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Doctor of Natural Sciences

Interplay between *Drosophila importin-a2, β* and *kelch* during oogenesis and early embryogenesis

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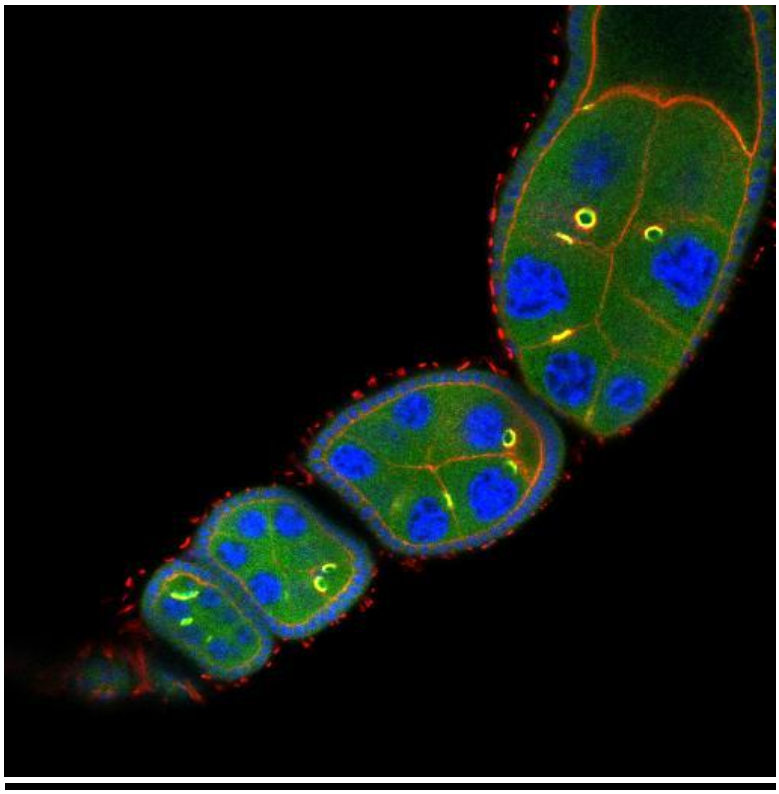

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Dedicated to my loving parents…….

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Zusammenfassung

Diese Arbeit beschreibt das Zusammenspiel von *Importin-α2 (imp-α2), kelch* und *Importin-β (imp-β)* im Verlauf der Oogenese und Embryonalentwicklung der Fruchtliege *Drosophila* melanogaster. Insbesondere untersuchten wir, welche Rolle Imp-α2 bei der Lokalisation von Kelch an den Ringkanal (RC) während der Oogenese spielt.

Imp-α2 ist entscheidend an der Bildung des RC beteiligt. Im Weibchen der *imp-α2* Mutanten sind die RCs verstopft und der Transport von Zytoplasma aus den Nährzellen in die Oocyte dadurch verhindert. In den Eikammern wird Kelch synthetisiert, kann aber an RCs nicht binden und die Öffnung bewirken. Kelch-Mutanten zeigen einen ähnlichen RC-Verschluß. In Wildtyp-Fliegen ist Kelch sehr stark an RCs assoziert. Imp-α2 jedoch verbleibt im Zytoplasma. Weitere Untersuchungen zeigten, dass keine direkte Interaktion zwischen Kelch und Imp-α2 besteht. Das könnte darauf hinweisen, dass ein Mechanismus existiert, durch den Imp-α2 mit Hilfe eines Faktors wirkt, der die Funktion von Kelch reguliert (Gorjánácz *et al*., 2002).

Ziel des ersten Teils dieser Studien ist es, die Interaktion zwischen *imp-α2* und *kelch* sowie zwischen *kelch u*nd *imp-β* zu untersuchen. Durch den Einsatz eines sensibilisierten Hintergrundes konnten wir zeigen, dass genetische Interaktion zwischen *imp-α2* und *kelch* stattfinden kann. Noch stärker erwies sich die Interaktion zwischen *imp-β* und *kelch*, da wir genetische und physische Interaktion nachweisen können. Die Analyse der Verteilung von Imp-α2 im Wildtyp und kel^{Δ} Ovarien läßt eine gegenseitige Abhängigkeit von Imp-α2 und Kelch bei ihrer zellulären Lokalisation vermuten. Untersuchungen am Confocalen Mikroskop zeigten, dass Kelch-Protein in Embryonen des Präblastodermstadiums nachweisbar ist. Kelch ist während der Mitose mit den Centrosomen und der Spindel assoziert, obwohl sein Verteilungsmuster normalerweise verschieden ist von Imp-α2, jedoch während der Anaphase überlappt. Da beide Proteine in den Kernen während der Mitose auftreten, kann man vermuten, dass sie miteinander interagieren. Es läßt uns daher schließen auf eine neue Rolle für Kelch bei den Mitosen während der frühen Embryonalentwicklung.

Die weiteren Untersuchungen zeigten, dass Imp-$\alpha 2^{\Delta IBB}$, das nicht an Imp-β binden kann, die Oogenese blockiert, während Imp-$\alpha 2^{NLSB-}$, das nicht in der Lage ist, an ein NLS-tragendes Cargo-Protein zu binden, es erlaubt, die Oogenese in mutanten *imp-α2* Weibchen vollständig durchzulaufen, danach aber die Kernteilung in Embryonen arretiert. Diese Befunde lassen vermuten, dass Imp-α2 spezifische Funktionen in bestimmten Prozessen ausübt, wie RC-Assembly und Mitose. Genetische Interaktionen zwischen Kelch und Imp-β konnte mit Hilfe des rezessiven $imp\text{-}\beta^{RE34}$ Allels, dessen Bindungsaffinität zu Imp-α2 verhindert ist, nachgewiesen werden. Heterozygote $kel^{\Delta}/imp\text{-}\beta^{RE34}$ Weibchen, ähnlich wie $imp\text{-}\alpha 2^{D14}/imp\text{-}\beta^{RE34}$ Fliegen, legen Eier, deren Entwicklung in der frühen Embryogenese angehalten wird. Pull-down Experimente zeigten, dass Kelch und Imp-β physisch interagieren können. Gestützt auf unsere Ergebnisse, schlagen wir ein Modell vor, in welchem Imp-β der Mediator zwischen Imp-α2 und Kelch während der Oogenese ist, und dass Bindung von Imp-α2 an Imp-β Kelch freisetzen kann, das dann in der Lage ist, an RCs zu assozieren. Zusammenfassend kann man sagen, dass diese Untersuchungen einen möglichen Mechanismus aufzeigen, durch welchen Imp-α2 die Lokalisation von Kelch an RCs steuert und Kelch eine neue Rolle während der frühen Embryonalentwicklung zuweist.

Der zweite Teil dieser Arbeit befasst sich mit 2D Gelanalysen des Kelch-Proteins, um den Grad der Phosphorylierung im Wildtyp und in $imp\text{-}\alpha 2^{D14}$ Ovarien zu untersuchen. Wir konnten einen Anstieg der Phosphorylierung von Kelch in $imp\text{-}\alpha 2^{D14}$ Ovarien nachweisen, worauf das Fehlen der nicht-phosphrylierten Isoform hinweist Dieser Befund lässt vermuten, dass die Phosphorylierung von Kelch von Imp-α2 abhängt und schließlich die Lokalisation von Kelch an RCs beeinflusst.

Der dritte Teil dieser Arbeit befasst sich mit der Identifizierung eines weiteren, in Wechselwirkung mit *imp-α* tretenden Proteins, nämlich *chickadee (chic)*. Erste Untersuchungen dieses Gens zeigen, dass eine genetische Interaktion zwischen *imp-α2* und *chic* sowie zwischen *imp-β* und *chic* stattfindet. Wir können nachweisen, dass *chic* eine Rolle in der frühen Embryonalentwicklung spielt, da es an Spindeln und Centrosomen während der mitotischen Teilungen assoziert ist. Weiterhin weist das Ergebnis darauf hin, dass verschiedene Komponenten des Prozesses, die zur RC-Bildung führen, auch während der Mitose aktiv sein können.

Summary

This work describes the interplay between *importin-α2 (imp-α2), kelch* and *importin-β (imp-β)* during two developmental periods of the fruit fly *Drosophila* namely, oogenesis and early embryogenesis. In particular, we emphasize on the role played by Imp-α2 in localizing Kelch to the ring canals (RC) during oogenesis.

Imp-α2 is critically involved in RC assembly. In mutant *imp-α2* females, the RCs are occluded and dumping of nurse cell cytoplasm into the oocyte is prevented. In the egg chambers, Kelch is synthesized but unable to bind RCs and mediate their opening. *kelch* mutations produce similar RC occlusion. In wild-type, Kelch strongly decorates RCs, yet Imp-α2 remains in the cytoplasm. Further analyses reveal no direct interaction between Kelch and Imp-α2, suggesting a mechanism by which Imp-α2 acts upon a factor regulating Kelch function (Gorjánácz *et al*., 2002).

The first part of this study focuses on the interactions that take place between *imp-α2* and *kelch* as well as *kelch* and *imp-β*. Using a sensitized background we were able to show that genetic interaction could take place between *imp-α2* and *kelch.* Moreover, the interaction between *imp-β* and *kelch* is even stronger because we can detect interaction genetically and physically. Analysis of the distribution of Imp-α2 in wild type and kel^{Δ} ovaries indicated an interdependence of Imp-α2 and Kelch in their cellular localization. Confocal analysis showed that the Kelch protein can be detected in preblastodermic embryos. Kelch was found to decorate the centrosomes and the spindle during mitosis although its pattern of distribution is generally distinct from that of Imp-α2 but overlaps during anaphase. The occurrence of both proteins in the nuclei during mitosis suggests that they may to interact. Hence, we suggest a new role for Kelch in mitosis during early embryogenesis.

Further analysis showed that Imp-$\alpha2^{\Delta IBB,}$ which is unable to bind Imp-β, blocks oogenesis whereas Imp-$\alpha2^{NLSB-}$, which is able to bind an NLS bearing cargo protein, allows oogenesis to fully proceed in mutant *imp-α2* females but subsequently arrests nuclear division in embryos, indicating that Imp-α2 exerts specific functions in distinct processes, such as RC assembly and mitosis. Genetic interaction between Kelch and Imp-β was detected by using the recessive $imp\text{-}\beta^{RE34}$ allele whose binding affinity for Imp-α2 is affected. Heterozygous $kel^{\Delta}/imp\text{-}\beta^{RE34}$ females, similar to $imp\text{-}\alpha2^{D14}/imp\text{-}\beta^{RE34}$, produced eggs whose development was arrested during early embryogenesis. Moreover, pull-down assays showed that Kelch and Imp-β can physically interact. Based on our results we propose a model, wherein Imp-β could be the mediator between Imp-α2 and Kelch during oogenesis and that binding of Imp-α2 to Imp-β could release Kelch which will be able to gain access to RCs. In conclusion, this study reveals a possible mechanism through which Imp-α2 controls the localization of Kelch to RCs and a new role played by Kelch during early embryogenesis.

In the second part of the work, 2D gel analysis of the Kelch protein was performed to investigate the degree of phosphorylation in wild type and $imp\text{-}\alpha2^{D14}$ ovaries. We observed an increased phosphorylation of Kelch in $imp\text{-}\alpha2^{D14}$ ovaries as indicated by the absence of non-phosphorylated isoform which suggests that phosphorylation of Kelch may depend on Imp-α2 and ultimately may affect the localization of Kelch to the RCs.

The third part of this work deals with the identification of another interactor of *imp-α2* namely*, chickadee (chic).* Preliminary work on this gene demonstrates that a genetic interaction takes place between *imp-α2* and *chic* as well as *imp-β* and *chic.* We also show that Chic plays a role in early embryogenesis by decorating the spindles and centrosomes during mitotic divisions. This result further points out that several components of the process leading to RC formation may also act during mitosis.

Introduction

A. The Fruit Fly *Drosophila melanogaster*: Developmental Aspects

1. Life cycle:

Drosophila melanogaster is a small, common fly found near unripe and rotted fruit. It has been in use for over a century to study genetics and lends itself well to behavioural studies. Thomas Hunt Morgan was the preeminent biologist studying *Drosophila* early in the 1900's. Morgan was the first to discover sex-linkage and genetic recombination, which placed the small fly in the forefront of genetic research. Due to its small size, ease of culture and short generation time, geneticists have been using *Drosophila* ever since. It is one of the few organisms whose entire genome is known and many genes have been identified. Though Morgan was not inclined towards development *Drosophila* turned out to be an ideal system to study development. A large number of techniques, some of which include microinjections, immunohistochemical methods and biochemical analysis of different tissues of *Drosophila* have been developed which provide a deeper insight into the development aspects of Drosophila.

Drosophila melanogaster exhibits complete metamorphism, meaning the life cycle includes an egg, larval (worm-like) form, pupa and finally emergence (eclosure) as a flying adult (Figure.1). The metamorphosis occurs in two stages wherein the larva transforms into pupa first and then the pupa into the imago (adult). The larval stage includes 3 instars, first, second and the third.

The Drosophila eggs are small, oval in shape with two filaments called the dorsal appendages at the anterior side and a micropyle through which the sperm enters. The larval cells don't divide right from the first instar until the pupal stage but only increase in size. Hence the overall increase in the larval size from 1st instar to 3rd instar. This increase in the size of larval cells occurs due to repeated rounds of DNA replication without any cell division (called endoreplication). They become large, banded chromosomes and are termed as polytene chromosomes. Polytene chromosomes are usually found in the larvae, where it is believed that these many-replicated chromosomes allow much faster larval growth than if the cells remained diploid as each cell now has many copies of each gene, it can transcribe at a much higher rate than with only two copies in diploid cells. The only cells that divide at this stage are the imaginal cells, which contribute later to the formation of adult structures. The larval tissues undergo autolysis or remodelling during the pupal stage whereas the imaginal cells differentiate to give rise to an adult fly. The adult fly can live for 30-40 days and the males become sexually active after 4 hrs of eclosion and the females will lay eggs only after 3 days.

Females can lay up to100 eggs/day and virgin females lay eggs which are sterile and few in number.

The time from egg to adult is temperature-dependent. The above cycle is for a temperature of 25°C. The higher the temperature, the faster the generation time, whereas a lower (18°C) temperature causes a longer generation time.

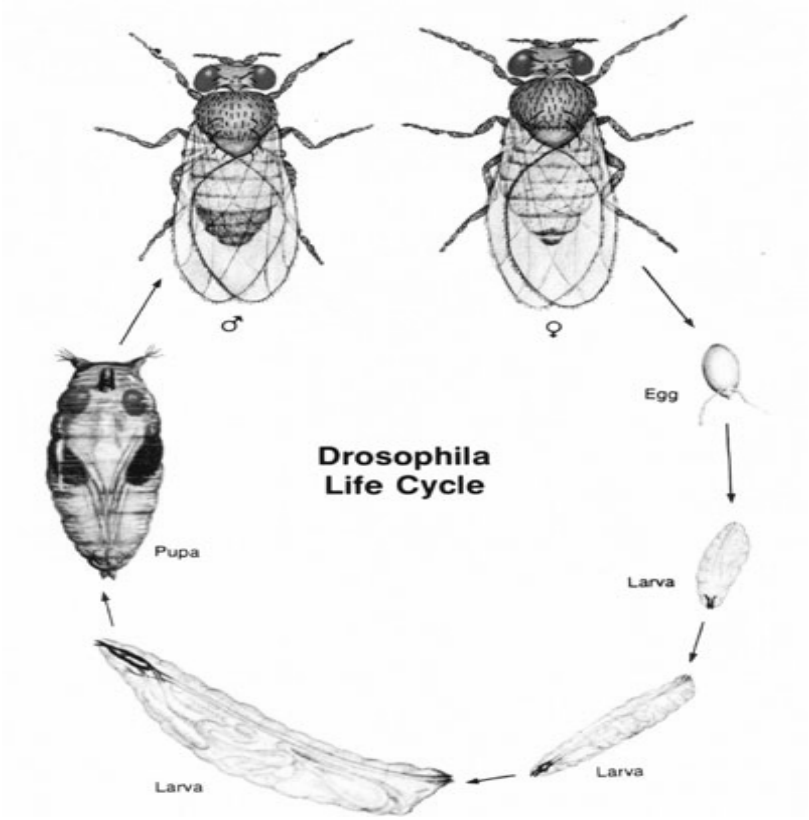


Figure 1. ***Drosophila*** **Life cycle**

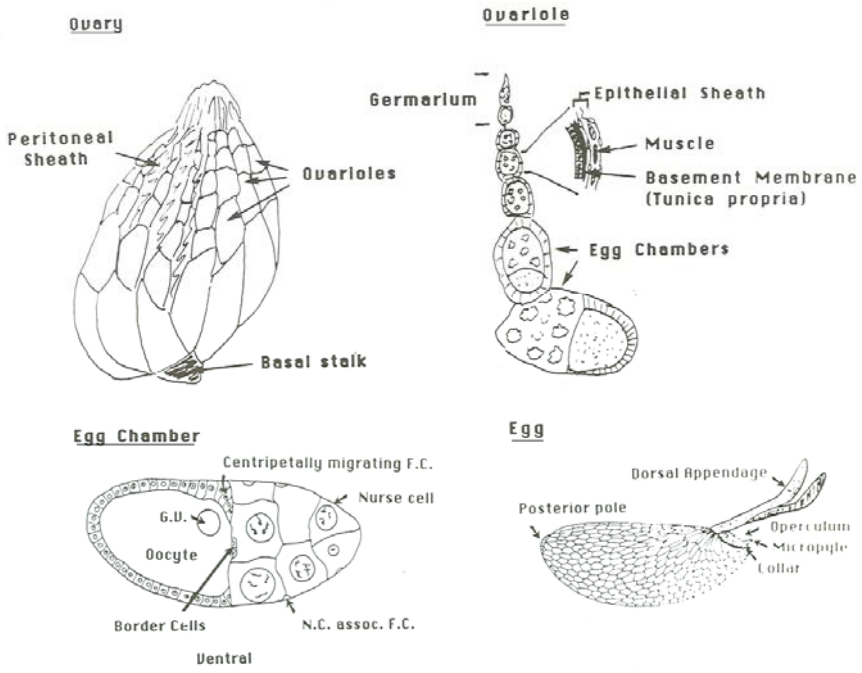


Figure 2. **Structure of ovary, ovariole, egg chamber and an egg.**

2. *Drosophila* oogenesis:

The development of a single stem cell into a mature egg involving a series of cellular processes is termed as oogenesis.

A female *Drosophila* has a pair of ovaries (Figure2) and each ovary contains 16-18 ovarioles, each representing an independent egg assembly line. At the anterior tip of every ovariole is the germarium which is made of somatic and germline stem cells whose progeny are subsequently organised into egg chambers (follicles). Egg chambers bud off from the germarium and continue developing as they move posteriorly within the ovariole.

Egg chambers are surrounded by basement membrane (tunica propria) .The integrity of the ovariole derives from a thin covering of muscles sandwiched between two epithelial layers, which are together called the epithelial sheath. The ovarioles are held together by a network of connective tissue called the peritoneal sheath which aid in piping in oxygen though tracheoles located in it.

Oogenesis takes almost a week and is divided into 14 stages (Figure 3). Stage one is budding of the egg chamber from the germarium and stage 14 is the mature egg.

2.1 Stem cells and Cyst Formation:

Each egg chamber begins development at the far anterior end of the germarium where the germline stem cells lie. These germline stem cells and produce a 16-celled germ line cyst. Cyst formation initiates when one of the stem cells at the tip of the germarium divide asymmetrically to produce a cystobalst. This cystoblast undergoes four more mitotic divisions with incomplete cytokinesis to form a cyst of 16 cells interconnected by cytoplasmic bridges known as the ring canals. The orientation of these divisions is controlled by the fusome, a branched structure composed of a continuous endoplasmic reticulum surrounded by cortical cytoskeletal components and microtubules.

The lineage of individual cystocytes can be partially determined from the number of ring canals that remains in each cell. The first two cells retain four canals, the next two retain three canals, the next four retain two canals and the remaining eight cells have just one canal. One of these 16 cells will differentiate into the oocyte, while the other cells become polyploidy nurse cells. All 16 cells enter a long S phase that constitutes the premeiotic S phase of the oocyte. As development proceeds the synchrony of the 16 cells is lost as the oocyte is the only cell within the cyst that undergoes meiosis. It arrests during the prophase I before exiting the germarium and does not continue until the mature egg is laid and activated.

2.2 Cyst Envelopment:

Region 2 is the large middle portion of the germarium that comprises the 16-celled cysts. Cysts start getting surrounded with follicle cells as they proceed posteriorly .This process of envelopment is slow as the cysts are not completely enclosed by the follicle cells in the anterior region of germarium termed as 2a. Hence the cysts in 2a region are still directly in contact with the neighbouring cysts as compared to region 2b.Initially the cysts are lens shaped but they become more rounded as the reach the end of region 2, after which the inwardly moving pre-interfollicular stalk cells separate out the stage-1 egg chamber. Before this separation the egg chambers constitute the region 3.

2.3 Oocyte Determination and Differentitaion:

Since the oocyte is the cell which always contains four ring canals it is understood that the initial cell (cystoblast) always becomes the oocyte.During the cystocyte divisions, a complex cytoskeletal transformation leads to the production of an assymetrical ring canal complex spanningall 16 cells. A flow of cytoplasmic constituents commences in region 2a. To facilitate and regulate the intercellular transport, the ring canal diameter increases, the disappearance of spindle remnants and development of extensive microtubule bundles takes place.

2.4. Egg Chamber Growth:

The development of the oocyte and the nurse cells continues to diverge. The oocyte nucleus transcribes actively in germirial cysts but becomes repressed when the egg chambers leave the germarium. The chromosomes of the ooycte condense into a karysome by stage 3. The germinal vesicle remains large and develops an endobody.

Nurse cells also undergo changes in their nuclear organization. After the initial round of endoreplication, nurse cell chromosomes remain somatically paired.

2.5 Vitellogenesis:

Yolk Production:

The eggs of Drosophila contain large amounts of yolk during stages 8 through 10. The major constituents of yolk are Protein-containing particles, glycogen-rich particles, and lipid droplets. The yolk supplies nutrients to the developing embryo. Vitellogenesis (the yolk precursor proteins are termed as vitellogenins) makes an extreme synthetic demand on adult females.

2.6. Follicle cell migrations:

a. Migration over the oocyte:

At the start of stage 9, almost all follicle cells move posteriorly towards the oocyte. Only about 50 cells remain over the nurse cells and flatten themselves to cover the nurse cells completely. To accommodate the migrating cells, the cells at the posterior end become columnar in shape.

b. Border Cell Migration:

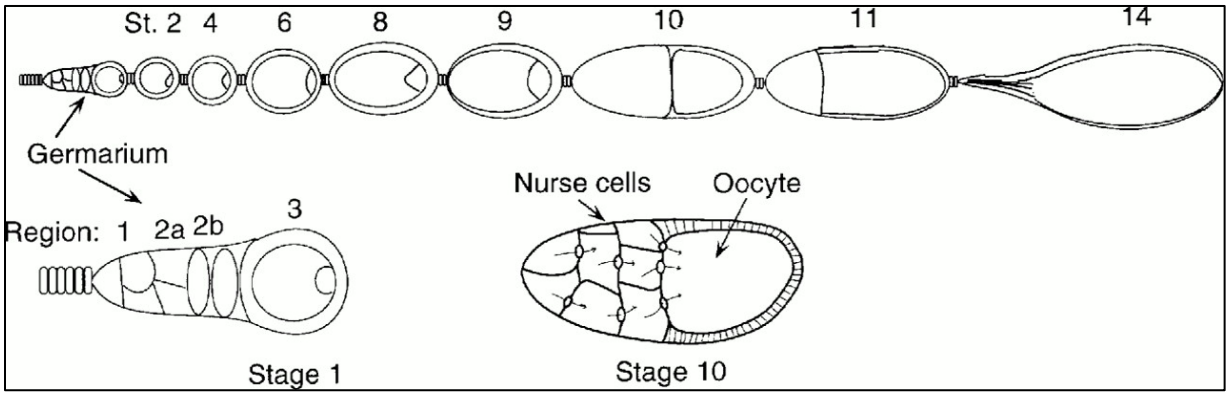
When the posterior movement of follicle cells is taking place, a group of six to ten cells called the border cells lag behind at the anterior end. They travel directly towards the anterior end of the oocyte and pass at least three nurse cell junctions without making an incorrect choice. The border cells remain associated with each other when they migrate. After their arrival, the border cells remain at the anterior end of the oocyte, although they move dorsally during stage 10B.They play a role in producing the micropyle.

2.7. Nurse Cell-Oocyte Transport:

Throughout oogenesis, the nurse cells provide a nutrient stream through the ring canals to the steadily growing oocyte. Ribosomes and mitochondria which are synthesized in the nurse cells are transported to the oocyte. The process of transport occurs in two phases, the first one takes place upto stage 10 and the second between stages 10B and 12 of egg chamber development. The first phase corresponds to a slow phase of transport involving a selective transfer of RNA transcripts and proteins, among which are the determinants for axis formation and early embryonic development. A second non-selective phase (also called dumping) of transfer results in a rapid discharge of the cytoplasmic content of the nurse cells into the oocyte, leaving behind the nuclei in the apoptotic nurse cell remnants. This process of transport nearly doubles the volume of the oocyte.

2.8. Eggshell formation:

The shell of the egg of the *Drosophila* is strong to support the egg in a hostile environment. The outer covering of the oocyte is made up of the vitelline membrane, the wax layer, the inner chorion layer, the endo chorion and the exochorion.



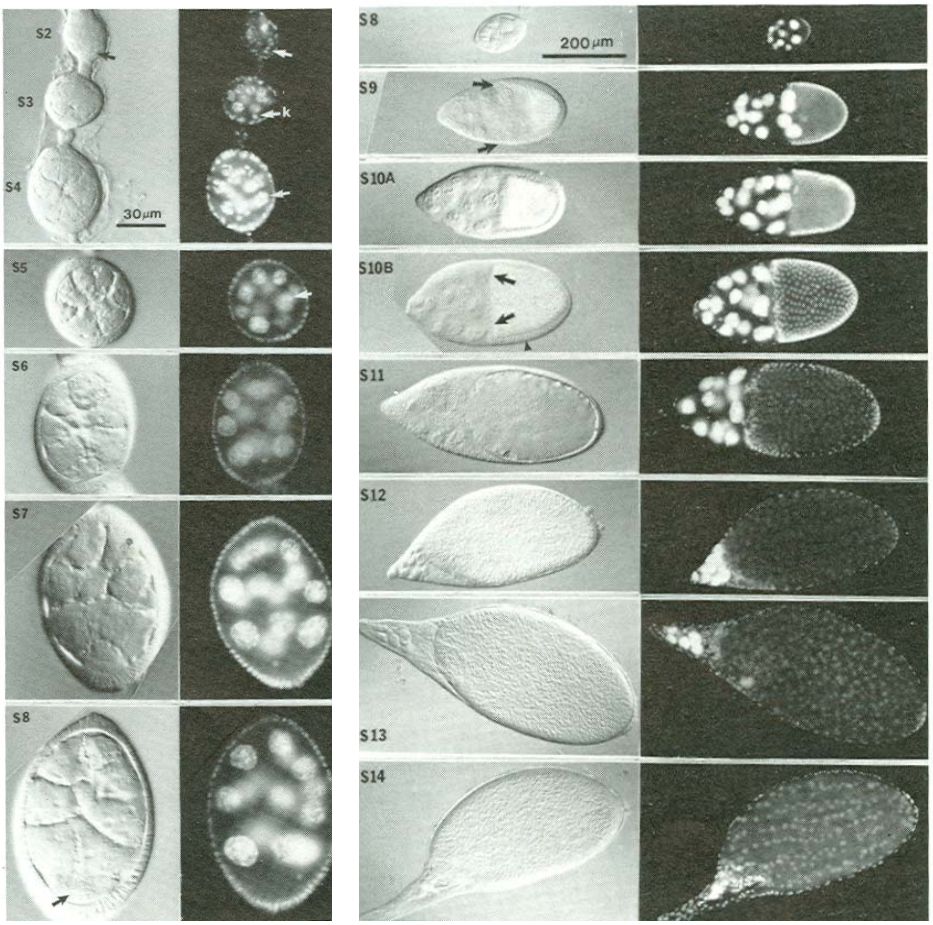


Figure 3. Stages of *Drosophila* Oogenesis (the Development of *Drosophila melanogaster* Vol I by Bate and Martienez Arias)

3. Drosophila Embryogenesis: Embryonic Mitotic cycles:

Embryonic development proceeds through a continuous and complex series of stages (Figure 4). After fertilization, the zygotic nucleus undergoes a series of mitotic divisions. Each cycle begins with the start of interphase and concludes at the end of mitosis. During the first 13 zygotic division cycles, most of the Drosophila embryo is synctial, with the exception of the pole cells that form in cycle 10 (the pole plasm located at the posterior end of the embryo contains osmophilic polar granules and numerous mitochondria that play a crucial role in the formation of the pole cells), the nuclei divide in a common cytoplasm, without cytokinesis. This syncytial period comprises two phases: preblastoderm and syncytial blastoderm. The first nine mitotic cycles constitute the preblastoderm cycles, during which the dividing nuclei lie deep in the interior of the egg.

A majority of the nuclei reach the embryo surface in cycle 10, leaving behind a population of the so-called yolk nuclei. These yolk nuclei remain in the interior, cease dividing after mitosis 10, and become polyploid. During the next four cycles (called the syncytial blastoderm cycles), the nuclei that reach the egg surface divide less synchronously than earlier, even though they still occupy the same cytoplasm. Entrance into mitoses 10, 11, 12, and 13 usually propagates through the nuclei on the embryonic surface as two wave fronts that originate near the poles of the egg and meet at the eggs equator. Thus, the nuclei near the equator are the last to enter mitosis during each cycle.

Cycle 14 is the first cycle with a long interphase, at least four times longer than the interphase of cycle 13. During this cycle, membranes grow inward between the blastodermal nuclei, creating an epithelial monolayer of approximately 6000 blastodermal cells surrounding a multinucleate and polyploidy yolk cell. Gastrulation begins immediately after cellularization concludes, thus the cellular blastoderm is a brief transient phase. Cells in different regions of

the embryo remain in interphase 14 for different times. One after the other, in a precise and reproducible sequence, groups of cells called “mitotic domains” synchronously enter mitosis. The first cells enter mitosis 14 while gastrulation is still in progress. Before hatching occurs, most somatic cells undergo two more mitoses of cycles 15 and 16 which also occur in a spatial and temporal fashion.

B. Importins- structure and function:

Transport of macromolecules, such as proteins and RNA, can take place between intracellular compartments or between cells and involves a selective passage mediated by nucleopores in the nuclear membrane between the cytoplasmic and nuclear compartments. Although a direct transport of macromolecules between cells is relatively rare, it can occur through specific canals. One of the best known examples deals with the transport of macromolecules between the nurse cells and the oocyte during *Drosophila* oogenesis.

In eukaryotic cells, the nuclear envelope separates the nucleus from the cytoplasm and allows a selective passage of components synthesized in the cytoplasm to enter the nucleus and, conversely, the transport of components synthesized in the nucleus into the cytoplasm. The transport of proteins from the cytoplasm to the nucleus includes three major players, Importin-α (Imp-α), Importin-β (Imp-β), and Ran. In the cytoplasm the Imp-α adaptor protein recognizes cargo proteins containing a Nuclear Localization Signal (NLS) peptide and forms a heterotrimeric complex with Imp-β. Following import into the nucleus through the Nuclear Pore Complex, the cargo/Imp-α /Imp-β complex is dissociated by Ran-GTP. Ran-GTP binds to Imp-β and forms a Imp-β/Ran-GTP complex. The other complex which is Imp-α/CAS/Ran-GTP and the Imp-β/Ran-GTP complex are recycled to the cytoplasm and dissociated when Ran-GTP is converted to Ran-GDP by Ran’s intrinsic GTPase activity. The

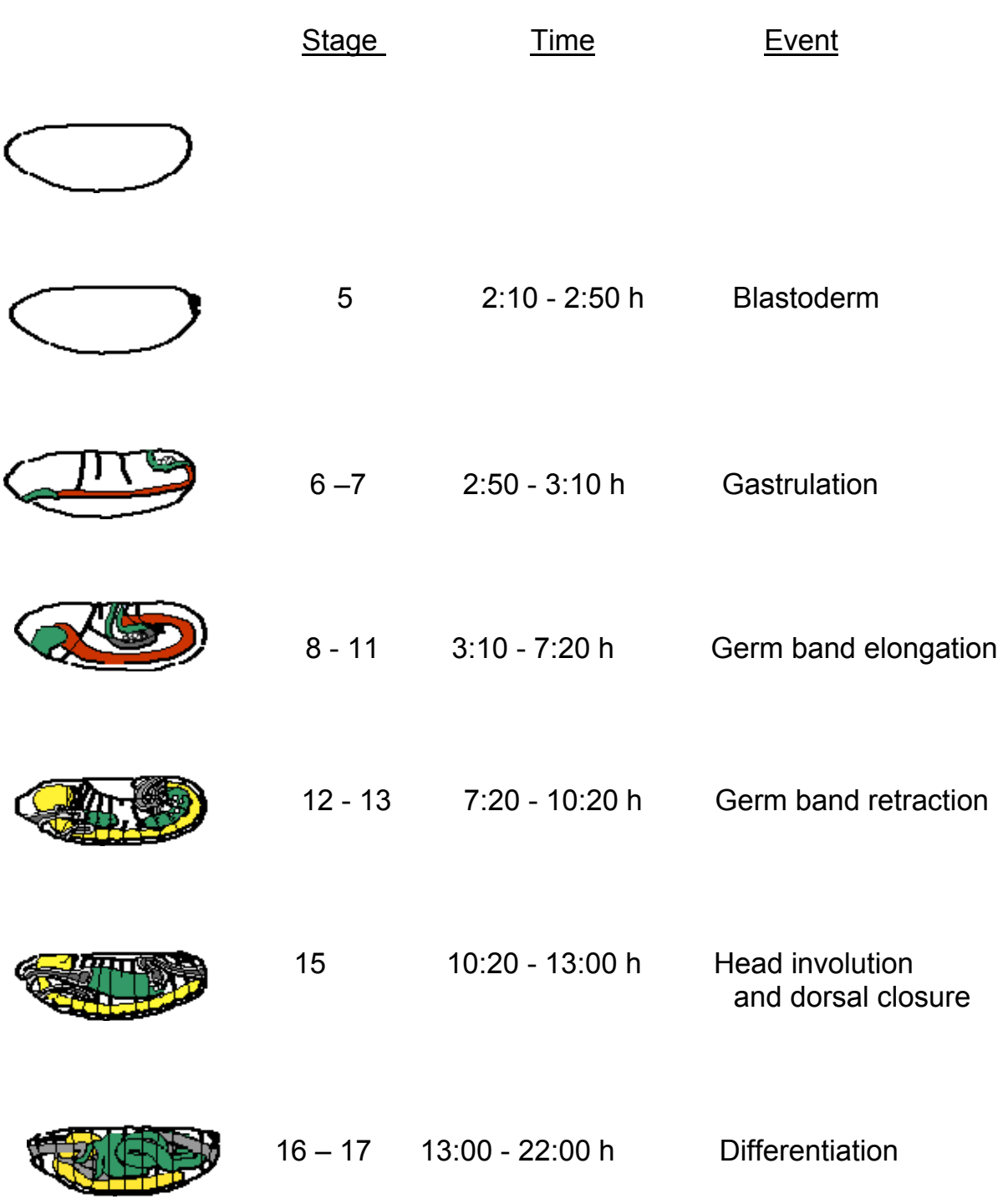


Figure 4. Stages of *Drosophila* Embryogenesis

RanGTP/RanGDP concentration gradient between the nucleus and the cytoplasm orchestrates the release of the cargo and the export of the Imp proteins to the cytoplasm.

Imp-α is also known to exert other functions (Gorjánácz, *et al*.,2002).

In vitro studies show that Ran-GTP elicits spontaneous microtubule polymerization and spindle assembly in cell-free *Xenopus* egg extracts (Carazo-Salas *et al.,* 1999; Kalab *et al.,* 1999; Wilde and Zheng, 1999; Zhang *et al.,* 1999). Recently, the effect of Ran was found to be mediated by Imp-α (Gruss *et al.,* 2001) and Imp- β Nachury *et al.,* 2001; Wiese *et al.,* 2001), which bind to and sequester the microtubule-associated proteins TPX2 and NuMA, respectively. TPX2 and NuMA become released from the Importins by Ran-GTP and can thus promote spindle assembly and centriole formation (Gruss *et al.,* 2001; Wiese *et al.,* 2001). In this way, Imp-α, Imp- β and Ran regulate together a key step in mitosis through molecular interactions resembling those in nuclear transport. Similar interactions may explain the involvement of Ran and Imp- β in nuclear envelope assembly following mitosis, in both *Xenopus* egg extract (Hetzer *et al.,* 2000) and early embryos of *Drosophila* (Tirián *et al.,* 2000; Timinszky *et al.,* 2002)

Importinα-2 displays critical functions during oogenesis and spermatogenesis in *Drosophila*. During spermatogenesis it showed a cell cycle dependent nuclear accumulation similar to its distribution during embryogenesis. However, during oogenesis, Importin α2 plays an essential role in the completion of the ring canals that link the nurse cells to the oocyte. Throughout oogenesis, the nurse cells provide a nutrient stream through the ring canals to the steadily growing oocyte. This process occurs in two phases, the first one takes place upto stage 10 and the second between stages 10B and 12 of egg chamber development. The first phase corresponds to a slow phase of transport involving a selective transfer of RNA transcripts and proteins, among which are the determinants for axis formation and early embryonic development. A second non-selective phase of transfer results in a rapid discharge of the

cytoplasmic content of the nurse cells into the oocyte, leaving behind the nuclei in the apoptotic nurse cell remnants.

A null mutant, *imp α2*^{D14} (*D14*) (Gorjánácz, *et al*., 2002) carrying an interstitial deletion in the *imp-α2* gene is recessive viable but causes sterility in both males and females. The egg chambers in *D14* ovaries display a dumpless phenotype characterized by partially occluded ring canals, whose morphology resembles that of *kelch* ring canals. However, by contrast to the Kelch protein that is a component of the inner rim of ring canals, the Imp-α2 protein forms no visible association with the ring canals. In wild-type egg chambers, Imp-α2 is diffusely distributed in the cytoplasm of the nurse cells and accumulates along the subcortical matrix layer in the oocyte. When the known components of the ring canals were examined in the *D14* mutant ring canals, the Kelch protein was found to be missing from the ring canals although it is produced in similar amount as in wild type ovaries suggesting a strong role of Imp-α2 in localizing the Kelch protein to the ring canals.

Imp-α2 (located in the 31A1-31A2 interval of 2L chromosome) is an adaptor protein (Figure 5.) that binds proteins containing NLS motif and Imp-β. It can be divided into three structural units: a central NLS-binding (NLSB) domain with 10 Armadillo (Arm) repeats, a positively charged N-terminal (IBB) domain that binds to either Importin-β or its own NLSB domain, and a C-terminal CAS/Cse1p-binding (CASB) domain (Chook and Blobel, 2001; Matsuura and Stewart, 2004). The ARM repeats stack to form a right-handed superhelix onto which the NLS peptides become associated at two sites on the concave groove. Each ARM repeat consists of three helices, H1, H2, and H3, with H3 orientated towards the concave side of Imp-a (Andrade et al., 2001; Coates, 2003). All the residues interacting with the NLS motif of the cargo substrate are located on the H3 helices of Imp-a (Conti et al., 1998; Fontes et al., 2000; Kobe, 1999).

C. Kelch: Structure and Function

Intercellular bridges between the nurse cells and oocyte of a Drosophila egg chamber were first described as large pores in the cell membranes (King, 1970).Electron microscopic examination showed that these bridges are cylindrical in shape with a diameter of 1μmor less when first formed in the germarium and then increase to 7-10μm in a mature egg chamber.

Kelch (located in the 36E5-36E6 interval of 2L chromosome) was isolated as a female sterile mutation (Xue and Cooley, 1993) affecting cytoplasm transport. *kelch* gene product is observed to be a component of the ring canals.

The *kelch* gene is found to encode an unusual transcript containing two open reading frames (ORF1 and ORF2) separated by a single UGA stop codon. Two protein products are formed from the *kelch* mRNA: a short protein from ORF1 (76kDa) and a longer protein from both ORF1 and ORF2 (160kDa) (due to partial suppression of the UGA codon.

It is shown that the shorter Kelch protein can fully rescue the *kelch* mutant phenotype. This short form consists of four domains, (Figure 6) the N-terminal region (NTR) which is necessary for accurate timing of Kelch deposition onto the ring canals, the BTB/POZ domain mediating the dimerization and the function of Kelch, the intervening region (IVR) needed for the functioning of Kelch and the Kelch repeat domain (KREP) containing six tandem repeats, which is needed for the localisation to the ring canals.

Recent works (Reed J.Kelso, *et al*., 2002) show that the Kelch phosphorylation at Y^{627} is critical for the ring canal assembly and the in vitro binding to F-actin. Since Y^{627} is within the actin binding domain in the fifth kelch repeat, its phosphorylation may simultaneously disrupt the actin-binding domain for the assembly of ring canals. The phosphorylated form of Kelch displays a lower affinity for binding F-actin in vitro. Dynamic phosphorylation-dephosphorylation of Kelch homodimers can mediate reversible cross binding of actin filaments, a function which plays a critical role during the rapid growth of ring canals after

stage 6. Genetic evidence shows that Kelch phosphorylation is accomplished by the Src 64 /or Tec 29.

Egg chambers from homozygous *kelch* females are defective in both the early which is a slow phase of nurse cell cytoplasm transport and the late which is a rapid nurse cell cytoplasm streaming into the oocyte (Xue and Cooley, 1993). Some nurse cell cytoplasm enters the oocyte, but transport is incomplete, resulting in an undersized oocyte and persistence of degenerated nurse cells at the anterior of the oocyte. Homozygous *kelch* mutant female flies do not usually lay any eggs as the small eggs degenerate in the ovary. In *kelch* mutants, the ring canals have a normal morphology until the time when kelch would normally reach all of the ring canals. After this time, the ring canals become disorganized. *Kelch* mutant ring canals are obstructed by disordered actin. Hence Kelch is required to maintain the organization of the actin filaments during the expansion of the ring canal rather than being required for the assembly.

D. chickadee:

In *Drosophila*, (Cooley et al, 1992) the force responsible for final cytoplasm transport to the oocyte is actin filament dependent. When the egg chambers were incubated in actin inhibitors such as cytochalasin the flow of cytoplasm into the oocyte stopped (Gutzeit, 1986).Two sets of actin microfilaments in nurse cells are found which are candidates for producing contractile force. The subcortical filamentous actin that is present in the nurse cells throughout oogenesis. Just before final nurse cell regression begins, a second, cytoplasmic network of actin-containing filaments is also found in each nurse cell. Examination of the cytoplasmic networks during nurse cell regression has led to the hypothesis that they are responsible for the contractile force that squeezes cytoplasm into the oocyte (Gutzeit, 1986).

One of the genes that falls in the class of mutants that disrupts the nurse cell cytoplasm flow is *chickadee,* which is in the 26A interval of the second chromosome. The phenotype of

Figure 5. Structure of Imp-α2 (Gorjánácz, *et al*., 2006)

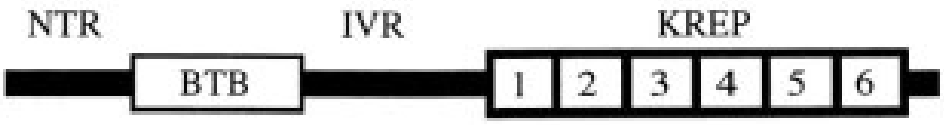


Figure 6. Structure of kelch (Robinson and Cooley,1997)

chickadee egg chambers includes an absence of nurse cell cytoplasmic actin networks. The nurse cell nuclei in *chickadee* egg chambers become dramatically rearranged after stage 10. In wild-type egg chambers, the nuclei remain near the center of each nurse cell, even in stage 11 while very rapid flow of cytoplasm is occurring. In *chickadee* egg chambers, however, nuclei are positioned normally in early stage 10 but then become irregularly spaced so that large, nucleus-free gaps are present. In older chickadee egg chambers, the nuclei of the four nurse cells directly attached to the oocyte form a cluster, and individual nuclei often appear to stretch partially into the oocyte through the ring canals. This suggests that the nurse cell nuclei are not properly held in position during *chickadee* nurse cell regression, and when flow starts, they are pushed into the ring canals, thus blocking the path to the oocyte and this could be the cause for the disruption of cytoplasm transport.

E. Aim of the Project:

The *importin-α2* gene (*imp-α2*), one of the three *imp-α* genes occurring in the Drosophila genome, plays a critical role during oogenesis. Inactivation of *imp-α2* leads to a maternal effect phenotype characterized by the formation of short eggs resulting from a failure to transfer the nurse cell cytoplasm into the oocyte during the ultimate stage of oogenesis. This failure ensues from the occlusion of ring canals linking the nurse cells between them and with the oocyte. In particular the Kelch protein, an oligomer actin organizer, is unable to become associated with the ring canals. Mutations in the *kelch* gene similarly result in a failure to dump nurse cell cytoplasm into the oocyte. Confocal microscopy shows that in wild type egg chambers Kelch is normally associated with the inner rim of the ring canals. There is no direct physical interaction between Kelch and Imp-α2, as revealed by immuno-precipitation, pull down assays or yeast two hybrid studies.

Based on the above considerations, Kelch appears to be an obvious candidate for being transported to the ring canals by Imp-α2. However, the absence of NLS or a stretch of basic residues suggests that Kelch may not directly bind to Imp-α2. Thus, we can envisage that Imp-α2 transports Kelch to the ring canals through an unknown intermediary component which is able to link Kelch to Imp-α2.

Imp-α2 may independently carry a Kelch–binding factor to the ring canals or mediate its complexing with Kelch in the cytoplasm, in this way indirectly targeting Kelch onto the assembling ring canal. It is also possible that Imp-α2 exerts an anti-aggregation activity on a component which binds Kelch and sequesters it in the cytoplasm.

Hence the aim of my project was to study the mechanism through which the interaction between Imp-α2 and Kelch could be explained.

Materials and Methods

A. Materials:

1. Fly strains (*Drosophila melanogaster*)

- *Oregon R* (Linsley and Grell, 1968) was used as a wild type stock.
- The *w; cn[1]/CyO, w; chic[221] cn[1]/CyO; ry[506]* (Verheyen and Cooley, 1994), *w ; imp-β[e02473]/CyO* (Gene Disruption Project members Exelixis, 2005), and *Sco/CyO; nanos-gal4* strains were obtained from Bloomington Stock Center, and the *w; imp-β [RE34] /CyO* strain (Erdeleyi et al., 1997) from J. Szabad (Szeged, Hungary). The *y w ; imp-α2[D14]/y+CyO* (Török et al., 1995), strain as well as the *y w; imp-α2[D14]/ y+CyO, UAS-imp-α2[NLSB-]/ TM6, UbX* (Gorjánácz et al., 2006), *y w; imp-α2[D14]/ y+CyO; UAS-imp-α2[ΔIBB] /TM6 UbX* (Gorjánácz et al., 2006 , and *yw ; imp-α2[D14]/ y+CyO; UAS-imp-α2[CASB-]/UAS-imp- α2[CASB-]* (Gorjánácz et al., 2006) transgenic lines were maintained in our laboratory.

2. Antibodies and Reagents for Immunolocalization and Microscopy

- Antibodies used were: mouse monoclonal anti-Kelch (Developmental Studies Hybridoma Bank), rabbit polyclonal anti-Importin-*α2* (Török et al., 1995), rabbit polyclonal anti-Importin-*β* (Erdeleyi et al., 1997) provided by J. Szabad (Szeged, Hungary). rat monoclonal anti-Tubulin alpha (Serotec), and mouse poly-monoclonal anti-Lamin (gift by H.Saumweber). Secondary antibodies were: Alexa-488 conjugated goat anti-rabbit IgG, Alexa-488 conjugated goat anti-mouse IgG, Cy3-conjugated goat anti-rabbit IgG, Cy3-conjugated goat anti-mouse IgG, Cy3-conjugated goat anti-rat IgG, Cy5–conjugated goat anti-rabbit IgG, and Cy5-conjugated goat anti-mouse IgG from Invitrogen; alkaline phosphatase-conjugated anti-mouse IgG and Alkaline phosphatase-conjugated anti-rabbit

IgG from Sigma. F-actin was stained with Alexa fluor 546 Phalloidin (1µg/ml.) from Molecular Probes and DNA with Dapi (100µg/ml).

3. Products, Kits and Core Facilities

- QIAfilter Plasmid Midi Kit (QIAGEN)
- Perfect Prep spin mini kit (5 PRIME)
- Ready Prep 2-D cleanup kit (BIO-RAD)
- Quick–change site directed mutagenesis kit (Stratagene)
- Restriction enzymes Enzymes (Roche, New England Biolabs, Fermentas, Promega)
- Primers (Operon)
- Sequencer (DKFZ core facility)
- 2-D Gel Electrophoresis using 7 cms strips (Amersham), Novex IEF gels (Invitrogen) and IPGphor (GE Healthcare)- DKFZ core facility
- Nikon Imaging Center (University of Heidelberg)
- VANEDIS Drosophila injection service

B. Methods:

Immunohistochemical Procedures:

1. Ovary staining

1. a. Dissection:

Prior to dissection adult female flies were fed on yeast overnight. Usually a good supply of yeasts enables the growth of the ovaries which swell the abdomen and make them readily visible. Dissection was performed on ice, in PBS saline solution. Two pairs of forceps were

used for the dissection of the abdomen. One pair was held on the thorax to keep the fly still and the other pair was used to pull out the ovaries which form rounded transparent bodies. Ovaries might be attached to the gut and have to be carefully separated from this organ. Then the peritoneal sheath of the ovaries is opened up to release the ovarioles.

1. b. Fixation, staining and analysis of dissected tissues:

After dissection, the tissues were fixed for 10 min in a 1:1 solution 4% paraformaldehyde (PFA): n-heptane in a 1.5ml Eppendorf tube. The fixed tissues were rapidly washed 4 times in PBS just by repetitive inversion of the tube followed by two more washes for 10 min each with PBT (1x PBS containing 0.1 % Tween 20).Then the tissues were blocked with PBS + Triton X100 (1x PBS containing 1% Triton X100) for 2h. After blocking, the samples were incubated with primary antibodies overnight at 4 °C in BBT incubation buffer (BBT: 1x PBS containing 0.05% Triton X100, 1.5% BSA and 5% normal goat serum). After the incubation the samples were washed three times 30 min with washing buffer (1x PBS containing 1.5%BSA, 0.1%Tween 20, and 1% Triton X100), and finally incubated for 2 hr at room temperature with the secondary antibodies in tubes wrapped with aluminium foil to prevent light illumination.. Finally, the tissues were washed 3 times for 30 min in washing buffer, mounted in elvanol and analysed with a Nikon 3-Laser C1S1 confocal microscope.

To visualise F-actin and DNA the samples were stained with 1µg/ml Alexa fluor 546 phalloidin and (100µg/ml) DAPI (4,6- diamidino-2- phenylindole), respectively.

2. Embryo Staining

2. a. Collecting embryos:

Wild type embryos were collected in a large cage containing about 2 to $4x10^4$ flies on Petri dishes implemented with the normal growing medium with additional fresh yeast paste and dry yeast. Mutant embryos were collected in small cages with Petri dishes prepared from apple juice, which usually provided a large yield of laid eggs.

- Normal growing media:

Normal growing medium consist of water, agar, corn meal, soy flour, yeast, malt extract, treacle, nipagin, propionic acid and phosphoric acid. To prepare 40 litres of medium, 320g of agar was first dissolved in water boiling in a thermaline boiling pan and then 3,200ml of malt extract and 880ml of treacle solution was added. Then 720 g of yeast, 3200 g of corn meal and 400g of soy flour solution was added, and the medium was again boiled with the lid closed and simmered for 2 h. After it cooled 250ml each of propionic and phosphoric acids were added at a temperature below 50° C and stirred well. Then the medium was filled in vials/bottles which were covered with gauze and allowed to dry for 2 hrs. The vials/bottles were plugged, left overnight at room temperature and stored at 18° C.

The same media was also poured on plates made of large Petri dishes (145/20mm)

- Apple juice plates:

Apple juice plates comprise of agar, apple juice, water, sucrose, nipagin and charcoal. 22.5 g agar was dissolved into 750 ml of double distilled water by boiling in a microwave oven. Afterwards, the mixture was put into a thermostat set at 60° C. In another flask, 33g

of sucrose and 3 g nipagin was added to 250ml apple juice and the mixture was boiled until homogenous and also kept at 60° C in a thermostat. After 30 min, both the solutions were mixed and a pinch of charcoal was added before pouring into small (60/15mm) Petri dishes.

2. b Egg preparation and fixation:

Freshly collected eggs were dechorionated in 4% bleach (12% Na-hypochlorite stock solution) for 4 min. Embryos were then rinsed with water followed by a 0.2% Triton X100 wash. A thorough wash with water was repeated and then the embryos were fixed.

Fixation was done in three distinct ways depending on the reactivity of the primary antibodies:

Methanol Fixation:

The washed embryos were fixed in a 1:1 methanol: n-heptane solution and vigorously hand shaken for 45 seconds. Following phase separation the interphase was first removed with a glass pipette Pasteur then the upper phase and finally the lower phase leaving the embryos attached to the wall of the tube. Fresh methanol was added and the tube was fixed on a rotor turning at 30 rpm for 5 min. The wash was repeated three times followed by two 5 min wash in 1:1 methanol: EGTA(0.5M. p^H 7.2). The embryos were then washed twice with methanol, suspended in methanol and stored at -20° C.

Fast Formaldehyde Fixation:

The embryos were transferred into 1:1 37% formaldehyde: n-heptane solution, moderately shaken for 15 sec and rotated for 5 min. The lower formaldehyde phase was removed and methanol was added. The embryos were further shaken for 15 sec. Fixed embryos sedimented at the bottom of the tube whereas debris remained in the interphase. The

following steps were performed according to the procedure described for the methanol fixation. .

Slow Formaldehyde Fixation:

The embryos were fixed in 1:1 4% formaldehyde: n-heptane solution, moderately shaken for 15 sec and rotated on a vertical rotor for 20 min. The following steps were similar to those described in the fast formaldehyde fixation.

2.c. Embryo rehydration and staining procedures:

In order to rehydrate embryos, solutions with serial dilutions of Methanol:1% Tween20 in PBT were used. Embryos were successively transferred in solutions of methanol:1% Tween20 in PBT in decreasing methanol concentrations of 8:2, 6:4, 4:6, 2:8, and finally in 0,1% Tween20 in PBS. At each step the embryos were agitated for 10 min on a vertical rotor at room temperature. This procedure was followed by 1 h incubation at room temperature in PBT.

Following rehydration the embryos were treated with blocking buffer containing 5% NGS (1.5%BSA, 0.1% Tween20+ 0.2% Triton x100, 250mM NaCl in 1x PBS) for a minimum period of 2 h. The embryos were then incubated overnight at 4°C with primary antibodies diluted in blocking buffer with 5% normal goat serum. The embryos were washed 6 fold 20 min in blocking buffer and again blocked for 2 hrs in blocking buffer containing 5% NGS. After blocking, the secondary antibodies were added for 2 hr at room temperature in darkness. Embryos were washed 6 fold 20 min in blocking buffer and 3 fold 10 min in PBT. Thereafter, the embryos were incubated with 1µg/ml DAPI in PBT for 1hr at room temperature and finally washed 3 fold 20 min with PBT before being mounted in elvanol

on a microscope slide. The embryos were analysed with a Nikon 3-Laser C1S1 confocal microscope.

3. Staining Procedures:

Ovary and Embryonic Staining	Primary antibodies and Reagents (dilution)	Secondary antibodies (dilution)
Kelch/Imp-*α2*/F-actin/DNA	Mouse anti-Kelch Ab (1/10) Rabbit anti-Imp-*α2* Ab (1:400) Phalloidin (1/40) Dapi (1/200)	Cy5 anti-mouse IgG (1:400) Alexa-488 anti-rabbit IgG (1:400)
Imp-α2/F-actin/DNA	Rabbit anti-Imp-*α2* Ab (1:400) Phalloidin(1/40) Dapi (1/200)	Alexa-488 anti-rabbit IgG (1:400)
Kelch/F-actin/DNA	Mouse anti-Kelch Ab (1/10) Phalloidin(1/40) Dapi (1/200)	Alexa-488 anti-mouse IgG (1:400)
Kelch/α-Tubulin/DNA	Mouse anti-Kelch Ab (1/10) Rat anti-α-Tubulin Ab (1/400) Dapi (1/200)	Alexa-488 anti-mouse IgG (1:400) Cy3 anti-rat (1:400)
Imp-β/Lamin/DNA	Rabbit anti-Imp-β Ab (1:2000) Mouse anti-Lamin Ab (1:10) Dapi (1/200)	Alexa-488 anti-rabbit IgG (1:400) Cy3 anti-mouse (1:400)

Genetic Approaches:

Crossing schemes:

1. Identification of genetic interaction through gene dosage:

Usually heterozygous combinations of recessive mutations in two independent genes give rise to a normal wild-type phenotype. However, if the gene products act in the same biological

pathway, it is possible that the combination of heterozygous mutations may result in a visible phenotype, suggesting a genetic interaction between the two genes.

For this purpose we used interstitial deletions in two genes, for example *imp-α2*^{D14} (or *D14*) and *kel*$^{\Delta}$ and determined whether egg formation or egg development may depend on a gene dosage between *imp-α2* and *kelch*.

Example: *kel*^{DE1}*/CyO* females carrying a deletion of *kel* were crossed with *imp-α2*^{D14}*/CyO* males carrying an interstitial deletion within *imp-α2*. Both genes are located on the second chromosome. Non-Curly *kel*$^{\Delta}$*/imp-α2*^{D14} females, which display an apparent wild-type phenotype, were selected and further crossed to wild type males to examine their ability to lay eggs and, then, to determine the viability of the laid eggs

♂ *imp-α2*^{D14}*/CyO* X ♀ *kel*$^{\Delta}$*/CyO*

↓

♀ *kel*$^{\Delta}$*/imp-α2*^{D14} X ♂ *WT*

If the egg viability and the number of laid eggs were similar to those of wild-type females, it is possible to use the *kel*$^{\Delta}$*/imp-α2*^{D14} combination as a sensitized background and to introduce modified *imp-α2* transgenes to test their effects. Such transgenes containing a *UAS*-promotor should be driven by a germ line-specific *Gal4* driver. To express the *UAS*-constructs in ovaries the *nanos::Gal4*^{VP16} enhancer trap line was used.

A stock was made by mating flies carrying *kel*$^{\Delta}$ and *p-{nos-Gal4}*, as indicated below.

♂ *kel*$^{\Delta}$*/CyO* *X* ♀ *Sco/CyO; p-{nos-Gal4}*

↓

kel^{Δ}/Sco; p-{nos-Gal4}/+ X kel^{Δ}/CyO; p-{nos-Gal4}/+

↓

Select for ♂♀ kel^{Δ}/CyO; p-{nos-Gal4}/ p-{nos-Gal4}

kel^{Δ}/CyO; p-{nos-Gal4}males and females were maintained as a stock and were used to introduce transgenes carried on either the X or 3rd chromosome.

Example:

kel^{Δ}/CyO; p-{nos-Gal4} X y w; imp-α2^{D14}/ y+CyO; P{w^{+}, UAS:: imp-α2$^{\Delta IBB}$}/TM6, UbX

↓

Select for phenotypically wild type ♀

kel^{Δ}/ imp-α2^{D14}; P{w^{+}, UAS:: imp-α2$^{\Delta IBB}$}/ p-{nos-Gal4}

The females of the above genotype were then mated with wild type males to check for egg viability and egg laying capacity.

2. Egg Laying

2-3 days old females with a specific genotype were crossed with wild type males and the eggs were collected for three successive periods of 12 hours.

The egg laying capacity was calculated by counting the total number of eggs laid divided by 12 h and the number of females in the tube.

Egg laying = total number of eggs laid/female/hour

The capacity of egg laying is a measure of oogenesis

3. Egg viability

Egg viability was measured as the percentage of eggs giving rise to first instar larvae. For this purpose about 20 3-days old females carrying the required genotype were crossed with the

same number of wild-type males. The eggs were collected for about 12 h, the flies removed from the tube. 200 eggs were aligned on a small agar plate and were further incubated for a period of 24 h at 25°C.

The egg viability of a particular genotype was calculated by counting the total number of eggs that hatched over the total number of eggs laid.

$$\text{Egg viability (\%)} = \frac{\text{Total number of hatched eggs}}{\text{Total number of laid eggs}} \times 100$$

Egg viability is a measure of embryonic development

Biochemical Analyses:

1. Western Blot analysis

1. a. Sample preparation:

- Protein extracts from *Drosophila* embryos

Protein extracts from *Drosophila* embryos were prepared by homogenizing 10µl of dechorionated eggs in 100µl of 5X SDS buffer (0.313 M Tris-HCl (pH6.8 at 25°C), 10% SDS, 0.05% bromophenol blue, 50% glycerol) using a motor-driven homogenizer. Then the tissues were boiled for 5 min and aliquots were loaded on an SDS-polyacrylamide gel.

- Protein extracts from *Drosophila* ovaries

Dissected ovaries in 1X PBS were transferred to an Eppendorf tube and the PBS overlay was aspirated. Then a solution containing 2 M DTT (20X reducing agent), 5X SDS buffer(0.313 M Tris HCl (pH6.8 at 25°C), 10% SDS, 0.05% bromophenol blue, 50% glycerol), Benzonase (DNase) and protease inhibitor was added to the tube. The samples were boiled on a thermomixer at 98°C for 5 min and centrifuged for 5 min at room temperature. The

supernatant was transferred with a Pasteur pipette in a new tube and diluted 10 times by adding an appropriate amount of PBS, 5X SDS buffer and DTT before loading onto the SDS-polyacrylamide gel.

1. b. SDS polyacrylamide gel electrophoresis and Western Blot:

The proteins were separated on 7 or 10% SDS-polyacrylamide gels. After electrophoresis, proteins contained in the gel were electro-transferred to an Immobilon-P polyvinylidenfluoride (PVDF) membrane (Millipore Corp., Bedford, MA) using a Multiphore semidry transfer apparatus (LKB Instruments, Inc., Bromma, Sweden).

	Dilution used
Primary Antibody (raised in)	
Kelch (mouse)	1:10
Importin-*α2* (rabbit)	1:500
Importin-β(rabbit)	1:2000
P40 (rabbit)	1:20,000

The filter was then probed with primary and secondary antibodies diluted in the I-blocking buffer (I-block= 0.2% +0.1% tween 20).The secondary antibodies were alkaline phosphatase tagged and the chemiluminescent system which uses CSPD (Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]decan}-4-yl) phenyl phosphate) as a substrate was used to develop the blot.

2. GST pull down

Full-length wild-type *ketel* cDNA and the modified cDNA corresponding to the $ketel^{RE34}$ sequence were subcloned into pGEX6P2 (Pharmacia). The $ketel^{RE34}$ allele is a partially recessive revertant of the dominant $Ketel^{D}$ mutation (Erdeleyi et al., 1997; Timinsky et al., 2002). The $Ketel^{D}$ allele contains a nucleotide modification that changes the Proline residue at position 466 into a leucine, or $P^{466}L$, whereas the $ketel^{RE34}$ allele contains another

nucleotide change that substitutes an aspartic acid at position 725 by an asparagine, or $D^{725}N$. Therefore the Imp-β^{RE34} protein contains two amino acid substitutions: $P^{466}L$ and $D^{725}N$. The second substitution partially abrogates the original genetic dominance of *Ketel*^{D} or *imp-*β^{P446L}. Recombinant proteins were synthesized in vitro using the TNT T7 Coupled Reticulocyte Lysate System (Promega) in the presence of unlabeled amino acids. GST-fusion proteins, GST-Imp-β and GST-Imp-β^{RE34} expressed in *E. coli* were purified with glutathione sepharose (Pharmacia) and washed with binding buffer [20 mM HEPES (pH 7.8), 10% glycerol, 300 mM NaCl, 0.1% sodium deoxycholate, 0.1% NP40 and 0.1% Triton X100] containing a cocktail of protease inhibitors (Complete EDTA free from Roche; 1:50 dilution). Wild-type or mutant Imp-β recombinant proteins (10% of the reaction volume) were added to this mixture (in 1 ml) and incubated for 3 hours at room temperature. The beads were washed six times (10 min each) with binding buffer, boiled in loading buffer, and the proteins were separated by electrophoresis on 8% or 12% SDS-polyacrylamide gels. After transfer to a polyvinylidene difluoride (PVDF) membrane, the bound proteins were detected by Western blotting using an S-protein Alkaline Phosphatase conjugate (Novagen).

3. 2-D Gel Electrophoresis

2D gel electrophoresis was done to detect the different isoforms of proteins using the kit prescribed by the Amersham using 7 cm strips.

3. a. Sample Preparation:

10 pairs of ovaries were dissected in 1X PBS, dissolved in 25 µl Lysis buffer (with appropriate 0.5% IPG buffer based on the IP of the protein), incubated for 30 mins at RT on shaker. Then 100 µl of rehydration buffer (along with 5µl/ml 4M DTT) was added and incubated for another 30 mins.

3. b. Loading of sample on IPG strips, isoelectric focussing and equilibration:

125 µl of sample was loaded on 7 cm strips placed in a strip holder. The strip holder was positioned on IPGphor (GE Healthcare) and left overnight at 30V.

After loading overnight the focussing of the sample was done for another 3 and a half hr. The program used was as follows:

Step 1:	300V	30 mins	0.15 kVhs
Step 2 Gradient :	1000V	30 mins	0.17 kVhs
Step 3 Gradient:	5000V	1h 30 mins	3.00 kVhs
Step 4:	5000V	30 mins	2.50 kVhs
		3h 30 mins	8.00 kVhs

After the IPGphor reached 8.00 kVhs, the IPG strips were first equilibrated in equilibration buffer (LDS buffer diluted 4 fold with water) containing 10mg DTT/ml and then with 25mg iodacetamid /ml in equilibration buffer.

3. c. Mounting of sample and 1D gel electrophoresis:

After equilibration, the IPG strips were slid into the ZOOM gel wells and a 1 dimensional SDS PAGE was performed using MES (2-(*N*-morpholino)ethanesulfonic acid**)** electrophoresis buffer.

Molecular Biology:

1. In vitro mutagenesis

1. a. *importin-α2*

A series of seven modified *imp-α2* transgenes containing nucleotide modifications leading to specific amino acid substitutions were constructed. Seven modifications in the N-terminal coding domain of *imp-α2* were performed and lead to substitution on potentially phosphorylated serine residues or nearby amino acid residues. For this purpose in vitro

mutagenesis of the *imp-α2 K9* cDNA (Török et al., 1995) cloned into the EcoRI site of the pBluescript-II-SK(+) vector was performed by by using the PCR-based Quick-Change Site-Directed Mutagenesis Kit (Stratagene). The sequence of each construct was determined to confirm the introduced modification (DKFZ core facility) and the absence of PCR-induced errors. The constructs were then sub-cloned into the Acc1 and Not1 cloning sites of pUASp2 transformation vector (Rorth, 1998) kindly provided by P. Rorth. The following PCR primers were used to generate the transgenes. Bold and underlined letters indicate the introduced nucleotide substitutions.

Amino acid substitution in *imp-α2*	Primer
$S^{17}A$	5' CTCCTACAAGGCCAAC**GC**CATTAACACGCAGGAC 3'
$S^{17}E$	5'GGCTCCTACAAGGCCAAC**GAG**ATTAACACGCAGGACTC 3'
$S^{23}E$	5'CATTAACACGCAGGAC**GA**ACGCATGCGCCGCC 3'
$S^{23}A$	5' CATTAACACGCAGGAC**G**CACGCATGCGCC 3'
$K^{39}E$	5' GCGCAAGTCCAAA**G**AGGAGGACCAGATGTTC 3'
$M^{25}R$	5' CAGGACTCACGCA**G**GCGCCGCCATGAG 3'
$S^{37}E$	5'GACCATCGAGCTGCGCAAG**GAG**AAAAAGGAGGACCAG 3'

1. b. ***kelch***

Similarly six modified *kel* transgenes were synthesized. A full-length *kel* cDNA obtained from Berkeley Drosophila Genome Project inserted between the EcoRI and XhoI sites of a pOT2 vector served as a template for introducing nucleotide changes in specific codons by using the PCR-based Quick-Change Site-Directed Mutagenesis Kit (Stratagene).The modified cDNAs were fully sequenced in the DKFZ core facility. The constructs containing the expected modifications were then sub-cloned into the EcoRI site of the pUASp2 transformation vector, as described above. The following PCR primers were used to generate the modified sequences. In a subset of the transgenes three glutamine residues at positions 31

to 33 were substituted by either three glutamic acid, leucine, or arginine residues. Two tyrosine residues were also substituted by alanine residues at positions 134 or 624.

Amino acid substitution in *kel*	Primer
$Q^{31, 32,33}E$	5' CAACAACAACCAGCAG**G**AG**G**AG**G**AACAACAACAGGGTCAGAAC 3'
$Q^{31, 32, 33}L$	5' CAACAACAACCAGCAGC**T**GC**T**GC**T**ACAACAACAGGGTCAGAAC 3'
$Q^{31, 32, 33}R$	5' CAACAACAACCAGCAGC**G**GC**G**GC**G**ACAACAACAGGGTCAG 3'
$N^{26}D$	5' GCAATGGCAACAGCAAC**G**ACAACAACCAGCAGC 3'
Y134 A	5' GGCACTGTGGGGCAG**GC**CAGCAATGAACAGC 3'
Y627 A	5' GTTCAGTGGCAGACATGTCA**GC**TTGCCGGCGAAATGC 3'

An unmodified *kel* cDNA was also inserted into the EcoRI and BamHI sites of the pBluescript-II-SK(+) vector. The same sites were used to further sub-clone the *kel* cDNA into a pUASp2 vector.

2. P-element mediated transformation

In vitro mutagenized *imp-α2* constructs were microinjected along with Δ2–3 transposase helper plasmid into w^{1118} syncytial blastoderm embryos according to standard techniques (VANEDIS Drosophila injection service). Transformed flies containing the $P\{w^+, UAS::imp\text{-}\alpha2^{K39E}\}$ and $P\{w^+, UAS::imp\text{-}\alpha2^{S37E}\}$ identified by the red eye coloration of the eyes were individually crossed with *y w; Sp/ y*$^+$ *CyO; Sb/TM6,Ubx* flies and for each construct three to five independent third chromosomal insertions were selected and further introduced by crossing into a heterozygous $imp\text{-}a2^{D14}$ genetic background. y w; imp-$\alpha2^{D14}$/y+ CyO; $P\{w^+, UAS::imp\text{-}\alpha2^{K39E}\}$ or $P\{w^+, UAS::imp\text{-}\alpha2^{S37E}\}$ /TM6, Ubx stocks were established.

Bioinformatic tools

1. HUSAR

The Husar programme is available through the DKFZ facility)

- Mapsort –displays cut sites and fragments
- Map –shows restriction map and translations

2. Double digest finder

The programme is provided by New England Biolabs

The reaction conditions amenable to DNA double digestion by two restriction enzymes were selected through the double digest finder programme.

3. Tm calculator

The melting temperature of a primer was calculated using the Stratagene software for designing suitable primers.

4. BLAST bl2seq

The NCBI program was used to align two or more nucleotide sequences.

5. ExPASy Tools

The 2D gel analysis software Melanie was used to analyse the spots after isoelectric focussing.

Results

A. Interdependence of Imp-α2 and Kelch in their cellular localization:

By comparison to wild type ovaries, the development of *kel* and *imp-α2* egg chambers is arrested due to the occlusion of the ring canals preventing the discharge of the cytoplasnic content from the nurse cells into the growing oocyte (Xue and Cooley, 1993; Gorjánácz et al., 2002; 2006). Although the few eggs that are laid or those remaining in the ovaries display apparently normal dorso-ventral and antero-posterior polarities, both mutant eggs are shorter in length than normal and exhibit a basket shape, characteristic of a dumpless phenotype. The occlusion of the ring canals affects the growth of the mutant oocyte, whose shape is frequently shrunken (Gorjánácz et al., 2002).

As shown in Figure 7, we found that the Kelch protein was absent from the ring canals in *imp-α2*^{D14} egg chambers but present in the cytoplasm (Gorjánácz et al., 2002), indicating that Imp-α2 plays a role in the association of Kelch with the ring canals. Conversely we examined the distribution of Imp-α2 in *kel*$^{\Delta}$ ovaries and found that the absence of Kelch markedly altered the pattern of Imp-α2 distribution in nurse cells. In particular, we found that Imp-α2 accumulated in nuclei, predominantly in the non-chromosomal nuclear space (Fig. 8). These findings indicate that the retention of Imp-α2 in the cytoplasm depends on Kelch. Reciprocally, the association of Kelch with the ring canals requires Imp-α2 (Gorjánácz et al., 2006).

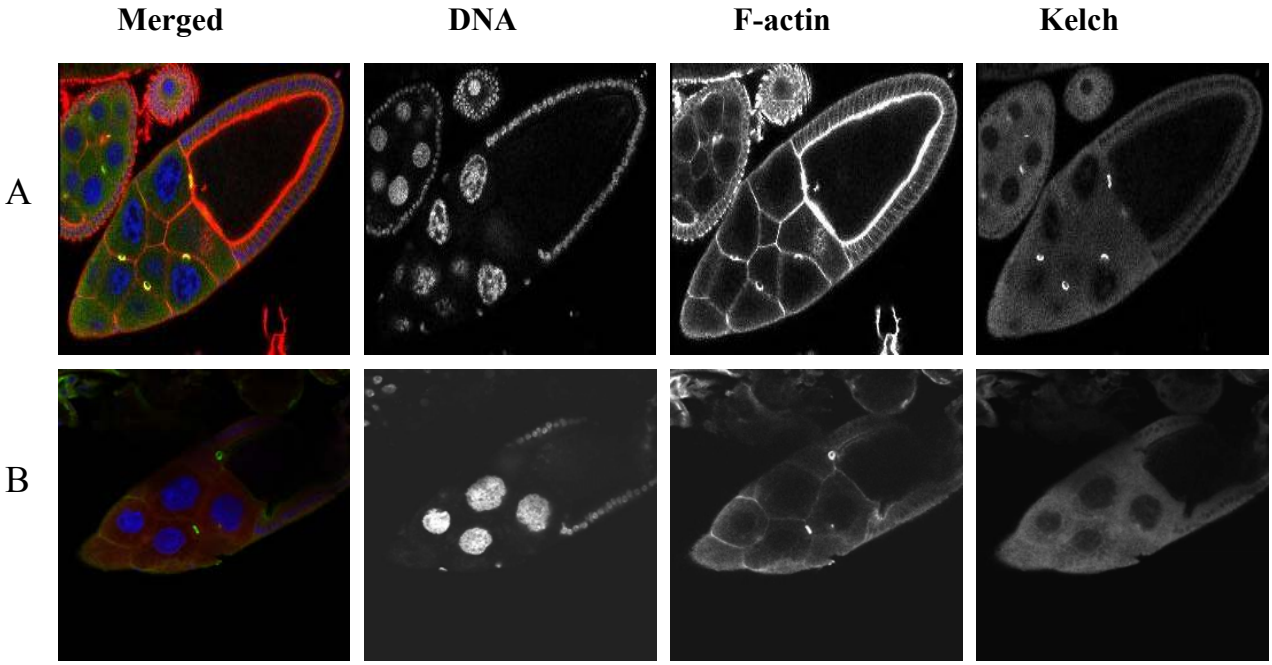


Figure 7. Kelch is delocalized in mutant imp-α2 egg chambers. (A) Wild type and (B) *imp-α2*^{D14} ovaries were attained for DNA, F-actin, and Kelch. Although Kelch is normally synthesized in *imp-α2*^{D14} ovaries, no binding to the ring canals could be detected.

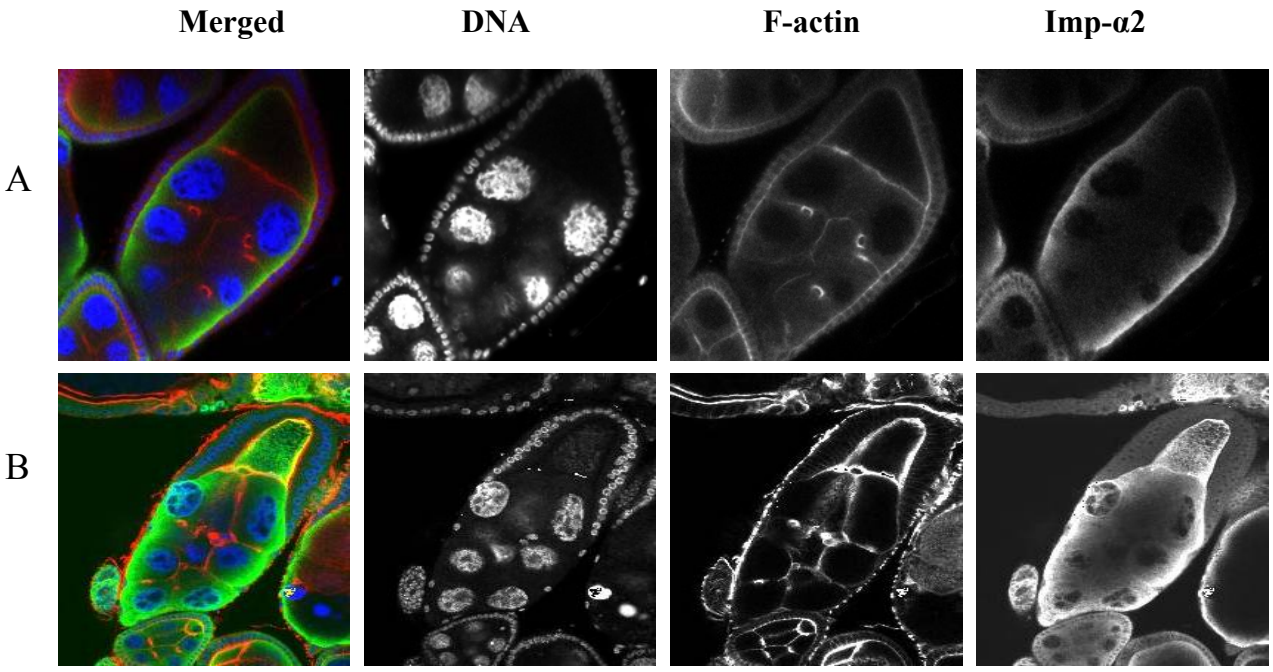


Figure 8. Nuclear accumulation of Imp-α2 in *kel*$^{\Delta}$ germ cells. (A) Wild type and (B) mutant *kel*$^{\Delta}$ ovaries were stained for DNA, F-actin, and Imp-α2. In wild type egg chambers Imp-α2 staining is predominantly detected in the cortical region of the nurse cells and remains undetectable in their nuclei, whereas in *kel*$^{\Delta}$ Imp-α2 markedly accumulates in the non-chromosomal nuclear space.

B. Genetic interaction between *kelch* and *imp-α2:*

To confirm the interaction between Imp-α2 and Kelch (involving either a direct or indirect contact between both proteins), we investigated whether a genetic interaction could be detected between both genes.

For this purpose we used interstitial null deletions, namely *imp-α2*^{D14} and *kel*$^{\Delta}$, and determined whether egg formation or egg development may be affected by a reduced gene dosage of *imp-α2* and *kel*. In a first series of crosses we determined whether the heterozygous combination *imp-α2*^{D14} +/+ *kel*$^{\Delta}$ (or *imp-α2*^{D14}/*kel*$^{\Delta}$) could block egg chamber development or cause embryonic arrest. We found that *imp-α2*^{D14}/*kel*$^{\Delta}$ females laid as may eggs as heterozygous *imp-α2*^{D14}/+ or *kel*$^{\Delta}$/+ females, and that these eggs were similarly viable (Table 1).

Although we were unable to detect any genetic interaction in a hemizygous combination, we further examined whether hemizygosity would provide a sensitive background when mutated *imp-α2* transgenes were expressed. Previous analyses have shown that the heterozygous combination between the *imp-α2*^{D14} and *imp-β*^{RE34} alleles resulted in embryonic lethality (Viragh, Szlanka, Kiss, and Mechler, personal communication). The *imp-β*^{RE34} allele is a partially recessive allele of the dominant *Ketel*^{D} mutation (Szabad et al., 1989) and encodes a protein with two amino acid substitutions (Viragh, Szlanka, Kiss, and Mechler, personal communication). By expressing a series of modified *imp-α2* transgenes (Figure 11) in *imp-α2*^{D14}/*kel*$^{\Delta}$ females we found that expression of the *P-{UAS-imp-α2*^{NLSB}*}* transgene driven by *p-{Gal4-nos}* exerted no effect on oogenesis but fully blocked embryonic development in the laid eggs (Table 2). In contrast, the expression of *P-{UAS-imp-α2*$^{\Delta IBB}$*}* in transheterozygous *imp-a2*^{D14}/*kel*^{D} females resulted in a block of egg formation. Furthermore expression of *P-{UAS-imp-α2*^{CASB}*}* in *imp-α2*^{D14}/*kel*$^{\Delta}$ females reduced moderately egg production but exerted

no effect on the development of the laid eggs. These data point out a dual function for both *kelch* and *imp-α2* during two different periods of *Drosophila* development. On the one hand, the deletion of the IBB domain, which allows the binding of Imp-α2 to Imp-β or to itself (Chook and Blobel, 2001; Matsuura and Stewart, 2004), produced a dominant effect on egg formation in $imp\text{-}\alpha2^{D14}/kel^{\Delta}$ females. On the other hand, inactivation of the NLSB domain exerted no affect on oogenesis but radically affected embryogenesis. No effect on egg laying and embryonic development was detected when the transgenes were expressed in either heterozygous $imp\text{-}\alpha2^{D14}$ or kel^{Δ} females, further indicating that a genetic interaction between both genes could only be noticed when gene dosage is decreased.

The finding that the expression of the $P\text{-}\{UAS\text{-}imp\text{-}\alpha2^{NLSB}\}$ transgene arrested embryonic development in eggs laid by transheterozygous $imp\text{-}a2^{D14}/kel^{D}$ females indicates that *kel* function is also required during embryogenesis. Therefore we investigated the spatio-temporal pattern of Kelch distribution during early embryogenesis, focusing our attention on early mitotic events.

C. Kelch distribution during early embryogenesis:

To obtain more information on the role of Kelch during embryogenesis, early wild type embryos were stained for Kelch, α-tubulin, and DNA. Confocal analysis of preblastoderm embryos revealed that Kelch accumulated in the nuclei during mitosis and could be detected in association with the centrosomes and the mitotic spindle from pro-metaphase to telophase. Kelch was however excluded from the nucleus during prophase. During pro-metaphase Kelch was predominantly detected in association with the centrosomes and the pericentrosomal polar microtubules. This pattern remained identical during metaphase and anaphase A but changed during anaphase B. At this stage, Kelch strongly decorated the elongating polar microtubules between the equatorial plate and the pole ward moving chromosomes, and more

moderately the centrosomes. These data suggest that Kelch may contribute to the process of chromosome-to-pole movements by acting on microtubule depolymerisation.

begins to accumulate on the centrosomes. (C) During metaphase Kelch co-localizes with tubulin on the mitotic spindle and the centrosomes. (D) When the chromosomes initiate their moving from the equatorial plate towards the poles in anaphase-A, Kelch predominantly caps the poles of the spindle. (E) When the chromosomes move from away from the equatorial plate during anaphase-B or early telophase, Kelch is moderately associated with the centrosomes and in a more pronounced manner with the subequatorial regions containing polar microtubules.

Table 1. *imp-α2^{D14}/kel^{Δ}* females laid as may eggs as heterozygous *imp-α2^{D14}*/+ or *kel^{Δ}*/+ females.

Females	Egg Viability* (%)	Egg laying
+/+	100	2.8
kel^{Δ}/+	70	2.9
imp-α2^{D14}/kel^{Δ}	79	2.5
imp-α2^{D14}/+	89	1.5
imp-α2^{D14}/kel^{Δ}; P-{nos-Gal4}	85	1.7

For each genetic combination 200 eggs were collected over a period of 12 hours and their viability was tested after 24h

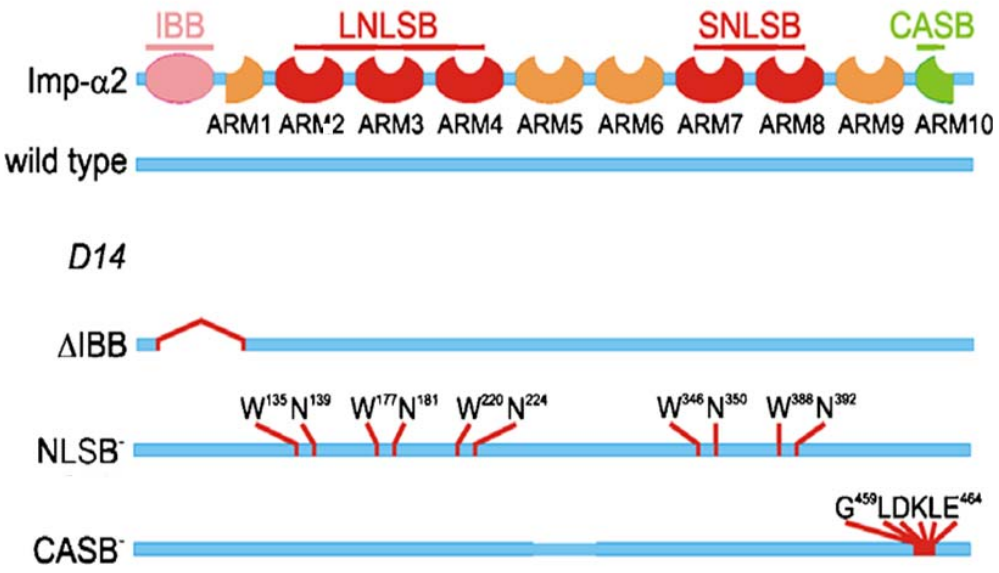


Figure 9. Imp-α2 structures and transgenes. (adapted from Gorjánácz *et al*., 2006). Diagram of the Imp-α2 protein containing a N-terminal Imp-β binding domain (IBB), 10 Armadillo

repeats (ARM1-10), two large (LNLSB) and short (SNLSB) Nuclear Localization Signal Binding domains, and CAS-binding domain. Positions of the amino acid residues substituted by alanine residues are indicated for each tissue. The different *imp-α*2 cDNA constructs are fused to a UAS promoter, inserted in a *P-{UASp2} P*-element and used to transform flies.

Table2. Genetic interaction between the IBB- and NLSB-domains of *imp-α2* and *kelch*.

Females	Egg viability (%)	Egg laying
imp-α2^{D14}/ kel^{Δ}	79	2.5
imp-α2^{D14}/ kel^{Δ}; P-{UAS-imp-α2^{NLSB}}/P-{nos-Gal4}	0	2.6
imp-α2^{D14}/ kel^{Δ}; UAS-imp- α2^{CASB}}/P-{nos-Gal4}	82	1.3
imp-α2^{D14}/ kel^{Δ}; P-{UAS-imp-α2^{ΔIBB}}/P-{nos-Gal4}	N.D	0
imp-α2^{D14}/+; P-{UAS-imp-α2^{NLSB}}/P-{nos-Gal4}	20	N.D
kel^{Δ} /+ ; P-{UAS-imp-α2^{NLSB}}/P-{nos-Gal4}		

For each genetic combination 200 eggs were collected over a period of 12 hours and their viability tested after 24h

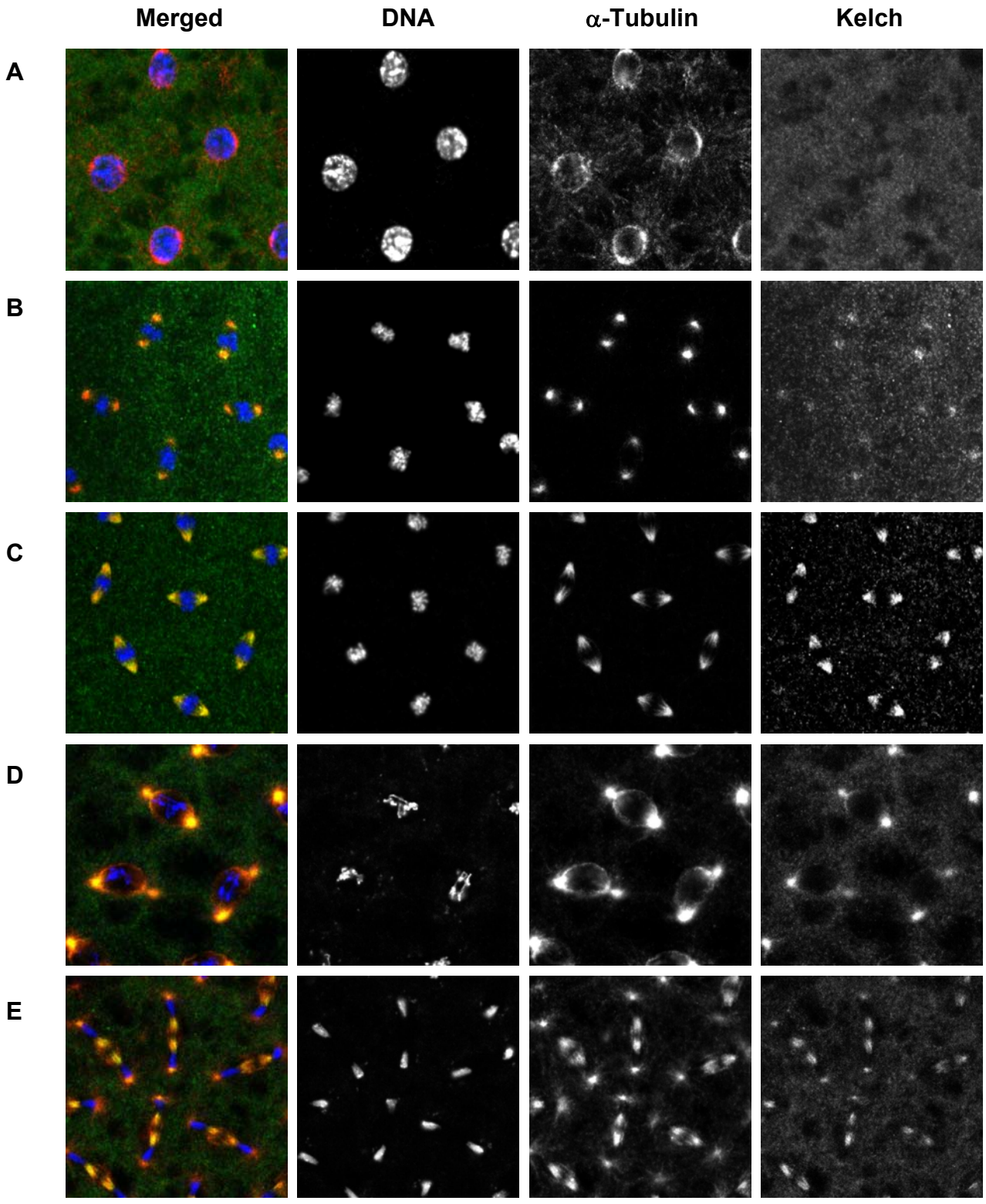


Figure 10. Kelch associates with polar microtubules and centrosomes during mitosis. Wild type embryos were stained for DNA, α-tubulin and Kelch. (A) During interphase (data not shown) and prophase Kelch is excluded from the nucleus. (B) In pro-metaphase Kelch

begins to accumulate on the centrosomes. (C) During metaphase Kelch co-localizes with tubulin on the mitotic spindle and the centrosomes. (D) When the chromosomes initiate their moving from the equatorial plate towards the poles in anaphase-A, Kelch predominantly caps the poles of the spindle. (E) When the chromosomes move from away from the equatorial plate during anaphase-B or early telophase, Kelch is moderately associated with the centrosomes and in a more pronounced manner with the subequatorial regions containing polar microtubules.

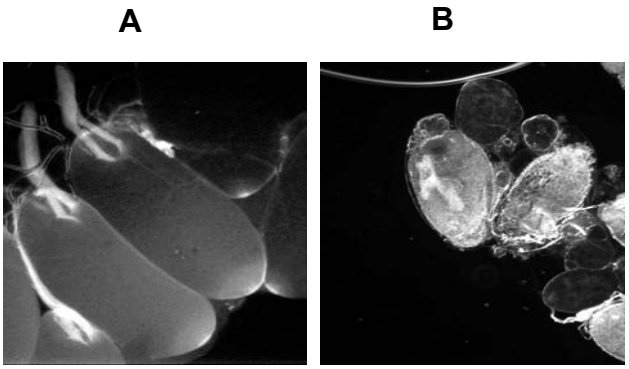


Figure 11. Dumpless phenotype of eggs laid by *imp-a2^{D14}/kel$^{\Delta}$; P-{UAS-imp-a2$^{\Delta IBB}$}/P-{nos-Gal4}* females. By comparison to (A) wild type (B) *imp-a2^{D14}/kel$^{\Delta}$; P-{UAS-imp-a2$^{\Delta IBB}$}/P-{nos-Gal4}* females were unable to lay eggs. These eggs, which accumulated at the lower extremity of each ovariole, were shorter and smaller in size than normal, and their dorsal appendages were reduced in length, flattened, and fused.

D. The IBB-domain of Imp-α2 contributes to *kel* function during oogenesis:

The genetic interaction between Imp-α2 and Kelch detected in *imp-α2^{D14}/kel^{Δ}; P-{UAS-imp-α2^{ΔIBB}}/P-{nos-Gal4}* females prompted us to further examine their ovaries. The mutant females were unable to lay eggs indicating a defect in oogenesis. Visual inspection of dissected ovaries revealed immediately that the mutant ovaries were smaller than wild type. Moreover the lower extremity of each ovariole was packed with shorter than normal eggs displaying deformed egg appendages, which were reduced in length, flattened and frequently fused. The rows of blocked egg chambers remaining in the ovaries resembled to those present in homozygous *imp-α2^{D14}* or *kel^{Δ}* females and displayed a basket structure characteristic of a dumpless phenotype (Figure 11).

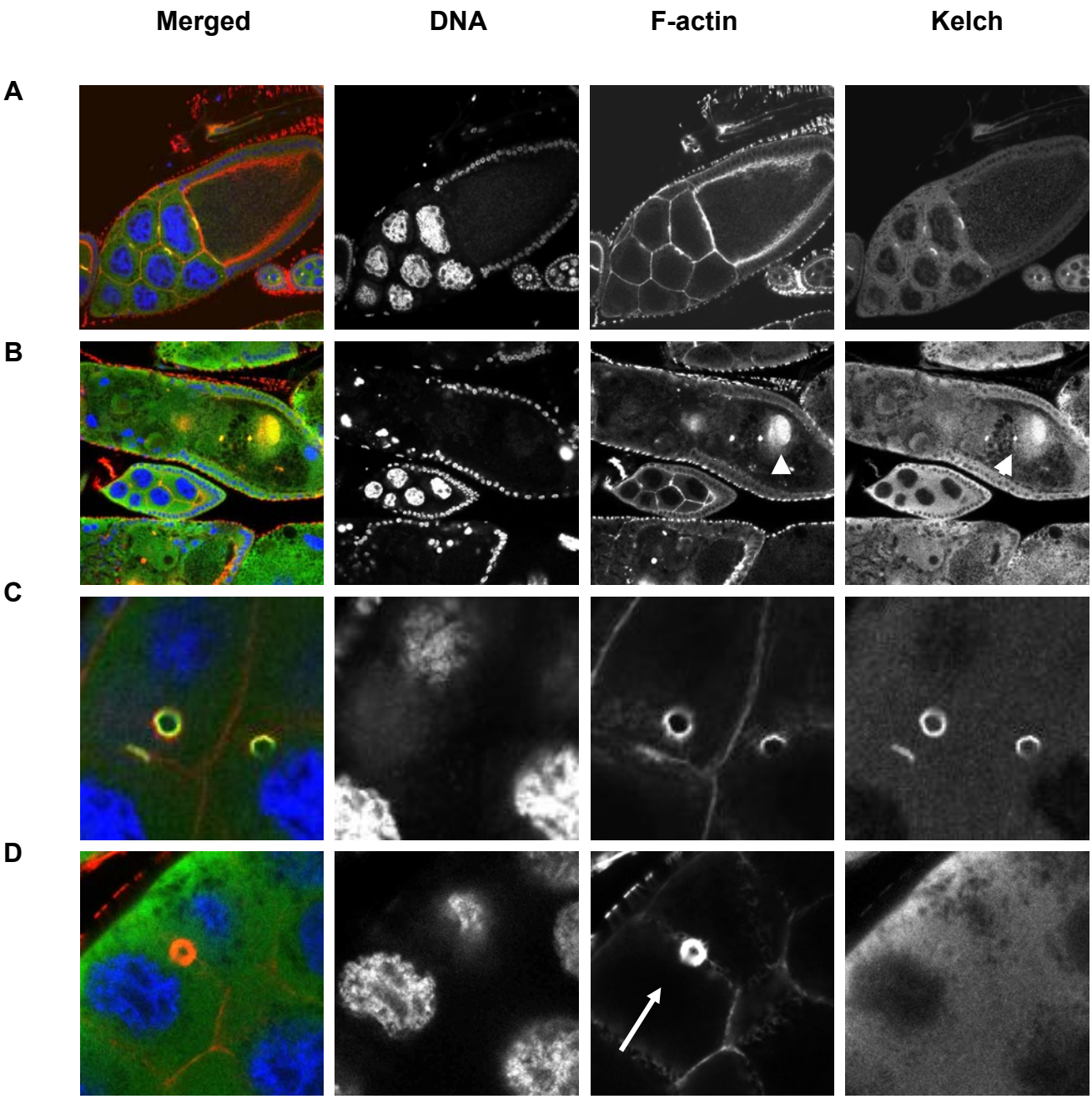


Figure 12. The IBB domain of Imp-α2 plays a critical role in Kelch function during oogenesis. (A and C) Wild type and (B and D) *imp-a2*^{D14}*/kel*$^{\Delta}$*/;P-{UAS-imp-a2*$^{\Delta IBB}$*}/P-{nos-Gal4}* egg chambers were stained for DNA, F-actin and Kelch. By comparison to wild type, the lumen of the ring canals in mutant egg chambers (arrows) is occluded with F-actin and unable to bind Kelch. The nuclei of the mutant vitellogenic egg chambers are frequently fragmented and F-actin and Kelch are included in large amorphic masses (arrowheads).

Staining of *imp-a2^{D14}/kel$^{\Delta}$; P-{UAS-imp-a2$^{\Delta IBB}$}/P-{nos-Gal4}* ovaries for F-actin, DNA and Kelch revealed that the egg chambers could reach a relatively large size. The mutant egg chambers contained fragmented nurse cell nuclei on their anterior half (Figure 12A). The F-actin cytoskeleton normally separating the nurse cells was missing and no ring canals could be detected. Kelch and F-actin accumulated in heterogeneous masses in the posterior centre of the egg chambers. Interestingly we noticed that the follicle cells, which synthesized comparable amounts of Kelch protein as in wild type, were able to form a nearly normal layer of elongated cells over the posterior half of the mutant egg chambers. However no follicle cells could be detected over the anterior half of the mutant egg chambers. This observation suggests that the follicle cells covering the posterior half of the egg chamber may contribute to the formation of the truncated chorion, as seen in Figure 11, and that the absence of anterior follicle cells may result in the formation of basket-shaped eggs.

In previtellogenic egg chambers, the structure of the egg chambers, as revealed by F-actin staining, resembled that of wild type. However, the ring canals were small and occluded by F-actin filaments. No Kelch protein decorated them (Figure 12B). All together our data support the idea that the IBB domain of Imp-α2 plays an important role in the binding of Kelch to the ring canals. Furthermore, our data indicate that this domain may play a less critical role in the somatic follicle cells which acquired a normal palisadic organisation. However, the extension of the follicle cells is blocked over the anterior half of the egg chambers.

E. The progression of mitosis is arrested in *imp-α2^{D14}/kel$^{\Delta}$;P-{UAS-imp-α2^{NLSB-}}/P-{nos-Gal4}*embryos

Examination by confocal microscopy of methanol fixed *imp-a2^{D14}/kel$^{\Delta}$* embryos expressing the *P-{UAS-imp-a2^{NLSB-}}* revealed a mitotic block occurring predominantly during the first nuclear division (Figure 13) and less frequently during the second nuclear mitosis. All

mutant embryos displayed the same phenotype indicating that the expression of a transgene producing an Imp-α2 protein in which the NLSB-domain is inactivated was toxic in *imp-a2^{D14}/kel$^{\Delta}$* embryos. This toxicity affected only embryonic mitosis but had no effect on meiosis, which was normally completed. As shown in Figure 14, female meiosis appeared to be successful as indicated by the three outer haploid meiotic nuclei, which after their segregation from the female pronucleus, became reorganised into the characteristic bouquet of condensed chromosomes localised under the egg cortex. Interestingly we observed a particularly strong enrichment of Kelch in both wild-type and mutant polar bodies whereas the amount of Kelch associated with the mitotic spindle was markedly reduced in *imp-α2^{D14}/kel$^{\Delta}$;P-{UAS-imp-α2^{NLSB-}}/P-{nos-Gal4}*embryos by comparison to wild-type.

Further characterization of the arrested nuclei in *imp-a2^{D14}/kel$^{\Delta}$; P-{UAS-imp-a2^{NLSB-}}/P-{nos-Gal4}* preblastoderm embryos revealed that the production of an Imp-α2 protein with an inactive NLSB domain resulted in a variety of mitotic defects. All nuclei in the first or second division displayed a strong metaphase arrest. Severe defects in centrosome maturation could also be noticed (data not shown) and different types of spindle defects. For example we observed tetrapolar spindles, (Fig. 15aB) anastral spindles with reduced amount of chromatin (Fig. 15aC), tripolar and apolar spindles with one axis (Fig. 15D and E), spindles with multiple chromatin aggregates (Fig. 15aF) reflecting chromosomes missegregation, and formation of a secondary spindle axis (Fig. 15aG). In addition we observed split centrosomes with one centrosome attached and the other split (Fig. 15aH). The majority of the spindles exhibited an enlarged barrel shape suggesting an excessive accumulation of microtubules. Although the mutant embryos were haploid for Kelch, the amount of Kelch protein associated with the spindles was markedly reduced by comparison to wild-type.

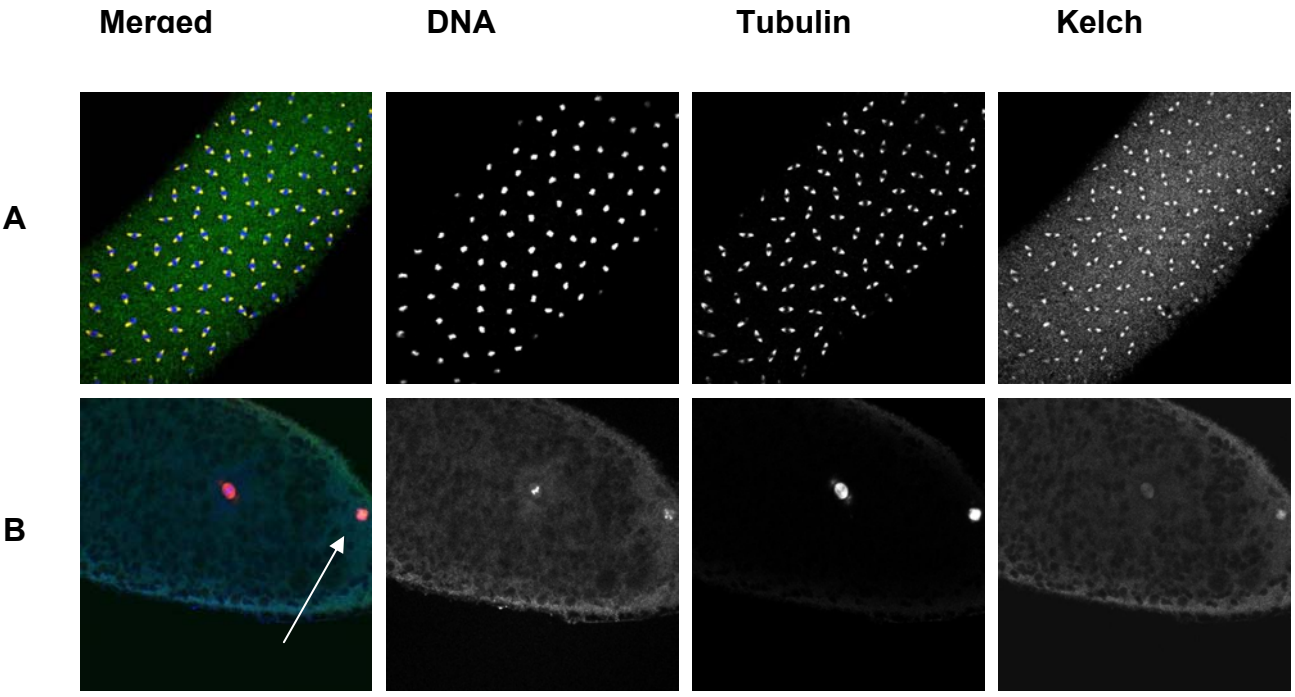


Figure 13. The NLSB domain of Imp-α2 interacts with Kelch during the first mitotic division. (A) OregonR and (B) *imp-α2^{D14}/kel^{Δ}; P-{UAS-imp-α2^{NLSB-}}/P-{nos-Gal4}* embryos collected for 2h were stained for DNA, α-tubulin and Kelch. Wild type embryos display numerous mitotic figures, whereas the development of the mutant embryos was blocked during the first mitotic division at the metaphase stage. (B) metaphase arrested zygotic nucleus and arrows to the polar body nucleus.

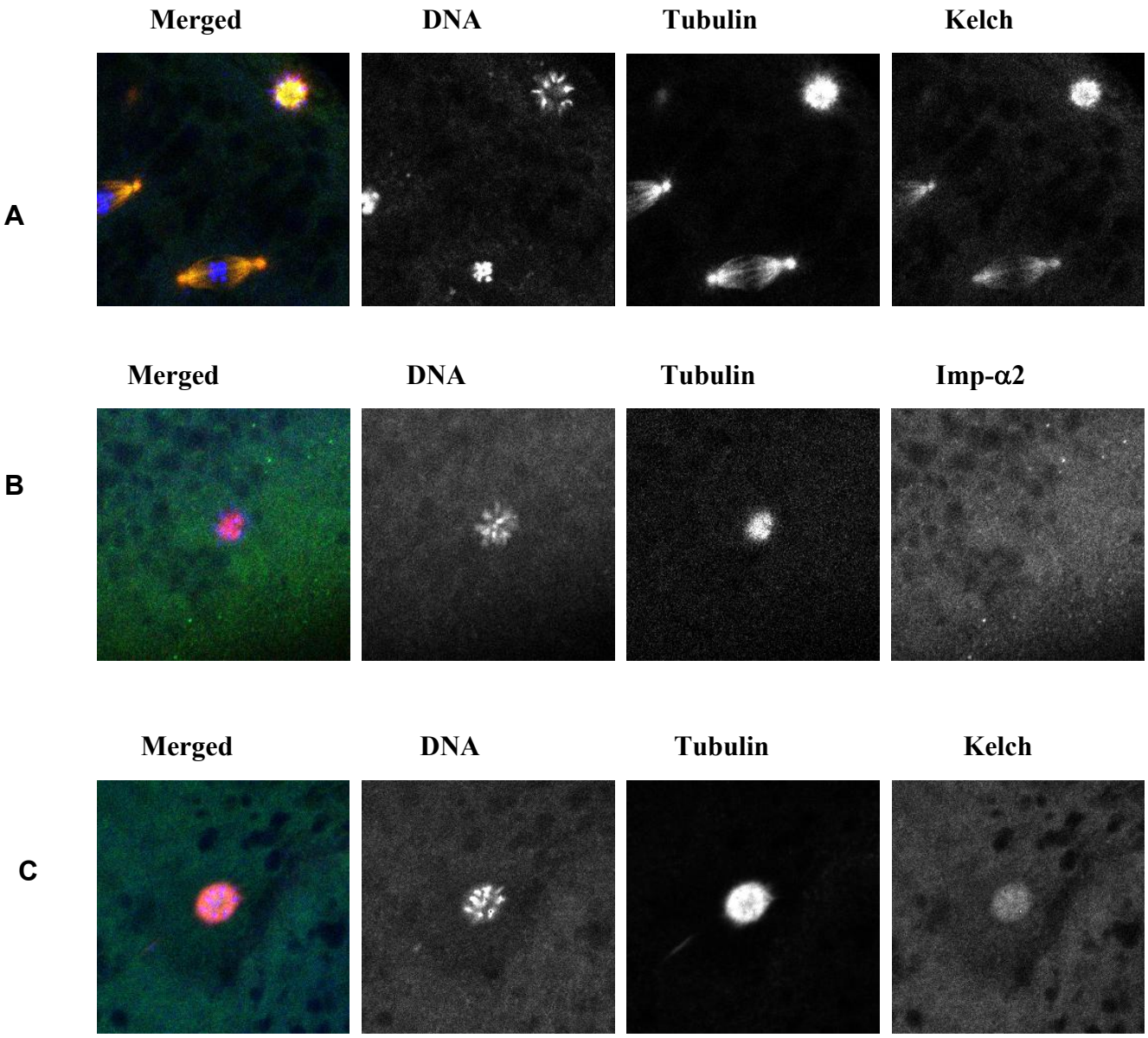


Figure 14. The three polar body nuclei fused normally in *imp-α2^{D14}/kel^{Δ}; P-{UAS-imp-α2^{NLSB-}}/P-{nos-Gal4}* embryos. Embryos (0-2 hr) were collected from either (A, B) Oregon R or (C) *imp-α2^{D14}/kel^{Δ}; P-{UAS-imp-α2^{NLSB-}}/P-{nos-Gal4}* females. Embryos were stained for DNA, α-Tubulin and Imp-α2 and Kelch.

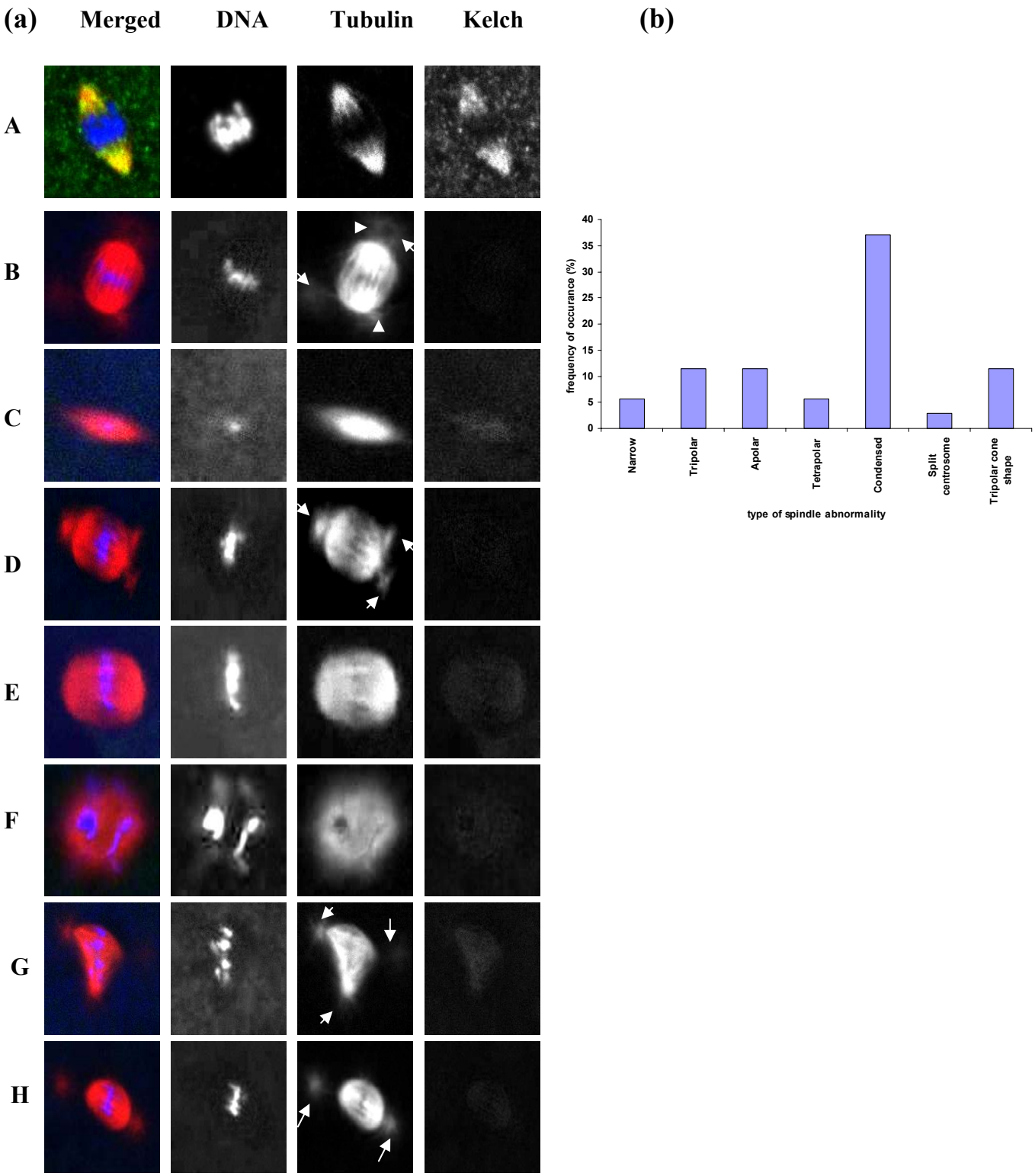

(a)
Merged
DNA
Tubulin
Kelch
A
B
C
D
E
F
G
H
(b)
frequency of occurance (%)
0
5
10
15
20
25
30
35
40
Narrow
Tripolar
Apolar
Tetrapolar
Condensed
Split centrosome
Tripolar cone shape
type of spindle abnormality

Figure 15. **Mitotic phenotypes observed in *imp-a2^{D14}/kel$^{\Delta}$; P-{UAS-impa2^{NLSB-}}/P-{nos-Gal4}* embryos.** *(a)* 0-4 h eggs were collected and the embryos were stained for DNA, α-tubulin and Kelch. Compared to (A) wild type embryos, Kelch staining was extremely or nearly absent in (B-H) mutant embryos. Different types of defects were seen. (B) spindle in tetrapolar spindle with four centrosomes and chromosomes aligned at the metaphase plate, (C) anastral and small narrow spindle with reduced amount of chromatin, (D) tripolar spindle and chromosomes aggregated at the metaphase plate, (E) large barrel shape spindle with chromosomes at the equator albeit without centrosome , (F) condensed spindle with several aggregations of chromosomes (G) tripolar cone shaped spindle with three centrosomes and aligned chromosomes, (H) spindle displaying one normally attached centrosome and one split centrosome on the opposite pole of the spindle. *(b)* Graph representing the type and frequency of f spindle abnormalities found in *imp-a2^{D14}/kel$^{\Delta}$; P-{UAS-impa2^{NLSB-}}/P-{nos-Gal4}* embryos.

F. Expression of Kelch, Imp-β and Imp-α2 in ovaries of different genotypes

As the interaction between Kelch and Imp-α2 or Imp-β could only be detected in conditions of haplo-insufficiency, it is important to control the relative level of expression for each investigated protein. For this purpose wild-type and mutant females were selected and proteins were extracted for a single pair of ovaries. The proteins were resolved on a 7% SDS polyacrylamide gel and transferred a PVDF membrane. The protein blot was then probed with antibodies raised against Imp-β, Kelch, and Imp-α2. The wild-type Imp-β protein and the Imp-β^{RE34} variant have a molecular mass of ~100 kDa in mass, the Kelch protein of ~77 kDA), the wild-type Imp-α2 protein as well as the Imp-$\alpha2^{NLSB-}$ and Imp-$\alpha2^{CASB-}$variants have the same molecular mass of ~58-60 kDa whereas the Imp-$\alpha2^{\Delta IBB}$ protein, which contains a deletion of the IBB domain, has a detectable lower molecular mass of ~ 55 kDa (Gorjánácz et al., 2006).

As shown in Figure 18, the relative amounts of the Imp-β and Kelch proteins corresponded to the genotype of the flies with the exception of Imp-β^{RE34} (lane 2) whose amount appeared to be higher that in wild-type (lane1). In mutant *kel*$^{\Delta}$*/kel* ovaries no Kelch protein could be detected (lane 7). Similarly no Imp-α2 protein could be detected in mutant *imp-a2*^{D14}*/imp-a2*^{D14} ovaries (lane 8), confirming that the *kel*$^{\Delta}$ and *imp-α2*^{D14} alleles were null mutations. The amount of Imp-α2 proteins in the transgenic ovaries was similar to that in wild-type, *kel*$^{\Delta}$*/imp-β*^{RE34} (lane 4), or *kel*$^{\Delta}$*/imp-β*^{2473} (lane 5) ovaries. In contrast the amount of Imp-$\alpha2^{\Delta IBB}$ protein was moderately reduced, as previously observed by Gorjánácz et al. (2006). These data show that similar amount of proteins are produced in the different genotypes and indicate that the observed genetic interactions are independent from the level of transcription/translation or the stability of the mutated Imp-α2 proteins. In this regard we

can infer that the Imp-α2$^{\Delta IBB}$ protein exerts a dominant toxic effect in a combined haplo dosage of *imp-α2* and *kel*.

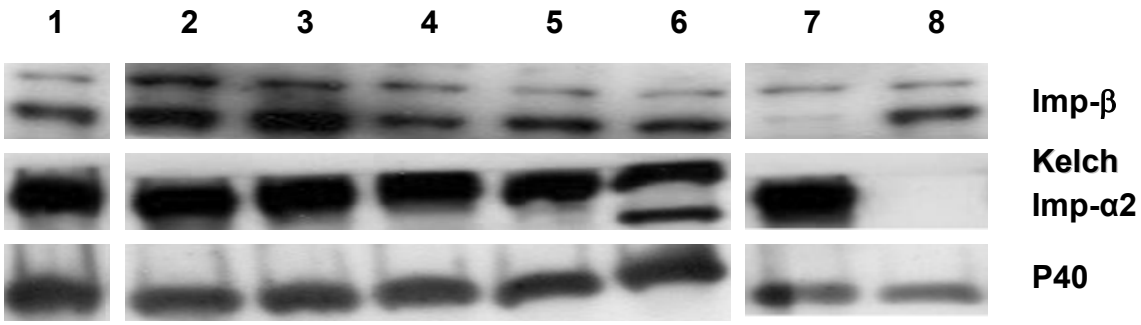


Figure 18. Western Blot analysis of ovarian extracts of different genotypes.

(1) Wild type,

(2) *kel*$^{\Delta}$*/imp-β*^{RE34},

(3) *kel*$^{\Delta}$*/imp-β*^{2473},

(4) *imp-a2*^{D14}*/kel*$^{\Delta}$*; P-{UAS-imp-a2*$^{NLSB-}$*}/P-{nos-Gal4},*

(5) *imp-a2*^{D14}*/kel*$^{\Delta}$*; P-{UAS-imp-a2*$^{CASB-}$*}/P-{nos-Gal4},*

(6) *imp-a2*^{D14}*/kel*$^{\Delta}$*; P-{UAS-imp-a2*$^{\Delta IBB}$*}/P-{nos-Gal4},*

(7) *kel*$^{\Delta}$*/kel*$^{\Delta}$

(8) *imp-a2*^{D14}*/imp-a2*^{D14}

Proteins extracted from one ovary for each genotype were loaded on a 7% polyacrylamide gel and transferred to a PVDF membrane. The protein blot was probed with affinity-purified antibodies raised against Kelch, Imp−β, and Imp-α2. Anti-P40 antibodies were used as a control.

G. Genetic interaction between *kelch* and *imp-β*

Previous genetic studies revealed a robust genetic interaction between *imp-α2* and *imp-β* (Virag et al, submitted) This interaction was obtained with *imp-β*^{RE34}, an EMS-induced recessive revertant of the dominant female sterile mutant *Ketel*. The *Ketel* gene encodes the Imp-β protein. In contrast, combinations of *imp-α2*^{D14} with other recessive mutations of *imp-β* (such as *imp-β*^{2473}) that resulted from the insertion of *P*-element transposons, were ineffective. Sequence analysis of the mutated *imp-β* genes showed that the dominant Ketel mutation is caused by a substitution of a proline by a leucine at residue position 446 and the recessive *imp-β*^{RE34} contains a second substitution of an aspartic acid by an asparagine at residue position 725. As Kelch and Imp-α2 are unable to directly interact, we suspected that an additional component may act as a mediator between both proteins. Imp-β could be an obvious candidate for mediating an interaction between Imp-α2 and Kelch. To ascertain this hypothesis we first investigated whether a genetic interaction could be detected between alleles of *imp-β* and *kelch.*

For this purpose we used the *imp-β*^{RE34} and *imp-β*^{2473} alleles, which were previously tested with *imp-α2* (Viragh et al., submitted) in heterozygous combinations with *kel*$^{\Delta}$. We analysed the viability of eggs laid by *kel*$^{\Delta}$*/imp-β*^{2473} and *kel*$^{\Delta}$*/imp-β*^{RE34} females. As shown in Table 3, the viability of eggs laid by *kel*$^{\Delta}$*/imp-β*^{2473} females was unaffected but strongly reduced in the case of *kel*$^{\Delta}$*/imp-β*^{RE34}. This result is similar to the data obtained with eggs laid by *imp-α2*^{D14}*/imp-β*^{RE34} and indicates that *kelch* may genetically interact *with imp-β* during embryogenesis.

Although the egg laying capacity of $kel^{\Delta}/imp\text{-}\beta^{RE34}$ females was reduced by comparison to that of $kel^{\Delta}/imp\text{-}\beta^{2473}$ females, both females laid morphologically normal eggs. This finding indicates that oogenesis proceeded normally in these females and no block occurred at this stage. Examination of their ovaries revealed no striking disturbance. However, the reduction in egg viability in $kel^{\Delta}/imp\text{-}\beta^{RE34}$ females suggests that first that *kelch* may also interact with *imp-β* during oogenesis but the interaction, albeit negative, may only exert an effect on the rate of egg maturation and egg laying without causing a noticeable morphological defect.

H. Kelch physically interacts with Imp-β

The finding of a genetic interaction between *kelch* and *imp-β* prompted us to analyze whether both proteins may physically interact between them.

For this purpose full-length wild type Imp-β-GST and Imp-β^{RE34}-GST fusion proteins were produced in bacteria, extracted and purified on a glutathion-Sepharose 4B beads. Following several washed the Imp-β-GST and Imp-β^{RE34}-GST beads were added to protein extracts from 0-2 hr OregonR embryos. Following incubation for4 one hour at room temperature, the bound Imp-β complexes were separated by centrifugation, washed several times in buffer. The bound Imp-β-complexes were released by treatment with recombinant TEV protease. The released proteins were separated by SDS polyacrylamide gel electrophoresis and the Imp-β and Kelch proteins were detected on a Western blot by using anti-Kelch and anti-Imp-β antibodies. As shown on Figure 19, the Kelch protein was recovered in association with both wild type Imp-β and the mutant Imp-β^{RE34} protein.This data indicates that Kelch and Imp-β could physically interact.

Table 3. Genetic interaction between *kelch* and *imp-β*.

Females	Egg Viability (%)*	Egg Laying (eggs/female/hr)
$kel^{\Delta}/imp\text{-}\beta^{2473}$	76.5	2.69
kel^{Δ} $imp\text{-}\beta^{RE34}$	2	1.36

*For each genetic combination 200 eggs were collected over a period of 12 hours and their viability tested after 24h.

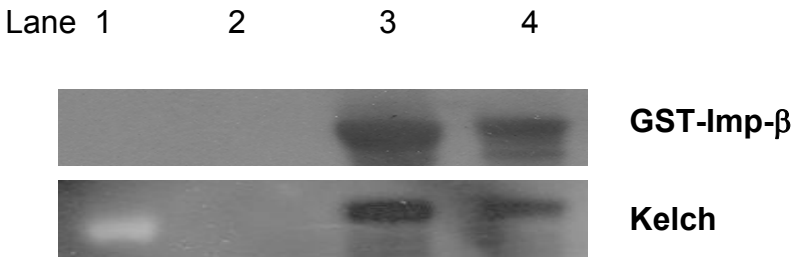


Figure 19. Physical interaction between Kelch and Imp-β.

Lane (1) marker (2) GST-alone (3) wild type GST-Imp-β (4) GST-Imp-β^{RE34}

I. Imp-β localization is independent from Kelch

As the intracellular distribution of Imp-α2 is altered in kel^{Δ} mutant egg chambers, we investigated the distribution of Imp-β in $imp\text{-}a2^{D14}$ and kel^{Δ} ovaries. The information gained from this analysis may indicate whether Imp-β acts downstream or upstream of Kelch. We isolated ovaries of wild type OregonR females as well as ovaries from and stained them for DNA, lamin as a maker of the nuclear membrane and Imp-β. As shown in Figure 20) Imp-b was uniformly distributed in the cytoplasm of nurse cells in wild-type egg chamber as well as both types of mutant egg chambers and decorated moderately the outer layer of the nuclear membrane. As the localization of Imp-b remained unaffected in the mutant egg chamber we concluded that Kelch exerts no influence on the localisation of Imp−β. This result would rule out the possibility that Imp-β acts downstream of Kelch.

J. Model

A hypothetical model of the interaction between Kelch, Imp-α2 and Imp-β could be proposed on the basis of our analysis. This model may essentially be applied to the formation of the ring canals because the function of these three proteins is independent of Ran-GTP. During embryogenesis Ran-GTP plays a crucial role during mitosis due to its specific nuclear localisation in association with the nuclear membrane and the condensed chromatin. Ran-GTP is known to critically regulate Imp-α and Imp-β functions in vertebrate cells.

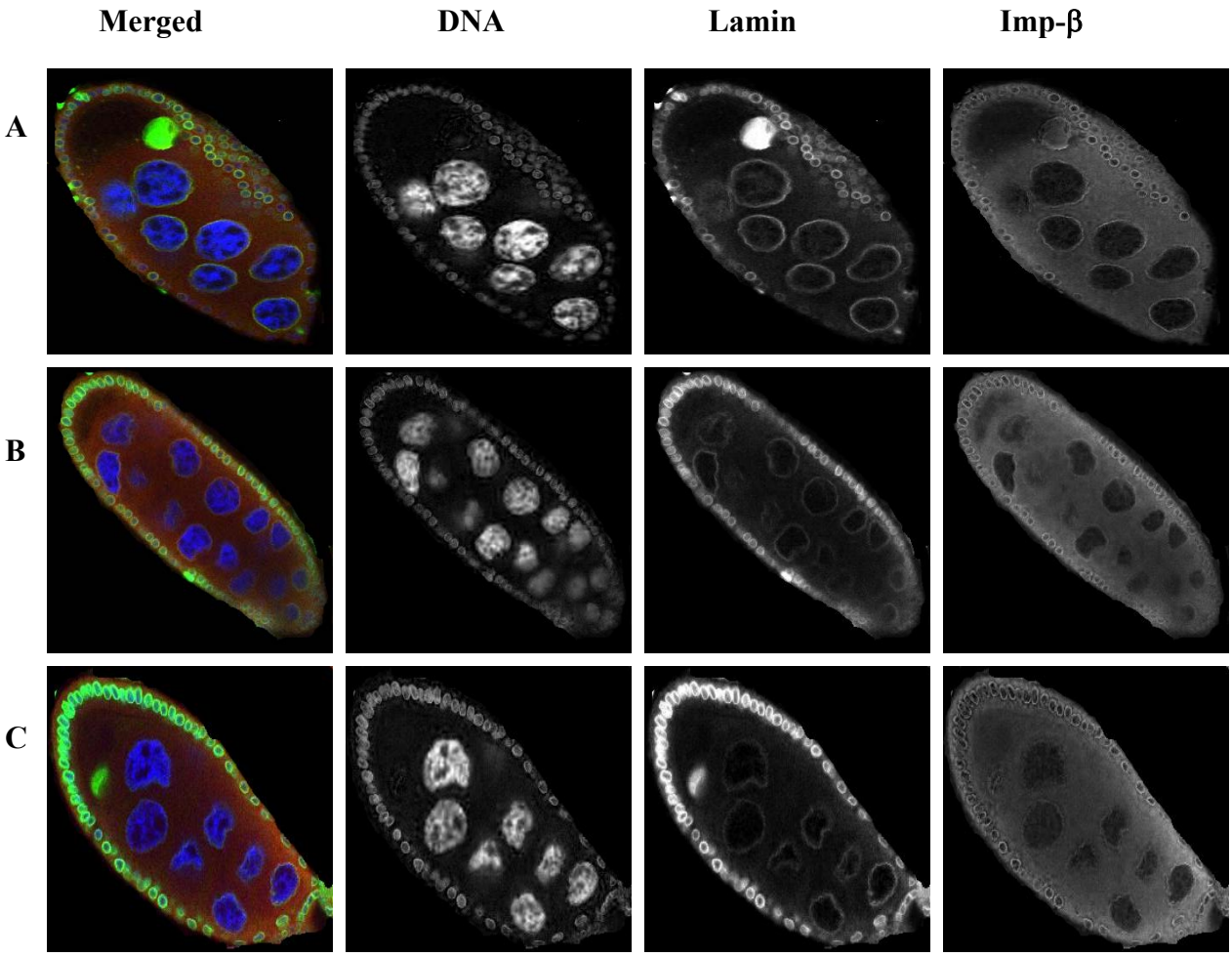
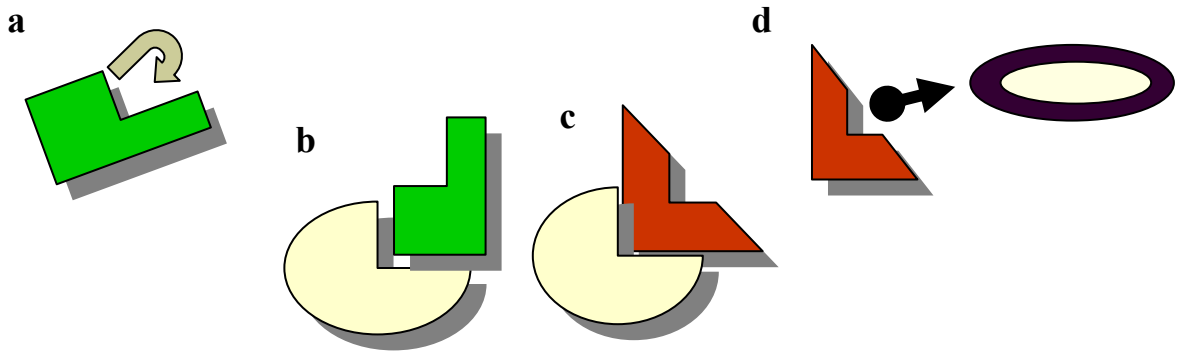


Figure 20. **Imp-β localisation is independent from Kelch**. (A) Wild type, (B) *imp-a2*^{D14}, and (C) *kel*$^{\Delta}$ egg chambers. Imp-β is uniformly distributed in the cytoplasm of the nurse cells and decorates the outer face of the nuclear membrane in the nurse cells and follicle cells. Note that the Imp-β distribution in mutant egg chambers is similar to that of wild type.

(A) In wild type ovaries



(B) In *imp-α2*^{D14} ovaries

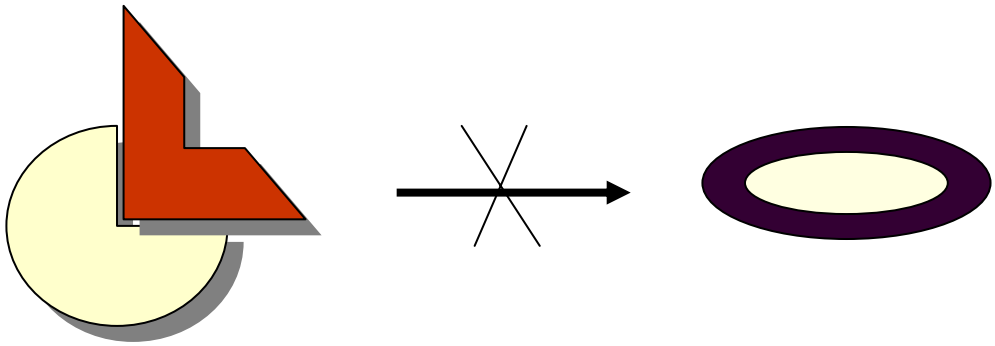


Figure 21. Model predicting the interaction between Imp-α2, Imp-β and Kelch during ring canal formation

(A) In wild type ovaries (a) The IBB domain of Imp-α2 can bind to itself through the NLSB domain, or to another Imp-α2 protein. (b) Imp-α2 may also bind to a fraction of Imp-β. (c) However Imp-β can also interact with Kelch. (d) When Imp-α2 binds to Imp-β, Kelch may become free and is able to bind to ring canals.

(B) In mutant *imp-α2*^{D14} egg chambers, the Kelch protein remains bound to Imp-β and is thus unable to associate with the ring canals.

On the basis of our results we would propose a model of interaction between the three components that we have yet so far studied. This model essentially deals with the ovarian period of development and ring canal formation, but can also be applied in a slightly different way to embryonic development. During both developmental phases both Imp-α2 and Imp-β proteins remain dissociated one from the other. Only a small fraction of Imp-β could be recovered in Imp-α2-immuno-complexes. Therefore we consider that both proteins are normally dissociated in the cytoplasm. In wild type conditions the IBB domain of Imp-α2 can bind to the NLSB domain or bind to Imp-β. We predict that since Kelch can bind directly to Imp-β, the binding of the IBB domain of Imp-α2 to Imp-β would release Kelch. In this way Kelch can then become associated with the ring canals. When Imp-α2 is absent in *imp-α2*^{D14} ovaries, there is no competitor to bind to Imp-β and Imp-β remains bound to Kelch, which remains unable to become associated with the ring canals. In this hypothetical model we assume that Imp-β may be acting as the mediator between Imp-α2 and Kelch.

Preliminary Results

K. Excessive phosphorylation of Kelch in *imp-α2^{D14}* ovaries

The Kelch protein is known to be a target of kinases ( ). Therefore we investigated the level of Kelch phosphorylation in wild-type and mutant *imp-α2^{D14}* ovaries. To examine Kelch phosphorylation in the *Drosophila* ovary, we performed 2D electrophoresis of ovarian protein extracts from wild type and *imp-α2^{D14}* backgrounds (Figure 22). When wild-type ovary lysates were treated with phosphatase inhibitors, three Kelch isoforms were detected. When the *imp-α2^{D14}* ovaries were treated with phosphatase inhibitors, only one isoform was detected. There was also a small shift towards the anode in the case of *imp-α2^{D14}* ovaries when compared with the internal standards (P40) indicating an increased phosphorylation. The increased phosphorylation and the presence of only a single isoform in *imp-α2^{D14}* ovaries indicate that the degree of phosphorylation of Kelch may depend upon Imp-α2 and ultimately affect its localizing to the ring canals.

L. The *chickadee (chic)* gene may also interact with *imp-α2*

L.1. Genetic interaction between *chic* and *imp-α2*

The *chic* gene plays a critical role in the formation of ring canals during oogenesis ( ). Like mutations in *kel* and *imp-α2*, mutations in *chic* display a maternal effect resulting in the occlusion of the ring canals during egg chamber development. Therefore we investigated

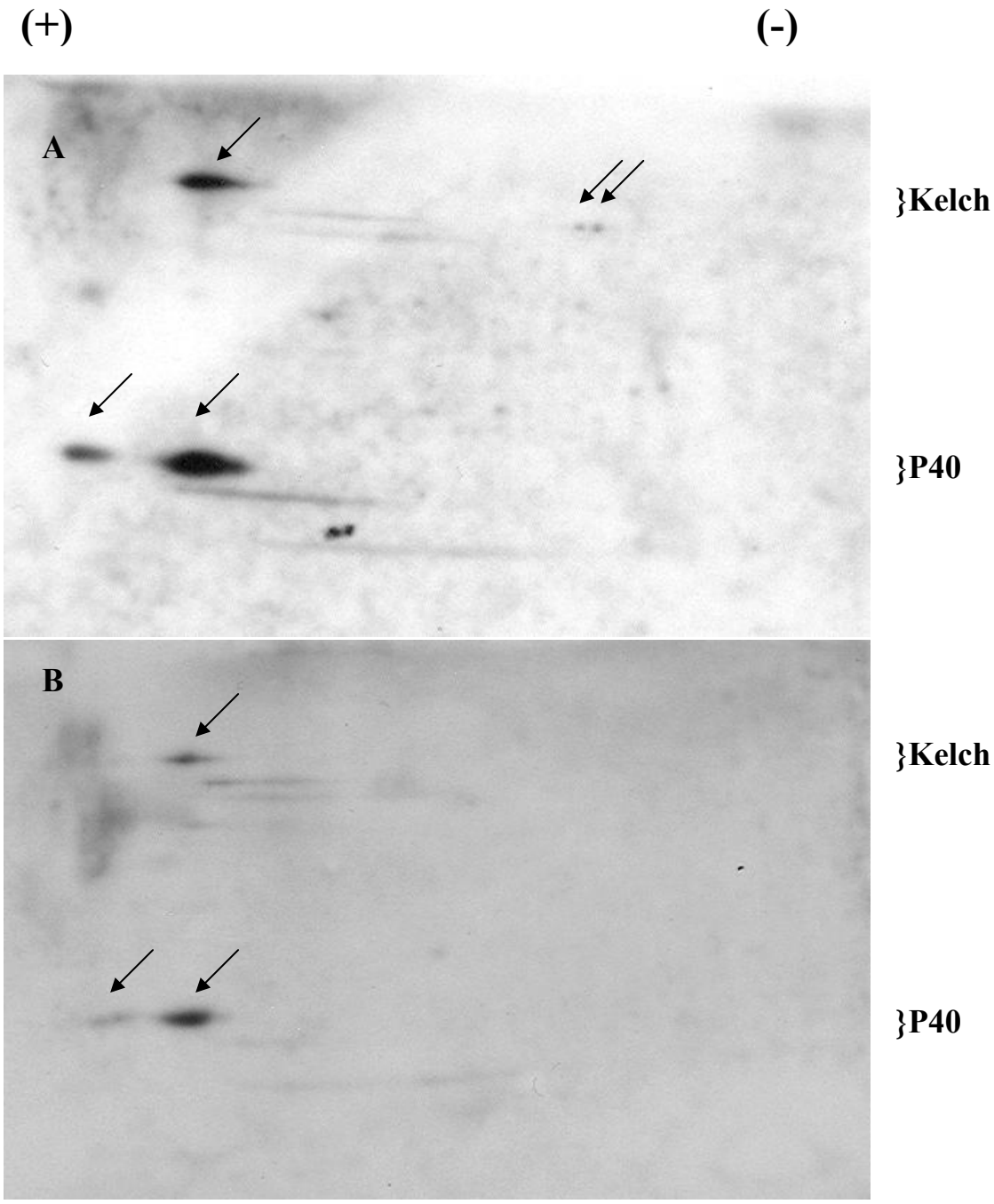


Figure 22. 2D gel-electrophoresis of Kelch. Protein extracts of (A) wild type and (B) *imp-α2*^{D14} ovaries was performed in the presence of phosphatase inhibitors. In the wild type protein extract three immuno-reactive Kelch spots could be detected whereas only one spot is detected in *imp-α2*^{D14} ovaries. A moderate shift towards the anode, suggesting a higher degree of phosphorylation, could also be noticed as compared to the internal P40 standard.

whether we could detect a genetic interaction between null mutations of *imp-α2* and *chic,* similar to what we have uncovered with *kel*. Like in the previous crosses, we used interstitial *imp-α2*^{D14} and *chic*$^{\Delta}$ and determined whether egg formation or egg development may depend on a gene dosage between *imp-α2* and *chickadee.* In the first series of crosses we combined *imp-α2*^{D14} and *chic*$^{\Delta}$and determined whether they would give rise to a block in oogenesis or embryogenesis. We found that *chi*$^{\Delta}$*/imp-α2*^{D14} females laid as many eggs as heterozygous *chic*$^{\Delta}$/+ *or imp-α2*^{D14} /+, and that these eggs were similarly viable (Table 4).

Since we were unable to obtain evidence for a genetic interaction in hemizygous combination we examined the conditional expression of the mutated *imp-α2* transgenes using hemizygosity as a sensitive background (Table 5).

Results of the crosses using modified *imp-α2* transgenes showed that the combination of *-imp-α2*$^{NLSB-}$ in a *chic*$^{\Delta}$*/imp-α2*^{D14} background generated females, which laid a significantly reduced number of eggs. No similar effect was detected with *imp-α2*$^{CASB-}$ or *imp-α2*$^{\Delta IBB}$ indicating that like in the case of *kel* a deletion of the NLSB domain in imp-α2 may exert a toxic, albeit more moderate, in combination with *chic* during oogenesis.

L.2. Genetic interaction between *chic* and *imp-β*

The weak interaction detected between *chick* and *imp-α2*$^{NLSB-}$ incited us to examine whether we could detect an interaction between *chic* and *imp-β.* Therefore we examined the viability of eggs laid by *chic*$^{\Delta}$*/imp-β*^{2473} and *chic*$^{\Delta}$*/imp-β*^{RE34} females and found that the viability of eggs laid by *chic*$^{\Delta}$*/imp-β*^{RE34} females was strongly reduced (Table 6). These results show that *chic* may also genetically interacts *with imp-β* during embryogenesis.

Table 4. *chic$^{\Delta}$/imp-α2^{D14}* females laid viable eggs

Females	Egg Viability (%)	Egg laying*
+/+	100	2.8
chic$^{\Delta}$/+	79	N.D.
chic$^{\Delta}$/imp-α2^{D14}; +	75	N.D.

Eggs were collected over a period of 12 hours and their viability tested after 24h. * Number of eggs laid per hour per female. N.D. Not determined.

Table 5. Genetic Interaction between *imp-α2* and *chic*

Females	Egg Viability (%)	Egg laying
chic$^{\Delta}$/imp-α2^{D14}; P-{nos-Gal4}	77	N.D.
chic$^{\Delta}$ imp-α2^{D14}; UAS-imp-α2^{NLSB-}/P-{nos-Gal4}	46	N.D
chic$^{\Delta}$/imp-α2^{D14}; UAS-imp-α2^{CASB-}/P-{nos-Gal4}	86	N.D
chic$^{\Delta}$/imp-α2^{D14}; UAS-imp-α2$^{\Delta IBB}$/P-{nos-Gal4}	98	N.D

Eggs were collected over a period of 12 hours and their viability tested after 24h. N:D: Not determined

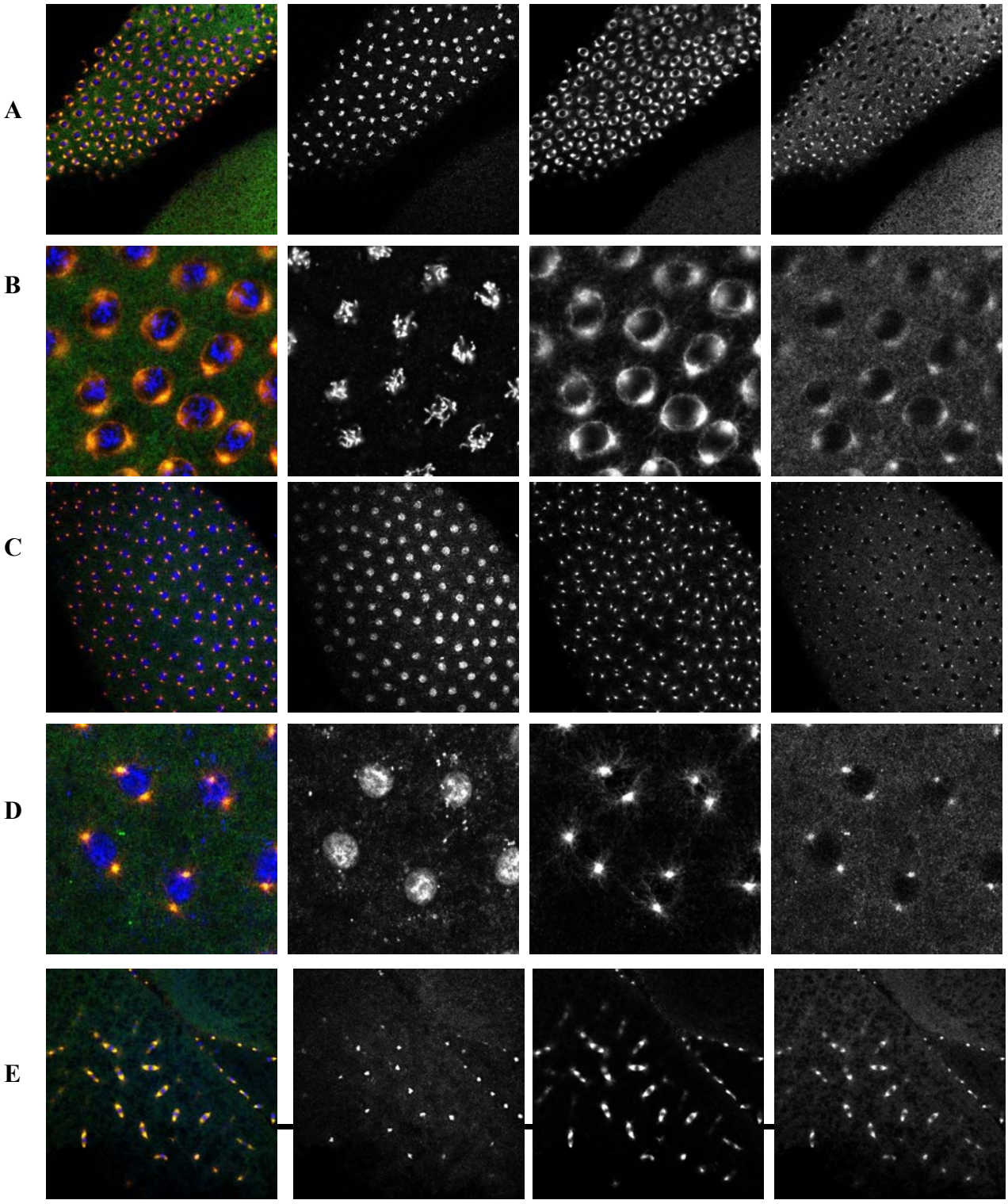
Table 6. Genetic interaction between chic and *imp-β.*

Females	Egg Viability (%)	Egg Laying
chic$^{\Delta}$/imp-β^{2473}	67.5	N.D
chic$^{\Delta}$/imp-β^{RE34}	7	N.D

Eggs were collected over a period of 12 hours and their viability tested after 24h. N.D. Not determined.

M. Chickadee is a component of the centrosomes and the polar microtubules

As genetic interactions could be detected between *chic* and either *imp-α2* or *imp-β* and appeared to affect embryogenesis, we stained wild type embryos for DNA, α-tubulin and Chic. Confocal analysis of preblastoderm embryos (Figure 23) showed that Chic decorates specifically the centrosomes during interphase and prophase and then becomes predominantly localised to the polar microtubules of the spindle during metaphase and anaphase. This localisation is highly reminiscent of that of Kelch and bears also similarity with that of Imp-α2, indicating that these proteins may conjointly function during mitosis in preblastoderm embryos.


Merged
DNA
Tubulin
Chickadee
A
B
C
D
E

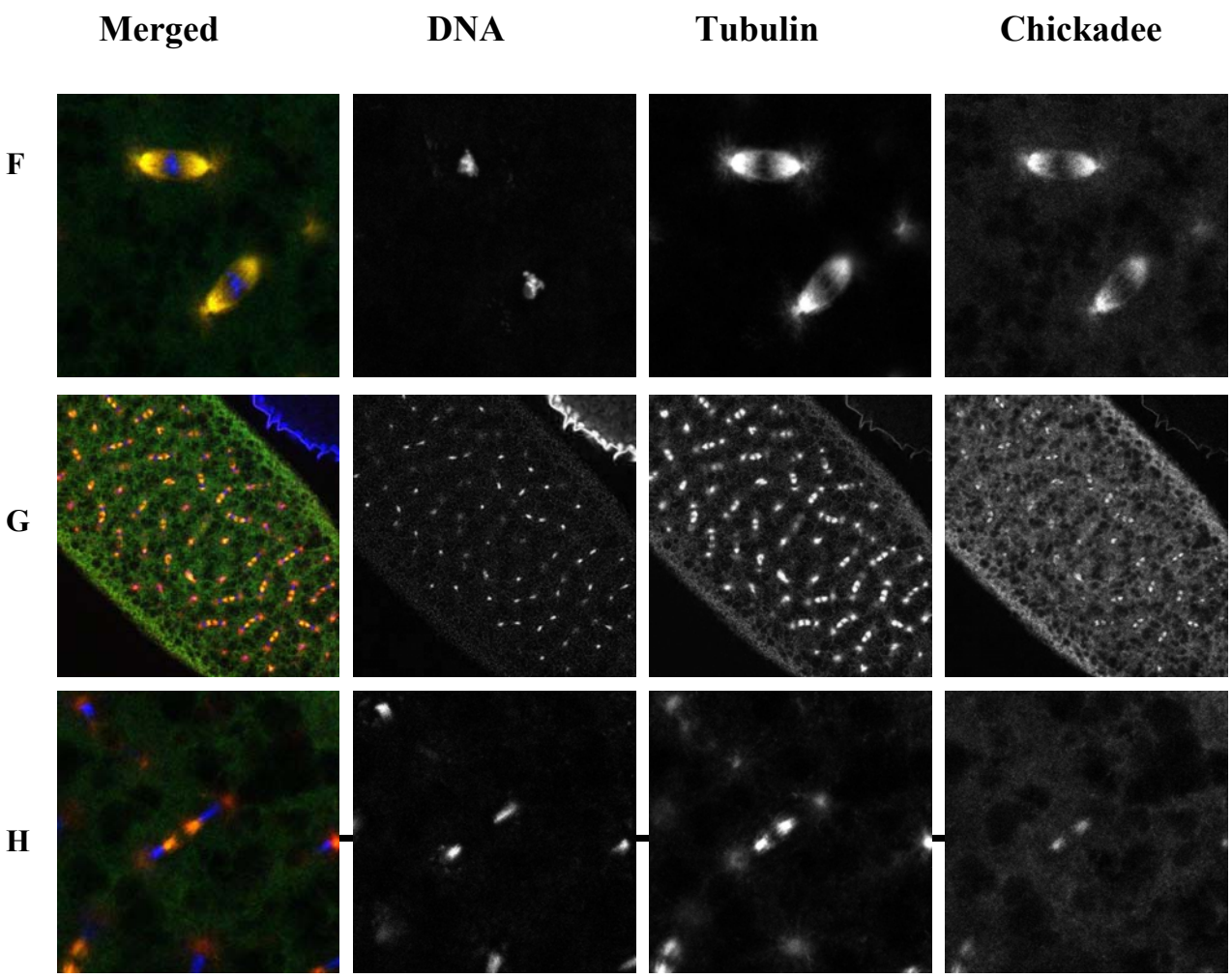


Figure 23.Chickadee distribution during mitosis in preblastoderm embryos.

Wild type embryos were stained for DNA, α-tubulin and Chickadee (A) Interphase, (C) prophase, (E) metaphase, and (G) telophase. (B, D, F, and H are enlargements of A, C, E, and G, respectively

Discussion

The *Drosophila* ovary is a favorable model system to study cellular morphogenesis. The ovary is the single largest organ in the female fly and the oocyte is the single largest cell. The ovary is not essential for survival and hence allows extensive manipulation. A single ovary contains every stage of development from stem cell to mature egg. Each egg chamber contains both somatic and germline cells which assists easy comparison. The development of a single stem cell into a mature egg involves almost every cellular process that can occur during development- from cell cycle control and fate specification to cell polarisation and epithelial morphogenesis. Hence this is a system that appears simple but can provide insights applicable to more complex processes. Therefore, oogenesis has become a very powerful system for investigating many aspects of cell and developmental biology. (Rebecca Bastock and Daniel St Johnston., 2008)

A. Kelch an overview:

The localization of the Kelch protein (Douglas N. Robinson *et.al*., 1994) to the ring canal begins in the region 3 of the germarium. This suggests that Kelch is not required for the initial establishment of the ring canal but is required to maintain the organization of the actin filaments during the expansion of the ring canal. Kelch mutant ring canals are highly disorganized and have additional actin filaments that extend into the canal partially obstructing cytoplasm transport.

Kelch is a multidomain protein and a member of a superfamily of proteins defined, in part, by the presence of six 50–amino acid kelch repeats (KREPs) .Kelch has three major domains: NTR, BTBIVR and the KREP. To investigate the mechanism by which Kelch organizes the ring canal actin cytoskeleton, myc epitope–tagged domains were expressed in the ovaries

(Douglas N. Robinson *et.al*.1997). Two domains, the BTB and the KREP are also found in diverse non-kelch proteins whereas the NTR domain is not present in other kelch family members.

NTR is the N-terminal region domain which is not required for Kelch's organizational activity but is specifically required for regulating the timing of the protein localization. Removal of the NTR (myc: ΔNORF1) produced a protein that had severe dominant-negative effects in egg chamber morphology and ring canal stability. Firstly myc: ΔNORF1 is localized to the ring canals in early stages of oogenesis (region 1) and secondly this mutant allele also displays a loss of nurse cell membrane integrity resulting in aberrant transport of nurse cell nuclei into the oocyte. The BTB domain is insufficient to localize to the ring canals but is capable of mediating dimerization. When the KREP domain was deleted, (ORF1-R) the protein produced was unable to bind to the ring canals and needed to interact with the amino terminal of an intact Kelch ORF1 protein to localize. Hence, the KREP domain is both necessary and sufficient for localization to the ring canals.)

B. Src/Tec signalling and Kelch :

Based on sequence similarity to galactose oxidase, the KREP domain is predicted to fold into a sixbladed β-propeller (Bork and Doolittle, 1994; Adams et al., 2000). In *Limulus* the KREP domain is present in at least three scruin proteins, each of which contains two KREP domains (Way et al., 1995). The KREP domains of α-scruin each form an F-actin binding domain that allows α–scruin to act as an actin filament–cross-linking protein (Tilney, 1975; Bullitt et al., 1988; Sanders et al., 1996; Sun et al., 1997). Recent work showed (Reed J.Kelso *et al*., 2002) that phosphorylation of Kelch is necessary for normal filament organization and that a major

cytoskeletal target of Src 64 (a D*rosophila* homolog of Src signalling at the ring canal is the actin-cross-linking protein Kelch.

Src family protein tyrosine kinases were first identified as transforming proteins in avian tumor viruses. Src kinases play a role in many cellular functions, including regulation of proliferation, differentiation, and F-actin organization. Mutations in the *Src 64,* produce flies which are fully viable but the females are sterile. The female sterility of *Src64* mutant flies is due to a specific defect in ring canal growth. Ring canals from *Src64* mutant egg chambers are smaller than wild-type ring canals. In addition, the intense phosphotyrosine labeling present in wild-type ring canals is eliminated in *Src64* mutants. All other aspects of ring canal morphology appeared to be normal in *Src64* mutants. (Hudson & cooley, 2002).

The actin-binding affinity of Kelch is regulated by phosphorylation. When the tyrosine residue in the position 627 in the 5th KREP repeat of Kelch was changed to alanine (Kel^{Y627A}) it resulted in a phenotype identical to *Src64.* Kel^{Y627A} is a non-phosphorylatable form of Kelch. Furthermore, when the phosphorylation state of Kelch in *Src64* mutant ovaries was examined, only the non-phosphorylated form was detected. These observations indicated that first, Kelch phosphorylation is downstream of Src64 activity, either directly or indirectly and second, Kelch appears to be the only significant cytoskeletal effector of Src64 at ring canals, as expression of KelY627A reproduces all of the phenotypes associated with loss of *Src64* function. The similar phenotype of *Tec29* germline clones suggests that it could function as a signaling intermediate between Src64 and Kelch. (Reed J.Kelso *et al*., 2002)

We performed a 2D electrophoresis of ovary lysates from wild type and *imp-α2^{D14}* backgrounds to examine the Kelch phosphorylation states. There was a major difference seen in the number of isoforms of Kelch in both wild type and *imp-α2^{D14}*. A shift towards the anode was also noticed in *imp-α2^{D14}* ovaries when compared to wild type. Though these

results need to be confirmed this is an indication of changes in Kelch phosphorylation states when Imp-α2 is inactivated.

An additional important question for future research is the identification of the kinase (s) and phosphatase(s) responsible for balancing the phosphorylation/dephosphorlytion state of Kelch.

C. Regulation of Kelch by Importin-α2:

In eukaryotic cells, synthesis of RNA and protein is confined to the nucleus and the cytoplasm, respectively. The nucleocytoplasmic transport essentially includes three major players, Imp-α, Imp-β, and Ran. In the cytoplasm the Importin-α adaptor protein recognizes cargo proteins containing a Nuclear Localization Signal (NLS) peptide and forms a heterotrimeric complex with Importin-β. These complexes then translocate into the nucleus and the RanGTP/RanGDP concentration gradient between the nucleus and the cytoplasm orchestrates the nuclear import and export of macromolecules.

However, the Importins along with Ran exert some other functions in eliciting microtubule polymerization, spindle assembly and nuclear envelope assembly following mitosis.

One of the critical functions displayed by Importin-α2 during oogenesis deals with the completion of the ring canals. The egg chambers in *imp-α2*^{D14} ovaries display a dumpless phenotype. When the known components of the ring canals were examined in the *imp-α2*^{D14} mutant ring canals, the Kelch protein was found to be missing from the ring canals although it is produced in similar amount as in wild type ovaries. Imp-α2 protein forms no visible association with the ring canals but is responsible for the localization of Kelch to the ring canals.

The structural similarity between *kelch* and *imp-α2*^{D14} ring canals as well as the absence of Kelch, from *imp-α2*^{D14} mutant ring canals indicate that the obstruction of the ring canals in *imp-α2*^{D14} and *kelch* may have a common cause. Hence it can be inferred that the organization of actin filaments and associated proteins in the ring canals depends on Kelch association with these structures. Since Kelch is present in the cytoplasm of *imp-α2*^{D14} nurse cells, we can conclude that the *imp- α2* gene exerts no control on the expression of *kelch* gene. However, the *imp-α2* function, and thus the Imp-α2 protein, is required for the correct localization of Kelch on the Ring Canals. Therefore Kelch is a downstream target of Importin-α2 and Imp-α2 plays a role in the Kelch access to the ring canals.

However, the distribution of Imp-α2 in *kel*$^{\Delta}$ egg chambers was also found to be altered. Imp-α2 was associated with the nuclear membrane of the nurse cells along with the cytoplasm of nurse cells and the cortex of the oocyte, suggesting that a full localization of Imp-α2 in the cytoplasm may depend on Kelch.

Based on the above considerations, Kelch appears to be an obvious candidate for being transported to the ring canals by Imp-α2. However, the absence of Nuclear Localisation Signal or a stretch of basic residues suggests that Kelch may not directly bind to Imp-α2. Thus, we can envisage that Imp-α2 mediates the transportation of Kelch to the ring canals through an unknown intermediary component which is able to link Kelch to Imp-α2. Recently a subset of KREP proteins was shown to function as substrate adaptor proteins (Andrew M.Hudson and Lyn Cooley, 2010). Their results also further confirm that BTB-Kelch proteins function as substrate adaptors for Cullin-RING ligase. Hence, Kelch functions as an adaptor protein and could possibly bind to the intermediary protein which binds both Kelch and Imp-α2.

D. Imp-β could be the mediator between Imp-α2 and Kelch during oogenesis:

Imp-α2 may independently carry a Kelch–binding factor to the ring canals or mediate its association with Kelch in the cytoplasm, in this way indirectly targeting Kelch onto the assembling ring canal. It is also possible that Imp-α2 exerts an anti-aggregation activity on a component which binds Kelch and sequesters it in the cytoplasm.

The model we propose, shows that Imp-α2 competes with Kelch to bind to Imp-β. Just the way Imp-α2 binds to Imp-β through its IBB domain during the nucleocytoplasmic transport We expect the same kind of binding of both proteins during oogenesis. Our results show a direct physical interaction between Imp-β and Kelch. Therefore we propose that Imp-β can bind both Imp-α2 and Kelch.

The phenotype that was detected in the ovaries derived from females with *P-{UAS-imp-α2$^{\Delta IBB}$}* transgene expressed in *imp-α2^{D14}/kel$^{\Delta}$* background could be explained by the inability of Imp-β to bind to Imp-α2. These females bear ovaries with degenerating egg chambers with occluded ring canals. This phenotype could be explained by the above proposed model. Since the Imp-β binding domain is deleted in this trangene, Imp-β can no longer bind to Imp-α2 due to the inability of Imp-β to release Kelch. When Kelch is bound to Imp-β it is unable to localize to the ring canals and hence the ring canals remain occluded with actin. The genetic combination *imp-α2^{D14}/kel$^{\Delta}$; P-{UAS-imp-α2$^{\Delta IBB}$}* is a hemizygous condition for both *kelch* and *imp-α2*, i.e there is still one wild type copy of each of the genes available. This could explain why the egg chambers were able to develop until a certain period of time and then eventually degenerate. This phenotype is similar to that of *imp-α2^{D14}* females with occluded ring canals.

In the case of *imp-α2^{D14}/kel$^{\Delta}$;UAS-impa2^{NLSB}/p-{nos-Gal4}* females, oogenesis was not blocked but the development of the laid eggs was arrested at early stages. This result is expected according to the model proposed. The NLSB domain of Imp-α2 is the domain through which cargo proteins bind to Imp-α2. In these females the IBB domain is not mutated and facilitates the binding of Imp-β to Imp-α2. Kelch protein is free to localize to the ring canals and perform its function of clearing the lumen of the ring canals. Hence there is no occlusion of ring canals and complete oogenesis takes place. These females are normal at this stage of development.

E. Gene dosage (threshold) is responsible for the ovarian phenotype:

It was previously reported that the *impa2^{NLSB-}* and *imp-α2^{CASB-}* mutant proteins expressed in *imp-α2^{D14}* homozygous background were unable to undertake normal oogenesis. The eggs that were laid were in similar amount and shape, as in case of *imp-α2^{D14}* flies whereas the *imp-α2^{D14}/imp-α2^{D14}; imp-α2$^{\Delta IBB}$/p-{nos-Gal4}* females were able to lay nearly the same number of eggs as wild type females though only 14% of them displayed normal morphology and 86% showed eggs with fused dorsal appendages (Gorjánácz *et al*., 2006).

In our case, an ovarian phenotype can be seen in *imp-α2^{D14}/kel$^{\Delta}$* hemizygote carrying the *imp-α2$^{\Delta IBB}$* transgene and embryonic phenotypes were shown by the *impa2^{NLSB}* and *imp-α2^{CASB}* mutant proteins expressed in the same hemizygous background.

In *imp-α2^{D14}/ imp-α2^{D14}; imp-α2$^{\Delta IBB}$ / p-{nos-Gal4}* females, wild type Imp-α2 is completely absent and the only copy of Imp-α2 present is from the transgene which is defective in the IBB domain. But the amount of Kelch is present in similar amount as in wild type flies. Imp-β binds both Kelch and Imp-α2 in a particular threshold amount thereby regulating their functions. In *imp-α2^{D14}/imp-α2^{D14}; imp-α2$^{\Delta IBB}$/p-{nos-Gal4}* flies the binding of Imp-β to

Imp-α2 is impaired resulting in the binding of Imp-β to Kelch. But as all of Kelch is unable to be bound by Imp-β some amount of Kelch can be associated to the ring canals. This amount of free Kelch is sufficient to prevent occlusion of the ring canals and to complete oogenesis .This could suggest that the *imp-α2*$^{\Delta IBB}$. In the case of *imp-α2*^{D14}*/kel*$^{\Delta}$*; P-{UAS-imp-α2*$^{\Delta IBB}$*}* females even the amount of Kelch is reduced to half. Hence there is no free Kelch unbound to Imp-β which could reach the ring canals and perform it's function. This means the *imp-α2*$^{\Delta IBB}$ transgene becomes toxic in the presence of one copy of Imp-α2.

In *imp-α2*^{D14}*/ imp-α2*^{D14}*; UAS-impa2*$^{NLSB-}$*/p-{nos-Gal4} and imp-α2*^{D14}*/ imp-α2*^{D14}*; UAS-impa2*$^{CASB-}$*/p-{nos-Gal4}* females the amount of wild type Imp-α2 protein is reduced to zero. The *impa2*$^{NLSB-}$ transgene that is introduced carries point mutations in the NLSB domain and *impa2*$^{CASB-}$ transgene in the CASB domain. Both the transgenes have a normal IBB domain which could facilitate the binding of Imp-β. In these flies Imp-β can bind both Kelch and the trangenes. But since there is no wild type copy of Imp-α2, Imp-β cannot bind much to Imp-α2 and hence is mostly bound to the Kelch thereby not letting Kelch free to bind to the ring canals.

In our case we have introduced these transgenes in a *imp-α2*^{D14}*/kel*$^{\Delta}$ background, in which the amount of Kelch and Imp-α2 is reduced to half. Imp-β can bind to both the wild type copy of Imp-α2 and the abnormal protein made by the transgenes. Since the amount of Imp-α2 is more than Kelch, Imp-β is mostly bound to Imp-α2 and hence Kelch is free to bind to the ring canals. Therefore, these females are able to complete oogenesis.

With the above considerations we propose that a particular phenotype that is seen could be due to the cumulative effect of all the genes and their dosages.

F.Embryonic Phenotype of *kel$^{\Delta}$ / imp-α2^{D14}; UAS-impa2^{NLSB-}/p-{nos-Gal4}:*

kel$^{\Delta}$ / imp-α2^{D14}; UAS-impa2^{NLSB-}/p-{nos-Gal4} females could manage to complete oogenesis but the eggs that were laid by them could not hatch. This was due an arrest in the early development of the egg. Various mitotic defects were noticed. The model that we propose is not sufficient to explain the defects seen during embryogenesis because various other components like Ran are involved in this process. However, Kelch could a major role during embryogenesis along with Imp-β and Imp-α2. Various spindle defects include excessive formation of spindles and centrosomes thereby changing the morphology of a spindle. It is known that Imp-β and Imp-α2 bind to the spindle assembly factors and keep them sequestered until they are needed during the spindle assembly. It could be possible that Kelch somehow plays a role in regulating the release of these factors. When the amount of Kelch is reduced in *kel$^{\Delta}$ / imp-α2^{D14}; UAS-impa2^{NLSB-}/p-{nos-Gal4}* females the regulation of these factors is disturbed and an untimely release happens. The excessive activity of these factors could be the reason for the malformed spindles. Kelch could also play an essential role in the metaphase to anaphase transition as most of the spindles were arrested at metaphase. Further investigations of mutation in the domains causing arrest during early embryogenesis could provide further information on the role played by Kelch.

Examination of these eggs revealed that only mitosis was affected but not meiosis. The inactivation of both Kelch and Imp-α2 caused no obstruction to the female meiosis which was indicated by the occurrence of polar bodies in the eggs laid by these mutant females. Interestingly, we observed a particularly strong enrichment of Kelch in both wild-type and mutant polar bodies whereas the amount of Kelch associated with the mitotic spindle was markedly reduced in *imp-α2^{D14}/kel$^{\Delta}$; P-{UAS-imp-α2^{NLSB-}}/P-{nos-Gal4}*embryos by

comparison to wild-type. Therefore, Kelch probably plays a role only in mitosis during early embryogenesis.

G. Ring canal proteins in Embryogenesis:

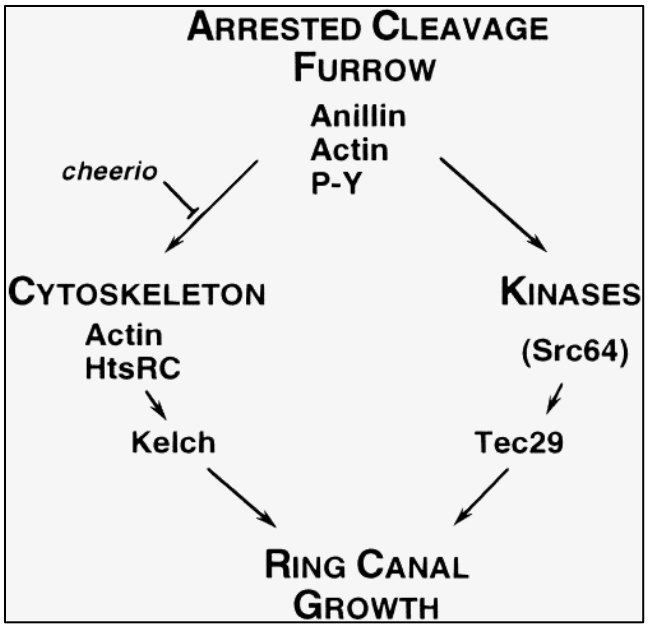
Based on our data and previous findings, it is possible that the proteins that play a major role during oogenesis could also also play an essential role during later stages of development.

Imp-α2 plays a critical role in both oogenesis and embryogenesis. Since the inactivation of the genes encoding these proteins cause sterility and don't lay eggs it is difficult to study their role during later stages. In our work, we have found two such genes *kelch* and *chickadee* whose products could be very critical in the nuclear divisions of the egg. Both the proteins are found to decorate the spindles and might be responsible for managing the spindle dynamics. These results need to be confirmed and further work has to be done to find out the exact domains that are needed for this function. Such studies could be also be achieved by changing the gene dosages.

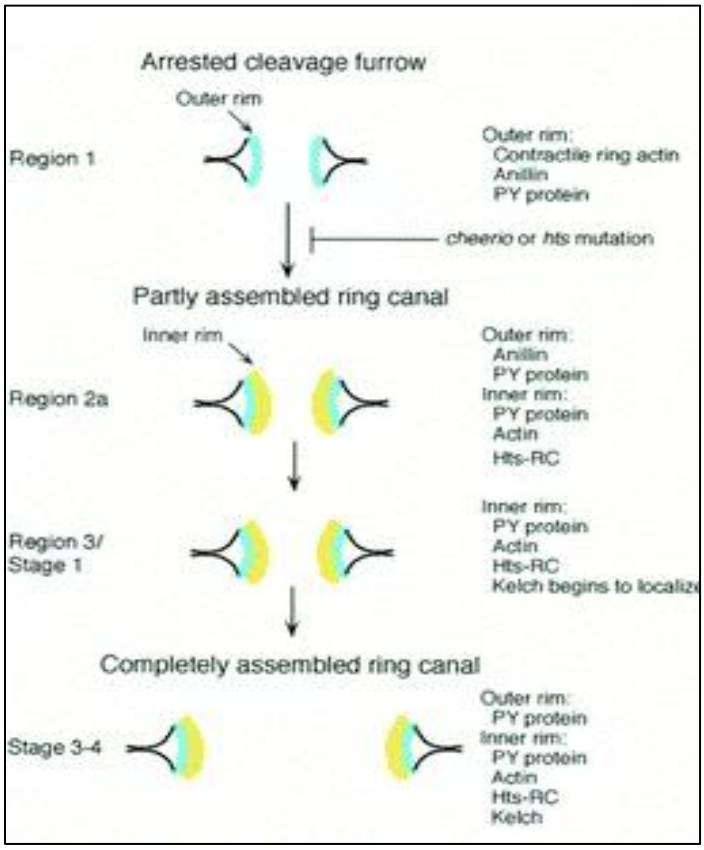
Appendix

Ring canal assembly and function:

The nurse cells and the oocyte of each egg chamber are sibling germline cells derived from a single precursor through four rounds of mitosis. The mitotic cleavage furrows in these divisions halt mysteriously before daughter cells are separated. These arrested cleavage furrows are further transformed into ring canals with a lining of actin cytoskeleton. These actin structures provide a unique opportunity to study the function of the cytoskeleton and its associated proteins because of their nearly perfect ring shape. Any disturbance in either the assembly or growth of the ring canals are revealed in the morphology of the ring canal and are visible with a light microscope. Assembly of wild type ring canals occurs through the sequential addition of a number of cytoskeletal proteins to the arrested cleavage furrows (figure 21). Two parallel pathways (Lynn Cooley, 1998) have been suggested for the assembly of the ring canal proteins (*figure 20*). Actin filaments accumulate along with HtsRC protein, followed by the addition of Kelch proteins. Before the accumulation of actin, proteins that are recognized by antibodies to phosphotyrosine appear. During cytokinesis, the contractile ring contains the cleavage furrow protein anillin (Field & Alberts 1995) and contractile actin filaments (Robinson *et al.,* 1997).After the third mitotic division, at least one protein immunoreactive with anti-phosphotyrosine antibodies localizes to the outer rims of nascent ring canals. After the fourth mitotic division and the movement of the 16 celled cyst into region 2a of the germarium, a product of *hu-li tai shao (hts)* gene, called hts-RC, and additional actin filaments localize to the cleavage furrow membrane, forming a robust actin-rich inner rim (Warn *et al.,* 1985, Robinson *et al*.1994). In region 3 (stage 1 egg chambers), Kelch proteins begin to the inner rims of the ring canals (Xue & Cooley 1993, Robinson et al 1994). A parallel pathway of actin recruitment involves Src and Tec kinases.



***Drosophila* ovarian ring canal development** (lynn cooley, 1998)



Pathway of ring canal assembly (Robinson & Cooley, 1997)

Bibliography

Adams, J., R. Kelso and L. Cooley (2000). "The kelch repeat superfamily of proteins: propellers of cell function." Trends Cell Biol **10**(1): 17-24.

Adams, R. R., A. A. Tavares, A. Salzberg, H. J. Bellen and D. M. Glover (1998). "pavarotti encodes a kinesin-like protein required to organize the central spindle and contractile ring for cytokinesis." Genes Dev **12**(10): 1483-94.

Andrade, M. A., C. Petosa, S. I. O'Donoghue, C. W. Muller and P. Bork (2001). "Comparison of ARM and HEAT protein repeats." J Mol Biol **309**(1): 1-18.

Bastock, R. and D. St Johnston (2008). "Drosophila oogenesis." Curr Biol **18**(23): R1082-7.

Baum, J. S., E. Arama, H. Steller and K. McCall (2007). "The Drosophila caspases Strica and Dronc function redundantly in programmed cell death during oogenesis." Cell Death Differ **14**(8): 1508-17.

Berg, C. A. (2005). "The Drosophila shell game: patterning genes and morphological change." Trends Genet **21**(6): 346-55.

Bolkan, B. J., R. Booker, M. L. Goldberg and D. A. Barbash (2007). "Developmental and cell cycle progression defects in Drosophila hybrid males." Genetics **177**(4): 2233-41.

Bonaccorsi, S., M. G. Giansanti and M. Gatti (2000). "Spindle assembly in Drosophila neuroblasts and ganglion mother cells." Nat Cell Biol **2**(1): 54-6.

Braybrook, C., G. Warry, G. Howell, A. Arnason, A. Bjornsson, G. E. Moore, M. T. Ross and P. Stanier (2001). "Identification and characterization of KLHL4, a novel human homologue of the Drosophila Kelch gene that maps within the X-linked cleft palate and Ankyloglossia (CPX) critical region." Genomics **72**(2): 128-36.

Buszczak, M. and L. Cooley (2000). "Eggs to die for: cell death during Drosophila oogenesis." Cell Death Differ **7**(11): 1071-4.

Chook, Y. M. and G. Blobel (2001). "Karyopherins and nuclear import." Curr Opin Struct Biol **11**(6): 703-15.

Conti, E., C. W. Muller and M. Stewart (2006). "Karyopherin flexibility in nucleocytoplasmic transport." Curr Opin Struct Biol **16**(2): 237-44.

Cooley, L. (1998). "Drosophila ring canal growth requires Src and Tec kinases." Cell **93**(6): 913-5.

Cooley, L. and W. E. Theurkauf (1994). "Cytoskeletal functions during Drosophila oogenesis." Science **266**(5185): 590-6.

Cooley, L., E. Verheyen and K. Ayers (1992). "chickadee encodes a profilin required for intercellular cytoplasm transport during Drosophila oogenesis." Cell **69**(1): 173-84.

Djagaeva, I., S. Doronkin and S. K. Beckendorf (2005). "Src64 is involved in fusome development and karyosome formation during Drosophila oogenesis." Dev Biol **284**(1): 143-56.

Dobbelaere, J., F. Josue, S. Suijkerbuijk, B. Baum, N. Tapon and J. Raff (2008). "A genome-wide RNAi screen to dissect centriole duplication and centrosome maturation in Drosophila." PLoS Biol **6**(9): e224.

Dodson, G. S., D. J. Guarnieri and M. A. Simon (1998). "Src64 is required for ovarian ring canal morphogenesis during Drosophila oogenesis." Development **125**(15): 2883-92.

Donaldson, T. D., M. A. Noureddine, P. J. Reynolds, W. Bradford and R. J. Duronio (2004). "Targeted disruption of Drosophila Roc1b reveals functional differences in the Roc subunit of Cullin-dependent E3 ubiquitin ligases." Mol Biol Cell **15**(11): 4892-903.

Emanuele, M. J., W. Lan, M. Jwa, S. A. Miller, C. S. Chan and P. T. Stukenberg (2008). "Aurora B kinase and protein phosphatase 1 have opposing roles in modulating kinetochore assembly." J Cell Biol **181**(2): 241-54.

Giansanti, M. G., E. Bucciarelli, S. Bonaccorsi and M. Gatti (2008). "Drosophila SPD-2 is an essential centriole component required for PCM recruitment and astral-microtubule nucleation." Curr Biol **18**(4): 303-9.

Giarre, M., I. Torok, R. Schmitt, M. Gorjanacz, I. Kiss and B. M. Mechler (2002). "Patterns of importin-alpha expression during Drosophila spermatogenesis." J Struct Biol **140**(1-3): 279-90.

Giot, L., J. S. Bader, C. Brouwer, A. Chaudhuri, B. Kuang, Y. Li, Y. L. Hao, C. E. Ooi, B. Godwin, E. Vitols, G. Vijayadamodar, P. Pochart, H. Machineni, M. Welsh, Y. Kong, B. Zerhusen, R. Malcolm, Z. Varrone, A. Collis, M. Minto, S. Burgess, L. McDaniel, E. Stimpson, F. Spriggs, J. Williams, K. Neurath, N. Ioime, M. Agee, E. Voss, K. Furtak, R. Renzulli, N. Aanensen, S. Carrolla, E. Bickelhaupt, Y. Lazovatsky, A. DaSilva, J. Zhong, C. A. Stanyon, R. L. Finley, Jr., K. P. White, M. Braverman, T. Jarvie, S. Gold, M. Leach, J. Knight, R. A. Shimkets, M. P. McKenna, J. Chant and J. M. Rothberg (2003). "A protein interaction map of Drosophila melanogaster." Science **302**(5651): 1727-36.

Goldfarb, D. S., A. H. Corbett, D. A. Mason, M. T. Harreman and S. A. Adam (2004). "Importin alpha: a multipurpose nuclear-transport receptor." Trends Cell Biol **14**(9): 505-14.

Golic, K. G. and M. M. Golic (1996). "Engineering the Drosophila genome: chromosome rearrangements by design." Genetics **144**(4): 1693-711.

Gorjanacz, M., G. Adam, I. Torok, B. M. Mechler, T. Szlanka and I. Kiss (2002). "Importin-alpha 2 is critically required for the assembly of ring canals during Drosophila oogenesis." Dev Biol **251**(2): 271-82.

Gorjanacz, M., I. Torok, I. Pomozi, G. Garab, T. Szlanka, I. Kiss and B. M. Mechler (2006). "Domains of Importin-alpha2 required for ring canal assembly during Drosophila oogenesis." J Struct Biol **154**(1): 27-41.

Goshima, G., R. Wollman, S. S. Goodwin, N. Zhang, J. M. Scholey, R. D. Vale and N. Stuurman (2007). "Genes required for mitotic spindle assembly in Drosophila S2 cells." Science **316**(5823): 417-21.

Guarnieri, D. J., G. S. Dodson and M. A. Simon (1998). "SRC64 regulates the localization of a Tec-family kinase required for Drosophila ring canal growth." Mol Cell **1**(6): 831-40.

Gupta-Rossi, N., S. Storck, P. J. Griebel, C. A. Reynaud, J. C. Weill and A. Dahan (2003). "Specific

over-expression of deltex and a new Kelch-like protein in human germinal center B cells." Mol Immunol **39**(13): 791-9.

Hudson, A. M. and L. Cooley (2002). "Understanding the function of actin-binding proteins through genetic analysis of Drosophila oogenesis." Annu Rev Genet **36**: 455-88.

Hudson, A. M. and L. Cooley (2010). "Drosophila Kelch functions with Cullin-3 to organize the ring canal actin cytoskeleton." J Cell Biol **188**(1): 29-37.

Hynes, R. O. and Q. Zhao (2000). "The evolution of cell adhesion." J Cell Biol **150**(2): F89-96.

Jackson, S. M. and C. A. Berg (1999). "Soma-to-germline interactions during Drosophila oogenesis are influenced by dose-sensitive interactions between cut and the genes cappuccino, ovarian tumor and agnostic." Genetics **153**(1): 289-303.

Johansen, J. and K. M. Johansen (2009). "The spindle matrix through the cell cycle in Drosophila." Fly (Austin) **3**(3): 213-20.

Kelso, R. J., A. M. Hudson and L. Cooley (2002). "Drosophila Kelch regulates actin organization via Src64-dependent tyrosine phosphorylation." J Cell Biol **156**(4): 703-13.

Kussel, P. and M. Frasch (1995). "Pendulin, a Drosophila protein with cell cycle-dependent nuclear localization, is required for normal cell proliferation." J Cell Biol **129**(6): 1491-507.

Lee, B. J., A. E. Cansizoglu, K. E. Suel, T. H. Louis, Z. Zhang and Y. M. Chook (2006). "Rules for nuclear localization sequence recognition by karyopherin beta 2." Cell **126**(3): 543-58.

Lee, T., A. Lee and L. Luo (1999). "Development of the Drosophila mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast." Development **126**(18): 4065-76.

Lonhienne, T. G., J. K. Forwood, M. Marfori, G. Robin, B. Kobe and B. J. Carroll (2009). "Importin-beta is a GDP-to-GTP exchange factor of Ran: implications for the mechanism of nuclear import." J Biol Chem **284**(34): 22549-58.

Lu, N., D. J. Guarnieri and M. A. Simon (2004). "Localization of Tec29 to ring canals is mediated by Src64 and PtdIns(3,4,5)P3-dependent mechanisms." EMBO J **23**(5): 1089-100.

Mahowald, A. P. (1971). "The formation of ring canals by cell furrows in Drosophila." Z Zellforsch Mikrosk Anat **118**(2): 162-7.

Mai, A., S. K. Jung and S. Yonehara (2004). "hDKIR, a human homologue of the Drosophila kelch protein, involved in a ring-like structure." Exp Cell Res **300**(1): 72-83.

Malik, H. S., T. H. Eickbush and D. S. Goldfarb (1997). "Evolutionary specialization of the nuclear targeting apparatus." Proc Natl Acad Sci U S A **94**(25): 13738-42.

Maresca, T. J., A. C. Groen, J. C. Gatlin, R. Ohi, T. J. Mitchison and E. D. Salmon (2009). "Spindle assembly in the absence of a RanGTP gradient requires localized CPC activity." Curr Biol **19**(14): 1210-5.

Mason, D. A., D. E. Stage and D. S. Goldfarb (2009). "Evolution of the metazoan-specific importin alpha gene family." J Mol Evol **68**(4): 351-65.

Mathe, E., H. Bates, H. Huikeshoven, P. Deak, D. M. Glover and S. Cotterill (2000). "Importin-alpha3 is required at multiple stages of Drosophila development and has a role in the completion of

oogenesis." Dev Biol **223**(2): 307-22.

Matsuura, Y. and M. Stewart (2004). "Structural basis for the assembly of a nuclear export complex." Nature **432**(7019): 872-7.

Mayer-Jaekel, R. E. and B. A. Hemmings (1995). "Role of protein phosphatase 2A in Drosophila development." Semin Cancer Biol **6**(4): 249-56.

Mazzalupo, S. and L. Cooley (2006). "Illuminating the role of caspases during Drosophila oogenesis." Cell Death Differ **13**(11): 1950-9.

McNeil, G. P., F. Smith and R. Galioto (2004). "The Drosophila RNA-binding protein Lark is required for the organization of the actin cytoskeleton and Hu-li tai shao localization during oogenesis." Genesis **40**(2): 90-100.

Millward, T. A., S. Zolnierowicz and B. A. Hemmings (1999). "Regulation of protein kinase cascades by protein phosphatase 2A." Trends Biochem Sci **24**(5): 186-91.

Minakhina, S., R. Myers, M. Druzhinina and R. Steward (2005). "Crosstalk between the actin cytoskeleton and Ran-mediated nuclear transport." BMC Cell Biol **6**: 32.

Minestrini, G., A. S. Harley and D. M. Glover (2003). "Localization of Pavarotti-KLP in living Drosophila embryos suggests roles in reorganizing the cortical cytoskeleton during the mitotic cycle." Mol Biol Cell **14**(10): 4028-38.

Minestrini, G., E. Mathe and D. M. Glover (2002). "Domains of the Pavarotti kinesin-like protein that direct its subcellular distribution: effects of mislocalisation on the tubulin and actin cytoskeleton during Drosophila oogenesis." J Cell Sci **115**(Pt 4): 725-36.

Nicolai, M., C. Lasbleiz and J. M. Dura (2003). "Gain-of-function screen identifies a role of the Src64 oncogene in Drosophila mushroom body development." J Neurobiol **57**(3): 291-302.

O'Reilly, A. M., A. C. Ballew, B. Miyazawa, H. Stocker, E. Hafen and M. A. Simon (2006). "Csk differentially regulates Src64 during distinct morphological events in Drosophila germ cells." Development **133**(14): 2627-38.

Perrimon, N. (1998). "Creating mosaics in Drosophila." Int J Dev Biol **42**(3): 243-7.

Pintard, L., J. H. Willis, A. Willems, J. L. Johnson, M. Srayko, T. Kurz, S. Glaser, P. E. Mains, M. Tyers, B. Bowerman and M. Peter (2003). "The BTB protein MEL-26 is a substrate-specific adaptor of the CUL-3 ubiquitin-ligase." Nature **425**(6955): 311-6.

Prag, S. and J. C. Adams (2003). "Molecular phylogeny of the kelch-repeat superfamily reveals an expansion of BTB/kelch proteins in animals." BMC Bioinformatics **4**: 42.

Prag, S., A. De Arcangelis, E. Georges-Labouesse and J. C. Adams (2007). "Regulation of post-translational modifications of muskelin by protein kinase C." Int J Biochem Cell Biol **39**(2): 366-78.

Qi, H., U. Rath, D. Wang, Y. Z. Xu, Y. Ding, W. Zhang, M. J. Blacketer, M. R. Paddy, J. Girton, J. Johansen and K. M. Johansen (2004). "Megator, an essential coiled-coil protein that localizes to the putative spindle matrix during mitosis in Drosophila." Mol Biol Cell **15**(11): 4854-65.

Ratan, R., D. A. Mason, B. Sinnot, D. S. Goldfarb and R. J. Fleming (2008). "Drosophila importin alpha1 performs paralog-specific functions essential for gametogenesis." Genetics **178**(2): 839-50.

Robinson, D. N., K. Cant and L. Cooley (1994). "Morphogenesis of Drosophila ovarian ring canals." Development **120**(7): 2015-25.

Robinson, D. N. and L. Cooley (1997). "Drosophila kelch is an oligomeric ring canal actin organizer." J Cell Biol **138**(4): 799-810.

Robinson, D. N. and L. Cooley (1997). "Examination of the function of two kelch proteins generated by stop codon suppression." Development **124**(7): 1405-17.

Robinson, D. N. and L. Cooley (1997). "Genetic analysis of the actin cytoskeleton in the Drosophila ovary." Annu Rev Cell Dev Biol **13**: 147-70.

Robinson, D. N., T. A. Smith-Leiker, N. S. Sokol, A. M. Hudson and L. Cooley (1997). "Formation of the Drosophila ovarian ring canal inner rim depends on cheerio." Genetics **145**(4): 1063-72.

Rorth, P. (1998). "Gal4 in the Drosophila female germline." Mech Dev **78**(1-2): 113-8.

Roulier, E. M., S. Panzer and S. K. Beckendorf (1998). "The Tec29 tyrosine kinase is required during Drosophila embryogenesis and interacts with Src64 in ring canal development." Mol Cell **1**(6): 819-29.

Ryder, E., M. Ashburner, R. Bautista-Llacer, J. Drummond, J. Webster, G. Johnson, T. Morley, Y. S. Chan, F. Blows, D. Coulson, G. Reuter, H. Baisch, C. Apelt, A. Kauk, T. Rudolph, M. Kube, M. Klimm, C. Nickel, J. Szidonya, P. Maroy, M. Pal, A. Rasmuson-Lestander, K. Ekstrom, H. Stocker, C. Hugentobler, E. Hafen, D. Gubb, G. Pflugfelder, C. Dorner, B. Mechler, H. Schenkel, J. Marhold, F. Serras, M. Corominas, A. Punset, J. Roote and S. Russell (2007). "The DrosDel deletion collection: a Drosophila genomewide chromosomal deficiency resource." Genetics **177**(1): 615-29.

Ryder, E., F. Blows, M. Ashburner, R. Bautista-Llacer, D. Coulson, J. Drummond, J. Webster, D. Gubb, N. Gunton, G. Johnson, C. J. O'Kane, D. Huen, P. Sharma, Z. Asztalos, H. Baisch, J. Schulze, M. Kube, K. Kittlaus, G. Reuter, P. Maroy, J. Szidonya, A. Rasmuson-Lestander, K. Ekstrom, B. Dickson, C. Hugentobler, H. Stocker, E. Hafen, J. A. Lepesant, G. Pflugfelder, M. Heisenberg, B. Mechler, F. Serras, M. Corominas, S. Schneuwly, T. Preat, J. Roote and S. Russell (2004). "The DrosDel collection: a set of P-element insertions for generating custom chromosomal aberrations in Drosophila melanogaster." Genetics **167**(2): 797-813.

Sabri, N., P. Roth, N. Xylourgidis, F. Sadeghifar, J. Adler and C. Samakovlis (2007). "Distinct functions of the Drosophila Nup153 and Nup214 FG domains in nuclear protein transport." J Cell Biol **178**(4): 557-65.

Santamaria, A., S. Nagel, H. H. Sillje and E. A. Nigg (2008). "The spindle protein CHICA mediates localization of the chromokinesin Kid to the mitotic spindle." Curr Biol **18**(10): 723-9.

Silverman-Gavrila, R. V., K. G. Hales and A. Wilde (2008). "Anillin-mediated targeting of peanut to pseudocleavage furrows is regulated by the GTPase Ran." Mol Biol Cell **19**(9): 3735-44.

Silverman-Gavrila, R. V. and A. Wilde (2006). "Ran is required before metaphase for spindle assembly and chromosome alignment and after metaphase for chromosome segregation and spindle midbody organization." Mol Biol Cell **17**(4): 2069-80.

Sokol, N. S. and L. Cooley (1999). "Drosophila filamin encoded by the cheerio locus is a component of ovarian ring canals." Curr Biol **9**(21): 1221-30.

Sokol, N. S. and L. Cooley (2003). "Drosophila filamin is required for follicle cell motility during

oogenesis." Dev Biol **260**(1): 260-72.

Soltysik-Espanola, M., R. A. Rogers, S. Jiang, T. A. Kim, R. Gaedigk, R. A. White, H. Avraham and S. Avraham (1999). "Characterization of Mayven, a novel actin-binding protein predominantly expressed in brain." Mol Biol Cell **10**(7): 2361-75.

Stogios, P. J. and G. G. Prive (2004). "The BACK domain in BTB-kelch proteins." Trends Biochem Sci **29**(12): 634-7.

Szabad, J., M. Erdelyi, G. Hoffmann, J. Szidonya and T. R. Wright (1989). "Isolation and characterization of dominant female sterile mutations of Drosophila melanogaster. II. Mutations on the second chromosome." Genetics **122**(4): 823-35.

Szlanka, T., L. Haracska, I. Kiss, P. Deak, E. Kurucz, I. Ando, E. Viragh and A. Udvardy (2003). "Deletion of proteasomal subunit S5a/Rpn10/p54 causes lethality, multiple mitotic defects and overexpression of proteasomal genes in Drosophila melanogaster." J Cell Sci **116**(Pt 6): 1023-33.

Takahashi, M., F. Takahashi, K. Ui-Tei, T. Kojima and K. Saigo (2005). "Requirements of genetic interactions between Src42A, armadillo and shotgun, a gene encoding E-cadherin, for normal development in Drosophila." Development **132**(11): 2547-59.

Thomas, J. H. and E. Wieschaus (2004). "src64 and tec29 are required for microfilament contraction during Drosophila cellularization." Development **131**(4): 863-71.

Tilney, L. G., M. S. Tilney and G. M. Guild (1996). "Formation of actin filament bundles in the ring canals of developing Drosophila follicles." J Cell Biol **133**(1): 61-74.

Timinszky, G., L. Tirian, F. T. Nagy, G. Toth, A. Perczel, Z. Kiss-Laszlo, I. Boros, P. R. Clarke and J. Szabad (2002). "The importin-beta P446L dominant-negative mutant protein loses RanGTP binding ability and blocks the formation of intact nuclear envelope." J Cell Sci **115**(Pt 8): 1675-87.

Torok, I., D. Herrmann-Horle, I. Kiss, G. Tick, G. Speer, R. Schmitt and B. M. Mechler (1999). "Down-regulation of RpS21, a putative translation initiation factor interacting with P40, produces viable minute imagos and larval lethality with overgrown hematopoietic organs and imaginal discs." Mol Cell Biol **19**(3): 2308-21.

Toure, A., R. Mzali, C. Liot, L. Seguin, L. Morin, C. Crouin, I. Chen-Yang, Y. G. Tsay, O. Dorseuil, G. Gacon and J. Bertoglio (2008). "Phosphoregulation of MgcRacGAP in mitosis involves Aurora B and Cdk1 protein kinases and the PP2A phosphatase." FEBS Lett **582**(8): 1182-8.

Velentzas, A. D., I. P. Nezis, D. J. Stravopodis, I. S. Papassideri and L. H. Margaritis (2007). "Apoptosis and autophagy function cooperatively for the efficacious execution of programmed nurse cell death during Drosophila virilis oogenesis." Autophagy **3**(2): 130-2.

Verheyen, E. M. and L. Cooley (1994). "Profilin mutations disrupt multiple actin-dependent processes during Drosophila development." Development **120**(4): 717-28.

Villanyi, Z., A. Debec, G. Timinszky, L. Tirian and J. Szabad (2008). "Long persistence of importin-beta explains extended survival of cells and zygotes that lack the encoding gene." Mech Dev **125**(3-4): 196-206.

Villanyi, Z., B. Papp, S. Szikora, I. Boros and J. Szabad (2008). "The DRE motif is a key component in the expression regulation of the importin-beta encoding Ketel gene in Drosophila." Mech Dev **125**(9-10): 822-31.

Walker, D. L., D. Wang, Y. Jin, U. Rath, Y. Wang, J. Johansen and K. M. Johansen (2000). "Skeletor, a novel chromosomal protein that redistributes during mitosis provides evidence for the formation of a spindle matrix." J Cell Biol **151**(7): 1401-12.

Warn, R. M., H. O. Gutzeit, L. Smith and A. Warn (1985). "F-actin rings are associated with the ring canals of the Drosophila egg chamber." Exp Cell Res **157**(2): 355-63.

Warn, R. M., L. Smith and A. Warn (1985). "Three distinct distributions of F-actin occur during the divisions of polar surface caps to produce pole cells in Drosophila embryos." J Cell Biol **100**(4): 1010-5.

Wiese, C., A. Wilde, M. S. Moore, S. A. Adam, A. Merdes and Y. Zheng (2001). "Role of importin-beta in coupling Ran to downstream targets in microtubule assembly." Science **291**(5504): 653-6.

Xue, F. and L. Cooley (1993). "kelch encodes a component of intercellular bridges in Drosophila egg chambers." Cell **72**(5): 681-93.

Yudin, D. and M. Fainzilber (2009). "Ran on tracks--cytoplasmic roles for a nuclear regulator." J Cell Sci **122**(Pt 5): 587-93.

Curriculum Vitae

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CAREER PROFILE

2007 -2010 Doctoral Student at the German Cancer Research Center, Heidelberg, Germany

2006 Nov- Dec Research Assistant at the National Institute of Immunology, New Delhi, India

2004 - 2006 Master of Science in Molecular and Human Genetics from Banaras Hindu University, Varanasi, India (8.41 CGPA out of 10:0)

2001 - 2004 Bachelor of Science with Triple major in Genetics (Biotechnology) , Zoology, and Biochemistry, Osmania University, India (86.28%)

ACHIEVEMENTS AND FELLOWSHIPS

- International PhD fellowship from German Cancer Research Center (DKFZ), Germany; 2007-2010.
- Selected for Junior Research Fellowship by the Council of Scientific and Industrial Research, Government of India in December 2006.
- Availed Department of Biotechnology Scholarship in M.Sc from Banaras Hindu University; 2004-2006
- Availed Shraddha Memorial Trust Scholarship for being the topper in M.Sc in the first year; 2004-2005
- Secured **257** score out of 300 in **TOEFL**, given in May 2006.

COURSES AND CONFERENCES ATTENDED WITH PRESENTATIONS

- ✓ Attended the prestigious **Wellcome Trust course on Advanced *Drosophila* Genetics and Genomics**, held at the Wellcome trust genome Campus, Hinxton, UK from 9th- 23rd August 2009. (Talk)
- ✓ Attended the **All India Cell Biology** meet in Varanasi, India in Decemeber 2007.

Publications

Erika Virágh, Mátyás Gorjánácz, István Török, Tolga Eichhorn, **Sowjanya Kallakuri**,Tamás Szlanka, István Kiss and Bernard M. Mechler (2010), **Fine-tuned balance of Imp-α2 and Ketel coordinates the mitotic divisions in the preblastoderm Drosophila embryo,** submitted to Genetics.

Kallakuri S., Viragh E., Szlanka T., Kiss I., and Mechler B.M., **Interplay between *Drosophila importin-α2,-β* and *kelch* during oogenesis and early embryogenesis,** manuscript in preparation.

Kallakuri S., Szlanka T., Viragh E., Kiss I., and Mechler B.M. (2009), **Interplay between *Drosophila importin-α2* and *kelch* during oogenesis and early embryogenesis,** *21st European Drosophila Research Conference, November 18-21 2009, Nice Acropolis, France*

Kallakuri S., Szlanka T.,Viragh E., Mechler B.M.(2009), **Interplay between *Drosophila importin-α2* and *kelch* during oogenesis and early embryogenesis,** *15th Regional Drosophila Meeting, August 27-28 2009 , Münster, Germany*

Kallakuri S., Szlanka T.,Viragh E.,Mechler B.M.(2008), **Interplay between *Drosophila importin-α2* and *kelch* during oogenesis and early embryogenesis,** *13th Graduate Seminar dkfz. PhD Students Retreat, July 22-27 2008, Landesakademie für Jugendbildung, Weil der Stadt, Germany*

Kallakuri S., Szlanka T., Viragh E., Kiss I., and Mechler B.M. **(2009), Interplay between *Drosophila importin-α2,-β* and *kelch* during oogenesis and early embryogenesis**, *33rd All India Cell Biology Conference and International Workshop on Cell Cycle regulation, December 10-13 2009, Hyderabad, India*

Kallakuri, S., Szlanka, T., Viragh, E., Kiss, I., and Mechler, B.M. (2010), ***imp-β* may act as a mediator between *imp-α2*** a**nd *kelch* during *Drosophila* oogenesis and early embryogenesis,** *51st Annual Drosophila Research Conference, April 7-11 2010,Washington, DC*