Ph.D. thesis

Paralog-Specific Cooperation between *importin-α2* and *importin-β/Ketel* in Spindle Assembly during Drosophila Early Nuclear Divisions

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1. Introduction

The active import of macromolecules from the cytoplasmic compartment to the nucleus is carried out by the importins under the regulation of Ran. Imp-α mediates the binding of cargo proteins to Imp-β through their NLS sequences by forming a ternary complex in the cytoplasm. The Imp-α/Imp-β complex involved in the spatial-temporal coordination of mitotic processes as well, regulating the activity of spindle assembly factors SAFs/MAPs. The regulatory effect of the Ran-system on mitosis operates in all eukaryotic organisms, from yeast to human.

The fruit fly has one *imp-β* and three *imp-α* genes. The *imp-β* and *imp-α3* are mainly involved in nucleo-cytoplasmic transport of somatic cells. The *imp-α1* and *imp-α2* have specific role in spermatogenesis and oogenesis, respectively. The evolution of specific functions for different *imp-α* genes may be driven by the unique requirements of gametogenesis. However, the role of each *imp-α* gene during embryogenesis is not yet understood. The loss off function mutation of *imp-α2* leads to female-sterility characterized by the occlusion of the ring canals, and over-expression of *imp-α1* or *imp-α3* failed to rescue this phenotype. Moreover, amongst a series of mutant *imp-α2* transgenes generated, only the expression of $imp\text{-}\alpha2^{SNLSB-}$ or $imp\text{-}\alpha2^{\Delta IBB}$ resulted in matured eggs. However, the development of these embryos was arrested. This prompted us to further investigate the role of the *imp-α2* during early embryogenesis to clarify its contribution for these mysterious processes. My thesis focuses on the *imp-α2* functions, in cooperation with $imp\text{-}\beta^{Ketel}$, during the early development of *Drosophila melanogaster* embryo.

2. Specific aims

The goal of our work was to study the roles played by importins during the syncytial divisions in the Drosophila embryo. In order to clarify the role of imp-α paralogs and the functions of the Imp-α2/Imp-β^{Ketel} complex during rapid cell divisions of early embryogenesis, we performed genetic interaction analysis between each *imp-α* gene and *imp-*β^{Ketel}, and used the methods of cell biology, molecular biology and *in silico* protein modeling.

1. To analyze the phenotype of *imp-*$\alpha 2^{D14}$ and recessive alleles of *imp-*β^{Ketel}.
2. To analyze the molecular-genetic and biochemical background of the *imp-*$\alpha 2^{D14}$*/imp-*$\beta^{KetRE34}$ interaction, using DNA sequencing, *in silico* protein modeling and pull down experiments.
3. To determine the paralog specific function of *imp-α2* by genetic analysis and immune-cytology.
4. To analyze the embryonic phenotype of *imp-α2* mutant transgenes.
5. To characterize the NLS-dependent regulation of mitotic factors by Imp-α2
6. To determine the regulatory functions of Imp-α2/Imp-β^{Ketel} complex using cell biological methods.

3. Methods

1. Genetic interaction analysis
2. Embryo viability test
3. Embryo fixation and immune-histochemistry
4. DNA techniques (sequencing, cloning, *in vitro* mutagenesis)

5. Protein expression
6. GST pull down experiments and Western blot
7. Protein modeling
8. Confocal laser scanning microscopy
9. Image processing

4. Results

1. First we set up a genetic analysis with *imp-α2* and *imp-β^{Ketel}*. We combined *imp-α2* null allele *imp-α2^{D14}*, with six different recessive *imp-β^{Ketel}* alleles (*imp-β^{KetRP13}*, *imp-β^{KetRX13}* and *imp-β^{KetRE34}* – revertant of the dominant female sterile *imp-β^{KetD}* –,as well as *imp-β^{Ketc02473}*, *imp-β^{Kete02657}* and *imp-β^{Kete03750}* – *piggyBac* insertions) and examined the viability of eggs laid by heterozygous females of each combination. Eggs produced by trans-heterozygous *imp-α2^{D14}/imp-β^{KetRE34}* females were lethal, whereas eggs laid by all other heterozygous females developed normally. The embryo-lethality could be rescued by expressing the wild type *imp-α2* or *imp-β^{Ketel}* transgenes. This indicates that *imp-α2* and *imp-β^{Ketel}* interaction is critical during embryogenesis.

2. We identified a second mutation in *imp-β^{KetRE34}*, a D725N substitution, which located in the Imp-α-binding domain of Imp-β^{Ketel}. The *in silico* analysis showed no difference between the binding affinities of Imp-β^{Ket725N} and Imp-β^{Ketel} to the IBB domain of Imp-α2. GST pull down experiments showed similar results. The D725N substitution forms new intra-molecular interactions which probably weaken the dominant negative character of *imp-β^{KetD}* allele by stabilizing its abnormally opened structure.

This structural modification in Imp-$\beta^{KetRE34}$ is not sufficient to restore the wild type Imp-β^{Ketel} molecule binding flexibility, which is showed by the strengthened binding affinity of Imp-$\beta^{KetRE34}$ to both RanGTP and RanGDP, the probable cause of the semi dominant character of this allele. Mutations decreasing the level of RanGTP (loss of function mutation in *Bj1/RCCI*) suppressed, while mutations increasing the level of RanGTP (loss of function mutation in *RanGAP*) enhanced the semi-dominant phenotype of Imp-$\beta^{KetRE34}$.

3. Introducing classical imp-α alleles into *imp-*$\beta^{KetRE34}$/+ or ovarian-specific RNA silencing the members of the *imp-α* gene family on the same genetic background showed that only the *imp-*$\alpha2^{D14}$/*imp-*$\beta^{KetRE34}$ and the *imp-α2i/imp-*$\beta^{KetRE34}$ combinations resulted in strong embryonic lethality. Moreover, expressing *UTR*$^{\Delta}$ transgenes producing similar amount of Imp-α1, Imp-α2, and Imp-α3 each, only the *UTR*$^{\Delta}$*-imp-α2* was able to restore the embryonic viability of eggs laid by *imp-*$\alpha2^{D14}$/*imp-*$\beta^{KetRE34}$ females. The amount of Imp-α2 is apparently high in the 0-2hs embryo cytoplasm. During the rapid syncytial divisions Imp-α2 associates to the spindle MTs while in the inter-phase it accumulates in the nucleus. The results of genetic analysis and the cell cycle dependent localization of Imp-α2 together reveal that imp-α2 has a paralog-specific role in the pre-blastoderm stage nuclear divisions of *Drosophila* embryo.

4. The analysis of mutant Imp-α2 transgenes (*imp-*$\alpha2^{NLSB^-}$, *imp-*$\alpha2^{SNLSB^-}$, *imp-*$\alpha2^{\Delta IBB}$, *imp-*$\alpha2^{CASB^-}$) was carried out on *imp-*$\alpha2^{D14}$/*imp-*β^{Ketel} null sensitized background using different recessive *imp-*β^{Ketel} alleles (*imp-*$\beta^{KetRX13}$, *imp-*$\beta^{KetRP13}$, *imp-*$\beta^{Ketc02473}$, *imp-*$\beta^{Kete02657}$, *imp-*$\beta^{Kete03750}$). Inactivation of the NLSB or SNLSB domain of *imp-α2* in combination

with decreased dosage of Imp-β^{Ketel}, completely block the embryonic development. We detected the antimorphic effect of *imp-α2$^{NLSB^-}$* transgene also in *imp-α2^{D14}*/+ females, and this effect was enhanced when gene dosage of *imp-β^{Ketel}* was reduced. Altogether, our data emphasize the importance of NLSB domain during the mitosis in the syncytial embryo, and the NLS-dependent co-immunoprecipitation of mitotic factors, like lamin, CP190 and ISWI with Imp-α2 further support this. In contrast, the expression of Imp-α2$^{\Delta IBB}$ exerted no deleterious effect on embryo development, which can be explained on the basis of the lack of IBB domain, which prevents it's binding to Imp-β^{Ketel}, whereas the other three Imp-α2 proteins contain an intact IBB domain and were able to physically interact with Imp-β^{Ketel}. In the lack of CASB domain, which stabilizes the Imp-α2 closed conformation, the opened structured Imp-α2^{CASB-}/Imp-β^{Ketel} complexes could be formed and could bind to the mitotic factors as well.

5. The genetic interactions observed between *imp-α2^{D14}*and *imp-β^{KetRE34}*, as well as between *imp-α2$^{NLSB^-}$* or *imp-α2^{SNLSB-}* and *imp-β^{Ketc02473}*, reveal that the concentration of the functional Imp-α2/Imp-β^{Ketel} complexes should be above a threshold level in the developing embryo. While expressing *imp-α2$^{NLSB^-}$* or *imp-α2$^{SNLSB^-}$* alleles on *imp-β^{Ket0}* heterozygous background reduces the amount of functional complexes, the *imp-β^{KetRE34}* allele, as inferred from pull down experiment and mitotic phenotypes, appears to decrease the stability of the NLS-protein/Imp-α2/Imp-β^{Ketel} ternary complex.

6. Embryos laid by *imp-α2^{D14}/imp-β^{KetRE34}* and *imp-α2^{D14}/Ket^{c02473}; nos-Gal4-imp-α2$^{NLSB^-}$* females displayed a wide range of abnormality. Their development was predominantly blocked during the very first mitotic divisions in cycle 1-4 and, we detected abnormalities both in the

number and the organization of the metaphase-like structures. Examination by confocal microscopy of embryos revealed numerous mitotic defects: uncontrolled free asters, overgrowth of well-focused or unfocused spindles, spindle-fusions and, less frequently, narrow spindles. We observed robust spindles with condensed and regularly aligned chromatin at the equator. We also detected discrete, non-condensed aggregates of chromatin mostly in the multipolar and the narrow spindles, which contained smaller amount of chromatin. Our data suggest that phenotypes of overgrowth spindles and free asters could result from the activity of factors that trigger a persistent MT formation in the spindle area, because in the absence of sufficient amount of functional Imp-α2/Imp-β^{Ketel} the SAFs could not be kept inactive.

7. The nuclear envelope structure, delineated by lamin, was abnormal in the embryos of *imp-α2*^{D14}*/imp-*$\beta^{KetRE34}$ and *imp-α2*^{D14}*/imp-*$\beta^{Ketc02473}$*;nos-Gal4-imp-α2*$^{NLSB^-}$ females, showing high concentration of lamin-stained vesicles at the spindle poles and nearly lacking the spindle envelope at the equator belt. In the multipolar spindles the continuous lamin-stained structures at the periphery of the spindles suggests that nuclear envelope could be formed around chromatids pulled out from the metaphase plate. Less frequently, we detected large masses of DNA and lamin aggregates encapsulated by an apparently continuous and particularly thick layer of lamin. Occasionally, we observed large lamin structures devoid of chromatin, which indicates that the factors released from Imp-α2/Imp-β^{Ketel} complexes could form nuclear envelope independently of chromatin. Our findings indicate that the Imp-α2/Imp-β^{Ketel} regulates the nuclear envelope assembly in the *Drosophila* embryo and the NLS-dependent association of lamin with

Imp-α2 further support this hypothesis.

8. The observed abnormalities in the centrosomin stained embryos indicate that the Imp-α2/Imp-β^{Ketel} complexes contributes to the centrosome dynamics and biogenesis independently of spindle formation. The majority of the mutant spindles contained either no centrosome or a single one, probably as a consequence of centrosome release driven by strong astral microtubule polymerizing activity. The occurrence of extra centrosomes associated with one spindle and the duplicating free centrosomal structures in the cytoplasm indicate that the centrosomes frequently replicate independently of the nuclear cycle. The detected centrosomes in the non-fertilized eggs of *imp-α2*^{D14}*/imp-β*^{Ketc02473}*; nos-Gal4-imp-α2*$^{NLSB^-}$ mutant females suggest a new possible function of Imp-α2/Imp-β^{Ketel} complex, i.e. it may regulate the factors of centriole-biogenesis as well.

5. Summary

1. Imp-α2 and Imp-β^{Ketel} specifically cooperate *in vivo* to essentially regulate the early development of Drosophila embryo.
2. The mitotic function of *imp-α2* can not be performed by *imp-α1* or *imp-α*3
3. The Imp-α2 shows a cell cycle-dependent dynamic distribution on the spindles.
4. The negative regulation of mitotic factors occurs through the NLS domain of Imp-α2.
5. The amount of maternally provided Imp-α2/Imp-β^{Ketel} functional complexes is critical during the syncytial divisions in the Drosophila embryo.
6. The Imp-α2/Imp-β^{Ketel} complex regulates:
   - spindle dynamics
   - chromatin condensation
   - nuclear envelope brake down/reassembly
   - centrosome dynamics and biogenesis

   during the early nuclear divisions in Drosophila embryos.

6. Publications

Publication used for the thesis:

Virágh E, Gorjánácz M, Török I, Eichhorn T, Kallakuri S, Szlanka T, Kiss I, Mechler BM. Specific Cooperation Between Imp-α2 and Imp-β/Ketel in Spindle Assembly During Drosophila Early Nuclear Divisions. G3 (Bethesda). 2012 Jan;2(1):1-14.
IF: the exact number of Impact Factor will be issued in July of 2013

Other Publications:

Willemsen M. H, Nijhof B., Fenckova M., Nillesen W. M., Bongers E M., Castells-Nobau A., Asztalos L., **Viragh E.** van Bon BW, Tezel E., Veltman J.A., Brunner H.G., de Vries B.B., de Ligt J., Yntema H. G., van Bokhoven H., Isidor B., Le Caignec C., Lorino E., Asztalos Z., Koolen D.A.,Vissers L.E., Schenck A., Kleefstra T. *GATAD2B* loss-of-function mutations cause a recognizable syndrome with intellectual disability and are associated with learning deficits and synaptic undergrowth in *Drosophila.* Journal of Medical Genetics, accepted: 2013. Apr 20.
IF: 6,365

van Bon BW, Oortveld MA, Nijtmans LG, Fenckova M, Nijhof B, Besseling J, Vos M, Kramer JM, de Leeuw N, Castells-Nobau A, Asztalos L, **Viragh** E, Ruiter M, Hofmann F, Eshuis L, Collavin L, Huynen MA, Asztalos Z, Verstreken P, Rodenburg RJ, Smeitink JA, de Vries BB, Schenck A.CEP89 is required for mitochondrial metabolism and neuronal function in man and fly.Hum Mol Genet. 2013 Apr 10. [Epub ahead of print]
IF: 7,636

D. Chen, A. Ahlford, F. Schnorrer, I. Kalchhauser, M. Fellner, **E. Virágh**, I. Kiss, A.-C. Syvänen, and B. J. Dickson. High-resolution high-throughput SNP mapping in *Drosophila melanogaster.* (2008) Nature Methods (4):323-9.
IF: 13.651

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IF: 28,751

T. Szlanka, L. Haracska, I. Kiss, P.Deák, E. Kurucz, I. Andó, **E. Virágh** and A. Udvardy. Deletion of proteasomal subunit S5a/Rpn10/p54 causes lethality, multiple mitotic defects and overexpression of proteasomal genes in *Drosophila melanogaster.* (2003) J Cell Sci. 116 (Pt 6): 1023-33.
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I.Kiss, A. Fodor, T. Timár, S. Hosztafi, P. Sebők, T. Török, **E. Virágh** and M. Berényi. (1988) Biological activity of precocene analogues on *Locusta migratoria*. Experientia 44, 790-792.
IF:1,147

Cumulative IF: 64,8