Introduction

Cell division

Cell division is the fundamental process that allows metazoan organisms to grow and to reproduce. Through a sequence of steps, the DNA gets first duplicated, and the replicated genetic material from a parent cell is then equally distributed to two daughter cells. The proper segregation of the genetic material is crucial for the survival of the organism. Chromosomal loss during meiosis leads to death in early embryogenesis or to birth defects, and segregation defects during mitosis can promote cancer (Kelly and Gilliland, 2002). Despite some subtle differences, mitosis is remarkably similar across organisms.

From its microscopic appearance, textbooks traditionally divide the cell cycle and mitosis into distinct steps: interphase, prophase, metaphase, anaphase and telophase. Interphase is the "holding" stage between two successive cell divisions. Some 90 percent of a cell's time in the normal cell cycle is spent in this phase. During interphase, the cell increases in size. The DNA of chromosomes is replicated, and the centrosomes are duplicated (for illustration see Figure 1). When entering the first mitotic stage, prophase, the chromatin condenses into discrete chromosomes. The nuclear envelope breaks down and spindles form at opposite "poles" of the cell (Figure 1). Prophase is followed by metaphase, during which the chromosomes attach to spindle microtubules via their kinetochores and are aligned at the metaphase plate (a plane that is equally distant from the two spindle poles). During anaphase, the sister chromatids split and move to opposite ends of the cell. Thereafter the two sets of daughter chromosomes arrive at the poles of the spindle and decondense during telophase. A new nuclear envelope reassembles around each set, giving rise to the formation of two nuclei. The last

step in cell division takes place during cytokinesis, the cytoplasm is divided in two by a contractile ring that ultimately seperates the two daughter cells.

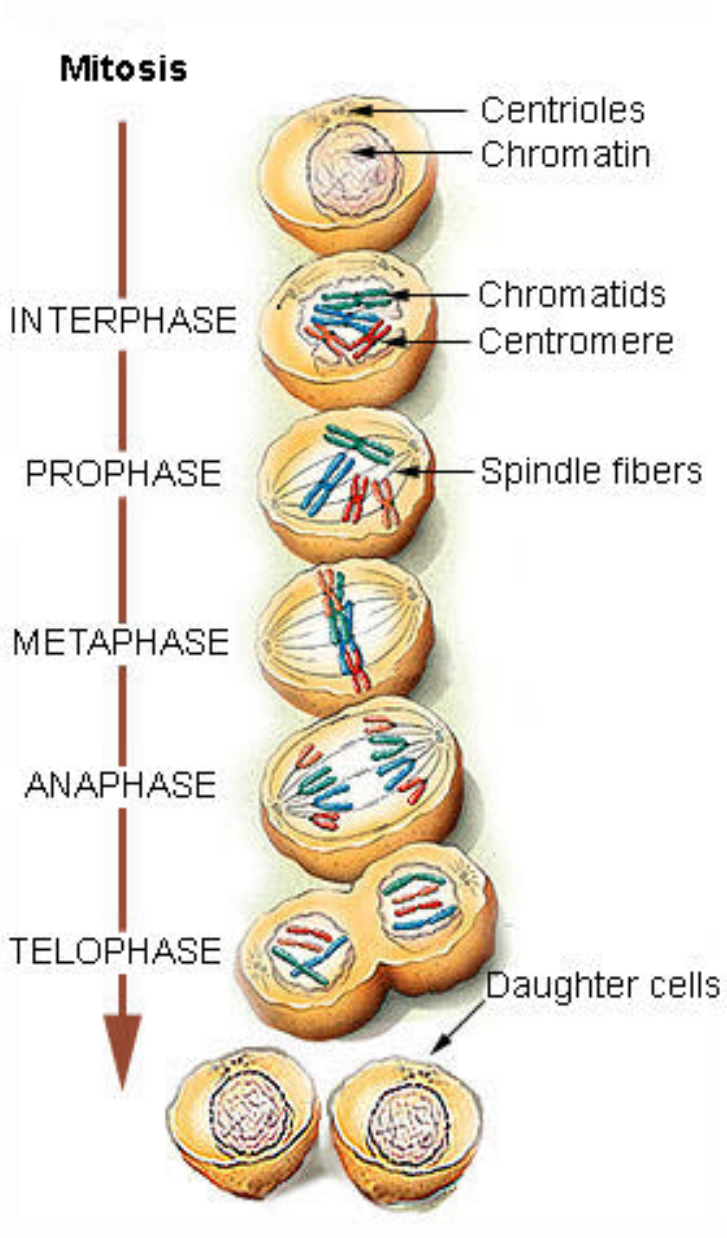

Figure1. The cell cycle: The division of a cell into two daughters occurs during M-phase of the cell cycle. M-phase consists of nuclear division (mitosis) and cytoplasmic division (cytokinesis).

The *Xenopus laevis* extract system and the cell cycle

The first insight into the cell cycle came from autoradiographic studies in eukaryotic cells showing that DNA replication occurs during a restricted part of interphase, S phase (Howard and Pelc, 1953). This work led to the division of the cell cycle into S phase and M phase, with the gap before S phase being called G1 and after S phase G2 (Mitchison and Carter, 1975). Later on, genetic screens in yeast identified several cell cycle mutants (Hartwell *et al*., 1974) and their analysis eventually lead to the checkpoint concept. At different points in the cell cycle, the cell "checks" if an earlier event, such as S phase, has been properly executed before proceeding to later events such as mitosis. Use of cell cycle extracts from amphibian allowed recapitulation of the cell cycle *in vitro* (Lohka and Masui, 1983). The ability to deplete and purify certain components from these complex cell-free extracts also opened a way for the biochemical analysis of these steps. This allowed the purification of maturation-promoting factor (MPF), a factor that promotes the onset of M phase and is a complex of cdc2 kinase and cyclin B (Lohka *et al*., 1988). This work established the fudamental role of cyclin dependent kinases (Cdks) for the cell cycle. In general these kinases are dependent on proteins called cyclins, named according to their cycle of synthesis and degradation in each cell cycle.

Early amphibian embryos have been used to study the cell cycle since they are easily available and faithfully recapitulate the rapid synchronous cell cycles seen in early embryogenesis. This is best exemplified by the *Xenopus laevis* extract systems. The eggs are large and easy to manipulate. Proteins are stockpiled in the eggs and no proteins, except cyclins, need to be translated until the midblastula transition. They can be prepared arrested in different phases of the cell cycle. The protein stocks allow biochemical purification of factors and extracts can be easily manipulated by biochemical methods. As in other systems,

the cell cycle in the early development of *Xenopus* is controlled by the activation and inactivation of the maturation-promoting factor (MPF).

The development of a frog starts with oogenesis. Immature oocytes are arrested with intact nuclear envelopes. Stimulation with progesterone induces completion of meiosis I and entry into meiosis II. Here, the oocytes are arrested at metaphase of meiosis II with stable cyclin B and high levels of cyclinB/cdc2 kinase activity. The ability of the anaphase promoting complex/cyclosome (APC), an E3 ubiquitin ligase, to trigger cyclin B destruction and metaphase exit is blocked in eggs by the action of cytostatic factor (CSF). Fertilization causes a transient increase in the cytoplasmic calcium concentration leading to CSF inactivation (Daar *et al*., 1991). The mediator of the CSF activity is Emi1, an inhibitor of the APC activator cdc20 (Reimann and Jackson, 2002)..

Three different kinds of extract can be prepared: First, a simple crushing of the eggs maintains the metaphase arrest in meiosis II in the cytosol which is released from the eggs. These extracts are called M-phase or CSF extracts. Second, addition of calcium and inhibition of protein synthesis activates the eggs to enter S-phase or interphase. Since no new cyclins are translated in the extracts they will arrest in interphase. Third, if eggs are activated by an electro pulse prior to crushing they continue to cycle through up to 12 cell cycles. The time of one cell cycle varies between 35 and 75 minutes depending on the individual extract (Murray and Kirschner, 1989).

CSF extracts can be used to investigate bipolar spindle assembly in vitro: proteins in the extract will form bipolar spindles upon addition of demembranated sperm heads (Shamu and Murray, 1992). Sperm heads consist of chromatin and a pair of centrioles (originating from the sperm basal body). As the sperm is introduced into the extract, the chromatin decondenses and the centrioles recruit pericentriolar material to form a centrosome. The centrosomes nucleate microtubule asters leading to a “half spindle” with one aster connected to the

sperm chromatin. Two of these half spindles can then fuse to form one real spindle (Shamu and Murray, 1992)

The mitotic spindle

Microtubules and dynamic instability

To divide chromosomes properly and with high accuracy, the cell uses a complex protein superstructure, the mitotic spindle. The mitotic spindle is a highly dynamic structure consisting of α and β tubulin monomers. These monomers are 55 kD proteins, that bind both to GTP and to each other in a head to tail manner. Upon polymerization, the GTP bound to the β-tubulin is hydrolyzed and GDP remains in the polymer until depolymerization, while the GTP bound to α-tubulin is never exchanged (Walker *et al*., 1988). The α/β monomers form long protofilaments which associate laterally to form cylindrical polymers of usually 13 protofilaments (Evans *et al*., 1985).

More than 30 years ago, the demonstration of reversible self-assembly of tubulin started the investigation of microtubule dynamics *in vitro* (Weisenberg, 1972). Electron microscope studies showed that microtubules are polar structures (Amos and Klug, 1974). The two ends of a microtubule show different polymerization rates. Measurement of their rate of directional growth can be used as a polarity indicator to determine their orientation with respect to a nucleation site (Borisy, 1978). The faster polymerizing end is termed the plus end whereas the slower polymerizing end is referred to as the minus end (Borisy, 1978). Within the protofilament, β-tubulin is at the plus end, and α-tubulin at the minus end. The length distribution of fixed MTs showed that although a population of microtubules exhibits a bulky steady state, a single microtubule never exists in a steady state but persists in prolonged phases of polymerization and depolymerization that interconvert infrequently (Figure 2) (Mitchison and Kirschner, 1984a). This led to the postulation of the dynamic instability model

according to which the behavior of microtubules can be described by four parameters: the polymerization rate, the depolymerization rate, the frequency of catastrophes (transition between growing state and depolymerizing state) and the rescue frequency (transition between depolymerization and polymerization). The existence of dynamic instability was confirmed by real-time analysis of single MT polymerization dynamics using dark field microscopy (Walker *et al*., 1988).

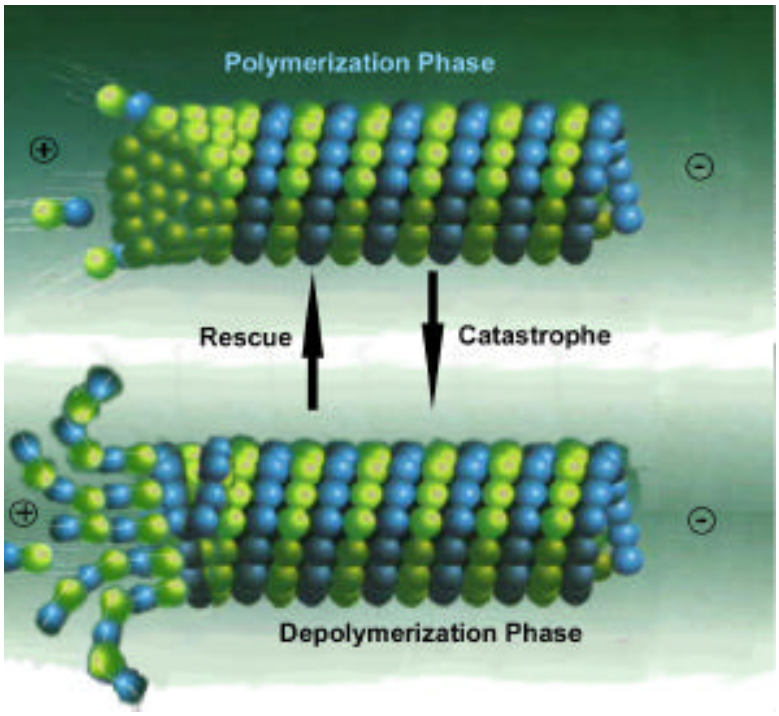

Figure 2. Microtubule dynamic instability: Polymerizing MTs infrequently transit to depolymerization phase (catastrophe). Depolymerization is characterized by the rapid loss of GDP-tubulin subunits and oligomers from the MT end. Depolymerizing MTs can also infrequently transit back to polymerization phase (rescue). (adapted from Inoue and Salmon 1995)

Electron microscopy revealed that polymerizing and depolymerizing microtubule ends look like. Growing microtubules begin as a two dimensional sheet stabilized by a GTP cap (Figure 2). Eventually this sheet zips up into a blunt ended tube, which is also stabilized at first by the GTP cap. However, once the GTP is hydrolyzed, the high compression of the naturally-curving end causes protofilaments to peel off the tube and dissolve immediately into subunits (Arnal *et al*., 2000).

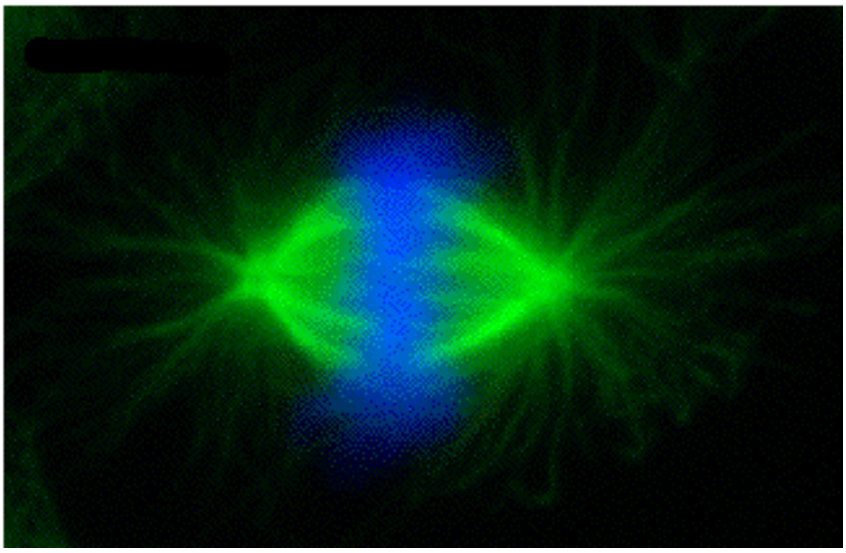

Figure 3. The mitotic spindle: Mitotic spindle microtubules shown in green and chromatin in blue (Walczak and Mitchison, 1996).

Centrosomes

During interphase the cytoplasmic microtubule cytoskeleton radiates out from a single microtubule organizing centre (MTOC), which in animal somatic cells is the centrosome. Centrosomes were named during the late 1800s by Theodor Boveri, on the basis of their central position in the cell. At the time, the centrosome had been observed as a small focus of phase-dense material, surrounded by a larger region of less phase density. The densely staining structures are centrioles; the larger region of less dense material is the surrounding pericentriolar material. Each centrosome has two centrioles that lie at right angles to one another and in close proximity at one end. Although as will be discussed in more detail later, centrosomes are not essential for microtubule nucleation (Binarova *et al*., 2000; Khodjakov *et al*., 2000; Mazia, 1984; Megraw *et al*., 2001; Szollosi *et al*., 1972) they are the main sites of microtubule nucleation in most cells. When cells are treated with drugs that depolymerize microtubules and are then washed to remove the drugs, microtubules nucleate exclusively from centrosomes. Sperm centrioles recruit pericentriolar material upon entry into the egg, it is necessary for their ability to nucleate microtubules. Experiments in *Xenopus laevis* showed that the γ-tubulin protein, a member of the tubulin superfamily that binds to sperm centrioles, is essential for them to gain nucleation capacity (Stearns and Kirschner, 1994). γ-Tubulin containing protein complexes were isolated from *Xenopus laevis* egg (Zheng *et al*., 1995) or *Drosophila* embryo (Oegema *et al*., 1999) extracts. They appear as a ring-like structure by electron microscopy. These so called γTuRCs (γ-tubulin ring complexes) nucleate microtubules *in vitro* and bind to MTs in an end-specific manner (Zheng *et al*., 1995). Centrosomes and associated astral microtubules seem to be important for spindle positioning in the mitotic cell cycle (Doe and Bowerman, 2001; Segal and Bloom, 2001). During mitosis, centrosome nucleate at least five times more microtubules than during interphase (Paoletti and Bornens, 1997). The change in nucleation capacity that accompanies the G2/M transition has been termed centrosome maturation. The increase of nucleation capacity of centrosomes is accompanied

by an increase in the γ-tubulin level at centrosomes. Studies using GFP-tagged γ-tubulin indicate that γ-tubulin recruitment in mammalian cells is a sudden event that occurs during the G2/M transition (Khodjakov and Rieder, 1999). The increase in γ-tubulin recruitment during centrosome maturation might be the cause of the gain in nucleating activity. Additionally it has been proposed that phosphorylation is part of the maturation process (Ohta *et al*., 1993).

When incubated in the presence of Ran GTP in M-phase extracts centrosomes gain a higher capacity to nucleate microtubules compared to a control experiment (Carazo-Salas *et al*., 2001). Carazo-Salas therefore postulated that Ran GTP might trigger centrosome maturation in *Xenopus* M-phase extracts (Carazo-Salas *et al*., 2001).

MAPs and spindle dynamics

In a living cell, MTs don't assemble as naked polymers, but with numerous bound proteins which regulate microtubule dynamics and the shape of the MT cytoskeleton. These proteins are classified as MAPs (microtubule associated proteins) and motor proteins, which use energy from ATP hydrolysis to move unidirectionally along MTs or, conversely to move MTs relative to their fixed position.

Some of the so far described microtubule associated proteins (MAPs), which bind and stabilize microtubules, are MAP1, MAP2, MAP4, tau and chTOG, the human homologue of the Xenopus protein XMAP215. MAP1, MAP2 and tau are neuronal, structural MAPs and strongly decrease the catastrophe frequency and promote rescue frequency and polymerization when added in recombinant form to *in vitro* assembled tubulin (Trinczek *et al*., 1995). XMAP215 increases MT polymerization selectively on plus ends but does not effect catastrophes. Recently, it has been shown that XMAP215 is necessary to rescue the ability of

salt stripped centrosomes to nucleate microtubules (Popov *et al*., 2002). Additional experiments with purified components also suggest that the stabilising activity of XMAP215 might be counterbalanced by XKCM1. XKCM1 is a Xenopus kinesin-like protein, believed to be a plus end directed motor based on its homology to other motors (Walczak *et al*., 1996b). It increases MT catastrophes by 4 fold. In mitotic extracts depleted of XKCM1 the microtubule length increases dramatically, the opposite effect from that when XMAP215 is depleted, indicating opposing functions of the two proteins. The activation or inactivation of counteracting proteins such as XMAP215 and XKCM1 is thought to be part of the mechanism which regulates microtubule dynamics during the cell cycle.

Regulation of MAPs and motor proteins

At the onset of mitosis the microtubule cytoskeleton undergoes a dramatic rearrangement. During interphase long and stable microtubules emanate from the centrosome, which is attached to the nucleus (Figure 4). During mitosis however the catastrophe rate increases 7-10 fold leading to short, unstable microtubules forming the metaphase spindle (Figure 3) and (Inoue and Salmon, 1995). Although the molecular details of these changes are not yet understood in detail, it is generally accepted that the cell-cycle dependent modification of MAPs and motor activities plays an essential role in this process.

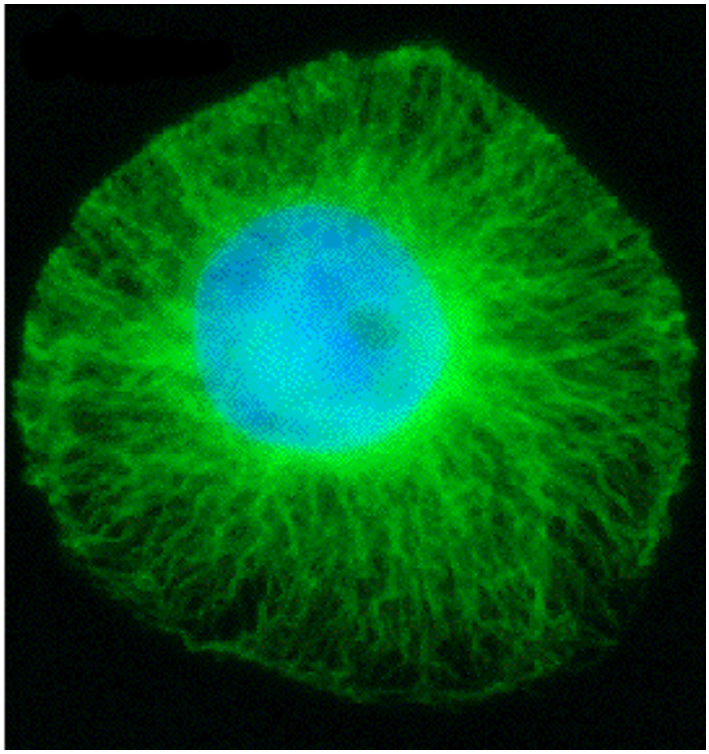

Figure 4. The microtubule cytoskeleton during interphase. Tubulin shown in green and chromatin in blue (Walczak and Mitchison, 1996).

The onset of mitosis is regulated by cdc2 and cyclin B. It had been observed that cyclin B undergoes rapid nuclear entry at the beginning of mitosis (Gallant and Nigg, 1992; Lehner and O'Farrell, 1990). It is also known that the kinase activity of cdc2 is tightly regulated by phosphorylation. However, a cdc2 mutant that cannot be phosphorylated does not cause premature entry into mitosis, even though it exhibits high levels of kinase activity (Jin *et al*., 1996). These data suggested that entry into mitosis might be regulated by nuclear import of cdc2/cyclin B in addition to phosphorylation. Indeed conditions that led to constitutive nuclear localization of cyclin B, such as addition of an extra nuclear localization signal (NLS), caused premature mitosis when the kinase activity of cdc2 is elevated at the same time (Jin *et al*., 1998). Taken together, these observations suggested that the regulation of MPF kinase activity and the regulation of its subcellular localization both contribute to proper control of the G2-to-M transition. One can imagine that the activity of other proteins than cyclin

B are regulated by their subcellular localization. Similarly, some proteins from the nucleus might play an important role in the mitotic cytoplasm and might be stored in the nucleus during interphase to avoid premature interaction with cytoplasmic components during interphase. Several microtubule associated protein (MAPs), as well as motor proteins, are present in the nucleus during interphase and might be regulated in this way. Using *in vitro* movement assays and non-hydrolyzable ATP analogs researchers were able to purify a protein from squid giant axons and named it kinesin from Greek "*kinein*" to move (Vale *et al*., 1985). Further studies led to the discovery of a large number of proteins that are related in structure to kinesin and constititute the kinesin superfamily of motor proteins.

Since these proteins are very diverse in their properties, and the combination of their activities is critical for MT organization and thus of interest for the work presented here, a short description of a number of them will be given. The first member of the mitotic kinesin-like protein (MKLP1) family was identified from CHO cells and therefore named CHO1. CHO1 is present in interphase centrosomes and nuclei and becomes associated with the mitotic spindle (Sellitto and Kuriyama, 1988). As chromosomes move toward the poles, the CHO1 protein shifts to the spindle midzone, and eventually concentrates into a bright spot at the midbody. CHO1 can interact both with microtubules and actin. It is probably involved in membrane fusion during the terminal phase of cytokinesis (Kuriyama *et al*., 2002).

Another member of the kinesin family of motor proteins, XCTK2 was isolated with antibodies raised against the conserved sequence in the kinesin motor domain. The protein cosediments with microtubules and anti-XCTK2 antibodies inhibit bipolar spindle formation (Walczak *et al*., 1997). During interphase XCTK2 persists in the nucleus, localizes to the spindle during mitosis, and reaccumulates in the nucleus at cytokinesis. XCTK2 is a minus end directed motor protein, and is thought to cross link microtubules and contribute to spindle integrity (Walczak *et al*., 1998).

XMAP310 was isolated by cosedimentation with polymerised microtubules (Andersen and Karsenti, 1997a). It localizes to the nucleus in interphase and prophase. During metaphase it relocalizes to spindle MTs and remains in areas with high MT density throughout anaphase. During cytokinesis it starts to reaccumulate in the nucleus. XMAP310 increases rescue frequency in MT dynamics experiments. Purified XMAP310 can bundle microtubules as revealed by electron microscopy (Andersen and Karsenti, 1997b).

The tetrameric motor protein Eg5 is a protein of the kinesin family and is present both in the nucleus and the cytoplasm during interphase but only small amounts of Eg5 are expressed during interphase. A fraction of it is also associated with microtubules. Eg5 is most concentrated on the mitotic and meiotic spindle and on the poles in particular (Houliston *et al*., 1994).

XKCM1 is another protein of the kinesin family with a C-terminal motor domain and is present as a soluble pool during interphase (Walczak *et al*., 1996a). This soluble pool persists throughout mitosis. However, a portion of XKCM1 localizes to the centromeric region of the chromosomes and to the centrosomal region of the spindle. XKCM1 is critical for mitotic spindle assembly. Depletion of the protein from *Xenopus* M-phase extracts suppressed catastrophe frequency, resulting in structures with abnormally long microtubules. Loss of endogenous XKCM1 from only the centromeres caused a misalignment of chromosomes on the metaphase plate without affecting global spindle structure (Walczak *et al*., 2002).

Xklp2 is a 160 kDa kinesin-like protein with an N-terminal motor domain. It was first identified from a *Xenopus laevis* oocyte cDNA library. It forms dimers and localizes to microtubule minus ends. Xklp2 was shown to be involved in centrosome separation (Boleti *et al*., 1996). The independent binding of the motor domain to microtubules is mediated by TPX2 (targeting protein for Xklp2), and its localization to the minus end is dynein dependent. The C-terminus of Xklp2 binds

to TPX2 whereas a domain N-terminal to the TPX2 binding site is critical for Xklp2's ability to dimerise (Wittmann *et al*., 1998).

TPX2 is an 87kD protein and was shown to be nuclear during interphase, bound to spindle poles in metaphase and associated with midbodies in telophase. It is a highly charged molecule (18.7% strongly basic and 14.7% strongly acidic amino acid residues). TPX2 has an isoelectric point of 9.5. TPX2 is highly conserved in human, frog, chicken, mouse and probably other vertebrates. No homologues in other eukaryotes, such as yeast or nematodes, have been identified. TPX2 was first found to function in spindle pole formation (Wittmann *et al*., 2000). The work presented in this thesis, as well as the work performed during the time frame of this thesis, shows that TPX2 is essential for chromatin induced microtubule assembly.

Spindle Bipolarity

During metaphase the chromosomes are aligned at the metaphase plate in the middle of the two spindle poles (Figure 3). They are attached to the microtubules via kinetochores (Compton, 2000). Kinetochores are the points of attachment for the spindle fibers on the centromere of chromosomes during mitosis or meiosis. The spindle microtubules can be divided into three separate classes, defined by the position of the microtubule plus end: Interpolar microtubules directly connect the two spindle poles, kinetochore microtubules connect the kinetochore with the spindle pole and astral microtubules, which form asters reaching outward from the poles to the cell cortex, stabilize the spindle orientation with respect to the cell cortex and the underlying actin matrix (Sharp *et al*., 2000b). Cross-linked kinetochore microtubules form a tight bundle referred to as the kinetochore fiber. Kinetochore fibers show uniform microtubule spacing of 50-100 nm along their entire length from the kinetochore to the pole (McEwen *et al*., 1997; Rieder and Borisy, 1981). Many electron microscopy studies have reported microtubule

cross-linking material within spindle microtubule bundles (Rieder and Bajer, 1977).

As mentioned above, the dynamic microtubule network is organized into the mitotic spindle by motor proteins. Antiparallel, interpolar microtubules can be cross-linked and bundled by motor proteins which bind to two microtubules at the same time (Figure 5 in green, yellow and orange). The two spindle poles, which are connected by these MT bundles, can be moved towards or apart from each other by motor proteins. Two different kinds of motors are known that apply different forces on the spindle. The BimC family of motors forms homotetramers (Figure 5 in yellow), with motor-domains on two antiparallel microtubules (Roof *et al*., 1992). Since BimC proteins are plus end directed motors they push the two spindle poles apart. Proteins which cross link MT, and push the spindle poles together, like Ncd in *Drosophila,* belong to the KinC family and they have two C-terminal motor domains binding two different microtubules (Figure 5 in orange). One of the two motor domains is not active. In this way the motor uses one of the microtubules as a cargo and moves it with respect to the other. Experiments with both frog egg extracts and human cells indicate that KinC motor proteins contribute to the focusing of microtubule ends at spindle poles (Mountain *et al*., 1999; Walczak *et al*., 1998). KinC motor proteins can push spindle poles together or apart depending whether they have either a minus end directed motor domain or a plus end directed motor domain (Figure 5 in orange) and (Sharp *et al*., 2000a). Forces which push spindle poles together and apart balance each other. Disturbing the motor balance disrupts the mitotic spindle. Either monopolar spindles or two separate asters will be formed. When the plus end directed motor activity of Eg5, a BimC motor, is inhibited by the drug monastrol the two poles collapse and form one big aster instead of a spindle with two poles (Mayer *et al*., 1999). Another protein pulling the spindle poles towards the cell cortex is dynein (Figure 5 in green). A fraction of dynein together with its activator dynactin forms a large multiprotein complex at the cell cortex consisting of dense actin filaments. Dynein is a minus end motor and moves microtubules towards the cell cortex (Sharp *et al*., 2000a).

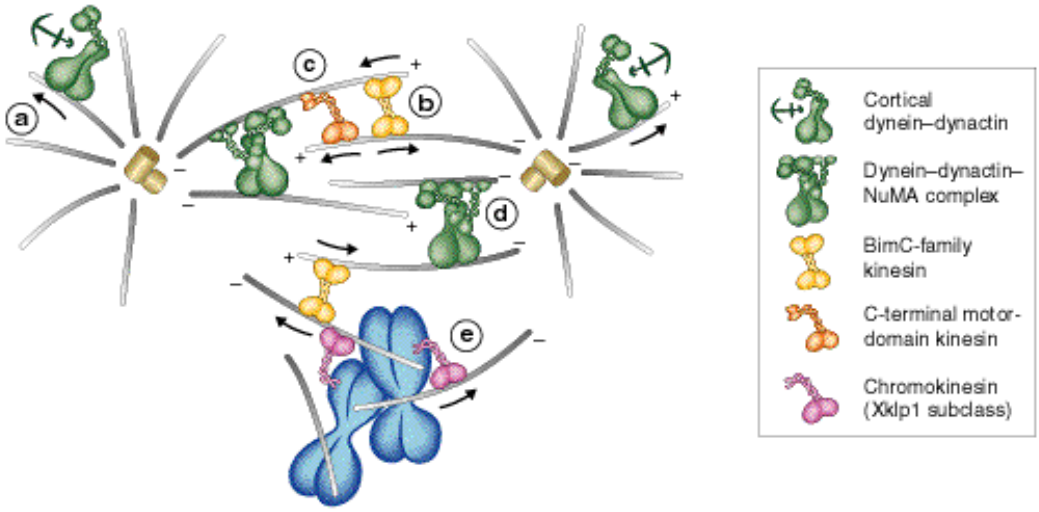

Figure 5. Mechanism of spindle bipolarity: Centrosomes are pulled apart by cortical dynein (a) and plus-end-motors of the BimC family sliding MTs apart thereby also sorting MTs into antiparallel arrays (b). Minus end directed motors of the C-terminal motor domain family push spindle poles together (c). Cytoplasmic dynein and other spindle pole proteins cross link MTs and focus spindle poles (d). Chromosome associated, plus-end-directed kinesins push the spindle poles away from the chromosomes (e). Arrows indicate forces applied on the MT lattice. (modified from Wittmann and Desai 2001)

The pole microtubules are focused by minus end directed motors like dynein and the KinC family of kinesin related proteins (Figure 5 in green and orange). Injection of antibodies against Eg5 into cultured cells and immunodepletion of Eg5 from *Xenopus* M-phase extracts disrupt spindle poles leading to MT bundles with split ends (Gaglio *et al*., 1996; Heald *et al*., 1997). These minus end directed motors cross link and slide one microtubule relative to another and thereby focus spindle poles. Dynactin and NuMA have been shown to be essential for dynein to focus spindle poles (Merdes *et al*., 1996). NuMA is a nuclear protein during interphase. In mitosis it forms a complex with dynein and dynactin. It is brought to the pole by dynein and is a component of the postulated “spindle matrix” at the spindle pole in *Xenopus laevis* mitotic extracts (Dionne *et al*., 1999; Gaglio *et al*., 1995).

The assembled mitotic spindle is not a rigid structure but highly dynamic. Also, the microtubule polymer undergoes a rapid turnover of monomers. In addition, the whole population of spindle microtubules undergoes a poleward movement termed “flux” (Heald *et al*., 1996). This movement of individual microtubules to the pole has first been demonstrated by photoactivation of caged fluorescent tubulin and more recently by fluorescent speckle microscopy (Mitchison, 1989; Waterman-Storer *et al*., 1998). It was concluded that microtubules polymerize at the plus end in the central spindle, thereby adding new subunits to the polymer. At the same time, subunits leave the polymer at the minus end, close to the spindle pole.

Forming a spindle

Two different, but not necessarily mutually exclusive models for the driving force underlying the assembly of the mitotic spindle have been proposed. The “search and capture” model predicts that dynamic microtubules randomly encounter kinetochores and are thus captured and stabilized against depolymerization. Once attached to a microtubule aster the chromosomes move poleward, favoring attachment by other microtubules from the same aster (Maney *et al*., 2000). Since at the time of nuclear envelope breakdown both centrosomes are attached to the nucleus and close to each other, the kinetochores connected with one aster also capture microtubules from the opposite pole leading to the bipolar spindle. The second model describes a local stabilization of microtubules in the vicinity of the chromosomes and is also termed the “chromatin effect”. It is important to note that whereas the search and capture model involves centrosomes as nucleation sites for MT the local stabilization model postulates nucleation independent of centrosomes as also being critical. The relevance of this model is highlighted by a number of observations.

In higher plant cells, there are neither centrioles nor classical microtubule organizing centers (MTOCs) and yet proper bipolar spindles are formed

(Binarova *et al*., 2000; Mazia, 1984). Meiotic divisions in mouse, *Xenopus* and human happen without centrosomes (Szollosi *et al*., 1972). Although plant cells and germ cells can divide without centrosomes it was believed that somatic cells did require centrosomes. However, inactivation of both centrosomes by laser surgery in somatic mammalian cells had no effect on spindle formation (Khodjakov *et al*., 2000). After this demonstration that centrosomes are neither essential for microtubule nucleation nor for the formation of a bipolar spindle it was proposed that they would be necessary for the proper orientation of the bipolar spindle with respect to the cell body. Evidence that that might not be the case was provided by a mutant fly line. *Drosophila* centrosomin (cnn) null mutants form spindles without centrosomes in cultured cell and cnn mutant flies grow normally and form adult flies, although these adults are sterile. Although the orientation of the spindle in neuroblast cells in cnn mutant flies is altered in every fifth cell, this has no apparent consequence for the insect (Megraw *et al*., 2001). Therefore, centrosome are neither essential for MT nucleation nor for spindle formation but are involved in spindle orientation.

The chromatin effect

A key experiment leading to the proposal of the local stabilization model was performed by Eric Karsenti in the early 1980s. He injected either centrosomes or karyoplast nuclei devoid of centrosomes into the cytoplasm of *Xenopus laevis* frog eggs arrested in metaphase of meiosis II. The centrosomes did not induce aster formation in these eggs whereas the crude nuclei did induce the formation of a mitotic spindle (Karsenti *et al*., 1984a; Karsenti *et al*., 1984b). Direct proof that chromatin can induce spindle formation came from experiments performed by Heald and co workers (Heald *et al*., 1996; Hyman and Karsenti, 1996). Heald showed that beads coated with chromatin directed the assembly of bipolar spindles in *Xenopus laevis* M-phase egg extracts in the absence of microtubule nucleating centrosomes. It was concluded that chromatin changes the local state of the cytoplasm, favoring microtubule nucleation and stabilization. Microtubules nucleated by chromatin are then bundled by cross linking motors, either in a

parallel or an antiparallel fashion, defining the single axis and leading to the bipolar spindle whose ends are focused by dynein, dynactin and NuMA (Heald *et al*., 1996; Heald *et al*., 1997). Interestingly, either chromatin beads alone, or beads together with two centrosomes form bipolar spindles. In contrast, when only one centrosome is present, a monopolar spindle is formed, indicating that centrosomes provide dominant sites for pole formation (Heald *et al*., 1997).

Nuclear-cytoplasmic transport

The ability of chromatin to induce spindle formation led to experiments revealing a function for the machinery which facilitates nuclear-cytoplasmic transport in spindle assembly. In this paragraph nuclear-cytoplasmic transport is introduced in order to provide a backround for understanding the function of some proteins of the transport machinery during spindle assembly.

During interphase the nucleus separates DNA replication, transcription and splicing from translation. The nuclear envelope (NE), a double membrane that is continuous with the ER, separates the cytoplasm from the nucleus. The NE is penetrated by nuclear pore complexes (NPCs), which mediate transport of macromolecules between the two compartments. The NPCs allow diffusion of small molecules, and can accommodate active transport of very large particles as large as ribosomal subunits. Active transport is a selective process triggered by specific transport signals. The first proof for the existence of such transport signals was obtained through a study of nuclear accumulation of nucleoplasmin (Dingwall *et al*., 1982). Nucleoplasmin is a pentameric nuclear protein from *Xenopus laevis* and consists of a core domain and a tail. Intact nucleoplasmin rapidly enters the nucleus after being injected into the cytoplasm. When all the tails are removed the residual core remains pentameric but fails to enter the nucleus. In contrast, the detached tails show rapid nuclear accumulation, indicating that the tails contain some signal for nuclear accumulation. The first nuclear import signal identified was in simian virus 40 (SV40) large-T antigen

(Kalderon *et al*., 1984). The import signals from nucleoplasmin and from SV40 are now called the classical nuclear localization signals (NLS). The SV40 large T antigen has the sequence PKKKRKV and is termed a monopartite NLS. The nucleoplasmin has the sequence KRPAATKKAGQAKKKK and is termed a bipartite NLS (Mattaj and Englmeier, 1998). The field of nucleo-cytoplasmic transport was greatly advanced by the development of an in vitro assay based on permeablilized cells (Adam *et al*., 1990). Using this assay researchers were able to identify soluble factors essential for protein import. The proteins identified were importin α (Adam and Adam, 1994; Gorlich *et al*., 1994), importin β (Adam and Adam, 1994; Gorlich *et al*., 1995b) and two constituents of the RanGTPase system, namely Ran itself (Melchior *et al*., 1993; Moore and Blobel, 1993) and nuclear transport factor 2 (NTF2) (Moore and Blobel, 1994; Paschal and Gerace, 1995).

Importin β turned out to be the founding member of a family of related transport receptors. Transport receptors bind their cargo on one side of the nuclear envelope (NE), translocate to the other side, release their cargo, and finally return to the original compartment to mediate the next round of transport. Consequently, the transport machinery needs to be able to distinguish the nucleus from the cytoplasm. This information is provided by the distribution of the two states of the small GTPase Ran, bound to GTP or GDP (Figure 6 and 7) and (Gorlich *et al*., 1996b; Izaurralde *et al*., 1997). Like other small GTPases, Ran needs regulators for its activity. The regulators either stimulate Ran to hydrolyze GTP, or to release the resulting GDP and rebind GTP. RCC1 is the nucleotide exchange factor of Ran and since GTP is more abundant in the cell RCC1 loads Ran with GTP. RCC1 is a chromatin bound protein, which therefore leads to a high concentration of RanGTP in the proximity of chromatin in the nucleus (Bischoff and Ponstingl, 1995). The hydrolysis of Ran GTP is catalyzed by RanGAP (Bischoff *et al*., 1994). Its GTPase activation potential is stimulated roughly 10-fold by RanBP1 (Bischoff *et al*., 1995). Both RanGAP and RanBP1 are cytoplasmic proteins that deplete RanGTP from the cytoplasm. X-ray

structures of RCC1 with or without Ran, as well as of RanGAP with Ran have revealed the atomic details of the Ran system. RCC1 facilitates GTP exchange via displacement of the P loop of Ran, which contributes a major part to the nucleotide binding energy (Renault *et al*., 2001; Renault *et al*., 1998). The structure of a RanBP1 domain (actually from RanBP2, a related protein) bound to Ran shows that RanBP1 facilitates GTP hydrolysis by displacing the C-terminal extension and the acidic helix, which otherwise folds back onto the surface of Ran. Removing this helix allows RanGAP to bind Ran. Unlike GAPs of other GTPases, RanGAP does not facilitate hydrolysis solely by stabilizing the Ran GTPase in an active conformation by providing an arginine residue to the catalytic center (Seewald *et al*., 2002; Vetter *et al*., 1999).The transport through the NPC is mediated by transport receptors and adaptor molecules (Figure 6 and 7). Importin β, the classical import receptor, binds different adaptor molecules: e.g. importin α and Snurportin1 (Gorlich *et al*., 1995a). Importin α binds to nuclear localization signals (NLS) (Figure 7). During import importin α binds both the NLS of a cargo molecule and importin β via the importin β binding domain (IBB) (Figure 7). The crystal structure of importin α bound to the targeting signal of the SV40 large T antigen has been solved (Conti *et al*., 1998). Importin α consists of ten helical repeats known as armadillo (ARM) motifs. Each ARM motif has three α helices. The overall structure of the protein has the shape of a corkscrew. The concave surface of importin α is shaped into a shallow and extended groove, which is lined by a ladder of conserved tryptophane and asparagine residues and surrounded by acidic amino acids. The c-*myc* and nucleoplasmin NLS peptides bind along the spine of the surface groove in an almost fully extended conformation. Two copies of the monopartite NLS of c-*myc* bind to the two binding sites, the large site being at the N-terminal half of importin α and the small binding pocket at the C-terminal half of the protein. It is believed that only binding to the large site takes place under physiological conditions. Nucleoplasmin spans the whole surface groove and binds to both pockets (Conti and Kuriyan, 2000). In the nucleus the trimeric import complex encounters a high concentration of RanGTP, which binds importin β, thereby releasing the cargo

and importin α (Figure 7). The crystal structures of Importin β, complexed to either RanGTP or to the IBB domain of importin α, suggests that the IBB-bound conformation of Importin β has to undergo considerable conformational changes to allow Ran binding. In addition, there is a partial overlap between the IBB and Ran-binding site on Importin β, which together explains why Ran displaces importin α from β.

Conversely to the situation in protein import, exportin-1, also called Crm1, binds to its cargo, leucine rich nuclear export signal (NES) containing proteins, together with RanGTP in the nucleus (Figure 6). After translocation of this export complex to the cytoplasm, the GTP on Ran is hydrolyzed with the help of RanGAP and RanBP1 and the complex disassembles, thereby releasing the cargo (Fornerod *et al*., 1997) and (Figure 6).

The receptors involved in transport have to be recycled after cargo release. Ran is translocated back into the nucleus by NTF2 (Figure 6) and (Ribbeck *et al*., 1998), whereas importin α is recycled back into the cytoplasm as a trimeric complex with RanGTP and CAS (Figure 7) and (Kutay *et al*., 1997).

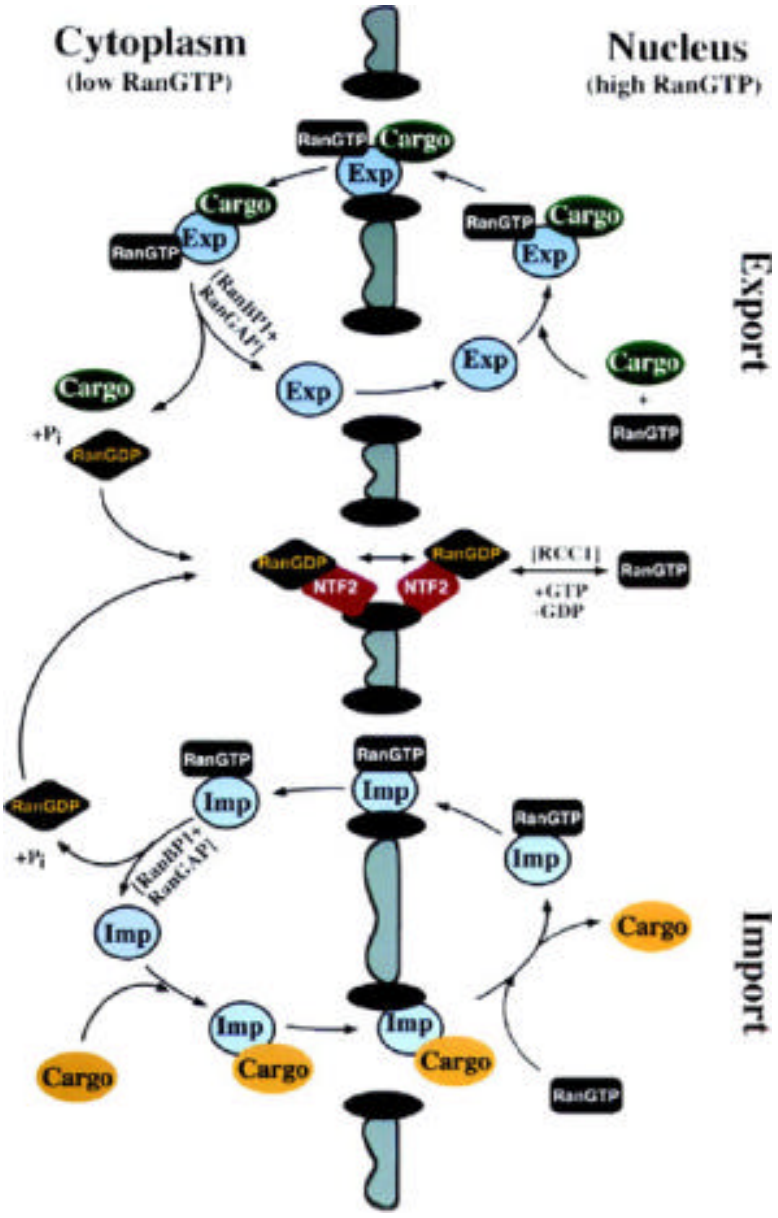

Figure 6. The Transport cycle of importins (Imp) and exportins (Exp). The importin bound cargo translocate into the nucleus. The complex dissociates at high concentrations of Ran GTP in the nucleus. Export cargos leave the nucleus in a trimeric complex with Ran GTP and export receptor. Ran is recycled by NTF2. (modified from Görlich and Kutay 1999)

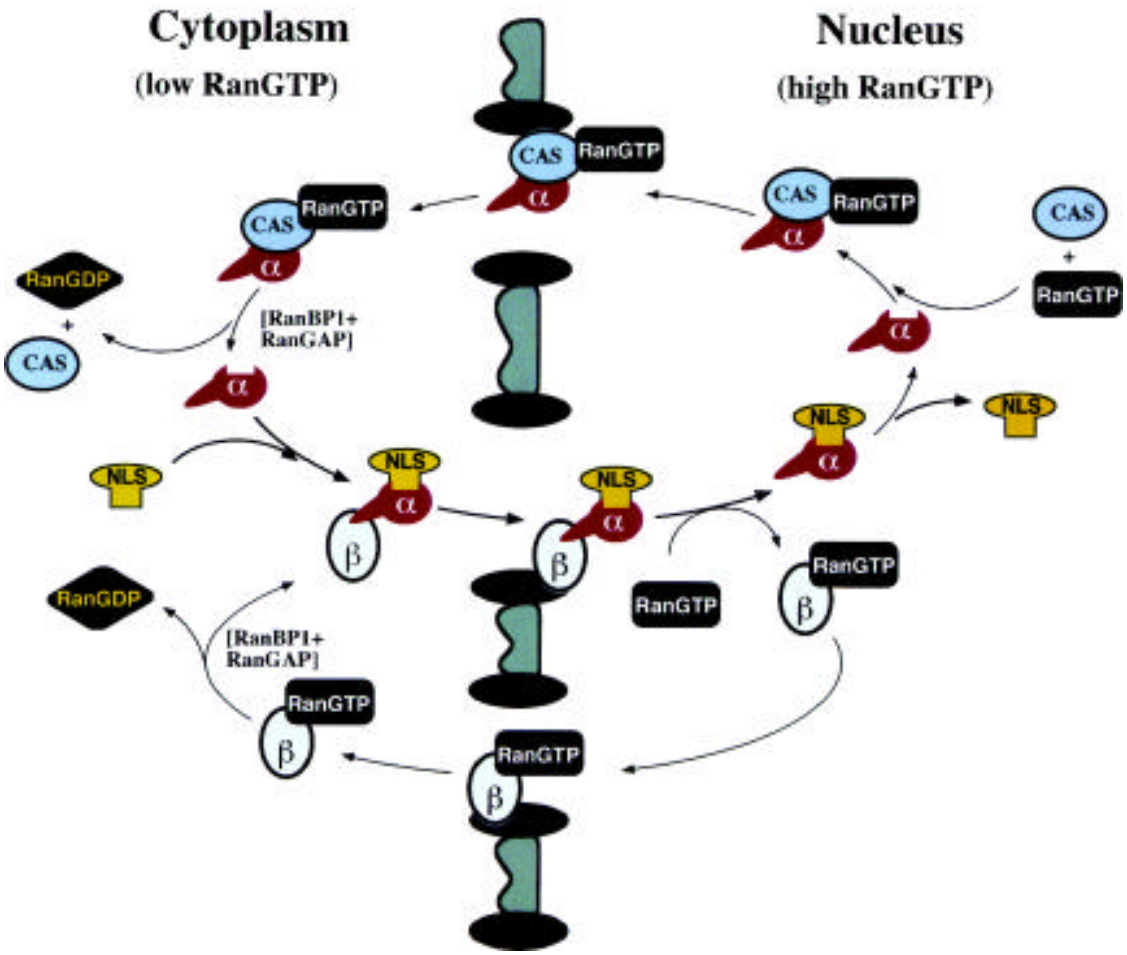

Figure 7. Transport cycle of importin α. NLS containing cargo molecules are imported into the nucleus by importin α (α) and importin β (β). importin α is recycled in a trimeric complex with CAS and Ran GTP (Görlich and Kutay 1999).

Ran's function in spindle formation

It was eventually established that the small GTPase Ran is critical for the chromatin effect. A potential role for Ran in cell cycle progression and mitosis had indeed been suggested by numerous experiments. A screen that was designed to identify temperature sensitive cell cycle mutants in mammalian cells identified the *ts*BN2 cell line, a mutant BHK (baby hamster kidney) cell line that contained the temperature-sensitive allele of RCC1 (Nishitani *et al.*, 1991). Shifting *ts*BN2 cells to the restrictive temperature triggered RCC1 degradation,

Ran GTP depletion and G1 cell cycle arrest. Another phenotype was observed in these cell lines: When *ts*BN2 cells were first arrested in S phase by DNA-replication inhibitors, they entered mitosis prematurely when shifted to the restrictive temperature. Both the full activation of cdc2-cyclinB kinase and premature chromosome condensation were observed in these cells. This indicated a function for Ran in both G1/S transition and S/M checkpoint control, and it suggested that RanGTP serves as a signal to delay G2/M transition. This was confirmed by the over-expression of Ran mutants defective in GTP hydrolysis (RanG19V/ RanQ69L), which arrests cells at the G1/S transition (Ren *et al*., 1994). Similarly, fission *S. pombe* mutant pim1^{-}, the yeast homologue of RCC1, has been described in which the onset of mitosis is uncoupled from DNA replication (Matsumoto and Beach, 1991). However, it was speculated that the loss of RCC1 might have led to the G1 arrest because of the effect on nucleo-cytoplasmic transport. Altered levels of the Ran GTP/GDP ratio will block protein import and mRNA export, which will ultimately shut off protein synthesis in the cytoplasm. This might affect protein complexes required for the cell cycle progression.

The other consequence of RCC1 loss is to override the DNA replication checkpoint at the S/M transition. Again one way to explain this effect would be the disruption of nuclear transport. Mitotic entry is initiated by the Cdc25C phosphatase, which dephosphorylates Cdc2/cyclin B. Cdc2/cyclin B and Cdc25C are both shuttling between the nucleus and the cytoplasm with a predominantly cytoplasmic localization at equilibrium (Jackman *et al*., 2002). Just before mitosis, these proteins accumulate in the nucleus. Loss of RCC1 might cause a premature accumulation of Cdc2/cyclin B Cdc25C in the nucleus, thereby overriding the checkpoint.

Furthermore, studies in *Xenopus* egg extracts and in somatic mammalian cells showed that a mutant Ran protein with a point mutation Ran T24N, which irreversibly binds and inhibits RCC1, causes an interphase arrest (Clarke *et al*., 1995). Overexpression of RanBP1 in somatic mammalian cells results in spindle

polarisation defects (Guarguaglini *et al*., 2000). Injection of anti-RanBP1 antibodies into mitotic cells delays metaphase and anaphase completion, interferes with chromosome segregation to the poles in anaphase and stabilises mitotic spindles against nocodazole induced microtubule depolymerization (Guarguaglini *et al*., 2000). For review see: (Moore, 2001).

Evidence for Ran being important for cell cycle control came also from budding yeast. Overexpression of Ran GEF in *S. cervisiae* suppresses a class of α-tubulin mutations that otherwise display excess nuclear and cytoplasmic microtubules and cell cycle arrest (Kirkpatrick and Solomon, 1994). Furthermore, overproduction of Ran or RanBP1 in budding yeast caused increased rates of chromosome non-disjunction and sensitivity to benomyl, a microtubule depolymerising drug (Ouspenski *et al*., 1995). A mutant allele of budding yeast RanBP1 causes substantial defects in progression through mitosis (Ouspenski, 1998). In *Schizosaccharomyces pombe* a strain harbouring a point mutation of Ran was isolated which is normal in nucleocytoplasmic transport, but whose microtubule cytoskeleton is defective, resulting in chromosome missegregation and abnormal cell shape. Interestingly, this mutation can be partially suppressed by overexpression of Mal3p, a microtubule associated protein, suggesting a function for fission yeast Ran in microtubule stability (Fleig *et al*., 2000).

However, all these effects of Ran on cell cycle might still have been explained by indirect effects, based on Ran's well established functions in nucleocytoplasmic transport.

Direct evidence for Ran's role in spindle assembly eventually came in 1999, when five groups reported that the addition of Ran in its GTP bound state triggers microtubule assembly in *Xenopus* M-phase egg extract in the absence of chromatin (Carazo-Salas *et al*., 1999; Kalab *et al*., 1999; Ohba *et al*., 1999; Wilde and Zheng, 1999; Zhang *et al*., 1999). Addition of demembranated sperm nuclei to metaphase-arrested *Xenopus* extract triggers the aggregation of active

nucleating material around its basal body and the formation of a functional centrosome that becomes a microtubule aster. In the absence of added sperm nuclei microtubule asters never form. Ran GTP assembles spindle structures without sperm nuclei. At first Ran GTP triggers microtubules to form aster-like structures which, after longer incubation times, become organized spindle-like structures. The centres of these asters or spindle poles contain γ-tubulin and NuMA proteins usually found in the pericentriolar material. γ-tubulin as well as XMAP215 are essential for Ran dependent aster formation (Wilde and Zheng, 1999). Carazo-Salas et al. proposed that the localization of RCC1 increases Ran GTP levels in the vicinity of chromatin during spindle assembly around DNA beads. Indeed inhibiting RCC1 with the Ran T24N mutant blocks spindle formation around DNA beads, supporting a role of RCC1 in mediating the chromatin effect. Also consistent with this prediction, increasing Ran GTP throughout a *Xenopus laevis* M-phase extract uncouples microtubule assembly from chromatin beads. When Ran GTP levels are mildly increased spindle assembly is enhanced and small bead clusters, normally incapable of forming spindles, now support spindle assembly (Carazo-Salas *et al*., 1999). Ran-induced spindle pole-like structures disappeared when dynein was inhibited (Ohba *et al*., 1999; Wilde and Zheng, 1999), suggesting that Ran promotes nucleation of microtubules whose organization into poles requires dynein. It was shown that Ran GTP also affects microtubule dynamics, it increases the rescue frequency and reduces catastrophe frequency therefore stabilizing microtubules (Carazo-Salas *et al*., 2001). The amount of Eg5, a plus end directed motor, moving to the plus end of MTs increased in the presence of Ran GTP giving a possible explanation for Ran's ability to generate spindle-like structures (Wilde *et al*., 2001). Recently a RanGTP gradient around chromosomes in mitosis and high Ran GTP concentration within the nucleus during interphase has been shown using FRET (fluorescence resonance energy transfer) based biosensors (Kalab *et al*., 2002). Based on Ran's binding partners and regulation of nuclear transport factor-cargo interactions, two chimeric biosensor probes that exhibit intramolecular FRET between cyan fluorescent protein (CFP) and yellow

fluorescent protein (YFP), modulated by the Ran nucleotide state were used and a steep concentration difference between nuclear and cytoplasmic Ran-GTP was established, providing evidence for a Ran-GTP gradient surrounding chromosomes throughout the cell cycle. Two other papers report the binding of Ran to chromatin directly both in *Xenopus laevis* eggs as well as in egg extracts (Bilbao-Cortes *et al*., 2002; Hinkle *et al*., 2002). This may serve to keep RanGTP in the vicinity of chromosomes to further increase the RCC1-dependent RanGTP gradient around the chromatin. Taken together, the data showed that the centrosome-independent, chromatin-dependent pathway of spindle assembly depends on Ran. However, it was neither known which molecules were the downstream targets of Ran in spindle assembly nor if proteins of the nucleocytoplasmic transport machinery played a role in MT assembly.

Aim of the project

This PhD project had the goal to characterize the function of the small GTPase Ran in spindle assembly and centrosome activation. A number of studies showed that the small GTPase Ran in its GTP bound state has four effects on the microtubule cytoskeleton in *Xenopus* M-phase egg extracts: 1. It induces microtubule nucleation. 2. Ran GTP increases the capacity of centrosomes to nucleate microtubules. 3. Ran GTP induces spindle like structures. 4. Ran GTP stabilizes microtubules.

How does Ran do all that? Which are the downstream molecules of Ran in any of these processes? How are those molecules regulated? First we wanted to identify the downstream target of Ran in microtubule nucleation. The process of Ran mediated microtubule assembly was approached first since it was a necessary step for all the effects observed and was relatively easy to monitor in our experimental system, *Xenopus* M-phase egg extracts. During the time of the thesis the microtubule associated protein TPX2 was identified as a target molecule of Ran in spindle formation. We then wanted to know how TPX2 can promote microtubule assembly and how it is regulated.

Using *Xenopus* M-phase extracts we first characterised the function of molecules of the import machinery in spindle formation. After the identification of the target molecule TPX2 we characterized its regulation by importin α. The major interaction site of TPX2 with importin α was identified and a mutant form of TPX2 with greatly decreased affinity for importin α was generated. This allowed a detailed analysis of both how TPX2 promotes microtubule assembly and how it is the regulated by importin α. With this knowledge of how Ran mediates microtubule assembly we could then approach the question of how Ran induces spindle-like structures.

In a parallel approach we tried to understand how Ran induces increase of microtubule nucleation by centrosomes. We also examined whether Ran affects the microtubule cytoskeleton in organism which does not have a TPX2 homologue, *Drosophila melanogaster*.