Discussion

A message from chromatin

The cytoskeleton undergoes a dramatic change at the transition from interphase to mitosis. Long stable microtubules become short and interchange frequently between the growing and shrinking states. It is thought that the change in microtubule dynamics is ultimately due to the activity of cdc2 kinase, which phosphorylates a large number of mitotic target proteins, among them MAPs. Some MAPs, like stathmin/Op18 (Andersen *et al*., 1997), are regulated by phosphorylation, and this regulation contributes to the changes in the properties of the cytoskeleton. In mitosis, not only do several kinases become active, but also the nuclear envelope breaks down, mixing the nuclear and the cytoplasmic compartments. It has been demonstrated that RanGTP has a major influence on microtubule dynamics and nucleation (Carazo-Salas *et al*., 2001; Wilde *et al*., 2001). It is likely that several effector proteins of microtubules are segregated from their target molecule, tubulin, and stored in the nucleus during interphase and thereby kept inactive. The nuclear envelope breakdown would than bring all these factors back to their site of action, triggering processes ultimately leading to the construction of a mitotic spindle and chromosome segregation. This is a particular intriguing model since Ran GTP produced by chromatin bound RCC1 acts as a spatial signal of where the DNA is located. It had been long thought that chromatin behaves during metaphase similar to a coffin at a funeral. It is the focus of attention but does not do anything itself. Early experiments however (Carazo-Salas *et al*., 1999; Heald *et al*., 1996; Karsenti *et al*., 1984b) showed that chromatin can trigger the formation of a spindle. It was then shown that Ran GTP is both required for chromatin-mediated spindle assembly (Carazo-Salas *et al*.,

1999; Guarguaglini *et al*., 2000; Kalab *et al*., 1999; Ohba *et al*., 1999; Wilde and Zheng, 1999)], as well as being sufficient to induce spindle-like structures in M-phase *Xenopus* extracts (Carazo-Salas *et al*., 1999). The mechanistic details of Ran's function in microtubule assembly remained unknown. The aim of this study was to identify targets of Ran in mitotic spindle assembly and to characterize the mechanism of their regulation.

Oliver Gruss identified TPX2 as the downstream target of Ran in microtubule assembly (Gruss *et al*., 2001). Recombinant TPX2 protein induces microtubule assembly in *Xenopus* M-phase extracts (Figures 13 and 15). Chromatin beads are not able to assemble spindles in extracts when TPX2 is removed (Figure 17). These two experiments together demonstrate that TPX2 is both required and sufficient to induce microtubule assembly mediated by Ran GTP. Taken together the data supports a model where chromosomes release a signal cascade resulting in microtubule nucleation as follows: Chromosome bound RCC1 produces Ran GTP and Ran GTP then releases TPX2, which is then free to nucleate microtubules. It is likely that other Ran dependent processes function in parallel resulting in the full chromatin effect, which is the assembly of a mitotic spindle around chromatin beads. Calculation of a Ran GTP gradient around chromosomes in cells, based on the enzymatic activities of the components of the Ran system and their concentration and diffusion constants led to the prediction that the distribution of Ran GTP in a gradient around chromatin would only be possible in larger cells, like oocytes and embryonic cells, not in smaller, somatic cells (Gorlich *et al*., 2003). On the other hand TPX2 is also critical for chromosome induced microtubule assembly in somatic cells as shown by experiments where TPX2 was depleted from HeLa cells using RNA interference technology (Gruss *et al*., 2002). Direct visualisation of the Ran gradient (Kalab *et al*., 2002) suggests that factors not considered in the theoretical analysis (Gorlich *et al*., 2003) may affect the shape and size of the Ran gradient. The release of TPX2 in the vicinity of chromatin and the inhibition of TPX2 by importin α in the cell periphery might nevertheless be particularly important in large cells, like oocytes and embryonic cells, to prevent ectopic microtubule assembly.

In small somatic cells an on off switch activating TPX2 during mitosis and inactivating it during interphase might be sufficient. Possibly, interaction with import receptors could also be essential for the efficient inhibition of TPX2 activity in the interphase cytoplasm. Ectopic microtubule nucleation during interphase by newly translated TPX2 might be prevented by efficient binding of import receptors to TPX2 and its segregation into the nucleus thereafter. TPX2 would then only be activated following nuclear envelope breakdown at the beginning of mitosis. As long as it has been not directly proven by experiments we will not know whether a Ran GTP gradient exists around chromatin in all different cell types and it might very well be that different cell types utilize TPX2 regulation to different extents.

TPX2 and importin α interaction

When BSA coupled to NLS peptide was added to *Xenopus* M-phase extract microtubule assembly was observed (Figure 8). Addition of the importin β binding domain (IBB), which competes with importin α for binding, also induced MT assembly (Figure 8). RanGTP induced asters did not form in the presence of excess importin α (Figure 11). These results strongly suggest that importin α acts as an inhibitor of Ran induced microtubule assembly. As illustrated in Figure 40 TPX2 forms a complex with importin α/β analogous to complexes formed during protein import into the nucleus. TPX2 binds to importin α via an NLS signal, since a point mutant of importin α, which is no longer able to bind NLS substrates, the "ED" mutation, does not inhibit TPX2 induced MT asters (Figure 11). We showed here that a site centred around amino acid 284 in TPX2 is critical for this interaction between importin α and TPX2 (Figure 25). TPX2 binds indirectly through importin α to importin β (Figure 21). Upon encountering RanGTP the complex is disassembled. RanGTP binds to importin β and displaces it from importin α and the bound cargo. The remaining complex of TPX2 and importin α is much less stable. The export receptor for importin α, CAS, probably further disassembles the TPX2-importin α complex. CAS binds importin α together with

RanGTP, and this interaction is incompatible with cargo binding of importin α (Kutay *et al*., 1997). It has been shown for other NLS cargos that RanGTP accelerates the dissociation of β from α-NLS cargo 450 fold and that CAS and RanGTP accelerates the dissociation of the NLS cargo from importin α 40 fold (Gilchrist *et al*., 2002). Although we did not measure affinities the binding experiments of importin α or importin α/β to TPX2 show that importin α binds only stable to TPX2 in a complex with importin β (Figure 21). This shows that importin α and β bind cooperatively to the TPX2 NLS. This data supports a model according to which RanGTP is produced by the chromatin bound RCC1 in the vicinity of the chromosomes. The concentration of RanGTP further away from the place of its production is much lower due to the proteins which help hydrolyse Ran bound GTP: RanBP1 and RanGAP. The release of TPX2 from α and β will occur primarily around the chromosomes.

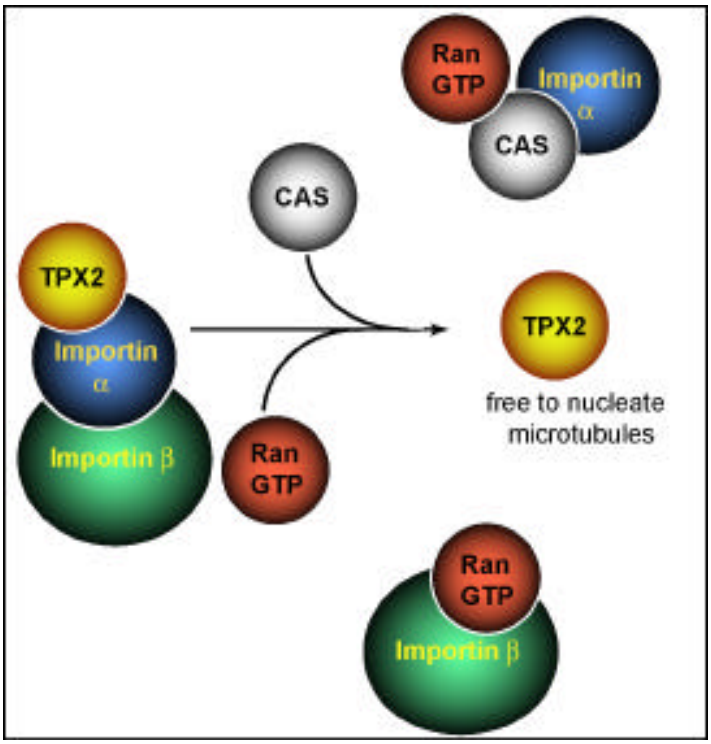

Figure 40. Model for TPX2 release from importin α.

RanGTP binds to the import receptor importin β and the export receptor CAS, influencing their association with importin α. The net result is the release of TPX2 from importin α. Free TPX2 can then function in microtubule assembly.

The mechanism of TPX2 action in microtubule assembly

There are two major classes of model to explain TPX2's function in microtubule assembly: 1. TPX2 might promote microtubule assembly by stabilising existing microtubule polymers. 2. TPX2 might nucleate microtubules *de novo*.

The fact that the average length of microtubules nucleated by centrosomes in *Xenopus* M-phase extracts depleted of TPX2 was not decreased (Gruss *et al.*, 2002), would argue against a function of TPX2 in stabilising microtubules, although we have not carefully measured microtubule dynamics when TPX2 was

either added or depleted from *Xenopus* M-phase extract. Addition of excess recombinant TPX2 induced microtubule assembly in *Xenopus* M-phase extracts (Figure 15), suggesting that TPX2 can nucleate microtubules. Additionally, TPX2 was able to induce small aster like structures when incubated in a minimal system consisting of tubulin, TPX2 and buffer (Figure 27). Taken together these observations clearly favour the model that TPX2 functions in nucleation rather than in stabilization of microtubules.

The question then becomes, how does TPX2 nucleate microtubules? Although microtubules can self-assemble *in vitro* from high concentrations of purified tubulin subunits, microtubuless are nucleated *in vivo* at relatively low tubulin concentrations. Under these conditions, the initiation of new microtubule ends is kinetically limiting. In order to start polymerization from tubulin monomers requires the formation of a nucleation seed of about 12-15 dimers (Fygenson *et al*., 1995). The formation of this seed is the rate limiting step in the nucleation reaction. At low tubulin concentration the time required to form this stable nucleus is infinite and no elongation occurs (Mitchison and Kirschner, 1984a). Above a tubulin concentration of 20 µM seed formation is not limiting anymore and free nucleation occurs spontaneously. Any factor which catalyses the formation of a nucleating seed can therefore serve as a microtubule nucleator. The main nucleator in the cells is thought to be the γ-tubulin ring complex and it probably only assembles microtubules at centrosomes (Schiebel, 2000), it was therefore not expected that other proteins like TPX2 can also nucleate microtubules. But two lines of evidence argue against microtubule nucleation only at centrosomes and only by γ-tubulin. First, in a variety of cells microtubules are not anchored at the centrosome. For instance in migrating newt lung cells, 80-90% of the microtubules are not bound to the centrosome (Waterman-Storer and Salmon, 1997) In epithelial cells microtubules form bundles parallel to the apico-basal axis (Bacallao *et al*., 1989). Several cases of centrosome independent microtubule nucleation have been reported for example in tissue culture cells (Rodionov and Borisy, 1997) and *Xenopus* M-phase extracts (Heald *et al*., 1996). Second, γ-

tubulin seems not to be essential for MT nucleation. Genetic studies in *Drosophila melanogaster* showed that γ-tubulin mutants can still form microtubule asters on centrosomes (Sampaio *et al*., 2001). When γ-tubulin was depleted from *C. elegans* embryos by siRNA techniques microtubules were still nucleated by centrosomes during mitosis and the aster size increased after nuclear envelope breakdown (Hannak *et al*., 2002). This suggests that there are other molecules besides γ-tubulin which can nucleate microtubules. One candidate which was reported to nucleate microtubules is XMAP215 (Popov *et al*., 2002), but even when both ZYG-9, the *C.elegans* homologue of XMAP215, and γ-tubulin were depleted from *C.elegans* embryos microtubules were still nucleated in mitosis (Hannak *et al*., 2002). Taken together, γ-tubulin might be the main nucleator of microtubules in cells but there have to be other factors which can promote microtubule assembly. One of these factors might be TPX2 since it was able to induce microtubule assembly both in extracts as well as in a minimal system of buffer, tubulin and TPX2 (Figures 15 and 27). It would be interesting to deplete both γ-tubulin and TPX2 by siRNA techniques in HeLa cells to see whether this blocks microtubule nucleation completely.

Another reason why there might be several factors which can nucleate microtubules is that the nucleators are active at different times or places. TPX2 activity is restricted to M-phase and the activity of other microtubule nucleators is also likely to be under cell-cycle control. XMAP215 activity has been proposed to be diminished in mitosis by cell-cycle dependent phosphorylation (Vasquez *et al*., submitted). These observations could reflect a more general phenomenon by which specific nucleators would only be required for certain periods during the cell cycle, or at certain locations in the cell to which microtubule formation needs to be targeted.

It has been shown that RanGTP-mediated microtubule assembly, a reflection of chromatin-induced microtubule formation, was abolished in *Xenopus* M-phase egg extract depleted of either γ-tubulin or XMAP215 (Wilde *et al*., 2001). Thus, TPX2, XMAP215 and γ-tubulin are all necessary for Ran-induced nucleation of

stable microtubule structures in these mitotic extracts. Since TPX2 can nucleate microtubules *in vitro* (Figure 27) it raises the question why it is dependent on γ-tubulin and XMAP215 in *Xenopus* M-phase extracts? It might be that many different activities are required in parallel for microtubule nucleation in *Xenopus* M-phase extracts. The protein concentration in an extract is much higher than in the experiments performed in buffer, therefore the competition for binding to tubulin is much higher. Also, the affinity of proteins involved in microtubule nucleation might be altered by regulators in an extract. It might be that whereas the only limiting step in microtubule nucleation in buffer is the formation of a microtubule seed, the nucleation process in extracts is a collection of structurally distinct steps. One step might be the templating of a tube containing thirteen protofilaments, the function proposed for the γ-tubulin ring complex (Moritz and Agard, 2001). A second might be the formation of tubulin oligomers that are stable enough to allow further addition to the growing tube. Protofilament stabilisation may occur before as well as after tube formation and stabilisation during these two processes may not utilise the identical mechanism (Schiebel, 2000).

TPX2, the target of Ran in MT nucleation

The work presented in this thesis identified the site on TPX2 that is critical for binding to importin α both in buffer and in *Xenopus* M-phase extracts. Mutating two amino acids in the full-length protein did not alter the function of the protein in microtubule assembly but abolished the interaction between TPX2 and importin α *in vitro* (Figure 25). Furthermore the mutant TPX2 protein was insensitive to importin α's inhibitory effect and thus to regulation by Ran in *Xenopus* extracts, resulting in a constitutively active form of TPX2 (Figure 26). This shows that lack of interaction between importin α and TPX2 is sufficient to short cut regulation of microtubule assembly. No other factor must be activated by Ran in order to form asters. Other groups have shown that the microtubule associated protein NuMA

can promote microtubule assembly and suggested that it is also activated by Ran (Nachury *et al*., 2001; Wiese *et al*., 2001). Our data shows that regulation of TPX2 by importin α alone is critical for Ran dependent microtubule assembly. Three other lines of evidence suggest that TPX2 is the only microtubule nucleator inhibited by importin α. First RanGTP is not able to induce microtubule assembly in extracts depleted of TPX2 (Figure 16) showing that TPX2 release is both necessary and sufficient for this early stage of spindle assembly. Second extracts which have been passed over an importin α column, and thereby depleted of the aster forming activity, do not have reduced amounts of NuMA, indicating that NuMA can not bind importin α with a high affinity. Third in the attempts to purify an activity able to nucleate microtubules in *Xenopus* extract only one peak activity was found in the fractions: TPX2 (Gruss *et al*., 2001). Although it should be kept in mind that the starting material for the purification, HeLa nuclear extracts, probably does not contain large amounts of NuMA due to technical reasons.

Regulation of TPX2 by importin α

TPX2 induces the formation of microtubule asters and bundles of microtubules in a minimal system consisting of tubulin and TPX2 (Figure 27). Importin α completely inhibited the ability of TPX2 to induce the formation of microtubules in this minimal system (Figure 29). On the other hand neither microtubule bundling by TPX2 nor the formation of microtubule aggregates was inhibited by importin α. This suggest that TPX2 has two different modes of interaction with tubulin. One which promotes bundling and which is unaffected by importin α and another one which promotes microtubule nucleation by TPX2, which is inhibited by importin α. First, TPX2 functions on tubulin dimers or oligomers and might promote their assembly into small, relatively stable intermediates, which are competent to

elongate microtubules. It is only this function in the TPX2-mediated microtubule assembly process which is inhibited by importin α: seeds can still form, but they cannot nucleate microtubule formation (Figure 29 and 41). Second, TPX2 can bundle microtubules. This results in arrays of parallel microtubules and likely also contributes to the aster-like structures observed upon microtubule assembly in the presence of TPX2. In the minimal system TPX2 would first promote the assembly of aggregate seeds and interconnect these seeds by its bundling activity. Subsequently the connected seed structures would grow out microtubules in different direction leading to an aster like structure (Figure 29 and 41). The two different tubulin binding sites could for example bind either to the microtubule cap promoting nucleation, or a different site in TPX2 could bind to a polymerised tubulin tube. In this example the binding site for the microtubule cap would be inhibited by importin α. TPX2 might promote microtubule assembly in *Xenopus* M-phase extracts in the same way it does in the minimal system although tubulin aggregates have not been observed in extracts.

In living cells TPX2 binds spindle microtubules and preferentially to spindle poles. Its binding to both microtubules and Xklp2 results in targeting of the complex to microtubules where dynein activity mediates the movement of both TPX2 and Xklp2 towards microtubule minus ends at the spindle poles (Wittmann *et al*., 1998; Wittmann *et al*., 2000). Initially it has been reported that reduced amounts of TPX2 lead to unfocused spindle poles, suggesting that Xklp2 and TPX2 together focus the spindle ends. Since TPX2 is able to bundle microtubules it might stabilize spindle poles by interconnecting and bundling microtubule arrays at the minus end. Interestingly the bundling activity which might be required further away from the chromatin with a lower level of RanGTP is not affected by importin α (Figure 31).

When TPX2 was depleted from HeLa cells by siRNA astral microtubules emanating from the centrosomes still formed but they did not connect to the chromosomes (Gruss *et al*., 2002), suggesting that two kinds of microtubules exist performing distinct functions. The two classes of microtubules either

emanate from chromatin and are dependent on TPX2 or are formed by centrosomes. It should be interesting to find out whether these different classes of microtubules can be distinguished by any means and if so whether Ran induced spindles only contain one class.

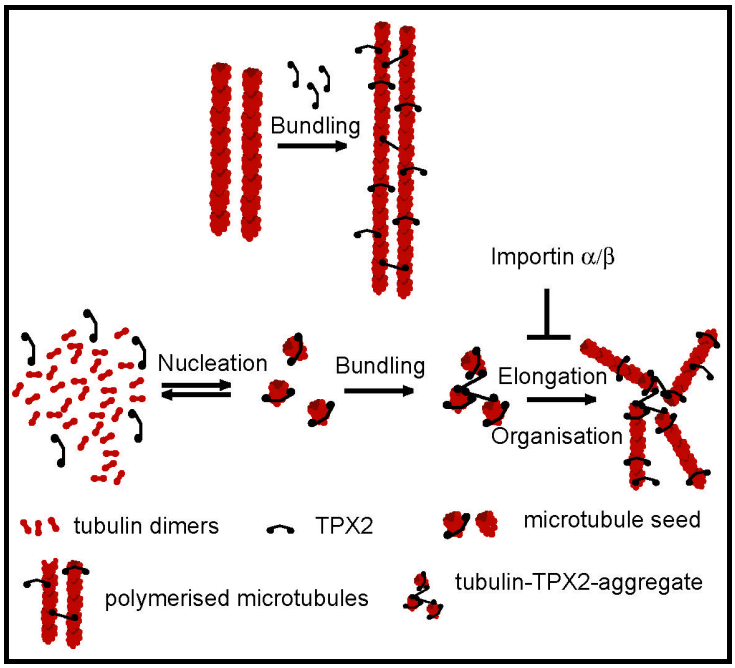

Figure 41. A Model of the activities of TPX2 in microtubules Nucleation and Bundling.

Microtubules are assembled in the absence of TPX2, e.g. by the action of taxol. Small intermediates ("seeds") are stabilized and microtubules elongate from these seeds. Added TPX2 can interact with a polymerized microtubule at several sites and promote parallel bundling. The bundling activity is not inhibited by importin α/β. (upper part of the figure) Microtubules are assembled in the presence of TPX2. TPX2 initially nucleates small seeds, which are eventually bridged with one another by TPX2 reflecting TPX2 bundling activity. From these clusters of seeds, microtubules can grow in all directions and organized, aster-like structures are formed. The function of TPX2 in generating seeds which are competent for further elongation is inhibited by importin α/β (lower part of the figure).

Ran induced spindle organization

When added to *Xenopus laevis* M-phase extract RanGTP induces the organization of microtubules into focused arrays (Figures 11 and 34). On the other hand TPX2, which is required for Ran induced MT nucleation forms aster like structures when added to M-phase extracts (Figure 15). The ΔNLS mutant version of TPX2 allowed us to induce aster formation in the presence of importin α. Under these conditions no spindle like structures formed even in the presence of RanGTP. We conclude that Ran induced spindle formation is a two step process. The first step involves aster formation by TPX2, the second step is spindle formation mediated by one or several so far unknown factors (Figure 35). Both steps are mediated by RanGTP and can be inhibited by importin α. One protein which has been reported to be involved in the process and which is possibly regulated by Ran is the tetrameric motor Eg5 (Wilde *et al*., 2001). Eg5 is present both in the nucleus and the cytoplasm during interphase (Houliston *et al*., 1994), suggesting that it is not completely segregated from tubulin and its function probably not regulated by transport receptors. In the presence of RanGTP the amount of Eg5 moving to the plus ends of microtubules was increased (Wilde *et al*., 2001). This might possibly be due to an Eg5 binding regulator which is subsequently regulated by Ran. Computer modelling approaches indicate that for the assembly of a mitotic spindle both minus and plus end directed motors are necessary (Nedelec *et al*., 2003), suggesting that a factor which bridges a minus and a plus end directed motor could be the target molecule of Ran in the second step in spindle assembly.

TPX2 in centrosome activation

Centrosomes are the main microtubule nucleation centre in the living cell. It was long known that this ability to nucleate microtubules increases dramatically at the transition between interphase and mitosis. Rafael Carazo-Salas showed in 2001 that RanGTP also leads to an increase of microtubule nucleation by sperm-centrosomes in mitotic extracts (Carazo-Salas *et al*., 2001). In this study, we wanted to investigate whether this phenomenon is regulated by Ran in the same way as spindle assembly. We therefore incubated sperm centrosomes in *Xenopus* M-phase extract either with or without RanQ69LGTP, reisolated them, and assayed for their ability to nucleate microtubules in buffer. The centrosomes were more active upon incubation with RanQ69L indicating that Ran has an effect on centrosome activation (Figure 36) and (Carazo-Salas *et al*., 2001). Analogous to the mechanism in spindle assembly centrosome activation was inhibited by importin α (Figure 37). Moreover, we demonstrated that TPX2 is necessary for Ran to activate centrosomes (Figure 38). Probably TPX2 gets released from importin α by Ran GTP in the extract and then binds to centrosomes causing the increased microtubule nucleation activity. This is supported by experiments performed with the kinase aurora A in *Xenopus* M-phase extract. Aurora A has been shown to be essential to establish and maintain the bipolar spindle as well as for centrosome activation (Hannak *et al*., 2001; Roghi *et al*., 1998). The two known target molecules for aurora A which have been described so far are Eg5 and TPX2 (Kufer *et al*., 2002). It has also been shown that free TPX2, in the presence of microtubules, causes autophosphorylation and activation of aurora A (Tsai *et al*., 2003). The activation of aurora A by TPX2 is essential for spindle assembly (Tsai *et al*., 2003). TPX2 might therefore be essential for centrosome maturation because aurora A, which is necessary for this process (Hannak *et al*., 2001), needs to be activated by TPX2. Direct evidence for a possible role of TPX2 in centrosome function was provided recently by an experiment where TPX2 has been depleted from HeLa

cells by siRNA techniques. The TPX2 depleted cells showed multiple spindle poles and centrosome destabilization (Garrett *et al*., 2002).

Ran impairs spindle formation in *Drosophila melanogaster*

Injection of two different mutants of Ran into *Drosophila* syncytial embryos affected microtubule organization and cell division (Figure 39), indicating a function for Ran in spindle organization *in vivo* in *Drosophila*. However, since the cells underwent multiple cell cycles during the experiment it is difficult to rule out that the defects on cell division are not caused by a protein import defect. However siRNA experiments in *C. elegans* showed a role for components of the Ran system during the first cell divisions in worms (Askjaer *et al*., 2002). When Ran was depleted centrosomes detach from nuclei to early and are not properly positioned. Whereas centrosomal microtubules are still formed no proper spindle could be wound when Ran was depleted (Askjaer *et al*., 2002). Also depletion of the *C.elegans* homologues of importin α and β disrupted spindle formation (Askjaer *et al*., 2002). This suggests that there is a conserved function of the proteins of the Ran system in spindle assembly in organisms which do not have a TPX2 homologue such as *C. elegans* or *Drosophila*. Remarkably, no homologue of NuMA has been found in *Drosophila* either. Since Ran acts similarly in flies as it does in *Xenopus laevis* other proteins probably fullfill the function of TPX2 and NuMA in these species. One candidate is the *Drosophila* abnormal spindle pole protein Asp. Asp localizes like TPX2 to the spindle poles during metaphase, to the midbody during telophase and to the nucleus in interphase in meiotic larval neuroblast cells (Wakefield *et al*., 2001). Similar to TPX2, Asp is required for aggregation of MTs into spindle poles (Wakefield *et al*., 2001). Asp, when it is phosphorylated by Polo kinase, is also necessary and sufficient to promote microtubule nucleation by centrosomes in *Drosophila* extracts (do Carmo Avides *et al*., 2001).