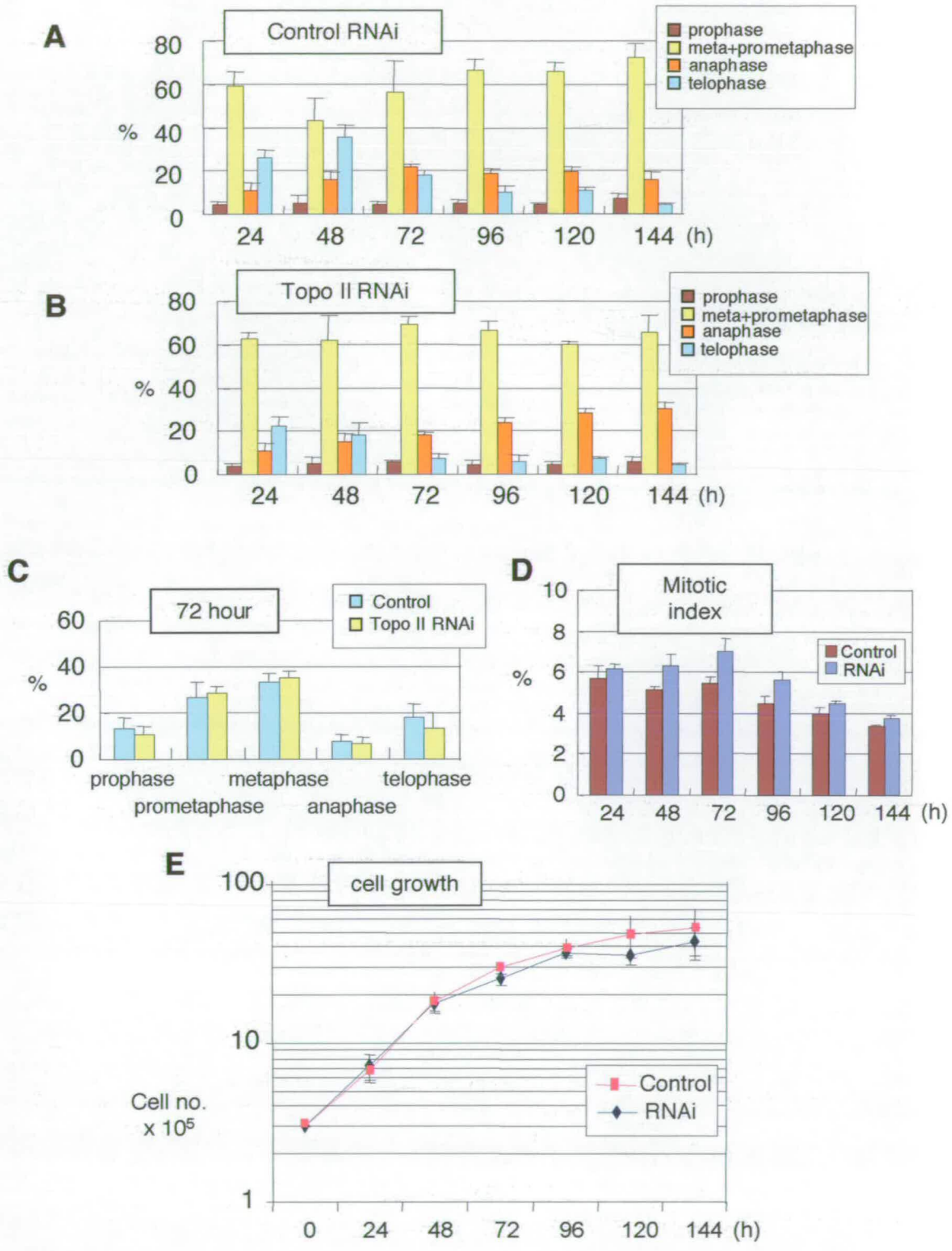


Figure 6.3 Effect of Topo II RNAi on cell growth and mitosis

6.6 Topo II function is required for sister chromatid segregation in *Drosophila*

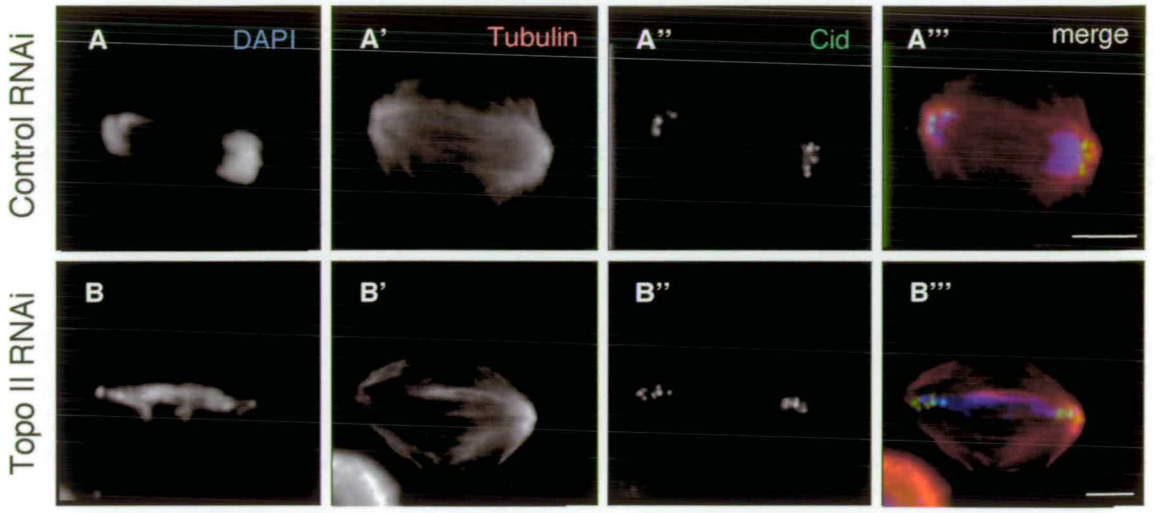
In control cells, the sister chromatids separated normally to the spindle poles at anaphase (Figure 6.4A). However, TopoII-depleted cells in anaphase showed extensive chromosomal bridges (Figure 6.4B). A similar phenotype was observed during the analysis of Topo II mutants in fission and budding yeasts, and inhibitor studies in metazoan cells. The percentage of the anaphase cells with chromosomal bridges increased starting 48 hours after addition of dsRNA, and more than 90% of anaphase and telophase cells showed this phenotype after 72 hours (Figure 6.4C). Despite these massive defects in sister chromatid segregation, the mitotic spindle appeared relatively normal in these cells (Figure 6.4B').

6.7 Topo II is not required for centromere/kinetochore assembly or function

A number of studies have shown that Topo II-alpha is concentrated at centromeres during prometaphase and metaphase in mammalian cells (Christensen et al., 2002; Floridia et al., 2000; Spence et al., 2002; Tavormina et al., 2002), and one study showed that kinetochore structure was apparently abnormal in cells treated with Topo II inhibitors (Rattner et al., 1996). In order to determine the status of kinetochores in Topo II-depleted cells, the cells were stained with anti-Cid/CENP-A antibody. Cid is the *Drosophila* homolog of CENP-A, which is present at the centromere throughout

the cell cycle. The results showed that Cid localized to the centromeres normally even when Topo II levels were significantly decreased.

In control cells, sister kinetochores were observed to migrate toward the spindle poles in a compact line perpendicular to the spindle axis (Figure 6.4A"). In most of the Topo II-depleted cells, the sister kinetochores still separated and moved to the spindle poles in anaphase even though the sister chromatids could not be resolved properly (Figure 6.4B-B'"). However, the sister kinetochores sometimes appeared to be distributed in a cluster whose long axis was parallel to the spindle axis (Figure 6.4B"). This pattern of distribution is reminiscent of the behavior of kinetochores in Topo II mutants of S. pombe (Funabiki et al., 1993). It may reflect a 'tethering' effect, where kinetochores migrate towards the poles until they are halted by trailing chromatin as a consequence of the failure of sister chromatid arms to separate due to unresolved interlocks generated at the end of DNA replication.



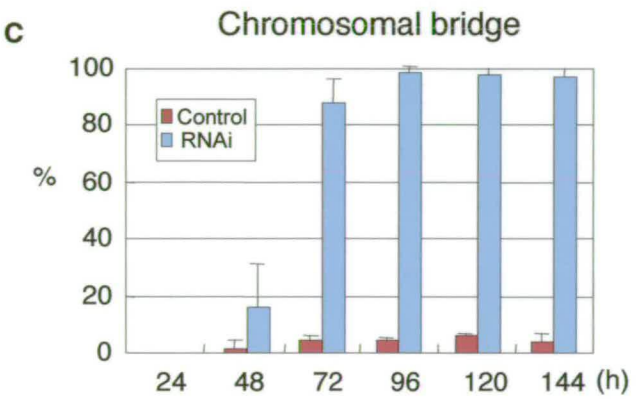


Figure 6.4 Topo II function is required for sister chromatid segregation

Anaphase in Topo II-depleted cells is characterized by the presence of massive chromatin bridges, however sister centromeres usually manage to disjoin and move towards the spindle poles. (A) Control RNAi, normal anaphase cell. (B) Topo II RNAi, anaphase cell with the bulk of the chromatin stretched out between the separating kinetochores. (C) Statistical analysis of the lagging chromosome phenotype. Panels A-B, DAPI staining for DNA. (A'-B'), anti-tubulin shows the mitotic spindle. (A''-B''), staining for Cid/CENP-A shows the position of kinetochores. (A'''-B'''), merged image (DAPI is blue, Tubulin is red, Cid is green). The bars represent 5 micrometers.

6.8 Topo II function is required for normal mitotic chromosome morphology but not histone H3 phosphorylation

One of the unanswered questions about Topo II function is whether the protein has a role in mitotic chromosome structure. The drugs and antibodies used to inhibit Topo II function in previous published studies may not necessarily block the function of Topo II as a structural element. The dsRNAi method could avoid this problem by generating cells where the protein was effectively absent. Many of the chromosomes in Topo II-depleted cells were morphologically abnormal, often having a mass of chromatin without defined sister chromatids (Figure 6.5B).

Phosphorylation of histone H3 on serine[10] has been proposed to correlate directly with chromosome condensation (Bradbury, 1992; Gurley et al., 1978; Wei et al., 1998). However, a previous study in our laboratory showed that there was a poor correlation between chromatin condensation and levels of phosphorylated histone H3 in *Drosophila* chromosomes (Adams et al., 2001c). Analysis of the phosphorylation state of histone H3 in cells depleted of Topo II showed that there were no obvious defects in histone H3 phosphorylation, even in the most abnormal looking chromosomes (Figure 6.5B).

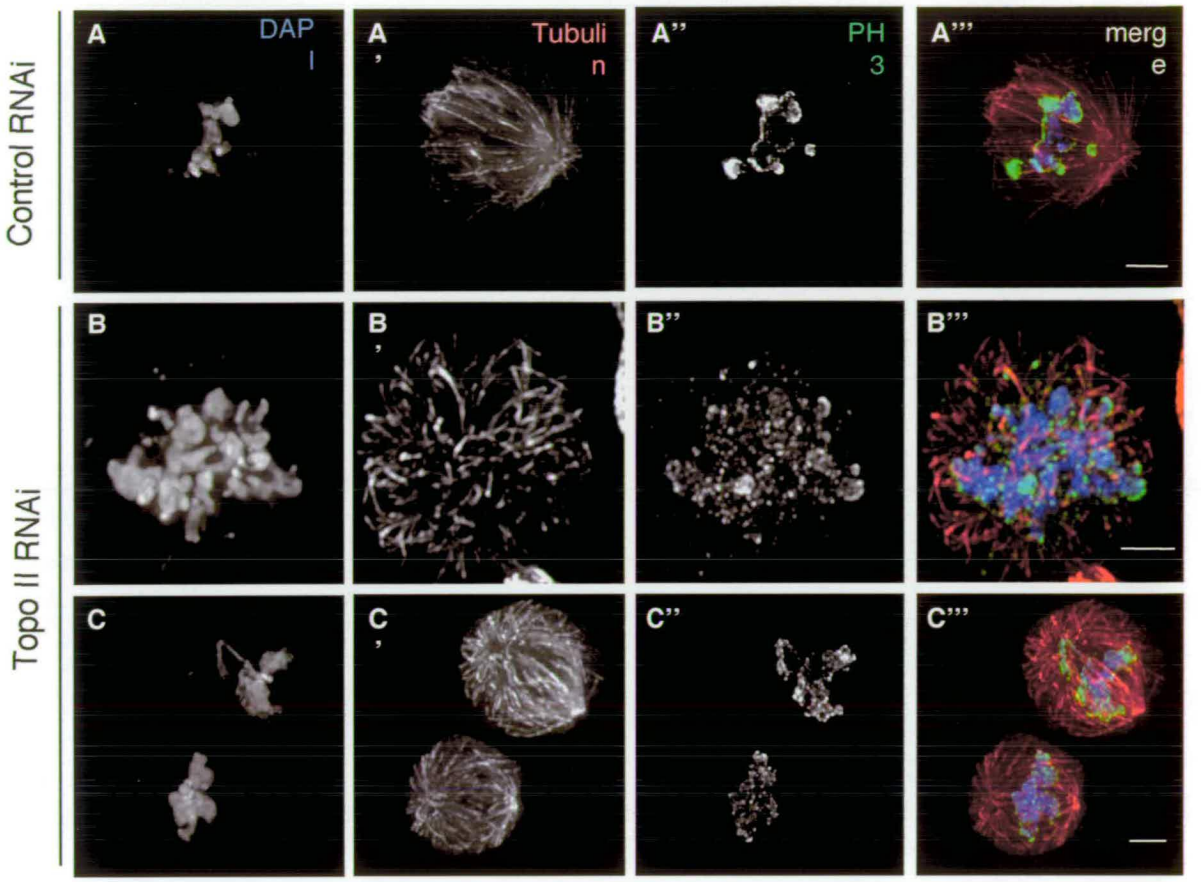


Figure 6.5 Topo II depletion does not affect histone H3 phosphorylation on serine[10]

All images are from cultures at 72 hours after addition of Topo II dsRNA. (A) Control RNAi Metaphase cell. (B) Prometaphase cell with abnormal chromosome morphology viewed parallel to spindle axis. (C) Two metaphase cells, one of which has a highly elongated chromosome arm extending to one spindle pole. Both are viewed nearly perpendicular to the spindle axis. (A-C) DAPI staining for DNA. (A'- C') anti-tubulin shows the mitotic spindle. Panels A'', B'', histone H3 phosphorylated on serine[10] is stained with specific antibody. (A'''- C'''), merged images (DAPI is blue, Tubulin is red, histone H3 is green. The bars represent 5 micrometers.

6.9 The condensin subunit Barren localizes normally in Topo II depleted cells

Topo II has been proposed to associate with Barren, one of the non-SMC condensin subunits (Bhat et al., 1996). Furthermore, in *barren* homozygous null embryos the centromeres disjoin at the metaphase-anaphase transition but sister chromatids remain highly entangled. This phenotype is reminiscent of the yeast Topo II mutants (Funabiki et al., 1993). I therefore considered whether the defects in chromosome morphology observed following depletion of Topo II could be due to effects on condensin targeting or function.

In control cells, Barren was distributed all along the chromosome arms in early mitosis, then appeared to become selectively concentrated at or near centromeres during metaphase (Figure 6.6A). The levels of chromosome associated Barren protein fell significantly during anaphase (Figure 6.6B).

The localization of Barren protein on mitotic chromosomes was normal even in cells with chromosomal bridges (Figure 6.6E). In all of these studies, no significant difference was observed between the levels of Barren staining on mitotic chromosomes in cells exposed to control or Topo II-specific dsRNA.

Figure 6.6 Localization of Barren is normal in Topo II depleted cells

The condensin subunit Barren associates normally with mitotic chromosomes in Topo II-depleted cells. (A, B) Control RNAi, normal metaphase and anaphase cells. (C, D) Topo II RNAi, metaphase cells with a highly elongated chromosome arm extending to one spindle pole. (E) Topo II RNAi, anaphase cell with the bulk of the chromatin stretched out between the separating kinetochores. (A-E) DAPI staining for DNA. (A'-E') Anti-tubulin shows the mitotic spindle. (A"-E") staining for Barren shows the position of the condensin complex. (A"'-E"') Merged images (DAPI is blue, Tubulin is red, Barren is green). The bars represent 5 micrometers.

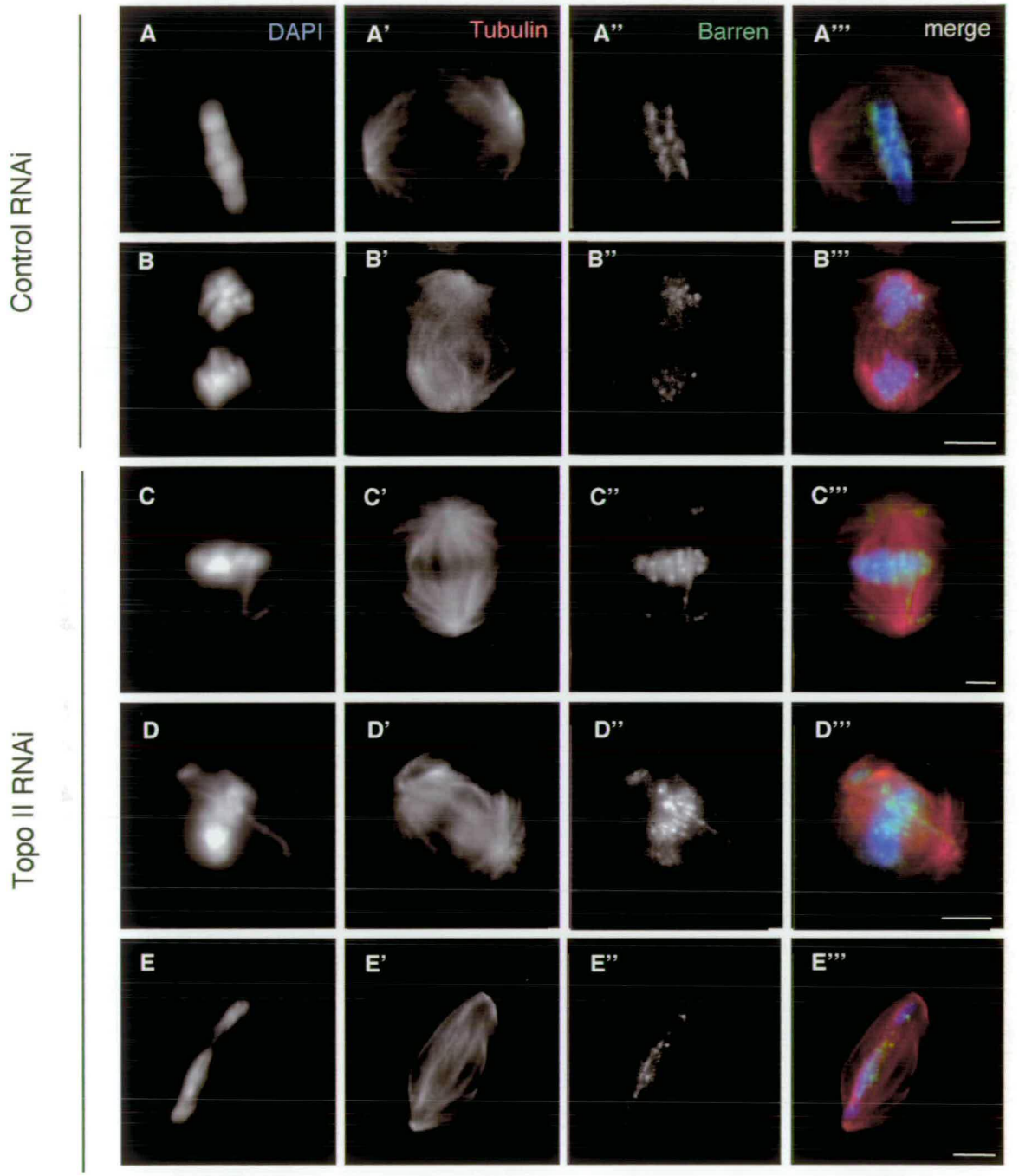


Figure 6.6 Localization of Barren is normal in Topo II depleted cells

6.10 Topo II is not required for the localization of INCENP and Aurora B to chromosomes

It has been shown that the chromosomal passenger proteins INCENP and Aurora B are required for assembly of mitotic chromosomes with a normal morphology (Adams et al., 2001c). The chromosomal factor required for the targeting of INCENP to chromatin remains unknown. Topo II, which like INCENP becomes concentrated in centromeres during prometaphase and metaphase, could be one candidate for performing this function.

In control cells, INCENP localized to centromere at metaphase (Figure 6.7A") and transferred to the central spindle at anaphase (Figure 6.7B"). The analysis of the distribution of INCENP and Aurora B in cells depleted of Topo II revealed no obvious defects. In the majority of cells, INCENP/Aurora B transferred normally to the central spindle at the metaphase/anaphase transition even though sister chromatids did not disjoin normally (Figure 6.7F-F"", Figure 6.8B-B""). However, in some anaphases INCENP localization was aberrant. INCENP distributed both along the segregating chromatids and parallel to the spindle microtubules (Figure 6.7E-E"'). The reason for this is unknown. It could be a result of defective release of sister chromatid cohesion in these cells.

Figure 6.7 localization of INCENP in Topo II depleted cells

The chromosomal passenger protein INCENP is localized normally in Topo II-depleted cells with the exception of a few highly abnormal anaphase cells. (A) Control RNAi, normal prometaphase cell. (B) Control RNAi, normal telophase cell. (C) Topo II RNAi, prometaphase cell with abnormal chromosomal morphology. (D) Topo II RNAi, late prometaphase cell with abnormal chromosomal morphology. (E) Topo II RNAi, abnormal cell with INCENP stretched along the chromosome arms. (F) Topo II RNAi, anaphase cell with massive chromatin bridging, but INCENP located normally on the central spindle.

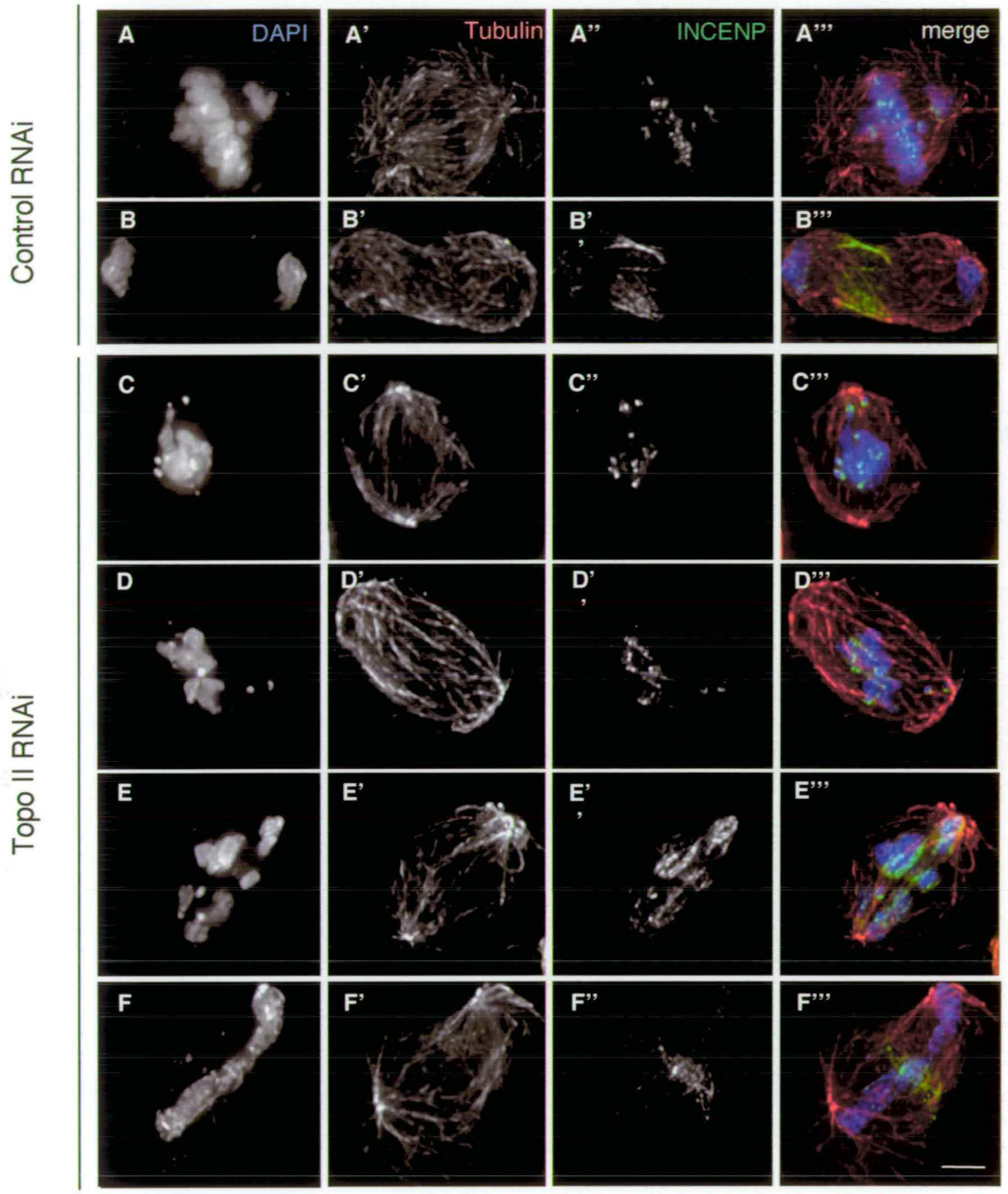


Figure 6.7 localization of INCENP in Topo II depleted cells

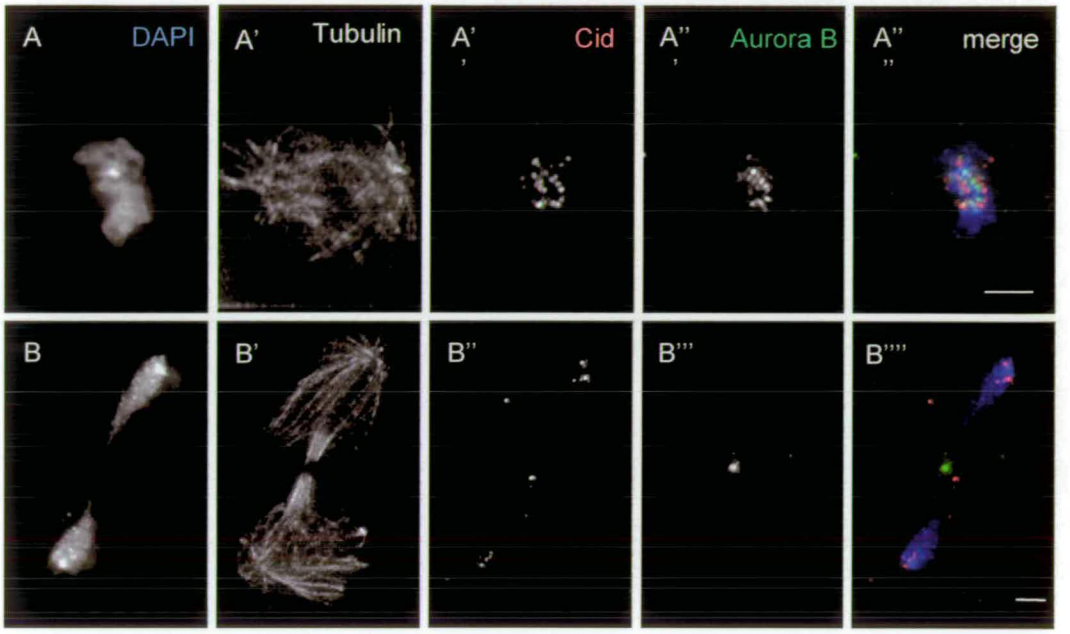


Figure 6.8 Localization of Aurora-B in Topo II depleted cells
The chromosomal passenger protein Aurora-B is localized normally in Topo II depleted cells. (A-A'''') Topo II RNAi, metaphase cell with abnormal chromosome morphology but normal Aurora-B staining at centromeres. (B-B'''') Topo II RNAi, cell in cytokinesis with lagging chromatin trapped in the intercellular bridge chromosome nondisjunction and anaphase bridging. (A-B) DAPI staining for DNA. (A'-B') Anti-tubulin shows the mitotic spindle. (A''-B'') Staining for Cid/CENP-A shows the position of kinetochores. (A'''-B''') Staining for the chromosomal passenger Aurora-B. (A''''-B'''') Merged image. The bar represents 5 micrometers.

6.11 Topo II is required for formation of a compact metaphase plate

Analysis of metaphase cells following Topo II depletion revealed a novel and unexpected result: in many metaphase cells, one or more chromosome arms extended outwards from the compact mass of chromosomes. They were stretched towards the spindle poles (Figure 6.9B, C). This phenotype was first observed at 48 hours, when Topo II levels had begun to fall significantly, and was maximal at 96 hours. Around 25% of metaphases had one or more protruding chromosome arms at 96 hours after Topo II dsRNA-treatment (Figure 6.9D).

In many cases, the protruding chromosome had a V- or J-shaped appearance (Figure 6.9C). This is similar to anaphase chromosomes in which the centromere leads towards the poles and the arms trail backwards. Immunostaining using Cid as centromere marker showed that the precocious movement of the centromere towards the pole could not be the explanation for this phenomenon. In fact, in the great majority of cases, the centromere of the protruding chromosome was localized normally at the metaphase plate (Figure 6.9B", C").

According to the description, the presence of chromosome arms protruding towards the spindle pole cannot be due to centromere activity. However, it was possible that some other specialized structure feature of the chromosome arms might be responsible for this localization. In order to address this question, I performed fluorescence in situ hybridization (FISH) to identify the chromosomal components involved in the protruding arm phenotype.

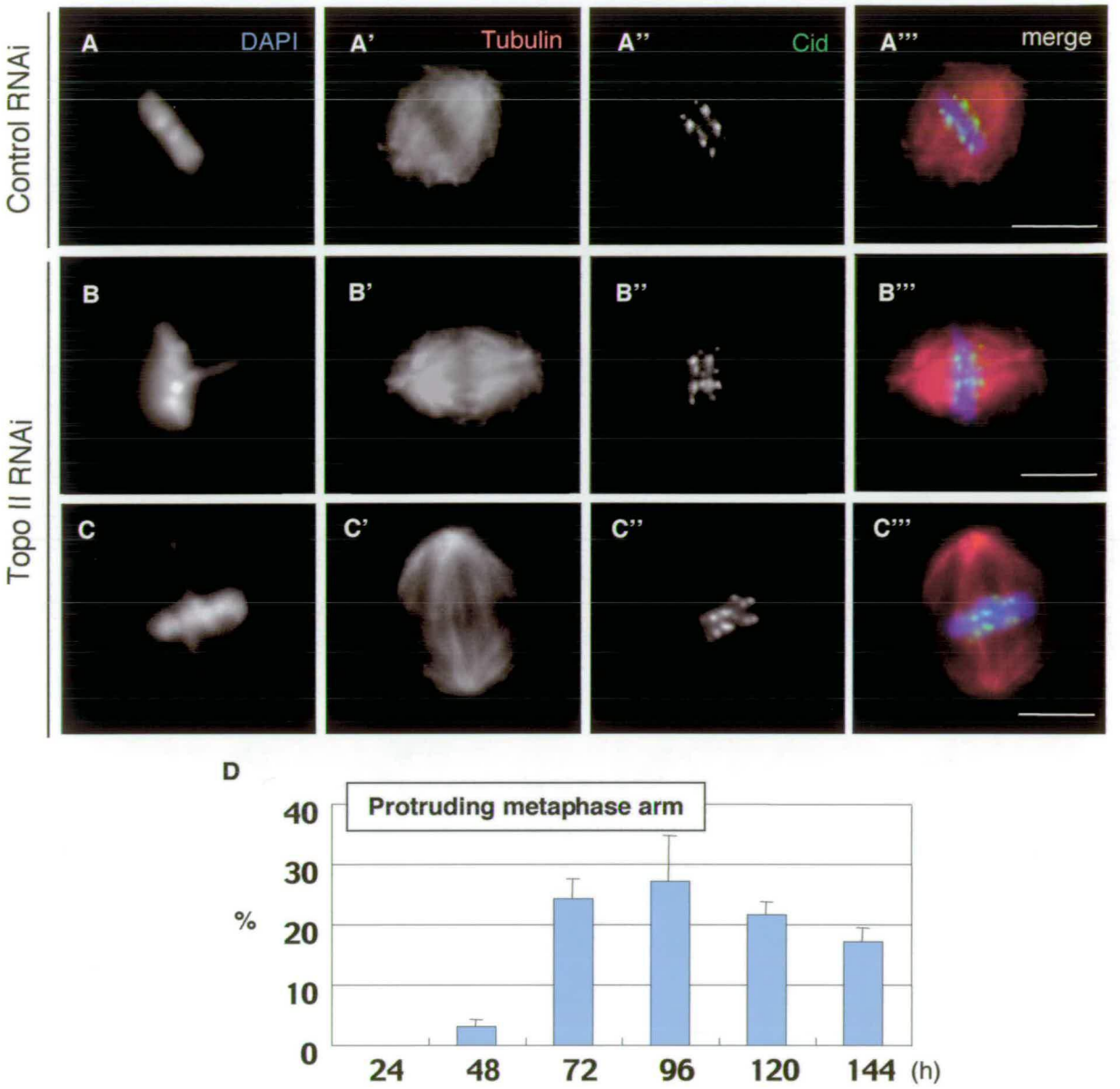


Figure 6.9 Topo II is required for formation of a compact metaphase plate

Many metaphases in Topo II-depleted cells have an unusual phenotype in which one or more chromosome arm(s) becomes highly elongated and stretches towards the spindle pole. (A) Control RNAi, normal metaphase cell. (B) Topo II RNAi, metaphase cell with a highly elongated chromosome arm extending to one spindle pole. (C) Topo II RNAi, metaphase cell with J-shaped chromosome arms extending to spindle poles. (D) Statistical analysis of the protruding arm phenotype. Panels A-C, DAPI staining for DNA. (A'- C') Anti-tubulin shows the mitotic spindle. (A''- C'') Staining for Cid/CENP-A shows the position of kinetochores. (A'''- C''') Merged images (DAPI is blue, Tubulin is red, Cid is green). The bars represent 5 micrometers.

6.12 Identification of protruding chromosome arms in Topo II RNAi metaphase cells

D. melanogaster has four pairs of chromosomes, X/Y, 2, 3, 4 (Figure 6.10). The X and fourth chromosomes are acrocentric. The sizes of the X, 2L, 2R, 3L, 3R are very similar. Chromosome 4 is only about 1/5 of those chromosome arms.

The size of the protruding chromosome arm rules out chromosome 4. As the S2 cell line is female, there is no Y. Therefore, possible candidates of the protruding arms were X, 2L, 2R, 3L, 3R.

Design of probes for FISH

In order to identify the chromosomal arms, BAC clones from X, 2L, 2R, 3L and 3R chromosome arms were used to made probes for a FISH experiment (Figure 6.10).

Labeling of the probes

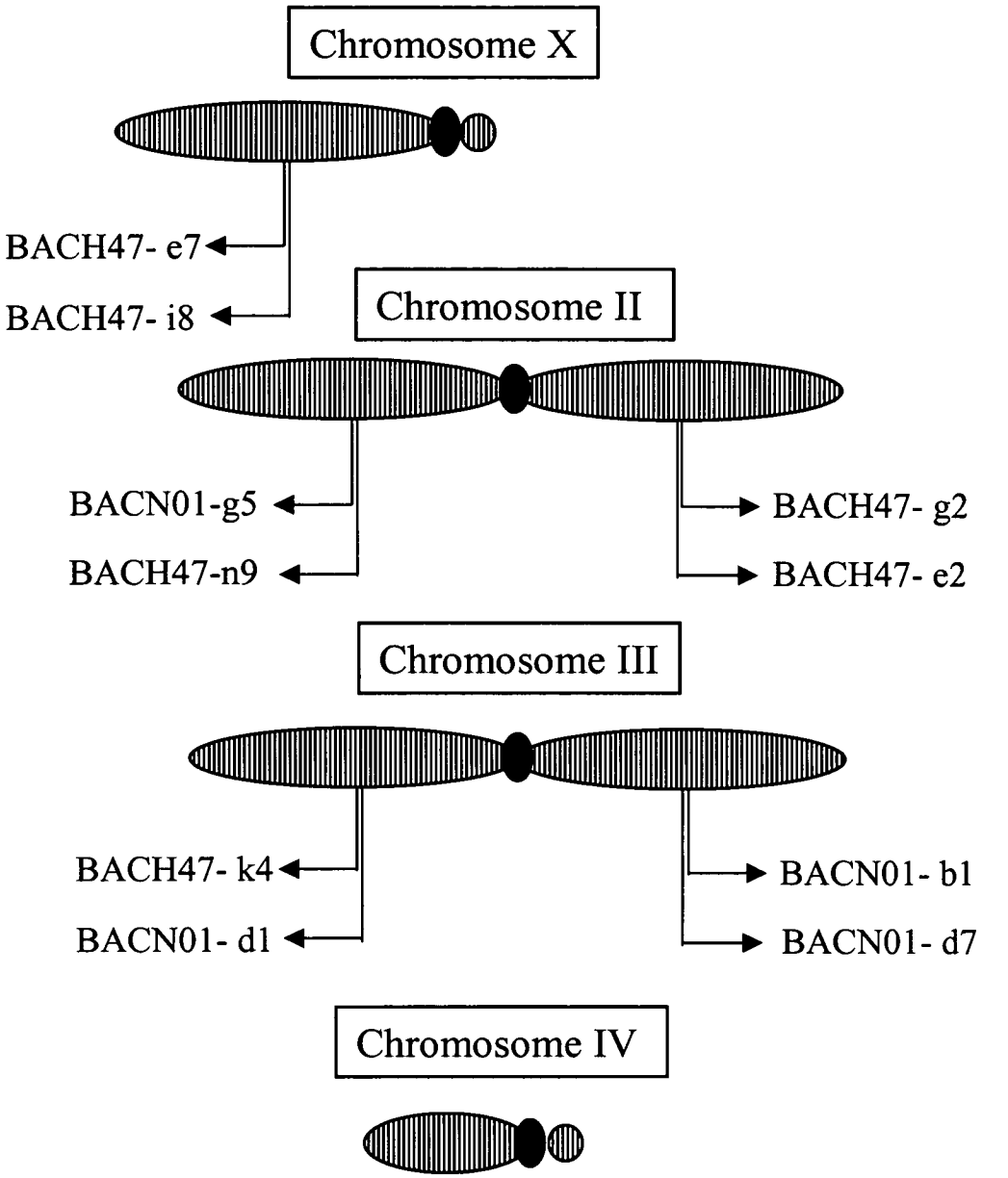
The labeling procedure used employed digoxigenin-labeled nucleotides, with detection by rhodamine-conjugated anti-digoxigenin (Cat. No. 1207750, Roche). The BAC clones are very big in size (around 75-150Kb). In order to increase the efficiency of the labeling, I needed to obtain smaller fragments. Different restriction enzymes (and different combinations of enzymes) were used to digest these clones to reduce their length. However, I did not find any enzyme that could digest these clones to a reasonable range of sizes. Finally, I found that the sizes of the clones could be reduced to an ideal range, from around 500bp to 2kb, after sonication

(Figure 6.11).

The protruding chromosome arms could be X or either arm of chromosome 3

The FISH experiments revealed that the protruding chromosome arm could be the X (39 out of 48 metaphase cells scored), or either arm of chromosome 3 (26 out of 39 metaphase cells scored)(Figure 6.12A, B). Chromosome 2 was never observed to stretch towards the pole (0 out of 22 metaphase cells scored)(Figure 6.12C). There is no common feature shared by these three arms (X, 3L and 3R) that readily explains their association with the spindle pole.

Despite the lack of a centromere close to the spindle pole, the protruding chromosome arm appeared to be under tension. This was most clearly seen when the heterochromatic probes were used in the FISH experiments. The centromeric heterochromatin could be occasionally observed in the protruding arm, and, it was often abnormally elongated (compare signals from the protruding chromosome and its homolog, Figure 6.12D). In the absence of evidence for a kinetochore actively pulling the arm, this is most consistent with the arm being somehow trapped at the pole, and then the combined forces of kinetochore congression to the metaphase plate, chromatin condensation and the "polar wind" (presumably due to chromokinesins) actively trying to push/pull the arm away from the pole.



BACH47-e7 10A-10A	BACH47-n9 30C5-30D	BACH47-g2 50C5-50C12	BACH47-k4 71A-71A	BACN01-b1 90C7-90C10
BACH47-i8 10A-10A	BACN01-g5 29F8-30A	BACH47-e2 50A1-50A5	BACN01-d1 71C-71C	BACN01-d7 91A-91A

Figure 6.10 Design of BAC clones from X, 2L, 2R, 3L and 3R chromosome arms

Schematic representation of positions of BAC clones used as probes for FISH. Two BAC clones were chosen from each chromosomal arm of X, 2L, 2R, 3L, 3R.

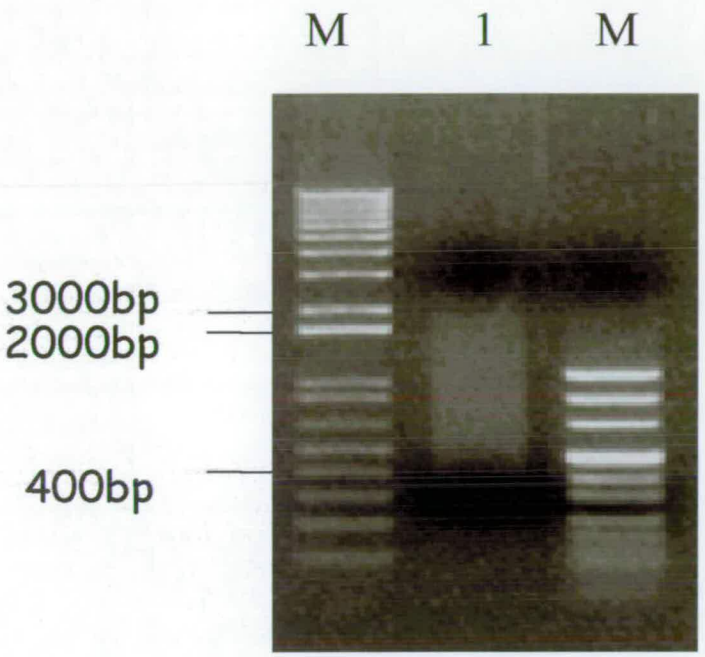


Figure 6.11 Gel electrophoresis of BAC clone DNA after sonication

The purified BAC clone DNA was sonicated to reduce the length before labeling as FISH probes. The length of BAC clone DNA was reduced to sizes that ranged from 400bp to 2000bp.

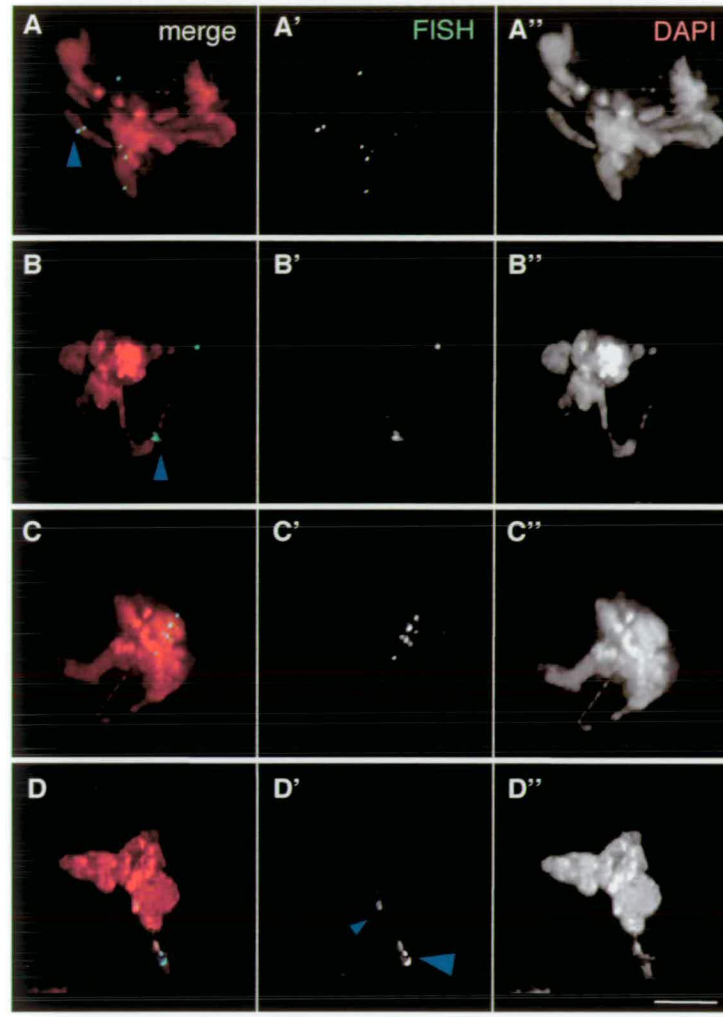


Figure 6.12 Identification of the chromosomal component in the protruding arm phenotype

Analysis by FISH at 72 hours after the addition of Topo II dsRNA using the following probes. (A) Euchromatic chromosome 3 probe (BACH47K04). (B) Euchromatic chromosome X probe (BACH47E07). In A and B, arrowheads point to FISH signal in protruding arm. (C) Euchromatic chromosome 2 probe (BACHN09). (D) Heterochromatic chromosome X probe (359 satellite). Large arrowhead points to extended FISH signal in protruding arm. Small arrowhead points to condensed FISH signal in metaphase plate. In all merged images, the probe is green and the DNA is red. The bar represents 5 micrometers.

Chapter 7

Discussion and Conclusion

The role of DNA topoisomerase II (Topo II) in mitotic chromosome structure has been controversial for well over a decade. Evidence supporting a role for Topo II in chromosome architecture, includes analysis of *cs* mutants in *S. pombe* (Uemura et al., 1987), studies of chromosome condensation *in vitro* in *Xenopus* egg extracts (Adachi et al., 1991), and inhibitor and antibody microinjection studies in *Drosophila* and mammalian cells (Buchenau et al., 1993; Gorbsky, 1994). However, Topo II is not required for the nocodozole-induced condensation of the rDNA locus in *S. cerevisiae* (Lavoie et al., 2002). Drug studies have suggested that Topo II activity is not involved in generating mitotic chromosomes with two morphological distinct chromatids (Andreassen et al., 1997). The evidence from FRAP experiments for a high mobility of Topo II associated with chromosomes still does not argue about whether the enzyme has an architectural role in chromosomes (Christensen et al., 2002). To date, there is no described mutant in *Drosophila* Topo II. Unlike vertebrates, *Drosophila* expresses a single Topo II isoform. I used RNAi to deplete Topo II in *Drosophila* culture cells.

7.1 Topo II is not required to inactivate a G2 checkpoint in *Drosophila* cells

In mammals, entry into mitosis is proposed to be regulated by a Topo-dependent checkpoint that monitors the level of catenation of sister chromatids (Clarke and Gimenez-Abian, 2000; Downes et al., 1994). This checkpoint involves signaling through the ATR kinase following inactivation of Topo II by ICRF-193 (Deming et al., 2001). In this study, the levels of Topo II fell substantially by 48 hours following exposure of the cells to dsRNA and became essentially undetectable after 72 hours. However, there was no significant difference in mitotic index between cultures exposed to control or Topo II-specific dsRNA, suggesting that Topo II depletion did not induce a significant G2 delay or arrest in these cells. This implied that *Drosophila* S2 cells do not have a checkpoint mechanism that couples mitotic entry or progression with Topo II action. There is no substantial increase in any particular phase of mitosis in cells following Topo II depletion (a slight increase in the fraction of anaphase cells). Cells progress through mitosis with normal kinetics.

More than 90% of anaphase and telophase cells have chromosomal bridges by 72 hours following exposure to Topo II-specific dsRNA, indicating that Topo II is essential for normal chromosome segregation. This result is consistent with studies in yeast. The absence of Topo II function does not block mitotic progression, but produces a 'cut' phenotype (cells attempt to cleave through a mass of lagging chromatin) (Holm et al., 1985; Uemura et al., 1986).

7.2 Topo II is not required to assemble functional kinetochores

Inhibition of Topo II activity using drugs was previously shown to cause abnormalities in kinetochore structure (Rattner et al., 1996). This suggested that Topo II might have an essential role in centromere/kinetochore structure and function (Bachant et al., 2002). However, our results showed that there was no key function for Topo II in kinetochore assembly or function. We found that Cid/CENP-A (centromere-specific histone H3 subtype) targeted to chromosomes normally when Topo II was depleted. Kinetochores interacted with microtubules and migrated towards the spindle poles in anaphase in Topo II-depleted cells (with chromosomal bridges).

In some Topo II-depleted cells, the centromeres were distributed in a cluster whose long axis was parallel to the spindle axis. This might be result from the high levels of residual catenation of sister chromatids. It is possible that the centromeres moved as far towards the poles as they could before they were stopped by the tangled chromatin trailing behind them.

7.3 Topo II is required for complete chromatin condensation and chromosome morphology

Chromosome morphology was abnormal in Topo II-depleted cells. In some metaphase cells, the chromosomes appeared as a mass of condensed chromatin that lacked well-defined sister chromatids. This could be the direct consequence of the loss of Topo II activity, or due to interference with targeting of other factors that are essential for mitotic chromosome morphology. Such factors might include the chromosomal passenger proteins INCENP and Aurora B or the condensin complex (Hirano and Hirano, 2002). Depletion of INCENP/Aurora B by using RNAi resulted in production of chromosomes with an irregular morphology (Adams et al., 2001c). Aurora B has also been shown to be required for targeting the condensin subunit barren to chromosomes (Giet and Glover, 2001). RNAi depletion of condensin subunits impairs the early stages of chromosome condensation in *C. elegans* embryos, although condensation appeared to be normal by metaphase (Hagstrom et al., 2002). Our results showed that Barren, INCENP and Aurora B target normally to mitotic chromosomes in Topo II-depleted cells. Thus the observed abnormalities in chromosome morphology are unlikely to be due to indirect effects of the RNAi on condensin or chromosomal passenger function. However, in some anaphases, INCENP localization was aberrant. INCENP was distributed both along the segregating chromatids and parallel to the spindle microtubules. This could be a result of defective release of sister chromatid cohesion in these cells. There is normally a strong correlation between sister chromatid pairing and INCENP binding: when chromatid cohesion is released, INCENP is no longer bound (Vagnarelli and Earnshaw, 2001).

In eukaryotes, phosphorylation of histone H3 at serine[10] is a hallmark of mitotic prophase when chromosome condensation is started (Hendzel et al., 1997). However, the state of phosphorylation of histone H3 is indistinguishable in control and Topo II-depleted cells, even though the mitotic chromosomes appeared to be less condensed in Topo II-depleted cells than in control cells.

7.4 Topo II is required for a compact metaphase alignment of the chromosomes

The most interesting phenotype observed in this study was that the chromosomes failed to form a compact metaphase plate in about 25% of Topo II-depleted metaphase cells. These cells had one or more chromosome arms protruding a substantial distance from the plate. These protruding arms stretched along the long axis of the spindle towards one of the spindle poles. In many case, the protruding chromosome had a V- or J-shaped appearance, similar to anaphase chromatids in which the centromere leads towards the poles and the arms trail backwards.

However, this was not a pseudo-anaphase configuration as, in most cases, the centromeres were clustered normally at the metaphase plate, and no Cid staining was observed on the protruding arm. Thus the protruding chromosomes were not due to abnormal kinetochore-based movement towards the spindle pole.

Analysis by FISH revealed that the chromosomal component involved in this

phenotype is not completely random. The arms of chromosomes 3 and X could be found near the poles of metaphase cells. Chromosome 2 was never observed to stretch towards the pole.

7.5 Possible models to explain the origin of the protruding arms in Topo II-depleted cells

The protruding chromosome arms could be chromosomal regions that exhibit a persistent failure in condensation. If this morphology were due to a failure in condensation, the arm would be expected to be oriented randomly or extruded laterally out of the spindle because of the action of the "polar wind" (Carpenter, 1991). When chromosome arms are severed with a laser, they are rapidly ejected from the vicinity of the spindle pole (Rieder et al., 1986). This is due to the action of plus-end-directed chromokinesin motors associated with the chromatin as well as collisions with elongating microtubules (Funabiki and Murray, 2000). However, our results showed that the protruding arms extended along the spindle from the metaphase plate to the vicinity of one spindle pole.

A second possibility is that the protruding arms move towards the pole because they in fact have active kinetochores that lack detectable Cid/CENP-A or concentrations of Barren. However, the protruding chromosomes derive from three different chromosome arms and have a range of morphologies. This means that were they to exist, the putative kinetochores would have to occupy a number of different positions on the long arms of chromosomes X and 3. It is unlikely that neocentromeres could be

formed at many different positions in such short-term cultures.

A third possibility is that the arms protrude from the metaphase plate towards one pole because they are physically trapped there and cannot retract back to the metaphase plate. This could explain the morphology of the J-shaped chromosomes if the point of entrapment was close to the pole: chromokinesins would try to move the short and long arms of the J away from the pole. This is consistent with the highly elongated appearance of these protruding arms, which would be stretched as they were pulled away from the pole by the associated motors (Funabiki and Murray, 2000).

Topo II is a major component of mitotic chromosomes and is generally considered to be a chromatin protein. However, Topo IIα can also be detected as a salt-stable component of centrosomes in human cell lines (Barthelmes et al., 2000). Our results suggested that this centrosomal Topo II could have a role in enabling the arms of metaphase chromosomes to release from the vicinity of the centrosomes and assume their condensed structure at the metaphase plate.

7.6 Summary

In this part of the thesis, we used double stranded RNA interference to analyze the roles of Topo II in *Drosophila* cells. The results revealed that the enzyme is essential in sister chromatid separation at anaphase. Topo II is not essential for assembly of a functional kinetochore, but has a role in establishment of a normal mitotic

chromosome morphology. Most surprisingly, in the absence of Topo II, one or more chromosome arms were frequently trapped in the vicinity of the spindle pole during metaphase.

Chapter 8

Materials and Methods

Antibodies

The antibodies used in this thesis are listed in the following table.

Antibody	Concentration	Reference/Sourcce
Rabbit anti-DmINCENP R801-1	1:500	Adams et al., 2001c
Mouse anti-α tubulin B512	1:2000	Sigma
Rabbit anti-phosphorylated histone3	1:500	Upstate Biotechnology
Chicken anti-Cid	1:1000	Blower and Karpen, 2001
Rabbit anti-DmAurora B R963	1:500	Adams et al., 2001c
Rabbit anti-Barren	1:1000	Bhat et al., 1996
Mouse 22C10	1:200	Gift from Jarman Lab
Anti-rabbit conjugated-Texas Red	1:200	Jackson ImmunoResearch
Anti-rabbit conjugated-FITC	1:200	Jackson ImmunoResearch
Anti-mouse conjugated-Texas Red	1:200	Jackson ImmunoResearch
Anti-mouse conjugated-FITC	1:200	Jackson ImmunoResearch
Anti-chicken conjugated-Cy5	1:200	Jackson ImmunoResearch
Anti-rabbit IgG-HRP	1:10,000	Jackson ImmunoResearch
Anti-mouse IgG-HRP	1:10,000	Jackson ImmunoResearch
Anti-mouse beta-galactosidase	1:200	Promega
Anti-rabbit beta-galactosidase	1:10,000	Cappel

Primers

The primers used in this work are listed in the following table.

Name	Sequence
Incenp1	TAAAGGCATGTTATCAAATGCTGACGCGCG
Incenp2	AGTATCCTGGCCATTTTAAAG
Incenp3	AGCAGCAGAAACAGCTGAGCA
Incenp4	CTCGAAGATCCGCCCGTTGCA
Incenp5	AGGCGTTCGAGAATGCGGCAA
Incenp6	CAATTCCACGTCCACAAAAAC
Incenp7	CACGAAGACGACTCGACAGAC
Incenp8-r	AGCTGGATATTAGTGTCAGAGTCGCAGTGA
Incenp1-1	TCGCAATTGCCGCCATTTGAA
Incenp5-1	ATGCGCGTGGAGGCGTTCGAG
TopoII-RNAi5'	TAATACGACTCACTATAGGGATGGAGAACGGAAACAAGGCC
TopoII-RNAi3'	TAATACGACTCACTATAGGGTTTGTTGCCGTTTAAGAAGAC

8.1 Genetic procedures

Drosophila husbandry

Fly stocks were maintained at 18°C or room temperature on standard cornmeal-agar medium ('Dundee Food'-agar glucose, brewers yeast, maize, live yeast, nipagin, propionic acid, prepared by Swann media kitchen staff).

Ethylmethane Sulfate (EMS) mutagenesis

All experiments using this reagent were performed in the fume hood. Everything that came in contact with EMS was neutralized in denaturing solution (1 M NaOH, 5% mercaptoacetic acid) for 24 hours before disposal.

Before carry out the EMS experiment, the denaturing solution was prepared freshly. Disks of 3 MM Whatman paper were cut to fit the bottom of an empty fly bottle. 1 ml of EMS/sucrose solution (30 mM sucrose, 30mM EMS) was added to the paper disc in the bottle. Young males (3-5 days old) were kept in an empty bottle to starve for six hours before transferring to the EMS containing bottles. The males were left to feed on EMS in the fume hood overnight with the light on. The following mornings, the males were removed and left to rest for 24 hours before mating. Males should only be left to mate for 4-5 days maximum and then removed because the mutagen can affect the gonial stem cells and males have to be discarded before these have the opportunity to become mature sperm.

8.2 Genomic DNA extraction from adult flies

25 adult flies were collected and frozen in 200 µl of lysis buffer (100 mM Tris-HCl pH9, 100 mM EDTA, 1% SDS) at -20°C overnight, then homogenised using a hand-held homogeniser. A 200 µl lysis buffer was added to the homogenised solution and was then incubated at 70°C for 30 minutes, then 150 µl of 8 M potassium acetate pre-cooled on ice were added. The mix was incubated on ice for 20 minutes, then centrifuged twice at 13,000 rpm, 15 minutes each, at 4°C to obtain a clear supernatant. 0.9 volumes of cold isopropanol was added to the supernatant and the DNA was then precipitated for at least 1 hour at -20°C. The DNA was pelleted at 13,000 rpm for 15 minutes at 4°C, washed in 70% ethanol, then air dried. The genomic DNA was resuspended in 50 µl TE. The DNA was extracted once with an equal volume of phenol-chloroform at 13,000 rpm for 5 minutes and once with chloroform, then precipitated in 2.5 volumes cold 100% ethanol and 1/10 volume 3 M sodium acetate for at least 1 hour at -20°C. The DNA was pelleted at 13,000 rpm at 4°C for 15 minutes, washed in 70% ethanol and air dried, then resuspended in 100 µl TE.

8.3 Preparation of DNA from a single embryo for PCR

Single embryos of the desired genotype were collected in 0.5 ml tubes (1 per tube). The embryos were homogenized in 10 µl of Gloor-Engel's extraction buffer (10 mM Tris pH 8.2, 1 mM EDTA, 25 mM NaCl, 200 µg/ml proteinase K freshly diluted from a frozen 20 mg/ml stock) by using a pipette tip. The homogenate was incubated at 37°C for 30 minutes, moved to 95°C for 2 minutes, then stored at 4°C.

8.4 Agarose gel electrophoresis

Unless otherwise stated in the text, all agarose gels were made up of 1% agarose with 0.3 μg/ml ethidium bromide in 1xTAE buffer (40 mM Tris-acetate, 1mM EDTA). The gels were also run in 1xTAE buffer. DNA bands were revealed under UV light.

8.5 Preparation of protein extracts from embryos

Embryos were collected over a fixed period on grape-juice agar plates with yeast paste smeared on top. A paintbrush and water was used to wash out the embryos into a sieve. The embryos were washed under tap water to remove the yeast.

For dechorionation, the sieve containing the embryos was placed in a beaker containing 50% hypochlorite bleach for 4 minutes. After this treatment, the embryos were fully rinsed in tap water.

Embryos were placed in a fresh 0.5 ml microcentrifuge tube. 50 μl of 1X SDS-PAGE sample buffer was added and the embryos were homogenized for 5 seconds three times with a sonicator. After homogenization, the mixture was placed at 100°C for 5 minutes. A hole was made in the tube to prevent pressure causing the cap to burst open. The heated sample was spun at 13,000 rpm for 3 minutes at 4°C. The supernatant was removed to a fresh tube. The sample was ready for loading on an SDS-PAGE gel or stored at -20°C.

Isolation of homozygous embryos

The balancer chromosome CyO-Kr-GFP was used to distinguish homozygous embryos. *Kruppel* (Kr) is a gap gene expressed in the developing embryo (Knipple et al., 1985). GFP is expressed under the control of the *Kruppel* promotor. The GFP signal appears in the middle of the 10 hour-old embryos (Casso et al., 2000). Embryos from strains carrying balancer CyO-Kr-GFP were collected over a fix of period. The embryos were aged of 12 hours, then dechorionated. The homozygous embryos (GFP-negative) were selected using a dissecting microscope with a fluorescence illuminator that enabled visualization of the GFP.

8.6 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The apparatus used for electrophoresis of protein samples was Hoefer Scientific Instruments 'Vertical Slab Gel Unit' rigs.

Two glass plates were separated by 2 spacers, one at either end, clamped together, then set up onto the apparatus. 1 ml of the resolving (Low) acrylamide gel was poured in between the glass plates from the top and allowed to polymerize for 3 minutes, then the rest of the resolving gel was poured. After pouring the resolving gels, 1 ml of butanol saturated with dH_2O was layered over the top to prevent oxidation and to help form an even interface between the resolving and stacking gels. Resolving gels were allowed to polymerize for 20 minutes at room temperature. The butanol was poured off, then washed with dH_2O, and the stacking gel poured on top.

A plastic comb was immediately inserted into the top of the stacking gel to form the wells, and the gel was allowed to polymerize for 20 minutes at room temperature.

After polymerization, the comb was removed and the wells were washed with 1X running buffer (25 mM Trizma base, 250 mM glycine, 0.1% SDS). Protein samples were boiled for 5 minutes and loaded into the wells. 1X running buffer was poured into the upper and lower buffer chambers. Gels were run at 5 mA for 16 hours at room temperature.

8.7 Transfer of SDS-PAGE gels

Proteins electrophoresed in SDS-PAGE gels were transferred to nitrocellulose membranes in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol and 0.0375% SDS) using a Bio-Rad transfer apparatus according to the manufacturers instruction. Electro-transfer was performed at 4°C, 400 mA for 5 hours. After transfer, the membrane was washed with dH_2O to remove methanol which may interfere with the subsequent Ponceau-S (Sigma) staining. The membrane was stained with Ponceau-S (0.2% Ponceau-S in 3% TCA) for 5 minutes at room temperature with shaking. The Ponceau-S was rinsed off with dH_2O to visualize the proteins and the position of the molecular weight markers on the filter was marked with a needle. The membrane was then ready for immunoblotting or stored at 4°C.

8.8 Immunoblotting and ECL

The membrane was blocked for 1 hour in 5% dried skimmed milk in PBS-0.1%Tween-20 at room temperature with shaking.

Primary antibodies were incubated with the membrane in PBS-0.1%Tween20/2.5% milk for 1 hour at room temperature or overnight at 4°C. The membrane was washed 4 x 10 minutes with PBS-0.1%Tween20/2.5% milk. The appropriate HRP-conjugated secondary antibodies were used at a concentration of 1:10,000 diluted in PBS-0.1%Tween20/2.5% milk, then incubated with the membrane for 1 hour at room temperature. The membrane was washed 4 x 10 minutes with PBS-0.1%Tween-20/2.5% milk, then rinsed with PBS-0.1%Tween20.

To develop the blot, equal volumes of Enhanced Chemiluminescence (ECL, Amersham) reagents 1 and 2 were mixed, then poured over the membrane which was placed on cling-film. The mixture was allowed to set for 1 minute and was then removed by covering with 3MM Whatman filter paper. The membrane was then wrapped up with Saran wrap and placed in an autoradiography cassette. The mounted membrane was then exposed to Kodak film in a dark room for a varying exposure times (usually 10 seconds, 30 seconds, 1 minute), in order to detect any signal. Kodak films were developed in a Konica SRX-101A developer.

8.9 Immunostaining of embryos

Embryos were prepared as described in section 10.5 as far as the dechorionation stage.

Fixing the embryos

Taxol treatment (option)

If the embryos were to be stained for tubulin, taxol treatment is necessary before fixation. 1 ml of buffer B (5 μM taxol, 10 mM K phosphate buffer (1 M K_2HPO_4, 1 M KH_2PO_4 pH 6.8), 45 mM KCl, 45mM NaCl) and 5 ml of heptane were added to a glass vial containing the embryos and shaken for 30 seconds.

Fixation

The aqueous layer (containing the taxol) was quickly removed and 3 ml of 4% paraformaldehyde was then added for fixation. Fixation was carried out by gentle cyclic inversion over 30 minutes to 1 hour.

Devitellinisation of embryos

The aqueous layer was completely removed as was most of the heptane. 3ml of fresh heptane and 3ml of methanol were added to the vial, which was shaken vigorously for 1 minute. The successfully devitellinised embryos will sink to the bottom of the vial. The embryos which have not been devitellinised will remain at the interface. The heptane was aspirated (with the undevitellinised embryos) and then the methanol. Fresh methanol was added to the vial and left for 10 minutes. The embryos were then rehydrated by washing in PBS/0.1% TWEEN 20 twice. They were then ready for the antibody staining procedure or could be stored for up to a week at 4 °C.

Antibody staining of embryos

Blocking

The embryos were incubated in 10% FBS/PBS/0.1% TWEEN-20 for 30 minutes. This step prevents the antibody from non-specific interaction with reagents used in the preparatory steps.

Antibody incubation

The embryos were incubated in the primary antibodies diluted in PBS/0.1% TWEEN 20 to their working concentration for overnight at 4℃. After the incubation, the embryos were washed three times in PBS/0.1% TWEEN 20 for at least 10 minutes.

The secondary antibodies were likewise diluted to their working concentration in PBS/0.1% TWEEN 20 and incubated for 2 hours at room temperature. The embryos were washed with PBS/0.1% TWEEN 20 to remove unbound secondary antibody.

DNA staining and mounting the embryos

After the embryos have been rinsed in PBS, they were incubated in 1 μg/ ml DAPI for 5 minutes, rinsed in PBS and transferred to a drop of mounting medium (Vectashield H-100, Vector Laboratories) with the help of a fine, slightly wet paintbrush. The embryos were covered with a 20x20mm coverslip and the slides sealed with nail vanish to prevent drying out.

8.10 Immunostaining of S2 cells

Cells were transferred onto sterile poly-Lysine treated slides and left to attach for 20

minutes. Slides were centrifuged for 15 minutes at 4000 rpm before fixation. Cells were fixed in 4% paraformaldehyde in cytoskeleton buffer (CB: 1.1 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 137 mM NaCl, 5 mM KCl, 2 mM MgCl, 2 mM EGTA, 5 mM Pipes, 5.5 mM glucose, pH 6.1) for 10 minutes at room temperature. They were permeabilized in 0.1% Triton X-100 in CB ,and then rinsed in PBS. Cells were blocked for 30 minutes at room temperature in PBS+10% FBS.

Primary antibody incubation was done in PBS+1% FBS for 1 hour at 37°C, followed by 4 x 10 minute washes in PBS at room temperature. Secondary antibody incubation was done in the same way as primary antibody incubation. DNA was counterstained with 0.1 μg/ml DAPI for 5 minutes at room temperature and rinsed with PBS. Slides were mounted in Vectashield mounting medium (VECTRA) and sealed using nail varnish. Rabbit polyclonal anti-DmINCENP Rb801-1 (Adams et al., 2001) was diluted 1:500 for use in immunofluorescence. The anti- α -tubulin antibody used in the immuofluorescence was mouse mAb B512, used at 1:2000 (Sigma-Aldrich).

8.11 Fluorescence *in situ* hybridization

Preparation of probes from BAC clones

Drosophila euchromatic BAC clones were obtained from the MRC UK HGMP RESOURCE CENTER, Cambridge (BACH47E07, 10A-10A –X chromosome; BACHN09, 30C5-30D –chromosome arm 2L; BACH47E02, 50A1-50A5 –chromosome arm 2R; BACH47K04, 71A-71A –chromosome arm 3L;

BACN01B01, 90C7-90C10 –chromosome arm 3R. BACs were reduced to a ~200 bp length by sonication. Probes were prepared from the sonicated DNA using DIG-high Prime DNA Labeling and Detection Starter Kit II (Roche). After removing excess nucleotides (ProbeQuant G-50 Micro Columns, Amersham), the quality and quantity of each probe was determined according to the protocol of the kit.

Preparation of slides

$1x10^6$ of RNAi-treated cells were transferred to poly-lysine slides and left for 10 minutes at room temperature. Cells were fixed in 4% paraformaldehyde for 5 minutes at room temperature then in methanol-acetic acid (1:3) at –20°C for 20 minutes, air dried and then aged for 2 days.

Hybridization

300 µg of each Digoxigenin-dUTP labeled probe was ethanol–precipitated with 20 µg human cot-1 DNA (sonicated to 200 bp length, Roche) and resuspended in 40 µl hybridization mix per slide (50% deionized formamide, 2 X SSC, 5% dextran sulphate). Probes were denatured at 85°C water bath for 10 minutes. Aged slides were rehydrated in 2 X SSC and digested with 100 µg /ml RNAse A for 1 hour at 37°C, then dehydrated for 5 minutes each in 70% and 100% ethanol. After air drying, the slides were denatured for 5 minutes at 80°C in 50% deionized formamide, 2 X SSC, washed for 5 minutes on ice in 2 X SSC, and dehydrated for 5 minutes each at 0°C in 70%, 95% and 100% ethanol. After air drying, the denatured probes were applied to the slides, covered with coverslips and slides were incubated in a humid box at 37°C overnight. After hybridization, the slides were washed with 2 x 5minutes at 42°C in 50% deionized formamide, 2 X SSC and 5 minutes in 50%deionized

formamide, 0.1 X SSC, then 5minutes each in 2 X SSC, PBS at room temperature. The slides were blocked for 1 hour in 10% FBS in PBS, then rinsed with PBS and incubated in rhodamine-conjugated sheep-anti-digoxigenin (used at 1:200, Roche) for 1 hour at 37°C. The slides were washed 3 x 10minutes in PBS and stained for 5 minutes with DAPI and washed for 10 minutes in PBS. Slides were mounted in Vectashield mounting medium (VECTRA) and sealed using nail polish.

8.12 Large scale preparation of plasmid DNA

Plasmid DNA was prepared using the QIAfilter midiprep kit (Qiagen), following the manufacturer's instructions. DNA was extracted from 100 ml of *E. coli* culture grown to stationary phase, eluted in 500 µl of distilled water and stored at -20°C.

8.13 Amplification of DNA by the Polymerase Chain Reaction

Specific regions of DNA were amplified using the polymerase chain reaction (PCR). Template DNA was either plasmid DNA or genomic DNA using specifically designed primers manufactured commercially. A typical PCR reaction using plasmid as template was as follows:

10x Polymerase Buffer ($MgCl_2$ plus*)	5µl
10mM dNTPs (dATP, dCTP, dTTP, dGTP)	5µl
Oligonucleotide primer 1 (10 pmol/µl)	1µl
Oligonucleotide primer 2 (10 pmol/µl)	1µl

Template DNA (10ng/μl)	1μl
DNA polymerase (2U/μl)	0.5μl
Sterile distilled water	36.5μl

* $MgCl_2$ concentrations were adjusted as required to optimize reaction conditions.

All PCR were carried out in a Biometra thermalcycle reactor or a DNA Engine Gradient Cycler (Genetic Research Instrumentation Ltd) programmed according to the length of the desired product and the annealing temperature of the oligonucleotide primers being used. A typical cycling program is shown below:

30 cycles of step1-step3:

Step 1	Denaturation	94°C for 20 seconds
Step2	Primer Anneaaling	40-65 °C for 1 minutes
Step 3	Extension	72°C for 1 minutes#
Step 3	Final Extension	72°C for 10 minutes

This time was extended by one minute for every kb over 1kb of length of the desired product.

8.14 Purification of PCR Products

DNA fragments generated by PCR were purified to remove oligonucleotide primers, unincorporated nucleotides, polymerases and salts using the QIAquick PCR purification kit (Qiagen), following the manufacturer's guidelines. Purified DNA was eluted in 30μl of distilled water and stored at -20°C.

8.15 DNA Sequencing

Genomic DNA to be sequenced was firstly amplified using PCR with suitable primers and the resulting DNA fragment was purified using the QIAquick PCR purification kit. Sequencing reactions were then performed using a dRhodamine terminator cycle sequencing kit (Perkin Elmer) in a Hot Lid reactor. A reaction mix was as follows:

Template DNA	100 ng of PCR product
Terminator mix	4 µl
Primer	1.6 pmol
Distilled water	make-up volume to 10 µl

Twenty-five cycles as described below were performed:

Step 1: 96°C for 30 seconds

Step 2: 50°C for 15 seconds

Step 3: 60°C for 4 minutes

10µl of distilled was then added into the reaction mix.

Samples were run in department on an ABI PRISM 377 DNA Sequencer (ICMB sequencing center) and the resulting DNA sequence studied using the computer program Gene Jockey II.

8.16 Double strand RNA interference

Preparation of dsRNA

Two fragments from the 5' end of DmTopo II fused to the T7 RNA polymerase promoter were used as PCR primers. The EST clone LD24716 was used as a PCR

reaction template. The PCR products were cleaned (about 700-bp) by using a MoBio UltraClean PCR Clean-up DNA Purification Kit (cat. 12500-100, from CAMBIO). This template was then used for the transcription reaction. Ambion MEGAscript T7 kit (cat. 1334) was used to synthesize double strand RNA. After the transcription reaction, the RNA was precipitated with LiCl. The pellet was washed twice in 70% ethanol, then resuspended in 100 µl nuclease free dH_2O. The tube containing the RNA was placed at 65°C for 30 minutes to denature the secondary structure of RNA, then placed in beaker containing 200ml of 65°C H_2O and left on bench to cool to room temperature (usually about 1 and half hours). To test if the double strand RNA was annealed properly, 1µl of the double-stranded RNA was run on regular agarose gel. If the double-stranded RNA was annealed properly, it ran like double-stranded DNA and was seen as a clean band.

RNA interference

Experiments were performed in six-well plates. Cells were diluted to a concentration of 10^6/ml by adding DES Serum-free expression medium (Invitrogen cat. No. Q510-01). 1 ml of cells was put in each well. 30 µg of dsRNA were then added to the wells and incubated for 1 hour at room temperature. Finally, 2 ml of Schneider's *Drosophila* medium (GibcoBRL cat. no. 11720-034) with 10% FBS were added to each well. The plates were incubated at 27°C.

At each time point, cells from experiments and controls were collected and processed for immunoblotting and immunofluoresence. For immunoblotting, 10^6 cells were collected by centrifugation, resuspended in 50 µl sample buffer, sonicated, and boiled for 5 minutes. For immunostaining, cells were prepared as described in section 8.10.

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RNAi analysis reveals an unexpected role for topoisomerase II in chromosome arm congression to a metaphase plate

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Summary

DNA topoisomerase II (Topo II) is a major component of mitotic chromosomes and an important drug target in cancer chemotherapy, however, its role in chromosome structure and dynamics remains controversial. We have used RNAi to deplete Topo II in *Drosophila* S2 cells in order to carry out a detailed functional analysis of the role of the protein during mitosis. We find that Topo II is not required for the assembly of a functional kinetochore or the targeting of chromosomal passenger proteins, nonetheless, it is essential for anaphase sister chromatid separation. In response to a long-running controversy, we show that Topo II does have some role in mitotic chromatin condensation. Chromosomes formed in its absence have a 2.5-fold decrease in the level of chromatin compaction, and are morphologically abnormal. However, it is clear that the overall programme of mitotic chromosome condensation can proceed without Topo II. Surprisingly, in metaphase cells depleted of Topo II, one or more chromosome arms frequently stretch out from the metaphase plate to the vicinity of the spindle pole. This is not kinetochore-based movement, as the centromere of the affected chromosome is located on the plate. This observation raises the possibility that further unexpected functions for Topo II may remain to be discovered.

Key words: Mitosis, Topoisomerase, Chromosome segregation, Chromosomal passengers, Condensin, Heterochromatin

Introduction

Type II DNA topoisomerases (Topo II) are essential enzymes that modulate the topology of DNA. Topo II function is necessary for virtually all processes involving double stranded DNA, including replication, transcription, recombination and decatenation of sister chromatids prior to anaphase of mitosis (Wang, 1996).

Yeasts and *Drosophila* have a single isoform of Topo II. No *Drosophila* Topo II mutant has been described to date. Yeast Topo II mutants die in mitosis when anaphase fails as the result of a large mass of unresolved lagging chromatin (DiNardo et al., 1984; Uemura and Yanagida, 1984; Holm et al., 1985). In *Saccharomyces pombe*, this phenotype was called 'cut' [cell untimely torn (Uemura and Yanagida, 1986)].

Vertebrates have two type II topoisomerases [α and β (Drake et al., 1989)], either of which can complement yeast Topo II function (Adachi et al., 1992; Jensen et al., 1996), but which have different locations in mitosis. Topo IIα associates with chromosomes from metaphase to telophase, whereas Topo IIβ remains mainly cytosolic until anaphase onset, when low levels of the protein associate with the chromatids (Christensen et al., 2002). There is an ongoing controversy over whether Topo II is concentrated more in axial regions (Earnshaw and Heck, 1985; Gasser et al., 1986; Tavormina et al., 2002; Maeshima and Laemmli, 2003), or spread diffusely throughout the chromatid arms (Hirano and Mitchison, 1993; Swedlow et al., 1993; Christensen et al., 2002).

Topo IIα is enriched at centromeres in prometaphase and metaphase, and a number of studies suggested that the protein may have a role in regulating kinetochore structure (Rattner et al., 1996; Christensen et al., 2002) and/or centromeric cohesion (Bachant et al., 2002). This view has been strengthened by studies of two different human centromeres. A conserved Topo II cleavage site was found near the Y chromosome centromere, and this was absent from two inactive derivatives of this centromere (Floridia et al., 2000). Subsequent functional dissection of the X centromere identified a minimal region of <50 kb that was found in all active centromere derivatives. This region contained a strongly preferred cleavage site for Topo II (Spence et al., 2002).

The function of Topo II has long been controversial, particularly concerning its role, if any, in regulating mitotic chromosome structure. Topo II is required for the final stages of chromosome condensation in fission yeast (Uemura et al., 1987), *Xenopus* cell-free extracts (Adachi et al., 1991) and in *Drosophila* syncitial embryos (Buchenau et al., 1993). However, it is not required for the nocodazole-induced condensation of rDNA in *Saccharomyces cerevisiae* (Lavoie et al., 2002) and drug studies have suggested that Topo II activity is not involved in generating mitotic chromosomes with two morphologically distinct chromatids (Andreassen et al., 1997).

We have used RNAi to successfully deplete Topo II from *Drosophila* cells. This has enabled us to show that in the absence of Topo II, mitotic chromosomes can condense,

although the detailed structures produced are not entirely normal. Thus, Topo II is not essential for mitotic chromatin condensation, but it may have a role in the establishment of chromosome architecture. Topo II is not essential for centromere/kinetochore assembly or function, but it is required for sister chromatid separation at anaphase. Quite surprisingly, Topo II is required for the formation of a compact metaphase plate. This latter result raises the possibility that further unexpected functions for Topo II may remain to be discovered.

Materials and Methods

dsRNA interference

dsRNAi experiments were performed as described previously (Adams et al., 2001). Two fragments from the 5′ end of DmTopo II fused to the T7 RNA polymerase promoter were used as PCR primers. A random human intronic sequence was used as control dsRNA as described previously (Adams et al., 2001). The EST clone LD24716 was used as a PCR reaction template. The PCR fragments obtained (about 700 bp) were used as templates for RNA synthesis using the Megascript kit (Ambion). dsRNAi experiments were performed in six-well plates. At each time point, experimental and controls cells were collected for scoring, immunoblotting and immunofluorescence.

Immunofluorescence staining and immunoblotting

For immunoblotting, cells were collected by centrifugation, resuspended in SDS sample buffer, boiled for 5 minutes and sonicated. For immunostaining, cells were transferred onto poly-lysine-treated slides and left to attach for 20 minutes. Slides were centrifuged for 15 minutes at 4000 rpm before fixation. Cells were fixed in 4% paraformaldehyde in cytoskeleton buffer (CB: 1.1 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 137 mM NaCl, 5 mM KCl, 2 mM MgCl, 2 mM EGTA, 5 mM Pipes, 5.5 mM glucose, pH 6.1) for 10 minutes at 37°C. They were permeabilised in 0.1% Triton X-100 in CB for 5 minutes and then rinsed in PBS. Cells were blocked for 30 minutes at room temperature in PBS+10% FBS. Antibody incubations were performed in PBS+1% FBS for 1 hour at 37°C, followed by four 10-minute washes in PBS at room temperature. DNA was stained with 0.1 μg/ml DAPI for 5 minutes at room temperature and rinsed with PBS. Slides were mounted in Vectashield mounting medium (Vectra) and sealed using nail varnish.

Antibodies

The primary antibodies used were as follows. Anti-α-tubulin (mouse mAb B512, used at 1:2000; Sigma-Aldrich); anti-phosphorylated histone H3 (rabbit polyclonal IgG, used at 1:500, Upstate Biotechnology); anti-DmINCENP rabbit polyclonal R801, used at 1:500 (Adams et al., 2001); anti-DmAuroraB R963, used at 1:500 (Adams et al., 2001); anti-Cid chicken polyclonal serum used at 1:200 (Blower and Karpen, 2001); anti-Topo II (rabbit polyclonal antibodies used at 1:500, a gift from Neil Osheroff, Vanderbilt University School of Medicine, Nashville, Tennessee and Donna Arndt-Jovin Dept. of Molecular Biology MPI for Biophysical Chemistry Goettingen, Germany); anti-Barren [rabbit polyclonal used at 1:1000 (Bhat et al., 1996)].

All fluorescently conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used according to the manufacturer's instructions.

Fluorescent in situ hybridisation (FISH)

1×10^6 RNAi-treated cells were transferred to poly-lysine-coated slides and left for 10 minutes at room temperature. Cells were fixed in 4% paraformaldehyde for 5 minutes at room temperature then in methanol-acetic acid (1:3) at –20°C for 20 minutes, air dried and then aged for 2 days.

Various heterochromatic probes were used: X-chromosome 359 satellite chromosome 2 AACAC satellite and chromosome 3 dodecasatellite (Abad et al., 1992). Euchromatic BAC clones were obtained from the MRC UK HGMP resource centre, Cambridge, UK (BACH47E07, 10A-10A –X chromosome–; BACHN09, 30C5-30D –chromosome 2L–; BACH47E02, 50A1-50A5 –chromosome 2R–; BACH47K04, 71A-71A –chromosome 3L–; BACN01B01, 90C7-90C10 –chromosome 3R–.

BACs were reduced to ~200 bp in length by sonication. Probes were prepared from the sonicated DNA using DIG-high Prime DNA Labelling and Detection Starter Kit II (Roche). After removing excess nucleotides (ProbeQuant G-50 Micro Columns, Amersham), the quality and quantity of each probe was determined according to the protocol of the kit. For hybridisation, 300 μg of each digoxigenin-dUTP-labelled probe was ethanol-precipitated with 20 μg human cot-1 DNA (sonicated to 200 bp length; Roche) and resuspended in 40 μl hybridisation mix per slide (50% deionised formamide, 2× SSC, 5% dextran sulphate). Probes were denatured at 85°C for 10 minutes. The aged cells on the slides were rehydrated in 2× SSC and digested with 100 μg/ml RNAse A for 1 hour at 37°C, then dehydrated for 5 minutes each in 70% and 100% ethanol. After air drying, the cells were denatured for 5 minutes at 80°C in 50% deionised formamide, 2× SSC, washed for 5 minutes on ice in 2× SSC, and dehydrated for 5 minutes each at 0°C in 70%, 95% and 100% ethanol. After air drying, the denatured probes were applied to the slides, covered with coverslips and slides were incubated in a humid box at 37°C overnight. After hybridisation, the slides were washed for 2× 5 minutes at 42°C in 50% deionised formamide, 2× SSC and 5 minutes in 50% deionised formamide, 0.1× SSC, then 5 minutes each in 2× SSC, PBS at room temperature. The cells were blocked for 1 hour in 10% FBS in PBS, then rinsed with PBS and incubated in sheep anti-digoxigenin-rhodamine (used at 1:200, Roche) for 1 hour at 37°C. The slides were washed 3× 10 minutes in PBS and stained for 5 minutes with 0.1 μg/ml DAPI and washed for 10 minutes in PBS. Slides were mounted in Vectashield mounting medium (Vectra) and sealed using nail polish.

Microscopy

Imaging was performed using a Zeiss Axioplan 2 or an Olympus IX-70 microscope controlled by Delta Vision SoftWorx (Applied Precision, Issequa, WA, USA). Image stacks were deconvolved, quick-projected and saved as tiff images to be processed using Adobe Photoshop.

Measurement of chromatin density

In order to quantify DNA staining density the ten central sections of an image stack were deconvolved and projected using an averaging algorithm. The total integrated intensity of a 20×20 pixel box was measured at the appropriate wavelengths using the Data Inspector tool. For each metaphase cell analysed, three measurements were taken on the chromosomes within the cell and three of the background outside the cell. Values were corrected by subtracting the background of the appropriate wavelength.

Results

Effect of Topo II RNAi on cell growth and mitosis

Immunofluorescence analysis of Topo II in S2 cells shows that the enzyme is localised diffusely on the chromosomes, but also accumulates in specific chromosomal regions, some of which apparently coincide with centromeres (Fig. 1C,E). Following the addition of specific dsRNA to *Drosophila* S2 cells, Topo II

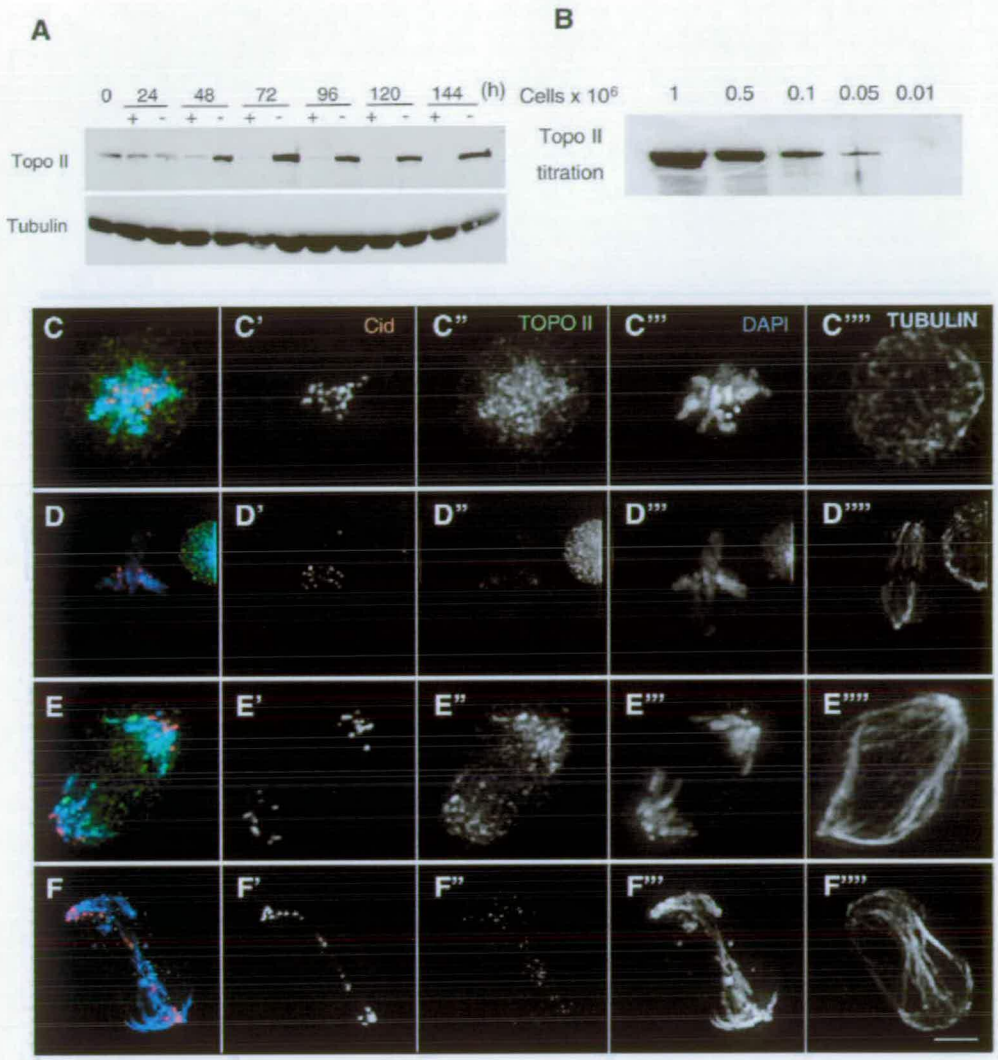


Fig. 1. Efficient depletion of Topo II in *Drosophila* S2 cells using RNAi. (A) Immunoblots: (upper) Topo II levels begin to fall by 48 hours after the addition of dsRNA and become undetectable by 72 hours (1×10^6 cells loaded per lane); (lower) loading control (anti-tubulin). –, control RNAi; +, Topo II RNAi treated. (B) The anti-Topo II antibody used can detect Topo II in 5×10^4 cells, but not 1×10^4 cells. (C-F) Immunofluorescence analysis showing Topo II depletion at the 72nd hour after treatment; (C,E) controls, (D,F) treated cells, (C,D) prometaphase, (E,F) anaphase. In all merged images, DAPI is blue, Cid is red and Topo II is green. Scale bar: 5 μm.

levels were severely reduced by 48 hours and the protein became undetectable by both immunoblotting and immunofluorescence after 72 hours (Fig. 1A,D,F). A titration experiment revealed that the antibody used in this study could detect Topo II from 5×10^4 cells, but not from 1×10^4 cells (Fig. 1B). Overall, we estimate that levels of the protein in the culture fell by at least a factor of 20 in response to the dsRNA treatment. The protein did not re-accumulate in the cells even by 144 hours after addition of dsRNA (Fig. 1A).

There was no significant difference in mitotic index between cultures treated with control or Topo II-specific dsRNA (Fig. 2D). Both populations displayed a slight decline in mitotic index over the course of the experiment, presumably because the medium was depleted of essential ingredients.

Detailed analysis of the distribution of mitotic phases in cells following Topo II RNAi revealed surprisingly few differences between the Topo II-depleted cells and cells treated with a control dsRNA. If prometaphases and metaphases were grouped together (this was because spindles are oriented randomly in these small non-adherent cells, and it is frequently difficult to distinguish between metaphase and prometaphase), few differences were observed between experimentals and controls (Fig. 2A,B). If we scored only cells where the spindle axis was perpendicular to the optical axis (where metaphases and prometaphases could be distinguished), again no reproducible difference was seen (Fig. 2C). In fact, the only significant difference seen in these experiments was a slight increase in the percentage of anaphase cells in the 96-, 120- and 144-hour time points (Fig. 2B). The frequency of cells in cytokinesis was also elevated relative to controls, and >85% of these cells had chromatin bridges across the midbody (data not shown).

These observations reveal that *Drosophila* S2 cells do not have a checkpoint that arrests the cell cycle in response to loss of Topo II function, and that progress through mitosis is not delayed significantly by lack of Topo II activity.

Topo II function is required for normal mitotic chromosome morphology but not histone H3 phosphorylation

One of the most persistent questions about Topo II function is whether the protein has a role in mitotic chromosome structure. This has been difficult to answer, because the drugs and antibodies used to inhibit Topo II function in previous published studies might not necessarily block the function of Topo II as a structural element in chromosomes. Furthermore, chromosome architecture is extremely dependent on specimen preparation conditions and is difficult to assess quantitatively. We have avoided these problems by, firstly, generating cells where the protein is effectively absent, and secondly, using a

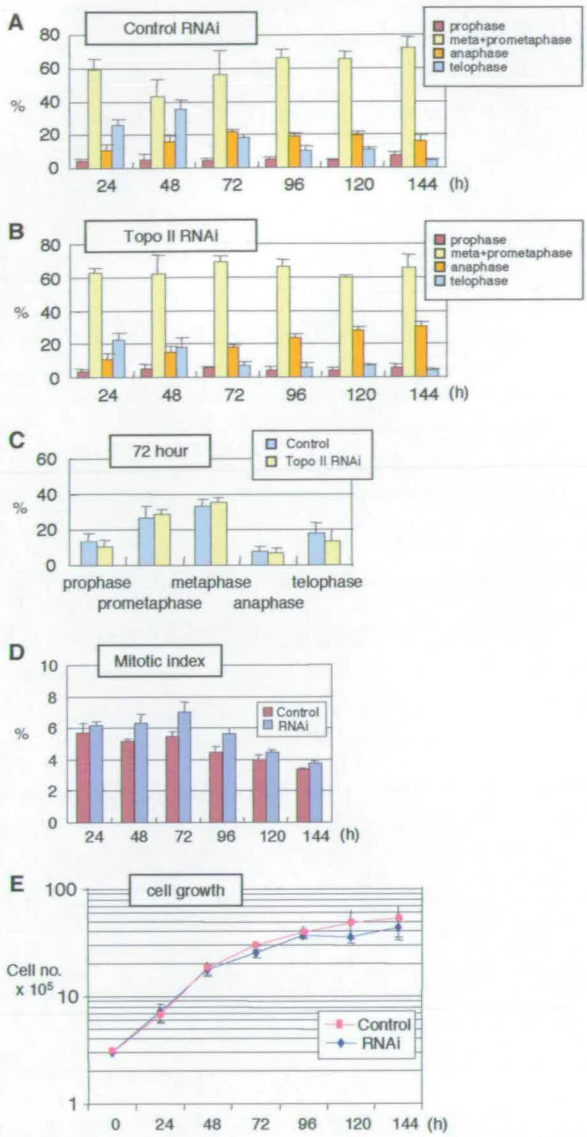


Fig. 2. (A,B) Scoring of mitotic cells in the different phases of mitosis reveals only slight differences between cells following control RNAi treatment (using dsRNA corresponding to a human intronic sequence; A) and Topo II RNAi (B). The Topo II RNAi causes a significant increase in the fraction of anaphase cells seen at later times. (C) The percentage of cells in each stage of the cell cycle at various times after treatment. There is no significant difference in any of the mitotic phases when control and Topo II RNAi-treated cells (selected because the spindle is viewed from the side and the metaphase plate can be unambiguously identified) are scored at 72 hours post addition of dsRNA. (D) Mitotic index. There is no significant difference in mitotic index between control and Topo II RNAi-treated cultures over the course of the experiment. (E) Cell growth curves of control and Topo II RNAi-treated cultures. These data are from three independent experiments. In each experiment, more than 2000 cells were scored at every time point. The mitotic index is determined by observing the DNA and spindle staining.

quantitative assay to examine whether the degree of chromatin condensation within mitotic chromosomes is normal in the absence of Topo II.

We performed a quantitative analysis of the amount of DAPI-stained DNA in a defined volume on image stacks (see Materials and Methods for details). A previous analysis had shown that this method was sensitive enough to detect the increases in chromosome condensation that normally occur from prophase through metaphase, and the subsequent decondensation that begins in anaphase (Adams et al., 2001). The present analysis showed that in the absence of Topo II, the chromatin was roughly 2.5-fold less condensed than normal (DAPI density, 2.15±0.68 versus 5.09±0.87 (arbitrary units); n=15). In addition to this difference in condensation, many of the chromosomes in Topo II-depleted cells in prometaphase were morphologically abnormal, often having a mass of chromatin without defined sister chromatids (Fig. 3B,C). The change in chromatin compaction following loss of Topo II did not correlate with any obvious abnormalities in histone H3 serine[10] phosphorylation, even in the most abnormal looking chromosomes (Fig. 3B,C).

It is now widely accepted that mitotic chromosome condensation is regulated by the condensin complex (Hirano and Hirano, 2002), one component of which, the non-SMC subunit Barren, has been proposed to interact functionally and physically with Topo II (Bhat et al., 1996). It was therefore possible that the alterations of chromatin packaging and morphology seen in the absence of Topo II might be due to effects on condensin targeting or function. However, we found that the behaviour of Barren is apparently unaffected by the depletion of Topo II: the protein becomes selectively concentrated at or near centromeres during metaphase before its levels decline significantly during anaphase (Fig. 4).

These experiments reveal that although the distribution of condensin appears normal and some degree of mitotic chromosome condensation can occur in the absence of Topo II, the enzyme does contribute both to the extent of chromatin condensation and to the apparent quality of the structures produced.

Topoisomerase II is required for formation of a compact metaphase plate

In many metaphase cells observed following Topo II depletion, one or more chromosome arms extended outwards from the compact mass of chromosomes, often stretching towards the spindle pole (Fig. 5B,C; examples of this characteristic configuration are also shown in Fig 3C, Fig. 4C,D and Fig. 6A-D). This phenotype was first observed at 48 hours, when Topo II levels had begun to fall significantly, and it was maximal at 96 hours, when roughly 25% of metaphases had one or more protruding chromosome arms (Fig. 5D).

In many cases, the protruding chromosome had a V- or J-shaped appearance, resembling a chromatid engaged in anaphase A poleward movement. However, precocious movement of the centromere towards the pole cannot be the explanation for this phenomenon. In the great majority of cases, when the centromere of the protruding chromosome was identified by immunostaining for CENP-A/Cid (Fig. 5B") it was positioned normally at the metaphase plate. Immunolocalisation of the condensin subunit Barren also

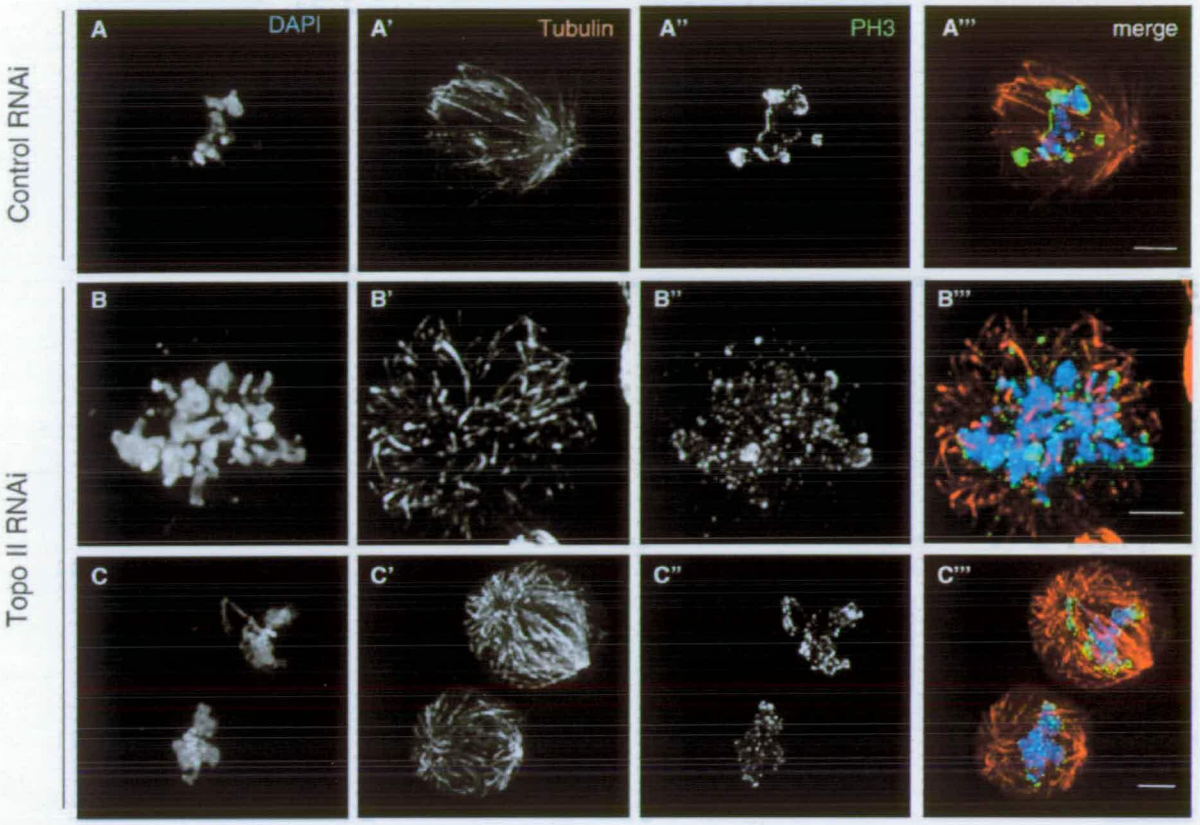


Fig. 3. Topo II depletion causes abnormalities in chromosome structure, but does not affect histone H3 phosphorylation on serine[10]. All images are from cultures at 72 hours after addition of Topo II dsRNA. (A-A''') Control RNAi-treated metaphase cell. (B-B''') Prometaphase cell with abnormal chromosome morphology viewed parallel to spindle axis. (C-C''') Two metaphase cells, one of which has a highly elongated chromosome arm extending to one spindle pole. Both are viewed nearly perpendicular to the spindle axis. (A-C) DAPI staining for DNA; (A'-C') anti-tubulin shows the mitotic spindle; (A'',B'') histone H3 phosphorylated on serine[10] is stained with a specific antibody; (A'''-C''') merged images (DAPI is blue, tubulin is red, histone H3 is green. Scale bar: 5 μm.

allowed us to visualise the normal metaphase alignment of the centromeres (Fig. 4C''). In this case, the entire elongated chromosome arm had detectable levels of the condensin subunit but the centromere-enriched staining for Barren was found on the metaphase plate. We could also observe INCENP staining all along the protruding chromosome arm (data not shown).

These observations reveal that the movement of the chromosome arm towards the spindle pole cannot be due to centromere activity, but it was possible that some other specialised structural feature of the chromosome arms, for example, the rRNA locus, might be responsible for this positioning. We therefore performed FISH experiments using BAC clones to identify the chromosome arm(s) involved in this unusual morphology. This analysis revealed that the protruding chromosome arm could be the X, or either arm of chromosome 3 (Fig. 6A,B). These three chromosome arms share no obvious feature that could explain this association with the spindle pole. Chromosome 2 was never observed to stretch towards the pole (n=22, Fig. 6C).

Despite the lack of a centromere close to the spindle pole, the protruding chromosome arm appeared to be under tension. This was most clearly seen when we used heterochromatic probes in our FISH experiments. Occasionally we could observe centromeric heterochromatin in the protruding arm, and when we did, it was often abnormally elongated (compare signals from the protruding chromosome and its homologue, Fig. 6D). In the absence of evidence for a kinetochore actively pulling the arm, this is most consistent with the arm being somehow trapped at the pole, and then the combined forces of kinetochore congression to the metaphase plate, chromatin condensation and the 'polar wind' (presumably primarily chromokinesins) actively trying to push/pull the arm away from the pole.

Topo II is not required for centromere/kinetochore assembly or function, but is required for sister chromatid segregation

A number of studies have shown that Topo IIα in mammalian cells is concentrated at centromeres during prometaphase and metaphase (Floridia et al., 2000; Spence et al., 2002; Christensen et al., 2002; Tavormina et al., 2002), and one study showed that kinetochore structure was apparently abnormal in cells treated with Topo II inhibitors (Rattner et al., 1996). However, all aspects of centromere behaviour examined were

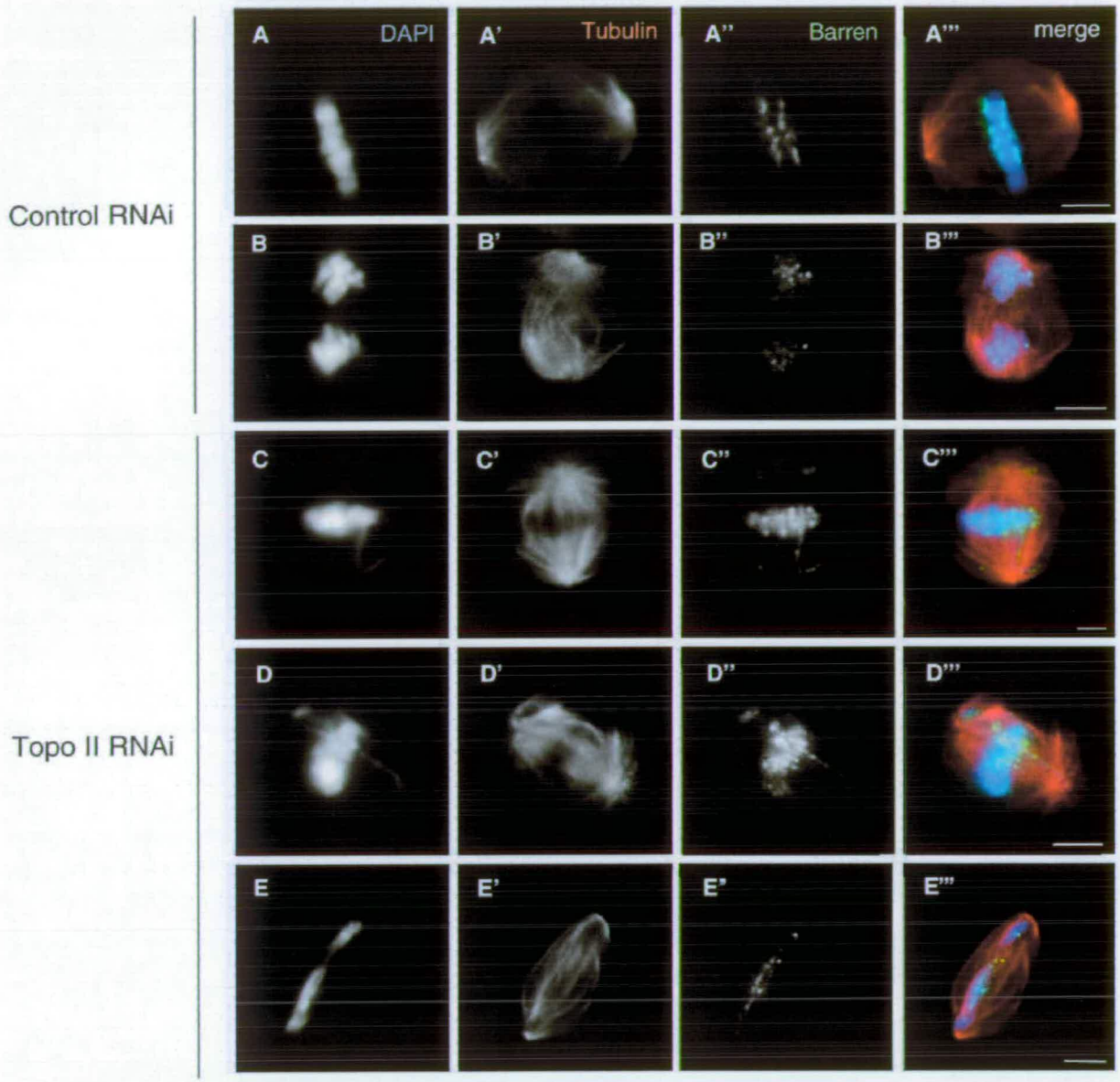
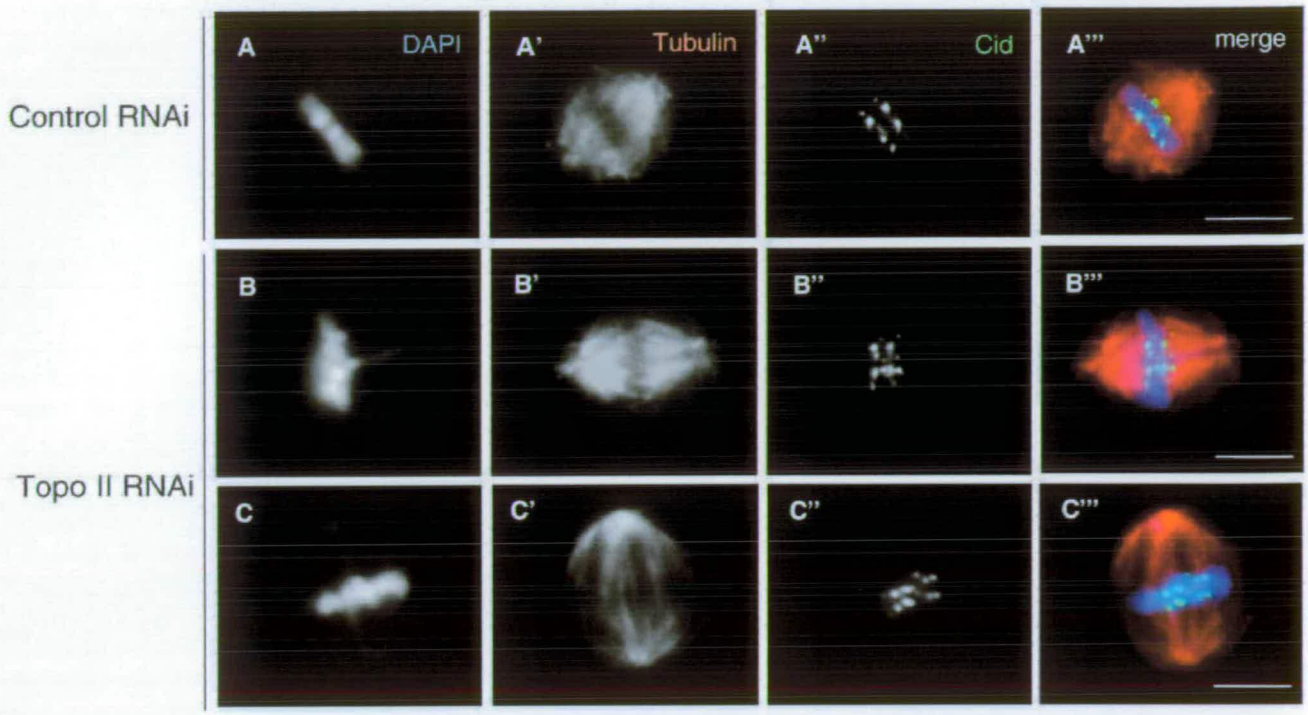


Fig. 4. The condensin subunit Barren associates normally with mitotic chromosomes in Topo II-depleted cells. (A,B) Control RNAi-treated normal metaphase and anaphase cells. (C,D) Topo II RNAi-treated metaphase cells with a highly elongated chromosome arm extending to one spindle pole. (E) Topo II RNAi-treated anaphase cell with the bulk of the chromatin stretched out between the separating kinetochores. (A-E) DAPI staining for DNA. (A'-E') anti-tubulin shows the mitotic spindle; (A''-E'') staining for Barren shows the position of the condensin complex; (A'''-E''') merged images (DAPI is blue, tubulin is red, Barren is green). Scale bar: 5 μm.

normal in Topo II-depleted *Drosophila* S2 cells. These included the ability to assemble a kinetochore (detected with Cid/CENP-A – Fig. 1D, Fig. 5B,C), targeting of chromosomal passenger proteins (Fig. 7), binding of spindle microtubules and migration towards the spindle poles (Fig. 8B''; see also Fig. 1F).

Since the chromosomal passenger proteins INCENP, Survivin and Aurora-B are essential for numerous chromosomal functions during mitosis, we examined their targeting in some detail in cells depleted of Topo II. No obvious defects were observed (Fig. 7). Furthermore, in the majority of cells, INCENP/Aurora B transferred normally to the central spindle at the transition to anaphase even though sister chromatids did not disjoin normally. However, in some anaphases INCENP localisation was aberrant, with the protein distributed both along the segregating chromatids and parallel to the spindle microtubules (Fig. 7E). The reason for this is unknown, but it could be a result of defective release of sister chromatid cohesion in these cells.

Despite the apparently normal centromere/kinetochore function virtually all anaphase and telophase cells depleted of Topo II (72 hours after addition of dsRNA) exhibited



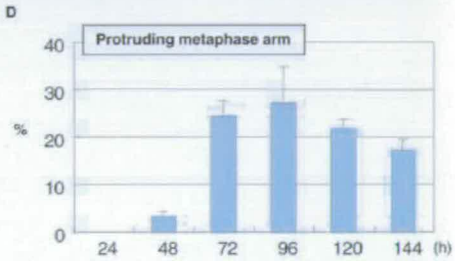


Fig. 5. Many metaphases in Topo II-depleted cells have an unusual phenotype in which one or more chromosome arm(s) becomes highly elongated and stretches towards the spindle pole. (A) Control RNAi-treated normal metaphase cell. (B) Topo II RNAi-treated metaphase cell with a highly elongated chromosome arm extending to one spindle pole. (C) Topo II RNAi-treated metaphase cell with J-shaped chromosome arms extending to spindle poles. (D) Statistical analysis of the protruding arm phenotype. (A-C) DAPI staining for DNA; (A'-C') anti-tubulin shows the mitotic spindle; (A"-C") staining for Cid/CENP-A shows the position of kinetochores; (A'"-C'") merged images (DAPI is blue, tubulin is red, Cid is green). Scale bar: 5 μm.

abnormalities in chromosome segregation (Fig. 8B,C). Anaphase and telophase cells had normal mitotic spindles with centromeres approaching the poles, but sister centromeres were linked by massive chromatin bridges (Figs 1F, 4E, 7F, 8B). This led to failures in cytokinesis, and the number of binucleate cells in cultures also increased fivefold (data not shown). This is in agreement with previous genetic analyses of Topo II function in yeasts, and with inhibitor studies in metazoan cells (DiNardo et al., 1984; Holm et al., 1985; Uemura and Yanagida, 1986; Gorbsky, 1994).

In control cells, kinetochores migrated towards the spindle poles in a compact line perpendicular to the spindle axis (Fig. 8A"). In contrast, kinetochores of Topo II-depleted cells in anaphase appeared to be distributed in a linear fashion along the spindle axis (Fig. 8B"; see also Fig. 1F'). This was reminiscent of the behaviour of kinetochores in *top2* mutants of *S. pombe* (Funabiki et al., 1993), and may reflect a 'tethering' effect, where kinetochores migrate towards the poles until they are halted by unresolved catenations in the trailing chromatin.

Thus Topo II is required for efficient sister chromatid segregation at anaphase, but this is probably primarily because of a requirement for decatenation of chromatid arms. Topo II appears not to be required for either centromere or kinetochore assembly or function.

Discussion

The role of DNA topoisomerase II (Topo II) in mitotic chromosome structure and dynamics has been controversial for well over a decade, at least partly because functional studies to date have involved the use of antibodies and drugs that may not have produced a straightforward null phenotype. We have used RNAi to deplete *Drosophila* cultured cells of their single Topo II isoform. This analysis has revealed that the function of Topo II differs in several substantial ways from that predicted by previous studies. For example, Topo II is not required for inactivation of a checkpoint that monitors chromatin catenation in G2, or for the assembly or function of kinetochores, however, it is required for alignment of the chromosomes at a compact metaphase plate. With regard for one of the most contentious issues, we found that Topo II is required for mitotic chromatin to reach a normal level of compaction, but not for the global process of mitotic chromosome condensation.

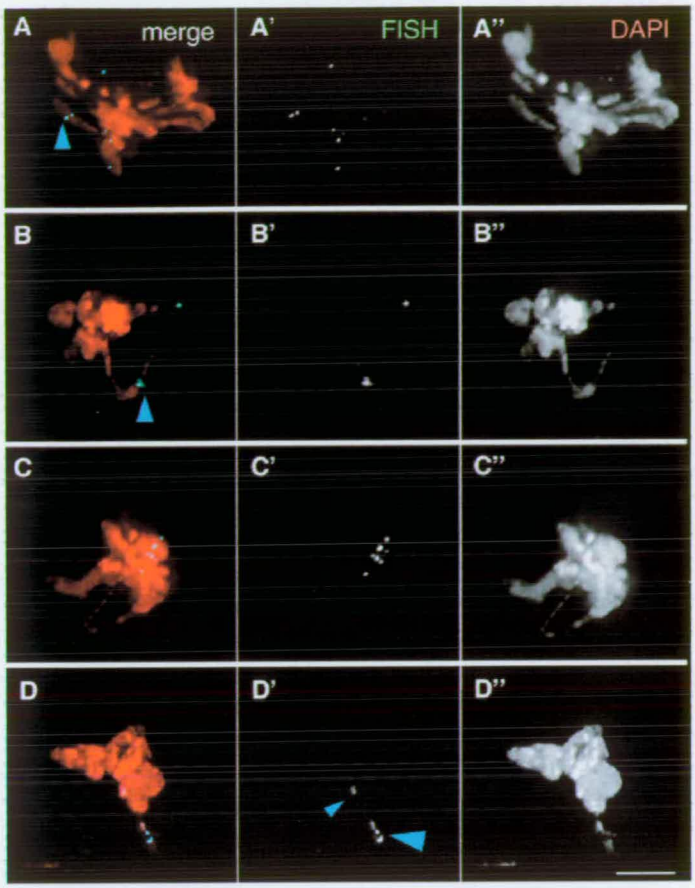


Fig. 6. Identification of the chromosomal component in the protruding arm phenotype. Analysis by FISH at 72 hours after the addition of Topo II dsRNA using the following probes. (A) Euchromatic chromosome 3 probe (BACH47K04); (B) Euchromatic chromosome X probe (BACH47E07). In A and B, arrowheads indicate FISH signal in protruding arm. (C) Euchromatic chromosome 2 probe (BACHN09); (D) Heterochromatic chromosome X probe (359 satellite); large arrowhead points to extended FISH signal in protruding arm; small arrowhead points to condensed FISH signal in metaphase plate. In all merged images, the probe is green and the DNA is red. Scale bar: 5 μm.

Topo II is not required to inactivate a G2 checkpoint or to assemble functional kinetochores in *Drosophila* cells

In mammals, entry into mitosis is thought to be regulated by a Topo II-dependent checkpoint that monitors the level of catenation of sister chromatids (Downes et al., 1994; Clarke and Gimenez-Abian, 2000). This checkpoint involves signalling through the ATR kinase following inactivation of Topo II by ICRF-193 (Deming et al., 2001).

The results of the present study reveal that *Drosophila* S2 cells do not appear to have a checkpoint mechanism that couples mitotic entry or progression with Topo II activity. Cells traversed mitosis with relatively normal kinetics, even though mitotic events were grossly abnormal, particularly in the closing stages. We have noted previously that S2 cells have weak checkpoints controlling behaviour in mitosis (see Adams et al., 2001), and the possibility remains that other cell types might have this checkpoint.

Previous studies suggested that Topo II could have an essential role in kinetochore structure (Rattner et al., 1996; Bachant et al., 2002). Our results identify no key function for Topo II in kinetochore assembly or function. Following Topo II depletion we observed normal chromosomal targeting of the centromere-specific histone H3 subtype Cid/CENP-A. Furthermore, kinetochores interacted with microtubules and migrated towards the spindle poles in anaphase. The different patterns of kinetochore distribution observed in control and Topo II-depleted anaphases probably reflect the high levels of residual catenation of sister chromatids.

Topo II is required for complete chromatin condensation and a normal chromosomal morphology

Chromosome morphology did not look normal following Topo II depletion. Local levels of chromatin condensation were roughly 2.5-fold less in the Topo II-depleted cells, and many metaphases had a mass of condensed chromatin that lacked well-defined sister chromatids. This phenotype could be either a direct consequence of the loss of Topo II activity, or due to interference with targeting of factors required for proper mitotic chromosome morphology. These include the condensin complex (Hirano and Hirano, 2002) and the chromosomal passenger proteins INCENP and Aurora-B.

Depletion of the chromosomal passenger proteins has previously been shown to result in production of dumpy, irregularly shaped chromosomes (Adams et al., 2001), possibly because Aurora-B is required both for chromosome targeting of Barren, a non-SMC subunit of the condensin complex (Giet and Glover, 2001), and for phosphorylation of histone H3 on serine[10] (Gurley et al., 1978; Bradbury, 1992; Adams et al., 2001; Murnion et al., 2001). However, INCENP and Aurora-B target normally to mitotic chromosomes in Topo II-depleted cells.

Our results show that Topo II is also not required for the normal targeting of Barren to chromosomes, however we cannot exclude a secondary role for Topo II in condensin function. For example, Topo II-mediated decatenation activity might be required for the resolution of the two sister chromatids during condensin-mediated chromosome condensation (Holm, 1994; Steffensen et al., 2001).

Topo II is required for a compact metaphase alignment of the chromosomes

The most surprising and novel phenotype observed in this study was that the chromosomes failed to form a compact metaphase plate in about 25% of metaphase cells. Instead, these cells had one or more chromosome arms protruding a substantial distance from the plate, typically stretched along the long axis of the spindle towards one of the poles. Analysis by FISH revealed that the chromosomal component involved in this phenotype is not entirely random, although any of the arms of chromosomes 3 and X could be found near the poles of metaphase cells. In contrast, neither arm of chromosome 2 was ever observed to protrude from the plate in this way.

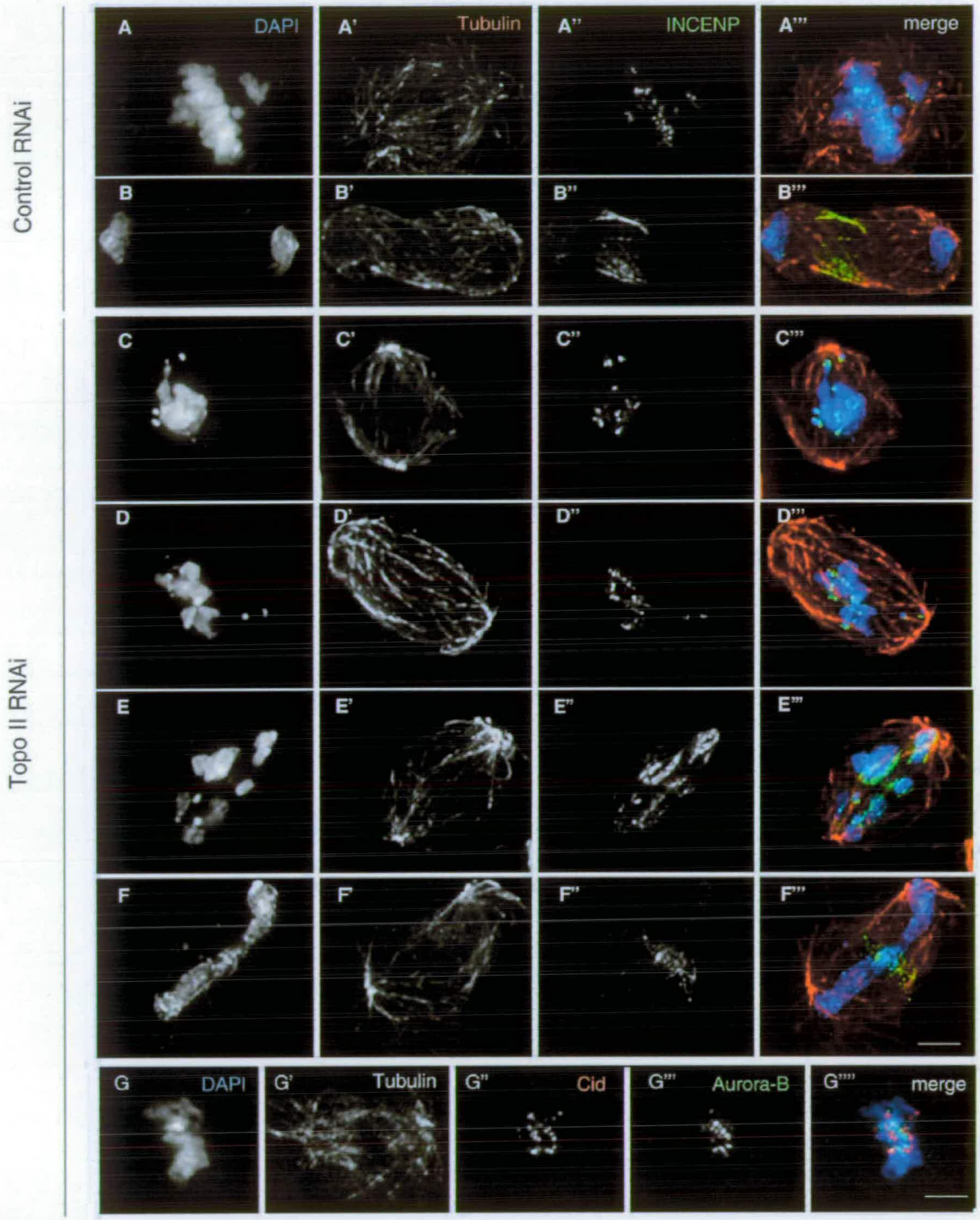


Fig. 7. The chromosomal passenger protein INCENP is localised normally in Topo II-depleted cells with the exception of a few highly abnormal anaphase cells. (A) Control RNAi-treated normal prometaphase cell. (B) Control RNAi-treated normal telophase cell. (C) Topo II RNAi-treated prometaphase cell with abnormal chromosomal morphology. (D) Topo II RNAi-treated late prometaphase cell with abnormal chromosomal morphology. (E) Topo II RNAi-treated abnormal cell with INCENP stretched along the chromosome arms. (F) Topo II RNAi-treated anaphase cell with massive chromatin bridging, but INCENP located normally on the central spindle. (G) Topo II RNAi-treated late prometaphase cell with abnormal chromosomal morphology: Aurora-B localises normally on the centromeres. (A-G) DAPI staining for DNA; (A'-G') anti-tubulin shows the mitotic spindle; (A''-F'') staining for INCENP; (G'') staining for Cid shows the positions of the kinetochores; (G''') staining for the chromosomal passenger Aurora B. In A'''-F''' DAPI is blue, tubulin is red, INCENP is green, and in G'''' DAPI is blue, Cid is red, Aurora-B is green. Scale bar: 5 μm.

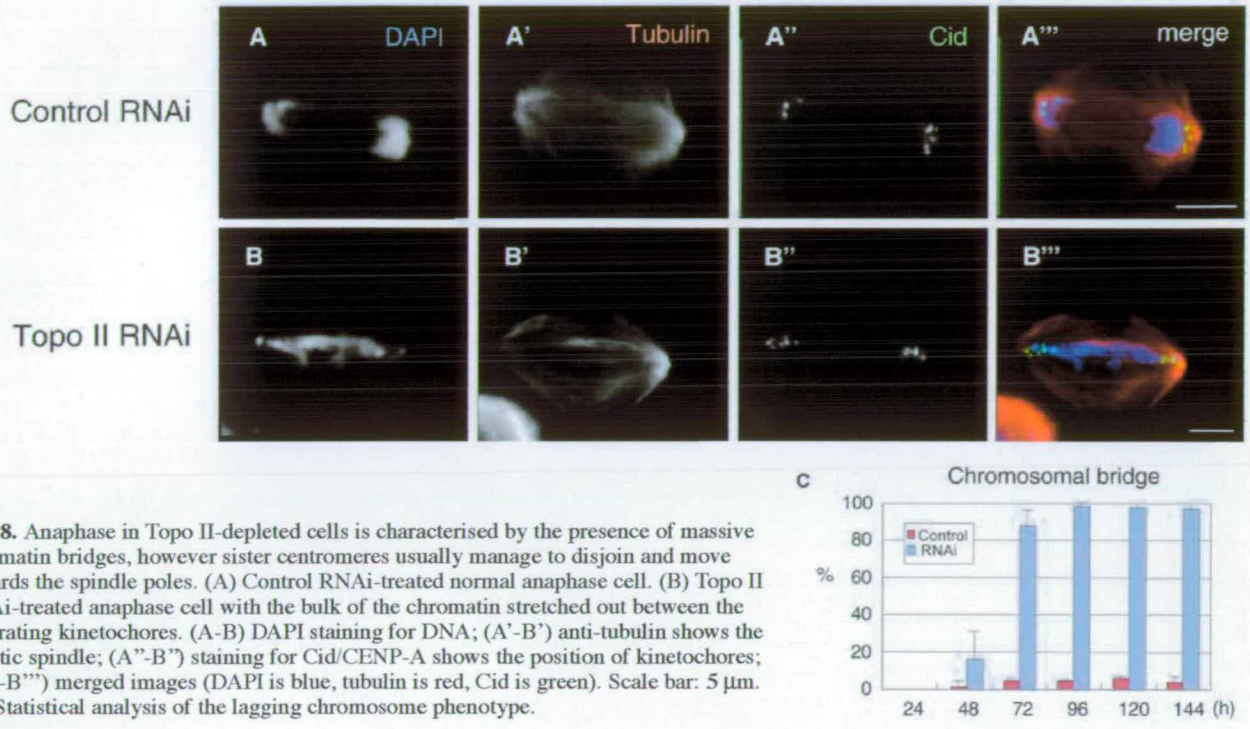


Fig. 8. Anaphase in Topo II-depleted cells is characterised by the presence of massive chromatin bridges, however sister centromeres usually manage to disjoin and move towards the spindle poles. (A) Control RNAi-treated normal anaphase cell. (B) Topo II RNAi-treated anaphase cell with the bulk of the chromatin stretched out between the separating kinetochores. (A-B) DAPI staining for DNA; (A'-B') anti-tubulin shows the mitotic spindle; (A''-B'') staining for Cid/CENP-A shows the position of kinetochores; (A'''-B''') merged images (DAPI is blue, tubulin is red, Cid is green). Scale bar: 5 μm. (C) Statistical analysis of the lagging chromosome phenotype.

The protruding chromosomes were not precociously separated sister chromatids moving to the pole. In almost every case, the centromeres were clustered normally at the metaphase plate, and no Cid staining was observed on the protruding arm. Furthermore, in favourable instances, the two sister chromatids could be observed. Thus, this phenomenon does not appear to represent abnormal kinetochore-based movement towards the spindle pole.

We have considered three models to explain the origin of the protruding arms in Topo II-depleted cells. First, they could be chromosomal regions that exhibit a persistent failure in condensation. If this was the case, we would expect the arm to be oriented randomly or extruded laterally out of the spindle because of the action of the 'polar wind' (Carpenter, 1991). For example, when chromosome arms are severed with a laser, they are rapidly ejected from the vicinity of the spindle pole (Rieder et al., 1986) as a result of the action of plus-end-directed chromokinesin motors associated with the chromatin as well as collisions with elongating microtubules (Funabiki and Murray, 2000). This is inconsistent with our observation that the stretched arms almost invariably have at least one region in close proximity to a spindle pole.

A second model suggests that the protruding arms move towards the pole because they do in fact have active kinetochores that lack detectable Cid/CENP-A. As the protruding chromosomes derive from three different chromosome arms and have a whole range of morphologies, this hypothesis would require the unlikely possibility of the efficient formation of neocentromeres at a number of different positions on the long arms of chromosomes X and 3.

A third possibility is that the arms are physically trapped close to the pole and cannot retract back to the metaphase plate. This could explain the morphology of the J-shaped chromosomes if the point of entrapment was close to the pole: chromokinesins would try to move the short and long arms of the J away from the pole. This is consistent with the highly elongated appearance of these protruding arms, which would be stretched as they were pulled away from the pole by the associated chromokinesin motors [see the stretched chromosomes in Funabiki and Murray (Funabiki and Murray, 2000)].

It is remarkable that the protruding arms consistently extend towards one pole of the mitotic spindle. Although Topo II is a major component of mitotic chromosomes and is generally considered to be a chromatin protein, one recent study has shown that Topo IIα can be detected as a salt-stable component of centrosomes in a number of human cell lines (Barthelmes et al., 2000). Although that study did not propose a definitive function for Topo II at the centrosome, our results suggest that this centrosomal Topo II could have a role in enabling the arms of metaphase chromosomes to detach from centrosomes and assume their classical condensed structure at the metaphase plate.

Conclusions

A detailed phenotypic analysis of the role of Topo II in mitotic events has confirmed that the enzyme has essential roles in sister chromatid separation at anaphase. The enzyme is not, as was expected, essential for assembly of a functional kinetochore, but it does appear to have a role in the establishment of a normal mitotic chromosome morphology. Most surprisingly, in the absence of Topo II, one or more chromosome arms are frequently trapped in the vicinity of the spindle pole during metaphase. It will be a challenge for future

experiments to test whether Topo II can detach DNA from entanglements with other polymers in addition to DNA.

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