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

LÁSZLÓ TIRIÁN

UNIVERSITY OF SZEGED
FACULTY OF MEDICINE
DEPARTMENT OF BIOLOGY

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PUBLICATIONS

I. László Tirián, Jaakko Puro, Miklós Erdélyi, Imre Boros, Bernadett Papp, Mónika Lippai and János Szabad, The Ketef\textsuperscript{D} dominant negative mutations identify maternal function of the Drosophila importin-\(\beta\) gene required for cleavage nuclei formation.
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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 Kete\textit{\textsuperscript{L}} gene encodes the Drosophila homologue of importin-\(\beta\).
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III.
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Abbreviations

IBB importin-β binding domain
Fs dominant female sterile mutation
fs recessive female sterile mutation
GDP guanosine diphosphate
GST glutathion-S-transferase
GTP guanosine triphosphate
$K^+$ wild type Ketel transgene
$K^D$ transgene with the KetelD allele
KetelD dominant mutant allele of the Ketel gene
kettleR 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 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 lead to dominant female sterility.
- We elucidated a new function for Drosophila importin-β. Cytological analysis of the KetelD 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 alleles is due to combined activities of the KetelD encoded protein and the maternally provided partner present only in the egg cytoplasm.
2. INTRODUCTION

Early embryogenesis of Drosophila and the generating of \textit{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 \textit{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 \textit{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 lengthen 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 (\textit{mel}) mutations, a special class of female sterile (\textit{fs}) mutations, provided major contribution to our knowledge about the genes contributing to the regulation of embryogenesis in \textit{Drosophila melanogaster} (Wieschaus 1996). Analysis of the \textit{mel} mutations promoted, among others, the understanding of cell differentiation along the anterior-posterior and the dorsal-ventral coordinates of the \textit{Drosophila} embryo (Anderson and Nüsslein-Volhard 1986; Nüsslein-Volhard et al. 1987).

Females homozygous for a \textit{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-\( \beta \), 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 factor 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 hydrolyzed (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. Dissociation 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). Histone 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 multicellular eucaryotic organism. We revealed that *Ketel* is preferentially expressed in mitotically active cells (Lippai et al. 2000; Tirian et al. 2000). The *Ketel<sup>P</sup>* 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* alleles: The four *Ketel^D* alleles were isolated in a screen for dominant female-sterile mutations. The twenty-seven recessive *ketel* alleles were generated through second mutagenesis of the *Ketel^D* mutations (Szabad et al. 1989; Erdélyi et al. 1997). The *ketel'/ketel'* homo- and the *ketel'/--* hemizygotes were produced by crossing *y/y; ketel'/y^+CyO* or *y/y; ketel'^X32/y^+CyO* females with *y/Y; ketel'/y^+CyO* males. (The *ketel'^X32* 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'/ketel'* or *ketel'/--*) 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'^X32/y^+CyO* females with *y/Y; Ketel'^D'/y^+CyO* males. For explanation of the genetic symbols throughout the text see Lindsley and 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^Dl* 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^Dl* 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^Dl* eggs. (cNLS = classic nuclear localization signal.) A sample of about 200 pl cNLS-PE containing *Ketel^Dl* 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 LEISS 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$^+$/-, (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 oligonucleotides:

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+KetelD. The exchange of the single nucleotide was confirmed with sequencing. The KpnI-HindIII fragment from pBS- KetelD 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 KetelD transgenic lines with the in vitro synthetised mutant allele. The fertility of the females carrying the KetelD 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 \textit{in vitro} nuclear protein import assay: Drosophila importin-\(\beta\) 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 \(\mu\)g/ml digitonin. The coverslips were washed three times in permeabilization buffer without digitonin. Coverslips were incubated as indicated with each 20 \(\mu\)l 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 \(\mu\)g/ml creatine kinase) and Ran mix (3 \(\mu\)M RanGDP, 150 nM Rn1a1p, 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-\(\alpha\) (IBB core pentamer). Import reaction samples contained 0.24 \(\mu\)M fluorescein-labeled IBB core pentamer. In the indicated reactions 1.2 \(\mu\)M Drosophila importin-\(\beta\), 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 \(\mu\)l 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^X13, a ketel null allele. (In ketel^X13 ~ 9/10th of the 3' end of the ORF was deleted.) In practice, pole cells of the embryos from a cross between ketel^X13/Bc Gla females and ketel^X32/Cyo males were transplanted into host embryos that derived from wild type (+/+ ) females and Fs(1)KI237/Y males. Fs(1)KI237 (= 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 It bw/It bw males. (The ketel^3 and ketel^2 -carrying chromosomes are labeled with the It and the bw recessive marker mutations (Lindsley and Zimm 1992, Erdélyi et al. 1997).

Follicle cell mosaics: Follicle cell clones homozygous for ketel^X13 were generated through mitotic recombination. In practice, ketel^X13 pr/Bc Gla adult females were mated with Fs(2)Ugra/Bc Gla males. [Three independent ketel^X13 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^X13 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^X13/Ugra and the control It bw/Ugra adult females were tested for offspring production. Whether the ketel^X13/ketel^X13 homozygous follicle cells can support egg development was decided by comparing the frequencies of follicle cell mosaicism in the ketel^X13/Ugra and the It bw/Ugra control females (Szabad et al. 1989).
Wing and tergite mosaics: Clones homozygous for the ketel\textsuperscript{Xl3} allele were induced, through mitotic recombination, in f\textsuperscript{6a}/Y; ketel\textsuperscript{Xl3}f\textsuperscript{+} ck larvae (See Figure 6.). The f\textsuperscript{+} symbol stands for a forked\textsuperscript{+} transgene in the 30B cytological region. It compensates effects of the X-linked f\textsuperscript{6a} mutation (P. Martin and A. Garcia-Bellido personal communication). The f\textsuperscript{6a}, 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\textsuperscript{Xl3}. The ck twin clones served as reference in analysis of the f clones. Wings and abdomens of the f\textsuperscript{6a}/Y; ketel\textsuperscript{Xl3}f\textsuperscript{+} 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-\textsuperscript{AUG-\beta}gal) 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 3\textsuperscript{rd} chromosome. Flies homozygous for any of the transgenes are viable and fertile. \beta-galactosidase activities of transgene homozygotes were studied in adult, embryonic and larval stages of development according to standard procedures.
4. RESULTS

The Kete\textsuperscript{p} mutant phenotype suggests NE related function of the normal Ketel gene product

The four Kete\textsuperscript{p} dominant female sterile mutations of Drosophila melanogaster emerged following EMS mutagenesis (Szabad et al. 1989). The Kete\textsuperscript{p}/+ females and the males are fully viable and male fertility is normal. However, the Kete\textsuperscript{p}/+ females are sterile. Development of the egg primordia is normal in all the Kete\textsuperscript{p}/+ females and the meiotic divisions are indistinguishable from wild type (J. Puro unpublished). The Kete\textsuperscript{p}/+ females deposit normal numbers of normal looking so-called Kete\textsuperscript{p} eggs. All the Kete\textsuperscript{p} 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 Kete\textsuperscript{p} eggs, suggesting impact nuclei (Figure 3A). Severe Kete\textsuperscript{p}-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 Kete\textsuperscript{p} 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 gonomic 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 Kete\textsuperscript{p} mutant phenotype is identical for the four Kete\textsuperscript{p} alleles.

The Kete\textsuperscript{p} egg cytoplasm prevents cleavage nuclei formation

Since the Kete\textsuperscript{p} mutations have been known to be gain-of-function type (Szabad et al. 1989), the above described defects are most likely brought about by Kete\textsuperscript{p}-encoded mutant gene products. To elaborate the above possibility we carried out egg cytoplasm injections. (1) First a sample of \textasciitilde 40 pl Kete\textsuperscript{p} 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
anterior structures were entirely missing following the injection of ~300 pl Ketel<sup>p</sup> 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 KetelP 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 KetelP egg cytoplasm, the cNLS-PE substrate readily entered the cleavage nuclei, implying that the KetelP-encoded molecules do not prevent nuclear import of the cNLS-PE substrate (Figure 4A and D).

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 KetelP 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 KetelP-related defects are brought about by mutant KetelP-encoded gene product.

**The KetelP alleles are very strong antimorph mutations**

To find out whether the KetelP-encoded products participate in the same process as the wild type counterpart (i.e. the KetelP alleles are antimorph mutations; Muller 1932) or they disrupt a process in which the normal Ketel gene products are not involved (i.e. the KetelP alleles are neomorphs), we constructed females that, in addition to a KetelP allele carried two
or three normal Ketel gene copies and studied whether dominant female sterility can be overcome in the Ketel$^{P1+/+}$ and in the Ketel$^{P1+/+/+}$ females. Two systems were analyzed.

1. In the XXTp(2;Y)G; Ketel$^{P1}$/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$^{P2}$/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$^{P2}$/CyO females during the three week test period. [The rate of offspring production was 5.6x10$^3$ offspring/(female x day)]. Of the 56 offspring 13 females carried the Ketel$^{P2}$ allele and were sterile. Analysis of the Ketel$^{P2+/+}$ females showed the strong antimorph nature of Ketel$^{P2}$ and implies involvement of the Ketel$^{P2}$-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$^P$ alleles: every egg remained white and not a single offspring descended from the 1196, 1341 and 945 XXTp(2;Y)G; Ketel$^{P}$/CyO females that carried the Ketel$^P$, the Ketel$^{P3}$ and the Ketel$^{P4}$ 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. 5 A and B and Table 1) and analyzed their effects on both ketel* and Ketel* 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$^{P2}$-associated dominant female sterility. In about 1% of the eggs deposited by the K*; Ketel$^{P2+/+}$ females embryogenesis progressed to the stage of embryonic cuticle formation and even a few offspring developed from the K*; Ketel$^{P2+/+}$ females. However, the rate of offspring production was as low as 2-4x10$^3$ 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$^{P2+/+}$ 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$^0$ allele. Red line indicates the position of the transition present in three of the four Ketel$^0$ 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 (Tirán 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 \(K^*/X; Ketel^P/+\) and \(K^*/K^*; Ketel^P/+\) 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 \(K^*/X; Ketel^P/+\) 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

<table>
<thead>
<tr>
<th>(Ketel^D) allele</th>
<th>Male partner</th>
<th>Genotype of tested females and the features of offspring production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(K^*/X; Ketel^P/+) females</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tested</td>
</tr>
<tr>
<td>(Ketel^{D1})</td>
<td>(XY; +/+)</td>
<td>233</td>
</tr>
<tr>
<td></td>
<td>(K^*/Y; +/+)</td>
<td>201</td>
</tr>
<tr>
<td>(Ketel^{D2})</td>
<td>(XY; +/+)</td>
<td>237</td>
</tr>
<tr>
<td></td>
<td>(K^*/Y; +/+)</td>
<td>172</td>
</tr>
<tr>
<td>(Ketel^{D3})</td>
<td>(XY; +/+)</td>
<td>188</td>
</tr>
<tr>
<td></td>
<td>(K^*/Y; +/+)</td>
<td>167</td>
</tr>
<tr>
<td>(Ketel^{D4})</td>
<td>(XY; +/+)</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>(K^*/Y; +/+)</td>
<td>365</td>
</tr>
</tbody>
</table>

\(^1\) 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 $KeteP$ alleles, irrespectively whether the $K^+/X$; $KeteP^+/+$ females were mated with wild type or with $K^+/Y$; +/+ males (Table 1). The $K^+/K^+$; $KeteP^+/+$ females were also sterile when mated with wild type males (Table 1). However, when mated with $K^+/Y$; +/+ males, the $K^+/K^+$; $KeteP^+/+$ and the $K^+/K^+$; $KeteP^3/+ $ females yielded a few offspring (Table 1). About 50% of the progeny females carried the $KeteP^0$ allele. Results of the transgene experiments confirmed (i) the highly toxic nature of the $KeteP^0$-encoded gene products and (ii) that three of the $KeteP^0$ 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 $KeteP^0$ mutations:** The maternal dowry supports life of the $KeteP^0^/-$ hemizygous individuals up to the second instar stage. We isolated DNA from $KeteP^0^/-$ hemizygous larvae and amplified fragments of the $Ketel$ mutant alleles with PCR. Sequencing of the fragments revealed a single nucleotide difference between the $KeteP^D3$ 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 Xhol site. Restriction digestion of the appropriate PCR fragments from the DNA of the $KeteP^0^1^/-$, $KeteP^0^2^/-$ and $KeteP^0^4^/-$ hemizygous individuals revealed the same nucleotide exchange in the $KeteP^D1$ and the $KeteP^D4$ allele. The in vitro exchange of the above nucleotide in a transformation vector (see figure 5C) confers female sterility in transgenic $KeteP^0^+/+$ 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 $KeteP^D2$ allele is not known yet.

**The $ketel'$ alleles reveal zygotic requirement of the $Ketel$ gene:** We generated, through second mutagenesis of the $KeteP^0$ alleles, 27 loss-of-function $ketel'$ alleles. Twenty-five of the 27 $ketel'$ alleles are recessive zygotic lethal mutations. The most severe $ketel'/ketel'$ homo- and the $ketel'^1/-$ hemizygous combinations, including $ketel'^D3^/-$, bring about death during second larval instar. (The – symbol stands for $ketel'^D2^/$, 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" alleles, it represents most likely the complete loss-of-function mutant phenotype. The zygotic lethal nature of the ketel" alleles clearly shows zygotic requirement of the Ketel gene.

When paternally derived the KetelD mutations behave as the zygotic lethal ketel" alleles: the KetelD/ketel" and the KetelD/- larvae also perish during second larval instar and can not be distinguished from the ketel"/- ones.

Germ line chimeras without functional Ketel gene revealed important features of Ketel gene function: To decide whether short life of the ketel"/- hemizygotes is made possible by the normal Ketel gene products provided by the +/- mothers, we constructed germ line chimeras with normal soma and ketel"/- 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 KetelD eggs.

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

<table>
<thead>
<tr>
<th>Genotype of the germ line cells</th>
<th>Number of chimeras</th>
<th>The rate of egg production</th>
<th>Genotype of the germ line cells</th>
<th>Number of chimeras</th>
<th>The rate of egg production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bc Gla/Cy Roi</td>
<td>3</td>
<td>9.3 ± 6.7^1</td>
<td>Bc Gla/Cy Roi</td>
<td>2</td>
<td>7.7 ± 5.3^2</td>
</tr>
<tr>
<td>KeteP(H)/Bc Gla</td>
<td>4</td>
<td>6.8 ± 4.5</td>
<td>KeteP(D)/Bc Gla</td>
<td>2</td>
<td>5.9 ± 3.8</td>
</tr>
<tr>
<td>KeteP(H)/KeteP(D)</td>
<td>3</td>
<td></td>
<td>KeteP(D)/KeteP(D)</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

^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 keteP(H)/Bc Gla females and keteP(H)/Bc Gla females and KeteP(D)/Cy Roi males in the KeteP(D)/- chimeras.
- The keteP(D) 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"/- 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"/- 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^D/- germ line cells (Table 2). Like in the ketel^-/+ germ line chimeras, the Ketel^D/- 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^-/+ 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^XI3/Ugra larvae for the generation - through mitotic recombination – of ketel^XI3/ketel^XI3 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^XI3/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^XI3/Ugra females with a reduced rate as compared to the control females (Table 3).

Table 3. Characteristics of the ketel^XI3 homozygous follicle cell clones.

<table>
<thead>
<tr>
<th>Specimen1 Females</th>
<th>Egg production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen</td>
<td>Tested</td>
</tr>
<tr>
<td>Control</td>
<td>132</td>
</tr>
<tr>
<td>Ketel^XI3</td>
<td>379</td>
</tr>
</tbody>
</table>

1 = It bwl/Fs(2)Ugra (as control) and ketel^XI3/Fs(2)Ugra females were irradiated as 3rd instar larvae and the developing adult females were tested.
The ketel" 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" homozygous wing and tergite cells. The ketel" homozygous clones were induced through mitotic recombination in f\(^{66}/Y;\) ketel" Ick young third instar larvae. Most of the forked homozygous clones were homozygous for ketel". As summarized in Table 4, frequencies and sizes of the different types of clones were similar in the ketel" and in the control experiments in both the wings and the tergites.

### Table 4. Characteristics of the ketel" homozygous wing and tergite clones.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Wings</th>
<th>Tergites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Screened</td>
<td>Types of clones</td>
</tr>
<tr>
<td></td>
<td>f/ck f f ck</td>
<td>f Ck</td>
</tr>
<tr>
<td>Control</td>
<td>20 52 11 18</td>
<td>6.6 ±1.7 6.4 ±1.9</td>
</tr>
<tr>
<td>ketel&quot;</td>
<td>20 45 13 10</td>
<td>6.2 ±1.5 6.6 ±2.2</td>
</tr>
</tbody>
</table>

**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\(^6\) clones by mitotic recombination. The \(f\) clones are homozygous for the ketel\(^6\) 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. To understand effects of the Ketel mutations on nuclear protein import, we prepared cytosol from ovaries of both Ketel+/+ and wild type females. All the four Ketel 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+/+-derived cytosol preparations supported nuclear import of the cNLS-PE substrate just as efficiently as the wild type ovary cytosol showing that the Ketel-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 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+/+ 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 immunocytoology 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.
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 characterize 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 revealed 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^P_*) dominant female sterile mutations and their *ketel^r* revertant alleles (Szabad et al. 1989; Erdélyi et al. 1997). The *Ketel^P_* alleles are gain-of-function type and bring about dominant female sterility by inhibiting the commencement of embryogenesis. The *Ketel^P_*-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 (Tirian 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 revealed 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^P_*-associated dominant female sterility showing that the normal and the *Ketel^P_*-encoded mutant gene products participate in the same pathway. The slight rescue of *Ketel^P_*-associated dominant female sterility implies the dominant negative nature of the *Ketel^P_* mutations, i.e. the *Ketel^P_*-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, Görlisch 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 KetelP and ketel' mutant phenotypes (Tirian 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 elucidated.

**The possible mode of action of the KetelP-encoded proteins**

Embryogenesis fails to commence in the KetelP eggs deposited by the KetelP/+ females: the KetelP-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 KetelP 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 KetelP egg cytoplasm exert deleterious effects through the prevention of cleavage nuclei formation. Toxic effects of the KetelP-encoded molecules can be an outcome of arrested nuclear protein import. To elaborate this possibility, we prepared cytosol from ovaries of the KetelP/+ females and studied their effects on nuclear protein import. Unexpectedly the KetelP 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 KetelP or wild type ovary cytosol. Consistent with this observation, the KetelP egg cytoplasm did not prevent import of the cNLS-PE molecules into nuclei of interphase nuclei of wild type cleavage embryos. Knowing that the KetelP 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 KetelP-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 importina/β heterodimer at least in yeast (Görlich and Kutay 1999). It is also possible that although the KetelP-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 KetelP mutations become apparent at the end of cleavage mitosis when the NE reassemble and daughter nuclei form. Remarkably, the KetelP cytosol did not disrupt HeLa cell nuclei and, along with this observation, Drosophila wild type interphase cleavage nuclei remained intact in presence of the KetelP 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 KetelP 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 KetelP 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 KetelP-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-dits 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\(^P\)-encoded molecules is also supported by persistence of the MT bundles and asters seen in the Ketel\(^P\) 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\(^P\) mutations should help to understand the role of importin-\(\beta\) in the process. The knowledge of the position of three Ketel\(^P\) mutations enables us to clarify the molecular basis of the dominant Ketel effects.

The Ketel\(^P\) alleles are dominant negative mutations

Four observations show the gain-of-function nature of the Ketel\(^P\) mutations and that mutant gene products bring about the Ketel\(^P\)-related defects. (1) The Ketel\(^P\) 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\(^P\)/+ females, the +/+ clones appear with reduced frequencies and with several days delay due to perdurance of the Ketel\(^P\)-encoded mutant gene products in the +/+ cells (Szabad et al. 1989). (3) The gain-of-function features of the Ketel\(^P\) alleles can be eliminated during second mutagenesis and loss-of-function ketel\(^f\) recessive alleles are generated (Szabad et al. 1989; Erdélyi et al. 1997). (4) The deleterious effects of three of the four Ketel\(^P\) 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\(^P\)-associated maternal-effect lethality clearly shows expression of the Ketel gene during embryogenesis.

Analysis of the Ketel\(^P\)/+/+/+ females revealed the strong antimorph nature of the Ketel\(^P\) mutations and imply involvement of the normal and the Ketel\(^P\)-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 Tomaf the mutations, that identify the αtubulin67C gene of Drosophila,
represent a typical example; Máthé et al. 1998.) Although the KetelD 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 KetelD eggs versus death during
second larval instar of the ketel' 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'/-- 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'/-- hemizygotes up to second larval instar when they perish.

The KetelD-related defects are brought about following interaction of the KetelD-encoded
molecules with maternally provided partner(s): In understanding the mode of KetelD action,
the following facts should be considered. (1) The Ketel maternal dowry and the "toxic" nature
of the KetelD egg cytoplasm imply deposition by the KetelD/+ mothers both the normal and the
KetelD-encoded mutant gene products into the egg cytoplasm. (2) Lethality of the ketel' homo-
and hemizygotes implies expression of the normal Ketel and the KetelD alleles during
development. Yet the KetelD-encoded mutant gene products exert their deleterious effects only
on cleavage embryos. (3) When paternally derived, the KetelD alleles behave as the ketel' loss-
of-function alleles: they do not disturb but also can not support cell functions. We interpret the
deleterious effects of the KetelD mutations on cleavage embryos by interaction of the KetelD-
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 KetelD-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 KetelD 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\(^P\)-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 (Tirian 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-\(\beta\)** 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-\(\beta\) 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\(^{X13}\)/ 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


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