DEVELOPMANTAL AND MOLECULAR GENETICS OF *KETEL*, THE DROSOPHILA HOMOLOGUE OF IMPORTIN-β

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II. Mónika Lippai, László Tirián, Imre Boros, József Mihály, Miklós Erdélyi, István Belecz, Endre Máthé, János Pósfai, Adam Nagy, Andor Udvardy, Efrosyni Paraskeva, Dirk Görlich and János Szabad, The *Ketel* gene encodes the Drosophila homologue of importin-β.

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Abbreviations

IBB	importin-β binding domain
Fs	dominant female sterile mutation
fs	recessive female sterile mutation
GDP	guanozine diphosphate
GST	glutathion-S-transferase
GTP	guanozine triphosphate
K^+	wild type *Ketel* transgene
K^D	transgene with the $Ketel^D$ allele
$Ketel^D$	dominant mutant allele of the *Ketel* gene
$ketel^R$	recessive mutant allele of the *Ketel* gene
mel	maternal effect lethal mutation
MT	microtubule
NE	nuclear envelope
NLS	nuclear localization signal
NPC	nuclear pore complex
NTF2	nuclear transport factor 2
PE	phycoerythrin
Ran	Ras like nuclear protein
RanBP	Ran binding protein
RanGAP	GTPase activating protein for Ran
RCC1	regulator of chromatin condensation 1 (nucleotide exchange factor for Ran)

1. SUMMARY

Four dominant female sterile (Fs) mutations and their recessive alleles identify the *Ketel* gene of *Drosophila melanogaster*. Comparison of the deduced amino acid sequence of the Ketel protein revealed 61% identity and 78% similarity with human importin-β, an essential component of nuclear protein import. The Ph.D. dissertation covers developmental genetics and molecular functions of the *Ketel* gene. Our results show that:

- *Ketel* is an essential gene with both zygotic and maternal functions.
- Ketel is a functional homologue of importin β. It docks nuclear proteins to the nuclear pore complexes (NPC) of digitonin permeabilized HeLa cells and –in the presence of Ran and energy supply- imports nuclear proteins into the nuclei.
- We studied expression of the *Ketel* gene both by *in situ* hybridizations, Western blotting and by a *Ketel* promoter driven *lacZ* reporter gene and learned that the *Ketel* gene is preferentially expressed in mitotically active cells.
- We cloned three of the four $Ketel^{D}$ alleles and found a single amino acid exchange between the wild type and the mutant proteins.
- We generated several wild type and mutant transgenes. The wild type transgenes rescue both zygotic lethality and – to some extent – female sterility. The mutant transgenes leed to dominant female sterility.
- We elucidated a new function for Drosophila importin-β. Cytological analysis of the $Ketel^{D}$ eggs and cytoplasm injections suggest a role of *Ketel* in the assembly of cleavage nuclear envelope.
- We propose evidence for the presence of a maternal partner present in the egg cytoplasm. The embryonic lethality associated with the $ketel^{r}$ alleles is due to combined activities of the $Ketel^{D}$ encoded protein and the maternally provided partner present only in the egg cytoplasm.

2. INTRODUCTION

Early embryogenesis of Drosophila and the generating of *Ketel*^{D} mutations

Before commencement of the embryogenesis, the egg cytoplasm must contain all the components essential for early embryogenesis. Since there is little if any zygotic gene expression during early embryogenesis, the majority of the embryogenesis controlling factors are deposited into the egg cytoplasm during oogenesis, and apparently their synthesis is under maternal control. The maternal genes engaged in the initiation of embryogenesis represent some of the *maternal-effect* genes.

The exact mechanism of the initiation of embryogenesis is yet poorly understood. Many developmental biologists have focused their efforts on model systems in which embryonic development is easier to study than in mammals. The fruit fly *Drosophila melanogaster* is an excellent experimental organism for studying maternal effects. Following completion of the meiotic divisions, the first 13 mitotic cycles are nuclear divisions that occur synchronously in a shared cytoplasm. The cleavage cycles oscillate rapidly between the S and M phases without detectable gap phases. DNA replication and mitosis take only about 8-10 min during cycles 1-10 and gradually lenghten to 21 min by cycle 13. The speed of the cleavage cycles is facilitated by maternal stockpiles of mRNAs and proteins (Wieschaus 1996). The zygotic genome is not transcribed before cycle 11, and transcription of most genes does not begin until cellularization begins (Orr-Weaver 1994).

Genetic dissection - gene identification and characterization through mutations – has been a useful approach to identify genes responsible for – among others – maternal effects. Genetic dissection implies first identification and understanding the function of single genes and the subsequent reconstruction of the process. Mutations that block the initiation of embryogenesis are likely to identify genes with functions required during commencement of embryogenesis. Analysis of maternal effect lethal (*mel*) mutations, a special class of female sterile (*fs*) mutations, provided major contribution to our knowledge about the genes contributing to the regulation of embryogenesis in *Drosophila melanogaster* (Wieschaus 1996). Analysis of the *mel* mutations promoted, among others, the understanding of cell differentiation along the anterior-posterior and the dorsal-ventral coordinates of the *Drosophila* embryo (Anderson and Nüsslein-Volhard 1986; Nüsslein-Volhard et al. 1987).

Females homozygous for a *mel* mutation deposit normal-looking and fertilized eggs.

However, embryogenesis does not commence or leads to the formation of abnormal embryos. 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 and, in fact about 90% of the *fs* mutations are weak (hypomorph) alleles of genes with essential zygotic functions (Schüpbach and Wieschaus 1989). 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 and go undetected in screens for *mel* mutations.

Genes with both early embryonic and zygotic functions may in principle be identified by dominant female sterile (*Fs*) mutations that disrupt the maternal-effect, however permit zygotic functions, i.e. females carrying an *Fs* mutation can 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). One of the genes identified by *Fs* mutations is the *Ketel* gene. Females carrying any of the four dominant $Ketel^{D}$ alleles deposit normal numbers of normal-looking eggs. Although the eggs are fertilized, embryogenesis is terminated shortly after fertilization in the $Ketel^{D}$-derived eggs suggesting involvement of the *Ketel* gene in commencement of embryogenesis.

To elucidate molecular function of the *Ketel* gene we isolated recessive $ketel^{r}$ alleles by reversion of the $Ketel^{D}$ alleles. Each of the four $Ketel^{D}$ alleles were reverted during second mutagenesis. The revertant alleles emerge upon elimination of the $Ketel^{D}$–encoded function. The $ketel^{r}$ revertant alleles are recessive zygotic lethal mutations with lethality during second instar of larval life (Erdélyi et al. 1997). The $ketel^{r}$ homozygotes perish without any characteristic morphological defects providing no clues about function of the normal *Ketel* gene product. Death of the mutant loss-of function $ketel^{r}$ zygotes shows zygotic requirement of the normal *Ketel* gene product. Since the Ketel gene products are synthetized under maternal genetic control the larval lethality does not mean that Ketel is not required until second instar of larval life.

We cloned the *Ketel* gene and learned, that it encodes importin-β, an essential component of nuclear protein import (Lippai 1999, Lippai et al. 2000, Tirian et al. 2000).

A short review of nucleocytoplasmic transport

Nucleus is the defining feature of eukaryotic cells. The chromosomal DNA is located and transcribed in the nucleus. Since translation happens exclusively in the cytoplasm the mRNA, tRNA molecules and ribosomal subunits must be exported from the nucleus to the cytoplasm. The nuclear proteins, on the other hand, are synthesized in the cytoplasm and are imported into the nucleus. The macromolecules can pass the nuclear envelope only through the NPCs. NPCs are visible pores in the nuclear envelope having a molecular weight of 125 MDa and comprised of approximately 100 different types of proteins. The selective transport requires specific transport mechanisms for macromolecules through the NPCs. Of the processes across the NPCs, the mechanism of nuclear protein import is best understood so far. Import into the nucleus proceeds by several distinct pathways. Most of the known pathways require a member of the importin-β superfamily as import facor and Ran as determinant of the transport direction. (Görlich and Kutay 1999)

The importin β superfamily

The best characterised nuclear transport receptors have a molecular weight of 90-130 kD and an acidic isoelectric point (pH 4.6-5.9) and a Ran binding domain at their N terminus (Görlich et al. 1997). The importin β superfamily is composed of at least 21 family members in human from which nine are proven transport receptors (see Görlich and Kutay 1999; Lippai et al. 2000). Members of the importin-β superfamily recognize different nuclear import or export signals. They bind or release them depending on the nucleotide state of Ran, a small Ras like GTPase.

Ran has a very low intrinsic nucleotide exchange and GTPase activity. RanGAP, (GTPase activating protein for Ran) and the RanBP1 (Ran binding protein 1) facilitate the GTPase activity of Ran. RanGAP and RanBP1 are cytoplasmic, while the RCC1 (the only known nucleotide exchange factor for Ran) is chromatin-bound. Localisation of the regulatory proteins of the Ran GTPase cycle indicate that the conversion of RanGTP to RanGDP is restricted to the cytoplasm, whereas the formation of RanGTP from RanGDP is restricted to the nucleus (Figure 1). The resulted steep RanGTP gradient – what is believed to be a driving force for nuclear transport processes - with a high nuclear concentration of RanGTP in the nucleus and a very low level in the cytoplasm is a marker for a nuclear or a

cytoplasmic environment which the Ran-binding site of importin-β like transport receptors can "sense" (Koepp and Silver 1996; Melchior and Gerace 1998, Tirián et al. 2001).

Import receptors bind their cargoes in the absence of RanGTP and release them in the nucleus when binding RanGTP (Figure 1).
Conversely export receptors bind their cargoes in presence of RanGTP and release them in the cytoplasm when the GTP is hydrolized (Figure 1). One transport circle requires the export and subsequent hydrolysis of a RanGTP molecule, which can enter the nucleus in GDP bound form with its own transporter NTF2 (Figure 1).
The only known energy requiring step of the nucleocytoplasmic transport processes is the maintaining of the RanGTP gradient.

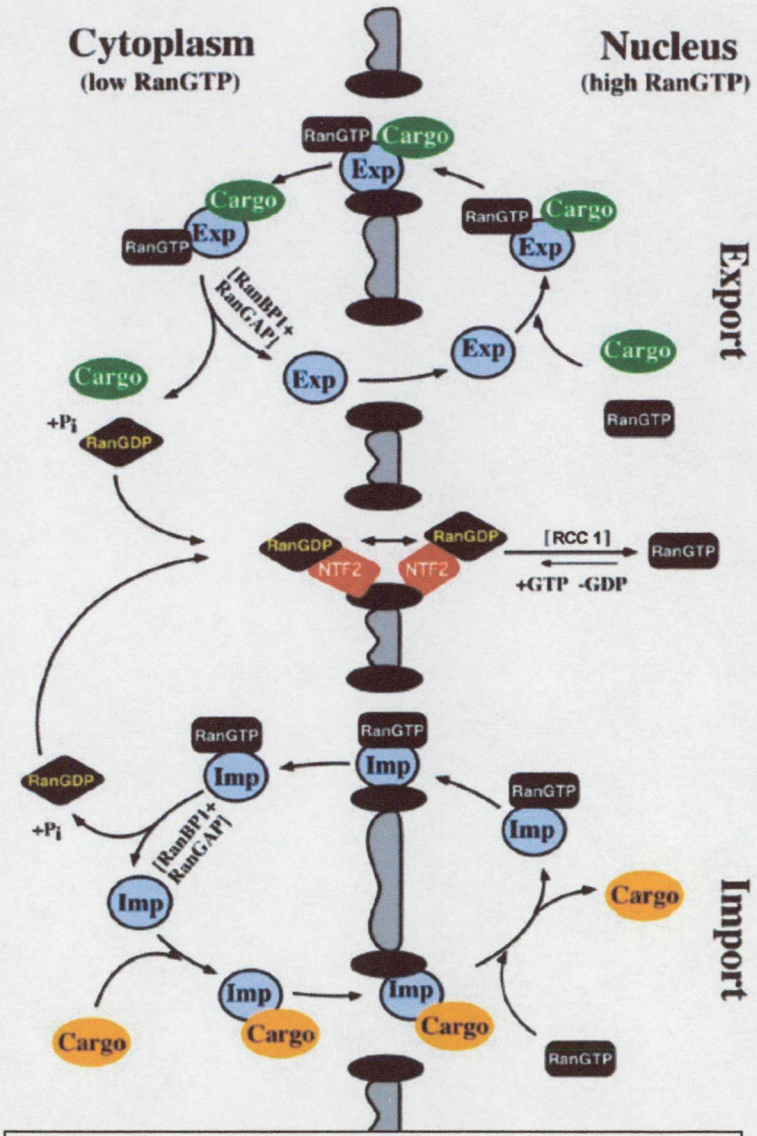

Figure 1. Nucleocytoplasmic transport and the generation of the RanGTP gradient. See text for details. (Adapted from Görlich and Kutay, 1999)

The importin-β dependent protein import pathways

importin-β, the founding member of the importin-β superfamily directs the transport of proteins containing several different nuclear localisation signals (NLS) either alone or in combination with other import factors.

Several NLS sequences, e.g. those present in the human immunodeficiency virus Tat and Rev proteins (Truant and Cullen 1999), the human T-cell leukemia virus type 1 Rex protein (Palmeri and Malim 1999), the T-cell protein tyrosine phosphatase (Tiganis et al. 1997), the

yeast cytoplasmic shuttle protein Nab2 are transported with importin-β alone. NLSs found in all of these proteins are rich in arginine residues rather than in the lysines that are more commonly found in classical, importin-α dependent types of NLSs.

importin-β transports several cargoes using adapter molecules. The best known example is importin-α, which can bind the lysine rich classical NLS (cNLS) sequences like the monopartitive cNLS of the SV40 T antigen or the bipartitive cNLS of nucleoplasmin. The N-terminal part of importin-α interacts via its importin-β binding domain (IBB domain) with the β-subunit (Török et al. 1995; Görlich et al. 1995a). importin-β targets the complex to the NPCs through the GLFG and FXFG repeats of the nucleoporins (Görlich et al. 1995b; Iovine et al. 1995). The transfer of the trimeric NLS-protein/importinα/β complex through the NPC happens through mostly unknown mechanisms. Dissociacion of the complex requires GTP-bound Ran. The binding of RanGTP to the importin-β on the nucleoplasmic site of the NPC induces a conformational change in the molecule and it results in the dissociation and subsequent recycling of importin-β to the cytoplasm, whereas importin-α and the cNLS containing protein enter the nucleoplasm. importin-α is subsequently exported with CAS, a member of the importin-β superfamily (Görlich and Kutay 1999).

Three other less characterized adaptors functioning together with importin-β were described recently. Snurportin (Huber et al. 1998) is the import receptor for the 5'-2,2,7-terminal trimethylguanosine cap containing U1, U2, U4 and U5 spliceosomal ribonucleoproteins. The transport mechanism is similar to that with importin-α. Snurportin is exported after the import cycle with CRM1 an export receptor belonging to the importin-β superfamily. XRIPα (Xenopus Replication Protein A Importer α) together with importin-β is required for import of the Replication Protein A in Xenopus oocytes (Jullien et al. 1999). Histon H1 is imported with the importin-β/importin-7 heterodimer (Jäkel et al. 1999). Within the heterodimer, however, importin-7 resembles an adapter molecule, although it can function alone as a complete import receptor for some ribosomal proteins.

Most of the results about the function of importin-β discussed so far came from *in vitro* biochemical studies done on digitonin permeabilized HeLa cells and from molecular genetic studies on yeast. Results of the studies suggested that importin-β is a ubiquitinously expressed protein required for life of every eucaryotic cell.

Our study was the first describing the expression pattern of importin-β in a multicelluler eucaryotic organism. We revealed that *Ketel* is preferentially expressed in mitotically active cells (Lippai et al. 2000; Tirian et al. 2000). The $Ketel^{D}$ mutations revealed new function of importin-β. Beside the well known zygotic function in nuclear protein import we showed that importin-β is required for nuclear envelope (NE) assembly during the cleavage divisions in Drosophila (Tirian et al. 2000).

3. MATERIALS AND METHODS

The *Ketel^D* and *ketel^r* alleles: The four *Ketel^D* alleles were isolated in a screen for dominant female-sterile mutations. The twenty-seven recessive *ketel^r* alleles were generated through second mutagenesis of the *Ketel^D* mutations (Szabad et al. 1989; Erdélyi et al. 1997). The *ketel^r/ketel^r* homo- and the *ketel^r/–* hemizygotes were produced by crossing *y/y*; *ketel^r/y$^+$CyO* or *y/y*; *ketel^{rX32}/y$^+$CyO* females with *y/Y*; *ketel^r/y$^+$CyO* males. (The *ketel^{rX32}* allele is a small deficiency that removes the *Ketel* and a few neighboring loci; Erdélyi et al. 1997.) The *y$^+$CyO* balancer chromosome carries a *y$^+$* transgene (Timmons et al. 1993). Head skeleton and ventral setae of the descending *y/y* (or *y/Y*); *ketel^r/ketel^r* (or *ketel^r/–*) zygotes are yellow and thus the homo- and the hemizygous larvae can be separated from the heterozygous non yellow (*y$^+$CyO*) sibling larvae in which the chitin structures are dark. The *Ketel^D/–* hemizygotes were produced by crossing *y/y*; *ketel^{rX32}/y$^+$CyO* females with *y/Y*; *Ketel^D/y$^+$CyO* males. For explanation of the genetic symbols throughout the text see Lindsley ans Zimm (1992). The Drosophila cultures were kept on 25°C.

Cytoplasm injections: Two types of cytoplasm injections were carried out. (1) A sample of about 40 pl *Ketel^{D1}* egg cytoplasm (~ 0.4% total egg volume) was injected on one side into the presumptive head region at 70% egg length and 70% egg diameter of wild type embryos. The antennal and the maxillary sense organs, characteristic landmark structures derive from the chosen blastoderm region (Jürgens et al. 1986). The donor and the recipient embryos were less than 30 min old. In another set of cytoplasm injections the wild type embryos received ~300 pl *Ketel^{D1}* egg cytoplasm. Cuticles of the developing embryos and larvae were analyzed (Jürgens et al. 1986, Wieschaus and Nüsslein-Volhard 1986). As control, cytoplasm samples of less than 30 min old wild type embryos were injected. (2) A small sample of the red fluorescent cNLS-phycoerythrin (cNLS-PE; 20 μg/ml; Cserpán and Udvardy 1995) was first injected into *Ketel^{D1}* eggs. (cNLS = classic nuclear localization signal.) A sample of about 200 pl cNLS-PE containing *Ketel^{D1}* cytoplasm was subsequently injected into wild type cleavage embryos. In the control the cNLS-PE substrate solution was first injected into newly deposited wild type eggs and the cNLS-PE containing wild type egg cytoplasm was subsequently injected into wild type cleavage embryos. Import of the cNLS-PE substrate into the cleavage nuclei was followed in a ZEISS LSM410 confocal microscope. The injections were done on 20 °C.

The *Ketel*^{D}/+/+ and the *Ketel*^{D}/+/+/+ females: We constructed *Ketel*^{D}/+/+ and *Ketel*^{D}/+/+/+ females which, in addition to the *Ketel*^{D} allele, carried two and three normal *Ketel* gene (+) copies. Egg and progeny production of the females was monitored. The extra *Ketel* gene copies (+) were introduced through the *Tp(2;Y)G* chromosome or through one of the *Ketel*$^{+}$ (K^{+}) transgenes.

The *Tp(2;Y)G* chromosome. For production of *XXTp(2;Y)G*; *Ketel*^{D}/+ females, *XXTp(2;Y)G*; *Df(2L)Sd68, pr/CyO, pr* females were mated with *XY*; *Ketel*^{D}/*CyO, pr* males. [In the *Tp(2;Y)G* chromosome the 36B5-C1 to 40F segment of the second chromosome - including the *Ketel* and the *purple* (*pr*) loci - was transposed onto a Y chromosome. The *Df(2L)Sd68* deficiency removes both the *Ketel* and the *pr* loci.] The *XXTp(2;Y)G*; *Ketel*^{D}/*CyO, pr* females were mated with *XTp(2;Y)G*; *pr/pr* males. As determined in a cross between *XXTp(2;Y)G*; *Df(2L)Sd68, pr/CyO, pr* females and *XY*; *pr/pr* males, 39% of the progeny females are *XXTp(2;Y)G*.

The *Ketel*$^{+}$ (K^{+}) transgenes. We constructed three different types of *Ketel*$^{+}$ (K^{+}) transgenes. The first type included the entire 22 kb fragment shown on Figure 5. The second type covered a 13.8 kb *Xba* genomic fragment. In the third type a 4.0 kb *Xba – BamHI* genomic fragment – including the *Ketel* promoter and the 5' segment of the *Ketel* coding region - was combined with a 2.3 kb cDNA fragment that corresponded to the rest of the transcribed part of the *Ketel* gene . The above sequences were cloned into the *CaSpeR* vector with the *mini-white* marker gene and germ line transformants were generated by standard procedures. The K^{+} transgene-carrying flies have light to orange yellowish eyes on the *white* genetic background. The K^{+} transgenes were used for the construction of (i) K^{+}; *ketel*^{r}/–, (ii)K^{+}; *Ketel*^{D}/– as well as (iii)K^{+}; *Ketel*^{D}/+ and (iv) K^{+}/K^{+}; *Ketel*^{D}/+ zygotes. Viability and fertility of the females were tested.

Identification of the Ketel^{D} mutations and generation of *Ketel*^{D} transgenes: We isolated DNA from *Ketel*^{D}/- hemizygous larvae and amplified fragments of the *Ketel* mutant alleles with PCR. Sequencing of the fragments revealed a single nucleotide difference between the *Ketel*^{D3} allele and the wild type sequence. This transition results in a missense mutation, the CCC (Pro) codon in the 445th position turns to CUC (Leu). The mutation resulted at the same time in a new XhoI site. Restriction digestion of the appropriate PCR fragments from the DNA of the *Ketel*^{D1}/-, *Ketel*^{D2}/- and *Ketel*^{D4}/- hemizygous individuals revealed the same nucleotide exchange in the *Ketel*^{D1} and the *Ketel*^{D4} allele. For generation of the *Ketel*^{D}

transgene that carries the *Ketel*^{D} dominant mutation we cloned the KpnI-HindIII fragment of the wild type Ketel cDNA into pBS+ Vector (pBS+KetelWT) (see Figure 2.).

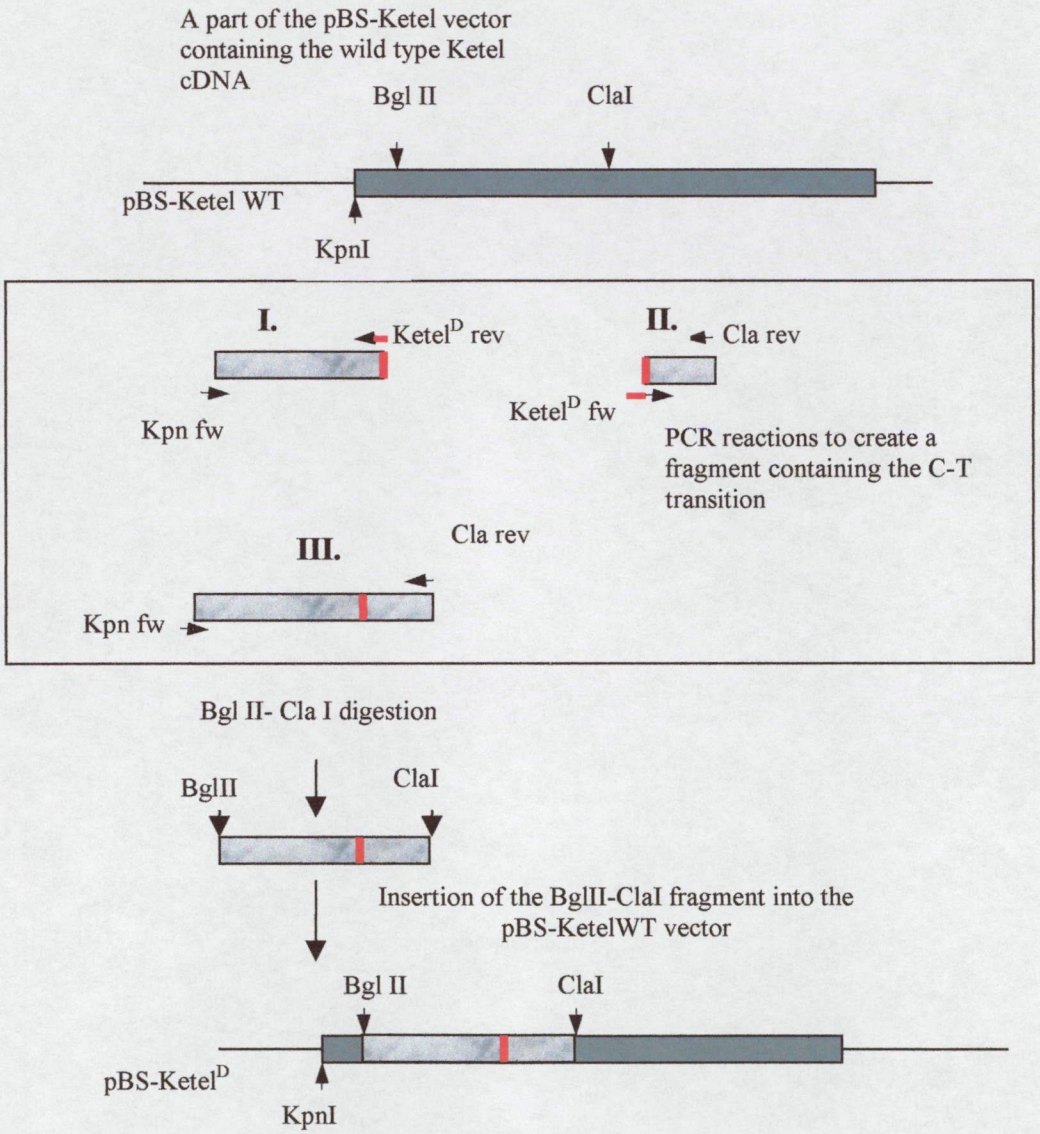

Figure 2. in vitro generation of a DNA Fragment containing the KetelD encoded transition. See text for details

We generated the *Ketel*^{D} mutation with PCR mutagenesis using the oligonicleotides:

Kpn fw: **5'CTGGCTGCTTTGGGTACCGAGAACA3'**

Ketel^{D} rew: **5'ATAGCCGCCTCGAGAATTATAT3'**

Ketel^{D} fw: **5'ATATAATTCTCGAGGCGGCTAT3'**

Cla rew: **5'GCTCCGTCCGATCGATCAGTGGTCTCC3'**

In the first step we generated two fragments, one from the KpnI site to the mutation (PCR reaction I. on Figure 2) and a second from the mutation to the ClaI (PCR reaction II., Figure 2) site using pBS+KetelWT as template. Then we used the two overlapping DNA fragments of PCR reactions I. and II. as templates and the two flanking oligonucleotide primers for a next reaction which resulted in a KpnI-Cla fragment carrying the point mutation (PCR reaction III., Figure 2). This fragment was digested with BglII and ClaI and the resulting 604 bp long fragment was exchanged in the pBS+KetelWT vector, resulting in pBS+Ketel^{D}. The exchange of the single nucleotide was confirmed with sequencing. The KpnI-HindIII fragment from pBS- Ketel^{D} was exchanged in the *CasPeR* transformation vector containing the third type of wild type *Ketel* transgene (Figure 5). Following germ line transformation we isolated three Ketel^{D} transgenic lines with the *in vitro* synthetised mutant allele. The fertility of the females carrying the *Ketel*^{D} transgenes was tested.

Production of the Ketel protein in bacteria and the generation of anti-Ketel polyclonal antibodies: A pGEX-Ketel plasmid was constructed first by the insertion of the BamHI-EcoRI fragment of the *Ketel* cDNA into the corresponding sites of a pGEX4T-1 expression vector and GST-Ketel fusion protein was produced in *E. coli*. The fusion protein consisted of the glutathione-S-transferase (GST) moiety fused in frame with the 147-884 amino acid encoding segment of the Ketel protein (Lippai, 1999). The GST-Ketel fusion protein was purified by affinity chromatography on a glutathion-agarose column and used for immunization of rabbits for the production of anti-Ketel polyclonal antibodies following standard protocols. After several boosts, the crude sera were analyzed for the presence of anti-Ketel antibody by Western blots. Two rabbits produced good titers of anti-Ketel sera by virtue of their ability to recognize the Ketel protein in *E. coli* extracts from strains with pGEX-Ketel but not in the control bacterial extracts.

For production of a nearly full length Ketel protein, we cloned the cDNA into the pET-His3A expression vector generating the pET-His-Ketel expression vector. The His-tagged Ketel protein, with amino acids 4-884, was purified by a Ni-chelating column and used for preparation of a Ketel protein affinity column.

The anti-Ketel antibody was purified in two steps: first on a protein-A and on a Ketel protein affinity column afterwards. The affinity purified anti-Ketel antibody was used both in Western blots and also in confocal microscopy for the detection of Ketel protein. For Western blots protein extracts were prepared from embryos, larvae and adults as well as from different

organs of late third instar larvae. For laser scanning microscopy ovaries were dissected, fixed and treated with antibodies. The Ketel protein was detected by the affinity purified polyclonal anti-Ketel rabbit antibody that was made visible by a goat anti-rabbit rhodamin-labeled secondary antibody (Jackson Laboratories Inc.). The NE was made visible with a primary monoclonal anti-lamin mouse antibody (Harel et al. 1989; Paddy et al. 1996), and a fluorescein-labeled anti-mouse secondary antibody (Jackson Laboratories Inc.). Optical sections were generated in a Zeiss LSM 410 confocal microscope.

The *in vitro* nuclear protein import assay: Drosophila importin-β cDNA was cloned into the SphI-XmaI sites of pQE30 expression vector (Qiagen), expressed with an NH_2-terminal His tag and purified, on nickel-NTA agarose, followed by chromatography on a Superdex 200 gel filtration column.

The nuclear protein import assay was conducted as follows. Permeabilized HeLa cells were prepared by a modification of a published 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 min in the same buffer containing 60 μg/ml digitonin. The coverslips were washed three times in permeabilization buffer without digitonin. Coverslips were incubated as indicated 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 RanGDP, 150 nM Rna1p, 300 nM NTF2, 150nM RanBP1). Nuclear import of a fluorescent substrate was monitored in optical sections. The substrate was the pentamer of a fusion protein in which the nucleoplasmin core domain was combined with the IBB domain from importin-α (IBB core pentamer). Import reaction samples contained 0.24 μM fluorescein-labeled IBB core pentamer. In the indicated reactions 1.2 μM Drosophila importin-β, Ran and an energy regenerating system were added. Reactions were stopped after 5 min by fixation in 3% paraformaldehyde (w/v) in PBS, washed in PBS, water, and mounted with 2 μl of vectorshield mounting medium (Vector).

The digitonin-permeabilized HeLa cell system was also used to follow nuclear import of the cNLS-phycoerythrin (cNLS-PE; Cserpán and Udvardy 1995) substrate in presence of cytosol samples prepared from ovaries of wild type and *Ketel*^{D}/+ females.

Germ line chimeras: We constructed germ line chimeras through the transplantation of pole cells, ancestors of the germ line (Illmensee 1973). In the chimeras functionally normal soma surrounded the germ line cells that were hemizygous for *ketel*^{rX13}, a *ketel*^{r} null allele. (In *ketel*^{rX13} ~ 9/10th of the 3' end of the ORF was deleted.) In practice, pole cells of the embryos from a cross between *ketel*^{rX13}/*Bc Gla* females and *ketel*^{rX32}/*CyO* males were transplanted into host embryos that derived from wild type (+/+) females and *Fs(1)KI237/Y* males. *Fs(1)K1237* (= *ovo*^{D1}) is a dominant female sterile mutation that disrupts function of the germ line cells without affecting the soma (Komitopolou et al. 1983; Busson et al. 1983). The eclosing *K1237/+* females were mated with *lt bw/lt bw* males. (The *ketel*^{rX13} and *ketel*^{rX32}-carrying chromosomes are labeled with the *lt* and the *bw* recessive marker mutations (Lindsley and Zimm 1992, Erdélyi et al. 1997).

Follicle cell mosaics: Follicle cell clones homozygous for *ketel*^{rX13} were generated through mitotic recombination. In practice, *ketel*^{rX13} *pr*/*Bc Gla* adult females were mated with *Fs(2)Ugra*/*Bc Gla* males. [Three independent *ketel*^{rX13} *pr* lines were recovered following meiotic recombination to remove possible second site lethal mutations that were induced during (i) the EMS treatment to induce the *Ketel*^{D1} mutation and (ii) the X-ray treatment when the *ketel*^{rX13} revertant allele was generated (Szabad et al. 1989; Erdélyi et al. 1997). The three lines gave identical results in the clonal analysis experiments and hence pooled data are presented in Tables 3 and 4. *Fs(2)Ugra* (= *Ugra*) is a dominant female sterile mutation that disrupts follicle cell function without affecting the germ line cells (Szabad et al. 1989, Szabad et al. 1991).] Early third instar larvae were X-irradiated for the induction of mitotic recombination by 1500 R of X-rays (150 kV; 0.5 mm Al filter, 1000 R/min). The eclosing *ketel*^{rX13}/*Ugra* and the control *lt bw*/*Ugra* adult females were tested for offspring production. Whether the *ketel*^{rX13}/*ketel*^{rX13} homozygous follicle cells can support egg development was decided by comparing the frequencies of follicle cell mosaicism in the *ketel*^{rX13}/*Ugra* and the *lt bw*/*Ugra* control females (Szabad et al. 1989).

Wing and tergite mosaics: Clones homozygous for the *ketel*^{rX13} allele were induced, through mitotic recombination, in *f*^{36a}/Y; *ketel*^{rX13}/*f*$^{+}$ *ck* larvae (See Figure 6.) . The *f*$^{+}$ symbol stands for a *forked*$^{+}$ transgene in the 30B cytological region. It compensates effects of the X-linked *f*^{36a} mutation (P. Martin and A. Garcia-Bellido personal communication). The *f*^{36a}, *ck* symbol represent a cell marker mutation that allow recognition of the different types of clones (Lindsley and Zimm 1992). Mitotic recombination was induced in young third instar larvae 72-80 hours after egg deposition (1500R, 150 kV, 0.5 mm Al filter, 1000 R/min). Following mitotic recombination, the majority of the *forked* (*f*) clones are homozygous for *ketel*^{rX13}. The *ck* twin clones served as reference in analysis of the *f* clones. Wings and abdomens of the *f*^{36a}/Y; *ketel*^{rX13}/*f*$^{+}$ *ck* males were mounted and analyzed for clones in a compound microscope. Types, frequencies and clone sizes were recorded.

The *Ketel-lacZ* reporter gene: We constructed a reporter gene in which a 1378 bp upstream segment of the *Ketel* gene between positions -1336 and + 42 was combined with the *E. coli lacZ* gene. (See the corresponding sequence under the accession number AJ002729 in the EMBL Nucleotide Sequence Database.) The 1378 bp fragment contains the entire *Ketel* promoter with the transcription start site (Lippai et al. 2000). The 1378 bp segment a was cloned into the *pP(CaSpeR-AUG-βgal)* P-element transformation vector (Thummel et al. 1988). Four germ line transformant lines of the reporter gene construct were generated using the *CaSpeR* vector with the *mini-white* marker gene. Two of the transgenes are inserted into the X, two into the 3rd chromosome. Flies homozygous for any of the transgenes are viable and fertile. β-galactosidase activities of transgene homozygotes were studied in adult, embryonic and larval stages of development according to standard procedures.

4. RESULTS

The *Ketel*^{D} mutant phenotype suggests NE related function of the normal *Ketel* gene product

The four *Ketel*^{D} dominant female sterile mutations of *Drosophila melanogaster* emerged following EMS mutagenesis (Szabad et al. 1989). The *Ketel*^{D}/+ females and the males are fully viable and male fertility is normal. However, the *Ketel*^{D}/+ females are sterile. Development of the egg primordia is normal in all the *Ketel*^{D}/+ females and the meiotic divisions are indistinguishable from wild type (J. Puro unpublished). The *Ketel*^{D}/+ females deposit normal numbers of normal looking so-called *Ketel*^{D} eggs. All the *Ketel*^{D} eggs are normally fertilized as revealed by the presence of sperm tail in the egg cytoplasm (Figure 3C). Like in wild type, the nuclei are well contoured in the newly deposited *Ketel*^{D} eggs, suggesting impact nuclei (Figure 3A). Severe *Ketel*^{D}-related defects appear 6-7 minutes after fertilization, when the female and the male pronuclei become juxtaposed (Figure 3C). In wild type, the daughter centrosomes separate and move around the perimeter of the male pronucleus to the opposite pole and organize the first mitotic spindle (Figure 3D, F). In six-to-seven minute old fertilized *Ketel*^{D} eggs the female and the male pronuclei are poorly contoured and suggest NE defects (Figure 3C). As a rule, disorganized masses of microtubules (MT) form instead of the gonomeric spindle . The MT mass appears as a prominent sperm aster and persists for several minutes (Figure 3E, G). The centrosome replicates, however the daughter centrosomes can not separate. The centrosomes may replicate 2-3 times but instead of separation they organize rudimentary asters of MTs along with a general decay of the egg cytoplasm (Figure 3I). The *Ketel*^{D} mutant phenotype is identical for the four *Ketel*^{D} alleles.

The *Ketel*^{D} egg cytoplasm prevents cleavage nuclei formation

Since the *Ketel*^{D} mutations have been known to be gain-of-function type (Szabad et al. 1989), the above described defects are most likely brought about by *Ketel*^{D}-encoded mutant gene products. To elaborate the above possibility we carried out egg cytoplasm injections. (1) First a sample of ~ 40 pl *Ketel*^{D} egg cytoplasm was injected into one side of the presumptive head region of each of 59 wild type cleavage embryos. The corresponding head structures were invariably missing at the site of injection. (In the control, where wild type cytoplasm

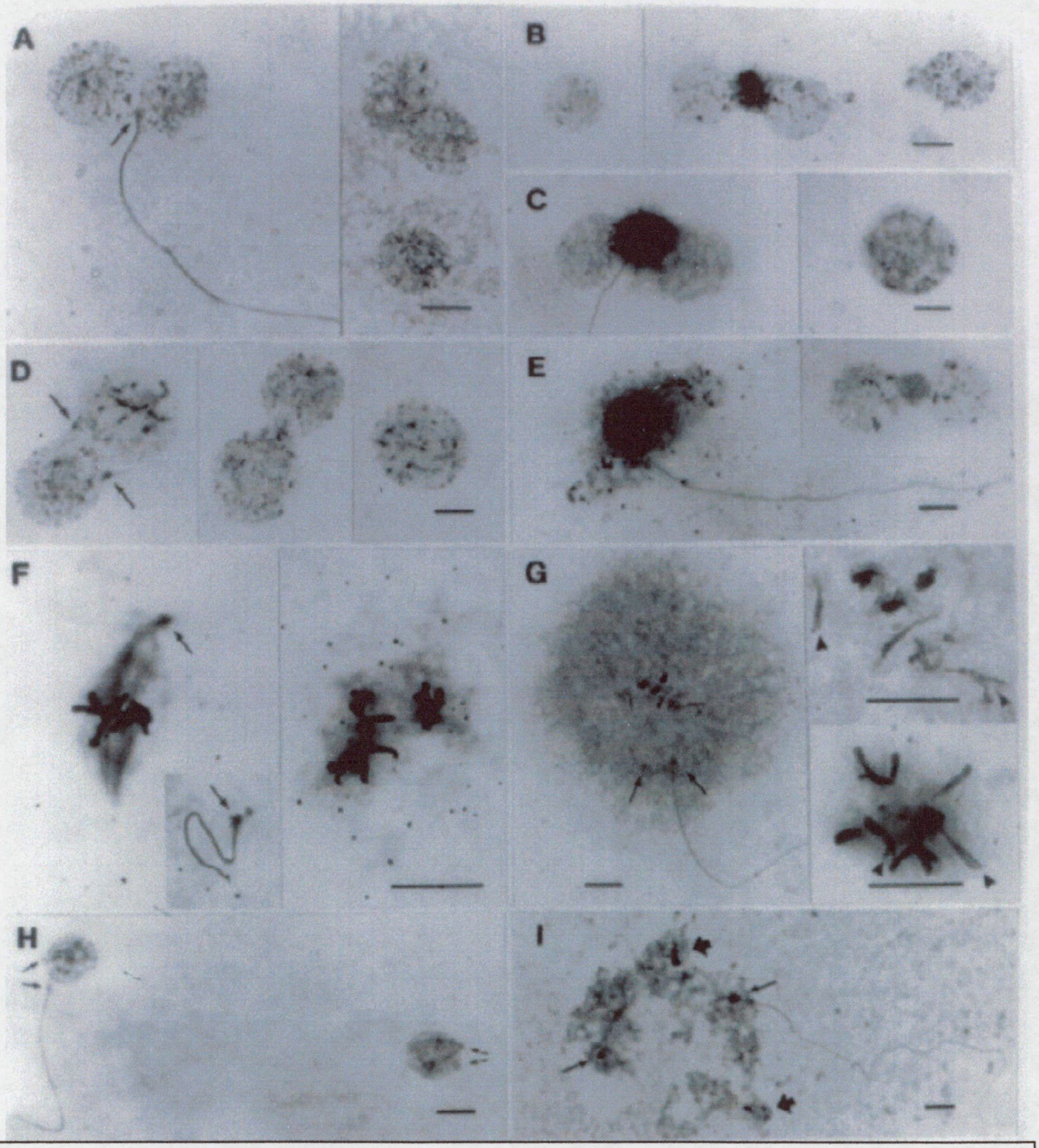

Figure 3. The first embryonic division cycle in Feulgen-Giemsa stained egg squashes of wild type (A, D, F, H) and *Ketel*^{D1}/+ females (B, C, E, G, I). See text for details.

anterior structures were entirely missing following the injection of ~300 pl *Ketel*^{D} egg cytoplasm per wild type embryo (44 embryos).

(2) In the second set of experiments, a small sample of cNLS-phycoerythrin (cNLS-PE) solution was first injected into newly deposited *Ketel*^{D} and (as control) into wild type eggs. A sample of the cNLS-PE containing egg cytoplasm was subsequently injected into wild type cleavage embryos and fate of the red fluorescent cNLS-PE substrate was followed in a laser scanning microscope. Whether the cNLS-PE was introduced in wild type or in *Ketel*^{D} egg cytoplasm, the cNLS-PE substrate readily entered the cleavage nuclei, implying that the *Ketel*^{D}-encoded molecules do not prevent nuclear import of the cNLS-PE substrate (Figure 4A and D).

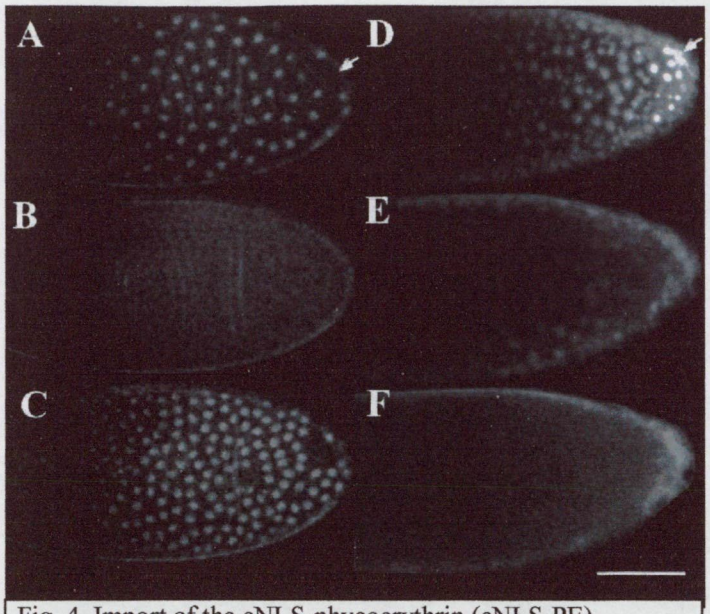

Fig. 4. Import of the cNLS-phycoerythrin (cNLS-PE) fluorescent substrate into nuclei of wild type cleavage Drosophila embryos. The cNLS-PE substrate was co-injected with wild type (A-C) or with *Ketel*^{D1} (D-F) egg cytoplasm at positions shown by the arrows. Import of the cNLS-PE molecules into the nuclei was followed in a laser scanner microscope. Photographs A and D, B and E and C and F were taken 15, 22 and 29 minutes following cytoplasm injections, respectively. Scale bar = 100 μm.

In the control the cNLS-PE substrate was essentially homogeneously distributed during mitosis in the egg cytoplasm (Fig. 4B). The cNLS-PE substrate highlighted the nuclei – that doubled is numbers - upon onset of the next interphase (Fig. 4C). Following *Ketel*^{D} egg cytoplasm injections some nuclei appeared normal, however many small nuclei-looking structures appeared (Fig. 4D). Both the small and the normal sized nuclei entered mitosis (with some delay as compared to control), however nuclei did not form at the end of mitosis as indicated by the homogeneously distributed cNLS-PE substrate in the egg cytoplasm (Fig. 4F). Results of the cytoplasm injection experiments clearly showed that the *Ketel*^{D}-related defects are brought about by mutant *Ketel*^{D}-encoded gene product.

The *Ketel*^{D} alleles are very strong antimorph mutations

To find out whether the *Ketel*^{D}-encoded products participate in the same process as the wild type counterpart (i.e. the *Ketel*^{D} alleles are antimorph mutations; Muller 1932) or they disrupt a process in which the normal Ketel gene products are not involved (i.e. the *Ketel*^{D} alleles are neomorphs), we constructed females that, in addition to a *Ketel*^{D} allele carried two

or three normal *Ketel* gene copies and studied whether dominant female sterility can be overcome in the *Ketel^{D}*/+/+ and in the *Ketel^{D}*/+/+/+ females. Two systems were analyzed.

(1) In the *XXTp(2;Y)G*; *Ketel^{D}*/*CyO* females the *Tp(2;Y)G* chromosome carried a normal *Ketel* gene as part of the second chromosome transposed onto a Y chromosome. The females were mated with *XTp(2;Y)G*; +/*CyO* males. About 1% of the eggs of the 446 *XXTp(2;Y)G*; *Ketel^{D2}*/*CyO* females turned brown indicating the progression of embryogenesis to the stage of embryonic cuticle formation. (An analysis of the cuticles of the deceased embryos revealed gross cell death apparently without any preference to the different body regions; J. Szabad unpublished.) In addition, 56 offspring descended from the total of 466 *XXTp(2;Y)G*; *Ketel^{D2}*/*CyO* females during the three week test period. [The rate of offspring production was 5.6×10^{-3} offspring/(female x day)]. Of the 56 offspring 13 females carried the *Ketel^{D2}* allele and were sterile. Analysis of the *Ketel^{D2}*/+/+ females showed the strong antimorph nature of *Ketel^{D2}* and implies involvement of the *Ketel^{D2}*-encoded and the normal *Ketel* gene products in the same process. The *Tp(2;Y)G* chromosome did not reduce sterility imposed by the other three *Ketel^{D}* alleles: every egg remained white and not a single offspring descended from the 1196, 1341 and 945 *XXTp(2;Y)G*; *Ketel^{D}*/*CyO* females that carried the *Ketel^{D1}*, the *Ketel^{D3}* and the *Ketel^{D4}* alleles, respectively.

(2) The *Ketel^{+}* transgenes: To prove that the cloned gene is indeed *Ketel*, we generated three different types of altogether 21 *Ketel^{+}* (*K^{+}*) transgenes (Fig. 5A and B and Table 1) and analyzed their effects on both *ketel^{r}* and *Ketel^{D}* mutations. Nine of the twelve tested *K^{+}* transgenes brought about full rescue of lethality associated with the loss-of-function *ketel^{rX13}*/– genotype: the *K^{+}*; *ketel^{rX13}*/– flies developed with the expected frequencies and were fully fertile, showing that the cloned gene is *Ketel* (Table 1; *ketel^{rX13}* is a null allele).

Type I and every of the three tested type III *K^{+}* transgenes brought about slight rescue of the *Ketel^{D2}*-associated dominant female sterility. In about 1% of the eggs deposited by the *K^{+}*; *Ketel^{D2}*/+ females embryogenesis progressed to the stage of embryonic cuticle formation and even a few offspring developed from the *K^{+}*; *Ketel^{D2}*/+ females. However, the rate of offspring production was as low as $2\text{-}4 \times 10^{-3}$ offspring/(female x day), as compared to the about 50 offspring/(female x day) control value. (It should be noted that cuticle did not develop and offspring did not derive in/from tens of thousands of eggs deposited by *Ketel^{D2}*/+ females.)

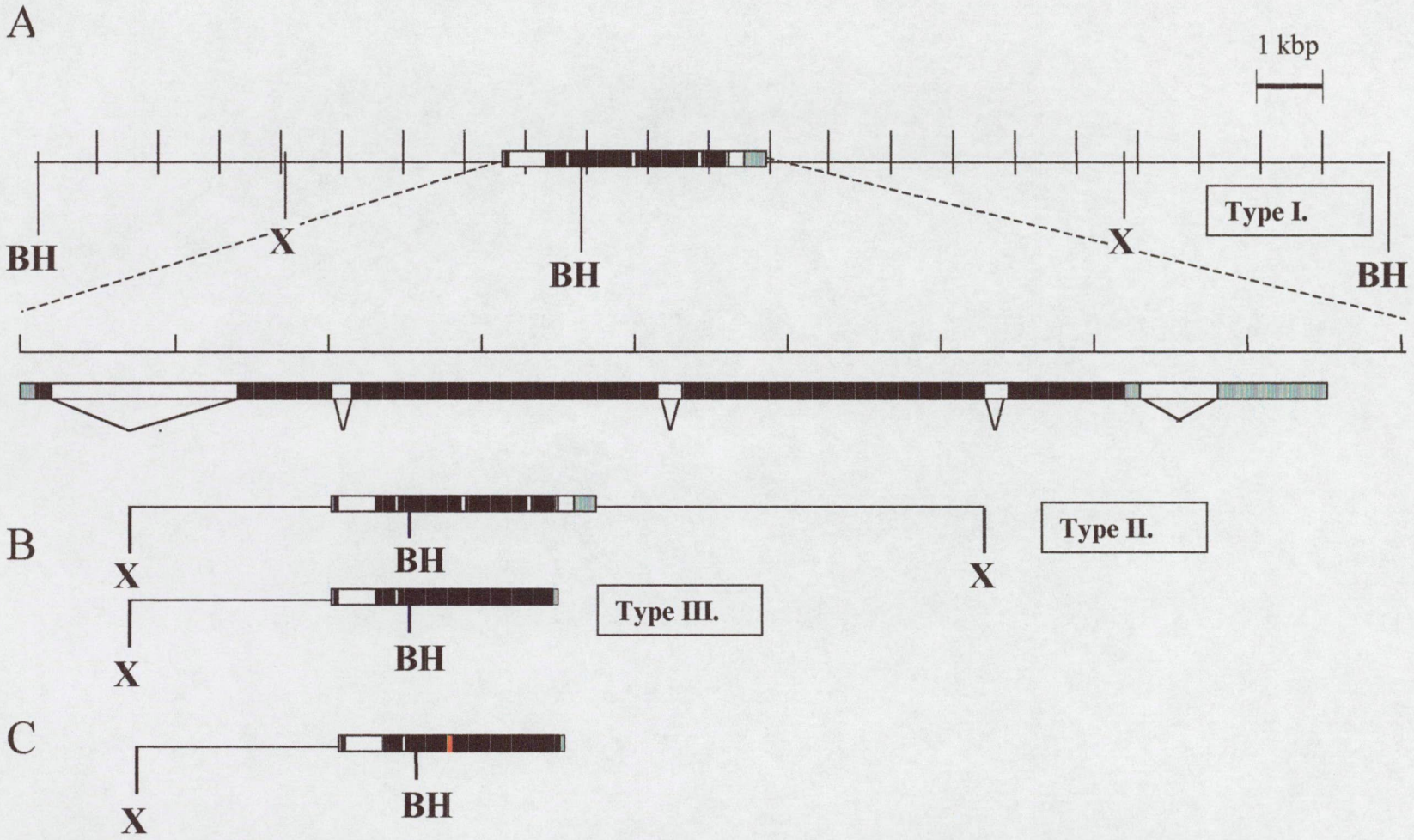

Figure 5. The 38E1.2-3 cytological region comprising the *Ketel* gene (A). The box on the line below represents the *Ketel* mRNA encoding region. Detailed genomic map of the *Ketel* mRNA encoding region is shown below. Exons, introns and the untranslated 5' and 3' regions are indicated by black, white and dotted boxes, respectively. (B). Structure of two of the three K^+ transgenes. Type I comprises the entire 22 kb genomic fragment shown on (A). Type II is the 13.8 kb XbaI genomic fragment. Type III contains the XbaI - BamHI 4.0 kb genomic fragment combined in frame with a 2.3 kb BamHI – EcoRI segment of the cDNA. (C) The transgene with the $Ketel^D$ allele. Red line indicates the position of the transition present in three of the four $Ketel^D$ alleles.The indicated restriction sites are as follows: BH = BamHI; X = XbaI.

One copy of the K^+ transgenes had no effects on the other three *Ketel*^{D} mutations. However, two copies of the K^+ transgenes brought about slight reduction of female sterility when sperm with two normal *Ketel* gene copies fertilized the eggs (Tirián et al. 2000). The slight rescue of the K^+ transgenes on *Ketel*^{D}-associated dominant female sterility clearly shows that (i) the cloned gene is *Ketel* and (ii) the normal and the *Ketel*^{D}-encoded gene products participate in the same process and hence the *Ketel*^{D} alleles are strong antimorph mutations.

In the second set of experiments we made use of three types of altogether seven *Ketel*$^+$ (K^+) transgenes linked to the X and to the third chromosomes (see Figure 5). Since effects of the transgenes were very similar, results related to only one of the X-linked K^+ transgenes is presented. The transgene carries a 22 kb genomic fragment including the promoter and the structural parts of the *Ketel* gene. Flies homozygous for the transgene are fully viable and fertile. We constructed both K^+/X; *Ketel*^{D}/+ and K^+/K^+; *Ketel*^{D}/+ females in which the wild type:*Ketel*^{D} ratios were 2:1 and 3:1, respectively. One group of the females was mated with wild type (X/Y; +/+), the another group with K^+/Y; +/+ males (Table 1). As in case of the *Tp(2;Y)G* chromosome, a slight reduction of female sterility was apparent in the K^+/X; *Ketel*^{D2}/+ females: cuticle developed in about 1% of the eggs and a few offspring descended (Table 1). Apparently offspring production of the K^+/X; *Ketel*^{D2}/+ females significantly increased when they were mated with K^+/Y; +/+ males showing contribution of paternal rescue of the mutant phenotype. Female sterility was further reduced in the K^+/K^+; *Ketel*^{D2}/+ females, especially when they were mated with K^+/Y; +/+ males (Table 1).

Table 1. Effects of an X-linked *Ketel*$^+$ K^+) transgene on *Ketel*^{D}-related dominant female sterility

Ketel^D allele	Male partner	Genotype of tested females and the features of offspring production							
		K^+/X; *Ketel*^{D}/+ females				K^+/K^+; *Ketel*^{D}/+ females			
		Tested	Offspring	Test period[1]	Rate of offspring production[2]	Tested	Offspring	Test period[1]	Rate of offspring production[2]
Ketel^{D1}	*XY*; +/+	233	0	25.1	-	250	0	19.6	-
	K^+Y; +/+	201	0	26.0	-	217	21	14.4	6.7×10^{-3}
Ketel^{D2}	*XY*; +/+	237	2	17.8	0.5×10^{-3}	261	13	12.7	3.9×10^{-3}
	K^+Y; +/+	172	11	19.6	3.3×10^{-3}	169	15	16.0	5.5×10^{-3}
Ketel^{D3}	*XY*; +/+	188	0	19.5	-	179	0	22.8	-
	K^+Y; +/+	167	0	17.8	-	163	15	11.1	8.3×10^{-3}
Ketel^{D4}	*XY*; +/+	168	0	27.2	-	433	0	27.5	-
	K^+Y; +/+	365	0	26.9	-	449	0	25.6	-

[1] Average test period per female (days). [2] Offspring/(female x day).

As expected, one copy of the K^+ transgene did not overcome female sterility brought about by the other three *Ketel*^{D} alleles, irrespectively whether the K^+/X; *Ketel*^{D}/+ females were mated with wild type or with K^+/Y; +/+ males (Table 1). The K^+/K^+; *Ketel*^{D}/+ females were also sterile when mated with wild type males (Table 1). However, when mated with K^+/Y; +/+ males, the K^+/K^+; *Ketel*^{D1}/+ and the K^+/K^+; *Ketel*^{D3}/+ females yielded a few offspring (Table 1). About 50% of the progeny females carried the *Ketel*^{D} allele. Results of the transgene experiments confirmed (i) the highly toxic nature of the *Ketel*^{D}-encoded gene products and (ii) that three of the *Ketel*^{D} alleles are very strong antimorph mutations. Furthermore the paternal rescue of embryonic lethality shows expression and function of the zygotic *Ketel* gene during embryogenesis.

Identification of the *Ketel*^{D} mutations: The maternal dowry supports life of the *Ketel*^{D}/- hemizygous individuals up to the second instar stage. We isolated DNA from *Ketel*^{D}/- hemizygous larvae and amplified fragments of the *Ketel* mutant alleles with PCR. Sequencing of the fragments revealed a single nucleotide difference between the *Ketel*^{D3} allele and the wild type sequence. This transition results in a missense mutation, the CCC (Pro) codon in the 445th position turns to CUC (Leu). The mutation resulted at the same time in a new XhoI site. Restriction digestion of the appropriate PCR fragments from the DNA of the *Ketel*^{D1}/-, *Ketel*^{D2}/- and *Ketel*^{D4}/- hemizygous individuals revealed the same nucleotide exchange in the *Ketel*^{D1} and the *Ketel*^{D4} allele. The in vitro exchange of the above nucleotide in a transformation vector (see figure 5C) confers female sterility in transgenic *Ketel*^{D}/+/+ flies, giving thus an evidence, that the Pro-Leu exchange at the 445th position is responsible for the dominant female sterility. The importance of the proline residue is supported by the observation that proline is conserved among the importin-β proteins in all examined multicellular eucaryotic organisms.
The position of the mutation in the *Ketel*^{D2} allele is not known yet.

The *ketel*^{r} alleles reveal zygotic requirement of the *Ketel* gene: We generated, through second mutagenesis of the *Ketel*^{D} alleles, 27 loss-of-function *ketel*^{r} alleles. Twenty-five of the 27 *ketel*^{r} alleles are recessive zygotic lethal mutations. The most severe *ketel*^{r}/*ketel*^{r} homo- and the *ketel*^{r}/– hemizygous combinations, including *ketel*^{rX13}/–, bring about death during second larval instar. (The – symbol stands for *ketel*^{rX32}, a small deficiency that removes the *Ketel* and

a few adjacent loci; Erdélyi et al. 1997.) The mutant larvae become sluggish and decease within a day without any apparent morphological defect. Since second larval instar death is the most severe defect associated with the *ketel*^{r} alleles, it represents most likely the complete loss-of-function mutant phenotype. The zygotic lethal nature of the *ketel*^{r} alleles clearly shows zygotic requirement of the *Ketel* gene.

When paternally derived the *Ketel*^{D} mutations behave as the zygotic lethal *ketel*^{r} alleles: the *Ketel*^{D}/*ketel*^{r} and the *Ketel*^{D}/– larvae also perish during second larval instar and can not be distinguished from the *ketel*^{r}/– ones.

Germ line chimeras without functional *Ketel* gene revealed important features of *Ketel* gene function: To decide whether short life of the *ketel*^{r}/– hemizygotes is made possible by the normal *Ketel* gene products provided by the +/– mothers, we constructed germ line chimeras with normal soma and *ketel*^{r}/– female germ line cells that lacked functional *Ketel* gene (Table 2). The chimeras deposited normal-looking eggs. Although the eggs were fertilized, embryogenesis did not commence inside them due to the lack of cleavage nuclei formation following fertilization, and the defects were indistinguishable from those described for the *Ketel*^{D} eggs.

Table 2. Features of the germ line chimeras with normal soma and mutant germ line.

The *ketel*^{rX13}/– germ line chimeras			The *Ketel*^{D1}/– germ line chimeras		
Genotype of the germ line cells	Number of chimeras	The rate of egg production[1]	Genotype of the germ line cells	Number of chimeras	The rate of egg production[1]
Bc Gla/Cy Roi	3		*Bc Gla/Cy Roi*	2	
Ketel^{rX13}/*Bc Gla*	4	9.3 ± 6.7[2]	*Ketel*^{D1}/*Bc Gla*	2	7.7 ± 5.3[2]
Ketel^{rX32}/*Cy Roi*	3		*Ketel*^{rX32}/*Cy Roi*	2	
Ketel^{rX13}/*ketel*^{rX32}	2	6.8 ± 4.5	*Ketel*^{D1}/*ketel*^{rX32}	3	5.9 ± 3.8

[1] = Egg/(chimera x day). Average and standard deviation over a ten day test period.
[2] = For the chimeras with *Bc Gla* and/or *Cy Roi* chromosomes.
Notes.
- The donor embryos derived from a cross between *ketel*^{rX32}/*Bc Gla* females and
- *ketel*^{rX13}/*Cy Roi* males in the *ketel*^{rX13}/– chimeras and
  ketel^{rX32}/*Bc Gla* females and *Ketel*^{D1}/*Cy Roi* males in the *Ketel*^{D1}/– chimeras.
- The *ketel*^{rX32} allele is a small deficiency that removes the *Ketel* and a few adjacent loci (Erdélyi et al. 1997).

Analysis of the germ line chimeras revealed three features of *Ketel* gene requirement. (1) Function of the *Ketel* gene is not required in the female germ line since the *ketel*^{r}/– cells are viable and are sources of normal-looking eggs. (2) Since embryogenesis does not commence in eggs of the above chimeras, development of the *ketel*^{r}/– larvae to the second larval instar

must be supported by the Ketel maternally dowry present in the egg cytoplasm. (Maternal support of embryogenesis is a rather general phenomenon in Drosophila; for a recent review see Szabad 1998.) (3) The identical phenotype seen in eggs of the above germ line chimeras and in the *Ketel^{D}* eggs shows that the *Ketel^{D}*–encoded mutant gene products impede function of the normal *Ketel* gene products, i.e. the *Ketel^{D}* alleles are dominant negative mutations.

To further clarify function of the *Ketel^{D}* alleles, we constructed germ line chimeras with normal soma and *Ketel^{D1}*/– germ line cells (Table 2). Like in the *ketel^{r}*/– germ line chimeras, the *Ketel^{D1}*/– germ line cells allowed proliferation of the female germ line cells and were sources of normal looking eggs. However, embryogenesis did not commence in their eggs due to the lack of cleavage nuclei formation.

Function of the *Ketel* gene is not required in the follicle cells: The normal-looking eggs that derive from mosaic egg primordia with *ketel^{r}*/– germ line cells and normal follicle cells may develop because (i) function of the *Ketel* gene is not required in the germ line, or (ii) the follicle cells compensate *Ketel* gene function absent in the germ line. To distinguish between the above possibilities, we generated mosaic egg primordia in which some or all the enveloping follicle cells lacked *Ketel* gene function and the germ line cells were normal. To produce the latter type of mosaic egg primordia, we X-irradiated *ketel^{rX13}/Ugra* larvae for the generation - through mitotic recombination – of *ketel^{rX13}/ketel^{rX13}* follicle cell clones. Egg and progeny production of the eclosing females was monitored. Results of the experiment can be summarized as follows (Table 3). (1) Similar frequencies of the *ketel^{rX13}/Ugra* and the *+/Ugra* control females were mosaic. (2) The two types of mosaic females produced eggs with similar rates. However, the larvae hatched form eggs of the *ketel^{rX13}/Ugra* females with a reduced rate as compared to the control females (Table 3).

Table 3. Characteristics of the *ketel^{rX13}* homozygous follicle cell clones.

Specimen[1]	Females			Egg production			
	Tested	Mosaic	Frequency of mosaicism (%)	Eggs deposited	Egg/(female x day)	Hatched	Egg hatch rate (%)
Control	132	16	12.1	37	0.29	18	48.6
Ketel^{rX13}	379	43	11.3	80	0.23	23	28.8

[1] = *lt bw/Fs(2)Ugra* (as control) and *ketel^{rX13}/Fs(2)Ugra* females were irradiated as 3rd instar larvae and the developing adult females were tested.

The *ketel*^{null} homozygous clones are fully viable on the wings and the tergites: To further characterize requirement of the *Ketel* gene in another cell types of the soma, we analyzed clones of *ketel*^{rX13} homozygous wing and tergite cells. The *ketel*^{rX13} homozygous clones were induced through mitotic recombination in *f*^{36a}/Y; *ketel*^{rX13}/*f*$^{+}$ *ck* young third instar larvae. Most of the *forked* homozygous clones were homozygous for *ketel*^{rX13}. As summarized in Table 4, frequencies and sizes of the different types of clones were similar in the *ketel*^{rX13} and in the control experiments in both the wings and the tergites.

Table 4. Characteristics of the *ketel*^{rX13} homozygous wing and tergite clones.

Specimen	Wings						Tergites					
	Screened	Types of clones			Clone size[1]		Screened	Types of clones			Clone size[2]	
		f/*ck*	*f*	*ck*	*f*	*Ck*		*f*/*ck*	*f*	*ck*	*f*	*ck*
Control	20	52	11	18	6.6 ± 1.7	6.4 ± 1.9	20	42	12	16	4.4 ± 2.6	4.0 ± 1.9
Ketel^{X13}	20	45	13	10	6.2 ± 1.5	6.6 ± 2.2	20	52	12	9	4.2 ± 2.0	4.2 ± 2.0

Notes
[1] = the average number of cell divisions required to reach the observed clone size.
[2] = the average number of bristles included in a clone.

Several of the *f* clones included as many as 70-100 cells. As much as seven rounds of cell divisions are required following mitotic recombination to reach clones of that size. Features of the *f* clones revealed that *Ketel* gene function is not required for life and function of the wing imaginal disk and the abdominal histoblast cells.

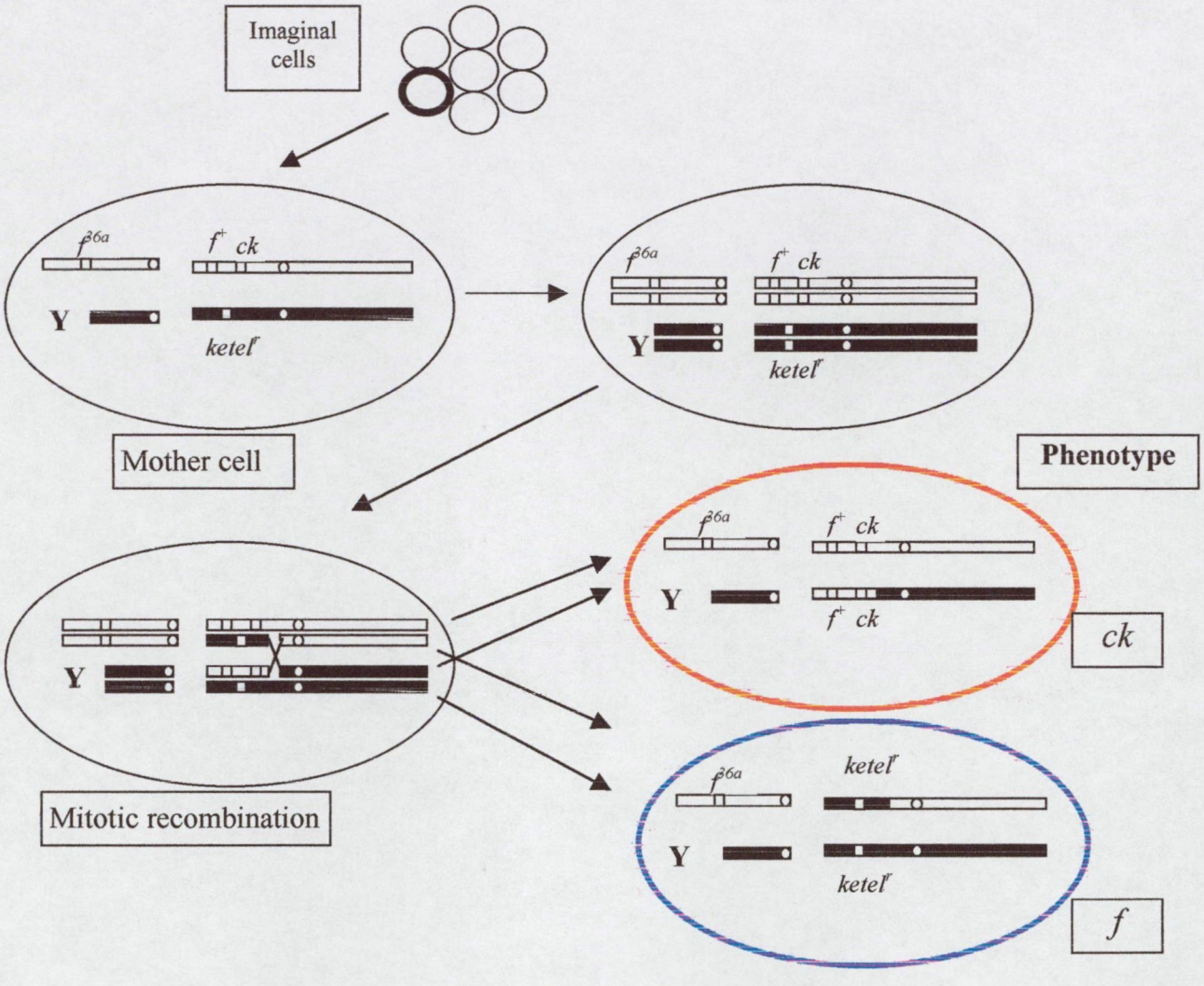

Figure 6. Generation of homozygous *ketel*^{0} clones by mitotic recombination. The *f* clones are homozygous for the *ketel*^{0} allele.

The Ketel protein supports nuclear protein import

To decide whether the Ketel protein does indeed function as importin-β we monitored (i) the docking on the cytoplasmic surface of the NE and (ii) import into nuclei of digitonin-permeabilized HeLa cells of a fluorescent labeled nuclear substrate in the presence of the Ketel protein and other components of the nuclear protein import apparatus (see Materials and Methods). As shown on Figure 7B, the substrate docked on the NE in presence of the Ketel protein, and when Ran and an energy source were added the substrate was imported into the nuclei (Fig. 7B and D). The permeabilized HeLa cell experiments clearly showed that the Ketel protein molecules function as importin-β: they support docking and import of nuclear proteins into the nuclei.

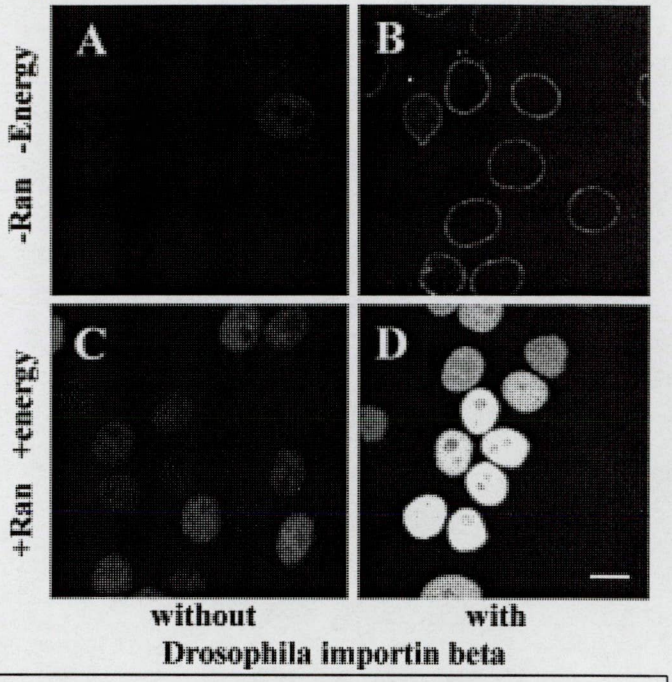

Figure 7. Ketel protein functions as importin-β: it promotes the docking on the NE and nuclear import of a fluorescent import substrate as detected in optical sections. Without addition of Ran and energy supply (A) or with Ran and energy supply (C) only background signals appear due to residual components in the digitonin-permeabilized HeLa cells. When Ketel protein is added and no energy supply the substrate docks on the cytoplasmic surface of the NE (B). When, however, Ran, energy supply and Ketel protein are added the substrate is imported into the nuclei (D). Scale bar = 10 μm.

To understand effects of the *Ketel*^{D} mutations on nuclear protein import, we prepared cytosol form ovaries of both *Ketel*^{D}/+ and wild type females. All the four *Ketel*^{D} mutations were included in the present study. The cytosol preparations were used in the permeabilized HeLa cell assay and import of the cNLS-PE substrate was monitored (see Materials and Methods). In presence of the wild type ovary cytosol the cNLS-PE substrate entered nuclei of HeLa cells within a few minutes. Surprisingly, the *Ketel*^{D}/+-derived cytosol preparations supported nuclear import of the cNLS-PE substrate just as efficiently as the wild type ovary cytosol showing that the *Ketel*^{D}-encoded mutant molecules do not interfere with import of the cNLS-PE substrate. Note that when injected into wild type cleavage embryos, traces of the *Ketel*^{D} egg cytoplasm prevent the formation of cleavage nuclei at the end of mitosis. However, nuclei of the digitonin-permeabilized HeLa cells remained intact for at least four hours in presence of the *Ketel*^{D}/+ derived ovary cytosol.

Expression pattern of the *Ketel* gene: To study expression pattern of the *Ketel* gene, we detected both *Ketel* mRNA and Ketel protein during oogenesis and embryogenesis. Some *Ketel* mRNA, as detected with the Digoxigenin-labeled *Ketel* cDNA, is present in nurse cells of the stage 9 egg primordia. The concentration of *Ketel* mRNA becomes rather high by stage 10 (Figure 8A) when dumping of the *Ketel* mRNA into the oocyte cytoplasm begins. Beyond stage 11 the *Ketel* mRNA is homogeneously distributed in the oocyte cytoplasm (Fig. 8B). The *Ketel* gene appears to be ubiquitously expressed in every blastoderm cell (Fig. 8C) and, as far as it can be deduced from the staining patterns, also during later stages of embryogenesis (Fig. 8D-F). The *Ketel* gene seems to be intensively expressed in the central nervous system and in the larval gonads (Fig. 8E and F). The larval gonads include both ovaries and testes since the gonads possess intensive staining in every of the embryos.

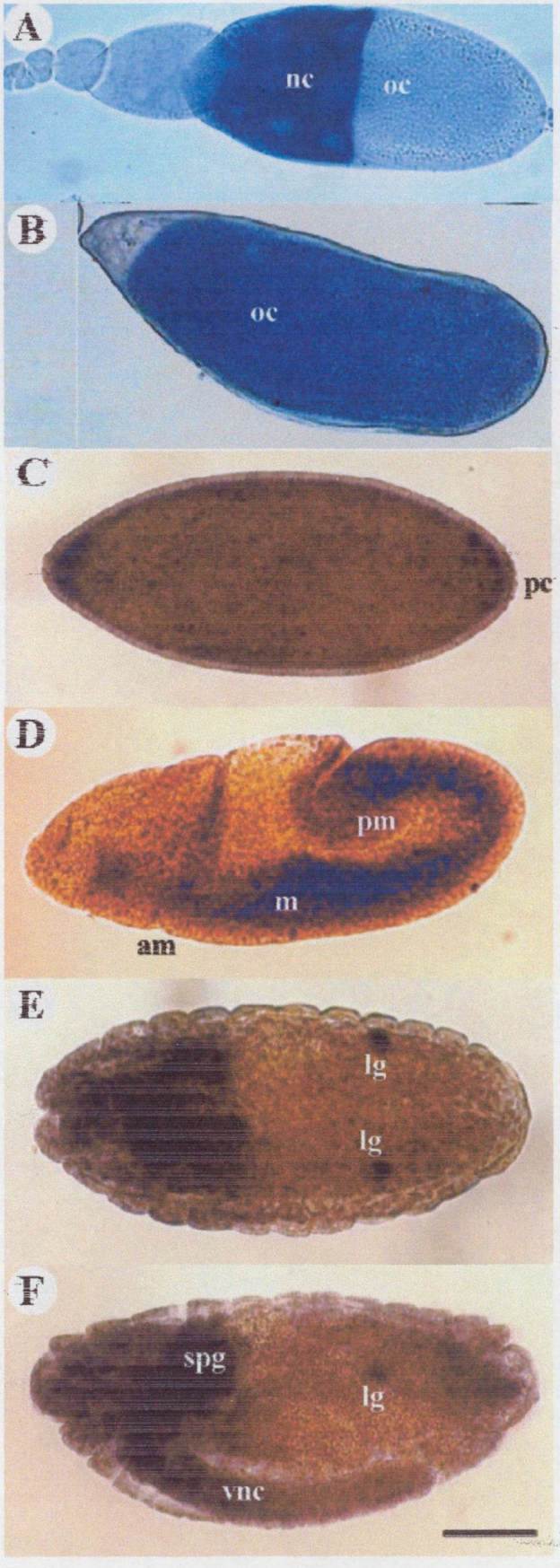

Figure 8. *In situ* hybridizations for the detection of Ketel mRNA during oogenesis (A and B) and different stages of embryogenesis: cellular blastoderm stage 5 (C), stage 8 (D), stage 14 (E) and stage 17 (F) embryos. Lateral (C, D and F; anterior left and dorsal up) and dorsal (E) views. The abbreviations are as follows: nurse cells (nc) oocyte (oc), pole cells (pc), anterior midgut primordium (am), posterior midgut primordium (pm), mesoderm (m), larval gonad (lg) supra oesophageal ganglion (spg), ventral nerve cord (vnc) (embryos were staged as described in Campos-Ortega and Hartenstein 1997). Bar = 50 µm.

We also followed *Ketel* gene expression through the detected Ketel protein with the affinity purified polyclonal anti-Ketel antibody. Localization of the Ketel protein was also followed in the course of oo- and embryogenesis by immunocytology and confocal microscopy. The Ketel protein is first detectable in nurse cells during stage 8 of oogenesis (Fig. 9 A). By stage 10 the nurse cells contain large quantities of the Ketel protein. The protein is cytoplasmic with pronounced accumulation in the NEs (Fig. 9A and C). Nurse cells dump their Ketel protein contents into the oocyte cytoplasm from stage 11 of oogenesis. The follicle cells also contain Ketel protein (Fig. 9A). Cytoplasm of a newly deposited egg contains stockpiles of the Ketel protein. During cleavage divisions the Ketel protein is present throughout the cleavage cycles. It is cytoplasmic and shows accumulation in the NE (Fig. 9D and E).

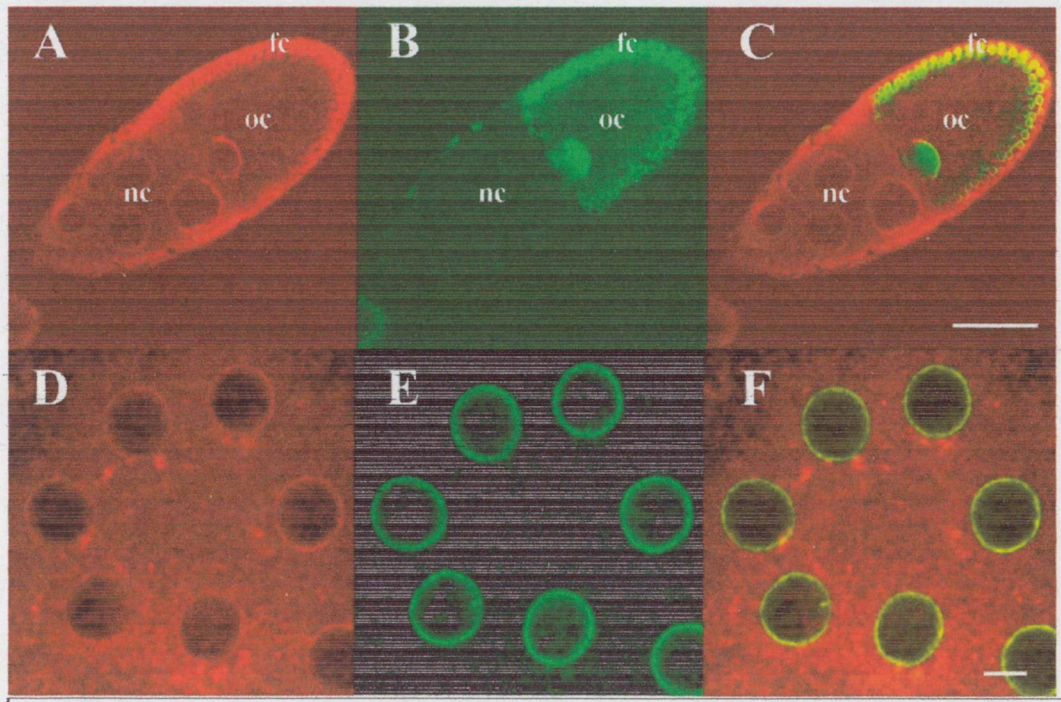

Figure 9. Distribution of the Ketel protein, as detected in optical sections, in a stage ten egg primordium and in an interphase cleavage embryo. The Ketel protein is shown in red (A and D), the nuclear lamina appears in green (B and E). Merged signals are shown on C and F where yellow coloration results form superimposition of green and red signals. Abbreviations are as follows: nc = nurse cells; oc = oocyte and fc = follicle cells. Scale bar = 50 μm for A-C and 5 μm for D-E.

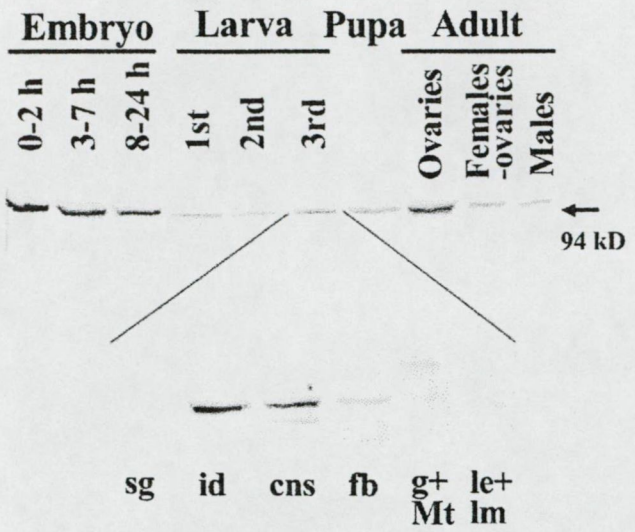

Figure 10. Western blot analysis to detect Ketel protein with a polyclonal anti-Ketel antibody. Equal amounts of protein samples were loaded in the different slots. With respect to third instar larval organs, the central nervous system (cns) did not include the ring gland and the larval gonads were removed from the fat body (fb) sample. The further letter codes stand for the following organs: sg = salivary glands; id = imaginal discs; g + Mt = gut and Malpighian tubules; le + lm = larval epidermis with the overlaying larval musculature.

To study the protein amount during development we performed a developmental Western analysis. The anti-Ketel antibody detected a single 97 kD protein band on Western blots with extracts prepared from different developmental stages (Fig. 10). The Ketel protein is abundant in the ovaries, in the newly deposited eggs, throughout embryogenesis and is present throughout all stages of development. However, when compared e.g. to ovaries, the relative Ketel protein concentration was rather low in larvae and adult females from which the ovaries were removed. To clarify the low Ketel protein content we dissected different organs from late third instar larvae and subjected to Western blot analysis. As shown on Fig 10, while e.g. the imaginal discs contained significant amounts of the Ketel protein, there were no detectable amounts of Ketel protein present in a number of larval tissues including the salivary glands, gut, Malpighian tubules, larval epidermis with the overlaying larval musculature.

To charachterise further the expression pattern of the *Ketel* gene, we generated four transgenes in which the *Ketel* promoter regulated expression of a *lacZ* reporter gene, and analyzed β-gal activities in embryos as well as in different larval and adult tissues. The reporter gene is expressed in the ovaries and its expression pattern in the ovaries and during embryogenesis is identical with that detected by RNA in situ hybridizations and by the anti-Ketel antibody. The reporter gene is also expressed in the testes and sperm pump. However, the reporter gene is not expressed in the bulk of the adult tissues. (Some β-gal activities appeared in the central nervous system. The blood-producing organ could not be included in the reporter gene analysis due to its intrinsic β-gal expression.) In third instar larvae the

reporter gene is intensively expressed in all the imaginal discs, in the larval gonads, in the imaginal ring cells of salivary glands and in the ring gland (Fig. 11). There was no β-gal activity present in most larval cells, including the salivary glands, fat body, larval epidermis, larval musculature and Malpighian tubules (Fig. 11).

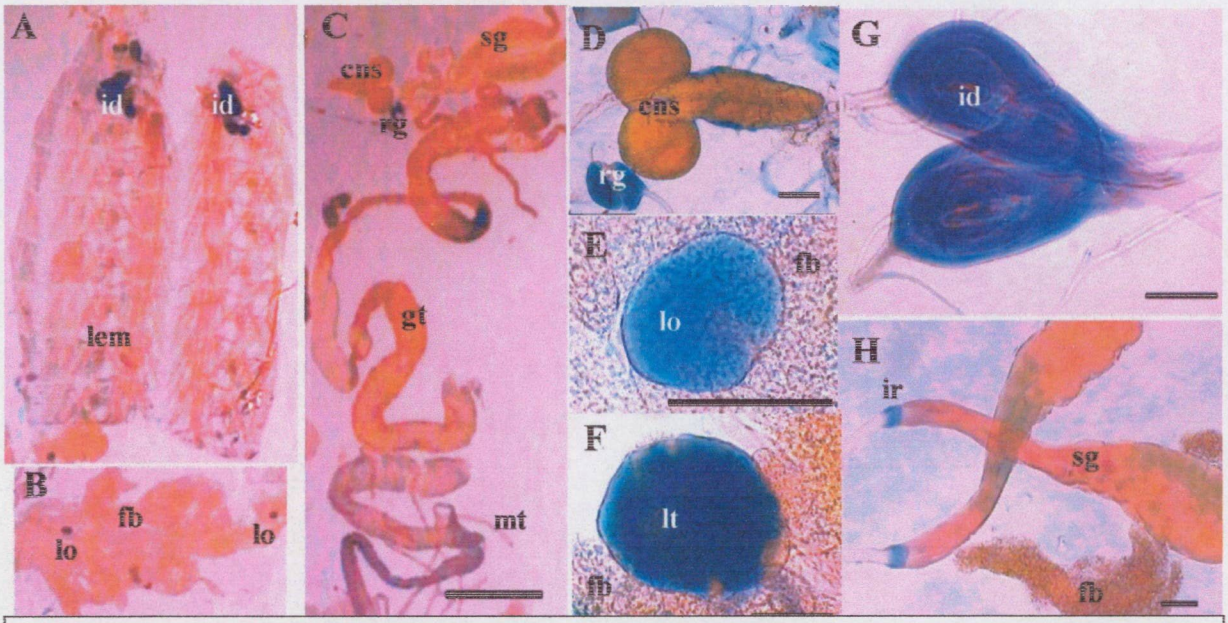

Figure 11. Expression pattern of a *Ketel* promoter-operated *lacZ* reporter gene in different tissues of late third instar larvae. Blue staining corresponds to expression of the *Ketel* gene as reveled through activities of β-galactosidase molecules. The reporter gene is expressed in all the imaginal discs (id), in the larval ovaries (lo) and testes (lt), in imaginal rings of the salivary glands (ir) and in the ring gland (rg). The reporter gene is not expressed in the larval epidermis and the overlaying muscles (lem), in the fat body (fb), in the salivary glands (sg), the central nervous system (cns), the gut (gt) and the Malpighian tubules (mt). (The slight staining in different sections of the digestive tract is due to endogenous β-galactosidase activities.) Scale bar = 1 mm on A-C and 100 μm on D-H.

5. DISCUSSION

The *Ketel* gene encodes the Drosophila homologue of importin-β

The *Ketel* gene was identified by four EMS-induced *Fs(2)Ketel* (= $Ketel^D$) dominant female sterile mutations and their $ketel^r$ revertant alleles (Szabad et al. 1989; Erdélyi et al. 1997). The $Ketel^D$ alleles are gain-of-function type and bring about dominant female sterility by inhibiting the commencement of embryogenesis. The $Ketel^D$-encoded gene products prevent cleavage nuclei assembly at the end of mitosis by, as it appears, disrupted NE formation/function and suggest a NE-related function of the normal *Ketel* gene product (Tirián et al. 2000). Most of the loss-of-function $ketel^r$ alleles are zygotic lethal mutations that cause death during second larval instar showing zygotic requirement of the *Ketel* gene (Erdélyi et al. 1997). To understand molecular *Ketel* gene function and by way of it novel aspects of the initiation of embryogenesis, we cloned the *Ketel* gene. Genomic Southern, developmental Northern and Western analyses reveled that the single copy *Ketel* gene encodes a single type of 3.6 kb mRNA and synthesis of the corresponding 97 kD Ketel protein. (Lippai et al. 2000)

To show that the cloned gene is indeed *Ketel*, we generated different types of K^+ transgenes. As the transgenes bring about rescue of $ketel^r$-associated lethality it is safe to conclude that the cloned gene is *Ketel*. Furthermore, the K^+ transgenes slightly reduce $Ketel^D$-associated dominant female sterility showing that the normal and the $Ketel^D$–encoded mutant gene products participate in the same pathway. The slight rescue of $Ketel^D$–associated dominant female sterility implies the dominant negative nature of the $Ketel^D$ mutations, i.e. the $Ketel^D$–encoded molecules impede function of the normal Ketel gene products.

Comparison of amino acid sequences of the Ketel protein revealed strong homology with human importin-β, a component of nuclear protein import: in the two protein sequences 60% of the amino acids are identical and 78% are of similar nature.

importin-β is a major component of nuclear protein import and has been known from biochemical studies in which components of nuclear protein import were identified (Adam and Adam 1994, Chi et al. 1995, Görlich et al. 1995a and b, Imamoto et al. 1995, Radu et al. 1995). The *Ketel* gene does indeed encode the Drosophila importin-β since the Ketel protein possesses characteristic features of importin-β. In absence of an energy source the Ketel protein produced in bacteria supports docking of a IBB-nucleoplasmin core fusion protein on the NE of digitonin

permeabilized HeLa cells (Figure 7B). When energy source is provided, the nuclear protein is imported into the nucleus(Figure 7D).

The Ketel protein is cytoplasmic and is not present in every cell type

As other members of the importin-β family, the Ketel protein is largely cytoplasmic (Görlich et al. 1995a) with pronounced accumulation in the NE (Figure 9A and D). As predicted by features of the *Ketel*^{D} and *ketel*^{r} mutant phenotypes (Tirián et al. 2000), the Ketel protein is produced and is dumped into the oocyte cytoplasm during oogenesis and cleavage embryos make use of the Ketel maternal dowry. Surprisingly however, the *Ketel* gene does not appear to be expressed in the fully differentiated larval cells. Apparently the Ketel protein appears to be present largely in mitotically active cells. The relation between *Ketel* gene expression and mitosis remains to be elucided.

The possible mode of action of the *Ketel*^{D}–encoded proteins

Embryogenesis fails to commence in the *Ketel*^{D} eggs deposited by the *Ketel*^{D}/+ females: the *Ketel*^{D}-encoded mutant gene products prevent formation of the first zygotic nuclei. Instead of cleavage nuclei formation MT bundles and asters persist. As results of cytoplasm injections revealed, the defects are brought about by "toxic" components in the *Ketel*^{D} egg cytoplasm that "poison" wild type cleavage embryos through the blocking of cleavage nuclei formation following cleavage mitoses.

When injected into wild type cleavage embryos, traces of the *Ketel*^{D} egg cytoplasm exert deleterious effects through the prevention of cleavage nuclei formation. Toxic effects of the *Ketel*^{D}-encoded molecules can be an outcome of arrested nuclear protein import. To elaborate this possibility, we prepared cytosol from ovaries of the *Ketel*^{D}/+ females and studied their effects on nuclear protein import. Unexpectedly the *Ketel*^{D} cytosol preparations did not prevent nuclear import of the cNLS-PE substrate. In fact, the cNLS-PE molecules were equally efficiently imported into the nuclei in presence of the *Ketel*^{D} or wild type ovary cytosol. Consistent with this observation, the *Ketel*^{D} egg cytoplasm did not prevent import of the cNLS-PE molecules into nuclei of interphase nuclei of wild type cleavage embryos. Knowing that the *Ketel*^{D} alleles are strong dominant negative mutations the above results may be surprising. A number of possibilities may come to light to explain the former observation. Perhaps although the *Ketel*^{D}-encoded molecules block function of the normal ones, the cNSL-PE substrate is imported into the nuclei via another nuclear protein import route powered by unidentified

components of the ovary cytosol. The existence of parallel import pathways is well established. E.g. the human ribosomal protein L25 is imported through at least four pathways (Jäkel and Görlich 1998). However, the fact that both Kap95p (the importin-β) and Cse1p (the importin α exporter) coding genes of yeast are essential suggests that there is no other way of cNLS import as the importinα/β heterodimer at least in yeast (Görlich and Kutay 1999). It is also possible that although the *Ketel*^{D}-encoded molecules do not participate in nuclear protein import they do not interfere with import function of the normal Ketel molecules. Interference of the two types of proteins may come into light once the importin-β molecules perform a function other than nuclear protein import.

Indeed, the deleterious effects of the *Ketel*^{D} mutations become apparent at the end of cleavage mitosis when the NE reassemble and daughter nuclei form. Remarkably, the *Ketel*^{D} cytosol did not disrupt HeLa cell nuclei and, along with this observation, Drosophila wild type interphase cleavage nuclei remained intact in presence of the *Ketel*^{D} egg cytoplasm. As the digitonin-permeabilized HeLa cells do not divide they are inadequate to detect defects associated with NE assembly. It appears as if the *Ketel*^{D} mutations identify a novel function of importin-β required during reassembly of the NE at the end of mitosis. Perhaps importin-β is not only engaged in nuclear protein import but is also structural component of the NPCs, as Corbett and Silver (1997) proposed, and the *Ketel*^{D} mutations identify the nucleoporin function of the gene.

Nuclear envelope dis- and reassembly

In higher eukaryotes the nuclei disassemble upon the initiation of mitosis. The process is under the control of p34^{cdc2}, the cyclin-B-dependent mitotic kinase (Gant and Wilson 1997; Marshall and Wilson 1997; Collas 1998). Phosphorylation of the lamins, the nucleoporins and the integral nuclear membrane proteins are important events of NE disassembly. Some of the disassembled nuclear membrane complexes disperse in the cytoplasm, others are stored in vesicles (Marshall and Wilson 1997). In Drosophila NE disassembly is complete only opposite the spindle poles. The nuclear pore complexes (NPCs) and the nuclear lamina dissociate from the rest of the nuclear membrane and so-called spindle envelopes form (FOE et al. 1993).

Formation of nuclei, as studied mostly in Xenopus egg extracts, begins with reassembly of the NE and the process was proposed to occur in the following major steps (Sutovsky et al. 1998; Zhang and Clarke 2000). The process begins during telophase when RanGDP associates with chromatin (Zhang et al. 1999). The chromatin-associated Ran-GDP promotes binding of

membrane vesicles to chromatin and recruit RCC1 (regulator of chromatin condensation) the guanine nucleotide exchange factor for Ran and promotes the association of nucleoporins (Goldberg et al. 1997; Gant et al.1998). RCC1 generates Ran-GTP from RanGDP, and RanGTP (most probable RanGTP hydrolysis itself) causes fusion of the vesicles and formation of double nuclear membrane (Gant et al.1998; Zhang and Clarke 2000, Hetzer et al. 2000). Formation of the NE with NPCs establishes condition for resumed nuclear protein import and the formation of functional nuclei. The process takes place *in vitro* where NE forms from egg cytoplasm extract components over the demembranated sperm chromatin (Burke and Gerace 1986) in a process is similar to NE assembly around the sperm chromatin during male pronucleus formation following fertilization (Sutovsky et al. 1998). As described recently by Zhang and Clarke (2000), functional NEs form over Sepharose beads loaded with RanGDP in Xenopus egg extract in the absence of DNA or chromatin.

importin-β (the Ketel protein) has been known to interact with importin-α, Ran, and a number of nucleoporins during nuclear protein import, and the regions of interactions have been determined (Kutay et al. 1997; Wozniak et al. 1998). In case of human importin-β, as in other members of the importin-β, the Ran binding domain resides at the N terminal region. Truncated importin-β molecules lacking the N terminal sections and thus can not bind Ran, can associate with NPCs and have a dominant negative effect on nuclear protein import as determined in the digitonin permeabilized HeLa assay system (Kutay et al. 1997): the NPC binding domain slightly overlaps the Ran binding domain and resides towards central regions of the protein. The importin-α binding domain is located towards the C terminus.

Abnormal interactions between the *Ketel*^{D}-encoded molecules and components of the nuclear transport process may lead to the failure of cleavage nuclei formation. In fact, dominant negative mutations in both Ran and RCC1 have been known to disrupt NE formation. The *RanT24N* (substitution of Thr at position 24 by Asn) dominant negative mutant allele of Ran encodes a protein that is defective in nucleotide binding and profoundly disrupts NE assembly in the *Xenopus laevis* egg extract system (Dasso et al. 1994; Zhang and Clarke 2000). Similarly to *RanT24N*, the nonhydrolyzable guanosine 5'-triphosphate analogues have also been known to inhibit NE assembly through disrupted function of Ran that has been known to play key role in NE integrity and exit from mitosis (Demeter et al. 1995; Maculay and Forbes 1996). The *pim1-d1ts* mutant allele of RCC1 of *Schizosaccharomyces pombe* prohibits the reestablishment of the NE following mitosis (Kornblut et al. 1994). Mutations in a number of nucleoporins have

also been reported to lead to nuclear fragmentation in yeast (reviewed in Corbett and Silver 1997).

The disturbing effects on NE organization of the *Ketel*^{D}-encoded molecules is also supported by persistence of the MT bundles and asters seen in the *Ketel*^{D} eggs (Tirián et al., 2000). We interpret the persistence of the MT bundles and asters by leakage of RanGTP form the nuclei through the inappropriately assembled NE. RanGTP has recently been reported to stabilize microtubule asters and promote spindle assembly (for reviews see Nishimoto 1999; Kahana and Cleveland 1999; Azuma and Dasso 2000). Evidently several types of molecules participate in NE dis- and reassembly upon the onset and towards the end of mitosis and the *Ketel*^{D} mutations should help to understand the role of importin-β in the process. The knowledge of the position of three *Ketel*^{D} mutations enables us to clarify the molecular basis of the dominant *Ketel* effects.

The *Ketel*^{D} alleles are dominant negative mutations

Four observations show the gain-of-function nature of the *Ketel*^{D} mutations and that mutant gene products bring about the *Ketel*^{D}-related defects. (1) The *Ketel*^{D} egg cytoplasm is toxic: when injected into wild type embryos it prevents formation of cleavage nuclei. (2) Following the induction of +/+ clones (through mitotic recombination) in the germ line of *Ketel*^{D}/+ females, the +/+ clones appear with reduced frequencies and with several days delay due to perdurance of the *Ketel*^{D}-encoded mutant gene products in the +/+ cells (Szabad et al. 1989). (3) The gain-of-function features of the *Ketel*^{D} alleles can be eliminated during second mutagenesis and loss-of-function *ketel*^{r} recessive alleles are generated (Szabad et al. 1989; Erdélyi et al. 1997). (4) The deleterious effects of three of the four *Ketel*^{D} alleles can be reduced by the addition of normal (+) *Ketel* gene copies: a few offspring derive form the *Ketel*^{D2}/+/+/+ females. Interestingly, mating of the *Ketel*^{D2}/+/+/+ females with males that provided two wild type *Ketel* genes significantly increased the rate of offspring production (as compared to the wild type males that provided only one *Ketel* gene). In fact, the *Ketel*^{D1}/+/+/+ and the *Ketel*^{D3}/+/+/+ females yielded a few offspring only when their male partners provided two normal *Ketel* gene copies during fertilization. The paternal rescue effect on *Ketel*^{D}-associated maternal-effect lethality clearly shows expression of the *Ketel* gene during embryogenesis.

Analysis of the *Ketel*^{D}/+/+/+ females revealed the strong antimorph nature of the *Ketel*^{D} mutations and imply involvement of the normal and the *Ketel*^{D}-encoded mutant gene products in the same process (Muller 1932). Since products of the antimorph mutations impede action of

the normal gene products they are also called dominant negative mutations. The gain- and the loss-of-function mutant phenotypes are expected to be identical in case of the strong antimorph mutations. (The *Tomaj*^{D} the mutations, that identify the *αTubulin67C* gene of Drosophila, represent a typical example; Máthé et al. 1998.) Although the *Ketel*^{D} alleles are very strong dominant negative mutations, the gain- and the loss-of-function mutant phenotypes are not identical: failure of the commencement of embryogenesis in the *Ketel*^{D} eggs versus death during second larval instar of the *ketel*^{r} homo- and hemizygotes. When, however, the Ketel gene product is removed from the eggs (in eggs of the germ line chimeras with normal soma and *ketel*^{r}/– hemizygous germ line cells) the gain- and the loss-of-function *Ketel* mutant phenotypes become identical: the lack of cleavage nuclei formation. Obviously, the normal Ketel gene products - as part of the maternal dowry provided by the +/– mothers - support life of the *ketel*^{r}/– hemizygotes up to second larval instar when they perish.

The *Ketel*^{D}-related defects are brought about following interaction of the *Ketel*^{D}-encoded molecules with maternally provided partner(s): In understanding the mode of *Ketel*^{D} action, the following facts should be considered. (1) The Ketel maternal dowry and the "toxic" nature of the *Ketel*^{D} egg cytoplasm imply deposition by the *Ketel*^{D}/+ mothers both the normal and the *Ketel*^{D}-encoded mutant gene products into the egg cytoplasm. (2) Lethality of the *ketel*^{r} homo- and hemizygotes implies expression of the normal *Ketel* and the *Ketel*^{D} alleles during development. Yet the *Ketel*^{D}-encoded mutant gene products exert their deleterious effects only on cleavage embryos. (3) When paternally derived, the *Ketel*^{D} alleles behave as the *ketel*^{r} loss-of-function alleles: they do not disturb but also can not support cell functions. We interpret the deleterious effects of the *Ketel*^{D} mutations on cleavage embryos by interaction of the *Ketel*^{D}–encoded molecules with a maternally provided partner present in the egg cytoplasm but absent from the somatic cells. The complexes not only inhibit cleavage nuclei formation but also impede activities of the complexes composed from normal Ketel molecule(s) and the maternally provided partner. During cellular stages of development, when the maternally provided partner is absent, the *Ketel*^{D}-encoded gene products well may be present but can not exert their deleterious activities in the absence of an appropriate partner and consequently the cells survive and function normally. Thus the *Ketel*^{D} mutations identify maternal function of a zygotically essential gene and the identified function if manifested following interaction with a maternally

provided partner. The maternally provided component that interacts with the Ketel and the *Ketel*^{D}–encoded molecules remains to be identified.

Requirement and expression patterns of the *Ketel* gene indicate parallel nuclear protein import pathways: It was generally believed that *Ketel* gene function was required in every cell for the import of cNLS-containing nuclear proteins. Surprisingly cells in the female germ line, follicle epithelium, wing and abdominal histoblasts are viable and function normally without *Ketel* gene (Tirián et al., 2000). The following possible explanations may be considered to account for viability of the above cells.

(1) Perdurance of the normal *Ketel* gene products in clones of cells without functional *Ketel* gene is unlikely the reason for survival of the cells for the following reasons. In female germ line chimeras the pole cell derived normal *Ketel* gene products are expected to decay and/or dilute between pole cell formation and egg production. Even if survived it is unlikely that they can support the immense biochemical activities associated with egg cytoplasm production. It is also rather improbable that perdurance of the normal *Ketel* gene products support life of the wing disc cells over as much as seven rounds of cell divisions following the induction of mitotic recombination.

(2) Non-autonomy of the lack of *Ketel* gene function (i.e. the supply with normal *Ketel* gene products of the mutant cells by the normal cells) is also unlikely since the 94 kD Ketel protein lacks signal sequences required for excretion and uptake from and into cells.

(3) Most likely parallel nuclear import pathways substitute lost *Ketel* gene function and proteins normally imported through the importin-β route are imported to the nucleus through another system. The human ribosomal protein L23a, for example, can be imported through at least four routes (Jäkel and Görlich 1998). Similarly transportin, a member of the importin-β superfamily, recognizes and assists import of rather different types of proteins into the nucleus (reviewed in Görlich and Kutay 1999). However, function of the *Ketel* gene must be essential in some cell type(s) since the *ketel*^{rX13}/– hemizygotes die during second larval instar. "Focus" i.e. the cell type in which function of the *Ketel* gene can not be replaced remains to be elucidated.

(4) It is also possible that the *Ketel* gene is not expressed in the studied cell types. To clarify this possibility, we constructed a reporter gene in which the *Ketel* promoter operated the *lacZ* reporter gene. In adult females β-gal activities appeared in the ovaries including the female germ line and the follicle cells. Expression patterns of the reporter and the *Ketel* genes - as

determined by *in situ* hybridizations and Western blot analysis - were in harmony (see Figure 8, 10 and 11).

In harmony with the genetic data, the egg cytoplasm was loaded with β-gal and there was β-gal activity present in virtually every cell of the cleavage and cellular embryos. However, except minor β-gal activities in the brain, no other cells in the adult females possessed β-gal activities. In late third instar larvae β-gal activity appeared in imaginal discs, larval gonads, imaginal rings of the salivary glands and in the ring gland. However, the other larval tissues did not possess β-gal activity. It appears thus that the *Ketel* gene is expressed, with exception of the ring gland cells, in certain types of the mitotically active cells. (The neuroblast cells in the central nervous system, and the histoblast cells, for example, do not possess β-gal activity.) However, the role of the Ketel protein in cell cycle progression remains to be elaborated. On the other hand, non-expression of the Drosophila importin-β coding (*Ketel*) gene in the mitotically quiescent cells raises question about import of cNLS-containing nuclear proteins in e.g. the larval cells.

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7. LITERATURE CITED

ADAM, E. J. H., and S. A. ADAM, 1994 Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope. J. Cell Biol. 125: 547-555.

ADAM, S. A., R. STERNE-MARR and L. GERACE, 1990 Nuclear import in permeabilized mammalian cells requires soluble cytoplasmic factors. J. Cell Biol. 111: 807-816.

ANDERSON, K.A. and NÜSSLEIN-VOLHARD, C., 1986 Dorsal group genes of *Drosophila*. pp. 177-194 in: *Gametogenesis and the Early Embryo*, edited by J.G. Gall, New York.

AZUMA, Y., and M. DASSO, 2000 The role of Ran in nuclear function. Current Opinion in Cell Biol. 12: 302-307.

BURKE, B., and L. GERACE, 1986 A cell free system to study reassembly of the nuclear envelope at the end of mitosis. Cell 44:4 639-652.

BUSSON, D., M. GANS, K. KOMITOPOULOU and M. MASSON, 1983 Genetic analysis of three dominant female sterile mutations located on the X chromosome of Drosophila melanogaster. Genetics 105: 309-325.

CAMPOS-ORTEGA, J. A., and V. HARTENSTEIN, 1997 The Embryonic Development of Drosophila melanogaster, Springer, Berlin.

CHI, N. C., E. J. H. ADAM and S. A. ADAM, 1995 Sequence and characterization of cytoplasmic nuclear protein import factor p97. J. Cell Biol. 130: 265-274.

COLLAS, P., 1998 nuclear envelope disassembly in mitotic extracts require functional nuclear pores and a nuclear lamina. J. Cell Sci. 111: 1293-1303.

CORBETT, A. H., and P. A. SILVER, 1997 Nucleocytoplasmic transport of macromolecules. Microbiol. Mol. Biol. Reviews 61: 193-211.

CSERPÁN, I., and A. UDVARDY, 1995 The mechanism of nuclear transport of natural or artificial transport substances in digitonin permeabilized cell. J. Cell Sci. 108: 1849-1861.

DASSO, M., T. SEKI, Y. AZUMA, T. OHBA and T. NISHIMOTO, 1994 A mutant form of the Ran/TC4 protein disrupts nuclear function in Xenopus laevis egg extracts by inhibiting the RCC1 protein, a regulator of chromosome condensation. EMBO J. 13: 5732-5744.

DEMETER, J., M. MORPHEW and S. SAZER, 1995 A mutation in the RCC1-related protein pim1 results in nuclear envelope fragmentation in fission yeast. Proc. Natl. Acad. Sci. USA 28: 1436-1440.

ERDÉLYI, M., and J. SZABAD, 1989 Isolation and characterization of dominant female sterile mutations of Drosophila melanogaster. I. Mutations on the third chromosome. Genetics 122: 111-127.

ERDÉLYI, M., E. MÁTHÉ and J. SZABAD, 1997 Genetic and developmental analysis of mutant Ketel alleles that identify the Drosophila importin-β homologue. Acta Biol. Hung. 48: 323-338.

FOE, V. E., G. M. ODELL and B. A. EDGAR, 1993 Mitosis and morphogenesis in the Drosophila embryo: point and counterpoint. In: The Development of Drosophila melanogaster, edited by M. BATE and A. MARTINEZ-ARIAS, pp. 149-300, Cold Spring Harbor Laboratory Press, New York.

GANT, T. M., and K. L. WILSON, 1997 Nuclear assembly. Annu. Rev. Cell Dev. Biol. 13: 669-695.

GANT, T. M., M. W. GOLDBERG and T. D. ALLEN, 1998 Nuclear envelope and nuclear pore assembly: analysis of assembly intermediates by electron microscopy. Current Opinion in Cell Biol. 10: 409-415.

GOLDBERG, M. W., C. WIESE, T. D. ALLEN and K. L. WILSON, 1997 Dimples, pores, star-rings, and thin rings on growing nuclear envelopes: evidence for structural intermediates in nuclear pore assembly. J. Cell Sci. 110: 409-4020.

GÖRLICH, D., and U. KUTAY, 1999 Transport between the cell nucleus and the cytoplasm. Ann. Rev. Cell Dev. Biol. 15: 607-660.

GÖRLICH, D, F. VOGEL, A. D. MILLS, E. HARTMANN and R. A. LASKEY, 1995a Distinct functions for the two importin-subunits in nuclear protein import. Nature 377: 246-248.

GÖRLICH, D, S. KOSTKA, R. KRAFT, C. DINGWALL, R. A. LASKEY, E. HARTMANN and S. PREHN, 1995b Two different subunits of importin-cooperate to recognize nuclear localization signals and bind them to the nuclear envelope. Current Biol. 5: 383-392.

GÖRLICH, D, M. DABROWSKI, F. R. BISCHOFF, U. KUTAY, P. BORK, E. HARTMANN, S. PREHN and E. IZAURRALDE, 1997 A novel class of RanGTP binding proteins. J. Cell Biol. 138: 65-80.

HAREL, A., F. ZLOTKIN, S. NAINUDEL-EPSZTEYN, N. FEINSTEIN, P. A. FISHER and Y. GRUENBAUM, 1989 Persistence of major nuclear envelope antigens in an envelope-like structure during mitosis in Drosophila melanogaster embryos. J. Cell Sci. 944: 463-470.

HETZER, M, D. BILBAO-CORTE'S, T. C WALTHER, O. J. GRUSS, I. W. MATTAJ, 2000 GTP Hydrolysis by Ran Is Requiredfor Nuclear Envelope Assembly. Molecular Cell 5 1013–1024

HUBER, J., U. CRONSHAGEN, M. KADOKURA, C. MARSCHALLY, T. WADA, T. SEKINE and R. LÜHRMANN, 1998 Snurportin 1, an m3G-cap-specific nuclear import receptor with a novel domain structure. EMBO J. 17: 4114-4126.

ILLMENSEE, K., 1973 The potentialities of early gastrula nuclei of D. melanogaster. Production of their imago descendants by germ line transplantation. Wilhelm Roux Archiv. 171: 331-343.

IMAMOTO, N., T. SHIMAMOTO, S. KOSE, T. TAKAO, T. TACHIBANA, M. MATSUBAE, T. SEKIMOTO, Y. SHIMONISHI and Y. YONEDA, 1995 The nuclear pore targeting complex binds to nuclear pores after association with a karyophile. FEBS Lett. 368: 415-419.

IOVINE, M. K., J. L. WATKINS and S. R. WENTE, 1995 The GLFG repetitive region of the nucleoporin Nup116p interacts with Kap95p, an essential yeast nuclear import factor. J. Cell Biol. 131: 1699-1731.

JÄKEL, S., and D. GÖRLICH,1998, importin-β, transportin, RanBP5 and RanBp7 mediate nuclear import of ribosomal proteins in mammalian cells. EMBO J. 17: 4491-4502.

JÄKEL, S., W. ALBIG, U. KUTAY, F. R. BISCHOFF, K. SCHWAMBORN, D. DOENECKE and D. GÖRLICH, 1999 The importin-β/importin-7 heterodimer is a functional nuclear import receptor for histone H1. EMBO J. 18: 2411-2423.

JULLIEN, D., D. GÖRLICH, U. K . LAEMMLI and Y. ADACHI, 1999 Nuclear import of RPA in Xenopus egg extracts requires a novel protein XRIPa but not importin-α. EMBO J. 18: 4348-4358.

JÜRGENS, G., R. LEHMANN, M. SCHARDIN and C. NÜSSLEIN-VOLHARD, 1986 Segmental organisation of the head in the embryo of Drosophila melanogaster. Roux's Arch. Dev. Biol. 195: 359-377.

KAHANA, J. A. and D. W. CLEVELAND, 1986 Beyond nuclear transport: Ran-GTP as a determinant of spindle assembly. J. Cell Biol. 146: 1205-1209.

KOEPP, D.M. and P.A. SILVER, 1996 A GTPase controlling nuclear trafficking: running the right way or walking RANdomly? *Cell* 87, 1-4.

KOMITOPOULOU, K., M. GANS, L. M. MARGARITIS, F. KAFATOS and M. MASSON, 1983 Isolation and characterization of sex-linked female-sterile mutants in Drosophila melanogaster. Genetics 105: 897-920.

KORNBLUT, S., M. DASSO and J. NEWPORT, 1994 Evidence for a dual role for TC4 protein in regulating nuclear structure and cell cycle progression. J. Cell Biol. 125: 705-719.

KUTAY, U., E. IZAURRALDE, F. R. BISCHOFF, I. W. MATTAJ and D. GÖRLICH, 1997 Dominant-negative mutants of importin-β block multiple pathways of import and export through the nuclear pore complex. EMBO J. 16: 1153-1163.

LINDSLEY, D. L., and G. G. ZIMM, 1992 The Genome of Drosophila melanogaster. Academic Press, San Diego and London.

LIPPAI, M., 1999 The Ketel gene encodes a Drosophila importin-β homologue Ph.D. Thesis

LIPPAI, M., L.TIRIÁN, I. BOROS, J. MIHÁLY, M. ERDÉLYI, E. MÁTHÉ, I. BELECZ, J. PÓSFAI, A. NAGY, A. UDVARDY, E. PARASKEVA, D. GÖRLICH and J. SZABAD, 2000 The Ketel gene encodes the Drosophila homologue of importin-β1. Genetics, 156: 1889-1900.

MACAULAY, C., and D. J. FORBES, 1996 Assembly of the nuclear pore – biochemically distinct steps revealed with NEM, GTPγS, and BAPTA. J. Cell Biol. 132: 5-20.

MARSHALL, I. B., and K. L.WILSON, 1997 Nuclear envelope assembly after mitosis. Trends in Cell Biol. 7: 69-74.

MÁTHÉ, E., I. BOROS, K. JÓSVAY, K. LI, J. PURO, T.C. K AUFMAN and J. SZABAD, 1998 The Tomaj mutant alleles of αTubulin67C reveal a requirement for the encoded maternal specific tubulin isoform in the sperm aster, the cleavage spindle apparatus and neurogenesis during embryonic development in Drosophila. J. Cell. Science 111: 887-896.

MELCHIOR, F., and L. GERACE, 1998 Two-trafficking with Ran. Trends in Cell Biol. 8: 175-179.

MULLER, H.J., 1932 Further studies on the nature and causes of gene mutations. Proc. 6th Int. Cong. Genetics, Brooklin Botanic Gardens U.S.A., 1: 213-255.

NISHIMOTO, T., 1999 A new role of Ran GTPase. Biochem. Biophys. Res. Comm. 262: 571-574.

NORVELL, A., R. L. KELLEY, K. WEHR and T. SCHUPBACH, 1999 Specific isoforms of squid, a Drosophila hnRNP, perform distinct roles in Gurken localization during oogenesis. Genes Develop. 13: 864-876

NÜSSLEIN-VOLHARD, C., FRONHÖFER, H.G. and LEHMANN, R., 1987. Determination of anteroposterior polarity in *Drosophila*. *Science* 238, 1675-1681.

ORR-WEAVER, T.L., 1994 Developmental modification of the *Drosophila* cell cycle. *Trends Genet.* 10, 321-327.

PADDY, M. R., H. SAUMWEBER, D. A. AGARD and J. W. SEDAT, 1996 Time-resolved, in vivo studies of mitotic spindle formation and nuclear lamina breakdown in Drosophila early embryos. J. Cell Sci. 109: 591-607.

PALMERI, D. and M.H. MALIM, 1999 importin β can mediate the nuclear import of an arginine-rich nuclear localization signal in the absence of importin α. *Mol. Cell. Biol.* 19, 1218-1225.

RADU, A., G. BLOBEL and M. S. MOORE, 1995 Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporin. Proc. Natl. Acad. Sci. USA 92: 1769-1773.

SCHÜPBACH, T. and E. WIESCHAUS, 1989 Female sterile mutations on the second chromosome of *Drosophila melanogaster*. 1. Maternal effect mutations. *Genetics* 121, 101-117.

SUTOVSKY, P., C. SIMERLY, L. HEWITSON and G. SCHATTEN, 1998 Assembly of nuclear pore complexes and annulate lamellae promotes normal pronuclear development in fertilized mammalian oocytes. J. Cell Sci. 111: 2841-2854.

SZABAD, J., M. ERDÉLYI, G. HOFFMANN, J. SZIDONYA and T. R. F. WRIGHT, Isolation and characterization of dominant female sterile mutations of Drosophila melanogaster. II. Mutations on the second chromosome. Genetics 122: 823-835.

SZABAD, J., 1998 Genetic requirement of epidermal and female germ line cells in Drosophila in the light of clonal analysis. Int. J. Dev. Biol. 42: 257-262.

SZABAD, J., V. A. JURSNICH and P. J. BRYANT, 1991 Requirement for cell-proliferation control genes in Drosophila oogenesis. Genetics 127: 525-533.

THUMMEL, K. S., A. M. BOULET and H. D. LIPSHITZ, 1988 Vectors for Drosophila P-element-mediated transformation and tissue culture transfection. Gene 74: 445-456.

TIGANIS, T., A.J. FLINT, S.A. ADAM and N.K. TONKS (1997). Association of the T-cell protein tyrosine phosphatase with nuclear import factor p97. *J. Biol. Chem.* 272, 21548-21557.

TIMMONS, L., E. HERSPERGER, E., WOODHOUSE, J. XU, L. Z. LIU and A SHEARN, 1993 The expression of the Drosophila awd gene during normal development and in neoplastic brain tumors caused by lgl mutations. Dev. Biol. 158: 364-379.

TIRIÁN, L., M. ERDÉLYI, J. PURO, I. BOROS, E. MÁTHÉ, M. LIPPAI and J. SZABAD, 2000 The KetelD dominant negative mutations identify maternal functionof the Drosophila importin-β gene required for cleavage nuclei formation. Genetics, 156: 1901-12

TIRIÁN L., M. LIPPAI and J. SZABAD, 2001 Ran, Jolly Joker a sejtben. Biokémia (in press)

TÖRÖK I., D. STRAND, R. SCHMITT, G. TICK, T. TÖRÖK, I. KISS and B. M. MECHLER, 1995 The *overgrown hematopoietic organs-31* tumor supressor gene of *Drosophila* encodes an importin-like protein accumulating in the nucleus at the onset of mitosis. *J. Cell Biol.* 129, 1473-1489.

TRUANT, R., and B. R. CULLEN, 1999 The arginine-rich domains present in human immunodeficiency virus type 1 tat and rev function as direct importin-beta-dependent nuclear localization signals. Mol. Cell Biol. 19: 1210-1217.

WIESCHAUS, E., and C. NÜSSLEIN-VOLHARD, 1986 Looking at embryos. In "Drosophila a Practical Approach". (D. B. ROBERTS ed.), pp. 199-227, I.R.L. Press, Oxford.

WIESCHAUS, E., 1996 Embryonic transcription and the control of developmental pathways. Genetics 142: 5-10.

WOZNIAK, R. W., M. P. ROUT and J. D. AITCHISON, 1998 Karyopherins and kissing cousins. Trends in Cell Biol. 8: 184-188.

ZHANG, C., M. HUGHES and P. R. CLARKE, 1999 Ran-GTP stabilizes microtubule asters and inhibits nuclear assembly in Xenopus egg extracts. J. Cell Sci. 112: 2453-2461.

ZHANG, C., and P. R. CLARKE, 2000 Chromatin-independent nuclear envelope assembly induced by Ran GTPase Xenopus egg extracts. Science 288: 1429-1432.