Specific Cooperation Between Imp-α2 and Imp-β/Ketel in Spindle Assembly During Drosophila Early Nuclear Divisions

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ABSTRACT The multifunctional factors Imp-α and Imp-β are involved in nuclear protein import, mitotic spindle dynamics, and nuclear membrane formation. Furthermore, each of the three members of the Imp-α family exerts distinct tasks during development. In Drosophila melanogaster, the imp-α2 gene is critical during oogenesis for ring canal assembly; specific mutations, which allow oogenesis to proceed normally, were found to block early embryonic mitosis. Here, we show that imp-α2 and imp-β genetically interact during early embryonic development, and we characterize the pattern of defects affecting mitosis in embryos laid by heterozygous imp-α2014 and imp-βKetRE34 females. Embryonic development is arrested in these embryos but is unaffected in combinations between imp-βKetRE34 and null mutations in imp-α1 or imp-α3. Furthermore, the imp-α2014/imp-βKetRE34 interaction could only be rescued by an imp-α2 transgene, albeit not imp-α1 or imp-α3, showing the exclusive imp-α2 function with imp-β. Use of transgenes carrying modifications in the major Imp-α2 domains showed the critical requirement of the nuclear localization signal binding (NLSB) site in this process. In the mutant embryos, we found metaphase-arrested mitoses made of enlarged spindles, suggesting an unrestrained activity of factors promoting spindle assembly. In accordance with this, we found that Imp-βKetRE34 and Imp-βKed bind a high level of RanGTP/GDP, and a deletion decreasing RanGTP level suppresses the imp-βKetRE34 phenotype. These data suggest that a fine balance among Imp-α2, Imp-β, RanGTP, and the NLS cargos is critical for mitotic progression during early embryonic development.

KEYWORDS Drosophila Importins genetic interaction mitosis spindle formation

The Ran pathway plays a central role in interphase cells by mediating and regulating the nucleocytoplasmic protein transport (Görlich et al. 1996; Izaurralde et al. 1997; Stewart 2007). During mitosis, it regulates spindle assembly, metaphase chromosome alignment, and nuclear envelope (NE) assembly (Barazani et al. 2001; Caudron et al. 2005; Zhang and Clarke 2000). In all these processes, the same basic mechanism is operational (Dasso 2001): Importin-β (Imp-β) binds to Importin-α (Imp-α) and induces a conformational change opening the NLS-binding site of Imp-α (Harreman et al. 2003; Kobe 1999). The NLS-bearing proteins, as cargos for the nuclear import (Mans et al. 2004; Pemberton and Paschal 2005) or spindle assembly factors (SAF), and other proteins regulating the dynamics of mitosis (Gruss et al. 2001; Nachury et al. 2001; Wiese et al. 2001) are bound to the Imp-α/Imp-β heterodimer. RanGTP present at a high concentration in the nucleus and distributed along a concentration gradient around the mitotic chromosomes binds to Imp-β and dissociates the complex, thereby liberating the bound proteins (Görlich et al. 1996; Walczak and Heald 2008). The RCC1/RanGEF, which mediates the exchange of the Ran-bound GDP for GTP, is associated with the chromatin
and hence responsible for the high RanGTP concentration in the interphase nucleus or around the mitotic chromosomes (Nemerget et al. 2001). Therefore, the liberation of NLS-bearing proteins occurs in the nucleus or near the chromatin (Bastiaens et al. 2006).

The regulation of the SAF activity by the Ran system during mitosis occurs in all eukaryotic organisms, from plants (Jeong et al. 2005; Pay et al. 2002) and yeast (Feig et al. 2000; Sato and Toda 2007) to humans (Li and Zheng 2004; Moore et al. 2002), and it also takes place in eggs like those of Drosophila and Xenopus, in which large amounts of SAFs and other mitotic proteins are deposited. These factors, including TPX2 (Gruss et al. 2001; Schatz et al. 2003; Vos et al. 2008), NuMa (Meredes et al. 1996; Nachury et al. 2001; Wiese et al. 2001), and NuSAP in the frog (Raeamakers et al. 2003; Ribbeck et al. 2007), as well as Mars in the fruit fly (Tan et al. 2008), are kept inactive under strict spatial and temporal control as normal activation is fatal to the embryo. Furthermore, the respective binding affinities of the various SAFs toward the NLS-binding domain of Imp-α appear to be critical to the mitotic process (Hodel et al. 2006; Riddick and Macara 2005). Interestingly, in Drosophila, where the first 13 rounds of synchronous mitoses take place in a syncytium, the Anillin and Peanut proteins, which are needed to keep the spindles separated, also appear to be regulated by Imp-α/Imp-β and Ran (Silverman-Gavrila and Wilde 2006). Furthermore, the Ran system is required for the assembly and integrity of the NE in eukaryotic organisms (Askaer et al. 2002; Ryan et al. 2003; Timinszky et al. 2002; Zhang and Clarke 2000). In addition, the Ran pathway exerts a critical role in centrosome duplication (Di Fiore et al. 2004), as Ran localizes to centrosomes, partly in the GTP-bound form (Keryer et al. 2003).

Phylogenetic studies of higher eukaryotes indicated that the imp-α genes could be classified in three conserved clades designated as α1, α2, and α3, whereas the imp-β gene is unique (Goldfarb et al. 2004; Hogarth et al. 2006; Köhler et al. 1997; Köhler et al. 1999; Malik et al. 1997). The first member of the imp-α gene family identified in Drosophila is imp-α2 (Török et al. 1995), and genetic analysis shows that a loss-of-function mutation in this gene leads to female sterility characterized by the occlusion of the ring canals linking the nurse cells to the oocyte. This occlusion prevents the transfer of cellular components at the time of nurse cell dumping into the oocyte and results in the formation of short basket-type eggs (Gorjánácz et al. 1997). The formation of short basket-type eggs (Gorjánácz et al. 1997) at the time of nurse cell dumping into the oocyte and results in the occlusion of cellular components that are transferred. The Ran pathway prevents the transfer of cellular components to the oocyte.

MATERIALS AND METHODS

Fly stocks

Flies were maintained at 25° on standard cornmeal-yeast-agar medium. Crosses were performed using standard genetic techniques. The following imp-α2 stocks and transgenic lines were used: imp-α2D14 (Török et al. 1995), P[imp-α2/+, P[UAS-imp-α2pDNA] (Gorjánácz et al. 2002), P[UAS-imp-α2i188], P[UAS-imp-α2NLSb−], P[UAS-imp-α2NLSb−], and P[UAS-imp-α2CAS−] (Gorjánácz et al. 2006). The mutant lines imp-βKetRE34, imp-βKetR13, and imp-βKetR13 (Erődelyi et al. 1997) and P[imp-β+] (Lippai et al. 2000) were kindly provided by J. Szabad. The third chromosomal ovarian driver line P[gal4VP16-nos.UTR]CG6325-MVD1, or nos-Gal4VP16, the P-element insertion lines w1118;RanGapEP1173/CyO and y w; P[Eg2]2/RanGapEP212763 and the deficiency Df(3L)w5.4/TM6, Tb were obtained from the Bloomington Drosophila Stock Center (Indiana University). The stocks w1118, imp-β02473/CyO, w1118, imp-β02657/CyO and w1118, imp-β03759/CyO (Thibault et al. 2004) were provided by Exelixis, Harvard Medical School. The RNAi gene silencing constructs P[imp-α1i28921], P[imp-α2i24625], P[imp-α2i23265], P[imp-α2i24665], P[imp-α3i36104], and P[imp-α3i38210] (Dietzl et al. 2007) were obtained from the Vienna Drosophila RNAi Center. The lines w1118; Df(3L)αS1 ca/TM6B uncovering the imp-α1 gene (Ratan et al. 2008) and imp-α3i3777/TM3(KR-GFP), Sb, as well as flies carrying the UTR3-imp-α constructs P[UTR3-imp-α1], P[UTR3-imp-α2], and P[UTR3-imp-α3] inserted on the second chromosome (Mason et al. 2003), were kindly provided by R. J. Flemming. A nos-Gal4VP16, P[UAS-imp-α2i24465] chromosome was generated by meiotic recombination. Recombinants were selected according to their stronger eye color and verified by PCR using primer pairs specific for each transgene. The pUASp2-based plasmid carrying the 2z-tagged imp-α2 sequences (see below), were microinjected along with the Δ23 transposable helper plasmid into w1118 syncytial blastoderm embryos according to standard techniques, and stable lines were generated.

Embryo viability

Females with different allele combinations were collected as virgins, and 20–30 of them were mated to 30 Oregon-R males. Eggs were collected from 3–6-day-old mothers for 12 hr on fresh apple juice plates (22.5 g agar boiled in 750 ml distilled water, mixed with 25 g sucrose and 250 ml apple juice) supplemented with charcoal, at 25° under 60% relative humidity. The plates were incubated at 25° for 23 hr. The numbers of laid eggs. All experiments were repeated three times.

DNA sequencing of imp-βKetRE34

Genomic DNA was isolated from a single homozygous imp-βKetRE34 second instar larva according to Goor et al. (1993), and 1 μl of the
preparation was used for PCR reaction in a 25 \mu l volume. A region (376–4122 bp, according to FlyBase numbering) of imp-\(\beta\) gene, covering the whole coding sequence, was PCR-amplified in two overlapping reactions using High-Fidelity PCR Master Kit (Roche Applied Science). For the first segment, we used the forward primer 376 (5’-TCTATACCCGACAGGCGACGATTC-3') starting 83 bp before the ATG translation initiation site and the reverse primer 2632 (5’-TATGCTCTGTTGATAACGGCCTCG-3’), whereas for the second segment, ending 134 bp after the termination codon, we used the forward primer 2424 (5’-CTTAAAGCCGCTGGTTAGAACGAAAG-3’) and the reverse primer 4122 (5’-CAAGATCGACGACATTGTGGTTCC-3’). The amplified products were separated on an agarose gel and isolated with the help of a QIAGEN DNA Purification Kit according to the manufacturer’s instruction, and sequenced on an ABI 3730XL DNA sequencer.

**Embryo fixation and immunohistochemistry**

Eggs were collected on apple juice plates for 2 hr at 25° and incubated for additional 2 hr. After dechorionization in 4% bleach for 4 min, the eggs were rinsed consecutively in H\(_2\)O, 0.2% Triton X-100, and H\(\text{\textsubscript{2}}\)O. For methanol fixation, the embryos were shaken vigorously for 45 sec in a 1:1 mixture heptane:methanol at room temperature and rinsed three times in methanol for 5 min each. Fast formaldehyde fixation and immunostaining of either methanol- or formaldehyde-fixed embryos were performed according to standard procedures (Rothwell and Sullivan 2000). Primary antibodies used were rat anti-\(\alpha\)-tubulin YLI1/2 (1:400, Serotec), rabbit anti-centrosomin [1:200 (Heuer et al. 1995), kindly provided by T. C. Kaufman], mouse anti-lamin Dm0 [1:30, (Paddy et al. 1996), a gift of H. Saumweber], and rabbit anti-phosphohistone (1:500, Santa Cruz Biotechnology). Secondary antibodies Alexa Fluor 488 anti-mouse (1:300) and Alexa Fluor 488 anti-rabbit (1:500) were purchased from Invitrogen and Cy3 anti-rat (1:400) [1:50, (Paddy et al. 1996), a gift of H. Saumweber]) antibodies.

**Protein A tagging of Imp-\(\alpha\)2 proteins**

In the first step, wild-type and NLSB\(^\text{\textsuperscript{\texttt{b}}}\) mutant imp-\(\alpha\)2 cDNAs (Gorjánácz et al. 2006) were PCR-amplified with the forward primer 5’-ATAAAGATGCGACCGCCGACAGGACGAAAC-3’ and the reverse primer 5’-CCCAAGCTCTAGGTGATACGAAAGC-3’. Both primers were purchased from Invitrogen and G3 anti-rat (1:400) from Jackson ImmunoResearch Laboratories. DNA was stained with DAPI. The samples were examined with a Nikon C1Si-CLEM confocal laser scanning microscope of the Nikon Imaging Center at the University of Heidelberg.

**GST-pulldown experiments**

Bacterially expressed and purified His-Ran\(^\text{\textsuperscript{\texttt{b}}}\)N (RanGDP form of Ran) or His-Ran\(^\text{\textsuperscript{\texttt{Q86L}}}\) (RanGTP form of Ran) proteins of Xenopus (kindly provided by I. W. Mattaj) were diluted to a final concentration of 10 \(\mu\)M in a protein extract of 2-hr-old embryos. Embryonic extracts were prepared in IP buffer (10 mM Tris\(\text{\textsubscript{\texttt{PH 7.5}}\), 50 mM KCl, 0.1% Tween 20, protease inhibitors}) at a concentration of 0.2 g embryo/ml as described before (Máthé et al. 2000). In a 0.5 ml tube, 0.4 ml aliquots of the above mixtures were incubated with 80 \(\mu\)l of a suspension made of GST-Imp-\(\beta\), GST-Imp-\(\beta\)\(^{\text{KetD}}\), or GST-Imp-\(\beta\)\(^{\text{KetRE34}}\) fusion proteins bound to Glutathione Sepharose beads for 60 min at room temperature. The beads were then centrifuged and washed several times with binding buffer. The proteins bound to IgG Sepharose beads were eluted with 50 mM Tris, pH 7.5, and 2 mM MgCl\(\text{\textsubscript{2}}\). The purified proteins were separated on SDS-PAGE, stained with Coomassie Brilliant Blue, and then proteins in selected bands were identified by MALDI spectroscopy in the Department of Proteome Analysis by M. Schnölzer at the German Cancer Research, Heidelberg.

**In vitro mutagenesis and expression of mutant proteins in bacteria**

Wild-type imp-\(\beta\) cDNA (Lippai et al. 2000), kindly provided by J. Szabad, was cloned in pBluescript II SK(+) vector, and mutant constructs were generated using the PCR-based QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. In the first step, the P\(^{\text{\texttt{D25N}}}\) mutation of the dominant female sterile imp-\(\beta\)\(^{\text{KetD}}\) allele was generated with PCR primers (bold letters indicate the introduced nucleotide substitutions) 5’-CGACG TATTTTGATATAATTCTCGAGGGCGCTATCAACG-3’ and 5’-CGTTGATAGCGCTGGAAATTATATGCCAAATACTCGCG-3’. In the second step, the D\(^{\text{\texttt{D25N}}}\) mutation of imp-\(\beta\)\(^{\text{KetRE34}}\) allele was introduced using the P\(^{\text{\texttt{D446L}}}\) construct as template with the primers 5’-GGTTCTGTCTGGTTTCCGAAATATTTGGCTTGGAC-3’ and 5’-GCTCAAGCGAATATTTCGCCGAAAGCGACAGAAACC-3’. Both constructs were sequenced to confirm the absence of PCR-induced errors. Sequences containing the full-length open reading frame of the imp-\(\beta\)\(^{\text{KetD}}\) and imp-\(\beta\)\(^{\text{KetRE34}}\) alleles and the wild-type imp-\(\beta\) coding sequence were cloned in frame into the pGEX-4T-2 expression vector (GE Healthcare Life Sciences) to create GST-fusion constructs. Fusion proteins were expressed in BL21-CodonPlus cells (Stratagene) at room temperature and purified on Glutathione Sepharose beads (GE Healthcare Life Sciences) according to the manufacturer’s instructions.
RESULTS

Genetic analysis of the imp-α2 and imp-β interaction

To determine whether any combination between mutations in the imp-α2 and imp-β genes could result in a synthetic phenotype, we first combined six different recessive imp-β alleles (Table 1) with the interstitial deficiency imp-α2014 (Gorjánácz et al. 2002; Török et al. 1995) and examined the viability of the eggs laid by heterozygous females of each combination. In all experiments, mutant females were crossed to wild-type males, and the hatched larvae were scored as a percentage of the total laid eggs. As shown in Table 1, eggs produced by trans-heterozygous imp-α2014/imp-βKetRE34 females are lethal, whereas eggs laid by all other heterozygous females develop normally.

Genomic P[imp-α2+]/P[imp-β+] transgenes or a P[UAS-imp-α2+RNAi] transgene driven by nos-Gal4VP16 could significantly restore embryonic development of eggs laid by heterozygous imp-α2014/imp-βKetRE34 females (Table 2), indicating that the observed interaction involved no second site mutation carried on either the imp-α2014 or the imp-βKetRE34 chromosome. Further, we tested whether RNAi silencing of imp-α2 would also produce a similar phenotype with imp-βKetRE34. Expression of the P[imp-α2+RNAi] construct (Dietz et al. 2007) driven by nos-Gal4VP16 apparently exerted no effect on a wild-type background, but it reduced embryonic viability in eggs laid by heterozygous imp-α2014 females (supporting information, Table S1). We obtained an even more dramatic effect when P[imp-α2266Δ] was expressed in females homozygous for wild-type imp-α2 but heterozygous for imp-βKetRE34. Only ~3% of the eggs were viable. These data show that a significant reduction of imp-α2 expression in the ovary in combination with imp-βKetRE34 resulted in high percentage of lethality of embryos laid by these females, indicating that the coordinated action of the Imp-α2 and Imp-β proteins is critical during embryogenesis.

The imp-βKetRE34 allele is an EMS-induced recessive revertant of the P446L substitution-characterized, dominant negative female-sterile imp-βKetD (Erdélyi et al. 1997; Lippai et al. 2000; Timinszky et al. 2002; Tirián et al. 2000). As no apparent defect could be detected in the combination between imp-α2014 and either imp-βKetRP13 (P-element-induced recessive revertant) or imp-βKetRX13, which is an X-ray–induced null allele of imp-βKetD (Lippai et al. 2000), we presumed that imp-βKetRE34 should carry an additional intragenic mutation.

Therefore, we determined the nucleotide sequence of the coding region in imp-βKetRE34. Besides the nucleotide modification leading to the substitution P446L, we found an additional nucleotide change resulting in the substitution of an aspartic acid at position 725 by an asparagine. Therefore, the D725N substitution could be a good candidate for the mutation that partially abrogates the dominance of imp-βKetD, although we could not exclude that an additional mutation may have taken place in the promoter or UTR region. Such a mutation could affect the synthesis of the Imp-βKetRE34 protein and weaken the dominant negative phenotype.

In silico analysis of the molecular structure of Imp-βD725N

As the D725N substitution is located in the Imp-α–binding domain of Imp-β, we performed an in silico analysis through docking of the IBB domain of Imp-α2 on Imp-βD725N to determine whether the substitution would markedly change the interaction between both Imp proteins. We found no alteration in the binding affinity between these molecules (Table S2). Further analysis of the modeled structure revealed that residue N725 located in the Helix B of HEAT repeat 16 forms an intramolecular polar interaction with residue E773 in Helix B of HEAT repeat 17 (Figure S1). This interaction allows less sliding of the B helices in the repeats 16 and 17 along each other, stabilizing in this way their relative positions. Imp-β is known to go through extensive conformational changes during its binding cycle (Conti et al. 2006), displaying the closest conformation when bound with the IBB domain of Imp-α. For homology modeling, we used the Protein Data Bank (PDB) data of the human Imp-β captured in association with the IBB domain of human imp-α [PDB code 1QGK, (Cingolani et al. 1999)]. It is possible that the D725N substitution might fix a closed structure by making the conformation of the Imp-βKetRE34 less prone to be open. This could compensate, at least to some extent, for the opening effect of the P446L substitution of the dominant negative Imp-βKetD reported earlier (Timinszky et al. 2002) and may thus reduce Imp-βKetRE34 toxicity.

imp-α2 critical function during early embryonic development

To determine whether the other two members of the imp-α gene family would interact with imp-β, we combined classical alleles and

Table 1 Viability of eggs laid by mutant females

| Female Genotype | Egg Viability (%) | SD | n |
|-----------------|------------------|----|---|
| imp-α2014/+     | 89               | 2.49 | 285 |
| imp-βKetRE34/+  | 61               | 9.50 | 234 |
| imp-α2014/imp-βKetRE34 | 0 | 0.00 | ~10,000 |
| imp-βKetRP13/+  | 79               | 3.23 | 120 |
| imp-α2014/imp-βKetRX13 | 80 | 9.97 | 300 |
| imp-βKetRP13/+  | 85               | 5.24 | 150 |
| imp-α2014/imp-β   | 85               | 9.29 | 276 |
| imp-βKetRX13/+  | 93               | 2.82 | 100 |
| imp-α2014/imp-β02473 | 90 | 5.56 | 290 |
| imp-β0257/+     | 83               | 1.41 | 100 |
| imp-α2014/imp-β02657 | 90 | 2.88 | 150 |
| imp-β03750/+    | 92               | 6.24 | 275 |
| imp-α2014/imp-β03750 | 94 | 3.44 | 175 |

For the origin of mutant imp-β alleles, see Materials and Methods. n, number of embryos scored.

Table 2 Increased imp-α2 or imp-β gene dosage restores viability of eggs laid by imp-α2014/imp-βKetRE34 females

| Female Genotype | Egg Viability (%) | SD | n |
|-----------------|------------------|----|---|
| imp-α2014/imp-βKetRE34 | 0 | 0.00 | ~10,000 |
| imp-α2014/imp-βKetRE34, P[UAS-imp-α2/2i34266}nos-Gal4VP16 imp-α2014/imp-βKetRE34; P[imp-α2+]/+ | 38 | 7.77 | 280 |
| imp-α2014/imp-βKetRE34; P[imp-β-1]/+ | 46 | 2.30 | 340 |
| imp-α2014/imp-βKetRE34; P[imp-β-1]/+ | 30 | 2.12 | 172 |

n, number of embryos scored.

Table 3 Effects of reduced gene dosages of the three imp-α genes on embryo viability in combination with imp-βKetRE34

| Female Genotype | Egg Viability (%) | SD | n |
|-----------------|------------------|----|---|
| imp-βKetRE34/+  | 61               | 9.50 | 234 |
| imp-α2014/imp-βKetRE34 | 47 | 2.36 | 290 |
| imp-βKetRE34/+; Df(3L)α151/+ | 33 | 7.07 | 181 |

Deletion Df(3L)α151 uncovers the imp-α1 gene. n, number of embryos scored.
RNAi knockdowns of imp-1 or imp-3 with a heterozygous imp-β<sub>KetRE34</sub>. As shown in Table 3, the deficiency Df(3L)α1S1, uncovering imp-α1 (Ratan et al. 2008), moderately reduced egg viability (47%, compared with 61% for eggs laid by imp-β<sub>KetRE34</sub>/+ females). The imp-α<sub>3</sub>1/2ο mutant allele producing a polypeptide of 131 residues (Mason et al. 2003) decreased the egg viability to 33%. RNAi silencing of either imp-α1 or imp-α3 resulted in very similar hatching ratios (40% and 43%, Table S3). These observations indicate that a decrease of the maternal “dowry” of either Imp-α1 or Imp-α3 produced only a moderate reduction of embryonic viability in the imp-β<sub>KetRE34</sub>/+ background. In contrast, in combination with imp-β<sub>KetRE34</sub>, a decrease in Imp-α2 caused either by a deletion or by RNAi silencing resulted in strong embryonic lethality.

As the amount of Imp-α2 is apparently higher in eggs than the amount of Imp-α1 or Imp-α3, the overall NLS-binding capacity is mainly ensured by Imp-α2. Therefore, a decrease in the expression of imp-α2 results in a stronger reduction of this capacity than does a decrease in the expression of imp-α1 or imp-α3, and it could also have a stronger effect on embryonic development. This prompted us to examine whether the effect on hatching rates was independent of the relative amount of the different Imp-α proteins deposited in the eggs. For this purpose, we tested transgenes producing similar amounts of Imp-α, in which the 5’ and 3’ UTR sequences of the various imp-α cDNAs were removed and contained an initiation AAAATG consensus sequence (Cavener 1987) inserted at the 5’ extremity of the coding region (Mason et al. 2003). As shown in Table 4, only P[UTR<sup>i</sup>3-imp-α2] was able to restore embryonic viability in an imp-α<sub>2</sub>0/+ imp-β<sub>KetRE34</sub> background. The rescue was, however, lower (21%) than with a full P[imp-α<sub>2</sub>DN<sup>i</sup>] construct (38%, Table 2), indicating the relative importance of UTR sequences in the expression of the imp-α2 gene. Accordingly, in control experiments, we found that the P[UTR<sup>i</sup>3-imp-α2] expressed in the germ line of imp-α<sub>2</sub>0/+ females resulted in 37% embryonic viability, whereas a full-length cDNA construct yielded 85% viability (detailed data not shown).

These data indicate that, during early embryonic development when the maternally deposited proteins are the limiting factors, Imp-α2 acts critically in cooperation with Imp-β and could not be substituted with Imp-α1 or Imp-α3.

### Specific substitutions in the NLSB domain of Imp-α2 produce embryonic lethality in combination with a reduced imp-β gene dosage

As a strong interaction between imp-α2 and imp-β was detected in eggs laid by imp-α<sub>2</sub>0/+ imp-β<sub>KetRE34</sub> females, we determined which domain of imp-α2 should be altered to produce embryonic lethality when the imp-β gene dosage is reduced. Heterozygous combinations of imp-α<sub>2</sub>0/+ with distinct imp-β<sub>0</sub> alleles, including the interstitial deficiency imp-β<sub>KetRE33</sub>, and the P-element–induced imp-β<sub>KetRE13</sub> mutation (Erdélyi et al. 1997), as well as the piggyBac insertion mutations imp-β<sub>IBB2</sub>0, imp-β<sub>IBB3</sub>0, and imp-β<sub>IBB5</sub>0 (Thibault et al. 2004), should provide us with a sensitized genetic background for testing four previously modified imp-α2 constructs (Gorjánácz et al. 2006).

In imp-α<sub>2</sub>0/+ homozygous background the NLS<sup>−</sup>-construct (in which the conserved W and N residues of the major and minor NLS sites are substituted by A) and the CASB<sup>−</sup>-construct (in which the six GLDKLE residues of the CAS nuclear export factor binding site are replaced similarly) exert a toxic effect during oogenesis. In contrast, the SNLSB<sup>−</sup>-construct, which contains substitutions of A in the conserved W and N residues of the small NLS site, and the ΔIBB construct, in which the Imp-β–binding domain is deleted, restore oogenesis but block embryogenesis under the same conditions (Gorjánácz et al. 2006). All four mutated imp-α2 cDNA constructs contain a UAS promoter. These cDNA constructs were expressed in an imp-α<sub>2</sub>0/+ imp-β<sub>0</sub> background driven by nos-Gal4<sup>P16</sup>, Viability of the eggs laid by these females was measured.

We found that the expression of both P[UAS-imp-α<sub>2</sub>SNLSB–] and P[UAS-imp-α<sub>2</sub>ΔIBB<sup>−</sup>] completely blocked embryonic development in eggs laid by all sensitized trans-heterozygous females with exception of

### Table 4 Effects of the three different P(UTR<i>i</i>-imp-α) constructs on the viability of eggs laid by imp-α<sub>2</sub>0/+ imp-β<sub>KetRE34</sub> females

| Female Genotype | Egg Viability (%) | SD  | n   |
|-----------------|------------------|-----|-----|
| imp-α<sub>2</sub>0/+ imp-β<sub>KetRE34</sub> | 0.00 ± 0.00 | ~10 000 |
| imp-α<sub>2</sub>0/+ imp-β<sub>KetRE34</sub>, nos-Gal4<sup>P16</sup>/+ | 0.00 ± 0.00 | 1426 |
| imp-α<sub>2</sub>0/+ imp-β<sub>KetRE34</sub>, nos-Gal4<sup>P16</sup>/+ | 2.08 ± 0.00 | 355 |
| imp-α<sub>2</sub>0/+ imp-β<sub>KetRE34</sub>, nos-Gal4<sup>P16</sup>/+ | 0.00 ± 0.00 | 1350 |

On average, 100–300 embryos were scored in each experiment.

**Table 5 Expression of mutant imp-α2 with an inactive NLS-binding domain strongly reduced egg viability laid by heterozygous imp-α<sub>2</sub>0/+ imp-β<sub>0</sub> females**

| Female Genotype<sup>a</sup> | NLS<sup>−</sup> | SNLSB<sup>−</sup> | CASB<sup>−</sup> | ΔIBB |
|-----------------------------|----------------|----------------|----------------|------|
| +/+                         | 97 ± 1.73       | 89 ± 4.94       | 93 ± 4.24       | 94 ± 2.08 |
| imp-α<sub>2</sub>0/+        | 20 ± 2.32       | 76 ± 4.94       | 86 ± 0.00       | 87 ± 5.85 |
| imp-α<sub>2</sub>0/+ imp-β<sub>KetRE13</sub> | 2 ± 1.12        | 0 ± 0.00        | 90 ± ND         | 85 ± ND |
| imp-α<sub>2</sub>0/+ imp-β<sub>KetRE13</sub> | 23 ± 9.19       | 32 ± 4.04       | 93 ± ND         | 91 ± ND |
| imp-α<sub>2</sub>0/+ imp-β<sub>IBB2</sub>0 | 0 ± 0.00        | 0 ± 0.00        | 91 ± ND         | 87 ± ND |
| imp-α<sub>2</sub>0/+ imp-β<sub>IBB5</sub>0 | 0 ± 0.00        | 1 ± 1.12        | 87 ± ND         | 83 ± ND |
| imp-α<sub>2</sub>0/+ imp-β<sub>IBB5</sub>0 | 0 ± 0.00        | 2 ± 1.76        | 84 ± ND         | 79 ± 1.54 |

<sup>a</sup> The genotype of second chromosome is given in the first column, and the corresponding mutated domains in the P[UAS-imp-α2] transgenes located on the third chromosome are shown in the subheading. All transgenes were expressed by the nos-Gal4<sup>P16</sup> driver on the third chromosome.
those with imp-βKetRE34, indicating that this P-element–induced allele is a hypomorph. In contrast, the expression of P[UAS-imp-α2Imm] and P[UAS-imp-α2Casb−] exerted no deleterious effect on embryonic development (Table 5). The ineffectiveness of P[UAS-imp-α2Imm] can be explained by a lack of the IBB domain, which prevents binding to Imp-β, whereas the other three Imp-α2 proteins contain an intact IBB domain and were able to physically interact with Imp-β. Interestingly the antimorphic effect of P[UAS-imp-α2NLSB−] could already be detected in imp-α2D14/- females, and this effect was enhanced when the imp-β gene dosage was reduced. Altogether, our data indicate that the NLSB domain, albeit not the CASB domain, mediates the genetic interaction between imp-α2 and imp-β. We further conclude that the cooperation between Imp-α2 and Imp-β requires the binding of one or several NLS-containing factors involved in the regulation of early embryonic mitosis.

**Concurrent reduction in specific imp-α2 and imp-β gene activity blocks mitosis in early embryos**

To determine more precisely when the developmental arrest takes place in eggs laid by imp-α2D14/imp-βKetRE34 trans-heterozygous females or imp-α2D14/imp-βKetRE34; nos-Gal4170, P[UAS-imp-α2NLSB−]/+ females (hereafter, NLSB− denotes nos-Gal4170P[UAS-imp-α2NLSB−/]), we collected eggs for 2 hr, and aged them for 2 hr before fixation. The embryos were then stained to visualize α-tubulin and DNA. Their development was predominantly blocked during the very first mitotic divisions in cycle 1 to 3 (Figure 1A). As a control, a 1-hr-old wild-type embryo is shown (Figure 1B). In the mutant embryos, all identified nuclei consisted of metaphase-like structures with chromatin in the center and enlarged masses of microtubules organized at both poles (Figure 1, C–F). Centrosomes were variable in number and organization. In addition, arrays of regularly spaced centrosomal structures (Figure 1, D and F) indicate that the cycle of centrosome replication was less hampered than the mitotic cycle, a characteristic also observed in other mutations affecting mitosis (Belecz et al. 2001; Zhang et al. 2009). Both types of mutant embryos displayed a wide range of abnormalities, among which the formation of free asters was one of the most frequent phenotypes (Figure 1, D and F) detected in about three-quarters of the laid eggs. We found also embryos with microtubules filling the ooplasm and giving rise to a cobweb of free asters (Figure 2D and Figure S2, B and C). Some embryos contained up to 50–60 free asters.

The importance of the cooperation between imp-α2 and imp-β was confirmed by comparing the number of mitotic figures between arrested embryos, including those derived from imp-α2D14/+; NLSB−/+ or imp-βKetRE34/+ single mutant females (80% and 39% lethality, respectively), and the embryos laid by imp-α2D14/imp-βKetRE34; NLSB−/+ or imp-α2D14/imp-βKetRE34 females (100% lethality). Whereas 95–100% of the embryos from double mutants exhibited fewer than 16 spindles, 35–45% of the lethal embryos from the single mutants displayed from 129 to 4,000 spindles in the ooplasm (Figure S3). Moreover, about 15% of 4- to 6-hr-old arrested embryos from the single mutants were able to partially cellularize, but their development was blocked due to the accumulation of mitotic defects. Our data further point out that embryonic development was arrested significantly earlier in eggs derived from females carrying specific mutations in both imp-α2 and imp-β genes than in those derived from females heterozygous for only one of them, emphasizing the synergy taking place between these two genes.

**Mitotic progression requires interaction between Imp-α2 and Imp-β**

Examination by confocal microscopy of embryos derived from imp-α2D14/imp-βKetRE34 and imp-α2D14/imp-βKetRE34; NLSB−/+ females revealed numerous mitotic defects, which are characterized by a mitotic block during the early nuclear divisions (Figure 2). Essentially no interphase nucleus could be detected in these embryos. In general, the majority of the mitotic figures consisted of considerably enlarged spindles. The most frequently detected type of mitotic abnormality was the occurrence of fatty spindles made of large masses of microtubules originating from both poles and widening out at the equator. In these structures, the chromatin was aligned at the equator, forming a metaphase plate. The barrel-shape spindles, which were smaller than the fatty spindles, might be a form of fatty spindle at the beginning of its growth. We also observed multipolar spindles containing discrete aggregates of chromatin and unfocused spindles in which the chromatin was fragmented in small aggregates. The multipolar spindles
and the partially fused spindles at one of the poles might represent remnants of incompletely divided nuclei or might result from repli-
cated centrosomes (*vide infra*). Furthermore, we detected narrow
spindles, which were present in about 15% of the mutant embryos.
These spindles contained significantly smaller amounts of microtu-
bules, as well as reduced or indetectable amounts of chromatin.

To further characterize the mitotic arrest, we examined the
distribution of phospho-histone staining in chromatin and found
that, by comparison to wild-type mitosis in which the level of
phospho-histone staining was high in condensed chromatin before
chromatid separation and lower after chromatid separation (Figure S4,
A and B), the level of staining in the mutant embryos was relatively
high and equally homogenous in the chromatin aligned on the meta-
phase plate in fatty spindles (Figure S4C), as well as on the dispersed
chromatin spots detected in multipolar spindles (Figure S4D). In
contrast, we frequently observed DNA aggregates negatively stained
for phospho-histone at the periphery of the spindles (data not shown)
or in narrow spindles, which might contain a reduced number of chromatids (Figure S4E). These data indicate that the process of chro-
matin condensation occasionally becomes dysregulated in the mutant
embryos.

Similarly, we examined the fate of the nuclear envelope in arrested
mitosis of mutant embryos laid by *imp*-α2*β*GαRE34 and *imp*-α2*β*KetRE34
females (Figure 3). In mitosis taking place in wild-type embryos, the staining of the nuclear envelope with
anti-lamin Dm0 antibodies showed that the interphase nucleus was
surrounded by a continuous membrane (Figure 3A), which became
broken at the onset of mitosis over both spindle poles. During meta-
phase, the lamin staining decorated remnants of the nuclear envelope
forming a wide belt around the nucleus equator (Figure 3B). In tele-
phase, the nuclear envelope reassembled on the surface of each group
of separated chromatids, and the lamin staining capped the forming
nuclei (Figure 3C).

In embryos laid by both types of mutant females, we found
a distinct pattern of lamin staining. In the fatty spindles with
a conspicuous metaphase plate, we found a high concentration of
positively stained lamin dots or membrane vesicles capping both poles
of the spindle. The lamin-stained vesicles were nearly absent from the
equator belt (Figure 3D). The vesicles were detected in a relatively
high concentration at the spindle pole harboring a large aster (Figure
3E). In a multipolar spindle, we found that the lamin staining formed
an apparently continuous stratum around the chromatin located at
one of the poles (Figure 3F), suggesting that a nuclear envelope could
be formed when chromatids were pulled from the metaphase plate to
one of the spindle poles. Furthermore, we detected less frequently
large masses of DNA aggregates encapsulated by an apparently con-
tinuous layer of lamin, whose thickness was particularly large (Figure
S5, A and A1). We also observed embryos filled with relatively large,
positively stained lamin vesicles essentially devoid of DNA (Figure S5,
B and B1). These data indicate that a concomitant decrease in *imp*-α2
and *imp*-β gene activity resulted in the fragmentation of the nuclear
envelope into small vesicles, which essentially accumulated at the

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**Figure 2** Spindle abnormalities in embryos derived from *imp*-α2*β*GαRE34 and *imp*-α2*β*KetRE34, NLSB*+/- females. (A–D) Wild-type and mutant embryos stained for α-tubulin (green) and DNA (blue). (A) Mitotic spindles in wild-type embryos at metaphase and anaphase. (B, C) Categories of spindle abnormalities found in embryos derived from (B) *imp*-α2*β*GαRE34 and (C) *imp*-α2*β*KetRE34, NLSB*+/- females. (D) Formation of aster networks found in both genotypes. Scale bar: 10 μm. (E) Frequency of spindle defects in embryos from both types of mutant females. Female genotypes are displayed at the upper right corner. At least 200 spindles were scored for both genotypes.
Imp-α2 forms complexes with ISWI, CP190, and lamin through the NLSB domain

As mutations in the NLSB domain of Imp-α2 resulted in a strong embryonic phenotype in an imp-α2Δ204/imp-βΔ2473 background, we performed an analysis to identify partner proteins that would specifically bind to the NLSB domain of Imp-α2. For this purpose, we overexpressed wild-type and NLSB− zz-Imp-α2-tagged proteins (Rigaut et al. 1999) in fly ovaries, purified the Imp-α2 complexes by affinity chromatography, eluted the bound proteins, and separated them by SDS-PAGE. In this way, we isolated a relatively large series of protein bands, which were specifically recovered in association with the wild-type zz-Imp-α2 protein, albeit absent among the NLSB− zz-Imp-α2 complexes (Figure 5). These protein bands were excised from the gel, submitted to trypsin digestion, and the nature of the peptides was identified by mass spectrometry. Among the proteins specifically interacting with the NLSB domain of Imp-α2, we identified three protein factors known to be involved in mitosis, which include the ISWI protein acting in spindle assembly, (Yokoyama et al. 2009), CP190 involved in centrosome formation (Oegema et al. 1995), and lamin Dm0 associated with the nuclear envelope (Lenz-Böhme et al. 1997). These data indicate that the Drosophila Imp-α2 protein can bind through its NLSB domain with specific factors regulating mitosis.

Both Imp-βKetD and Imp-βKetRE34 proteins bind RanGTP and RanGDP with high affinity

The mitotic arrest occurring in imp-α2Δ204/imp-βΔ2473; NLSB−/+ could be explained by the dominant toxic effect of the NLSB− construct being unable to bind factors promoting spindle assembly. However, the Imp-βKetRE34 deleterious effect could not be directly attributed to Imp-α2 but to modifications occurring in Imp-β that would affect the binding of factors to imp-α2. The marked enlargement of spindles in mitotically arrested embryos derived from imp-α2Δ204/imp-βKetRE34 females suggests an abnormally high level of active factors involved in spindle assembly. This may indicate an alteration of RanGTP/GDP affinity for the Imp-βKetRE34 protein, similar to that found in the original Imp-βKetD mutant protein (Timinszky et al. 2002). Therefore, we examined the affinity of Imp-βKetRE34 for mutant Ran proteins His-RanQ69L and His-RanT24N locked in the GTP- and the GDP-bound forms, respectively (Klebe et al. 1995). For this purpose, we performed a GST-pulldown experiment with Imp-β- and mutant Imp-βKetD or Imp-βKetRE34 proteins. The full-length coding For this purpose, we performed a GST-pulldown experiment with Imp-β- and mutant Imp-βKetD or Imp-βKetRE34 proteins. The full-length coding
domain of the corresponding cDNAs were cloned in frame with a GST sequence of the pGEX-4-T-2 expression vector. The bacterially synthesized fusion proteins were purified on Glutathione Sepharose beads and mixed with Xenopus His-RanT24N or His-RanQ69L proteins, as well as crude proteins extracted from 0- to 120-min-old Drosophila embryos. After washes with binding buffer, the bound Ran proteins were separated by SDS-PAGE and detected by Western blotting with anti-Ran antibodies. As shown in Figure 6 (left panel), the Imp-βKetRE34 and Imp-βKetD proteins were able to bind higher amounts of RanGDP than the wild-type Imp-β. Similarly, Imp-βKetRE34 and Imp-βKetD displayed a higher binding affinity for RanGTP than Imp-β (right panel). These data are in contrast with previous results showing that Imp-βKetD might have a weaker affinity toward RanGTP than Imp-β (Timinszky et al. 2002), but as is shown in the upper panels, we used equal amounts of Imp-β proteins in the reaction mix. The high affinity of RanGTP and RanGDP for Imp-βKetD and Imp-βKetRE34 suggests that both mutant proteins could be prone to dissociate from Imp-α2, thus inducing a release of the cargo proteins carried by Imp-α2, or it may prevent their binding to Imp-α2. Consequently, the activity of factors involved in spindle assembly may be permanently enhanced, resulting in the formation of enlarged spindles and a metaphase arrest.

**Reduction in the level of RanGTP can restore viability of imp-βKetRE34 eggs**

Because a higher RanGTP/GDP affinity for Imp-βKetRE34 may favor its dissociation from Imp-α2 and thus the activation of spindle assembly factors, we tested whether mutations affecting the level of RanGTP/GDP might modify the semidominant lethality in imp-βKetRE34. As shown in Table 6, the viability of eggs laid by imp-βKetRE34/+ females reached 61%. When we combined imp-βKetRE34 with Df(3L)w5.4, which uncovers the B/I/RCC1 sequence encoding the Ran guanosine exchange factor (RanGEP), viability of eggs laid by imp-βKetRE34/+; Df(3L)w5.4/+ females was strongly enhanced, reaching 90%. When we combined imp-βKetRE34 with loss-of-function mutations in RanGap, which encodes the RanGTPase-activating protein, we reduced the viability of the eggs laid by imp-βKetRE34/RanGapEP1173 or imp-βKetRE34/RanGapEY21763 females to 27% and 28%, respectively. These data showed that a reduction in the level of RanGTP was able to compensate the imp-βKetRE34 mutation, presumably by allowing a functional interaction between Imp-α2 and Imp-β. In contrast, when RanGTP level was increased by reducing the GTPase activity of RanGap, the dysfunction of the Imp-βKetRE34 protein was enhanced. Alone, the RanGapEP1173 or RanGapEY21763 mutation in heterozygous conditions exert no significant decrease in embryo viability.

**DISCUSSION**

This study reveals that Drosophila imp-α2 plays a specific role in early embryogenesis and cannot be substituted with imp-α1 or imp-α3. Furthermore, the cooperation between imp-α2 and imp-β is strictly required to regulate the organization of microtubules, centrosomes, and the nuclear envelope throughout mitosis in preblastoderm-stage Drosophila embryos.
imp-α2 protein bands present in the Imp-α2zz purified fraction but absent from the NLSB zz fraction were excised, digested with trypsin and subjected to mass spectrometry. The following proteins were identified in the selected bands: (1) CP190, (2) ISWI, and (3) lamin Dm0.

Figure 6 Imp-β^KetD^ and Imp-β^KetRE34^ bind RanGDP and RanGTP with a higher affinity than wild-type Imp-β. His-RanT^™N^ (left panel) and His-RanQ^™O^L^ (right panel) proteins, representing the GDP- and GTP-bound forms, respectively, were expressed in bacteria, purified, and subsequently added to wild-type embryonic protein extract. Aliquots of both mixtures were incubated with GST-imp-β, and either GST-Imp-β^KetD^ or GST-Imp-β^KetRE34^ fusion proteins immobilized on Glutathione Sepharose beads. Proteins bound to the beads were analyzed by SDS-PAGE and immune-detected on Western blot with anti-Ran and anti-Imp-β antibodies.

Balanced cooperation between Imp-α2 and Imp-β is essential to enable rapid synchronous nuclear divisions in the Drosophila syncytial embryo

The Ran-regulated cooperation between Imp-α2 and Imp-β controls the activity of a variety of NLS-containing SAF proteins. Imp-α2 and Imp-β are synthesized during oogenesis, stored in relatively large amounts in Drosophila and Xenopus eggs (Görlich et al. 1994; Gruss et al. 2001; Jans et al. 2000). During the first 2 hr of Drosophila embryogenesis, essentially no zygotic gene expression takes place and the rapid synchronous divisions within the syncytial cytoplasm are exclusively driven by maternally deposited gene products (Foe et al. 1993). Even when the amount is reduced by half the maternal input of Imp-α2 and Imp-β proteins is sufficient to drive mitotic divisions.

The findings of a genetic interaction between, on the one hand, imp-α2^K2D14^ and Imp-β^KetRE34^ and, on the other hand, imp-α2^K2SNLSB^- or imp-α2^K2SNLSB^- and null alleles of imp-β suggest that a critical threshold of functional Imp-α2/Imp-β complexes should be maintained to sustain mitosis during early embryogenesis. One gene dosage of imp-α2^K2SNLSB^- or imp-α2^K2SNLSB^- alleles reduced the amount of functional complexes, while imp-β^KetRE34^, as inferred from pulldown experiments and mitotic phenotype of the genetic interactions, appears to decrease the stability of the NLS-protein/Imp-α2/Imp-β ternary complex. In embryos from imp-α2^K2D14^/imp-β^KetRE34^ transheterozygous females, the Imp-β^KetRE34^ and wild-type Imp-β proteins display a 1:1 ratio (data not shown) and compete for a reduced amount of Imp-α2 (50% of normal level). Analysis of the docking of the IBB domain of Imp-α2 on Imp-β^KetRE34^ and wild-type Imp-β by computer modeling revealed no striking difference in their binding affinities for the IBB domain. This result supports the assumption that the binding affinity of Imp-β^KetRE34^ or wild-type Imp-β for a full-length Imp-α2 is similar. Therefore, the amount of functional Imp-β and Imp-α2 complexes in embryos laid by imp-α2^K2D14^/imp-β^KetRE34^ females should be reduced to a subthreshold level, inadequate to regulate early embryonic mitosis.

An analogous argumentation could be used for explaining the lethality of embryos expressing imp-α2^K2SNLSB^- or imp-α2^K2SNLSB^- in an imp-α2^K2D14^/imp-β^K2^ background. In these embryos the mutations affecting the NLS-binding domain of Imp-α2 dominantly blocked the binding of specific cargos to the Imp-α2/Imp-β complexes but should not prevent the formation of these complexes, as the IBB domain remains intact. It is also possible that NLS-mutated Imp-α2 proteins through their intact IBB domain induced a preferential binding to the NLS-domain of intact Imp-α2 proteins and thus prevented the binding of cargos to the Imp-α2/Imp-β complex, resulting in a 100% arrest of embryonic development.

All these data point out the importance of the NLSB domain in the regulation of the rapid nuclear divisions taking place in syncytial embryos and suggest that critical SAF proteins bind to the Imp-α2/Imp-β complex through their NLS sequences. Moreover our finding

| Table 6 | Mutations affecting the RanGTP concentration modify the imp-β^KetRE34^ phenotype |
|---------|-----------------------------------------------|
| Female Genotype | Egg Viability (%) | SD | n |
| imp-β^KetRE34/+ | 61 | 9.50 | 234 |
| imp-β^KetRE34/+; Df(3L)w5.4/+ | 96 | 2.33 | 310 |
| imp-β^KetRE34/RanGap^{F1173} | 27 | 6.55 | 728 |
| imp-β^KetRE34/RanGap^{F21763} | 28 | 4.72 | 395 |
| RanGap^{F1173}/+ | 92 | 6.92 | 150 |
| RanGap^{F21763}/+ | 81 | 3.05 | 150 |

Deletion Df(3L)w5.4 uncovers B71/RCC1 gene (encoding RanGAP). n, number of embryos scored.
that SAF are specifically recovered in association with the NLS-binding domain of Imp-α2 further strengthens our assumption.

**Molecular nature of the mutant Imp-β proteins**

Compared with the original imp-βKetRE34 allele, we found that imp-βKetRE34 contains a second site mutation (substitution D725N) potentially responsible for the partial suppression of the dominant female sterile phenotype of imp-βKet. However, it is possible that mutations reducing the expression of the dominant negative Imp-βKet protein (e.g. mutations in the promoter or the UTRs) could also weaken the dominant negative phenotype, but the occurrence of a third site mutation could be considered negligible.

Furthermore, the intragenic mutation resulting in S17T substitution, which fully suppresses the dominance of imp-βKet (Timinszky et al. 2002) indicates that a second site substitution could be sufficient to change the neomorphic function of imp-βKet.

**Specific role of Imp-α2**

Each of the three Imp-α proteins displays distinct, but partly overlapping, roles in development (Hogarth et al. 2006; Mason and Goldfarb 2009). These specific Imp-α functions may be driven during the evolution of Metazoa by unique requirements in gametogenesis (Geles and Adam 2001; Hogarth et al. 2006; Mason and Goldfarb 2009). While Drosophila homologous mutants of imp-α1 and imp-α2 grow to adulthood, both females and males remain sterile, indicating specific roles of Imp-α1 and Imp-α2 in spermatogenesis (Girrè et al. 2002; Mason et al. 2002; Ratan et al. 2008) and oogenesis (Gorjánácz et al. 2002; Mason et al. 2002; Ratan et al. 2008), respectively. The imp-α3 null mutant is zygotic lethal (Mason et al. 2002; Mathé et al. 2000), and although it is also expressed in spermatids, testes and ovaries (Girrè et al. 2002; Hogarth et al. 2006; Mathé et al. 2000), its function is basically devoted to nuclear transport (Chan et al. 2008; Fang et al. 2001).

The specific roles of the three Imp-α proteins during early embryogenesis of Drosophila have not been examined. The data of the UTR3-imp-α experiments clearly show a specific role of Imp-α2 in the syncytial divisions of Drosophila embryos. This finding is in accordance with previous data showing that transgenes carrying mutations in the SNLSB and IIB domains of Imp-α2 were able to rescue the dumbless phenotype of imp-α2D14 homozygous females but were unable to sustain embryogenesis of the rescued eggs (Gorjánácz et al. 2006). Similar observations were reported in other metazoans ranging from C. elegans to human: in cooperation with Imp-β, the Imp-α2 orthologs act as regulators of mitotic spindle assembly (Askjaer et al. 2002; Nachury et al. 2001; Ribbeck et al. 2007; Schatz et al. 2003). Our analysis also indicates that Imp-α2 contributes to the regulation of mitosis in the Drosophila syncytial embryo.

The requirement for large amounts of Imp-α2 protein in Drosophila eggs could be explained by the need of a sufficient supply to regulate the rapid synchronous mitotic events taking place during early embryogenesis that lead to the formation of 6000 nuclei in about 2.5 hr (Foe et al. 1993). When the nuclei become cellularized, the following cell divisions occur at a much-reduced pace and can occur in the absence of Imp-α2, leading to the formation of fully viable, albeit sterile adults (Gorjánácz et al. 2002; Török et al. 1995) that display defects in muscle patterning and organization of the neuromuscular junction (Mosca and Schwarz 2010a, 2010b). In normal eukaryotic cells, some of the factors involved in mitosis are imported to the nucleus to be sequestered from the mitotic apparatus before the breakdown of the nuclear envelope (Kisurina-Evgenieva et al. 2004; Raemaekers et al. 2003; Walczak and Heald 2008). In Drosophila eggs, these “shuttling” proteins are deposited in an amount large enough to form thousands of spindles and should become available for nuclear import according to the number of dividing nuclei. These proteins should be stored inactive in the cytoplasm until they are needed in the nucleus. Binding to Imp-α2 through their NLS sequence could be a plausible solution for this problem. In this respect, the CP190 protein that can be recovered in association with Imp-α2 constitutes a good example, as this protein shuttles between the nucleus and the centrosomes in a cell cycle–specific manner (Kellogg and Alberts 1992; Oegema et al. 1995, 1997). Interestingly, no CP190 was found in the nuclei of Drosophila cleavage embryo prior to cycle 10, but it was detected in the cytoplasm and at centrosomes (Frasch et al. 1986). Moreover CP190 is involved in axial expansion of the nuclei along the anterior-to-posterior axis of the embryo (Chodagam et al. 2005). We suppose that prior to cycle 10, the binding of CP190 with Imp-α2 may be responsible for keeping it inactive in the cytoplasm located beyond a certain distance from centrosomes.

**Imp-α2 and Imp-β regulate the mitotic processes in the syncytial embryo**

The role of Importin-β in spindle assembly was demonstrated in Xenopus egg extract (Nachury et al. 2001). Although the Drosophila imp-β is a well-characterized gene, identified through its dominant imp-βKet mutation causing female sterility (Erdélyi et al. 1997; Lippai et al. 2000), its involvement in spindle assembly has not been clearly shown. The development in the eggs from imp-βKet/+ females was blocked at the first cleavage division, the gonomeric spindle failed to form, and disorganized masses of microtubules were observed (Tirian et al. 2000). However, when purified Imp-βKet protein was injected into wild-type syncytial Drosophila embryos, the NE formation was blocked, but neither spindle nor spindle envelope defects could be detected (Timinszky et al. 2002). Although the spindle abnormalities detected in the analysis of imp-βKetRE34 bear no direct relationship with the imp-βKet phenotype, it clearly shows the critical role of imp-β in the process of spindle formation.

Our data suggest that overgrown spindles could result from the activity of factors that trigger a persistent microtubule formation in the spindle area, because the absence of functional Imp-α2/Imp-β complexes prevents the sequestration of SAFs and further hampers other factors to foster mitotic progression. Therefore, mitosis was predominantly blocked at the metaphase to anaphase transition.

The observed abnormalities of chromosome condensation, alignment, and separation in embryos developing from imp-α2D14/imp-βKetRE34 and imp-α2D14/imp-β12473, NLsB−/+ females could be a secondary consequence of the spindle defects. However, the finding that the fly ortholog of ISWI was coimmunoprecipitated with Imp-α2 in a NLsB domain-dependent manner suggests that altered concentration of free NLs-bearing proteins could also have a direct effect on the above processes in the mutant embryos. This hypothesis is supported by the recent finding that the ISWI protein, a chromatin-remodeling ATPase (Brown et al. 2007; Corona et al. 1999; Siriaco et al. 2009), was also identified as a RanGTP-dependent MAP required for chromosome segregation and anaphase microtubule stabilization in the Xenopus egg and Drosophila S2 cells (Yokoyama et al. 2009).

The lamin proteins associated with the internal side of the NE play a central role in the nuclear organization by binding nuclear membrane components and DNA (Melcer et al. 2007). Reducing the level of Imp-α in C. elegans embryos (Geles et al. 2002) or elevating its concentration in Xenopus egg extract (Adam et al. 2008) results in
lamin mislocalization or lamin B accumulation in distinct patches on the surface of the chromatin. Our finding that lamin forms aggregates in defective Imp-α2/Imp-β embryos points out that in vivo the Imp-α2/Imp-β complex prevents lamin to form spontaneous aggregates, an intrinsic property of lamin dimers detected earlier in vitro (Moir et al. 1991).

Histone H2A-mediated attachment of lamin to chromosomes is an important step in nuclear lamina assembly (Mattout et al. 2007). However, in extracts of Xenopus egg, the assembly of the NE was observed on RanGTP-coated beads even in the absence of chromatin (Zhang and Clarke 2000). It is not clear how Ran directs NE assembly, but the mechanism involves the Importins (Brittle and Ohkura 2005; Clarke and Zhang 2008). The formation of spherical lamin Dm0 structures without DNA, which were detected in the mutant embryos, indicates that the factors released from Imp-α2/Imp-β complexes could form NE independently of chromatin.

Altogether, the comparison of the normal lamin pattern (Paddy et al. 1996; Walker et al. 2000; and this study) with that detected in embryos defective for Imp-α2/Imp-β and the finding of the association of lamin Dm0 with Imp-α2 highlight the importance of RanGTP–Imp-α/Imp-β pathway in the organization of NE assembly. In conclusion, our work shows that Imp-α2 and Imp-β specifically cooperate in vivo to essentially regulate spindle dynamics and events related to mitosis during the early nuclear divisions in Drosophila embryos.

ACKNOWLEDGMENTS

We thank Robert J. Fleming, János Szabad, the Vienna Drosophila RNAi Centre (VDRC), and the Bloomington and the Exelixis Collection of Harvard Medical School Stock Centers for providing the fly stocks. We thank Thomas C. Kaufman, János Szabad, and Harald Saumweber for kindly providing antibodies for cnn, Ketel, and fl. We are grateful to Iain W. Mattaj for purification of laminDm0 with Imp-RanQ69 and His-RanT24N proteins. We also thank the Nikon Imaging Center (U. Engel and C. Ackermann) at the University of Heidelberg for the possibility to use the laser scanning microscope facilities. We are grateful to Dorothée Albrecht, Gabriele Robinson, and Rolf Schmitt for technical assistance. This work was supported by the German Research Foundation (DFG)-Hungarian Academy of Sciences (MTA) Collaboration Program (UNG 436 113/81/0–6), the Hungarian Scientific Research Fund (OTKA K69279), and GARC P302/11/640 (to B.M.M.).

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Communicating editor: B. J. Andrews