Material and Methods

Chemicals

2-Mercaptoethanol	Sigma-Aldrich (Steinheim, Germany)
Acetic Acid	Molecular Probes (Göttingen, Germany)
Acrylamide/Bisacrylamide, 37.5:1 (30% w/v) (Protogel®)	National Diagnostics (Hull, UK)
Alexa Fluor®) 546 C5 maleimide	Molecular Probes (Göttingen, Germany)
Ampicillin, Binotal®	Grünenthal (Aachen, Germany)
APS (ammonium peroxodisulfate)	Sigma (St. Louis, USA)
ATP (adenosin 5'-trisphosphate)	Sigma-Aldrich (Steinheim, Germany)
Bacto agar Gibco BRL	(Eggenstein, Germany)

BH_3O_3 (boric acid)	Merck (Darmstadt, Germany)
Bio-Rad protein assay	Bio-Rad (München, Germany)
Bromophenol blue (3',3'',5',5''-tetrabromophenolsulfonephtalein)	Sigma (St. Louis, USA)
$CaCl_2$	Merck (Darmstadt, Germany)
Complete EDTA-free Protease Inhibitor	Roche (Mannheim, Germany)
Coomassie brilliant blue G-250	Sigma-Aldrich (Steinheim, Germany)
Creatine phosphate	Roche (Mannheim, Germany)
Cysteine	Sigma-Aldrich (Steinheim, Germany)
Cytochalasin B	Sigma-Aldrich (Steinheim, Germany)
DAPI (4',6-diamidino-2-phenyindole)	Sigma-Aldrich (Steinheim, Germany)
DMSO (dimethyl sulfoxid)	Merck (Darmstadt, Germany)
DTT (1,4-dithio-L-threitol)	Merck (Darmstadt, Germany)
EDTA (1-(4-aminobezyl)ethylenediamine-N,	Sigma-Aldrich (Steinheim,

N, N', N'-tetraacetic acid)	Germany)
EGTA (ethylene glycol-bis(2-aminoethyl)-N, N, N', N'-tetraacetic acid)	Sigma-Aldrich (Steinheim, Germany)
GDP (guanosine 5'-diphosphate)	Sigma-Aldrich (Steinheim, Germany)
Glutaraldehyde (50% aqueous solution)	Sigma-Aldrich (Steinheim, Germany)
Glycerol (87% aqueous solution)	Merck (Darmstadt, Germany)
Glycine	Merck (Darmstadt, Germany)
GTP (guanosine 5'-triphosphate)	Merck (Darmstadt, Germany)
GTPγS (guanosine 5' –γ-thio]triphosphate)	Merck (Darmstadt, Germany)
HCl (hydrochloric acid, 37%)	Sigma-Aldrich (Steinheim, Germany)
HEPES (4-(2-hyroxyethyl-)piperazine-1-ethansulfonic acid)	Sigma-Aldrich (Steinheim, Germany)
Imidazole (1, 3-diaza-2,4-cyclopentadiene)	Sigma-Aldrich (Steinheim, Germany)

Immersion oil	Leica (Bensheim, Germany)
IPTG (isopropyl β-D-thiogalactopyranoside)	Sigma-Aldrich (Steinheim, Germany)
K_2HPO_4	Merck (Darmstadt, Germany)
Kanamycin	Serva (Heidelberg, Germany)
KCl	Merck (Darmstadt, Germany)
KAc (potassium acetate)	Merck (Darmstadt, Germany)
$MgCl_2$	Merck (Darmstadt, Germany)
MgAc (magnesium acetate)	Merck (Darmstadt, Germany)
$Na_2B_4O_7x10\ H_2O$ (sodium borate, borax)	Merck (Darmstadt, Germany)
NaCl	Merck (Darmstadt, Germany)
NLS-peptide (CGGGPKKKRKVED)	Sigma GenoSys (London, UK)
Paraformaldehyde	Sigma-Aldrich (Steinheim, Germany)
PIPES (piperazine-1,4-bis(2-ethanesulfonic acid))	Sigma-Aldrich (Steinheim, Germany)
PMSF (phenylmethylsulfonyl fluoride)	Sigma-Aldrich (Steinheim, Germany)

	Germany)
Poly-L-Lysine solution	Sigma (St. Louuis, USA)
SDS (sodium dodecylsulfate)	Serva (Heidelberg, Germany)
Sephadex G-50	Amersham (Freiburg, Germany)
Sucrose	Merck (Darmstadt, Germany)
TALONTMresin	Clontech (Palo Alto, USA)
TEMED Tris (tris-(hydroxymethyl)-aminoethane) (N,N,N' ,N'-tetramethyl-ethylendiamine)	Merck (Darmstadt, Germany)
Triton X-100 (t-octylphenoxypolyethoxyethanol)	Sigma-Aldrich (Steinheim, Germany)
Vectashield® mounting medium H-1000	Vector Laboratories (Burlingame, USA)

Commonly used buffers, solutions and media

All solutions were prepared with double de-ionised water. Solutions were sterile filtered and, unless otherwise indicated (in brackets), stored at room temperature.

Blotting buffer	25mM Tris base 192 mM Glycine
Borate buffer pH 7.6	300 mM NaCl 50 mM $Na_2B_4O_7$x10 H_2O adjusted to pH 7.6 with boric acid
Borate buffer pH 8.5	300 mM NaCl 100 mM $Na_2B_4O_7$x10 H_2O adjusted to pH 8.5 with boric acid
BRB80	80 mM Pipes, pH 6.8, 1 mM $MgCl_2$, 1 M EGTA, pH adjusted to 6.8 with KOH, sterile filtered and stored at 4°C.
CSF-XB (Extract Buffer)	2 mM $MgCl_2$ and 5 mM EGTA pH 7.7, adjusted to pH 7.7. CSF-XB with protease inhibitors: add 100 µl each of leupeptin, pepstatin, aprotinin at 10 mg/ml, check pH 7.7.
Dejellying solution	2% Cysteine (w/v) in 0.25xMMR adjusted to pH 7.8 with 5N NaOH
Energy mix (-20°C)	100 mM Creatine phosphate 5 mM GTP 5 mM ATP 0.5 mg/ml Creatin

	kinase
Sample buffer	(6x, -20°C) 0.6% (w/v) Bromophenol blue 12% (w/v) SDS 60% (v/v) glycerol 300 mM Tris, pH 6.8
LB agar (autoclaved)	1.5% Bacto agar in LB medium
LB medium (autoclaved)	1% (w/v) Bacto tryptone 0.5% (w/v) Bacto yeast extract 170 mM NaCl adjusted to pH 7.6 with 5N NaOH
MMR (20°C)	100 mM NaCl 10 mM $MgCl_2$ 20 mM $CaCl_2$ 1 mM EDTA 50 mM HEPES, pH 8.0
PBS (phosphate buffered saline)	130 mM NaCl 100 mM Na_2HPO_4, pH 7.0
PCR buffer	20 mM TrisCl, pH 8.8 10 mM KCL 10 mM $(NH_4)SO_4$ 2 mM $MgSO_4$ 1% (v/v) Triton X-10 1 mg/ml BSA
Phosphate buffer pH 6.0	100 mM Na_2HPO_4, pH 6.0
Resolving gel (12%) solution	(4°C) modified for other percentages 375 mM Tris, pH 8.8 12% (w/v)

	Acrylamide/Bisacrylamide 0.1% (w/v) SDS
Running buffer (5x)	303 g Tris base 1.44 kg Glycine 50 g SDS H_2O added to a final volume of 10 l
Stacking gel (3%) solution (4°C)	125 mM Tris, pH 6.8 3% (w/v) Acrylamide/Bisacrylamide 0.1% (w/v) SDS
Staining solution	0.2% (w/v) Coomassie brilliant blue G-250 in methanol : acetic acid : water (5:1:4)
XB (Extract Buffer)	100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 M potassium HEPES pH 7.7, 50 mM sucrose, pH adjusted to 7.7 with KOH.
20x XB salt	2 M KCl, 20 mM $MgCl_2$, 2 mM $CaCl_2$, sterile filtered at 4°C.

Commonly used materials

0.2 ml reaction tubes (Thermo Tube™)	PEQLAB (Erlangen, Germany)
0.5 ml micro tubes	Sarsted (Numbrecht, Germany)
1.5 ml reaction tubes	Eppendorf (Hamburg, Germany)
5 ml columns	MoBiTech (Göttingen, Germany)
Aluminium foil	Conresco (Minden, Germany)
Bottle top filters, 0.22 µm pore size	Millipore (Molsheim, France)
Costar® Transfer Pipettes	Corning Costar Corporation (Cambridge, USA)
Coverslips (11 mm diameter)	Menzel-Gläser (Braunschweig, Germany)
Filter paper	Whatman (Maidstone, UK)
General glass ware	Schott (Nürtingen, Germany)
General plastic ware	Greiner (Nürtingen, Germany)
Microcon® YM-10 centrifugal filter units	Amicon Millipore (Eschborn, Germany)
Microlance™ sterile needles	Becton Dickinson (Temse, Belgium)

Microscope slides	Menzel-Gläser (Braunschweig, Germany)
Millex®-GV 0.22 µm filter units	Millipore (Molsheim, France)
Multitest slides 10-well	ICN Biomedicals (Aurora, USA)
Nail polish	Manhattan (Stuttgart, Germany0
Plastic cuvettes	Ratiolab (Dreieich, Germany)
Spectra/Por® Membrane, MWCO 3.5 kDa	Spectrum Laboratories (Broadwick, USA)
Syringes	Becton Dickinson (Temse, Belgium)
Ultracentrifuge tubes	Beckman (Palo Alto, USA)

Methods

Nucleoplasmin binding assay

Radioactive labelled nucleoplasmin was generated by *in vitro* transcription-translation in Reticulocyte lysate (Promega, Heidelberg, Germany). *In vitro* translated nucleoplasmin (5µl) was incubated with protein A-tagged importin α or ED bound to IgG Speharose or empty IgG Sepharose (10µl) in 200 µl PBS for 2 hours at 4°C. IgG Sepharose was washed three times with PBS and bound proteins eluted with SDS sample buffer without DTT. Bound and unbound

(corresponding volumes) nucleoplasmin was analysed by SDS-PAGE and autoradiography.

Xenopus laevis M-phase extract preparation

Xenopus laevis female frogs, previously injected with 100 units of pregnant mare serum gonadotropin (PMSG) were injected 16-18 hours prior to collecting eggs with 1000 units of human chorionic gonadotropin (HCG) (Sigma Chemical Co., St. Louis, MO). Frogs were placed in individual boxes in MMR at 16°C. Eggs were collected and extensively washed in MMR and subsequently first for 5 min in cysteine solution (10 g in 500 ml 0.25x MMR) to dejelly them, then in XB and finally in CSF-XB. Eggs were then packed for 30 s at 900 rpm then 1 min 30 s at 2000 rpm in a clinical centrifuge, then centrifuged at 10200 rpm at 15°C for 17 min in a HB4 rotor to crush the eggs. The cytoplasmic layer was collected using a needle and syringe via side puncture and cytochalasin D and protease inhibitors were added. After this step extracts were kept on ice (Murray, 1991).

Microtubule assembly assay in *Xenopus* M-phase extracts

10 µl of *Xenopus* M-phase extract (Murray, 1991) was supplemented with rhodamine tubulin (Hyman *et al*., 1991) and either 15 µM importin α or buffer was added. Thereafter 160 nM TPX2 wild-type or mutant protein was added and incubated for 25 min at 20 ºC. 1.5 µl samples were fixed and squashed (Sawin and Mitchison, 1991). Typically 1.5 µl of reaction were pipetted with a cut tip and laid on a slide, and a 1.5 µl droplet of fix solution (0.3 ml formaldehyde 37%, 0.6 ml 80% glycerol, 1 ml 1xMMR, 1 µl 10 mg/ml Hoechst) was laid on top of the extract. The resulting fixed drop was squashed carefully laying a coverslip on top of it.

For spin down experiments reactions were diluted with 1 ml BRB80 (80 mM K-PIPES, pH 6.8, 1 mM EGTA, and 1 mM $MgCl_2$) containing 10% glycerol, 0.25% glutaraldehyde and 0.1% Triton X-100 and subsequently centrifuged (HB4 rotor, 12,000 rpm, 12 min, 16°C) through a 25% glycerol cushion in BRB80 onto coverslips as described (Sawin and Mitchison, 1991). The coverslips were fixed in –20C methanol, incubated twice for 10 min in 0.1% $NaBH_4$ in PBS. Image accquisition and analysis was on a Leica confocal microscope. Reactions were quantified by counting all structures from at least three samples. Histograms represent mean values and error bars standard deviation. TPX2 was depleted from M-phase extracts as described. Depleted extract was reconstituted with 20 nM wild-type TPX2 or mutant TPX2.

Depletion and reactivation of *Xenopus* M-phase extracts

For depletion of NLS proteins, importin α or the ED mutant were bound to IgG Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) to a concentration of 10 mg/ml, blocked with BSA (10 mg/ml), and washed with ten volumes of CSF buffer (Murray, 1991). Freshly prepared M phase extracts were incubated 1:1 (v/v) with the resin on a rotating wheel for 90 min in the presence of 20 µM RanQ69L GTP in 0.5 ml Mobicol columns (MoBiTec, Göttingen, Germany). The Sepharose was removed by centrifugation at 1500 g and the extracts frozen. Depletion was routinely checked by Western blotting.

Immunodepleting TPX2

For Immunodepletions, 7µg of antibody was bound to 25 µl of protein A-conjugated Dynabeads 280 (Dynal). The antibody was mixed with the beads in a total volume of 100-200 µl PBS containing 0.1% Triton X-100 and incubated for at least 1 h on a rotating wheel. The beads were then washed once with PBS, 0.1% Triton X-100, once with 0.5 M NaCl in PBS, 0.1% Triton X-100, and three

times with CSF-XB (10 mM K-Hepes, pH 7.7, 50 mM sucrose, 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 5 mM EGTA). Between the washing steps the beads were retrieved on a magnet and at the end the buffer was removed as much as possible. 100µl extract was added to the beads and pipetted up and down carefully until the beads were completely resuspended. The extract was then placed on ice for 90 min and mixed by pipetting once or twice during this time. The beads were retrieved on a magnet on ice for ~10 min and the recovered extract was used in the experiments.

Cloning of TPX2 expression constructs

Xenopus TPX2 fragments were PCR-amplified and cloned into the Nco I and Bam HI sites of the zzpQE60 expression vector, which encodes two N-terminal IgG binding domains of *Staphyloccocus aureus* protein A (Gruss *et al*., 2001). In the TPX2 mutant constructs the following amino acids were changed to alanines: **124**: K124, K126; **284**: K284, R285; **550**: K550, K551; **h315**: K315, R316. Mutations were inserted by site-directed mutagenesis and the mutated regions of the ORF were sequenced.

Expression and purification of recombinant proteins

TPX2 full-length protein and fragments was cloned into Nco I / Bam HI sites of pQE60. TPX2 was expressed in *E.coli* BL21pRep4. Bacteria were grown at 37°C to an OD_{600} of 0.8, diluted 1:1 with ice cold medium and 4% ethanol, and grown for 30 min at 18°C. After induction of protein expression using 0.3 mM IPTG, bacteria were grown overnight at 18°C. Purification on TALON (Clonetech, Palo Alto, CA, USA) was as described by the manufacturers. For binding assays, *Xenopus* TPX2 was further purified on a Mono S column (Pharmacia). Importin α and the ED mutant of importin α were expressed and purified as described

(Gorlich *et al*., 1994; Gruss *et al*., 2001). RanQ69L was expressed, purified and loaded with GTP as described (Weis *et al*., 1996).

Preparation of chromatin beads

Bluescript plasmid containing a 5-kb insert of non-coding *Drosophila* DNA was linearized, biotinylated and coupled to streptavidin magnetic beads in a reaction containing 50 mM Tris-HCl, pH 8, 1 M NaCl, 2 mM EDTA, and an immobilization activator available from Dynal, Oslo. 0.6 µg beads were incubated in 100 µl interphase extract for 2 hours at 20°C before addition of 0.5 volumes of fresh mitotic extract. After an additional 30 min incubation, beads were retrieved on ice and resuspended in 200µl fresh mitotic extract containing 0.2 mg/ml fluorochrome-labelled tubulin.

TPX2-importin α binding assay

900 pmol of bacterially expressed zz-tagged *Xenopus* TPX2 was prebound to 10 µl IgG Sepharose in buffer A (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 5 mM $MgCl_2$, 10 mM β–mercaptoethanol, 0.01% Triton X-100) for 1 hr at 23 ºC. Beads were washed 5 times with 500 µl buffer A and 150 pmol importin α was added and incubated for 1 hr at 23ºC. After binding, beads were washed as before and bound protein eluted with 100 µl 1M $MgCl_2$, 50 mM Tris-HCl (pH 7.5) precipitated with acetone and separated by SDS-PAGE (Laemmli, 1970) followed by Coomassie blue staining.

Assays for *in vitro* microtubule assembly

Porcine tubulin was purified and labelled with tetramethylrhodamine or FITC (Hyman *et al*., 1991; Mitchison and Kirschner, 1984b). Microtubule assembly

reactions consisted of 9 µl BRB80 (80 mM Pipes, 1 mM K-EGTA, 1 mM $MgCl_2$, pH 6.8) and 5, 10, 15 or 20 µM tubulin containing 10% rhodamine-labelled tubulin and 1 mM GTP. Samples were supplemented with either buffer or TPX2 protein at the concentration specified in the text, incubated for 12 min at 37 ^{o}C and fixed, spun down and analysed as described (Wittmann *et al*., 1998).

For importin α/β inhibition of TPX2 induced microtubule nucleation, importin α or the ED mutant was added to 1 µM together with 1 µM importin β to the nucleation assay that was processed as described above at room temperature to prevent protein precipitation. Numbers of aggregates with or without associated microtubules in a given field were counted. Error bars are standard errors of the mean.

To test the bundling activity of TPX2, microtubules were first polymerised from a solution of 40 µM tubulin in BRB80 in the presence of 10 µM Taxol (paclitaxel, Sigma) and 1 mM GTP for 10 minutes at 37°C. Microtubules were then diluted 1:40 in BRB80, 10 µM Taxol, 1 mM GTP and further incubated for 10 minutes at 37°C. To exclude effects on microtubule polymerization, the diluted microtubules were put on ice, mixed with buffer or with 0.2 µM TPX2 or 0.2 µM TPX2 and both 5 µM importin α and β, incubated for 5 minutes on ice and fixed and processed as described (Wittmann *et al*., 1998).

Microtubule co-pelleting assay

0.8 µM of purified recombinant TPX2 protein was incubated for 30 min with or without 10 µM both importin α and β at 37°C in 50 µl of BRB80 containing 4 mM $MgCl_2$, 4 mM ATP, 4 mM GTP, 0.4 µg/µl porcine brain tubulin, 100 mM NaCl and 20 µM Taxol. After centrifugation at 37°C on a BRB80 sucrose cushion (BRB80, 10% sucrose, 10 µM Taxol, 2 mM GTP) at 100,000 g for 20 minutes, the pellets were resuspended in SDS sample buffer (Laemmli, 1970), and separated by

SDS-PAGE followed by Western blotting probing for the protein A tag with secondary antibody (Giet and Prigent, 2001).

Electron Microscopy

Recombinant TPX2 (0.8 µM) was incubated with purified 5 µM porcine brain tubulin for 10 min at 37 °C in BRB80 containing 5 mM $MgCl_2$. Reactions were spotted on copper grids for electron microscopy, washed twice with BRB80 and shock-frozen as described (Dubochet *et al*., 1985). To visualise zz-tagged TPX2, the reactions were performed in the presence of 5 nm gold-labelled goat anti-mouse antibodies (BBI international, 1:10 dilution). Images were taken on a CM-120 (Biotwin) electron microscope.

Western blotting and antibodies

Proteins were separated by SDS-PAGE (Laemmli, 1970), transferred to nitrocellulose and decorated with antibodies against human TPX2 (Gruss *et al*., 2002), which were visualised with secondary antibodies coupled to horseradish peroxidase (Amersham).

Activation and re-purification of sperm centrosomes

Sperm nuclei (3.3 µl, 2 x 10^7 per ml) were incubated for 30 min at room temperature in 25 µl of precleared (4°C, 10,000g, 9 min) *Xenopus* M-phase extract containing 10 µM nocodazole together with buffer, 30 µM Ran Q69L GTP or 10 µM Ran BP1 and 10 µM Ran GAP. Sperm were re-purified by diluting the sperm-extract mix with three volumes of SuNaSp buffer (50 mM HEPES pH 7.5, 250 mM sucrose, 75 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine and 10

µg/ml protease inhibitors (leupeptide, pepstatin, aprotinin, phenylmethylsulphonyl fluoride), layering the mix on top of 1 ml of 25% glycerol-SuNaSp and centrifuging at 4°C for 3 min at 18,900g. Re-purified sperm centrosomes were resuspended in 6 µl of 25% glycerol-SuNaSp, and 1.2 µl of re-purified sperm were then added to 9 µl of 20 µM tubulin containing 10% rhodamine-labelled tubulin and 1 mM GTP (Sigma); the mix was then incubated at 37°C for 10 min. Samples were fixed and spun as described above. Images were acquired using a confocal microscope. Aster fluorescence was determined by determining the total aster grey-level intensity and subtracting the backround fluorescence using a specifically written macro for the program Scion image 1.62a (Carazo-Salas *et al*., 2001) Total aster fluorescence was considered as an estimate of microtubule mass.

Drosophila embryo injection

Embryos (0-1 hr) were devitellinized manually and microinjected as described previously (Su *et al*., 1998), with 5 mg/ml peptide in PBS. Estimated injection volume was between 2% and 5% of the total volume of the embryo. Injected embryos were aged at 18°C for 30 min previously (Su *et al*., 1998). Ran wildtype or mutants was injected at 6 mg/ml in PBS.