# FUNCTIONAL ANALYSIS OF INCENP, A CHROMOSOMAL PASSENGER PROTEIN

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## Declaration

I hereby declare that this thesis was composed entirely by myself, that the work presented in it is my own, except where explicitly stated otherwise, and that the work has not been submitted for any other degree or professional qualification.

Fiona MacIsaac

20 February 2007

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## Abstract

The chromosomal passenger proteins are a group of proteins that display a distinctive pattern of localisation through mitosis. In human cells this group currently comprises six proteins; Survivin, Aurora-B, Aurora-C, INCENP, TD-60 and Borealin. The functions of the chromosomal passenger proteins include chromatin modification, correction of kinetochore-microtubule attachment errors, some aspects of the spindle assembly checkpoint, maintenance of a stable bipolar spindle and completion of cytokinesis.

To try and understand the functions of INCENP, various truncations have been expressed. The N-terminus of INCENP has previously been shown to be essential for targeting to the centromere when expressed ectopically. I have carried out a yeast two-hybrid screen in order to identify proteins that interact with this region of INCENP. This screen identified a number of potential INCENP interactors. I have further analysed one of these proteins, FLJ14346. I have shown that it has a dynamic localisation during mitosis, associating with the spindle poles and spindle microtubules, the midbody during cytokinesis and then with the centrosomes during interphase. This protein binds INCENP, Borealin and TD-60. The interaction with TD-60 is of particular interest since no direct interaction has yet been shown between TD-60 and the other chromosomal passenger proteins.

In a separate study, performed as a collaboration between several labs, we characterised the role of INCENP in male meiosis in *Drosophila melanogaster*. This study revealed that INCENP was required for the maintenance of sister chromatid cohesion in meiosis. INCENP and Aurora-B partially colocalised with MEI-S332, a protein that is known to be required for the maintenance of sister chromatid cohesion in meiosis. My data presented in this thesis shows that INCENP binds MEI-S332 *in vitro*. We show that MEI-S332 is

phosphorylated by Aurora-B *in vitro* and that mutation of the Aurora-B phosphorylation site leads to unstable association of MEI-S332 with the centromere.

## Publications

The work presented in this thesis has led to the following publication:

Resnick TD, Satinover DL, MacIsaac F, Stukenberg PT, Earnshaw WC, Orr-Weaver TL, Carmena M. **INCENP and Aurora B Promote Meiotic Sister Chromatid Cohesion through Localization of the Shugoshin MEI-S332 in *Drosophila*.** Dev Cell. 2006 Jul;11(1):57-68.

## List of Figures

## List of Tables

## Abbreviations

| | |
|---|---|
| 3-AT | 3-amino-1,2,4-triazole |
| aa | amino acid |
| ACA | anti-centromere antibodies |
| AD | activation domain |
| APC | anaphase promoting complex |
| BIR | baculovirus IAP repeat |
| BLAST | basic local alignment search tool |
| bp | base pair(s) |
| BSA | bovine serum albumin |
| Budding yeast | *Saccharomyces cerevisiae* |
| *C. elegans* | *Caenorhabditis elegans* |
| CB | cytoskeleton buffer |
| cDNA | complementary DNA |
| CENP | centromere protein |
| Chicken | *Gallus gallus* |
| CLAP | chymostatin, leupeptin, antipain, pepstatin A |
| CMV | cytomegalovirus |
| C-terminus | carboxy-terminus |
| DAPI | 4',6-diamidino-2-phenylindole |
| DEPC | diethylpyrocarbonate |
| DBD | DNA binding domain |
| dCTP | deoxycytidine-5'-triphosphate |
| DIC | differential interference contrast |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| dNTPs | deoxyribonucleotide-5'-triphosphate |

| | |
|---|---|
| *Drosophila* | *Drosophila melanogaster* |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethylene glycol-bis-(β-aminoethyl ether)-N, N, N', N'-tetraacetic acid |
| EST | expressed sequence tag |
| FBS | fetal bovine serum |
| Fission yeast | *Schizosaccharomyces pombe* |
| FITC | fluorescein isothiocyanate |
| GAP | GTPase activating protein |
| GEF | guanine exchange factor |
| GFP | green fluorescent protein |
| GST | glutathione S transferase |
| GTP | guanosine-5'-triphosphate |
| GTPase | GTP hydrolysing enzyme |
| H3K4diMe | Histone H3 dimethylated on Lysine 4 |
| H3K9triMe | Histone H3 trimethylated on Lysine 9 |
| HeLa | Henrietta Lacks |
| HEPES | N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] |
| HRP | horseradish peroxidase |
| IAP | inhibitor of apoptosis |
| IPTG | isopropyl β-D-thiogalactopyranoside |
| kb | kilobase(s) |
| kDa | kilodalton |
| LB | Luria-Bertani medium |
| MOPS | 3-(N-Morpholino)-propanesulfonic acid |
| mRNA | messenger ribonucleic acid |
| NMR | nuclear magnetic resonance |

| | |
|---|---|
| N-terminus | amino-terminus |
| OD | optical density |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| PIPES | piperazine, N, N'-bis-[2-ethanesulfonic acid] |
| PMSF | phenylmethylsulfonyl fluoride |
| RNA | ribonucleic acid |
| RNAi | RNA interference |
| RNase | ribonuclease |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| *S. cerevisiae* | *Saccharomyces cerevisiae* |
| *S. pombe* | *Schizosaccharomyces pombe* |
| SBP | streptavidin binding peptide |
| SDS | sodium dodecyl sulphate |
| TAE | tris acetate EDTA |
| TAP | tandem affinity purification |
| TCA | trichloroacetic acid |
| TEV | tobacco etch virus |
| TrAP | triple affinity purification |
| Tris | tris (hydroxymethyl)aminomethane |
| TRIzol | total RNA isolation reagent |
| UV | ultraviolet |
| X-α-GAL | 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside |
| X-β-GAL | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |
| *Xenopus* | *Xenopus laevis* |

Three-letter and one-letter codes will be used to identify amino acids.

## Table of contents

# I Introduction

# 1. Introduction

## *1.1. The Cell*

The cell is the smallest unit of life. All living things from unicellular organisms such as bacteria and yeasts to multicellular organisms such as humans are made up of cells. Viruses are smaller, and are not made up of cells, but are not considered truly alive because they do not metabolise on their own and cannot replicate on their own.

Cells can only arise by replicating their contents, including importantly their DNA, into two new cells. In eukaryotes, the process by which cells coordinate their growth with DNA replication and cell division is called the cell cycle. All contemporary organisms arose from a common ancestor. It is therefore possible to study simple "model" organisms such as yeast, which are amenable to growth and manipulation in the laboratory, in order to learn about the general principles of cell biology. These principles can then be tested in more complex organisms such as humans.

## *1.2. The Cell Cycle*

Understanding how the cell cycle is controlled is an important aspect of cell biology and has important implications in medicine, especially for research on cancer, a disease characterized by uncontrolled cell division. The cell cycle is the process by which cells duplicate their contents and divide into two daughter cells. The cell cycle is divided into four stages; $G_1$ phase, S phase, $G_2$ phase and M phase (Figure 1.1). S-phase & M-phase are the active phases where there are discrete functional landmarks. The cell replicates its DNA in S-phase and segregates it in mitosis. $G_1$ and $G_2$ are the first and second gap phases, respectively, in which the cell monitors various criteria before moving to the next stage of the cell cycle. Checkpoints at specific points of the cell cycle

ensure that the cell has correctly completed the previous phase and that the external environment is suitable for the cell to progress to the next stage. Defects in these checkpoint machineries can lead to cancer (reviewed by (Kastan and Bartek, 2004)).

$G_1$ allows the cell time to grow after dividing and monitor its environment before making the decision to proceed into the next cell cycle. There are two checkpoints that control transit from $G_1$ into S-phase. The restriction point ensures the cell has increased in mass and monitors environmental conditions and the $G_1$ DNA damage checkpoint ensures there is no DNA damage before it is replicated. The restriction point monitors the cell's environment (nutrients, growth factors). The restriction point is defined as the point after which the cell cycle will proceed into S phase and through the cell cycle even if mitogenic factors are withdrawn. Some cells can exit the cell cycle during $G_1$ into a specialized state called $G_0$.

Cells in multicellular organisms enter $G_0$ and differentiate in order to stop dividing and perform specialized functions. In some cases cells that are in $G_0$ can re-enter the cell cycle.

During S-phase the cell replicates its DNA and centrosomes, and cohesion between the sister chromatids is established.

$G_2$ provides a safety margin between DNA synthesis and mitosis to ensure DNA replication is complete before the cell divides. A $G_2$ DNA damage checkpoint ensures cells do not enter mitosis with damaged or unreplicated DNA. Before entry into mitosis there may also be a checkpoint to ensure the cells have replicated their centrosomes.

During mitosis the replicated chromosomes are segregated into two daughter cells and at the end of mitosis two daughter cells are formed in a process called cytokinesis. Mitosis and cytokinesis together make up M-phase. In most instances cytokinesis follows mitosis, however there are exceptions. In some specific cases where cytokinesis does not occur, single cells containing

multiple nuclei form, e.g. fungi, slime moulds and the early *Drosophila* embryo. Of all the stages of the cell cycle, mitosis is the most visually dramatic since this is when the chromosomes condense and become visible and segregate. Although the process of mitosis has been studied for over 100 years there are still many questions that remain unanswered.

In sexually reproducing organisms, cells may also undergo meiosis in order to produce haploid cells, called gametes, which can fuse to produce new diploid cells. Meiosis will be explained in detail later (see section 1.7).

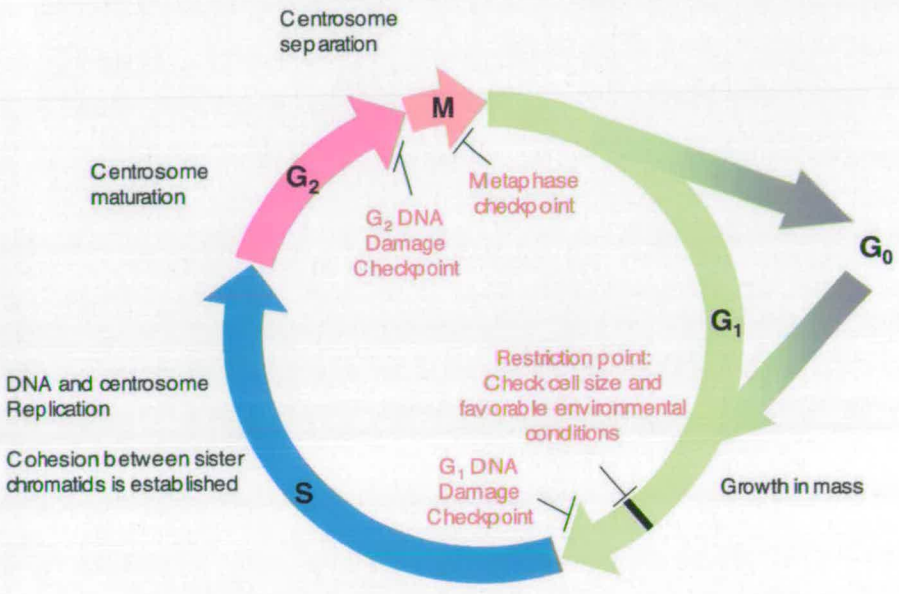


Figure 1.1: The eukaryotic cell cycle.
The arrows indicate the relative lengths of each of the four phases of the cell cycle in cultured cells. The checkpoints are indicated in red text.

## *1.3. Regulation of the cell cycle by cyclin dependant kinases*

Cyclin dependant kinases (CDKs) are a family of serine/threonine kinases that regulate key transitions through the cell cycle and are activated by forming complexes with activating subunits called cyclins (recently reviewed in

(Malumbres and Barbacid, 2005)). CDKs are also regulated by phosphorylation and by binding of inhibitory subunits. Cyclins are regulated by ubiquitin-mediated proteolysis (Glotzer et al., 1991; Hershko et al., 1991). Different cyclin-CDK complexes are active at different phases of the cell cycle (Cross, 1988; Hadwiger et al., 1989; Nash et al., 1988). CDKs trigger their effects by phosphorylating key substrates, which in turn drive the cell cycle forward.

Progression through the cell cycle is under the control of the checkpoint machinery, which delays the cycle in response to checkpoint activation (Murray and Kirschner, 1989; Painter and Young, 1980; Weinert and Hartwell, 1988). Many checkpoints work by inhibiting CDK activity.

## *1.4. Mitosis*

Mitosis, first described by Flemming in 1882, is divided into five distinct stages (Figure 1.2): prophase, prometaphase, metaphase, anaphase and telophase (Flemming, 1965).

Prophase is defined by the onset of chromosome condensation, which results in the formation of two condensed sister chromatids paired along their length. Duplicated centrosomes begin to separate, forming two radial asters. In late prophase cyclin B suddenly accumulates in the nucleus.

The beginning of prometaphase is defined by nuclear envelope breakdown. During prometaphase, chromosomes begin to attach to microtubules emanating from the separating centrosomes. This stage of the cell cycle is characterised by the presence of $APC^{Cdc20}$ and active cyclin B-CDK1. The activity of the APC (anaphase promoting complex) is under the control of the spindle assembly checkpoint (see section 1.5.4).

Metaphase is defined as the point at which all the chromosomes have attained a bipolar attachment to the mitotic spindle. When all the kinetochores are correctly attached, the spindle assembly checkpoint is silenced, permitting

the full activation of the APC. This leads to the destruction of Securin, which leads to separation of sister chromatids, and destruction of cyclin B, whose destruction is necessary for mitotic exit, nuclear envelope reformation and cytokinesis (Pollard and Earnshaw, 2002).

During anaphase the sister chromatids are separated first by moving towards the poles (anaphase A) and secondly by movement of the poles apart from each other (anaphase B).

During telophase, the nuclear envelope begins to reform on the surface of the chromatin, which begins to decondense. In parallel the mitotic spindle disassembles. At this stage $APC^{cdc20}$ is replaced with $APC^{cdh1}$, which remains active through the subsequent $G_1$ phase until passage of the restriction point.

At the final stage of M-phase, called cytokinesis, a contractile ring of actin and myosin forms on the cell cortex and constricts the equator of the cell, dividing the cytoplasm of the cell into two, and eventually forming a thin intercellular bridge containing a dense array of microtubules called the midbody (reviewed by (Pines and Rieder, 2001)).

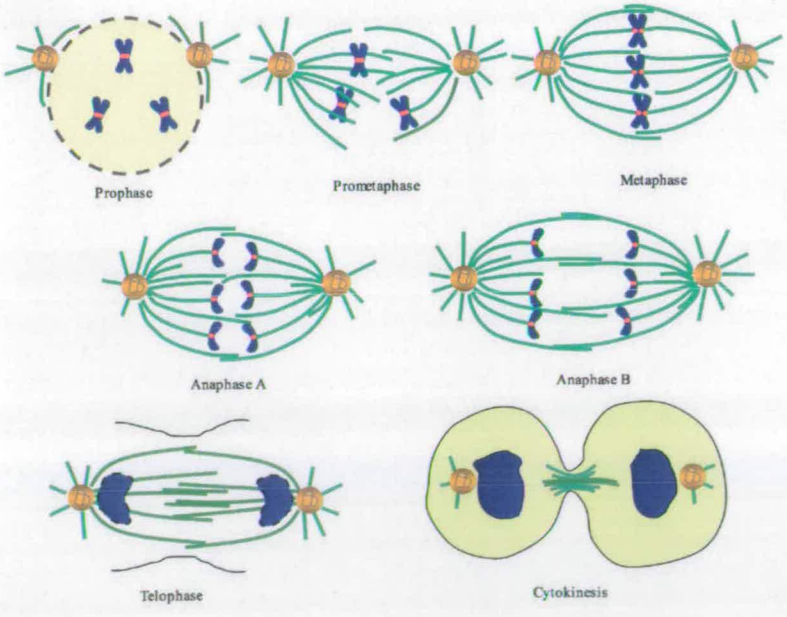


Figure 1.2: The stages of mitosis.
Microtubules are shown in green, DNA is shown in blue and kinetochores of condensed chromosomes are shown in red.

## *1.5. The Mitotic Apparatus*

### 1.5.1. Mitotic chromosome formation

In eukaryotes, DNA compaction is essential to ensure that the chromosomes are segregated properly during anaphase. In order to efficiently segregate, the two sister chromatids must be resolved from each other, and sister chromatid cohesion must be maintained until anaphase onset.

In order to form mitotic chromosomes, cells must compact their DNA 10,000 fold in length compared to naked DNA. The first level of compaction is achieved by wrapping the DNA around the core histones to form nucleosomes. The nucleosomes are further compacted into a 30nm fibre by interactions involving the N-terminal tails of core histones plus linker histone H1 (recently reviewed in (Robinson and Rhodes, 2006). Together these two levels of compaction account for about a 40-fold compaction compared to naked DNA. Levels of compaction above this, including the condensation of the mitotic chromosomes, are poorly understood. The condensin complex can introduce positive supercoils into circular DNA *in vitro* (Kimura and Hirano, 1997; Kimura et al., 1999) and was shown to be essential for chromosome condensation *in vitro* in *Xenopus* egg extracts (Hirano and Mitchison, 1994). The condensin complex was therefore thought to be responsible for compacting mitotic chromosomes. However, more recent studies have shown that when subunits of the condensin complex are depleted by genetic rather than biochemical means, mitotic chromosomes can still achieve an almost normal level of compaction (Hagstrom et al., 2002; Hudson et al., 2003; Steffensen et al., 2001). Therefore additional factors must be required for the compaction of mitotic chromosomes. Interestingly, condensin may be required for maintaining the structural integrity of chromosomes (Hagstrom et al., 2002; Hudson et al., 2003).

Sister chromatid cohesion is achieved by two mechanisms. During DNA replication the sister chromatids become intertwined (catenated). This intertwining is resolved by topoisomerase II. Decatenation is normally completed before anaphase and is required for the separation of sister chromatids (DiNardo et al., 1984). Cell-cycle-regulated sister chromatid cohesion is provided by the Cohesin complex which creates a protein-mediated linkage between sister chromatids. The Cohesin complex is loaded onto the replicated sister chromatids during S-phase and has been suggested to form a ring around the two sister molecules of DNA (Gruber et al., 2003; Uhlmann and Nasmyth, 1998). In metazoans, Cohesin is lost from the chromosome arms in a manner dependant on Aurora-B and PLK1 phosphorylation during prophase (Gimenez-Abian et al., 2004; Losada et al., 2002). Cohesin remains at the centromere until the metaphase–anaphase transition. Cleavage of the Sccl/Mcd1 subunit of the Cohesin complex by Separase triggers sister chromatid separation (Hauf et al., 2001; Uhlmann et al., 2000).

### 1.5.2. The Centromere

The centromere is a specialised chromosomal region that is necessary for chromosome segregation during mitosis and can be recognised cytologically as the primary constriction of mitotic chromosomes. The centromere is the final point of attachment of the sister chromatids and is therefore involved in the regulation of sister chromatid cohesion. The centromere acts as a platform on which the kinetochore forms during mitosis (Craig et al., 1999; Pluta et al., 1995).

Human centromeres are made up of large tandem arrays of repetitive DNA called α-satellite DNA, 0.3 to several megabases in length. These arrays are composed of tandemly repeated units of 171 base pairs, which are arranged into higher order repeats (reviewed by (Masumoto et al., 2004)). For a time, it

was thought that α-satellite DNA specified the location of the centromere since normal human centromeres all contain α-satellite DNA. However, analysis of neocentromeres shows that kinetochores can assemble on DNA that is not usually centromeric, and which does not consist of α-satellite DNA (du Sart et al., 1997). This suggests that α-satellite DNA may not be necessary for kinetochore formation. However, it is essential for the *de novo* formation of human artificial chromosomes when naked DNA is transfected into HT1080 cells (reviewed by (Masumoto et al., 2004)).

There are dramatic divergences in the sequence of centromeric DNA between organisms. Since there does not seem to be a requirement for specific centromeric DNA sequences, there must be some other mechanism that specifies kinetochore formation. One hypothesis is that kinetochore formation is epigenetically controlled. Epigenetic inheritance is the transmission of information from the mother cell to the daughter cell without the information being encoded in the nucleotide sequence. Epigenetic information is typically encoded by DNA methylation and histone modifications. Different combinations of modifications have been suggested to form a "histone code". Such covalent modifications generate another level of genetic regulation by influencing binding sites for specific proteins that recruit factors that affect transcriptional activation or silencing.

One common characteristic of all centromeres in all organisms studied so far is the replacement of histone H3 with CENP-A. CENP-A is a histone variant, which replaces Histone H3 at the inner kinetochore plate of active centromeres (Sullivan et al., 1994; Warburton et al., 1997). Analysis of chromatin fibres from human and *Drosophila* revealed that CENP-A nucleosomes are interspersed with Histone H3-containing nucleosomes (Sullivan and Karpen, 2004). In three dimensions, it is thought that the CENP-A containing nucleosomes face towards the kinetochore, whereas the Histone H3 containing nucleosomes are on the

inner side of the chromatid where the sister chromatids are joined (Figure 1.3) (Blower et al., 2002). Studies looking for epigenetic marks that are associated with CENP-A containing nucleosomes found H3K4diMe-containing nucleosomes, an epigenetic mark usually associated with euchromatin. This has been taken to indicate that centromeres have a special type of chromatin, termed centrochromatin (Sullivan and Karpen, 2004).

In organisms with complex regional centromeres (such as *S. pombe*, *Drosophila* and humans) CENP-A-containing chromatin is usually flanked by heterochromatin, called pericentric heterochromatin that is marked by the presence of high levels of H3K9triMe. During mitosis, this pericentric heterochromatin is also hypoacetylated on H3 and H4 (Sullivan and Karpen, 2004). The H3K9triMe modification is important for the recruitment of HP1/Swi6 (Jacobs et al., 2001). HP1 together with the RNAi machinery promotes heterochromatin formation (reviewed by (Pidoux and Allshire, 2005). Formation of pericentric heterochromatin is essential for the recruitment of Cohesin to pericentric regions (Bernard et al., 2001; Guenatri et al., 2004).

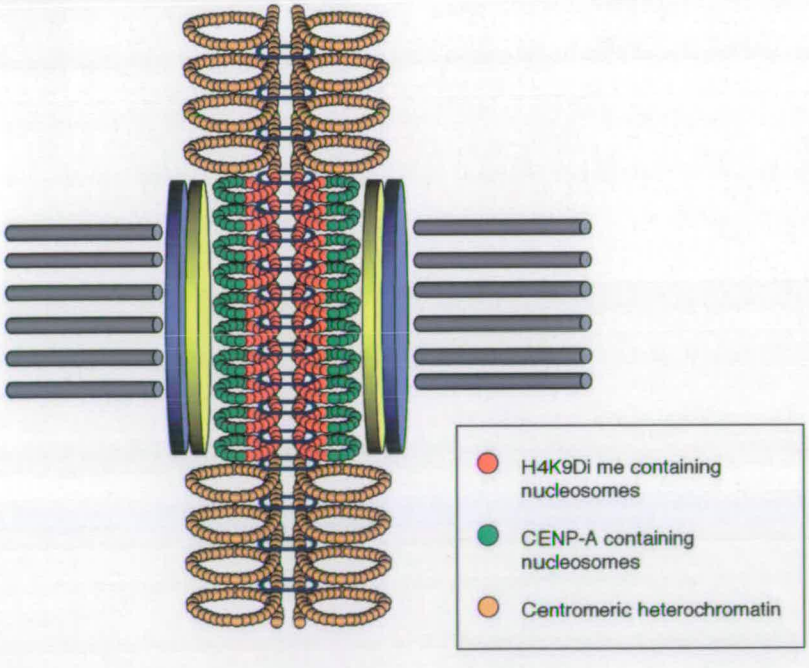


Figure 1.3: 3D organization of chromatin at the centromeres.
The blocks of CENP-A and H3 containing chromatin are orgainsed in such a way that the CENP-A containing nucleosomes are adjacent to the kinetochore. The inner and outer kinetochore plates are shown as yellow and purple disks respectively, the microtubules are shown in grey and the Cohesin rings are indicated in blue.

### 1.5.3. The Kinetochore

The kinetochore is a proteinaceous structure that forms on the centromeric DNA during mitosis. It is the point where the spindle microtubules attach to the chromosome and it regulates the movement of chromosomes during anaphase. The kinetochore is also involved in the spindle assembly checkpoint.

The structure of the kinetochore was first described in the 1960s using electron microscopy (Brinkley and Stubblefield, 1966; Jokelainen, 1967). The heterochromatin underlying the kinetochore shows up as an electron dense mass. In conventional thin-section electron microscopy, the kinetochore is seen as a trilaminar plate, which consists of an electron-dense outer plate separated by an electron-translucent interzone from a second electron-dense inner plate (Figure 1.4). However this interzone is smaller or absent using more recently developed methods of cryo-fixation (McEwen et al., 1998). The inner plate is associated with the surface of the centromeric heterochromatin and contains both proteins and DNA. These proteins are constitutively localised at the centromere throughout the cell cycle. The outer plate is formed mostly of proteins (Cooke et al., 1993). Microtubule plus-ends terminate at the outer plate and in their absence a fibrous corona is seen (Brinkley and Stubblefield, 1966; Jokelainen, 1967)(See Figure 1.4).

The outer kinetochore assembles during mitosis and is highly dynamic. The components of the outer kinetochore change depending on whether microtubules are bound.

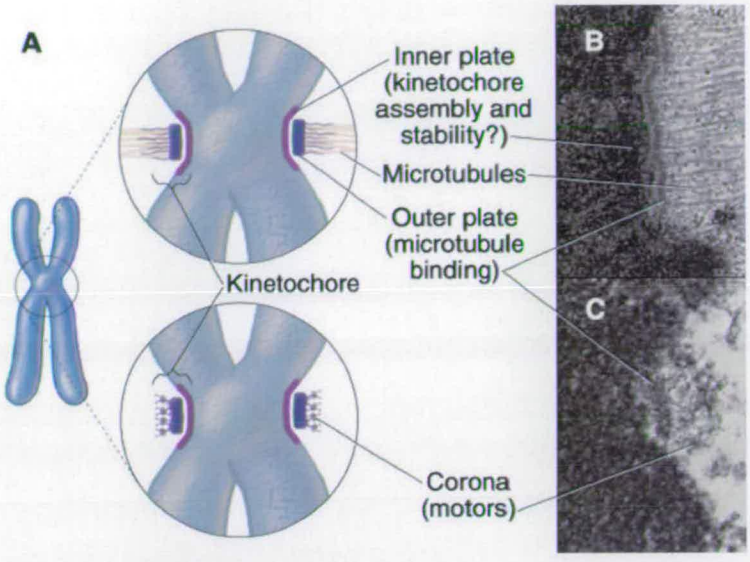


Figure 1.4: Structure of the kinetochore.
(A) Drawing illustrating the primary constriction of a mitotic chromosome and the trilaminar plate of the kinetochore. (B) Electron micrograph of a kinetochore with attached microtubules and without (C). (from (Pollard and Earnshaw, 2002)).

### 1.5.4. Spindle assembly checkpoint and Kinetochore microtubule attachments

A variety of mechanisms help the cell to biorient its chromosomes. During prometaphase, microtubules display what is know as "dynamic instability" (Mitchison and Kirschner, 1984). Microtubules grow and shrink until they come in contact with a kinetochore. This process is known as "search and capture", and is probably guided by a gradient of Ran-GTP or another factor, which emanates from the chromosomes (Kalab et al., 2002; Li and Zheng, 2004c). Microtubule bundles can also be generated by nucleation at the kinetochore. These microtubules grow outwards, are captured by astral microtubules and transported to the poles (Maiato et al., 2004).

Chromosomes that are attached via one kinetochore to a spindle pole are said to be mono-orientated or monotelically attached. Once the sister kinetochore is captured by the opposite spindle pole, then the chromosome is said to be bi-orientated or amphitelically attached. During the process of

kinetochore – microtubule attachment, errors can occur. These must be corrected before chromosome segregation, or aneuploid progeny are produced (Figure 1.5). Chromosome capture is a stochastic process, and it is therefore necessary for the cell to delay the onset of anaphase until all the kinetochores are biorientated.

The spindle assembly checkpoint is an evolutionarily conserved process that helps to ensure the fidelity of chromosome segregation by preventing cell cycle progression until all the chromosomes make proper bipolar attachments. A single unattached chromosome is sufficient to activate the spindle assembly checkpoint by producing a "wait anaphase" signal that emanates from the kinetochore (Rieder et al., 1995). The "core" proteins involved in the spindle assembly checkpoint were discovered in yeast. These include Mad1, Mad2, Mad3 (BubR1 in vertebrates), Bub1, Bub3 and Mps1 (Hoyt et al., 1991; Li and Murray, 1991; Weiss and Winey, 1996). Other proteins involved in the spindle assembly checkpoint have been discovered in higher eukaryotes. These include CENP-E, Aurora-B, Zw10, and Rod (reviewed by (Musacchio and Hardwick, 2002)).

There is much debate in the literature as to whether the error that the cells sense is due to lack of attachment or lack of tension. It was shown that laser ablation of a single unattached kinetochore overcomes the mitotic checkpoint. This suggests that it is lack of attachment that is important (Rieder et al., 1995). However, in meiosis, experiments using micromanipulation on insect spermatocytes showed that when tension was exerted on kinetochores of an unpaired chromosome, the spindle checkpoint was silenced (Li and Nicklas, 1995). It seems that the cells may monitor both the attachment of microtubules to kinetochores and the tension that is exerted across the sister kinetochores when chromosomes are biorientated (reviewed by (Pinsky and Biggins, 2005)).

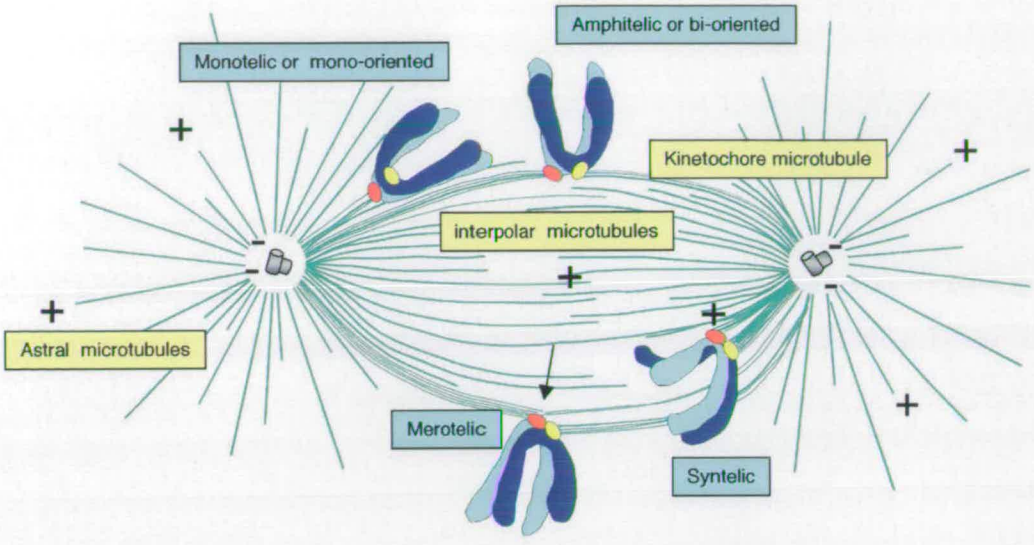


Figure 1.5: The mitotic spindle and kinetochore – microtubule attachments.
Sister kinetochores are shown in red and yellow. Note the kinetochore that is attached to both spindle poles (arrow). The polarity of the microtubules are shown with the minus end at the centrosome. The blue boxes show the various types of attachments between the kinetochore and the spindle. The yellow boxes shown the different subsets of spindle microtubule.

Syntelically attached chromosomes are attached by both kinetochores to the same spindle pole and therefore are not under tension (Figure 1.5). Syntelic attachments are not necessarily errors, and may simply reflect an early stage of chromosome attachment that must be corrected before anaphase in order to avoid aneuploidy. Inhibition or depletion of Aurora-B results in an increase in syntelic attachments and bypass of the spindle assembly checkpoint under conditions where tension within the spindle is compromised (Hauf et al., 2003). Similarly, the yeast Aurora kinase, Ipl1, is required to destabilise incorrect kinetochore-microtubules attachments (Tanaka et al., 2002) and to maintain the spindle assembly checkpoint in the absence of tension (Biggins and Murray, 2001). It is possible that the checkpoint does not directly sense a lack of tension. The chromosomal passenger complex may act as part of a tension sensitive mechanism, which creates unattached kinetochores and therefore activates the spindle assembly checkpoint.

Aurora-B may act in concert with the microtubule-depolymerising Kinl kinesin, MCAK, to destabilise kinetochore-microtubule attachments that are not under tension by creating unattached kinetochores (see section 1.9.2).

It has been proposed that CENP-E is the attachment sensitive sensor at the kinetochore that transduces the signal from microtubule capture to the spindle assembly checkpoint components (Mao et al., 2005). However, yeasts lack CENP-E yet they have fully functional checkpoints.

The spindle assembly checkpoint inhibits the APC by binding to the cdc20 subunit of the APC (Hwang et al., 1998; Kim et al., 1998). The APC is a multisubunit E3 ubiquitin ligase. A key target of the APC is Securin, a protein that binds Separase and inactivates it, thereby blocking cleavage of the Cohesin subunit Scc1. Separase is kept inactive both by its association with Securin and by phosphorylation by CDK1/Cyclin B. Both Cyclin B and Securin are targets of the APC. They are ubiquitinated by APC and degraded via the 26S proteasome (reviewed by (Nasmyth, 2005)).

A merotelic attachment is formed when one (or both) kinetochore(s) is attached to microtubules from opposite spindle poles (Figure 1.5). This attachment error is not detected by the spindle assembly checkpoint. The merotelically-attached kinetochore(s) cannot move because it is being simultaneously pulled towards both spindle poles. However, the kinetochore and its attached microtubules are under normal tension. Although merotelic attachments are the most common source of aneuploidy in vertebrate cultured cells (Cimini et al., 2001), there seem to be mechanisms that correct these errors. One mechanism that functions before anaphase onset to reduce the number of microtubules connected to the wrong pole may involve Aurora-B (Cimini et al., 2003a). A second mechanism that functions during anaphase to move the chromatid to the correct pole is based on the ratio of correct to incorrect attachments and could be as simple as a 'tug of war' where the

strongest side wins (Cimini et al., 2001; Cimini et al., 2003a; Salmon et al., 2005).

### 1.5.5. Centrosomes and the mitotic spindle

The centrosomes are the major site for microtubule organisation in most animal cells. In most cases the centrosome consists of two barrel shaped centrioles surrounded by a matrix of proteins called the pericentriolar material.

Centrosome duplication is unusual because it takes more than one complete cell cycle. In early $G_1$ have a single centrosome, made up of a mother and a daughter centriole. The mother centriole was assembled at least two cell cycles previously and the daughter was assembled in the preceding S-phase. The mother has subdistal appendages at the distal end of the centriole. During $G_1$ the mother and daughter move apart slightly. In early S phase, the two centrioles begin a process by which they each form daughter centrioles. The daughter centriole goes through a process of maturation, acquiring subdistal appendages, and is biochemically equivalent to the mother centriole by the end of $G_2$. In prophase, the two centrosome pairs separate and help organise the bipolar spindle during mitosis (Figure 1.6). After cytokinesis, each daughter cell inherits one centrosome pair (recently reviewed in (Delattre and Gonczy, 2004)).

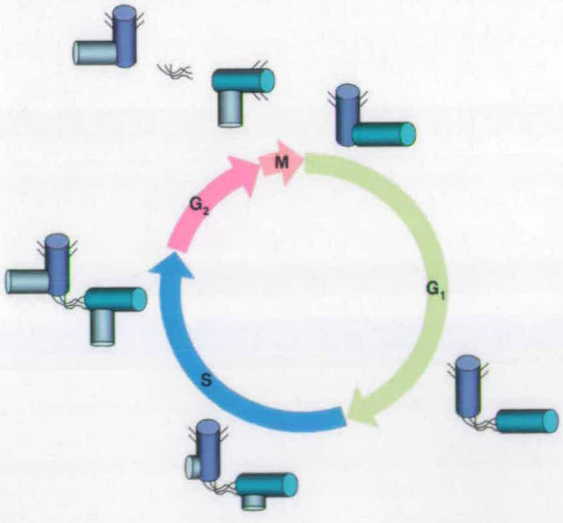


Figure 1.6: The centrosome duplication cycle. In early $G_1$ the cell has a single centrosome made up of a mother centriole (purple) and a daughter centriole (green). The mother has subdistal appendages at the distal end of the centriole (shown as black lines) and the daughter centriole acquires subdistal appendages by the end of G2. The newly forming centrioles, which will become the daughter centrioles in the next cell cycle, are shown in light blue.

Three classes of microtubules are present in the mitotic spindle (Figure 1.5). Astral microtubules project from the spindle poles towards the cell cortex. They help to position the spindle within the cell by interaction of the microtubule plus ends with components at the cell cortex. Kinetochore microtubules have their minus ends at the spindle poles and their plus ends attached to the kinetochores. Human kinetochores typically bind about 20 microtubules each. These microtubules tend to form a microtubule bundle called a kinetochore fibre. Kinetochore microtubules control the movement of the chromosomes during mitosis. Interpolar microtubules emanate from the spindle poles toward the cell equator. Some form interdigitating antiparallel bundles, which prevent the spindle from collapsing on itself and, together with pulling by cortical dynein on astral microtubules, contribute to anaphase B movement through the action of plus end directed kinesins. During anaphase, an organised spindle midzone forms by bundling and stabilisation of these overlapping microtubules (recently reviewed in (Kline-Smith and Walczak, 2004)).

## *1.6. Cytokinesis*

Cytokinesis is the division of the cell into two at the end of mitosis. In animals, this process is carried out by a contractile ring composed of myosin II and actin filaments. Constriction of the actomyosin ring results in a cleavage furrow as the cell begins to pinch in two.

The astral microtubules as well as the spindle midzone microtubules have been implicated in positioning of the cleavage furrow, possibly by delivering a signal that originates elsewhere in the cell. In many systems, astral microtubules elongate towards the equatorial cortex at anaphase, apparently triggering furrow ingression when they touch the cortex. The relative importance of the class of microtubule required to induce furrowing depends on the cell

type. In most cells, the cleavage furrow forms perpendicular to the axis of chromosome segregation, midway between the reforming nuclei (Eggert et al., 2006).

What is the nature of the signal that is delivered to the cell cortex and initiates furrowing? The small GTPase RhoA is thought to be a key regulator determining the site of the cleavage furrow. Several RhoA regulators, including Ect2 (a GEF for RhoA) and MgcRacGAP, accumulate at the cleavage furrow. RhoA regulates both myosin II activation, via ROCK and citron kinase, and polymerisation of actin (Glotzer, 2005).

Furrow ingression is achieved by force generation via the actomyosin ring and an increase in the plasma membrane surface area. After the cleavage furrow has fully ingressed, an intercellular bridge packed with bundled microtubules is formed. The final step of cytokinesis involves delivery of membrane vesicles in order to fully separate the two daughter cells (Gromley et al., 2005).

## *1.7. Meiosis*

Meiosis is a specialised form of cell division that occurs in sexually reproducing organisms. Meiosis involves two rounds of cell division – Meiosis I and Meiosis II without an intervening S phase (Figure 1.7). During meiosis I homologous chromosomes pair and recombine. The paired homologues are held together by structures on chromosome arms called chiasmata that represent the sites where recombination occurred. During meiosis I, and in contrast to mitosis, sister chromatids remained paired and kinetochores of sister chromatids attach to the same pole. Cohesion is destroyed between sister chromatid arms at the onset of anaphase I allowing segregation of homologues. Cohesion at centromeres persists until anaphase II onset, so sister chromatids remain paired. Meiosis II is similar to mitosis in many ways; cohesion between

sister centromeres is destroyed at metaphase II, and sister chromatids are segregated (Petronczki et al., 2003).

The Cohesin complex is required to hold sister chromatids together until anaphase in mitosis and anaphase II in meiosis. In mitosis, centromeric Sccl is cleaved by Separase at anaphase onset (Uhlmann et al., 1999). In meiosis, Sccl is replaced by Rec8. In Meiosis I, Rec8 is cleaved along the chromosome arms but not at centromeres, allowing separation of homologues, but keeping the sister chromatids together thanks to cohesion at the centromere. Centromeric cohesion persists until anaphase II onset when sister chromatids separate (recently reviewed in (Watanabe, 2005a), . Recent studies have established an important role for the MEIS-332/Shugoshin family of proteins in protecting centromeric cohesion in mitosis and meiosis (Katis et al., 2004; Kerrebrock et al., 1992; LeBlanc et al., 1999; Marston et al., 2004; Moore et al., 1998).

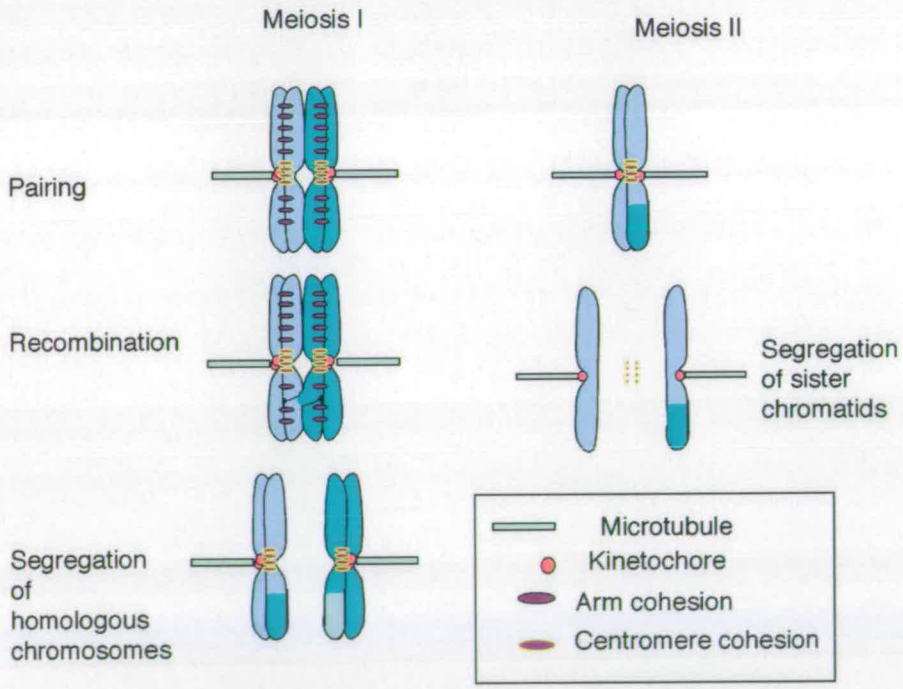


Figure 1.7: The stages of meiosis.

## *1.8. Chromosomal passenger proteins*

The chromosomal passenger proteins are a group of proteins that display a distinctive pattern of localisation through the cell cycle. They start accumulating in the nucleus in G2. From prophase to metaphase, they are concentrated at the inner centromere/pairing domain, until the onset of anaphase where they transfer to the spindle midzone. In late anaphase, they are found at the equatorial cortex and central spindle which becomes the midbody during cytokinesis (Adams et al., 2001a; Vagnarelli and Earnshaw, 2004).

Their dynamic localisation during mitosis to the chromosomes and then to the cytoskeleton was thought to reflect a potential role in coordination of chromosomal and cytoskeletal events (Earnshaw and Bernat, 1991). Subsequent studies have found that the chromosomal passenger proteins are key regulators of mitotic events and that their precisely choreographed movement through mitosis is important for their proper function during mitosis. In line with their dynamic localisation and expected role in multiple aspects of mitosis, many studies in various organisms using diverse techniques have revealed that interfering with chromosomal passenger function leads to multiple defects in mitosis. This group of proteins currently comprises six proteins in mammals: INCENP, Aurora-B, Aurora-C, Survivin, TD-60 and Borealin. A complex is known to form compromising Aurora-B, INCENP, Survivin and Borealin (see chapter 4, figure 4.1). The active component of this complex is Aurora-B, a serine-threonine kinase.

### 1.8.1. Aurora-B

Aurora-B is a serine-threonine kinase, belonging to the Aurora kinase family, which is conserved in all eukaryotes. Yeasts have a single Aurora kinase (Ipl1 in *S. cerevisiae* and Ark1 in *S. pombe*). *Drosophila* has two Aurora kinases

(Aurora-A and Aurora-B). There are three Aurora kinases in mammals: Aurora-A, -B, and -C. Aurora-A kinases are believed to be involved in spindle assembly, whereas Aurora-B and C kinases are chromosomal passenger proteins. Aurora-C kinases are highly expressed in testis and some cancer cell lines and are discussed further below.

Aurora-B kinase activity and expression levels peak in mitosis (Bischoff et al., 1998). Another chromosomal passenger, INCENP, has been shown to be both a substrate and an activator of Aurora-B. Aurora-B phosphorylates INCENP in the conserved IN-box (the Aurora-B-binding domain). This phosphorylated form of INCENP then enhances the activity of the kinase, forming a positive feedback loop (Bishop and Schumacher, 2002; Honda et al., 2003; Sessa et al., 2005). There are conflicting reports as to whether Survivin stimulates Aurora-B kinase activity (Bolton et al., 2002; Chen et al., 2003; Honda et al., 2003).

The kinase activity of Aurora-B is important for the function of the chromosomal passenger complex. Studies using kinase dead mutants or small molecule inhibitors show that the kinase activity is essential for chromosome congression, spindle checkpoint arrest induced by microtubule drugs, and cytokinesis (Ditchfield et al., 2003; Hauf et al., 2003; Murata-Hori et al., 2002b; Murata-Hori and Wang, 2002a). The kinase activity of Aurora-B is not required for its localisation to centromeres. In cells expressing low levels of exogenous kinase-dead Aurora-B and cells treated with the Aurora-B inhibitor, ZM447439, the Aurora-B is able to localise to the centromeres. However, in cells overexpressing kinase-dead Aurora-B the chromosomal passenger complex is mislocalised presumably due to a dominant negative effect (Ditchfield et al., 2003; Honda et al., 2003).

A consensus phosphorylation motif for Ipl1, the *S. cerevisiae* Aurora kinase has been described as: (R/K)X(T/S)(I/L/V) (Cheeseman et al., 2002). In

metazoans, the phosphorylation sites that have been mapped are usually preceded by one or two basic residues [$(R/K)_{1-3}X(S/T)$] (Meraldi et al., 2004).

### 1.8.2. Aurora-C

Although initial results suggested that Aurora-C localised to centrosomes in anaphase and telophase, it has now been shown to be a chromosomal passenger protein (Li et al., 2004a; Sasai et al., 2004). It is highly expressed in testis, ovary, placenta, lung, and other tissues (Yan et al., 2005), but is also detectable in cell lines from different human tissues at a lower level than that of Aurora-B (Sasai et al., 2004). Aurora-C binds Survivin (Yan et al., 2005) and INCENP (Li et al., 2004a). INCENP binds and activates Aurora-C via the IN-box (Li et al., 2004a). Aurora-C competes with Aurora-B for binding to INCENP, however INCENP binds preferentially to Aurora-B (Sasai et al., 2004). Depletion of Aurora-C caused multinucleation in HeLa cells similar to that seen when Aurora-B is depleted (Sasai et al., 2004). Aurora-C could partially rescue the multinucleation phenotype induced by Aurora-B depletion (Sasai et al., 2004).

### 1.8.3. INCENP

INCENP (Inner Centromere Protein) was first discovered in a screen using monoclonal antibodies made against the mitotic chromosomal scaffold (Cooke et al., 1987). Analysis of the INCENP protein did not reveal any distinct motifs other than a coiled-coil region (Mackay and Earnshaw, 1993). INCENP homologues are found in all eukaryotes and are characterised by a conserved domain called the IN-box (Adams et al., 2000). Aurora-B binds INCENP via the IN-box and this binding leads to partial activation of the kinase. Aurora-B then phosphorylates the IN-box and the binding of this phosphorylated form fully activates the kinase (Bishop and Schumacher, 2002; Honda et al., 2003; Sessa et al., 2005).

To try to understand the function of INCENP, various truncations have been expressed and this has revealed INCENP to be a complex multidomain protein.

The N-terminus of INCENP has been thought of as the centromere targeting domain since it was shown that the N-terminal 47 amino acids were necessary for centromere targeting (Mackay and Earnshaw, 1993). The N-terminal 68 amino acids (in chicken, *Gallus gallus*) were sufficient to target GFP to centromeres (Ainsztein et al., 1998). $INCENP_{1-68}$-GFP is targeted first to the centromeres and subsequently to the spindle midzone. This region of INCENP contains two evolutionarily conserved motifs (see Chapter 3, Figure 3.2). The motif comprising residues 32-44 is necessary for targeting to the centromere and the motif comprising residues 52-62 is required for targeting to the midbody (Ainsztein et al., 1998). Recent reports have shed new light on the mechanism of how the chromosomal passengers localise to the centromere (see section 1.9.3).

Is centromere targeting of INCENP required for its subsequent transfer to the midzone? Mutation of residues 32-44 which abolishes centromere targeting of chicken INCENP also abolished targeting to the midbody (Ainsztein et al., 1998). It may be that a priming event at the centromere such as a post-translational modification is necessary to release the chromosomal passenger complex from the centromere, and/or it may just be that the position of the chromosomal passenger proteins adjacent to growing midzone microtubules at the centromere is needed to target the chromosomal passenger proteins to the spindle midzone. However, two deletion mutants, Borealin $_{1-141}$ and the C-terminal coiled-coil region of Survivin, can localise to the spindle midzone without prior centromere localisation (Gassmann et al., 2004; Lens et al., 2006).

Targeting of the chromosomal passenger complex may occur via growing midzone microtubules since $INCENP_{48-85}$ binds to β-tubulin and this interaction is required to target INCENP to the equatorial cortex. In cells treated with taxol,

which suppresses microtubule dynamics, INCENP accumulates at the microtubule plus ends where the β-tubulin subunit is exposed (Wheatley et al., 2001a). This also fits with studies on Aurora-B-GFP showing that centromeric Aurora-B was delivered to the midzone microtubules. Photobleaching centromeric Aurora-B-GFP signal caused loss of the Aurora-B-GFP signal at the spindle midzone. In contrast, delivery of cytoplasmic Aurora-B to the equatorial cortex was unaffected (Murata-Hori and Wang, 2002c). In *S. cerevisiae*, dephosphorylation of INCENP by Cdc14p is necessary and sufficient for spindle transfer (Pereira and Schiebel, 2003). However, this has not yet been demonstrated in mammalian cells, which express more than one Cdc14 isoform.

### 1.8.4. Survivin

Survivin belongs to the IAP (Inhibitor of Apoptosis) family of proteins and contains a single BIR domain (Ambrosini et al., 1997). Survivin exists as a dimer *in vitro* (Klein et al., 2006). The crystal structure reveals an N-terminal zinc-binding BIR domain where the two molecules dimerise flanked by C-terminal α-helices which protrude outwards at 110° from each other (Chantalat et al., 2000; Verdecia et al., 2000).

Survivin has recently been implicated in the targeting of the chromosomal passenger complex to centromeres, for details see section 1.9.3 on targeting of the chromosomal passenger proteins to the centromere (Klein et al., 2006; Vader et al., 2006a; Vong et al., 2005). The presence of an intact Survivin BIR domain is necessary (but not sufficient) for centromere targeting and proper spindle checkpoint function in the absence of endogenous Survivin. In contrast, the C-terminal coiled-coil is necessary and sufficient for binding to Borealin, spindle midzone localisation, and execution of cytokinesis in the absence of endogenous Survivin (Lens et al., 2006).

### 1.8.5. Borealin

Borealin was discovered in a proteomics screen for proteins associated with histone-depleted human mitotic chromosomes (Gassmann et al., 2004). Concurrently, the *Xenopus* homologue of Borealin (Dasra-B) and a distant paralogue (Dasra-A) were found in an expression screen for proteins that bound to metaphase chromosomes (Sampath et al., 2004). Sequence analysis of Borealin and Dasra-B did not reveal significant homology to any known functional domain or other protein families (Gassmann et al., 2004; Sampath et al., 2004).

Borealin binds directly and robustly to Survivin, and to a lesser extent to INCENP. Borealin also dimerises *in vitro* (Gassmann et al., 2004), and *in vivo* (Klein et al., 2006). Indeed, Borealin has been suggested to stabilize the interaction between Survivin and INCENP *in vivo* and all the endogenous Survivin in mitotic colcemid-blocked cells is associated with Borealin (Gassmann et al., 2004; Vader et al., 2006a). Immunoprecipitation of Borealin brings down the complete chromosomal passenger complex. The N-terminal half of the protein (aa1-141) is necessary and sufficient to bind Survivin and has a dominant negative effect in cells. This portion of Borealin is able to target to the spindle midzone, but not the centromere. Its expression in cells prevents targeting of the other chromosomal passenger proteins to the centromere. Both halves of Borealin are necessary for INCENP binding, whereas either half on its own was able to dimerise (Gassmann et al., 2004). Therefore it may be that the N-terminal half binds Survivin but is no longer able to stabilise the Survivin-INCENP interaction at the centromere, whilst the whole complex may be necessary to stabilise the interaction with the centromere.

Borealin does not appear to be required for Aurora-B kinase activity *in vitro* and depletion of Borealin does not affect the phosphorylation of Histone H3 *in vivo* (Gassmann et al., 2004). Interestingly, depletion of Borealin caused problems in maintaining a bipolar spindle during anaphase. Live cell imaging

showed that cells formed a normal metaphase plate with a bipolar spindle but upon entry into anaphase, sister chromatids segregated in different directions to ectopic poles (Gassmann et al., 2004).

CSC-1 (Chromosome Segregation and Cytokinesis defective family member) was identified in a genetic screen for mutations affecting chromosome segregation and cytokinesis in *C. elegans* (Romano et al., 2003). The authors were unable to find homologues in species other than *Caenorhabditis briggsae* at the time. However, manual alignment of Borealin proteins with CSC-1 revealed a weak similarity (Gassmann et al., 2004; Sampath et al., 2004). Both CSC-1 and Borealin bind directly to Survivin / Bir-1, however, CSC-1 requires zinc to bind Survivin whereas Borealin does not. Unlike Borealin, CSC-1 does not bind directly to INCENP *in vitro* (Romano et al., 2003).

No homologue of Borealin has yet been found in yeast and it has been suggested by Vader *et al.* (Vader et al., 2006b) that since Bir1p (the Budding yeast Survivin homologue) is considerably larger than its counterpart in higher organisms, it could have diverged during evolution into two polypeptides in higher organisms.

### 1.8.6. TD-60

TD-60 was first described as a chromosomal passenger protein based on its localisation using a human autoantibody (Andreassen et al., 1991; Martineau-Thuillier et al., 1998). Although the localisation has been known for more than a decade, the gene encoding TD-60 has only recently been cloned. TD-60 appears to be a guanosine nucleotide exchange factor (GEF) for Rac1. The GTPase cycle is shown in figure 1.8. Sequence analysis revealed that it is a member of the RCC1 family and contains seven RCC1 domain repeats (Mollinari et al., 2003). However, unlike RCC1, which is a GEF for Ran, TD-60 may serve this role for Rac1.

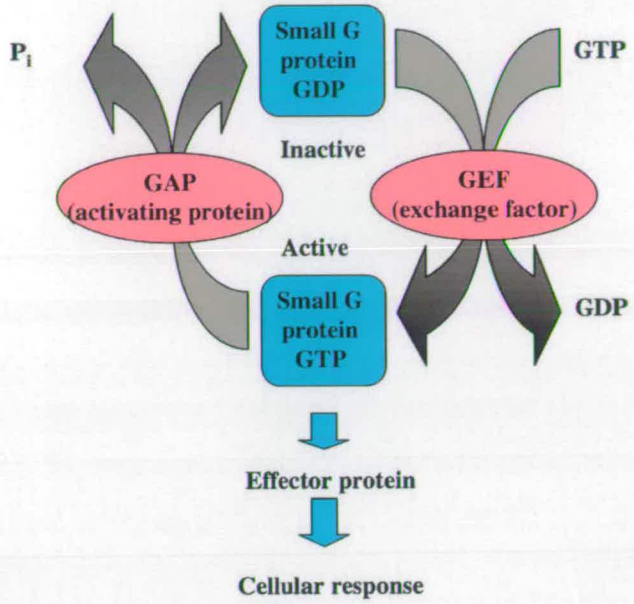


Figure 1.8: The GTPase cycle

Interestingly, in contrast to depletion of the other chromosomal passenger proteins, depletion of TD-60 activated the spindle checkpoint and prevented progression from prometaphase to metaphase, suggesting a possible role for TD-60 in kinetochore microtubule attachment. In line with this, TD-60 can associate directly with microtubules *in vitro* and when depleted, cells showed smaller, more disorganised spindles. It has been suggested that TD-60 may regulate microtubule dynamics (Mollinari et al., 2003).

Survivin and Aurora-B are dependent on TD-60 for their localisation (Mollinari et al., 2003). Given that TD-60 and the other chromosomal passenger proteins are dependent on one another for their localisation it will be of interest to establish if a direct complex forms between TD-60 and the chromosomal passenger complex at any stage of the cell cycle. It currently appears that when cells are arrested as a result of microtubule depolymerization, that no association of TD-60 and the chromosomal passenger complex is seen (Gassmann et al., 2004).

TD-60 has been shown to bind the small G protein Rac1, binding preferentially to the nucleotide free form, which is characteristic of a GEF (Guanosine nucleotide Exchange Factor), however GEF activity has not yet been proven (Mollinari et al., 2003). Downstream effectors of Rac1 include Pak1 and IGQGAP, both of which are implicated in microtubule dynamics (Banerjee et al., 2002; Daub et al., 2001; Fukata et al., 2002).

### 1.8.7. Chromosomal passenger complex

Four of these proteins: INCENP, Aurora-B, Survivin, and Borealin are now known to form a complex (Adams et al., 2001b; Gassmann et al., 2004; Kaitna et al., 2000; Wheatley et al., 2001b). There may also be a subcomplex containing Aurora-B and INCENP. This subcomplex may phosphorylate H3 on serine 10, but its other biological functions are not known (Gassmann et al., 2004). Other forms of the chromosomal passenger complex may also exist. The chromosomal passenger complex and its various roles throughout the cell cycle will now be discussed.

## *1.9. Functions of the chromosomal passenger complex*

### 1.9.1. Chromosome condensation and cohesion

Serine 10 of Histone H3 is phosphorylated during mitosis, a modification that is conserved in all eukaryotes analysed. This modification has been shown to be dependant on Ipl1 in yeast (Hsu et al., 2000), and Aurora-B in metazoans (Adams et al., 2001b; Crosio et al., 2002; Ditchfield et al., 2003; Giet and Glover, 2001; Hauf et al., 2003; Honda et al., 2003; Hsu et al., 2000; Murnion et al., 2001; Speliotes et al., 2000). Phosphorylation of Histone H3 at serine 10 coincides with the onset of chromosome condensation (Wei et al., 1998).

However, there are conflicting reports as to whether H3 phosphorylation affects condensation (Hsu et al., 2000; Jelinkova and Kubelka, 2006; Wei et al., 1999) and whether depletion of chromosomal passenger proteins results in defects in chromosome condensation (Adams et al., 2001b; Giet and Glover, 2001; MacCallum et al., 2002). Therefore the role of Histone H3 phosphorylation and chromosomal passenger proteins in chromosome condensation remains controversial.

A possible function of Aurora-B may be the recruitment of Condensin. Condensin does not localise properly in *S. pombe* cells depleted of Ark1 (Aurora-B) or Bir1 (Survivin) (Morishita et al., 2001; Petersen and Hagan, 2003). The same is seen in cells expressing a kinase-dead Aurora-B mutant, in Aurora-B depleted *Drosophila* cells, and *C. elegans* embryos, at least at metaphase (Giet and Glover, 2001; Hagstrom et al., 2002; Kaitna et al., 2000). However there are conflicting reports as to whether Aurora-B depletion affects recruitment of Condensin. In cells treated with hesperadin, an Aurora-B inhibitor, condensin localises properly (Hauf et al., 2003). Interestingly, INCENP localisation is dependant on condensin in chicken cells (Hudson et al., 2003).

Epigenetic modifications of chromatin are thought to regulate the binding of chromatin proteins and therefore affect gene expression and heterochromatin formation etc. HP1, a protein involved in heterochromatin formation, binds methylated Histone H3 Lysine 9 (H3K9Me). When cells enter mitosis, HP1 leaves the chromosome arms. The mechanism by which this happens was recently suggested to involve phosphorylation of methylated Histone H3K9 on Serine 10 by Aurora-B, a modification that happens only during mitosis. It was reported that this modification is sufficient to disrupt the interaction of Histone H3 and the chromodomain of HP1 (Fischle et al., 2005; Hirota et al., 2005). However, later experiments using both the HP1$\alpha$ chromodomain and the full length HP1$\alpha$ protein showed that phosphorylation was necessary, but not sufficient, to trigger dissociation (Mateescu et al., 2004; Terada, 2006).

Furthermore, it was shown that an H3 peptide methylated on lysine-9, phosphorylated on Serine-10 and acetylated on Lysine-14 (K9meS10pK14Ac) no longer bound HP1α. Therefore it seems that acetylation of Lysine 14 may also be required to dissociate HP1 from the chromosome arms during mitosis. A fraction of HP1α, but not -β or -γ, remains bound to the pericentromeric heterochromatin (Hayakawa et al., 2003; Minc et al., 1999), where it has important functions during mitosis (Obuse et al., 2004; Pidoux and Allshire, 2005). The C-terminal region, including the chromoshadow domain, is necessary for targeting of HP1α to the pericentromeric heterochromatin (Hayakawa et al., 2003). The pericentromeric heterochromatin is hypoacetylated during mitosis (Dunleavy et al., 2005; Sullivan and Karpen, 2004), and this may allow HP1α to bind specifically at the centromere.

INCENP has been shown to bind specifically an H2A variant, H2A.Z (Rangasamy et al., 2003). This histone variant is targeted to pericentric heterochromatin during early mouse development and may be important for the formation of facultative heterochromatin (Rangasamy et al., 2003). It may be involved in establishing a specialized chromatin structure that promotes HP1α binding at pericentric heterochromatin (Fan et al., 2004; Suto et al., 2000 3). Interestingly, when H2A.Z was depleted, this caused chromosome segregation defects. However, H2A.Z was not enriched at the centromere in the mouse and monkey cell lines used in this study (Rangasamy et al., 2004). INCENP has also been shown to bind HP1α and depletion of HP1α also causes chromosome segregation defects (Ainsztein et al., 1998; Obuse et al., 2004).

The chromosomal passenger proteins may also play a role in sister chromatid cohesion. INCENP and Survivin localisation is dependant on Cohesin (Morrison et al., 2003; Sonoda et al., 2001) and loss of INCENP from the inactive centromere of a dicentric chromosome correlates with loss of sister chromatid cohesion (Vagnarelli and Earnshaw, 2001). The chromosomal

passenger proteins clearly play a role in sister chromatid cohesion in meiosis. *Drosophila incenp* mutants have severe defects in sister chromatid cohesion in meiosis I (Resnick et al., 2006). In *C. elegans*, AIR-2 (Aurora-B) has been shown to regulate the release of cohesion on chromosome arms in meiosis (Kaitna et al., 2002; Rogers et al., 2002). Aurora-B also contributes, together with Plk1, to release of cohesion between sister-chromatid arms in mitotic prophase/prometaphase (Gimenez-Abian et al., 2004; Losada et al., 2002).

### 1.9.2. Chromosomal passenger proteins and kinetochore function

The chromosomal passenger proteins have been shown to be important for chromosome alignment and segregation in many different organisms from yeast to mammals, using a variety of techniques including temperature sensitive alleles, RNAi, mouse knockouts, dominant negative mutants and small molecule inhibitors (Adams et al., 2001b; Carvalho et al., 2003; Chan and Botstein, 1993; Cutts et al., 1999; Ditchfield et al., 2003; Francisco et al., 1994; Gassmann et al., 2004; Hauf et al., 2003; Honda et al., 2003; Kaitna et al., 2000; Kim et al., 1999; Mackay et al., 1998; Mollinari et al., 2003; Murata-Hori and Wang, 2002a; Romano et al., 2003; Sampath et al., 2004; Schumacher et al., 1998; Uren et al., 2000).

These defects in chromosome alignment suggested that the chromosomal passenger proteins are required for chromosome bi-orientation. Indeed, it was discovered first in *S. cerevisiae,* that Ipl1 was required for activating the spindle checkpoint in response to lack of tension, but not lack of attachment (Biggins and Murray, 2001). The same phenotype has also been observed in vertebrate cells (Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003; Kallio et al., 2002; Lens et al., 2003; Murata-Hori and Wang, 2002a; Petersen and Hagan, 2003).

Studies in *S. cerevisiae* first showed that Ipl1 and Sli15 are required for the correction of syntelic attachments and the authors proposed that biorientation and therefore tension may cause a separation between the Ipl1/Sli15 and its substrates, thereby affecting kinetochore-microtubule attachments (Tanaka et al., 2002). It has been suggested that the chromosomal passenger proteins do not directly inhibit the APC. They can however communicate lack of tension to the checkpoint machinery, possibly by creating unattached kinetochores.

In vertebrates, Aurora-B has been shown to regulate the localisation and kinesin activity of MCAK, a centromere associated kinesin (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004).

MCAK depletion in *Xenopus* extracts has been shown to affect chromosome alignment and anaphase A movement (Maney et al., 1998; Walczak et al., 2002). When sister kinetochores are not under tension (mono-oriented or syntelically attached), MCAK and Aurora-B are concentrated at the inner centromere. This allows Aurora-B to phosphorylate MCAK, thus deactivating its kinesin activity and stabilising microtubule attachments. Once the sister kinetochores are biorientated and under tension there is a tension-dependant separation of Aurora-B and MCAK (Andrews et al., 2004; Lan et al., 2004). In *Xenopus* egg extracts, the chromosomal passenger complex is important for chromatin-induced microtubule stabilisation via a pathway that is distinct from the Ran GTP pathway and involves MCAK (Sampath et al., 2004).

Another protein that is present at the inner centromere and which may be important for MCAK function at the centromere is ICIS (inner centromere Kinl stimulator). ICIS coimmunoprecipitates with MCAK, INCENP and Aurora-B in *Xenopus* extracts. Its localization to the inner centromere is dependant on MCAK and it stimulates MCAK activity *in vitro*. ICIS may stimulate MCAK by improving its processivity by anchoring it to the microtubule. ICIS binds microtubules independently of MCAK. ICIS may therefore be important for the

relocalisation of MCAK by bringing MCAK along with it on the depolymerising microtubule (Ohi et al., 2003). The precise relationship between Aurora-B, INCENP, MCAK and ICIS remains to be resolved.

An important factor that has not been studied in detail yet is the correlation between the level of INCENP on the centromere as seen by immunofluorescence and the attachment status of the kinetochore (Vagnarelli and Earnshaw, 2004). Aurora-B kinase activity is also important for the correct localisation of CENP-E and dynein to the kinetochore, but they have not been shown to be substrates of Aurora-B (Murata-Hori and Wang, 2002a).

### 1.9.3. Targeting of the chromosomal passenger proteins to the centromere

The exact mechanism of how the chromosomal passenger proteins are targeted to the centromere is not yet fully understood. There are some clues however. In mammalian cells, ubiquitinating and deubiquitinating enzymes regulate chromosome alignment and segregation. Ubiquitination of Survivin, by Ufd1, is required to target Survivin, and therefore the other chromosomal passenger proteins to the centromere. Thereafter, deubiquitination, by hFAM, is required to dissociate Survivin from centromeres (Vong et al., 2005). Histone deacetylase inhibitors (e.g. FR901228) cause a delay in phosphorylation of Ser10 and Ser28, concomitant with a decrease in pericentromeric Aurora-B in premitotic cells. However, the reason why this occurs is not known (Robbins et al., 2005). The *S. cerevisiae* Survivin homologue, Bir1p, binds the centromere protein Ndc10 (Yoon and Carbon, 1999), which is also a substrate of Ipl1 (Biggins et al., 1999). However, the mammalian homologue of Ndc10 has yet to be identified.

Exogenous truncated forms of INCENP expressing only the N-terminal region have previously been shown to localise to centromeres. As a result, the INCENP N-terminus was thought to mediate targeting of the complex to

centromeres (Ainsztein et al., 1998). Survivin has recently been implicated in the targeting of the complex to centromeres. In an elegant study carried out by Vader *et al.* (Vader et al., 2006a), it was shown that Survivin binds the N-terminus of INCENP and mediates the targeting of the complex to the centromere. They showed that the N-terminus (aa1-47) of INCENP only targets to the centromere in the presence of Survivin. Furthermore, a Survivin:INCENP$_{47-920}$ fusion protein (lacking the centromere targeting domain) was able to target to the centromere and to the spindle midzone, even in the absence of Borealin or endogenous Survivin, or INCENP, albeit not as efficiently as the endogenous proteins since a significant portion of the chimera localised to chromatin throughout mitosis (Vader et al., 2006a). Borealin, which has also been shown to bind the same region of INCENP, is important for stabilising the interaction between INCENP and Survivin (Gassmann et al., 2004; Vader et al., 2006a). Furthermore a complex of INCENP$_{1-58}$, Survivin and Borealin is able to target to centromeres and Borealin was shown to bind directly to double stranded DNA *in vitro* (Klein et al., 2006). In reality there may be redundant mechanisms for the centromere targeting of the chromosomal passengers via Borealin and/or Survivin as they both bind to the N-terminus of INCENP. Survivin may be important for the initial targeting of the complex, but the whole complex may be important to stabilise the interaction with the centromere at physiological levels.

CENP-A is phosphorylated by Aurora-B at a motif that is similar to the sequence motif that is phosphorylated on Histone H3 by Aurora-B, but at slightly different times during mitosis. CENP-A is phosphorylated on Serine 7 in early prophase after the completion of Histone H3 phosphorylation and dephosphorylated in early anaphase before completion of Histone H3 dephosphorylation, suggesting that the modifications on the two proteins serve different functions (Zeitlin et al., 2001a). The phosphorylation is Aurora-A dependant and also dependant on Aurora-B after late prophase, therefore it was

proposed that CENP-A is sequentially phosphorylated first by Aurora-A in early prophase and then by Aurora-B after late prophase. The initial phosphorylation of CENP-A by Aurora-A was also suggested to be required to concentrate Aurora-B, and presumably the other chromosomal passenger proteins at centromeres (Kunitoku et al., 2003). Non-phosphorylatable or phosphomimic mutants caused a delay in cytokinesis. The localisation of INCENP, Aurora-B and PP1γ1 (all of which are necessary for cytokinesis) were examined by immunofluorescence. In CENP-A-S7A expressing cells, PP1γ1 was found to be inefficiently targeted to the midbody and the localisation of INCENP and Aurora-B were normal whereas in CENP-A-S7E expressing cells, INCENP and Aurora-B were detectable on chromosomes during anaphase and PP1γ1 failed to concentrate at the midbody (Zeitlin et al., 2001b). Therefore CENP-A dephosphorylation might be required to release the chromosomal passenger proteins from the centromere.

### 1.9.4. Translocation from the centromere to the spindle midzone

In mouse, CDK1 phosphorylates INCENP at Thr59 and Thr388 (Thr412 in human). Phosphorylation of Thr388 is required for binding of INCENP to PLK1, recruitment of PLK1 to the centromere, and for progression from metaphase to anaphase (Goto et al., 2006). Interestingly, translocation of the chromosomal passenger proteins to the spindle midzone in *S. cerevisiae* is signalled by dephosphorylation of a residue, phosphorylated by Cdc28/Clb2 (CDK/Cyclin B), within the coiled-coil of INCENP (Pereira and Schiebel, 2003). Although the same has not been demonstrated in higher eukaryotes, this mechanism is likely to be conserved since release of Aurora-B and INCENP onto the spindle midzone was blocked by non-degradable Cyclin-B in *Drosophila* (Parry et al., 2003) and in vertebrate cells (Murata-Hori et al., 2002b). Transfer

to the spindle midzone is also dependant on dynamic microtubules (Murata-Hori et al., 2002b; Wheatley et al., 2001a).

Studies using Aurora-B-GFP show that there is a rapid turnover of Aurora-B at centromeres, which is dependant on kinase activity and to a lesser extent on dynamic microtubules. In contrast, there is a much slower turnover of Aurora-B at the midzone and midbody. It was shown that centromeric Aurora-B was delivered to the midzone microtubules, whereas cytoplasmic Aurora-B was delivered to the equatorial cortex (Murata-Hori and Wang, 2002c).

### 1.9.5. Anaphase and Cytokinesis

The chromosomal passenger proteins have been shown to be essential for cytokinesis in a number of different organisms from worms to mammals, using a variety of techniques including RNAi, mouse knockouts and dominant negative mutants (Adams et al., 2001b; Cutts et al., 1999; Eckley et al., 1997; Fraser et al., 1999; Gassmann et al., 2004; Giet and Glover, 2001; Honda et al., 2003; Kaitna et al., 2000; Mackay et al., 1998; Romano et al., 2003; Severson et al., 2000; Tatsuka et al., 1998; Terada et al., 1998; Uren et al., 2000; Woollard and Hodgkin, 1999).

Experiments performed with mitotic heterokaryons show that INCENP localisation correlates with furrowing and that INCENP is found at the cleavage furrow even before myosin II (Eckley et al., 1997).

Interestingly the two following dominant negative INCENP mutants fail at different stages of cytokinesis. CENP-$B_{1-158}$:$INCENP_{43-839}$ fusion caused cells to fail completion of cytokinesis, leaving cells with a large intercellular bridge containing a prominent midbody. These cells proceeded through the next cell cycle without furrow regression (Eckley et al., 1997). In contrast, in cells transfected with $INCENP_{1-405}$, the furrow regressed (Mackay et al., 1998).

Several Aurora-B substrates have been shown to be important for cytokinesis. MgcRacGAP is phosphorylated by Aurora-B and this phosphorylation was reported to modify its activity from Rac/Cdc42 to RhoA (Minoshima et al., 2003), although this result has proven difficult for others to reproduce (Takeshi Urano, personal communication). Furthermore, the non-phosphorylatable form of MgcRacGAP fails cytokinesis. MKLP1 is a plus end directed motor protein, which can slide one microtubule over another (Nislow et al., 1992). MKLP1/ZEN-4 is phosphorylated by Aurora-B during anaphase and cytokinesis. A non-phosphorylatable mutant failed to complete cytokinesis (Guse et al., 2005). In *C. elegans* Aurora-B (AIR-2) is required to recruit ZEN-4 (MKLP1) to the spindle midzone (Severson et al., 2000).

MKLP2, another mitotic kinesin, binds to and is required to recruit Aurora-B to the spindle midzone (Gruneberg et al., 2004). Interestingly MKLP2 also binds CDC14a, the yeast homologue of which has been shown to be involved in triggering spindle transfer of the chromosomal passenger proteins in *S. cerevisiae* (Gruneberg et al., 2004; Pereira and Schiebel, 2003).

Other Aurora-B substrates include the intermediate filament proteins (Vimentin, Desmin, and GFAP) (Goto et al., 2003; Kawajiri et al., 2003) as well as myosin II, a motor protein (Murata-Hori et al., 2000). It has been shown that a non-phosphorylatable Vimentin mutant caused multinucleation (Goto et al., 2003).

Another protein, EVI5 has been shown to bind Aurora-B INCENP and Survivin *in vivo* (Faitar et al., 2006). EVI5 has a TBC domain suggestive of Rab GTPase activating protein (GAP) activity, exists in complexes with both alpha- and gamma-tubulin and has been shown to stabilize the anaphase-promoting complex inhibitor emi1 (Eldridge et al., 2006; Faitar et al., 2005).

## *1.10. Aims*

Mitosis has been studied for many years and still fascinates scientists today. Mitotic segregation of chromosomes into daughter cells is a fundamental process for life. There are many things to learn about how eukaryotic cells can segregate their complement of paired sister chromatids with such precision and what the mechanisms are that underlie these processes. When these processes go wrong, causing defects in chromosome segregation, they can lead to chromosomal loss or rearrangements and cancer. In order to understand these processes better the Earnshaw lab studies proteins critical to proper chromosome segregation in eukaryotic cells.

The chromosomal passenger proteins have emerged as key regulators of mitosis, coordinating chromosomal and cytoskeletal events. One of their roles is regulating kinetochore - microtubule attachments at the centromeres. In order to fully understand this aspect of their function, it was important to investigate how the chromosomal passenger proteins are targeted to the centromere in mitosis.

The role of the chromosomal passengers in meiosis is much less studied than in mitosis. The chromosomal passenger proteins may have additional functions in meiosis or they may have functions that operate in both systems, but have not been observed in mitosis because of redundant mechanisms. Investigation of the role of the chromosomal passenger in meiosis may reveal as yet undiscovered aspect to their function. The aim of this thesis is to further understand the role of the chromosomal passenger protein, INCENP, at the centromere in mitosis and meiosis.

My hypothesis is that protein-protein interactions influence the targeting of chromosome passenger proteins and these interactions in turn recruit additional proteins required for segregation. I have addressed this hypothesis in two ways. In the first part I will describe a yeast two-hybrid screen that I carried out in order to identify new protein-protein interaction at the centromeres using a portion of

INCENP that has previously been shown to target to the centromeres. In the second part I will describe work that was carried out, as part of a collaboration, in which we looked at the role chromosomal passenger proteins in sister chromatid cohesion and their interaction with MEI-S332, a centromere specific protein involved in sister chromatid cohesion.

# II Materials and methods

# 2. Materials and Methods

## *2.1. Molecular biology techniques*

### 2.1.1. cDNA synthesis

Total messenger RNAs were prepared from HeLa S3 cells by Reto Gassmann, using TRIzol reagent (Invitrogen). cDNA was synthesised from 5 μg of total mRNA using oligo (dT) and Superscript II Reverse Transcriptase (Invitrogen).

### 2.1.2. Cloning PCR products

Coding sequences were amplified from HeLa cDNA, plasmid DNA or using annealed synthetic oligo nucleotides using the appropriate primer pairs (see table 1). Thermal cycling was carried out on a T3000 thermocycler (Biometra) or on a Robocycler gradient 40 (Stratagene) for gradient PCRs, according to the conditions recommended by the polymerase manufacturer. PCR products were cloned into appropriate vectors (pBluescript II SK, Stratagene; pEGFPN1, pEGFPC1, pGADGH, pGBKT7, Clontech; pGEX-4T-3, Amersham; pET-22b pET30a, Novagene; pRSETA, B, C pCDNA3α, Invitrogen) using standard molecular biology techniques described below or ligated into pGEMT®-T Easy (Promega) or pCR2.1 (Invitrogen) and then subcloned into the appropriate vectors.

Table 1: Cloning of coding sequences.

| Coding sequence | Vector | Restriction sites | Primer pair[a] 5′–3′ |
|---|---|---|---|
| Gg INCENP 1-68 | pGBKT7 | EcoRI BamHI | Forward GGG GAA TTC ATG GCG GTG GCA ACG GGC Reverse CGC GGA TCC TCA CTT CCT CCT CTT CCG |
| Gg INCENP 1-85 | pGBKT7 | EcoRI BamHI | Forward GGG GAA TTC ATG GCG GTG GCA ACG GGC Reverse CGC GGA TCC TCA TAA TCT CCT CCT GCC |
| Gg INCENP 1-105 | pGBKT7 | EcoRI BamHI | Forward GGG GAA TTC ATG GCG GTG GCA ACG GGC Reverse CGC GGA TCC TCA GAG TCG CTG AGA ATC |
| Gg INCENP 1-135 | pGBKT7 | EcoRI BamHI | Forward AA GGA TCC CAT ATG GGG ACG ACG GCC C Reverse CGC GGA TCC TCA AAC CTG GGA TCT TGT C |
| Hs INCENP 1-69 | pGBKT7 | NdeI Sal I | Forward CC GCT CGA GCC CAT ATG GGG ACG ACG GCC CCA Reverse CCC GTC GAC TCA TCT CTT CTT CCG T |
| | pEGFPN1 | BamHI Sal I | Forward CC GCT CGA GCC CAT ATG GGG ACG ACG GCC CCA Reverse CCC GTC GAC GCT CTC TTC TTC CGT |
| Hs INCENP 1-101 | pGBKT7 | NdeI Sal I | Forward CC GCT CGA GCC CAT ATG GGG ACG ACG GCC CCA Reverse CCC GTC GAC TCA GAG GCG TCG GGA GCT CAG |
| | pEGFPN1 | BamHI Sal I | Forward CC GCT CGA GCC CAT ATG GGG ACG ACG GCC CCA Reverse CC CGT CGA CCC GAG GCG TCG GGA GCT CAG |
| HsINCENP 1-69 CEN[b] | pGBKT7 | NdeI Sal I | Forward CC GCT CGA GCC CAT ATG GGG ACG ACG GCC CCA Reverse CCC GTC GAC TCA TCT CTT CTT CCG T |
| | pEGFPN1 | BamHI Sal I | Forward CC GCT CGA GCC CAT ATG GGG ACG ACG GCC CCA Reverse CCC GTC GAC GCT CTC TTC TTC CGT |
| HsINCENP 1-101 CEN[b] | pGBKT7 | NdeI Sal I | Forward CC GCT CGA GCC CAT ATG GGG ACG ACG GCC CCA Reverse CCC GTC GAC TCA GAG GCG TCG GGA GCT CAG |
| | pEGFPN1 | BamHI Sal I | Forward CC GCT CGA GCC CAT ATG GGG ACG ACG GCC CCA Reverse CC CGT CGA CCC GAG GCG TCG GGA GCT CAG |
| FLJ14346 | pEGFPC1 | EcoRI KpnI | Forward G GGG AAT TCC ATG GCG GCG CAG GGC GTA GGG CC Reverse CC CGG TAC CGG GGT GCT GCC CCG TGT AGG |
| FLJ14346 | pGEX4T3 | EcoRI XhoI | Forward GG AAT TCC ATG GCG GCG CAG GGC GTA Reverse |

| | | | |
|---|---|---|---|
| | pEGFPC1<br>pTrAPN1[c] | EcoRI<br>XhoI/SalI | CCG CTC GAG CTA GGT GCT GCC CCG TGT |
| Mei-S332 | pOT2 | | from BDGP Clone ID LD13774 |
| DmAurora-B | pOT2 | | from BDGP Clone ID LD39409 |
| DmINCENP | PGEX4T3 | | (Adams et al., 2001b) |
| Dm MEI-S332[d] | pGEX | EcoRI<br>XhoI | Forward<br>G GGG AAT TCG ATG GGA TCC AAA GTG GAG<br>Reverse<br>CCG CTC GAG CTT TTT GGC CTT GGC CTT |
| GFP Mei-S332 | pJL9 | | (Lee et al., 2004) |
| GFP MEI-S332[S124,125,126A] | pJL9 | | MEI-S332 phospho-site mutants were generated using PCR mutagenesis in pGEX and a subcloned using SpeI & BlpI into pJL9 |

[a] Forward and reverse primers are 5'-3'. Start and stop codons are highlighted.
[b] The amino acid sequence of the centromere targeting domain was randomized (see Chapter 3, Figure 3.2). Two synthetic oligos were annealed, amplified by PCR and then cloned into full length HsINCENP. This was used as a template for pFM104, pFM106, pFM108, pFM110 (see section 2.1.3 below).
[c] For detail of how this plasmid was constructed and a map of this plasmid (see section 2.1.4 below).
[d] This construct has a point mutation E267G.
Gg (*Gallus gallus*)
Hs (*Homo sapiens*)
Dm (*Drosophila melanogaster*)
BDGP (Berkeley *Drosophila* Genome Project)

### 2.1.3. Construction of INCENP CEN mutation

In order to construct the mutation in the centromere targeting domain I made use of the fact that HsINCENP has an EcoRI site following the domain. The mutation in centromere targeting domain of HsINCENP was made by annealing the following two oligos:

Forward: CCG CTC GAG ATG GGG ACG ACG GCC CCA GGG CCC ATT CAC CTG CTG GAG CTA TGT GAC CAG AAG CTC ATG GAG TTT CTC TGC AAC ATG G

Reverse: G GAA TTC TCT GGT CTC GAA CTC GGC CAT GAT CCA TTG CTC GCG CTC AAG CTC CAC CAA GTC CTT ATT ATC CAT GTT GCA GAG AAA CTC C

The annealed product was amplified by PCR using the following primers
Forward: CCG CTC GAG ATG GGG ACG
Reverse: GGA ATT CTC TGG TCT CGA AC

The PCR product was digested with XhoI and EcoRI, cloned into pBluescript II SK HsINCENP, and used as a template for PCR.

### 2.1.4. Construction of pTrAP vectors

We constructed plasmids (pTrAP) containing a series of three small tags (Figure 2.1). These constructs were constructed in the same way as the INCENP CEN mutation as follows:

For pTrAP-N1 the following oligos were annealed:

Forward: GTC GAC GCT AGC ATG CAT CAC CAT CAC CAT CAC ATG GAC GAG AAG ACC ACC GGC TGG CGG GGC GGC CAC GTG GTG GAG GGC CTG GCC GGC GAG CTG GAG CAG CTG CGG GCC AGG CTG GAG

Reverse: GCT GTC CAT GTG CTG GCG TTC GAA TTT AGC AGC AGC GGT TTC TTT GCC GCC GCC GCC GGG CTC CCG CTG GCC CTG AGG GTG GTG CTC CAG CCT GGC CCG CAG CTG CTC CAG CTC GCC GGC

The annealed products were amplified by PCR using the following primers

Forward: GTC GAC GCT AGC ATG CAT C

Reverse: GTC GAC GGA TCC GCT GTC CAT GTG CTG GCG

The PCR product was cloned (NheI/BamHI) into the NheI and Bgl II sites of pEGFPC1 to make the following plasmid.

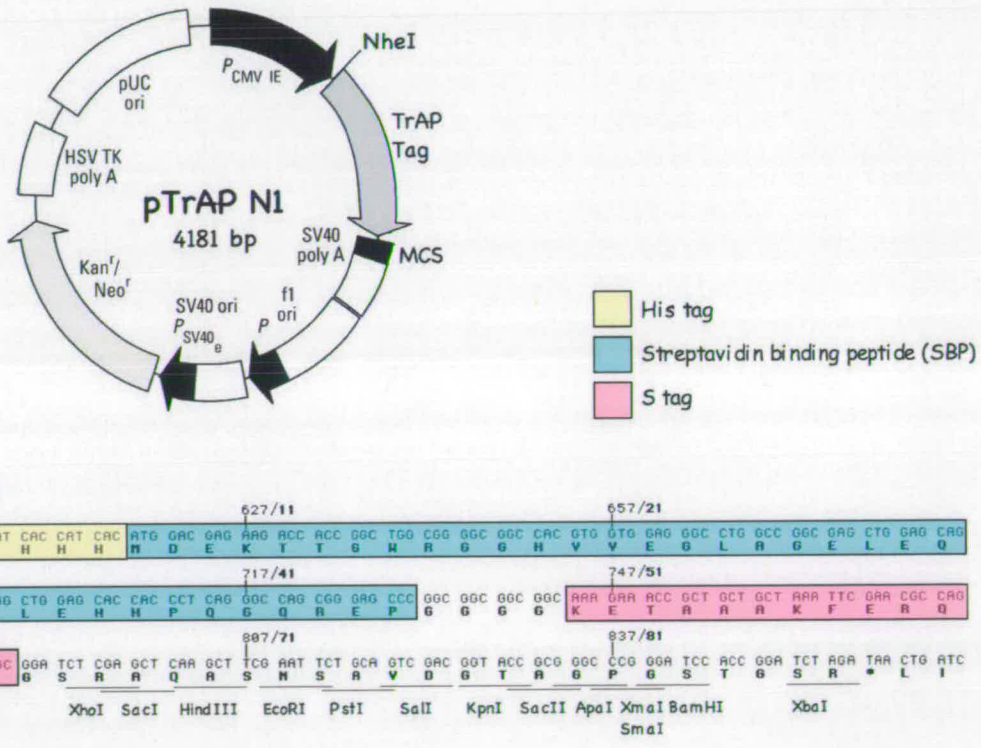





Figure 2.1: pTrAPN1 map.

### 2.1.5. Restriction digestion, electrophoresis, purification and ligation of DNA

PCR products and plasmid DNA were digested with the appropriate restriction enzymes and buffers (New England Biolabs) in the presence of 0.1mg/ml BSA for 2 - 16 hours at the recommended temperature. Where appropriate the vector backbone was dephosphorylated in the presence of calf intestinal phosphatase (New England Biolabs). DNA fragments in 1x gel loading buffer (0.04% Bromophenol Blue, 5% glycerol) were separated on 0.7 – 3% agarose gels, depending on the size of the fragment, containing 0.5 μg/ml Ethidium Bromide in TAE (40mM Tris Acetate, 1 mM EDTA) and visualised on a UV transilluminator. 1Kb or 100bp ladders (New England Biolabs) were used as a standard DNA size marker. DNA fragments were excised from the agarose gel and purified using the QIAquick gel extraction kit (Qiagen). DNA fragments were then ligated for 16 hours at 4°C or 2 hours at room temperature using T4 DNA ligase.

### 2.1.6. Preparation of competent *E. coli*

*E. Coli* (TOP10 or DH5α) cells were grown overnight in LB (Luria-Bertani) media. After approximately 16 hours the culture was diluted 1:200 into LB and incubated with shaking at 37°C until the culture had grown to an $OD_{600nm}$ 0.5. The culture was transferred to a 250 ml centrifuge bottle and chilled on ice for 10 minutes. The cells were centrifuged in a pre-chilled rotor for 5 minutes at 3836 g at 4°C. The supernatant was discarded and the cells were resuspended in 40 ml TbfI buffer (30 mM Potassium Acetate, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15 % glycerol (v/v), pH 5.8) per 100 ml of culture and incubated on ice for 5 minutes. The cells were centrifuged at 1881 g for 10 minutes at 4°C. The supernatant was discarded and the cells were resuspended in 4 ml TbfII buffer (10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$, glycerol 15 % (v/v), KOH to pH

6.5) and incubated on ice for 15 minutes. 100 μl aliquots were chilled rapidly on dry ice or snap frozen in liquid nitrogen and stored at - 80 °C. TbfI and TbfII solutions were filter sterilized and pre-chilled to 4°C. After growing to the correct OD all steps were carried out at 4°C.

**LB**

Per litre :

To 950ml of deionised $H_2O$, add:

Tryptone 10g

Yeast extract 5g

NaCl 10g

Dissolve and adjust the pH to 7.0 with NaOH. Adjust the volume of the solution to 1L and sterilise by autoclaving.

### *2.1.7.* Transformation of competent *E. coli*

100 μl aliquots of competent cells were thawed on ice and mixed with ligation reactions. The cells were incubated for 20 min on ice, heat shocked for 90 seconds at 42 °C, then chilled on ice for 1 min. 1 ml LB was then added and the cells were incubated on a shaking platform at 37 °C for 1 h. After pelleting and resuspending in 100 μl LB, cells were spread on LB agar plates containing the appropriate antibiotic. Where appropriate blue/white selection was used to screen for colonies that contained an insert by spreading 50 μl of 2% X-β-GAL and 10 μl 1M IPTG on the agar plates prior to plating out the bacteria.

### 2.1.8. Amplification and Extraction of plasmid

After transformation of plasmids into competent *E. coli,* single colonies were picked and grown in LB containing the appropriate antibiotic for 16 hours at 37°C. Plasmid DNA was isolated from these cultures using miniprep, midiprep, or maxiprep plasmid kit (Qiagen), which is based on the alkaline lysis method (Sambrook and Russell, 2001).

### 2.1.9. Sequencing

All constructs were sequenced using the BigDye® Terminator v3.1 cycle sequencing reaction system. Sequencing reactions typically contained 250 μg of plasmid DNA, 3.2 pmoles of the appropriate primer, 2 μl of BigDye reaction mix (Applied Biosystems). DNA sequencing was performed by the SBS sequencing service, Ashworth laboratories, University of Edinburgh.

## *2.2. Yeast protocols*

### 2.2.1. Yeast strains used

Y187: MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met$^-$, URA3 : : $GAL1_{UAS}$ -$GAL1_{TATA}$ –lacZ, MEL1

AH109: MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::$GAL1_{UAS}$ -$GAL1_{TATA}$ -HIS3, $GAL2_{UAS}$ -$GAL2_{TATA}$ -ADE2, URA3 : : $MEL1_{UAS}$- $MEL1_{TATA}$ –lacZ, MEL1

### 2.2.2. Yeast media and Agar

**100X Amino acid stocks**

Table 2: 100X Amino acid concentrated stock solution.

| Amino Acid | Final concentration | 100x stock (g/500ml) |
|---|---|---|
| Adenine | 20mg/ml | 1 g/500 ml (add 2ml of 5M NaOH to dissolve) |
| Arginine | 20mg/ml | 1 g/500 ml |
| Histidine | 20mg/ml | 1 g/500ml |
| Leucine | 60mg/ml | 3 g/500 ml |
| Lysine | 30mg/ml | 1.5 g/500 ml |
| Methionine | 20mg/ml | 1 g/500 ml |
| Phenylalanine | 50mg/ml | 2.5 g/500 ml |
| Threonine | 200mg/ml | 10 g/500 ml |
| Tryptophan | 20mg/ml | 1 g/500 ml |
| Tyrosine | 30mg/ml | 1.5 g/500 ml (add 3ml of 5M NaOH to dissolve) |
| Uracil | 20mg/ml | 1 g/500ml |

**10x Dropout solution**

The above 100x amino acid stocks were mixed to get the appropriate 10x dropout solutions. They were then filter sterilized and stored at 4°C.

**SD medium (1 L)**

6.7 g Yeast nitrogen base without amino acids

20 g Agar (for plates only)

The volume was made up to 850 ml (or less depending if 3-AT, kanamycin or X-α-GAL were added) with water and autoclaved. The medium was then allowed to cool to 55°C, glucose was added to 2% (50 ml of a 40% sterile stock solution) and 100ml of the appropriate sterile 10x Dropout solution was added.

For plates containing:

3-AT

2.5 ml of a 1 M stock was added after autoclaving if necessary in a total volume of 1litre.

Kanamycin

1ml of a 50 mg/ml stock solution (50 μg/μl final) was added if where indicated in a total volume of 1L.

X-α-GAL

2 ml of X-α-GAL (20 mg/ml) was added in a total volume of 1 litre.

**YPDA medium (500 ml)**

10 g Difco peptone

5 g Yeast extract

10 g Agar (for plates only)

The volume was made up to 467.5 ml and autoclaved. The medium was then allowed to cool to 55°C and glucose was added to 2% (25 ml of a 40% sterile stock solution) and Adenine hemisulphate solution to 0.003% (7.5ml of a 0.2% solution).

### 2.2.3. Small scale yeast transformation

25 µl of yeast for each transformation was scraped into an eppendorf tube, resuspend in 1 ml of sterile water and centrifuged at 11300 g in a microcentrifuge for 5 seconds. The cell pellet was resuspend in 1 ml of 100 mM Lithium Acetate and incubated for 5 minutes at 30°C. The volume representing a single transformation was placed in separate microcentrifuge tubes and centrifuged at 11300 g in a microcentrifuge for 5 seconds. The supernatant was removed and the following components were added on top of the cell pellet in the following order (240 µl PEG 3350 (50% w/v), 36 µl 1 M Lithium Acetate, 50 µl Salmon sperm DNA (2 mg/ml), 5 µl plasmid DNA (1 mg/ml), 20 µl sterile deionised water). The cell pellet was resuspended by vortexing for 1 minute in the transformation mix and then incubated at 42°C for 20 minutes. The cells were centrifuged at 11300 g in a microcentrifuge for 10 seconds and the supernatant was removed. The pellet was gently resuspended in 200 µl of sterile deionised water. The cells were then plated on SD/-Tryptophan plates for PGBKT7 plasmids and SD/-Leucine plates for pGADGH or PTD1-1 plasmids and incubated at 30°C[from (Gietz and Woods, 2002)]

### 2.2.4. Preparation of protein extract from yeast

2 ml of yeast culture was grown for 16 hours overnight at 30°C in selective media (SD/-Trp). 8 ml of YPDA was added and incubated at 30°C for 6-7 hours until the $OD_{600nm}$ was 0.8. 5 ml of this culture was centrifuged at 1881

g for 1 min at 4°C, resuspended in sterile water containing 0.1 mM PMSF and centrifuged again at 11300 g for 1 minute at 4°C and resuspended in 100 µl of sample buffer. The same volume of glass beads was then added and the samples were bead beaten for 2 minutes. 20 µl of each sample was loaded on a 17% polyacrylamide gel.

### 2.2.5. Small scale yeast mating protocol

One colony of each mating type (i.e. AH109 and Y187) was placed in a 1.5 ml microcentrifuge tube containing 0.5 ml of 2x YPDA. The tube was vortexed for 1 minute to resuspend the cells and incubated at 30°C overnight with shaking at 200 rpm. 100 µl was then spread on SD/-Leu/-Trp to select for diploids and incubated at 30°C for 3-5 days until colonies were visible.

### 2.2.6. Library Screening

50 ml of SD/-Trp was inoculated with AH109 pGBKT7 INCENP 1-105 and incubated at 30°C with shaking at 250 rpm for 24 hours until the $OD_{600nm}$ was 0.8. The cells were centrifuged at 1000 g for 5 minutes. The supernatant was decanted and the cell pellet was resuspended in the residual liquid by vortexing. A frozen aliquot of the library culture was thawed in a room temperature (21°C) in a water bath and then gently vortexed. 10 µl of this was taken to titer the library. The entire AH109 pGBKT7 INCENP 1-105 culture was combined with the 1 ml of library culture in a 2 L sterile flask. 45 ml of 2X YPDA/Kanamycin was added and the mixture was swirled gently. The library tube was rinsed out with a further 2 ml of 2X YPDA/Kanamycin and the volume was made up to 50 ml with 2X YPDA/Kan. The culture was incubated for 24 hours with gentle swirling (40 rpm).

The 10 µl library aliquot was diluted to 1ml in YPDA/Kanamycin in an eppendorf tube and gently mixed by vortexing (dilution A). Then 10 µl of dilution

A was diluted in YPDA/Kanamycin in an eppendorf tube and gently mixed by vortexing (dilution B). 50 µl (B1) and 100 µl (B2) of dilution B were plated onto SD/-Leu plates, in order to assess the titer of the library.

After incubating for 24 hours, the mating mixture was transferred to a sterile centrifuge bottle and centrifuged at 1000 g for 10 minutes. The mating flasks were rinsed twice with 50 ml 1X YPDA/Kanamycin. The rinses were combined and used to resuspend the first pellet. This was then centrifuged again at 1000 g for 10 minutes. The pellet was then resuspended in 10 ml of 1X YPDA/Kan. The total volume of the resuspended cells was 12.5 ml. 100 µl of a 1:10000, 1: 1000, 1:100 and 1:10 dilution of the mating mixture was plated on SD/-Leu, SD/-Trp and SD/-Leu/-Trp to check the mating efficiency. The remaining mating mixture was spread on 90 mm plates, 80 µl per plate, containing SD/-Leu/-Trp/-His + 50 µg/µl Kanamycin + 2.5 mM 3-AT. The plates were incubated for 8 days at 30°C.

### 2.2.7. Isolation of plasmids from yeast

Yeast cultures were grown overnight and 1.5 ml of culture was centrifuged in a microfuge tube. The supernatant was resuspended in 250 µl of Qiagen P1 buffer (50 mM TrisCl pH8, 10 mM EDTA, 100 µg/ml RNase A) along with 250 µl of glass beads and vortexed for 5 minutes. The rest of the plasmid purification was carried out according to the manufacturers instructions for the Qiagen miniprep kit.

### 2.2.8. Colony hybridization

Nitrocellulose membranes were incubated in 1 X SSC (0.15M NaCl, 15mM Sodium citrate pH7) and then dried on whatman 3MM paper. Colonies were lifted from yeast onto nitrocellulose membranes (Hybond-N) by placing the membranes face down on the plate for 5 minutes. The membrane was then

transferred face up onto a sheet of 3MM paper that was soaked in denaturing solution (1.5 M NaCl, 0.05 M NaOH) for 2 minutes, then onto 3MM paper that was soaked in neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl pH8) for 2 minutes, then onto 3MM paper that was soaked in washing solution (0.2 M Tris-HCl pH8.5, 2 X SSC) for 2 minutes. The membranes were then dried on 3MM Whatman paper and crosslinked with UV (0.12 joules). A HindIII-SacI fragment was cut out from pGADGH-colony-266 plasmid for the C1QBP probe and a BglII-SacII fragment was cut out from pGADGH-colony-331 for the RPS15 probe. The membranes were prehybridised in Church buffer (0.5 M $Na_2HPO_4$,7% SDS) and 20μg/ml salmon sperm DNA for 1 hour at 65°C. Random priming and incorporation of radiolabel $\alpha$-$^{32}$P-dCTP was performed using the Megaprime DNA labelling kit (Amersham) according to manufacturer's instructions. The labelled probe was hybridised to the membranes in Church buffer/20μg/ml salmon sperm DNA overnight at 65°C. Membranes were then washed in 40 mM $Na_2HPO_4.H_2O$, 1% SDS at 65°C for 10 minutes. The membrane was washed until the desired radioactive signal, monitored with a Geiger counter, was reached (usually 10 cpm). Finally, membranes were exposed to photographic film (Kodak Biomax MS) at –80°C for the appropriate length of time depending on the strength of the signal.

## *2.3. Biochemistry and Cell Biology techniques*

### 2.3.1. Expression and purification of recombinant proteins

Bacterial expression plasmids (e.g. pGEX) were transformed into *E. coli* BL21 cells. A culture was grown for 16h at 37°C in LB and then diluted 1:100 into 500 ml of LB and grown at 37°C in LB to an $OD_{600nm}$ of 0.5 in 200 μg/ml Ampicillin. Protein expression was induced by addition of IPTG to a final concentration of 1 mM and an additional 600 μg/ml Ampicillin was added. Cells

were grown for 3 hours at 37°C and harvested by centrifugation at 6000 g for 15 minutes. Cells were lysed in 20 ml Lysis buffer (50 mM Tris pH8, 400 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 1 mM PMSF, 1 μg/ml CLAP). 20 mg of Lysozyme was added and the lysate was incubated on ice for 30 minutes. The lysate was sonicated 3 x 20 second pulses and then centrifuged 14100 g for 15 minutes. The supernatant was incubated with 500 μl Glutathione Sepharose High Performance beads (Amersham) for 30 minutes at 4°C, mixing end over end. The beads were centrifuged at 500g for 5 minutes and washed three times with lysis buffer and once with PBS.

### 2.3.2. *In vitro* binding assays

Full length proteins were labelled with [$^{35}$S]-methionine expressed from cDNAs cloned in pOT2, pBluescript or, pCDNA3 vectors, using a reticulocyte lysate coupled transcription/translation system (Promega). For each binding reaction *in vitro* translated proteins were added to binding buffer (PBS, 5 mM EGTA, 0.1% triton, 0.5 mM PMSF, and 1 μg/ml CLAP [chymostatin, leupeptin, antipain, and pepstatin A]) and mixed with GST-Protein-X or GST alone bound to glutathione sepharose beads. Samples were incubated for 90 minutes at 4°C. The supernatant was precipitated with TCA and the beads were washed three times with binding buffer. Bound and unbound fractions were separated by SDS-PAGE, and the proteins were visualized using a phosphorimager (STORM 860) with ImageQuant software (Amersham biosciences).

### 2.3.3. SDS-PAGE

Protein samples were resuspended in 1x Sample buffer (50 mM Tris-HCl pH6.8, 15% Sucrose, 2 mM EDTA, 3% SDS, 1.43 M β-mercaptoethanol) and boiled for 5 minutes at 95°C. SDS-PAGE was carried out using the Tris-Glycine buffer system (Sambrook and Russell, 2001).

### 2.3.4. Coomassie Blue staining

For coomassie blue staining polyacrylamide gels were stained in 1x Coomassie [0.25% (w/v) Coomassie Brilliant Blue, 50% methanol, 10% Acetic acid] for 10 minutes and then put in Slow Destain [10% methanol, 7% acetic acid] until bands were visible.

### 2.3.5. Immunoblotting

Following SDS-PAGE, proteins were transferred onto nitrocellulose membranes (Amersham pharmacia) in transfer buffer (0.2 M Glycine, 25 mM Tris, 0.1% SDS, 20% Methanol) at 220 mAmpV for 150 minutes. Membranes were rinsed with $dH_2O$ and stained with Ponceau S (0.1% (w/v) Ponceau S, 5% Acetic acid) to visualise transferred proteins. Membranes were then rinsed in PBS. Membranes were blocked in 5% milk / PBS / 0.05% Tween 20 and then incubated with the primary antibody (see table 3) in blocking buffer for 1 hour at room temperature. After washing in 5% milk / PBS / 0.05% Tween 20, the membrane was incubated with secondary antibody in blocking buffer. After washing in PBS / 0.05% Tween 20 the HRP signal was revealed by enhanced chemiluminescence (Amersham Pharmacia).

### 2.3.6. Antibody production

Polyclonal antibodies, R1789 and R1790, were generated against full length FLJ14346 with an N-terminal GST tag. The fusion protein was expressed in *E. coli* BL21 cells and purified as described above. The purified protein was run on a 12.5% polyacrylamide gel. The gel was washed in $dH_2O$ and stained with aqueous coomassie Blue (0.5% coomassie Blue, 25 mM Tris, 250 mM Glycine) and destained (25 mM Tris, 250 mM Glycine). The protein band was

excised from the gel with a razor blade, cut into small pieces and ground to a fine powder by freezing in liquid nitrogen and grinding with a pestle and mortar. The powder was resuspended in PBS and this mixture was injected into two male rabbits (Diagnostics Scotland).

### 2.3.7. Indirect immunofluorescence microscopy

HeLa JW cells were grown on Poly-L-lysine coated coverslips then fixed in 4% Paraformaldehyde / 1X cytoskeleton Buffer (137 mM NaCl, 5 mM KCl, 1.1 mM $Na_2HPo_4$, 0.4 mM $KH_2Po_4$, 2 mM $MgCl_2$, 2 mM EGTA, 5 mM PIPES, 5,5 mM Glucose, pH6.1). Cells were permeabilized with 0.15% (v/v) Triton-X 100 in cytoskeleton buffer.

For gamma-tubulin staining coverslips were fixed with paraformaldehyde as above and washed in cytoskeleton buffer and then put in cold methanol (chilled in -20 °C freezer) for at least 10 minutes and washed again with cytoskeleton buffer.

Coverslips were then blocked in PBS/1%BSA/0.1% Azide for 30 minutes at 37 °C. Primary antibodies were diluted in PBS/BSA and applied to the coverslips for 30 minutes at 37°C. Coverslips were washed three times in PBS/BSA and then incubated with the appropriate secondary antibody coupled to FITC, Texas Red or Cy5, (See table 3) for 30 minutes at 37 °C. Coverslips were washed again three times in PBS/BSA and then mounted on glass slides with vectashield containing 0.5 μg / ml DAPI (Vector laboratories). Images were captured on a Zeiss Axioplan 2 using a 100x objective or on an Olympus IX-70 microscope controlled by Deltavision SorfWorx software (Applied Precision) using a 100x objective. After deconvolution, image stacks were quick projected and saved as tiff files.

Table 3: Antibodies used for indirect immunofluorescence and immunoblotting.

| Antigen | Species | Source | Dilution for Immuno-fluorescence | Dilution for Immuno-blotting |
|---|---|---|---|---|
| Myc | Mouse | Cell Signalling, 9B11 | | 1:1000 |
| alpha-tubulin | Mouse | Sigma (clone B512) T5168 | | 1:4000 |
| α & β-tubulin | Sheep | Cytoskeleton ATN02 | | 1:30 |
| GFP | Rabbit | Molecular probes, A11122 | | 1:200 |
| FLJ14346 | Rabbit | **R1789** | 1:500 | 1:200 |
| FLJ14346 | Rabbit | **R1790** | 1:500 | |
| ACA | Human | **NR, GS, BM** | 1:1000 1:200 | |
| RnAim-1 (Aurora-B) | Mouse | Translab (clone 6) 611082 | 1:500 | |
| γ-tubulin | Rabbit | Sigma, T3559 | 1:1000 | |
| INCENP | Rabbit | **R1186** | 1:500 | |
| INCENP | Mouse | Upstate (clone 58-217) 05-940 | 1:50 | |
| Streptavidin Binding peptide | Mouse | **Clone 20** | 1:200 | |

Antibodies from the Earnshaw lab are in bold.

| Secondary Antibodies | Species | Source | Dilution for Immuno-fluorescence | Dilution for Immuno-blotting |
|---|---|---|---|---|
| anti-rabbit, HRP conjugated | Donkey | Amersham Pharmacia, NA934 | | 1:10000 |
| anti-mouse, HRP conjugated | Sheep | Amersham Pharmacia NA931 | | 1:10000 |
| anti-rabbit, FITC conjugated | Goat | Jackson Laboratories 111-096-045 | 1:200 | |
| anti-rabbit, Texas Red conjugated | Goat | Jackson Laboratories 111-076-045 | 1:200 | |
| anti-mouse, FITC conjugated | Goat | Jackson Laboratories 115-096-062 | 1:200 | |
| anti-mouse, Texas Red conjugated | Goat | Jackson Laboratories 115-076-062 | 1:200 | |
| Anti-human, Cy5 conjugated | Goat | Jackson Laboratories 109-176-127 | 1:200 | |
| Anti-rabbit, Cy5 conjugated | Goat | Jackson Laboratories 111-176-045 | 1:200 | |

### 2.3.8. Cell culture

HeLa JW cells were grown in RPMI 1640 medium supplemented with 5% (v/v) FBS, 100 U / ml penicillin, 100 μg / ml streptomycin and 300 mg/ml L-glutamine at 37°C in 5% $CO_2$ in a humid incubator.

### 2.3.9. Transient transfection of Hela cells and generation of stable cell lines

Plasmids were transfected into HeLa JW cells by electroporation or by Fugene 6 (Roche). For electroporation cells were resuspended in Optimem (Invitrogen) at $5 \times 10^6$ cells / ml. 400 μl of this suspension was added to each electroporation cuvette (0.4 cm electrode gap; Biorad) on ice, and mixed gently with 10 μg of plasmid DNA. Cells were electroporated at 0.28 kV and 950 μF using the Gene Pulser II Electroporation system. Cells were then plated out on polylysine coverslips for immunoflouresnece or in tissue culture flasks. For transfection with Fugene6 (Roche), Hela JW cells were seeded on polylysine coverslips at $0.5 \times 10^5$ cells per well of a 12-well dish and transfected according to the manufacturer's instructions.

# III Results

# 3. Yeast Two Hybrid Screen for Proteins that Interact with the N-terminus of INCENP

## *3.1. Introduction*

The most common methods currently employed to screen for novel protein-protein interactions are two-hybrid analysis and affinity purification of tagged proteins.

The yeast two-hybrid system was first described by Fields and Song (Fields and Song, 1989) and has been used extensively since then to identify novel protein-protein interactions (Hannon et al., 1993), to test known proteins for interaction, and identify domains or amino acids required for a protein-protein interaction (Li and Fields, 1993). It is a very sensitive technique, since the initial interaction is amplified and can therefore detect weak or transient protein interactions, which may not be biochemically detectable. Yang *et al.* (Yang et al., 1995) proposed that weak interactions with dissociation constants above 70 μM could be detected, although this is dependant on the reporter used (Estojak et al., 1995). Furthermore, this method can detect proteins of relatively low abundance. The other main benefit of the yeast two hybrid system is that it is a eukaryotic *in vivo* assay therefore proteins are more likely to be folded properly and post-translationally modified than they are following bacterial expression. However, expressing vertebrate proteins in yeast does not guarantee that they will be correctly modified.

The other most commonly used method to screen for novel protein interactions is affinity purification followed by mass spectrometry. This technique involves expressing a fusion protein containing the protein of interest and an affinity tag, such as the streptavidin binding peptide (Keefe et al., 2001; Wilson et al., 2001). For a review of affinity tags see (Terpe, 2003). The fusion

protein, along with any proteins that bind to it, can then be purified by binding to streptavidin.

The TAP (tandem affinity purification) tag has been used extensively in budding yeast to purify protein complexes (Gavin et al., 2006). The TAP tag encodes the CBP (Calmodulin binding peptide), a TEV protease cleavage site and Protein A. The TAP method involves fusion of the TAP tag to the target protein and introduction of the construct into the host cell. The TAP tagged protein plus any proteins bound to it are first purified on an IgG matrix. Any unbound proteins are washed away then TEV protease is added. TEV cleavage releases the protein complex from the beads. A second affinity step using Calmodulin-coated beads purifies the complex further removing further contaminants and the TEV protease. Finally the protein complex is eluted with EGTA (Rigaut et al., 1999).

This method has some benefits over the two-hybrid system. The purification is carried out from vertebrate cell extracts. The proteins are therefore more likely to be correctly post-translationally modified. This technique identifies proteins interacting with their partners in their normal cellular compartment unlike the GAL4 based two-hybrid system in which the proteins are targeted to the nucleus. For this reason the TAP tag method has a low false negative rate. However, it is less sensitive than two-hybrid (kD < 50 nM compared to 50 μM, (Piehler, 2005)) therefore low abundance proteins may not be identified. Some complexes are unstable and may disassemble during the purification procedure, others may be insoluble. One of the main benefits of the yeast two-hybrid system is that it should normally detect direct interactions.

Since we wanted to identify proteins that bound directly to the N-terminus of INCENP we first chose to carry out a yeast two-hybrid screen.

### 3.1.1. Principle of the GAL-4 based yeast two hybrid system

In the GAL4–based yeast two-hybrid system, a bait gene is expressed as a fusion to the GAL4 DNA–binding domain (DBD), and another gene (the prey) or a library is expressed as a fusion to the GAL4 activation domain (AD). If the bait and prey proteins interact the DNA-BD and the AD are brought close together. This activates transcription of the reporter genes such as ADE2, HIS3, lacZ or MEL1, as shown in Figure 3.1. Transcriptional activation of the ADE2 and HIS3 reporter genes can be selected for by growth on medium lacking adenine (SD/-Ade) and/or lacking histidine (SD/-His). Activation of the lacZ reporter genes can be assayed by colorimetric assays of β-galactosidase activity (Fields and Song, 1989). Similarly, Activation of the MEL1 reporter genes can be assayed by colorimetric assays of α-galactosidase activity (Aho et al., 1997).

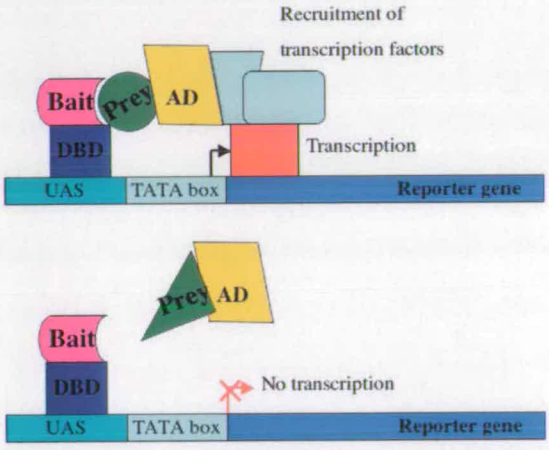

Figure 3.1: The principle of the yeast two-hybrid system.

### 3.1.2. Using the INCENP N-terminus as a bait for two-hybrid screening

To try to understand the function of the INCENP protein, various truncations have been expressed. These reveal INCENP to be a complex multidomain protein. Specific domains of INCENP are required for association

with microtubules, chromosomes, centromeres and the central spindle (Ainsztein et al., 1998; Mackay et al., 1998). Interestingly, centromere targeting of INCENP is normally required for its subsequent transfer to the midbody. The domain that has been shown to be essential for targeting of INCENP to the centromere and the central spindle is contained in the N-terminal 68 amino acids. $INCENP_{1-68}$-GFP can target to the inner centromere and subsequently to the central spindle. This domain contains two evolutionarily conserved motifs (Figure 3.2A-B, (Ainsztein et al., 1998)). Residues 32-44 are necessary for targeting to the centromere (Figure 3.2C, (Ainsztein et al., 1998)) and residues 52-62 are required for targeting to the midbody (Ainsztein et al., 1998). In agreement with these results a recent paper has shown that $HsINCENP_{1-47}$ is sufficient to target to centromeres (Klein et al., 2006). We therefore wanted to discover how INCENP is targeted to the centromere.

Some proteins such as CENP-B are targeted to the centromere via recognition of a specific sequence by a DNA-binding domain (Pluta et al., 1992). INCENP is likely to be targeted to the centromere by binding to another protein(s) since $INCENP_{1-68}$ does not contain any putative DNA binding domains. We therefore wanted to identify proteins that bind the N-terminus of INCENP. I have tested several different constructs and used the smallest construct which contains amino acids 1-68 but which is not transcriptionally active as a bait for a two-hybrid screen.

Since this screen was started there have been several publications concerning the targeting of the chromosomal passengers to the centromere (see section 1.9.3). It was shown that the N-terminus (aa 1-47) of INCENP only targets to the centromere in the presence of Survivin. Furthermore a Survivin:$INCENP_{47-920}$ fusion protein (lacking the centromere targeting domain) was able to localise to the centromere and to the spindle midzone, even in the absence of endogenous Borealin, Survivin or INCENP (Vader et al., 2006a). In addition, a complex of $INCENP_{1-58}$, Survivin and Borealin could target to

centromeres and Borealin was shown to bind directly to double stranded DNA *in vitro* (Klein et al., 2006). Although Survivin may be required for the initial loading on the centromere, the stabilization of the complex at centromeres until anaphase onset may involve more than one interacting factor, especially when the proteins are expressed at physiological levels.

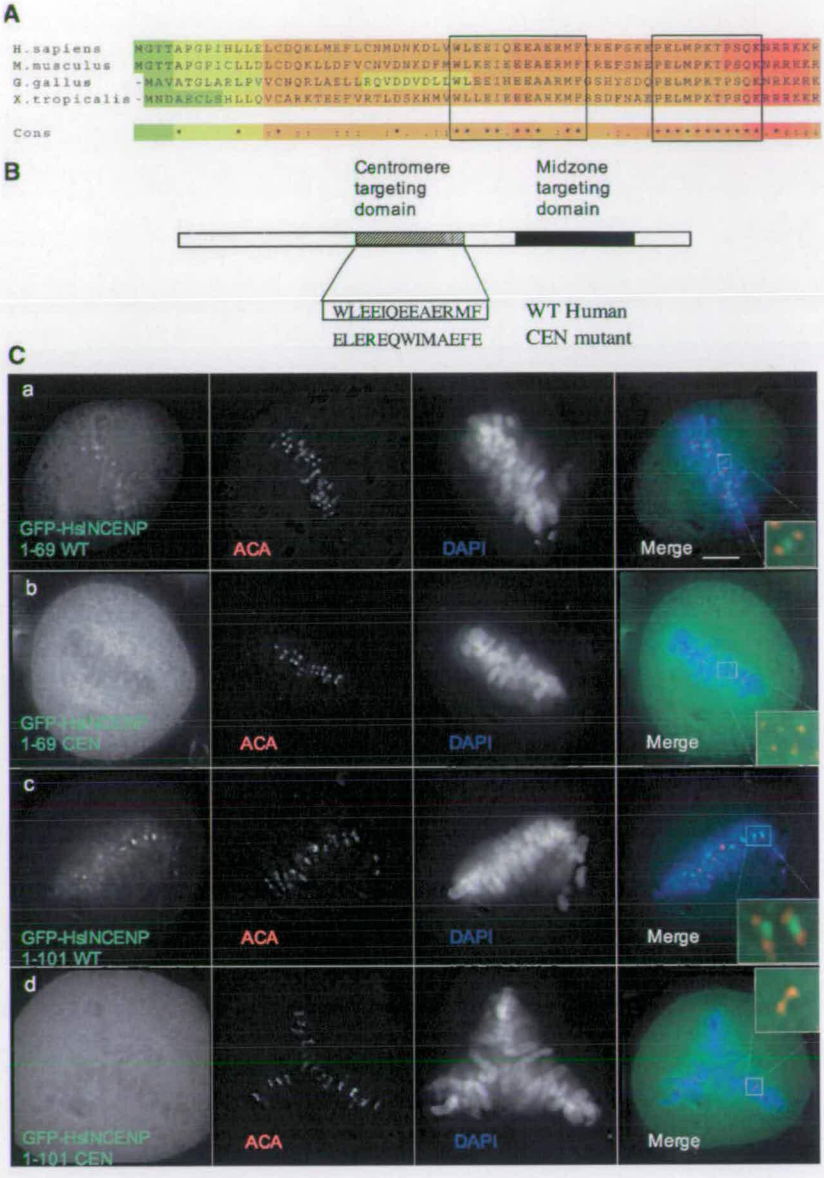


Figure 3.2: The centromere targeting domain of INCENP contains an evolutionarily conserved motifs required for centromeres targeting.
(A) Sequence alignment of the N-terminal sequences of INCENP from human, mouse, chicken, and *Xenopus*. (* = Conserved amino acids, : = Conservative substitution, . = semi-conservative substitution). (B) Schematic representation of the amino-terminal region of human INCENP and mutanted sequence. The hatched region shows the centromere–targeting motif. The filled region shows the midzone targeting domain. The boxed sequence below the bar corresponds to the wild-type sequence for the centromere-targeting motif. Below is the mutagenised sequence, arrived at by randomization of the amino acid sequence shown in the box. (C) HeLa cells expressing INCENP constructs fused to GFP fixed and stained with ACA (human autoimmune serum BM) and DAPI. Transfected $HsINCENP_{1-69}$-GFP and $INCENP_{1-101}$-GFP concentrate at the inner centromere. In contrast, $HsINCENP_{1-69}$-CEN-GFP and $HsINCENP_{1-101}$-CEN-GFP fail to localize at inner centromeres. Images were taken using an Olympus IX-70 microscope. These are single sections (scale bar is 5 μm) and all pictures are at the same magnification.

## 3.2. *Results*

### 3.2.1. Constructing and testing the bait plasmids

Before beginning the screen, it was important to verify that the bait used for the screen was suitable. Therefore several tests were carried out on various N-terminal INCENP fragments from *Gallus gallus* (chicken). Chicken INCENP was used despite the fact that the screen was against a human cDNA library since chicken $INCENP_{1-68}$ was shown by Ainzstein *et al.*, to target to centromeres in human (HeLa) cells.

Four regions of the N-terminus of INCENP were amplified and cloned into pGBKT7. These encoded amino acids 1-68, 1-85, 1-105 and 1-135 of chicken INCENP. These plasmids were then tested to find out which was best suited for use as a bait plasmid in the yeast two-hybrid screen.

It was essential to test that the bait protein would not autonomously activate the reporter genes. Therefore, yeast strain AH109 transformed with the pGBKT7 plasmids were mated with yeast strain Y187 transformed with the empty library plasmid, pGADGH. These diploids were then tested for activation of the HIS3 and ADE2 reporter genes by growth on media lacking histidine or adenine respectively (Figure 3.3), and for activation of the MEL1 reporter gene by a colour change to blue on media containing X-α-GAL (Figure 3.4). 3-amino-1,2,4-triazole (3-AT) is a competitive inhibitor of the yeast HIS3 protein and can be used to inhibit low levels of leaky expression from the HIS3 reporter gene, which is seen in some strains, including AH109 (Durfee et al., 1993). However, too much 3-AT can kill freshly transformed cells. To assess the optimum 3-AT concentration a series of SD/-Leu/-Trp/-His plates containing a range of 3-AT concentrations (0, 2.5, 5, 7.5, 10, 12.5 and 15 mM) were used.

All strains grew on SD/-Leu/-Trp media (which selects for diploids) as expected. This shows that yeast have been transferred efficiently when they

were replica plated. Only the positive control (AH109 pGBKT7-p53 x Y187 pTD1-1) grew strongly on all plates. Very weak background growth was observed on the SD/-Leu/-Trp/-His plate (which selects diploids which are able to activate the HIS3 reporter gene) for all the other plasmids as expected because of the leaky expression from the HIS3 reporter. However, this very weak growth is not visible in this figure. This background growth was not seen on plates containing 2.5 mM (or higher concentrations) of 3-AT. On plates containing SD/-Leu/-Trp/-Ade (which selects diploids able to activate the ADE2 reporter gene) only the positive control grew. This indicated that none of the INCENP fusion proteins activated the HIS3 or ADE2 reporter genes and that 2.5 mM 3-AT was the optimum concentration to use in order to suppress leaky expression from the HIS3 reporter in AH109 x Y187 diploids.

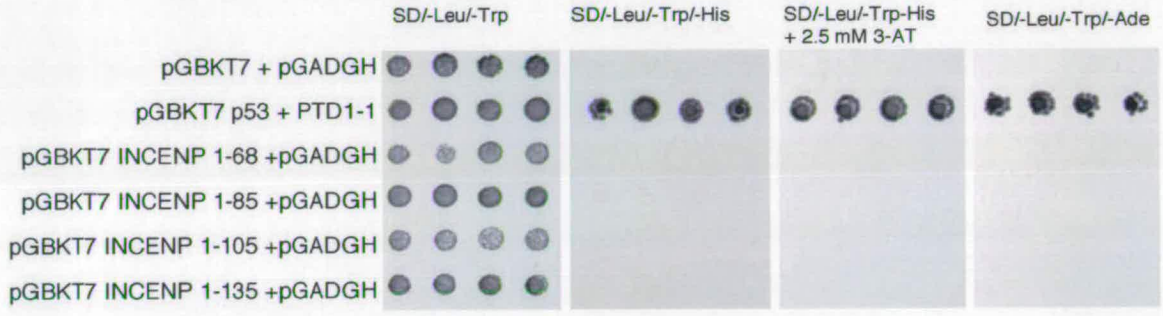


Figure 3.3: Activation of reporter genes by expression of fusion proteins.
Yeast strain AH109 containing the pGBK T7 plasmids indicated were mated with yeast strain Y187 containing pGADGH or pTD1-1 and plated onto SD/-Leu/-Trp plates to select for diploids. Individual colonies were replica plated onto SD/-Leu/-Trp, SD/-Leu/-Trp/-His, SD/-Leu/-Trp/-His + 2.5 mM 3-AT, SD/-Leu/-Trp/-Ade and incubated for 3 days at 30°C. pGBKT7–p53 x PTD1-1 was used here as a positive control and pGBKT7 x PGADGH as a negative control.

Plates containing SD/-Leu/-Trp + X-α-GAL were used to test for activation of the MEL1 reporter gene. The MEL1 reporter gene encodes α-galactosidase, a secreted enzyme, which can be assayed directly on plates using X-α-GAL, a chromogenic substrate, in the medium. Figure 3.4 shows a weak activation of

the MEL1 reporter gene by the fusion protein expressed by the pGBKT7 $INCENP_{1-68}$, and pGBKT7 $INCENP_{1-85}$ plasmids.

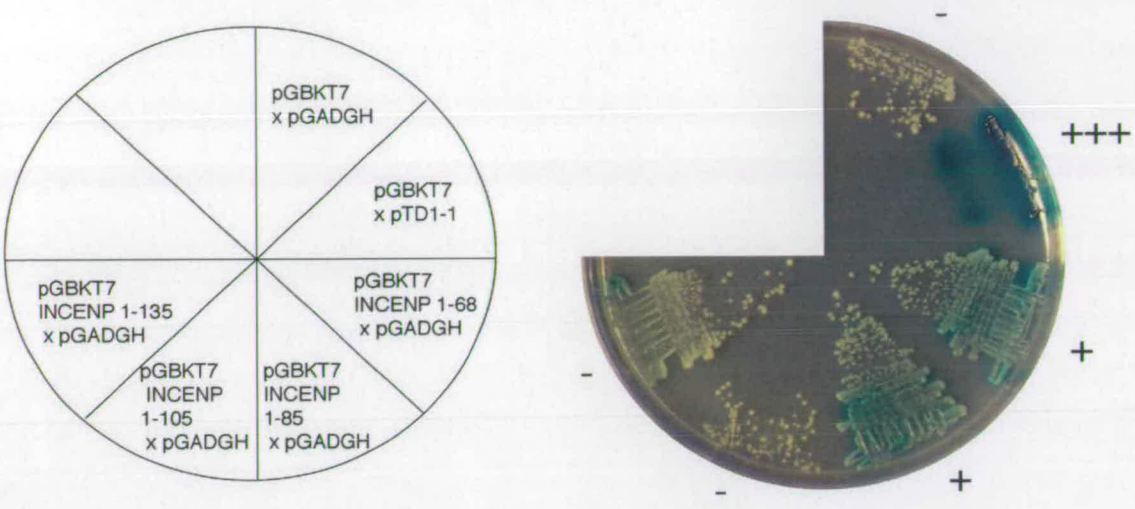


Figure 3.4: Activation of MEL1 reporter gene by expression of fusion proteins.
Yeast strain AH109 containing the pGBK T7 plasmids indicated were mated with yeast strain Y187 containing pGAD GH or pTD1-1 and plated onto SD/-Leu/-Trp plates to select for diploids and then streaked onto SD/-Leu/-Trp+X-α-GAL plates. pGBKT7–p53 x PTD1-1 was used here as a positive control and pGBKT7 x PGADGH as a negative control.

To test if any of the bait plasmids were toxic to the yeast cells, the $OD_{600nm}$ of the cultures was measured over a period of 9 hours (Figure 3.5). The doubling times (Table 4) show that the expression of these fusion proteins did not significantly increase the doubling time and it therefore was concluded that these fusion proteins were not toxic to the cells. The equation of the line was calculated using a function in Microsoft Excel which calculates the exponential trendline for a given set of values, given in the table in the form of $y=ce^{bx}$, where c is the OD at time 0, b is a constants, e is the base of the natural logarithm, x is the time in hours and y is the OD. The doubling time is therefore natural log of 2 divided by b (ln2/b, See Table 4).

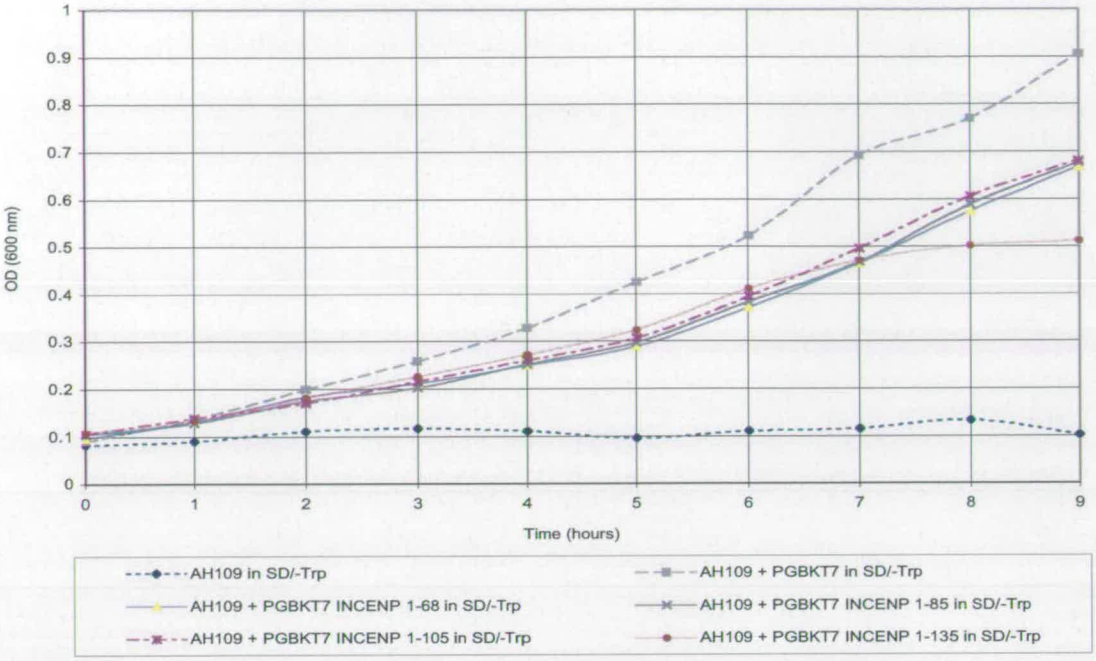


Figure 3.5: Comparison of growth curves of yeast strain expressing fusion proteins.
AH109 containing pGBKT7 INCENP 1-68, 1-85, 1-105 and 1-135 compared with the empty yeast strain (negative control) and AH109 containing empty pGBKT7 vector (positive control) in the same medium.

Table 4: Doubling times for yeast strain AH109 containing pGBKT7 plasmids.

| Yeast strain and plasmid | Equation of the line | Doubling time (hours) |
|---|---|---|
| AH109 | $Y=0.1e^{0.0149x}$ | 46.5 |
| AH109 + pGBKT7 | $Y=0.1e^{0.2299x}$ | 3.0 |
| AH109 + pGBKT7 INCENP 1-68 | $Y=0.1e^{0.1885x}$ | 3.7 |
| AH109 + pGBKT7 INCENP 1-85 | $Y=0.1e^{0.1898x}$ | 3.7 |
| AH109 + pGBKT7 INCENP 1-105 | $Y=0.1e^{0.1942x}$ | 3.6 |
| AH109 + pGBKT7 INCENP 1-135 | $Y=0.1e^{0.1843x}$ | 3.8 |

It was then important to test that the fusion proteins were expressed and were of the expected size. Figure 3.6 shows that all the fusion proteins were expressed at similar levels and that they were migrating at their expected size.

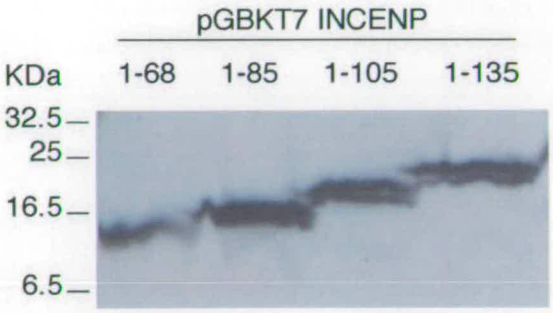


Figure 3.6: Expression of fusion proteins in yeast strain AH109. Protein samples were prepared and western blotting carried out as described in section 2.2.4.

We decided to carry out the screen using the pGBKT7 $INCENP_{1-105}$ plasmid since the fusion protein was not toxic to the yeast cells (Figure 3.5), was expressed (Figure 3.6), did not activate the HIS3 or ADE2 reporter genes (Figure 3.3) and was the smallest construct that did not activate the MEL1 reporter gene (Figure 3.4). We decided to perform our initial screen on the lower stringency selection media (SD/-Leu/-Trp/-His) so that no interactions were missed and used 2.5 mM 3-AT to suppress the background expression from the HIS3 promoter.

### 3.2.2. Screening the library

A Clontech pretransformed human HeLa cDNA library was chosen. The original library was from Hannon et al., 1993, prepared from exponentially growing HeLa cells (Hannon et al., 1993). This library was transformed into yeast strain Y187, serving as a mating partner for yeast strain AH109. AH109 contains four reporter genes, unlike many other reporter strains that have only one. These four reporter genes - HIS3, ADE2, MEL1 and LacZ – under the control of three distinct, although related, promoters are all recognised by the GAL4 DNA binding domain (Figure 3.7). This reduces the number of false positives, such as proteins that interact with the UAS or TATA box sequences. The use of AH109 also has the advantage of having two stringency options for

the yeast two-hybrid screen. Proteins that have a strong interaction with the bait protein can be identified using both the HIS3 and ADE2 reporter genes. On the other hand, selection using the HIS3 reporter alone can be used to identify a broad range of protein–protein interactions including relatively weak interactions.

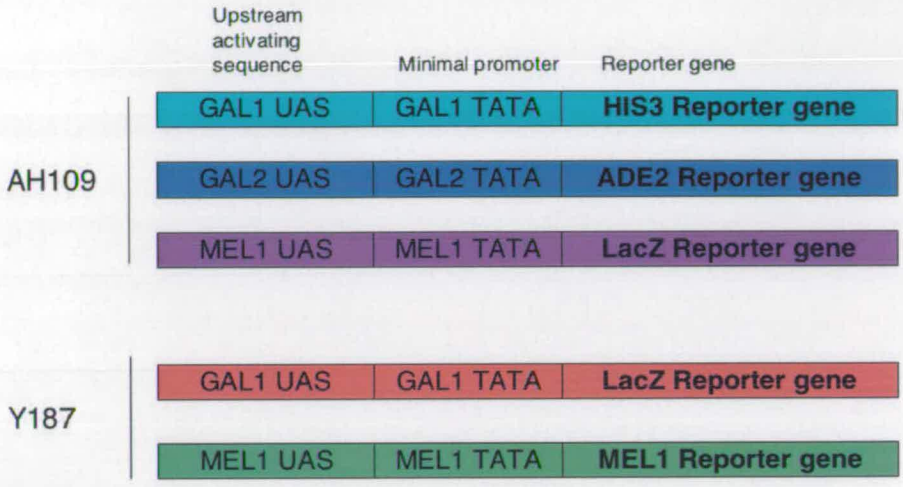


Figure 3.7: Reporter genes present in yeast strains AH109 and Y187.

pGBKT7 INCENP$_{1\text{-}105}$ was transformed into AH109, grown in liquid culture and then mated with an aliquot of the library as described in Chapter 2, section 2.2.6. The mating mixture was spread on 90 mm plates, 80 µl per plate, containing SD/-Leu/-Trp/-His + 50 µg/µl Kanamycin + 2.5 mM 3-AT. The plates were incubated for 8 days at 30°C. The library titer was determined as describe in Section 2.2.6 and calculated as follows:

No of colonies/volume plated x dilution factor = colony forming units (cfu)/ml

Dilution B1 $687 / 0.05 \times 10^{-4} = 1.374 \times 10^{8}$cfu/ml

Dilution B2 $2 / 0.1 \times 10^{-4} = 1.652 \times 10^{8}$cfu/ml

Average $1.513 \times 10^{8}$cfu/ml

The viability of each mating partner and the diploids were determined as described in Section 2.2.6 and calculated as follows:

Cfu x1000ul/ml / volume plated x dilution factor = cfu/ml

Y187 (library) $115 \times 1000 / 100 \times 10^{-3} = 1.15 \times 10^{6}$ cfu/ml

AH109 (bait) $1348 \times 1000 / 100 \times 10^{-4} = 1.348 \times 10^{8}$ cfu/ml

Diploids $341 \times 1000 / 100 \times 10^{-2} = 3.41 \times 10^{5}$ cfu/ml

Mating efficiency was then calculated from the viability of the limiting mating partner (which should be the library strain) and the viability of the diploids:

Cfu/ml for diploids/cfu/ml for limiting partner (Y187 library) x 100 = % diploids

$3.41 \times 10^{5} / 1.15 \times 10^{6} \times 100 = 29.65\%$

The number of clones screened was calculated from the viability of the diploids:

Diploids (cfu/ml) x volume plated (ml)

$3.41 \times 10^{5} \times 12.5 = 4.26 \times 10^{6}$ clones screened

The original cDNA library in E. coli had $6 \times 10^{6}$ independent clones and an estimated 93% of them had inserts, therefore the number of clones available to screen was $5.6 \times 10^{6}$. I screened $4.26 \times 10^{6}$ clones. Therefore 76% of the library was screened.

### 3.2.3. Results of the initial screen library screen

After 8 days 535 putative interactors were picked and re-plated onto SD/-Leu/-Trp/-His + 2.5 mM 3-AT. The growth was noted for each colony after a further 8 days. Table 5 shows the growth of colonies picked from library screening plates on SD/-Leu/-Trp/-His + 2.5 mM 3-AT.

Table 5: Results of initial screen.

| Growth on SD/-Leu/-Trp/-His + 2.5mM 3-AT | Number of Colonies |
|---|---|
| Did not regrow | 2 |
| Very weak growth | 5 |
| Weak growth | 10 |
| Moderate growth | 11 |
| Good growth | 507 |

**Secondary screen**

All of the 533 colonies that regrew on SD/-Leu/-Trp/-His + 2.5 mM 3-AT media were replica plated onto the following plates to test the putative interactors using all three reporter genes. SD/-Leu/-Trp/-His+2.5 mM 3-AT + X-α-GAL (for the selection of diploids which are able to transcriptionally activate the HIS3 reporter gene. The X-α-GAL causes yeast colonies that are able to transcriptionally activate the MEL1 reporter gene, to turn blue). SD/-Leu/-Trp/-His/-Ade + X-α-GAL (to select for diploids which are able to transcriptionally activate the HIS3, ADE3 and MEL1 reporter genes. The colonies were sorted into 6 categories (Table 6) according to their growth and color on the various media described above.

Table 6: Phenotype of colonies from yeast two-hybrid screen.

| Category | Phenotype | Number of days after plating that colonies turned blue | No of colonies |
|---|---|---|---|
| 1 | His+, Ade+, Mel1+ | 2 | 55 |
| 2 | His+, Ade+, Mel1+ | 3 | 118 |
| 3 | His+, Ade+, Mel1+ | 4 | 65 |
| 4 | His+, Ade+, Mel1-/+ | 5 | 127 |
| 5 | His+, Ade+, (Mel1-/+ or Mel1-)* | 5 | 66 |
| 6 | His+, Ade+, Mel1- or His-, Ade-, Mel1- or His-, Ade+, Mel1- or His+, Ade-, Mel1- | 5 | 104 |

Mel1 phenotype was score as either Blue= +, Blue/White = +/- or White = -
*Blue/White on one either the Adenine or Histidine selection and white on the other

238 colonies that were $HIS3^+$, $ADE3^+$ and $Mel1^+$ were analysed further (category 1,2 and 3).

### 3.2.4. Isolation of plasmid DNA from putative interactors

Since the initial library clones may contain more than one library plasmid these colonies were restreaked twice onto SD/-Leu/-Trp + X-α-GAL. This allowed segregation of the library plasmids while maintaining selective pressure of the interacting library plasmid by picking single blue colonies to restreak. The phenotype was then retested by plating onto SD/-Leu/-Trp/-His/-Ade + X-α-GAL.

Plasmid DNA was isolated from Mel+ Ade+ His+ colonies and sequenced. Sequences were identified by running them through BLAST (Basic Linear Alignment Search Tool) programs at http://www.ncbi.nlm.nih.gov/BLAST/ (Altschul et al., 1990; McGinnis and Madden, 2004). Sequences were then first compared with the NCBI nr (non-redundant) protein database using BLASTX (translates nucleotide into protein sequence in all 6 reading frames and

compares them to the protein databases). Any sequences that did not match anything in the protein database were compared with the NCBI nr or EST nucleotide database using BLASTN.

Many of the plasmids that were first identified were (C1QBP complement component 1, q subcomponent binding protein) also known as p32 (subunit of pre-mRNA splicing factor SF2) and hyaluronic acid-binding protein. (NP_001203.1). Many were also ribosomal protein S15 (gene name RPS15 NP_001009.1).

Therefore we decided that yeast colony hybridization would be carried out in order to identify the colonies containing plasmids encoding these genes. I then isolated plasmids from the remaining colonies (see table 7).

Table 7 Results of colony hybridisation and sequencing.

| **Protein match (Accession No.)** | **Function** | **Number of clones** |
|---|---|---|
| BRG-1 (NP_003063.2) | Chromatin remodeling factor | 4 |
| SAP30 (NP_003855.1) | Histone Deacetylase complex sununit | 2 |
| MGC5306 (NP_077021.1) | Unknown function | 1 |
| C7ORF24 (NP_076956.1) | Unknown function | 1 |
| FLJ14346 (NP_079305.2) | Unknown function | 4 |
| c-myc (NP_002458.2) | Transcription factor | 5 |
| AATF (NP_036270.1) | Transcription factor | 1 |
| eIF3 (NP_003746.2) | Translation | 1 |
| 40S Ribosomal protein S15 (NP_001009.1) | Translation | 4 |
| 40S Ribosomal protein S26 (NP_001020.2) | Translation | 99 |
| P32 (NP_001203.1) | component of the serum complement system;<br>hyaluronic acid-binding protein<br>pre mRNA Splicing factor associated protein | 44 |
| NAD(P)H dehydrogenase (NP_000895.1) | oxidation of NADH or NADPH | 2 |

| Nucleotide match | (Accession No.) | Number of clones |
|---|---|---|
| OK/SW-CL.16 | (BAB93516.1) | 3 |
| FLJ12845 (matches only to 3' UTR) | (AK022907.1) | 2 |
| Yg80h02.r1 (match to only one EST) | (R52455.1) | 4 |
| UI-H-FH1-bfg-n-10-0-UI.s1 NCI_CGAP_FH1 (match to 3' UTR of Hoxc8) | (BU618342.1) | 3 |
| Not yet identified | | 57 |
| **Total** | | **238** |

### 3.2.5. Retesting interactions in the yeast Two Hybrid system.

We chose to investigate further the following proteins identified in the screen – BRG1, SAP30, C7ORF24, MGC5306 and FLJ14346. The first two of these (BRG1 and SAP30) are known chromosomal proteins and are therefore good candidates for proteins that interact with INCENP. The other three proteins were uncharacterized at the time of doing the screen and were therefore chosen for further analysis.

Three of the interactors from the screen contained sequences that only matched ESTs but no proteins in the NCBI databases. The other interactors included proteins involved in transcription (c-Myc, AATF, eIF3, p32), translation (S15, S26) and Metabolism (NAD(P)H) and were not followed up.

The library plasmids identified were reintroduced back into yeast (strain Y187) and mated with yeast containing various chicken INCENP fragments (Figure 3.8). All five putative interactors showed an interaction with chicken INCENP fragments 1-105, 1-85 and 1-68.

Since the library I screened was a human cDNA library I also checked that these proteins also interacted with the equivalent fragments (1-101 and 1-69) of human INCENP, which correspond to chicken INCENP 1-68 and 1-105).

As a control to focus on interactors with a possible role in targeting INCENP to centromeres, a plasmid was constructed in which the amino acids of the centromere targeting motif of human INCENP were randomized as previously reported by Ainsztein et al (1998) for chicken INCENP (see section 2.1.3 and Figure 3.2). This mutation in chicken INCENP is unable to target to the centromere. Therefore any interactor that binds the WT protein but not the mutant protein is a good candidate for the protein, which targets INCENP to the centromere.

The library plasmids identified were reintroduced back into yeast (strain Y187) and mated with yeast containing these human INCENP fragments, in strain AH109. All five putative interactors showed an interaction with the WT versions of human INCENP fragments 1-101 and 1-69 (Figure 3.9, see sectors with HsINCENP 1-69 WT and HsINCENP 1-101 WT).

In this figure we can also see the effect of the mutation in the centromere targeting domain on the interactions. All five interactors showed some growth when tested against with the mutated centromeres targeting domain (Figure 3.9, See sectors with HsINCENP 1-69 CEN and HsINCENP 1-101 CEN). On the plate showing the interactions of C7ORF24 with the various INCENP fragments, the interaction with the WT INCENP fragments is stronger than with the CEN mutation. However when this experiment was repeated the difference between the CEN mutation and the WT was not obvious.

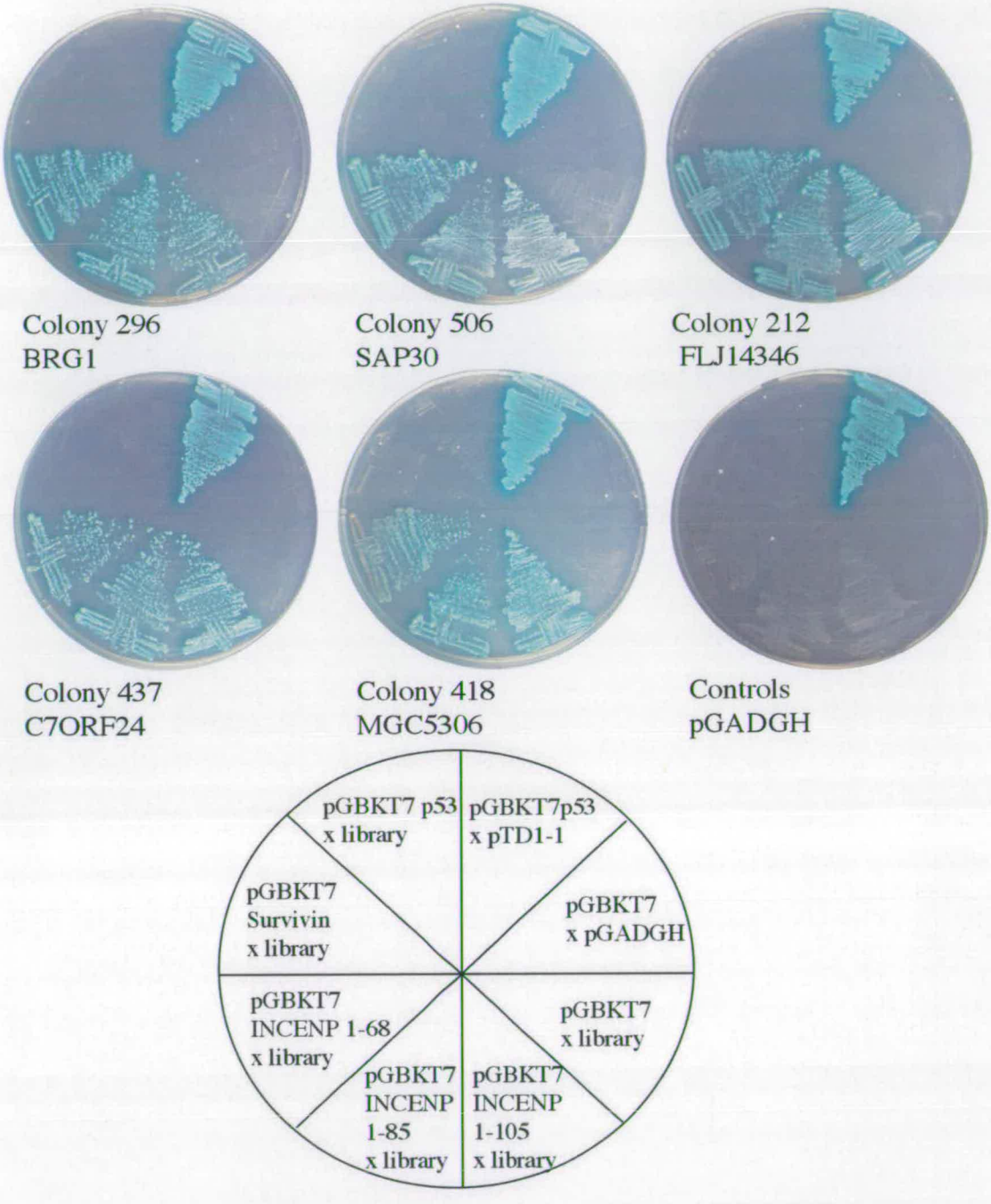


Figure 3.8: Yeast two-hybrid library plasmids interact with chicken INCENP 1-68.
Plasmids from the colonies indicated were transformed into yeast strain Y187 and mated with the opposite mating type AH109 containing a plasmid (pGBKT7) encoding INCENP fragments, Survivin or p53. Diploids were plated on SD/-Leu/-Trp/-His/-Ade + X-α-GAL and incubated for 3 days at 30°C.

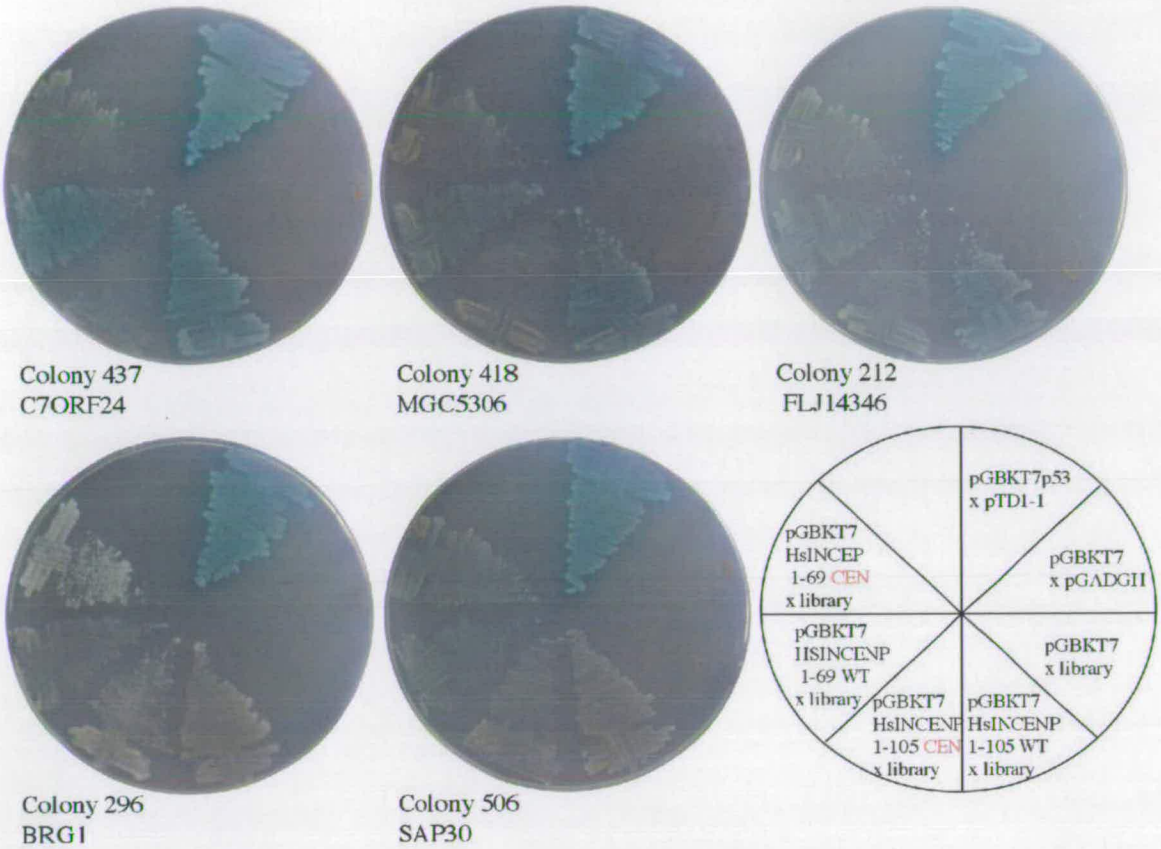


Figure 3.9: Yeast two-hybrid library plasmids interact with human INCENP 1-69
Plasmids from the colonies indicated were transformed into yeast strain Y187 and mated with the opposite mating type AH109 containing a plasmid (pGBKT7) encoding human INCENP fragments. Diploids were plated on SD/-Leu/-Trp/-His/-Ade + X-α-GAL and incubated for 3 days at 30°C.

## 3.3. Discussion

The yeast two hybrid system is a powerful technique for the identification of novel protein-protein interactions and has been used in many high throughput and genome wide screens (Giot et al., 2003; Li et al., 2004b; Schwikowski et al., 2000; Stanyon et al., 2004).

However the yeast two-hybrid system is not without its drawbacks. The highly sensitive nature of the assay can lead to generation of false positive results. Therefore any interactions identified using this system should be verified by an independent means such as GST pulldowns. Proteins may interact in this system but may not normally interact *in vivo* because they are not present in the same cell type or cellular compartment or during the same stage of the cell cycle. Alternatively, two-hybrid interactions may occur between cloned domains that are not normally accessible in the native protein. The purified poly $A^+$ RNA used to construct the MATCHMAKER cDNA library was not treated with DNase and may therefore contain a small amount of genomic DNA (<1%). Several of the plasmids from the screen contained sequences that did not correspond to known protein coding regions and may have resulted from contaminating genomic DNA (e.g. introns or other non-coding DNA).

Several proteins known to bind the N-terminus of INCENP (Survivin, Borealin, β-tubulin) were not found in this screen. Although this is an *in vivo* system, it is still possible that interactions might not be picked up because they are mediated by post-translational modifications, which may not occur in the yeast nucleus especially for human proteins. Some interactions are only detected when one partner is the bait (fused to the DBD) and the other is the prey (fused to the AD) presumably because the DBD or AD hinder the interaction. In this screen only 76% of the library was screened. This could also be a reason why known interactors were not identified.

Below, I will discuss several of the known proteins identified in the two-hybrid screen.

MGC5306 was also identified in a yeast two-hybrid screen with DNA polymeraseβ. It was shown to be nuclear in interphase and depletion of the protein by siRNA resulted in apoptosis and S-phase arrest (Wang et al., 2004)

Interestingly, this screen identified BRG1 and SAP30. These proteins are involved in chromatin remodelling and histone deacetylation respectively, and it is possible that interactions with INCENP may link them to functions at centromeres.

SAP30 was isolated as part of the Sin3/HDAC complex but may be present in only a subset of Sin3/HDAC complexes (Zhang et al., 1998). Interestingly the centromeric region is hypoacetylated (Dunleavy et al., 2005; Sullivan and Karpen, 2004) and there is evidence that interfering with HDAC activity causes problems in mitosis. Histone deacetylase inhibitors have been shown to affect the pericentromeric targeting of Aurora-B (Robbins et al., 2005) and HP1/Swi6 (Ekwall et al., 1997; Taddei et al., 2001) and histone hyperacetylation prevents sister chromatid separation (Cimini et al., 2003b). mSin3-associated protein, mSds3, has been shown to be essential for pericentric heterochromatin formation and chromosome segregation in mammalian cells (David et al., 2003). Thus there is a link between HDAC complexes and heterochromatin formation at the centromere. Heterochromatin formation is in turn important for the establishment /maintenance of cohesion, since HP1/Swi6 has been shown to recruit Cohesin to the centromere (Nonaka et al., 2002).

HP1 is a protein that plays an important role in heterochromatin formation. In interphase it is enriched in heterochromatin. During prophase all HP1 isoforms dissociate from chromosome arms. While HP1β and HP1γ are delocalised from the chromatin during mitosis, HP1α associates with the

pericentromeric heterochromatin during mitosis (Hayakawa et al., 2003). Binding of HP1 is regulated by histone modifications. HP1 is known to bind H3K9me3 via its chromodomain (Bannister et al., 2001). HP1 dissociates from the chromosome arms concurrent with H3S10 phosphorylation however it was recently shown that this is not sufficient to dissociate HP1 from the chromosome arms, since HP1 can still bind the dual modification H3K9Me3S10p (Terada, 2006). However an additional modification of H3, acetylation on lysine 14, is enough to prevent HP1 binding. Acetylation of H3K14 generally increases during mitosis (Mateescu et al., 2004). However, the hypoacetylated state of the chromatin at the centromere may prevent the delocalization of HP1$\alpha$ from that region during mitosis. HDAC complexes must therefore concentrate at the centromere during or prior to mitosis to keep the centromeric heterochromatin hypoacetylated. Following that idea we can speculate that the putative interaction between INCENP and SAP30 could target the HDAC complex to the centromere, thus leading to hypoacetylation and therefore binding of HP1$\alpha$.

BRG1's function at the centromere is more obscure. In past years it was thought that the role of SWI/SNF complexes was to activate gene expression by increasing accessibility to transcriptional machinery. However, it is becoming apparent that SWI/SNF complexes have a general role in facilitating DNA access to factors involved in various processes e.g. replacement with histone variants (Henikoff and Dalal, 2005) and enhancing access of histone tails to HDAC and methyltransferases, leading to heterochromatin and gene silencing (Nielsen et al., 2002; Pal et al., 2003; Sif et al., 2001). BRG1 could facilitate binding of HP1 by enhancing access of histone tails to HDAC and methyltransferases, leading to heterochromatin formation and gene silencing. Indeed SAP30 antibodies have been shown to co-immunoprecipitate BRG1 (Kuzmichev et al., 2002), and BRG1 interacts directly both *in vivo* and *in vitro* with mSin3A (Sif et al., 2001). Furthermore, BRG1 has been shown to interact

directly with HP1α and this interaction is important for HP1α mediated transcriptional repression (Le Douarin et al., 1996; Nielsen et al., 2002).

BRG1 is phosphorylated and inactivated during mitosis (Muchardt et al., 1996; Sif et al., 1998). The inactivation of this complex might be important for transcriptional inhibition during mitosis and condensation of mitotic chromosomes and would argue against a role for active BRG1 during mitosis. However a small proportion or subcomplex may be active at the centromere during mitosis /or may be important prior to mitosis for centromere function.

Some reports have suggested a role for this complex in centromere function. The human SWI/SNF-B (PBAF) complex contains BRG-1 and is related to the yeast Rsc complex. PBAF has been shown to localise to the centromere/kinetochore of mitotic chromosomes (Xue et al., 2000). In yeast the Rsc complex is important for centromere function. It has been shown to be required for establishment of cohesion (Baetz et al., 2004; Huang et al., 2004) and kinetochore function (Hsu et al., 2003; Tsuchiya et al., 1998).

There appears to be a complex network of protein interactions involved with centromere function with a spatial and temporal sequence of events yet to be elucidated (Figure 3.10). HP1α seems to be central to this network and has also been shown to bind INCENP directly (Ainsztein et al., 1998). HP1α binds to a region of INCENP located just C-terminal to the region used in my two-hybrid screen.

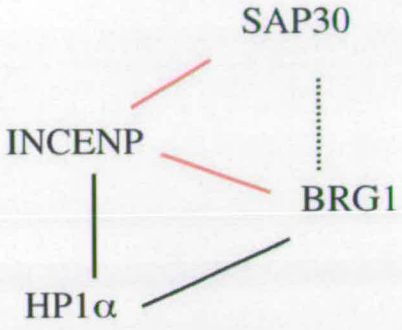


Figure 3.10: Potential interactions of INCENP with chromatin remodeling proteins.
Dotted lines refer to interactions identified by Immunoprecipitation and continuous lines refer to interactions identified by yeast two-hybrid. Black lines refer to interactions described by Nielsen et al., 2002 and Ainsztein et al., 1998. Red lines refer to interactions found in this study.

Although this screen has identified several known proteins of interest as discussed above, I have focused my attention on one unknown protein (FLJ14346). Initial characterisation of this novel protein showed it to have very interesting localisation during mitosis. Subsequently, analysis of the DNA sequence of the plasmids recovered from the screen, which contained DNA matching regions of the FLJ14346 coding sequence, revealed that the FLJ14346 was not in frame with the upstream GAL4 domain. However, because of its localisation during mitosis we decided to characterise this protein further. I will discuss the initial characterisation of FLJ14346 in the next chapter.

# IV Results - Characterization of an INCENP interacting protein identified by yeast two-hybrid

# 4. Characterization of an INCENP interacting protein identified by yeast two-hybrid

## *4.1. Introduction*

The chromosomal passenger proteins show a dynamic localization during mitosis. In keeping with this dynamic behavior, they perform multiple functions at various sites at different stages of mitosis and meiosis. These include chromatin modification, correction of kinetochore-microtubule attachment errors, some aspects of the spindle assembly checkpoint, maintenance of a stable bipolar spindle, regulation of sister chromatid cohesion and completion of cytokinesis (Resnick et al., 2006; Vagnarelli and Earnshaw, 2004) (see introduction section 1.9).

Aurora-B, INCENP, Survivin and Borealin form a complex in mitotic cells (Gassmann et al., 2004). Figure 4.1 shows a schematic representation of how the chromosomal passenger complex may be assembled. Aurora-B, Borealin and Survivin bind directly to INCENP (Gassmann et al., 2004; Wheatley et al., 2001b). Borealin and Survivin have been shown to bind the N-terminal 58 amino acids of INCENP, which contains the conserved centromere targeting motif (Klein et al., 2006). Both Survivin and Borealin have been shown to dimerise *in vivo* and bind each other (Gassmann et al., 2004; Klein et al., 2006). Aurora-B on the other hand binds INCENP near the C-terminus of INCENP (Adams et al., 2000; Kaitna et al., 2000) and also binds Survivin (Wheatley et al., 2001b).

Aurora-B and INCENP have been shown to form a subcomplex. The function of this subcomplex is not yet clear. It may be present in interphase cells or it may have a specific function such as phosphorylation of Histone H3, since H3 phosphorylation was unaffected by Borealin RNAi (Gassmann et al., 2004).

TD-60, a putative GEF for Rac1, is a chromosomal passenger protein but has not as yet been shown to be a part of the chromosomal passenger complex, however it is dependant on the other chromosomal passenger proteins for its localisation. Therefore, it is likely that it binds the chromosomal passenger complex at some time during mitosis. (Gassmann et al., 2004; Mollinari et al., 2003)

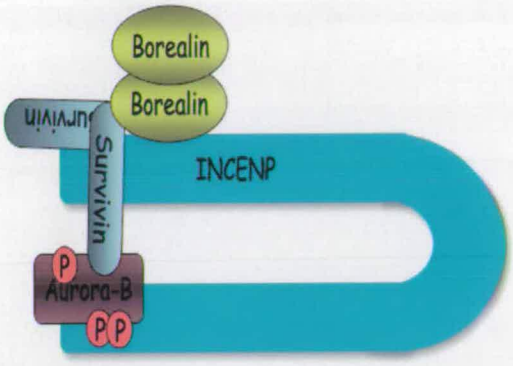


Figure 4.1: Schematic drawing of the chromosomal passenger complex.

My yeast two-hybrid screen for proteins interacting with the N-terminus of INCENP identified FLJ14346 (see chapter 3) as well as several other proteins including - MGC5306, C7ORF24, SAP30 and BRG1. Initial characterization of FLJ14346 revealed an interesting localization that merited further investigation. Here I report the initial characterization of this protein. Sequence analysis and database searches revealed that FLJ14346 is conserved in *Deuterostomes*. The sequence does not contain any identifiable conserved domains that might give a clue to its function. Humans contain a recently diverged paralogue that is not found in other closely related species. FLJ14346 has a dynamic localization though mitosis. Although the antibody staining and the localization of the GFP-tagged protein are not in complete agreement, they both show this protein on the spindle and the spindle poles and associated with the midbody during cytokinesis. Interestingly, FLJ14346 binds to Borealin and TD-60, as well as INCENP *in vitro* thus potentially bridging the gap between the chromosomal passenger complex and TD-60.

## 4.2. *Results*

### 4.2.1. FLJ14346 is conserved in *Deuterostomia*

The human FLJ14346 gene (NCBI Gene ID: 80097, ENSEMBL gene ID ENSG00000152082) is located on chromosome 1. The gene has 3 exons encoding a basic (pI 10) protein of 158 amino acids with a predicted molecular weigh of 16 kDa. Secondary structure predictions, which were done in collaboration with Dr. Dietlind Gerloff, suggest that it has four alpha helices at its N-terminus (Figure 4.2).

No conserved domains or known motifs were detected in FLJ14346. The third predicted alpha helix which contains a region in which there is a potential cyclin substrate recognition site (KILV), a nuclear receptors box motif (ILVDLLK) and a Class III PDZ domain binding motif (VDLL) (blue line in Figure 4.2). These potential binding sites are conserved in FLJ14346 homologues from different vertebrates. The cyclin substrate recognition sites interact with cyclins and thereby increase phosphorylation by cyclin/cdk complexes. Cyclin inhibitors also use these cyclin substrate recognition sites. The nuclear receptor box motif confers binding to nuclear receptors and PDZ domains are protein – protein interaction domains (www.elm.eu.org/links.html) (Puntervoll et al., 2003).

Multiple sequence alignment analysis shows that the N-terminal region containing the predicted alpha helical region is more conserved than the C-terminus. The most distantly related species, from human, in which I could detect an FLJ14346 homologue, was *Strongylocentrotus purpuratus* (purple sea urchin), which, like humans, are *deuterostomes*. The name *deuterostome* means "second mouth" and comes from the fact that during development the first opening, the blastopore, becomes the anus while in *protostomes*, such as *Drosophila,* it becomes the mouth.

Humans have two gene paralogues, both on chromosome 2 (position 2q21.1) about 1.3 megabases apart (ENSEMBL Gene IDs: ENSG00000152082

and ENSG00000173272). The two proteins are 96% identical at the protein level. There appears to have been a gene duplication event rather than a mistake in the building of the genome sequence since ESTs can be found matching both proteins. This indicates that both proteins are expressed. This gene duplication must have happened fairly recently in evolutionary history since even the length and sequence of the introns is highly conserved (intron 1 - 92%, intron 2 - 96%). Furthermore, all closely related species examined lack a second copy of this gene.

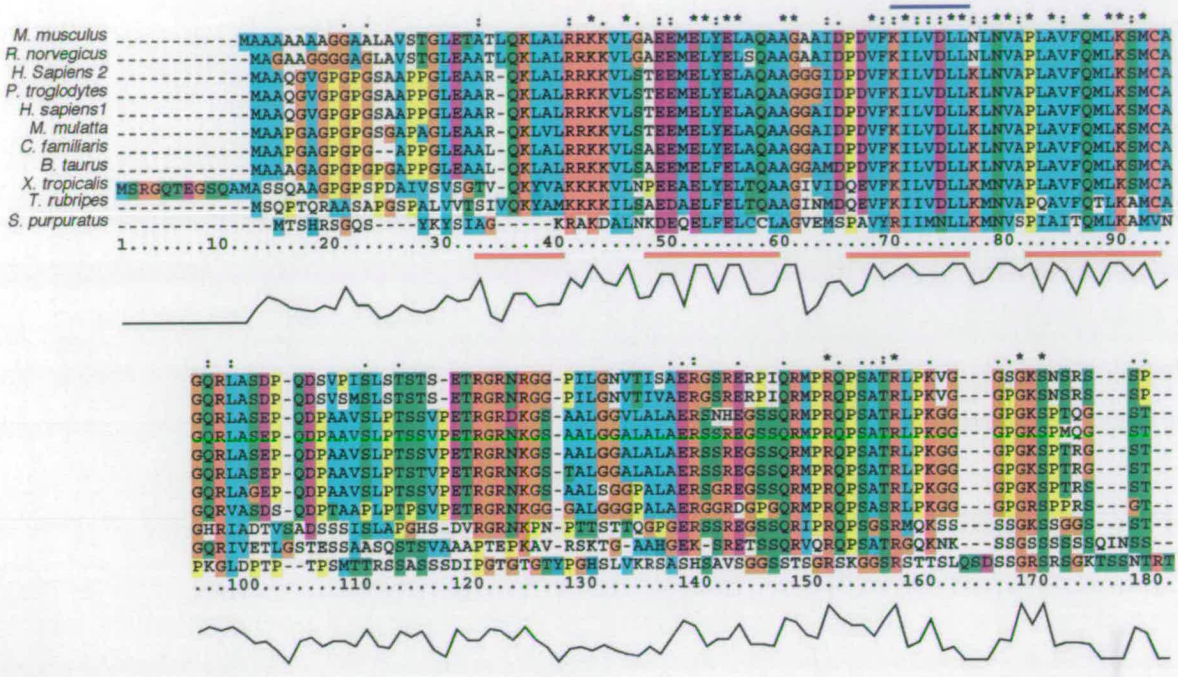


Figure 4.2: FLJ14346 is conserved in *deuterostomia*.
Multiple sequence alignment (clustalx) and secondary structure prediction of FLJ14346 proteins. NCBI/TrEMBL/ENSEMBL accession codes: *Homo sapiens*-1 (Human), NP_079305.2 [cDNA, chromosome 2]; *Homo sapiens*-2 (Human), AAH63024.1 [cDNA, chromosome 2], corrected in exon one by alignment with BM785188.1, BX426980.2, BQ932997.1, BX396153.2, BX344982.2, BM788236.1, BM767080.1 [ESTs]; *Pan troglodytes* (Chimpanzee), ENSPTRP00000021341 [pred. Protein, chromosome 2B] corrected in exon one by alignment with genomic sequence [17989170–17989059, chromosome 2B]; *Macaca mulatta* (Rhesus monkey), ENSMMUP00000017846 [pred. protein, Scaffold 105762, chromosome unknown]; *Mus musculus* (Mouse), NP_083630.1 [cDNA, chromosome 16]; *Rattus norvegicus* (Rat), XP_213563.1 [pred., chromosome 11]; *Canis familiaris* (Dog), XP_534764.2 [pred., chromosome 26]; *Bos taurus* (Cow), (XP_584206.2 [cDNA, chromosome 17] corrected in one exon by alignment with ESTs [DN526576.1, CO874736.1, CO877609.1, CB452177.1]; *Xenopus tropicalis* (African clawed frog), CAJ81722.1 [cDNA, chromosome unknown, scaffold 7]; *Takifugu rubripes* (Pufferfish), GENSCANSLICE00000014030 [exons 1+ 2 pred. Protein, scaffold 27, chromosome unknown] and NEWSINFRUP00000136542 [exons 3 pred. Protein, scaffold 27, chromosome unknown]; *Strongylocentrotus purpuratus* (Purple sea urchin), XP_792939.1 [pred. Protein, chromosome unknown]. Secondary structure alpha helices underlined in red predicted by Psirpred, Sam-t99, Profsec programmes. A cluster of conserved motifs is highlighted by a blue line.

### 4.2.2. GST-FLJ14346 interacts with Borealin, TD-60 and INCENP

In order to verify the yeast two-hybrid results and confirm whether FLJ14346 is capable of binding directly to INCENP, I cloned FLJ14346 into pGEX-4T3 for *in vitro* binding studies.

We investigated whether GST-FLJ14346 bound to INCENP and other chromosomal passenger proteins *in vitro*. Bacterially expressed GST-FLJ14346 was assayed for binding to *in vitro*-translated chromosomal passenger proteins INCENP, Aurora-B, Survivin, Borealin and TD-60 (Figure 4.3). Bound and unbound fractions were run on a polyacrylamide gel and visualised using a phosphorimager (Figure 4.3A). In Figure 4.3A it can be seen that GST alone does not pull down any of the chromosomal passenger proteins. In contrast, GST-FLJ14346 can bind to INCENP, Borealin and TD-60. GST-Borealin, used here as a positive control, interacts with INCENP, Survivin and itself as has been previously shown (Gassmann et al., 2004). Quantitation of these results is shown in Figure 4.3B. GST-Borealin was not tested against TD-60 or Aurora-B since Gassmann et al., showed that Borealin does not bind directly to them. Equal loading of the GST proteins is shown in Figure 4.3A'.

These experiments showed that GST-FLJ14346 can interact directly with INCENP *in vitro*. Furthermore, the observation that GST-FLJ14346 binds to two other chromosomal passenger proteins Borealin and TD-60 opens interesting insights for the function of this new protein.

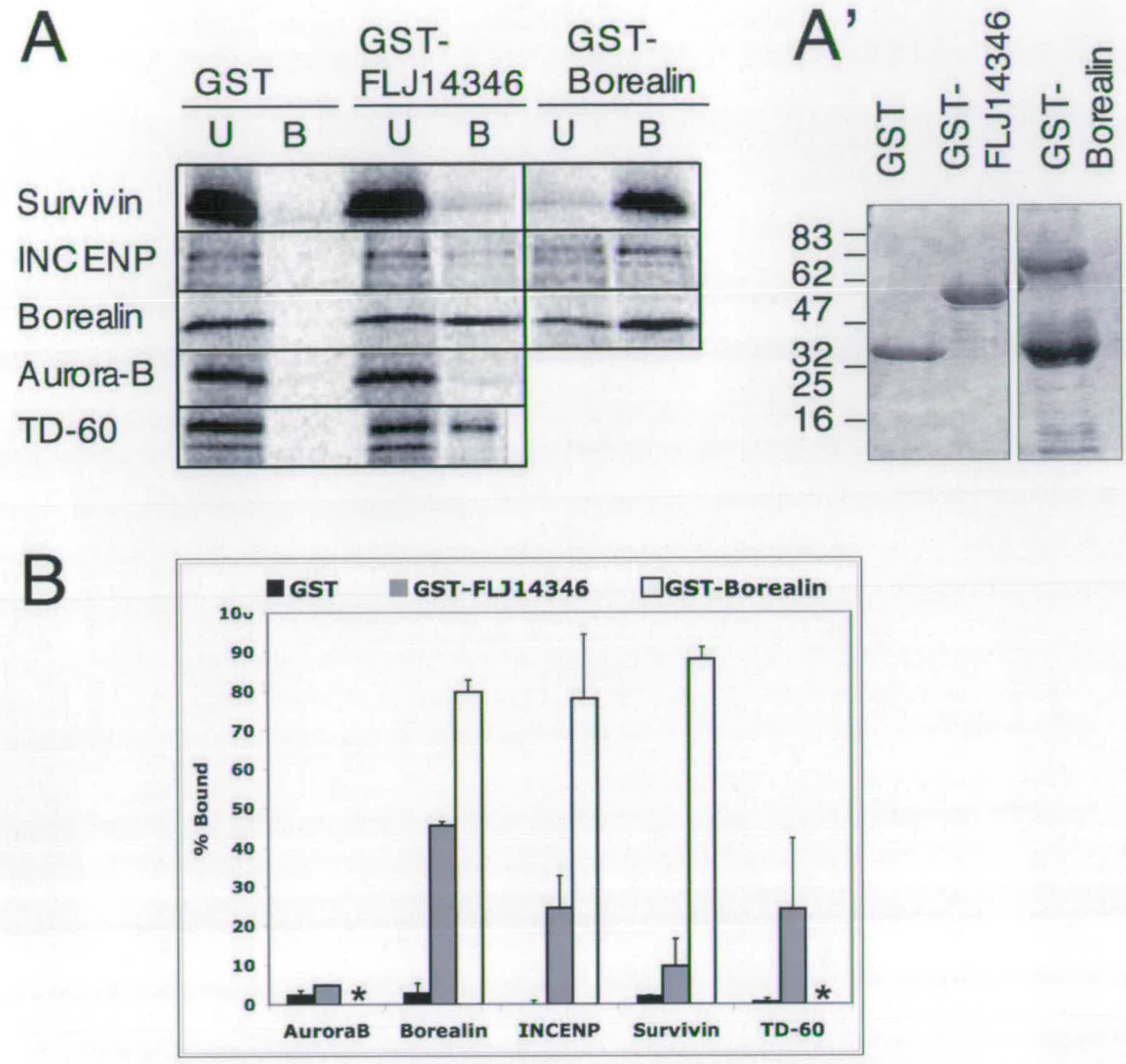


Figure 4.3: GST-FLJ14346 interacts with INCENP, Borealin and TD-60 *in vitro.*
(A) Proteins were translated in the presence of [$^{35}$S]-Methionine and incubated with bacterially expressed GST, GST-FLJ14346 or GST-Borealin bound to glutathione sepharose beads. Bound (B) and Unbound (U) fractions were run on SDS-PAGE gels and the proteins were visualized on a phosphorimager. (A') Equal loading of GST, GST-FLJ14346 and GST-Borealin, used in the binding experiments was checked by staining with Coomassie brilliant blue. (B) Quantification of binding experiments shown in A ($n \geq 2$). Error bars show the Standard deviation. * = Not done.

### 4.2.3. Localization of GFP-FLJ14346

In order to analyse the cellular localization of FLJ14346 I first tagged the protein with GFP by cloning it into the vector pEGFP-C1, which produces an N-terminal GFP-FLJ14346 fusion. The tagged protein was expressed in HeLa cells by transient transfection (Figure 4.4).

We found that GFP-FLJ14346 has a dynamic localization during mitosis. During prometaphase it localized to centrosomes and kinetochores (Figure 4.4A, see inset). It then localized to the spindle poles and along microtubules during metaphase, also accumulating at kinetochores (Figure 4.4B & 4.4B'). Figure 4.4B' shows a single optical section showing the accumulation of GFP-FLJ14346 at kinetochores. During anaphase GFP-FLJ14346 localized to the spindle poles and spindle midzone and then moved to the midbody during cytokinesis (Figure 4.4C&D). GFP-FLJ14346 localized to the centrosomes and nucleus during interphase (Figure 4.4E). When the GFP tag was put on the other end of the protein (FLJ14346-GFP), this caused the protein to mislocalise giving a diffuse cytoplasmic staining.

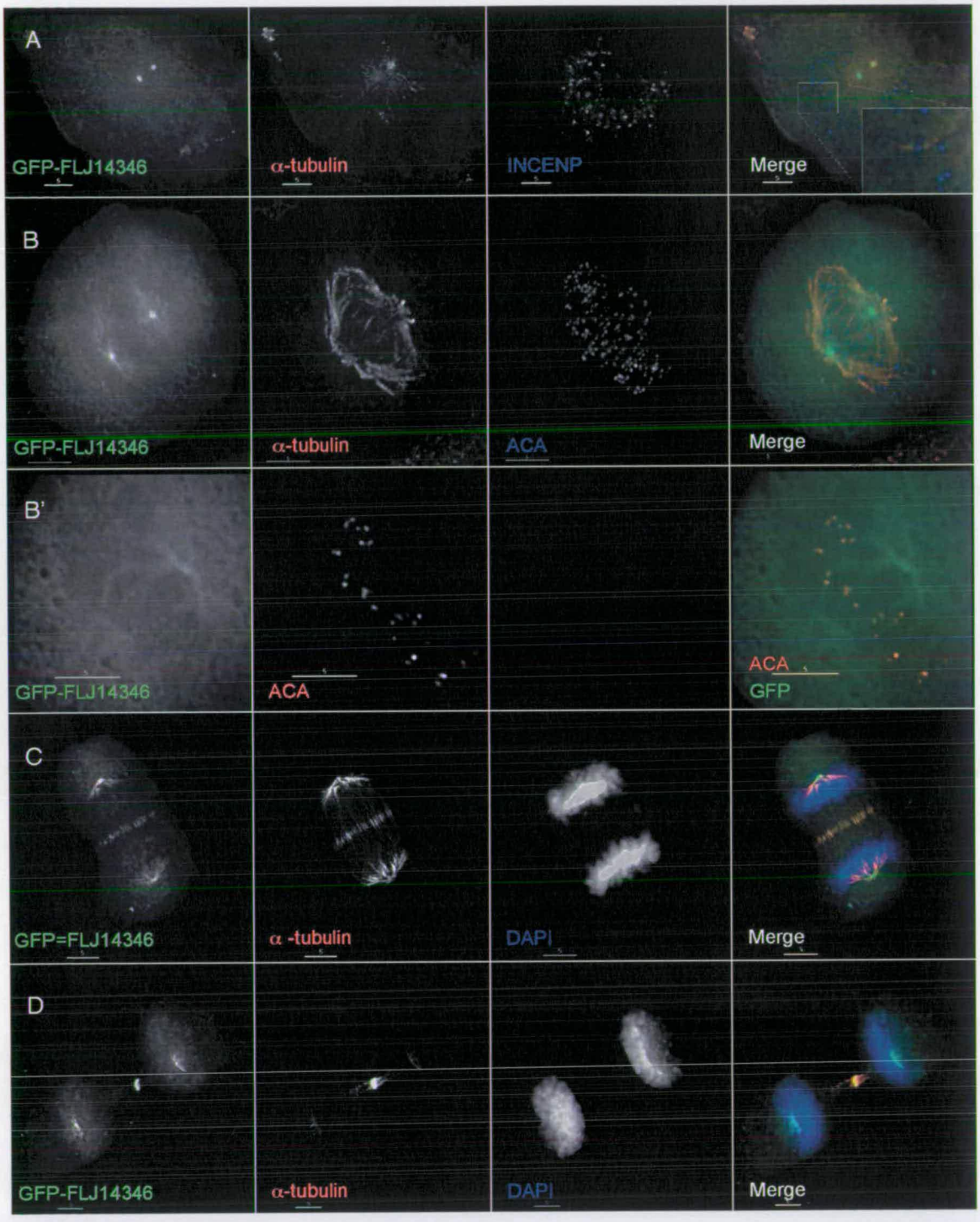


Figure 4.4: Localization of GFP tagged FLJ14346.
HeLa cells were transiently transfected with pEGFPC1 FLJ14346, fixed and stained with DAPI, INCENP antibody, γ-tubulin antibody and α-tubulin antibody, ACA (anti-centromere antibody). Scale bar is 5 μm. Imaged using an Olympus IX-70 microscope [Paola Vanerelli assisted me in taking these pictures].

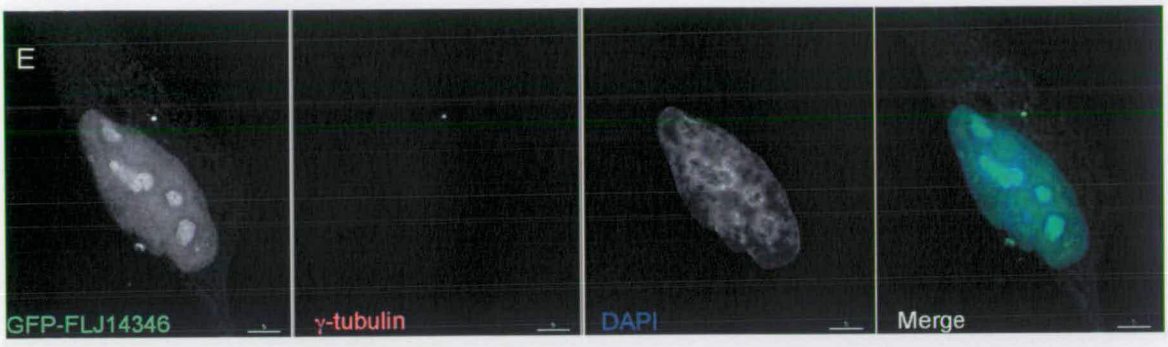


Figure 4.4 (cont.): Localization of GFP tagged FLJ14346.
HeLa cells were transiently transfected with pEGFPC1 FLJ14346, fixed and stained with DAPI, INCENP antibody, γ-tubulin antibody and α-tubulin antibody, ACA (anti-centromere antibody). Scale bar is 5 μm. Imaged using an Olympus IX-70 microscope [Paola Vanerelli assisted me in taking these pictures].

### 4.2.4. Characterisation of FLJ14346 antibodies

To determine the localization of endogenous FLJ14346 I expressed and purified GST-FLJ14346 from bacteria (Figure 4.5) and used it to immunize two rabbits.

Attempts to affinity-purify specific antibodies from these sera were not successful (data not shown). By western blot, one of the sera, R1789, recognized a single band of 16 kDa in HeLa cells. This is the expected size of the endogenous FLJ14346 protein (Figure 4.6). Serum R1789 recognized both the endogenous protein and the tagged protein in cells expressing GFP-FLJ14346 or TrAP-FLJ14346 (Figure 4.6), whereas the preimmune serum did not recognize either of these bands.

Indirect Immunofluorescence using serum R1789 showed that endogenous FLJ14346 is present on the spindle poles throughout mitosis (Figure 4.7). During metaphase it is highly concentrated at the spindle poles and is also present along the spindle microtubules (Figure 4.7A). During anaphase it is seen on each half spindle on the interpolar microtubules behind the chromosomes and extending towards the spindle midzone (Figure 4.7C). During cytokinesis the antibody stains a region of the midbody flanking the

region where the chromosomal passengers are located (Figure 4.7E&G). In interphase the antibody stains the centrosomes (Figure 4.7I).

A second antibody (R1790) raised against FLJ14346 shows an identical localization pattern (Figure 4.7B, D, F, &H) suggesting that they both recognize the same protein.

In order to look more closely whether endogenous FLJ14346 localised to the kinetochore, images were taken using an Olympus IX-70 microscope using deconvolution software (Figure 4.8). In prometaphase FLJ14346 is concentrated on the spindle poles (Figure 4.8A, B, C &D) and is present along the microtubules emanating from the spindle poles. Figure 4.8A (inset) shows a single optical section showing FLJ14346 on the microtubules. Endogenous FLJ14346 does not appear to accumulate at the kinetochore, as was seen with the GFP-FLJ14346 (Figure 4.8B & C). During anaphase FLJ14346 begins to accumulate on each half of the spindle behind the chromosomes (Figure 4.8E & F), flanking the region where the chromosomal passengers are located (Figure 4.8E). During cytokinesis FLJ14346 is located where there are dense microtubule bundles flanking the region where the chromosomal passengers are located (Figure 4.8G & H).

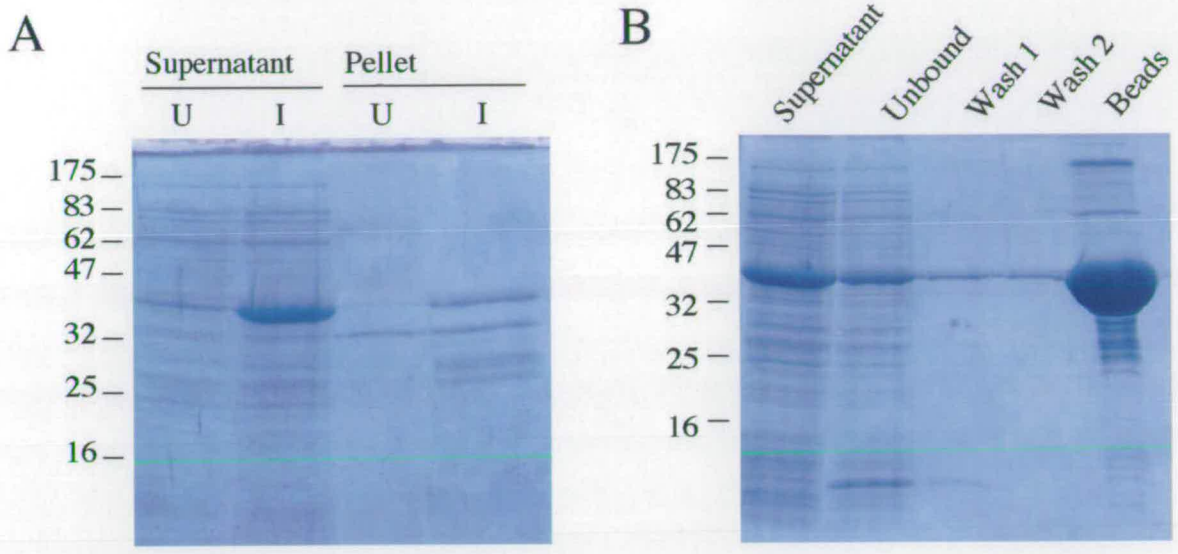


Figure 4.5: Expression and purification of GST-FLJ14346 from *E. coli.*
[A] Coomassie stained acrylamide gel showing the expression of GST-FLJ14346 of in *E. coli.* Uninduced (U) and induced (I) samples were taken and the equivalent of 1/20$^{th}$ of 1ml of culture was run on the gel. [B] Coomassie stained acrylamide gel showing the purification of GST-FLJ14346. The equivalent of 1/20$^{th}$ of 1ml of culture was run on the gel for supernatant, unbound and wash fractions. An aliquot of the purified protein bound to sepharose beads was taken and the equivalent of 12ml of culture was loaded on the gel.

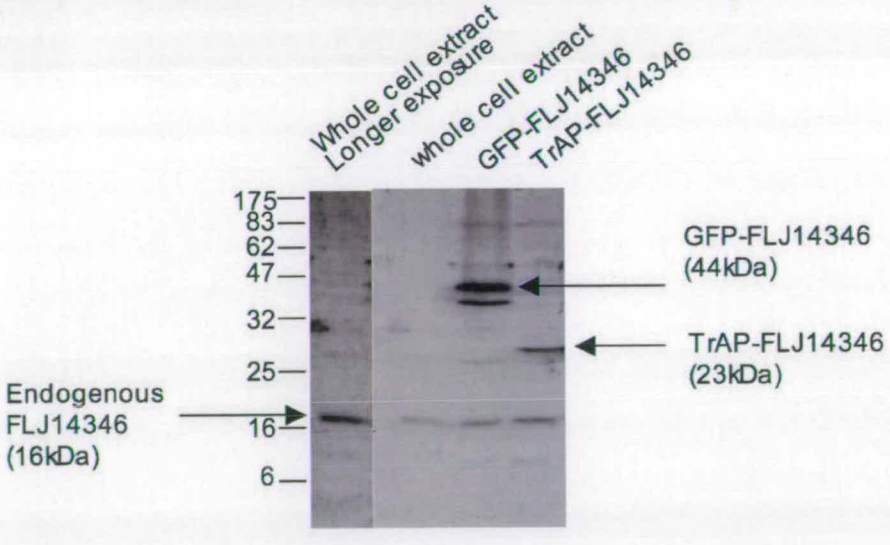


Figure 4.6: Anti-FLJ14346 (R1789) serum recognizes endogenous and tagged forms of FLJ14346.
Western blot of whole cell extract from untransfected HeLa cells, GFP-FLJ14346 expressing HeLa cells or TrAP-FLJ14346 expressing HeLa cells. Samples were run on a 12.5% polyacrylamide gel (2x10$^5$ cells per lane) and transferred to nitrocellulose membrane. Anti-FLJ14346 (R1789) was used at 1:200 in PBS / 5%milk / 0.25% Tween 20.

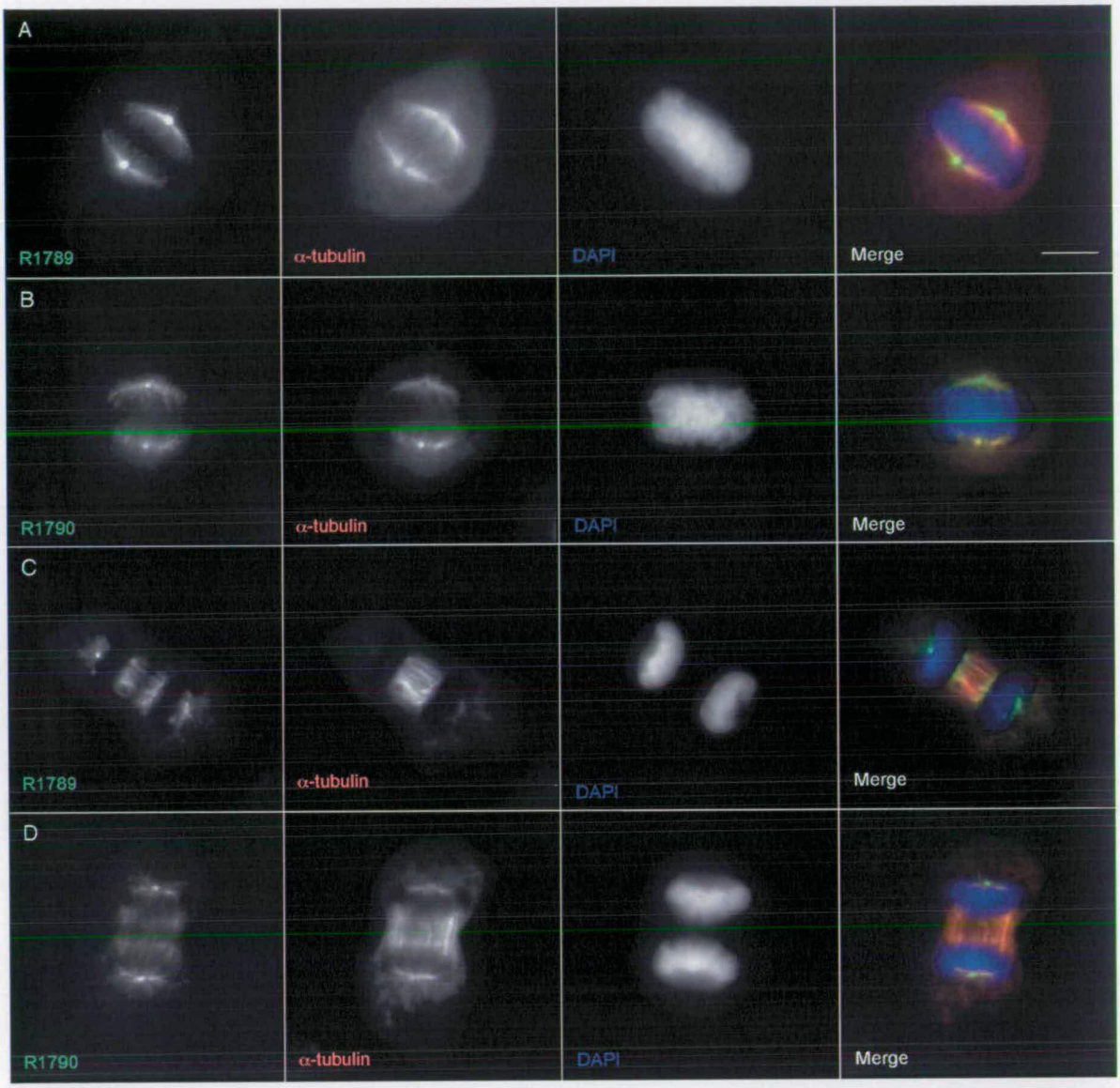


Figure 4.7 A-D: Both FLJ14346 antibodies (R1789 and R1790 serum) show an identical localization pattern.
Cells were fixed and stained with R1978 serum, DAPI, α-tubulin antibody and imaged on a Zeiss Axioplan 2 microscope. Scale bar is 10 μm and all pictures are at the same magnification.

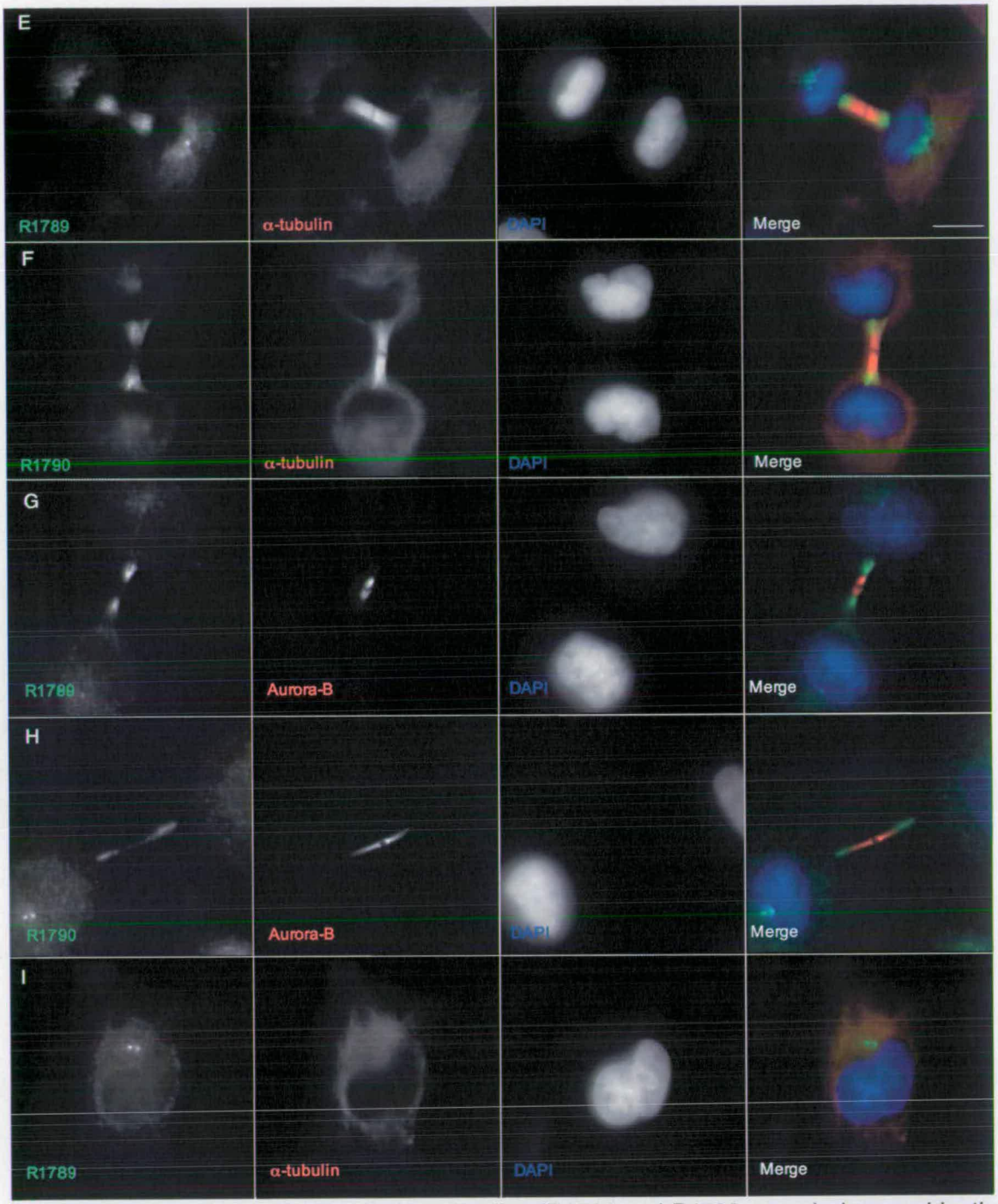


Figure 4.7 (cont.) E-H: Both FLJ14346 antibodies (R1789 and R1790 serum) show an identical localization pattern.
Cells were fixed and stained with R1978 and R1790 serum, DAPI, Aurora-B antibody and imaged on a Zeiss Axioplan 2 microscope. Scale bar is 10 μm and all pictures are at the same magnification.

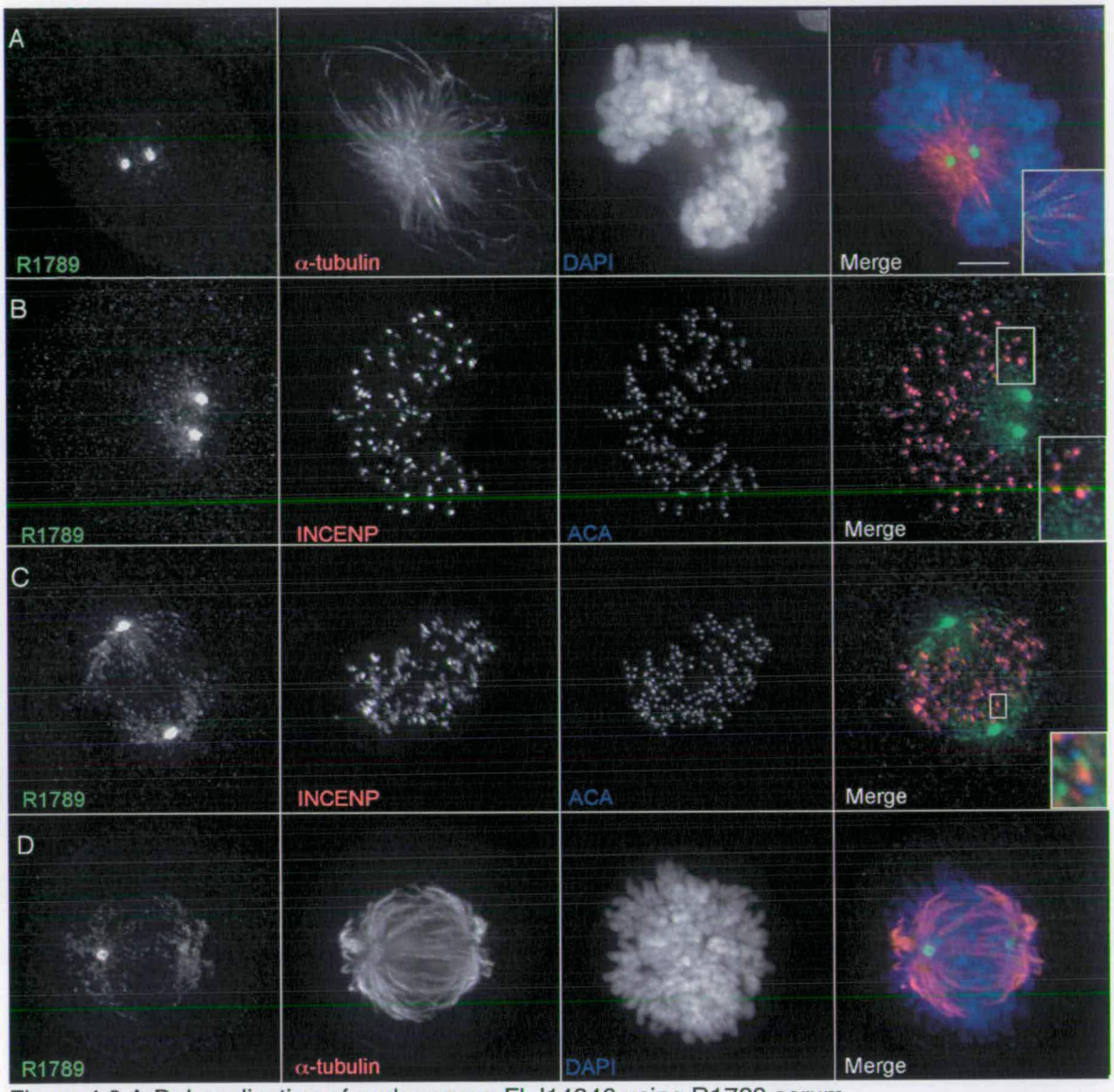


Figure 4.8 A-D: Localisation of endogenous FLJ14346 using R1789 serum.
Cells were fixed and stained with R1790 serum, DAPI, Aurora-B antibody and α-tubulin antibody and imaged using an Olympus IX-70 microscope. Scale bar is 5 μm and all pictures are at the same magnification. [Mar Carmena took these pictures].

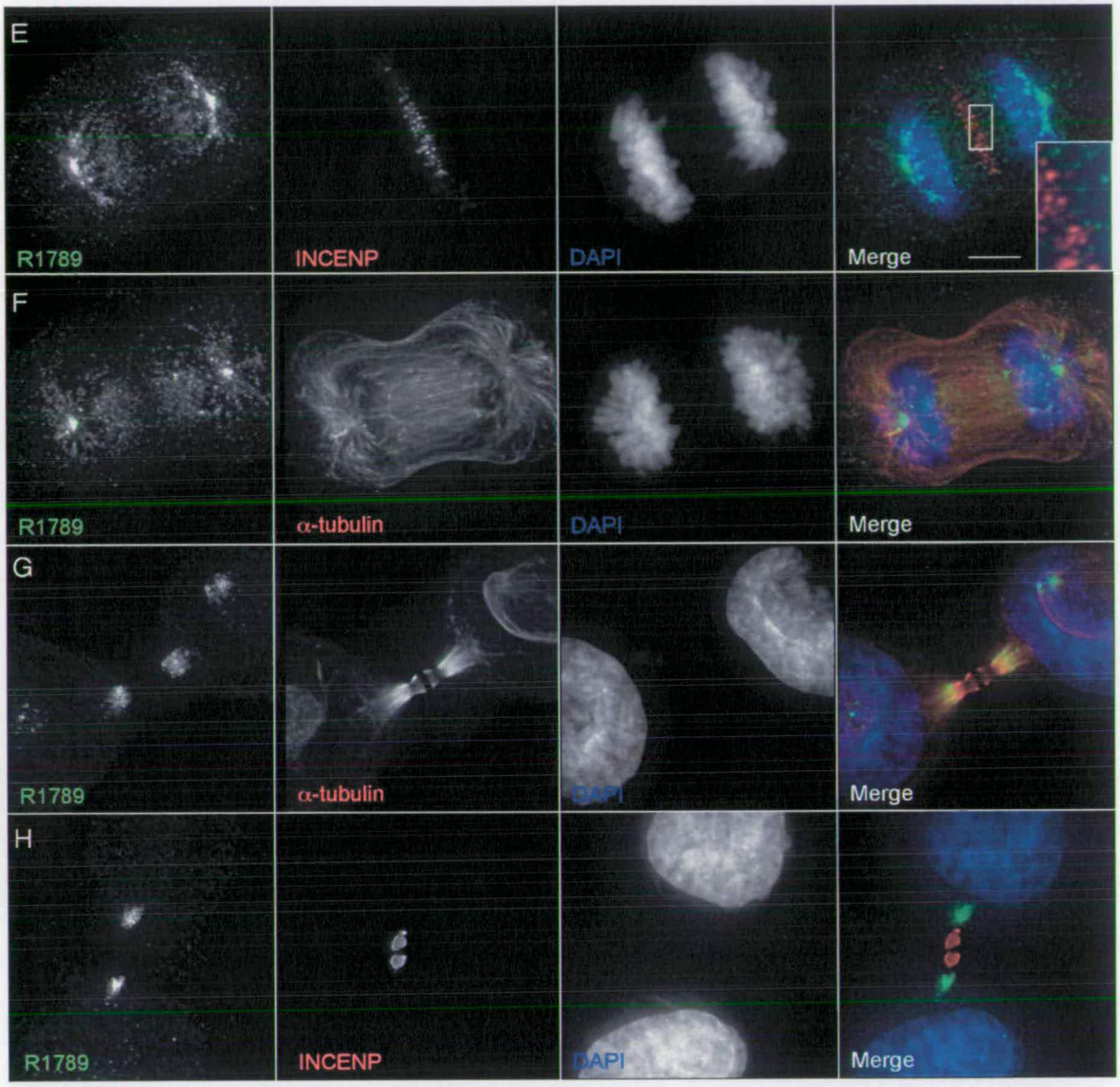


Figure 4.8 (cont.) E-H: Localisation of endogenous FLJ14346 using R1789 serum.
Cells were fixed and stained with R1790 serum, DAPI, Aurora-B antibody and α-tubulin antibody and imaged using an Olympus IX-70 microscope. Scale bar is 5 μm and all pictures are at the same magnification. [Mar Carmena took these pictures].

There are some discrepancies between the localization of the GFP tagged protein and the antibody staining (See table 8 below, differences are highlighted in red).

Table 8: Comparison of localization of GFP-FLJ14346 and antibody staining.

| Cell cycle stage | Localisation of GFP-FLJ14346 | Localisation of Anti-FLJ14346 antibodies |
|---|---|---|
| Interphase | Centrosomes<br>Nucleus | Centrosomes |
| Prometaphase/Metaphase | Spindle poles<br>Microtubules<br>Kinetochores | Spindle poles<br>Microtubules |
| Anaphase | Spindle poles<br>Spindle midzone | Spindle poles<br>interpolar microtubules behind the chromosomes |
| Cytokinesis | middle of the midbody | outer zone of the midbody |

These discrepancies could be caused by a number of factors:

The tagged protein may not localize correctly since the tag is large (26 kDa) compared to the size the protein (16 kDa). The tag could interfere with its proper localization.

The tagged protein may not localize properly because it is overexpressed. Overexpressed GFP tagged proteins often accumulate in the nucleus and nucleolus (Gassmann et al., 2004). The accumulation of the GFP-tagged proteins at the kinetochore may reflect a movement of the protein towards the microtubules plus ends.

The antibody may also recognize a paralogue of FLJ14346 (AAH63024.1), which may localise differently. Although not included in the present study due to lack of time, in the future, it will be important to clone and tag the paralogue (see multiple sequence alignment section 4.2.1).

FLJ14346 within some structures may not be accessible to these antibodies. This could be the case when the protein is in the midbody and the

spindle midzone, where there are dense arrays of microtubules (Saxton and McIntosh, 1987).

### 4.2.5. Localisation of TrAP tagged FLJ14346

To address some of these problems I constructed a plasmid (pTrAP) containing a series of three small tags (7 kDa in total)-His tag, S-tag and SBP tag (see section 3.1.4 for a plasmid map).

The Streptavidin Binding Peptide (SBP) was isolated from a random peptide library screen for peptides that bound Streptavidin. It has a high affinity for Streptavidin (Kd = 2.5 nM) and can be eluted under native conditions with biotin (Wilson et al., 2001; Keefe et al., 2001). The S-tag is a 15 amino acid fragment of RNase A which binds with high affinity (Kd = 0.5μM) to the S-protein also derived form RNase A (Karpeisky et al., 1994). Polyhistidine tags are widely used to purify protein and these proteins can be eluted under native conditions with imidazole (Terpe, 2003).

These small tags can be used to visualize the localization of a fused protein since antibodies against some of them were available to us and working by indirect immunofluoresence. Furthermore all three tags can be used for affinity purification of the protein in the future.

I first tagged the protein with this triple tag by cloning it into the vector pTrAP-N1, which produces an N-terminal His-S-tag-SBP-FLJ14346 (TrAP-FLJ14346) fusion. The tagged protein was expressed in HeLa cells and a stable cell line was established. We found that TrAP-FLJ14346 has as a localization that is identical to the antibody staining (Figure 4.9). During metaphase it localized to spindle poles and along microtubules during metaphase (Figure 4.9A & B). During early anaphase TrAP-FLJ14346 localized on the spindle poles and along the microtubules (Figure 4.9C). By late anaphase TrAP-FLJ14346 was still on the spindle poles and on each half spindle on the

interpolar microtubules behind the chromosomes and extending towards the spindle midzone (Figure 4.9D). During cytokinesis TrAP-FLJ14346 is on the centrosomes and the outer zone of the midbody (Figure 4.9E).

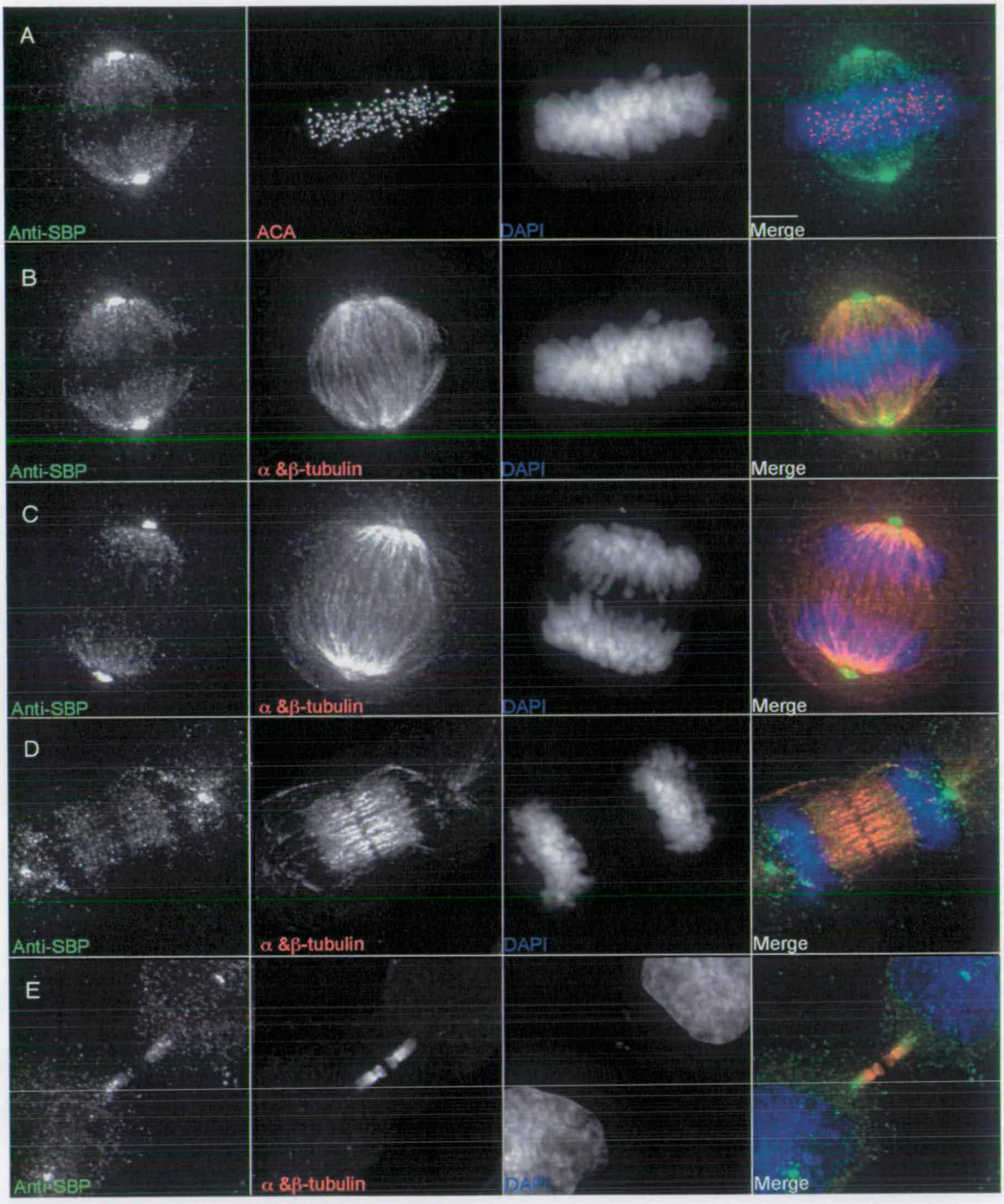


Figure 4.9: Localisation of TrAP FLJ14346.
HeLa stable cell lines expressing pTrAPN1 FLJ14346, fixed and stained with R1789 serum, DAPI, α- & β-tubulin antibody and ACA. Scale bar is 5 μm and all pictures are at the same magnification. Imaged using an Olympus IX-70 microscope. [Mar Carmena took these pictures].

## *4.3. Discussion*

### 4.3.1. FLJ14346 sequence analysis

The human FLJ14346 is a 16 kDa protein, which is conserved in *Deuterostomia*. Secondary structure predictions suggest that it has four alpha helices at its N-terminus. Multiple sequence alignment revealed the predicted alpha helical region is more conserved than the C-terminus. There are several conserved binding motifs within the third predicted alpha helix (a potential cyclin substrate recognition site, a nuclear receptors box motif and a Class III PDZ domain binding motif).

In humans, FLJ14346 has a paralogue (Accession No. AAH63024.1). This gene duplication must have happened fairly recently in evolutionary history since closely related species do not have a second gene. It would also be interesting to clone and tag the paralogue and see if it also binds members of the chromosomal passenger complex.

Although sequence analysis has not given us any clues as to the function of FLJ14346, the secondary structure analysis may be useful when designing deletion constructs. It may be interesting to express the highly conserved alpha helical N-terminus and the less conserved C-terminus separately to see which half is responsible for binding, localization. There are several conserved putative binding sites (cyclin substrate recognition site, nuclear receptors box motif and a Class III PDZ domain binding motif) all of which overlap with each other. To find out if these are real binding sites it will be important to mutagenise this region.

### 4.3.2. FLJ14346 binds INCENP, Borealin and TD-60

FLJ14346 is a novel protein that was identified by yeast two-hybrid as a potential INCENP interactor. I used the GST pulldown method to verify the

interaction between INCENP and FLJ14346 identified by yeast two-hybrid. I also examined whether FLJ14346 bound any of the other chromosomal passenger proteins and found it also bound Borealin and TD-60 in this assay.

Interaction of FLJ14346 with INCENP and Borealin is weaker compared to the positive control used in this experiment, Borealin, which binds INCENP, Survivin and itself (Gassmann et al., 2004). The binding of FLJ143467 to the chromosomal passenger proteins may reflect a transient or weak interaction. As discussed in chapter 3, the yeast two-hybrid system is highly sensitive and therefore interactions picked up by yeast two-hybrid system are not always detectable by other means. Robust binding may require the presence of another protein such as Borealin or TD-60, or a post-translational modification. INCENP, for example, is required to target PLK1 to the centromere but PLK1 binds only to INCENP that has been phosphorylated by CDK1 (Goto et al., 2005). Borealin has been shown to bind the N-terminal 58 amino acids of INCENP (Klein et al., 2006) therefore a complex may form containing INCENP, FLJ14346 and Borealin. However, due to the fact that the coding regions of FLJ14346 from the two-hybrid screen were in the wrong frame, it will be crucial to confirm these interactions *in vivo*.

Interestingly, FLJ14346 also bound TD-60. TD-60, a putative GEF for Rac1, is a chromosomal passenger protein that colocalises with the chromosomal passenger complex at all stages of mitosis (Martineau-Thuillier et al., 1998). There is co-dependance between TD-60 and the other members of the chromosomal passenger complex for their localization (Gassmann et al., 2004; Mollinari et al., 2003). However, no direct or indirect interaction has been shown between TD-60 and any other member of the chromosomal passenger complex. FLJ14346 may provide a link between TD-60 and the chromosomal passenger complex but may do so during a limited period during the cell cycle or in specific compartments within the cell.

### 4.3.3. FLJ14346 has a dynamic localization during mitosis

We have studied the localization of FLJ14346 using GFP-tagged and TrAP-tagged protein and made two antibodies against FLJ14346. There were some discrepancies observed between the localization of the GFP tagged protein and the staining of the endogenous protein with our antibodies. The large size of the GFP tag or overexpression could have caused the protein to mislocalise. In order to resolve this problem, I tried using a smaller tag and making a stable cell line expressing TrAP-FLJ14346 in order to select a cell line that had a lower expression level. This gave the same localization as was seen with the antibody suggesting that the antibody and the TrAP-FLJ14346 staining are both correct. Overexpression of an untagged version of the protein or the TrAP tagged protein should tell us if overexpression alone causes the protein to accumulate at the kinetochores, spindle midzone and midbody.

The antibody may also recognize the paralogue of FLJ14346 (AAH63024.1) since the two proteins are 96% identical that the protein level, which may localise differently. In the future, it may be interesting to clone this paralogue and look at its localisation.

The antibody, both to the FLJ14346 protein and the TrAP tag, may not have access to some structures such as the midbody. There are new technologies emerging such as small fluorescent tags (12aa), which work by binding of a dye molecule to a tetracysteine motif. This may be useful for looking at structures such as the midbody (Martin et al., 2005).

FLJ14346 localizes to centrosomes during interphase and the spindle poles during mitosis. It localises along spindle microtubules during prometaphase and metaphase. It then associates with each half of the spindle behind the chromosomes, flanking the region where the chromosomal passenger proteins are located. During anaphase it is on the spindle poles and is associated with the outer part of the midbody during cytokinesis.

FLJ14346 is a novel conserved protein that binds INCENP, TD-60 and Borealin. Analysis of its localization has revealed a dynamic distribution throughout mitosis. Much work is still needed to find out more about the function of this protein.

# V Results - The chromosomal passenger INCENP promotes the maintenance of sister-chromatid cohesion in meiosis through MEI-S332

# 5. The chromosomal passenger INCENP promotes the maintenance of sister-chromatid cohesion in meiosis through MEI-S332.

## *5.1. Introduction*

The chromosomal passenger proteins have been shown to play essential roles in mitosis and cytokinesis, and their dynamic distribution in mitosis has been well documented, for review see (Vagnarelli and Earnshaw, 2004) and section 1.9. However, much less is known about the function of the chromosomal passengers in meiosis.

Cohesion plays an important role in both mitosis and meiosis. In mitosis, sister chromatids must be held together in order for them achieve bipolar attachment (Watanabe, 2005a). Cohesion between sister chromatids is maintained by the Cohesin complex. At the onset of anaphase Scc1/Rad21 is cleaved by Separase allowing the sister chromatids to segregate to opposite poles (Hauf et al., 2001; Uhlmann et al., 2000). In mitosis cohesion is lost first from the arms in prophase and then from the centromere at anaphase onset. In meiosis cohesion is also lost in a stepwise manner. Centromeric cohesion but not arm cohesion between sister chromatids persists through anaphase of meiosis I. Separase is activated at the metaphase I – anaphase I transition where it cleaves Rec8, a meiosis specific Rad21 paralogue (Parisi et al., 1999), along the arms but not at the centromere where cohesion is protected by MEI-S332/shugoshin until anaphase II (see discussion, section 5.3). MEI-S332 leaves centromeres at metaphase II, and sister chromatids separate at anaphase II when Separase is again activated (Wang and Dai, 2005; Watanabe and Kitajima, 2005b).

The chromosomal passenger proteins have been shown to play a role in the regulation of arm cohesion in meiosis I. In *C. elegans* AIR-2 (Aurora-B) has

been shown to regulate the release of chromosome cohesion in meiosis (Kaitna et al., 2002; Rogers et al., 2002). The localization of the chromosomal passenger proteins to the equatorial axes between paired arms of homologous chromosomes in *C. elegans* has been shown to be dependant on the formation of chiasmata (Rogers et al., 2002). Depletion of AIR-2 by RNAi prevented separation of homologue pairs in meiosis I and sister chromatids in meiosis II. Rogers *et al.* showed that AIR-2 phosphorylated REC-8 *in vitro* and in AIR-2 depleted cells, REC-8 was not removed and therefore the homologues were not segregated (Rogers et al., 2002). Depletion of GLC-7$\alpha$,$\beta$ (PP1) phosphatase, which acts antagonistically to AIR-2, caused a decrease of chromosomal REC-8 and premature separation of sister chromatids at anaphase I. Based on the fact that in both budding yeast and human cells phosphorylation of Scc1/Rad21 by Plk1 leads to more efficient degradation of Scc1/Rad21 by Separase (Alexandru et al., 2001; Hauf et al., 2005; Uhlmann et al., 2000), Rogers *et al.* proposed that phosphorylation of REC-8 by AIR-2 promotes its cleavage.

During mitosis chromosomal passenger proteins undergo a dramatic change of localisation at the metaphase-anaphase transition, leaving the chromatin and binding the microtubules of the central spindle. In budding yeast this is regulated by dephosphorylation of INCENP by Cdc14 (Pereira and Schiebel, 2003). However, in meiosis in mouse, passenger proteins are retained on the centromere at the metaphase I – anaphase I transition (Parra et al., 2003; Tang et al., 2006a). In *D. melanogaster*, INCENP shows a similar pattern of distribution in male meiosis (Resnick et al., 2006). INCENP localisation at centromeres in meiosis correlates with the maintenance of sister centromere cohesion. This led us to investigate a possible role of the chromosomal passenger complex in this process.

In both *C. elegans* and mouse meiosis the chromosomal passenger proteins localise to chromosome arms distal to chiasmata (Kaitna et al., 2000; Parra et al., 2003; Rogers et al., 2002; Tang et al., 2006a). In *C. elegans* the

chromosomal passenger proteins have a role in the release of sister chromatid arm cohesion and resolution of chiasmata. In a typical meiosis I, where homologue segregation requires recombination and formation of chiasmata (Watanabe, 2005a), a defect in the function of the chromosomal passenger complex may result in unresolved chiasmata and homolog non-disjunction. This effect may obscure any further defect in sister centromere cohesion. In order to overcome this problem we studied the roles of the chromosomal passenger complex in *Drosophila melanogaster* male meiosis, in which there is no recombination. The work presented in this chapter is part of this study (Resnick et al., 2006).

Using viable mutations in *INCENP*, Resnick *et al.* used a combination of genetic and cytological techniques to show that disruption of INCENP function leads to premature loss of sister chromatid cohesion in meiosis (Resnick et al., 2006). One protein known to be required for the maintenance of sister chromatid centromere cohesion is MEI-S332 (Kerrebrock et al., 1992). Resnick *et al.* showed that INCENP/Aurora-B partially colocalises with MEI-S332 and that INCENP and/or Aurora-B function are required for the stable localisation of MEI-S332 on centromeres in mitosis and meiosis (Resnick et al., 2006).

The data presented here show that INCENP binds to MEI-S332 *in vitro*. We also showed that MEI-S332 is a substrate of Aurora-B kinase *in vitro* and that when the phosphorylation site for Aurora-B on MEI-S332 is mutated, MEI-S332 is no longer stably associated with the centromere.

The experiments described here were carried out in collaboration with Mar Carmena and David L. Satinover (University of Virginia).

## 5.2. Results

### 5.2.1. MEI-S332 associates directly with DmINCENP *in vitro*

Since DmINCENP and/or DmAurora-B have been shown to be required for the stable localisation of MEI-S332, we investigated whether DmINCENP and MEI-S332 were able to bind *in vitro*. Bacterially expressed GST-DmINCENP was assayed for binding to *in vitro*-translated MEI-S332, DmAurora-B, or a mixture of both proteins (Figure 5.1). These experiments show that there is a direct interaction between GST-DmINCENP and MEI-S332. Interestingly more MEI-S332 was bound when both MEI-S332 and DmAurora-B were added together in the GST pulldown.

### 5.2.2. MEI-S332 is phosphorylated by Aurora-B *in vitro*

We next tested whether MEI-S332 was a substrate for Aurora-B kinase *in vitro*. GST-MEI-S332 was incubated with recombinant bacterially expressed *Xenopus* Aurora-B/INCENP and was phosphorylated at levels comparable to the test substrate MBP, Myelin Basic protein. MEI-S332 could also compete away label from MBP (Figure 5.2A). We concluded that MEI-S332 is an excellent *in vitro* substrate for Aurora-B.

There are three potential Aurora-B phosphorylation sites in the MEI-S332 protein. They contain two or more consecutive serine residues (S98/S99, S124/S125/S126, S138/S139). Our collaboraor David Satinover used site directed mutagenesis to mutate these serines to alanines. GST tagged mutant proteins were expressed and purified from *E. coli*. When these mutant proteins were used as substrates in kinase assays, only one of the three mutated regions MEI-S332$^{S124,125,126A}$ (Mei-S332 124AAA) affected phosphorylation in the kinase assay (Figure 5.2B). The major Aurora-B phosphorylation site/s on MEI-S332 is

therefore in the region between S124 and S126. There may also be other phosphorylation sites on MEI-S332 since the phosphorylation of MEI-S332$^{S124,125,126A}$ (MEI-S332 124AAA) was not reduced to zero.

### 5.2.3. MEI-S332$^{S124,125,126A}$ does not stably associate with centromeres in mitosis.

In order to analyze the role of Aurora-B phosphorylation of MEI-S332 *in vivo*, we studied the effect of the GFP-tagged MEI-S332$^{S124,125,126A}$ phosphorylation mutant (MEI-S332-124AAA) in transiently transfected S2 cells. We found high levels of centromeric wild-type GFP-MEI-S332 (Figure 5.3A) in 94% of prometaphase/metaphase cells (Figure 5.3E; n >400 per experiment). In contrast, only 33.3% of prometaphase/metaphase cells showed high levels of GFP-MEI-S332-124AAA mutant protein at centromeres (Figure 5.3D & E). 66% of cells expressing this mutant version showed reduced signal at centromeres (Figure 5.3B & C, arrow; Figure 5.3E). Quantification of the intensity of fluorescence showed that kinetochores in cells showing high levels of mutant MEI-S332 do so at a similar level to wild type, whereas kinetochores with lower levels of mutant protein show up to a fifteen-fold reduction in fluorescence (Figure 5.3F). This difference is not due to differences in protein levels, caused by mutation making the protein unstable (Figure 5.3G).

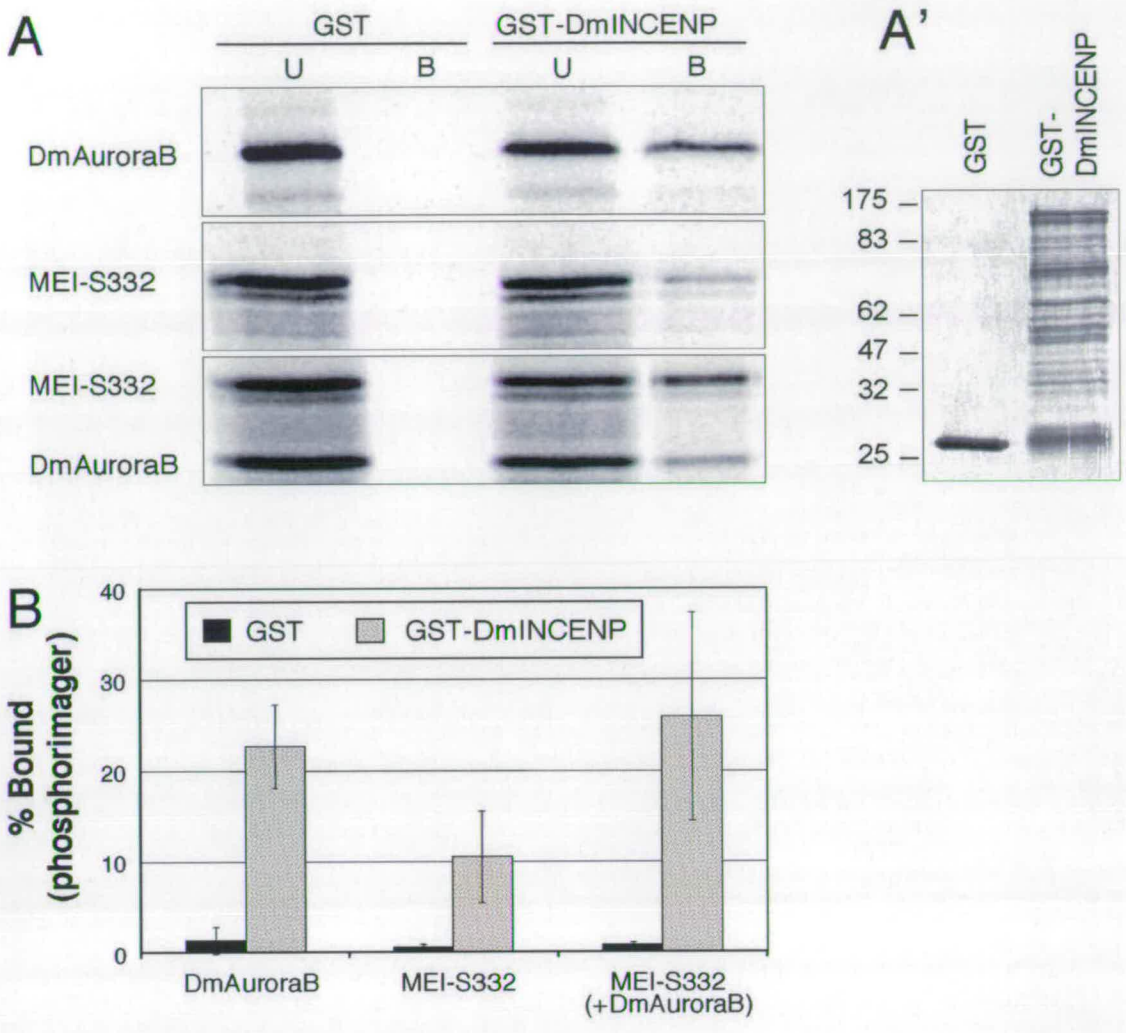


Figure 5.1: DmINCENP interacts directly with MEI-S332 *in vitro.*
[A]. Proteins were translated in the presence of [$^{35}$S]-methionine and incubated with bacterially expressed GST-DmINCENP or GST bound to glutathione sepharose beads. Bound (B) and unbound (U) fractions were separated by SDS-PAGE, and the proteins were visualized using a phosphorimager. (A') Equal loading of GST, GST-DmINCENP used in the binding experiments was checked by staining with Coomassie brilliant blue. [B] Quantification of the binding experiment shown in A. The bars represent the percentage of total protein bound to or GST (black) or GST-DmINCENP (grey). The error bars indicate the standard deviation ($n \geq 3$).

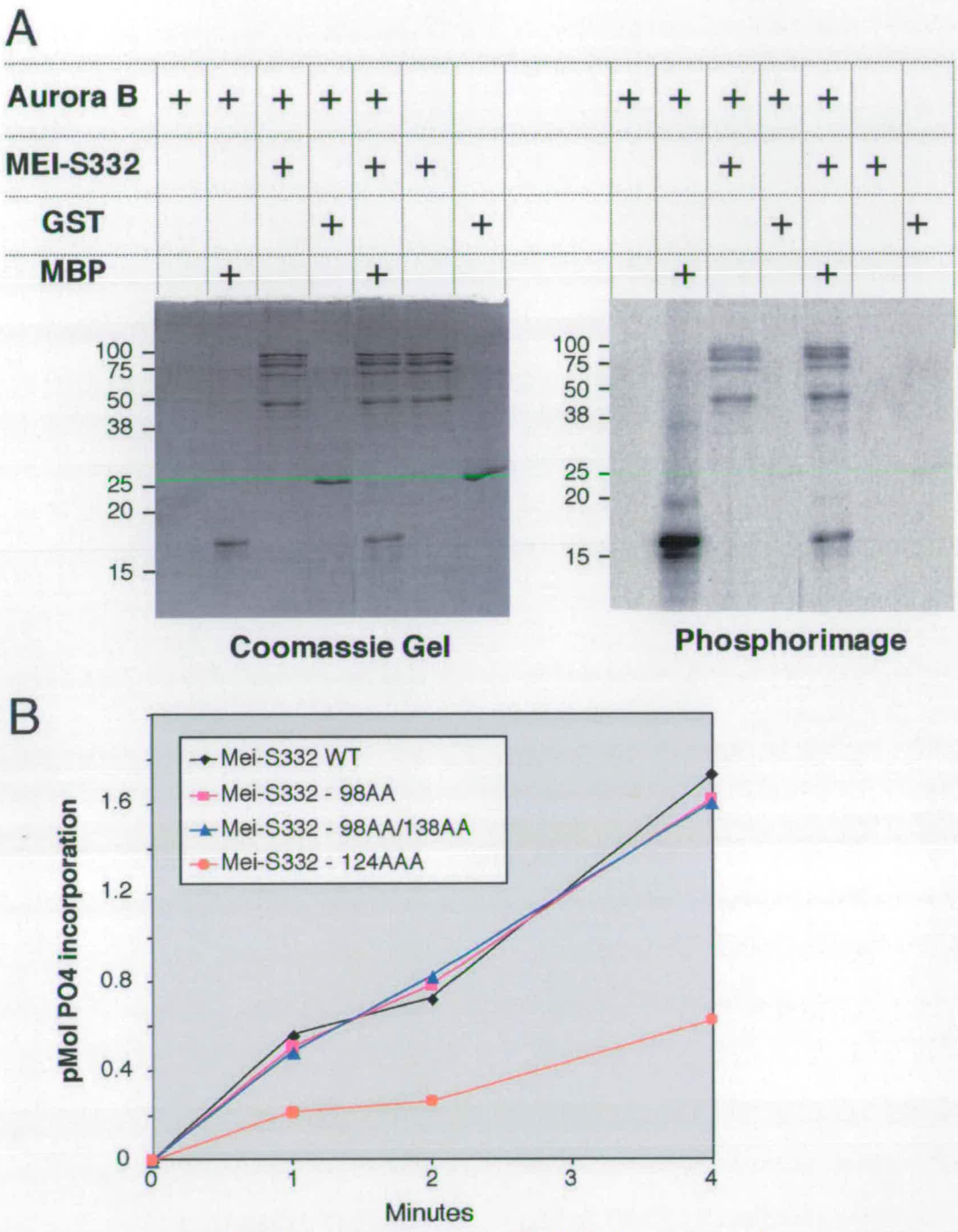


Figure 5.2: Aurora-B/INCENP phosphorylates MEI-S332 *in vitro* on at least one serine between residues 124-126.
(A) Recombinant Aurora-B/INCENP complex was incubated with 32P-ATP and the indicated substrate for 1 minute and incorporation of phosphate onto the proteins was visualized by autoradiography (right) and protein loading analyzed by Coomassie Blue stain (left). MBP-Myelin Basic Protein, GST-Glutathione S-transferase. (B) Time course of Aurora-B/INCENP kinase activity (assayed as in A) using WT MEI-S332 or the indicated phospho-site mutant. [These experiments were carried out by David Satinover].

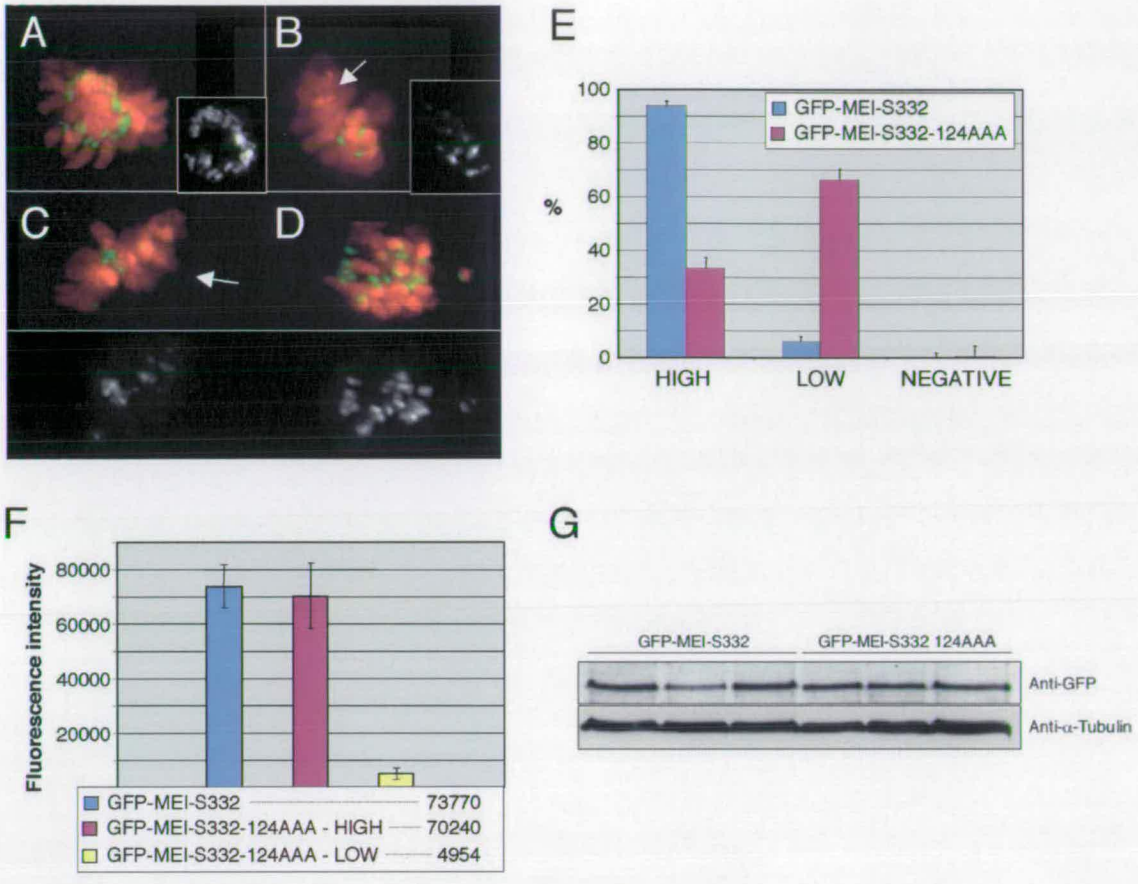


Figure 5.3: MEI-S332-124AAA phosphorylation mutant does not stably associate with centromeres in mitosis.
(A) High level of centromeric GFP-MEI-S332 in metaphase. (B) Reduced level of the phosphorylation mutant GFP-MEI-S332-124AAA on metaphase centromeres (arrow). (C & D) Microscope field showing a prometaphase cell with high levels of centromeric GFP- MEI-S332-124AAA and a metaphase cell with very reduced levels of mutant protein in most centromeres (arrow). In A-C the GFP staining alone is shown in the black and white panels. (E) Percentage of cells transfected with GFP-MEI-S332 or GFP-MEI-S332-124AAA showing normal levels of GFP signal on kinetochores (HIGH category), lower than normal (LOW category) or no signal (NEGATIVE). (F) Quantification of the kinetochore fluorescence in cells transfected with GFP-MEI-S332 vs. GFP-MEI-S332-124AAA showing HIGH or LOW fluorescence levels. (G) Western blot showing levels of expression of the GFP-tagged proteins in three different transfection experiments. $1x10^6$ cells were loaded per lane were run on a 12.5% polyacrylamide gel [These experiments were carried out by Mar Carmena (A-F) and Fiona MacIsaac (G)].

## 5.3. Discussion

Analysis of *Drosophila incenp* mutants has revealed a role for INCENP in centromeric cohesion in meiosis (Resnick et al., 2006).. INCENP and Aurora-B have been shown to be required for the stable localisation of MEI-S332 on centromeres in mitosis and meiosis (Resnick et al., 2006).. Presumably the failure to recruit MEI-S332 to centromeres in meiosis leads to defects in centromeric cohesion after the metaphase I - anaphase I transition. However, INCENP must also be required for some other MEI-S332 independent functions since *incenp* mutants show defects in both meiotic divisions, whereas *mei-s332* mutants have defects mainly in meiosis II (Resnick et al., 2006).

Here we have shown that INCENP binds directly to MEI-S332 *in vitro*. Interestingly this interaction is strengthened in the presence of Aurora-B. It may be that Aurora-B phosphorylates INCENP, MEI-S332 or both proteins and that this stabilises the INCENP - MEI-S332 interaction. Another possibility is that binding of Aurora-B helps to stabilise the complex. It is also possible that MEIS-S332 could affect Aurora-B kinase activity and Aurora-B then regulates cohesion via its kinase activity.

MEI-S332 belongs to a family of proteins called shugoshins (Sgo) that have recently been shown to be essential for the maintenance of centromeric cohesion during anaphase I in meiosis by protecting Rec8 from cleavage by Separase (Katis et al., 2004; Kitajima et al., 2004; Marston et al., 2004; Rabitsch et al., 2004). Some species, such as fission yeast, have two shugoshin proteins (Sgo1 and Sgo2) whereas other species (*D. melanogaster* and budding yeast for example) have only Sgo1. In fission yeast, Sgo1 is expressed only during meiosis and is required for centromeric protection during meiosis I, whereas Sgo2 is ubiquitously expressed and plays a role in both meiosis and mitosis (Kitajima et al., 2004; Rabitsch et al., 2004). In organisms with a single

shugoshin protein, it is required during mitosis and meiosis (Katis et al., 2004; Kerrebrock et al., 1992; LeBlanc et al., 1999; Marston et al., 2004; Moore et al., 1998). Therefore, it is thought that the function of Sgo1 in budding yeast has been split been two divergent proteins in fission yeast. However, to date no Rec8 homologue has been identified in *Drosophila* so it is not clear what MEI-S332 is protecting during the first meiotic division. It is possible that a Rec8 like protein exists in *Drosophila* but has not been identified yet or that that Rad21 may take over the role that Rec8 performs.

Several recent papers have shown that Sgo1 recruits PP2A to the centromeres in fission yeast meiosis and human mitosis (Kitajima et al., 2006; Riedel et al., 2006; Tang et al., 2006b). Phosphorylation of Scc1 by PLK1/Polo/Cdc5 enhances its cleavage by Separase (Alexandru et al., 2001). Rogers *et al.* proposed that phosphorylation of REC-8 in meiosis by Aurora-B also promotes its cleavage by Separase (Rogers et al., 2002). Phosphorylation of Cohesin subunits SA1/2 by PLK1 and aurora-B leads to dissociation of Cohesin from chromosome arms during prophase in mitotic cells (Hauf et al., 2005). Kitajima *et al.* have suggested that at least part of the role of Sgo1 is to recruit PP2A in order to counteract the action of mitotic kinases (such as PLK1 and Aurora-B) at the centromere and thereby maintaining centromeric cohesion (Kitajima et al., 2006).

A recent paper has shown that phosphorylation of INCENP by CDK1 is required to target PLK1 to centromeres in human cells (Goto et al., 2006). Phosphorylation by Polo kinase is required to release MEI-S332 from centromeres at anaphase II onset (Clarke et al., 2005). Therefore, since INCENP stays on the centromere after the metaphase I – anaphase I transition, perhaps INCENP binds Polo and prevents the phosphorylation of MEI-S332 by Polo until the metaphase II – anaphase II transition when INCENP leaves the centromere.

Here we have shown that MEI-S332 can bind INCENP, is phosphorylated by Aurora-B *in vitro* between Serine 124 and Serine 126 and that a non-phosphorylatable mutant (MEI-S332$^{S124,125,126A}$) does not stably associate with centromeres in mitosis. The role of MEI-S332 in mitosis is not yet clear. MEI-S332 is not required for organism viability since null mutants of *mei-S332* are fully viable. However, its overexpression can lead to organism lethality and cell death. The ratio of anaphase to metaphase figures is increased in *mei-S332* mutants and decreased when MEI-S332 is overexpressed (LeBlanc et al., 1999; Moore et al., 1998). The role of MEI-S332 in mitosis is therefore subtle but significant and its regulation by the chromosomal passengers may be yet another important role of the complex in ensuring accurate chromosome segregation.

# VI Conclusions and Perspectives

# 6. Conclusions and Perspectives

In recent years the chromosomal passenger proteins have emerged as key regulators of mitosis controlling events that include chromatin modification, kinetochore microtubule attachment, sister chromatid cohesion and the completion of cytokinesis. The work presented in this thesis has focused on the role of the chromosomal passenger protein INCENP, and its roles and interactions in mitosis and meiosis. These studies have lead to a fuller understanding of the roles of INCENP in maintaining sister chromatid cohesion in meiosis and identified several potential INCENP interactors.

## *6.1. The chromosomal passengers and the centromere*

I have used a yeast two-hybrid screen in order to identify factors that may target the chromosomal passenger complex to the centromere. It has since emerged that Survivin can play a key role in targeting the complex to the centromere and that this targeting is regulated by ubiquitination. In the screen, I did not find an interactor that no longer bound INCENP when the centromere targeting domain was mutated, therefore the same analysis should be repeated with Survivin. It will be interesting to find the factor that binds Survivin at the centromeres and how this binding is regulated by ubiquitination.

## *6.2. FLJ14346*

The results of the yeast two-hybrid screen identified several interesting putative interactors. One of the proteins identified, FLJ14346, was further characterised since it had an interesting localisation when expressed as a GFP fusion. I also made an antibody against FLJ14346 that showed a similar, although not identical, localisation to the GFP fusion. The localisation of a second fusion protein, TrAP-FLJ14346, was identical to the antibody staining.

Therefore, it is likely that the antibody recognises the endogenous FLJ14346 protein.

### 6.2.1. FLJ14346 linking the chromosomal passenger proteins with TD-60

We used GST-pulldowns to verified the yeast two-hybrid interaction with INCENP and found that FLJ14346 also bound Borealin and TD-60. TD-60, a putative GEF for Rac1, behaves like the known members of the complex but has not been shown to bind directly to members of the chromosomal passenger complex. FLJ14346 could provide a link between TD-60 and the complex. Many important questions concerning TD-60 remain. These include whether the protein acts upstream or downstream of Aurora-B or on parallel pathways and whether TD-60 is truly a GEF for Rac1 since binding to Rac1, but not GEF activity, has been shown until now. It will also be important to identify the downstream effectors of Rac1 and to find if any of these effectors are regulated by the chromosomal passenger complex.

### 6.2.2. Future functional analysis of FLJ14346

In order to characterise the function of FLJ14346, RNAi should be carried out alone and in conjunction with RNAi of the chromosomal passenger proteins. This will allow us to explore the functional relationships between the chromosomal passenger proteins and FLJ14346. Expression of FLJ14346 deletions and GST pulldowns would also be useful in order to identify the domains responsible for interaction with INCENP, Borealin and TD-60, and also for the localisation of the protein throughout the cell cycle.

It may also be interesting to do crystallographic studies or NMR on FLJ14346 since it is easy to purify from bacteria and sequence analysis did not reveal any clues as to its possible function. The crystal or NMR structure might

shed some light on its function if the protein was found to adopt a known protein fold.

## *6.3. Other proteins identified in the screen*

Other known proteins identified in the yeast two-hybrid screen included BRG1, a chromatin-remodelling factor, and SAP30, a subunit of some classes of HDAC (histone deacetylase complexes). It is possible that interactions with INCENP may link them to functions at centromeres.

Two of the unknown proteins, MGC5306 and C7ORF24, gave no interpretable results when tagged with GFP. GFP-MGC5306 was nuclear in interphase but I never saw transfected mitotic cells. GFP-C7ORF24 formed aggregates in mitotic cells. As a result they were not pursued further. Due to time constraints it was too late to begin a detailed analysis of these proteins, but in the future it will be interesting to visualise these proteins with a smaller tag or by making specific antibodies.

### 6.3.1. SAP30

Hypoacetylation is important for recruitment of HP1 to the centromere and therefore for heterochromatin formation. During mitosis HP1α associates primarily with the pericentromeric heterochromatin. Binding of HP1 is regulated by modifications at two residues of histone H3 - phosphorylation of Serine 10, by Aurora-B, and Acetylation of K14. Histone H3 K14 must be deacetylated for HP1α to bind to the centromere. HDAC complexes must therefore concentrate at the centromere during or prior to mitosis to keep the centromeric heterochromatin hypoacetylated. HP1 is in turn important for the establishment and maintenance of cohesion. We can speculate that the putative interaction between INCENP and SAP30 could target the HDAC complex to the centromere, thus promoting hypoacetylation and therefore binding of HP1α.

### 6.3.2. BRG1

SWI/SNF complexes have a general role in facilitating access through chromatin to factors involved in various DNA or Histone modifications. For example, BRG1 could facilitate binding of HP1 by enhancing access of histone tails to HDACs and methyltransferases, leading to heterochromatin formation and gene silencing.

Intriguingly BRG1 binds directly to HP1α and mSin3A, a subunit of the same HDAC complex as SAP30. The human SWI/SNF-B (PBAF) complex contains BRG-1 and is related to the yeast Rsc complex. PBAF has been shown to localise to the centromere/kinetochore of mitotic chromosomes. In budding yeast, the Rsc complex is important for establishment of cohesion and kinetochore function.

Given the fact that INCENP also binds HP1α and also the functions of the chromosomal passenger complex in sister centromere cohesion and kinetochore function; the relationship between INCENP, HP1α and these novel putative INCENP interactors merits further investigation.

## *6.4. The chromosomal passenger proteins in sister chromatid cohesion*

In other studies during my Ph.D., we have characterised the role of INCENP in the maintenance of sister chromatid cohesion in meiosis in *D. melanogaster*, as part of a collaboration between several labs (William Earnshaw's, Terry Orr-Weaver's and Todd Stukenberg's). We showed that INCENP was required for the maintenance of sister chromatid cohesion in meiosis. I showed INCENP binds directly to MEI-S332 and our collaborators showed that MEI-S332 is phosphorylated by Aurora-B *in vitro.* Mutation of the

Aurora-B phosphorylation site to a nonphorylatable form leads to unstable association of MEI-S332 with the centromere. It would also be interesting to make a phosphomimic mutant to see what happens to this mutant protein and the chromosomal passenger proteins.

In our paper on the interactions between MEI-S332 and the chromosomal passengers, we presented a model to try to explain a number of recent findings suggesting that INCENP might integrate the function of several important protein kinases during mitosis and meiosis. We proposed that INCENP binding to POLO kinase prevents POLO from phosphorylating MEI-S332 until anaphase II. This is important, because POLO phosphorylation of MEI-S332 promotes its release from kinetochores. One way to test this hypothesis would be to do an *in vitro* kinase assay and compare the phosphorylation of MEI-S332 in the presence and absence of INCENP.

## *6.5. Conclusion*

In this study we have studied proteins involved in mitosis in order to further understand this process. The first aim of this project was to identify the proteins that target INCENP to the centromeres. Although, we were not successful in finding the protein that recruits INCENP to the centromeres, we did however identify a novel conserved protein that may have important functions during mitosis. The second aim was to study the role of INCENP in recruiting proteins involved in centromere cohesion. To this end we identified an interaction with a protein known to regulate sister chromatid cohesion.

# VII References

# 7. References


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