The role of importin-β in nuclear envelope assembly

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Publications

I. Gyula Timinszky*, László Tirián*, Ferenc T. Nagy, Gábor Tóth, András Perczel, Zsuzsanna Kiss-László, Imre Boros, Paul R. Clarke and János Szabad, The importin-β P446L dominant negative mutant protein loses RanGTP binding ability and blocks the formation of intact nuclear envelope.
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Abbreviations

APA: aster promoting activity

BSA: bovine serum albumin

DHCC: 3,3'-dihexyloxacarbocyanine iodide

EMS: ethyl methane sulfonate

Fs mutation: dominant female sterile mutation

Gal4: yeast derived transcription activator binding to UAS

GFP: green fluorescent protein

GST: glutathione S-tranferase

I178D importin-β: mutant importin-β deficient in nucleoporin binding

IBB: importin-β binding domain of importin-α

mAb414: monoclonal anti-nucleoporin antibody

MAP: microtubule associated protein

MT: microtubule

NE: nuclear envelope

NES: nuclear export signal

NLS: nuclear localization signal

NPC: nuclear pore complex

NTF2: nuclear translocation factor 2

Nup: nucleoporin

P446L-imp-β: P446L mutant Drosophila importin-β

RanBP1: Ran binding protein 1

RanGAP: Ran GTPase activating protein

RanQ69L: GTPase deficient form of Ran

RCC1: regulator of chromatin condensation 1

TRITC: Tertramethylrhodamine isothiocyanate Isomer R

UAS: upstream activator sequence

Summary

Three of the four independently induced *Ketel*^{D} dominant negative female sterile mutations, that identify the Drosophila importin-β gene, originated due to $C^{4114} \rightarrow T$ transition and the concurrent replacement of proline^{446} by leucine (P446L-imp-β). The above-mentioned *Ketel*^{D} mutations abolish RanGTP binding of P446L-imp-β and result in increased RanGDP binding ability. P446L-imp-β does not exert its dominant negative effect on nuclear protein import and has basically no effect on mitotic spindle-related functions and chromosome segregation. However, it interferes with nuclear envelope (NE) assembly during the mitosis-to-interphase transition, revealing a novel function of importin-β. Along elucidating the mode of action of P446L-imp-β we studied *in vitro* NE assembly on Sepharose beads. While Drosophila embryo extracts support NE assembly over Sepharose beads coated with Ran, NE assembly – like in the *Ketel*^{D} eggs – does not take place in extracts supplied with exogenous P446L-imp-β. NE also forms over importin-β coated beads. Surprisingly, when immobilized to Sepharose beads P446L-imp-β as efficiently recruit NE vesicles as normal importin-β. The discrepancy in behavior of cytoplasmic and bead bound P446L-imp-β appears to be related to its increased microtubule (MT) binding ability as compared to normal importin-β. While wild type importin-β is able to bind to MTs and the binding ceases upon RanGTP interaction, P446L-imp-β cannot be removed from the MTs by RanGTP. P446L-imp-β, like normal importin-β, binds some types of the nucleoporins that have been known to be required for NE assembly at the end of mitosis. It appears that the inhibitory effect of P446L-imp-β on NE assembly is caused by sequestering to the MTs some of the nuclear pore complex (NPC) proteins (nucleoporins) required for NE assembly.

Introduction

Maternal effect genes

Before the commencement of embryogenesis in the animal kingdom, the egg cytoplasm contains all the components essential for early embryogenesis. The rate of zygotic gene expression during early embryogenesis varies from species to species but there must be a stockpile of mRNAs and proteins present in the egg for proper start of embryogenesis. The majority of the embryogenesis controlling factors are deposited into the egg cytoplasm during oogenesis, and their synthesis is apparently under control of the maternal genome. The maternal genes engaged in the initiation of embryogenesis represent the maternal-effect genes, and the phenomenon that maternally-provided molecules control early embryogenesis has been known as maternal effect.

Genetic dissection – gene identification and characterization through mutations – has been a useful approach to identify genes responsible for maternal effect. Genetic dissection implies first the identification and understanding of the function of single genes and the subsequent reconstruction of the process in which the gene plays role. Mutations that block the initiation of embryogenesis are likely to identify genes with functions required during the commencement of embryogenesis. However, the true maternal effect genes are rare: it is rather common that early embryogenesis is governed by genes that have both maternal and zygotic functions (Szabad, 1998). The complete loss-of-function (amorph) mutations in genes with both maternal and zygotic functions result in zygotic lethality and hence do not allow readily analysis of the maternal function.

Genes with both early embryonic and zygotic functions may be identified by dominant female sterile (*Fs*) mutations that although disrupt maternal-effect, permit zygotic functions, i.e. females carrying an *Fs* mutation develop to normal but sterile adults. To study the role of maternal control of early embryogenesis, our group used *Fs* mutations which allow the formation of normal-looking eggs but arrest development at or shortly after fertilization (Erdélyi and Szabad, 1989; Szabad et al., 1989).

About 40% of the EMS-induced *Fs* mutations isolated in our laboratory identify genes with essential functions during the commencement of Drosophila embryogenesis (Erdélyi and Szabad, 1989; Szabad et al., 1989). *Ketel*^{D} is one of the *Fs* identified genes and, as it was

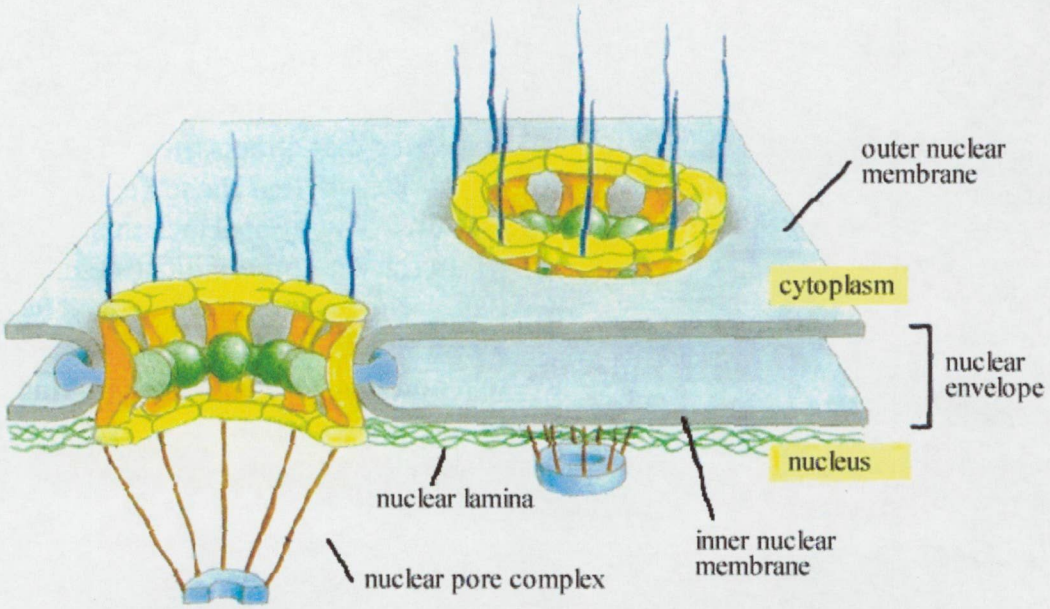

Figure 1. Scematic drawing of the nuclear envelope and the nuclear pore complex. (Adapted from Alberts et al., 1998.)

shown, it encodes the Drosophila homologue of importin-β (Lippai et al., 2000; Tirián et al., 2000). Importin-β is a component of the nuclear protein import machinery (reviewed by Görlich and Kutay, 1999).

The nucleus and nuclear protein import

The defining feature of the eukaryotic cell is that their chromosomes are enclosed inside the nucleus by the NE (Figure 1). The NE is made up of two parallel membranes the outer of which is the continuation of the endoplasmic reticulum. Beneath the internal membrane, there is a meshwork of proteins called the nuclear lamina that has functions in replication, transcription and nuclear organization. The mRNAs have to pass the NE to reach the cytoplasmic ribosomes thus they have to be exported from the nucleus, while several proteins including those engaged in replication, gene expression regulation or support of chromatin structure have to be imported into the nucleus. The gates that are responsible for the immense transport across the NE are the NPCs (Figure 1.). NPCs are the largest known protein complexes within the cell. The NPCs are composed from more than 20 different NPC proteins also known as nucleoporins. The NPC has an estimated mass of 125 MDa. It is a cylindrical structure embedded in the NE and is about 120 nm wide and about 40 nm long. It has an

about 40 nm wide inner channel which contains a proteinaceous material referred to as the 'central plug' (Burke and Ellenberg, 2002). The central plug is responsible for the selectivity of the transport across the NE: smaller than 30-40 kDa molecules can cross the NPCs by free diffusion. For larger molecules the NPCs are impassable unless they carry a nuclear localization signal (NLS) that is a short sequence of basic amino acids required for entry of the nucleus or a nuclear export signal (NES) required for exit from the nucleus. The NLS and NES are recognized by different transportins that are able to actively transport the molecules across the NE. Importin-β is such a transportin involved in the "classical" import of nuclear proteins (Görlich and Kutay, 1999).

During a "classical" import cycle, importin-β interacts – directly or through an importin-α adapter molecule – with the NLS containing nuclear protein (cargo) in the cytoplasm (Figure 2). After formation of the import complex it docks on the cytoplasmic side of an NPC. During translocation through the NPC, importin-β interacts with a number of nucleoporins. Import of the NLS-containing nuclear protein is completed on the nuclear surface of the NPC, where the import complex disassembles due to the interaction of importin-β with RanGTP, a small nuclear, Ras-related G protein (Azuma and Dasso, 2000). While the nuclear protein stays inside the nucleus, the importin-β-RanGTP complex returns to the cytoplasm where importin-β is released from RanGTP following an interaction of RanGTP with RanGAP (RanGTP-ase activating protein) and RanBP1 (Ran binding protein; Bischoff and Görlich, 1997; Azuma and Dasso, 2000). Upon activity of RanGAP and RanBP1, RanGTP is converted to RanGDP that is subsequently imported to the nucleus where it is converted to RanGTP by RCC1 (regulator of chromatin

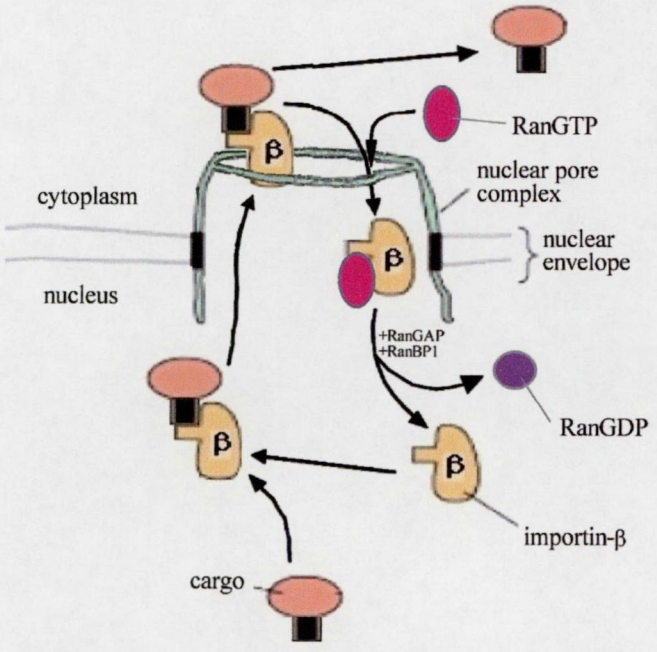

Figure 2. Schematic illustration of a nuclear protein import cycle. (See text for details.)

condensation), the only known guanine nucleotide exchange factor for Ran (Figure 2) (Renault et al., 2001). The driving force of the nuclear transport process is Ran: while its GDP-bound form is prevalently cytoplasmic, RanGTP is basically nuclear. Existence of the different Ran forms in the cytoplasm and inside the nucleus is the consequence of the nuclear localization of RCC1 and the cytoplasmic localization of RanGAP and RanBP1 (Görlich and Kutay, 1999).

The *Ketel*^{D} phenotype

Although the so-called *Ketel*^{D} eggs (that are deposited by the *Ketel*^{D}/+ females) appear normal and are fertilized, cleavage nuclei do not form inside. The *Ketel*^{D} egg cytoplasm is very toxic: when injected into wild type cleavage embryos it hinders formation of cleavage nuclei (Tirián et al., 2000). Surprisingly however, when injected into wild type cleavage embryos the *Ketel*^{D} egg cytoplasm does not prevent nuclear protein import. Furthermore, nuclear proteins are imported into nuclei of digitonin-permeabilized HeLa cells in the presence of ovary extracts of the *Ketel*^{D}/+ females (Lippai et al., 2000).

Thus the *Ketel*^{D} gene product, although very toxic, does not block nuclear protein import but interferes with some mitotic event. Since the *Ketel*^{D} mutations are of dominant negative nature (implying that the mutant gene product interferes with the wild type gene product and that they act in the same process; Tirián et al., 2000). The block of a mitotic process by *Ketel*^{D} implies that importin-β is involved in mitosis.

The stages of mitosis

Mitosis is the most dynamic cellular event that requires the perfect orchestration of several sequential events (for a review see Burke and Ellenberg, 2002). There are distinct steps through mitosis as was revealed by light microscopy. One of the first visible changes is the condensation of chromatin during the prophase of mitosis. The NE that encloses the chromosomes is a basic problem for cell division because the MTs remain cytoplasmic throughout the cell cycle. To achieve segregation of the chromosomes the MTs have to either get inside the nucleus (that happens e.g. in yeast) or the NE has to break down such that the chromosomes will be accessible for the MT-composed spindle apparatus, as it happens in most of the multicellular organisms. NE breakdown is preceded by phosphorylation of NE

proteins by the M-phase promoting factor. Phosphorylation abolishes protein-protein interactions and leads to the disassembly of the NE. The nuclear lamins, constituents of the nuclear lamina, are also phosphorylated, the nuclear lamina disassembles and the nuclear lamins diffuse into the cytoplasm. At the same time disassembly of NPCs takes place as well. The MTs tear down the NE and with the first holes appearing in the NE, the cytoplasmic molecules enter the nucleus (Beaudouin et al., 2002). NE breakdown labels the transition from prophase to prometaphase. Much of the NE components become part of the mitotic endoplasmic reticulum (reviewed in Burke and Ellenberg, 2002).

As soon as the NE breaks down, MTs form the mitotic spindle and – along with the motor proteins – align the chromosomes into the equatorial plane in metaphase. In the meanwhile, both Ran and importin-β were shown to be involved in mitotic spindle assembly (Wiese et al., 2001; Nachury et al., 2001; Gruss et al., 2001). There are several proteins with – as reffered to aster promoting activities (APA) – that are responsible for proper spindle organization. Importin-β participates in the process of spindle assembly by binding the soluble APAs and depositing them on the MTs near the chromatin (Figure 3). It has been shown that GTP bound Ran associated with the chromatin is responsible for detaching the APAs from importin-β just like in the case of nuclear protein import. By detaching APAs from importin-β, APAs can initiate and promote spindle assembly in the vicinity of chromatin.

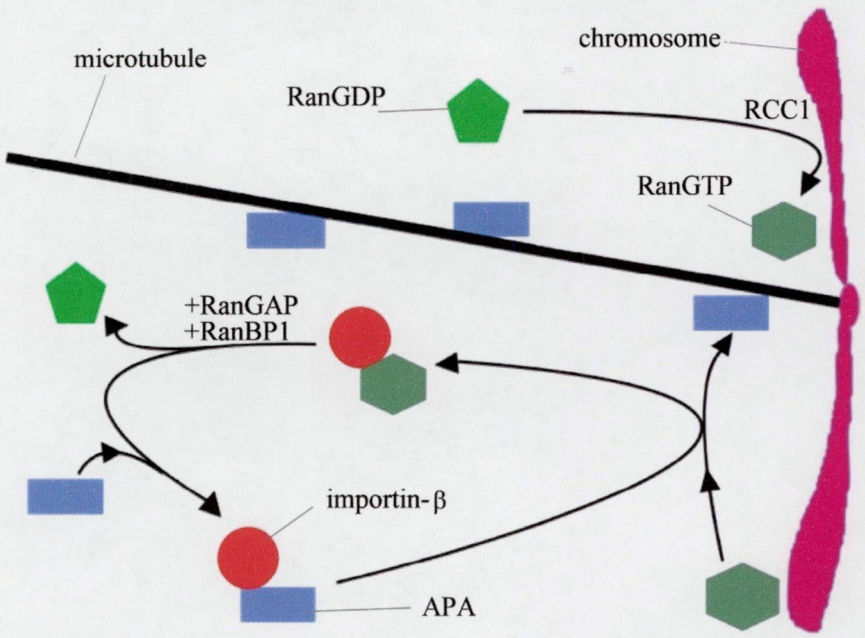

Figure 3. The schematic drawing of mitotic spindle assembly. (See text for details.)

In anaphase, the chromosomes are segregated to the two opposite poles in the cell and the mitotic spindle disassembles.

NE reassembly that has also been shown to be a Ran dependent process starts in late

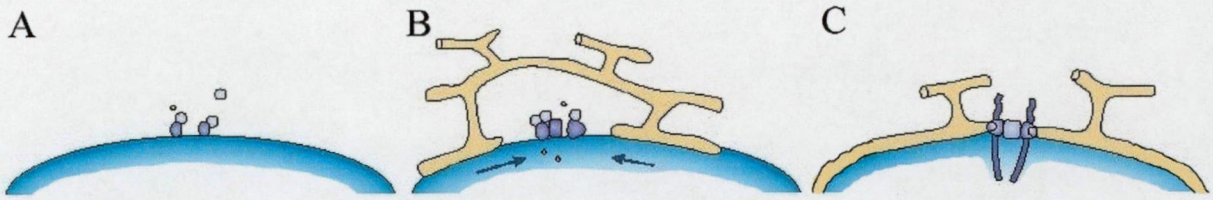

Figure 4. The schematic drawing of NE assembly. Nucleoporins are grey, chromatin is blue, endoplasmic reticulum is yellow. See text for details. (Adapted from Burke and Ellenberg, 2002)

anaphase. Nucleoporins and NE components bind the chromosomes (Figure 4A); the NE components flatten (Figure 4B) and fuse to form a continuous NE over the chromosomes (Figure 4C). Ran is required for the NE vesicle binding to chromatin and the fusion of NE vesicles (Hetzer et al., 2002). (Due to the fractionation techniques to obtain NE components for *in vitro* assays the endoplasmic reticulum fragments and forms vesicles, later in the text NE components are referred as NE vesicles.) The NPCs reassemble and nuclear protein import resumes. The restart of nuclear protein import is responsible for the bloating of the NE to regain its well-known globular form.

It is obvious that Ran is a general regulator of nuclear and chromosome related processes. In interphase RanGTP is within the nucleus and in mitosis RanGTP is concentrated around the chromosomes thus RanGTP is a marker of chromosome position throughout the cell-cycle (Hetzer et al., 2002). It has also been shown that importin-β is not exclusively involved in nuclear protein import but also has further mitotic functions (Wiese et al., 2001; Nachury et al., 2001; Gruss et al., 2001). Our aim was to identify the *Ketel*^{D}-associated mitotic defect: whether it is exerted during mitotic spindle assembly or during some other mitotic event.

Identification of the *Ketel*^{D}-blocked mitotic event

There are four independently isolated *Ketel*^{D} alleles and three of those carry the same single $C^{4114}\rightarrow T$ transition that was proved to be responsible for the *Ketel*^{D}-associated mutant phenotype (Timinszky et al., 2002). The consequence of the $C^{4114}\rightarrow T$ transition is the replacement of proline^{446} by leucine. The mutant importin-β with the proline^{446} to leucine change is called P446L-importin-β (P446L-imp-β).

To elucidate the mitotic event that the P446L-imp-β blocks, bacterially expressed, purified P446L-imp-β protein was injected into wild type cleavage embryos.

Sophisticated techniques have been developed to follow cell components through mitosis

after the discovery of the green fluorescent protein (GFP) (for reviews see Prasher, 1995; Matz et al., 2002). GFP is a naturally fluorescent protein that emits green light when excited with long-wave ultraviolet or with blue light. GFP was identified in the jellyfish *Aequoria victoria* and is responsible for the green bioluminescence when the jellyfish is mechanically stimulated. GFP can be fused to proteins (when so-called chimeric proteins are generated) like tubulin, histone, nuclear lamin, centrosomin or to targeting signals like NLS to label specific structures within the cells. The GFP tagged proteins are encoded by transgenes and can be examined in living cells using fluorescent or laser-scanning microscopes.

Results of the experiments described in the present dissertation show that P446L-imp-β does not exert its dominant negative effect through (i) blocking nuclear protein import in cleavage Drosophila embryos, or (ii) through preventing mitotic spindle formation and chromosome segregation. It appears that P446L-imp-β induces death of the Drosophila embryos by preventing NE reassembly at the end of mitosis. Because the P446L-imp-β blocks mitotic event through the block of NE reassembly, and because the *Ketel*^{D} alleles are dominant negative, the wild type importin-β must be involved in NE reassembly.

P446L-imp-β sequesters NE assembly factors to the MTs

NE assembly can be directly studied *in vitro* using Ran coated Sepharose beads. If the Ran coated beads are incubated in Xenopus egg extracts functional NE forms over the beads (Zhang and Clarke, 2000). We show that NE assembles over the Ran coated beads when incubated in wild type Drosophila egg extracts and also that P446L-imp-β blocks NE assembly over Ran coated Sepharose beads. Although, P446L-imp-β can recruit NE vesicles and some Nup's when immobilized to Sepharose beads.

It has also been established that P446L-imp-β – though the amino acid replacement resides in the presumptive nucleoporin and importin-α binding domain of importin-β (Timinszky et al., 2002) – interacts with both importin-α and nucleoporins. But unlike normal importin-β, P446L-imp-β cannot bind RanGTP (Timinszky et al., 2002). It appears that the inhibitory effect of P446L-imp-β on NE assembly stems from its altered RanGTP binding ability. We published evidence that wild type importin-β can bind the MTs both directly and indirectly through binding some types of the MT associated proteins (MAPs) (Tirián et al., 2003). MT binding in both cases decreases upon importin-β/RanGTP interaction. P446L-imp-β binds

MTs with higher affinity than normal importin-β and because P446L-imp-β does not bind RanGTP it remains attached to MTs and sequesters some types of the Nup's required for NE assembly.

Materials and Methods

Production of the *Ketel*^{D}-encoded protein in bacteria

Normal importin-β was produced as described in Lippai et al., 2000. For production of the P446L-imp-β protein in *E. coli*, the expression vector containing the *Ketel* cDNA with the P446L mutation was generated by replacing of the *Bgl*II – *Cla*I section in the pQE30 expression vector containing the wild type *Ketel* cDNA (the *Bgl*II – *Cla*I section includes the $C^{4114}\rightarrow T$ transition). Construction of the pQE30 expression vector with the wild type *Ketel* cDNA was described in Lippai et al., 2000.

The expressed proteins included an NH_2-terminal His tag that allowed purification on a nickel-NTA agarose column (Qiagen). Unlike the normal importin-β, over 95% of the P446L-imp-β protein were present in inclusion bodies. To produce functional P446L-imp-β, we dissolved the inclusion bodies in 6 M guanidine hydrochloride in TNM buffer (50 mM Tris pH=7.5, 300 mM NaCl, 5 mM $MgCl_2$ and 5% glycerol) as used during purification of the normal importin-β. Renaturation of P446L-imp-β was achieved through a 6 to 0 M decreasing guanidine gradient in TNM buffer and eluted with 0 to 0.5 M imidazole gradient in TNM buffer. The eluted protein was dialyzed against 0.1xTNM buffer overnight. Two hundred µl aliquots of the dialyzed protein solution were lyophilized and stored at –70°C. When used the aliquots were dissolved in 20 µl H_2O. To test biological activities of the purified P446L-imp-β we (i) injected it into wild type, histone-GFP or lamin-GFP expressing cleavage embryos, (ii) used in the nuclear import assay system with digitonin-permeabilized HeLa cells and (iii) used in solutions to study importin-β–Ran interactions.

Injections into cleavage embryos

To visualize effects of the P446L-imp-β molecules on cleavage embryos, we carried out the following injection experiments. (1) We injected into wild type embryos ca. 200 pl/egg (~ 2% total egg volume) from a solution that contained wild type or P446L-imp-β (1.2 µM) and a fluorescent nuclear protein import substrate (0.24 µM). The fluorescent substrate was a pentamer of a fusion protein in which the nucleoplasmin core domain was combined with the importin-β-binding domain from importin-α (IBB core pentamer; Lippai et al., 2000). (2) The wild type or P446L-imp-β solutions were injected into embryos in which the chromatin was

labeled by histone-GFP protein (Clarkson and Saint, 1999). (3) The wild type or P446L-imp-β solutions were co-injected with a 1% solution of the 170 kDa red fluorescent TRITC (Tertramethylrhodamine isothiocyanate Isomer R)-dextrane. (4) Wild type or P446L-imp-β was injected into cleavage embryos in which an UAS-tubulin-GFP construct (Grieder et al., 2000) was driven by a nanos-Gal4 driver. (For a review on the UAS-Gal4 system see Duffy, 2002.) (5) The wild type or P446L-imp-β solution (1.2 μM) was also injected into cleavage embryos in which a UAS-lamin-GFP construct was driven by a nanos-GAL4-VP16 driver (Van Doren et al., 1998). (The UAS-lamin-GFP coding transgene was kindly provided by Dr. N Stuurman; see the FlyBase website http://flybase.bio.indiana.edu). Following injections, fate of the injected embryos was followed through optical sections in a ZEISS LSM410 confocal microscope. The injections were done on 20°C.

Nuclear protein import assay

The nuclear protein import assay was conducted as follows. Digitonin-permeabilized HeLa cells were prepared by a modified protocol (Adam et al., 1990). Briefly, HeLa cells were grown on coverslips to 50-80% confluence, washed in ice-cold permeabilization buffer (20 mM HEPES-KOH pH 7.5, 110 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose, 0.5 mM EGTA) and permeabilized for 15 minutes in the same buffer containing 60 μg/ml digitonin. The coverslips were washed three times in permeabilization buffer without digitonin. Coverslips were incubated with each 20 μl of import reaction. The import buffer contained 2 mg/ml nucleoplasmin core (to block non-specific binding), 20 mM Hepes/KOH pH 7.5, 140 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose, 0.5 mM EGTA. Where indicated, reactions were supplemented with an energy regenerating system (0.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, 50 μg/ml creatine kinase) and Ran mix [3 μM Ran-GDP, 150 nM RanGAP (= Rna1p from yeast), 300 nM NTF2, 150 nM RanBP1]. Nuclear import of the IBB core pentamer was monitored in optical sections. Import reaction samples contained 0.24 μM fluorescein-labeled IBB core pentamer and 1.2 μM wild type or P446L-imp-β protein, and – where indicated – Ran mix and an energy regenerating system were added. Reactions were stopped after 5 minutes by fixation in 3% paraformaldehyde (w/v) in PBS, washed in PBS, water, and mounted with 2 μl of vectorshield mounting medium (Vector).

Importin-β and tubulin were detected in digitonin-permeabilized HeLa cells - following a 3% paraformaldehyde fixation - by affinity purified anti-Ketel primary antibody and an anti-tubulin monoclonal antibody DM 1A (Sigma) and FITC and Texas Red-conjugated secondary antibodies (Jackson Laboratories), respectively.

Binding assays and immunoprecipitations

Binding assays were carried out as described in Hughes et al., 1998. Briefly, the Sepharose beads covered with GST-Ran loaded with GDP or GST-RanQ69L (a RanGTP frozen form of Ran) loaded with GTP were incubated with wild type and *Ketel*^{D} egg extracts as well as with bacterially expressed wild type and P446L-imp-β proteins. The beads were recovered, washed, the bound proteins were separated by SDS–PAGE and immunostained using affinity-purified anti-Ketel antibody (Lippai et al., 2000). The anti-Ketel antibody is equally efficient in recognizing the wild type and the P446L-imp-β proteins. The concentration of wild type and P446L-imp-β proteins was 0,3 μM. Protein concentrations of the egg extracts were adjusted to 18 mg/ml.

For immunoprecipitations we incubated Protein-A-agarose beads with the polyclonal anti-Ketel antibody and after washing the anti-Ketel beads were given to wild type or *Ketel*^{D} egg extracts. When indicated an energy regenerating system (0.5 mM ATP, 0.5 mM GTP, 20 mM creatine phosphate, 100 μg/ml creatine kinase) and 3 μM of wild type or P446L-imp-β were added. The precipitated Ran was detected by Western blot.

Enzymatic assays

Labeling of Ran with [γ-^{32}P]GTP and GTPase assays were performed essentially as described in Görlich et al., 1996. Concentrations of the proteins were as follows: Ran 0.3 μM, wild type and P446L-imp-β 1 μM, RanBP1 0.4 μM, Drosophila RanGAP 25 nM. Hydrolysis of Ran[γ-^{32}P]GTP to RanGDP and the release of [γ-^{32}P] was measured in a liquid scintillation counter 2 minutes after bringing the components together. For measuring of nucleotide exchange activity of RCC1 on Ran human Ran protein was loaded with [^{3}H]GTP or [^{3}H]GDP. Protein concentrations in the reactions were as follows: Ran 0.3 μM, wild type and P446L-imp-β 1 μM, RCC1 30 nM. The exchange of labeled GTP or GDP to unlabeled GDP was measured 2, 3 and 4 minutes in a liquid scintillation counter after the components were

brought together.

In vitro nuclear envelope assembly

NE assembly around (i) RanGDP-loaded glutathione-Sepharose beads or (ii) Protein-A-Sepharose beads loaded with anti-Ketel antibody was monitored after staining with 3,3'-dihexyloxacarbocyanine iodide (DHCC, a fluorescent dye that labels phospholipid membranes) or immunostaining with the mAb414 monoclonal anti-nucleoporin antibody (BAbCO) essentially as described in (Zhang and Clarke, 2000) with the following modifications. Extracts from 0-3 hours old Drosophila embryos were prepared as follows. Eggs were dechorionated in Clorox and thoroughly rinsed in KHM buffer on ice (Zhang and Clarke, 2001). The eggs were either immediately homogenized or frozen in liquid nitrogen. After homogenization the procedure was identical to that described for the isolation of HeLa cell extract (Zhang and Clarke, 2001).

To prepare importin-β and P446L-imp-β coated beads, we loaded Protein-A-Sepharose beads with anti-Ketel antibody by incubating 60 µl of bead in 500 µl KHM solution containing ~50 µg/ml affinity purified anti-Ketel polyclonal antibody for 2 hours at room temperature on a rotating platform. When indicated the anti-Ketel loaded beads were loaded with wild type or with P446L-imp-β by an overnight incubation in 3 µM importin-β solutions. The beads were washed in KHM buffer and stored frozen at –70C°.

Images were taken either in a Zeiss Axiovert fluorescent microscope with a cooled CCD camera or with a Zeiss Axiovert confocal microscope. Images were processed either with Improvision Openlab and Adobe Photoshop software or Zeiss LSM and Corel Photopaint software.

In vitro tubulin binding assay

Microtubule preparation was carried out according to Cullen et al. (1999) with some modifications. To obtain the highest ovary to body ratio, Drosophila whole extracts were prepared from female fruit-flies fed for 3 days to be at the top of their egg laying period. Flies were homogenized in PEM buffer (50mM Pipes pH 6.8, 5 mM EGTA, 5 mM $MgSO_4$) containing a cocktail of protease inhibitors (Bestatine, Leupeptin, Pepstatin in 1 µg/ml working concentration) and 1 mM dithiothreitol. The homogenate was incubated at 0°C for 30

minutes and spun at 120,000 g at 4°C for 30 minutes. A final concentration of 20 μM of taxol (paclitaxel, Sigma) and 10 μM of tubulin and if mentioned a final concentration of 3 μM of RanQ69L was added to the supernatant before incubation at 36°C for 30 minutes to polymerize MTs. The MTs and associated proteins were pelleted by spinning at 80,000 g at 36°C for 30 minutes through a 30% sucrose cushion in PEM buffer.

In an other set of experiments a final concentration of 10 μM of tubulin and 20 μM of taxol (paclitaxel, Sigma) in PEM buffer containing 1 mg/ml BSA (bovine serum albumin) was incubated at 36°C for 30 minutes to polymerize MTs. When mentioned, recombinant proteins were added to the tubulin solution before the incubation period. A final concentration of 0.5 μM of importin-β and 3 μM of RanQ69L or RanGDP was used. The MTs and associated proteins were pelleted by spinning at 80,000 g at 36°C for 30 minutes through a 30% sucrose cushion in PEM buffer.

The supernatant and pellet fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. The proteins were detected by an ECF Western blotting kit (Amersham Pharmacia) using Molecular Probes Typhoon 8600 optical scanner and the bands were analyzed using Molecular Probes ImageQuant 5.2 software.

Results

P446L-imp-β can dock on the NPC but does not support nuclear protein import

As described earlier, the wild type Drosophila importin-β molecules support import of NLS-containing substrates into nuclei of digitonin-permeabilized HeLa cells (Lippai et al., 2000). Interestingly, ovary extracts of the *Ketel*^{D}/+ females that contains both wild type and P446L-imp-β, as efficiently support nuclear protein import as ovary extracts of wild type (+/+) females (Lippai et al., 2000). Two feasible possibilities seem to account for the above phenomena. (i) It well may be that although P446L-imp-β molecules do not participate in nuclear protein import, they do not prevent function of the wild type importin-β molecules to accomplish their function. (ii) Perhaps P446L-imp-β supports nuclear protein import. To determine which of the two possibilities is true we analyzed behavior of P446L-imp-β in the nuclear protein import assay. As illustrated on Figure 4, in presence of only the fluorescent nuclear substrate and importin-β or P446L-imp-β nuclear import complexes form and dock on the cytoplasmic surface of digitonin-permeabilized HeLa cell nuclei (Figure 5A and C). The higher cytoplasmic background in case of P446L-imp-β is most likely the consequence of the altered structure of the P446L-imp-β molecules leading to association of the import cargo/P446L-imp-β with some cytoplasmic structures. In presence of normal importin-β and when further components of nuclear import are added (i.e. Ran,

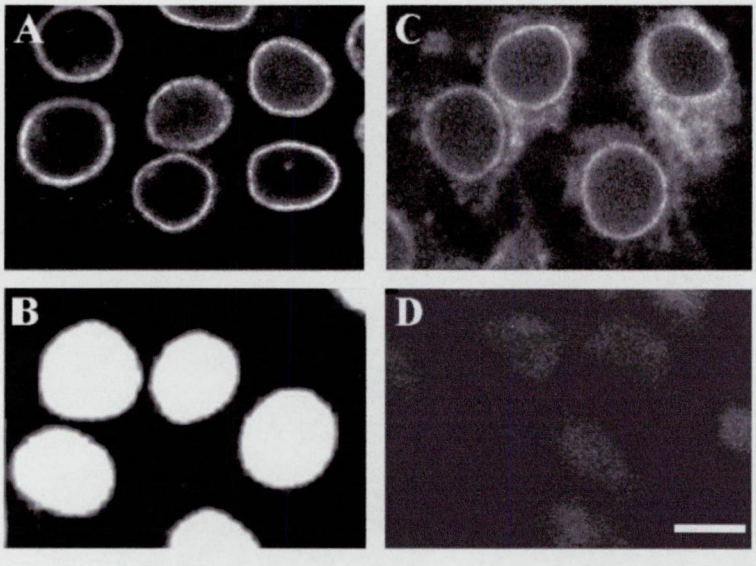

Figure 5. Nuclear import complexes form and dock on the NE of the digitonin-permeabilized HeLa cells following the addition of either wild type (A) or P446L-imp-β (C) in the presence of the fluorescent IBB-nucleoplasmin fusion protein. Upon addition of the import mixture (the fluorescent IBB-nucleoplasmin fusion protein, Ran, NTF2, RanGAP, RanBP1 and energy supply) and wild type importin-β, nuclear import complexes form and enter the nuclei (B). However, when P446L-imp-β is added along with the import mixture, the HeLa cell nuclei are not highlighted by fluorescent signal suggesting that import complexes do not form (D). Scale bar = 10 μm.

NTF2, RanGAP, RanBP1 and energy supply) the nuclear proteins are imported into the nuclei (Figure 5B). In case of P446L-imp-β, however, import complexes do not form (Figure 5D) as revealed by the absence of fluorescent signal in or around the HeLa cell nuclei.

Results of the above experiments show that P446L-imp-β does not support nuclear protein import. However – as the nuclear import assays with ovary extracts of *Ketel*^{D}/+ females revealed (Lippai et al., 2000; Tirián et al., 2000) – it does not hinder nuclear import accomplished by the normal importin-β molecules. Apparently, effects of P446L-imp-β are manifested only during mitosis and the digitonin-permeabilized non dividing HeLa cells are inappropriate tools to reveal mitotic defects (Timinszky et al., 2002).

P446L-imp-β loses affinity to RanGTP but binds RanGDP

An altered Ran binding ability of P446L-imp-β is suggested based on the fact that the P446L-imp-β molecules are unable to accomplish nuclear protein import in the digitonin-permeabilized HeLa cells (Figure 5D). To examine the above possibility, we tested the binding of wild type and P446L-imp-β to different GST-Ran fusion proteins in pull down assays (Figure 6A). As the pull down assays revealed, RanGDP binds significantly higher amounts of importin-β from *Ketel*^{D} egg extracts than from wild type Drosophila egg extracts. Since Ran is mainly in its GDP-bound form in cytoplasmic extracts, the above result suggests increased RanGDP binding affinity of P446L-imp-β as compared to wild type importin-β. Conversely, RanQ69L binds higher amounts of importin-β from wild type egg extracts than from *Ketel*^{D} extracts, suggesting reduced binding ability of P446L-imp-β to RanGTP (RanQ69L is a GTP loaded GTPase deficient mutant Ran protein). As negative control we used GST protein alone which showed only background binding levels with both P446L-imp-β and wild type importin-β. (The *Ketel*^{D} egg extracts contain most likely 50% wild type and 50% P446L-imp-β because they are deposited by *Ketel*^{D}/+ females, the extracts are not suitable to examine the RanGTP binding ability of P446L-imp-β.) To confirm the reduced affinity of P446L-imp-β to RanGTP, we measured the amount of the pulled down importin-β from solutions containing purified wild type or P446L-imp-β. As Figure 6A shows wild type importin-β binds strongly to RanQ69L, but P446L-imp-β shows only background binding to RanQ69L.

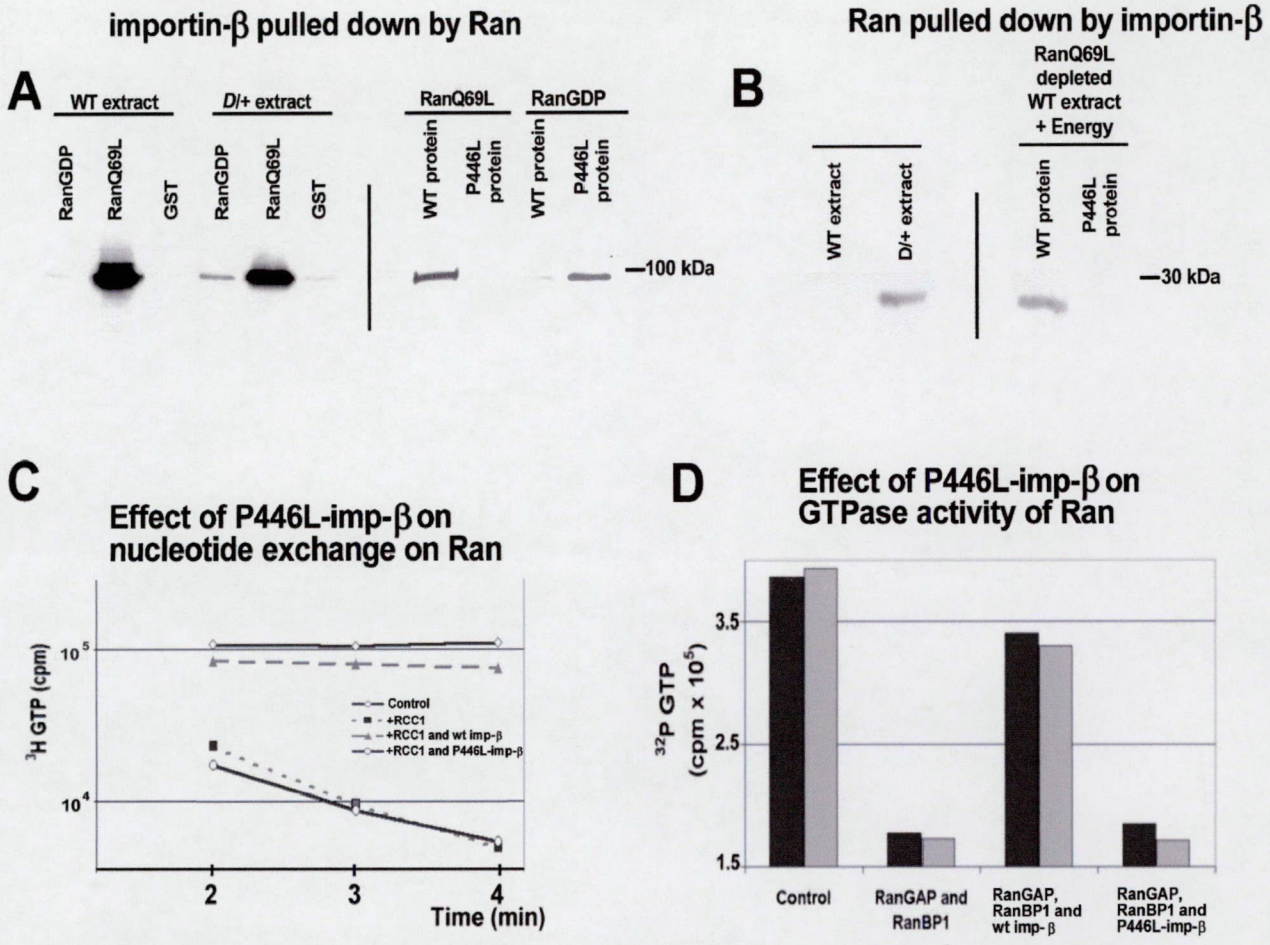

Figure 6. RanGDP removes higher amounts of importin-β from extracts of *Ketel*^{D} eggs than from extracts of wild type eggs (WT). RanQ69L binds higher amounts of importin-β protein from WT egg extract than from extract of *Ketel*^{D} eggs. As negative control GST was used (A, left). RanQ69L removes high amounts of purified WT importin-β but not purified P446L-imp-β. At the same time RanGDP removes higher amounts of purified P446L-imp-β as compared to the purified WT importin-β (A, right). More Ran is precipitated with the anti-Ketel antibody from extracts of the *Ketel*^{D} eggs than from extracts of WT eggs. (B, left). However, if an energy-regenerating system and 3 μM (10 times the endogenous importin-β concentration in the extract) purified wild type or P446L-imp-β is added to WT egg extract, Ran is precipitated from the extract supplemented with WT importin-β (B, right). Wild type importin-β inhibits both exchange of the labeled GTP from Ran (C) and GTP hydrolysis (D), whereas P446L-imp-β has no effect on both nucleotide exchange and GTP hydrolysis. In (C) time course of nucleotide exchange is shown on semi logarithmic scale, and on (D) results of the reactions performed in duplicates.

To further support the altered binding of P446L-imp-β to Ran, we carried out immunoprecipitations with the polyclonal anti-Ketel antibody. The amount of precipitated endogenous Ran was higher from *Ketel*^{D} egg extracts as compared to wild type egg extracts (Figure 6B). However if an energy regenerating system and purified wild type or P446L-imp-β is added to the wild type extract, more Ran is precipitated from the extract supplemented with the wild type importin-β (Figure 6B). The shift in Ran binding ability following energy supply is consistent with the ability of wild type importin-β to bind RanGTP and the inability

of P446L-imp-β to do so. Results of the described experiments are further supported by the enzymatic assays described below.

Importin-β has been known to inhibit both GTP hydrolysis on Ran and the exchange of RanGTP catalyzed by RCC1. We studied, in solutions, the effects of the purified importin-β and P446L-imp-β on nucleotide exchange and GTP hydrolysis on Ran. Wild type importin-β inhibits both GTP nucleotide exchange and GTP hydrolysis (Figure 6B, C), whereas the *Ketel*^{D} encoded P446L-imp-β has no effect on the above mentioned processes (Figure 6C, D) showing that indeed P446L-imp-β can not bind to RanGTP. Neither wild type nor P446L-imp-β has significant effect on nucleotide exchange from RanGDP (data not shown). In conclusion, the failure of functional NE formation may be the consequence of the altered RanGTP binding ability of P446L-imp-β (Timinszky et al., 2002).

P446L-imp-β inhibits formation of intact NE when injected into wild type cleavage embryos

Cytoplasm of the *Ketel*^{D} eggs is exceedingly toxic: when injected into wild type cleavage embryos the *Ketel*^{D} egg cytoplasm prevent formation of nuclei at the end of mitosis (Tirián et al., 2000). To determine whether the P446L-imp-β molecules (produced and purified from *E. coli* cells) possess the same effect as the *Ketel*^{D} egg cytoplasm, we injected small volumes of P446L-imp-β solution along with a fluorescent nuclear substrate into wild type cleavage Drosophila embryos. As illustrated on Figure 7A and D, the nuclear substrate entered the cleavage nuclei irrespectively whether wild type or P446L-imp-β protein solutions were injected. It is important to note that P446L-imp-β did not disrupt the NE. Similarly, ovary extracts of the *Ketel*^{D}/+ females did not block accumulation of the fluorescently-labeled import substrates into digitonin permeabilized HeLa cells, and nuclei remained intact for at least four hours in the presence of P446L-imp-β (Lippai et al. 2000). During the upcoming mitosis the fluorescent substrate was homogeneously distributed in the egg cytoplasm around the site of injection indicating disassembly (that is partial in Drosophila; Foe et al., 1993) of the NE and the concomitant release of the fluorescent substrate into the egg cytoplasm (Figure 7B, E). Following termination of mitosis, in the control the fluorescent substrate entered the newly forming nuclei that doubled in numbers (Figure 7C). In case of P446L-imp-β, however, the fluorescent substrate remained homogeneously distributed at the site of injection (Figure

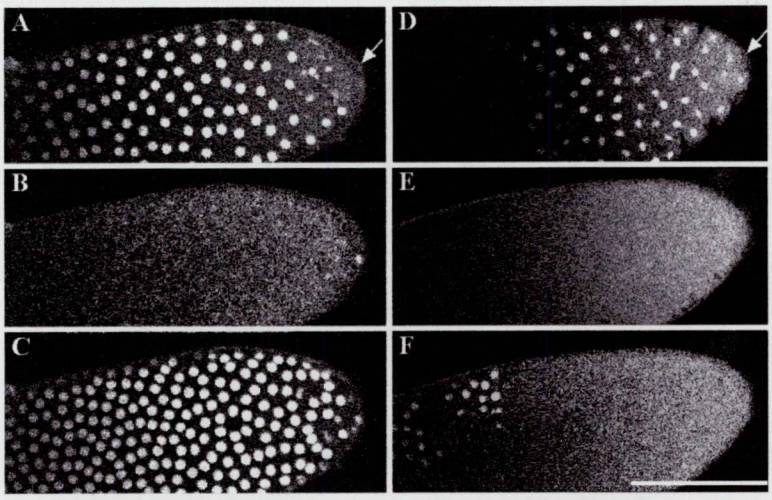

Figure 7. Effects of the wild type (A-C) and the P446L-imp-β (D-F) following their injection – along with a fluorescent nuclear substrate – into wild type cleavage embryos. Arrows show the site of injection. Import of the fluorescent nuclear substrate into the nuclei was followed in a laser-scanning microscope. The (A) and (D), the (B) and (E) and the (C) and (F) photographs were taken at roughly identical stages of the cleavage cycles. The few nuclei shown on (F) appeared following diffusion of the fluorescent substrate away from the site of injection. Scale bar = 100 μm.

7F). Should intact NE form, the high molecular weight fluorescent substrate would be (i) either excluded – in case of the absence of nuclear protein import – from the newly forming nucleus leaving a dark outline of the nucleus or (ii) it is re-imported – in case of resumed nuclear protein import – in which case the nuclei would be highlighted by the fluorescent substrate, as seen in the control experiment (Figure 7C). Since neither of the expected versions occurred, it appears that P446L-imp-β prevents formation of intact cleavage nuclei. Evidently, the P446L-imp-β exerts the same toxic effect on wild type cleavage embryos as the *Ketel*^{D} egg cytoplasm and is very efficient in abolishing function of the wild type importin-β molecules, which observation confirms the dominant negative nature of the *Ketel*^{D} mutations.

One possible explanation for the failure of cleavage nuclei formation is that the P446L-imp-β molecules induced decay of the chromatin around which the NE would have assembled. To clarify the "chromatin decay" possibility, we injected purified P446L-imp-β into the posterior end of cleavage embryos that expressed GFP–tagged histone highlighting the chromatin. Fate of the chromatin was followed through two rounds of cleavage divisions – through optical sections in a laser scanner microscope - in the injected embryos. As time lapse recording of the optical sections revealed, the P446L-imp-β molecules did not hinder the upcoming chromosome segregation (Figure 8D-F) that proceeded the basically the same way as chromosome segregation in the anterior part of the embryo that were free of P446L-imp-β (Figure 8A-C). While chromatin – highlighted by histone-GFP – persists at the posterior end of the embryo and form aggregates, further cleavage cycles are accomplished at the anterior end (data not shown), that acts as "internal control". Results of the histone-GFP experiments

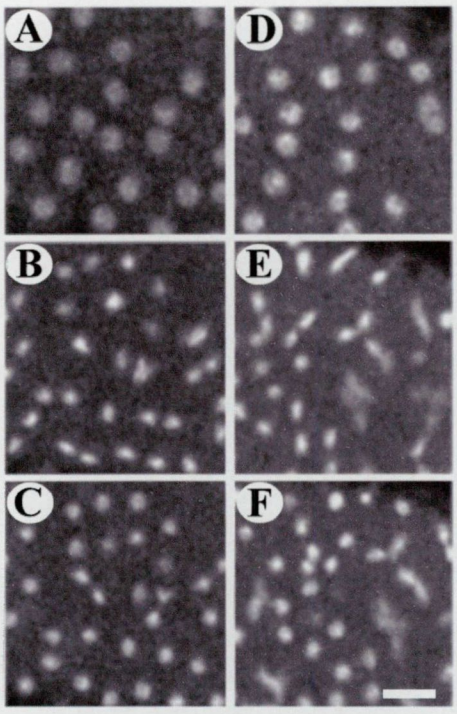

Figure 8. Effects of P446L-imp-β on cleavage chromatin following its injection into wild type cleavage embryos expressing histone-GFP. ~200 pl P446L-imp-β solution (1.2 μM, approximately the endogenous importin-β concentration) was injected into the posterior end of a wild type cleavage embryos in which histone-GFP highlighted chromatin. Chromatin organization was followed in a laser-scanning microscope. Optical sections from the anterior (A-C) and the posterior (D-F) regions of the same embryo are shown. While the anterior section was devoid of P446L-imp-β, P446L-imp-β was present at the posterior region. A and D represent interphase chromatin following P446L protein injection. B and E show segregating chromosomes. C and F show chromatin during the upcoming interphase. Note that the nuclei doubled in number, the chromosomes segregate both at the anterior (control) and at the posterior ("experimental") regions of the embryo. Scale bar = 20 μm.

ruled out the possible decay of chromatin as the reason for the failure of cleavage nuclei formation in the presence of P446L-imp-β. Although chromosomes always segregated, the distance between the chromatin blocks did not grow subsequently, leading to the eventual formation of structures that appeared as chromatin aggregates.

To examine whether the formation of chromatin aggregates is the consequence of persisting or abnormally organized mitotic spindles, we injected wild type and P446L-imp-β into tubulin-GFP expressing Drosophila cleavage embryos and monitored behavior of the mitotic spindles. As Figure 9A-D shows the injection of wild type importin-β has no affect on mitotic spindle formation, shape and disassembly. Following the injection of P446L-imp-β (Figure 9E-H) mitotic spindles form normally, spindle elongation and disassembly are not affected. However, the

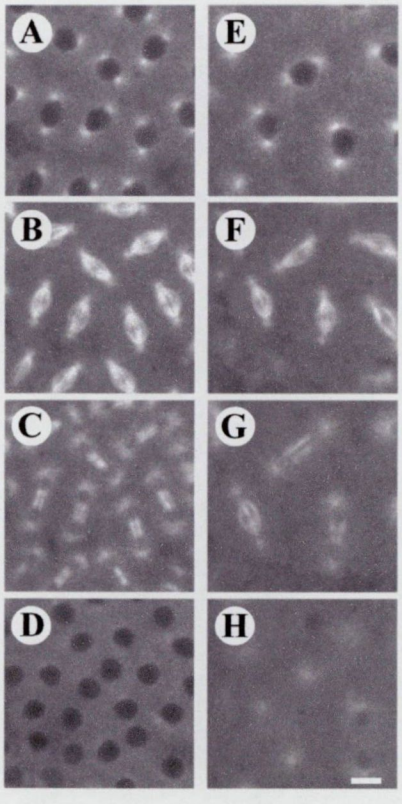

Figure 9. Effect of P446L importin-β on cleavage mitotic spindle organization. Wild type (A – D) or P446L-imp-β (E – H) solution was injected into cleavage Drosophila embryos expressing tubulin-GFP fusion protein. While mitotic spindle assembly, elongation and disassembly is not affected by the injected wild type (A – C) and P446L (E – G) importin-β, the tubulin-GFP protein is homogeneously distributed in the site of P446L-imp-β injection indicating the failure of NE assembly (H). Tubulin-GFP is excluded from the nuclei and appears as dark holes on the optical sections (D). Scale bar = 10 μm.

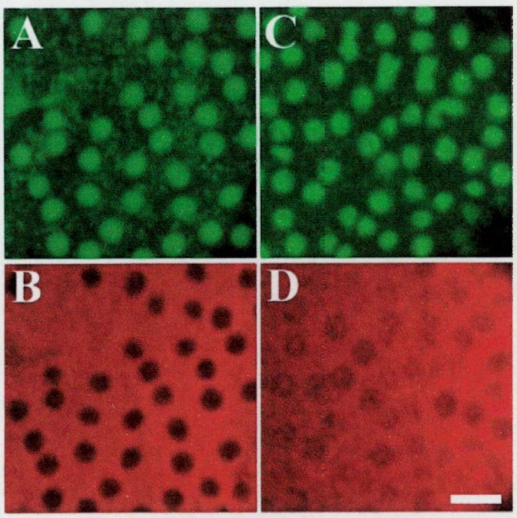

Figure 10. Localization of the chromatin (as revealed by GFP-tagged histone; A and C) and the red-fluorescent 170 kDa TRITC-dextrane (B and D) in cleavage embryos injected with wild type (A and B) or with P446L-imp-β (C and D). Following the injection of wild type importin-β, the TRITC-dextrane is excluded from the nuclei that form following mitosis (B). However, following the injection of P446L-imp-β, the TRITC-dextrane is not excluded from the region of the chromatin following mitosis (D), an indication of the absence of functional NE. The vague contour of the chromatin (D) is due to the chromatin itself excluding some of the TRITC-dextrane. Note that the chromatin morphology is hardly affected (C). Scale bar = 20 μm.

homogenous distribution of the GFP-tubulin (Figure 9H) indicates that NE failed to assemble since GFP-tubulin is not excluded from the space where nuclei should have formed. Failure of the chromosomes to move apart during interphase is most likely the consequence of the failure of NE formation.

To elucidate the possibility of failure of NE assembly, we conduced two further sets of injection experiments. (1) To test whether or not intact NE forms around the chromatin in the presence of P446L-imp-β, we co-injected a high molecular weight red-fluorescent dextrane with P446L-imp-β into histone-GFP expressing cleavage embryos. If intact NE is assembled around the chromatin the dextrane is expected to be excluded from the nuclei that should appear as black holes on the optical sections. If, however functional NE fails to form around the chromatin the dextrane is expected to possess an almost

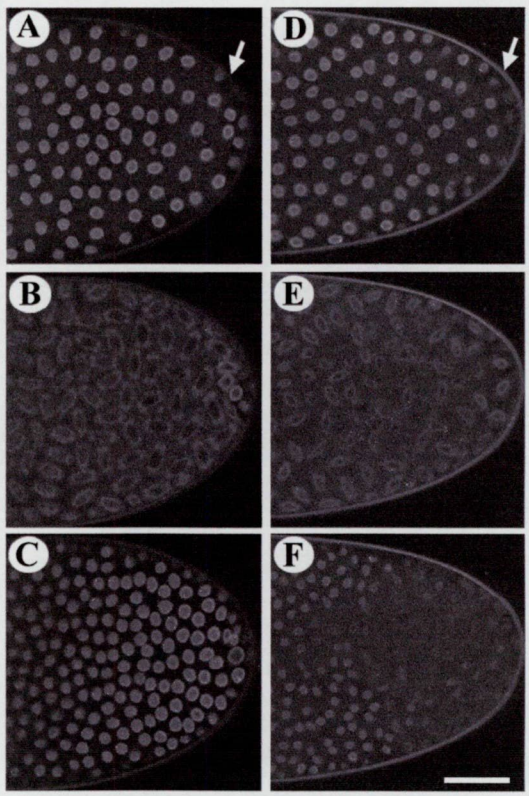

Figure 11. Lamin-GFP expressing cleavage embryos were injected at the posterior end (arrows) with wild type (A-C) or with P446L-imp-β (D-F). A and D show localization of lamin-GFP during interphase after injection. The lamin-GFP molecules highlight the NE. Embryos are in metaphase in B and E, and the spindle envelopes are not affected (B and E). During the upcoming interphase, in the embryos that were injected with normal importin-β nuclear lamina re-forms (C). No nuclear lamina assembles at the site of injection of the P446L-imp-β revealing the failure of intact NE formation (F). Scale bar = 50 μm.

homogenous distribution. Following co-injection of wild type importin-β and the red-fluorescent dextrane, the red and the green (chromatin-derived) signals were clearly separated: the green signal originated from the inside of the interphase cleavage nuclei and the red signal form the cytoplasm showing the formation of cleavage nuclei and hence functional NE (Figure 10A, B). In case of P446L-imp-β and red-fluorescent dextrane co-injections the dextrane-derived signal was basically homogeneously distributed (Figure 10D) even though the histone-GFP highlighted chromatin resembled normal interphase chromatin (compare Figure 10A, C). Results of the above experiment are in agreement with the failure of functional NE formation in presence of the P446L-imp-β.

(2) To visualize the effect of P446L-imp-β induced NE defect, we injected wild type (as control) or P446L-imp-β solutions into cleavage embryos in which lamin-GFP highlighted the lamin lining of the internal NE surface. Most of the lamin is phosphorylated upon the entry into mitosis and the residual lamin-GFP molecules faintly show the so-called spindle envelope. (Cleavage mitosis in Drosophila is an intermediate between closed and open mitosis; Foe et al., 1993) Upon entering the upcoming mitosis the lamin molecules re-enter the nucleus and highlight the NE. It is to be expected that if the P446L-imp-β molecules prevent NE assembly there will be no lamin-GFP signal outlining the NE at the site of injection. As Figure 11A-C show following the injection of wild type importin-β the lamin-GFP molecules highlight the NE during the upcoming interphase. Upon entry to mitosis, most lamin molecules diffuse into the cytoplasm and only some remain attached to the spindle envelope (Paddy et al., 1996; Figure 11B). Following chromosome segregation the NE reassemble as pictured by formation of the green fluorescent lamin lining (Figure 11C). When P446L-imp-β is injected into the lamin-GFP expressing cleavage embryos, the lamin disappears during mitosis as in the control experiment showing that mitosis is not affected until late anaphase (Figure 11D-F). In presence of the P446L-imp-β molecules, however, the lamin lining never re-forms showing the failure of intact NE assembly. The above injection experiments show that P446L-imp-β interfere with the formation of intact cleavage nuclei and the defect is manifested during the mitosis to interphase transition through the prevention of intact NE assembly. Since there are plenty of normal importin-β molecules in the P446L-imp-β embryos and yet effect of P446L-imp-β is deleterious, the *Ketel*^{D} mutations must be dominant negative in action on NE assembly and impede function of the normal importin-β

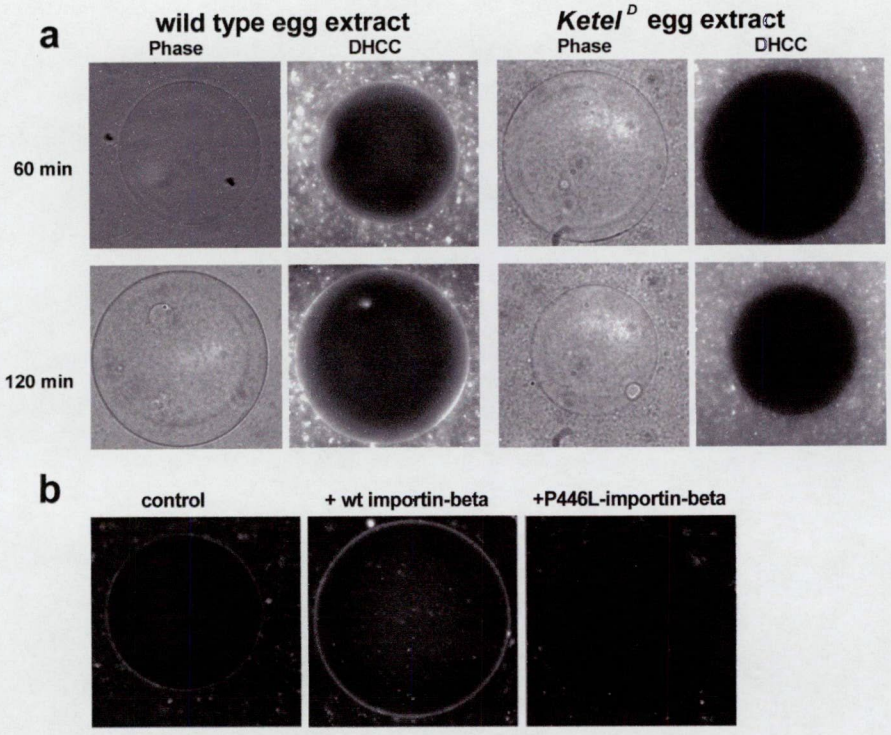

Figure 12. Effect of the *Ketel*^{D}-encoded P446L-imp-β on NE assembly around Ran-coated Sepharose beads. Glutathione-Sepharose beads were loaded with RanGDP and incubated in extract prepared from 0-3 hours old Drosophila embryos. After 60 or 120 minutes of incubation the samples were diluted 20-fold by PBS and stained with the membrane labeling dye DHCC. The beads were examined in either a fluorescent (a) or in a confocal microscope (b). (a) The bright halo around the beads indicates NE assembly around the bead. Note that NE did not assemble in *Ketel*^{D} egg extracts even during 120 minutes. (b) While the addition of wild type Drosophila importin-β facilitated NE assembly in the wild type egg extract, the mutant P446L-imp-β inhibited NE assembly.

molecules and thus reveal a novel role of importin-β required during NE assembly at the end of mitosis. The novel importin-β function is distinct from both its role in nuclear protein import and the recently described function in mitotic spindle assembly (Wiese et al., 2001; Nachury et al., 2001; Gruss et al., 2001).

P446L-imp-β inhibits NE assembly over Ran coated Sepharose beads

Ran loaded Sepharose beads initiate the assembly of functional NE *in vitro* not only in extracts of Xenopus eggs (Zhang and Clarke, 2000) or mammalian mitotic cells (Zhang and Clarke, 2001) but also in extracts of wild type Drosophila embryos (Tirián et al. 2003). In fact the wild type Drosophila embryo extract as efficiently support NE assembly around the Ran coated beads as the Xenopus egg extracts: NE assembled within 60 minutes as revealed by the fluorescent halo around the beads (Figure 12a). However, when the Ran coated beads were

incubated in *Ketel*^{D} egg extracts, NE did not form even during a two-hour incubation period (Figure 12a). When 3 μM exogenous wild type Drosophila importin-β was added (to reach the importin-β concentration in Xenopus eggs) to the wild type Drosophila embryo extract, complete NE assembled within 15 minutes (Figure 12b). To test the dominant negative effect of P446L-imp-β on NE assembly, we incubated Ran-coated Sepharose beads in wild type extracts into which 3 μM purified P446L-imp-β was added. P446L-imp-β prevented the formation of NE (Figure 12b). The above experiments demonstrated that (i) the wild type Drosophila embryo extracts support NE assembly *in vitro* around Ran-coated Sepharose beads, (ii) wild type importin-β accelerates NE assembly and (iii) P446L-imp-β – in either extracts of the *Ketel*^{D} eggs or exogenously added to wild type extract – block NE assembly in dominant negative fashion, and thus results of the *in vitro* and the *in vivo* experiments – described in Tirián et al. (2000) and Timinszky et al. (2002) – are in agreement.

NE assemble over importin-β-coated Sepharose beads

It was shown recently that NE assembles over importin-β coated Sepharose beads in Xenopus egg extract (Zhang et al., 2002). It was revealed furthermore, that Sepharose beads coated with the 45-462 truncated form of importin-β that cannot bind RanGTP and importin-α but bind Nup's are capable of recruiting NE components, and also that Sepharose beads coated with I178D importin-β that cannot bind Nup's is deficient in stimulating NE assembly (Zhang et al., 2002). To study if P446L-imp-β is able to recruit NE components we coated the Sepharose beads with wild type or with P446L-imp-β by (i) chemically coupling to BrCN activated-Sepharose and also (ii) through a polyclonal anti-Ketel antibody to Protein-A-Sepharose. Results of the two types of couplings were essentially identical. Because the BrCN activated-Sepharose beads bind DHCC, and strong background fluorescence emerged, only results of the antibody coupling experiments are presented (Figure 13). NE did not assemble over the control Protein-A Sepharose beads in extracts of 0-3 hour old wild type Drosophila embryos (Figure 13a). When the Protein-A-Sepharose beads were coated with the anti-Ketel antibody, NE assembled around the beads within the first 10 minutes of incubation (Figure 13b). NE assembly was even faster and more intensive over those beads that had been coated with anti-Ketel-Protein-A and preloaded with wild type importin-β (Figure 13c). The most likely explanation for the accelerated NE assembly as compared to the Ran coated beads is the

high importin-β concentration readily available over the bead surface such that the importin-β molecules can bind vesicles with NE components.

To our surprise, identical result emerged following the incubation in wild type embryo extracts of those anti-Ketel-Protein-A-Sepharose beads that had been preloaded with P446L-imp-β (Figure 13d). The result shows that unlike *in vivo* and *in vitro* over the Ran-coated beads where P446L-imp-β block NE assembly, when immobilized to the Sepharose beads P446L-imp-β induces NE assembly.

We also determined – through immunostaining – whether the above-described beads recruited some of the nucleoporins by making use of the mAb414 anti-nucleoporin antibody. Apparently when immobilized to beads wild type and P446L-imp-β molecules equally efficiently recruit nucleoporins as it appeared following immunostaining (Figure 14) or Western blot analysis (data not shown). It should perhaps be noted that the mAb414-based staining was much more intensive over the importin-β as compared with the Ran beads (Figure 14).

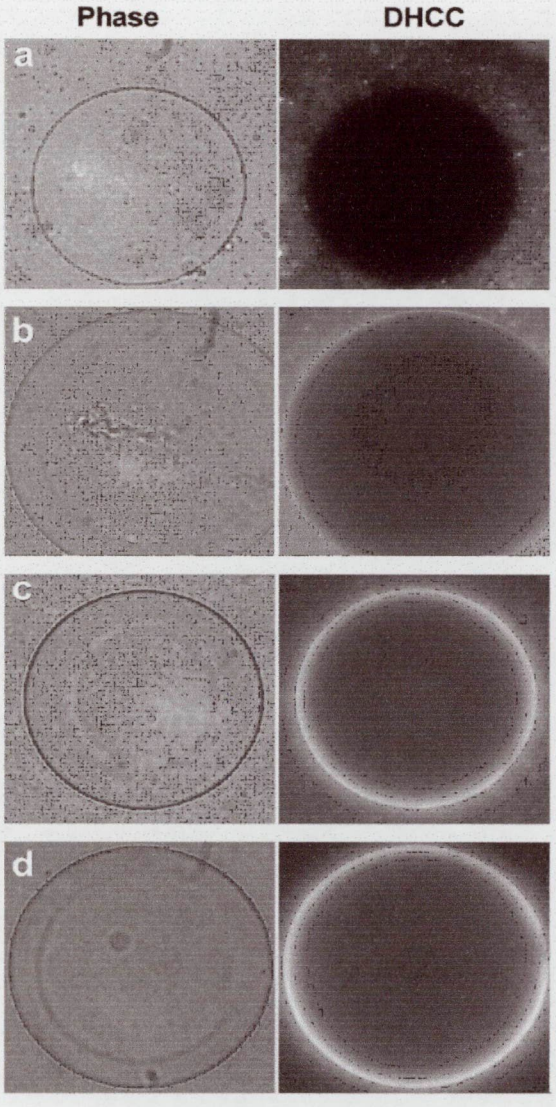

Figure 13. NE assembly around wild type importin-β and P446L-imp-β coated beads. Beads with different types of coats were incubated for ten minutes in extract prepared from wild type Drosophila embryos and stained with DHCC for the presence of NE. NE does not assemble over Protein-A coated (control) Sepharose beads (a). NE assembles over beads coated with anti-Ketel antibody (b). High amounts of NE materials assembly over the beads coated with wild type Drosophila importin-β (c) or with P446L-imp-β (d).

Mislocalization of P446L-imp-β on MTs

A plausible explanation for the unexpected behavior of P446L-imp-β, i.e. it inhibits NE assembly *in vivo* and *in vitro* on Ran-coated Sepharose beads and yet supports NE assembly when directly bound to Sepharose beads, is

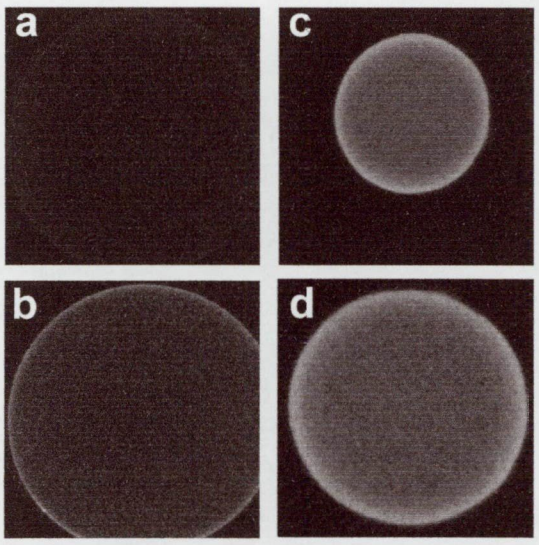

Figure 14. Nucleoporins are embedded in the NE that form over the Sepharose beads coated with wild type importin-β or P446L-imp-β. Beads with different types of coats were incubated for one hour in extract prepared from wild type Drosophila embryos and immunostained with the mAb414 anti-nucleoporin antibody. There is no indication of Nup's over the Protein-A coated (control) Sepharose beads (a). The presence of Nup's is apparent over the Ran coated beads (b). Along with the high amount of NE material (as shown on Figure 2c and d), intensive Nup signal emerged from both the importin-β (c) and the P446L-imp-β coated beads (d).

that the P446L-imp-β molecules cannot localize correctly in the egg extract and also in the *Ketel*^{D} eggs. The dominant negative nature of P446L-imp-β becomes understandable if P446L-imp-β binds factors required for NE assembly (as shown by its ability to recruit nucleoporins when bound to the Sepharose beads) and while mislocalizes it sequesters components required for NE assembly. To find out whether P446L-imp-β is indeed mislocalized inside the cells, we analyzed the distribution of wild type and P446L-imp-β molecules in digitonin-permeabilized HeLa cells that are routinely used for analysis of nuclear protein import. (We could not use Drosophila embryos since there is a huge stockpile of maternally provided importin-β in the egg cytoplasm, a condition that does not allow detection of difference in importin-β distribution in wild type and *Ketel*^{D} eggs; Lippai et al., 2000). Following permeabilization by digitonin, the soluble cytoplasmic factors including those involved in nuclear protein import are washed away from the HeLa cells. Nuclear protein import is analyzed following the re-addition of purified wild type or P446L-imp-β along with other components of nuclear protein import. We used immunostaining to detect wild type or P446L-imp-β in the digitonin-permeabilized HeLa cells. It should be noted that the polyclonal anti-Ketel antibody equally efficiently recognizes wild type and P446L-imp-β. In the presence of nuclear protein and in the absence of Ran and energy regenerating system, wild type and P446L-imp-β are uniformly distributed in the cytoplasm, although wild type importin-β is slightly more abundant around the NE as P446L-imp-β (Figure 15a and 15b). However, in presence of Ran and energy regenerating system there is a major difference between localization of the wild type and the P446L-imp-β molecules: while wild type importin-β localize exclusively to the NE (Figure 15c), being engaged in nuclear protein import, P446L-imp-β is completely cytoplasmic, showing mislocalization of P446L-imp-β

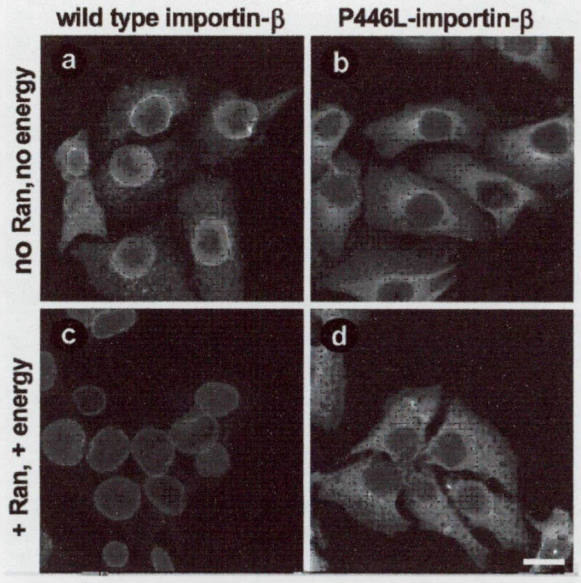

Figure 15. The localization of wild type importin-β and P446L-imp-β in digitonin permeabilized HeLa cells. In the absence of a "Ran mixture" and an energy regenerating system both the wild type importin-β (a) and P446L-imp-β (b) localize to the cytoplasm with slight accumulation over the NE. (The "Ran mixture" is composed from Ran, RanBP1, NTF2 and RanGAP.) In the presence of "Ran mixture" and an energy regenerating system, while wild type importin-β binds exclusively to the NE (c), P446L-imp-β molecules remain cytoplasmic (d). Bar 10 μm.

(Figure 15d; Tirián et al. 2003).

Since distribution of importin-β and P446L-imp-β in the digitonin permeabilized HeLa cells was reminiscent of the MT cytoskeleton, we double stained the digitonin permeabilized HeLa cells with anti-Ketel and anti-tubulin antibodies and analyzed the cells in a confocal microscope. Results of the staining show localization of P446L-imp-β to the MTs in the presence of RanGTP (Figure 16). Note that MTs were present despite the fact that the above experiments were carried out on 4°C, a condition that leads to disassembly of the MTs, implying that P446L-imp-β may stabilize the MTs.

To examine if there is a difference in MT binding ability of wild type and P446L-imp-β, cytoplasmic extracts were prepared from wild type and *Ketel*^{D}/+ females. The cytoplasmic extracts of *Ketel*^{D}/+ females most likely contain 50% wild type and 50% P446L-imp-β. The extracts were supplemented with 10 μM tubulin to reach the critical concentration required for tubulin polymerization and MT formation was induced by 20 μM taxol. Two conditions were

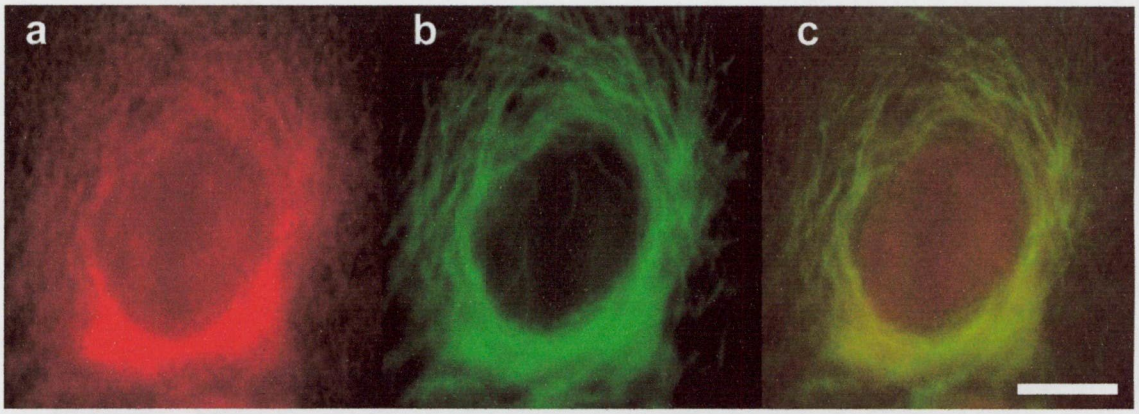

Figure 16. P446L-imp-β co-localizes with the MTs in digitonin permeabilized HeLa cells. P446L-imp-β and "Ran mixture" and energy regenerating system were added to digitonin permeabilized HeLa cells, fixed after five-minute incubation and stained with (a) the anti-Ketel (red) and with (b) the anti-tubulin antibody (green). Note accumulation of P446L-imp-β over the MTs (yellow) on the merged image (c). Bar 10 μm.

examined. In the first one, no RanQ69L was added to the extract to mimic cytoplasmic conditions farther from the chromatin. In the second condition, RanQ69L was added to a final concentration of 3 μM to mimic conditions near the chromatin where Ran exists in its GTP bound state (Kalab et al., 2002). Addition of RanQ69L removed a significant amount of importin-β from the MTs in both wild type and *Ketel*^{D}/+ extracts (Figure 17A). However, even in wild type extract 12% of importin-β remains MT bound in the presence of RanQ69L. Thus MT-importin-β binding seems to originate from a RanGTP sensitive pool and an insensitive pool that may come from direct binding of importin-β to the MTs.

To investigate if importin-β does indeed bind MTs directly, MTs were polymerized in the

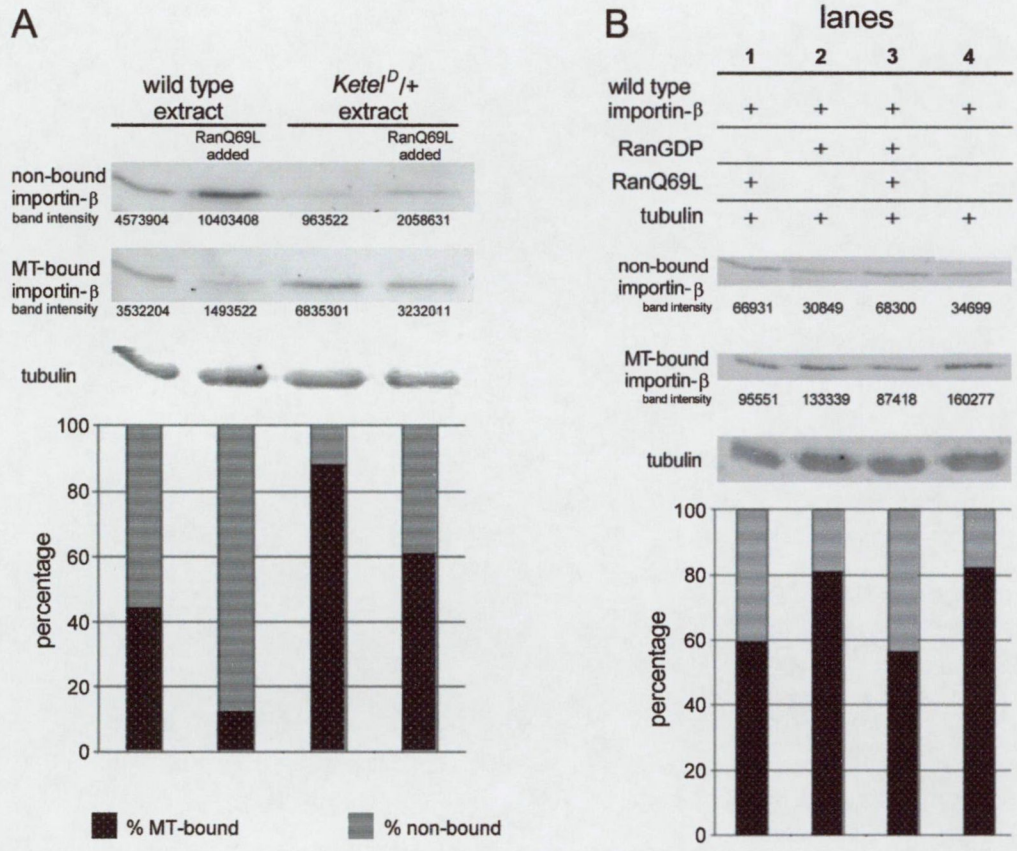

Figure 17. The *in vitro* assembled MTs pull-down importin-β in a RanGTP-dependent way. MTs were polymerized in wild type and *Ketel*^{D}/+ extracts (panel A) or in the presence of wild type purified importin-β (panel B) under conditions indicated and subsequently centrifuged through a 30% sucrose cushion. Importin-β in the supernatant and the MT pellet was detected by Western blot using anti-Ketel antibody. Polymerized MTs were detected using anti-tubulin antibody. The ratio of non-bound and MT-bound importin-β under different conditions was calculated by ImageQuant software and is shown in the bottom diagrams. Importin-β-MT interaction decreases in the presence of RanQ69L (panel A and B). Significantly more importin-β is MT bound in the *Ketel*^{D}/+ extract (panel A). The increased amount of importin-β is not merely the result of the inability of P446L-imp-β to bind RanGTP for more importin-β is MT bound in the *Ketel*^{D}/+ extract than in the wild type extract even in the absence of RanQ69L (panel A); P446L-imp-β has higher MT binding ability than wild type importin-β.

presence of purified wild type importin-β. (Results of the experiments with purified P446L-imp-β are not shown because it forms aggregates and is pelleted in the absence of MTs under the present experimental conditions.) If the reaction mixture contains only tubulin and wild type importin-β, importin-β is present exclusively together with the pelleted MTs irrespective of being alone or together with RanGDP or RanQ69L (data not shown). When the mixture is supplied with 1 mg/ml BSA to decrease unspecific binding of importin-β, 82% of importin-β appears in the pellet bound to the MTs (Figure 17B). [BSA does not bind to the MTs under any conditions examined and no importin-β was found in the pellet without polymerized MTs (data not shown).] The presence of RanGDP does not change the amount of MT bound importin-β (81%). RanQ69L decreased importin-β binding to MTs resulting in 59% MT bound importin-β. If both RanGDP and RanQ69L are present the amount of MT bound importin-β (56%) is equal to that observed if RanQ69L is present alone that is in line with the observations that importin-β preferentially binds RanGTP. The experiments with the recombinant wild type importin-β reveal that importin-β can bind MTs directly and the interaction decreases upon RanGTP binding. The binding of purified importin-β to the MTs is much stronger than that observed in the wild type extract. It is most likely the consequence of the "naked" MTs compared to the extract where the MTs are highly decorated with MAPs and also the extract certainly contains several soluble interacting partners for importin-β that affects availability of importin-β for MTs. Of course, the results of the extract are expected to be closer to the *in vivo* conditions as results with purified importin-β. The RanGTP insensitive pool (12%) observed in the wild type extract well may be the result of the inherent ability of importin-β to bind MTs even in the presence of RanQ69L (Figure 17B). The RanGTP sensitive pool, on the other hand, can be the combination of both direct and MAP directed binding of MTs. The binding of importin-β to certain types of MAPs either directly or through importin-α is not very surprising since several proteins including NuMA, TPX2, Eg5 have been shown to interact with importin-β (Gruss et al., 2001; Nachury et al., 2001; Wiese et al., 2001).

Observations summarized on Figures 15 and 16 suggest that P446L-imp-β bind stronger to MTs than normal importin-β. Indeed, both in the absence and in the presence of RanQ69L, more importin-β is bound to the MTs from the *Ketel*^{D}/+ extract than from the wild type extract (Figure 17A). Note that the *Ketel*^{D}/+ extract contains wild type and P446L-imp-β, as

well, thus the increased signal from the *Ketel*^{D}/+ extract must come from the higher affinity of P446L-imp-β to MTs. Interestingly the altered binding ability of P446L-imp-β is not merely due to not interacting with RanGTP and that way not releasing certain MAPs since the amount of MT bound importin-β is higher in the *Ketel*^{D}/+ extract than in the wild type extract even in the cytoplasm mimicking condition where Ran is in GDP bound form. If 50% wild type importin-β content of the *Ketel*^{D}/+ extract is considered and that it has the same affinity toward MTs as observed in the wild type extract, all the P446L-imp-β is likely to be MT bound under both conditions. The observations support the hypothesis that the increased MT binding ability of P446L-imp-β together with the intact Nup binding ability counts for the *Ketel*^{D}-associated mutant phenotype, i.e. P446L-imp-β interferes with NE assembly by sequestering certain NE assembly factors onto the MTs.

Discussion

Proline 446 is a functionally important position in importin-β

Importin-β was originally identified along elaborating the mechanism of nuclear protein import (for a review see Görlich and Kutay, 1999). During the past years the interacting partners - most importantly importin-α, Ran and some of the Nup's - were identified and its molecular structure has been elucidated (Vetter et al., 1999; Cingolani et al., 1999; Lee et al., 2000). Along genetic dissection of the commencement of embryogenesis our group identified the *Ketel* gene - by $Ketel^{D}$ dominant female sterile mutations - that encodes the Drosophila homologue of importin-β (Lippai et al., 2000). The $Ketel^{D}$ mutations block embryogenesis in dominant negative fashion, however not through the inhibition of nuclear protein import but by preventing NE reassembly at the end of cleavage mitosis suggesting involvement of importin-β in the process (Tirián et al., 2000). The fact that in three of the four independently isolated $Ketel^{D}$ mutations the same $C^{4114}\rightarrow T$ transition and the concurrent replacement of Pro^{446} by Leu is the basis of dominant female sterility underlines the importance of Pro^{446} in importin-β function (Timinszky et al. 2002).

P446L-imp-β loses RanGTP binding ability

Experiments with digitonin-permeabilized HeLa cells show that P446L-imp-β does participate in formation of the nuclear import complexes and in their docking on the cytoplasmic surface of the NE, it does not support import of the complexes into the nuclei in the presence of Ran, energy source, RanGAP and RanBP1. In fact, the import complexes do not form in the presence of the latter components. Apparently the main structural domains of P446L-imp-β are intact (binds importin-α, Nups and Ran) but the interaction with Ran is altered. Indeed, we found that the binding of wild type and P446L-imp-β to Ran are very different: the P446L-imp-β can not bind to RanGTP to which the wild type importin-β binds strongly. Instead P446L-imp-β shows elevated affinity to RanGDP to which wild type importin-β shows very little affinity. It is noteworthy that a single amino acid exchange outside the classical Ran binding domain can change Ran binding ability so dramatically. The change in Ran binding ability is the source of the $Ketel^{D}$-associated dominant female sterility. However, the $Ketel^{D}$-associated dominant negative effect is not manifested via nuclear protein import but rather through the prevention of cleavage nuclei formation – revealing a novel

importin-β function.

The P446L-imp-β exerts its toxic effect at the end of mitosis

The injection experiments into wild type cleavage embryos revealed that P446L-imp-β does not inhibit nuclear protein import: when co-injected with P446L-imp-β, a fluorescent nuclear substrate readily entered the nuclei. Furthermore, although the cleavage nuclei enter mitosis and the chromosomes segregate normally, intact NE never forms in the presence of P446L-imp-β. Failure of NE assembly in the presence of P446L-imp-β is revealed by the following observations. (1) The homogenous distribution of (i) a fluorescent nuclear substrate, (ii) the high molecular weight dextrane and (iii) the GFP-tubulin. (2) Absence of the nuclear lamina lining. Thus the mutant P446L-imp-β reveals a novel importin-β function required during the mitosis to interphase transition, a function distinct from the already known functions of importin-β in nuclear protein import and in mitotic spindle assembly (Görlich and Kutay, 1999; Wiese et al., 2001; Nachury et al., 2001; Gruss et al., 2001).

P446L-imp-β possesses altered Ran binding properties: it does not bind RanGTP but shows elevated affinity to RanGDP. A series of experiments showed that altered RanGTP-RanGDP balance leads to very similar phenotype in yeast i.e. arrest in mitosis to interphase transition (Sazer and Nurse, 1994; He et al., 1998). Results of enzymatic assays show that the altered Ran binding ability of P446L-imp-β does not interfere with the GTP hydrolysis and nucleotide exchange on Ran, and thus it is unlikely that the *Ketel*^{D}-related defects are consequences of distorted Ran metabolism. Most probably importin-β is a downstream effector of Ran in NE assembly just like in nuclear protein import and mitotic spindle assembly.

Importin-β is an essential component in targeting NE assembly factors to the chromatin

Drosophila egg extracts as effectively support NE assembly over Ran or importin-β coated Sepharose beads as Xenopus egg extracts thus make the bead system feasible to study NE assembly *in vitro*. Applicability of the bead system is also supported by the following observations. (i) The importin-β coated beads initiate fast and efficient assembly of NE around the beads and (ii) NE assembly does not take place over Ran coated beads in Xenopus egg extracts from which importin-β had been depleted (Zhang et al., 2002).

Although functional NE assemble in both the demembranated sperm chromatin and in the Sepharose bead *in vitro* NE assembly systems (Lohka and Masui, 1983; Zhang and Clarke, 2000) it is not clear whether the process proceeds through the same mechanism. In the chromatin system, NE assembly is believed to progress through the same steps as *in vivo*. The process of NE assembly is divided into distinct stages (Burke and Ellenberg, 2002; Hetzer et al., 2002). It begins during anaphase when the nucleus-derived vesicles bind to the chromosomes, fuse and flatten. Nucleoporins incorporate next in a stepwise fashion into the forming NE until functional NPCs form. Nuclei form following the resumed import of nuclear proteins through the NPCs. It appears that components of the Ran system are involved in the membrane fusion event but not in vesicle targeting (Hetzer et al., 2001). The fact that Ran coated beads attract vesicles raises the question as to what extent does the Ran coated Sepharose system represent NE assembly *in vivo*. It was hypothesized recently that NE assembly proceeds in opposite fashion in the Ran coated Sepharose system as over chromatin (Hetzer et al., 2002): NE vesicle targeting is based on interaction between importin-β and the Nup's, that are embedded in the NE vesicles, and the subsequent fusion of the vesicles. Our *in vivo* results – based on injection of P446L-imp-β into wild type cleavage embryos – support the model that NE assembly starts with importin-β dependent vesicle targeting, since macromolecules persist in the chromatin area that would not be awaited if intact, continuous NE would form over the chromatin. Thus, an importin-β mediated event preceding vesicle fusion occurs in NE assembly at least in the cleavage Drosophila embryos. The former proposition is supported by the finding that there is a high RanGTP concentration on the chromatin (Kalab et al., 2002; Bilbao-Cortes et al., 2002) that is mimicked by Ran bound to the Sepharose beads in the *in vitro* bead system. It well may be that in a living cell both Ran dependent and Ran independent vesicle targeting contribute to NE assembly, although in different cell types the contribution of the two mechanisms may be different.

Identical to the *in vivo* findings, P446L-imp-β prevents NE assembly over the Ran coated beads. The P446L-imp-β action is dominant negative since it blocks NE formation the Drosophila egg extracts whether prepared from $Ketel^{D}$ eggs or from wild type embryos into which P446L-imp-β was added and contained both normal importin-β and P446L-imp-β. Apparently the inhibitory effect of P446L-imp-β is only slightly reduced in eggs of females carrying one $Ketel^{D}$ (P446L-imp-β coding) and up to as many as eight normal importin-β

coding *Ketel* gene copies (our unpublished results). The inhibitory effect of P446L-imp-β is best explained by the binding of factors present in limited numbers that are involved in targeting NE vesicles to chromatin (Tirián et al., 2003). Possible targeting factors may be some of the Nup's, many of which are known to interact with importin-β (Shah et al., 1998; Bayliss et al., 2000; Ben Efraim and Gerace, 2001), or a thus far unknown protein that functions as a link between NE vesicles and importin-β.

Unexpectedly, when bound to Sepharose beads P446L-imp-β as efficiently induces NE assembly as normal importin-β. It is rather unlikely that P446L-imp-β prevent NE assembly by dragging away the targeting factor(s) since Ran bound to Sepharose beads can itself interact with NE vesicles. As soon as NE vesicles are attracted over the beads by Ran or by wild type importin-β, the vesicles can fuse in the vicinity of Ran (Hetzer et al., 2001) and the beads are covered by a continuous double membrane. However, if the targeting factors and/or the NE vesicles would be sequestered by P446L-imp-β and anchored in the cytoplasm (i.e. not available for NE assembly), the toxic effect of P446L-imp-β becomes understandable.

P446L-imp-β is anchored to the MTs and sequesters factors required for NE assembly

It appears that the structure to which P446L-imp-β is anchored are the MTs since a number of observations suggest that MTs are involved in regulation of importin-β function. (i) The nuclear import of parathormone related protein was shown to be MT dependent (Lam et al., 2002). (ii) Most of the cytoplasmic importin-α coalign with MTs and microfilaments (Gorjanacz et al., 2002; Smith and Raikhel, 1998). (iii) The KIF5B and KIF5C microtubular-based motor proteins bind to importin-β (Mavlyutov et al., 2002). (iv) Taxol has an inhibitory effect on nuclear protein import in digitonin permeabilized HeLa cells (our unpublished data). (v) Under the rare experimental conditions when both intact NE and mitotic spindles are present at the same time (following the injection into cleavage Drosophila embryos of the p25 protein, that disrupts MT function (Hlavanda et al., 2002), protein import is inhibited into the nuclei (Tirián L., unpublished data).

We set out to clarify features of importin-β-MT interaction hoping to understand the mode of dominant negative action of P446L-imp-β. Figure 17 presents evidence that importin-β binds MTs both directly and through MAPs. The effect of RanQ69L on MT and importin-β

interaction shows that both type of binding is Ran sensitive. Assuming that RanQ69L abolishes cargo/MAP binding of importin-β, the 44% MT bound importin-β in the wild type extract in the absence of RanQ69L is mainly due to MAP directed binding. Whether the MT-importin-β binding has physiological significance or not remains to be elucidated. During interphase MTs may serve as routes directing importin-β with its cargo toward the nucleus. Experiments with the wild type extract suggest that when Ran is loaded with GTP in the vicinity of chromatin, importin-β is released from the MTs (Figure 17). During mitosis the MTs may function as depositories for factors involved in NE assembly that can be released upon disassembly of the mitotic spindle.

P446L-imp-β is strongly bound to MTs both in the absence and in the presence of RanGTP (Figure 17A). The elevated MT binding ability of P446L-imp-β is not merely due to its lost RanGTP binding ability and is likely to stem from both increased direct and indirect binding. (To our regret, instability of the purified P446L-imp-β under the experimental conditions did not allow us to gain more information on the mode of MT binding.) It appears thus that while P446L-imp-β attains increased MT binding ability it binds nucleoporins as normal importin-β and prevents NE assembly through sequestering certain factors required for the process (Tirián et al., 2003).

Still to solve

We identified a new importin-β function during mitosis that is importin-β is involved in NE reassembly at the end of mitosis. We showed that P446L-imp-β is MT bound and is able to sequester factors required for NE assembly. However, it remains to be revealed what NE assembly factors P446L-imp-β binds. The *Ketel*^{D} mutations are female sterile. However, the P446L-imp-β effects are not manifested in every cell. Why not? How is it possible that although the *Ketel*^{D} mutations are expressed in the mitotically active cells, the cells do not die? Why is the toxic effect of P446L-imp-β restricted to the cleavage embryos? The above questions are interesting and important. Hopefully, the better understanding of the mode of action of P446L-imp-β will bring us closer to the answers to the above questions.

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