How is expression of the importin-β encoding *Ketel* gene of *Drosophila* regulated?

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2008.

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ABBREVIATIONS

Act	actin
arm	armadillo
BEAF	Boundary Element Associated Factor
CF2-II	chorion factor 2
CFDD	Common Regulatory Factor for DNA Replication
CNS	central nervous system
croc	crocodile
dl	dorsal
DRE	DNA Replication-related Element
DREF	DNA replication-related element binding factor
ds	double stranded
Elf1	grainy head
en	engrailed
esg	escargot
ey	eyeless
fb	fat body
Fs	dominant female-sterile mutation
ftz	fushi-tarazu
GAL4	positive regulator of gene expression for the galactose-induced genes in *Saccharomyces cerevisie* and in Drosophila transgenes
GFP	green fluorescent protein
NPC	nuclear pore complex
PCR	polymerase chain reaction
ptc	patched
SDS	sodium-dodecyl-sulphate
sna	snail
Su(H)	suppressor of hairless
TG	transgene
Tub	tubulin
twi	twist
UAS	upstream activator sequence in *Saccharomyces cerevisie* and in Drosophila transgenes
Ubx	Ultrabithorax
vg	vestigial
X-gal	A clorless compound (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) that is broken to a blue compound (5-bromo-4-chloro-3-hydroxyindole) and galactose by β-galactosidase

INTRODUCTION

It has long been known that most of the factors that are required during early embryogenesis are deposited into the egg cytoplasm during oogenesis and are maternally provided (DeRenzo and Seydoux 2004; Tadros and Lipshitz 2005). The importance of maternal contribution is emphasized by the fact that there is very little, if any, zygotic gene expression during the initial cleavage divisions in Drosophila. At the end of the 1980's, along a genetic dissection of maternal effects in Drosophila, people in the so-called Szabad laboratory isolated 75 dominant female sterile (*Fs*) mutations (Erdélyi and Szabad 1989; Szabad et al. 1989). In 32 of the *Fs* mutations the *Fs*/+ females deposit normal-looking eggs, and although the eggs are fertilized, embryogenesis does not commence or ceases after a few abnormal cleavage divisions inside the eggs. The 32 *Fs* mutations identify 21 genes, suggesting that products of several genes are required for the commencement and/or the initial steps of embryogenesis.

One of the 21 genes is *Ketel*, which was identified by four *Ketel*^{D} *Fs* mutations (Szabad et al. 1989; Erdélyi et al. 1997). The *Ketel* gene was cloned in the Szabad laboratory (Lippai et al. 2000; Tirián et al. 2000) and turned out to encode the Drosophila homologue of importin-β. Importin-β is a protein that is an essential component of nuclear protein import (Fig. 1).

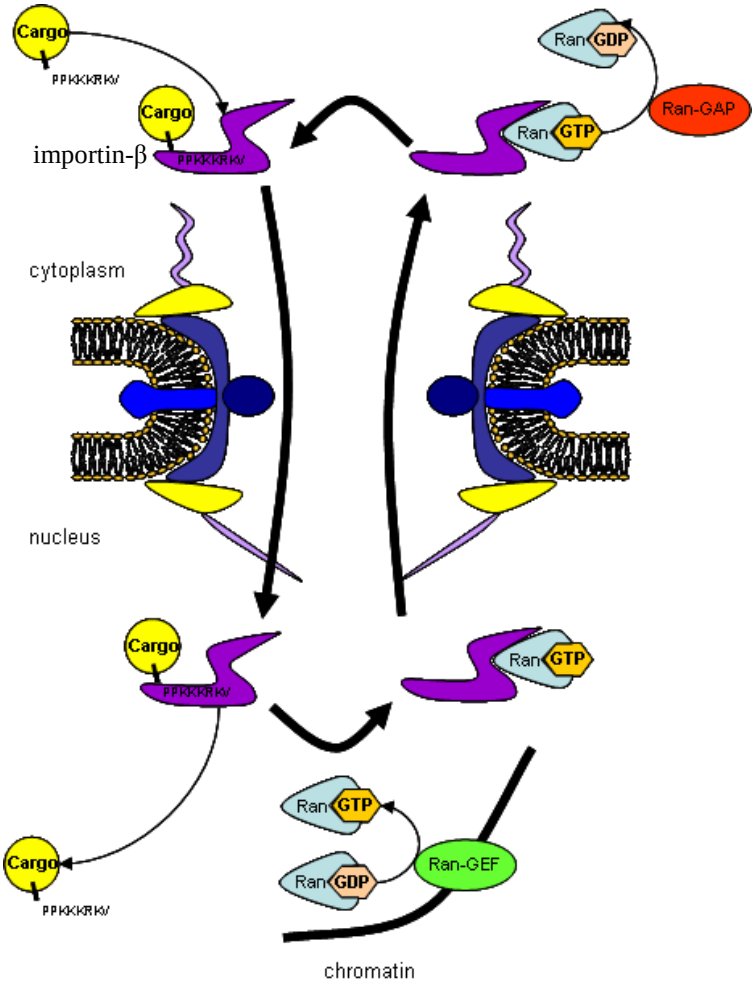


Figure 1. Mechanism of the nuclear protein import cycle. (Based on the http://www.steve.gb.com/science/protein targeting.html home page.) Cargo molecules bound to importin-β enter the nucleus through the nuclear pore complexes where they meet RanGTP and dissociate. While the cargo remains inside the nucleus to fulfill its function, the importin-β/RanGTP complex leaves the nucleus. Upon entering the cytoplasm, RanGTP becomes hydrolyzed to RanGDP following the action of Ran-GAP (Ran-GTP-ase Activator Protein) and also RanBP (The Ran-Binding Protein, not shown on the figure). While importin-β becomes free along with the GTP→GDP hydrolysis and is ready to pick up the next cargo, RanGDP is imported into the nucleus where it is converted to RanGTP through the only known and chromatin bound Ran-GEF nucleotide exchange factor, RCC1 (Regulator of Chromatin Condensation 1; see Görlich and Kutay 1999; Fried and Kutay 2003).

Analyses of the *Ketel*^{D} dominant negative female-sterile mutations lead to two important new findings. (1) Importin-β is engaged not only in nuclear protein import but is also an essential component during the reassembly of the nuclear envelope towards the end of mitosis (Tirián et al. 2000; Timinszky et al. 2002). (2) Analysis of the *Ketel*^{D} mutant phenotype suggested the involvement of importin-β in the formation of the spindle apparatus (Lippai et al. 2000; Tirián et al. 2003).

Second mutagenesis of the *Ketel*^{D} alleles lead to the induction of the so-called *ketel*^{revertant} alleles, some of which are complete loss-of-function (*ketel*^{null}) mutations, others are short deficiencies that remove the *Ketel* and a few of the adjacent loci (Szabad et al. 1989; Erdélyi et al. 1997). The *ketel*^{revertant} alleles are recessive zygotic lethal mutations: the *ketel*^{null} homo- or the *ketel*^{null}/– hemizygotes perish towards the end of the 2nd larval instar.

A number of enigmas emerged during the course of the former studies regarding *Ketel* gene expression regulation and importin-β function. The "difficult-to-understand" phenomena stimulated further research which kept me busy during the past four years and yielded the present dissertation. The obscure phenomena and the related questions are as follows:

(1) Analysis of a reporter gene, in which the *Ketel* gene regulatory sequences ensured the expression of the *LacZ* gene and the production of β-galactosidase, revealed the expression of the *Ketel* gene only in the diploid imaginal and not in the polytenic larval cells (Tirián et al. 2000). Results of Western blot analysis, that made use of an importin-β specific antibody, were in harmony with that of the reporter gene expression analysis (Lippai et al. 2000). Expression of the *Ketel* gene only in the diploid cells is puzzling since there is only a single importin-β coding gene in the Drosophila genome (Tirián et al. 2000) and nuclear protein import must also be going on in the metabolically vastly active polytenic larval cells. How do the polytenic cells import protein into their nuclei if all the known nuclear protein import mechanisms include importin-β or a closely related protein? Perhaps, through a not yet known nuclear protein import pathway? This would not be unusual since the human ribosomal protein L23a, for example, can be imported into the nuclei through at least four routes (Jäkel and Görlich 1998). However, function of the *Ketel* gene must be essential in at least some larval cell type(s) since zygotes without the *Ketel* gene die during the 2nd larval instar. Which are those cell types, i.e. the "focus of gene activity" in which function of the *Ketel* gene is essential?

(2) Zygotes without the importin-β encoding *Ketel* gene (the *ketel*^{null} homo- and the *ketel*^{null}/– hemizygotes) live for three days, up to the end of 2nd larval instar. It has been shown that the maternal dowry, provided by the *ketel*^{null}/+ heterozygous females, supports their relatively short life (Tirián et al. 2000). Assuming that function of the *Ketel* gene is required only in the diploid

cells, the death of the *ketel*^{null}/– larvae in 2nd larval instar is astonishing since larvae without diploid cells have been known to develop to the end of larval life (Szabad and Bryant 1982). Why do the *ketel*^{null}/– larvae not accomplish larval life?

(3) Clones of cells - that originated through mitotic recombination and become homozygous for a *ketel*^{null} allele - are fully viable and capable to differentiate normally in four different diploid cell types (Tirián et al. 2000). How can cells without importin-β live and differentiate normally?

(4) How is expression of the *Ketel* gene regulated? What sequences and mechanisms ensure expression of the *Ketel* gene in the diploid, dividing, and non-expression in the polytenic, non dividing larval cells?

To resolve the above rather perplexing observations we set out to localize the cell types in which function of the *Ketel* gene is essential and carried out the classical gynander-based focusing analysis (Bryant and Zornetzer 1973; Janning 1978). The experiment clearly showed that focus of *Ketel* gene action is either huge and/or is extended over large areas of the embryos. We then made use of the *Gal4*; *UAS* system (Brand and Perrimon 1993; Duffy 2002) and drove a *UAS-Ketel* transgene with different tissue-specific *Gal4* drivers on a *ketel*^{null}/– background and tested viability to adulthood of the *ketel*^{null}/– zygotes. The *Gal4*; *UAS* system revealed requirement of the *Ketel* gene in the embryonic ectoderm that is a rather large and wide spreading primordium in the blastoderm (Campos-Ortega and Hartenstein 1997). We also eliminated function of the *Ketel* gene by driving a *UAS-Ketel-RNAi* transgene - with different *Gal4* drivers - and inspected fate of the zygotes. Results of the RNAi experiments confirm largely the above conclusions.

Using GFP-tagged importin-β (encoded by a *ketel*^{GFP} allele; Karpova et al. 2006), we revealed that the maternally provided GFP-importin-β molecules persist up to the end of larval life and that the zygotic *Ketel* gene is expressed in every cell during early gastrulation. Although the *Ketel* gene is then turned off in the non-dividing larval cells, the already produced importin-β molecules persist long and carry out nuclear protein import throughout the subsequent stages of development. In fact, importin-β appears to be the longest lived type of proteins in the Drosophila cells (Villányi et al. 2008a).

To elucidate the mode of *Ketel* gene expression regulation, we made use of the so-called promoter analysis approach using *LacZ* reporter trangenes and determined which transcription factors and which transcription factor binding sites are engaged in the expression regulation of the *Ketel* gene, both in time and space (Villányi et al. 2008b).

As a result of a joint effort of a team, in which I had the pleasure to work, we answered the above questions and got to know the unusual mode of *Ketel* gene expression regulation.

MATERIALS AND METHODS

The *Ketel* mutant alleles

The *ketel*^{null}/– hemizygous larvae descended from a cross in which *y*/*y*; –/y^{+}*CyO* females were mated with *y*/*Y*; *ketel*^{null}/y^{+}*CyO* males, or vice versa. The *y*/*y* (and *y*/*Y*); *ketel*^{null}/– larvae appear *yellow* and can be selected conveniently as they do not carry the y^{+}*CyO* chromosome with the y^{+} gene. [The – symbol stands for a small deficiency (*ketel*^{rX32}) that removes *Ketel* and a few of the adjacent loci, *ketel*^{null} (= *ketel*^{rX13}) is a complete loss-of-function mutant allele (Erdélyi et al. 1997).] The *ketel*^{null}/– zygotes perish towards the end of the 2nd larval instar (Tirián et al. 2000). (For explanation of the genetic symbols see the FlyBase at http://flybase.bio.indiana.edu.)

The *ketel*^{GFP} allele, which encodes GFP-importin-β, was generated via the protein trap technique, which makes use of the random insertion of a P-element carrying the GFP-coding sequence without the start and the stop codes and a donor and an acceptor splice site at the two ends of the GFP exon (Morin et al. 2001; Karpova et al. 2006). Insertion of a "GFP exon" into the second intron of the *Ketel* gene leads to the production of full length, GFP-tagged importin-β under the control of the *Ketel* gene regulatory sequences (see Fig. 4). The GFP exon does not disturb the upstream and the downstream splicing events. Sequences for at least about 2 kb upstream from the +1 site in the *ketel*^{GFP} allele are as in wild type. Fluorescence emitted by GFP-importin-β molecules was detected in optical sections generated with an Olympus FV1000 confocal microscope or with an Olympus IX71 fluorescent microscope equipped with a cooled CCD camera.

The focus of *Ketel* gene function

To determine the cell type in which function of the *Ketel* gene is indispensable, i.e. the focus of the *Ketel* gene activity, we set out to generate *X*$\mathbf{X}^{\mathbf{TG}}$//*X*0, female//male mosaics (gynanders) in which the only functional *Ketel* gene was present in the transgene inserted into the $\mathbf{X}^{\mathbf{TG}}$ chromosome in the otherwise *ketel*^{null}/– zygotes. The *ketel*^{null}/– zygotes are viable and fertile in the presence of the $\mathbf{X}^{\mathbf{TG}}$ chromosome (Lippai et al. 2000). The *X* chromosome carried the *y* (*yellow* body), *w* (*white* eyes) and *f* (*forked* bristles) recessive marker mutations. The $\mathbf{X}^{\mathbf{TG}}$ chromosome carried, in addition to the transgene, wild type alleles of *y* and *f* and, thus, while the female (*X*$\mathbf{X}^{\mathbf{TG}}$) cells appear wild type and carry a functional *Ketel* gene, the male (*X*0) cells lack *Ketel* gene and display the mutant phenotypes. (A wild type allele of the *white* gene, the *mini-white* marker gene, was part of the transgene which ensured normal *Ketel* gene function; Lippai et al. 2000.) We isolated late 3rd instar gynander larvae, based on their y^{+} female and *y* male chitinous structures, small female and large male larval gonads and the slightly distorted shape

due to an overall reduced size of the male parts and determined whether they developed to adulthood or not.

The *X***X**$^{\mathbf{TG}}$//*X*0 mosaics were generated by crossing *y w f*/*y w f*; *ketel*^{null}/*Bc Gla*; +/+ females with **X**$^{\mathbf{TG}}$/*Y*; –/*SM5*; *Horka*^{D}/*TM3* males. Number and second chromosome composition of the offspring females and of the *X***X**$^{\mathbf{TG}}$//*X*0 mosaics were recorded. The *ketel*^{null}/*SM5*, the –/*Bc Gla* and the *Bc Gla*/*SM5* sibling females and gynanders served as "internal control" classes. *Bc Gla*, *SM5* and *TM3* stand for the so-called balancer chromosomes which are marked with dominant marker mutations (see the FlyBase). *Horka*^{D} rendered the **X**$^{\mathbf{TG}}$ chromosome unstable such that it might have been lost in the descendants during early embryogenesis (Szabad et al. 1995).

Tissue specific expression of a *UAS-Ketel* transgene

To determine the types of tissues in which expression of the *Ketel* gene rescues lethality of the *ketel*^{null}/– zygotes, i.e. in which the expression of the *Ketel* gene is necessary and sufficient, we crossed *ketel*^{null}/*Bc Gla*; *UAS-Ketel*/*UAS-Ketel* females with –/*SM5* males that carried a *Gal4* driver (Brand and Perrimon 1993; Duffy 2002; see also the FlyBase). The *Gal4* drivers we made use of are listed in the first column of Table 2. The *UAS-Ketel* transgene was constructed by Gyula Timinszky according to Brand and Perrimon (1993) and contained the structural part of the *Ketel* genomic sequence (Villányi et al. 2008a). The *UAS-Ketel* transgenes became inserted into the X, the 2nd or the 3rd chromosomes. When driven by a ubiquitously expressed *Gal4* driver (such as *α1Tub-Gal4)*, the *UAS-Ketel* transgenes efficiently rescue the *ketel*^{null}/– associated lethality. Since the *UAS-Ketel* transgenes carry the *mini-white*$^{+}$ marker gene, all the crosses were conducted on a *white* genetic background. Drivers located on the 2nd chromosome were recombined into the chromosome that carried a *ketel*^{null} allele. Viability to adulthood of the *ketel*^{null}/– offspring flies carrying both a *Gal4* driver and a *UAS-Ketel* transgene was monitored.

Tissue specific abolition of the *Ketel* gene function by the RNAi technique

To eliminate the function of the *Ketel* gene in specific cell types, we crossed females carrying a *Gal4* driver with males that carried a *UAS-Ketel-RNAi* transgene (Dietzl et al. 2007) and monitored the fate of those zygotes that carried a driver as well as the *UAS-Ketel-RNAi* transgene.

The life span of GFP-importin-β

To determine the life span of the maternally provided GFP-importin-β molecules, we crossed *y*/*y*; *ketel*^{GFP}/*y*$^{+}$*CyO* females with y/*Y*; +/+ males and screened the non-*yellow* (*y*/*y* or *y*/*Y*;

+/y^{+}*CyO*) larvae and pupae for the presence of GFP-importin-β. In such larvae and pupae the GFP-importin-β molecules must have been maternally provided through the egg cytoplasm.

The expression of the paternally derived *Ketel* gene

To determine when the paternally derived *ketel*^{GFP} allele (representing the *Ketel* gene) is first expressed during embryogenesis, we crossed wild type (+/+) females with *ketel*^{GFP}/*CyO* males, collected embryos for 30 minutes, aged them for different amounts of time and analyzed for the presence of GFP-importin-β through time lapse optical sections. The nuclei were highlighted by histone-RFP (Schuh et al. 2007).

***In silico* promoter analysis**

To reveal the sequences that may regulate expression of the *Ketel* gene, we analyzed an 1877 bp long region between the *Eco*RV (at -1471) and the *Pst*I (at +406) restriction sites, slightly upstream of the only ATG translation start code in the *Ketel* gene (Fig. 2). The promoter analysis was carried out with the TRES software (http://bioportal.bic.nus.edu.sg/tres/). To identify the sequences that may be engaged in the regulation of *Ketel* gene expression, we compared the corresponding promoter sequences in the following Drosophila species: *melanogaster, simulans, sechellia, yakuba* and *erecta* and focused attention on the evolutionarily conserved transcription factor binding sites. (See the FlyBase for the genome data.)

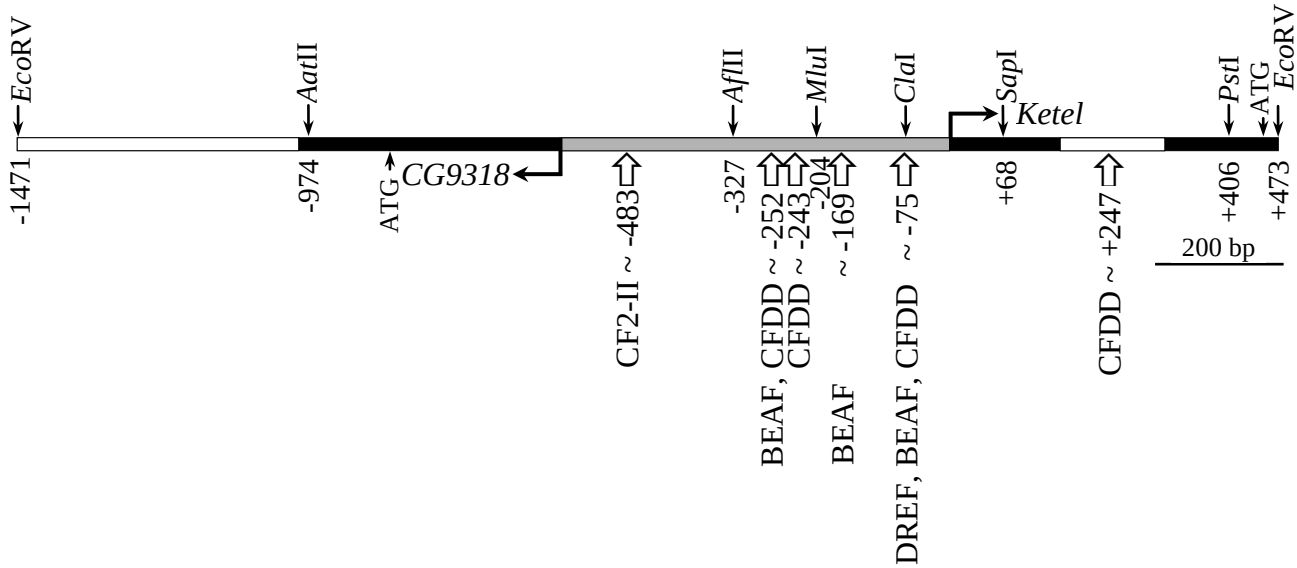


Figure 2. Landmarks around the 5' end of the Ketel gene in *Drosophila melanogaster*. (A) Bird-eye-view showing the restriction sites that were of importance in the present analysis, the binding sites of the transcription factors BEAF, CFDD, CF2-II and DREF, the transcription (↵ and ↱) as well as the translation start sites (ATG). Black, white and grey bars represent exons, introns and the sequence between the two genes, respectively.

Gel-shift experiments

To detect protein-DNA interactions, gel-shift assays were performed as described in Pápai et al. (2005). Briefly, 30-137 bp long fragments were generated by PCR. The fragments included binding sites for different transcription factors (see Fig. 8). The DNA fragments were gel purified and end-labelled by ^{32}P-γ-ATP using the T4 polynucleotide kinase. The 63 bp long PCR4 fragment was created as follows: a 93 bp long fragment was PCR amplified first, digested with *Vsp*I, gel purified and labelled with *Klenow* fill in using ^{32}P-α-ATP. The labelled fragments were separated from the unincorporated nucleotides by gel electrophoresis. Nuclear extracts were prepared from the S2 *Drosophila* cells as described by Dignam et al. (1983). The ^{32}P-labeled probe was incubated in 20 µl of reaction mixture containing 15 mM HEPES (pH 7.8), 60 mM KCl, 0.1 mM EDTA 1 mM dithiothreitol and 10% glycerol at room temperature for 30 min. Each reaction mixture contained 5,000-10,000 cpm ^{32}P-DNA, 3-5 µl nuclear extract (3-10 µg protein), 0.008 – 0.04 µg of poly(dIdC) competitor DNA to avoid unspecific DNA-protein interactions. Specific competitor DNA and anti-DREF antibody (a kind gift of Rafael Garesse) were added as indicated in the legend of Figure 10. The specific competitor DNA was dsDRE, a 30 bp long double-stranded oligonucleotide with the DRE motif (TATCGATA): 5'GTTATTAGATTTAAAAATTATCGATAGTTC3'. The DNA-protein complexes were electrophoretically resolved on a 5% non-denaturing polyacrylamide gel in 50 mM Tris-borate (pH 8.3) 1 mM EDTA at 4ºC. The gels were dried and autoradiographed on X-ray films.

Generation of *LacZ* reporter transgenes and analysis of their expression pattern

Transgenic lines, in which different segments of the *Ketel* gene promoter were combined with *LacZ*, were generated by standard P-element germ-line transformation using the *mini-white* marker gene (Spradling 1986). All the fly stocks carried the *white* marker mutation and were kept at 25^{o}C. The different *Ketel* promoter DNA fragments were first ligated to pKS Bluescript^{TM} plasmids and then transferred to a CaSpeR-AUG-β-gal vector (Thummel et al. 1988). During the construction of the so-called -74→+406 transgene, the insertion of the *Cla*I and *Pst*I restriction enzyme-digested region into the pKS Bluescript^{TM} plasmid created an inactive DRE motif (see Fig. 8). The -979→+406 transgene with a mutated DRE site was created through PCR mutagenesis using the following primer pairs such that the TATCGATA DRE motif was replaced by GCCAAGCGGC:

5'GGTTGCACATTTCCTACGCATTTA 3',

5'GCCGCTTGGCATTTTTAAATCTAATAACGTAT 3'

and

5'GCCAAGCGGCGTTCGAATAAAGCAATCG 3',

5'GCGACACCTATTCGATTGCTTTAT 3'

Altogether six different types of transgenes were generated, with 2-4 lines each. Chromosomal locations of the different lines of the different types of the transgenes were as follows: for -974→+406: X, 2nd and 2x3rd; for -327→+406: 2xX and 2x2nd; for -327→+68: X, 2x2nd and 3rd; for -204→+68: X, 2nd and 2x3rd; for -74→+406: X, 2nd and 2x3rd; for -974→+406 with a mutated DRE motif: 2xX (see Fig. 11).

To monitor β-galactosidase activity, X-gal staining was carried out on embryos and on late 3rd instar larvae as described in Ashburner (1989). For staining tissues, late 3rd instar larvae were "blown up" with the fixative and cut open along the dorsal midline with a fine scissors in PBS (Szabad et al. 1979). The organs were dissected and post-fixed for 10 min. in PBS containing 2% formaldehyde and 0.2% glutaraldehyde. The tissues were rinsed in PBS and stained in 1% X-gal containing buffer (5 mM $K_3Fe(CN)_6$, 5 mM K_4 $Fe(CN)_6$ 2 mM $MgCl_2$ in PBS) in dark at 37 °C. The organs were prepared and analyzed using a light microscope.

RESULTS

The focus of the *Ketel* gene function is large and/or widespread

To locate the tissue in which the function of the *Ketel* gene is indispensable (the focus), we attempted to generate $X\mathbf{X}^{\mathbf{TG}}$//*X*0 female//male mosaics in which the $X\mathbf{X}^{\mathbf{TG}}$ cells carried a *Ketel* gene (in the $\mathbf{X}^{\mathbf{TG}}$-linked transgene; Lippai et al. 2000), while the *X*0 cells did not. Principles of focusing are as follows: since the borderline separating the $X\mathbf{X}^{\mathbf{TG}}$ and the *X*0 tissues runs randomly in the different $X\mathbf{X}^{\mathbf{TG}}$//*X*0 mosaics, those gynanders are expected to develop to adulthood in which the focus is composed from $X\mathbf{X}^{\mathbf{TG}}$ cells. The *X*0 cells may include tissues where the function of the *Ketel* gene is not essential. Distribution of the $X\mathbf{X}^{\mathbf{TG}}$ (female) and the *X*0 (male) tissues in the surviving mosaics should thus, pinpoint the focus of *Ketel* gene action (see Bryant and Zornetzer 1973).

While 313 "internal control" sibling $X\mathbf{X}^{\mathbf{TG}}$//*X*0 gynanders (that carried a functional *Ketel* gene in a balancer chromosome) were recovered in the experiment, not a single mosaic of the expected type survived to adulthood (Table 1). Based on the frequencies of the sibling females and the "internal control" gynanders, formation of 23 $X\mathbf{X}^{\mathbf{TG}}$//*X*0; $ketel^{null}$/– mosaics was expected (Table 1). We also identified 41 gynanders as late 3rd instar larvae. Every one of them developed to adulthood and turned out to be of "internal control" type. Absence of the expected type of gynanders indicates a large focus and/or a small focus that spreads over large areas in the developing embryos (Bryant and Zornetzer 1973).

Table 1. Features of $X\mathbf{X}^{\mathbf{TG}}$//*X*0 female//male mosaic production

Genotype	Role in experiment	Female	$X\boldsymbol{X}^{\boldsymbol{TG}}$//*X*0 mosaic
$X\boldsymbol{X}^{\boldsymbol{TG}}$; *ketel*^{null}/*SM5* $X\boldsymbol{X}^{\boldsymbol{TG}}$; –/*Bc Gla* $X\boldsymbol{X}^{\boldsymbol{TG}}$; *SM5*/*Bc Gla*	"Internal control"	15,903	313 (1.9%)
$X\boldsymbol{X}^{\boldsymbol{TG}}$; *ketel*^{null}/–	Experimental	1,193	0

Notes
- $\boldsymbol{X}^{\boldsymbol{TG}}$ stands for an X chromosome that carries a functional *Ketel* transgene.
- The *SM5* and the *Bc Gla* balancer chromosomes carry a functional *Ketel* gene.

Focusing with the *Gal4*/UAS system

To determine the types of tissues in which the expression of the *Ketel* gene is indispensable, we generated *ketel*^{null}/– zygotes in which a set of *Gal4* drivers (of course, one in each experiment) ensured the expression of a *UAS-Ketel* transgene and tested whether the *Gal4*; *UAS-Ketel*; *ketel*^{null}/– zygotes survive to adulthood (Table 2).

Ubiquitous expression of the *UAS-Ketel* transgene (using the *α1Tub-Gal4* driver) overcame lethality of the *ketel*^{null}/– zygotes: the *α1Tub-Gal4*; *UAS-Ketel*; *ketel*^{null}/– zygotes developed to adulthood (Table 2). (Note that the *UAS-Ketel*; *ketel*^{null}/– and the *α1Tub-Gal4; ketel*^{null}/– control types of zygotes, just like the *ketel*^{null}/– ones, perish towards the end of the 2nd larval instar.) When expression of the *UAS-Ketel* transgene was driven by the *elav-Gal4*, the *esg-Gal4* or the *vg-Gal4* drivers, the *UAS-Ketel*; *ketel*^{null}/– zygotes developed to adulthood, with essentially the expected frequencies (Table 2). A common feature of these drivers is that they induce the expression of the GAL4 protein in the embryonic ectoderm, the progenitor cells for the epidermis and the nervous system (Table 2). Since expressing *Gal4* in the neuroectoderm (by *ey-Gal4*), in the embryonic central and peripheral nervous systems (by *arm-Gal4*), in the embryonic central nervous system (by *en-Gal4*, in a segmentally repeated pattern) or in the embryonic brain (by *ptc-Gal4*) only did not result in the survival of the *ketel*^{null}/– zygotes (Table 2), it may be concluded that zygotic expression of the *Ketel* gene in the epidermis primordial cells is necessary to accomplish development. [Production of GAL4 in the imaginal disc primordia (by *dll-Gal4*) or only in the presumptive mesoderm cells (by *twi-Gal4*) did not overcome lethality of the *UAS-Ketel*; *ketel*^{null}/– zygotes; Table 2]. Since none of the *elav-Gal4*, the *esg-Gal4* and the *vg-Gal4* drivers is expressed in e.g. the mesoderm-derived cells, it is hard to understand how, for instance, the *vg-Gal4*; *UAS-Ketel*; *ketel*^{null}/– zygotes survive to adulthood.

Table 2. Focusing with the *Gal4*; *UAS* system making use of a *UAS-Ketel* or a *UAS-Ketel-RNAi* transgene

Gal4 driver*	Gene represented by the *Gal4* driver	Expression pattern of the gene and/or the *Gal4* driver	Survival of the *Gal4*; *UAS-Ketel*; *ketel*^{null}/– zygotes to adulthood	Fate of the *Gal4*; *Ketel-RNAi* zygotes
*α1Tub-Gal4** (P{tubP-GAL4}LL7)	*αTub84B*	Ubiquitous expression of GAL4 (Lee and Luo 1999).	YES	–
*Act5c-Gal4** (P{Act5C-GAL4}25F01)	*Actin5C*	Ubiquitous expression of GAL4 (Ito et al. 1997).	–	Die in 2nd larval instar
*elav-Gal4** (P{GAL4-elav.L}3)	*elav*	In young embryos the elav transcripts are present in the dorsal ectoderm and the embryonic nervous system (Campos et al. 1987, Robinow and White 1988). The *elav*-Gal4 driver ensures GAL4 expression in the embryonic and the larval nervous system and in all the postmitotic neurons (Luo et al. 1994; Schuster et al. 1996; Sink et al. 2001).	YES	Die as pupae
*esg-Gal4** (*GAL4*$^{esg\text{-}NP5130}$)	*escargot*	The *esg* gene is expressed in the ectoderm, the neurectoderm, the embryonic nervous system, the presumptive imaginal discs, and the histoblasts (Whiteley et al. 1992*;* Hayashi et al. 1993). The *esg-NP5130* Gal4 enhancer trap line ensures GAL4 expression in the *esg* gene expression pattern (Goto and Hayashi 1999).	YES	Die as pupae
vg-Gal4 (P{vg-GAL4.B})	*vestigial*	The *vg* gene is expressed in the embryonic ectoderm, in the presumptive central nervous system and in the imaginal discs (Williams et al. 1991; Williams et al. 1994; Huang and Rubin 2000).	YES	Die as pupae
ey-Gal4 (*GAL4*$^{ey.PH}$)	*eyeless*	The *ey* gene is expressed in the embryonic central nervous system, the procephalic neurectoderm, a subset of the protocerebral neuroblasts, the embryonic acron and the eye-antennal disc primordia (Quiring et al.1994; Hazelett et al. 1998; Urbach and Technau 2003; Hirota et al. 2005). Expression of the *ey-Gal4* drivers reflects expression of the ey gene.	No	Die as pupae
*ptc-Gal4** (P{GawB}ptc559.1)	*patched*	The *ptc* gene is expressed in the embryonic/larval hindgut, the neuroblasts in the head, the labrum, the Malpighian tubules and the analia (Hooper and Scott 1989). The *pct-Gal4* driver ensures GAL4 expression in the embryonic brain (in a segmentally repeated pattern), and in the foregut visceral mesoderm (Speicher et al. 1994*;* Page 2002).	No	Die as pupae
*en-Gal4** (P{en2.4-GAL4}e16E)	*engrailed*	The *en* gene is expressed in parasegments 2-15, in certain groups of the neuroblasts, in a segmentally repeated pattern in the embryonic central nervous system (Mlodzik et al. 1990*;* Doe 1992; Namba et al. 1997). The EN protein is expressed in stripes in the posterior region of each segment in the developing embryo (Patel et al. 1989). The *en-Gal4* driver ensures GAL4 expression in the *en* gene expression pattern (Weiss et al. 2001).	No	–
*arm-Gal4** (P{GAL4-arm.S}11)	*armadillo*	In contrast to the uniform distribution of arm mRNA, the ARM protein is present in segmental stripes at the posterior half of the anterior compartments. In stage 13 embryos, the ARM protein is present in the central and in the peripheral nervous systems (Riggleman et al. 1990). The *arm-Gal4* driver ensures GAL4 expression in the *arm* gene expression pattern.	–	Viable
dll-Gal4 (*GAL4*$^{Dll\text{-}md23}$)	*distalless*	The *dll* gene is expressed during the extended germ band stage in the maxillary segment, the leg primordia, the labral segment and proboscis (Cohen et al. 1989; Simcox et al. 1991; Vachon et al. 1992). The *dll-Gal4* driver ensures GAL4 expression in the *dll* gene expression pattern (Lecuit and Cohen 1997; Gorfinkiel et al. 1997).	No	Viable
*twi-Gal*4 (P{GAL4-twi.G})	*twist*	The *twi* gene is expressed in the presumptive mesoderm (Thisse et al. 1987; Leptin 1991). The *twi-Gal4* driver ensures GAL4 expression in the *twi* gene expression pattern (Baylies and Bate 1996; Riechmann et al. 1997*;* Hacker and Perrimon 1998).	No	Viable

* Source: Bloomington Stock Center

Focusing with the RNAi system

To further elaborate on the requirement of the *Ketel* gene, we eliminated its function in specific tissue types using the RNAi technique. A set of *Gal4* drivers ensured tissue specific expression of a *UAS-Ketel-RNAi* transgene and the formation of an interfering RNA to silence *Ketel* mRNA and thus, gene function (Dietzl et al. 2007).

All over-expression of the *UAS-Ketel-RNAi* transgene by the *Actin5C-Gal4* driver lead to the elimination of zygotic *Ketel* gene function and death of the zygotes in late 2nd larval instar (Table 2). Features of the dying *Actin5C-Gal4*; *UAS-Ketel-RNAi* and the $ketel^{null}$/– larvae, which lack the zygotic *Ketel* gene, were essentially identical. This observation indicates complete abolition of the *Ketel* gene function via the RNAi technique and implies that the viability of zygotes lacking *Ketel* gene function is supported by the maternally provided importin-β protein pool and not by *Ketel* mRNA, at least not beyond the blastoderm stage when expression of many of the zygotic genes commence (Tadros and Lipshitz 2005). If the maternally provided *Ketel* mRNA molecules played some role beyond the blastoderm stage in the survival of the $ketel^{null}$/– zygotes up to the end of the 2nd larval instar, the *Actin5C-Gal4*; *UAS-Ketel-RNAi* larvae should die well before the end of the 2nd larval instar. The above consideration implies long persistence of the importin-β protein.

Elimination of the *Ketel* gene function in the embryonic ectoderm and in the presumptive nervous system (by driving the *UAS-Ketel-RNAi* transgene with the *elav-Gal4*, the *esg-Gal4* or the *vg-Gal4* drivers) resulted in lethality during pupal life and not towards the end of 2nd larval instar as it might have been expected. Analysis of late 3rd instar larvae did not reveal any apparent morphological abnormality: the imaginal discs, the central and the peripheral nervous systems appeared normal. The reason for the discrepancy between the observed and the expected phases of death well may be the difference in time between the expression of the zygotic *Ketel* genes, the ensuing *de novo* formation of importin-β and the destruction of the encoding *Ketel* mRNAs. The formed importin-β molecules can support life of e.g. the *elav-Gal4*; *UAS-Ketel-RNAi* larvae well beyond the end of 2nd larval instar, into pupal life. Assuming efficient RNAi action (as shown above), long persistence of the formed importin-β molecules well may account for the above difference.

Zygotes in which the *ey-Gal4* driver ensured expression of the *UAS-Ketel-RNAi* die as pupae due to the lack of most of the head capsule (Table 2, Fig. 3). The *ptc-Gal4*; *UAS-Ketel-RNAi* zygotes also die as pupae due to multiple head defects. Elimination of zygotic *Ketel* gene function by driving the *UAS-Ketel-RNAi* transgene with the *arm-Gal4*, the *dll-Gal4* or the *twi-Gal4* drivers had no effect on the viability of the zygotes. (Normal development of the *arm-Gal4*; *UAS-Ketel-RNAi*, *dll-Gal4*; *UAS-Ketel-RNAi* and the *twi-Gal4*; *UAS-Ketel-RNAi* zygotes can not be related to the lack of *Gal4* driver function since all three drivers ensured expression of

a *UAS-GFP* transgene and the formation of characteristic green fluorescence pattern.) Survival of the above zygotes must be attributed to the presence of functional importin-β molecules in those cells from which the *Ketel* mRNA molecules were removed by RNAi. The most likely explanation for the phenomenon is that the *Ketel* gene is expressed and importin-β is synthesized before the encoding *Ketel* mRNAs are eliminated and the importin-β molecules thus formed persist all through the rest of the development. [However, complete knock down of gene function through RNAi may not be equally efficient for the different drivers (Dietzl et al. 2007).]

Results of the RNAi experiments suggested long persistence of importin-β. To characterize the longevity of importin-β, we made use of a *ketel*^{GFP} allele which encodes the formation of GFP-importin-β and assumed that the two types of molecules possess comparable life spans.

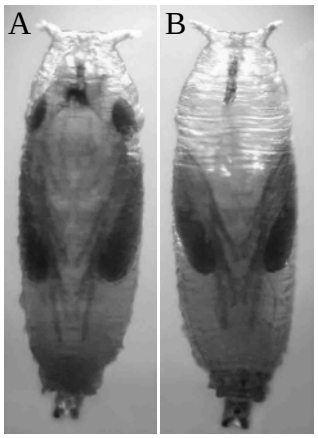


Figure 3. Ventral view of a wild type pupa (A) and a pupa in which the *ey-Gal4* driver ensured expression of the *UAS-Ketel-RNAi* transgene (B). While most head structures are missing in (B) the thorax and the abdomen are normal.

The maternally provided GFP-importin-β persists unusually long

It has been well established that importin-β is maternally provided and supports life of the *ketel*^{null}/– hemizygous larvae throughout the 1st and the 2nd larval instars (Tirián et al. 2000). To visualize the maternally provided importin-β, we made use of a *ketel*^{GFP} allele in which a GFP-coding exon became inserted into the second intron of the *Ketel* gene (Fig. 4). GFP disrupts importin-β near its N-terminus, in the RanGTP binding domain (reviewed in Görlich and Kutay 1999; Fried and Kutay 2003). Although the GFP tag reduces the function of GFP-importin-β, as shown by the lethality of the *ketel*^{GFP}/– hemizygous larvae, the GFP-importin-β molecules are not completely inactive since several of the *ketel*^{GFP}/– hemizygous larvae live up to pupariation, well beyond the *ketel*^{null}/– larvae, which all die by the end of the 2nd larval instar. The nuclear pore complex (NPC) binding ability of GFP-importin-β appears normal as shown by the characteristic bright spotted green fluorescent ring along the cytoplasmic surface of the nuclear envelope (see Figures 5 and 6).

To analyze the life span of the maternally provided GFP-importin-β, we crossed *ketel*^{GFP}/+ females with wild type males and sorted out +/+ larvae and pupae that completed embryogenesis

inside the egg cytoplasm produced by the *ketel*^{GFP}/+ females. (Egg cytoplasm of the *ketel*^{GFP}/+ females glows bright green.) The maternally provided GFP-importin-β molecules are present and delicately highlight the cytoplasmic surface of the nuclear envelope in all the larval cells (Fig. 5). Although the GFP-importin-β ring becomes fainter and fainter during larval development, it is clearly visible up to the end of larval life in all the larval and imaginal cells. Finally, the GFP-importin-β related signal becomes invisible within one hour following pupariation in midst of the strongly autofluorescent structures. Apparently at least some of the maternally-provided GFP-importin-β molecules live remarkably long, for at least five days after they were produced. Indirect evidences, presented in the above chapter on RNAi and cited in the discussion, indicate that wild type importin-β molecules also persist long.

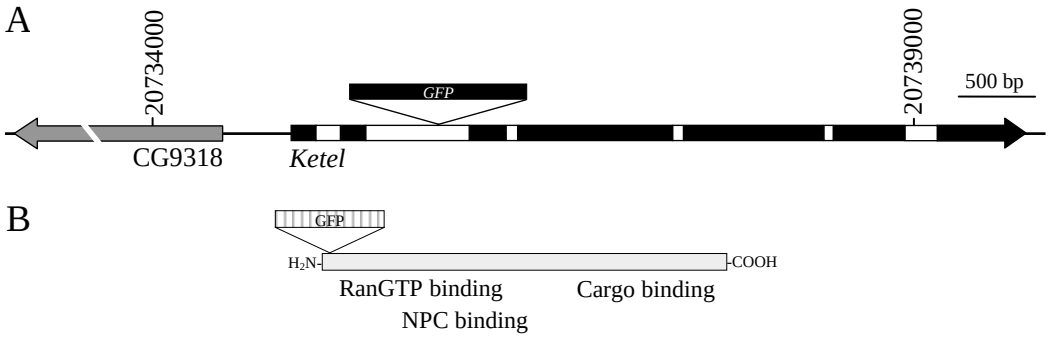


Figure 4. Organization of the *Ketel* gene (A) and the encoded importin-β (B). In (A) the eight-digit-numbers mark nucleotide positions in the Drosophila genome. Black and white boxes represent exons and introns, respectively. Insertion of a GFP-coding exon (between nucleotides 20735883 and 20735884) into the second intron led to the formation of a *ketel*^{GFP} mutant allele, which encodes GFP-importin-β. (B) The 884 amino acid long importin-β, as other members of the importin-β superfamily, contains three well-defined domains: an N-terminal RanGTP binding domain, a NPC binding domain which associates with specific components of the NPCs and a C-terminal cargo binding domain (reviewed in Görlich and Kutay 1999, Fried and Kutay 2003). In GFP-importin-β, a 238 amino acid long GFP tag is inserted between the 18th and the 19th amino acids.

Obviously, the maternally provided molecules ensure survival of the *ketel*^{null}/– larvae up to the end of the 2nd larval instar. However, as development proceeds and the larval cells increase in size, the importin-β molecules become diluted or some may decompose and can not sustain the life of *ketel*^{null}/– larvae anymore. To elucidate the fate of the maternally provided GFP-importin-β during development, we measured - in optical sections - the intensities of the bright halos around the nuclear perimeter of the Malpighian tubule cells. The signal intensities correlate with the GFP-importin-β concentrations (Fig. 5). Results of the analysis are summarized in Table 3 and indicate that although the fluorescence intensities (and thus local concentration of GFP-importin-β) decrease during enlargement of the nuclei, the total amount of the fluorescent

molecules remains constant over the nuclear surface indicating survival of the maternally provided GFP-importin-β molecules throughout larval life, at least of those associated with the NPCs.

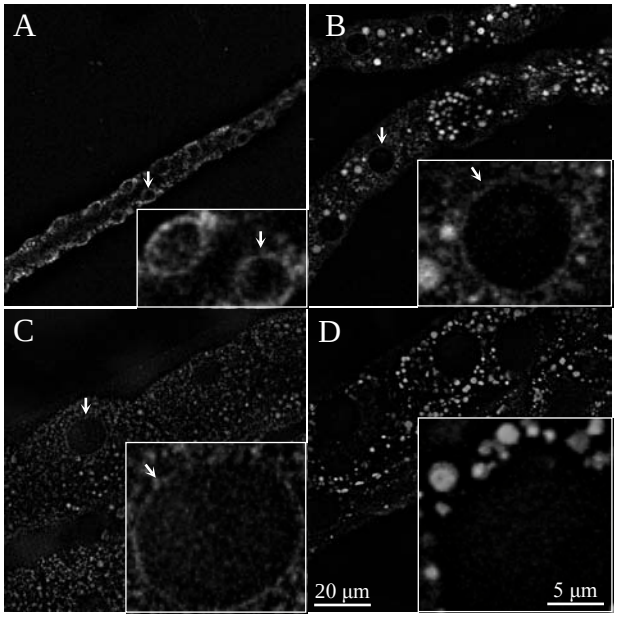


Figure 5. The maternally provided GFP-importin-β highlights the nuclear envelope in Malpighian tubule cells of 2nd (A), mid (B) and late 3rd instar larvae (C) but not in young pupae (D). White arrows point to the GFP-importin-β-highlighted nuclear envelope as it appears in optical sections. Inserts show four-fold magnification of nuclear regions. (Bright dots represent autofluorescent components that are not present in the nuclei.)

Table 3. The amount of GFP-importin-β around Malpighian tubule nuclei in wild type larvae that descended from *ketel*^{GFP}/+ females

Stage of larval development	Fluorescence intensity around the nuclear perimeter[a]	Diameter of nuclei (μm)	Fluorescence over surface of the nuclear envelope [b]
2nd instar	272 ± 26	4.0 ± 0.2	1088
Early 3rd instar	149 ± 20	8.1 ± 1.3	1192
Late 3rd instar	93 ± 13	12.0 ± 1.8	1116

[a] Signal intensity in the pixels, as determined with the ImageQuant software, in the bright halo around the perimeter of the nuclear envelope as compared to the background. Twenty-five nuclei (5 in 5 larvae) were analyzed at each stage. Average ± standard deviation.
[b] Calculated based on fluorescence intensity in the halo around the nuclear perimeter and assuming spherical shape of the nuclei.

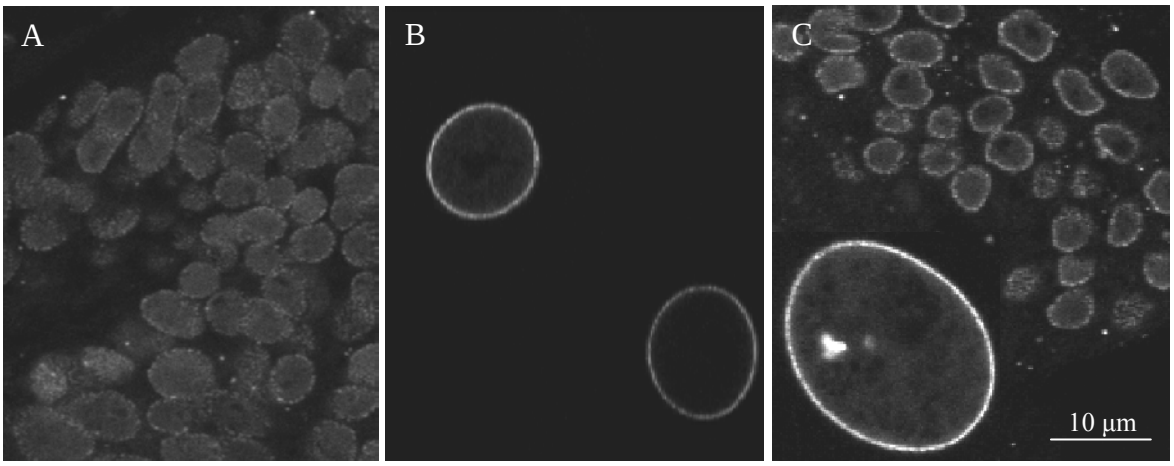


Figure 6. GFP-importin-β, encoded by the paternally derived *ketel*^{GFP} allele, appears as a bright halo around the nuclear envelope in optical sections. (A) Diploid cells in the ventral ganglion. (B) Polytenic cells in the Malpighian tubule. (C) Salivary gland with a giant nucleus and some of the diploid imaginal ring cells. (The organs were dissected from the same late 3rd instar larva.)

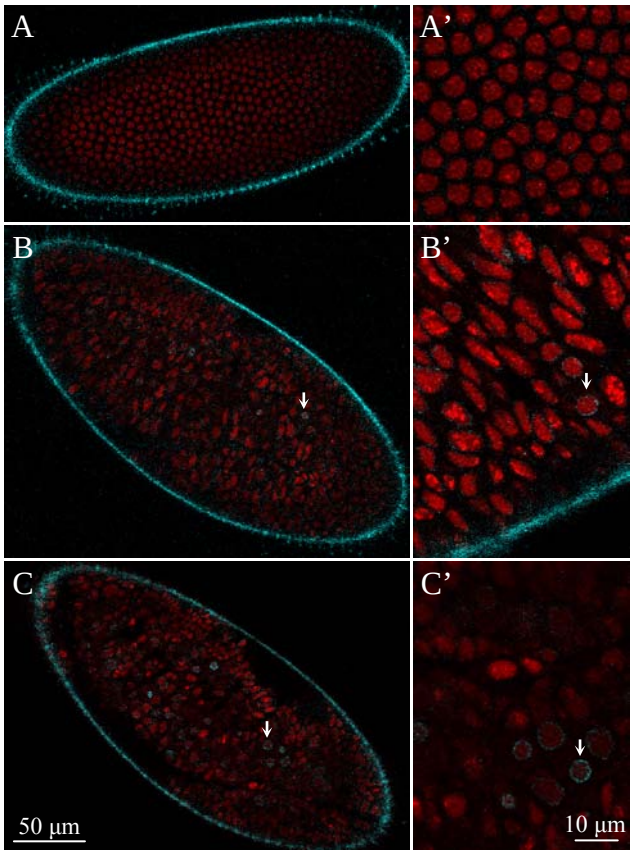


Figure 7. Expression of the zygotic Ketel gene begins during the onset of gastrulation. Zygotic expression of the *ketel*^{GFP} allele is revealed through the formation of GFP-importin-β and the appearance of a green halo (↓) around the nuclear envelope in the optical sections. The nuclei were highlighted in red by histone-RFP. The live embryos were 3, 4 and 6 hours old and were in the blastoderm (A and A'), in the germ band extension (B and B') and in the segmentation stage (C and C'). (The stages of development are described in the flymove.uni-muenster.de web site). Panels on the right side depict high magnification of some of the nuclei shown in the left panels. GFP-importin-β, just like wild type importin-β detected by immunostaining, highlights only the interphase nuclei.

Expression of the zygotic *Ketel* gene commences during early gastrulation

To determine when during development the expression of the *ketel*^{GFP} allele commences, we crossed wild type (+/+) females with *ketel*^{GFP}/+ males and monitored the appearance of GFP-importin-β around the nuclear envelope in the descending embryos. The nuclei were highlighted by histone-RFP (Schuh et al. 2007). We simultaneously monitored red and green fluorescence in optical sections on live embryos from late cleavage divisions throughout the subsequent stages of development for about six hours. There was no sign of GFP-importin-β before the cellular blastoderm stage (Fig. 7). The first albeit rather week GFP-importin-β related signal, the indicator of zygotic expression of the *ketel*^{GFP} allele, appeared as green dotted halos around the nuclear envelope of the interphase nuclei four hours following the commencement of embryogenesis, during early gastrulation. Since GFP-importin-β related green halos formed around all of the interphase nuclei, it is safe to conclude that the paternally derived *ketel*^{GFP} allele is expressed in every cell of the zygote.

***In silico* analysis of the *Ketel* gene promoter sequence**

Analysis of Expressed Sequence Tag data revealed a single transcription start site in the *Ketel* gene. The nearest transcription start site resides in the neighbouring *CG9318* gene, 604 bp upstream of the *Ketel* transcription start site (Fig. 2). To identify the sequences which may be engaged in the regulation of *Ketel* gene expression, we chose a 1944 bp long sequence delineated by two *Eco*RV sites: one in the first intron of the *CG9318* gene and the other in the second exon of the *Ketel* gene (Fig. 2). Two observations suggest that the chosen sequence contains all the elements needed for the normal expression of the *Ketel* gene. (1) The *Ketel*$^{+}$ transgenes in which importin-β production is controlled by the 1944 bp long sequence, fully rescue the lethality of the *ketel*^{null}/– zygotes (our unpublished result). (2) During embryogenesis the expression patterns are identical for the *Ketel* gene (as determined through *in situ* hybridizations to the *Ketel* mRNA) and for the reporter transgenes in which the *Aat*II - *Pst*I region control the expression of the *LacZ* gene (Lippai et al. 2000, Tirián et al. 2000). This observation also indicates that there are no regulatory sequences in the 497 bp long *EcoR*V - *Aat*II region and in the 67 bp long *Pst*I - *Eco*RV sites in the 2nd exon of the *Ketel* gene (Fig. 8).

Computer analysis of the analyzed region revealed several known transcription factor binding sites in the *Ketel* gene promoter (Fig. 8). We focused attention on those binding sites which are evolutionarily conserved in the corresponding regions in at least three Drosophila species among the following: *melanogaster, simulans, sechellia, yakuba* and *erecta*.

The *Ketel* promoter does not contain a TATA box. However, it contains a number of conserved motifs including the TATCGATA palindrome sequence known as the DRE motif (Hirose et al. 1993.) Part of the DRE motif is CGATA, one of the CFDD binding sites. (The other is

CAATCA.) The BEAF (Boundary Element-Associated Factor) transcription factor also binds to CGATA. DREF and BEAF have been known to compete for the DRE motif (Hart et al. 1999). The BEAF binding sites are present in special chromatin structures and a minimum of three BEAF binding sites are needed for their insulator function (Hart et al. 1997; Hayashi et al. 1997). The special chromatin structures function as boundary elements which, when appropriately spaced, interfere with communication between the enhancers and the promoter (Udvardy et al. 1985; Hart et al. 1997). There are three BEAF binding sites in the *Ketel* gene promoter, and they may well function as an insulator. There are four CFDD binding sites within the analyzed region. One of them is part of the DRE motif, one coincides with a BEAF binding site, one with an Elf1 binding site and one is freely available for CFDD binding (Fig. 8). The DREF, the CFDD and the BEAF binding sequences are all present in the *Ketel* gene homologues of the *D. simulans*, *D. sechellia* and *D. yakuba* species suggesting a conserved function of these *cis*-regulatory elements in the expression regulation of the *Ketel* gene. Moreover, the CFDD, the DREF and the BEAF binding sequences are also present in a number of DNA replication- and cell proliferation-related genes that have been known to be regulated by the DRE/DREF system (Hirose et al. 1996; Hayashi et al. 1997).

The gel-shift experiments

To locate the protein binding regions in the *Ketel* promoter and to confirm results of the computer analysis, we carried out gel-shift experiments in which different regions of the promoter were combined with protein extract prepared from nuclei of S2 cells and monitored changes in the mobility of the PCR-generated DNA fragments. We focused attention on the -327→-204 (with two CFDD binding sites), the -99→-37 (with a DRE motif) and the +204→+283 (with a CFDD binding site) regions (Fig. 9).

The -327→-264 fragment did not interact with nuclear proteins indicating that the bicoid and the crocodile binding sites within the region do not bind proteins isolated from S2 cells and, hence, the -327→-204 activator region identified in the previous experiment can be narrowed down to the -264→-204 region. The -284→-167 fragment bound nuclear proteins as two bands appeared with DNA-protein complexes inside. Since there are two CFDD binding sites within the -284→-167 region, the two bands most likely represent complexes in which a DNA fragment is associated with one or with two CFDD protein molecule(s). PCR sub-fragments were generated next to locate the protein binding regions within the -284→-167 fragment. Of the three sub-fragments, only the -254→-204 bound nuclear proteins. In fact, the banding patterns of the -284→-167 and the -254→-204 fragments were identical implying that the protein binding sites reside within the -254→-204 region which includes two CFDD binding sites (Fig. 9).

Figure 8. Landmarks between around the 5' end of the Ketel gene in *Drosophila melanogaster*. The 1944 bp long *Ketel* promoter sequence between the two EcoRV restriction sites. The bold letters correspond to the transcribed sequences. Upper case letters represent exons. The lower case, non-bold letters show the sequence between the two genes. The lower case, bold letters represent introns. The restriction sites are shaded, the translation sites are underlined and the transcription factor binding sites are framed with dashed lines.

To further locate the nuclear protein binding sites, we constructed the Δ -243→-204 fragment which includes the CFDD binding site around -252 but does not include the CFDD binding site around -243. Since only a single band appeared in the gel-shift, we concluded that the CFDD binding site around -252 does indeed bind a nuclear protein. It can also be concluded that the CFDD site at -243 also binds a nuclear protein, at least in the S2 cells.

Both the -190→-65 and the -99→-37 fragments interact with S2 cell nuclear proteins and bring about the formation of two bands each. Considering that the -99→-37 fragment is only 63 bp shorter than -190→-65 fragment, it may well be that the same types of proteins bind the two fragments. One of the DNA binding proteins is DREF, the other is CFDD as it was shown in the following competition experiment (Fig. 10).

Upon the addition of increasing amounts of cold dsDRE (a 30 bp long double-stranded olygonucleotide with the DRE motif inside) to the studied mixture, the DNA/protein band become rather faint indicating that the -99→-37 fragment does indeed bind DREF (lanes 3, 5 and 6 in Fig. 10). Moreover, a DREF-specific antibody brought about a supershift of the DNA/DREF complex (lane 7 in Fig. 10). Following the addition of dsDRE not only the DREF-related but also the other band faded away (lanes 3 and 6 in Fig. 10). Since CFDD also binds dsDRE, it is conceivable that the other band fades away since several of the CFDD molecules bind to the cold DRE motif in dsDRE. It is thus safe to conclude that the other band corresponds to CFDD (lanes 3 and 6 in Fig. 10). In fact, the sizes of the bands confirm the former assumption: DREF is an 80, whereas CFDD is 76 kDa protein (Hirose et al. 1996; Hayashi et al. 1997). Based on the above results, the presence of the Ap1 binding site around -156 can be excluded since no additional band appeared with the -190→-65 fragment. Thus the -190→-65 and the -99→-37 fragments clearly revealed the involvement of the CFDD and the DREF binding sites in the regulation of *Ketel* gene expression.

The +204→+283 fragment (not shown in Fig. 9A) is part of the first intron in the *Ketel* gene. As Figure 9B shows, the +204→+283 fragment binds some type of nuclear protein. There are five transcription factor binding sites in the +204→+283 region: Su(H) (suppressor of hairless), dl (dorsal), CFDD, Elf1 (= grainy head) and sna (snail; see Fig. 8). It appears that of the listed transcription factors CFDD binds the +204→+283 fragment for the following reasons: (1) Although well studied, Su(H), dl, Elf1 and sna have not been reported to be present in S2 cells. (2) Based on the approximate sizes of the DNA/protein complexes shown in Fig. 9, association of the snail, the Su(H), several forms of the dl and the Elf1 proteins (43, 66.9 111.6 and 143 kDa, respectively) with the +204→+283 fragment can be excluded. The most likely type of protein to bind the +204→+283 fragment is, thus, CFDD.

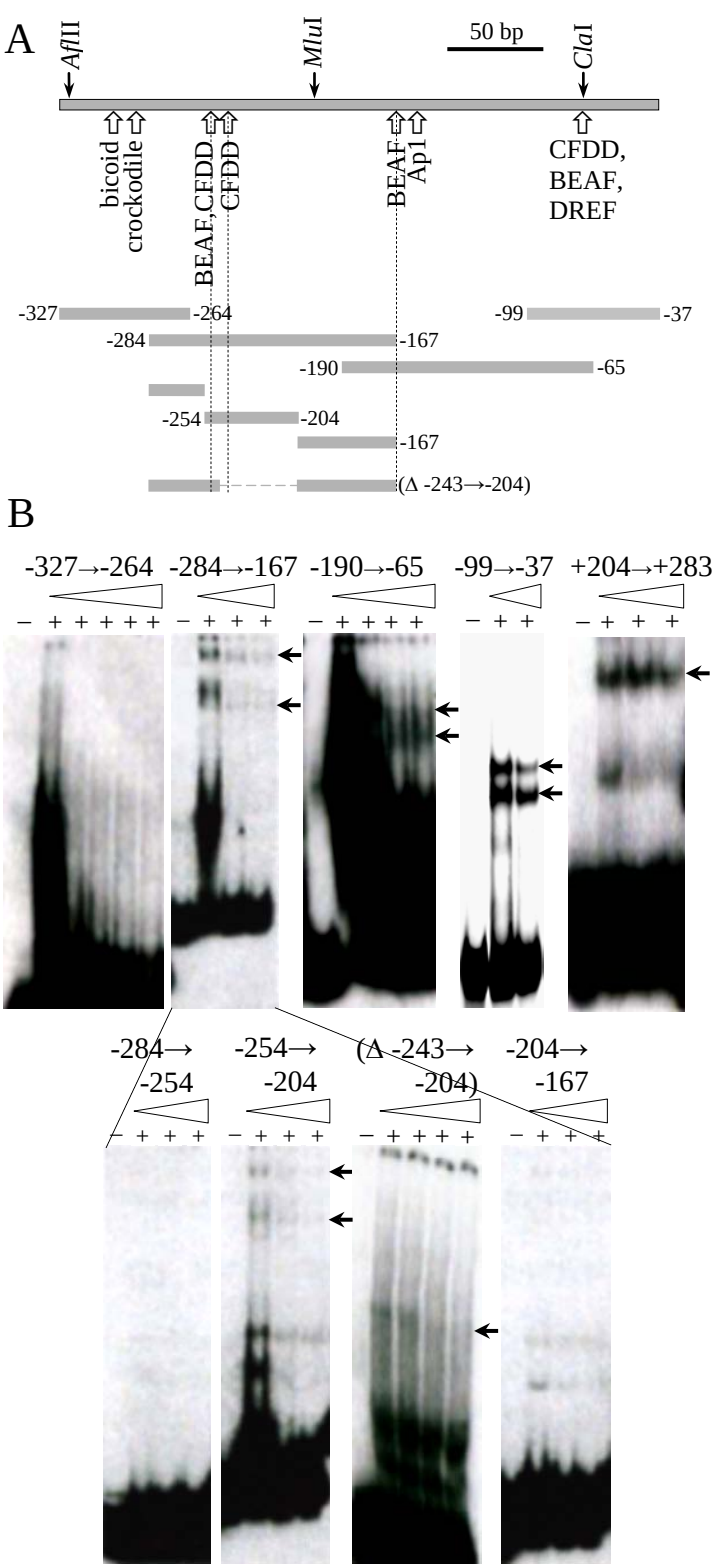


Figure 9. Detection of nuclear protein binding regions within the *Ketel* promoter by the electrophoretic mobility/gel-shift assay. In (A) the -327→-37 region of the *Ketel* gene promoter is shown with some of the predicted transcription factor binding sites. The PCR fragments were ^{32}P-labeled and used as probes in the assay. (B) Results of the assay. The +204→+283 fragment is part of the first intron in the *Ketel* gene and contains a putative CFDD binding site. The arrows point to bands with DNA-protein complexes. The ◁ symbol represents increasing concentrations of the poly(dIdC) competitor DNA. The – and the + symbols indicate the absence and the presence of nuclear protein extract prepared from S2 cells.

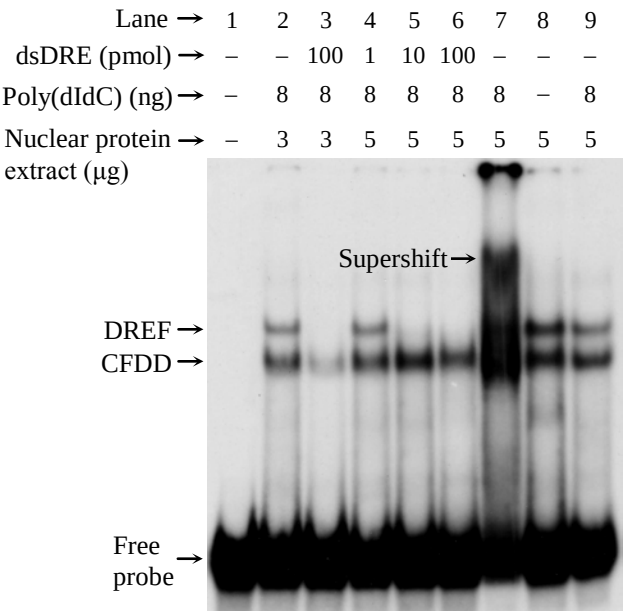
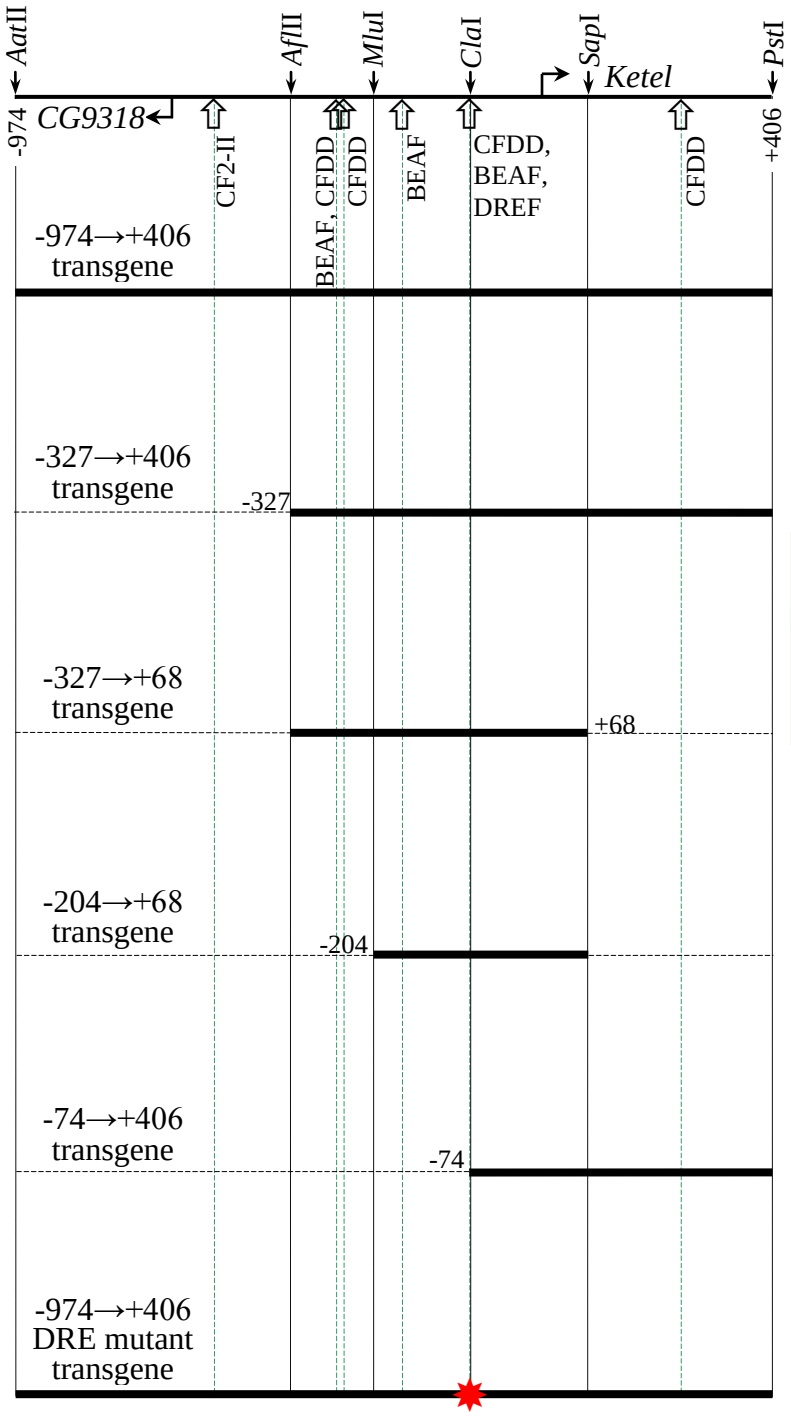


Figure 10. Identification of the transcription factors that bind the -99→-37 fragment with the DRE motif inside. The ^{32}P-labeled DNA probe was the 62 bp long -99→-37 fragment. In addition to the probe and the components listed in the heading of the figure, a 30 nucleotide long dsDRE competitor was added to some of the samples. A DREF-specific antibody was added into the sample represented in Lane 7. The antibody resulted in a supershift indicating the presence of DREF in the forming complexes. Several of the reaction mixtures contained poly(dIdC) competitor DNA to avoid unspecific DNA-protein interactions.

In summary, results of the gel-shift experiments are in harmony with predictions of the computer-based analyses and highlight those sequences in the *Ketel* gene promoter which are engaged in the regulation of gene expression and exclude those predicted binding sites which, although present in the *Ketel* promoter, do not have roles in the regulation of gene expression in the absence of corresponding transcription factors.

Deletion mapping with the *LacZ* reporter genes

To identify the promoter regions that ensure tissue specific expression of the *Ketel* gene, we constructed six different types of transgenes with a number of sub-lines each. Every transgene type carried a different segment of the promoter combined with the *LacZ* reporter gene. In practice, transgene homozygous males were crossed with wild type females and the *LacZ* expression pattern, as revealed through β-galactosidase activity, was analyzed in the descending gastrulating embryos, in dissected tissues of late 3rd instar larvae and in ovaries of the adult females. The staining pattern was determined in every line of the six different types of transgenes. It needs to be mentioned that the staining pattern was identical in all the sub-lines for every of the six different types of transgenes. Results of the experiments are summarized in Figure 11.


AatII
AflIII
MluI
ClaI
SapI
PstI
Ketel
-974
CG9318
+406
CF2-II
BEAF, CFDD
CFDD
BEAF
CFDD, BEAF, DREF
CFDD
-974→+406 transgene
-327→+406 transgene
-327
-327→+68 transgene
+68
-204→+68 transgene
-204
-74→+406 transgene
-74
-974→+406 DRE mutant transgene

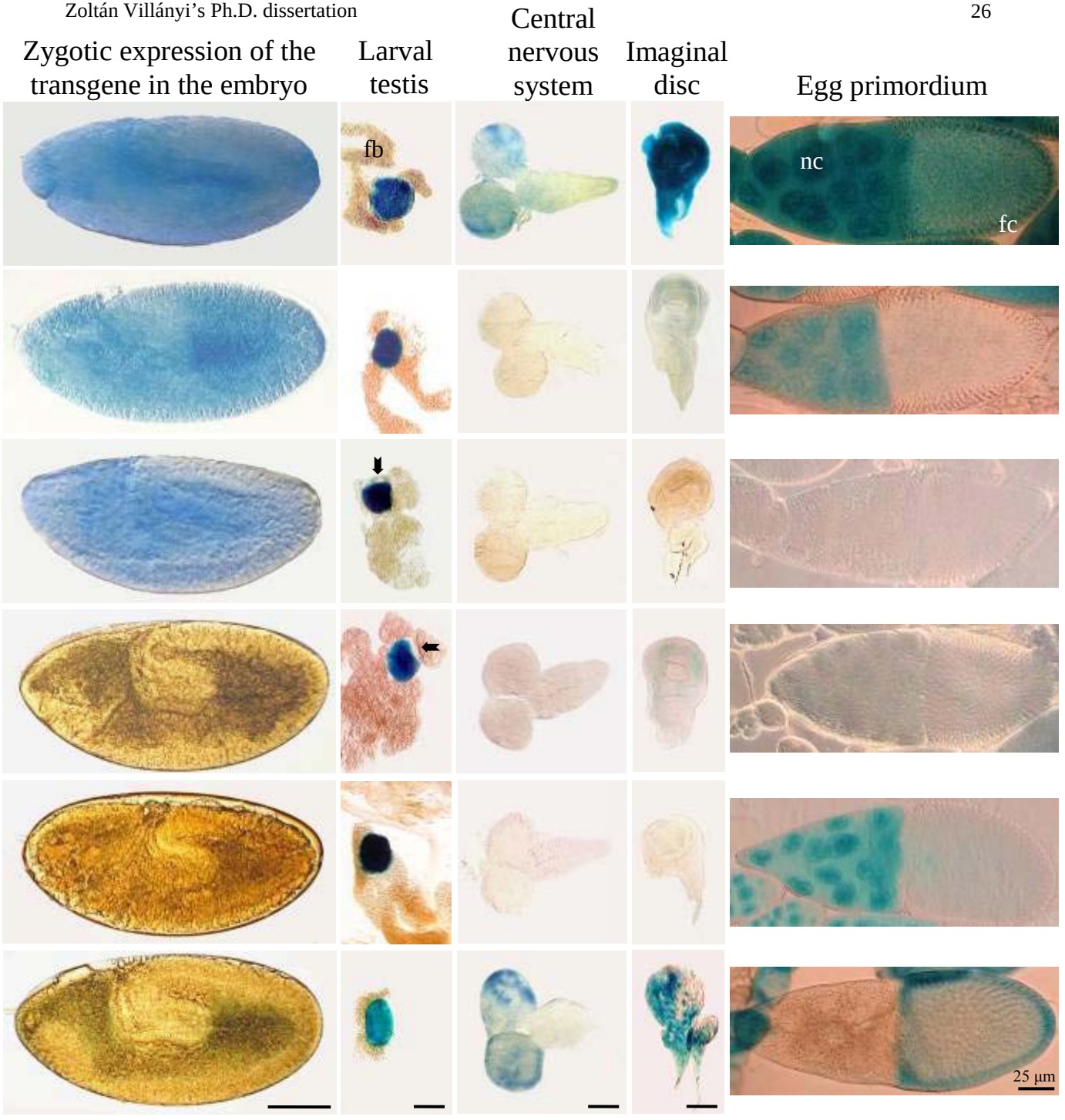

Zygotic expression of the transgene in the embryo
Larval testis
Central nervous system
Imaginal disc
Egg primordium
fb
nc
fc
25 µm

Figure 11. Detection of tissue-specific promoter activity with *LacZ* reporter transgenes. In the transgenes different parts of the *Ketel* promoter ensured the production of β-galactosidase. The promoter segments that were combined with the *LacZ* sequences appear as thick lines in the left panel of the figure. The restriction sites and the transcription factor binding sites appear as in Fig. 2. To prepare the stained specimen, wild type females were crossed with transgene carrying males and the descending young gastrulating embryos, different tissue types of late 3rd instar larvae and ovaries of the adult females were stained for β-galactosidase activity, i.e. the formation of blue pigment. The fb symbol stands for fat body, a typical larval tissue type. The ⬇ arrow in the larval testis panel points to the non-staining somatic cell groups in the larval testes. If otherwise not stated, the scale bars represent 100 μm.

When the -974→+406 fragment controlled the expression of the *LacZ* gene, β-galactosidase activity was apparent in every cell of the young gastrulating embryos, in the larval gonads, in the diploid cells of the central nervous system, in the imaginal discs as well as in the nurse and in the follicle cells of the developing egg primordia (see also Tirián et al. 2000). However, and in agreement with the previously published results, there was no indication of β-galactosidase activity in any type of the polytenic larval cells (see also Lippai et al 2000, Tirián et al. 2000; Villányi et al. 2008b). This result implies that the -974→+406 part of the *Ketel* gene promoter contains all the sequences that are necessary and sufficient for the characteristic expression pattern of the *Ketel* gene.

When the -327→+406 fragment controlled the expression of the *LacZ* gene, β-galactosidase activity was present in all the cells of the gastrulating embryos, in the larval gonads and in the nurse cells. However, cells in the imaginal discs, the larval CNS and the follicle epithelium did not stain. Apparently, the -974→-327 region contains cis-regulatory element(s) which ensure(s) *Ketel* gene expression in the imaginal discs, in the larval CNS and in the follicle cells. The lack of reporter gene expression in the former cell types is a common property of all the other transgene types which do not contain the -974→-327 region. Of the six transcription factors which have binding sites in the -974→-327 region (dorsal, tramtrack, fushi-tarazu, CF2-II, crocodile and bicoid; see Fig. 8), only CF2-II is present in the follicle cells (Shea et al. 1990). The other transcription factors are present and function during embryogenesis and thus their involvement in *Ketel* gene expression regulation is rather unlikely. (See the gene expression data base in the FlyBase). It appears thus that the CF2-II binding site around -483 is necessary for *Ketel* gene expression in all the diploid cells other than those in the gastrulating embryos.

When the -327→+68 fragment controlled the expression of the *LacZ* reporter gene, β-galactosidase activity was present in the early gastrulating embryos and in the germ line components of the larval gonads. Remarkably, the reporter gene was not expressed in the nurse cells, showing the presence of a cis-acting element in the +68→+406 region which is responsible for *Ketel* gene expression in the nurse cells. Of the eight transcription factors (Ultrabithorax,

hunchback, STAT, SUH, dorsal, CFDD, Elf1 and snail) which have binding sites in the +68→+406 region seven are not expressed in the nurse cells and, thus, only CFDD appears to be involved in the regulation of *Ketel* gene expression in the nurse cells and in the production of the maternal *Ketel* dowry.

When the -204→+68 fragment controlled the expression of the *LacZ* gene, β-galactosidase activity appeared only in the germ line components of the larval gonads and in none of the other studied tissues. Apparently, there are sequences within the -327→-204 region that are required for the commencement of zygotic *Ketel* gene expression during early gastrulation. The -327→-204 region contains a bicoid, a crocodile, a BEAF and two CFDD binding sites. Considering the expression pattern of the encoding genes and knowing that BEAF is an insulator element, the function of the CFDD binding sites is most probably essential in the regulation of *Ketel* gene expression. This conclusion is strongly supported by the results of the gel shift experiments.

When the -74→+406 fragment controlled the expression of the *LacZ* gene, β-galactosidase activity appeared in both the gonial and in the somatic cells of the larval gonads and, as expected, in the nurse cells of the egg primordia. The -74→+406 transgenes include the DRE motif (TATCGATA) to which DREF, CFDD and BEAF can bind. The expression patterns of the -74→+406 transgenes confirm the former conclusion: the sequences required for the maternal effect of the *Ketel* gene reside in the +68→+406 region.

To clarify the importance of the DRE motif around -74, we constructed a mutant transgene which covered the -974→+406 region and a mutation around -74: the TATCGATA DRE motif was replaced by GCCAAGCGGC. The mutation eliminated the expression of the reporter gene in the mid gastrulating embryos and also in the nurse cells. Apparently, the simultaneous presence of the DRE motif and the -204→-327 sequence is necessary for the commencement of zygotic *Ketel* gene activity during early gastrulation. Similarly, the concurrent presence of the DRE motif and the +68→+406 sequence is a prerequisite for the *Ketel* gene related maternal effect. However, the DRE motif around -74 is not required for *Ketel* gene expression in the diploid cells in late third instar larvae and in the follicle epithelium.

In summary, analysis of the different transgenic lines established a correlation between different regions of the *Ketel* gene promoter and tissue specific expression of the gene. (1) The -979→-327 region is indispensable for *Ketel* gene expression in the imaginal discs, in the diploid cells of the CNS and in the follicle epithelium. (2) The -327→-204 region is necessary for the commencement of zygotic *Ketel* gene expression during embryogenesis. (3) The ~~+68~~+406 region is needed for the *Ketel* gene related maternal effect. (4) The DRE motif around -74 is required for the zygotic expression of the *Ketel* gene during early gastrulation as well as in the nurse cells and, thus, for the *Ketel* gene related maternal effect.

DISCUSSION

It appeared formerly that the *Ketel* gene, which encodes importin-β in Drosophila, was expressed only in the dividing diploid imaginal and not in the non-dividing polytenic cells in late 3rd instar larvae (Tirián et al. 2000, Lippai et al. 2000). The observation raised the possibility that the function of the *Ketel* gene was not required in every cell and suggested the existence of a thus far unknown nuclear protein import mechanism that operates without importin-β. It was also difficult to understand how the diploid cells, in which the *Ketel* gene is expressed, can proliferate and function normally without the *Ketel* gene (Tirián et al. 2000). In any case, the function of the *Ketel* gene must be indispensable in at least some cell type(s) since larvae without the *Ketel* gene perish during mid larval life.

To identify the cell types in which the function of the *Ketel* gene is essential, we attempted to generate gynanders that carried a functional *Ketel* gene in their female but not in their male cells. Such gynanders do not survive to the end of larval life implying that the function of the *Ketel* gene is most likely required in a large group of cells (Bryant and Zornetzer 1973). This conclusion is supported by the observation that when the *elav-Gal4*, the *esg-Gal4* or the *vg-Gal4* drivers ensured expression of a *UAS-Ketel* transgene, zygotes that otherwise lack a functional *Ketel* gene survive to adulthood. The former drivers induce gene expression in the ectoderm, the largest germ layer which comprises about 72% of the blastoderm cells (3,600/5,000 cells; Campos-Ortega and Hartenstein 1997). In accordance with the former findings, elimination of *Ketel* gene activity in the primordial cells of the epidermis (by expressing a *UAS-Ketel-RNAi* transgene with the *elav-Gal4*, the *esg-Gal4* or the *vg-Gal4* drivers) led to death of the zygotes. How do e.g. the *esg-Gal4*; *UAS-Ketel*; *ketel*^{null}/– zygotes, in which the *Ketel* gene is expressed only in the ectoderm and the neuroectoderm but not in the other germ layers (Whiteley et al. 1992; Hayashi et al. 1993; Goto and Hayashi 1999), survive to adulthood? How do the entoderm- and the mesoderm-derived cells acquire importin-β? The most likely source of importin-β in those cells is the maternal importin-β dowry, which persists and functions in the entoderm- and in the mesoderm-derived cells throughout development. This proposition presumes a very long persistence of importin-β, a feature that is supported by the following:

(1) We report in the present thesis that the GFP-importin-β molecules can persist up to five days (Villányi et al. 2008a). The life span of the wild type importin-β molecules may be even longer than five days since the GFP-importin-β molecules are barely functional and are probably prone to faster degradation than wild type importin-β, due to the misfolding induced by the GFP tag. Yet, the GFP-importin-β molecules survive longer than expected, stabilized by their intimate association with the NPCs (Figures 5 and 6). It was suggested recently that being parts of large protein complexes, components of the anaphase promoting complex are stabilized; they persist unusually long and function in low concentrations (Pál et al. 2007a and b; Wehman et al. 2007).

The maternally provided importin-β molecules may be able to support the few cell divisions the entoderm- and the mesoderm-derived cells accomplish between the blastoderm stage and their final differentiation (Leptin 1995). Thereafter, they need to assist only in nuclear protein import in the protective milieu of the NPCs.

(2) When transplanted into host embryos, pole cells without the *Ketel* gene but with the maternally provided importin-β inside perform the usual 4-5 mitosis during larval life (Wieschaus and Szabad 1979), become functional oogonial cells, go through the cystocyte divisions and become sources of normal looking eggs. [However, when the eggs are fertilized embryogenesis does not commence inside (Tirián et al. 2000)]. The maternally derived importin-β molecules can thus support female germ line development for at least ten days.

(3) Elimination of the zygotic *Ketel* gene function in e.g. the *esg-Gal4*; *UAS-Ketel-RNAi* zygotes did not lead to death in mid larval life as in the $ketel^{null}$/– larvae but only during late pupal life. The prolonged life of the zygotes can be accounted for by the persistence of those importin-β molecules that form during the short period of time between synthesis and demolition of the *Ketel* mRNA molecules.

The finding that importin-β molecules persist long and carry out their functions in low concentrations provides an explanation for the normal behavior of clones of wing disc cells without the *Ketel* gene. Such cells can accomplish as many as seven rounds of cell divisions following the induction of mitotic recombination and becoming homozygous for a $ketel^{null}$ allele (Tirián et al. 2000). Perdurance of importin-β, inherited from the $ketel^{null}$/+ mother cell, sustains the life of the descending $ketel^{null}$ homozygous cells.

The $ketel^{GFP}$ allele encoded GFP-importin-β clearly showed that importin-β is present in every cell type, though in very different concentrations (Villányi et al. 2008a). Apparently, the non-dividing larval cells also make use of importin-β in nuclear protein import and, thus, there is no unknown mechanism of nuclear protein import to be discovered. However, the diploid cells contain a lot more importin-β as compared to the non-dividing larval cells. The low importin-β concentration in the non-dividing cells is reasonable since here the protein is engaged in the nuclear protein import only. (Precursor cells of the larval epidermis and the Malpighian tubules, for example, divide only two-to-three times following the blastoderm stage and become polytenic; Szabad et al. 1979; Janning et al. 1986). The imaginal disc cells remain diploid and keep on proliferating throughout larval and early pupal life (Brook 1998). Diploid cells need a higher importin-β concentration to accomplish three functions: nuclear protein import, formation of the spindle microtubule bundles and assembly of the nuclear envelope at the end of mitosis (Zhang and Clarke 2000; Nachury 2001; Wiese et al. 2001; Gruss 2001; Timinszky et al. 2002; Zhang et al. 2002; Tirián et al. 2003).

Apparently, as revealed by the analysis of the *ketel*^{GFP} encoded GFP-importin-β, the zygotic *Ketel* gene is expressed in every cell during early gastrulation (Fig. 7; Villányi et al. 2008a) in the same stage of development as the reporter gene in which the *Ketel* gene regulatory sequences control the expression of *LacZ* and the formation of β-galactosidase (Tirián et al. 2000; Lippai et al. 2000; Villányi et al. 2008b). However, the β-galactosidase molecules decompose in all the larval cells by late embryogenesis and leave the cells unstained for the rest of the larval development demonstrating the non-expressed status of the *Ketel* gene. Although the *Ketel* gene is not expressed in the larval cells, presence of GFP-importin-β clearly shows that there are importin-β molecules in the larval cells and they fulfill their function over a long period of time (Fig. 5).

To understand the mechanisms that ensure the characteristic expression pattern of the *Ketel* gene, we identified cis-acting control elements that are engaged in (1) the proper loading of the egg cytoplasm with the *Ketel* gene products, (2) the regulation of the all-over type of importin-β production during gastrulation and (3) controlling tissue specific expression of the *Ketel* gene during the later stages of development.

Computer analysis revealed several evolutionarily conserved transcription factor binding sites in the *Ketel* promoter of which, as described in the Result section, only the CF2-II, the CFDD, the DREF and perhaps the BEAF binding sites are of relevance. The CFDD, the DREF and the BEAF transcription factors have been known to be involved in the expression regulation of a number of genes engaged in cell cycle regulation (Hirose et al. 1993; Yamaguchi et al. 1995; Ohno et al. 1996). In fact, the CFDD binding sites are commonly present in the promoters of a number of DNA replication-related genes like PCNA and DREF (Hayashi et al. 1997). Since importin-β is required for spindle formation and nuclear envelope assembly, which are essential events in cell proliferation, it may not be surprising that the expression of the *Ketel* gene is regulated by the same transcription factors which control the expression of several genes engaged in cell cycle regulation.

The "active" transcription factor binding sequences within the *Ketel* gene promoter region were identified in gel-shift experiments, and the sequences that ensure tissue-specific expression of the *Ketel* gene were determined through the analysis of the expression patterns of *LacZ* reporter transgenes. It appears that the presence of an approximately 140 bp long sequence around the transcription start site is sufficient for a basic expression of the *Ketel* gene in the gonial cells (Fig. 12). Interestingly, none of the six different types of *LacZ* reporter transgenes are expressed in any polytenic larval cell types. One possible explanation could be the different modes of action of DREF in the larval and in the diploid cells: DREF does not displace BEAF from the DRE motif in the larval cells (see Hart et al. 1999) and, thus, an insulator can form which blocks transcription of the *Ketel* gene. [Three BEAF binding sites are necessary for the

formation of an insulator (Udvardy et al. 1985; Hart et al. 1997; Cuvier et al. 1998), and the promoter of the *Ketel* gene contains three BEAF binding sites, one of which is part of the DRE motif.] In the diploid cells, where DREF binds to the DRE motif and competes with BEAF (Hart et al. 1999), the insulator cannot form and, hence, there is no block to prevent expression of the *Ketel* gene. However, the above model is rather unlikely since when the DRE motif, and along with it one of the BEAF binding sites, is abolished the BEAF insulator cannot form. Yet, the *Ketel* gene is not expressed in the larval cells. The lack of *Ketel* gene expression in the larval cells can also be explained by the absence of CF2-II transcription factor in that cell type. CF2-II has been reported to be expressed in the follicle cells (Shea et al. 1990) and the CF2-II binding site around -483 seems to be the only candidate to control *Ketel* gene expression in the imaginal disc cells, in the neuroblasts and in the follicle cells (Fig. 12). Further studies are needed to ascertain whether this assumption is correct.

The simultaneous presence of two sequences is required for the expression of the *Ketel* gene in the nurse cells and for the loading of the egg cell cytoplasm with the *Ketel* gene products: a CFDD binding site in the first intron (around +247) and the DRE motif around -74 (Fig. 12). (Note that the importin-β-related maternal effect depends on the expression of the *Ketel* gene in the germ line components of the egg primordia; Tirián et al. 2000.) Removal of either of these sequences leads to an absence of *Ketel* gene expression in the nurse cells. Similarly, the concurrent presence of the DRE motif at -74 and the CFDD site(s) around -250 is necessary for the expression of the *Ketel* gene in every cell of the gastrulating embryo. Removal of any of these sequences abolishes *Ketel* gene expression during embryogenesis. It appears that cooperative binding of transcription factors to the DRE motif and to either of the CFDD recognition sites establishes favourable conditions for tissue-specific expression of the *Ketel* gene.

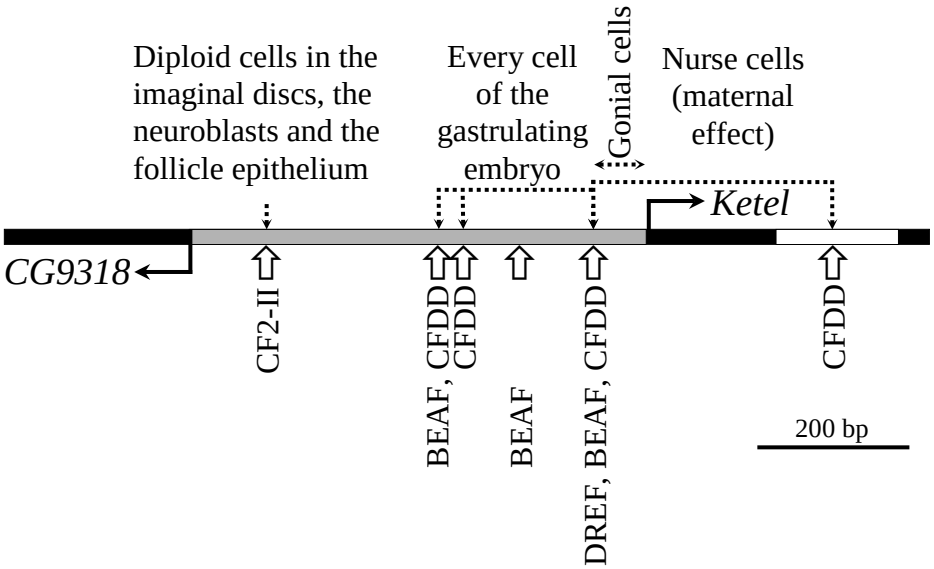


Fig. 12. Summary figure illustrating the transcription factor binding sites that are engaged in the control of *Ketel* gene expression in the different cell types

SUMMARY OF THE MAIN RESULTS

With the aid of a *ketel*^{GFP} mutant allele that encodes the formation of GFP-tagged importin-β and with *Ketel* promoter-*LacZ* fusion reporter transgenes we show in the present thesis that:

(1) Some of the maternally derived GFP-importin-β molecules persists up to pupariation. To our best knowledge, GFP-importin-β is the longest living molecule reported to function inside live Drosophila cells.

(2) The paternally-derived *ketel*^{GFP} mutant allele as well as the full length *Ketel* promoter driven *LacZ* reporter transgene is first expressed during early gastrulation, at a time when most of the maternally-provided mRNAs decay and expression of many of the zygotic genes commences. Apparently, the *Ketel* gene is expressed in every cell of the gastrulating embryos.

(3) GFP-importin-β clearly showed that the Ketel protein is present in the larval cells. However experiments with *Ketel* promoter *LacZ* fusion reporter transgenes revealed that the expression of the *Ketel* gene is blocked in the polythenic cells beyond gastrulation. The importin-β molecules present in the polythenic cells come from two sources: the maternal dowry and zygotic expression of the *Ketel* gene during early gastrulation. The long living importin-β persists in the larval cells and assist, very effectively, in nuclear protein import.

(4) The *LacZ* reporter transgenes delineated the sequences in the *Ketel* gene promoter, which ensure tissue specific *Ketel* gene expression. A CFDD site in the first intron, along with a DRE motif in the promoter, is responsible for the establishment of the importin-β maternal dowry during oogenesis. Another CFDD site, in collaboration with a DRE motif (both in the promoter), is indispensable for the commencement of gene expression in every cell during early gastrulation. Following the demolition of the DRE motif, the reporter gene expression was absent from both the germ line components of the egg primordia and from the gastrulating embryos. Apparently, the DRE motif interacts with both up- and downstream cis-regulatory elements while it controls tissue specific expression of the *Ketel* gene (Fig. 12).

(5) Function of a far upstream CF2-II binding site appears to be required for intensive *Ketel* gene expression in the diploid cells.

EPILOGUE

The present Ph.D. thesis and the Mechanisms of Development papers do not mean the end of the "Ketel story". The finding that the *ketel*^{null}/− hemizygous larvae perish in 2nd larval instar, through they were expected to reach the end of larval development, initiated an analysis of the dying larvae. It has turned out that they perish due to the absence of mitochondria. Apparently, the *ketel*^{null}/− hemizygous larvae rely on the maternally-derived mitochondria while they develop to the end of the 2nd larval instar. Since these mitochondria become worn out and decay with time and since there are no new mitochondria synthesized, the larvae die (Fig. 13.) It well may be that in absence of importin-β (in the *ketel*^{null}/− hemizygous larvae) some transcription factors are not imported into the nuclei and hence the gene expression pattern of the cells become altered such that there are no new mitochondria synthesized. To test the present hypothesis, we collected total mRNA samples from both *ketel*^{null}/− and, as control, from +/− hemizygous larvae. The mRNAs served as sources of cDNA synthesis and the cDNA pattern of the two types of larvae were compared in DNA chips. It has turned out that the two types of larvae differ in the expression pattern of only 30 genes. One-by-one analysis of the genes (by the RNAi technique) revealed that two genes have essential functions in regulation of mitochondria concentration in the cells: negative elongation factor and peroxiredoxin 6005.

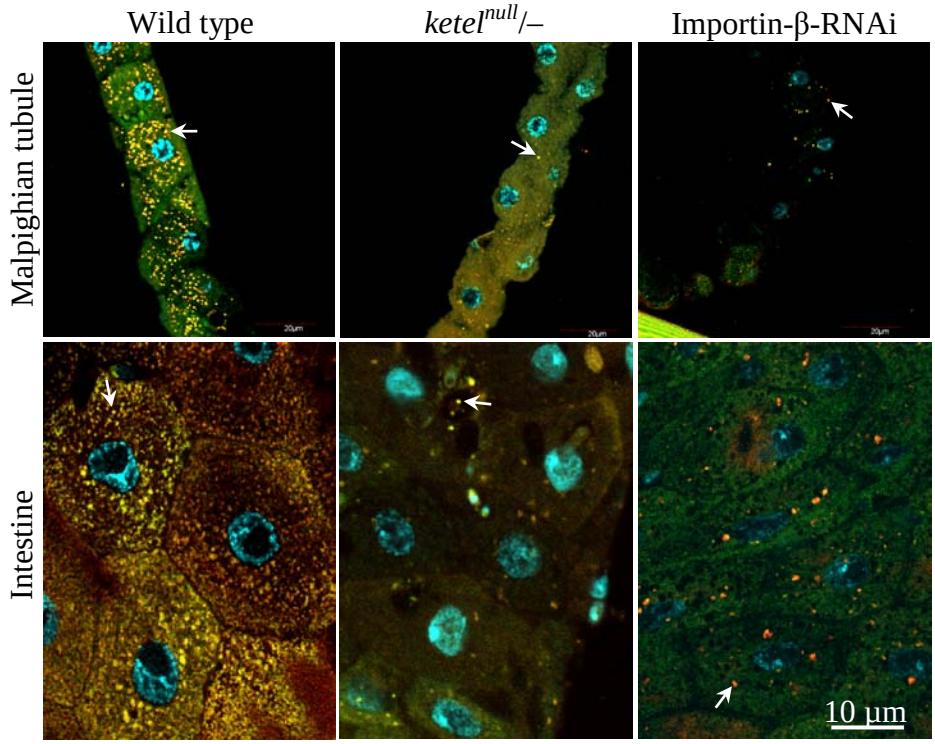


Figure 13. In absence of importin-β, the larval cells contain very few, though functional mitochondria. Optical sections of polytenic larval cells in the intestine and in a Malpighian tubule of wild type, importin-β lacking (*ketel*^{null}/–) and in importin-β-RNAi expressing larvae. The pictures represent merged optical sections recorded on 405 nm for the detection of Hoechst 33342 stained DNA in the nuclei, on 535 and on 595 nm to detect the inactive and the functional mitochondria by the stain JC1. The Arrows point to some of the functional mitochondria which appear in orange.

Analysis of the *ketel*^{null}/− larvae thus led to an understanding of the mechanism that controls mitochondria biogenesis, an essential feature of cellular energy supply. The manuscript, which will describe the "mitochindria chapter of the Ketel-story" is currently in preparation.

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ACKNOWLEDGEMENTS

I wish to thank my scientific supervisor Professor János Szabad for his encouragement, guidance and support throughout my Ph.D. studies. Many thanks for Professor Imre Boros for his guidance and for the great scientific conservations. The joint experimental work with Bernadett Papp, Alain Debec and Szilárd Szikora were of great pleasure. I wish to thank members of the Medical Biology Department: László Tirián, Gyula Timinszky, Imre Gáspár, György Seprényi, Dóra Tombácz for their help, scientific conversations and comments. For thought provoking conversations many thanks for Zsolt Boldogkői, István Belecz, Zsolt Venkei, Tamás Szalontai, Balázs Ördög, Mónika Lippai, Zsuzsa Sarkadi, Farkas Kerényi, Judith Tóth and Lídia Mészáros. I am grateful for the technical support of Gabriella Teleki, Kissné Ani, Kisapátiné Margó, Révész Kati, Magyarné Papdi Csilla, Muskó Pálné, Magyar Lászlóné, Piroska "néni" and Lajos "bácsi". Thanks for my wife and daughter and also for my sister and parents for their spiritual support. I wish to thank the Bloomington Drosophila Stock Center and Steven M. Cohen for the *Gal4* driver strains, Barry Dickson and his co-workers for the UAS-*Ketel-RNAi* transgene, Stefan Heidmann for the histone-RFP transgene.

Support for work came from several sources: Maternal-Effect and Embryogenesis Research Group of the Hungarian Academy of Sciences, three grants of the Hungarian Scientific Research Fund: OTKA T5537, OTKA 032540 and OTKA NI69180, Agency for Research Fund Management and Research Exploitation 3.2.1-2004-04-0411/3.0, an ECO-NET grant (ACI BCMS 2004-2007) from the French "Ministère des Affaires Etrangères" and the Graduate Student Program of the University of Szeged.