INAUGURAL-DISSERTATION

Submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
Rupert-Karl-Universität Heidelberg
for the degree of
Doctor of Natural Sciences

Presented by
Master of Science Anshuman Kelkar
Born in: Aizwal, India
2005

Oral Examination:

Functional Analysis of Sec61β,
A Component of the
Sec61 Protein Translocation Channel at the
Endoplasmic Reticulum

Referees:

Professor Dr. Bernhard Dobberstein

Professor Dr. Dirk Görlich

Acknowledgements

To make a list of people who I would like to thank would extend into several pages. So, although I will try to name a few, but I apologise right at the beginning to the people who I may have forgotten to name. I am sure I will remember the help of every one.

To being with I would of course like to thank Bernhard. I thank him not only for his support during my Phd, but also for his immense patience. I think I gave him some of the hardest time ever during our long discussion sessions. I also would like to thanks Dr. Dirk Görlich for being my second referee.

I would also like to thank the people in the lab past and present. It was great to have Martin, Jeannie, Sumudhu, Joanne and Steffen around in the lab; I think Martin was kind of my mentor during my early days in the lab. Mathias and Peter were like elder brothers of the lab who one could turn to when things got bad or when a quick advice, without necessarily involving Bernhard, was needed. It was always nice to talk to Oliver Gruss and to realise that combination of phosphorylation and translocation and Phd and Bernhard was hard not only for me. I thank Marc for asking really critical questions during the seminars, and having a quick chat between spins and a quick beer between experiments. Gerry and Ute were simply amazing to have around in the lab, to let out the steam or to exchange cake recipes. I enjoyed talking to Katya about science or about music or just anything else and of course we especially enjoyed receiving each others phone calls. Martha was an excellent recruit to the lab, and this is not only because of the great cookies she made. Sabrina, Kiran and Maria made excellent lab mates; it is pity that I cannot spend more time with them. Gaik and Sybille were always ready for a quick laugh, in-between doing excellent work.

There are some people who made coming to the lab worth it, friendship of these people I will cherish all my life. Christoph, Milan (and the enlarged Spasic family) and Sabina (in strictly alphabetic order), I really cannot thank you enough. I really do not know how I would have survived these years without you guys.

The atmosphere of ZMBH was made special due to some really cool people. Ute, Pawel, Fani, Anneli and Christian made the second floor a nice and friendly place. I owe it big time to Jörg, not only for his help with my experiments, but also some excellent advice about how to make the best cake. I also thank Renato Paro for his advice and his lab for a steady supply of flies (Michaela) and gossip (everyone else). Stephan taught me all he knew about fly crosses, which is why my crosses were never more than two generations. It was good to have cool guys like Marcus, Thomas and Pious around for science, *umzug* or beer as the need maybe. Fourth floor in general and the Schwappah lab in particular was a great place

to hang out, one got nice cell lines, could chat with interesting people and if lucky get invited for parties.

I would like to thank Christine for everything. Not to mention her critical reading of this manuscript at an equally critical phase of my Phd.

I especially thank Gauri for her trust and constant encouragement while I was writing my Phd. I would like to thank my parents for support and Pranoti for cheering me up whenever I was feeling a bit down. These people were far away, but it was a great feeling to talk to them and know that they are there for you.

Finally I thank the staff and technical support at the ZMBH for excellent service. I also thank DFG and SFB for financially supporting Bernhard, which indirectly supported my Phd.

1. ABSTRACT

Secretory and membrane proteins are translocated across or inserted into the ER membrane by an aqueous channel called the Sec61 complex. The Sec61 complex consists of three subunits, α subunit which forms the actual channel, and β and γ subunits which associate with the channel. The present study was aimed at investigating the function of Sec61β in *Drosophila* and characterization of Sec61β phosphorylation.

Germline clones of the Sec61β loss of function allele (sec61β^{P1}) lack Sec61β in the oocytes. Embryos which are formed from these oocytes show perturbations in the dorsal-ventral polarity suggesting changes in protein amounts and localization of Gurken, an EGFR ligand in the oocyte. In these oocytes amount of Gurken at the plasma membrane is drastically reduced. Gurken is also absent from the surrounding follicle cells. Gurken is localized to punctuate structures in the cytoplasm, which do not co-localize with ER. Localization of the plasma membrane protein, Yolkless, remains unchanged. Based on these observations it seems that Sec61β affects a post-ER step during trafficking of a subset of proteins to the plasma membrane. The defect can be indirect when Sec61β affects localization of proteins which play a role in Gurken traffic. Sec61β interacts with the exocyst complex and Gurken needs the exocyst for plasma membrane localization, hence lack of Sec61β may directly affect Gurken traffic. Ectopic expression of Sec61β in the *Drosophila* wings causes specific changes in the wing morphology and loss of wing veins. These phenotypes are similar to the phenotypes seen in mutants affecting EGFR signalling and trafficking of EGFR ligands. This indicates that Sec61β may affect biogenesis of a specific set of molecules during other developmental processes too.

Sec61β is phosphorylated by the cdc2 kinase during the M-phase of the cell cycle. Phosphorylation occurs in both human and the *Drosophila* protein at a highly conserved serine residue at a consensus cdc2 kinase phosphorylation site. Sec61β protein with mutation in this residue does not completely rescue the lethality caused by sec61β^{P1} allele. Ectopic expression of the mutant protein in the wing enhances the morphological changes seen by ectopic expression of the wild type protein. This indicates that phosphorylation of Sec61β may affect the functional properties of Sec61β. Sec61β seems to play an essential role during secretion and phosphorylation may represent an additional level of regulation.

Zusammenfassung

Der Sec61-Komplex vermittelt über eine wäßrige Pore die Translokation von sekretorischen Proteinen ins ER-Lumen sowie die Insertion von Membranproteinen in die ER-Membran. Der Komplex besteht aus einer α-Untereinheit, die den eigentlichen Translokationskanal bildet, sowie einer β- und einer γ-Untereinheit, die mit dem Kanal assoziiert sind.

Die vorliegende Untersuchung zielt auf die Analyse der Funktion von Sec61β in *Drosophila* sowie die Charakterisierung der Sec61β Phosphorylierung.

Keimbahnklone mit dem Funktionsverlust-Allel sec61β^{P1} besitzen kein Sec61β in den Oocyten. Die sich aus diesen Oocyten entwickelnden Embryonen zeigen Störungen der dorso-ventralen Polarität, die auf Veränderungen der Proteinmenge und -lokalisation von Gurken, einem EGF-Rezeptor Liganden, hinweisen. Die Proteinmenge von Gurken an der Plasmamembran ist in den Oocyten drastisch reduziert. Außerdem fehlt Gurken in den umliegenden Follikelzellen. Gurken befindet sich in punktartigen Strukturen im Cytoplasma, die nicht mit dem ER kolokalisieren. Die Lokalisation des Plasmamembranproteins Yolkless bleibt dagegen unverändert.

Ausgehend von diesen Beobachtungen scheint Sec61β bei einer Gruppe von Proteinen einen Transportschritt in einem post-ER-Kompartiment zu beeinflussen. Dieser Effekt könnte indirekt sein, falls Sec61β die Lokalisation von Proteinen beeinflußt, die eine Funktion beim Transport von Gurken besitzen. Sec61β interagiert mit dem Exocyst-Komplex und Gurken benötigt den Exocyst-Komplex für seine Lokalisation zur Plasmamembran. Demzufolge könnte das Fehlen von Sec61β einen direkten Transportdefekt von Gurken bewirken. Die ectopische Expression von Sec61b in Flügeln von *Drosophila* führt zu spezifischen morphologischen Veränderungen und zum Verlust von Flügeladern. Diese Phänotypen zeigen Ähnlichkeit zu den bei EGFR-Signaldefekten beobachteten und deutet auf einen Einfluß von Sec61β bei der Biogenese einer spezifischen Gruppe von Molekülen auch während anderer Stadien des Entwicklungsprozeß hin.

Im Verlauf der M-Phase des Zellzyklus wird Sec61β durch die cdc2-Kinase phosphoryliert. Die Phosphorylierung erfolgt sowohl beim humanen als auch beim *Drosophila* Protein an einem stark konservierten Serin innerhalb eines cdc2-Konsensus Motivs. Die Mutation dieser Position kann die Lethalität des sec61β^{P1} -Alles nicht vollständig komplementieren. Die ectopische Expression des mutierten Proteins in Flügeln verstärkt die morphologischen Veränderungen im Vergleich zur Expression des Wildtyp Proteins. Dies deutet darauf hin,

daß die Phosphorylierung von Sec61β die funktionellen Eigenschaften von Sec61β verändert. Sec61β scheint eine essentielle Rolle bei der Sekretion zu spielen und die Phosphorylierung könnte eine zusätzliche Regulationsmöglichkeit darstellen.

2. INTRODUCTION

2.1 The Secretory Pathway

Translocation of proteins across the membrane of the Endoplasmic Reticulum (ER) is the first step in the biogenesis of secretory and membrane proteins. The nascent chains of these proteins enter the ER via a protenaceous channel called the Sec61p complex and this constitutes the entry point into the secretory pathway. If the mature protein does not possess specific signals for retention in the ER the protein exits the ER from specialized regions and is transported to the Golgi complex. The protein then moves along the Golgi complex till it reaches the trans-Golgi region. From the trans-Golgi region the protein is either transported to endocytic organelles or to the plasma membrane.

Transport of proteins between these various compartments of the secretory pathway largely occurs via small vesicles that are generated at a donor compartment and fuse with a downstream acceptor compartment. Cytoplasmic coat proteins sculpt vesicles by locally deforming the donor membrane. Distinct sets of coats function at different steps in the secretory pathway: CopII coat is present on vesicles which travel from ER to the Golgi complex, CopI coat is present on the retrograde transport vesicles from Golgi to the ER, similarly other vesicle in the intra-cellular transport have distinct protein coats.

In polarised cells vesicles bearing cargos which are destined for the different regions of the cell such as apical or basal surfaces, are segregated at the trans-Golgi region (Mostov et al., 2000). A soluble complex of six proteins called the exocyst forms a tethering complex at defined regions of the plasma membrane and mediates fusion of the vesicles (Lipschutz and Mostov, 2002) (Figure 2.1)

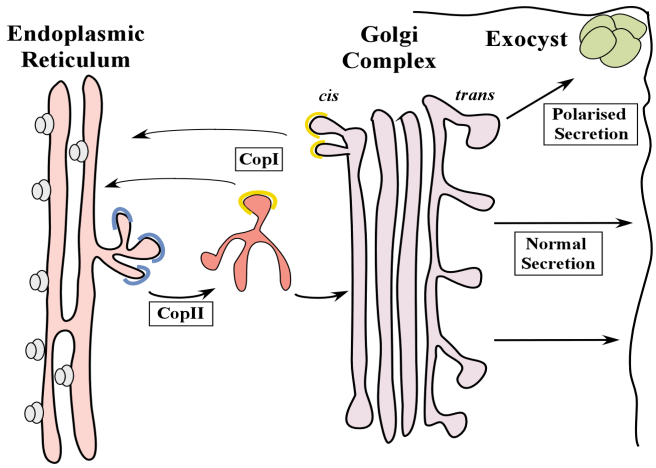


Figure 2.1 **The Secretory Pathway.** Secretory and membrane proteins enter the secretory pathway when the either get translocated across or inserted into the membrane of the Endoplasmic Reticulum. The proteins exit the ER via specialized exit sites and are packaged into CopII coated vesicles. These vesicle fuse with the Golgi complex and the protein travel from cis to trans-Golgi, again by specialized vesicles. From the trans-Golgi region the protein can either fuse with the Plasma membrane or are transported to other intra-cellular organelles.

2.2 Protein Targeting to the Endoplasmic Reticulum

The membrane and secretory proteins which are be targeted to the ER contain a short stretch of hydrophobic residues known as the signal sequence. As the synthesized signal sequence emerges from the ribosome, it is recognized by the Signal Recognition Particle (SRP). The SRP binds to the signal sequence and induces a transient retardation in protein synthesis. SRP then targets the ribosome, together with the nascent chain to the ER. This is mediated by the interaction of SRP with the SRP Receptor (SR), a heterodimeric integral membrane protein. SR catalyses the transfer of the ribosome and the nascent chain to the Sec61p translocation channel. This releases the block in protein synthesis, and the translation resumes with the newly synthesized chain being inserted into the ER. This process has been termed the SRP dependent co-translational translocation pathway (Johnson and van Waes, 1999).

In addition to the co-translational translocation, the Sec61p complex also receives protein for translocation after their complete synthesis in the cytoplasm. This pathway of translocation into the ER is called post-translational translocation. The fully translated protein is brought to the ER by as yet unidentified means. For the protein insertion into the ER, in yeast and in mammalian cells, besides the Sec61p translocon, additional proteins are required. This is the Sec62/63p complex consisting of Sec62, Sec63, Sec71 and Sec72. The post-translational targeting is presumably initiated when the hydrophobic domain of the protein to be targeted to the ER interacts with the Sec61 complex. The luminal domain of Sec62p activates the ATP bound Kar2p, the luminal chaperone, which is thought to bind to the translocated protein and mediate translocation into the ER (Rapoport et al., 1999) (Figure 2.2)

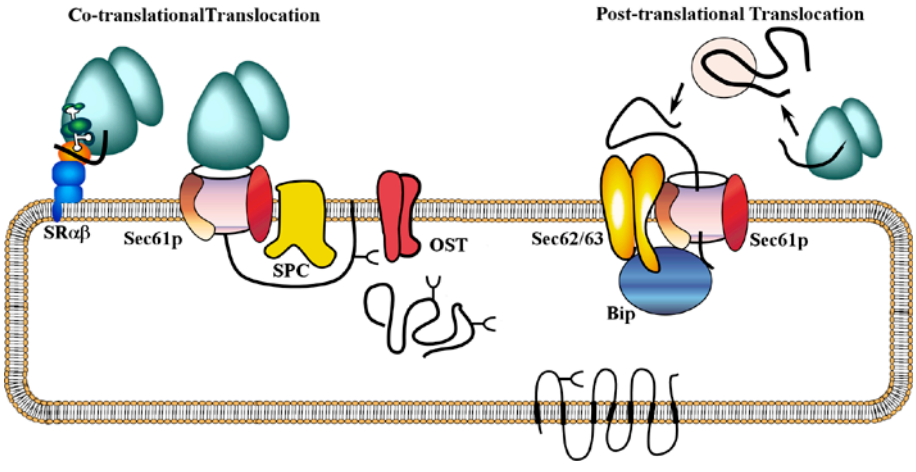


Figure 2.2 **Co-translation and Post-translational ER targeting pathways.** Proteins are targeted to the ER via two pathways. In the co-translational pathway protein are inserted during translation and in case of post-translational pathway the insertion occurs after the protein has been fully translated in the cytoplasm. The processes of signal sequence cleavage and glycosylation occurs co-translationally. Transport competent proteins are then packaged into transport vesicles.

2.3 The Translocation Process

Translocation across the ER membrane is mediated by a protenaceous channel called the Sec61p complex. The complex was initially identified in a yeast screen aimed at identification of genes which affect the translocation process (Deshaies et al., 1990). The role of the Sec61p complex in translocation of secretory and membrane proteins was biochemically defined in studies where fractionated ER proteins were reconstituted into liposomes and assayed for the ability to translocate proteins. It was found that Sec61p complex and SR receptor form the minimum translocational machinery (Görlich and Rapoport, 1993). The Sec61p complex performs a multitude of functions during the process of translocation. It binds ribosomes with high affinity (Kalies et al., 1994) and recognises functional signal sequences (Jungnickel and Rapoport, 1995). The Sec61p translocon allows secretory proteins to be transported completely across the membrane, whereas membrane proteins have lumenal domains translocated across the membrane, while others remain in the cytosol. Transmembrane segments, which anchor proteins in the membrane, need to be released laterally through the walls of the channel into the lipid phase.

2.3.1 The Sec61p Channel

Sec61p complex was characterized as the protein translocating channel at the ER when it was purified in a functional state (Görlich and Rapoport, 1993). The protein translocating activity of Sec61p consisted of three proteins which co-purified. These proteins tightly associated with each other and could be co-immuno-precipitated. The three subunits of the Sec61p protein translocation channel have been named as the α, β and the γ. All three proteins were identified as membrane proteins, Sec61α or its homolog in bacteria, SecY, consists of 10 transmembrane (TM) domains, Sec61β or the bacterial homolog SecG and the Sec61γ /SecE are single membrane spanning proteins (Görlich and Rapoport, 1993). Recently the crystal structure of the SecY complex from *Methanococcus jannaschii* has been solved and this has provided additional information to the actual translocation process and the role of the individual subunits (Van den Berg et al., 2004). It is presumed that the SecY complex represents the fundamental structure of the Sec61 protein translocation channel at the ER.

It is seen that SecY complex is formed of three subunits, the α, β and the γ. The α subunit which consists of ten TM segments forms the actual channel. The β and the γ subunits are associated with the channel on the periphery, in contact with the lipid bilayer. The complex has a roughly rectangular shape with the α subunit open on one side and the two smaller subunits surrounding the central channel. The 10 TMs of the α subunit are arranged

into two domains, TM1-5 and TM 6-10, which form a 'clam shell' hinged by a loop between TM5 and TM6 at the 'back' of the molecule. The two domains are held together by the γ subunit, which extends one TM diagonally across the interface between them and has a second α-helix that lies flat on the cytoplasmic surface of the membrane. The β-subunit consisting of a single TM seems to have minimal interactions with the channel. The side that is opposite the hinge (the front of the complex, where the month of the clamshell would be) is left free and most likely provides a site for exit of TM segment of membrane proteins during translocation. The ten helices of the α-subunit surround a large, water-filled cavity that has the shape of a funnel that opens on the cytoplasmic side and extends about halfway across the plane of the membrane (Figure 2.3).

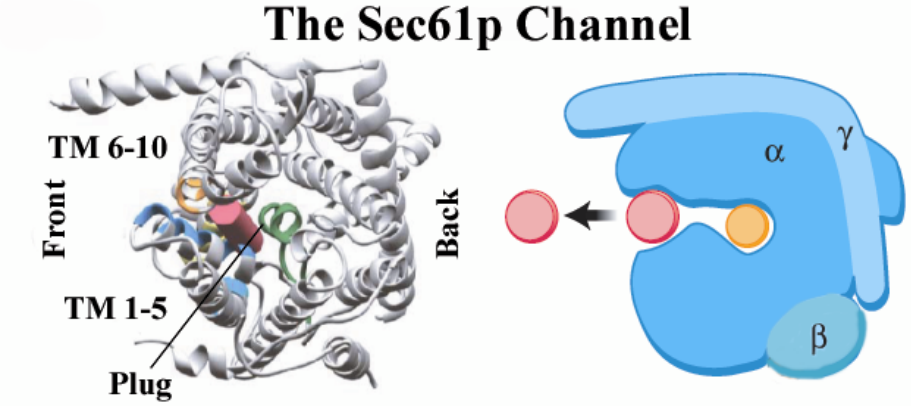


Figure 2.3 **The Sec61p Protein Translocation Channel**. X-ray crystal structure of the Sec61 protein translocation channel showing the transmembrane segments which constitutes the channel when viewed from the cytosolic side. The 10 TM segments of Sec61α form the hour-glass shaped translocation pore. The β and the γ subunits contribute one TM segment each which is present on three sides of the central pore. Also shown on the right is a schematic representation of the channel.

To understand the role of the individual components, the Sec61p complex was purified and reconstituted into liposomes. The re-cosntiution was also done after depletion of the individual subunits. These liposomes were analysed for the ability to translocate a subset of proteins. Sec61α and γ were found to be essential for translocation, where as Sec61β had no effect on translocation per-se (Görlich and Rapoport, 1993). Mutating Sec61α and γ was seen to be lethal (Deshaies et al., 1991). Mutating Sec61β, on the other hand had no effect on viability at normal temperatures (Panzner et al., 1995; Toikkanen et al., 1996). The exact role of Sec61β in protein translocation has not been elucidated.

2.3.2 Sec61β

Sec61β is a 13 KDa membrane protein with a transmembrane segment at the C-terminal region. The protein is anchored at the ER at the C-terminus and contains with a large N-terminal cytoplasmic segment and a few amino acids in the ER luminal (Figure 2.4).

Figure 2.4 **Sec61β**. Sec61β is a tail anchored protein of the ER, with a relatively long cytoplasmic segment and a few amino acids in the ER lumen.

2.3.2.1 Biochemical Characterization of Sec61β

Role in Protein Translocation

Liposomes containing purified Sec61p complex, along with TRAM and the receptor for the SRP were seen to be competent for translocation (Görlich and Rapoport, 1993). When Sec61β was immuno-depleted from the Sec61p complex before reconstitution, only a slight delay in translocation of substrate protein was observed (Kalies et al., 1998). However the effect was kinetic and increasing the translocation time was able to elevate the defect (Kalies et al., 1998).

Role in Ribosome binding

Dog pancreatic microsomes have been used to reconstitute the protein translocation (Walter et al 1981). Translating ribosome binds tightly to the microsome when it interacts with the Sec61p translocon, no other ribosome receptor is necessary for the ribosome to bind to the micosomes (Kalies et al 1994). Sec61β has been proposed to play a role in this ribosome binding to the microsomes. Purified cytoplasmic domain of Sec61β competes for ribosome binding to microsomal membranes stripped of endogenous ribosomes with puromycin and high salt (Levy et al., 2001).

Interaction with SPC

In biochemical studies with the microsomes it was also observed that the Sec61β was in proximity of the SPC25, a subunit of the signal peptidase complex, as determined by cross-linking using a cysteine specific cross-linker (Kalies et al., 1998). However the physiological significance of this association is not clear since the yeast knock-out does not show any accumulation of un-cleaved signal sequences (Panzner et al., 1995; Toikkanen et al., 1996).

Sec61β as a GEF

Purified Sec61β was seen to function as GTP exchange factor (GEF) for the β subunit of the SRP receptor (SRβ) (Helmers et al., 2003). SRβ is a GTPases and hydrolysis of GTP is thought to play a role in translocation (Fulga et al., 2001).

2.3.2.2 Phosphorylation of Sec61β

Sec61β has been reported to be phosphorylated both *in-vitro* and *in-vivo* (Gruss et al., 1999) It was seen that the α and the β isoforms of PKC are associated with dog pancreatic microsomes (Gruss et al., 1999). These forms of PKC are known to be responsive to Ca^{2+} and lipids (Goodnight et at 1995,(Azzi et al., 1992)). It was seen in this study that phosphorylation of Sec61β was stimulated by Ca^{2+} and diacylglycerol. It was thus suggested that these membrane associated kinases are most likely the same kinases which can phosphorylate Sec61β *in-vitro* (Gruss et al., 1999). Sequence analysis using phosphorylation site prediction programs however show the presence of additional putative sites which could be targets for other kinases besides the PKC (Blom et al 1999). The sites which are predicted are in the cytoplasmic domain of the protein. The kinase(s) which phosphorylate Sec61β *in-vivo* have not been identified. Also the actual site at which PKC or other *in-vivo* kinase(s) phosphorylates Sec61β has not been identified.

Phosphorylation by PKC is known to play a role in a variety of signal transduction cascades (Nishizuka, 1992). It has been suggested that phosphorylation of Sec61β by PKC or other kinases may have a role in modulating its function and provide a mechanism to make the process of translocation responsive to cellular or extra-cellular regulatory signals (Gruss et al., 1999).

2.3.2.3 Characterization of Sec61β function in Yeast and Mammalian Cells

Based on sequence comparison in yeast a homolog for Sec61β (Sbh1 or Seb1 in yeast) has being identified called Sbh2 or Seb2 (Panzner et al., 1995; Toikkanen et al., 1996). It was shown that yeast strain with both the genes mutated did not have any visible growth defect when grown under normal growth conditions. At higher temperatures (38.5^{o}C) the double knock-out strain had growth defects (Panzner et al., 1995; Toikkanen et al., 1996). Translocation of both membrane and secretory proteins was observed to be normal at permissive temperature (Panzner et al., 1995; Toikkanen et al., 1996)

Genetic studies in yeast revealed that over-expression of Sec61β could suppress the growth defect of the exocyst mutants (Toikkanen et al., 2003). The exocyst consists of a set of eight proteins initially characterized from yeast genetic screens designed to identify genes involved in secretion (Novick et al., 1980). The exocyst proteins form a 19.5S complex which localizes at the plasma membrane and preferentially localizes to regions of active cell expansion or areas of exocytosis. It is thought to provide directionality to the process of secretion by facilitating tethering of vesicles (Guo et al., 1999; Guo et al., 1997). In yeasts Sec61β can be immuno-precipitated with Sec10, a component of the exocyst, indicating a physical interaction of Sec61β with the exocyst (Toikkanen et al., 2003).

Over-expression of Sec10 in polarised mammalian MDCK cells resulted in an increase in delivery of protein which was targeted to the basolateral part of the cell as compared to apically targeted protein (Lipschutz et al., 2003). Over-expressed Sec10 was found to coimmuno-precipitate with Sec61β (Lipschutz et al., 2003). These interactions of the exocyst with a translocon component suggest that the translocon may have functions beyond translocation and this may be a way to co-ordinate the first and the last step of secretion (Guo and Novick, 2004).

2.4 Functional Characterisation of Sec61β in *Drosophila*

Sec61β is essential in *Drosophila*, since embryos homozygous for an allele with a P-element insertion before the transcription start site (sec61β^{P1}) are lethal (Valcarcel et al., 1999). The embryos die at the end of embryogenesis, at stage 17 and show defects in the deposition of the cuticle. The embryonal epidermal cells secrete cuticle at the end of stage 15 and stage 16, about 11 to 13 hours after egg laying (Campos-Ortega, 1985). The cuticle is composed of three different layers. The outer most layer is constituted by cross-linked hydrocarbons, called cuticulin. The layer below this is made up of the protein epicuticle and the lower most layer contains chitin fibers. sec61β^{P1} homozygous embryos show reduction in the thickness of the outer most layer and absence of the protein epicuticle in the second layer. These observations imply a defect in secretion of the cuticle proteins. However, Sec61β does not cause general trafficking defects since it is observed that sec61β^{P1} homozygous embryos have normal patterning which indicates normal Wingless secretion. This is also suggested from the finding that embryos reach stage 17 of development (Valcarcel et al., 1999).

The lethality associated with the sec61β^{P1} homozygous embryos occurs after the embryos have reached advanced stages in development. It is possible that maternal contribution is responsible for the embryos to development to this extent. This allele was used

for generation of the maternal germline clones causing depletion of Sec61β from the ovaries (Chou et al., 1993) preventing maternal contributions, produced embryos which died much before stage 17. Embryos from these sec61β^{P1} homozygous germline clones were also used to analyze phenotypes which occur early in development. The embryos showed a striking phenotype where the dorsal appendages are completely or partially fused (Valcarcel et al., 1999). This phenotype is reminiscent of reduced signalling by Epidermal growth factor receptor (EGFR), indicating ventralization of the embryo (Neuman-Silberberg and Schupbach, 1993). The mature egg exhibits clear asymmetry along the anterior-posterior and dorso-ventral axes. The dorsal side is marked by two anterior respiratory structures, the dorsal appendages. It has been reported that females homozygous for mutant Gurken alleles are sterile and produce eggs in which the position of the dorsal appendages is shifted dorsally so that a single appendage of reduced length is found at the dorsal midline. The only known EGFR ligand in the oocyte is Gurken. Mutants which cause reduced expression or cause mis-localization of the Gurken to regions other than the anterior-dorsal part of the oocyte also result in the dorsal appendage fusion (Nilson and Schupbach, 1999). The amount of Gurken released from the plasma membrane of the oocyte and the site of Gurken secretion from the oocyte in relation to the dorsal follicle cells determines the strength of the signal. An appropriate signal reaching the follicle cell results in generation of asymmetry in the developing oocyte. It was therefore predicted that the phenotype observed in the embryos from the sec61β^{P1} germline clones was due to changes in the biosynthesis of Gurken.

However, characterization of the phenotype in the sec61β^{P1} germline clones at molecular level was not performed. The phenotype observed in the embryos from the sec61β^{P1} germline clones could be due to changes in the amounts of synthesized Gurken, or the intra-cellular trafficking of Gurken.

2.4.1 Trafficking of Gurken during oogenesis

The biosynthesis of Gurken is initiated when the nurse cells transcribe the mRNA for Gurken and deposit it at the posterior part of the oocyte in close association with the oocyte nucleus. As the oocyte matures the nucleus migrates to the anterior-dorsal part of the oocyte and the Gurken mRNA which remains associated is also relocalized. During stage 10 of oogenesis local translation of the Gurken mRNA occurs at a distinct site of the oocyte. Gurken protein contains a classical signal sequence and is synthesized as a type I membrane protein in the ER. Gurken is transported out of the ER due to the presence of an ER resident protein called Star (Roth, 2003). Transport from ER to the Golgi complex is facilitated by Cornichon which

is thought to package Gurken into specialized CopII vesicles (Roth et al., 1995). As Gurken proceeds along the secretory pathway into the Golgi complex it is processed by member of the Rhomboid family of proteases, called Rhomboid-2, which cleaves Gurken within the transmembrane region. At the plasma membrane Gurken is released into the surrounding intercellular space. The surrounding follicle cells internalize the soluble Gurken by receptor mediated endocytosis when Gurken interacts with its cognate EGF receptor (Figure 2.4). Internalized Gurken induces the ras signalling cascade in these cells which in turn stimulates production of the membrane bound protease Rhomboid-1 allowing release of Spitz, another member of the EGF family. Activation of Spitz in the follicle cells triggers further signalling cascades, one consequence of these cascades is the reduced expression of a protein called pipe in the dorsal follicle cells. (Nilson and Schupbach, 1999; Roth, 2003).

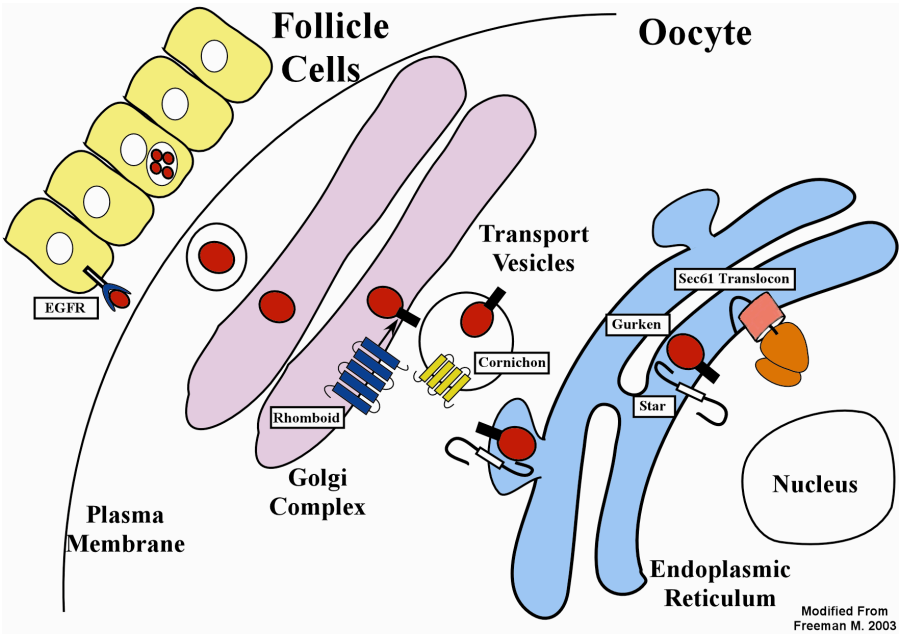


Figure 2.5 **The Trafficking of Gurken in Oocytes.** Gurken is synthesized as a Type I transmembrane protein in the ER. Star mediates export from the ER from presumably specialized exit sites. Gurken is then packaged into CopII vesicles by Cornichon. Rhomboid which has been seen to be localized to the Golgi cleaves Gurken within the transmembrane segment and the soluble Gurken generated is secreted outside the Oocyte. Soluble Gurken interacts with the Epidermal Growth Factor Receptor (EGFR) and gets internalized in follicle cells where it can initiate signalling.

2.4.2 Morphological changes in adult structures seen in sec61β^{P1} Lines

Organ specific depletion of Sec61β in flies produced distinct phenotypes in the eyes and the legs (Valcarcel et al., 1999). In the eyes approximately 5% of the ommatidia lacked photoreceptors or showed other morphological defects. These included differences in the number of rhabdomeres in the apical and basal sections of the eyes. This can be explained by defects in the differentiation of the photoreceptors which cause the rhabdomeres not to form over the entire apical-basal extent of the retina. Depletion of Sec61β during leg development resulted in severe size-reduction of the tarsal segments. The reduction in tarsal segment size often occurred distal to the area from where the protein was depleted.

2.5 Additional Protein Required for Translocation

However it is seen that in addition to the Sec61p complex other proteins in the ER are also essential in the process of protein translocation. Using reconstituted liposomes it was observed that for efficient translocation of a subset of proteins, with weak signal sequences, an additional protein called Translocating chain-associating membrane protein (TRAM), a multiple membrane spanning protein known to interact with the Sec61p complex, is necessary (Voigt et al., 1996). TRAM is also thought to play a role in release of the TM segments into the lipid bilayer (Heinrich et al., 2000). Translocon Associated Protein complex (TRAP), a heterotrimeric complex of membrane proteins, becomes necessary for translocation for another subset of proteins (Fons et al 2003). Among them is the Prion protein which is seen to attain different topologies depending on the repertoire of the proteins, like TRAP, present during the translocation process (Hegde et al., 1998). Ribosome Associated Membrane Protein 4 (RAMP4), another tail anchored protein bound to the translocon is seen to interact with the newly translocated nascent chain (Schroder et al., 1999), but the exact role of this interaction is not know.

2.6 Protein Maturation in the ER

ER is the site for post-translational modifications of the secretory and membrane proteins. Folding of the translocated protein in a biologically active conformation is initiated in the ER. Disulphide bond formation is one of the pre-requisite for proper folding of proteins containing cysteine residues. ER provides an oxidising environment favouring disulphide bond formation between cysteine residues within the same polypeptide or between different subunits (Hwang et al., 1992; Rietsch and Beckwith, 1998). Many secretory and membrane proteins are subjected asparagine-linked glycosylation. This involves transfer of a preformed branched chain of sugars of the composition $Glc_3Man_9GlcNA_2$ (where Glc is glucose, Man is mannose, and GlcNA is N-acetylglusosamine). These sugars are transferred to asparagine within the sequence motif NX(S/T) of the nascent polypeptide from a membrane-bound dolichol carrier catalysed by oligosaccharide-protein transferase (Kornfeld and Kornfeld, 1985). Many membrane and secretory proteins are expressed as oligomers. Oligomerization of multimeric protein complexes also happens to a large extent in the ER (Hurtley and Helenius, 1989). Many of these steps are intricately linked to the process of translocation and occur co-translocationally, when the folding enzymes and the chaperones associate with the nascent chain (Ellgaard et al., 1999).

Mechanisms exits in the ER which constantly monitor these post-translational modification and process of protein maturation. Only when the protein is in its biologically active state it is allowed to proceed along the secretory pathway. Aberrant proteins are retained in the ER and are subsequently transported back to the cytoplasm for degradation (Ellgaard et al., 1999).

2.7 Regulation of the Secretory Pathway

The process of secretion can respond to extra cellular cues (Balch, 1990; Beckers et al., 1989). GTPases and secondary messengers such as Ca^{2+} are known to play an important role in ER to Golgi complex transport (Beckers et al., 1989). In addition, experiments with inhibitors of phosphataes and kinases have indirectly provided evidence that the phosphorylation plays a role in regulating the process of ER to Golgi transport (Davidson et al 1992). Activity of Sec31p, responsible for packaging of proteins into CopII coats, is regulated by phosphorylation (Salama et al 1997). Studies have also identified CopI coat components as being phosphorylated, and phosphorylation has been suggested to regulate the function of these proteins (Sheff et al 1996). It is also reported that the last step of secretion, the exocytosis of proteins in specialized cells such as pancreatic acinar cells depends upon Ca^{2+}. Increases in intra-cellular levels of Ca^{2+} activate kinases and phosphatases such as the Ca^{2+} and calmodulin-dependent kinase or the Ca^{2+} and lipid-dependent kinase (Ito et al., 1997). During M-phase the vesicle tethering protein GP130 resiing on the Golgi-complex is phosphorylated which prevents docking of vesicles at the Golgi and contributes to a secretion block (Lowe et al., 1998a). Components of the ER are also phosphorylated by Protein Kinase C (PKC) which stimulates the process of protein translocation into the ER (Gruss et al., 1999).

2.8 Aim of the study

Sec61β is a sub-unit of the protein translocation channel at the ER. In *Drosophila*, Sec61β is essential for survival. Depleting Sec61β from *Drosophila* ovaries results in perturbations in the anterio-posterior patterning of the embryo. It has been proposed that one of the major determinants of the anterior-posterior axis formation is a signalling protein called Gurken, whose asymmetric localization in the oocyte generates the polarity during the axis formation. The study presented here aims to characterize the molecular basis of this phenotype. Investigation of the biosynthesis of Gurken with respect to protein amounts and intracellular localization constitutes the first part of the study.

Sec61β is also seen to be phosphorylated *in-vitro* and *in-vivo*. The kinase mediating *in-vivo* phosphorylation has not been identified. Sequence analysis of Sec61β predicts the cdc2 kinase as a likely candidate. Cdc2 kinase is a cell cycle dependent kinase which is active in the M-phase of the cell cycle. The second part of the work is aimed at characterizing cdc2 kinase mediated cell-cycle dependent phosphorylation of Sec61β *in-vivo* and identification of the exact residue at which phosphorylation occurs. This residue would be mutated in the Sec61β gene and transgenic flies harbouring this mutant gene of Sec61β would be generated. The physiological significance of phosphorylation would be tested by the ability of this mutated Sec61β to rescue the lethal loss of function allele of Sec61β.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

All standard chemicals were purchased from Sigma-Aldrich (Steinheim, Germany), Serva (Heidelberg, Germany), Merck (Darmstadt, Germany) or Gibco BRL (Eggenstein, Germany) unless otherwise mentioned.

3.1.2 Enzymes

Enzymes were purchased from New England Biolabs (Beverly, MA, USA), Roche Mannheim, Germany) or Amersham-Pharmacia (Uppsala, Sweeden).

3.1.3 Oligonuclotides

Name	Sequence (5'-3')
5'hsSec61β	ATGAACGAGTGTACTTGCC
3'hsSec61β	CATGCCTGGTCCGACCC
5'HA-hsSec61β	ATGTACCCATACGATGTTCCAGATTACGCTATGCCTGGTCCGACCC
5'hsSec61β S/D	CGTGGGCTCGTCGGGGCGAGACCCCAGCAAAGCAGTGG
3'hsSec61β S/D	CCACTGCTTTGCTGGGGTCTCGCCCCGACGAGCCCACG
5'hsSec61β S/A	CGTGGGCTCGTCGGGGCGCGCTCCCAGCAAAGCAGTGG
3'hsSec61β S/A	CCACTGCTTTGCTGGGAGCGCGCCCCGACGAGCCCACG
5'dmSec61β	ATGCCCGCTCCAGCCAGTT
3'dmSec61β	TTAAGAACGATTGTATTTGCC
5'dmSec61β S/D	CCGTGGGCAGCGGATCGCGAGACCCCAGCAAATTGTCGG
3'dmSec61β S/D	CCGACAATTTGCTGGGGTCTCGCGATCCGCTGCCCACGG
5'dmSec61β S/A	CCGTGGGCAGCGGATCGCGCGCCCCCAGCAAATTGTCGG
3'dmSec61β S/A	CCGACAATTTGCTGGGGGCGCGCGATCCGCTGCCCACGG
5'InvariantChain	GACCGGTACCATGGAGAAAAGGATCCTGGATGACCAGCGCG
3'InvariantChain	GCCGTCTAGATCACATGGGGACTGGG
5'Gurken	ATGATGCAAATCCCATTTAC

3'Gurken	TCAGCATCTGACAAAAAAGCGCTG

3.1.4 Vectors

Name	Source
pCMV-TnT	Promega
pUAST	Brand and Perrimon, 1993

3.1.5 Plasmids

Name	Description
pCMV-HA-hsSec61β^{WT}	Human HA-tagged wild type Sec61β cDNA for HeLa
pCMV-HA-hsSec61β$^{S/A}$	Human HA-tagged serine-alanine mutant Sec61β cDNA
pCMV-HA-hsSec61β$^{S/D}$	Human HA-tagged serine-aspartate mutant Sec61β cDNA
pCMV-IRES-Ii	Invariant Chain expression plasmid with the IRES element
pCMV-HA-dmSec61β^{WT}	*Drosophila* wild type Sec61β for expression in HeLa cells
pCMV-HA-dmSec61β$^{S/A}$	*Drosophila* serine-alanine mutant Sec61β for expression in HeLa cells
pCMV-HA-dmSec61β$^{S/D}$	*Drosophila* serine-aspartate mutant Sec61β for expression in HeLa cells for HeLa cells
pUAST-dmSec61β^{WT}	*Drosophila* wild type Sec61β cDNA for GAL4 activation
pUAST-dmSec61β$^{S/A}$	*Drosophila* serine-alanine Sec61β cDNA for GAL4 activation
pUAST-dmSec61β$^{S/D}$	*Drosophila* serine-aspartate Sec61β cDNA for GAL4 activation
pCMV-Gurken	Gurken plasmid for expression in HeLa cells
pCMV-Myc-Star	Myc-tagged Star plasmid for expression in HeLa cells
pCMV-HA-Rho	Ha-tagged Rho plasmid for expression plasmid in HeLa cells
pSup-Sec61β	Plasmid containing siRNA directed against 3'UTR of Sec61β

3.1.6 Antibodies

Antibody	Mono/polyclonal	Reference
cfSec61β	polyclonal	Görlich and Rapoport, 1993
dmSec61β	polyclonal	A. Kelkar (this study)
Cdc2	polyclonal	Upstate Biotech

cfL23a	polyclonal	Pool et. al., 2002
HA	polyclonal	Santa Cruz, USA
Invariant Chain	polyclonal	Lipp and Dobberstein, 1986
Gurken	monoclonal	Hybridoma Bank
Yolkless	monoclonal	Schonbaum et. at., 2000
Boca	polyclonal	Culi and Mann, 2003
Myc	monoclonal	

3.1.7 Secondary antibodies

Anti-rabbit/mouse IgG-Horseradish (HRP)-Conjugate (Sigma-Aldrich, Steinheim, Germany)
Anti-rabbit/mouse/rat-FITC/TR (Jackson Laboratories, USA)

3.1.8 Buffers, solutions and media

Solutions and buffers

All solutions were prepared according to Sambrook et al. 1991 (Sambrook, 1991).Solutions were made with double de-ionised water, sterile filtered and unless indicated otherwise stored at room temperature.

10xDNA buffer	0.1% (w/v) Bromophenolblue 50% (v/v) glycerol
Laemmli buffer (4x, -20˚C)	200 mM TrisHCl, pH 6.8 400 mM DTT/ 10% (v/V) βME 4% (w/v) SDS 0.2% (w/v) Bromophenolblue 20% (v/v) glycerol
10xPBS	27 mM KCl 17 mM KH_2PO_4 1.3 M NaCl 100 mM Na_2HPO_4, pH 7.0
PBST	PBS 0.05% (v/v) Tween 20
SDS-PAGE buffer (5x)	25 mM Tris base 192 mM Glycine 0.5% (w/v) SDS

Phosphorylation Buffer (4°C)	50 mM Hepes pH 7.8 120 mM KOAc 5 mM $Mg(OAc)_2$ 2 mM DTT 0.5 mM EGTA
Lysis Buffer for HeLa cells (4°C)	20 mM Hepes 150mM NaCl 5mM $MgCl_2$ 0.5mM EDTA 1% Triton X-100 10mM NaF 50mM Na_2VO_3 1X Clap Mix
Homogenization Buffer for HeLa cells (4°C)	50mM Tris base 25mM KCl 500 mM KOAc 1 mM $MgCl_2$ 250mM Sucrose
RM buffer for dog pancreatic microsomes (4°C)	20 mM Hepes 2mM $Mg(OAc)_2$ 50 mM KOAc 2mM DTT 250mM Sucrose
Phosphatase Inhibitors Used	10mM NaF 50mM Na_2VO_3 100nM Okadaic Acid

3.1.9 Cell Culture Reagents

Cell line

The cell line used in this study was HeLa, human cervical carcinoma cell line (ATCC, USA)

Media and Transfection

Dulbecco Modified Eagle's Medium (DMEM), DMEM methionine and cysteine free, DMEM phosphate free, Foetal Calf Serum, Penicillin/Streptomycin and Trypsin-EDTA were purchased from Gibco/Invitrogen, USA.

Transfection Reagent used was Lipofectamine 2000 from Invitrogen, USA.

3.1.10 Other Reagents

ECL (Western Blot Detection)	Amersham, UK
Mowiol (Mounting Medium)	Calbiochem, US

3.1.11 Drosophila Lines

Fly Line	Description	Reference
Sec61β^{P1}/Cyo	Sec61β with a P-element insertion	Valcarcel et. al. (1999)
FRTSec61β^{P1}/Cyo	Same as above, P-element with flanking FRT sites	Valcarcel et. al. (1999)
UAS-Sec61β Wild Type	GAL4 driven expression of the wild type Sec61β protein	A.Kelkar
UAS-Sec61β S/A	GAL4 driven expression of the S/A mutant	A.Kelkar
UAS-Sec61β S/D	GAL4 driven expression of the S/D mutant	A.Kelkar
Armadello-GAL4	Ubiquitous GAL4 expression	BDGP
Scalloped-GAL4	Wing Specific GAL4 expression	BDGP
Engrailed-GAL4	Wing Specific GAL4 expression	BDGP
Patched-GAL4	Wing Specific GAL4 expression	BDGP

3.2 Methods for DNA Manipulation

3.2.1 Standard Techniques

Standard DNA manipulation techniques were done according to Sambrook et al., 1991 (Sambrook, 1991). These included plasmid purification (both small scale and large scale), restriction digestion, agarose gel electrophoresis and ligation.

3.2.2 Cloning of cDNAs in Expression Vectors

Mammalian Expression

Human and *Drosophila* Sec61β cDNAs, wild type and the phosphorylation mutants containing only the coding region were cloned at the KpnI-NotI sites of the pCMV-TnT vector from Promega. Gurken cDNA was also cloned at this site. Plasmids encoding Rho and Star were a gift from Professor M. Freeman (Urban et al., 2002).

***Drosophila* Expression**

Drosophila Sec61β cDNA wild type and the phosphorylation mutants containing only the coding region were cloned at the KpnI-XhoI sites of the pUAST vector (Brand and Perrimon, 1993).

3.2.3 Site Directed Mutagenesis

The exchange of one amino acid to another was performed by synthetic oligonucleotide. The plasmid, containing desired gene, was purified by following the protocol mentioned in Qiagen purification kit. Oligonucleotide was designed containing the changed nucleotides in the middle and complementary to the flanking region. In addition a site for a restriction enzyme was also incorporated in this oligonucleotide. A reverse oligonucleotide of this oligonucleotide was also designed. These oligonucleotides were used for a PCR reaction:

Plasmid DNA	5-50 ng
Primer 1	125 ng
Primer 2	125 ng
dNTPs	1 ul (2.5mM)
Buffer	5 ul (10x reaction buffer)
pfu turbo polymerase	1 ul (2.5 units/ul)
H_2O	to add up 50 ul

Then the mixture was placed in a PCR machine with a program as follows:

95°C	1 min	
95°C	30 sec	(12-18 cycles)
55°C	30 sec	
68°C	1 min/kb DNA length	

After the reaction the mixture was then cooled in ice for 10 min. There after 1 ul *Dpn* I (10 unites/ul) was added to the mixture in order to cut the parental DNA and incubated for 1 hr in 37^{o}C, after which 1-2 ul of the mixture was transformed into DH5_ competent cells with appropriate antibiotic. Then the plasmids were isolated from the single colonies as mentioned before with phenol-chloroform treatment. The mutations were identified first with restriction digestion and then confirmed with DNA sequencing.

3.3 Methods for Standard Protein Biochemistry

3.3.1 SDS-Polyacrylamide Electrophoresis

SDS-gel electrophoresis allows protein separation according to their molecular weights. The anionic detergent SDS denatures the proteins and in addition inserts negative charges to the proteins. The separation occurs in an electric field. Depending on the molecular weight of the separating proteins, 7.5–15% acrylamide gels or 10-17% gradient gels were casted as follows (ratio acrylamide: bis-acrylamide 30: 0.8):

Stacking gel buffer	0.25 M Tris, pH 6.8 0. 0.2% (w/v) SDS
Separating gel buffer	1 M Tris, pH 8.5, 0.1% (w/v) SDS
Running buffer	25 mM Tris pH8 8.3, 250 mM glycine, 0.1% (w/v) SDS
Sample buffer	50 mM Tris pH 6.8, 3% (w/v) SDS, 10% (v/v) Glycerol, 0.02%

3.3.2 Western Blotting

For Western Blotting, proteins were first separated by SDS-PAGE and then transferred using a semi-dry blotter onto a nitrocellulose membrane (Protran BA85, Pore 0,45um, Schleicher&Schuell, Dassel, Germany). First three sheets of whatmann paper were soaked in Anode Buffer I and placed on the blotter, then three sheets equilibrated with Anode Buffer II, followed by the membrane in Anode Buffer II. The nitrocellulose membrane was placed on the top this stack, and on top this was placed the three more whatmann sheets equilibrated with the Cathode buffer. Transfer was done by applying current (1.5 times area of the membrane in mA) for 1 hour.

The blot was then blocked in PBS-T with 5% dry fat free milk for 1hour. After blocking, the blot was incubated in the same buffer containing the primary antibody for 3hours at room temperature or 4^{o}C overnight. The blot was washed three times (10 min each) with PBST, incubated with secondary, horseradish peroxidase coupled in PBS-T containing 5% milk for 20 min at room temperature. After this incubation, the blot was washed again at least three

times (10 min each) with PBST. The secondary antibody was then detected using the ECL Western-Blot Detection-Kit.

Western Blotting Transfer Buffers

Anode Buffer I	30mM Tris, 20% (v/v) Methanol
Anode Buffer II	300mM Tris, 20% (v/v) Methanol
Cathode Buffer	40mM 6-Aminohexanoic acid, 25mM Tris, 20% (v/v) Methanol, 0.01% (w/v) SDS

3.3.3 Visualization and Quantification of Radio-labelled Proteins

Radioactive proteins on dried gels or nitro-cellulose membranes were visualized by using a phosphoimager (Fuji Photo Film Co.). Quantification of radioactivity was done using the program MACBAS 2.0 (Fuji Photo Film Co.).

3.3.4 Silver Staining

After SDS-PAGE the gel was treated as follows:

1. Fix I	30% Ethanol, 10% Acetic Acid, 30 minutes
2. Fix II	30% Ethanol, 0.5M Sodium Acetate, 0.5% Glutaraldehyde, 0.2% Sodium bisulphate , 30 minutes
3. Washing	3 X 10 minutes
4. Impregnation	0.1% Silver Nitrate, 15-60 minutes
5. Developing	3% Sodium Carbonate, 0.02% Formaldehyde, till bands appear
6. Stop	1% Acetic Acid or 50mM EDTA

3.3.5 Preparation of Antigen for Immunization

Synthetic peptide was synthesized corresponding to eight residues from the N-terminal region of the dmSec61β, with an extra cysteine containing a sulfhydryl for coupling to keyhole limpet hemacyanin (KLH).

The coupling was done was follows:

1 KLH (Pierce) dissolved in 2ml of H2O (yields solution of 10mg/ml in 1XPBS)

2 Dissolved 14mg of Sulfo-SMCC (Pierce) in 75ul of DMSO

3 Added Sulfo-SMCC to KHL and incubated at 25^{o}C for 30 minutes.

4 Briefly centrifuged to remove aggregates

5 Loaded supernatant onto a G-25 de-salting column (Pharmacia, NAP/PD10) pre-equilibrated with 1XPBS. About 3ml of activated KLH was obtained.

6 Dissolved 5mg of cysteine containing peptide in 0.5ml 1XPBS

7 To the peptide added 5mg (0.75ml) activated KLH

8 Incubated at 25^{o}C for 2.5hours

9 Dialysed overnight against 1XPBS

The coupled peptide was mixed with Freuds complete adjuvant and injected in Rabbits. Subsequent steps in immunization and preparation of the serum were done according to Harlow and Lane, 1988 (Harlow, 1988).

3.4 Drosophila Handling and Genetic Methods

3.4.1 Drosophila Handling, standard fly food

Standard fly food:
10 l H_2O
80 g Agar-agar
180 g dry yeast
100 g soy-flour
220 g beet syrup
800 g cornmeal
24 g nipagin (methyl-4-hydroxybenzoate, Merck)
62.5 ml propionic acid (Sigma)

Fly stocks were raised on standard fly food and crossed at 25°C with 60-70% relative humidity, except when stated otherwise. Fly stocks were maintained at 18°C with 60-70% relative humidity.

3.4.2 P-element mediated Germline Transformation

Injection buffer:
5 mM KCl
0.1 mM NaH_2PO_4, pH 6.8

Bleach solution:
4% (v/v) HOCl (Roth) in ddH_2O

Acetic acid agar plates:
500 ml ddH_2O
12 g Bacto-agar
2-3 ml acetic acid

Transgenic flies carrying the gene of interest were generated by P-element mediated germ line transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Adult w^{1118} flies were allowed to lay eggs on acetic acid agar plates for 20-30 min at 25 °C. The embryos were recovered, dechorionized with bleach solution for 2 min and extensively washed with water. About 80-120 embryos were lined on an agar stripe and transferred onto a double-sided sticky tape (3M, Scotch) mounted on a coverslip. The embryos were dehydrated in a closed chamber containing Silica gel for 7 min and covered with Voltalef 10S oil (Lehmann & Voss & Co.). The appropriate pUASt constructs (9 µg) and the helper DNA pUChsΔ2-3 (3 µg) were

ethanol co-precipitated and dissolved in 30 µl injection buffer. Prior to the injection into the treated w^{1118} embryos, the DNA mixture was centrifuged for 5 min at 13.000 rpm/4°C and the supernatant was loaded in a Femtotip needle (Eppendorf). Microinjection was performed at 18°C with the Femtotip needle using an Eppendorf FemtoJet Microinjector at 200-500 hPa injection pressure. In general, about 300-400 embryos were injected per construct and kept in a humid chamber at 18°C until larvae hatched. Larvae were transferred onto standard fly food and kept at 25°C until the founder G_0-Generation hatched.

3.4.3 Establishing Transgenic Fly line and Mapping of the Integration site

The hatched founder G_0-Generation flies were crossed to w^{1118}; *BcGla/Cyo* virgins or males and progenies were then screened for the transformation marker *white*, i.e. pigmented eyes. Transformed flies were backcrossed to w^{1118}; *BcGla/Cyo* flies twice and stable homozygous lines were established. The crossings to the to w^{1118}; *BcGla/Cyo* line also allowed the mapping of the chromosomal integration site of the transgene, dependent on the distribution of the transgene and the markers on the progenies. At least 6 independently transformed fly lines per construct (two on each chromosome) were kept as stocks.

3.5 Special Methods Used in this Study

3.5.1 siRNA mediated reduction in Sec61β levels in HeLa cells

Double stranded oligonucleotides were designed against different regions of Sec61β the mRNA. The designed were based on the guide lines provide by the manufacturer which included a ScaI site in the hairpin region of the primers for identification.

Vector Construction

The pSuppressorNeo plasmid (IMG-800) was obtained from Imgenex, and vector DNA was digested with SalI and XbaI to generate compatible ends for cloning.

siRNA plasmid	Target Sequence on hsSec61β
pSUP 1	attctacacagaagattca
pSUP 2	tgttccagtattggttatga
pSUP 3	tgtatttatgttgcacattt
pSUP 4	aagtacactcgttcgtagatt
pSUP 5	aagtatagtgactatctgt

For the annealing reactions, 1 μg of each oligo (forward and reverse) were mixed with 2 μl of annealing buffer (Imgenex) and water to 10 μl. Primers were annealed at 95˚C for 10 minutes and slowly cooled to room temperature. Annealed oligos were ligated into the linearized pSuppressor vector. Ligation reactions were set up with 1 μl of linearized vector DNA (50 ng/μl), 1 μl of the insert DNA (100 ng/μl), 1 ul of 10x T4 DNA ligase buffer, 1 μl of T4 DNA ligase, 6 μl water, and incubated overnight at 16˚C. Clones were screened by ScaI digest, as a ScaI site was designed in the insert oligos.

Transfection

Plasmids were transfected into HeLa cells using Lipofectamine 2000 (Invitrogen), following the manufactures protocol. 3μg of the siRNA plasmid was used for transfection. After three days a second round of transfection was done with the same amount of plasmid. Cell lysate was then used for western blotting.

3.5.2 Pulse Analysis in HeLa cells

HeLa cells which were 80-90% confluent were grown in DMEM without methionine and cysteine (depletion medium) for 2 hours. At the end of this incubation 30 uCi/ml of ^{35}S labelled methionine and cysteine were added to the depletion medium. The cells were allowed to incorporate radioactivity usually for 10 minutes. The cells were then immediately placed on ice to prevent further incorporation. Cells were washed twice with ice cold PBS, and scraped of the plates in PBS and pelleted in centrifuge tubes. The cell pellet was lysed in 1% Triton X-100 containing lysis buffer and used for immuno-precipitation or mixed with lamelli buffer and applied to SDS-PAGE.

3.5.3 Ovarian Dissection and Immuno-Florescence

Ovaries from 2- to 4-day-old females fed on yeast for two days were dissected in PBS, and kept on ice. Fixation was done using 4 vol H_2O, 1 vol Buffer A (100mM potassium phosphate pH 6.8, 450 mM KCl, 150 mM NaCl, and 20 mM $MgCl_2$), and 1 vol 37% Formaldehyde in a 1:6 ratio with Heptane for 30 minutes. All antibody staining was carried out in PBS, containing 0.5% BSA, 0.1% Triton-X-100 and 5% normal goat serum. Staining with the primary antibodies was performed over-night at 4^{o}C. Incubation with the primary antibody was followed by washing using PBS containing 0.1% Triton-X 100 for two hours with repeated changes of the buffer. Appropriate secondary antibody was added and samples incubated for two hours. This was followed by washing with PBS containing Triton-X 100; finally the samples were washed in PBS, and mounted using Mowiol.

3.5.4 Immuno-fluorescence of Mammalian Cells

HeLa cells growing on coverslips were processed for immuno-fluorescence as follows. Coverslips were washed twice with PBS and fixed using 5% Formaldehyde in PBS for 30 minutes at room temperature. Incubated the coverslips with 0.1M Glycine in PBS for 1 minute at room temperature. Washed the coverslip with PBS and permeabilized the cells incubating the coverslips with 0.4% Triton X-100 in PBS for 5 minutes at room temperature. Washed the coverslip with PBS and incubated with blocking buffer, 5% BSA in PBS, for 30 minutes. Added the primary antibody, in blocking solution and incubated for 1 hour at 37^0C in a humid chamber. Washed the coverslip with PBS three times with each wash lasting 5 minutes. Added secondary antibody, in blocking solution and incubate for 1hour at 37^0C in a humid chamber. Washed the coverslip in PBS, three times, with each wash lasting 5 minutes and then once more in water. Mounted the coverslip on a glass slide using Mowiol.

For visualizing the GFP signal, cells after fixation were washed in PBS and directly mounted on slides using Mowiol.

3.5.5 Microscopy

Fluorescent images were recorded with a Leica confocal microscope (DMIRE2, 20x NA 0.7 water, HCX PL APO 63x NA 1.4-0.6 oil, laser at 405, 488, 543 or 633, as required). Digital photographs were processed with Photoshop (Adobe).

3.5.6 Preparation of *Drosophila* Wings for Analysis

Wings from two to three days old flies were dissected under a microscope. The dissected wings were immersed in absolute ethanol for 5-10 minutes. The wings were briefly dried on paper towels and laid on Hoyers medium and incubated for 1 hour at 60^oC for fixing and mounting.

Hoyers Medium:

Distilled Water	50ml
Arabic gum	30g
Choral Hydrate	200g
Glycerin	16ml

3.5.7 Preparation of Rough Microsomes (RMs)

Rough microsomes were prepared from canine pancreas as described by Blobel and Dobberstein. Rough microsomes were collected from the interphase between the 1.5 m and the 1.75 m sucrose step, diluted with rough microsome buffer (20 m m Hepes, pH 7.8; 2 m m

$Mg(OAc)_2$ 50 m m KOAc; 2 m m dithiothreitol and 10 µg·mL 1 phenylmethanesulfonyl fluoride), centrifuged for 60 min in a 45 Ti rotor (Beckman) at 100 000g and resuspended to a final concentration of 1membrane equivalent per µL, as defined by Walter et al., in rough microsome buffer containing 250 mM sucrose.

3.5.8 Preparation of EDTA/High Salt Treated Microsomes

For 10 000 eq RM adjusted RMs to 10 ml 1 eq/ul in RM buffer. Added 5 mls 1.95 M KOAc/15 mM Mg $(OAc)_2$/Protease Inhibitors and dounced for 20 minutes on ice. Loaded onto 7 ml high salt sucrose cushion and spun at 37,000rpm for 60 minutes at 4°C in a Ti50.2. Resuspended the pellet (High Salt Washed Microsomes) in 10 ml RM buffer by douncing and added the following components

Amount added	Final concentration
20.5g sucrose	2 M
3.9 ml 5M KOAc	650 mM
1.5 ml 500 mM EDTA	25 mM

Sample was kept on ice for 30 minutes. Divided into 6 SW40 tubes (5 ml each) and carefully over-laid with 2.7 ml 1.5M floatation cushion followed by 2.7 ml 1 M floatation cushion followed by 2.7 mls 0.25 M floatation cushion .Spun overnight at 38,000rpm at 4°C in a SW40 rotor.

Recovered floated EKRMs (1M sucrose phase) and added 2 volumes of RM buffer (-DTT). Spun in a Ti45/Ti50 rotor just to pellet the membranes for 1hour at 42,000rpm at 4°C. Take up pellet in 10 ml RM buffer.

3.5.9 Preparation of Membrane and Cytosolic Fraction from HeLa cells

HeLa cell which were 80-90% confluent were scraped off tissue culture plates and washed with ice cold PBS and resuspended in homogenization buffer. The cells were then lysed by douncing with a loose fitting dounce homogenizer. The cell breakage was confirmed using Trypan blue staining (Sigma-Aldrich, Steinheim, Germany) about 80% breakage was usually seen. The suspension was spun at 2000rpm in a bench top centrifuge for 10 minutes to pellet the un-lysed cells, nuclei, which remain undamaged and cellular debris. The supernatant was spun at 150,000g in a TL 100.1 rotor for 20 minutes at 4^oC using a bench top ultra-centrifuge. The pellet which contains the crude membrane fraction was resuspended in RM buffer. The supernatant which is free of membranes was used as the cytosolic fraction.

4. RESULTS

4.1 Phosphorylation of Sec61β

4.1.1 Phosphorylation of Sec61β during the Cell-Cycle

Sec61β has been reported to be phosphorylated both *in-vitro* in dog pancreatic microsomes and *in-vivo* in pancreatic cells and MelJuSo cells (Gruss et al., 1999). Isoforms of Protein Kinase C's (PKC) were identified as being responsible for phophorylation of Sec61β *in-vitro*. This study although did not identify the kinase which phosphorylates Sec61β *in-vivo*. However differences in the phosphopeptide maps obtained for Sec61β isolated under *in-vitro* or *in-vivo* conditions were reported. This could be interpreted as indicative of a second kinase, besides PKC, which phosphorylates Sec61β *in-vivo*. Phosphorylation site prediction algorithms predict in addition to the putative site for the PKC phosphorylation, a phosphorylation site for the cdc2 kinase (K/RSP**S**/**T**R/K/H). Serine at position 17 was predicted as the phosphorylated residue (Holmes and Solomon, 1996 Blom, 1999 #4958). The predicted cdc2 phosphorylation site is present at the N-terminal cytosolic part of Sec61β. The cdc2 phosphorylation site is conserved in human, Xenopus and *Drosophila* protein (Figure 4.1). The yeast homologs of Sec61β, Sbh1 and Sbh2 do not have this site. The focus of the present study was to test if cdc2 indeed was the kinase which phosphorylated Sec61β *in-vivo*.

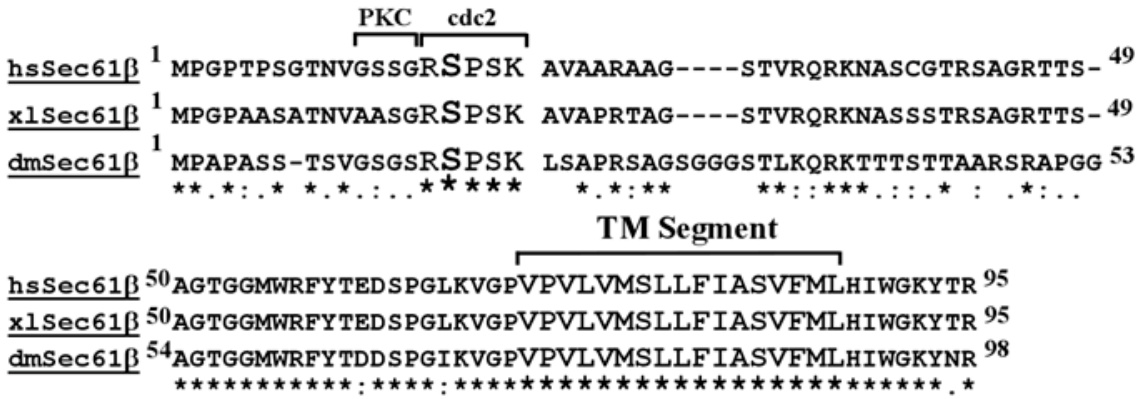


Figure 4.1 **Sequence Aligment of Sec61β.** Human, Xenopus and *Drosophila* Sec61β protein sequences are aligned to show the conservation of the cdc2 phosphorylation site. The serine predicted to be phosphorylated is enlarged. The PKC site in mammalian Sec61β is also indicated

Cdc2 kinase is a member of the cyclin-dependent kinases which is active during the M-phase of the cell cycle and mediates the progression through M-phase (Nurse et al., 1976). Cdc2 kinase is responsible for morphological changes in intracellular structures including the

ER during the M-phase (Glotzer et al 1990). The ER which exists as a tubular structure during the interphase gets fragmented into vesicles during the M-phase (Warren, 1993). *In-vitro* assays have implicated cdc2 kinase's role in this fragmentation (Dreier and Rapoport, 2000). M-phase is also characterised by block in the vesicular transport, which is in part mediated by the cdc2 dependent phosphorylation of vesicle tethering proteins at the Golgi complex (Lowe et al., 1998b).

In order to see if Sec61β is phosphorylated in the M-phase of the cell cycle when the cdc2 kinase is active, phosphorylation status of the Sec61β during the cell cycle was analysed. Cells are synchronised in the late G1-phase of the cell cycle using aphidicolin. Aphidicolin binds to the α-subunit of DNA polymerase I, prevents DNA replication and this induces a cell cycle arrest. The arrest at late G1 occurs after 16 hours of incubation with aphidicolin. The growth arrested cells are then released into the cell cycle by washing off the inhibitor and addition of fresh medium. The released cells proceed along the cell cycle and enter the M-phase after 12 hours. The cell cycle phase of these cells is confirmed by analysis of DNA content after labelling the DNA by propidium iodide (data not shown).

In-vivo ^{32}P ortho-phosphate labelling is used to determine the phosphorylation of Sec61β at different stages of the cell cycle following release from the aphidicolin induced block. The first time point at which cells are analyzed is the G1-phase, at 0 hours. Subsequent time points are taken at intervals of every four hours. The analysis was continued for 16 hours, which allow the cells to traverse through the M-phase. Cells are incubated with ^{32}P ortho-phosphate four hours prior to analysis to allow the incorporation of the label. A schematic representation of the labelling and harvesting procedure is shown in Figure 4.2A.

Cells are collected for analysis by scraping them off the tissue culture dishes, followed by lysis in 1% Triton X-100. 1/20 of the protein sample is applied directly to SDS-PAGE. From the other fraction Sec61β is immuno-precipitated; the immuno-precipitation reaction is performed in duplicate. The immuno-precipitated samples are also applied to a SDS-PAGE and transferred to nitrocellulose membrane and exposed to phosphoimager screen. The same nitrocellulose membrane is then used for western blotting.

It is observed that phosphorylated Sec61β can be detected in all stages of the cell cycle. The amount of protein immuno-precipitated is similar as determined by western blotting using antibody against Sec61β (Figure 4.2B). The amount of ^{32}P incorporated in immuno-precipitated Sec61β is quantified (Figure 4.2C). Two fold higher level of phosphorylation of Sec61β is observed in cells immuno-precipitated at 12 hours following release from the G1

block. At this time point cells are in the M-phase of the cell cycle. These data indicate that phosphorylation of Sec61β is stimulated during the M-phase.

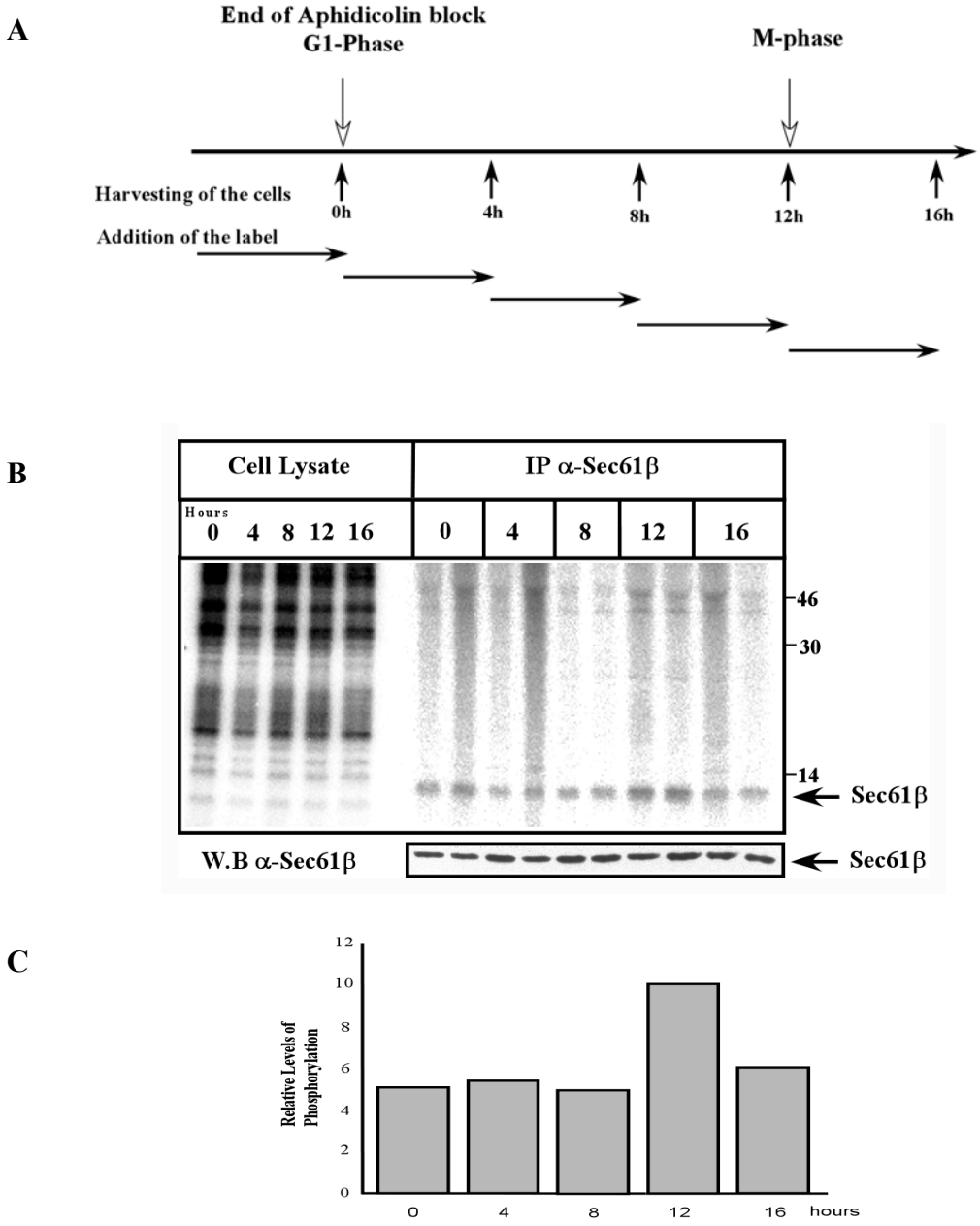


Figure 4.2 **Phosphorylation of Sec61β during the cell cycle.** A) HeLa cells were synchronized in the G1-phase of the cell cycle using Aphidicolin. After a 16 hour block cells were released into the cell cycle by washing off Aphidicolin. Cells were grown in the presence ^{32}P ortho-phosphate four hours prior to being harvested at the indicated time points. B) Harvested cells were lysed and 1/20 of the sample was directly applied to the SDS-PAGE where as the other fraction was used for immuno-precipitation using Sec61β antibody. Phosphatase inhibitors were present during lysis and also during immuno-precipitation. Protein after separation were transferred to a nitro-cellulose membrane and exposed to a phosphoimager screen. The same blot was then used for western blotting using antibody against Sec61β C) Label incorporated in the immuno-precipitated protein was quantified. Phosphorylation at G1 phase was given a random value and the levels of phosphorylation at other time points were compared against this.

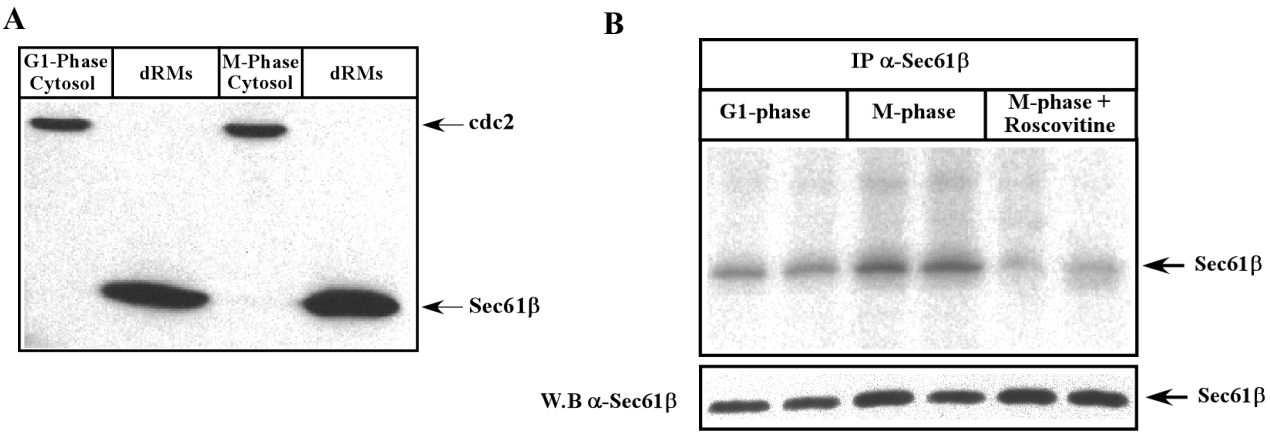
4.1.2 *In-vitro* assay for cdc2 kinase dependent phosphorylation of Sec61β

In order to investigate if the M-phase phosphorylation of Sec61β is due to cdc2 kinase, cytosolic extracts were used from cells arrested in the M-phase and the G1-phase and rough microsomes from dog pancreas (dRMs) as substrate (Walter and Blobel, 1983). To block the activity of cdc2 kinase a specific inhibitor of the cdc2 kinase called roscovitine was to be used. Roscovitine acts by competing for ATP binding to the cdc2 kinase (De Azevedo et al., 1997). However, incubation with roscovitine was toxic for the cells and thus *in-vivo* phosphorylation analysis could not be done. To circumvent this problem the above mentioned *in-vitro* assay system was developed which could recapitulate the Sec61β's phosphorylation reactions as occurring in cells.

Cdc2 along with its cognate cyclin partner, cyclin B1 forms a soluble complex in the M-phase. The cdc2 kinase also exists in the other cell cycle phases, but it is inactive due to absence of the cyclin partner. Cells were synchronized in the M-phase by using nocodazole which depolymerises microtubules and induces cell cycle arrest. These M-phase cells detached from the substratum were separated from non-mitotic cells by shaking them off the plates. G1-phase synchronization was achieved by using aphidicolin as described in the previous experiment. These synchronized cells were used to prepare cytosolic extracts which should contain the activity from the soluble cdc2 kinase/cyclin complex. The substrate, Sec61β, was present as part of rough microsomes which were prepared from dog pancreas (dRMs) as described (Walter and Blobel, 1983). dRMs represent relatively pure form of the ER and it had previously been shown that microsomal membrane proteins can be targeted for phosphorylation by soluble kinases *in-vitro* (Gruss et al., 1999). However, it cannot be ruled out that the microsomes may be contaminated with soluble cytosolic proteins. Using antibodies against cdc2 it is seen that the dRMs did not contain any detectable cdc2 kinase. The cytosolic extracts from both the G1 and the M-phase contained cdc2 kinase, however the cdc2 kinase seen in the G1 phase is the inactive form. Furthermore, the cytosolic extracts did not show the presence of Sec61β (Figure 4.3A).

dRMs were incubated with the cytosolic extracts and γ-^{32}P ATP, in addition roscovitine was added to the M-phase extract. After incubation for 20 minutes the reaction was stopped by addition of 20% TCA. The precipitated protein was solubilized using 1% SDS. Sec61β was immuno-precipitated, applied to SDS-PAGE, transferred to a nitro-cellulose membrane and exposed to a phosphoimager screen. The same nitro-cellulose membrane after phosphoimaging was used for western blotting using the Sec61β antibody. Sec61β is seen to be phosphorylated to different degree when incubated with either the G1 or M-phase extracts.

Western blot shows that similar amounts of protein are immuno-precipitated in all instances (Figure 4.3B). The amount of label incorporated in the immuno-precipitated protein was quantified. A two fold increase in the level of phosphorylation of Sec61β was observed when the microsomes were incubated with the M-phase extract as compared to the G1-phase extract. The increase in phosphorylation of Sec61β upon incubation with the M-phase extract is sensitive to roscovitine (Figure 4.3C). This indicates that the stimulation in Sec61β's phosphorylation seen during the M-phase is indeed due to activity of a roscovitine sensitive kinase.



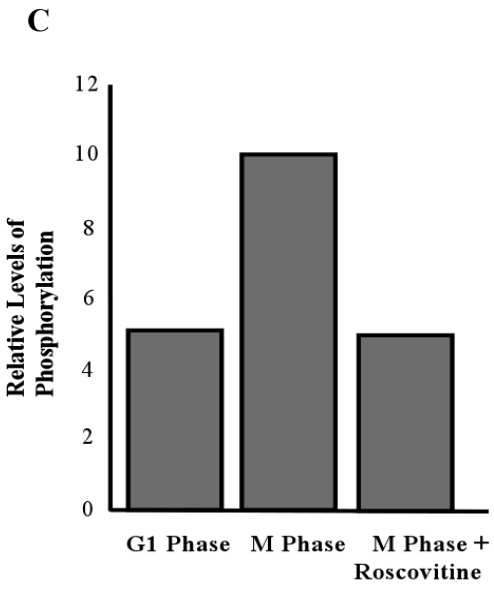


Figure 4.3 **Characterization of cdc2 kinase activity from cytosolic extracts from synchronized cells**. A) Cytosolic fraction was prepared from cells synchronized in the G1 phase using Aphidicoin or in the M-phase using Nocodazole and the RMs were prepared as described (Walter and Blobel, 1981). The samples were applied to SDS-PAGE, transferred to nitocellulose membrane and probed with antibodies against cdc2 and Sec61β. B) Cytosol prepared from G1-phase or M-phase synchronized cells was incubated with RMs in the presence of γ-^{32}P ATP. In addition the M-phase cytosol was incubated along with the cdc2 inhibitor roscovitine. After 20 minutes reaction and solubilization Sec61β was immuno-precipitated and applied to SDS-PAGE. The amount of precipitated protein was determined by western blotting using antibody against Sec61β. C) Immuno-precipitated protein was quantified using the Mac-Bas program used to scan the phosphoimager screen. Phosphorylation at G1 phase was given a random value and the levels of phosphorylation at other time points were compared against this.

4.1.3 Phosphorylation of Sec61β in EDTA-High Salt Washed Microsomes

Phosphorylation of Sec61β by the M-phase extract could not be blocked completely by roscovitine. Sec61β was also phosphorylated in the G1-phase. This indicates that either Sec61β is a substrate for an additional kinase which is active in both G1 and M-phase extracts or roscovitine is unable to completely inhibit the activity of the cdc2 kinase. These questions were addressed by using an *in-vitro* system where the kinase activity could be further characterized. This was done using dRMs which were washed with high salt and EDTA (EKRMs) instead of dRMs. This treatment should remove proteins which associate with the membrane by electrostatic interactions and dissociate ribosomes into their respective subunits. This treatment is known to remove the PKC isoforms which have been reported to be associated with the microsomes and phosphorylate Sec61β (Gruss et al., 1999). Washing the membranes with high salt would also eliminate probable membrane associated phosphatases which could dephosphorylate Sec61β during the course of the assay. In addition, purified cdc2 kinase instead of the cytosolic extracts was used for the phosphorylation assay. Recombinant human Cdc2 kinase and its positive regulatory subunit cyclin B1 are purifed as an active complex from Sf9 cells.

EKRMs were used for an *in-vitro* phosphorylation assay to test if purifed cdc2 kinase can phosphorylates Sec61β. In addition it was tested if staurosporine can inhibit the roscovitine insensitive kinase that phosphorylates Sec61β. Staurosporine, is an inhibitor of a broad range of serine/therionine kinases, which includes PKC (Meyer et al., 1989; Nakano et al., 1987). EKRMs and γ-^{32}P ATP were incubated together alone or with the following additions, cdc2 kinase, cdc2 kinase plus roscovitine, roscovitine alone and staurosporine alone. After incubation for 20 minutes the samples were solubilized, Sec61β immuno-precipitated and phosphorylation of the immuno-precipitated protein visualized. Sec61β is phosphorylated even when EKRMs alone are incubated with γ-^{32}P ATP. This phosphorylation, in the absence of any soluble kinase activity, was termed as endogenous phosphorylation. Staurosporine can inhibit the endogenous phosphorylation, where as roscovitine cannot. Sec61β is differentially phosphorylated when EKRMs were incubated with purified cdc2 kinase or cdc2 kinase and roscovitine (Figure 4.4A, upper panel). Similar amounts of protein were immuno-precipitated as determined by western blotting with antibody against Sec61β (Figure 4.4A, lower panel). Upon quantification of the immuno-precipitated protein, a two fold increase in the phosphorylation of Sec61β was observed in the presence of cdc2 kinase, as compared to EKRMs alone. Incubation with roscovitine prevented

this stimulation. Lack of detectable phosphorylation of Sec61β in the presence of staurosporine suggests that a kinase is present in EKRM that can phosphorylate Sec61β and is sensitive to staurosporine (Figure 4.4B).

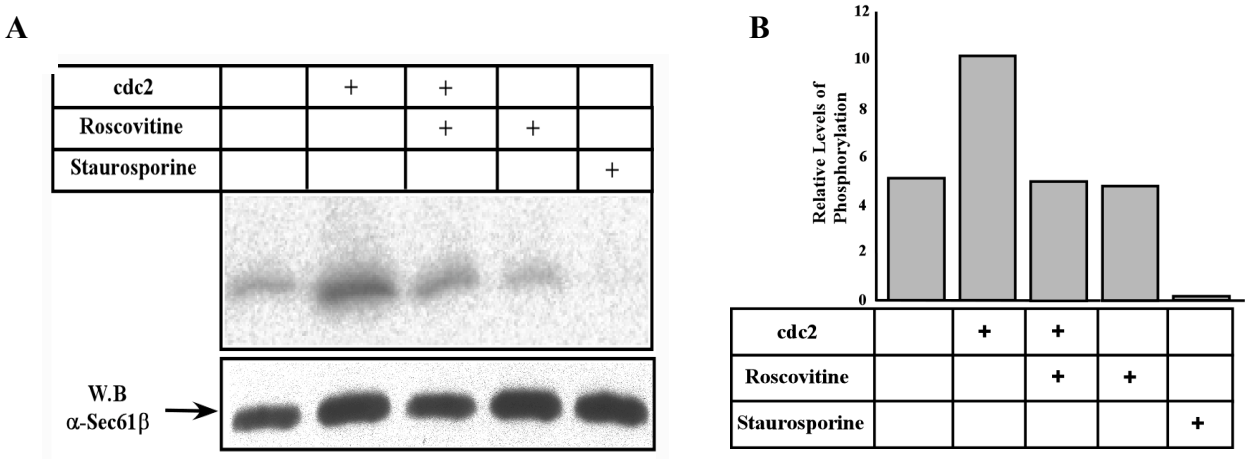


Figure 4.4 **Phosphorylation of Sec61β in EKRMs.** A) High-salt EDTA washed microsomes (EKRMs) were incubated with γ-^{32}P ATP alone or with the following additions, purified cdc2 kinase alone, cdc2 kinase and roscovitine, roscovitine alone, and staurosporine alone. These additions are shown on top of the upper panel. After 20 minutes reaction and solubilization Sec61β was immuno-precipitated and applied to SDS-PAGE. The amount of precipitated protein was determined by western blotting using antibody against Sec61β. B) Immuno-precipitated protein as seen in A was quantified. Phosphorylation in the presence of cdc2 kinase was given a value of 10 and the levels of phosphorylation in other lanes were compared against this.

In an independent experiment EKRMs after incubation with γ-^{32}P alone, or in the presence of cdc2 kinase, or cdc2 kinase and roscovitine were directly applied to an SDS page. The phosphorylated proteins were detected by phosphoimaging. It was seen that two major proteins of sizes 13KDa and 35KDa were phosphorylated in a roscovitine sensitive manner shown as Protein 1 and Protein 2 in Figure 4.5. The 13KDa protein was identified as Sec61β by immuno-precipitation (Figure 4.5). The identity of the other protein with approximate size 35kDa was not determined.

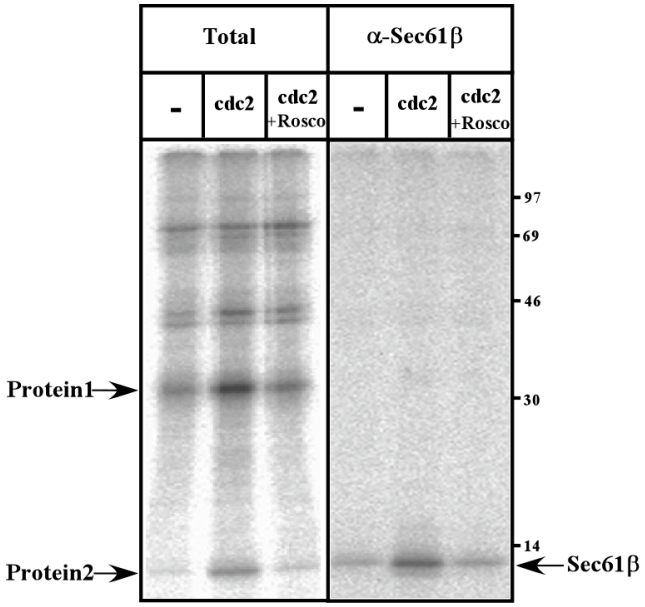


Figure 4.5 **EKRM proteins phosphorylated by cdc2 kinase.** High-salt EDTA washed microsomes were incubated with γ-^{32}P ATP alone or with the addition of purified cdc2 kinase and purified cdc2 kinase plus roscovitine. The reaction was stopped after 20 minutes by addition of 20% TCA and the precipitated proteins solubilized. 1/3 of the sample was either directly applied to SDS-PAGE, (Total) or from the remaining fraction Sec61β was immuno-precipitated (α-sec61β). The gel was analysed by phosphoimager.

Taken together these data indicate that purified cdc2 kinase can directly phosphorylate Sec61β in EKRMs. cdc2 kinase targets at least one additional ER membrane protein. Moreover, the kinase phosphorylating Sec61β independent of the cdc2 kinase is straurosporine sensitive and is associated with the ER membrane.

4.1.4 Phosphorylation of Sec61β in Reconstituted Proteoliposomes

In order to investigate if Sec61β as part of a purified Sec61 complex can be phosphorylated by the cdc2 kinase, Sec61p complex consisting of the α, β and γ subunits was purified from dog pancreas and reconstituted into liposomes (Sec61p-liposomes) (Görlich and Rapoport, 1993). An aliquot of the purified Sec61p complex was separated by SDS-PAGE and silver stained to estimate its purity. Three major bands representing the 38KDa α sub-unit, 13KDa β sub-unit and 7KDa γ subunit are visible (Figure 4.6).

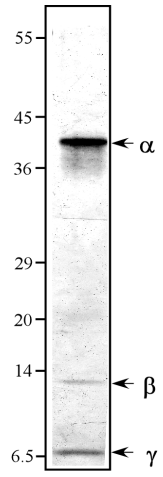


Figure 4.6 **Purified Sec61p Complex.** The Sec61p complex consisting of the α, β and the γ subunits was purified as described in Görlich and Rapoport, 1993. The sample was applied to a SDS-PAGE and proteins visualized by silver staining the gel. The protein markers and the subunits are indicated.

The Sec61p-liposomes were then used for *in-vitro* kinase assays in presence or absence of the HeLa cytosolic extracts derived from cells arrested in G1 or M-phase. The Sec61p-liposomes were incubated with γ-^{32}P ATP alone or with addition of G1-phase extract and M-phase extracts. The cell extracts were also incubated with γ-^{32}P ATP without the Sec61p-liposomes. After the reaction the samples were precipitated and characterized by SDS-PAGE and autoradiography. A large number of phosphorylated proteins are seen when the liposomes are incubated with either the G1-phase or the M-phase extracts (Figure 4.7, lanes 1 to 4). Most of these proteins are from the cell extracts since these phosphorylated proteins are not seen when the Sec61p-liposomes are incubated with γ-^{32}P ATP alone (lanes 5 and 6) or when the cell extracts are incubated with γ-^{32}P ATP alone in the absence of Sec61p-liposomes (lanes 7 to 10). Incubation of the Sec61p-liposomes with M-phase extract shows a prominent phosphorylated band of 13 KDa band which corresponding to Sec61β, this phosphorylation is sensitive to roscovitine (lanes 3 and 4). Upon incubation of the Sec61p-liposomes with γ-^{32}P

ATP alone or in addition G1-phase extract no phosphorylated protein is seen which can correspond to Sec61β (Figure 4.7)

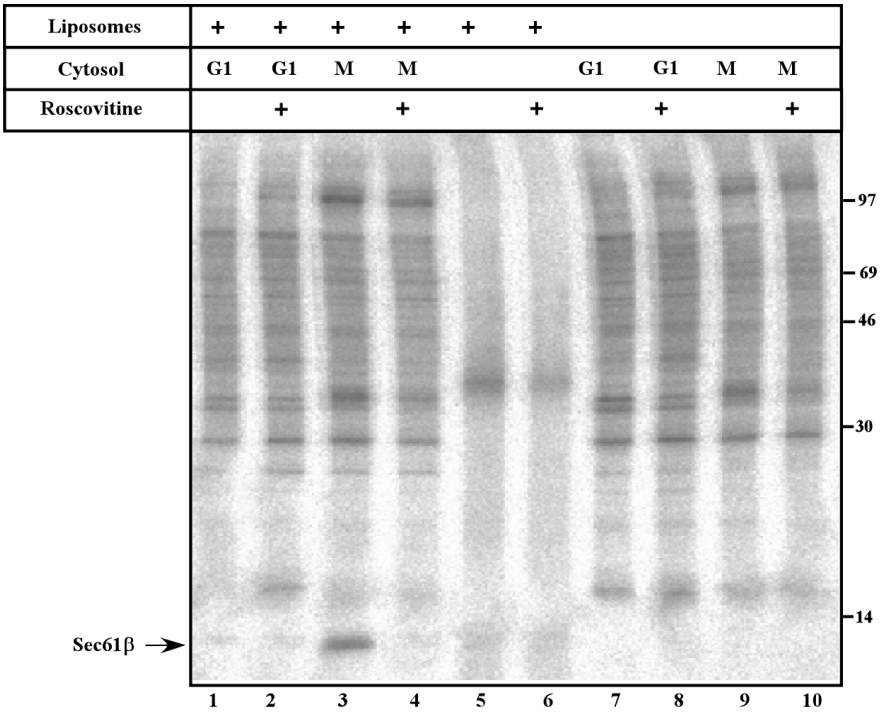


Figure 4.7 **Phosphorylation of Sec61β as part of Sec61p-liposomes.** Sec61p-liposomes were incubated with γ-^{32}P ATP alone, or with addition of the cytosolic extracts from G1 or M-phase arrested cells in the presence or absence of roscovitine. Incubations with γ-^{32}P ATP were also done with G1-phase and M-phase extracts without Sec61p-liposomes as control. The reaction was stopped after 20 minutes by addition of 20% TCA, precipitated proteins solubilized with 1%SDS and applied to SDS-PAGE.

This experiment indicates that G1-phase cytosol lacks the ability to phosphorylate Sec61β as part of the Sec61p-liposomes. Under these conditions the endogenous phosphorylation of Sec61β is also not observed. M-phase cytosol contains a roscovitine sensitive kinase that can phosphorylate Sec61β when present as part of the Sec61p-liposomes.

4.1.5 Identification of cdc2 kinase Phosphorylation Site in Sec61β

The predicted cdc2 phosphorylation site in Sec61β is the serine at position 17. In order to see if indeed this is the only residue which gets phosphorylated by the cdc2 kinase Sec61β protein lacking this serine was used for an *in-vitro* phosphorylation assay. Site directed mutagenesis was used to replace this serine to alanine. cDNAs were generated encoding for the N-terminal HA-tagged form of the wild type protein (HA-Sec61β^{WT}) and the serine to alanine mutant protein (HA-Sec61β$^{S/A}$). Presence of the HA-tag allowed discrimination between the recombinant and the endogenous Sec61β. Membranes from cells expressing the HA-tagged proteins were used for the phosphorylation assay.

The plasmids encoding the HA-tagged proteins were transfected into HeLa cells. 24 hours after transfection the cells were harvested and crude membrane and membrane free fractions were prepared. This preparation allowed γ-^{32}P ATP access to the membrane proteins. The membrane and the membrane free fraction were analysed by western blotting using antibodies against the HA tag and Sec61β to verify the expression of the HA-tagged proteins. A ribosomal protein, L23a was used as a loading control. It was seen that the HA-tagged proteins are present in the same fraction as the endogenous Sec61β (Figure 4.8A). It was also observed that the HA tagged protein is not detected by the Sec61β antibody, either in western blots or in immuno-precipitation (data not shown).

In order to see whether the HA-Sec61β could be phosphorylated by the cdc2 kinase the membrane fraction was incubated with purified cdc2 kinase and γ-^{32}P ATP. After the reaction the membrane were solubilized and immuno-precipitation was done using antibody against either the HA tag or Sec61β. The immuno-precipitated proteins were applied to a SDS-PAGE and phosphorylated protein visualized. It is seen that HA-Sec61β^{WT} gets phosphorylated when incubated with cdc2 kinase, where as HA-Sec61β$^{S/A}$ does not show any phosphorylation. Phosphorylation of the endogenous Sec61β remains unchanged (Figure 4.8B). This indicates that the cdc2 kinase indeed phosphorylates Sec61β at serine 17.

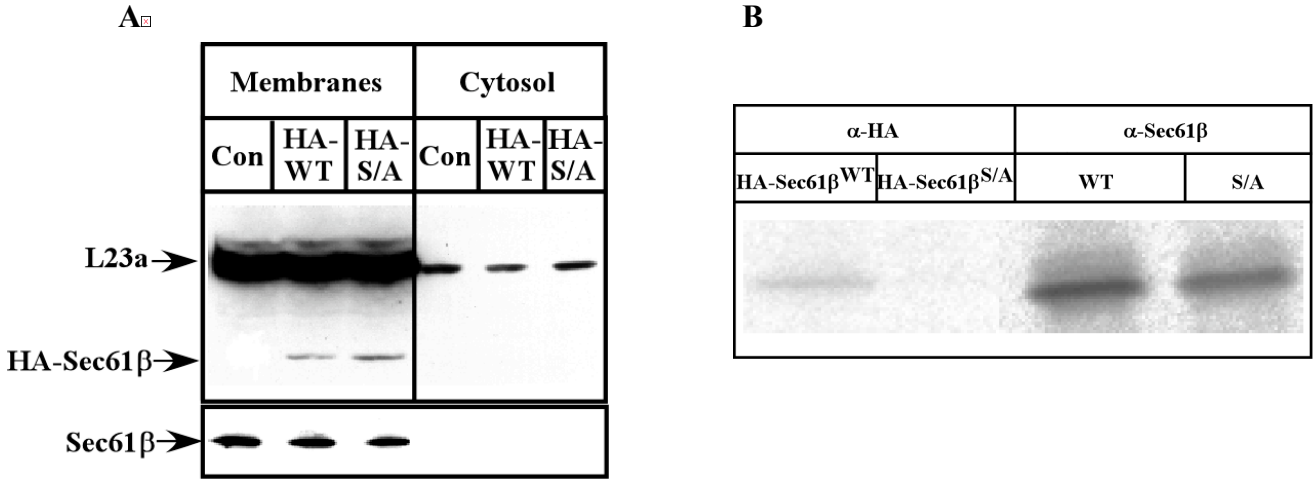


Figure 4.8 **Expression and Phosphorylation of Recombinant HA-tagged Sec61β. A)** HeLa cells were transfected with N-terminally HA-tagged constructs encoding for the wild type protein (HA-Sec61β^{WT}) or the serine 17 to alanine mutant (HA-Sec61β$^{S/A}$). 24 hours after transfection the cells were homogenized and membranes pelleted. Equivalent amounts from the membrane and cytosol fraction were applied to SDS-PAGE and probed with antibodies against L23a (a ribosomal protein), Sec61β or HA-tag. **B)** Membrane fractions from transfected HeLa cells were incubated with cdc2 kinase. The reaction was stopped after 20 minutes by addition of 20% TCA, precipitated proteins solubilized and Sec61β immuno-precipitated using either the antibody against Sec61β or the antibody against the HA tag. Immuno-precipitated proteins visualized by SDS-PAGE and phosphoimaging

4.2 Protein Translocation during M-phase

4.2.1 Membrane Protein Insertion During the M-phase

Cdc2 kinase is active during the M-phase and by phosphorylating different proteins affects a variety of physiological processes. The cdc2 kinase is seen to have at least two possible substrates at the ER. One of these proteins was identified as Sec61β. It is conceivable that the phosphorylation of Sec61β could affect a function which is related to the Sec61p translocon. In order to test this, the efficiency of insertion of a membrane protein during the M-phase was analysed.

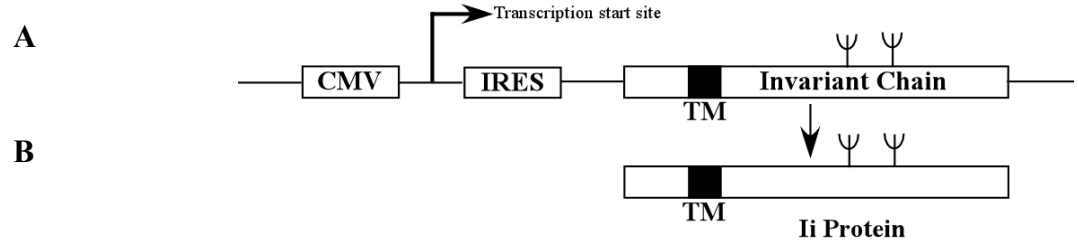
The reporter protein used was the Invariant chain (Ii), which is part of the MHCII complex. Ii protein gets glycosylated at two positions within the ER and this glycosylation can be used to monitor the process of insertion into the ER. Changes affecting membrane insertion of Ii would result in delay in glycosylation and would be reflected in accumulation of unglycosylated or single glycosylated protein.

The second step was to ensure translation of the reporter protein during the M-phase since majority of protein translation during the M-phase is blocked due to modification of members of the translation initiation machinery. During the M-phase the proteins which bind to eIF4E, the translation initiation factor, get phosphorylated, 4E-BP1 is hypo-phosphorylated and eIF4GII is hyper-phosphorylated (Cormier et al., 2003; Pyronnet et al., 2001). This modification inhibits the formation of the initiation complex at the 5' ends of the mRNA and thus prevents ribosome from initiating translation. The cell ensures translation of essential growth stimulatory and metabolic proteins such as cMyc and Ornithine de-carboxylase respectively, by including an Internal Ribosome Entry Site (IRES) in the 5' untranslated region of the mRNAs coding for these proteins. This allows ribosomes to initiate translation from within the mRNA and bypass the translational block (Vagner et al., 2001). The IRES element from the orinithine decarboxylase gene (Pyronnet et al., 2000) was cloned in front of the Ii coding plasmid thus allowing translation during the M-phase (Figure 4.9A).

HeLa cells which were 80% confluent were transfected with the plasmid encoding the Ii construct. Cells were kept in the transfection solution for three hours. At the end of this period the transfection solution was washed off and cells were allowed to recover by growing them in normal medium for 5 hours. Cells were then synchronized either in the M-phase using nocodazole or in the G1-phase using aphidicolin. After synchronization the cells were grown in medium lacking methionine and cysteine for two hours and then pulsed with ^{35}S labelled methionine/cysteine for 10 minutes. Cells were then lysed and 1/10 of the sample was directly

applied to SDS-PAGE and the other part was used for immuno-precipitation using an antibody against li. Samples after SDS-PAGE were exposed to phosphoimager screen to visualize the labelled proteins.

The unsynchronized cells show efficient translocation into the ER, as determined by fully glycosylated li protein and absence of the unglycosylated protein. On the other hand the cells synchronized in the M-phase show accumulation of the unglycosylated protein. The ratio of glycosylated to unglycosylated protein is about 1:1 (Figure 4.9B). This suggests that the insertion of a membrane protein into the ER is slightly delayed during the M-phase.



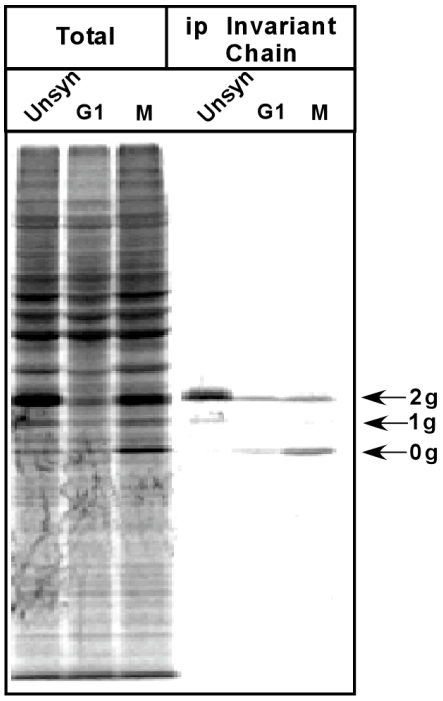


Figure 4.10 **ER Translocation of li during M-phase.** A) Schematic representation of the Invariant Chain expression construct. Between the CMV promoter and the coding region, an IRES element was inserted. B) Trasnlation and membrane insertion of Ii during M-Phase. HeLa cells were transfected with the Invariant chain expression construct. Cells were synchronized in the G1 phase by Aphidicolin and in the M-phase by Nocodazole or were left unsynchronized. The cells were grown for 2 hours in methionine/cysteine free medium and then pulsed with ^{35}S labelled methionine and cysteine for 10 minutes. The cells were lysed and 1/10 of the sample was directly applied to SDS-PAGE and from the remaining sample invariant chain was immuno-precipitated. The position of fully glycosylated (2g), single glycosylated (1g) and unglycosylated (0g) are indicated.

It is possible that the large number of physiological and morphological changes during the M-phase could have an indirect effect on the process of translocation. The present system was clearly inadequate to investigate role of the phosphorylation of Sec61β on translocation. It was therefore decided that the *Drosophila* system would be a better system to investigate the physiological significance of phosphorylation.

4.3 Analysis of Gurken Trafficking in Oocytes

4.3.1 The Sec61β loss of function allele

It has been previously been reported that knock out of sec61β in *Drosophila* is lethal (Valcarcel et al., 1999). Flies which are homozygous for an allele of Sec61β with a P-element insertion at the beginning of the transcription start site show embryonic lethality (sec61β^{P1}). This insertion results in a loss of function allele of Sec61β since the phenotype of this allele is similar to the phenotype obtained when the whole chromosomal region containing Sec61β is deleted (Valcarcel et al., 1999). This allele was used to study the function of Sec61β in Drosophila. The embryos homozygous for sec61β^{P1} allele are able to reach a later stage of development; this may be due to maternal contribution of the Sec61β mRNA in the oocyte (Valcarcel et al., 1999). In order to prevent the maternal contribution, flies having ovaries without Sec61β were generated using the FLP-dominant female sterile technique, defined as the germline clones of sec61β^{P1} (Chou et al., 1993; Valcarcel et al., 1999). These flies laid eggs with fused dorsal appendages indicating perturbations in the anterior-dorsal axis formation (Valcarcel et al., 1999).

4.3.2 Expression of Sec61β in wild type and sec61β^{P1} germline clones

To directly investigate the depletion of the Sec61β protein in germlines clones of sec61β^{P1}, western blot analysis was done on ovarian extracts from the homozygous germline clones and the ovarian extracts from wild type flies using an antibody generated against Sec61β. The amount of the Sec61β is drastically reduced in ovaries derived from the germline clones as compared to the wild type ovaries (Figure 4.11). A small amount of Sec61β observed in the germline clones which most likely reflects Sec61β in the follicle cells which do not originate from the germ cell lineage and retain normal amounts of Sec61β.

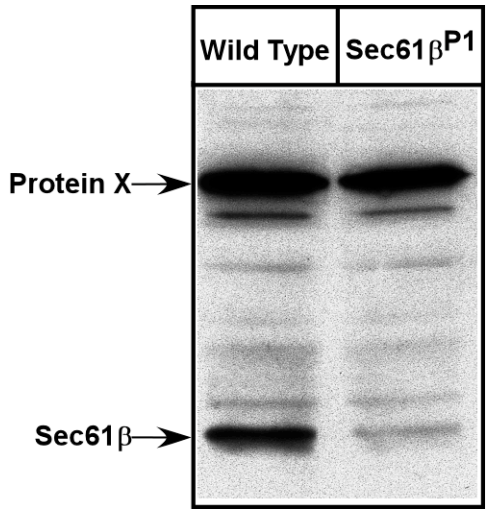


Figure 4.11 **Sec61β levels in ovaries from wild type and Sec61β^{P1} germline clones.** Ovaries were dissected from flies lysed in buffer containing 2% SDS. Western blotting was performed using Sec61β antibody. A cross reactive band indicated as Protein X serves as a loading control.

4.3.3 Localization of Gurken in Oocytes and Germline clones of sec61β^{P1}

It has been proposed that the observed dorsal appendage fusion phenotype in sec61β^{P1} germline clones could be due to reduced signalling by Gurken, a ligand for the Epidermal Growth Factor Receptor (EGFR) (Valcarcel et al., 1999). The signalling by the EGFR occurs when Gurken is secreted from the plasma membrane of oocyte and interacts with its receptors on the surrounding follicle cells (Nilson and Schupbach, 1999). To investigate the intracellular localization of Gurken in oocyte, a monoclonal antibody generated against Gurken was used for immuno-fluorescence analysis.

At stage 10 of oogenesis Gurken signals to the follicle cells at the anterio-dorsal boundary. At this stage Gurken mRNA and protein becomes localized to the anterio-dorsal end of the oocyte. Gurken signals to the follicle cells present at the anterior end of the oocyte in proximity of the regions where Gurken is seen to be accumulated (Figure 4.12A). Hence, localization of Gurken was done during the stage 10 of oogenesis.

In wild type egg chambers Gurken is localized on the anterio-dorsal region of the oocyte in close proximity to the nucleus (Figure 4.12B). Plasma membrane in these oocytes was delineated by staining for actin which underlines the plasma membrane. Distinct actin staining is seen in form of two bands which corresponds to the plasma membrane of the oocyte and the surrounding follicle cells. When this region of the oocyte is magnified, Gurken is found in two locations in the oocyte. Gurken co-localizes with the actin, indicating plasma-membrane localization. The Gurken is also seen in punctuate structures in the oocyte cytoplasm below the plasma membrane (Figure 4.12C). Using immuno-fluorescence the total amounts of Gurken in wild type oocytes and oocytes from the germline clones homozygous for sec61β^{P1} cannot be determined. However, the protein is still at the anterior-dorsal part of the oocyte in proximity to the nucleus (Figure 4.12D). However, co-staining with actin and magnified image shows that the amounts of Gurken at the plasma membranes are drastically reduced. Reduction in Gurken occurs in the part of the plasma membrane which is in direct apposition to the follicle cells. The cytoplasmic pool of Gurken on the other hand is still localized to the same region in the sec61β^{P1} germline clones as in the wild type oocytes (Figure 4.12E).

Gurken, after release from the oocyte plasma membrane is internalized by the adjacent follicle cells. In wild type egg chambers Gurken protein is also seen in area corresponding to the adjacent follicle in distinct punctuate structures (Figure 4.12F). In the sec61β^{P1} germline clones however, this signal is rarely observed (Figure 4.12G). The immuno-fluorescence images described here are representative of 20 oocytes which were examined.

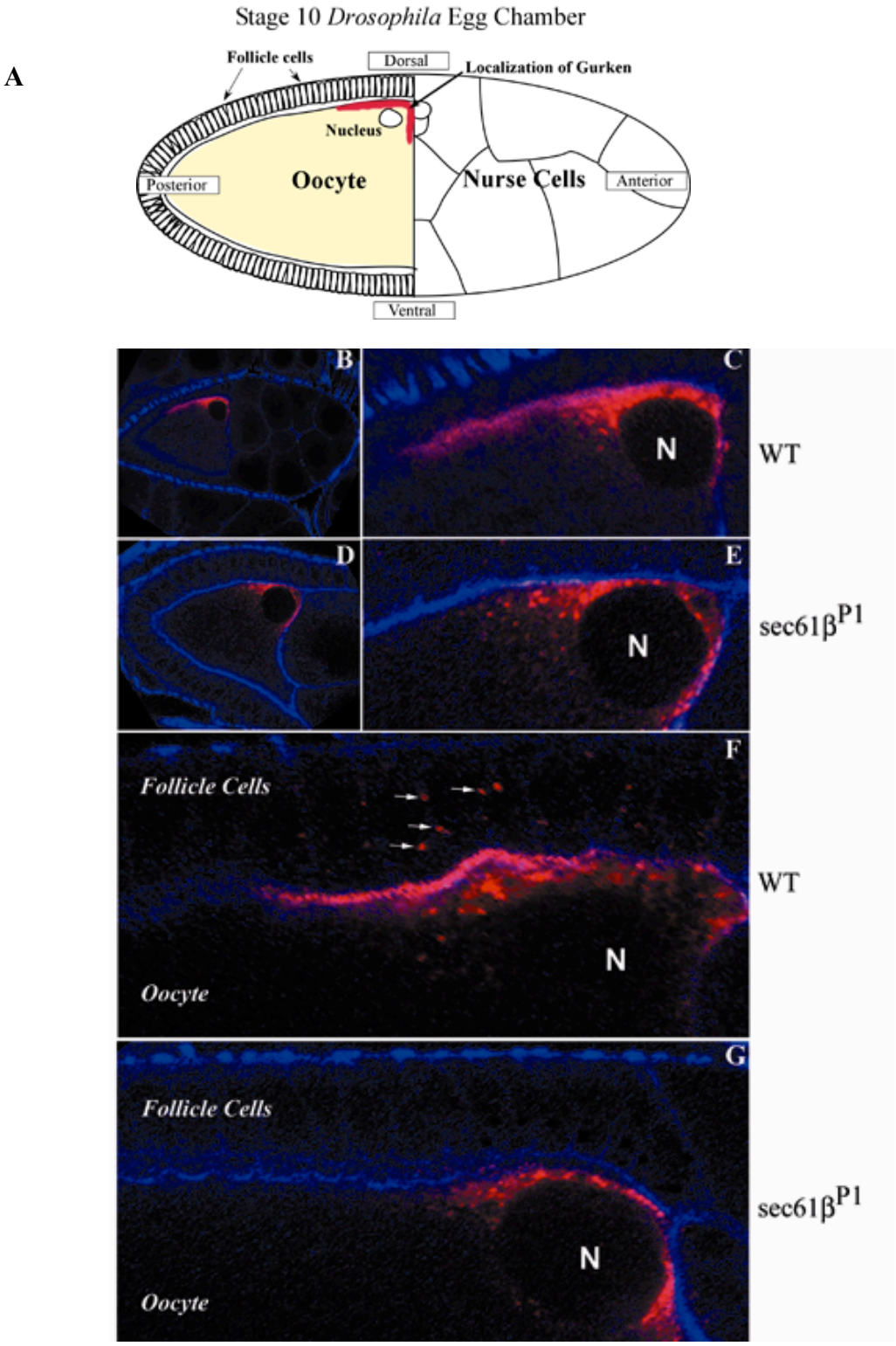


Figure 4.12 **Localization of Gurken Protein in Stage 10 Egg Chambers of Wild type and sec61β^{P1} Germline Clones.** A) Cartoon depicting the localization of Gurken in an egg chamber during stage 10 of oogenesis. In B-G egg chambers of the indicated genotype were stained for actin using labelled Phalloidin (blue) and Gurken (red). B is the wild type egg chamber at stage 10; Gurken appears at the anterior-dorsal postion. The region where Gurken is localized is magnified in C and co-stained with phalloidin. D and E are similar images of oocytes from sec61β^{P1} germline clones at low and high magnification respectively. Gurken is not seen at the plasma membrane to the same extent in these oocytes. F shows the anterior-dorsal region of the oocyte where Gurken is localized along with the follicle cells. Gurken in the follicle cells is indicated by arrows. G shows a similar region of an oocyte from sec61β^{P1} germline clone. N indicates the position of the nucleus.

These data indicate that Sec61β affects the trafficking of Gurken to the plasma membrane. The amount of Gurken released from the plasma membrane and internalized by the follicle cells is also reduced in sec61β^{P1} germline clones.

4.3.4 The Intracellular Localization of Gurken

Gurken is a type I membrane protein and the presence of the signal sequence suggests that the protein may enter the ER and proceed along the secretory pathway to reach the plasma membrane. To see if the intra-cellular localized Gurken protein is in the ER, egg chambers were stained with an antibody specific to the ER. An antibody raised against the Boca protein which has been previously characterized as an ER protein in oocytes was used (Culi and Mann, 2003). ER in stage 9-10 egg chambers appears in a diffused area below the plasma membrane and throughout the oocyte. Co-staining of Gurken with Boca revealed no co-localization either in the wild type egg chambers (Figure 4.13A and B) and egg chambers from the germline clones of sec61β^{P1} (Figure 4.13C and D). The cytoplasmic pool of the Gurken in the oocytes was clearly distinct from the ER.

These data indicate that the intra-cellular localization of Gurken, which is not affected by lack of Sec61β, does not correspond to the ER.

4.3.5 Gurken Signalling and Localization during Early Oogenesis

During the earlier stages of oogenesis (stages 6-9) the nucleus is localized to the posterior part of the oocyte, Gurken mRNA is associated with the nucleus and is consequently also present at the posterior part of the oocyte (Nilson and Schupbach, 1999). The ER at these stages is found to be distributed throughout the oocyte (Figure 4.13F). The Gurken protein in the wild type egg chambers at these stages localizes in punctuate cytoplasmic structures structures at the posterior part of the egg chamber (Figure 4.13G). Occasional overlap with ER is also seen (Figure 4.13H). Gurken staining is observed from the posterior follicle cells most likely represents the processed and internalized form of the protein. In sec61β^{P1} germline clones Gurken is also observed in cytoplasmic structures with occasional overlaps with ER (Figure 4.13I-L). However, no staining at either the plasma membrane or in the follicle cells is observed.

These data indicate that a defect in Gurken transport in sec61β^{P1} germline clones occurs already during early stages of oocyte development.

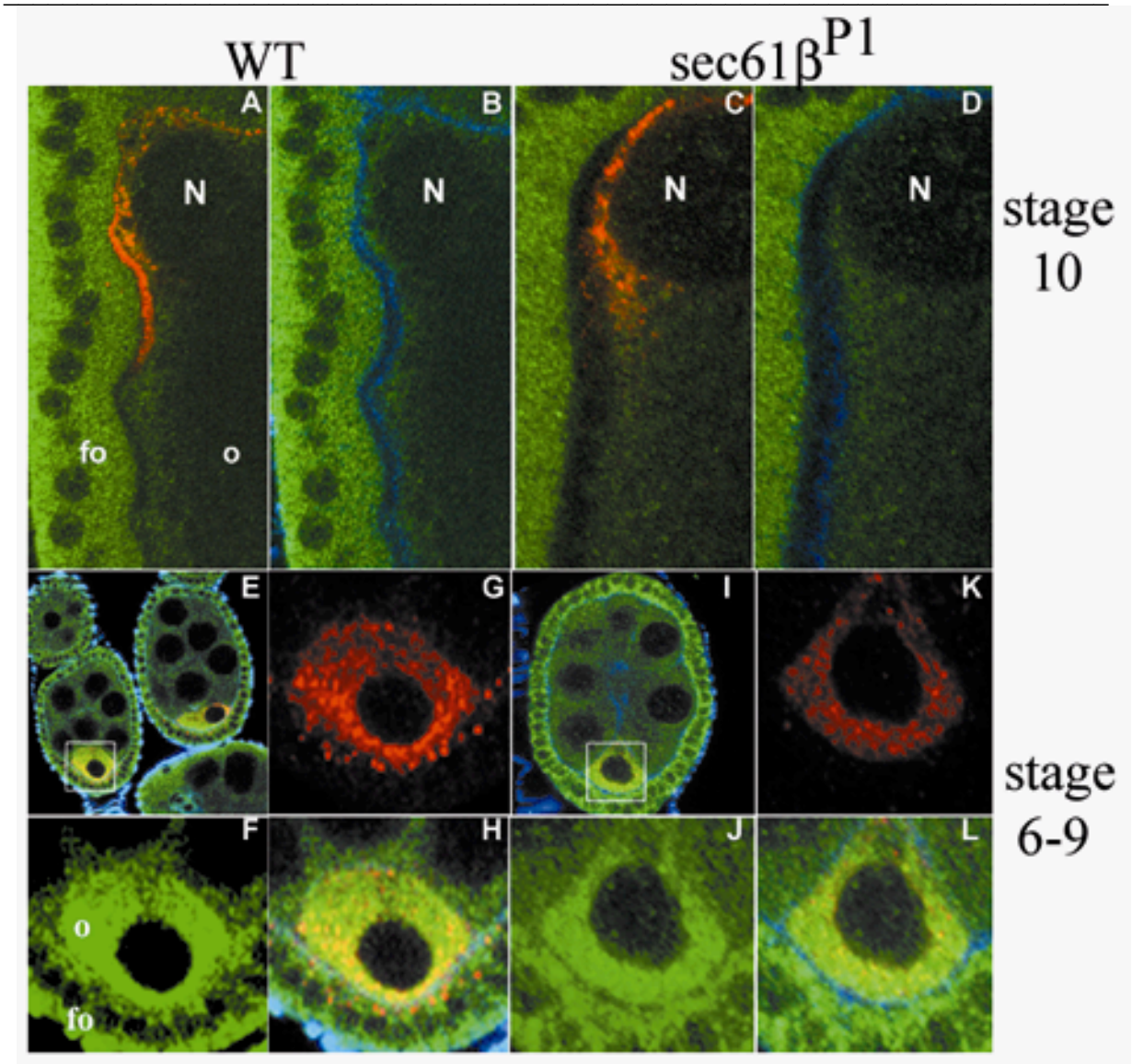


Figure 4.13 **Co-staining of Gurken and Boca in Egg Chambers during Early and Later Stages of Development.** Oocytes were stained with Boca (green) and Gurken (red) and Phalliodin (blue). In oocytes from stage 10 egg chambers (A-D) Boca stains a diffuse region below the plasma-membrane which indicates the ER in both the wild type (A and B) and the sec61β^{P1}germline clones (C and D), Gurken staining is observed either along the plasma membrane or in distinct puncta which does not co-localise with ER either in wild type oocytes (B) or the oocytes from sec61β^{P1}germline clones. Oocytes from younger egg chambers were similarly stained (E-L) and the boxed regions in E and I are magnified. Gurken appears diffused through out the oocyte in the wild type (G) and sec61β^{P1} germline clones (K). Additional staining is also seen in the surrounding follicle cells in wild type oocyte (G and H) and not in the oocyte from the germline clone (K and L). ER is also distributed through the oocyte in both the wild type oocytes (F) and those which were derived from the germline clones (J). Occasional overlap of staining between Gurken and ER is seen both the wild type oocytes (H) and those which were derived from the germline clones (L). N indicates the position of the nucleus. fo and o represent follicle cells and oocyte, respectively.

4.3.6 Localization of Yolkless in Wild type and Germline Clones of sec61β^{P1}

To see whether the intracellular and plasma membrane localization of another membrane protein is affected in the sec61β^{P1} germline clones the egg chambers were stained for Yolkless (Yl). Yl is a type I membrane protein, similar to Gurken which is expressed by the oocytes for the uptake of vitellogenins and yolk proteins (Schonbaum et al., 1995). Plasma membrane in these oocytes was delineated by staining for actin which underlines the plasma membrane. An antibody raised against the Boca protein which has been previously characterised as an ER protein in oocytes was used to stain the ER. In both the wild type egg chambers (Figure 4.14A and B) and sec61β^{P1} homozygous clones (Figure 4.14C and D) during stage 9-10 Yl is seen to co-localize with actin indicating localization at the plasma membrane.

These data indicate that depletion of Sec61β does not seem to affect a general transport pathway since Yl is correctly localized at the plasma membrane in sec61β^{P1} germline clones.

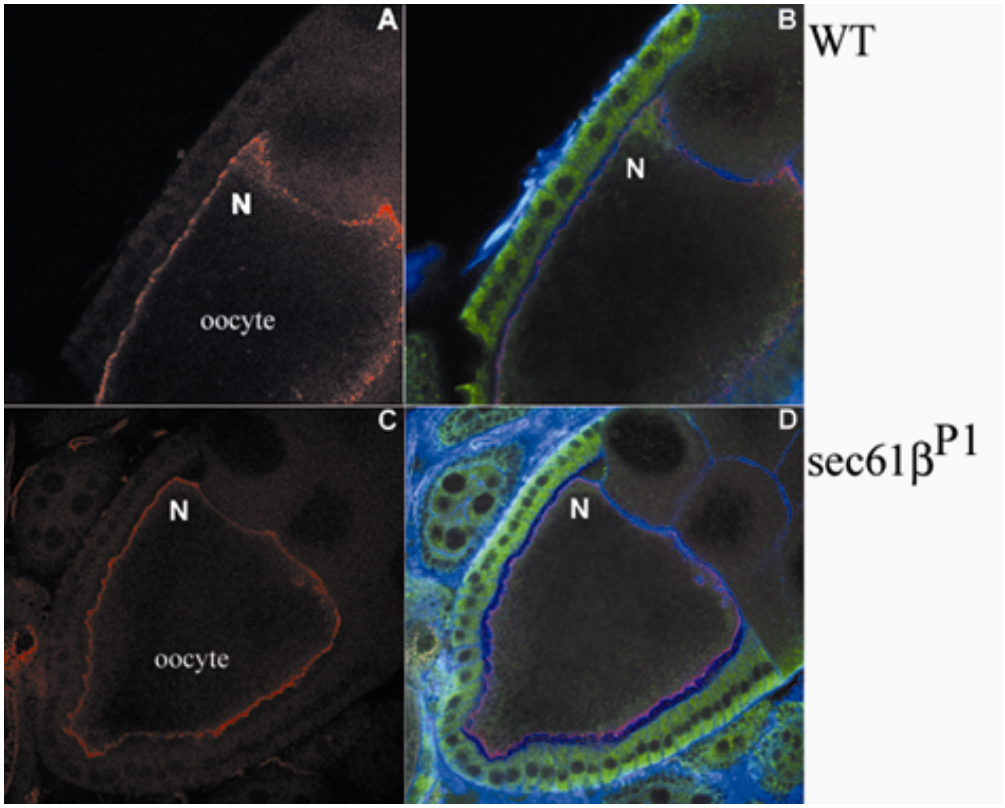


Figure 4.14 **Localization of Yolkless in stage 10 oocytes.** Oocytes from wild type egg chambers or from sec61β^{P1} germline clones were stained with Yolkless (red), Boca (green) and Phalloidin (Blue). In oocytes from wild type stage 10 egg chambers (A) or from sec61β^{P1} germline clones (C) Yl is at a peripheral location in the oocyte. In wild type oocytes (B) and oocytes from the germline clones (D) Yl co-localizes with actin indicating plasma membrane localization. Boca stains a diffuse region below the plasma membrane in oocytes from wild type egg chambers (B) and those from the germline clones (D) N indicates the position of the nucleus.

4.4 Molecular Characterization of Gurken traffic in Mammalian Cells

4.4.1 Reconstitution of Gurken Biosynthesis in Mammalian Cells

Experiments in the previous section show that lack of Sec61β in oocytes affects trafficking of Gurken to the plasma membrane. It seems that Gurken accumulates in a post-ER compartment. This is a surprising finding that lack of a component of the Sec61p protein translocation channel at the ER affects a step beyond the ER. In order to further characterize this defect and to determine at which stage of the secretory pathway the trafficking of Gurken is affected, HeLa cells were used to recapitulate Gurken traffic. Gurken is a type I membrane proteins and most likely enters the ER by signal-sequence dependent co-translational translocational pathway. Gurken is also glycosylated in the ER. Gurken transport to the plasma-membrane is governed by a series of proteins in the secretory pathway. The defect in trafficking could either be due to Sec61β directly affecting the transport of Gurken, or lack of Sec61β could affect localization of the regulatory proteins which would indirectly affect the plasma membrane traffic of Gurken.

Previous studies aimed at reproducing the trafficking of EGFR ligands in mammalian cells have shown that upon transfection into mammalian cells Spitz (an EGFR ligand) enters the ER. Exit from the ER is mediated by an ER chaperone, Star. Rhomboid (Rho) is localized to the Golgi complex and cleaves Spitz which is able to reach the Golgi. This facilitates the transport of Spitz out of the cell. From these experiments it is believed that complex trafficking pathway of the EGFR ligands as it occurs in different organs of *Drosophila* can be, to a large extent, recapitulated in mammalian cells in culture (Lee et al., 2001; Urban et al., 2002).

Analysis in HeLa cells allows the investigation of targeting and insertion of Gurken in the ER as indicated by glycosylation. Trafficking along the secretory pathway can be monitored by changes in the susceptibility of Gurken to endo-glycosidase H (endo H). ER specific N-glycosylation can be cleaved by endo H whereas glycosylation which occurs after the protein has left the ER is insensitive to endo H.

Gurken was transfected into HeLa cells, either alone or co-transfected with the regulatory proteins, Star and Rho. On western blot analysis of the whole cell lysate, Gurken is seen to migrate as a 45-47 KDa protein (Figure 4.15 A). Upon co-transfection with Myc-tagged Star the position of the band is unchanged. When HA-tagged Rho is transfected, Guken shows faster migration which most likely represents the intra-membrane cleaved form of Gurken. Gurken is glycosylated as seen by its sensitive to Endo-glycosidase H. Sensitivity

to EndoH is retained even when Star and Rho are tranfected along with Gurken (Figure 4.15A). Proteins samples from the transfected cells were also analysed by western blotting using antibodies against Myc (Figure 4.15B) and HA (Figure 4.15C) to detect the expression of Star and Rho in these cells respectively. Western blot with the anti-myc and anti-HA antibody shows additional bands of lower molecular weight the identity of these bands could not be determined.

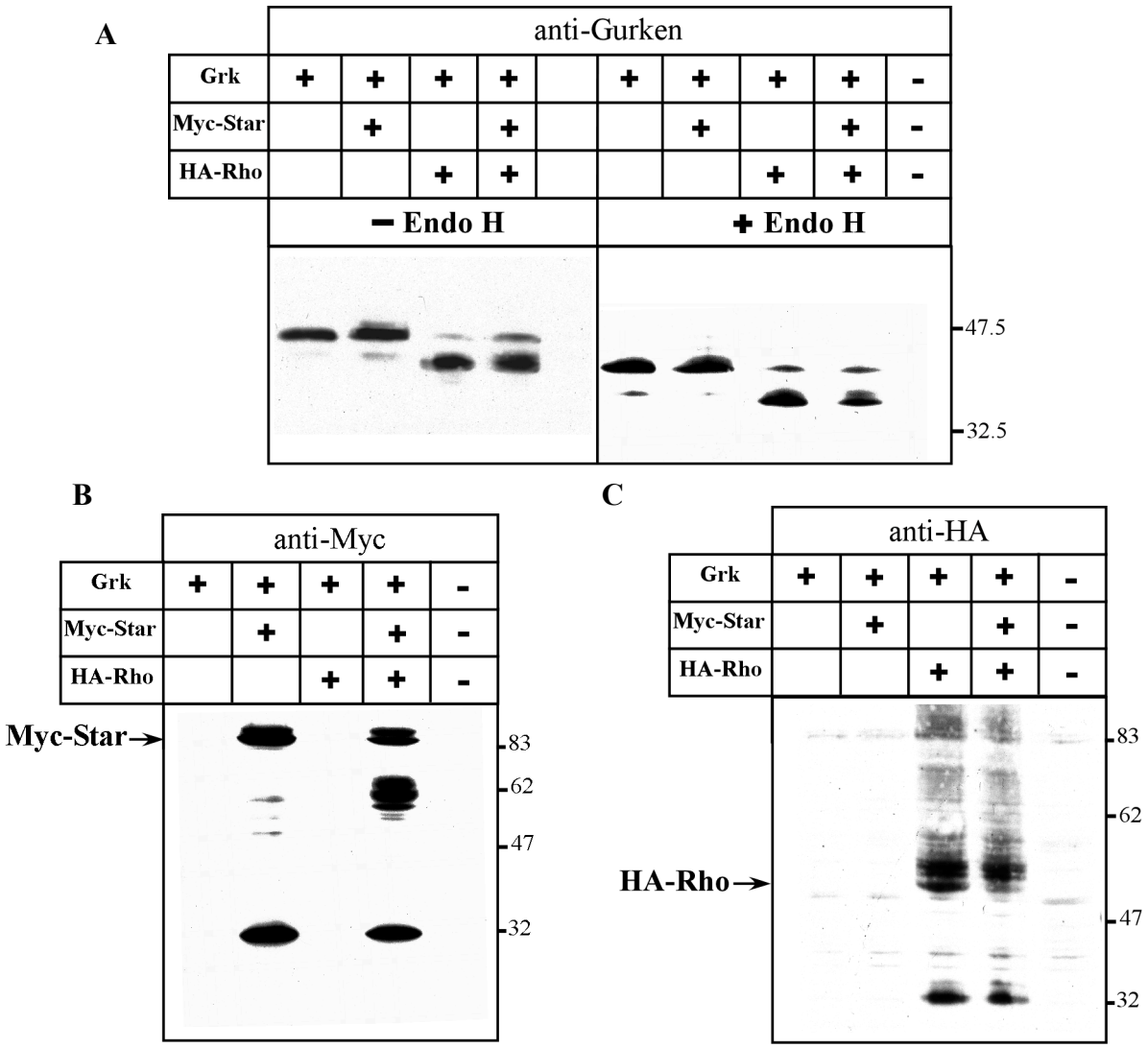


Figure 4.15 **Expression of Gurken, Star and Rho in HeLa cells.** Plasmid encoding Gurken protein alone or in combination with Myc tagged Star or HA tagged Rho were transfected into HeLa cells as indicated on top of the figure or left untransfected. 24 hours after transfection, aliquots of the cell lysates were applied to SDS-PAGE. A) Aliquots were applied to the gel directly and after being treated with EndoH. Western bloting was done using an antibody against Gurken. B) Aliquots were also probed with anti-Myc antibody to detect expression of Star or C) samples were probed with anti-HA antibody to detect expression of Rho. The arrows indicate the bands which appear at sizes expected for Star and Rho

These data indicate that Gurken is efficiently inserted into the ER since essentially all detected protein is glycosylated. Gurken is sensitive to endo H independent of other co-transfected proteins.

4.4.2 Reduced Sec61β Protein Levels by siRNA in Mammalian Cells

siRNA has recently been recently established as an efficient method of altering protein expression in mammalian cells. The technique involves introduction of double stranded RNA molecules complementary to the mRNA encoding the protein whose expression has to be altered. Presence of these double stranded RNA molecules triggers cleavage of the target mRNA by activation of specific exonucleases. This strategy was utilized to reduce the level of Sec61β in mammalian cells. Double stranded oligomers encompassing different parts of the Sec61β mRNA were designed following the published guidelines with respect to length, distance from the transcription start site and GC content of the primers (Imgenex). The oligomers were cloned into an expression vector under the control of RNA polymerase III promoter (pSuppressorNeo plasmid, Imgenex) was obtained and the resulting plasmids pSUP-Sec61β was transfected into mammalian cells. It was intended that the siRNA encoding plasmid remains in the cells for more than three days. Since HeLa cells are thought to lose the transiently transfected plasmids in two-three days, a second transfection was done on the third day and protein levels were analysed by western blotting on the fourth day. A scrambled nucleotide sequence was also cloned in the same vector and transfected in cells in a similar manner as a control.

At day 0 the level of Sec61β protein is the same in the cells transfected with the pSUP-Sec61β siRNA plasmid and those transfected with the control siRNA. After 4 days of transfection Sec61β levels are reduced in cells which were transfected with Sec61β siRNA plasmid (Figure 4.23). The levels of the protein remain unchanged cells transfected with the control siRNA. Thus, transfection with siRNA can result in drastic reduction in the levels of Sec61β. A cytosolic cross reactive band was used to normalise the amount of the protein loaded in each lane (Figure 4.23). Of the five sets of siRNA plasmids directed against different parts of the Sec61β mRNA (see materials and methods) one against the 5' UTR of the mRNA shows most efficient reduction in the level of Sec61β and was used for further experiments.

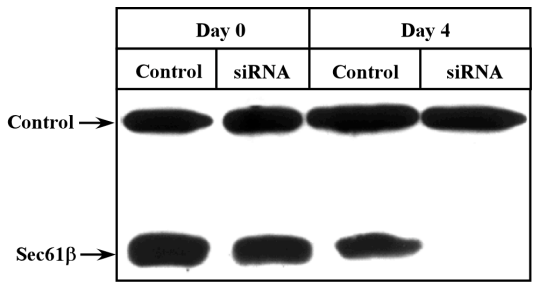


Figure 4.16 **siRNA mediated knock-down of Sec61β protein levels.** HeLa cells were transfected with the pSUP-Sec61β plasmid generating a double stranded RNA against Sec61β. After three days the same cells were re-transfected with the plasmids. On the fourth day, cells lysate was made and aliquots were probed with an antibody against Sec61β

4.4.3 Analyses of Gurken biosynthesis after Sec61β knock down

Gurken is inserted into the ER and is accessible for cleavage by Rho in HeLa cells. Transfection with the siRNA plasmid results in drastic reduction in protein levels of Sec61β. The next experiment was to investigate if the reduction in the level of Sec61β has any effect on the insertion of Gurken in the ER and accessibility to Rho dependent cleavage. Cells were transfected with Gurken, Rho and Star together, to recapitulate the Gurken traffic, with either the siRNA against Sec61β, pSUP-Sec61βor the control siRNA plasmid. After four days (which included a second transfection on day three) the steady state expression of the proteins in transfected cells was analysed using western blotting. The cells were also used to determine translocation of the Gurken by a pulse analysis using ^{35}S labelled methionine and cysteine, followed by immuno-precipitation.

Western blot analysis using the Gurken antibody shows that the different glycosylated/unglycosylated forms of Gurken appear in a similar ratio in cells transfected with the siRNA against Sec61β, pSUP-Sec61β or cells with a control siRNA (Figure 4.24A). After transfection with the pSUP-Sec61β Gurken remains sensitive to endoH and is efficiently cleaved by Rho as indicated by the lower molecular weight form. The pulse experiment shows that a majority of Gurken is glycosylated, and reduced level of Sec61β does not seem to affect the process (Figure 4.24B). The reduction in the level of Sec61β after transfection of the siRNA plasmid is confirmed by western blot analysis using an antibody against Sec61β (Figure 4.24C). The expression levels of Star and Rho were similar in cells with normal or reduced levels of Sec61β (data not shown).

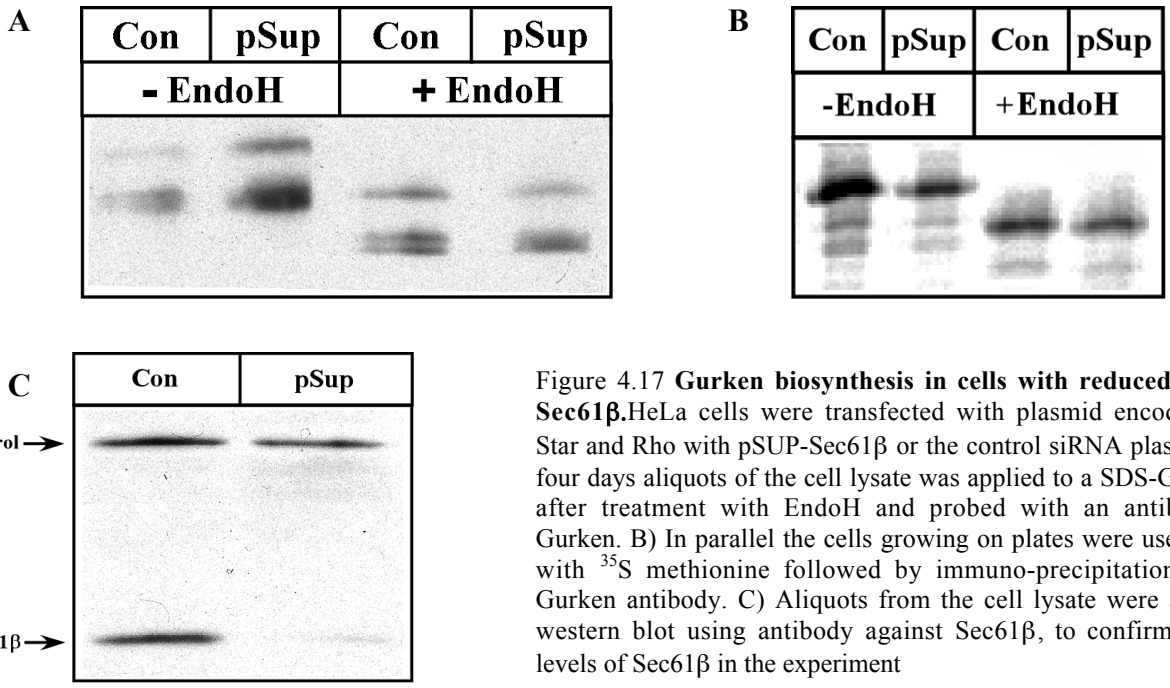


Figure 4.17 **Gurken biosynthesis in cells with reduced amounts of Sec61β.**HeLa cells were transfected with plasmid encoding Gurken, Star and Rho with pSUP-Sec61β or the control siRNA plasmid A) After four days aliquots of the cell lysate was applied to a SDS-Gel directly or after treatment with EndoH and probed with an antibody against Gurken. B) In parallel the cells growing on plates were used for a pulse with ^{35}S methionine followed by immuno-precipitation using anti-Gurken antibody. C) Aliquots from the cell lysate were also used for western blot using antibody against Sec61β, to confirm the reduced levels of Sec61β in the experiment

Data from these experiments indicate that Gurken translocation into the ER and the post-translational modifications with respect to glycosylation and Rho dependent cleavage can be recapitulated in mammalian cells. Gurken translocation into the ER as indicated by post-translational glycosylation and Rho dependent cleavage does not seem to be affected when level of Sec61β is drastically reduced.

4.5 Investigating the Physiological Relevance of Phosphorylation

4.5.1 Phosphorylation of dSec61β in HeLa Cells

Experiments mentioned in the previous section show that cdc2 kinase phosphorylates the human Sec61β at a serine residue. This serine residue is present as part of the predicted cdc2 kinase phosphorylation site and is conserved in *Drosophila*. This could indicate that Sec61β is also a substrate for the cdc2 kinase in *Drosophila*. In order to see if indeed the *Drosophila* Sec61β is phosphorylated by cdc2 kinase at the conserved serine residue, wild type form of the protein and protein lacking this serine were used for an *in-vitro* phosphorylation assay similar to the one described in section 3.1.5.

Site directed mutagenesis was used to replace the predicted serine to alanine. cDNAs were generated encoding for the N-terminal HA-tagged form of the wild type protein (HA-dSec61β$^{\mathbf{WT}}$) and the serine to alanine mutant protein (HA-dSec61β$^{\mathbf{S/A}}$). Presence of the HA-tag allowed discrimination between the *Drosophila* Sec61β and the endogenous human Sec61β. The plasmids encoding the HA-tagged proteins were transfected into HeLa cells. 24 hours after transfection the cells were harvested and crude membrane and membrane free fractions were prepared. This preparation allowed γ-^{32}P ATP access to the membrane proteins.

In order to see whether the HA-dSec61β could be phosphorylated by the cdc2 kinase the membrane fraction was incubated with purified cdc2 kinase and γ-^{32}P ATP. After the reaction the membrane were solubilized and immuno-precipitated using antibody against the HA tag to detect the *Drosophila* protein and with antibody against human Sec61βto detect the endogenous Sec61β. The immuno-precipitated proteins were applied to a SDS-PAGE and phosphorylated protein visualized. HA-dSec61β$^{\mathbf{WT}}$ gets phosphorylated when incubated with cdc2 kinase, where as HA-dSec61β$^{\mathbf{S/A}}$ does not show any phosphorylation (Figure 4.15). Phosphorylation of the endogenous Sec61β remains unchanged. This indicates that the cdc2 kinase indeed phosphorylates the *Drosophila* Sec61β at the predicted serine residue at position 16.

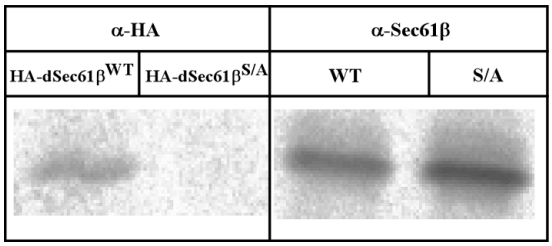


Figure 4.18 **Phosphorylation of Drosophila Sec61β and the phosphorylation mutant in HeLa cells.** Membrane fractions from HeLa cells transfected with either wild type HA-dSec61β^{WT} or phosphorylation mutant HA-dSec61β$^{S/A}$ were incubated with cdc2 kinase. The reaction was stopped after 20 minutes by addition of 20% TCA, precipitated proteins were solubilized and used for immuno-precipitation using the antibody against the HA tag to detect the *Drosophila* Sec61β and the antibody against human Sec61β to detect the endogenous Sec61β.

4.5.2 Rescue of Sec61β^{P1} allele by GAL-Sec61β

In order to investigate the physiological relevance of phosphorylation at position 16 by cdc2 kinase position the loss of function allele of Sec61β, sec61β^{P1} was employed. To begin with, the aim of the experiment was to see if the lethality associated with the loss of function allele can be rescued by a recombinant dSec61β which is expressed from a non-endogenous promoter. If this was indeed possible, then it was to be investigated if the recombinant dSec61βwith mutation in the cdc2 kinase phosphorylation site can rescue the lethality. Moreover, rescued flies would be carefully examined for the phenotype in different organs.

In this experiment Sec61β was expressed using the GAL4-UAS system. This system uses the yeast GAL4 activator from yeast which binds to specific binding sites at promoter regions of genes and activates transcription (Ptashne, 1988). GAL4 is believed to have no endogenous promoter in *Drosophila* (Brand and Perrimon, 1993). In *Drosophila* the system works as follows: a transgenic fly line is generated where the expression of the GAL4 transcription activator is driven by an endogenous *Drosophila* promoter, called a GAL4 driver. A second fly line is generated which is transgenic for a desired target gene. The target gene is cloned behind the GAL4 binding site, making the expression of the target gene GAL4 dependent. To activate the target gene, flies expressing GAL4 are crossed to flies carrying the GAL4-dependent target gene. In the progeny of this cross the target gene is expressed (Brand and Perrimon, 1993). For the present experiment, GAL4 was expressed under control of a promoter of a ubiquitous *Drosophila* gene called Armadillo (Arm-GAL4) since Sec61β is also believed to have a ubiquitous expression.

Transgenic fly line was made which expressed the wild type Sec61β under the control of the GAL4 upstream activating sequence (UAS-dSec61β^{WT}). Point mutations were made in the Sec61β cDNA which either caused serine to be replaced to alanine (UAS-dSec61β$^{S/A}$) to knock out the phosphorylation site, or serine to be replaced with aspartic acid (UAS-dSec61β$^{S/D}$) to conserve the negative charge of a phosphorylated protein and hence mimic a

constitutively phosphorylated protein. Transgenic lines were also generated with these dSec61β point mutants. Arm-GAL4 and UAS-Sec61β were combined in a single fly line which ensured continuous expression of Sec61β.

The sec61β^{P1} allele is maintained in a heterozygous condition by marking the chromosome containing the endogenous Sec61β with a Cyo balancer which makes the wings curly. Flies homozygous for sec61β^{P1} alleles are lethal and in addition the flies homozygous for Cyo balancer are also lethal. Thus, only flies containing one copy of the balancer and one copy of the sec61β^{P1} allele are able to survive.

The rationale of the rescue experiment was as follows; rescue by the Sec61β^{WT} transgene would be a control for the functionality of the strategy, which would indicate that recombinant Sec61β under the control of GAL4 can rescue the lethality. If phosphorylation of dSec61β at the serine residue is essential for the protein function the knock out allele, Sec61β$^{S/A}$ would not be able to rescue the lethality. Rescue by Sec61β$^{S/D}$ would strengthen the argument that Sec61β has to be phosphorylated at this position. The crossing scheme used for the rescue experiments with the three transgenic lines is described in Figure 4.15.

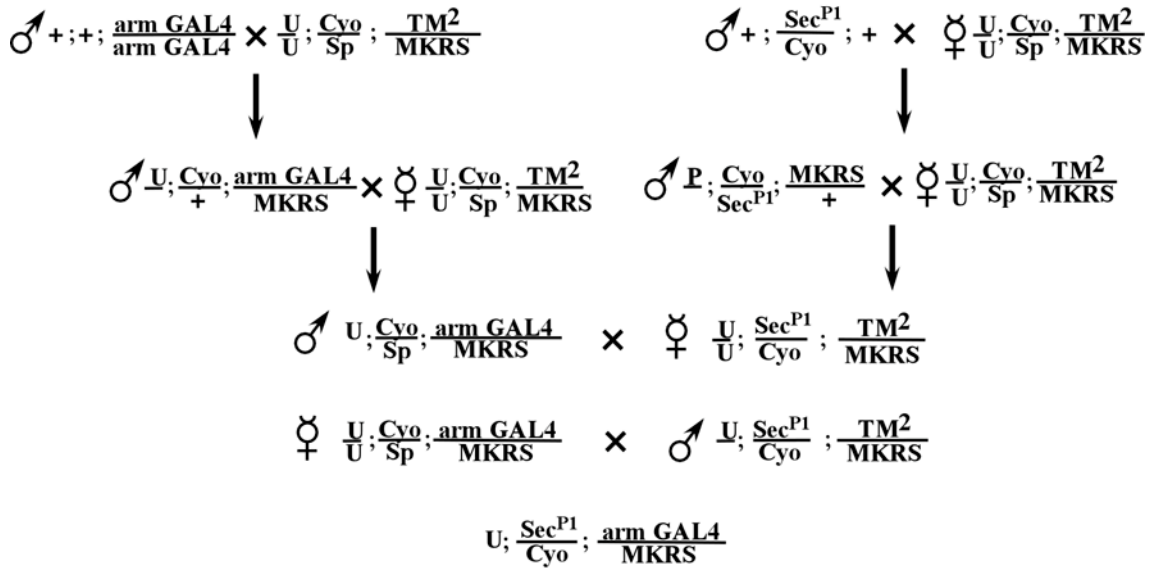


Figure 4.19 **Crossing scheme for GAL4-Sec61β rescue of Sec61β^{P1} homozygous flies.** UAS-Sec61β either wild type or the phosphorylation mutants (U), the GAL4 was under the control of the Armadillo promoter, (arm-GAL4) and sec61β^{P1} allele (Sec^{P1}) were combined in one fly line. Specific chromosomes were marked by different balancers as indicated.

The fly line with the Cyo marker, the transgene and the GAL4 driver was crossed to itself and the ratio of normal to curled wings in the next generation of flies was scored. Presence of flies with normal wings (not curled) in the progeny would indicate that they have lost the endogenous Sec61β. The fact that these flies survive would indicate that the transgene

can rescue the lethality since the only functional allele of Sec61β in these flies comes from the transgene, yet flies survive. On the other hand, if no flies with normal wings hatched this would mean that the transgene is unable to complement the loss of endogenous Sec61β.

It was seen that wild type Sec61β rescues the lethality, since the ratio of flies with curled to non-curled wings is close to 1 (Figure 4.20). This means that all flies with the transgene can survive. However, in the case of Sec61β$^{S/A}$ and Sec61β$^{S/D}$ the ratio is strongly biased in the favour of the curled wings. This indicates that although the rescue is still possible, the mutant transgenes cannot functionally complement the loss of function allele. Upon preliminary observation the rescued flies in any of the three cases did not show any morphological changes when compared with wild type flies.

	Curled Wings	**Normal Wings**	**Ratio**
Wild Type	50	46	**0.92**
Serine-Alanine	50	10	**0.2**
Serine-Aspartic Acid	50	6	**0.12**

Figure 4.20 **Rescue of the sec61β lethal allele by GAL4-Sec61β Transgenes.**

The amount of protein expressed from each of the transgene was equal as determined by western blotting using antibody against Sec61β (data not shown). This experiment indicates that the phosphorylation mutants of sec61β do not rescue the homozygous sec61β^{P1} embryos to the same extent as the wild type sec61β.

4.5.3 Over Expression of Sec61β in the Fly Wings

Manipulating levels of Sec61β in adult fly organs generates morphological changes (Valcarcel et al., 1999). Oocytes without Sec61β produce embryos with perturbation in the dorso-ventral axis, lack of Sec61β in developing eye results in reduced number of omatidia and lack of Sec61β during development of legs cause shortening of legs. In order to generate morphological changes in additional structures in the adult fly Sec61β was over-expressed in fly wings. It was previously observed in yeast that expression of Sec61β complements growth defects in the exocyst mutants (Toikkanen et al., 2003). The exocyst is a complex of proteins which is seen to be localized at the plasma-membrane and mediate directed vesicular fusion (Lipschutz and Mostov, 2002; Mostov et al., 2000). This indicates that even ectopically

expressed Sec61β may play a role in traffic to the plasma-membrane. Wing development is an extremely sensitive process and perturbations in morphological changes such as wing structure, vein delineation and hair polarity are easily visualized in adult wings. In many cases these morphological changes in the adult wings can be co-related to defects affecting specific signalling molecules during development of the wings. Ectopic expression in the wings was also utilized to test the differences in functional properties between the Sec61β wild type protein and the phosphorylation mutants of Sec61β.

Wing specific expression was achieved by using the GAL4-UAS system. Fly lines selected as source of GAL4 had the expression of GAL4 under the control of promoters of wing specific genes. The wing specific genes were Scalloped, Engrailed and Patched. The wings develop from a group of progenitor cells in larvae called imaginal discs where these genes have defined expression pattern. Cells in imaginal discs of the larvae can be co-related with regions in the adult wing. The expression pattern of the genes is shown in Figure 4.17. The wing imaginal discs are simple invaginated epithelial sacs composed of a convoluted columnar epithelium. A disc can be viewed as a series of circumferentially arranged regions. The outermost will have the dorsal and the ventral body wall structure, the notum, and the pleura. The next ring will make the dorsal and ventral hinge region, and the more centrally located wing pouch region will make the wing blade. The dorsal and the ventral discs are both divided into anterior (A) and posterior (P) compartments (Cohen, 1993).

Scalloped is expressed in a broad region in the wings. On the other hand engrailed and patched are expressed in the border defining the A and P compartments as shown in Figure 4.21. The adult structures which develop from the cells of the A/P boundary is the region of the wing blade between veins III and IV. In these experiments Sec61β and the mutant proteins were expressed in flies having wild type levels of endogenous Sec61β.

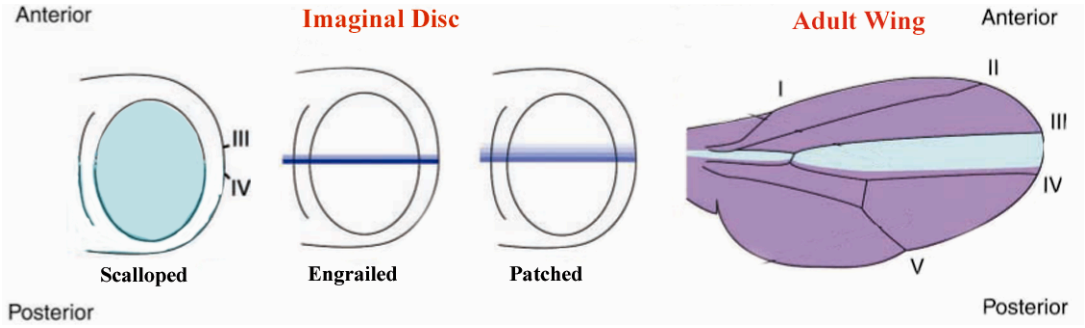


Figure 4.21 **Expression pattern of the GAL4 driven by promoters of different genes.** Scalloped is expressed in the anterior and the posterior compartment of the wing disc, where as engrailed and patched are expressed in the boundary of the anterior and posterior boundary. This, Sec61β driven by scalloped is expressed though out the adult wing blade, engrailed and patched cause expression in the middle domain of the wing blade as indicated.

When the UAS-Sec61β^{WT} is expressed using the Scalloped-GAL4 driver the wings become distorted and this is seen in close to 100% of the cases (Figure 4.22). These wings are characterised by reduced number and thickening of the veins, and loss of the hairs at the margin of the wings. Expression of UAS-Sec61β$^{S/A}$ and UAS-Sec61β$^{S/D}$ causes lethality.

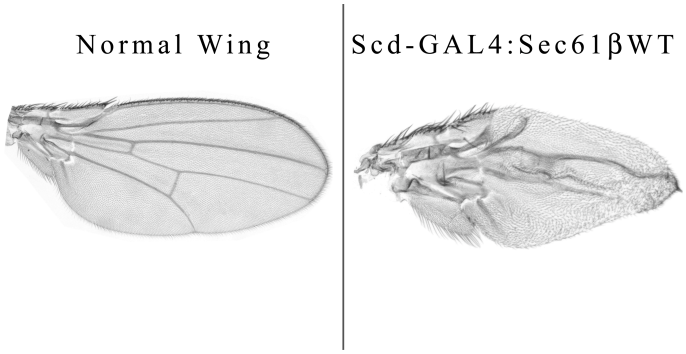


Figure 4.22 **Expression of Sec61β, using Scalloped driven GAL4.** Transgenic flies with UAS-Sec61βwere crossed with flies expressing GAL4 under the control of scalloped promoter. The F1 generation flies can express Sec61β through out the imaginal disc of the larvae and hence through out the adult wing. Two to four days after hatching the wings were fixed and mounted as described in materials and methods.

Engrailed driven expression of UAS-Sec61β^{WT} causes loss of vein structures from the wings (Figure 4.23). The affected veins are in the lower part of the wings, or in the ventral region, namely L4 and L5. The distance between these two veins is also reduced. Engrailed driven expression of the either of UAS-Sec61β$^{S/A}$ and UAS-Sec61β$^{S/D}$ also causes lethality. The larvae develop as far as the pupal stages, but eclosion is not observed.

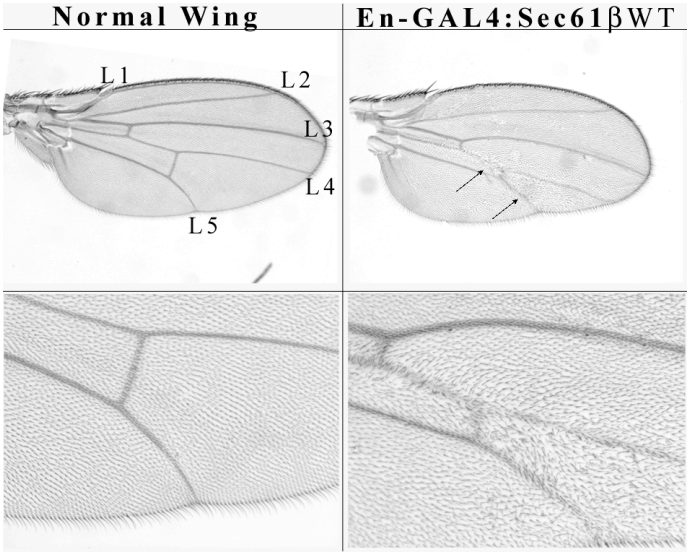


Figure 4.23 **Expression of Sec61β, using Engrailed driven GAL4.** Transgenic flies with UAS-Sec61β were crossed with flies expressing GAL4 under the control of scalloped promoter. The F1 generation flies can express the Sec61β in the boundary of the A/P compartments and in the regions between vein III and IV of the adult wing. Two to four days after hatching the wings were fixed and mounted as described in materials and methods.

Patched driven expression of UAS-Sec61β^{WT} does not result in any visible phenotype. On the other hand the UAS-Sec61β$^{S/D}$ produces a multiple hair phenotype in the region of the wing between veins L3 and L4. The wing cells under normal conditions produce one hair per cell and in a defined orientation. However under the over-expression conditions the cells produce

more than one hair and without any fixed orientation (Figure 4.20). Expression of the Sec61β$^{S/A}$ does not produce any viable flies, lethality occurs at the pupal stage.

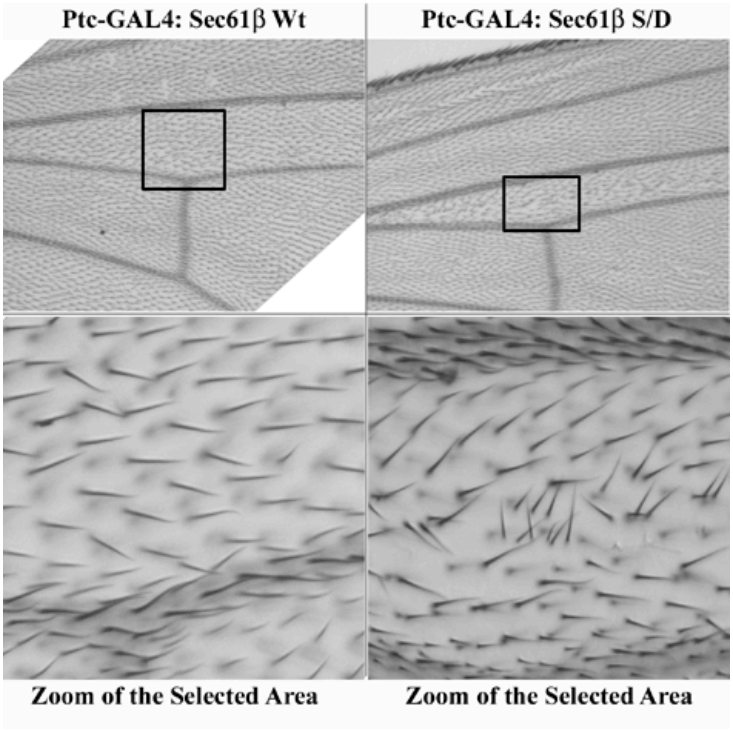


Figure 4.24 **Expression of GAL-4-Sec61β, using Patched driven GAL4.** Transgenic flies with GAL4-Sec61β were crossed with flies expressing GAL4 under the control of scalloped promoter. The F1 generation flies can express the transgene at the desired part of the wing. Two to four days after hatching the wings were fixed and mounted as described in materials and methods.

Based on the phenotypes generated from ectopic expression of the wild type Sec61β in the wings which results in reduced wing size and loss of vein structure, it can be suggested that signalling by EGFR pathway is affected (Guichard et al., 1999). Expression of the phosphorylation mutants of Sec61β from the same GAL4 drivers is more severe than expression of wild type protein. Expression of wild type form of Sec61β with the patched GAL4 driver had no effect, whereas the serine to aspartic acid mutant generates phenotype indicating loss in planar polarity during development of the wing veins (Adler, 2002). Based on these observations Sec61β seem to affect a broad range of developmental pathways during wing development and phosphorylation at the serine residue seems to affect the functional properties of Sec61β.

5. DISCUSSION

5.1 Summary

The present study was aimed at investigating the function of Sec61β, a component of the Sec61 protein translocation channel at the endoplasmic reticulum (ER), in *Drosophila*. Oocytes derived from the germline clones of the loss of function allele of Sec61β (sec61β^{P1}) do not synthesize Sec61βprotein. These oocytes show reduced amount of Gurken at the plasma membrane and in the surrounding follicle cells. Lack of Sec61β does not seem to affect trafficking of all proteins since localization of a plasma membrane protein, Yolkless, remains unchanged in these oocytes. Analysis of Gurken trafficking in mammalian cells shows that process of ER targeting and insertion of Gurken into the ER is not affected by reduced amounts of Sec61β. Ectopic expression of Sec61β in fly wings causes perturbations in wing morphology and vein delineation, suggesting that Sec61β may affect processes in other developmental pathways.

The second part of the study was to characterize the kinase responsible for the phosphorylation of Sec61β. Sec61β protein from humans and *Drosophila* is phosphorylated *in-vivo* by the cdc2 kinase at a conserved serine residue at the N-terminal cytoplasmic region. Sec61β is also phosphorylated by a second membrane associated straurosporine sensitive kinase. In *Drosophila*, Sec61β protein with the mutated serine can only partially rescue the lethality associated with homozygous loss of function allele, sec61β^{P1}. Ectopic expression of these phosphorylation mutant proteins in wings generate morphological changes distinct from those seen by ectopically expressing the wild-type protein. These experiments suggest that phosphorylation can affect the functional properties of Sec61β.

So far, protein translocation across the ER membrane has been studied using *in-vitro* biochemical assays with isolated microsomes or it has been studied in single cells such as yeasts or mammalian cell culture systems. However, these systems could not be used to test the function of proteins whose absence did not generate any phenotype. Sec61β is one such protein which does not seem to have a role in translocation per-se and is not essential in yeast. However, loss of Sec61β is lethal in *Drosophila*; thus indicating its vital role. The *Drosophila* system was therefore used for investigating the function of Sec61β. The present study is one of the first studies aimed at analysing the function of an ER translocation channel protein in a multi-cellular organism.

5.2 Analysis of the function of Sec61β during Drosophila Development

Function of Sec61β in *Drosophila* was studied in the following way. The level of Sec61β was manipulated in different organs by genetic techniques. Changes in the levels of Sec61β were manifested as morphological changes during development. These morphological changes are specific phenotypes which were used to identify the molecules affected by Sec61β.

In order to investigate the role of Sec61β during development of other organs, Sec61β was expressed in *Drosophila* wings. Expression of Sec61β in the wings did generate specific phenotypes. Although, the phenotypes obtained in the wings could not be directly co-related with the molecules affected, they allowed speculation about the molecules being affected.

5.3 Trafficking of Gurken to the Plasma membrane

Germline clones of sec61β^{P1}, the loss of function allele of Sec61β results in embryos with perturbations in the dorsal-ventral axis as indicated by fusion of the dorsal appendages (Valcarcel et al., 1999). This phenotype suggested reduced signalling by Gurken, which is an EGFR ligand in the oocyte. The localization and biosynthesis of Gurken was investigated using immuno-fluorescence analysis in ovaries derived from these clones.

In the germline clones of sec61β^{P1} amount of Gurken at the oocyte plasma membrane of the oocyte in direct apposition to the follicle cells is significantly reduced. The germline clones of sec61β^{P1} do not show any Gurken staining in the follicle cells. Localization of Gurken was also done in oocytes at stage 6-9 of oogenesis. At this stage Gurken mRNA is localized and translated in the posterior part of the oocyte and signals to the follicle cells in the posterior part of the oocyte. This signalling establishes the posterior axis of the oocyte (Nilson and Schupbach, 1999). Even at this stage the germline clones of sec61β^{P1} show lack of Gurken in the posterior follicle cells. Defect in plasma membrane localization of Gurken and the subsequent uptake by follicle cells occurs already at an early stage of oogenesis. However, disruption of Gurken signalling at this stage does not seem to generate an additional phenotype. In the germline clones of sec61β^{P1} the peri-nuclear, cytoplasmic localization of Gurken seemed unaltered. Gurken in the cytoplasm does not co-localize with the ER. It seems that in the germline clones of sec61β^{P1} Gurken is trapped in this cytoplasmic location and is not transported to the plasma membrane. This indicates defects in trafficking of Gurken. Moreover, this also indicates that the trafficking defect is from a post-ER compartment, probably during later steps of secretion.

Localization of Yolkless, a plasma membrane protein of the oocyte, was also examined. Yolkless is member of the LDL receptor family and is responsible for yolk uptake into the

oocyte (Schonbaum et al., 1995). Yolkless, which like Gurken synthesized as type I membrane protein, retains its plasma membrane localization in germline clones of sec61β^{P1}. This indicates that the defect in plasma membrane traffic in the germline clones of sec61β^{P1} does not result from a general block in transport of membrane proteins to the plasma membrane. It seems that specific subsets of proteins require Sec61β for plasma membrane traffic, and Gurken could be one of the proteins which belong to this subset.

5.3.1 Analysis of Gurken Traffic in Oocyte

Gurken traffic to the plasma membrane during the stage 10 of oogensis is tightly regulated. Two features characterize Gurken transport, firstly, the directionality during transport that allows differential plasma membrane localization and secondly, presence of a set of proteins which govern the process of transport. These two features make the trafficking of Gurken to the plasma membrane distinct from that of Yolkless. In the oocytes from the germline clones of sec61β^{P1} the Gurken was localized to cytoplasmic punctuate structures; the identity of these structures could not be determined.

The process of directed Gurken secretion during stage 10 of oogenesis begins with the localization of the Gurken mRNA at the anterio-dorsal end of the oocyte. Gurken is synthesized as a type I membrane protein and translocated into the ER. Gurken's exit from the ER is mediated by Star, an ER resident protein. Cornichon (Cni) mediates Gurken packaging into CopII vesicles, Gurken is proteolytically processed in the Golgi complex by members of the Rhomboid family of proteases (Brho) and is finally delivered to the plasma membrane in an exocyst dependent manner (Freeman, 2004; Murthy and Schwarz, 2004). Yolkless whose traffic to the plasma membrane is not affected by lack of Sec61β does not show localization of the mRNA to any specific region of the oocyte. Yolkless also does not need additional proteins like Star, Cni or Brho for its transport to the plasma membrane (Schonbaum et al., 1995; Schonbaum et al., 2000). It however does need the exocyst for plasma membrane localization (Murthy and Schwarz, 2004).

The defect in the plasma membrane transport of Gurken in germline clones of sec61β^{P1} can occur due to direct effect of Sec61β on the process of Gurken localization and traffic. On the other hand, it is also possible that lack of Sec61β in the oocytes affects the insertion or localization of Star, Brho or Cni or another unknown protein in the secretory pathway thus indirectly affecting Gurken transport to the plasma membrane.

5.3.1.1 mRNA Localization

Mutations in the 3' or 5' UTR of the Gurken mRNA which affect localization generate phenotypes similar to the one seen in the germline clones of sec61β^{P1} (Schupbach and Roth, 1994). It has also been reported that the Gurken mRNA and ER are closely associated during different stages of oogenesis (Saunders and Cohen, 1999). However, in the absence of Sec61β, the Gurken mRNA is still localized to the anterio-dorsal part of the oocyte similar to the localization in the wild-type oocytes (present study, not shown). The Gurken protein is also seen at the anterio-dorsal end. Thus, factors such as microtubule organization, local protein synthesis machinery and anchoring of the mRNA at the ER which are proposed to have a role in mRNA localization (Lopez de Heredia, 2004) do not seem to be affected by absence of Sec61β in the oocyte.

5.3.1.2 Regulation at the level of ER Translocation

Gurken is a type I membrane protein with a classical signal sequence. This suggests co-translational translocation, hence, any perturbations during translocation into the ER due to lack of Sec61β would result in reduced amounts of Gurken in the oocytes. Reduced translocation would have also caused reduced levels of Gurken from all the three location where Gurken is seen in the egg chamber. However, this does not seem to be the case, it is observed that in the clones of sec61β^{P1} the over-all Gurken amount in the oocyte is not drastically altered but the amount of Gurken at the plasma membrane is specifically reduced. This suggests that although Sec61β is part of the Sec61p translocation channel, the reduced amounts of Gurken at the plasma membrane do not seem to be due to a translocation defect. This observation is similar to what has previously been characterized in *in-vitro* assays where Sec61β was not essential for translocation per-se (Kalies et al., 1998). These observations suggest that if indeed Sec61β directly affects Gurken traffic it does so at a step beyond translocation into the ER.

5.3.1.3 ER exit

Once proteins have been made in the ER, they exit the ER via defined regions called ER exit sites. The organization of the ER exit sites in stage 9-10 Drosophila oocyte has recently been characterised (Herpers and Rabouille, 2004). Oocytes isolated from fly line expressing GFP-tagged Sec23, member of the CopII coat and a marker for the ER exit site, were observed using con-focal microscopy. It is seen that the oocyte consists of defined number of ER exit sites which are marked by the dSec23p. The Golgi complex is in close proximity of the exit

sites and these units are evenly distributed throughout the cytoplasm. It is also observed that only a subset of these units is used during Gurken transport, indicating presence of a specialized pathway for Gurken export out of the ER and trafficking to the plasma membrane. Thus, during Gurken secretion the ER exit contributes to the directionality in Gurken transport. Yolkless which is present all around the oocyte plasma membrane, on the other hand, shows no such preferences and is seen at all ER exit sites (Herpers and Rabouille, 2004; Schonbaum et al., 2000). Exit from a correctly positioned exit site allows the transport of Gurken to the plasma membrane. These observations suggest that different domains exist within the ER.

In the oocytes from germline clones of sec61β^{P1} failure to exit the ER would result in Gurken being retained in the ER. In these oocytes, however, Gurken does not co-localize with the ER marker. Indicating that process of ER exit, which ensures entry into the specialized secretory pathway, is not affected by lack of Sec61β. Moreover, Gurken is still seen in the anterior-dorsal with no signal from elsewhere in the oocyte, indicating that even in the absence of Sec61β Gurken does get inserted into the right domain of the ER.

5.3.1.4 Regulating the Proteins which Affect Gurken Traffic

A set of proteins have been identified which govern Gurken traffic to the plasma membrane. The ER chaperone Star is reported to facilitate Gurken exit from the ER (Freeman, 2004). Star is a type II membrane protein with un-commonly long cytoplasmic and luminal domains. (Pickup and Banerjee, 1999). Lack of Star at the ER in the absence of Sec61β could indirectly affect Gurken transport to the plasma membrane by preventing ER exit. The germline clones of Star affect oogenesis, with oocytes not developing beyond the stage1/2 (Pickup and Banerjee, 1999). This indicates that Star is involved in processes other then Gurken export. If Sec61β was indeed involved in Star biosynthesis it is likely that the phenotype in the oocyte would be more severe than what has been observed in the germline clones of sec61β^{P1}. Moreover, defects in the insertion or localization of Star due to lack of Sec61β, would affect the exit from the ER. The germline clones of sec61β^{P1} do not show any Gurken co-localizing with the ER.

Cni is responsible for packaging of Gurken in specialized COPII vesicles (Roth et al., 1995). Lack of Cni in the oocyte is also known to generate the dorsal appendage fusion phenotype. Intracellular localization of Gurken has not been reported in the oocytes lacking Cni, but Gurken is thought to accumulate in the ER. Sec61β can affect the translocation or localization of Cni thus indirectly affecting Gurken transport to the plasma membrane.

In the Golgi complex Gurken is cleaved by Brho. Brho dependent cleavage is essential for the protein to reach the plasma membrane and to be released from the plasma membrane for uptake by the follicle cells (Freeman, 2004). It has been shown in reconstituted trafficking experiments in *Drosophila* S2 cells that in the absence of Brho, Gurken is unable to be secreted and is trapped in the Golgi complex (Lee et al., 2001). The form of Gurken lacking the transmembrane segment can not compliment the loss of anterior-posterior polarity. These data indicate a dependency on Brho mediated cleavage for signalling (Queenan et al., 1999). Sec61β could also indirectly affect the plasma membrane transport of Gurken by mediating changes in the activity or localization of Brho in the oocyte. Lack of cleavage would prevent Gurken transport to the plasma membrane. Gurken would then accumulate in structures distinct from the ER.

Interestingly, in wild-type egg chambers Star, Cni and Brho mRNAs do not show a polarized localization, but are present all over the oocyte (Guichard et al., 2000; Pickup and Banerjee, 1999; Roth et al., 1995). Intracellular localization of the Star, Cni and Brho proteins in wild-type oocytes or oocytes from the germline clones of sec61β^{P1} could not be examined due to absence of suitable antibodies.

5.3.1.5 Interaction of Sec61β with the Exocyst

The observations that Sec61β can interact with the members of the exocyst provides an explanation as to how Sec61β, protein at the ER, can directly affect later steps of secretion. The exocyst plays a role in directed traffic by acting as a tethering complex at the plasma membrane. Exocyst is a complex of eight proteins which is known to be associated with the plasma membrane and is known to be localized to the regions of membrane addition (Lipschutz and Mostov, 2002; Mostov et al., 2000). Expression of Sec10, a component of the exocyst complex, caused increased delivery to the basolateral plasma membrane. Sec10 interacts with Sec61β and at least a subset of Sec10 was seen to be localized to the ER (Lipschutz et al., 2003). Over-expression of Sec61β (Sbh1) can suppress lethal exocyst mutations which indicates genetic interaction between Sec61β and the exocyst components. In yeast Sec61β can be co-immunoprecipitated with Sec15p and Sec8p, the components of the exocyst (Toikkanen et al., 2003). The exocyst is thought to function by capturing the vesicles bearing the cargo which has to be transported to a defined part of the plasma membrane from the trans-Golgi network and allowing fusion with the plasma membrane. However, accumulating evidence states that cargo identification could occurs early in the secretory pathway, possibly at the ER itself. It has been observed that during trafficking of the NMDA

receptors subunits to the plasma membrane, a complex of the NMDA receptor subunit, NR2; SAP102, a PDZ family protein, and Sec8 forms has been visualized at the ER (Sans et al., 2003). It is conceivable that Sec61β serves as a docking site at the ER for the exocyst subunits, alone or in complex with other proteins. This would allow early identification of the cargo and increase the efficacy of directed transport. Gurken, whose transport to the plasma membrane is affected when Sec61β is depleted, also requires the exocyst complex for traffic to the plasma membrane.

Germline clones of a hypomorphic allele of Sec5 ($Sec5^{E13}$) a subunit of the exocyst result in failure of Gurken traffic to the plasma membrane and accumulation of the protein in the cytoplasm of the oocyte (Murthy et al., 2003). The germline clone of sec5 shows accumulation of Gurken at locations other than the anterior-dorsal part of the oocyte, indicating a much severe loss of directionality in the Gurken trafficking.

In the germline clones of $sec61\beta^{P1}$ on the other hand, Gurken is still restricted to the anterio-dorsal end. It is possible that the phenotype observed in the germline clones of $sec61\beta^{P1}$ is due to the interaction of Sec61β with members of the exocyst complex. Lack of Sec61β during translocation and hence lack of interaction with the exocyst causes Gurken to be trapped in the late Golgi compartment and unable to be transported to the plasma membrane. Over a period of time Gurken is mis-localized to other parts of the oocyte in small amounts which are not sufficient to be detected by the antibody and hence are not seen by immuno-fluorescence.

Sec5 itself has been reported as being enriched at the anterio-dorsal and anterio-ventral corners of the oocyte (Murthy and Schwarz, 2004). Sec5 has also been suggested as one of the primary determinant of polarized secretion. Localization of Sec5 protein is not affected in the oocytes from the germline clones of $sec61\beta^{P1}$.

Figure 5.1 is a summary of mutations known to disrupt the directed transport of Gurken. Lack of Sec61β in the oocytes does not affect localization of the Gurken mRNA and the local translation of Gurken in the anterior-dorsal region of the oocyte. Gurken which is unable to be transported to the plasma membrane is seen in punctuate structures in the cytoplasm of the oocyte. The identity of these punctuate structures could not be determined. These structures were distinct from the ER. Co-localization of Gurken with a Golgi complex marker was not done since both the antibodies were of mice antibodies. However, Golgi is also seen in punctuate structures in the cytoplasm (this study, data not shown and (Herpers and Rabouille, 2004)). From this cytoplasmic location Gurken is unable to be transported to the plasma membrane. It need to be examined it Sec61β is responsible for this effect by either

directly affecting Gurken transport or indirectly by affecting the biosynthesis of proteins like Star, Cni and Brho, which would also cause the trafficking defects.

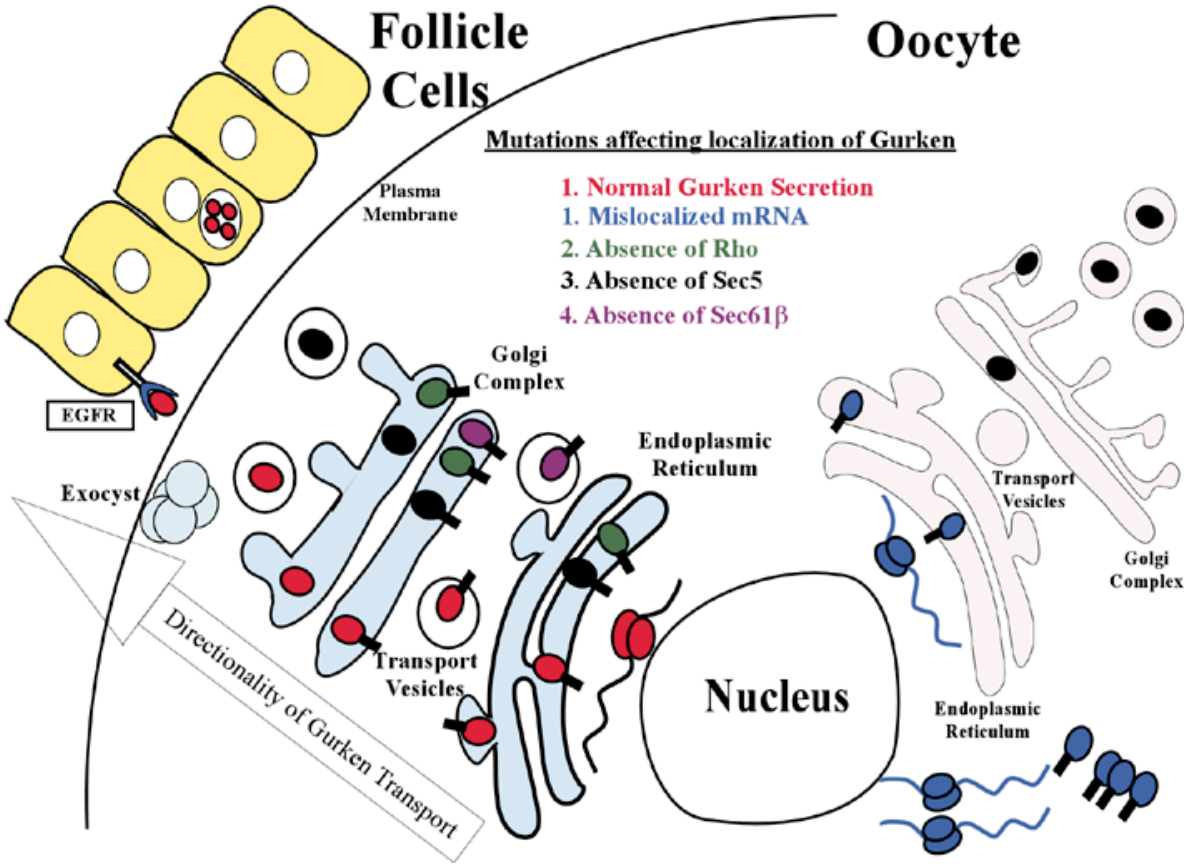


Figure 5.1 **Schematic representation of the mutations which affect biosynthesis of Gurken**. Mutations in the Gurken mRNA (shown in blue) result in localization of the Gurken protein all over the cytoplasm of the oocyte. Absence of proteolytic processing prevents secretion and Gurken is localized in the Golgi complex (shown in green). In absence of Sec5 Gurken is also seen all over the cytoplasm (black). In germline clones of sec61β^{P1}, Gurken is seen at the anterior-dorsal end, but plasma membrane traffic is prevented (purple). Normal Gurken secretion is shown in red.

5.3.2 Analysis of Gurken Traffic in Mammalians Cells in Culture

In order to understand the molecular mechanisms of Gurken traffic an alternate system was developed in HeLa cells. SiRNA was used to reduce the level of Sec61β and mammalian expression vectors to express Gurken, Star and Rho. This system allowed direct testing of effects of Sec61β depletion on targeting and insertion of Gurken in the ER. Trafficking along the secretory pathway could be monitored by reduced sensitivity to endo H. This system also

offered the possibility of investigating the biosynthesis of the proteins involved in Gurken transport such as Star and Rho under conditions when Sec61β levels were reduced. Biosynthesis of these proteins can be monitored by changes in Gurken localization and modification. Functionally active Star would result in export of Gurken from the ER making it insensitive to endo H. On the other hand, functionally active Rho would cause cleavage of Gurken and hence generate a lower molecular weight form of Gurken.

5.3.2.1 siRNA mediated reduction of Sec61β levels

siRNA against Sec61β was used to reduce the amount of Sec61β. siRNA constructs directed against different parts of the mRNA were tested for their ability to reduce the amounts of Sec61β. It was seen that the siRNA directed against the 5'UTR resulted in maximum reduction in the levels of Sec61β. This method however was not completely efficient since small amounts of Sec61β remained in the cells even after 4-5days of transfection. This included a second transfection after three days since HeLa cells tend to lose the transiently transfected plasmid after two to three doublings. Increasing the amounts of the siRNA did not result in further down-regulation of Sec61β amounts. Sec61β is fairly abundant protein in the cells and with a long half-life this could explain why siRNA did not result in removal of all the Sec61β protein from the cells.

The reduction in the amounts of Sec61β was monitored by western blotting using an antibody against Sec61β. Since total cell lysates were used for this purpose, it is possible that the amounts which are visible on western blot represent either the residual Sec61β from transfected cells or Sec61β from cells which were un-transfected and hence retain normal levels of the protein. To discount for this possibility immuno-fluorescence analysis was done using the Sec61β antibody on cells after co-transfection with siRNA plasmid and a GFP tagged protein as a marker for transfection. It was assumed that if a cell gets transfected with one plasmid it should be transfected with the other too. No difference in the level of Sec61β was seen in cells which were GFP positive containing the siRNA plasmid and non-GFP positive cells without the siRNA plasmid. These cells did show reduced amounts of Sec61β as determined by western blotting. The most probable explanation is that the antibody has a high affinity for Sec61β hence even the residual amounts of Sec61β are able to give a strong staining. These experiments were done by Martha Butkus, a summer student, under my supervision.

5.3.2.2 Biosynthesis of Gurken in HeLa cells

Gurken synthesized in mammalian cells migrates as a 45-47 KDa band on SDS-PAGE. Gurken is sensitive to Endo glycosidase H (EndoH), which cleaves the glycan chains added in the ER, indicating that the protein is retained in the ER. Upon co-transfection of Star, the Gurken is thought to exit the ER (Lee et al., 2001; Urban et al., 2002). However, no change in the sensitivity of Gurken to EndoH was observed in the present study. Additional transfection of Rho causes Gurken to migrate faster, which most likely represents the intra-membrane cleaved form. However, Gurken was still sensitive to EndoH. This indicates majority of Gurken is in the ER even when Star and Rho are present. It is interesting to note that Rho which has been reported to be localized to the Golgi-complex (Lee et al., 2001; Urban et al., 2002), can mediate the cleavage of ER localized Gurken. This can be explained due to the fact that during trafficking Rho has to pass through the ER where it can meet Gurken and cause cleavage. The intracellular localization of Gurken, Star or Rho was not tested.

In previous studies which investigated trafficking of EGFR ligands in mammalian cells, Cos1, an african green monkey cell line, was used (Lee et al., 2001; Urban et al., 2002). Where as in the present study HeLa cells were used since the siRNA plasmid had to be designed against the human Sec61β as the DNA sequence of the african green monkey Sec61β gene is not known. S2 cells have also been used to investigate Gurken trafficking however these cells did not incorporate ^{35}S methionine from the medium meant for pulse analysis and hence were not used.

The steady state expression of Gurken in cells having reduced level of Sec61β was tested by western blotting using an antibody against Gurken. The relative level Gurken which is glycosylated and un-glycosylated appears similar, which indicates that targeting and insertion of Gurken is not affected by reduced amounts of Sec61β. The expression of Brho or Star in cells with reduced Sec61β levels appears similar to wild-type cells. In the cells with either wild-type levels of Sec61β or cells with reduced levels of Sec61β, a majority of Gurken is cleaved by Brho. This indicates that the targeting and insertion of Brho is not affected by reduced amounts of Sec61β. Upon ^{35}S methionine pulse analysis in wild-type and cells with reduced amount of Sec61β it is observed that the amount Gurken which is glycosylated is similar. This indicates that the process of targeting of Gurken to the ER and post-translocational modification are unaffected by the reduced levels of Sec61β. These observations could be explained in two ways, Gurken or the other regulatory proteins do not need Sec61β for translocation into the ER. However, it is also possible that the amounts of Sec61β which remained in the cells after siRNA knock down were sufficient for translocation

of Gurken and the regulatory proteins into the ER. In the present experiments these two possibilities cannot be discriminated.

Features in Gurken secretion such as, synthesis as a type I membrane proteins and requirement of additional proteins during the trafficking to the plasma membrane, are shared by other members of the EGFR ligand family of which Gurken is a member. It is possible that Sec61β affects trafficking of these proteins as well. Hence it was interesting to examine if phenotypes observed in other organs could also be ascribed to defects in trafficking of signalling molecules or morphogens such as the EGFR ligands.

5.4 Role of Sec61β in trafficking of the EGFR ligands

It has previously been reported that generating clones of sec61β^{P1} in the eyes and legs caused morphological changes (Valcarcel et al., 1999). Clones of sec61β^{P1} in the eyes showed lack of photoreceptors in the ommatidia (Valcarcel et al., 1999). However, the R8 photoreceptor was not affected. The defect in photoreceptor development was more apparent in the apical part then in the basal part of the ommatidia. Drosophila eye development takes place by successive steps of induction, giving rise to the formation of different cell types at each round. The first step is the differentiation of the founder photoreceptor R8, followed by determination of seven other photoreceptors around R8. Formation of the founder photoreceptor R8 which is not affected in the eye clones does not require EGFR (Dominguez, 1998). Star, on the other hand, affects the development of R8 photoreceptor which indicates that it has roles independent of EGFR signalling (Kolodkin, 1994).

Clones of sec61β^{P1} in the legs result in severe size reduction in the tarsal segments (Valcarcel et al., 1999). Leg formation is induced by combined action of different mophogenetic signalling events. The first involves proximal-to-distal axis (PD) which is mediated by action of Wingless (Wg) and Dpp. The EGFR ligand Vein (Vn) is expressed in response to Wg and Dpp signalling. Vn, a secreted EGFR ligand, induces further signalling events in the leg disc. The size reduction in the tarsal segment occurred distal to the clonal area. It is possible that this is because of the mutant cells prevent a secreted factor or a signalling event to reach a more distal part of the leg. This would be consistent with defects in trafficking due to lack of Sec61β.

Based on the phenotypes in the eyes and legs and defects in Gurken secretion it is possible that the EGFR ligands belong to the subset of proteins which require Sec61β. Sec61β can either directly affect the trafficking of the lignds or affect the proteins which occur in the trafficking pathway. EGFR signalling performs a crucial role in development (Shilo, 2003).

The EGFR has a ubiquitous expression and the specific responses are controlled by regulated activation of the cognate ligands. Four activating ligands and one inhibitory ligand have been identified so far. Three of these ligands Spitz, Gurken and Keren, are produced as transmembrane precursors and need to be cleaved to generate the active secreted form (Neuman-Silberberg and Schupbach, 1993; Schweitzer et al., 1995; Urban et al., 2002). Vein, the fourth activating ligand on the other hand is produced as a secretory protein (Schnepp et al., 1998; Schnepp et al., 1996). These ligands all have been shown to require functioning of Star and Rho for signalling (Figure 5.2).

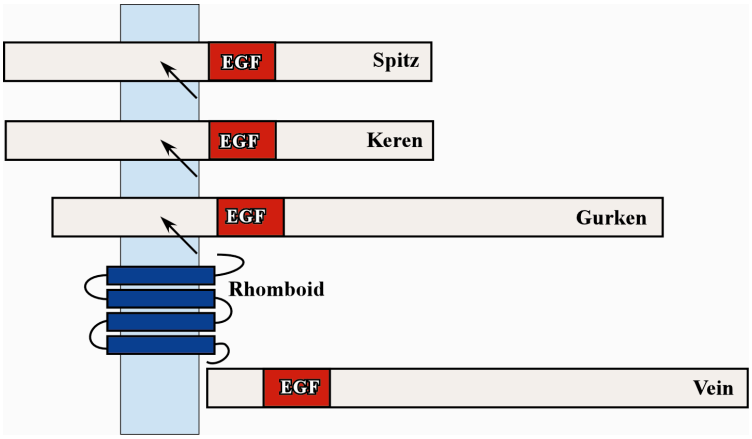


Figure 5.2 **Ligands of the EGFR** in *Drosophila*. Four ligands have been identified for the EGFR in Drosophila. Gurken is exclusively expressed in the oocyte. Spitz, Keren and Vein are known to regulate diverse developmental pathways Arrows represent intra-membrane cleavage. Vein, unlike the other three ligands is synthesized as a secreted molecule.

5.5 Role of Sec61β during Wing Development

In order to test if Sec61β has a function in other developmental pathways, Sec61β was ectopically expressed in the wing discs. It has been reported that over-expression of Sec61β in yeast can rescue the lethal mutations in the exocyst complex, indicating genetic complementation. It is possible that such a complementation also function in *Drosophila*. The UAS-GAL4 expression system used allowed expression of Sec61β at different developmental stages and in different regions of the wings. Sec61β was expressed either in all regions of the wing disc or in a specific region of the wing disc. Expression in the wing disc affected the adult wing, generating distinct phenotype with respect to wing morphology and vein delineation. Different morphological changes were observed depending on the region where Sec61β was expressed or depending on the level of expression. Wing development and vein delineation occurs by concerted action by a number of different signalling molecules, including the EGFR pathway. The phenotypes observed could result from perturbation in normal functioning of these signalling pathways.

5.5.1 Development of Wings

Wings originate from the wing discs which contains adult progenitor cells in the body of the larvae. The wing discs are divided into anterior (A) and posterior (P) compartments. The identity of the cells in the P compartment is imparted by the expression of the gene Engrailed (En) (Simmonds et al., 1995; Tabata et al., 1995). Under the control of En, cells of the posterior compartment synthesize Hedgehog (Hg), which is secreted into the A-compartment (Tabata and Kornberg, 1994). There Hh induces several target genes, including Patched and Decapentaplegic (Dpp). Dpp patterns the wing beyond the central domain (Lecuit et al., 1996). Dpp is expressed along the border between the A and the P compartment and patterns the wing beyond the central domain (Lecuit et al., 1996).

After being subdivided into A and P compartments, the wing imaginal disc is subsequently subdivided into Dorsal (D) and Ventral (V) compartments compartments along the dorsoventral axis, and later the DV border develops into the wing margin. The protein Apterous acts on the cells in the dorsal compartment, inducing the expression of the gene Fringe, which activates Notch receptor pathway at the DV border (Diaz-Benjumea and Hafen, 1994; Irvine and Wieschaus, 1994; Kim et al., 1995). Activated Notch induces Wingless (Wg) synthesis at the DV border where it functions as a morphogen to induce expression of target genes such as Distalless and Vestigial which organizes wing patterning (Neumann and Cohen, 1998).

Signalling by the EGFR pathway is required during early development for promoting wing-notum distinction. This is achieved by antagonistic activites of wingless and EFGR, high levels of Wg prevents EGFR signalling and vice versa. Thus EGFR signalling is restricted to the Notum and Wg to the wing region (Baonza et al., 2000; Wang et al., 2000). The EGFR ligand identified in the wing is called Vein (Vn). Vn in contrast to the other know ligands of the EGFR pathway, is not synthesized in a membrane bound form, but is secreted (Shilo, 2003).

The second aspect to wing development is the dileation of the wing veins. The veins are linear cuticular structures that appear distributed in a characteristic pattern in the wing blade. The veins confer structural rigidity to the wings and, in some case, serve as conducts for sensory axons, hemocites and tracheal cells. In *Drosophila*, there are four longitudinal veins (L2-L5), that span the length of the wing, and two transverse veins that connect the longitudinal veins. In addition, the wing contains a marginal vein encompassing the length of the anterior wing margin, and two incomplete longitudinal veins, one in the anterior (L1) and one in the posterior compartment (L6).

Experiments with loss of function alleles have shown that the EGF receptor, torpedo; EGFR ligand, Vein; Star and Rho are required at different stages of wing vein formation (Diaz-Benjumea and Garcia-Bellido, 1990; Garcia-Bellido and de Celis, 1992; Guichard et al., 1999; Queenan et al., 1997; Simcox, 1997). Characterization of the function of the individual proteins in vein formation revealed that Star and Rho act co-dependently, since over expression of Star alone does not show any phenotype, when expressed in the presence of Rho causes a strong ectopic vein phenotype. Strong ectopic Rho expression cannot generate any phenotype in wing clones lacking Star. Thus the two proteins collaborate to activate the EGFR pathway by a common molecular mechanism (Guichard et al., 1999). However the fact that vein is a secreted ligand suggests that it may not need the role of Rho. The Rho dependent phenotype observed could be explained by invoking the presence of yet unknown EGFR ligand functioning in the wing vein delineation.

5.5.2 Ectopic Expression Phenotypes

One of the GAL4 drivers used to express Sec61β was Apterous which causes expression early in development of the wings and is also known to cause high levels of expression from the GAL4 promoter. Apterous-GAL4 driven expression of Sec61β caused loss of complete wing structure. The wings were replaced by a mass of tissue where no clear structure could be identified (data not shown). The severity of the phenotype obtained by expression using the Apterous-GAL4 driver made interpretation difficult. Scalloped-GAL4 drives the expression of Sec61β predominantly in the dorsal region of the wing disc. It is seen that expression of Sec61β using the Scalloped driven GAL4 results in over-all reduction wing size, loss of a large number of veins and thickening of the remaining vein, L2. The phenotype seen after expression of Sec61β by Scalloped-GAL4 indicated that the initial steps of wing development which include formation of the wing blade and the wing to notum demarcation are unaffected. The phenotype was somewhat similar to that observed by ectopic expression of Rho in the dorsal region of the wing disc (Guichard et al., 1999). The expression of dominant negative form of the EGFR also resulted in a similar phenotype (Guichard et al., 1999). However the phenotype seen in case of over-expressed Sec61β is more pleiotropic, since the wing margins are also lost.

Engrailed-GAL4 driven expression of Sec61β results in loss of dorsal part of the L4 vein structure. Engrailed drives the expression of Sec61β in the A-P boundary in a much restricted region as compared to Scalloped driven expression. The specificity of this phenotype allowed speculation on the molecular events which were affected. Loss of Star or

Rho has been seen to cause similar loss of vein phenotype (Guichard et al., 1999). The ectopic expression phenotype is consistent with defects in trafficking since the regions where the morphological changes are seen are different than the regions where the Sec61β is expressed. Engrailed-GAL4 drives the expression of Sec61β at the A-P boundary but the vein which is affected is the L4. This is similar to the effect where Rho can activate EGFR dependent MAPK in cells adjacent to those expressing Rho (Guichard et al., 1999). Ectopic expression of Gurken without the transmembrane region using the Engrailed-GAL4 driver results in a phenotype which is similar to the one observed for Sec61β and has been interpreted as having been caused by activation of the EGFR signalling (Queenan et al., 1999).

These phenotypes also suggest that Sec61β acts in the EGFR signalling pathway at the same level as Vein, Rho and Star, which is upstream of EGFR. Ectopic expressing Sec61β in the wings and generating sec61β^{P1} clones in the eyes and legs resulted in distinct morphological changes. Based on these phenotypes it is possible that Sec61β affects either the biosynthesis of the EGFR ligands directly or the proteins like Star or Rho which are part of the cellular machinery transporting these ligands to the cell surface.

5.6 Cdc2 kinase mediated Phosphorylation of Sec61β

Sequence analysis of Sec61β shows presence of a site for phosphorylation by the cdc2 kinase. The cdc2 kinase is activated when it associates with its cognate cyclin partner, cyclin B1, which is synthesized in the M-Phase of the cell cycle. Activity of the cdc2 kinase allows the cell to traverse through the M-phase. *In-vivo* phosphorylation of Sec61β was seen to be two-fold stimulated when cells were in the M-phase of cell cycle. This two fold stimulation of Sec61β phosphorylation could be recapitulated *in-vitro* using M-phase synchronized cell extracts or purified cdc2 kinase and Sec61β being present in microsomes. This increase in phosphorylation was sensitive to roscovitine. Sec61β when part of liposomes which where reconstituted with purified Sec61p complex, was also phosphorylated by the M-phase cytosolic extract. The putative phosphorylation site predicted was serine at position 17; the consensus site for cdc2 phosphorylation is not seen elsewhere in Sec61β. Serine 17 was replaced to alanine. This mutation prevented cdc2 dependent phosphorylation of Sec61β. These data indicate that it is indeed the cdc2 kinase which phosphorylates Sec61β and it is the serine at position 17 which is the phosphorylated residue.

5.7 ER Dynamics during the M-Phase

The cdc2 kinase which is active during the M-phase of the cell cycle phosphorylates Sec61β. During M-phase the ER is characterised by morphological and physiological changes since ER is fragmented into vesicles. During the M-phase the process of secretion which starts with translocation of proteins into the ER is also blocked. In both these processes cdc2 kinase is thought to play a role. Sec61β is part of the ER and it is possible that phosphorylation of Sec61β could play a role in the over all structure of the ER. Sec61β is also part of the Sec61p translocation channel, so phosphorylation of Sec61β could also affect translocation of proteins into the ER. It was therefore investigated if phosphorylation of Sec61β by the cdc2 kinase affects any of these processes. The mammalian cells in culture were used to investigate the function of the ER during the M-phase of the cell cycle.

5.7.1 ER Fragmentation during M-phase

During interphase the ER is a continuous membrane system linked by tubular structures. This membrane system includes the nuclear envelope, the rough and the smooth ER, and the regions that contact other organelles, like the Golgi, vacuoles endosomes etc (Baumann and Walz, 2001). The idea that ER is a single membrane system is supported by experiments with fluorescent dye which cannot exchange between discontinuous membranes. (Terasaki and Jaffe, 1991). In another type of experiment GFP-tagged proteins were targeted either to the lumen or the membrane of the ER and the organization of the ER was observed (Subramanian and Meyer, 1997; Terasaki, 2000).

Since the ER is present as single organelle and in a single copy at the interphase in eukaryotic cells; a mechanism must exist during mitosis to divide it between the two daughter cells. In case of ER the mechanism involves fragmentation, dispersal and subsequent reassembly in each daughter cell. Evidences suggest that during mitosis the ER is seen to break down presumably by random scission process into differently sized vesicles (Warren, 1993).

The exact mechanism for ER breakdown is not known, but the process can be compared with the well studied mechanism of Golgi complex breakdown during M-phase. The process of Golgi breakdown during M-phase requires the cdc2 kinase (Lowe et al., 1998b). cdc2 kinase phosphorylates a Golgi protein, GM130, which interacts with p115 a vesicle tethering protein of CopII vesicles and mediate vesicle fusion (Lowe et al., 1998b). Phosphorylated GM130 is unable to bind to p115 and this prevents vesicle tethering and results in Golgi breakdown (Nakamura et al., 1997). GM130 is membrane protein anchoured

to the cytoplasmic side of the cis-Golgi membrane by its extreme C-terminus. GM130 interacts with p115 at its extreme N-terminus. The cdc2 phosphorylation site has been identified at the N-terminus (Nakamura et al., 1997). Two other components have also been implicated in the process of ER breakdown. These are the Mitogen-activated protein kinase kinase 1 (MEK1) and polo-like kinase 1 (Plk1) (Acharya, 1998, Sutterlin, 2001 #4985). Mek1 is a substrate for the cdc2 kinase, hence it could be considered as acting downstream of the cdc2 kinase (Colanzi, 2003). Mek1 is thought to phosphorylate another Golgi protein called GRASP55 which has been implicated in stacking of Golgi cisternae (Colanzi, 2003). Substrates for Plk1 have not been established.

Cdc2 kinase also has a role during ER breakdown. Evidence comes from *in-vitro* experiments using membranes from *Xenopus* oocytes which have directly implicated cdc2 kinase in conversion of tubular ER to vesicular ER (Dreier and Rapoport, 2000). In these experiments the membranes from *Xenopus* oocytes were either incubated in the presence of interphase extracts or M-phase extracts where cdc2 kinase was seen to be active. The formation of ER network was severely curtained (Dreier and Rapoport, 2000). However in case of ER no targets for cdc2 kinase have been identified so far.

The structure of Sec61β appears to be similar to that of GM130, both are single membrane spanning protein and anchored to the ER at the C-terminus, although the size of GM130 is much bigger. Interestingly, the phosphorylation in case of GM130 occurs at the N-terminus, similar to Sec61β. (Lowe et al., 2000). It is possible that phosphorylation of either Sec61β or the other unknown 35KDa protein which was also seen to be phosphorylated by cdc2 kinase is the target which is responsible for the ER breakdown.

Preliminary experiments were done to see if Sec61β has a role in the process of ER breakdown during M-phase. During M-phase ER appears distributed throughout the cytoplasm without any apparent organization (Barr, 2004). HeLa cells with normal amounts of Sec61β or HeLa cells where Sec61β levels were reduced by siRNA were co-transfected with a GFP-tagged ER marker and arrested in the M-phase using nocodazole. No difference in the localization of the GFP marker during M-phase was seen when amount of Sec61β was normal or reduced. SiRNA mediated reduction of Sec61β protein level is not complete; it is possible that the residual protein is sufficient for ER morphology. Over-expression of the mutant Sec61β which mimics the constitutively phosphorylated form of the protein did not cause any change in the morphology of the ER. Sec61 channel is thought to consist of four copies of the subunits, it is therefore possible that the recombinant protein could not complete

replace the endogenous protein in the complex and hence the complex with even a single functional copy was sufficient for cellular functions.

ER fragmentation is hard to visualize with standard fluorescence microscopy, hence we cannot exclude the possibility that Sec61β has a role in fragmentation or maintenance of the structure of the ER. It is however observed that the over all morphology of the ER in oocytes derived from the germline clones of sec61β^{P1} appears similar to that seen in the wild-type oocytes.

5.7.2 Sec61β Phosphorylation and Secretion during the M-phase

M-phase is characterized by block in protein translation and secretion. Since processes upstream of ER translocation and downstream of ER translocation are affected during the M-phase. It was tested if the process of ER translocation itself was affected during the M-phase. Sec61β whose phosphorylation is stimulated two fold during the M-phase by cdc2 kinase is part of the translocation channel. It is possible that changes in ER translocation could be due to phosphorylation of Sec61β. ER translocation was tested by expressing a membrane protein during the M-phase.

The block is protein synthesis during the M-phase phase occurs when cap-dependent translation of initiation is impaired. All nuclear-encoded eukaryotic mRNAs are modified at their 5' end with a structure termed cap (m7GpppN). The cap plays a key role in facilitating ribosome binding to the 5' end of the mRNA. The function of the cap structure is mediated by eIF4F, an initiation factor composed of three subunits, eIF4E, eIF4A and eIF4G. The activity of the eIF4E is modulated by a family of three 4E binding proteins, 4E-BPs. During the M-phase the 4E-BP1 is hypo-phosphorylated and eIF4GII is hyper-phosphorylated this most likely results in failure in formation of the complete initiation complex (Cormier et al., 2003). Inhibition of cap dependent translation in the M-phase ensures that capped, yet unspliced, mRNAs are not translated. These mRNA normally would be restricted to the nucleus in the interphase cells but since the nuclear envelope breaks down during M-phase, these get dispersed through out the cytoplasm (Cormier et al., 2003; Pyronnet et al., 2000; Pyronnet and Sonenberg, 2001).

However a certain sub-set of mRNAs continue to be translated even in the M-phase and are not affected by block in the cap dependent translation. These mRNAs have an alternative mode for recruitment of the 40S ribosomal subunit for initiation of translation. This process is called the internal entry of ribosomes and is mediated by sequences in the 5' region of the mRNA called the internal ribosome entry sites (IRES). IRESs originally were

identified from viruses, but increasingly are also found in many cellular genes. The viruses use IRES to ensure preferential translation of viral transcripts over cellular transcripts. This is mediated in case of picorna virus infection, when a virally encoded protease cleaves eIF4G, thus preventing the cap dependent translation initiation. On the other hand the translation of virally encoded transcripts continues due to presence of IRES element in the mRNA. The cellular mRNAs which have been shown to contain IRESs code for proteins with essential function. The first mRNA to be characterised was immunoglobulin heavy chain binding protein, Bip. Other mRNAs known to have IRES are c-myc, a growth stimulatory factor, and ornithine de-carboxylase, essential in nucleic acid metabolism (Pyronnet et al., 2000; Vagner et al., 2001).

The process of exocytosis/secretion is also blocked during M-phase. Studies using Vesicular Stomatitis Virus G (VSV-G) protein (Warren et al., 1983). MHC class I antigen and human growth hormone (Kreiner and Moore, 1990; Souter et al., 1993) showed that the proteins accumulated in the ER, and cannot not be transported out of the ER. Although the exact mechanism is not clearly established, strong evidence exists indicating that the block is due lack of formation of the Cop II vesicles at ER exit sites (Farmaki et al., 1999). Phosphorylation plays a role in transport from ER to the Golgi, as has been shown using inhibitory of protein phosphatase PP1A and 2A (Lucocq et al., 1991). Cdc2 kinase which is active in the M-phase although is believed to play a role in inhibition. Kinases down stream of cdc2 kinase such as Mek1, which are responsible for Golgi breakdown, are also implicated in ER to golgi block in transport (Colanzi, 2003; Warren, 1993; Woodman et al., 1993).

Invariant chain (Ii), a type II membrane protein, was the reporter protein whose insertion was monitored. To overcome the translational block during the M-phase the IRES element of ornithine de-carboxylase gene was cloned in the front of the Ii expressing construct. It was observed that upon a pulse analysis, Ii accumulated in an unglycosylated form. This delay in glycosylation could result from delay in insertion of Ii into the ER. Phosphorylation of Sec61β could affect the functions such as ribosome binding or GTP exchange. This could lead to changes in the insertion rates of proteins into the ER. It is also possible that Sec61β could be co-ordinating translocational events with post-translocational events like glycosylation. M-phase phosphorylation could disrupt this co-ordination which would then result in accumulation of the unglycosylated form of the protein.

In order to directly investigate the process of translocation, microsomes were isolated from M-phase and G1-phase synchronized cells. These microsomes were used for an *in-vitro* translocational assay using rabbit reticulocyte lysate for translation of the mRNA and dRMs

for translocation. However, upon incubation of the microsomes with the reticulocyte lysate the phosphorylation of Sec61β was lost. This was attributed to the presence of phosphatases in the reticulocyte lysate. Activity of these phosphatases was blocked using a mixture of phosphatase inhibitors, NaF, Na_2VO_3 and Okadaic acid. However, the amount of phosphate inhibitors needed to prevent de-phosphorylation of Sec61β proved inhibitory for translation of the reporter mRNAs. Thus, the experiment to investigate directly the role of Sec61β phosphorylation in translocation of reporter proteins could not be performed using the *in-vitro* system.

Since protein exit from the ER is blocked during the M-phase, preventing entry into the ER would ensure that no secretory or membrane proteins accumulate in the ER. Since most protein synthesis is inhibited during the M-phase, the block in translocation can be visualized as a "back-up" mechanism. However this block has to be specific since it should not affect translocation of proteins like Bip or other ER chaperones which are constantly translated during the cell cycle due to presence of an IRES element in the mRNA.

Phosphorylation of a component of the Sec61 ER translocation channel allows us to visualize the channel in a different light. So far it had been believed that the Sec61 channel is a pore which can translocate any protein having a signal-sequence without any discrimination. It is possible that phosphorylation of a translocon component constitutes a means of regulating the activity of the channel. It could make the channel and the process of ER translocation responsive to intracellular or extra-cellular signalling cues. In order to understand the physiological significance of phosphorylation the *Drosophila* system was used where cdc2 kinase also phosphorylates Sec61β at a serine residue.

5.8 Analysis of Sec61β phosphorylation in *Drosophila*

In addition to the bio-chemical analysis of the role of Sec61β phosphorylation in mammalian cells the *in-vivo* function of phosphorylation was tested in *Drosophila* using two different experimental systems. On one hand it was investigated if Sec61β with mutations in the phosphorylation site can rescue the lethality associated with the loss of function allele. On the other hand, the mutant proteins were ectopically expressed in the fly wings where it has been shown in the present study that Sec61β causes morphological changes. For these experiments Sec61β was expressed using the UAS-GAL4 system. The mutations generated were to replace the phosphorylated serine residue to alanine eliminating the phosphorylation site (UAS-Sec61β$^{S/A}$). Serine was also replaced to aspartic acid, which contain a negative charge and could mimic phosphorylation and act as a constitutively phosphorylated protein (UAS-

Sec61β$^{S/D}$). These mutant alleles together with the wild-type allele with the UAS promoter (UAS-Sec61β^{WT}) as a control were used to rescue the lethality associated with embryos homozygous for the sec61β^{P1} loss of function allele. The wild-type and the mutant proteins were also ectopically expressed in Drosophila wings using the UAS-GAL4 system in the presence of endogenous Sec61β.

5.8.1 Rescue of Homozygous sec61β^{P1} Embryos by Sec61β Mutant Alleles

Expression of the phosphorylation mutant alleles (UAS-Sec61β$^{S/A}$ UAS-Sec61β$^{S/D}$) or the wild-type allele (UAS-Sec61β^{WT}) was driven by a GAL4 driver which uses the promoter of the Armadello gene (Arm-GAL4). Since the Armadillo expression is ubiquitous in *Drosophila*, use of this driver ensured ubiquitous expression of the Sec61β alleles too. Sec61β is assumed to be expressed in all cells of *Drosophila*, which would be recapitulated by the Arm-GAL4 driven expression.

It is seen that the Gal4 driven UAS-Sec61β^{WT} can rescue the lethality associated with the homozygous sec61β^{P1}. This means that flies harbouring the UAS-Sec61β^{WT} allele without endogenous Sec61β gene are able to survive. This experiment is similar to the previous experiments where an allele of Sec61β with expression under the control of the tubulin promoter rescued the lethality associated with the homozygous sec61β^{P1} (Valcarcel et al., 1999). Using this system the mutant alleles, UAS-Sec61β$^{S/A}$ and UAS-Sec61β$^{S/D}$ were tested for their ability to rescue the lethality. It is seen that the mutant alleles can rescue the lethality but it is not to the same extent as that observed with the Sec61β^{WT} allele. A majority of flies can not tolerate the replacement of the endogenous Sec61β with the mutant alleles. The small amount of flies which did show rescue by the mutant alleles can be attributed to individual genetic differences which could either affect levels of expression, or presence of a second compensatory mutation. Flies harbouring the mutant alleles of Sec61β had growth disadvantages which affected the survival capacities. Flies rescued with these alleles did not survive beyond two generations, and hence could not be used for generating fly lines.

The flies which were rescued by either the wild-type allele or the mutant alleles showed no morphological changes in the organs examined. This is in contrast to observations where ectopic expression of the wild-type protein in the wings resulted in morphological. This observation can be explained in the following way. In the rescue experiments the endogenous copy of Sec61β is replaced by the UAS-Sec61β, where as the ectopic expression of Sec61β using wing specific drivers is in the presence of endogenous amounts of the Sec61β. It is known that different mammalian cell types, depending on the secretory function of the cell,

have different amounts of ER and hence different amounts of Sec61β expressed. Similarly in *Drosophila* it is possible that certain cell types, like wings, would have lesser amounts of Sec61β. Ectopic expression of Sec61β in these cell types would be tolerated to lesser levels as compared to cell types having already high levels of ER/Sec61β. In addition, the strengths of the GAL4-drivers used for the rescue or the ectopic expression are also different. Wing specific GAL4-drivers are known to cause much greater expression of the UAS-transgene as compared to the expression of the transgene driven by the ubiquitous Arm-GAL4 driver used for the rescue experiment.

5.8.2 Ectopic Expression of Sec61β Phosphorylation Mutants in Fly Wings

The wild-type and the mutant alleles of Sec61β were expressed in *Drosophila* wings using the UAS-GAL4 system. In contrast to the expression of the wild-type protein using the Scalloped-GAL4 or Engrailed-GAL4, expression of the phosphorylation mutants with the same drivers caused lethality. To rule out the possibility that the allels were differentially expressed, two independent transgenic lines corresponding to each mutant allele were used for these experiments. Since the expression of Sec61β in wings could not be directly tested, the transgenes were expressed in the embryos using a different GAL4 driver and the level of expression analysed by western blotting. The wild-type and the phosphorylation mutants alleles are expressed to similar levels.

Expression of wild-type Sec61β using the Patched-GAL4 results in no morphological changes in the wings, expression of the S/A mutant was lethal. Expression of Sec61β$^{S/D}$ resulted in viable flies and closer observation of the wings revealed differences in the patterning of the wing hairs in the regions where Patched is known to express. Wild-type wing has one hair per wing cell organised in a defined orientation with respect to the axis of the wing. When Sec61β was ectopically expressed using the Patched-Gal4 driver, it was seen that the one cell generated more then one hair and the orientation of the hairs was also disturbed. This observation indicated defects in the planar polarity during wing hair development.

5.8.3 Generation of Planar Polarity in *Drosophila* Wings

The wing is covered by an array of hairs which are polarised with each hair pointing towards the distal end of the wing. The wing hair is part of a single cell, and each wing cell is itself within the plane of the epithelium. The developing hairs are initiated at the distal-most part of

the cell and grow outwards from the cell leading to distal hair polarity (Wong and Adler, 1993). Genetic analysis in Drosophila has identified a 'core' group of tissue polarity genes which affect polarization of many structures including the wing hair and omatidia in the eye (Eaton, 2003). This group is composed of Frizzled (Fz, seven transmembrane domains), Dishevelled (Dsh, PDZ domain), Prickle-Spiney leg (Sple, LIM domain), Strabismus/Van Gogh (Vang/Stbm, PDZ domain), Flamingo (Fmi, seven transmembrane domains) and Diego (Dgo, ankyrin repeats) (Adler, 2002). Besides these a large number of other genes are known which affect planar polarity in wing hair, reviewed in Adler, 2002 (Adler, 2002).

It has been suggested that overall planar polarity in any body region could be organised by a discrete group of cells, perhaps by the secretion of a polarity morphogen. A gradient of a morphogen could provide a polarity vector to align cells (Lawrence, 1966). However none of the protein mentioned above can qualify for this role of a polarity morphogen. These proteins are believed to act downstream of the morphogen (Adler, 2002). During formation of the wing hair the distal edge of wing cells is marked by preferential accumulation of Fz and Dsh and the proximal edge has accumulation of Pk and Sple (Axelrod, 2001; Tree et al., 2002). Fmi and Dgo both accumulate at distal and proximal edges (Feiguin et al., 2001; Usui et al., 1999). The model which has been proposed is following, Pk accumulates first during development, although the exact mechanism for this is not known; however, accumulation of Pk promotes accumulation of Dsh and Fz on the distal side. Accumulation of Dsh and Fz on the distal side promotes the accumulation of Pk in the neighbouring cell which is present on the distal end (Adler, 2002). Formation of the hair itself requires stimulation of the actin and the microtubule cytoskeleton (Eaton et al., 1996). However the exact mechanism by which distal accumulation of Fz and Dsh triggers changes in actin and microtubules is not known.

A number of mutants have been characterised which perturb polarity in the hairs. These have been grouped into five classes depending on the orientation of the hairs. Figure 5.3 is a graphical representation of these mutant phenotypes (Adler, 2002).

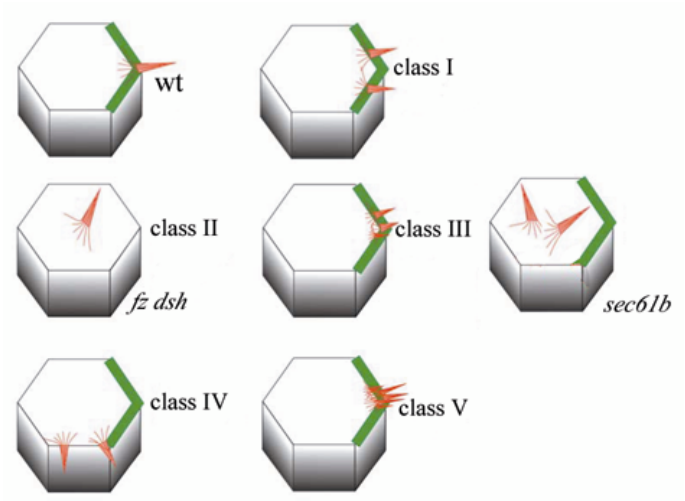


Figure 5.3 **Schematic representations of the mutations affecting Planar Polarity in Wing hairs**. The hair is shown in red. The distal edge of the hair forming cell with accumulation of Fz and Dsh is shown in green. The classes of mutations which affect the hair number or polarity are numbered from I till V. Phenotype obtained due to mutations in fz, dsh and sec61β are indicated. The mutants show independent mechanisms governing hair growth in the cell and hair growth at the distal edge of the cell.

When the phenotype obtained from the ectopic expression of the Sec61β$^{S/D}$ is compared with these mutant classes it is seen that Sec61β$^{S/D}$ phenotype is combination of multiple hairs with loss of polarity. This indicates that ectopic expression of the constitutively phosphorylated Sec61β could affect more than one pathway involved in planar polarity. One of the reasons for this phenotype can be defects in transport of the conceptualized polarity morphogen. The effect of the ectopic expression is non-cell autonomous, which means that the cells that express Sec61β$^{S/D}$ protein show the phenotype. This could indicate that the molecule affected is in the same cells where Sec61β$^{S/D}$ is expressed. At least two of the polarity genes (Fz and Fmi) are transmembrane proteins which have to be transported to distinct part of the cell. Mutants in which these protein are absent from the wings result in loss of polarity (Figure 5.3, class II). Gurken, which required Sec61β for plasma membrane transport, is also transported to a distinct part of the oocyte by polarized secretion. Sec61β may play a role during polarized and hence affect transport of proteins such as Fz and Fmi.

Disruption in the morphology of the ER, resulting in incorrect partitioning during cell division could be another factor which could explain the formation of multiple hairs. Morphology of the ER in imaginal disc cells with or without ectopic expression of Sec61b was not analysed. However, as discussed in the previous section, expression of the Sec61β$^{S/D}$ in mammalian cells did not result in visible morphological changes in the ER morphology.

5.9 Cdc2 kinase independent phosphorylation of Sec61β

In the *in-vivo* ortho-phosphate labelling experiment or *in-vitro* phosphorylation assays it was seen that Sec61β is phosphorylated in cdc2 kinase independent manner. This kinase activity is roscovtine insensitive and staurosporine sensitive. The kinase responsible for this phosphorylation event was not identified. It has been previously reported that Sec61β can be phosphorylated by the PKC family of kinases which were seen to be associated with the ER (Gruss et al., 1999). It is therefore possible that the cdc2 kinase independent phosphorylation is due to the one of the PKC homologs. However, two other ER membrane bound kinase have been identified which are known to phosphorylate substrates on the cytoplasmic site of the ER. These are the PERK and the IRE1 kinase, both are part of the pathways by which cell copes with ER stress (Chapman et al., 1998; Liu et al., 2002). These type I membrane kinases are activated in response to unfolded proteins in the ER. Upon activation they trigger adaptive responses, such as transcription induction and translational attenuation. Upon activation PERK phosphorylates translation initiation factor 2 (eIF2α) and phosphorylated eIF2α prevents initiation of translation (Chapman et al., 1998).

In order to test if phosphorylation of Sec61β is stimulated during periods of ER stress, cells were incubated with Tunicamycin. Tunicamycin interferes with glycosylation and hence causes unfolded proteins to accumulate in the ER triggering the activation of the IRE1 kinase and PERK (Chapman et al., 1998). No increase in phosphorylation of Sec61β was seen under these conditions (data not shown). This indicates that Sec61β may not phosphorylated by these kinases.

5.10 Prespectives

The present study addressed two aspects Sec61β function, role of Sec61β during *Drosophila* development and physiological relevance of Sec61β phosphorylation. Sec61β is required for the plasma membrane traffic of a specific set of proteins. Strong candidates for proteins which require Sec61β for plasma membrane traffic are morphogens such as the ligands of EGF receptor. Sec61β is phosphorylated by the cdc2 kinase and based on the complementation data from *Drosophila* it seems that phosphorylation by the cdc2 kinase does affect the functional properties of Sec61β. The question which needs to be addressed is: does phosphorylation of Sec61β play a role in the plasma membrane trafficking of the substrates? Sec61β is a part of the ER protein translocation channel hence it is possible that phosphorylation may affect this translocation. Mechanism of regulating translocation by

phosphorylation has been shown to be operative during chloroplast tranlocation. Phosphorylation of a subunit of the translocation complex of the outer chloroplast membrane, TOC34, prevents GTP binding and hence regulates translocation (Soll, 2004). However, from evidences obtained in the present study it cannot be concluded that such a mechanism exits during ER translocation. Although recent data have suggested that Sec61β can act as a GTP exchange factor (Helmers et al., 2003).

Ectopic expression of the wild type form of Sec61β protein in the *Drosophila* wings generates distinct phenotypes. Based on the phenotype it cannot be discriminated if this is a loss of function, gain of function or a new function of Sec61β. However, expression of the phosphorylation mutant of Sec61β in the wings results in stronger phenotypes. This could be interpreted as a dominant negative effect on the function of Sec61β,phosphorylation mechanistically affecting the trafficking of morphogens to the plasma membrane. It is possible that phosphorylation of Sec61β affects a completely different function of Sec61β which is independent of translocation or plasma membrane trafficking.

Signalling during development is brought about by a set of secretory and membrane proteins. These signalling molecules in addition to transcriptional regulation show regulation during the passage through the secretory pathway (Dudu, 2004; Stewart, 2002). This additional step of regulation becomes critical during development where emphasis is placed on the precise amounts and precise timing of secretory events. Sec61β seems to play such a regulatory role and phosphorylation of Sec61β could add another level of regulation.

6. **ABBREVIATIONS**

AD(T)P	adenosin di (tri)phosphate
A-P	Anterior-Posteroir
APS	Ammonium per sulphate
BDGP	Berkeley *Drosophila* Genome Project
bp	base pair
Bip	Heavy chain binding protein
Brho	Brother of Rhomboid
Cop	Coat Protein Complex
DMEM	Dulbecco' Modified Eagle Medium
DMSO	DimethylSulphoxide
DNA	deoxyribonucleic acid
dNTP	2'-deoxy nucleotide triphospahte
DTT	Dithiothreitol
D-V	Dorsal-Ventral
E. coli	Escherichia coli
EDTA	Ethylendiaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
EKRM	EDTA high salt washed Rough Microsomes
Endo H	Endoglycosydase H
ER	endoplasmatic reticulum
GFP	green fluorescent protein
GTP	Guanosine-5'-triphosphate
GTPase	guanosine-5'-triphosphatase
HA	Hemagglutinin
Ii	Invariant Chain
IRES	Internal Ribosome Entry Site
KDa	Kilo-Daltons
KLH	Keyhole Limpet Heme
M	molar
MAPK	mitogen activated protein kinase
NaF	Sodium Floride
Na_2VO_3	Sodium Vanadate
NEB	New England Biolabs
ORF	open reading frame
PAGE	polyacrylamid gel elctrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pkc	protein kinase C
Rho	Rhomboid

RM	Rough Microsomes
RNA	Ridonucleic acid
S2	Schneider Cells
S. cerevisiae	Saccharomyces cerevisiae
SDS	Sodiumdodecylsulfate
siRNA	Small interfering RNA
SR	SRP Receptor
SRP	Signal Recognition Particle
Ser	Serine
SPC	Signal peptidase complex
TCA	Trichloro Acetic acid
TEMED	tetrametyhylthylene
TM	transmembrane
Tris	Tris-(Hydroxymethyl)-Aminomethane
UAS	Upstream Activating Sequence
Vn	Vein
W.B	Western Blotting

7. **REFERENCES**

Acharya, U., Mallabiabarrena, A., Acharya, J.K., and Malhotra, A. (1998). Signalling via mitogen-activated protein kinase kinase (MEK1) is required for Golgi fragmentation during Mitosis. Cell *92*, 183-192.

Adler, P. N. (2002). Planar signaling and morphogenesis in Drosophila. Dev Cell *2*, 525-535.

Axelrod, J. D. (2001). Unipolar membrane association of Dishevelled mediates Frizzled planar cell polarity signaling. Genes Dev *15*, 1182-1187.

Azzi, A., Boscoboinik, D., and C., H. (1992). The protein kinase C family. Eur J Biochem *208*, 547-557.

Balch, W. E. (1990). Molecular dissection of early stages of the secretory pathway. CuurOpinCell Biol *2*, 634-641.

Baonza, A., Roch, F., and Martin-Blanco, E. (2000). DER signaling restricts the boundaries of the wing field during Drosophila development. Proc Natl Acad Sci U S A *97*, 7331-7335.

Barr, F. A. (2004). Golgi inheritance: shaken but not stirred. J Cell Biol *164*, 955-958.

Baumann, O., and Walz, B. (2001). Endoplasmic reticulum of animal cells and its organization into structural and functional domains. Int Rev Cytol *205*, 149-214.

Beckers, C. J., Block, M. R., Glick, B. S., Rothman, J. E., and Balch, W. E. (1989). Vesicular transport between the endoplasmic reticulum and the Golgi stack requires the NEM-sensitive fusion protein. Nature *339*, 397-398.

Brand, A. H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

Campos-Ortega, J. A., and Hartenstein, V. (1985). The Embryonic Development of Drosophila Melanogaster, Springer Verlag).

Chapman, R., Sidrauski, C., and Walter, P. (1998). Intracellular signaling from the endoplasmic reticulum to the nucleus. Annu Rev Cell Dev Biol *14*, 459-485.

Chou, T. B., Noll, E., and Perrimon, N. (1993). Autosomal P[ovoD1] dominant female-sterile insertions in Drosophila and their use in generating germ-line chimeras. Development *119*, 1359-1369.

Cohen, S. M. (1993). Imaginal Disc Development. In (New York, Cold Spring Harbor Laboratory Press), pp. 747-841.

Colanzi, A., Suetterlin, C., and Malhotra, V. (2003). Cell-cycle specific Golgi fragmentation: how and why? Curr Opin Cell Biol *15*, 462-467.

Cormier, P., Pyronnet, S., Salaun, P., Mulner-Lorillon, O., and Sonenberg, N. (2003). Cap-dependent translation and control of the cell cycle. Prog Cell Cycle Res *5*, 469-475.

Culi, J., and Mann, R. S. (2003). Boca, an endoplasmic reticulum protein required for wingless signaling and trafficking of LDL receptor family members in Drosophila. Cell *112*, 343-354.

De Azevedo, W. F., Leclerc, S., Meijer, L., Havlicek, L., Strnad, M., and Kim, S. H. (1997). Inhibition of cyclin-dependent kinases by purine analogues: crystal structure of human cdk2 complexed with roscovitine. Eur J Biochem *243*, 518-526.

Deshaies, R. J., Eun, A., Koch, J. A., Rothblatt, J. A., Sanders, S., Stirling, C., and Schekman, R. (1990). Soluble and membrane-associated factors required for protein translocation into the yeast endoplasmic reticulum. In Dynamics and biogenesis of membranes, J. A. F. Op den Kamp, ed. (Heidelberg, Springer-Verlag), pp. 327-341.

Deshaies, R. J., Sanders, S. L., Feldheim, D. A., and Schekman, R. (1991). Assembly of yeast Sec proteins involved in translocation into the endoplasmic reticulum into a membrane-bound multisubunit complex. Nature *349*, 806-808.

Diaz-Benjumea, F. J., and Garcia-Bellido, A. (1990). Behaviour of cells mutant for an EGF receptor homologue of Drosophila in genetic mosaics. Proc R Soc Lond B Biol Sci *242*, 36-44.

Diaz-Benjumea, F. J., and Hafen, E. (1994). The sevenless signalling cassette mediates Drosophila EGF receptor function during epidermal development. Development *120*, 569-578.

Dominguez, M., Wassermann, J., D., and Freeman, M. (1998). Multiple functions of the EGF receptor in Drosophila eye development. Curr Biol *8*, 1039-1048.

Dreier, L., and Rapoport, T. A. (2000). In Vitro Formation of the Endoplasmic Reticulum Occurs Independently of Microtubules by a Controlled Fusion Reaction. J Cell Biol *148*, 883-898.

Dudu, V., Pantazis, P., and Gonzalez-Gaitan M. (2004). Membrane traffic during embryonic development: epithelial formation, cell fate decisions and differentiation. Curr Opin Cell Biol *16*, 407-414.

Eaton, S. (2003). Cell biology of planar polarity transmission in the Drosophila wing. Mech Dev *120*, 1257-1264.

Eaton, S., Wepf, R., and Simons, K. (1996). Roles for Rac1 and Cdc42 in planar polarization and hair outgrowth in the wing of Drosophila. J Cell Biol *135*, 1277-1289.

Ellgaard, L., Molinari, M., and Helenius, A. (1999). Setting the standards: quality control in the secretory pathway. Science *286*, 1882-1888.

Farmaki, T., Ponnambalam, S., Prescott, A. R., Clausen, H., Tang, B. L., Hong, W., and Lucocq, J. M. (1999). Forward and retrograde trafficking in mitotic animal cells. ER-Golgi transport arrest restricts protein export from the ER into COPII-coated structures. J Cell Sci *112 (Pt 5)*, 589-600.

Feiguin, F., Hannus, M., Mlodzik, M., and Eaton, S. (2001). The ankyrin repeat protein Diego mediates Frizzled-dependent planar polarization. Dev Cell *1*, 93-101.

Freeman, M. (2004). Proteolyis Within the Membranes: Rhomboid Revealed. Nature Reviews: Molecular Cell Biology *5*, 188-197.

Fulga, T. A., Sinning, I., Dobberstein, B., and Pool, M. R. (2001). SRbeta coordinates signal sequence release from SRP with ribosome binding to the translocon. Embo J *20*, 2338-2347.

Garcia-Bellido, A., and de Celis, J. F. (1992). Developmental genetics of the venation pattern of Drosophila. Annu Rev Genet *26*, 277-304.

Görlich, D., and Rapoport, T. A. (1993). Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane. Cell *75*, 615-630.

Gruss, O. J., Feick, P., Frank, R., and Dobberstein, B. (1999). Phosphorylation of components of the ER translocation site. Eur J Biochem *260*, 785-793.

Guichard, A., Biehs, B., Sturtevant, M. A., Wickline, L., Chacko, J., Howard, K., and Bier, E. (1999). rhomboid and Star interact synergistically to promote EGFR/MAPK signaling during Drosophila wing vein development. Development *126*, 2663-2676.

Guichard, A., Roark, M., Ronshaugen, M., and Bier, E. (2000). brother of rhomboid, a rhomboid-related gene expressed during early Drosophila oogenesis, promotes EGF-R/MAPK signaling. Dev Biol *226*, 255-266.

Guo, W., Grant, A., and Novick, P. (1999). Exo84p is an exocyst protein essential for secretion. J Biol Chem *274*, 23558-23564.

Guo, W., and Novick, P. (2004). The exocyst meets the translocon: a regulatory circuit for secretion and protein synthesis? Trends Cell Biol *14*, 61-63.

Guo, W., Roth, D., Gatti, E., De Camilli, P., and Novick, P. (1997). Identification and characterization of homologues of the Exocyst component Sec10p. FEBS Lett *404*, 135-139.

Harlow, E., and Lane, D. (1988). Antibodies: A laboratory manual (New York, Cold Spring Harbour Laboratory Press).

Hegde, R. S., Voigt, S., and Lingappa, V. R. (1998). Regulation of protein topology by trans-acting factors at the endoplasmic reticulum. Mol Cell *2*, 85-91.

Heinrich, S. U., Mothes, W., Brunner, J., and Rapoport, T. A. (2000). The Sec61p complex mediates the integration of a membrane protein by allowing lipid partitioning of the transmembrane domain. Cell *102*, 233-244.

Helmers, J., Schmidt, D., Glavy, J. S., Blobel, G., and Schwartz, T. (2003). The beta-subunit of the protein-conducting channel of the endoplasmic reticulum functions as the guanine nucleotide exchange factor for the beta-subunit of the signal recognition particle receptor. J Biol Chem *278*, 23686-23690.

Herpers, B., and Rabouille, C. (2004). mRNA localization and ER-based protein sorting mechanisms dictate the use of transitional endoplasmic reticulum-golgi units involved in gurken transport in Drosophila oocytes. Mol Biol Cell *15*, 5306-5317.

Holmes, J. K., and Solomon, M. J. (1996). A predictive scale for evaluating cyclin-dependent kinase substrates. A comparison of p34cdc2 and p33cdk2. J Biol Chem *271*, 25240-25246.

Hurtley, S. M., and Helenius, A. (1989). Protein oligomerisation in the endoplasmic reticulum. Annu Rev Cell Biol *5*, 277-307.

Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992). Oxidized redox state of glutathione in the endoplasmic reticulum. Science *257*, 1496-1502.

Irvine, K. D., and Wieschaus, E. (1994). Cell intercalation during Drosophila germband extension and its regulation by pair-rule segmentation genes. Development *120*, 827-841.

Ito, K., Miyashita, Y., and Kasai, H. (1997). Micromolar and submicromolar Ca2+ spikes regulating distinct cellular functions in pancreatic acinar cells. Embo J *16*, 242-251.

Johnson, A. E., and van Waes, M. A. (1999). The translocon: a dynamic gateway at the ER membrane. Annu Rev Cell Dev Biol *15*, 799-842.

Jungnickel, B., and Rapoport, T. A. (1995). A posttargeting signal sequence recognition event in the endoplasmic reticulum membrane. Cell *82*, 261-270.

Kalies, K.-U., Görlich, D., and Rapoport, T. A. (1994). Binding of ribosomes to the rough endoplasmic reticulum mediated by the Sec61p-complex. J Cell Biol *126*, 925-934.

Kalies, K. U., Rapoport, T. A., and Hartmann, E. (1998). The beta subunit of the Sec61 complex facilitates cotranslational protein transport and interacts with the signal peptidase during translocation. J Cell Biol *141*, 887-894.

Kim, J., Irvine, K. D., and Carroll, S. B. (1995). Cell recognition, signal induction, and symmetrical gene activation at the dorsal-ventral boundary of the developing Drosophila wing. Cell *82*, 795-802.

Kolodkin, A., L., Pickup, A., T., Lin, D., M., Goodman, C., S., and Banerjee, U. (1994). Characterization of Star and its interactions with sevenless and EGF recpetor during photoreceptor cell development in Drosophila. Development *120*, 1731-1745.

Kornfeld, R., and Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. Ann Rev Biochem *54*, 631-664.

Kreiner, T., and Moore, H. P. (1990). Membrane traffic between secretory compartments is differentially affected during mitosis. Cell Regul *1*, 415-424.

Lawrence, P. A. (1966). The hormonal control of the development of hairs and bristles in the milkweed bug, Oncopeltus fasciatus, Dall. J Exp Biol *44*, 507-522.

Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H., and Cohen, S. M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. Nature *381*, 387-393.

Lee, J. R., Urban, S., Garvey, C. F., and Freeman, M. (2001). Regulated intracellular ligand transport and proteolysis control EGF signal activation in Drosophila. Cell *107*, 161-171.

Levy, R., Wiedmann, M., and Kreibich, G. (2001). In vitro binding of ribosomes to the beta subunit of the Sec61p protein translocation complex. J Biol Chem *276*, 2340-2346.

Lipschutz, J. H., Lingappa, V. R., and Mostov, K. E. (2003). The exocyst affects protein synthesis by acting on the translocation machinery of the endoplasmic reticulum. J Biol Chem *278*, 20954-20960.

Lipschutz, J. H., and Mostov, K. E. (2002). Exocytosis: the many masters of the exocyst. Curr Biol *12*, R212-214.

Liu, C. Y., Wong, H. N., Schauerte, J. A., and Kaufman, R. J. (2002). The protein kinase/endoribonuclease IRE1alpha that signals the unfolded protein response has a luminal N-terminal ligand-independent dimerization domain. J Biol Chem *277*, 18346-18356.

Lopez de Heredia, M. a. J., R.P. (2004). mRNA localization and the cytoskeleton. Curr Opin Cell Biol *16*, 80-85.

Lowe, M., Gonatas, N. K., and Warren, G. (2000). The mitotic phosphorylation cycle of the cis-Golgi matrix protein GM130. J Cell Biol *149*, 341-356.

Lowe, M., Nakamura, N., and Warren, G. (1998a). Golgi division and membrane traffic. Trends Cell Biol *8*, 40-44.

Lowe, M., Rabouille, C., Nakamura, N., Watson, R., Jackman, M., Jamsa, E., Rahman, D., Pappin, D. J., and Warren, G. (1998b). Cdc2 kinase directly phosphorylates the cis-Golgi matrix protein GM130 and is required for Golgi fragmentation in mitosis. Cell *94*, 783-793.

Lucocq, J., Warren, G., and Pryde, J. (1991). Okadaic acid induces Golgi apparatus fragmentation and arrest of intracellular transport. J Cell Sci *100 (Pt 4)*, 753-759.

Meyer, T., Regenass, U., Fabbro, D., Alteri, E., Rosel, J., Muller, M., Caravatti, G., and Matter, A. (1989). A derivative of staurosporine (CGP 41 251) shows selectivity for protein kinase C inhibition and in vitro anti-proliferative as well as in vivo anti-tumor activity. Int J Cancer *43*, 851-856.

Mostov, K. E., Verges, M., and Altschuler, Y. (2000). Membrane traffic in polarized epithelial cells. Curr Opin Cell Biol *12*, 483-490.

Murthy, M., Garza, D., Scheller, R. H., and Schwarz, T. L. (2003). Mutations in the exocyst component Sec5 disrupt neuronal membrane traffic, but neurotransmitter release persists. Neuron *37*, 433-447.

Murthy, M., and Schwarz, T. L. (2004). The exocyst component Sec5 is required for membrane traffic and polarity in the Drosophila ovary. Development *131*, 377-388.

Nakamura, N., Lowe, M., Levine, T. P., Rabouille, C., and Warren, G. (1997). The vesicle docking protein p115 binds GM130, a cis-Golgi matrix protein, in a mitotically regulated manner. Cell *89*, 445-455.

Nakano, H., Kobayashi, E., Takahashi, I., Tamaoki, T., Kuzuu, Y., and Iba, H. (1987). Staurosporine inhibits tyrosine-specific protein kinase activity of Rous sarcoma virus transforming protein p60. J Antibiot (Tokyo) *40*, 706-708.

Neumann, C. J., and Cohen, S. M. (1998). Boundary formation in Drosophila wing: Notch activity attenuated by the POU protein Nubbin. Science *281*, 409-413.

Neuman-Silberberg, F. S., and Schupbach, T. (1993). The Drosophila dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein. Cell *75*, 165-174.

Nilson, L. A., and Schupbach, T. (1999). EGF receptor signaling in Drosophila oogenesis. Curr Top Dev Biol *44*, 203-243.

Nishizuka (1992). Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C. Science *258*, 607-614.

Novick, P., Field, C., and Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell *21*, 202-215.

Nurse, P., Thuriaux, P., and Nasmyth, K. (1976). Genetic control of the cell division cycle in the fission yeast Schizosaccharomyces pombe. Mol Gen Genet *146*, 167-178.

Panzner, S., Dreier, L., Hartmann, E., Kostka, S., and Rapoport, T. A. (1995). Posttranslational protein translocation in yeast reconstituted with a purified complex of Sec proteins and Kar2p. Cell *81*, 561-570.

Pickup, A. T., and Banerjee, U. (1999). The role of star in the production of an activated ligand for the EGF receptor signaling pathway. Dev Biol *205*, 254-259.

Ptashne, M. (1988). How eukaryotic transcription activators work. Nature *335*, 779-782.

Pyronnet, S., Dostie, J., and Sonenberg, N. (2001). Suppression of cap-dependent translation in mitosis. Genes Dev *15*, 2083-2093.

Pyronnet, S., Pradayrol, L., and Sonenberg, N. (2000). A cell cycle-dependent internal ribosome entry site. Mol Cell *5*, 607-616.

Pyronnet, S., and Sonenberg, N. (2001). Cell-cycle-dependent translational control. Curr Opin Genet Dev *11*, 13-18.

Queenan, A. M., Barcelo, G., Van Buskirk, C., and Schupbach, T. (1999). The transmembrane region of Gurken is not required for biological activity, but is necessary for transport to the oocyte membrane in Drosophila. Mech Dev *89*, 35-42.

Queenan, A. M., Ghabrial, A., and Schupbach, T. (1997). Ectopic activation of torpedo/Egfr, a Drosophila receptor tyrosine kinase, dorsalizes both the eggshell and the embryo. Development *124*, 3871-3880.

Rapoport, T. A., Matlack, K. E., Plath, K., Misselwitz, B., and Staeck, O. (1999). Posttranslational protein translocation across the membrane of the endoplasmic reticulum. Biol Chem *380*, 1143-1150.

Rietsch, A., and Beckwith, J. (1998). The genetics of disulfide bond metabolism. Annu Rev Genet *32*, 163-184.

Roth, S. (2003). The origin of dorsoventral polarity in Drosophila. Philos Trans R Soc Lond B Biol Sci *358*, 1317-1329; discussion 1329.

Roth, S., Neuman-Silberberg, F. S., Barcelo, G., and Schupbach, T. (1995). cornichon and the EGF receptor signaling process are necessary for both anterior-posterior and dorsal-ventral pattern formation in Drosophila. Cell *81*, 967-978.

Rubin, G. M., and Spradling, A. C. (1982). Genetic transformation of Drosophila with transposable element vectors. Science *218*, 348-353.

Sambrook, J. (1991). Molecular Cloning: A Laboratory Manual., Vol 1, 3 edn (New York, Cold Spring Harbor Laboratory Press).

Sans, N., Prybylowski, K., Petralia, R. S., Chang, K., Wang, Y. X., Racca, C., Vicini, S., and Wenthold, R. J. (2003). NMDA receptor trafficking through an interaction between PDZ proteins and the exocyst complex. Nat Cell Biol *5*, 520-530.

Saunders, C., and Cohen, R. S. (1999). The role of oocyte transcription, the 5'UTR, and translation repression and derepression in Drosophila gurken mRNA and protein localization. Mol Cell *3*, 43-54.

Schnepp, B., Donaldson, T., Grumbling, G., Ostrowski, S., Schweitzer, R., Shilo, B. Z., and Simcox, A. (1998). EGF domain swap converts a drosophila EGF receptor activator into an inhibitor. Genes Dev *12*, 908-913.

Schnepp, B., Grumbling, G., Donaldson, T., and Simcox, A. (1996). Vein is a novel component in the Drosophila epidermal growth factor receptor pathway with similarity to the neuregulins. Genes Dev *10*, 2302-2313.

Schonbaum, C. P., Lee, S., and Mahowald, A. P. (1995). The Drosophila yolkless gene encodes a vitellogenin receptor belonging to the low density lipoprotein receptor superfamily. Proc Natl Acad Sci U S A *92*, 1485-1489.

Schonbaum, C. P., Perrino, J. J., and Mahowald, A. P. (2000). Regulation of the vitellogenin receptor during Drosophila melanogaster oogenesis. Mol Biol Cell *11*, 511-521.

Schroder, K., Martoglio, B., Hofmann, M., Holscher, C., Hartmann, E., Prehn, S., Rapoport, T. A., and Dobberstein, B. (1999). Control of glycosylation of MHC class II-associated invariant chain by translocon-associated RAMP4. Embo J *18*, 4804-4815.

Schupbach, T., and Roth, S. (1994). Dorsoventral patterning in Drosophila oogenesis. Curr Opin Genet Dev *4*, 502-507.

Schweitzer, R., Howes, R., Smith, R., Shilo, B. Z., and Freeman, M. (1995). Inhibition of Drosophila EGF receptor activation by the secreted protein Argos. Nature *376*, 699-702.

Shilo, B. Z. (2003). Signalling by the Drosophila epidermal growth factor receptor during development. Exp Cell Res *284*, 140-149.

Simcox, A. (1997). Differential requirement for EGF-like ligands in Drosophila wing development. Mech Dev *62*, 41-50.

Simmonds, A. J., Brook, W. J., Cohen, S. M., and Bell, J. B. (1995). Distinguishable functions for engrailed and invected in anterior-posterior patterning in the Drosophila wing. Nature *376*, 424-427.

Soll, J., and Schleiff, E. (2004). Protein Import into chloroplast. Nat Rev Mol Cell Biol *5*, 198-208.

Souter, E., Pypaert, M., and Warren, G. (1993). The Golgi stack reassembles during telophase before arrival of proteins transported from the endoplasmic reticulum. J Cell Biol *122*, 533-540.

Spradling, A. C., and Rubin, G. M. (1982). Transposition of cloned P elements into Drosophila germ line chromosomes. Science *218*, 341-347.

Stewart, B. A. (2002). Membrane trafficking in Drosophila wing and eye development. Semin Cell Dev Biol *13*, 91-97.

Subramanian, K., and Meyer, T. (1997). Calcium-induced restructuring of nuclear envelope and endoplasmic reticulum calcium stores. Cell *89*, 963-971.

Tabata, T., and Kornberg, T. B. (1994). Hedgehog is a signaling protein with a key role in patterning Drosophila imaginal discs. Cell *76*, 89-102.

Tabata, T., Schwartz, C., Gustavson, E., Ali, Z., and Kornberg, T. B. (1995). Creating a Drosophila wing de novo, the role of engrailed, and the compartment border hypothesis. Development *121*, 3359-3369.

Terasaki, M. (2000). Dynamics of the endoplasmic reticulum and golgi apparatus during early sea urchin development. Mol Biol Cell *11*, 897-914.

Terasaki, M., and Jaffe, L. A. (1991). Organization of the sea urchin egg endoplasmic reticulum and its reorganization at fertilization. J Cell Biol *114*, 929-940.

Toikkanen, J., Gatti, E., Takei, K., Saloheimo, M., Olkkonen, V. M., Soderlund, H., De Camilli, P., and Keranen, S. (1996). Yeast protein translocation complex: isolation of two genes SEB1 and SEB2 encoding proteins homologous to the Sec61 beta subunit. Yeast *12*, 425-438.

Toikkanen, J. H., Miller, K. J., Soderlund, H., Jantti, J., and Keranen, S. (2003). The beta subunit of the Sec61p endoplasmic reticulum translocon interacts with the exocyst complex in Saccharomyces cerevisiae. J Biol Chem *278*, 20946-20953.

Tree, D. R., Shulman, J. M., Rousset, R., Scott, M. P., Gubb, D., and Axelrod, J. D. (2002). Prickle mediates feedback amplification to generate asymmetric planar cell polarity signaling. Cell *109*, 371-381.

Urban, S., Lee, J. R., and Freeman, M. (2002). A family of Rhomboid intramembrane proteases activates all Drosophila membrane-tethered EGF ligands. Embo J *21*, 4277-4286.

Usui, T., Shima, Y., Shimada, Y., Hirano, S., Burgess, R. W., Schwarz, T. L., Takeichi, M., and Uemura, T. (1999). Flamingo, a seven-pass transmembrane cadherin, regulates planar cell polarity under the control of Frizzled. Cell *98*, 585-595.

Vagner, S., Galy, B., and Pyronnet, S. (2001). Irresistible IRES. Attracting the translation machinery to internal ribosome entry sites. EMBO Rep *2*, 893-898.

Valcarcel, R., Weber, U., Jackson, D. B., Benes, V., Ansorge, W., Bohmann, D., and Mlodzik, M. (1999). Sec61beta, a subunit of the protein translocation channel, is required during Drosophila development. J Cell Sci *112 (Pt 23)*, 4389-4396.

Van den Berg, B., Clemons, W. M., Jr., Collinson, I., Modis, Y., Hartmann, E., Harrison, S. C., and Rapoport, T. A. (2004). X-ray structure of a protein-conducting channel. Nature *427*, 36-44.

Voigt, S., Jungnickel, B., Hartmann, E., and Rapoport, T. A. (1996). Signal sequence-dependent function of the TRAM protein during early phases of protein transport across the endoplasmic reticulum membrane. J Cell Biol *134*, 25-35.

Walter, P., and Blobel, G. (1983). Preparation of microsomal membranes for cotranslational protein translocation. Methods Enzymol *96*, 84-93.

Wang, S. H., Simcox, A., and Campbell, G. (2000). Dual role for Drosophila epidermal growth factor receptor signaling in early wing disc development. Genes Dev *14*, 2271-2276.

Warren, G. (1993). Membrane partitioning during cell division. Annu Rev Biochem *62*, 323-348.

Warren, G., Featherstone, C., Griffiths, G., and Burke, B. (1983). Newly synthesized G protein of vesicular stomatitis virus is not transported to the cell surface during mitosis. J Cell Biol *97*, 1623-1628.

Wong, L. L., and Adler, P. N. (1993). Tissue polarity genes of Drosophila regulate the subcellular location for prehair initiation in pupal wing cells. J Cell Biol *123*, 209-221.

Woodman, P. G., Adamczewski, J. P., Hunt, T., and Warren, G. (1993). In vitro fusion of endocytic vesicles is inhibited by cyclin A-cdc2 kinase. Mol Biol Cell *4*, 541-553.