# Role of *Drosophila* LPPs in Germ Cell Migration and Survival

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To Ma
For everything

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# Summary

Lipids are versatile molecules that, in addition to providing a membrane barrier in cells, also play a pivotal role in regulating important signaling events that affect cell migration and survival.

Germ cells in *Drosophila* migrate during embryogenesis by utilizing a guidance signal provided by the spatio-temporal expression of two related and redundant genes called *wunen* (*wun*) and *wunen2* (*wun2*), together called as *wunens*. *wunens* encode catalytically active, membrane bound lipid phosphate phosphatase (LPP) enzymes that are expressed by both somatic cells and germ cells. On the basis of genetic and *in vitro* over-expression studies, it was hypothesized that gradients of lipid phosphates set up by expression of somatic Wunens guide germ cell migration and that germ cell Wunens are important for detection of lipid phosphate levels.

In this thesis, I investigated the role of Wunens in germ cells. Using fluorescent lipid phosphate analogs I demonstrated that germ cells internalize exogenous phosphatidic acid in a dephosphorylation and LPP dependent manner. In a second project I investigated whether Wunen mediated guidance is a contact dependent or an independent process. By substituting the endogenous Wunen expression by various segmentally repeated ectodermal and parasegmental stripes and using *Drosophila* germ cell behavior as a readout, I demonstrated the diffusible nature of the signal and defined its effective range.

This study demonstrates the ecto-enzyme activity of LPPs in dephosphorylation and internalization of an extracellular lipid phosphate in a functionally relevant cell type and at endogenous expression levels. In addition, this study also provides the first quantitative information of the effective range of a lipid phosphate signal *in vivo* which has implications for other cell types that migrate in response to extracellular lipid gradients.

# Zusammenfassung

Lipide sind vielseitige Moleküle, die, neben ihrer Funktion Membranbarrieren in Zellen herzustellen, auch eine zentrale Rolle in der Regulation von wichtigen Signalvorgängen spielen, welche die Zellmigration und das Überleben der Zelle beeinflussen.

Die Keimzellen in *Drosophila* wandern während der Embryogenese indem sie ein Leitsignal benutzen, das durch die räumlich-zeitliche Expression zweier verwandter und redundanter Genen bereitgestellt wird, genannt *wunen* (*wun*) und *wunen2* (*wun2*), zusammen *wunens* genannt. *wunens* codieren katalytisch aktive Membran-gebundene Lipid-Phosphat-Phosphatasen (*lipid phosphate phosphatase*, LPP), die von somatischen Zellen und Keimzellen exprimiert werden. Anhand von genetischen und *in vitro* Überexpressions-Studien, wurde angenommen, dass Phospholipid-Gradienten, die von der Expression somatischer Wunens gebildet werden, die Keimzellmigration steuern und dass Wunens in den Keimzellen für die Detektion des Phospholipidlevels wichtig sind.

Mit Hilfe von fluoreszierenden Phospholipidanalogen habe ich gezeigt, dass Keimzellen exogene Phosphatidsäure in einer Dephosphorylierungs- und LPP-abhängigen Weise aufnehmen. In einem zweiten Projekt habe ich untersucht, ob die Wunen-vermittelte Steuerung ein kontaktabhängiger oder –unabhängiger Prozess ist. Durch den Ersatz der endogenen Wunen-Expression mittels verschiedener segmentären Wiederholungen von ektodermalen und parasegmentären Streifen und der Verwendung des *Drosophila* Keimzellverhaltens als messbaren Parameter, habe ich die diffusionsfähige Eigenschaft des Signals und seinen effektiven Wirkungsbereich aufgezeigt.

Diese Studie demonstriert die ekto-enzymale Aktivität von LPPs im Zuge der Dephosphorylierung und Aufnahme von extrazellulären Phospholipiden in einem funktional relevanten Zelltyp und bei endogenen Expressionsleveln. Zusätzlich liefert diese Studie die ersten quantitativen Informationen über den effektiven Wirkungsbereich eines Phospholipidsignals *in vivo*, was folglich auch für andere Zelltypen relevant sein könnte, deren Migration als Reaktion auf einen extrazellulären Lipid-Gradienten erfolgt.

# Abbreviations

ABC ..... ATP binding cassette
aPKC ..... atypical protein kinase C
BSA ..... bovine serum albumin
Cer1P ..... ceramide 1 phosphate
CNS ..... central nervous system
CXCR..... chemokine receptor
DAG..... diacylglycerol
DGK..... diacylgylcerol kinase
Dri42 ..... differentially expressed in rat intestine 42
ECM ..... extracellular matrix
EGF ..... epidermal growth factors
EMT ..... epithelial to mesenchymal transition
*en* ..... *engrailed*
ER..... endoplasmic reticulum
ERK..... extracellular signal regulated kinase
*eve* ..... *even skipped*
FGF..... fibroblast growth factors
*foi*..... *fear of intimacy*
*fpps* ..... *farnesyl pyrophosphate synthase*
Gal4 ..... galactose 4 transcription factor
*gcl*..... *germ cell-less*
GM-CSF..... granulocyte-macrophage colony stimulating
GPCR ..... G-protein coupled receptor
*h* ..... *hairy*
*hkb* ..... *huckebein*
Hmgcr..... 3-hydroxy3-methyl glutaryl coenzyme A reductase
IGF..... insulin like growth factors
LDL ..... low density lipoprotein

*lola* ..... *longitudinal lacking*

LPA ..... lysophosphatidic acid

LPLAT ..... lysophospholipid acyl transferase

LPP ..... lipid phosphate phosphatase

*lrp* ..... *lipophorin receptor*

LTP ..... lipid transfer proteins

*mdr49* ..... *multi drug resistance 49*

*mid* ..... *midline*

MLVs ..... multilamellar vesicles

*moe* ..... *moesin*

msSGP ..... male specific somatic gonadal precursors

*mtlr* ..... *mitochondrial large ribosomal RNA*

MTO ..... microtubule organizing center

mTOR ..... mammalian target of rapamycin

NBD ..... 7-nitro-2-1-3-benzoxadiazol-4-yl amino dodecanoyl

*nos* ..... *nanos*

PA ..... phosphatidic acid

PAP ..... phosphatidate phosphohydrolase

PE ..... phosphatidyl ethanolamine

PI3K ..... phosphatidylinositol 3-kinase

*pgc* ..... *polar granule component*

PGC ..... primordial germ cell

PKC ..... protein kinase C

PLC ..... phospholipase C

PLD ..... phospholipase D

PMG ..... posterior midgut

POA ..... phosphonate

POPC ..... palmitoyl oleoyl phosphocholine

PS ..... parasegment

*pum* ..... *pumilio*

*qm* ..... *quemao*

S1P ..... sphingosine 1 phosphate

SDF ..... stromal cell derived factor

*serp* ..... *serpent*

SGP ..... somatic gonadal precursor

*shg* ..... *shotgun*

SK1 ..... sphingosine kinase 1

*slam* ..... *slow as molasses*

*tj* ..... *traffic jam*

TEV ..... tobacco etch virus

*tre1* ..... *trapped in endoderm1*

UAS ..... upstream activating sequence

ULVs. ..... unilamellar vesicles

*wun* ..... *wunen*

*wun2* ..... *wunen2*

βGGT1 ..... β subunit of geranylgeranyl transferase type 1.

# Contents

# Chapter 1
# Introduction

## 1.1 Cell migration

Migration is the one of the most interesting phenomenon observed in nature. It may be defined as the translocation of cells either single or in groups from one position to another, in order to perform specific functions. Free-living single cell organisms migrate in response to extracellular environmental cues, which may be in the form of a food source or a harsh environment. In multicellular organisms, cell migration takes place both during development as well for homeostatic maintenance. Cells can migrate individually or in a collective group depending on the type of developmental or homeostatic process that is triggered by extracellular chemotactic cues.

Whether for development or maintenance, abnormal cell migration events in a multicellular organism can have devastating consequences. Early cell migration defects during development may interfere with the proper formation of organs or tissues while defects in cell migration events later in life may lead to several pathological conditions like vascular diseases, chronic inflammatory diseases, mental retardation, metastasis of tumor cells and cancer. Thus, it is very important to understand and study the process of cell migration not only because it is a fascinating area of developmental biology but also because it will ultimately help us understand the anomalies that occur in the process leading to a disease state.

## 1.2 Models for studying cell migration

Cell migration has been studied extensively over the past years using cultured cells that can be easily manipulated and imaged. From decades of research, cell migration has emerged as a complex process that involves dynamic

interaction between the cell and the substratum to which the migrating cells adhere and move over. For the majority of migratory cells, the process of migration can be divided into three major steps:

First, for a cell to migrate it is essential for it to become polarized with a flat leading edge and a pointed lagging tail. Such polarity is generated and maintained by several polarity proteins like Rho family of GTPases, Par proteins, microtubule organizing center (MTOC) and the Golgi apparatus in addition to enzymes such as aPKC and PI3K that keep these molecules localized at the leading edge (Ridley et al., 2003). In many cases directional polarity in a migrating cell is triggered in response to chemoattractants that activate G protein coupled receptors (GPCRs) or tyrosine receptor kinases. Activation of effector molecules in turn changes the cytoskeletal machinery and adhesive properties of the cells (Chung et al., 2001).

In the second step of the process, the migrating cell expands at the leading edge by producing protrusions in the form of blebbing (Charras and Paluch 2008) or lamellipodia while integrins provide new adhesion contacts with the underlying substratum.

At the same time, the third step of adhesion disassembly (Webb et al., 2004) and contractions of the lagging tail helps the posterior part of the cell to retract. This retraction is controlled by accumulation of myosin II (Horwitz and Parsons 1999, Horwitz and Webb, 2003; Vicente-Manzanares et al., 2005). Although some variation based on the cell type, the developmental stage or the substratum over which the cells migrate exists, the basic principle underlying the process of migration remains the same.

Studies in the unicellular organism *Dictyostelium* and mammalian leukocytes and neutrophils in culture have contributed immensely to our present day knowledge in the field of cell migration due to chemotactic response (J. Franca-Koh et al., 2006; Van Haastert and Devreotes, 2004; Janetopoulos and Firtel, 2008). In spite of the ease of manipulation and imaging of cells on two dimension, cell migration *in vivo* is very different and far more challenging than on a two dimensional flat surface. Although, migration of cells in three-dimensional collagen gels can provide some aspects of a three dimensional environment, it is still very far from mirroring the exact conditions that a migrating cell may face *in vivo*. Often, cells *in vivo* are challenged with multiple and even conflicting signals which have to

be interpreted correctly in order to reach their final destination. Studying cell migration *in vivo* in a genetically tractable organism gives a far more holistic picture and helps us understand migration in a much broader perspective.

### 1.3 Gradient formation and migration *in vivo*

In order to migrate to the correct destination *in vivo*, a cell requires extracellular guidance cues either in the form of attractive or repulsive signals. Although these signals by themselves often have the potential to activate the migrating machinery in cells and polarize them (chemokinesis), the path of migration is generally provided by gradients of such guidance cues that a migrating cell can detect and respond to. Such gradients may be set up by the active secretion of an attractant or a repellant, which may either be a cell surface bound molecule or may involve an extracellular soluble short or long range signaling molecule (chemotaxis). Additionally cells *in vivo* could also migrate in response to gradients of extracellular matrix or signaling molecules that bind to extracellular matrix proteins (haptotaxis, Carter 1965). For example, fibronectin, an ECM molecule plays an important role in PGC migration in mouse (Fujimoto et al., 1985; Alverez-Buylla and Merchant-Larios, 1986).

Classically chemokines are substances that stimulate cell locomotion. Studies in developmental and disease processes have shown that a large variety of molecules can act as chemokines which may include growth factors like epidermal growth factors (EGF) receptor ligands, insulin like growth factors IGF-I and IGF-II, members of fibroblast growth factors (FGF) family and cytokines like granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukins and even ECM components (Carter 1967; Kohn et al., 1990; Liotta et al., 1991; Kohn et al., 1993; Sekido et al., 1993; Engebraaten et al., 1993; Arihiro et al., 2000). However in addition to these, several lipids have also been shown to have chemotactic activity. These include arachidonic acid and its related molecules, lysophosphatidic acid, diacylglycerol and phosphatidic acid (Hoffman et al., 1982; Quinn et al., 1988; Wright et al., 1988; Whatley et al., 1990; Zhou et al., 1995).

Chemotactic responses may be autocrine where the migrating cell itself is capable of producing and secreting a chemokine and activating itself or the

response may be paracrine where distant cells produce and secrete the chemokine molecule which then spreads in the inter-tissue space via haemolymph or blood or even bind to ECM to spread to different distances to elicit a chemotactic response. A migrating cell may carry receptors for such signaling molecules which when stimulated, trigger downstream effectors that causes cytoskeletal reorganization with the net effect of the cell migrating towards higher gradients of an attractant or away from a repellant. Additionally, destruction or sequestration of the attractant by the surrounding environment could also produce a gradient of the attractant that can have similar effects on cell migration. An example of this is seen in germ cells in zebrafish where SDF-1a acts as a chemoattractant, binds to CXCR4b receptor expressed in the germ cells and is important for their migration process (Doitsidou et al., 2002; Knaut et al., 2003). However, the spatio-temporal expression of CXCR7 another receptor, in the surrounding somatic cells internalizes SDF-1a and in turn provides a gradient of the chemoattractant that guides the migrating germ cells (Boldajipour et al., 2008).

### 1.4 Primordial germ cells – A model for *in vivo* migration

Primordial germ cells (PGCs) are one of the cell types that has been studied extensively as a model for directed individual cell migration in *Drosophila* and other organisms. PGCs are cells that give rise to sperm and eggs in the adult organism and hence have the potential to propagate a species by giving rise to the next generation.

Germ cells are found in all metazoans studied to date and particularly in bilaterians these cells are specified very early during development perhaps in order to protect the genome from somatic differentiation signals. In many bilaterians therefore, germ cells are specified distant from their final position in the fully developed embryo or adult. Germ cells in these species show a process of directed migration that is essential for them to reach their target destination in a fully developed embryo. In some metazoans including *Drosophila* and zebrafish, germ cells are specified by “pre-formation” that is the inheritance of a specialized maternal cytoplasm that is rich in maternal RNAs and proteins. In other metazoans

like mice, germ cells are formed by the induction of cells by surrounding somatic tissues (Extavour and Akam 2003).

## 1.5 Germ cell migration in *Drosophila*

### 1.5.1 PGC specification

The early *Drosophila* embryo consists of a syncytium of many nuclei in a single common cytoplasm (Sonnenblick 1941). A subset of maternally deposited proteins and RNAs are concentrated in the posterior pole of the developing *Drosophila* oocyte. This localized pre deposited pool of RNAs and proteins, called the pole plasm or germ plasm, is essential for the budding of the first germ cells (Williamson and Lehmann, 1996; Rongo and Lehmann 1996). Approximately 1.5 hours after egg laying and two hours prior to the cellularisation of somatic cells in the rest of the blastoderm, 10 nuclei at the posterior pole cellularise and undergo two cycles of cell division to give rise to around 40 pole cells (Lehmann 1992). While several maternally provided mRNAs give pole plasm its characteristics, germ cell formation requires *germ cell-less* (*gcl*) and *mitochondrial large ribosomal RNA* (*mtlrRNA*) (Santos and Lehmann 2004a). Regulation of gene expression and maintaining the identity of the PGCs throughout development is provided by the expression of *nanos* (*nos*), *pumilio* (*pum*) and *polar granule component* (*pgc*) that act as transcriptional and translational repressors (Santos and Lehmann 2004a).

### 1.5.2 PGC migration in *Drosophila*

Germ cells display a migratory nature very soon after they have been specified at the posterior pole although they remain together in a cluster (Jaglarz and Howard, 1995; Kunwar et al., 2008). During gastrulation this germ cell cluster is passively taken inside posterior midgut pocket (Sonnenblick 1941; Jaglarz and Howard 1994). This is where the germ cells start their process of active migration (Jaglarz and Howard 1995) at stage 10 (Hay et al., 1990; Warrior 1994). The process of germ cell migration and gonad formation in *Drosophila* may be divided into four steps: a) Transepithelial migration across the midgut, b) Reorientation of

germ cells on the dorsal side of the midgut, c) Migration of germ cells into the mesoderm and their association with the somatic gonadal precursor (SGP) cells and d) The compaction of germ cell and SGP clusters into gonad. These steps involve both attractive and repulsive signals and the molecular players involved in the processes are discussed below:

a) The transepithelial migration across the midgut

At late stage 9 the cluster of germ cells sitting inside the midgut pocket individualise and become polarized. They arrange themselves in a radial pattern with the leading edge of the cells facing the posterior midgut (PMG) epithelium, in order to undergo transepithelial migration (figure 1.1 A, B) (Kunwar et al., 2003; Kunwar et al., 2008). One of the genes required in germ cells at this stage is *trapped in endoderm 1* or Tre1, which is an orphan G-protein coupled receptor (GPCR). GPCRs such as CXCR4 affects migration of different cell types like germ cells and immune cells in organisms as distant as zebrafish and mouse (Doitsidou et al., 2002; Knaut et al., 2003; Molyneaux et al., 2003).

*tre1* RNA is maternally provided and is required in the germ cells themselves. In the absence of maternal *tre1*, germ cells remain inside the midgut pocket, ultimately failing to migrate across the PMG. Activation of Tre1 by a yet unknown ligand, causes redistribution of Gβ13f and Gγ1 subunits, Rho1, and the adherens junction component DE-cadherin to the lagging tail thus polarizing the germ cells (Kunwar et al., 2008) (Figure 1.1 C).

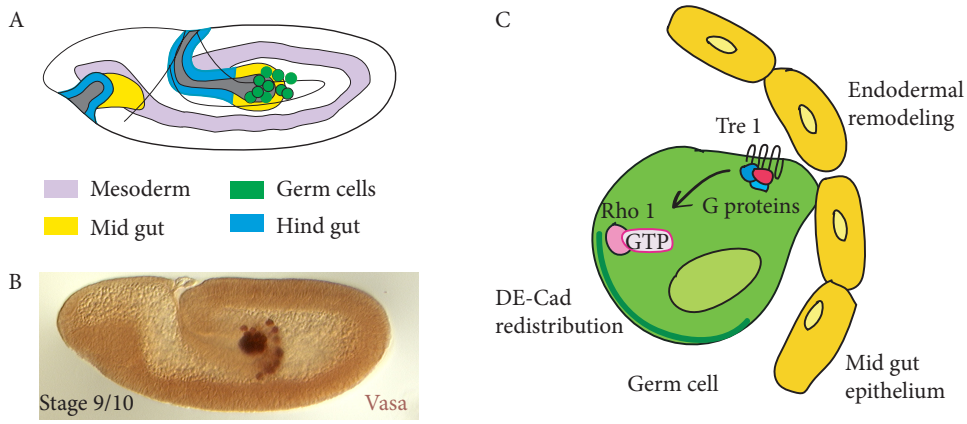


Fig 1.1: (A) Cartoon depicting the lateral view of a stage 10 *Drosophila* embryo with germ cells (green) inside the posterior midgut pocket (yellow), about to migrate through the epithelium onto the overlying mesoderm (pale violet), (adapted from Williamson and Lehmann 1996) (B) A wild type stage 10 *Drosophila* embryo stained against Vasa (brown) which marks the germ cells, (adapted from Starz-Gaiano and Lehmann 2001) (C) Cartoon depicting the major molecular players and events that occur at this stage of germ cell migration.

At the same time during embryonic development the midgut epithelium undergoes endodermal remodeling (Callaini et al., 1995; Jaglarz and Howard 1995), which is an intrinsic property of the PMG cells as part of an epithelial to mesenchymal transition (EMT) (Campbell et al., 2011). The germ cells take advantage of this process, in order to accomplish their transepithelial migration (Seifert and Lehmann 2012). The developmental stage of the endoderm dictates the timing of germ cell migration (Jaglarz and Howard 1994). In mutants such as *serpent* and *huckebein* where the midgut does not undergo remodeling or acquires a fate of the hindgut epithelium, the germ cells fail to migrate across to the basal side and are trapped in the midgut pocket (Reuter 1994; Warrior 1994; Jaglarz and Howard 1995; Moore et al., 1998).

b) Re-orientation on the dorsal side of the midgut

After the germ cells have crossed the midgut epithelium, the germ cells reorient themselves by migrating towards the dorsal side of the midgut in order to migrate into the overlying mesoderm. This movement is brought about by the expression of two related genes called *wunen* (*wun*) and *wunen2* (*wun2*), together called *wunens*, which encode for *Drosophila* lipid phosphate phosphatase (LPP) enzymes (Zhang et al., 1996; Zhang et al., 1997; Starz-Gaiano et al., 2001). These two genes have a dynamic spatio-temporal expression pattern in embryonic somatic cells and are also expressed in the germ cell themselves (Renault et al., 2002). The expression of *wunens* is thought to provide a repulsive signal to direct migrating germ cells (Zhang et al., 1997; Starz-Gaiano et al., 2001). I have discussed the role of Wunen mediated repulsion in germ cell migration in greater detail in the following sections.

c) Association of germ cells with the somatic gonadal precursor cells (SGPs)

Germ cells require somatic counterparts in order to form a fully functional gonad. This somatic counterpart called the somatic gonadal precursor (SGP) cells is specified in three bilateral clusters in parasegments (PS) 10, 11 and 12 in the dorsolateral mesoderm. They are a group of 25-35 cells that form at stage 11, around the same time as the germ cells start migrating towards the overlying mesoderm (Brookman et al., 1992; Warrior 1994) (Figure 1.1 A, B). An additional fourth SGP cluster also called the male specific SGPs (msSGPs) is specified in PS 13 in both males and females but is only maintained in males (De Falco et al., 2003). The germ cells migrate out of the midgut into the overlying mesoderm and split into two bilateral clusters. The SGPs associate with the germ cells as they migrate into the mesoderm ensheathing them with long cytoplasmic extensions. Once the germ cells have associated with the SGPs, the germ cells stop active migration.

The SGPs are thought to produce an attractant that the germ cells detect and migrate towards to associate with these clusters. The major molecular player involved in this process is 3- hydroxy 3-methylglutaryl coenzyme A reductase (Hmgcr) which is the rate limiting enzyme catalyzing the conversion of HmgcoA to mevalonate that is rapidly converted into cholesterol in mammals (Goldstein and Brown, 1990). In Hmgcr mutants some germ cells fail to leave the endoderm while the ones that do migrate across fail to associate with the SGPs, finally scattering in the entire mesoderm (Van Doren et al., 1998). This enzyme is expressed broadly in the mesoderm at stage 11 and by stage 12 its expression is restricted to the SGP clusters. Ectopic expression of Hmgcr in the ectoderm and the central nervous system, is capable of attracting germ cells to these locations confirming that the Hmgcr pathway is involved in the synthesis of a germ cell attractant (Van Doren et al., 1998).

Other enzymes downstream of *hmgcr*, in the mevalonate pathway, like farnesyl-diphosphate synthase (*fpps*) and *quemao* (*qm*) have similar embryonic expression patterns to *hmgcr* (Santos and Lehmann, 2004b). Loss of *fpps*, *qm* as well as mutants in the β subunit of geranylgeranyl transferase type 1 (βGGT1) show defects in germ cell migration (Santos and Lehmann, 2004b).

Secreted fission yeast M factor and budding yeast a-mating factors are also prenylated proteins that act as attractants (Christensen and Nielsen, 1997). The farnesylated a-type pheromone in *S.cerevisiae* binds to a G-protein coupled receptor in the α-cell to promote cytoskeletal rearrangements (McGrath and Varshavsky, 1989). It is believed that in *Drosophila* too, a geranlygeranyl modified protein is secreted by the SGPs via *mdr49* that codes for an ABC transporter, the yeast orthologue of which regulates the transport of farnesyl modified mating factors (Ricardo and Lehmann, 2009)(Figure 1.2 C). This yet unidentified isoprenylated secreted protein is believed to be the attractant for *Drosophila* germ cells that may act on Tre1, the GPCR involved in germ cell migration.

Beside *hmgcr*, a separate pathway is thought to act in parallel to get germ cells into the mesoderm. Slow as molasses (*slam*) is one of the earliest genes to be transcribed and is expressed during the time of cellularization of the blastoderm. Its expression becomes undetectable after stage 8 (Lecuit et al., 2002; Stein et al., 2002). But this early expression is thought to deposit a guidance molecule on the dorsal side of the midgut that persists till late stages. Although the nature of this guidance factor is unknown, loss of both *hmgcr* and *slam* has a more severe germ cell migration defects than each of the single mutants (Stein et al., 2002).

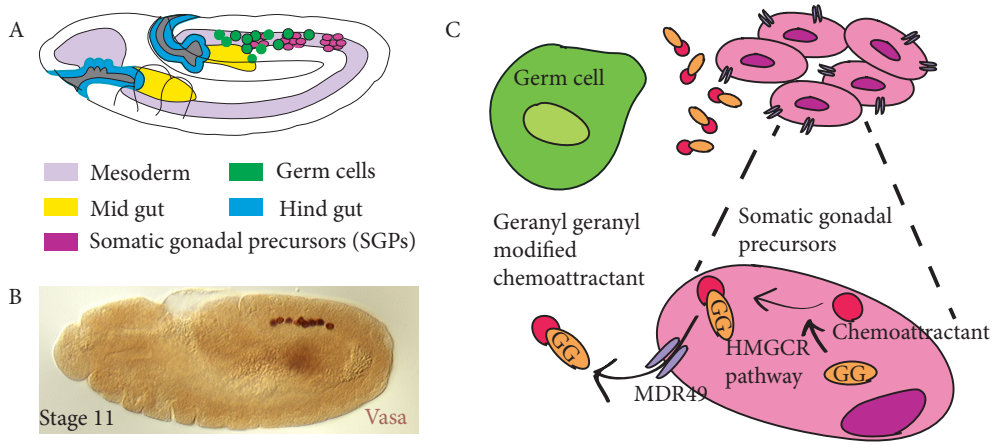



Fig 1.2: (A) Cartoon depicting the lateral view of a stage 11 *Drosophila* embryo with germ cells (green) that are migrating into the mesoderm (pale violet) and have started to associate with the somatic gonadal precursor cells (SGPs, magenta) which are also being specified in the mesoderm at this stage. (adapted from Williamson and Lehmann 1996) (B) A wild type stage 11 *Drosophila* embryo stained against Vasa (brown) which marks the germ cells (adapted from Starz-Gaiano and

Lehmann 2001). (C) Cartoon depicting the attractive signal that is secreted by the SGPs to attract germ cells.

d) Germ cell SGP coalescence

By embryonic stage 13 all germ cells have associated with and been ensheathed by the SGPs (Jenkins et al., 2003). These two cell types are found neatly lined up on either side of the embryo at this stage. The germ cells and SGPs then undergo a process of compaction to form a functional gonad (Figure 1.3 A, B). At stage 14 of embryonic development, the msSGP cluster fuse with the other anterior clusters, in the male embryos (Figure 1.3 C) while in females, this cluster undergoes cell death. In both cases by stage 15 a compact round gonad is formed (Van Doren et al., 2003; Casper and Van Doren, 2006). Defects in any of the stages of SGP association, ensheathment or compaction may affect gonad formation and ultimately be detrimental to the fertility of the organism.

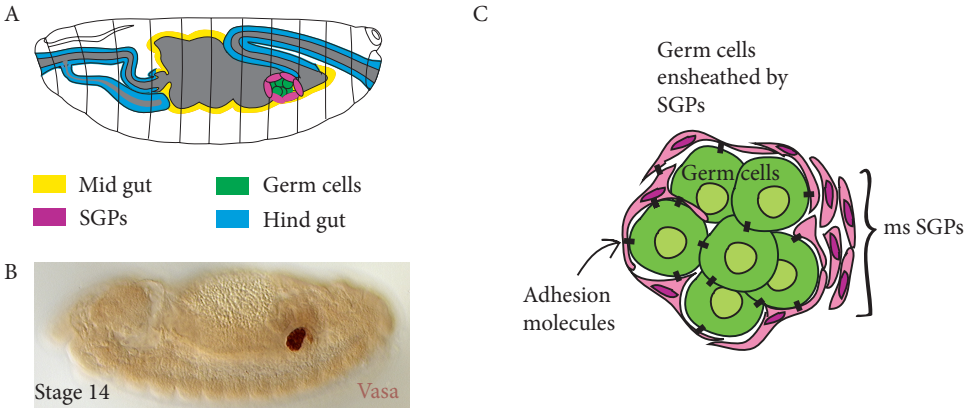



Fig 1.3: (A) Cartoon depicting lateral view of a stage 15 *Drosophila* embryo with germ cells (green) and SGPs (magenta) having associated and coalesced together to form a round compacted gonad (adapted from Williamson and Lehmann 1996) (B) A wild type stage 15 *Drosophila* embryo stained against Vasa (brown) which marks the germ cells (adapted from Starz-Gaiano and Lehmann 2001) (C) Cartoon depicting the SGPs ensheathing the germ cells and the role of adhesion molecules in keeping the gonad together.

Several genes affect ensheathment and compaction of the gonads including *traffic jam* (*tj*), which encodes a transcription factor (Li et al., 2003), *fear of intimacy* (*foi*), which encodes a zinc transporter (Van Doren et al., 2003) and

*shotgun* (*shg*), which encodes a *Drosophila* E-cadherin (Jenkins et al., 2003). *foi* is also known to affect DE-cadherin levels post transcriptionally in the gonads (Mathews et al., 2005). Although cell-cell adhesion is very important for the final compaction of the gonad, there are also adhesion independent mechanisms like the Slit/Robo pathway was recently identified in a gonad morphogenesis screen to be involved in *Drosophila* gonad compaction (Weyers et al., 2011). Two other transcription factors *longitudinals lacking (lola)* and *midline (mid)* have also been recently identified to regulate germ cell-SGP ensheathment and SGP cluster fusion independent of DE-cadherin (Tripathy et al., 2013 manuscript in preparation).

### 1.6 Importance of repulsion in germ cell migration – The role of Wunens

One of the major guiding factors that influence the migration of *Drosophila* germ cells has been uncovered by the analysis of two related genes called *wunen* (*wun*) and *wunen2 (wun2),* collectively often referred to as *wunens.* These two genes are located next to each other on chromosome 2R and are transcribed in opposite directions. *wun* and *wun2* were first identified in a deficiency screen (Zhang et al., 1996) and an overexpression screen (Starz-Gaiano et al., 2001) respectively.

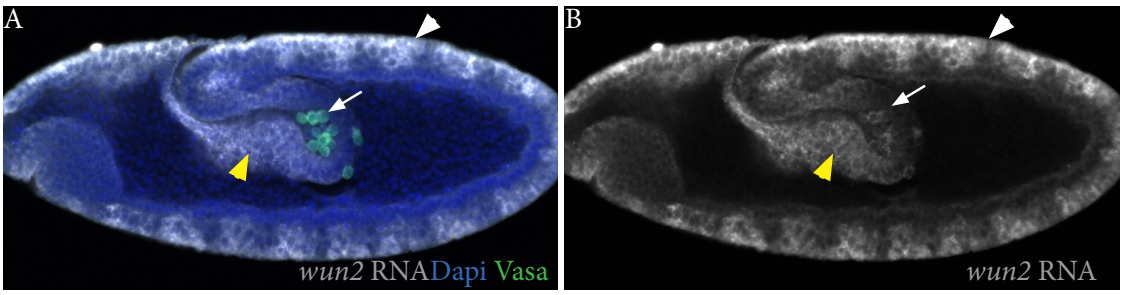



Figure 1.4: (A, B) Confocal image of the lateral view of a section through a stage 10 *Drosophila* embryo depicting germ cells (in green, stained against Vasa) present inside the midgut pocket about to cross the midgut epithelium. *wun2* RNA (B, grey) is detected in the ectoderm, CNS (white arrowhead), part of the midgut primordium (yellow arrowhead) and also in the germ cells themselves (arrow).

By *in situ* hybridization, both *wunens* have very similar expression pattern that includes the posterior midgut (Figure 1.4, yellow arrowhead) and part of the

hindgut at stage 10 (Figure 1.4 A, B). At stages 10, 11 and 12, expression is also observed in the central nervous system (CNS) (Figure 1.4, white arrowhead), and parts of the ectoderm (Zhang et al., 1996; Starz-Gaiano et al., 2001; Renault et al., 2002). By late stage 14, *wun2* expression becomes prominent in the heart cells that persist till stage 17 (Renault et al., 2002).

During their migration, germ cells do not enter embryonic tissues expressing Wunens. In addition, overexpression of Wunen in the tissues, which are normally attractive to germ cells, like the mesoderm, is sufficient to prevent germ cell entry, and germ cells often undergo cell death (Starz-Gaiano et al., 2001). In the absence of both *wunens* from the somatic cells alone, the germ cells survive and migrate but are lost in the posterior part of the embryo (Figure 1.5 A, B cartoon showing stage 13 lateral view and B, D showing stage 13 dorsal view in actual embryos). Live imaging of germ cells in *wun wun2 Z-* embryos showed normal germ cell motility but loss in bilateral sorting, which was rescued by expression of Wun2 in the CNS (Sano et al., 2005). Thus, this spatiotemporal dynamicity of Wunens is thought to provide a repulsive cue to the migrating germ cells.

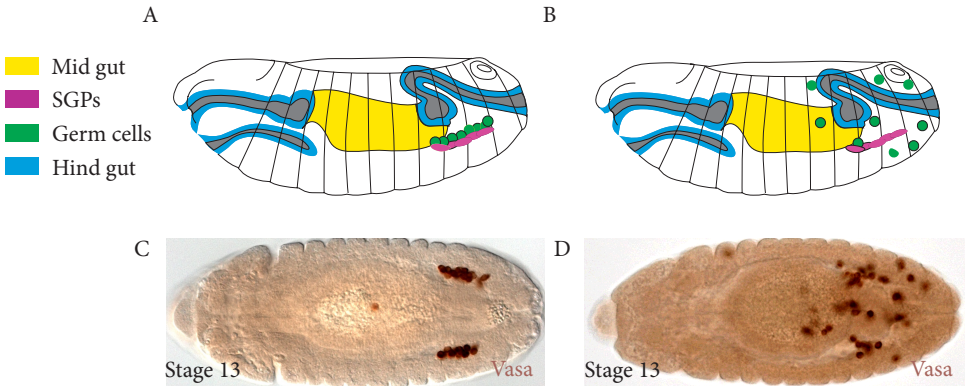



Fig 1.5: (A, B) Cartoons depicting the lateral view of a stage 13 wild type (A) and a *wun wun2 Z-* (B) embryo with germ cells (green) shown to be scattered (B) in the absence of somatic *wunens* while they line up with the SGPs (magenta) in the wild type (A). (C, D) Dorsal view of stage 13 wild type (C) and *wun wun2 Z-* (D) embryos stained against Vasa (brown) showing the position of germ cells.

Wunens are also provided in the germ cells maternally and intriguingly this maternal expression is essential for their survival (Renault et al., 2002, Figure 1.4 B arrow). In the absence of germ cell *wunens* (provided maternally), the germ cells fail to individualize, show delayed migration across the midgut and eventually the

majority of them die (Renault et al., 2010). However in the absence of *wunens* both maternally as well as zygotically, germ cells remain inside the midgut pocket and they never migrate outside. This demonstrates that germ cell Wunens function not only to keep the germ cells alive but contributes to their intrinsic motility and/or makes them competent to respond to extracellular cues. Expression of Wunens in the germ cells are also important for germ cell-germ cell repulsion which aids in the equal partitioning of the germ cells into two gonads (Renault et al., 2010).

Wunens are partially redundant in germ cells, such that absence of one of them only partially affects germ cell survival. Genetic rescue experiments have shown that functionally they are fully redundant, since either one can rescue a *wun wun2* maternal null phenotype (Renault et al., 2010). Genetic studies using different combinations of somatic and germ cell *wunens* has revealed a complicated dose sensitive relationship that is thought to underlie their mechanism of action (table 1.1).

| Germ cells | Somatic cells | Phenotype |
|---|---|---|
| + | + | Migration normal. Survival normal |
| + | - | Germ cells scatter in the posterior part of the embryo. Survival normal. |
| + | +++ | Germ cells die. |
| - | + | Germ cells die. |
| - | - | Failure of migration and germ cells remain in the gut. |
| +++ | + | Slight mismigration of germ cells. Survival normal. |

Table 1.1: The complex dose dependent relationship between the somatic and germ cell Wunens and their effect on germ cell migration and survival. The symbols used: + normal expression, - no expression, +++ overexpression.

## 1.7 *wunens* encode for *Drosophila* lipid phosphate phosphatases

Wunens belong to the family of *Drosophila* lipid phosphate phosphatases (LPPs). These are membrane bound enzymes formerly named phosphatidic acid phosphatase 2 (PAP-2), based on the two kinds of Phosphatidate

phosphohydrolase (PAP) activities found in mammalian cells. The first, PAP-1 requires $Mg^{2+}$ for its enzymatic activity, is soluble and is inhibited by N-ethylmaleimide. It is translocated form the cytosol to the ER where it converts phosphatidic acid (PA) to diacylglycerol (DAG). The second type, PAP-2 is characterised by Mg+2 independent enzymatic activity, is insoluble and insensitive to inhibition by N-ethylmaleimide (Jasinska et al., 1999). The latter was renamed lipid phosphate phosphatases (LPPs) due to their diverse substrate specificity.

### 1.8 Models of repulsion by Wunens

Several models were proposed by Zhang et al., 1997 to explain how Wunens may act at a molecular level to mediate their affect on germ cell migration and survival.

**a) Direct**: According to this model, Wunen acts as a membrane bound repulsive ligand while germ cells have a receptor that recognizes it and on contact are repelled away from Wunen expressing somatic cells.

**b) Recoil**: Alternatively, Wunen could act as a receptor for a ligand on the germ cells, the binding of which activates Wunen to release a repellant factor.

Both the above models require direct contact of the germ cells to the Wunen expressing somatic cells.

**c) Remote**: According to this model, Wunens may be constitutively active and produce a repellant independent of germ cell contact. Hence the expression of Wunen anywhere would render the environment unfavourable for germ cell migration.

It was later discovered that the expression of the catalytic dead forms of Wunen in the mesoderm had no effect on germ cell migration or survival and was also unable to rescue the loss of function in germ cells (Renault et al., 2004; Renault et al., 2010). Taking this catalytic activity together with the indispensible role of Wunens in germ cells (Renault et al., 2002) another model was proposed by Renault et al., 2004 to explain the molecular basis of Wunen action and to explain the dose sensitive relationship observed between germ cell and somatic cell Wunens.

**d) A contact independent gradient model**

According to this model, somatic cell Wunens dephosphorylate and destroy an extracellular lipid phosphate substrate, which is important for germ cell survival. In other words, germ cell and somatic cell Wunens compete for this extracellular pool of lipid phosphates. Expression of somatic Wunens depletes the extracellular environment of this lipid phosphate. This leads to the formation of an extracellular lipid phosphate gradient, with higher levels of lipid phosphate in regions lacking somatic Wunen expression. This extracellular lipid phosphate gradient is detected by germ cells and they move away from Wunen expressing somatic cells towards cells that do not express Wunens. When Wunens are expressed ectopically in regions where germ cells normally migrate, like the mesoderm, germ cells Wunens are possibly out competed by the somatic Wunen overexpression, which leads to germ cell death. In the complete absence of somatic Wunens, no lipid phosphate gradient exists and hence in the absence of the guidance cue, the germ cells mismigrate and are lost (Figure 1.6). To date, this model best explains the molecular basis of Wunen action in germ cell and somatic cells and hence we favor this model over the others.

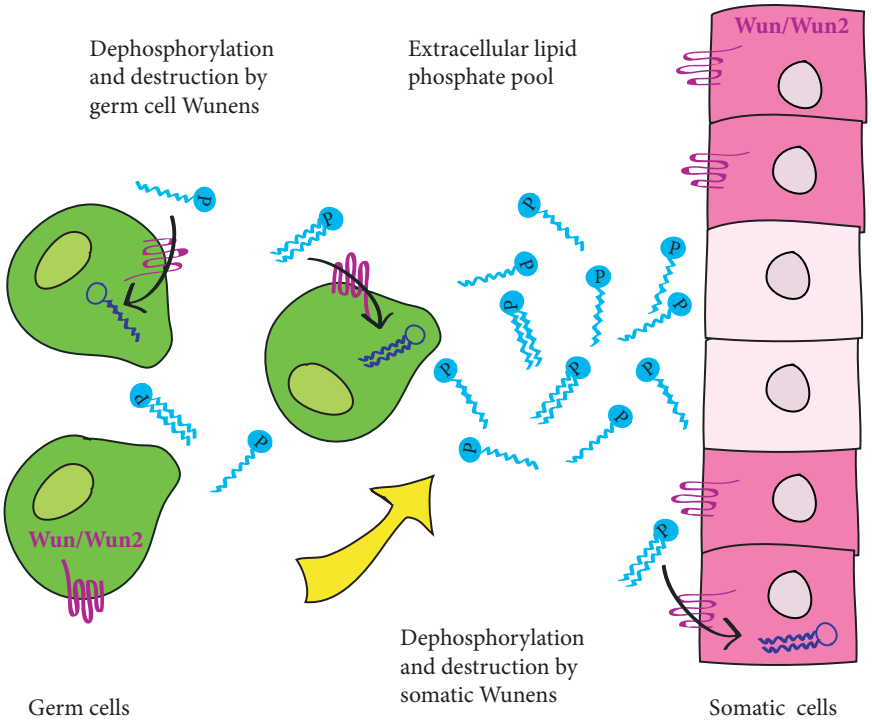


Fig 1.6: Soma and germ line competition model for germ cell migration and survival. Spatially restricted expression of Wunens (shown in magenta) in somatic cells provides a gradient of lipid phosphate attractant (shown in blue). The germ cells move to higher concentration of the lipid phosphate (direction of movement shown by yellow arrow). The germ cells themselves dephosphorylate and internalize the substrate, which is important for their survival. (Adapted from Renault et al., 2004; Renault et al., 2010)

This model suggests the establishment of extracellular lipid/lipid phosphate gradients by spatially and temporally restricted expression pattern of the Wunens. The model was further extended to include the action of germ cell Wunens that may act similarly to generate germ cell-germ cell repulsion that provides the signal to the germ cells to move out of the midgut pocket and disperse in the overlying mesoderm (Renault et al., 2010).

The gradient generated by the somatic expression of Wunens may be short or long range or in other words, it may affect cells in a contact dependent manner or act over several cell diameters i.e. in a contact independent manner. Although lipids have emerged as important signaling molecules over the past decade, there are only a few instances of lipids acting as long distance signaling molecules in development and much less is known in terms of the distances over which such lipids can act.

## 1.9. Lipid - cell interactions

Extracellular lipids could be present in the extracellular space either bound to lipoprotein particles or in membranous vesicles. Different cells have different mechanisms of internalization of exogenous lipids. Firstly, lipids could bind to membrane receptors for example specific lipoprotein receptors and internalize lipids via a non-endocytotic pathway for example, *Drosophila lipophorin receptor 1* and *2* (*lrp1* and *lpr2*) mediates uptake of fatty acids in oocytes and larval imaginal discs (Parra-Peralbo and Culi, 2011). Lipids can also be internalized by receptor-mediated endocytosis for example, scavenger receptors found on macrophages can bind to oxidized or acetylated LDL and internalize the lipids by endocytosis (Endemann et al., 1993; Abrams et al., 1992). Thirdly, enzymes like lipid phosphate phosphatases (LPPs) expressed on the plasma membrane could also function to

dephosphorylate and internalize extracellular lipid phosphates (Roberts and Morris, 2000).

### 1.10 Structure and function of LPPs

LPPs are characterized by six transmembrane α-helical domains linked by extramembrane loops with their N and C terminals facing the cytoplasm (Brindley and Waggoner, 1998; Sigal et al., 2004) and the mammalian enzymes have a N-glycosylation site at a single conserved residue in the extracellular loop between the third and the fourth α helix (Kai et al., 1996; Kai et al., 1997) (Figure 1.7). The structural predictions obtained from computer predicted a transmembrane topology model. Experiments using Dri42, a rat homolog of LPP3, indicated that the catalytic sites are within the second and third extracellular loop (Brindley and Waggoner, 1998; Kanoh et al., 1999; Stukey and Carman, 1997; Neuwald 1997), thus indicating towards a role of LPPs as ecto-enzymes that are capable of dephosphorylating exogenous lipid phosphates.

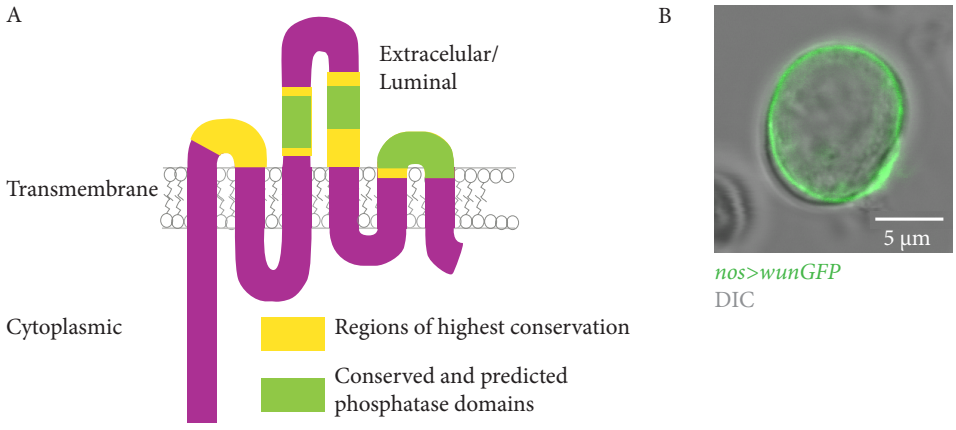



Figure 1.7: (A) Predicted structure lipid phosphate phosphatase enzymes. This class of enzymes has six trans membrane domains (magenta), with conserved regions (in yellow) and catalytic site exposed to the extracellular/luminal side (green) (B) A live *Drosophila* germ cell expressing GFP tagged Wun. Intense GFP expression is seen on the plasma membrane.

LPPs hydrolyze different lipid phosphates by cleaving the phosphomonoester bond. Although the *in vivo* substrates are still not clear the *in*

*vitro* substrates of LPPs include phosphatidic acid (PA), lysoPA (LPA), ceramide-1-phosphate (Cer1P) and sphingosine-1-phosphate (S1P), diacylglycerol pyrophosphate and N-oleoyl ethanolamine phosphate (Brindley et al., 1998). But in spite of the capacity of LPPs to dephosphorylate a broad range of lipid phosphates, it is more likely that LPPs are more specific *in vivo*. Out of the three mammalian LPPs, Wunens have the maximum similarity to mLPP3 and this is also reflected in the ability of mLPP3 to have the same effect *in vivo* as Wunens in terms of germ cell migration and survival (Ile et al., 2012). However mLPP1 cannot substitute the function of Wunens in *Drosophila* even though they all dephosphorylate the same range of substrates *in vitro* (Burnett and Howard 2003).

Insect cell lines when transfected with Wun2 acquired the capacity to internalize fluorescent analogs of phosphatidic acid (PA) presented in the extracellular medium. Catalytic dead versions of Wun2 did not show such uptake (Renault et al., 2004). This demonstrated that like its mammalian counterparts (Roberts and Morris 2000), overexpression of *Drosophila* LPPs could function in transfected cells to dephosphorylate and internalize externally supplied labeled lipids. Wunens are expressed in the embryonic germ cells and somatic cells and this expression is linked to germ cell survival and migration. Thus it is essential to understand if Wunens actually function to internalize extracellular lipids at their endogenous level and place of expression that could possibly provide a survival and migration signal in germ cells.

While it is certain that these enzymes are membrane bound, there has been some ambiguity regarding their plasma membrane or intracellular membrane localization and function. Their expression on plasma membrane would be consistent with their function as ectoenzymes. However, different tagged versions of Wunens showed different localization patterns in germ cells (Ratna Tripathy PhD thesis). A germ cell survival assay using a TEV (tobacco etch virus) protease cleavable recombinant variety of Wun2, provided the evidence that Wunens are required at the plasma membrane for germ cell survival (Ratna Tripathy, PhD thesis). If endogenous Wunens provide germ cells the ability to internalize extracellular lipid by dephosohorylating lipid phosphates, this would also support the plasma membrane localization and site of action of Wunens.

## 1.11 Role of bioactive lipids in survival and migration

Phospholipids like phosphatidlycholine, phosphatidylethanolamine, phosphatidlyserine, phosphatidylglycerol, phosphatidylinositol, are major structural components of cell membranes. But besides giving a structure and separating the different compartments inside a cell, lipids also act as important signaling molecules that affect cell migration, survival and proliferation.

a) Extracellular lipid mediators

The various *in vitro* substrates of LPPs have been implicated in regulating a range of cell behaviors. Extracellular lyso-phospholipase D (lyso PLD, also known as autotaxin) is a major source of LPA in mammals and is known to be an ecto-phosphodiesterase (Nakanaga et al., 2010). It has been implicated in metastasis and tumour progression (Tokumura, 2002). Extracellular PA has chemotactic effect on human neutrophils (Frondorf et al., 2010) in vitro.

External S1P is a stimulator of angiogenesis *in vivo* (English et al., 2002) and acts as an attractant for endothelial cells during blood clotting (English et al., 2000). It is crucial for lymphocyte trafficking (Matloubian et al., 2004) and interfering with S1P signaling can be used as a mechanism of immunosupression (Mandala et al., 2002). It is also required for the correct migration of the bilateral heart cell progenitors towards the midline in zebrafish embryos (Kupperman et al., 2000).

Exogenous Cer1P can stimulate cell division in rat fibroblasts (Gomez-Munoz et al., 1995) It is involved in inflammatory responses (Pettus et al., 2004) and can block the caspase 9/caspase 3 pathway and apoptosis in macrophages (Gomez-Munoz et al., 2004).

In vertebrates, LPA and S1P act as ligands for G protein coupled receptors (GPCRs) (Olivera and Spiegel, 1993; Hecht et al., 1996). Majority of the downstream consequences that occur depends upon the coupling of the GPCRs to different heterotrimeric G-proteins. Homologs of such vertebrate GPCRs for lipids have not been identified in flies (Kunwar et al., 2006). Therefore the mechanism of action of extracellular lipids on the migration of germ cells in flies may be different from what is known in vertebrates.

b) Intracellular lipid mediators

The different lipid phosphates as well as their dephosphorylated products can also act as signaling molecules inside cells.

PA is a major intermediate in phospholipid biosynthesis and levels of PA inside cells could potentially act as an indicator of the physiological state. PA is able to bind to several effector proteins directly, which have PA binding sites and which target other downstream effectors. For instance PA binds to sphingosine kinase 1 (SK1) which coverts sphingosine to sphingosine 1-phosphate (S1P) (Delon et al., 2004). In mammalian cells intracellular PA activates mTOR that affects cell survival (Fang et al., 2001).

Activation of SK1, either by phospholipase D (PLD) dependent or independent pathways, ultimately generates more S1P, which is a survival signal for the cell (Delon et al., 2004). It leads to an increase in actin stress fibre formation and protects against apoptosis (Pyne and Pyne, 2000; English et al., 2002). While intracellular ceramide has been found to induce cell differentiation, senescence in fibroblast cells (Venable et al., 1995) and cell death (Obeid et al., 1993) in several mammalian cells types.

The dephosphorylation product of PA is diacylglycerol (DAG), which has emerged as an important signaling molecule for cell migration and survival. The best characterized pathways, which lead to the production of DAG are through the activation of either tyrosine-kinase or G-protein coupled receptors via coupling to specific phospholipase Cs (PLCs), although activity of LPPs could potentially also generate intracellular DAGs. There are several effector molecules are activated by DAG. The most well known is protein kinase Cs (PKCs). Depending on the PKC isoforms activated, the effects on the cell may be pro or anti-proliferative or apoptosis. PKCs are also known to increase invasive migration of cancer cells and this property may be mediated via a direct interaction with integrins (Griner and Kazanietz 2007).

## 1.12 Aim of the study

Lipids and lipid modifying enzymes have emerged as important regulators of cell migration and survival both in cases of development and diseases. However, the high complexity and interconversion of different bioactive lipid phosphates and their products makes it difficult to understand this process in its entirety. In this work I have used the power of *Drosophila* genetics to understand some aspects of the function of a lipid-modifying enzyme affecting cell migration and survival *in vivo*. In particular, I have explored the role of Wunens in the two different cell types: germ cells and somatic cells, and their function with respect to germ cell migration and survival.

In the first chapter, I have investigated the role of Wunens in promoting lipid uptake. Using genetically labeled germ cells and somatic cells obtained from embryos, and fluorescently labeled lipid phosphate species, I have shown that expression of Wun2 is important for uptake of extracellular PA.

The second chapter describes how I have ectopically expressed Wunens in segmental patterns in *Drosophila* embryos and used germ cell migration as readout of the existence of an extracellular lipid phosphate gradient. I have measured the range of action of the lipid phosphate signal to assess the effective range of endogenous Wunen expression on the germ cell migration path.

# Chapter 2
# Results - I

## 2. Validating the uptake of extracellular lipids by germ cells in *Drosophila*

Earlier studies have shown that overexpression of Wun2 in Hi5 insect cell lines can show a dephosphorylation dependent uptake of 7-nitro-2-1,3-benzoxadiazol-4-yl amino dodecanoyl labeled phosphatidic acid (NBD-PA). This demonstrated for the first time that similar to mammalian counterparts, *Drosophila* LPPs like Wun2 can dephosphorylate and internalize labeled extracellular lipid phosphates (Renault et al., 2004). My aim was to determine if wild type *Drosophila* germ cells with endogenous levels of Wunens also function in a similar manner. In particular, I wanted to test whether *Drosophila* germ cells and somatic cells show uptake of exogenously supplied lipids and whether such uptake can be correlated with the level of Wunens.

### 2.1. Wild type *Drosophila* germ cells show uptake of exogenously supplied NBD labeled phosphatidic acid (NBD-PA)

*Drosophila* germ cells were genetically labeled by *nosGal4* driving *UAS nuclear dsRed* expression exclusively in the nuclei of these cells. Single cell suspensions obtained by embryo homogenization were incubated with NBD labeled phospholipids presented either as liposomes (POPC:NBD-PA in 5:1 molar ratio) or complexed to BSA, with a final concentration of 20µM NBD-PA. After incubation, the cells were washed to remove excess lipids and imaged immediately using confocal microscopy. The genetic labeling technique helped to identify the germ cells with red fluorescent nuclei in a population of unlabeled somatic cells while the cytoplasm of germ cells remained free to detect the green fluorescence of the internalized NBD labeled lipid. Since the NBD label is on the acyl chain of the

lipid, the dephosphorylated product still has the NBD label, which can therefore be detected inside the cells.

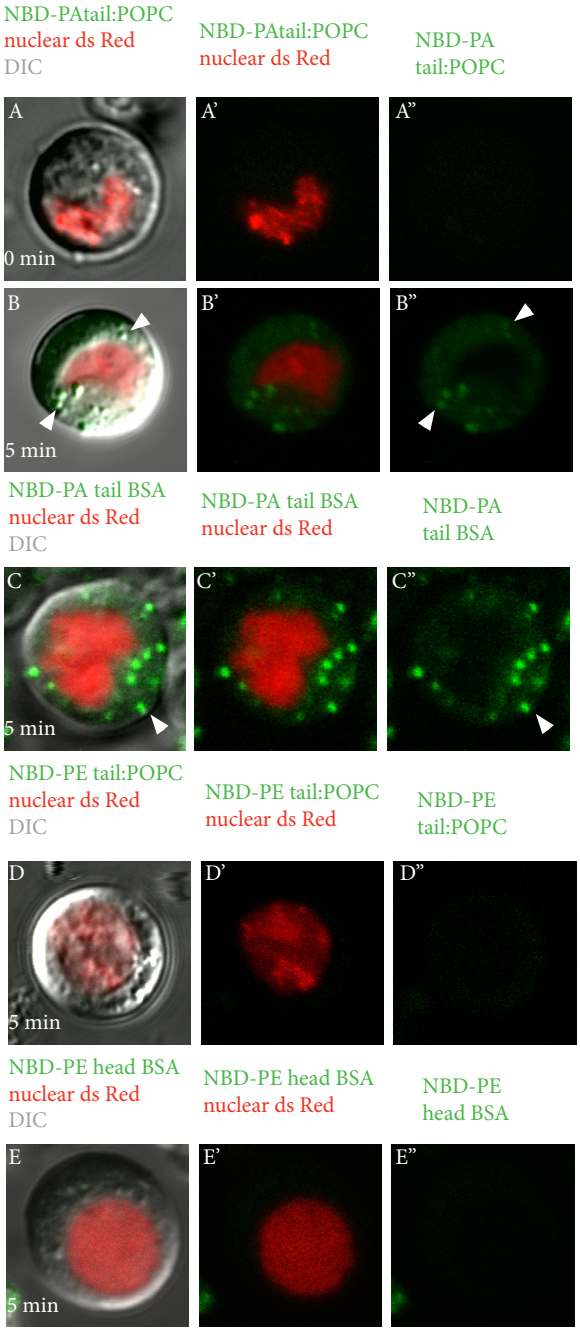



Figure 2.1: Wild type germ cells internalize NBD labeled phosphatidic acid (NBD-PA) but not NBD labeled phosphatidylethanolamine (NBD-PE). Wild type germ cell (A-E), that was not incubated with any lipid, shows no detectable fluorescence in the green channel (A –A''). On incubation with POPC:NBD-PA liposomes for 5 minutes germ cells show NBD fluorescence in the cytoplasm with some intense punctate structures (arrowheads B and B''). Germ cells presented with NBD-PA complexed to BSA show a similar uptake and distribution as NBD-PA presented in the form of liposomes (C – C'' arrowheads show intense fluorescent spots). Both tail labeled NBD-PE in liposomes (D – D'') and head labeled NBD-PE in BSA complex (E – E''), which are not LPP substrates are not internalized by wild type germ cells. All NBD labeled lipid substrates were used at a final concentration of 20µM.

Germ cells that were not incubated with any lipid, denoted as 0 minutes (Figure 2.1A – A'') showed no NBD fluorescence (Figure 2.1A''). Upon incubation of the single cell suspensions for 5 minutes with NBD labeled phosphatidic acid (NBD-PA) presented in POPC:NBD-PA liposomes, wild type germ cells accumulated NBD fluorescence in their cytoplasm (Fig 2.1B – B''). This fluorescence is distributed throughout the germ cell cytoplasm often with several bright puncta (Figure 2.1B'', arrowhead). In order to check if this uptake is dependent on the manner of substrate presentation, I also provided the cells with NBD-PA complexed to BSA. Under the same incubation conditions, germ cells show an uptake that is similar to that observed for NBD-PA provided in liposomes (Figure 2.1 C – C'')

To check whether the uptake is dependent on the ability of Wunens to dephosphorylate the lipid substrate, I provided two species of NBD labeled phosphatidyl ethanolamine (NBD-PE). The first has a label on the acyl chain whilst the second has the NBD label in the polar head group, which is a non-transferable lipid (a lipid that is not exchanged from the liposome to the plasma membrane). Membrane fraction studies have shown that LPPs do not to dephosphorylate phosphatidyl ethanolamine (Jamal et al., 1991). On incubation for 5 minutes with NBD-PE when presented either in liposomes or complexed to BSA, no green fluorescence was detected in the cytoplasm (Figure 2.1 D – E'''). This shows firstly, that the uptake of NBD labeled lipids is dependent on dephosphorylation and secondly, that the uptake is not due to bulk fusion of liposomes or uptake of BSA conjugated lipids.

## 2.2. Uptake of NBD-PA by *Drosophila* germ cells is dependent on the level of expression of Wun2

We next wanted to know if the uptake of NBD-PA is dependent on the expression of Wunens in germ cells. In order to answer this question I varied the level of expression of Wunens in the germ cells, incubated them with NBD-PA and checked for fluorescence in the cytoplasm of these cells. As shown previously, on incubation of wild type germ with POPC:NBD-PA liposomes for 5 minutes, green fluorescence is detected in the cytoplasm (Figure 2.2 A – A''). I used a *wun2* RNA null mutant to obtain DsRed labeled germ cells and on incubation of embryonic cells obtained from these embryos with NBD-PA, very little fluorescence was detected in the cytoplasm of these cells (Figure 2.2 B –B''). I then overexpressed Wun2 in the germ cells labeled with nuclear DsRed, and on incubation with NBD-PA, a large amount of green fluorescence was detected in the cytoplasm of these germ cells (Figure 2.2 C –C'').

To check whether the higher fluorescence detected in the germ cells on overexpression of Wun2 is because of an increased ability of these cells to bind, fuse or endocytose the liposomes, Wun2 overexpressing germ cells were incubated with NBD-PE tail (a non-LPP substrate). These germ cells showed no detectable fluorescence in their cytoplasm (Figure 2.2 D – D'').

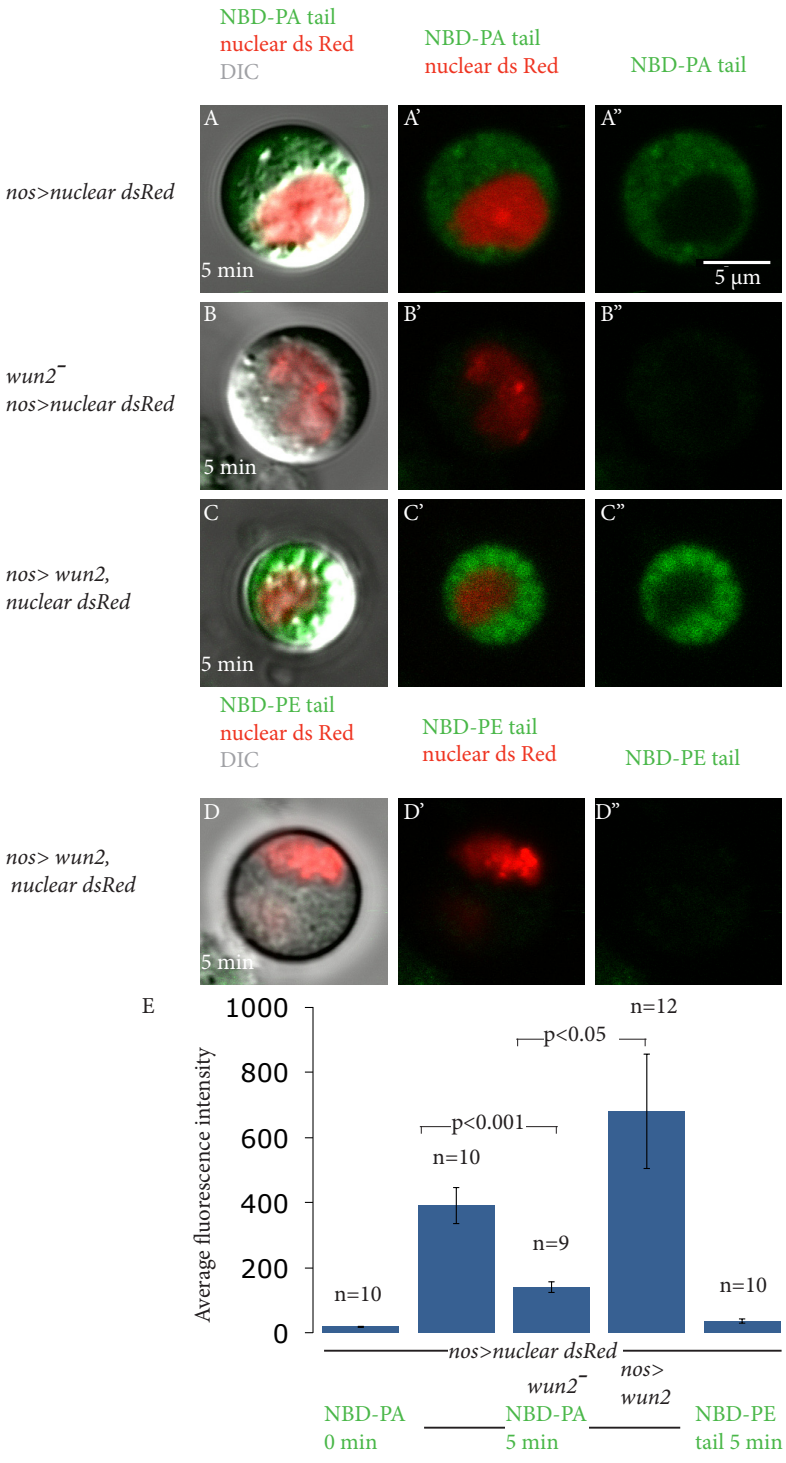



Figure 2.2: Uptake of NBD-PA by *Drosophila* germ cells is dependent on the level of expression of Wun2. Wild type germ cell (A – A'') on incubation with POPC:NBD-PA show fluorescence accumulation in the cytoplasm. Loss of Wun2 decreases this uptake of NBD-PA by germ cells (B – B''). Overexpression of Wun2 in germ cells increases the amount of NBD fluorescence in the cytoplasm (C – C''). This overexpression does not cause uptake of the non-LPP substrate NBD-PE (D – D''). The cytoplasmic fluorescence was quantified in wild type, Wun2 null and Wun2 overexpressing germ cells (E). All NBD labeled lipid substrates were used at a final concentration of 20µM.

To compare the relative levels of uptake between wild type, *wun2* null and *wun2* overexpressing germ cells, the fluorescent intensity of the cytoplasm in the green channel was quantified for at least 10 germ cells for each genotype or lipid (Figure 2.2 E). Basal level of fluorescence measured from cells with no incubation is similar to that of NBD-PE incubations supporting the fact that we do not observe uptake of NBD-PE. Overexpression of Wun2 significantly increases the uptake of PA by three fold as compared to Wun2 null germ cells. There is also a significant decrease in the

fluorescence intensity of Wun2 null germ cells as compared to wild type by almost two fold (Figure 2.2E).

The level of fluorescence observed for Wun2 null germ cells is slightly higher than the basal fluorescence level of wild type cells. We believe that this low level of fluorescence in Wun2 null germ cells is because these germ cells still carry a copy of Wun. Germ cells lacking both Wun and Wun2 die after stage 10 (Renault et al., 2010) hence it was not possible to use these germ cells for the lipid uptake analysis.

## 2.3. Lipid uptake by wild type *Drosophila* somatic cells correlates with the expression pattern of Wunens

According to the model of Wun/Wun2 mediated repulsion and survival, somatic and germ cell Wunens compete for the same extracellular lipid phosphate pool (Renault et al., 2004). We found that *in vivo* germ cell Wunens facilitate the uptake of extracellular lipid phosphates like NBD-PA. Our next question was, do somatic Wunens also function in a similar manner? To answer this I used a *wun2Gal4* driver to express nuclear *dsRed* in somatic cells endogenously expressing Wunens (Figure 2.3A). The cells labeled were the ones that express endogenous somatic Wunen because *wun2pGawB* is a p-element with Gal4 sitting in the promoter region of *wun2*. When the DsRed labeled cells were incubated with NBD-PA, green fluorescence accumulated in their cytoplasm. This supports the role of endogenous Wunens in the uptake of extracellular lipid phosphates in both germ cells and somatic cells (Figure 2.3 B – B'').

The next question was whether somatic cells that do not express Wunen, do not show lipid uptake. To answer this I used a *twistGal4* driver that is expressed in the mesoderm (Figure 2.3 C) because this is also a tissue where somatic *wunens* are not expressed (Renault et al., 2001). I used *UAS nuclear dsRed* to mark the nuclei of the mesoderm cells and obtained single cell suspension from these embryos. On incubation with NBD-PA, no fluorescence was detected for majority of the cells (78 % , n=54 cells) (Figure 2.3 D – D''). Unexpectedly however 22 % of the labeled cells showed large fluorescence accumulation in the cytoplasm (Figure 2.3 E – E'').

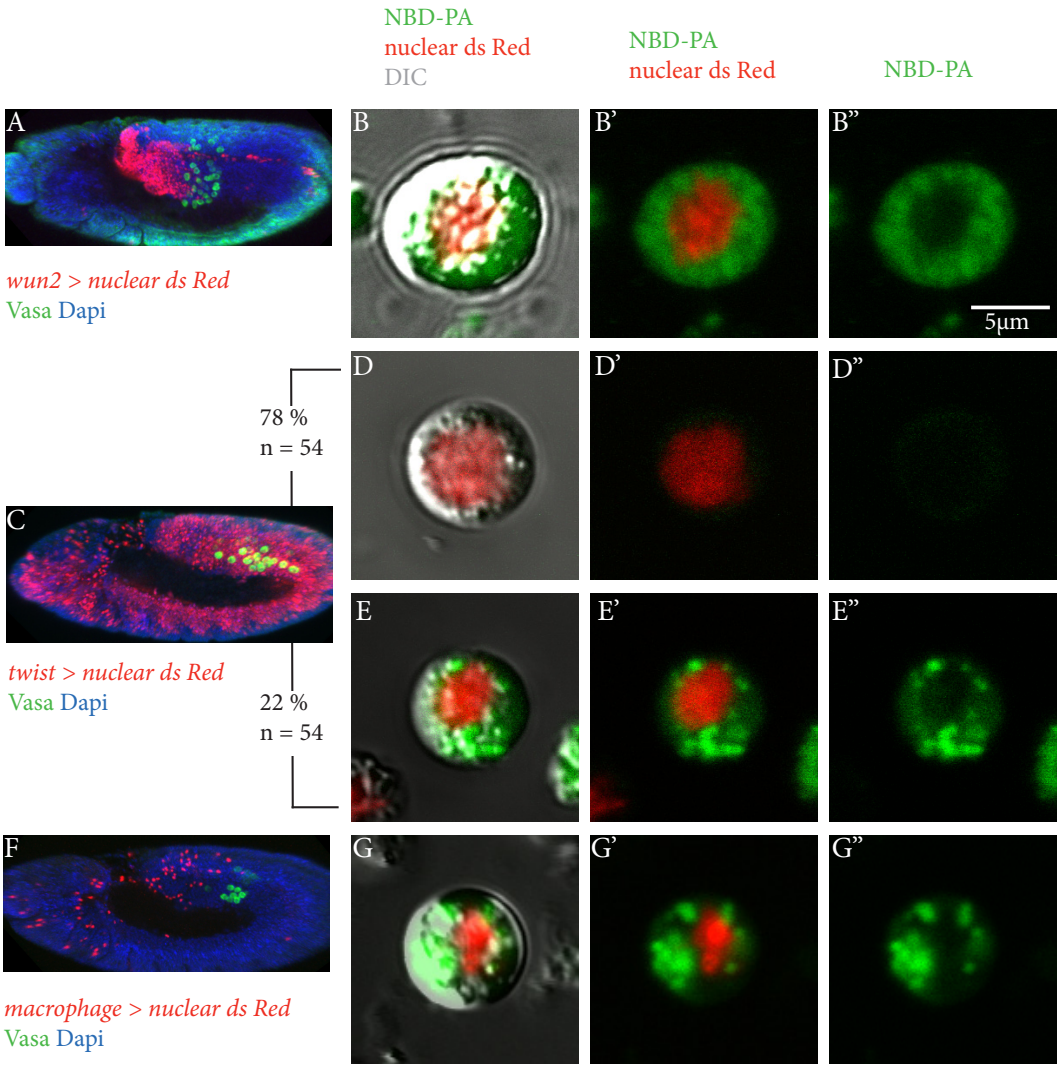



Figure 2.3: Lipid uptake by wild type *Drosophila* somatic cells correlates with the expression pattern of Wunens. Endogenous somatic *wun2* expressing cells marked by nuclear DsRed (A) show uptake of NBD-PA(B – B''). 78% of mesodermal cells marked by *twistGal4* (C), show no uptake (D – D'') while 22% of these cells show high NBD fluorescence (E – E''). The latter population of *twist* positive cells may be enriched in macrophages (F), which also show a very high NBD-PA uptake (G – G'').

One possibility is that these fluorescent cells are macrophages, which originate in the head mesoderm. Macrophages carry scavenger receptors that can bind to a large number of ligands including acetylated LDL (Abrams et al., 1992). I used a macrophage driver to label this cell population with nuclear DsRed (Figure 2.3 F) and used cells obtained from these embryos in similar assays to check if they take up NBD-PA. On incubation with NBD-PA, macrophages showed a large accumulation of NBD fluorescence in their cytoplasm, with the fluorescence

concentrated in large punctate structures (Figure 2.3 G –G''). This suggests that at least a fraction of this 22% of cells that internalize PA could be macrophages.

Thus we conclude that somatic cells that do not express Wun/Wun2 do not take up exogenously applied NBD-PA, with the exception of macrophages. However the latter are likely to internalize lipids via a *wun/wun2* independent mechanism as they do not express *wunens* (Renault et al., 2001).

### 2.4. Germ cells can take up LPA, S1P and Cer1P independent of Wunens

Lipid phosphate phosphatases are capable of dephosphorylating other substrates including lysophosphatidic acid (LPA) as well as sphingolipids like sphingosine 1 phosphate (S1P) and ceramide 1 phosphate (Cer1P) *in vitro*. However it is not known if LPPs can also use these substrates *in vivo*.

In order to check this I used fluorescently labeled lipids in complexed to BSA and incubated with wild type, *wun2* null and *wun2* overexpressing germ cells. After 5 minutes of incubation, NBD-S1P is detected in very bright puncta along with faint green fluorescence in the cytoplasm of wild type germ cells (Figure 2.4 A,A'). However, in contrast to what I observe with PA, in the absence of Wun2 NBD-S1P was readily internalized at similar levels to wild type. On over expression of Wun2 in the germ cells, they show bright fluorescent punctae similar to that observed for wild type, with no increase in the number of spots or intensity of green fluorescence.

Incubation of wild type germ cells with NBD labeled Cer1P showed accumulation of fluorescence around the nucleus (Figure 2.4 D,D' arrow) with parts of the cytoplasm devoid of fluorescence (Figure 2.4 D,E,F arrowheads). Similar to S1P, the level or pattern of fluorescence showed no difference either in the absence or overexpression of Wun2 (Figure 2.4 E,E').

Finally to test a third *in vitro* substrate of LPPs, I used Bodipy labeled LPA complexed to BSA and checked for lipid uptake. Wild type germ cells show NBD fluorescence in the cytoplasm with some intense puncta (Figure 2.4 G,G'' arrowheads). Similar to what was observed for S1P and Cer1P, changing the levels of Wun2 in the germ cells showed no difference in the level of fluorescence of

Bodipy LPA in the cytoplasm. (Figure 2.4 H – I, arrowheads show lipid accumulation in spots).

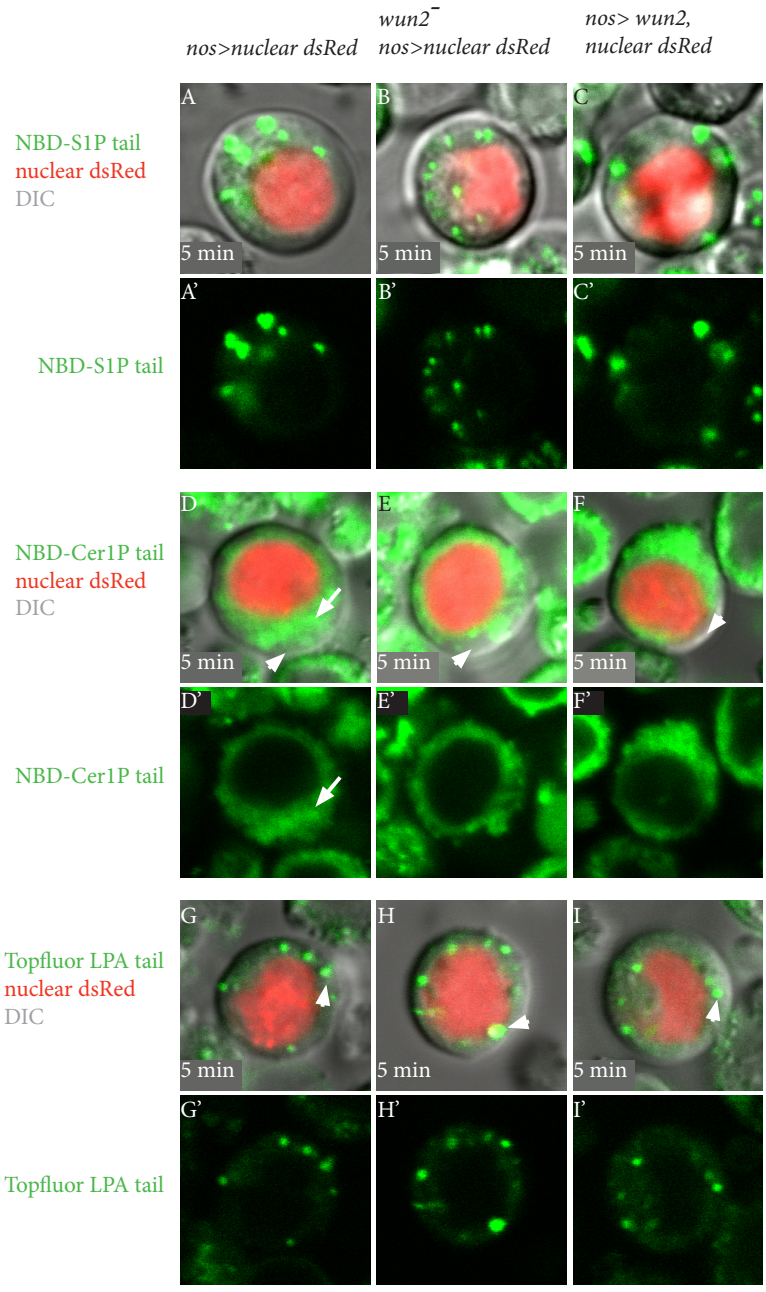



Figure 2.4: Germ cells can take up LPA, S1P and Cer1P independent of Wunens. Wild type germ cells show an uptake of different potential substrates of LPPs, including sphingosine 1 phosphate (A - C), ceramide 1 phosphate (D - F) and lysophosphatidic acid (G - I). NBD-S1P, fluorescence is detected in the form of bright punctate structures in the cytoplasm (A - C). Incubation with of NBD-Cer1P shows accumulation in the perinuclear region of the cytoplasm (D - F). Topfluor-LPA is present in the cytoplasm in addition to bright punctate structures (G - I). Loss of *wun2* (B,B',E,E',H,H') or overexpression of *wun2* (C,C',F,F',I,I') does result in any detectable difference in the level or pattern of accumulation of NBD fluorescence. All lipid substrates were used at a final concentration of 20µM except for NBD-S1P which was used at a concentration of 2 µM.

This shows that *Drosophila* germ cells are capable of taking up a variety of extracellular lipid phosphates like S1P, Cer1P and LPA but unlike PA, uptake of the other lipid phosphates is independent of Wun2 expression. The distinct distribution patterns of the different lipids in germ cells suggest that these lipids may be metabolized differently and hence end up in different intracellular compartments.

## 2.5. Germ cells are not highly endocytotic in nature and overexpression of Wunens do not increase the rate of endocytosis

I have shown that PA is internalized by germ cells in a Wun2 dependent manner, however it is possible that cells internalize lipids via other pathways. I wanted to make sure that the lipid internalization observed is contributed by dephosphorylation by Wunens alone and the lipids are not entering by another pathway for example by endocytosis. I therefore wanted to check whether a) wild type germ cells have a high rate of endocytosis and b) if overexpression of *wunens* affected endocytosis.

In order to answer to these questions, I used FM4-64 that binds to lipid membranes and intensely labels the plasma membrane in red fluorescence. FM4-64 fluoresce only when bound to lipid membranes while it does not fluoresce in aqueous solution. Through the appearance of intracellular puncta can this is used as a marker for endocytosis in a variety of eukaryotic cells including *Drosophila* (Tanaka and Nakamura, 2008; Harris and Tepass 2008). I made single cell suspensions from *Drosophila* embryos expressing *nos>moe GFP* to identify the germ cells in the green channel, suspended them in FM4-64 dye and imaged them immediately. Individual cells were imaged at single time points upon differing lengths of exposure to the dye.

In Figure 2.5 (A-F), the germ cells identified by their moeGFP signal (green) were imaged in the red channel (magenta) to visualize the FM4-64 which is seen exclusively on the plasma membrane at 0 minutes (Figure 2.5 A,A'). At 9 minutes of incubation, a small spot of labeled membrane is detected in the cytoplasm (Figure 2.5 B,B' arrow). Until 72 minutes of incubation in FM4-64, the labeled membrane spots do not increase in number (Figure 2.5 C,C' – E,E' arrows). Only after 106 minutes of incubation, are a few more FM4-64 positive spots detected (Figure 2.5 F,F' arrows). This shows that the endogenous rate endocytosis in wild type germ cells is very slow with an average of only one spot detected in the germ cells upto 72 minutes of incubation.

To compare the extent of membrane labeling in *Drosophila* germ cells to other cells, I looked at the non-GFP positive somatic cells in the same embryonic cell suspension. Several non-GFP cells showed membrane vesicles inside their

cytoplasm within 5 minutes of incubation in FM4-64. At 20 minutes of incubation extensive membrane staining is observed in non-GFP somatic cells. Beside the plasma membrane (Figure 2.5 G,G' arrow) and the nuclear membrane (Figure 2.5 G,G' yellow arrow), several small vesicular structures in the cytoplasm are stained.

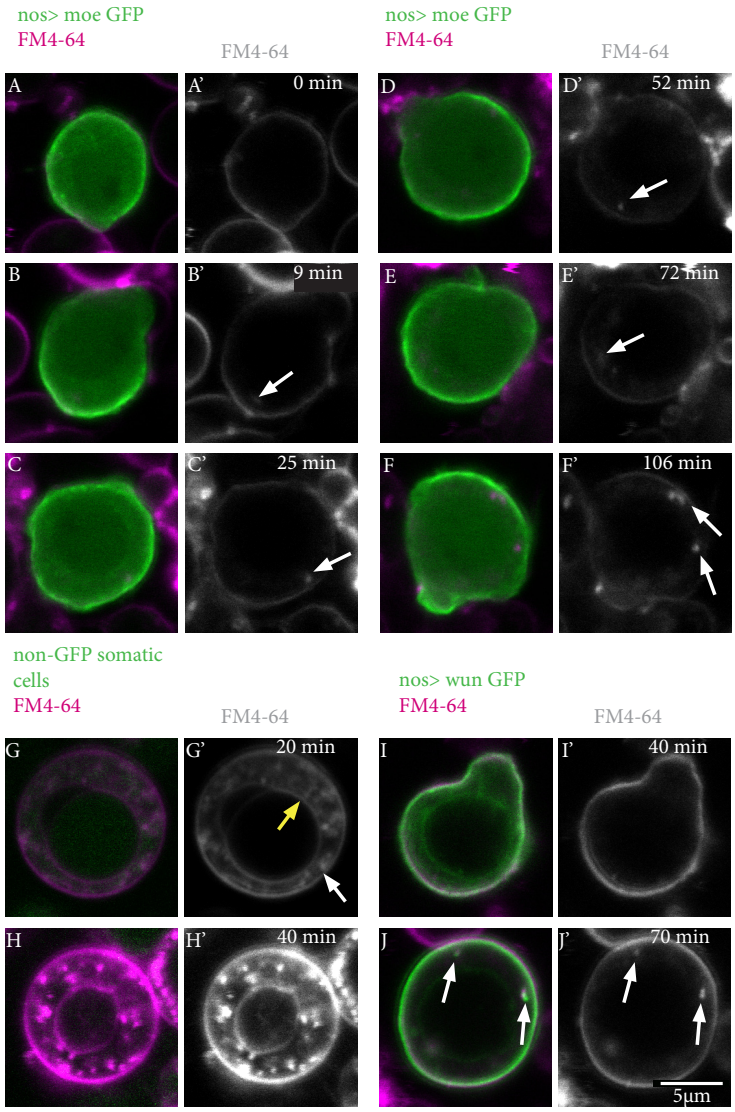



Figure 2.5: Germ cells are not highly endocytotic in nature and overexpression of Wunens do not increase the rate of endocytosis. Wild type germ cells (A – F) incubated with FM 4-64 show little cytoplasmic staining of intracellular vesicles over time. Somatic cells (G – H) show an intensive staining of the nuclear membrane (G'' yellow arrow) and other intracellular vesicles in addition to the plasma membrane (G'' white arrow). Overexpression of WunGFP does not increase the amount of intracellular vesicles as compared to wild type (I – J, arrows in J,J'' , for comparison see E' and I'). Note that different cells in the same experiment setting were used to capture the different time points.

At 40 minutes of incubation, a stronger labeling of the plasma membrane and intracellular membranes is observed in another somatic cell (Figure 2.5 H,H'). This shows that *Drosophila* germ cells and somatic cells have different rates of endocytosis.

We wanted to see if overexpression of Wun can cause an increase in endocytosis in the germ cells. For this I used *nos Gal4* to overexpress WunGFP in the germ cells of the embryos. I obtained single cell suspensions from these embryos and incubated them with FM4-64. After 40 minutes of incubation no

spots were detected in the FM4-64 channel while the plasma membrane labeling co-localized with the membrane WunGFP staining (Figure 2.5 I,I'). After 70 minutes of incubation a few small FM 4-64 spots were detected in the cytoplasm which were found to co-localize with Wun GFP positive spots (Figure 2.5 J,J' arrows).

This shows that over expression of Wun GFP in germ cells does not increase the number of FM4-64 positive spots in the cytoplasm as compared to wild type. Only after 70 minutes of incubation a few membrane vesicles are detected and this is comparable to our observation for wild type germ cells. Thus we conclude that wild type germ cells are not highly endocytotic in contrast to some of their somatic counterparts. Also overexpression of WunGFP in germ cells does not increase the rate of endocytosis. Therefore increase in uptake of NBD-PA by overexpression of Wun2 in the germ cells is not due to increase in the rate of endocytosis but rather it is a direct effect of Wun2 action on PA.

## 2.6. Presence of extracellular lipids does not increase endocytosis in wild type germ cells

I have shown that wild type germ cells have a very low level of endocytosis that does not increase on overexpression of Wun2. However it could be possible that incubation with lipids increases the endocytosis rate.

In order to investigate this, I co-incubated the different NBD labeled lipids used for my previous assays along with FM4-64 with wild type germ cells. I used MoeGFP labeled germ cells while the red channel was used to visualize the membrane staining. Because both MoeGFP and the NBD lipids fluoresce in the green channel, the internalization of the lipids was not visible. On incubation the plasma membrane of the germ cells were labeled with FM4-64 in all three cases (Figure 2.6 A'',B'' and C'' grey channel). However no increase in intracellular FM4-64 was detected in the presence of lipids (compare with Figure 2.5 B,C). This shows that PA, LPA and Cer1P uptake occurs independent of membrane internalization and that the presence of these lipids has no effect on the rate of endocytosis in germ cells.

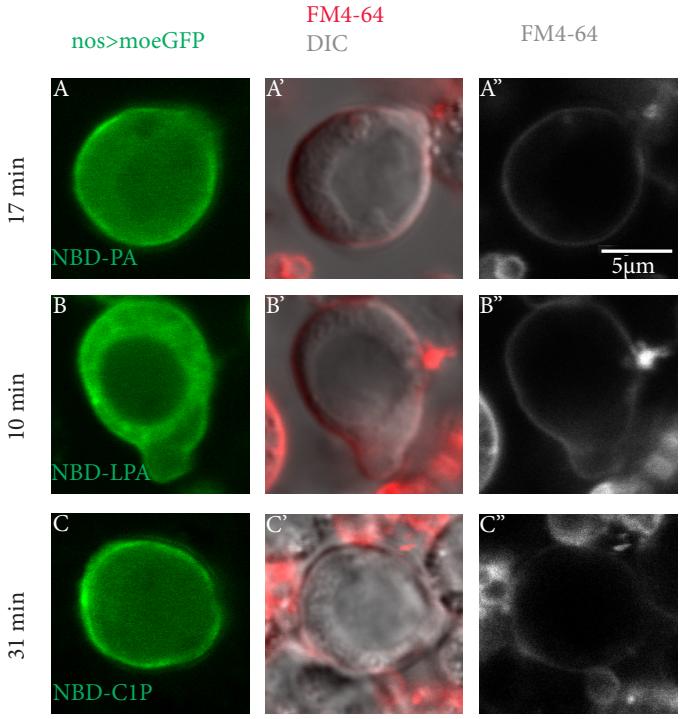



Figure 2.6: Presence of extracellular lipids does not increase endocytosis in wild type germ cells. FM4-64 (red, A',B',C' and grey, A'',B'',C'') remains confined to the plasma membrane of wild type germ cells marked by *nos>moe GFP* (green, A,B,C) when co-incubated with NBD-PA (green, A – A''), NBD-LPA (green, B – B'') and NBD-Cer1P (green, C – C''). Three different cells at three different time points show that the uptake of lipids inside the cytoplasm is not associated with membrane internalization. The NBD uptake in case of these germ cells is obscured by the signal from *nos>moe GFP* in the green channel although Bodipy LPA can still be detected in the form of a brighter perinuclear green in the background of green cytoplasm (B).

## 2.7. Intracellular punctae of internalised S1P does not colocalize with FM4-64 membrane labeling

Intense green punctae were observed in the cytoplasm for both PA and LPA but was more prominent for NBD-S1P. We particularly wanted to know if these bright NBD punctae colocalized with membrane labeling by FM4-64. Since MoeGFP germ cells obscured the NBD signal in the cytoplasm, I checked this by expressing nuclear *dsRed* in the germ cells. Although the nuclear DsRed would be detected in the same channel as the FM4-64, it would be spatially separate while the uptake of S1P could be visualized in the green channel.

Upon co-incubation with NBD-S1P and FM4-64, intense green puncta of NBD lipid was detected while the FM4-64 labeled the plasma membrane (Figure 2.7 A,A',A''). After 45 minutes of incubation the first FM4-64 positive spot was detected in the cytoplasm (Figure 2.7 B,B',B'' arrow). The latter did not co-localize with any of the intense green punctae of NBD-S1P (Figure 2.7 B,B' arrow heads). In fact the green spots of NBD-S1P are associated with distinct cytoplasmic structures visible in the DIC images (Figure 2.7 B' arrowheads). This association is observed

not only for S1P but also in case of NBD-PA and Bodipy-LPA (see Figure 2.1B,B'' arrowhead for NBD-PA and Figure 2.4 G-I arrowheads for Bodipy-LPA).

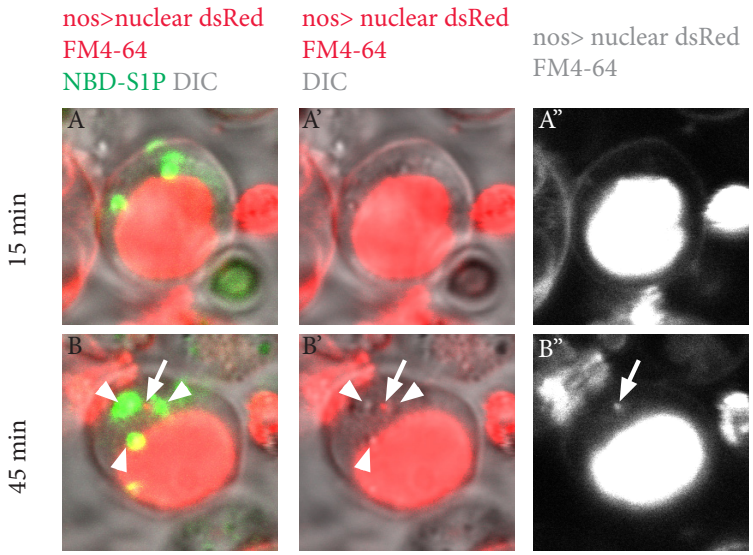



Figure 2.7: Intracellular punctae of internalised S1P does not colocalize with FM4-64 membrane labeling. Wild type germ cells marked with nuclear dsRed (red, A,A',B,B' and grey, A'',B'') shows an NBD-S1P (green) uptake in punctate structures in the cytoplasm (A,B arrowheads) which correlate with cytoplasmic structures in DIC (B' arrowheads) but does not co-localize with FM4-64 spot inside the cytoplasm (B,B',B''' arrow).

## 2.8. What is the identity of the punctate structures where the lipids accumulate?

In order to identify the structures where these labeled lipids ended up after being internalized, I used different live stains to visualize the subcellular organelles in live *Drosophila* germ cells.

I used ER-tracker Red (glibenclamide Bodipy) to stain the endoplasmic reticulum in germ cells marked with *nos>moe GFP*. Germ cells showed staining in the perinuclear region that did not reach the cell periphery (Figure 2.8 A). The round punctate structures that are obvious in the DIC images of the germ cells, do not correlate with the brightest spots of the ER staining (Figure 2.8 A',A''' arrow heads). But the labeling is strikingly similar to the uptake seen for NBD labeled Cer1P in germ cells (Figure 2.4 D,E,F). It is probable that after uptake Cer1P or its metabolite accumulates in the ER.

Next I used Bodipy labeled Ceramide as a marker to visualize the Golgi apparatus. Staining with Bodipy ceramide shows that the Golgi is like a network distributed throughout the cytoplasm. Although some regions are intensely stained (Figure 2.8 B'' arrows) these spots do not correlate with any specific structures in the DIC image (Figure 2.8 B'''). Therefore I conclude that that the bright puncta of PA, LPA and S1P are not part of the Golgi system.

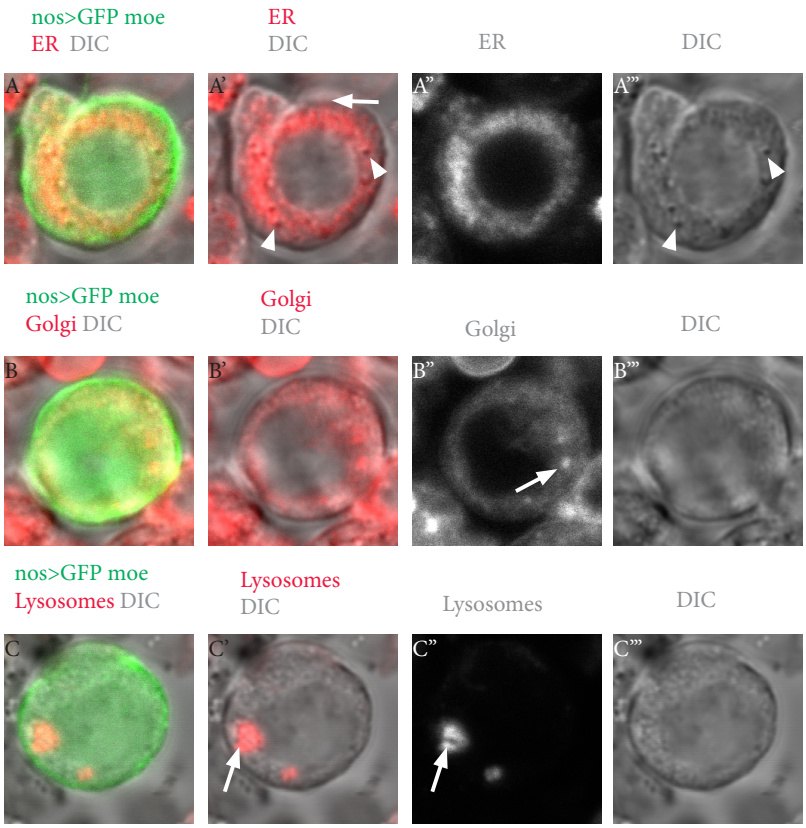



Figure 2.8: Intracellular compartments of wild type germ cells marked by *nos>moeGFP* (green, A,B,C) shows staining of the ER (red and grey, A – A''), Golgi (red and grey, B – B'') and Lysosomes (red and grey, C – C'') and their corresponding DIC images (A''',B''',C'''). Note that the intense fluorescence signal in each of these markers (arrows A',B'',C'') do not correlate with any prominent structure in the corresponding DIC images.

Next I wanted to check if these lipids were being degraded in the lysosomes and if the bright punctae corresponds to these organelles. For this I used the marker Lysotracker blue and stained wild type germ cells marked by *nos>moe GFP* (Figure 2.8 C). Lysotracker stained intensely in parts of the cytoplasm (Figure 2.8 C',C'' arrows) although these bright structures did not correlate to any specific structures in the corresponding DIC images (Figure 2.8 C'''). Since none of lipids

taken up by the germ cells showed an accumulation pattern similar to that of lysotracker, I conclude that the lipids were not entering the lysosomes to be degraded.

One other possibility was that the bright punctate structures were lipid droplets and the NBD labeled lipids were accumulating in the lipid droplets. To check this I stained wild type germ cells marked using *nos> moe GFP* with Nile red. Nile red is a lipophilic dye that penetrates the cell membrane and fluorescently labels phospholipids as well as neutral lipids like triacylglycerols (TAGs) and cholesterol esters (Greenspan et al., 1985). Thus Nile red marks the endomembrane system and especially sites of lipid accumulation, namely lipid droplets.

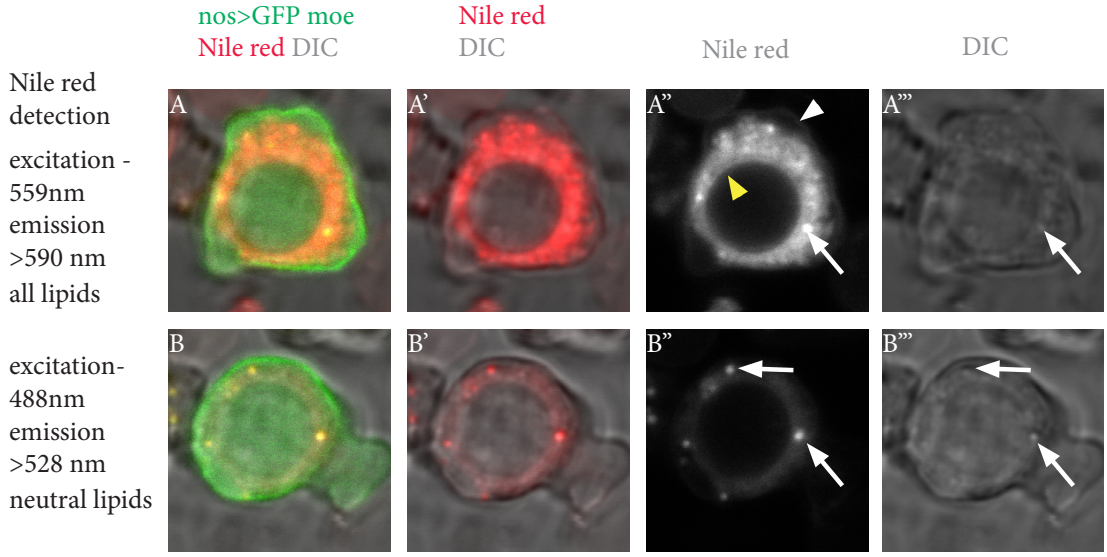



Figure 2.9: Nile red stain lipid droplets in wild type germ cells. Nile red staining of wild type germ cells marked by *nos>moeGFP* (green, A,B) shows the distribution of total lipids (red, A' and grey, A'') and neutral lipids in lipid droplets (red, B' and grey, B'') with their corresponding DIC images (A''',B'''). Note that lipids are detected on the plasma membrane (A'' white arrowhead), nuclear membrane (A'' yellow arrowhead) and lipid droplets (A'' arrow). Neutral lipid staining in lipid droplets appear as punctate structures which correspond with vesicle like structures in the DIC images (B'',B''' arrows).

Nile red fluoresces differently depending on the kind of lipid that it binds to (Greenspan et al., 1985). At an excitation wavelengths between 515 – 560 nm and emission wavelength > 590nm, all lipids are stained. When Nile red stained wild type germ cells were imaged with these settings, we detect the Nile red staining on

the plasma membrane, nuclear membrane and also what appears to be the ER or Golgi which surrounds the nucleus but does not reach the cell periphery (Figure 2.9 A - A''' white arrowhead showing plasma membrane and yellow arrowhead showing nuclear membrane). At an excitation wavelength between 450 – 500 nm and emission > 528 nm, Nile red fluorescence is detected only from neutral lipids like TAGs. When the same germ cell (Figure 2.9 A - A''') was imaged with these settings, the fluorescence was confined to bright punctate structures, which corresponds to the prominent structures in the DIC image of the cell (Figure 2.9 B – B''', arrows showing the bright spots of Nile red and the corresponding structure in the DIC image). Therefore the prominent structures in the DIC image are most likely lipid droplets and are the site of accumulation of NBD labeled PA, LPA and S1P in the germ cells.

## 2.9. NBD-S1P spots are lipid droplets in *Drosophila* germ cells

To confirm that the bright punctate structures seen in germ cells upon incubation with NBD-S1P corresponded to lipid droplets I co-stained these germ cells with Nile red. The germ cells were labeled with *nos>moe GFP* and therefore in the green channel I should detect both NBD-S1P with puncta as well as MoeGFP.

Wild type germ cells on incubation with NBD-S1P show uptake in the form of bright punctate structures (Figure 2.10 A,A',B,B') visible in spite of the fluorescence from the MoeGFP. Imaging the Nile red staining at an excitation of 488 nm and emission > 528 nm revealed only the lipid droplets that corresponded to the bright green puncta of internalized NBD-S1P (Figure 2.10 B-B''', arrow heads marking the bright NBD fluorescent puncta that correspond to the Nile red neutral lipid droplets and the prominent structures visible in DIC). Given that we observe similar punctate distribution in case of NBD-PA and Bodipy-LPA and their corresponding DIC structures, we believe that similar to S1P, PA and LPA also accumulate in lipid droplets in the germ cells.

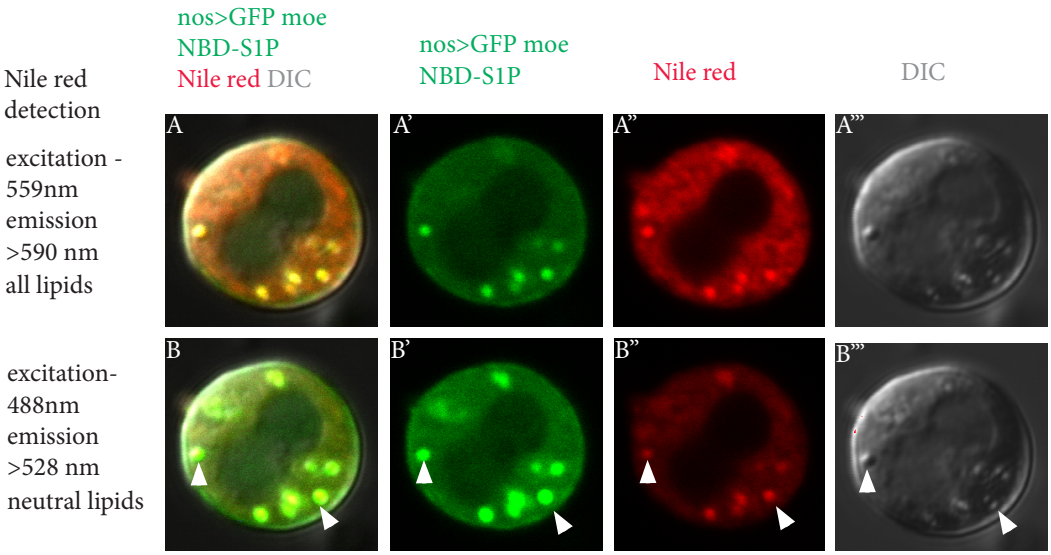



Figure 2.10: NBD-S1P spots are lipid droplets in *Drosophila* germ cells. Wild type germ cell marked by *nos>moeGFP* (green) incubated with NBD-S1P (green) and then stained with Nile red (red) shows punctate green uptake of S1P (A,A' and B,B' arrowheads) which co-localize with neutral red staining in lipid droplets (B'' arrowheads) and corresponds to prominent structures in the DIC images (B''' arrowheads). Nile red detects all lipids in A'' and only neutral lipids in lipid droplets in B''.

## 2.10. NBD-PA is not partitioned into the plasma membrane before dephosphorylation

I have shown that Wunens in *Drosophila* germ cells are involved in the dephosphorylation and uptake of extracellular NBD-PA in a substrate specific, non-endocytotic and fusion independent manner. We were interested to know whether NBD-PA is being partitioned into the plasma membrane before being dephosphorylated by Wunens. In earlier studies of lipid uptake with Chinese hamster fibroblasts, Pagano and Longmuir in 1984 had demonstrated that short chain non-dephosphorylatable analogs of fluorescently labeled PA, are transferred onto the plasma membrane where they become enriched but are not internalized. While Roberts and Morris in 2000 showed that this process of lipid internalization is dependent on the expression of mammalian LPPs on the plasma membrane and hypothesized that exogenous PA is incorporated in the outer leaflet of the plasma membrane before it is hydrolysed.

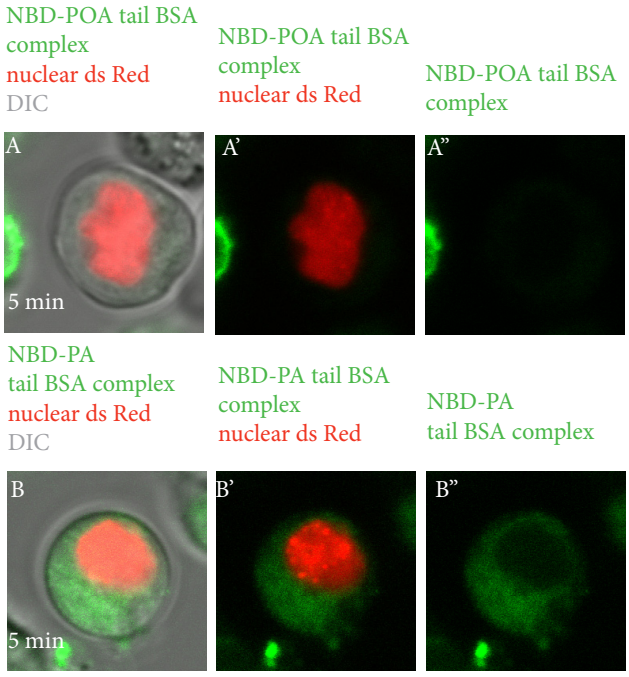



Figure 2.11: NBD-PA is not partitioned into the plasma membrane before dephosphorylation. Wild type germ cells marked using *nos> nuclear dsRed* on incubation with NBD labeled phosphonate (NBD-POA, green), a non-dephosphorylatable analog of PA, showing no uptake into germ cells and no accumulation on the plasma membrane (A – A'') while NBD labeled PA shows green fluorescence in the cytoplasm following the same duration of incubation (B – B'').

To determine whether Wunens dephosphorylate exogenous PA in a manner similar to its mammalian counterparts, I incubated wild type nuclear DsRed germ cells, with NBD labeled phosphonate analog (NBD-POA) complexed to BSA. This non-dephosphorylatable form of lipid was made as a control to the NBD-PA used for our previous lipid uptake experiments. After 5 minutes of incubation, no fluorescence was detected in the cytoplasm of the germ cells (Figure 2.11 A – A'') as compared to incubation with NBD-PA (Figure 2.11 B – B'') for the same duration. This shows that NBD-PA uptake observed in germ cells is due to dephosphorylation by Wunens and that non-dephosphorylatable forms cannot be internalized.

In contrast to previous observations by Pagano and Longmuir, we did not observe plasma membrane accumulation of NBD-POA. This indicates that spontaneous transfer of the extracellular PA to the outer plasma membrane of germ cells may not be essential for dephosphorylation by Wunens. However it is still possible that only a small fraction of NBD-POA is transferred to the plasma membrane, which is not detectable by laser scanning.

# Chapter 3
# Discussion - I

Lipid phosphates can be internalized by cells in different ways one of which is dephosphorylation by LPPs. LPPs are integral membrane enzymes that localize to the plasma membrane as well as to the intracellular membrane like the ER and Golgi. *In vitro* studies using membrane fractions of mammalian and *Drosophila* LPPs have shown that these enzymes are capable of dephosphorylating several phospholipids including PA, LPA, S1P and Cer1P. Overexpression of Wunens in insect cell lines caused uptake of extracellular fluorescently labeled PA (Renault et al., 2004).

Expression of Wunens in germ cells is essential for their survival (Renault et al., 2010). Our aim was to validate the role of Wunens in the uptake of extracellular lipid phosphates in their endogenous cell and level of expression. To achieve this, we genetically labeled the germ cells and presented embryo suspensions with fluorescent analogs of different lipid phosphates. To prove that the internalization is dependent on the Wun2, I manipulated the levels of Wun2 in the germ cells and demonstrated that germ cells internalize PA in a Wun dependent but endocytosis and fusion independent manner. Further using a dephosphorylatable analog of PA, I have highlighted the mechanism of internalization of PA by Wunens.

### 3.1. Germ cells and somatic cell Wunens show uptake of exogenous PA and supports the competition model of Wunen action

I have shown that germ cells internalize NBD-PA in a Wun2 dependent manner. Using a non-transferable lipid (head labeled NBD-PE), which cannot be exchanged from the liposomes/BSA to the plasma membrane of the cells, I showed that internalization of PA was due to its preferential transfer from the extracellular liposomes or BSA complex to the cell membrane and not due to bulk fusion of

liposomes. By using a non-LPP substrate (tail labeled NBD-PE), I demonstrated that it was Wunen mediated dephosphorylation of PA that is responsible for PA internalization (Figure 2.1, 2.2). Additionally, I showed that the majority of mesoderm cells, that have no endogenous Wunen expression show negligible uptake of NBD-PA as compared to somatic cells that do express Wun2 endogenously. This indicates that Wun2 expression in somatic cells functions in a manner similar to that in germ cells.

However a fraction of mesodermal cells show a robust accumulation of NBD-PA (Figure 2.3 E). I reasoned that Wunens or another LPPs could contribute to this fraction of mesoderm cells. *wun* and *wun2* are absent from the mesoderm by *in-situ* hybridization (Renault et al., 2001), however mesectodermal cells at stage 10 do express *wun2* (my unpublished observation). In addition, it is also possible that a subset of these cells are macrophages.

Macrophages are highly endocytic and also express scavenger receptors that have the capacity to bind and internalize acetylated LDL (Abrams 1992). It is therefore possible that macrophages internalize these POPC:NBD-PA liposomes via a non-dephosphorylation dependent but endocytic dependent pathway, which could involve scavenger receptors. The *twist* driver used to mark the mesoderm cells in the uptake assays labels the mesectodermal cells (my unpublished observation) and also the macrophages (compare expression pattern of DsRed in Figure 2.3 C and F). I believe that, the mesodermal cells that show internalization of NBD-PA could be a result of the combination of mesectodermal cells expressing Wun2 and the macrophages expressing scavenger receptors.

In order to check whether macrophages internalize lipids via a non-dephosphorylation dependent pathway, one could do similar lipid uptake assays using the non-dephosphorylatable analog of PA (NBD-POA), which is not internalized by germ cells. I expect macrophages to internalize NBD-POA as was observed in case of NBD-PA. Additionally one can check the rate of endocytosis in macrophages using membrane markers like FM4-64 used in this study. This would provide more evidence that the PA accumulation in macrophages is due to endocytosis of liposomes and not due to dephosphorylation.

According to the currently existing hypothesis of Wunen action on germ cell migration, germ cell and somatic cell Wunens compete for the same extracellular

lipid phosphate pool while internalization of this lipid phosphate is essential for germ cell survival (Renault et al., 2004). My results demonstrate that Wunen expressing somatic as well as germ cells internalize extracellular PA, while majority of the non-Wunen expressing cells (mesodermal) do not show such internalization. This supports the competition model of Wunens.

It is known that overexpression of Wunens in the mesoderm causes germ cell death (Starz-Gaiano et al., 2001). Whilst not tested in my thesis, I would expect that Wunen expression alone would be sufficient to transform non-uptaking cells into cells that internalize extracellular lipid phosphates. This would also provide an explanation for the germ cell death seen in overexpression of Wunens in the mesoderm (Starz-Gaiano et al., 2001) presumably because lipid phosphate internalization by mesodermal cells on Wunen overexpression out competes the internalization by wild type germ cells.

### 3.2. Neither Wunen dependent nor independent lipid internalization is associated with endocytosis

In order to rule out endocytosis as a means of lipid uptake in germ cells I used FM4-64 dye that is widely used as a plasma membrane and endocytosis marker in various cell types including *Drosophila* oocytes (Tanaka and Nakamura, 2008) and neuroectoderm cells (Harris and Tepass, 2008). I demonstrated that germ cells are not highly endocytic and the rate of endocytosis is not increased upon overexpression of Wun. This suggests that the increased internalization of PA observed on Wun2 overexpression in germ cells is due to increase in dephosphorylation and uptake and not due to increased membrane internalization (Figure 2.5). I further demonstrated using co-incubation studies that presence of extracellular lipid phosphates does not increase membrane internalization in wild type germ cells (Figure 2.6). This shows that although internalization of S1P, LPA and Cer1P is not dependent on the expression of Wunens in germ cells, they do not enter by binding to the plasma membrane and subsequent membrane internalization. Using co-incubation of NBD-S1P and FM4-64 I could also show that the rate membrane internalization is much slower than that of the lipid internalization.

There could be three possible interpretations to account for the observation that S1P, LPA and Cer1P are internalized by germ cells independent of Wun2 expression. Firstly, PA could be the only *in vivo* substrate for Wun2.

Secondly, the acyl chain length of the lipids might affect the ability of the substrate to be dephosphorylated. The chain lengths of free fatty acids present in *Drosophila* vary between carbon chain lengths $C_{12}$ and $C_{18}$ (Hammad et al., 2011) and the most abundant sphingosine and dihydrosphingosine long chain bases are $C_{14}$ and $C_{16}$ (Fyrst et al., 2004). While the NBD-$C_{12}$ PA and Bodipy-$C_{12}$ LPA we used, resembled the endogenous varieties found in *Drosophila*, we were limited by the commercial availability of the other labeled lipids. We tested NBD-$C_{18}$ S1P, and NBD-$C_6$ Cer1P in this study as these were the ones available. Using chain lengths that more closely resemble the endogenous lipids found in *Drosophila* in similar uptake experiments might reveal some differences in the internalization of Cer1P and S1P.

Thirdly, these lipids might be internalized by a dephosphorylation independent mechanism. To check if dephosphorylation is essential for internalization, one could use non-dephosphorylatable analogs of these lipids and check for internalization by germ cells. If similar internalization is observed to that of the phosphate varieties, it would suggest that these lipids are internalized via a pathway that is not dependent on dephosphorylation. S1P and LPA are known to bind to G-protein coupled receptors and in the presence of high substrate concentrations, these receptors are internalized (Gräler and Goetzl, 2004; Matloubian et al., 2004). However, to date G-protein coupled receptors for LPA and S1P have not been found in the fly genome. I have also shown that the distribution of internalized LPA, Cer1P and S1P does not match that of endocytic vesicles as judged by FM4-64 labeling which makes receptor binding and subsequent endocytosis less likely. If no internalization is observed, it would suggest the presence of enzymes other than Wunens in germ cells with the capacity to dephosphorylate these lipid phosphates. My findings support that PA is the most likely endogenous substrate for Wunens.

### 3.3. A non-dephosphorylatable analog of PA is not enriched on the plasma membrane

Using a NBD labeled non-dephosphorylatable analog of PA, I demonstrated that dephosphorylation is essential for the internalization of NBD-PA by germ cells (Figure 2.11). What is the mechanism of internalization of NBD-PA?

Firstly, PA could be transferred spontaneously to the outer layer of the plasma membrane independent of Wunens and subsequently dephosphorylated by Wunens and internalized. Secondly, dephophorylation by Wunens could be a pre-requisite for the transfer and internalization of PA by germ cells. Alternatively, an equilibrium might exist in the amount of PA in the plasma membrane and the extracellular space. A small percent of NBD-PA may be transferred spontaneously but dephosphorylation by Wunens tips this equilibrium. Subsequently more dephosphorylation by Wunens results in greater internalization of PA.

Experiments using a short chain phosphonate analog of PA (NBD-$C_6$POA) demonstrated that the lipid phosphonate analog accumulates on the plasma membrane (Pagano and Longmuir 1984). This suggested that these lipids undergo spontaneous transfer and subsequent dephosphorylation. Experiments using reconstitution of purified LPP1 in liposomes of defined lipid composition suggested that LPPs are preferentially active against substrates localized to the same membrane (Roberts and Morris 2000). However short chain lipid analogs are not representative of real lipids and could be spontaneously transferred, I therefore used a long chain NBD-$C_{12}$POA (non-dephosphorylatable analog) similar in structure to the PA used in my uptake studies. I did not observe any enrichment on the plasma membrane of germ cells, which implies that transfer of PA on the plasma membrane of germ cells is not a pre-requisite for dephosphorylation and internalization by Wunens. Although it is still possible that only a small fraction of NBD-POA is spontaneously transferred to the plasma membrane of the germ cells that is not detected by scanning.

The dephoshorylation product of PA is diacylglycerol (DAG). While PA cannot be flipped from the outer to the inner plasma membrane, DAG can be rapidly flipped inside. DAG could be then internalized by facilitated transfer from the plasma membrane to intracellular membranes either by specific or non-

specific lipid transfer proteins (LTPs) or by lateral diffusion to cytoplasmic membranes that are in close apposition to the plasma membrane.

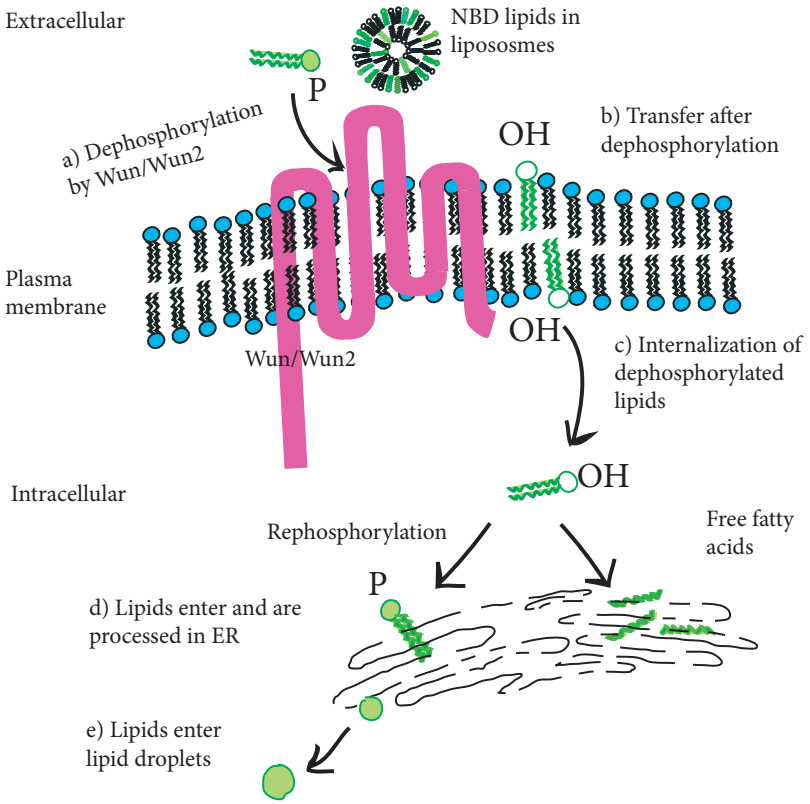



Figure 3.1: Model of lipid entry in *Drosophila* germ cells. (a – e) shows the sequence of events that occurs when germ cells are presented with lipids in the form of liposomes or complexed to BSA. Wunens on the surface dephosphorylates PA to DAG, which is transferred to the plasma membrane and spontaneously flipped inside. This internalized DAG is further processed in the ER and finally enters lipid droplets. Contrary to the earlier belief from studies in mammalian cells, I think dephosphorylation precedes transfer of the lipid in case of *Drosophila* Wunens in germ cells (Image adapted from Pagano and Longmuir, 1984 and Roberts and Morris, 2000).

The internalized DAG could then either be rephosphorylated to PA by diacylglycerol kinase (DGKs) or by itself act as a regulatory molecule. I have shown that exogenous PA internalized by Wunens, accumulate in punctate structures that I believe are lipid droplets. DAGs that are present in the intracellular membranes could be further broken down to free fatty acids or acylated to triacylglycerols, which could then enter the lipid droplets (Figure 3.1).

In addition to demonstrating the role of Wunens in dephosphorylation and internalization of PA, this study also provides evidence that the Wunens are functional at the plasma membrane of germ cells. This supports previous finding

that a functional Wun2 enzyme is required at the cell surface for germ cell survival in embryos (Ratna Tripathy, PhD thesis).

### 3.4. What is the functional consequence of PA internalization by Wunens?

I have shown that internalized S1P enters the lipid droplets (Figure 2.10). Based on the similarity of the punctate structures I believe that like S1P, internalized PA and LPA also accumulate in the lipid droplets.

Is there a link between lipid droplets and cell migration? In a recent study, accumulation of lipid droplets was linked to migration ability of cancer cells. A decrease in exogenous lipids caused a visible reduction in the lipid droplets and an 85% reduction in cell migration (Antalis et al., 2011). Chemotaxis is a highly energy intensive process and similar to cancer cells, *Drosophila* germ cells could also be utilizing lipid droplets as energy reserves to migrate. To gather more evidence to support this hypothesis one could check the role of enzymes that are important for the formation or break down of lipid droplets in germ cell migration and survival.

Dephosphorylation and internalization of exogenous PA by LPPs and their subsequent rephosphorylation may not be the only mechanism by which cells acquire PA. Several different enzymes that modify existing intracellular lipids could generate intracellular PA. Firstly, phospholipase D (PLD) can hydrolyze phosphatidylcholine (PC) to generate PA and free choline. Secondly, DGKs can phosphorylate DAG to produce PA and thirdly, by the action of lysophospholipid acyl transferases (LPLATs) which produces PA by acylation of LPA. While there is no evidence so far to suggest that either PLD or DGK is involved in germ cell migration, *Drosophila* LPLATs have been shown to be involved in germ cell migration and spermatid individualization with some evidence of genetic interaction between Wun2 and LPLATs (Steinhauer et al., 2009). This evidence suggests that either PA itself or its dephosphorylated product DAG could be the lipid that is important in the context of germ cell migration.

To gather more evidence for PA in *Drosophila* germ cell migration and survival, one can express a green fluorescent protein tagged PA sensor specifically in the germ cells and check for its localization in wild type and *wun wun2 M-Z-*

germ cells. A difference in the localization of the GFP signal in germ cells in the two genetic backgrounds would provide a direct link between germ cell Wunens and PA. The uptake experiments using germ cells shown in this thesis could be easily modified to check for effects on actin localization upon the addition of exogenous PA. Such experiments could also be extended to test whether PA can act as a chemoattractant by allowing germ cells to migrate on ECM coated coverslips in the presence of a point source of PA.

If one observes a positive effect of exogenous PA on germ cell migration, one could then investigate intracellular downstream targets of PA and DAG. For example, protein kinase C (PKC) is a widely studied DAG regulated enzyme that is involved in enhanced migration and invasion of cancer cells (Ng et al., 1999). In mammalian cells PA is known to activate mTOR (Fang et al., 2001) that in turn affects cell survival. PA can affect cytoskeletal structure (Exton 1997), and chemotaxis in human neutrophils via a S6K dependent pathway (Frondorf et al., 2010). Additionally PA is also known to stimulate several enzymes *in vitro* like PKC (Stasek Jr et al., 1993), phospholipase C γ (PLC γ), monoacyl glycerol acyltransferase (Brindley et al., 2002), sphingosine kinase (SK) and phosphatidylinositol 4-phosphate 5' kinase (Delon et al., 2004; Jenkins et al., 1994) as well as PLD itself, which can in turn produce more PA (Mazie et al., 2006) that affects motility in mammalian cells.

A good deal of evidence already exists to suggest that extracellular and intracellular PA is involved in the migration of different cell types. However, so far no link has been shown between dephosphorylation and internalization of PA by the action of LPPs like Wunens and the activation of downstream effectors of PA or DAG. I have demonstrated that expression of Wunens is important for the internalization of extracellular PA in an endocytosis independent and dephosphorylation dependent manner. This study highlights the possibility to utilize *Drosophila* germ cells in order to explore this link between lipid internalization by LPPs and to study the effect of the internalized lipid on migration and survival *in vivo.*

# Chapter 4
# Results – II

## 4. Investigating the existence and range of a lipid signal *in vivo*

Expression of somatic Wunens is essential for guiding germ cell migration and survival. In the absence of somatic Wunens germ cells are lost while overexpression of Wunens, causes germ cell repulsion and death. According to the model proposed by Renault et al., 2004, somatic Wunens dephosphorylate and destroy an extracellular lipid phosphate pool. This sets up gradients of extracellular lipids that the germ cells detect and would migrate towards.

Our aim was to determine whether Wunen mediated repulsion is a contact dependent or independent mechanism and to find evidence to support the existence of this extracellular lipid phosphate gradient and finally to measure the distance of action of this signal. Unlike proteins, endogenous lipids cannot be tagged and lipid stains specific for extracellular lipids do not exist. Thus, I used *Drosophila* germ cells as a read out of the extracellular lipid phosphate signal that is set up by spatially restricted expression of somatic Wunens.

### 4.1. Modeling contact dependent and independent mechanism of action

To test if somatic Wunen expression produces an extracellular lipid gradient I wanted to express somatic Wunens in parallel stripes in an otherwise *wun wun2 Z-* background and observe the response of the germ cells.

Case1: Contact dependent mechanism

If there is no extracellular lipid phosphate gradient and the germ cells need to be very close to or actually touch a somatic Wunen expressing cell to be repelled away then the germ cells at any given time point would be randomly distributed

within the interstripe: some germ cells could be present close to the stripe and others further away but overall there would be no particular pattern. (Figure 4.1A).

Case 2: Contact independent mechanism

On the other hand, if somatic Wunen expression translates into an extracellular lipid gradient, which the germ cells could detect, then they could position themselves midway in the interstripe. (Figure 4.1B).

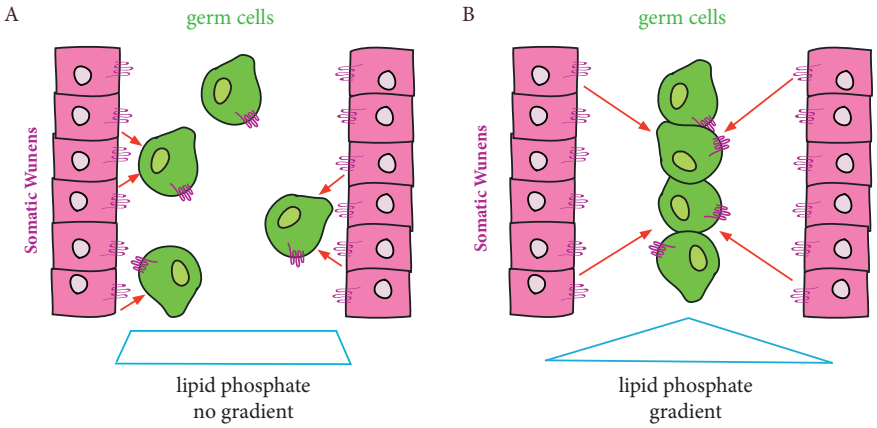



Figure 4.1: Modeling contact dependent and contact independent mechanism of Wunen repulsion. Models depicting a contact dependent (A) and a contact independent (B) mechanism of Wunen mediated repulsion. In the first case, no lipid phosphate gradient exists and some germ cells are found close to the somatic Wunen stripes. In the second scenario, a lipid phosphate gradient set up by the parallel somatic Wunen stripes is detected by germ cells and they position themselves in the middle of the interstripe.

## 4.2. Ectoderm expression of Wunens in a *wunwun2* zygotic null background does not kill germ cells and removes germ cells from the ectoderm

Ectopic expression of Wunens in the mesoderm, trachea or the gut in a wild type embryo causes germ cell death (Starz-Gaiano et al., 2001). I required a means of expressing these enzymes in domains that firstly, do not kill germ cells and secondly, offers a means of imaging the migration pattern of the germ cells. I believed that the ectoderm might be one of the best places to express Wunens because it is far from where the germ cells would normally be and therefore should have the least effect on germ cell survival. I tested several ectoderm drivers

and found two that fulfilled both our criteria. I overexpressed Wun2 in the ectoderm of wild type embryos using *engrailed Gal4* (*enGal4*) and *hairy Gal4* (*hGal4*) drivers. *engrailed* is a segment polarity gene that is expressed in 14 stripes in the ectoderm (Figure 4.2A,B) while *hairy* is a pair rule gene that is expressed in 7 broader stripes (Figure 4.2A). The position of the Gal4 drivers used in this study has been shown in a stage 11 *Drosophila* embryo cartoon (Figure 4.2A).

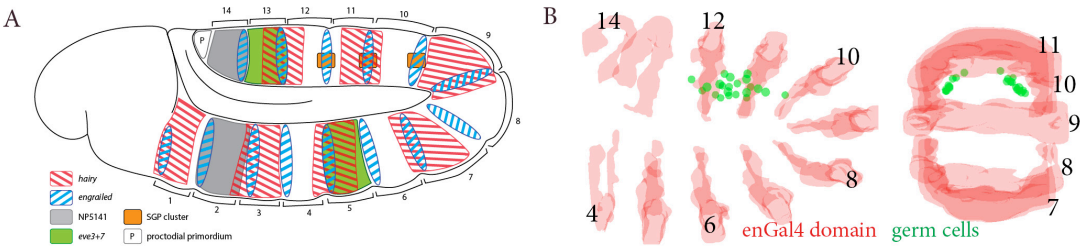



Figure 4.2: (A) A cartoon showing a stage 11 *Drosophila* embryo depicting the position of the ectoderm drivers and the somatic gonadal precursors. (B) A lateral and a posterior view of a segmented 3 D reconstructed wild type stage 11 embryo showing the position of germ cells (green) and the *en* stripes (red). Note that the stripes are restricted to the ectoderm (the outermost layer of the embryo) while the germ cells are present in the mesoderm (just below the ectoderm). The numbers denote the parasegment number that are marked by the *en* stripes.

In a wild type embryo, by stage 13 germ cells reach the two bilateral gonads (Figure 4.3A). In a *wun wun2 Z-* embryo, the germ cells are lost in the entire posterior of the embryo with some germ cells found in the ectoderm (Figure 4.3B, arrows showing germ cells in the ectoderm). On expression of Wun2 in the ectoderm using *enGal4* (Figure 4.3C) or *hGal4* (Figure 4.3D), I observe two things: Firstly, the total number of germ cells is not highly reduced (Average number of germ cells written for each genotype, Figure 4.3 A - D). Secondly, the number of germ cells in the ectoderm quantified from three dimensional reconstructions of fluorescently stained embryos in the same backgrounds (Figure 4.3E), showed that no germ cells were detected at the ectoderm when Wunens are expressed in the ectoderm using either *enGal4* or *hGal4* drivers (Figure 4.3C - D, the position of the ectoderm drivers are shown using dotted lines).

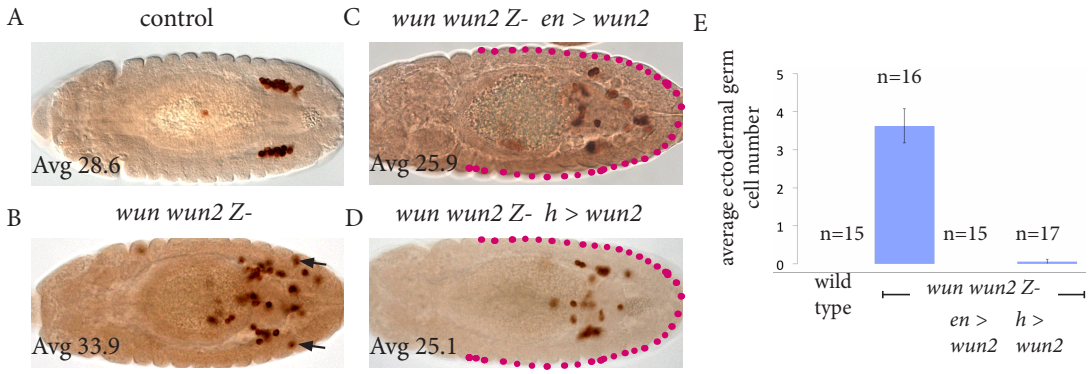



Figure 4.3: Ectoderm expression of Wunens in a *wunwun2* zygotic null background does not kill germ cells and removes germ cells from the ectoderm. Overexpression of Wun2 in the ectoderm using *engrailed* (*en*) (C) and *hairy* (*h*) (D) in a *wunwun2 Z-* background does not highly reduce germ cell number as compared to wild type embryo (A). Germ cells seen in the ectoderm in *wunwun2 Z-* embryos (arrows B) are absent when Wun2 is expressed in the ectoderm using either *h* or *en* drivers (C,D). Graph depicting the number of germ cells in ectoderm quantified from fluorescently stained 3-D reconstructed embryos (E).

## 4.3. Germ cells avoid Wun stripes and line up in the interstripe

To find out whether Wunen mediated repulsion is a contact dependent or independent process, I observed how the germ cells migrate when Wun2 is expressed in the ectoderm in stripes. I fixed and stained embryos that were a) wild type with control stripes, b) *wun wun2 Z-* with control stripes and c) *wun wun2 Z-* embryos with *en* or *h* stripes expressing Wun2.

In wild type stage 10 embryos, the germ cells are evenly spaced from each other on the basal side of the posterior midgut (endoderm) closest to the overlying mesoderm (Figure 4.4A). In *wun wun2 Z-* embryos the germ cells are not confined and scatter over the entire basal surface of the posterior midgut with some germ cells present at the *en* stripe (arrowheads Figure 4.4B). In *wun wun2 Z-* embryos with *en* driven Wun2, the germ cells already begin to align in the interstripe in a segmental fashion (Figure 4.4C).

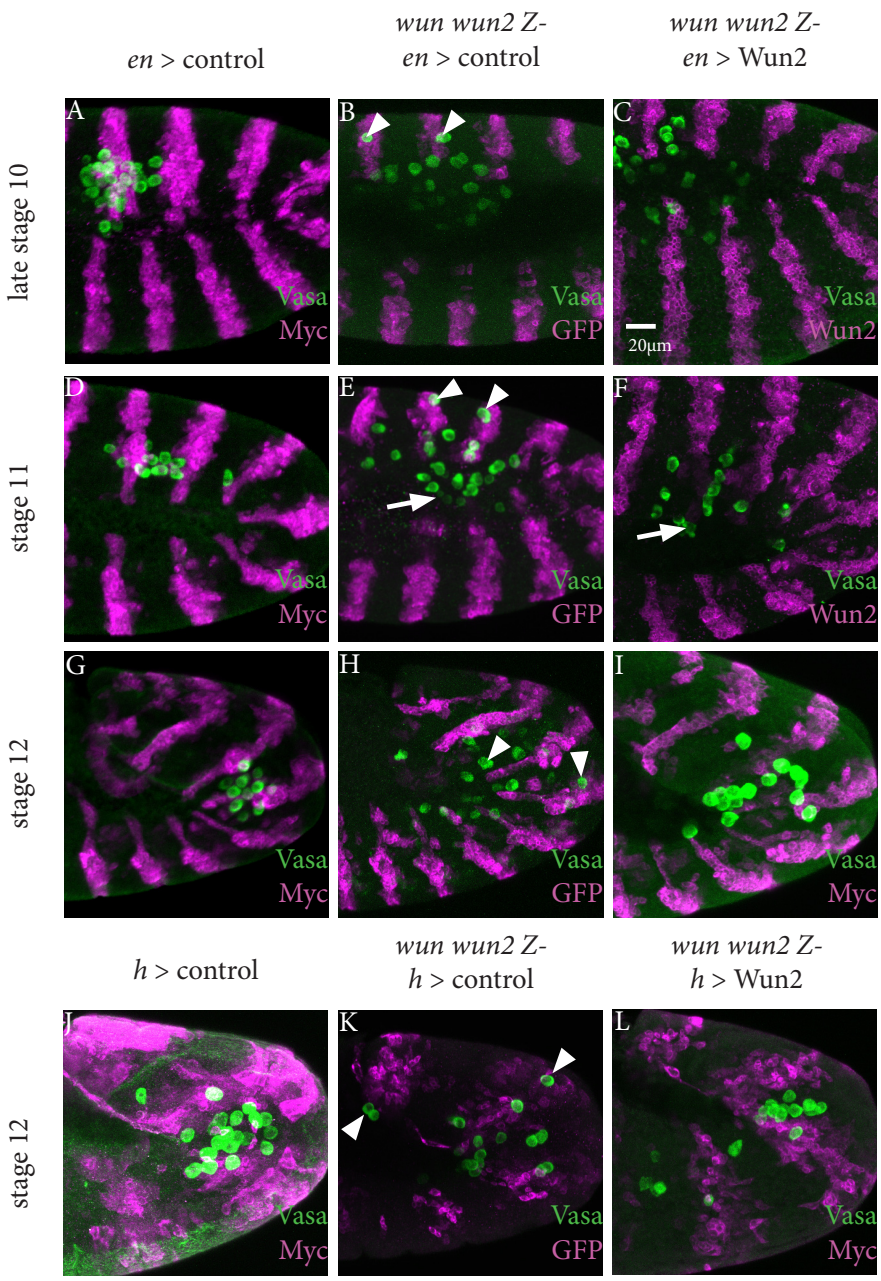



Figure 4.4: Germ cells avoid Wun stripes and line up in the interstripe. In a wild type embryo with stripes expressing a control protein (magenta), germ cells (green) are contained in the mesoderm which runs perpendicular to the stripes (A,D,G,J). In the absence of *wunens* from somatic cells (*wun wun2 Z-*) with stripes expressing a control protein (magenta), germ cells (green) are dispersed everywhere with no particular alignment with respect to the stripes (B,E,H,K). Some germ cells are present at the ectoderm (arrowheads B,E,H,K) while some remain on the midgut in the endoderm (arrow E). On expression of Wun2 (magenta) in the ectoderm in stripes using *en* or *h* driver in a *wun wun2 Z-* embryo, germ cells (green) align in the mesoderm, parallel to and between the stripes. No germ cells are found at the ectoderm while some germ cells may be present on the midgut in the endoderm (arrow F).

At stage 11 in wild type embryos, the germ cells enter and align with the mesoderm which runs perpendicular to segmental stripes (Figure 4.4D). In *wun wun2 Z-* embryos there is no clear alignment with the stripes and some germ cells remain on the midgut (arrow Figure 4.4E) while others enter the ectoderm (arrowhead Figure 4.4E). In *wun wun2 Z-* embryos with *en* driven Wun, germ cells

align in the underling mesoderm parallel to but between the Wun stripes (Figure 4.4F, arrow shows some germ cells remaining on the basal side of the midgut).

In wild type embryos at stage 12 the germ cells remain in the mesoderm and are loosely dispersed (Figure 4.4G,J). In *wun wun2 Z-* embryos the germ cells are scattered between ectoderm, mesoderm and endoderm (Figure 4.4H,K, arrow heads show germ cells in the ectoderm). In *wun wun2 Z-* embryos with *en* or *h* driven Wun2, germ cells avoid the ectoderm and majority of them remain in the mesoderm well aligned parallel to but between the stripes (Figure 4.4I,L).

I quantified the position of the germ cells in the endoderm, mesoderm and ectoderm relative to the overlying ectodermal stripes in wild type, *wun wun2 Z-* and *wun wun2 Z-* embryos with *en* driven Wun2 (Figure 4.5A, a representative embryo illustrating the germ cell distribution across the germ layers is given in Figure 4.5B).

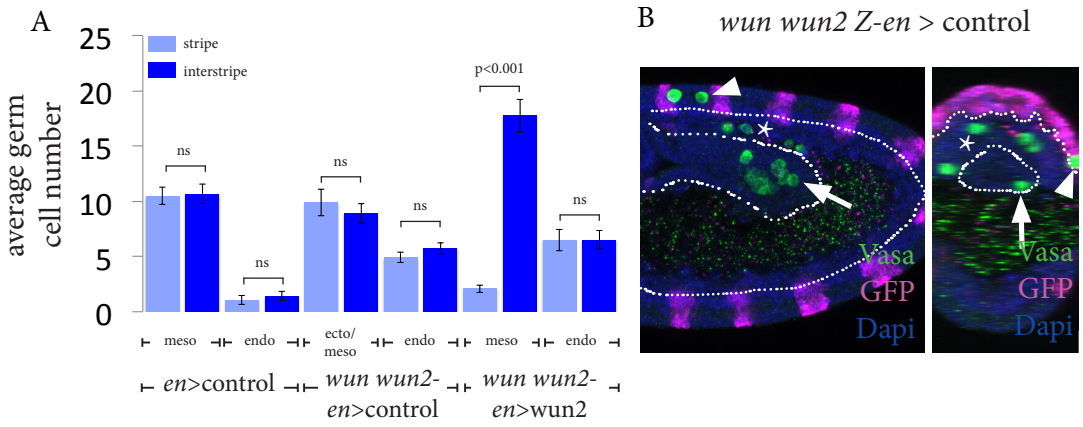



Figure 4.5: Majority of germ cells in a *wun wun2 Z- en*>Wun2 embryo are in the mesoderm below the interstripe. (A) Graph showing the number of the germ cells in the ectoderm, mesoderm and the endoderm which are in or below a stripe or in or below an interstripe. In wild type and *wun wun2 Z-* embryos, germ cells are distributed equally between stripe and interstripe. Overexpression of Wun2 in *wun wun2 Z-* embryos using *enGal4* causes a significant number of germ cells in the mesoderm to be in the interstripe while in the endoderm, the germ cells show no bias in their distribution. (B) 3D reconstructed sagittal (left) and transverse (right) views of a *wun wun2 Z-* embryo with *en* driven control stripes (magenta) with Dapi stained nuclei (blue) shows germ cells (green) that are in the different germ layers: ectoderm (arrowheads), mesoderm (asterix) and the endoderm (arrows). Boundaries between the different germ layers are indicated with dotted lines.

In wild type embryos the majority of germ cells are in the mesoderm and there is no difference in the number below a stripe and between the control stripes

(interstripe). In *wun wun2 Z-* embryos germ cells are in all 3 tissues, but again there is no difference in the number in or below the control stripes and in the interstripe. In *wun wun2 Z-* embryos with *en* driven Wun2, whilst the cells in the endoderm were equally distributed between the stripe and interstripe regions, the majority of germ cells in the mesoderm were in the interstripe. This indicates that the signal from the ectoderm is able to affect the position of the germ cells in the mesoderm but not in the endoderm.

### 4.4. Determining the minimum distance of Wun action

In a *wun wun2 Z-* several germ cells are found in the ectoderm already by stage 11 and 12 (Figure 4.4 E,H,K). On expression of Wun2 in stripes in the ectoderm using either *enGal4* or *hGal4* in such a background, I found that the entire ectoderm was devoid of germ cells (Figure 4.4 F,I,L and Figure 4.3E). This indicates that segmental Wun2 expression is sufficient to exclude germ cells from the entire ectoderm.

At late stage 11 and early stage 12, the distance between the *h* stripes that overlie the germ cells (between parasegments 9 and 11, Figure 4.2A) is 30 μm (6 measurements in 3 embryos, s.d.=3.7 μm) while for the *en* driver the distance between stripes that overlie the germ cells (between parasegments 10 and 11, and 11 and 12, Figure 4.2A) is 31 μm (8 measurements in 3 embryos, s.d.=3.5 μm) (Figure 4.6).

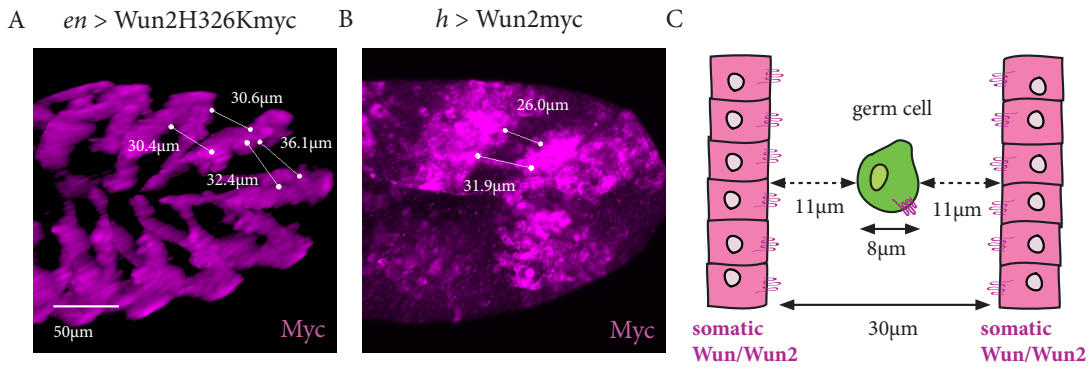



Figure 4.6: The minimum distance of Wun action is 11μm. Lateral view of 3D reconstruction of stage 12 embryos with *enGal4* (A) and *hGal4* (B) driving Wun2H326Kmyc and Wun2myc

respectively, stained against Myc (magenta) showing the distance measurements between the stripes at the ectoderm. (C) Rationale for the estimate that the minimum distance of Wun2 mediated repulsion is 11 µm, based on the ability of *h* or *en* driven Wun2 ectodermal stripes to prevent germ cells from entering the ectoderm in the interstripe, which spans over an average distance of 30µm.

Therefore Wun expressing ectodermal stripes can exert influence over the germ cells, in particular to exclude them from the ectoderm, over a distance of at least 11 µm (the distance between the *h* stripes minus the diameter of a germ cell divided by 2, Figure 4.6C) and indicates that repulsion is likely cell-contact independent.

### 4.5. Germ cells lie equidistant between the Wun stripes

If Wunen mediated repulsion is a cell-contact independent process and if the Wunen mediated signal provides positional information in the form of a gradient, then the germ cells should be able to sense their position relative to their neighboring Wunen expressing stripes and occupy the midpoint between them, i.e. the germ cells should lie equidistant from their surrounding stripes.

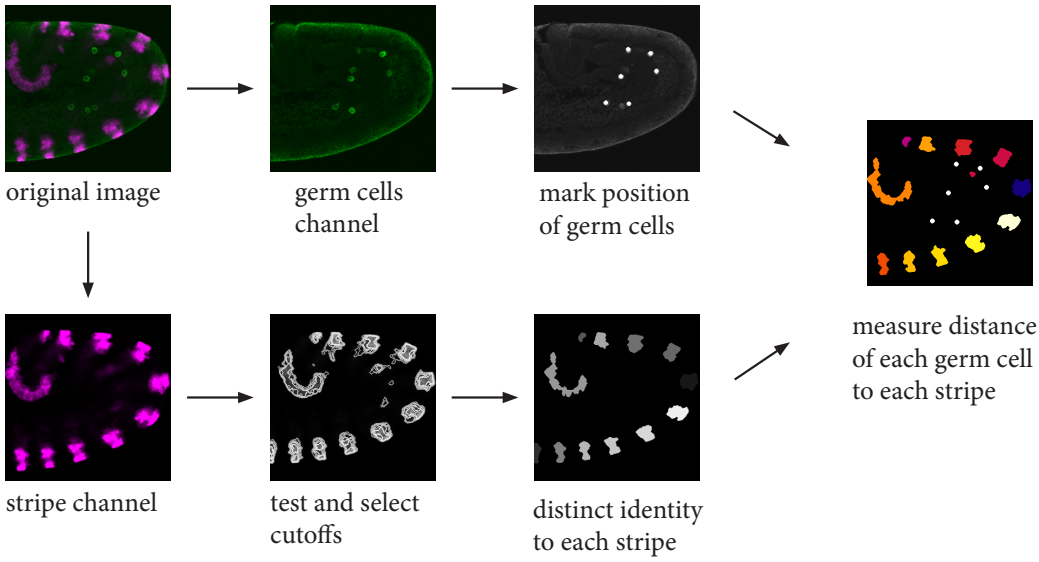



Figure 4.7: Workflow showing the steps in measuring the distance of the germ cells (green) to the stripes (magenta). The positions of the germ cells were marked manually. The *en* stripes were segmented using a thresholding method and each stripe was given a unique label. The distances of each germ cell to the two closest stripes were determined.

To test this hypothesis we determined the distances of the germ cells to the stripes that lie on either side of them (the closest and second closest stripes). We focused on the *en* driven stripes as their boundaries were better defined as compared to *h.* We segmented wild type, *wun wun2 Z-* embryos with control stripes, and *wun wun2 Z-* embryos with *en* driven Wun2 stripes using a thresholding method to define the stripe domains. Each stripe was assigned a unique label and the distance of each germ cell to the nearest point of its closest stripe and the nearest point of its second closest stripe was calculated (Figure 4.7).

We found that the distribution of the distances of the germ cells to their closest stripe was narrow in wild type embryos, with a median of 7 μm (Figure 4.8A). For *wun wun2 Z-* embryos the distribution was broader, as would be expected due to the scattered nature of the germ cells, with a median of 11 μm. For *wun wun2 Z-* with *en* driven Wun2 stripes the distribution was again broader but significantly shifted towards increased distances, with a median of 15 μm ($p<0.01$ by Mann–Whitney *U* test). For the second closest stripe the distributions overlapped with each other and the medians were relatively similar lying between 19 to 22 μm (Figure 4.8A).

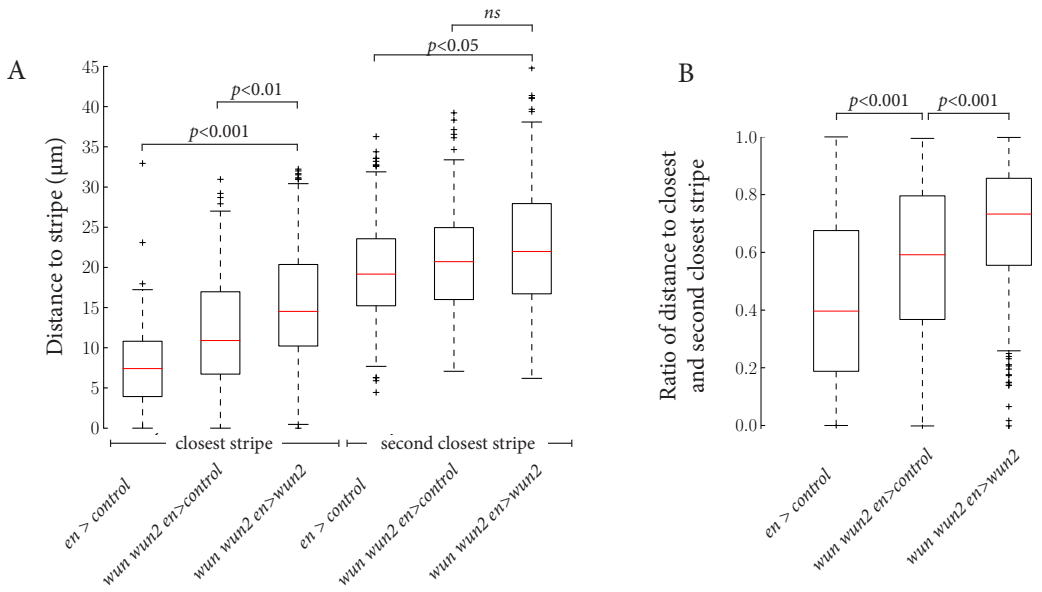



Figure 4.8: Germ cells lie equidistant between the Wun stripes. Box and whisker plots showing (A) the distribution of germ cell distances to its closest and second closest *en* stripe and (B) the ratio of the distance to closest and second closest stripe in wild type embryos with control stripes (*en>Gal4/UASwun2H326Kmyc*), *wun wun2 Z-* embryos with control stripes (*Df(2R)wunGL en>Gal4 / Df(2R)wunGL UASlazaroGFP*) and *wun wun2 Z-* with *en>* Wun2 (*wunCE UASwun2myc /*

*Df(2R)wunGL en>Gal4*). Outliers, defined as being more than one interquartile distance from the box, are indicated as crosses.

As a measure of the equidistance between neighboring stripes we used the ratio of the distance to the closest and second closest stripes (Figure 4.8B). A value of 1 indicates that a germ cell is perfectly equidistant. When we compared the distribution of ratios for the *wun wun2 Z-* embryos with Wunen stripes compared to either of the controls we found that there were significantly more germ cells with a ratio closer to 1 (Figure 4.8B, $p<0.001$). We conclude that germ cells can sense the stripes on either side of them, which is consistent with the presence of a diffusible signal.

## 4.6. Germ cells maintain their interstripe position without making cell-cell contact

Although the fixed tissue analysis argues for a diffusible signal, it remains possible that the germ cells find an equidistant position by migrating back and forth and making repeated contacts with the stripes on either side of them. To exclude this hypothesis we performed live imaging to monitor the path of the germ cells as they maintain this position.

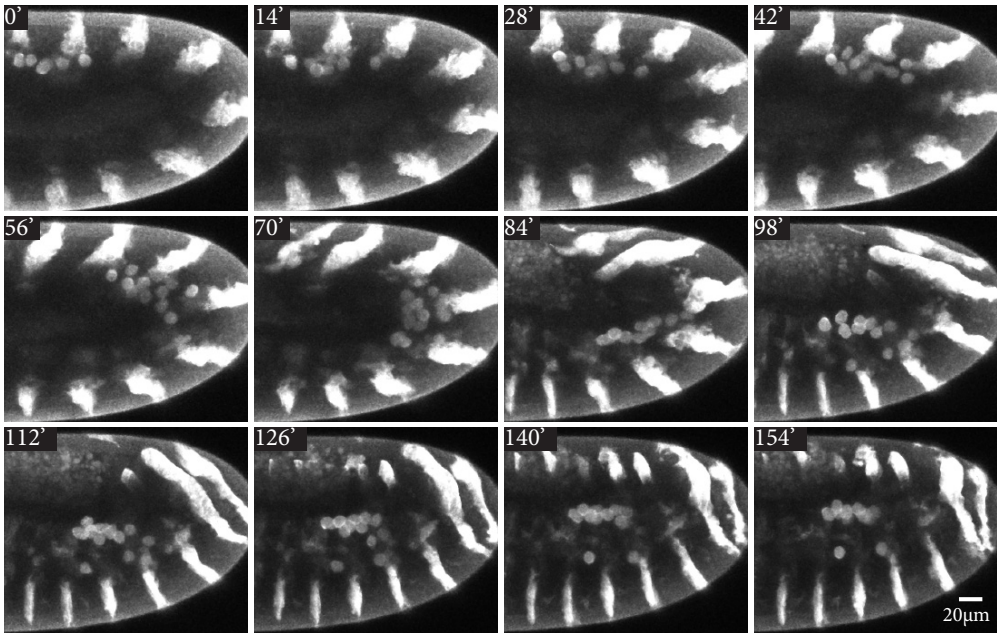


Figure 4.9: Germ cells are motile and remain in a plane perpendicular to the plane of the stripes but are present below and between stripes in *nos>moeGFP; en>Gal4/UAS moeGFP* embryos. Maximum intensity projection of lateral views of wild type embryo with germ cells labeled with eGFP fused to actin binding domain (*nos>moe GFP*) and stripes expressing moeGFP (*en>moeGFP*) imaged every 2 minutes with every 7th time point shown here. The lateral most confocal slices that would obscure the position of the germ cells have not been included in the projections. Germ cells in wild type embryos are motile but are restricted in their distribution in the mesoderm lying in a plane perpendicular to the stripes.

In wild type stage 11 embryos, the germ cells remain motile but make only short movements within the mesoderm, whilst the embryo as a whole undergoes germ-band retraction as visualized by the clockwise movement of the control stripes (Figure 4.9). The germ cells are restricted in their distributed in the mesoderm in a plane perpendicular to the ectodermal en stripes expressing control protein.

In *wunwun2 Z-* embryos, germ cells are still motile but they migrate slower as compared to wild type embryos at stage 10 (Sano et al., 2005). However later stages had not been examined hence I wanted to examine lateral view of the migration of germ cells at stage 11 and beyond to observe their motility and directionality.

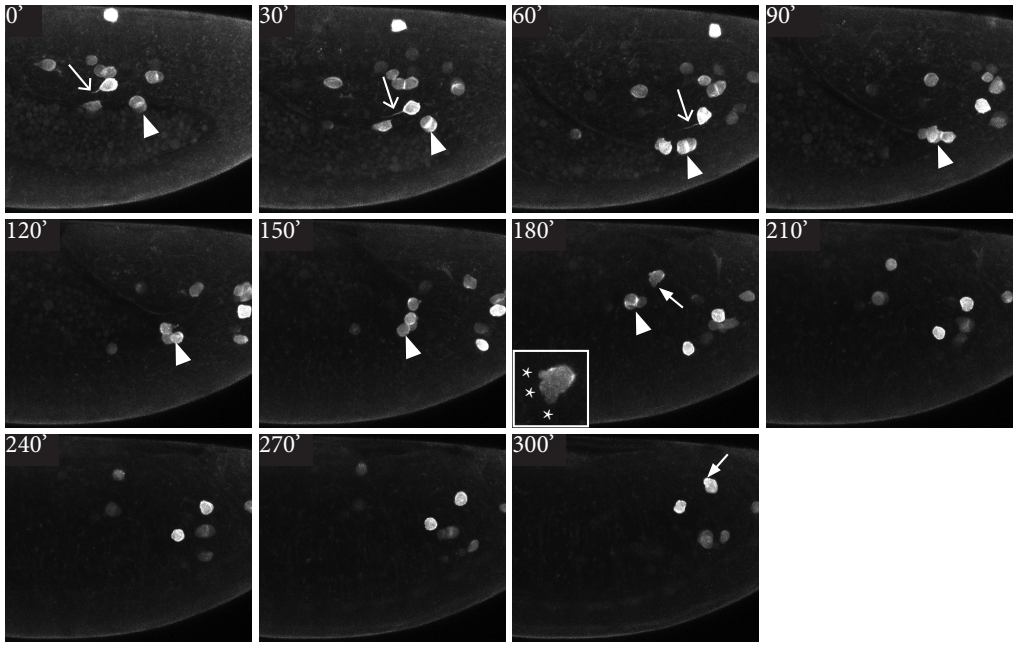



Figure 4.10: Germ cells are motile but show random movement in the absence of somatic Wunens in *nos>moeGFP; Df(2R) wunGL* embryo. Maximum intensity projection of lateral views of *wun wun2 Z-* embryo with germ cells labeled with eGFP fused to actin binding domain (*nos>moe GFP*) imaged every 3 minutes with every 10th time point shown here. Germ cells in *wun wun2Z-* embryos are

motile but some germ cells that stick to each other at stage 11 (0' arrowhead) remain attached for their entire migration duration (0' -180' arrowheads). A germ cell shows a prominent uropod at its tail end that lasts for about 60 minutes (0' - 60' open arrow). Another germ cell shows multiple pseudopods at its leading edge (180' arrow, germ cell enlarged to show pseudopods marked by asterix). At very late stages germ cells still show pseudopods (300' arrow).

The germ cells show motility but unlike those in wild type, several germ cells in *wun wun2 Z-* reach the ectoderm. One such germ cell show a long uropod at its tail end that persists over 60 minutes (Figure 4.10, open arrow). Some germ cells stick to each other and remain attached for the entire duration of migration (Figure 4.10 arrowhead). One germ cell showed multiple pseudopods at its leading edge (Figure 4.10, 180' arrow, enlarged germ cell with pseudopods marked by asterix). Germ cells still show pseudopods at very late stages (Figure 4.10, 300' arrow). In total this shows that germ cells are motile in *wun wun2 Z-* embryos, however their movement is not directed. This also suggests that in the absence of somatic Wunens, germ cells have no gradient to guide them which results in their random distribution.

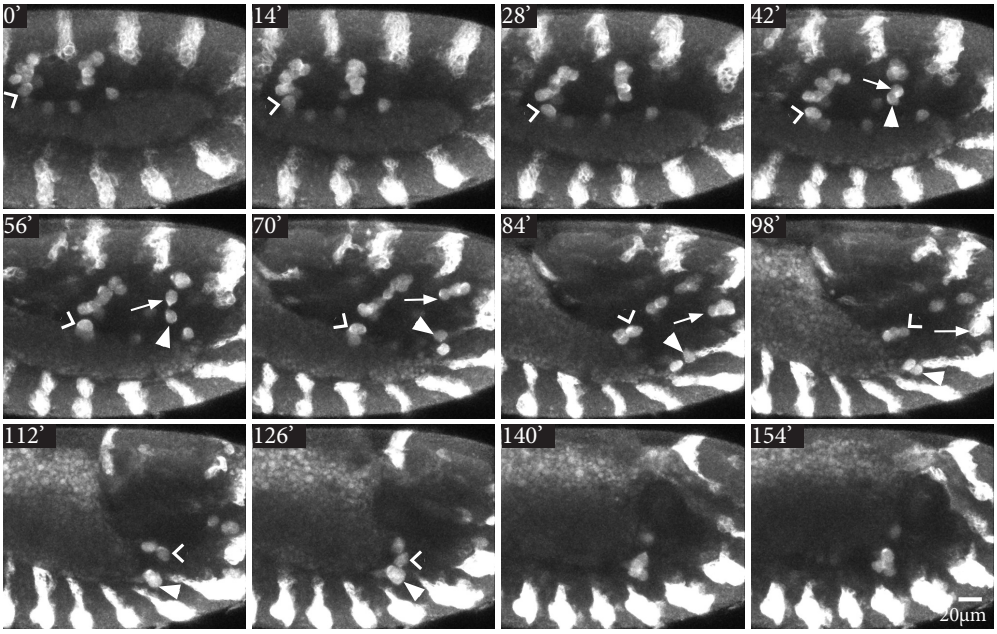



Figure 4.11: Germ cells are motile but remain restricted in a plane parallel to the plane of the stripes and never contacts the stripes in a *nos>moeGFP; Df(2R)wunGLen>Gal4/Df(2R)wunGLUASwunGFP* embryo. Maximum intensity projection of lateral views of *wun wun2 Z-* embryos with germ cells labeled with eGFP fused to actin binding domain (*nos>moe GFP*) and stripes expressing WunGFP (*en>wunGFP*) imaged every 2 minutes with every 7$^{th}$ time point shown here. The lateral most confocal slices that would obscure the position of the germ cells have not been included in the projections. Arrow indicates a germ cell that migrates to

join a cluster of germ cells that end up behind an *en* stripe, but this cluster does not move relative to stripe and does not join the presumed gonad cluster. Arrowhead indicates a neighboring cell that stays in a more central location and later joins the presumed gonad cluster. Open arrow indicates an extreme lateral cell that joins to form the presumed gonad cluster.

At stage 11 in *wun wun2 Z-* embryos with *en* driven Wunen stripes the germ cells are also motile as evidenced by dynamicity of the actin enrichments in the germ cells as well as the movement of the two germ cells marked with an arrow and an arrowhead away from each other (Figure 4.11). The germ cells align in a plane parallel to that of the stripes indicating that they are integrating a signal from Wunen expressing stripes on their either side. But the germ cells do not touch a stripe on one side and then on the other while aligning between them but maintain their interstripe position.

In *wun wun2 Z-* embryos with *en* driven Wun stripes some germ cells form a cluster in the position corresponding to the gonad. Several germ cells appear to take a shorter route to the gonad, by remaining close to the basal side of the midgut and joining the gonad cluster at stage 13 as the germ band finishes retracting (Figure 4.11 open arrow shows a germ cells that sits on the midgut and ultimately join the gonad cluster).

### 4.7. The maximum distance of Wunen repulsion

In *wun wun2 Z.-* embryos with *en*>wunGFP, the germ cells are confronted with stripes both anterior and posterior to their position. Thus the germ cells may not be able to move as far away as they would in case of a single stripe and this would lead us to underestimate the distance at which a germ cell could sense Wunen expressing somatic cells. Therefore I wanted to test how germ cells would respond to a single stripe.

I made use of a Gal4 containing P-element, *p{GawB}NP5141,* which drives expression in parasegments 2 and 14 (Figure 4.2A, Kuhnlein et al., 1998). The former domain is far enough anterior that it is unlikely to affect the germ cells whereas the latter, at stages 10-11, lies above but anterior to where the germ cells would normally migrate. In a *wun wun2 Z-* embroys, an average of 4.7 germ cells mis-migrate and enter parasegment 14 (Figure 4.12 A and C, 10 embryos

examined at stages 11 and 12). In contrast, when Wun is expressed in this domain no germ cells were located in this region (Figure 4.12B and D, 10 embryos examined at stages 11 and 12), most likely because the germ cells that would have entered this region have been repelled more posteriorly. The alternative explanation that these germ cells were eliminated by cell death is not supported because the total germ cell number in such embryos is not significantly less than in control embryos (Figure 4.12E).

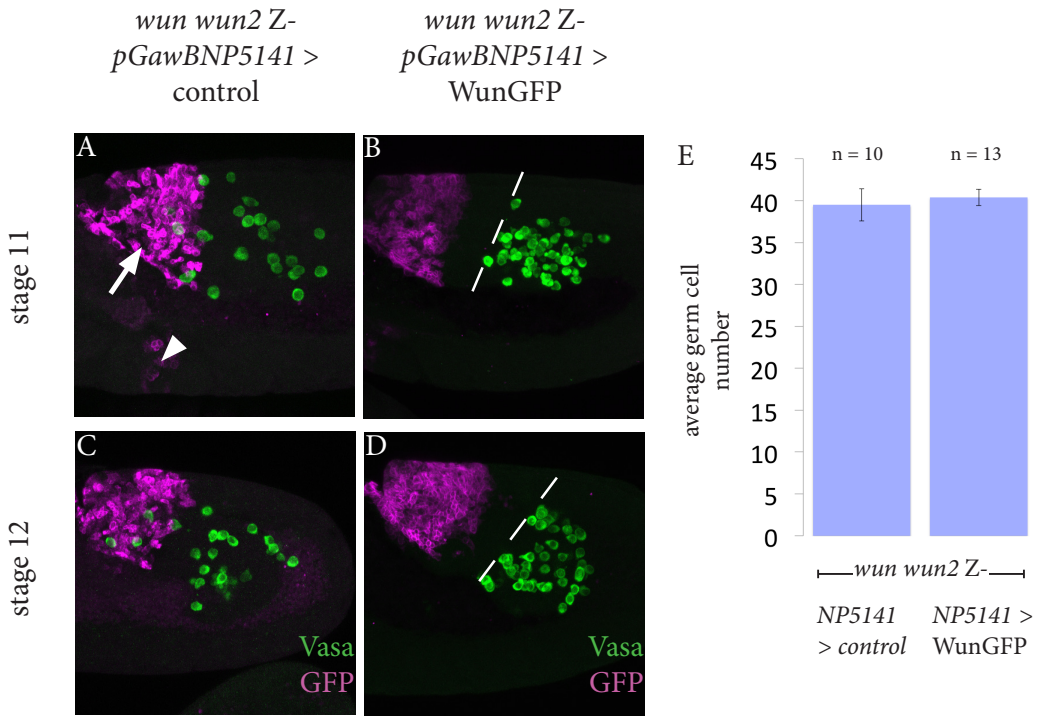



Figure 4.12: Determining the maximum distance of Wunen action. *wun wun2 Z-* embryos expressing a control protein in parasegments 2 and 14 using *pGawBNP5141* ( magenta, A arrowhead and arrow) at stages 11 (A) and 12 (B) shows germ cells (green) present in this domain. On overexpression of WunGFP using this driver in a *wunwun2 Z-* embryo, no germ cells enter parasegment 14 at stages 11 (B) and 12 (D). Graph shows the quantification of the number of germ cells at stages 11 and 12 in fluorescently stained 3D reconstructed embryos of *Df(2R)wunGLNP5141>Gal4/ Df(2R)wunGLUASlazGFP* and *Df(2R)wunGLNP5141>Gal4/ Df(2R)wunGLUASwunGFP*. Error bars represent standard error and *n* indicates the number of embryos scored.

Given that 4.7 germ cells mis-migrate and enter parasegment 14 in *wunwun2 Z-* embryos, I measured the distance between Wun expressing cells in parasegment 14 to the 5 nearest germ cells in *wunwun2 Z-* embryos with *p{GawB}NP5141* driven Wun. I found that these germ cells were located on

average 33 µm (s.d. = 8.8, 10 embryos with a total of 50 germ cells measured at stages 11 and 12) from the nearest point of Wun expression (Figure 4.12). Thus when faced with an expression domain of somatic *wunen* on one side with no restriction on the other, germ cells are repelled beyond 33 µm. This distance represents our estimate of the maximum range of influence of Wun expressing somatic cells on germ cells.

### 4.8. Small patches of Wun expressing tissue can also repel germ cells

The drivers used in the previous sections all result in expression in large stripes containing many hundreds of cells. I was interested to know whether small patches of cells would be sufficient to also cause repulsion. I therefore used a driver under the control of the promoter of the 3rd and 7th stripe of the *even skipped* (*eve*) pair rule gene (*eve3+7*), that expresses in parasegments 5 and 13 respectively (Figure 4.2A, Small et al., 1996). At stage 11 and 12, this driver gave small patches of expressing cells rather than a continuous stripe. Cells expressing within parasegment 5 are too far ventral to affect the germ cells and although parasegment 13 is slightly anterior to where the germ cells would normally migrate, several germ cells enter this region in a *wun wun2 Z-* background (Figure 4.12 A,C).

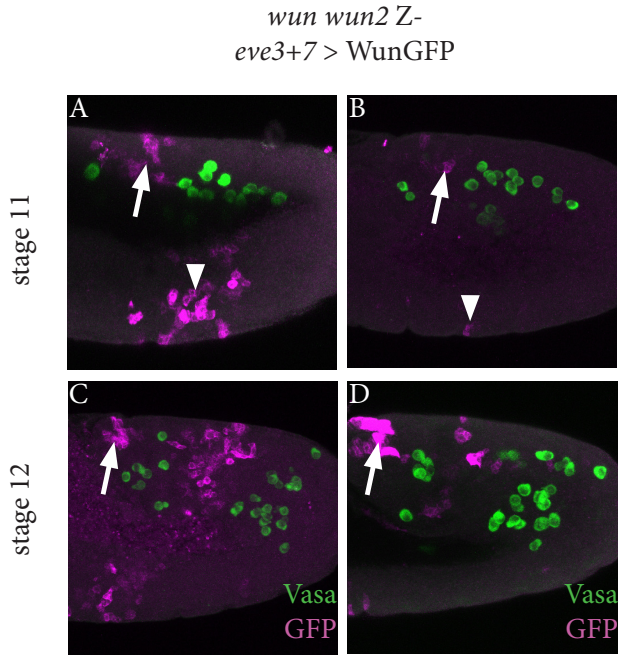



Figure 4.13: Lateral views of stage 11 (A,B) and stage 12 (C,D), of *Df(2R)wunGLeve3+7>Gal4/ Df(2R)wunGLUASwunGFP* embryos with *eve3+7>Gal4* expressing WunGFP (magenta) in parasegment 13 (arrow A,B) and parasegment 5 (arrowheads A,B). Germ cells (green) are absent in the parasegment 13 at stages 11 and 12 in case of prominent stripes (A,C) as well as patchy expression (C,D). Arrows in C,D represent additional expression in the proctodeal promordium.

When Wunen is expressed using *eve3+7>Gal4* in a *wun wun2 Z-* background, no germ cells enter the parasegment 13 region (Figure 4.13 A, B, arrows) and the expression of Wunen in distinct stripes (Figure 4.13 A,C) or smaller patches of cells in parasegment 13 (Figure 4.13 B,D), splits the migrating germ cell population into two distinct clusters anterior and posterior to this region of expression at stages 11 and 12 (Figure 4.13 A,B,C,D 18 embryos examined). This indicates that small expression domains of Wunens are sufficient to create a repulsion zone, which the germ cells avoid migrating into.

## 4.9 Ectopic Wun expression is sufficient to herd some germ cells to the gonad and affects SGP cluster occupancy

In *wun wun2 Z-* embryos with *en* or *h* driven Wun, a number of germ cells clustered together in regions equating to the positions of the embryonic gonads (Figure 4.3 C,D). I therefore examined how many germ cells were actually reaching the gonad in such backgrounds by staining embryos for the SGP marker, Eyes absent (Eya) (Figure 4.14). The SGPs arise as four clusters in the mesoderm at stage 11 in parasegments 10,11,12 and 13 (Cluster 13 is only retained in males embryos). By stage 13 all the SGP clusters are fully occupied as they line up before coalescing together at stage 14 into a round compact structure.

Loss of somatic Wunens or segmental Wun mis-expression does not affect SGP behavior (Figure 4.14A-L). At stages 14-16, control embryos contained on average 12 germ cells per gonad, whereas the gonads in *wun wun2 Z-* embryos contained on average just 1 germ cell (Figure 4.14M). Upon expression of *en* driven Wun, the number of germ cells reaching the gonad was slightly increased (an average of 4 germ cells per gonad) and greatly increased upon expression of *h* driven Wun (an average of 9 germ cells per gonad) (Figure 4.14M).

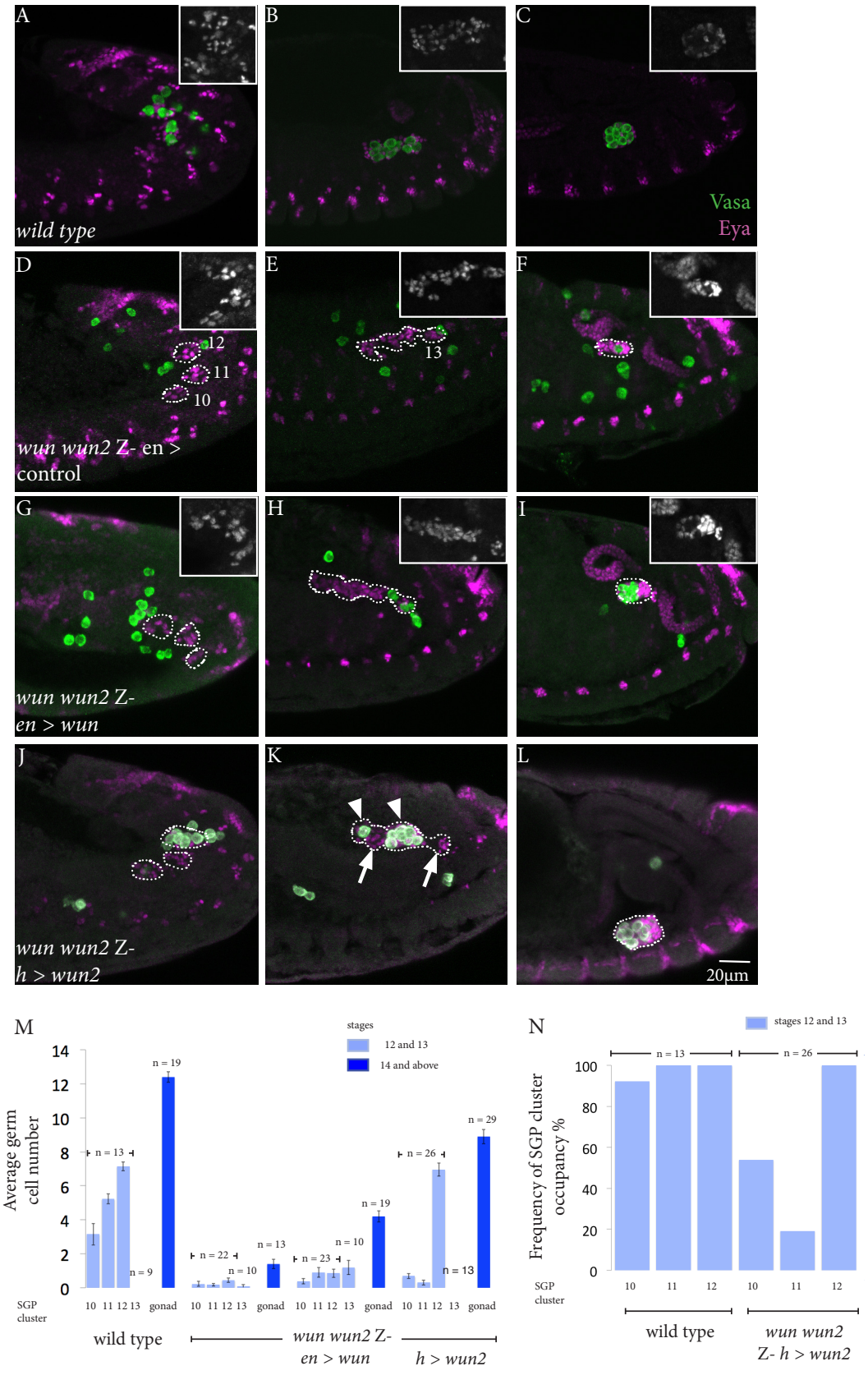



Figure 4.14: Ectopic Wun expression affects SGP cluster occupancy. (A-L) Lateral views of stage 12 (A,D,G,J), 13 (B,E,H,K) and 15 (C,F,I,L) embryos of genotype *nos>moeGFP ; en>moeGFP* (A-C),

*Df(2R)wunGL en>Gal4 / Df(2R)wunGL UASlazaroGFP* (D-F), *Df(2R)wunGL en>Gal4 / Df(2R)wunGL UASwunGFP* (G-I) and *wunCE UASwun2myc / Df(2R)wunGL ; h>Gal4 / +* (J-L) stained with antibodies against Vasa (green) to mark germ cells and against Eya (magenta) which marks the SGP nuclei but is also expressed in other tissues. Insets show SGP clusters in grey scale and numbers indicate the parasegment identity for each of the SGP clusters. (K) Arrows indicate unoccupied SGP clusters in parasegments 11 and 13, arrowheads indicate germ cells occupying SGP clusters in parasegments 10 and 12. (M) Graph showing average number of germ cells per SGP cluster in parasegments indicated (at stages 12 and 13) and per gonad (at stages 14-16) in fluorescently stained 3D reconstructed embryos of the genotypes described above. Error bars represent the standard error, *n* indicates number of gonads scored. (N) Graph showing the frequency of SGP cluster occupancy in *wunCE UASwun2myc / Df(2R)wunGL ; h>Gal4 / +* compared with sister control embryos. SGP cluster is scored as occupied based on presence of 1 or more germ cells. *n* indicates number of gonads scored.

It was intriguing as to why either *en* or *h* driven Wun should result in any rescue of germ cells to the gonad and why *h* should rescue better than *en* (Figure 4.14M, dark blue bars). One explanation is that *en* ectodermal domains lie directly above all SGP clusters whilst *h* ectodermal domains sit above two of the SGP clusters (parasegments 11 and the male specific cluster in parasegment 13) (Figure 4.2A). I therefore examined the distribution of the germ cells associating with the individual clusters whilst they remained clearly distinguishable (during stages 12 and 13) to see if this was being affected by the expression of Wun in the ectoderm.

In wild type stage 13 embryos, germ cells have already associated with each of the clusters of SGPs in parasegments 10-12 (Figure 4.14A,B,M). In *wun wun2 Z-* embryos, the few germ cells that do associate with the SGPs do not preferentially associate with any particular SGP cluster (Figure 4.14D,E,M). This situation is similar in *wun wun2 Z-* embryos with *en* driven Wun (Figure 4.14G,H,M). At later stages however increased numbers of germ cells are found at the gonad (Figure 4.14 I,M) this correlates well with our live imaging in which we see some germ cells cluster laterally in the region of the gonad relatively late (Figure 4.11).

In comparison, with *h* driven Wun in the same background I found that a large number of germ cells associated with the SGPs in parasegment 12 (Figure 4.14J,K). Indeed these SGPs contained at least 1 germ cell in 100% of cases examined (Figure 4.14M,N). The SGPs of parasegment 10 also had associated germ cells in 50% of cases, but the average number of associated germ cells was far less than for parasegment 12 (Figure 4.14M,N). In contrast, germ cells were very rarely associated with SGPs of parasegment 11 (Figure 4.14M,N).

This bias in association of germ cells with different SGP clusters is consistent with the expression of Wun in the ectodermal *h* stripes that lie over the SGP clusters in parasegments 11 and 13 repelling germ cells so that they associate with the SGPs in parasegment 12 and to a lesser extent parasegment 10. The distance of the ectodermal stripes to the underlying mesodermal SGP clusters is 12.3 μm (s.d.=1.2μm, averaged over 15 SGP clusters), which is well within our estimated of the range of Wun action on germ cells.

I conclude that the substitution of the intricate endogenous expression pattern of the *wunens* by ectopic domains is sufficient to herd a surprisingly large proportion of the germ cells to the gonad but with clear effects on the relative occupancy of SGP clusters.

## 4.10. Overexpression of Wunens in endogenous *wun2* domains does not affect germ cell survival and migration

Our estimate of the maximum range of influence of Wun expressing somatic cells is based on protein mis- and over-expression. To determine if over-expression has a disproportionate influence on the degree of repulsion, I over-expressed Wun and Wun2 under the control of the Gal4 containing *wun2* driver line, *p{GawB}wun2*$^{9\text{-}175}$ which recapitulates much of the endogenous expression pattern of *wun2* (Figure 4.15A,B).

I found that germ cell survival and migration were identical to that in control embryos (Figure 4.15C) indicating that over-expression of Wunens can be tolerated in Wun2 endogenously expressing cells and argues that spatial expression outweighs protein levels in determining the path of germ cell migration and level of survival. I conclude that our distance estimates based on Wun over-expression are likely relevant to the wild-type Wun levels.

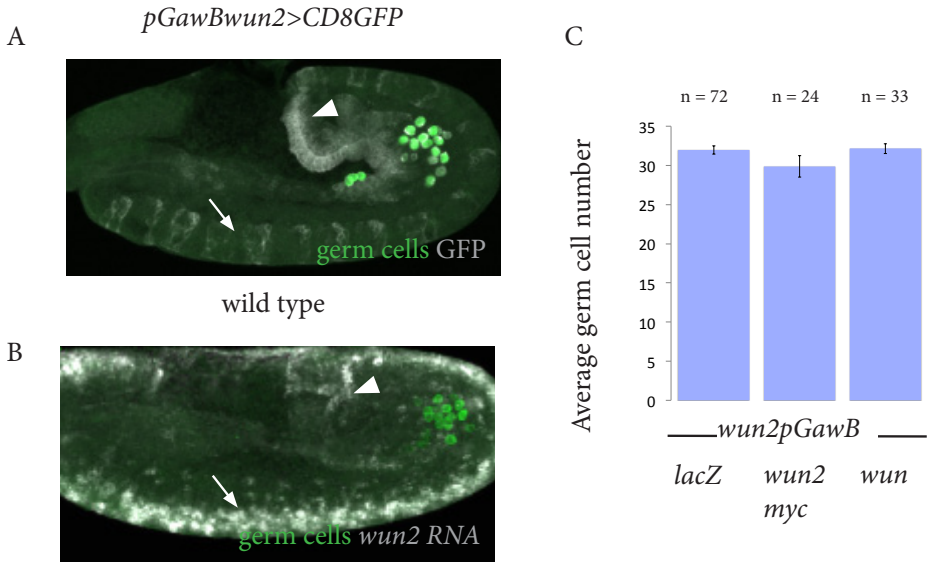



Figure 4.15: Lateral view of a wild type stage 11 embryo with p*{GawB}wun2* $^{9\text{-}175}$ expressing CD8GFP (A) and *in situ* hybridization pattern of *wun2* (B) showing expression in the CNS (arrow A,B) and part of the gut (arrowhead, A,B). Overexpression of Wun and Wun2 in a wild type embryo in the endogenous *wun2* domains does not affect germ cell survival as compared with a lacZ control (C). The level of expression of the control GFP protein is weaker than the RNA *in situ* expression pattern.

## 4.11. Migrating germ cells are within the range of influence of Wunens

To determine the extent to which the path of migrating germ cells can be explained by Wunen dependent repulsion we examined the position of germ cells in embryos fluorescently stained to highlight endogenous *wun2* RNA expression (Figure 4.16). We segmented the *wun2* expression domains and measured the distance of germ cells to their nearest Wun2 segment in stages 10, 11 and 12 embryos (Figure 4.16 A,B,D,E,G,H).

We find that germ cell distances are normally distributed with median values of 15.5, 15 and 12 µm at stages 10, 11 and 12 respectively. This decrease at later stages is largely due to the proximity of small patches of *wun2* positive cells in the lateral mesoderm and ectoderm. Since small patches of *wunen* positive cells can repel germ cells (Figure 4.13 A-D) we considered these areas functionally relevant.

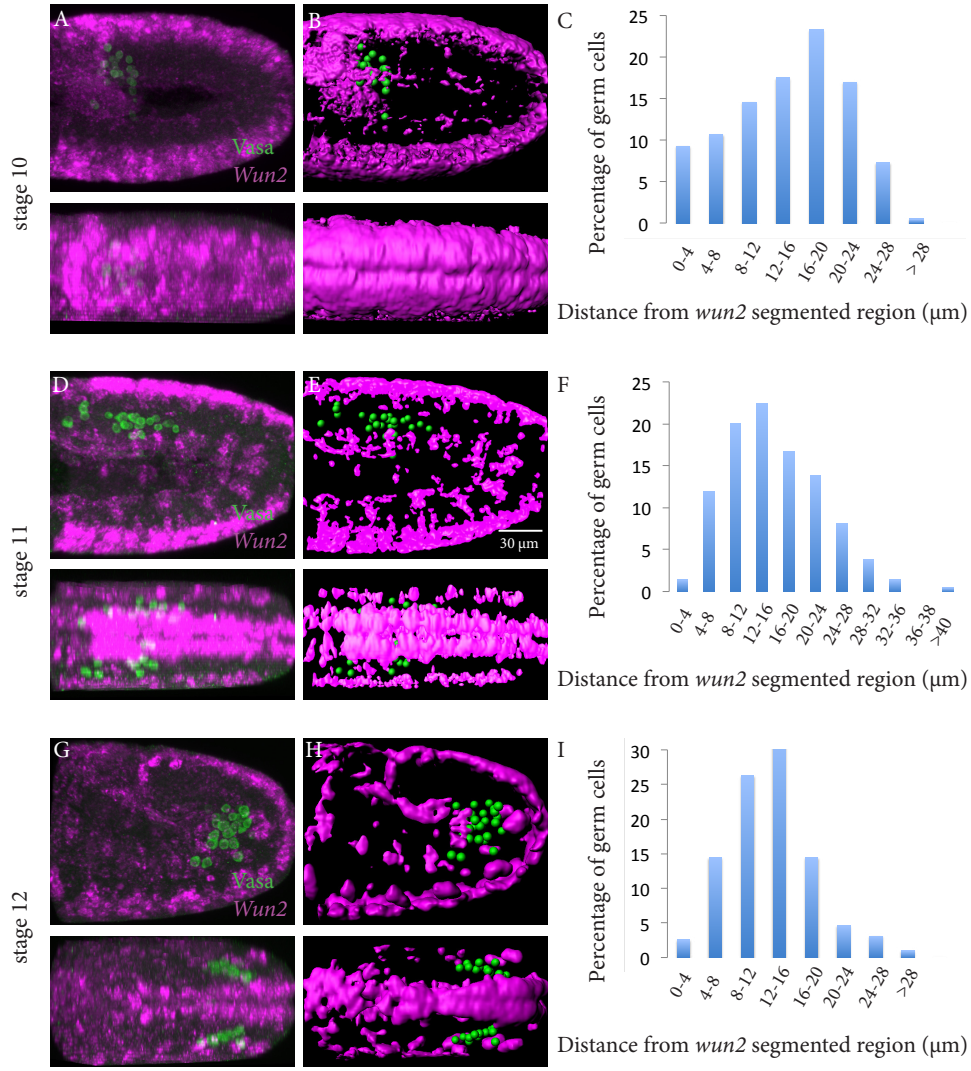



Figure 4.16: Lateral (top) and dorsal (bottom) views of 3D reconstructed wild type embryos stained for *wun2* mRNA expression (magenta) and Vasa to mark the germ cells (green) at stages 10 (A-B), 11 (D-E) and 12 (G-H). The position of germ cells and *wun2* expression pattern was segmented in Imaris (B,E,H) and the distance of each germ cell to its nearest segmented *wun2* expression was determined. Graphs showing the distribution of germ cell distances measured from 3D reconstructed and segmented *wun2 in situ* pattern at stages 10 (C), 11 (F) and 12 (I).

The experiment using the single stripe NP5141 driver demonstrated that germ cells can be affected up to a distance of 33 µm, therefore we considered this distance to be the effective range of Wunen action. From our distance distribution of germ cells from Wun2 segmented regions in wild type embryos, we find that overall more than 99 % of germ cells are located closer than 33 µm to their nearest *wun2* segment (Figure 4.16). We conclude that all germ cells are likely influenced by Wunen expression for the entire duration of their migration.

# Chapter 5
# Discussion – II

Extracellular lipids guide several important homeostatic or developmental processes like heart cell migration in zebrafish and T lymphocyte migration in mammals (Kupperman et al., 2000; Matloubian et al., 2004). However, the distances to which lipids act *in vivo* are not known. *Drosophila* germ cell migration is guided by the spatially restricted somatic expression pattern of two lipid phosphate phosphatase enzymes – Wunen (Wun) and Wunen2 (Wun2) (Zhang et al., 1996; Starz-Gaiano et al., 2001). This gives us an excellent system to study the distance to which lipids can signal *in vivo* to affect cell migration.

My aim was to determine whether Wunen mediated repulsion is a contact dependent or independent process. I used *Drosophila* genetics to precisely manipulate Wunen expression and the behavior of germ cells as a read out of the extracellular lipid phosphate signal. We found evidence to support the existence of a contact independent mechanism involving a lipid gradient. Additionally I used three-dimensional reconstructions of embryos, segmentation of Wunen expression domains and *in silico* measurements to define the distance to which the expression of Wunens can signal to germ cells.

## 5.1. Somatic Wunen expression produces a gradient of lipid phosphates that is instructive to the germ cells

In the absence of somatic Wunens germ cells although motile, are scattered in the entire posterior part of the embryo. It was previously known that germ cells avoid entering somatic tissues that express Wunens (Starz-Gaiano et al., 2001). Wunens are catalytically active lipid phosphate phosphatase enzyme, which are believed to dephosphorylate and destroy an extracellular lipid phosphate. According to the current model for Wunen action, germ cells would detect this lipid phosphate, migrate to higher concentrations and thus would migrate away from Wunen expressing tissues (Renault et al., 2004). However evidence for the

existence of extracellular lipid phosphate gradients is lacking and it is unclear whether germ cells detect this gradient or just absolute levels.

Absence of germ cells from the entire ectoderm when Wunens are expressed in *en* or *h* stripes in a *wun wun2 Z-* embryo implied that the signal is contact independent (Figure 4.3). No Vasa positive cell remnants, which are characteristic of dying germ cells (Sano et al., 2005), were observed in the ectoderm of these embryos. This indicated that the germ cells were not undergoing death upon ectoderm entry but in fact avoided entry altogether signaled by a contact independent mechanism of repulsion.

On expression of Wunen in stripes in the ectoderm in a *wun wun2 Z-* embryo, germ cells remain in the mesoderm and line up parallel to the stripe but remain below the overlying interstripe domain. Live imaging of these embryos proved that the germ cells do not touch *en* stripes on their either side in order to maintain their position in the interstripe. The ability of the germ cells to position themselves in the interstripe equidistant from stripes on either side implied that the germ cells were integrating a signal from Wunen expressing stripes and interpreting a gradient and not absolute levels of an extracellular lipid phosphate (Figure 4.8).

In *wun wun2 Z- en>wun* embryos, in contrast to the distribution of the germ cells in the mesoderm, I noticed that the germ cells in the endoderm are not biased in their distribution with respect to the overlying stripe or interstripe (Figure 4.5). This could either be because there exists a physical barrier between the mesoderm and endoderm that the Wunen signal cannot cross, or there is a maximum distance to which the signal can affect germ cells. Using a parasegment 14 driver I determined the maximum range of Wunen action as 33μm. Germ cells in the endoderm lie beyond this 33μm distance and hence are presumably not under the influence of Wunen stripes in the ectoderm.

## 5.2. Does the amount or the area of Wunen expression affect the distance of action of Wunens?

I have shown that all germ cells at stages 10, 11 and 12 are within 33μm of the nearest *wun2* segmented region (Figure 4.16). However I found that the

median value of germ cell distance at stage 12 is slightly lower than at stages 10 and 11. This I believe is due to the presence of lateral small ectodermal patches of Wun2 that are located closer to the germ cells at stage 12 than at any of the other stages. Using *eve37Gal4* I could demonstrate that small patches of Wunen expressing cells could also influence the distribution of germ cells (Figure 4.13). Therefore I believe that the small expression domains of endogenous *wun2* expression at stage 12 are significant.

However it is not known if the smaller patches of *wun2* expression affect the lipid gradients as much as larger areas of expression. One way of determining this would be by expressing Wun2 in smaller lateral patches in an embryo that is *wun wun2 Z-*. The average distance of the germ cells from the nearest segmented *wun2* expression domains would illustrate if the area of expression of *wun2* has an affect on the distance of Wun2 action.

In order to understand more about the nature of the lipid phosphate gradient that is produced by the expression of somatic Wunens, it is important to know if the level of expression of the enzyme has an effect on the range of the signal. I have shown that expression of one copy of *UAS*wun*GFP* in PS 14 using one copy of *NP5141pGawB* affects germ cells up to a distance of 33μm. In order to check if the distance of gradient detection by germ cells increases beyond 33μm one could increase the amount of Wun by using two copies of *NP5141pGawB* and *UASwunGFP* in a *wun wun2 Z-* background. Similarly one could check if a decreased enzymatic level causes a reduction in the distance of Wun action. This could be achieved by incorporating a temperature sensitive *UASGal80* in *wun wun2 Z-* embryos with *NP5141pGawB* expressing WunGFP and aging the embryos at normal and partially restrictive temperatures. If we observe a difference in the average closest distance in either cases, it would indicate that amount of Wun expression translates onto the amount of destruction of the extracellular lipid phosphate. This observation would also support our gradient model of lipid phosphate signal.

### 5.3. What is the nature of the lipid gradient?

Our data suggests that the germ cells can detect differences in the levels of extracellular lipid phosphates and position themselves accordingly. It is unlikely that lipid phosphates exist in isolation in the extracellular embryonic space then, in what physical form do these lipid phosphates exist in the embryos?

It is known that in human plasma and serum, S1P and LPA are bound to low and high density lipoproteins and albumin (Murata et al., 2000; Yatomi et al., 2000; Tigyi and Miledi, 1992). Similar protein binding partners could be present in *Drosophila* that would help the lipid phosphates to spread throughout the embryo. In vertebrates, both hematopoietic and vascular endothelial cells are the sources of S1P in plasma (Hla et al., 2008). Similarly in flies, specific cells could produce S1P and this intracellular S1P could likely be transported to the extracellular environment by the ATP-binding cassette (ABC) family of transporters like those in vertebrates, (Meer and Lisman, 2002), or by a specific sphingosine1-phosphate transporter.

Such protein bound or lipoprotein particles may be present uniformly in the entire embryo in a *wun wun2 Z-* embryo. Restricted somatic Wunen expressions could dephosphorylate the lipid phosphate and internalize the dephosphorylated lipid, thereby decreasing the amount of extracellular lipid phosphates in the lipid particles near the Wunen expressing somatic cells. This could produce differences in the extracellular distribution of lipid phosphate carrying particles that the germ cells can detect and hence migrate away from Wunen expressing somatic cells.

However lipid phosphates may not have to hitch a ride on lipoproteins or albumin but may be generated everywhere in the embryo, either by a membrane bound or secreted enzyme. For example, phospholipase D, an enzyme that can produce PA is expressed ubiquitously in *Drosophila* embryo (Renault et al., 2002). However, this PA would be generated intracellularly and has to be flipped or transported to the outside of the cell membrane for Wunens to dephosphorylate. Alternatively, a secreted lysophospholipase enzyme, like that of mammalian Autotaxin (Nakanaga et al., 2010) could also generate ubiquitous extracellular LPA in *Drosophila*. In both the cases, somatic expression of Wunens could destroy lipid phosphate and generate gradients that the germ cells can detect.

Such lipid phosphate gradients instead of being freely soluble, may be stabilized by binding to extracellular matrix (ECM) proteins. Germ cells would possibly detect the lipid phosphate and move towards higher concentrations of ECM bound extracellular lipid phosphate. Human monocytes show such haptotactic migration to gradients of PA and LPA bound to fibronectin-coated polycarbonate filters (Zhou et al., 1995). Recently haptotactic chemokine gradients formed by binding to heparan sulphate were found to be involved in interstitial dendritic cell migration (Weber et al., 2013).

The observation that the germ cells maintain their interstripe position without bouncing from stripe to stripe suggests that the lipid phosphate gradient is stable and maintains its shape as long as Wunens are expressed. To understand more about the stability of the gradient of extracellular lipid phosphate, one could express Wunens transiently in stripes and observe whether germ cells remain aligned in the interstripe region. If so, this would indicate towards a more stable immobilized gradient that might involve binding to ECM. On the other hand if germ cells loose their alignment this would indicate that the lipid phosphate is mobile and spreads as soon as Wunen expression is lost.

### 5.4. Are attractive signals acting together with Wunens for the germ cells to find the gradient?

This study demonstrates that a gradient of lipid phosphates set up by somatic Wunen expression determines the position of the germ cells. This implies that the germ cells would enter those regions that have the highest lipid phosphate and avoid entering the tissues close to Wunens. In that case the highest lipid phosphate should be deep in the endoderm, then why do germ cells leave the endoderm to migrate and enter the mesoderm?

Although we do not have a complete explanation for this observation, several possibilities exist. Firstly, the mesoderm may secrete additional cues that the germ cells are attracted towards and hence enter the mesoderm rather than stay on the endoderm. This factor could be provided by the Hmgcr pathway which when expressed ectopically can attract germ cells to ectopic locations (Van Doren et al., 1998). To test for such a contribution one could express Wunens in

ectodermal stripes in a *wun wun2 Z-* and an *hmgcr* null background and observe if germ cells can still enter the mesoderm and line up in the interstripe. If so, it would suggest that lipid phosphate gradients set up by the spatio-temporal expression of Wunens alone is sufficient for germ cells to find their destination.

Secondly, in a stage 11 wild type embryos, small amounts of *wun2* RNA can be detected in parts of the endoderm just before the germ cells migrate into the mesoderm (our observations). It could be possible that sufficient Wun2 protein levels exist that could function to repel germ cells away from the endoderm and aid them to enter the mesoderm.

Germ cell migration is a co-ordinated action of repulsive signal provided by expression of Wunens as well as attractive signals, which is provided by an unknown attractant produced by the Hmgcr pathway. Is there a link between the *hmgcr* pathway and *wunens*? *hmgcr* encodes HMGCoA reductase that converts 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate. Since flies are auxotrophs for cholesterol, the isoprenoid branch of the mevalonate pathway has been implicated in the production of a geranylgeranyl-pyrophosphate modified protein as the germ cell attractant (Santos and Lehmann, 2004). Whether this attractant is in fact a lipid phosphate and a substrate of Wunens remains an open question.

We have discovered that germ cells can detect the Wunen signal upto a distance of 33µm. In order to fully comprehend the entire picture of *Drosophila* germ cell migration it is essential to know how far the attractive signal of Hmgcr pathway can affect germ cells. To find this one can express *hmgcr* in the ectoderm in stripe domains in an *hmgcr* null background and observe whether the germ cells now migrate to the ectoderm and enter the stripes, which would argue for a long-range effect of *hmgcr*. Similarly *hmgcr* overexpression in *wun wun2 Z-* background might reveal connections between these two pathways.

# Chapter 6
# Conclusion

Cell migration is a fundamental requirement for morphogenesis. While correct cell migration is essential for wound healing and immune response in adults, aberrant migration events can cause mestastasis of cancer cells and ultimately death. Lipids are important signaling molecules that influence migration and survival of cells in different systems. The influence of the *Drosophila* lipid phosphate phosphatase enzymes Wun and Wun2 on germ cell migration and survival gives us a unique system to study the effect of lipid signals on cell survival and migration and test for the existence of extracellular lipid phosphate gradients.

Gradients of chemoattractants or chemorepellants are crucial for directed chemotactic guidance. This extracellular gradient of chemokines maybe produced either by secretion from target tissues alone or by a combination of a more general secretion and subsequent destruction in specific tissues, which translates onto a gradient that guides migrating cells. Experiments in cell culture have demonstrated that cells can undergo directed migration in response to gradients of lipid chemoattractants, for example, *Dictyostelium* cells can respond at a distance of approximately 70 μm away from a micropipette containing LPA (Jalink et al., 1993) and human neutrophils migrate in response to extracellular PA *in vitro* (Frondorf et al., 2010). However the existence of such lipid gradients although implied has remained illusive *in vivo*.

S1P is important for the movement of heart progenitor cells from bilateral positions to the midline in zebrafish (Kupperman et al., 2000), which involves a distance of approximately 100 μm. S1P also regulates the circulation of T-lymphocytes in mouse, in particular allowing T-cells to exit from lymph nodes that are several millimeters in length (Matloubian et al., 2004). In spite of these essential roles it is not always clear whether absolute levels or gradients of lipid phosphates are required. In case of the later, it is also challenging to estimate the distances over which such gradients affect cell behavior.

This study takes a novel approach to study and measure distance of action of an extracellular lipid gradient by using a defined and restricted spatial expression of a lipid phosphate phosphatase. This study has shown that extracellular lipid gradients can be identified *in vivo* using the behavior of migrating cells that respond to such signals. Expression of *Drosophila* lipid phosphate phosphatases can influence germ cells up to 33µm *in vivo*. Are such distances scaled up in larger embryos and tissues of other species? This remains an open question.

How do germ cells sense and read this extracellular gradient? Expression of germ cell Wunens is essential for their survival. While overexpression of LPPs in cell culture has implicated the internalization of exogenous lipids as being the critical function of LPPs, I have confirmed this as being the likely function *in vivo* by verifying this uptake in a relevant cell type at endogenous levels of LPP expression. I have shown that germ cells can internalize extracellular PA in a Wunen dependent manner, which accumulates in lipid droplets. Such accumulation could provide energy reserves for migrating germ cells or the intracellular DAG or PA could bind and activate downstream effectors that affects migration. But how the change in levels of extracellular lipids is translated into migration "towards" or "away from" or "death" signals inside the germ cells is still unknown.

Although extracellular and intracellular lipids are known to affect survival and migration in different cell types, there is no link yet between the dephosphorylation and internalization of lipids by LPPs and the activation of downstream PA or DAG effectors. Testing for such a link in *Drosophila* germ cells would be a useful starting point, which could provide tools and insight to see if this holds true in other migratory cell types that respond to extracellular lipid signals in other organisms.

# Chapter 7
# Materials and Method

**Materials**

**7.1. Fly stocks**

List of Gal4 and UAS transgenic fly lines and mutants used in this study and their references:

| Genotype | Source/Reference |
|---|---|
| *w;;nosGal4VP16* | Van Doren et al., 1998 |
| *twistGal4* | Bloomington stock center |
| *pGawB8-163* | Siekhaus et al., 2010 |
| *w;;hairyGal4[1J3]* | Bloomington stock center |
| *yw;P{en2.4-Gal4}e16EP{UAS-FLP1.D}JD1* | Bloomington stock center |
| *eve37* Gal4* | Small et al., 1996 |
| *pGawBNP5141* | DGRC Japan |
| *pGawBwun2$^{9-175}$* | Daria Siekhaus |
| *w;;p{w+ UAS RedStinger}6* | Bloomington stock center |
| *UAS GFPmoe* | Bloomington stock center |
| *w ;; p{w+ UASbetaglobin Wun2myc}5-25/TM3 p{ry+ ftz>lacZ}* | Starz-Gaiano et al., 2001 |
| *p{UASbetaglobin wun2mycH326K}/±TM3 Ser* | Starz-Gaiano et al., 2001 |
| *UASwunGFP* | Burnett and Howard, 2003 |

| | |
|---|---|
| *UASlazaroGFP* | Garcia-Murillas et al., 2006 |
| *UASlacZ* | Brand and Perrimon 1993 |
| *white-* | Bloomington stock center |
| *w;wun2ex60* | Renault et al., 2004 |
| *w;Df(2R)wunGL/CyO p{ry+ftz>lacZ}* | Zhang et al., 1996 |
| *wunCE/CyO p{ry+ ftz>lacZ}* | Zhang et al., 1996 |

### 7.2. Solutions and Chemicals

PBST - 10 ml of 10% Tween 20 was added to 100 ml of PBS and diluted using miliQ water to make a total volume of 1000ml.

HEPES - To make 1litre 1M HEPES buffer, 238.3 g of HEPES was dissolved in 500ml nanopure water. The pH was adjusted to 7.4 using NaOH pellets. The volume was finally made upto a litre. The final working concentration used was 10mM, which was made from 1M HEPES.

CALCIUM FREE SCHNEIDER'S MEDIUM (Sigma) - The powdered media was dissolved in 800ml of bidest water while gently stirring. The package was rinsed with a small volume of water and added to the 800 ml. 0.4g of sodim bicarbonate was added to the solution and stirred till it dissolved. The pH was adjusted to 9.2 (+/- 0.2) with sodium hydroxide and stirred for 10 min. The solution became turbid. The pH was then adjusted to 0.1- 0.3 pH units below the desired pH with HCl and the solution became clear. This was done because the pH usually rises during filtration. Once the pH was adjusted, the volume was made up to 1000ml. The media was sterilized immediately by filtration using a membrane with 0.22 microns porosity and aseptically dispensed into a sterile container and stored at 4°C.

HYBRIDISATION SOLUTION - The following components were used to make 50 ml of hybridisation solution and stored at -20°C:

| | | Final concentration |
|---|---|---|
| Formamide | 25ml | (50%) |
| SSC 20X | 12.5ml | 5X |
| tRNA (20mg/ml) | 5µl | 100µg/ml |
| Heparin | 50µl | 50µg/ml |
| Tween 20% | 0.5ml | 0.1% |

CELL ORGANELLE STAINS - To stain the different intracellular compartments of the germ cells we used the following vital dyes:

**Lysotracker Blue DND-22** (Invitrogen Molecular Probes) – This is fluorescent acidotropic probe used for labeling and tracking acidic organelles in live cells. This was used to mark the lysosomes in germ cells in *Drosophila*.

**Bodipy TR $C_5$ – ceramide complexed to BSA** (Invirogen Molecular Probes) - This marker selectively stains for the golgi apparatus in living cells.

**ER-Tracker™ Red (glibenclamide Bodipy TR)** (Invitrogen Molecular Probes) – This is dye with glibenclamide conjugated to Bodipy TR which helps it to bind to the sulphonylurea receptors of ATP sensitive potassium channels, prominent on the ER.

**Nile red** (Invitrogen Molecular Probes)– This is an uncharged phenoxazone dye that is used as a fluorescent stain for intracellular lipids. It fluoresces differently depending on whether it binds to neutral lipid droplets or phospholipids inside cellular compartments.

**FM4-64** (Invitrogen Molecular Probes) – This is a lipophilic styryl dye used widely as a marker for plasma membrane and vesiculation in various eukaryotic cell types. This binds to the outer layer of the plasma membrane and becomes intensely fluorescent.

### 7.3. Antibodies used for DAB, fluorescent staining and in situ hybridization of the embryos:

| Primary antibody | Raised in | Dilution used | Reference / source |
|---|---|---|---|
| Vasa | Rabbit | 1/10000 | Lehman lab |
| | Rat | 1/40 | DSHB |
| LacZ | Rabbit | 1/20000 | Cappel |
| | Mouse | 1/1000 | Promega Z378a |
| Myc | Rabbit | 1/500 | Abcam Ab9106 |
| GFP | Chicken | 1/1000 | Abcam Ab13970 |
| Wun2 | Rabbit | 1/100 | Andrew Renault |
| Eya | Mouse | 1/12 | DSHB |
| Dig:POD | Sheep | 1/250 | Roche |

| Secondary antibody | Raised against | Dilution used | Reference / source |
|---|---|---|---|
| Biotin | Rabbit | 1/500 | Jackson Immuno Research Laboratories |
| Alexa488 | Rat/Rabbit/Mouse/Chicken | 1/500 | Invitrogen |
| Cy5 | Rat/Rabbit/Mouse | 1/500 | Jackson Immuno Research Laboratories |
| Cy3 | Rabbit/Mouse/Chicken | 1/500 | Jackson Immuno Research Laboratories |

Dapi was used as a DNA marker and added with the secondary antibodies in a dilution of 1/1000.

### 7.4. Lipids

List of the lipids that were used for uptake studies are given in the Appendix.

## Methods

### 7.5. Fly Husbandry

All *Drosophila melanogaster* fly stocks were maintained at 18°C. They were reared on standard *Drosophila* food consisting of corn meal, agar and sugar in medium (25ml) and small (10ml) size vials. Stocks in use were kept at room temperature while crosses set were maintained at 25°C.

### 7.6. Setting up crosses

Virgin females required for setting up crosses were collected twice everyday within eight hours of emergence from pupae when stock or cross vials were kept at 25°C. If they were at 18°C then virgin females could be collected every 18 hours. Crosses were set in yeasted vials with males of the desired genotype and kept at 25°C.

### 7.7. Embryo collection

The fly stocks or crosses were set for egg laying on yeasted apple juice plates for overnight. The embryos were then fixed and stored in methanol at -20°C until use.

### 7.8. Embryo fixation and antibody staining

#### 7.8.1. Methanol fixation of embryos

For methanol fixation of embryos, a fixation mix containing 1.75ml PBS, 0.25ml 37% formaldehyde (4% final formaldehyde concentration) and 8ml n-Heptane was prepared and mixed in glass scintillation vials.

Apple juice plates containing embryos were covered with 50% bleach for 3min to dechorionate the embryos. The embryos were poured through sieves and washed thoroughly by squirting deionized water. The mesh carrying the embryos was removed, slightly dried and swirled in the fixation mix in scintillation vials. The vials were shaken on an orbital shaker for 20 min at 200 rpm. The lower PBS phase was removed with a glass pipette, 7ml of methanol was added and the vials shaken vigorously for 1 min to remove the vitelline membrane. The embryos that were fixed and de-vitellinised, settled at the bottom of the vial. All of the methanol and heptane together with the embryos that did not sink to the bottom was removed. The fixed embryos were washed twice with methanol, transferred into eppendorfs and stored in methanol at -20°C for later use.

### 7.8.2. Antibody staining of embryos

#### a) DAB staining of embryos

The embryos stored at -20°C in methanol were rehydrated twice in PBST. The embryos were blocked in 0.1% BSA in PBST for an hour on a nutator at room temperature. The embryos were incubated overnight with the required primary antibodies in 0.1%BSA in PBST at 4°C. The following day the embryos were washed with 0.1% BSA in PBST four times for 1min, 5min, 15min and 30 min. An appropriate Biotin secondary antibody in 0.1% BSA in PBST was added and placed on the nutator for 3 hours at room temperature. The embryos were rinsed four times with PBST at room temperature and incubated for 30 min in vectastain solution (10µl of solution A + 10 µl of solution B per ml of PBST). The embryos were rinsed four times in PBST for 1min, 5min, 15min and 30min and then stained with DAB solution (1µl of 30% hydrogen peroxide + 10 µl of 20mg/ml DAB per ml of PBST) and monitored for staining. Once staining was complete, DAB was replaced with PBST. The embryos were washed three times with PBST to remove extra DAB and mounted in 75% glycerol and the edge of the cover slips sealed with varnish. The slides were stored at 4°C or imaged immediately.

For making permanent slides, the embryos were dehydrated serially in 30%, 50%, 70% and 100% ethanol and finally washed in acetone. After complete removal of acetone, the embryos were mounted in epon resin and baked at 65°C overnight.

**b) Fluorescent staining of embryos**

For fluorescent antibody stainings, all the steps are identical to those described in the previous section with the exception that the secondary antibodies used here are labelled with a fluorophore and the steps of vectastain and DAB incubations are unnecessary. Incubation with the secondary antibodies and all the subsequent washes of the embryos were carried out in dark. The embryos were washed four times in PBST for 1min, 5min, 15min and 30min and mounted in aquamount. The slides were allowed to harden overnight at 4°C and imaged the following day.

**c) Benzyl benzoate mounting of embryos**

For imaging embryos through their entire volume, fluorescently stained embryos were first dehydrated in 50% methanol and then washed twice with 100% methanol. The embryos were mounted in a mixture of benzyl alcohol and benzyl benzoate (1:2 ratio). The edge of the coverslip was sealed using nail varnish and imaged immediately.

**7.9. Fluorescent In-situ hybridization and antibody staining**

Embryos to be used for in situ hybridization were fixed in 37% formaldehyde for 20 minutes. The rest of the fixation protocol was similar to what has been described earlier.

**a) Making of the riboprobes**

For making *wun2* riboprobe, *wun2* was PCR amplified using SP6 and T7 from a full-length cDNA clone (termed LD7) in pBluescript. This PCR amplified *wun2* was used for *in vitro* transcription using the Dig RNA labeling kit (Roche) and T7 RNA polymerase. For a total reaction volume of 10µl, the following components were added:

| | |
|---|---|
| PCR product | 2µl |
| 10X transcription buffer | 1µl |
| DIG-NTP mix | 1µl |
| RNase inhibitor | 0.5µl |
| Nuclease free water | 4.5µl |
| T7 polymerase | 1µl |
| Total | 10µl |

The reaction mixture was incubated at 37°C for 2 – 4 hours. After the reaction was over, the transcription product was run on a 1% agarose gel stained with SYBRSafe DNA stain (Invitrogen), to check the size of the riboprobes synthesized. After incubation, nuclease free water was used to make the volume up to 100µl. To this, 20µl of 4M LiCl, 10µl of 20mg/ml tRNA and 600µl of ethanol (RNase free) was added and incubated at -20°C overnight. The sample was centrifuged at 4 °C for 15 minutes and the supernatant was discarded. The pellet was washed in 70% ethanol, dried, resuspended in 150µl of hybridization solution, incubated at room temperature for 15 - 20 minutes and stored at -20°C.

**b) Fluorescent in situ hybridization and antibody staining**

*Day 1*

For fluorescent in situ hybridization, the embryos were rinsed in ice cold methanol (-20°C) and incubated in 1:1 methanol:PBST for 5 minutes. The embryos were rinsed twice with PBST and post fixed for 20 minutes in 5% formaldehyde in PBST. The formaldehyde was removed with several washes in PBST. Proteinase K was defrosted and used at a final concentration of 3µg/ml. The embryos were incubated in Proteinase K at room temperature for 8 minutes and then on ice for 45 minutes with intermittent mixing. Proteinase K was removed and the embryos washed twice in 2 mg/ml of glycine for 2 minutes. PBST washes were used to remove glycine and the embryos post fixed again in 5% formaldehyde for 20 minutes. Fixative was removed by several washes in PBST. The embryos were then incubated in 1:1 PBST:hybridization solution for 5 minutes and next in

hybridization solution for 5 minutes. This solution was replaced by pre-hybridisation solution (pre heated hybridization solution) and incubated at 56°C for at least 2 hours. The pre-hybridisation solution was replaced with 10µl probe + 90µl hybridization solution. The embryos were incubated at 56°C for at least 12 hours.

*Day 2*

Probe was removed and stored at -20°C to be reused later (can be reused upto two times more). Pre-warmed hybridization buffer was used to wash the embryos at 56°C for 2 hours with several wash changes. The embryos were then washed in 1:1 hybridisation solution:PBST on the nutator for 20 minutes. This was followed by several washes in PBST for upto 1 hour and blocked in 1% BSA in PBST for 1 hour. The embryos were incubated at 4 °C overnight with antiDig:POD (1:250) in addition to other primary antibodies required for antibody staining.

*Day 3*

Embryos were washed several times with PBST to remove primary antibodies and incubated in Tyramide staining solution (Invitrogen Molecular Probes) for 2 hours on shaker. The staining solution was washed off with PBST and the embryos blocked in 1% BSA in PBST for 1 hour. Secondary antibodies were added at this stage and kept on shaker for 2 hours or overnight. The embryos were washed with PBST several times to remove excess secondary antibody and mounted in aquamount or benzyl benzoate.

## 7.10. Methods in cell homogenate incubations

### 7.10.1. Preparation of the lipid substrate

#### a) Preparation of multilamellar vesicles (MLVs)

The liposomes were prepared by freeze thaw extrusion method. All glasswares to be used were first cleaned and rinsed with chloroform. The required volume of lipids (in chloroform) were transferred in a 50ml round bottom flask and diluted with the requisite amount of chloroform. The flask was connected to a rotary evaporator to evaporate the chloroform and obtain a thin layer of lipids at the

bottom of the glass flask. The pressure was first adjusted to - 0.2 bar and gradually lowered and maintained at - 0.5 bar for 1 hour. The flask was rotated constantly at approximately 80 rotations per min while the pressure was further lowered upto - 0.9 bar. After the chloroform was removed completely, the flask was detached and kept under high vacuum overnight to remove any residual fluid. An appropriate volume of preheated buffer (1mM HEPES, pH 7.6) was added to the dried lipid film and the flask was shaken at 180 rpm for 1-2 hours at 37°C till all the lipid film was resuspended in the aqueous buffer. The flask was then put through a freeze thaw cycle by alternately dipping it into liquid nitrogen and a water bath maintained at 40°C, 10 times each to generate large multilamellar vesicles (MLVs). The MLVs were then aliquoted into eppendorfs and snap frozen in liquid nitrogen and stored at - 80°C till use. The final proportion of NBD lipids to POPC was 1:5 molar ratio with a final concentration of NBD labeled substrate at 20μM.

**b) Making unilamellar vesicles (ULVs)**

The frozen MLVs were thawed at room temperature. An extruder was used to generate small unilamellar vesicles (ULVs) of a desired size. The MLVs were loaded into a glass Hamilton syringe and passed through a polycarbonate membrane of a 100nm pore size into another empty Hamilton syringe (from Avanti Polar Lipids). This was done 21 times so as to ensure most of the MLVs have been made into small ULVs of size 100 nm. Before loading the MLVs, the extruder setup was optimized using the same buffer as used to resuspend the liposomes, in order to minimize the loss of MLVs during the extrusion process. The ULVs thus generated were checked for uniformity in size by electron microscopy. The ULVs were stored at 4°C and used up within a week.

**c) Negative staining of the ULVs before checking on the electron microscope**

The ULVs prepared were diluted serially in 1:10, 1:100 and 1:1000 dilutions. Several carbon coated support films of 400# grid size were first made hydrophilic by 5min glow discharge under vaccum. A drop of the ULV preparation was then adsorped on the discharged support meshes. It was rinsed with water and stained

with 1% uranyl acetate in water for 1 min. The excess stain was drained using a filter paper. The sample was then mounted into the holder and placed inside the electron microscope to be observed and photographed.

**d) Making lipids complexed to BSA**

The required volume of lipids in chloroform was transferred into a glass round bottomed flask or a glass test tube. The chloroform was evaporated in a rotary evaporator as described in the previous section 5.1.a or using a nitrogen evaporator. The chloroform was evaporated completely until a thin film of lipid mixtures was left behind at the bottom of the glass flask or tube. This thin lipid film was resuspended in 1% fatty acid free BSA (Sigma life sciences). Equal molar ratios of lipids to BSA or slightly higher molar ratio of BSA was taken. The mouth of the glass tube or flask was closed tightly and shaken at 37 for 1-2 hours until the entire lipid flim is resuspended in the BSA solution. This lipid complexed to BSA was stored at 4°C and was used up within a week. The final concentration of lipid substrates used was 20μM.

### 7.10.2. Preparation of cell suspension

The embryos used for preparation of cell suspensions were obtained by crossing *w;;nos Gal4* or *w;wun2[EP2208ex60];nosGal4* to *UAS red stinger* males to label the germ cell nuclei red or *UASwun2myc;UASred stinger* males to label and overexpress Wun2 in the germ cells.

The cross of about 80 virgins and 40 males were set for laying overnight on yeasted apple juice plates. The embryos were dechorionated with 50% bleach as mentioned earlier. The embryos were washed well with deionized water, transferred into the glass dounce homogeniser and resusupended in appropriate volume of calcium free Schneider's medium. The embryos were homogenized using gentle but firm 8-10 slow strokes of a loose pestle. The cells were passed through the 40 μm filter directly after homogenization. The cells were then pelleted at 1200g for 3 min on a tabletop centrifuge and washed twice with PBS.

### 7.10.3. Conditions and method of cell suspension incubations

The cell pellet was resuspended in 150 µl of the freshly prepared ULVs in 1.5ml eppendorfs. The cells were incubated at room temperature (26°C) for 5minutes and centrifuged for 3min at 1200g. The supernatant was removed and cells resuspended and washed once with PBS. The cell pellet was finally resuspended in an appropriate volume of PBS. A drop of this cell suspension was put on a coverslip well made of silicon gaskets and sealed with a glass slide covering the top of the gasket wells. The cells were observed and imaged immediately using an inverted confocal microscope within the next one hour.

## 7.11. Microscopy and Image processing

### 7.11.1. Light microscopy

The vasa stained embryos (stages 13 and 14) were counted for germ cells using the 20X air objective and DIC on the Zeiss Imager Z1 microscope and also pictured with an AxioCamHRc (Zeiss) and the Axio Vision Rel. 4.6 software (Zeiss) to check the pattern of migration.

### 7.11.2. Confocal microscopy

Fluorescent antibody staining of embryos was imaged using the Zeiss upright confocal microscope at 40X and 60X magnification and the Olympus Fluoview 1000 microscope at 40X and 60X magnification using the Olympus Fluoview software Version 1.7a.

Wunen dependent labeled lipid substrate uptake was imaged using the Olympus Fluoview1000 microscope and Olympus Fluoview software Version 1.7a at 60X, 7.5 zoom magnification under comparable laser settings for all the different incubation experiments.

Live imaging of embryos was performed using the Olympus Fluoview 1000 microscope and Olympus Fluoview software Version 1.7a at 60X, oil objective, at comparable settings for the different genotypes, with an interval of 3 minute.

### 7.11.3. Electron microscopy

The ULVs made of NBD:POPC in 20mM HEPES buffer was negatively stained with uranyl acetate adsorbed on a glow discharged copper grid (400#) to check the homogeneity of preparation and the size distribution. The liposome preparation was imaged at 30,000 magnification in a Technai T12 Spirit TEM, at 120KB by a Gatan Ultrascan USC4000 and Digital Micrograph (Gatan).

**7.11.4. Image processing -** Images were processed using the following software:
Imaris 7.4.1
Fiji or Image J
Adobe InDesign CS5.5

### 7.11.5. Segmentation of ectodermal stripes

The positions of the germ cells were manually defined using the Point Picker plugin (Philippe Thévenaz, Biomedical Imaging Group, Swiss Federal Institute of Technology Lausanne) in ImageJ. To identify the stripes expressing the fluorescent marker, the fluorescence intensity was first standardized along the z-axis by determining the 90 percentile expression in each z-slice, fitting a line (slope + intercept) to these values and using the line fit to normalize fluorescence intensities. The normalized image stack was then thresholded at the 80, 85, 90, and 95 percentile. The resulting binary stack was then smoothed by 5 iterations of binary erosion in the x-y directions, followed by 3 iterations of binary dilation. Subsequently, all objects with a pixel volume of >1000 were labeled. The labeled image stacks were manually inspected to identify the appropriate threshold (85 in most cases). Oversegmentation was corrected by merging disjoint parts of the same stripe. Given the curated segmentation, the distance of each germ cell from

all stripes was determined using the distance_transform_edt() function from scipy.ndimage (http://www.scipy.org/).

To investigate the hypothesis that germ cells migrate to locations as far as possible from Wun2 expressing stripes, their distances to the closest and next closest stripes were compared in wild type embryos with *en* driven control stripes, *wun wun2* somatic null embryos with *en* driven control stripes, and *wun wun2* somatic null embryos with *en* driven *wun2* stripes. Since different germ cells of the same embryo are not statistically independent observations, statistical significance was assessed by Mann- Whitney *U-test* on embryo means. In addition, we produced 1000 boot-strap data sets by sampling embryos with replacement and measured the boot-strap distribution of the Kolmogorov-Smirnov statistic (maximal vertical difference of the cumulative histograms). If this statistic is consistently either positive or negative, the observations were considered to be robust across all embryos examined. The observations were statistically significant using both criteria.

# Contributions

The work described in this thesis was performed at the Max Planck Institute for Developmental Biology in Tübingen under the supervision of Dr. Andrew Renault. The following people have contributed to the work:

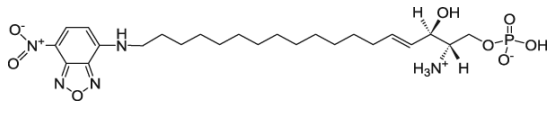
Andrew Renault
a) Movies shown in this thesis for *nos moe GFP; en>wunGFP* in *wunwun2 Z-* and *nos moe GFP; en>GFPmoe* embryos.
b) Segmentation and distance measurements for stages 11 and 12 of *wun2 in situ* pattern using Imaris software.

Richard Neher
a) Segmentation of the ectodermal stripes in wild type *en>* control, *wunwun2 Z- en >* control and *wunwun2 Z- en>wun2* embryos.
b) Equidistance measurements of germ cells to *en* stripes in the above mentioned backgrounds. The analysis scripts were written in python.

# Appendix

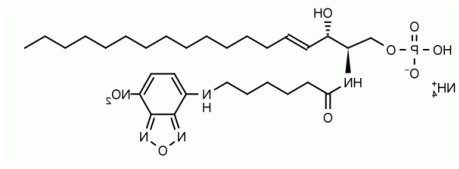
**List and structure of lipids used in the study:**

NBD Sphingosine-1-Phosphate (NBD-S1P)



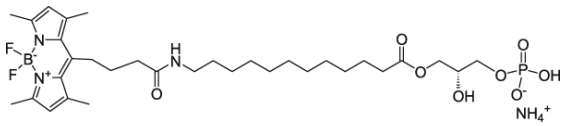
Mol Wt = 557.577
Exitation/ Emission
460nm/534nm
Source: Avanti

N-NBD Ceramide-1-Phosphate (NBD-Cer1P)



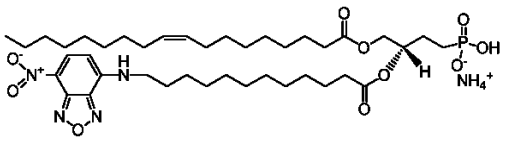
Mol Wt = 672.75
Exitation/ Emission
465nm/535nm
Source: Echelon

TopFluor Lyso PA (TF-LPA)



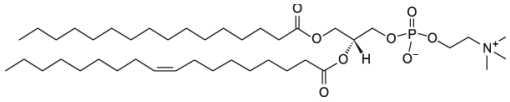
Mol Wt = 702.575
Exitation/ Emission
495nm/503nm
Source: Avanti

18:1-12:0 NBD Phosphonate Analog (NBD-POA)



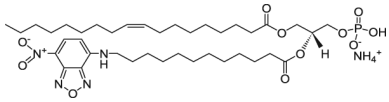
Mol Wt = 811.99
Exitation/ Emission
467nm/535nm
Source: Avanti

16:0 - 18:1 Phosphocholine



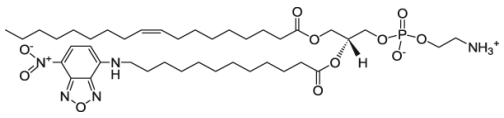
Mol Wt = 760.076
Source: Avanti

18:1-12:0 NBD Phosphatidic Acid (NBD-PA)



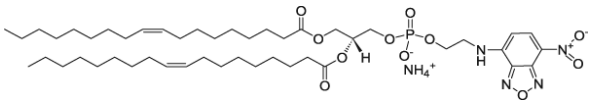
Mol Wt = 813.958
Exitation/
Emission
460nm/534nm
Source: Avanti

18:1-12:0 NBD Phosphatidylethanolamine (NBD-PE)



Mol Wt = 839.995
Exitation/
Emission
460nm/534nm
Source: Avanti

18:1 NBD Phosphatidylethanolamine (NBD-PE)



Mol Wt = 924.155
Exitation/
Emission
445nm/507nm
Source: Avanti

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